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

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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.

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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 persulfate
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BLAST	Basic Logical Alignment Search Tool
bp	Base-pair
BSA	Bovine serum albumin
ca.	<i>circa</i> (approximately)
CBB	Coomassie Brilliant Blue
cDNA	DNA complementary to a RNA transcript, synthesised from RNA by reverse transcription <i>in vitro</i>
dATP	Deoxyadenosine Triphosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMF	<i>N,N</i> -dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleotide 5' triphosphate
DTT	Dithiothreitol
dUTP	2'-Deoxyuridine 5'-Triphosphate
<i>E.coli</i>	<i>Eschericia coli</i>
EDTA	Ethylenediaminetetraacetic Acid
EFE	Ethylene forming enzyme
EIN	Ethylene insensitive
FW	Fresh weight
<i>g</i>	Acceleration due to gravity (9.8m s ⁻²)
g	Gram
GAA	Glacial Acetic Acid
GACC	1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid
GUS	<i>E. coli</i> β-glucuronidase
h	hour
IgG	Immunoglobulin G

IPTG	Isopropyl- β - <i>D</i> -thiogalactopyranoside (C ₉ H ₁₈ O ₅ S)
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	<i>p</i> -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	Polyvinylidene difluoride
PVP-40	Polyvinyl pyrrolidone
PVPP	Polyvinyl polypyrrolidone
RH	Relative Humidity
RNA	Ribonucleic acid
RNase	Ribonuclease
RO	reverse osmosis
RT-PCR	Reverse Transcriptase-polymerase chain reaction
s	second
SAM	S-adenosyl- <i>L</i> -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	<i>N,N,N',N'</i> -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
Asparagine	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
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1. INTRODUCTION

1.1. Background

Plants can experience many different stress conditions, both biotic and abiotic, during their life span that influence their growth rate and productivity (Yang *et al.*, 2000; Boudsocq and Lauriere, 2005; Yang *et al.*, 2006; Gallie *et al.*, 2009). Amongst these different types of stress, the effect of a suboptimal environment on plant growth is of particular interest, since globally the world is facing climate change, which is predicted to affect the growth of plants in their natural ecosystems (Chapin, 1991). The effect of climate change, due to the enhancement of the greenhouse gases, has increased the average global surface temperature of the earth by $0.6 \pm 0.2^\circ\text{C}$ over a period of 10 years (from 1990 to 2001). In addition, it has changed rainfall patterns, with prolonged heavy daily rainfall or prolonged drought in certain seasons which increases soil aridity due to a decrease in water availability (Chapin, 1991; Campbell *et al.*, 1997; Petit *et al.*, 1999; Woodward *et al.*, 2001; Hughes, 2003). Concern on water availability for plant productivity has received particular attention because agriculture is a major user of water resources (Chaves *et al.*, 2003) and the availability of water to plants during growth is a key factor determining plant growth and development (Palta, 2005; Chaves *et al.*, 2009).

Plants respond differently to water deficit and there are some plants which can tolerate low water availability, through various morphological adaptations, including alteration of growth rate and regulation of stomatal closure (de Souza *et al.*, 2003; Bohnert *et al.*, 2006; Chaves *et al.*, 2009; Duan *et al.*, 2009; Liu *et al.*, 2009; Quist *et al.*, 2009). Other plant species are more susceptible to a water deficit but the responses of all plants are controlled by complex mechanisms, involving water stress perception and signalling, which then alters the biosynthesis and the signalling of various plant hormones, including ethylene. These changes are proposed to drive the biochemical and morphological adaptations of plants to water deficit (de Souza *et al.*, 2003; Bohnert *et al.*, 2006; Chaves *et al.*, 2009; Duan *et al.*, 2009; Liu *et al.*, 2009; Quist *et al.*, 2009).

1.2. The Plant Hormone Ethylene

Ethylene, a simple unsaturated gaseous plant hormone, plays an important role in the regulation of many physiological responses of plants (Bleecker and Kende, 2000). Ethylene is produced by the majority of higher plants (Osborne *et al.*, 1996) and it is continuously present during the whole life cycle (Bleecker and Kende, 2000). The level of ethylene changes naturally throughout the different developmental stages of the plant and regulates various developmental processes, including embryo development (*eg.* HersHKovits *et al.*, 2009), breaking of seed dormancy and seedling emergence (*eg.* Geneve and Wood, 2006), control of hypocotyl elongation (*eg.* De Grauwe *et al.*, 2005), root initiation and elongation (*eg.* Takahashi and Inoue, 2008), cell elongation (*eg.* Pieriek *et al.*, 2009), shoot and leaf initiation (*eg.* Matsuo and Banno, 2008), leaf development, maturation and senescence (*eg.* Hunter *et al.*, 1999; Chen and McManus, 2006) through to flower and fruit development, maturation, senescence and abscission (Yang and Hoffmann, 1984; Abeles *et al.*, 1992; Liang *et al.*, 1996; Bleecker and Kende, 2000).

In addition to the role of ethylene in regulating plant cell development, the exposure of plants to various environmental cues, such as wounding, pathogen attack, flooding, drought, anoxia, ozone and light has also been shown to alter ethylene biosynthesis in plants (Yang and Hoffmann, 1984; Abeles *et al.*, 1992; Liang *et al.*, 1996; Bleecker and Kende, 2000; Chang *et al.*, 2008; Choudhury *et al.*, 2008) and so the hormone is proposed to be an important regulator of the response of the plant to these various stimuli. Ethylene biosynthesis is also altered by other hormonal cues, including jasmonic acid (Heredia and Cisneros-Zevallos, 2009), auxin (Pieriek *et al.*, 2009), abscisic acid (Rodrigous *et al.*, 2009); gibberellic acid (Fukao and Bailey-Serres, 2008), and by ethylene itself which can negatively or positively regulate its own biosynthesis (Riov and Yang, 1982; Inaba and Nakamura, 1986; Nakatsuka *et al.*, 1998; Chang *et al.*, 2008; Tang *et al.*, 2008). In addition, the balance between ethylene and these other hormones can mediate different responses by plants to these various environmental cues, in different tissues (Fukao and Bailey-Serres, 2008; Heredia and Cisneros-Zevallos, 2009).

Thus the evidence suggests that ethylene has a role in a wide range of physiological processes involving ethylene, not only in the regulation of developmental changes but also in the response of plants to hormonal and environmental cues. These responses are regulated by the level of ethylene biosynthesis, ethylene perception and ethylene signal transduction. These aspects will be discussed subsequently.

1.3. Ethylene Biosynthesis in Higher Plants

Ethylene-regulated processes are initiated by an alteration in ethylene biosynthesis (Kende, 1993). The biosynthetic pathway of ethylene, in higher plants, has been well characterised and reviewed (Yang and Hoffmann, 1984; Bleecker and Kende, 2000; Rzewuski and Sauter, 2008) (Figure 1.1.).

In higher plants, ethylene biosynthesis is initiated from the conversion of methionine (Met) to S-adenosyl-L-methionine (SAM or S-AdoMet), the precursor of ethylene biosynthesis (Yang and Hoffmann, 1984; Kende, 1993), by the enzyme S-adenosylmethionine synthetase (SAM synthetase or AdoMet synthetase). S-AdoMet is the major methyl donor used by plants for various biochemical pathways, including polyamines and ethylene biosynthesis (Wang *et al.*, 2002). AdoMet is converted into 1-aminocyclopropane-1-carboxylic acid (ACC), by the enzyme ACC synthase (ACS) (Yang and Hoffmann, 1984; Zarembinski and Theologis, 1994; Bleecker and Kende, 2000; Wang *et al.*, 2002; Kim *et al.*, 2004). In addition, ACS also produces 5-methylthioadenosine (MTA), which is converted to methionine, *via* a modified methionine cycle, which is also known as the 'Yang Cycle' and thus continuous ethylene synthesis can occur without any depletion of the methionine pool (Yang and Hoffman, 1984; Miyazaki and Yang, 1987; Bleecker and Kende, 2000). MTA is hydrolysed by MTA nucleoside, to produce adenosine and methylthioribose (MTR). MTR is phosphorylated by MTR kinase to produce MTR-phosphate (MTR-P), which is then converted into acireductone, by an isomerase and dehydratase-enolase-phosphatase. Reaction of acireductone with dioxygen, which is catalysed by Fe-acireductone dioxygenase (Fe-ARD), produces 2-keto-4-methylthiobutyrate (KMTB) which is then transaminated by the enzyme

transaminase to produce methionine (Yang and Hoffmann, 1984; Bleecker and Kende, 2000; Rzewuski and Sauter, 2008).

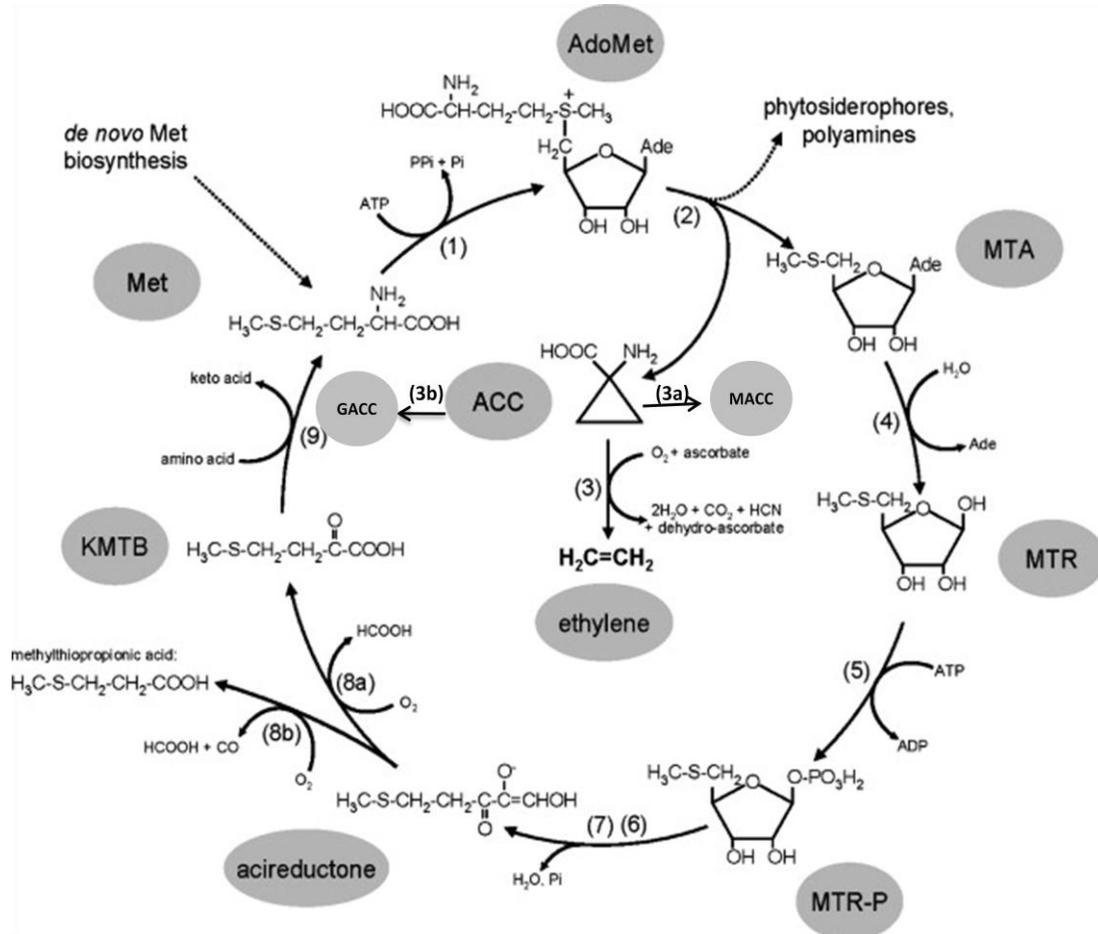


Figure 1.1 The ethylene biosynthetic pathway and the methionine cycle in higher plants (modified from Rzewuski and Seuter, 2008).

Met, L-Methionine; AdoMet, S-adenosyl-L-methionine; MTA, 5-methylthioadenosine; Ade, Adenosine; MTR, methylthioribose; MTR-P, MTR-phosphate; KMTB, 2-keto-4-methylthiobutyrate; ACC, 1-aminocyclopropane-1-carboxylic acid. The enzymes involved in these reactions are: 1. S-adenosylmethionine synthetase (SAM synthetase or AdoMet synthetase); 2. ACC synthase (ACS); 3. ACC oxidase (ACO; 3a. γ -Glutamyl ACC Transferase; 3b. N-Malonyl ACC Transferase; 4. MTA nucleoside; 5. MTR kinase; 6. isomerase; 7. dehydratase-enolase-phosphatase, 8a. Fe-acireductone dioxygenase (Fe-ARD); 8b. Ni-acireductone dioxygenase (Ni-ARD); and 9.transaminase.

Introduction

The key step in ethylene biosynthesis is the conversion of AdoMet to ACC, by the enzyme ACC synthase and the oxidative cleavage of ACC by the enzyme ACC oxidase into ethylene (Yang and Hoffmann, 1984; Zarembinski and Theologis, 1994; Bleecker and Kende, 2000; Wang *et al.*, 2002; Kim *et al.*, 2004). Each of these enzymes will be discussed subsequently. In addition, two ACC conjugates can also be formed: *N*-Malonyl-ACC (MACC) and γ -Glutamyl ACC (GACC) (Sarquis *et al.*, 1992). Conjugation of ACC into MACC, or GACC, is catalysed by *N*-Malonyl ACC Transferase or γ -Glutamyl ACC Transferase, respectively. This reaction is irreversible but conjugation may serve to reduce the availability of free ACC, and act as a mechanism to limit ethylene synthesis (Sarquis *et al.*, 1992).

Ethylene biosynthesis is a highly regulated process. The regulation of ethylene biosynthesis in normal growing conditions (or by environmental cues) is controlled at both the transcriptional, post-transcriptional and post-translational levels (Yamagami *et al.*, 2003).

1.3.1. S-adenosylmethionine Synthetase (SAM synthetase or AdoMet synthetase)

S-adenosyl-L-methionine synthetase (methionine adenosyltransferase or AdoMet synthetase; EC 2.5.1.6) is the enzyme catalysing the synthesis of *S*-adenosyl-L-methionine (SAM or AdoMet) from methionine and ATP, by the transfer of an adenosyl group from ATP to the sulfur atom of methionine. SAM synthetase is found in both prokaryotic and eukaryotic organisms (Tabor and Tabor, 1984) and is classified as a housekeeping enzyme (Fluhr and Mattoo, 1966), since SAM is not only the precursor of ethylene biosynthesis in plants (Yang and Hoffman, 1984; Kende, 1993), but it is also decarboxylated and used for the biosynthesis of polyamines (Miyazaki and Yang, 1987; Roeder *et al.*, 2009) (Figure 1.1.). Evidence exists that polyamine and ethylene biosynthesis are in metabolic competition for SAM, since the compound is a precursor for both polyamines and ethylene biosynthesis (Wi and Park, 2002; Tari *et al.*, 2006). Using transgenic plants harbouring antisense cDNAs of carnation ACS or ACO, Wi and Park (2002) demonstrated enhanced polyamine and decreased ethylene production in the transgenic lines. Similarly, Tari *et al.* (2006) showed an increased accumulation of

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Ca^{2+} and ethylene production in the roots of wheat (treated by Ca^{2+}). In their report, application of MGBG [methylglyoxal bis(guanylhydrazone)], an inhibitor of SAM decarboxylase (an enzyme involved in conversion of SAM into polyamines) increased accumulation of Ca^{2+} in the roots (Tari *et al.* (2006) indicating an indirect evidence of competition on SAM utilisation for ethylene and polyamine biosynthesis. In addition, SAM is an important substrate for many metabolite methyltransferases and a major methyl group donor in transmethylation reactions of proteins, carbohydrates, lipids and nucleic acids (Fluhr and Mattoo, 1996; Roeder *et al.*, 2009). Therefore, it is suggested that only a minor portion of SAM is used for ACC production so the formation of SAM is not widely considered to be a rate limiting step in ethylene biosynthesis (Yang and Hoffman, 1984; Peleman *et al.*, 1989).

SAM synthetase is encoded by a small multi-gene family in plants and it is differentially expressed during plant development and in response to environmental cues (Peleman *et al.*, 1992). For example, it is differentially expressed during senescence in carnation flowers (Woodson *et al.*, 1992) and in response to IAA treatment (Gomez-Gomez and Carrasco, 1998). Moreover, SAM synthetase is induced by environmental cues (which also induce ethylene production) such as salt stress (Espartero *et al.*, 1994), mechanical stimuli (Kim and Yang, 1994), water deficit stress (Mayne *et al.*, 1996), ozone exposure (Tuomainen *et al.*, 1997) and fungal and bacterial infections (Arimura *et al.*, 2002). These studies suggest that there may be some level of control of the gene family in tissues that produce ethylene either as part of development or in response to environmental cues.

1.3.2 ACC Synthase (ACS)

ACC synthase (*S*-adenosyl-l-methioadenosine lyase; EC 4.4.1.14) belongs to the aspartate aminotransferase enzyme family which requires pyridoxal-5'-phosphate- (PLP) as a cofactor (Li *et al.*, 2005; Barnes *et al.*, 2008). ACS is a cytosolic enzyme which is highly labile and can be inactivated by its substrate, SAM, during catalysis (Satoh and Esashi, 1986; Kim and Yang, 1992). In tomato, incubation of partially purified ACS with SAM inactivated the ACS enzyme by covalent linking of the aminobutyrate portion of SAM to the protein (Satoh and Yang, 1989). ACS has high

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substrate specificity and affinity for SAM (Kim and Yang, 1992) with a half-life ranging from 20 minutes to several hours (Spanu *et al.*, 1990; Kim and Yang, 1992; Kende, 1993).

Analysis of the four members of the aminotransferase enzyme super family reveals that 12 invariant amino acid residues are conserved amongst the aminotransferase enzyme families (Metha *et al.*, 1989) and ACS has the 12 conserved amino acid residues which are involved in the binding of the substrate (SAM) to the PLP (Li *et al.*, 2005; Barnes *et al.*, 2008). ACS is encoded by a multi-gene family in many plant species (Bleecker and Kende, 2000; Yamagami *et al.*, 2003; Murray and McManus, 2005). Many reports suggest that regulation of ethylene biosynthesis by ACS is controlled at the transcriptional and/or transcriptional levels (Yamagami *et al.*, 2003). Various ACS isoenzymes catalyse the same chemical reaction, but each member of the gene family is differentially expressed and regulated during plant growth and development and in response to different environmental stimuli (Woeste *et al.*, 1999; Wang *et al.*, 2002; Kim *et al.*, 2004; Tsuchisaka and Theologies, 2004; Yang *et al.*, 2004; Murray and McManus, 2005; Joo *et al.*, 2008). In *Arabidopsis*, there are nine members of the ACS multi-gene family and eight of them encode active enzymes, whilst one, *ACS1*, encodes for a catalytically inactive enzyme (Yamagami *et al.*, 2003; Joo *et al.*, 2008). The nine ACS genes in *Arabidopsis* are differentially expressed during plant development (Yamagami *et al.*, 2003; Tsuchisaka and Theologies, 2004). Using transgenic *Arabidopsis* expressing GUS reporter genes, Tsuchisaka and Theologies (2004) reported that all genes, except *ACS9* (which is expressed in mature plants), are expressed in the seedling particularly in the epidermal cell layer, guard cells and the vascular tissue. In *Arabidopsis* flowers, these authors found that only *ACS11* is expressed in the trichomes of the sepals, whilst *ACS1* is specifically expressed in the replum. Similar to *Arabidopsis*, seven members of the *ZmACS* gene family from maize are also differentially expressed during plant development. Differential expression of ACS multi-gene families has also been reported in rose (*Rosa hybrida*) flowers where three different ACS genes are differentially regulated during flower development and their expression differs between ethylene-sensitive and ethylene-insensitive mutants (Nun *et al.*, 2005). Only

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Rh-ACS3 is differentially expressed during flower maturation in ethylene sensitive and insensitive cultivars, whilst expression of *Rh-ACS1* and *Rh-ACS2* during flower maturation is similar (Nun *et al.*, 2005).

Evidence for the differential regulation of members of *ACS* multi-gene families by environmental cues is also emerging. For example, *ACS2* and *ACS6* in *Arabidopsis* are reported to be induced by various biotic and abiotic stresses (Wang *et al.*, 2004), whilst the expression of *ACS1* and *ACS5* is inhibited by wounding (Yamagami *et al.*, 2003). In addition, four *ACS* transcripts (*ACS2*, *ACS6*, *ACS7* and *ACS9*) are induced by hypoxia stress (Peng *et al.*, 2005). Differential expression of members of *ACS* multi-genes family by abiotic and biotic stresses have also been reported in other plant species, including citrus, cabbage and tobacco. In citrus fruit, *ACS1* and *ACS2* expression is induced by the pathogen *Penicillium digitatum* (Jose *et al.*, 2005), whilst in cabbage (*Brassica oleracea*) three genes (*ACS2*, *ACS6* and *ACS7*) are induced by ozone stress (Babula *et al.*, 2006). The expression of four *NT-ACS*s in tobacco has also been shown to be differentially induced by environmental cues, where *NT-ACS2* and *NT-ACS4* expression is induced by wounding, but induction of *NT-ACS4* occurs at a faster rate. In addition, *NT-ACS2* is also induced by cold and light, whilst the other two transcripts, *NT-ACS1* and *NT-ACS3*, are not induced by either wounding, cold or light (Ge *et al.*, 2000).

Members of the *ACS* gene families have also shown differential responsiveness to hormonal cues. A wide range of plant hormones have been reported to induce the expression of different *ACS* genes in many plant species. For example, exogenous application of indole-3-acetic acid (IAA) enhances expression of *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, and *ACS11*, in the roots of *Arabidopsis* (Yamagami *et al.*, 2003). Using transgenic lines of *Arabidopsis* harbouring the promoter regions of *ACS4*, *ACS5* and *ACS7* fused to a GUS reporter gene, Wang *et al.* (2005) reported that these three *ACS* transcripts were differentially induced by exogenously applied ethylene. In addition, exogenous application of IAA and jasmonic acid (JA) increased the promoter activity of *ACS4* and *ACS7*, but not *ACS5*. Moreover, the expression of *ACS7* was also induced by gibberellic acid (GA₃), abscisic acid (ABA), salicylic acid

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(SA) and brassinosteroid (BR) (Tang *et al.*, 2008). This evidence further supports the suggestion that certain members of the *ACS* gene families are differentially regulated during plant growth and development in addition to responding to different environmental and hormonal cues.

In addition to the significance of regulation of *ACS* gene transcripts to ethylene biosynthesis, it has also been suggested that ethylene biosynthesis can be regulated post-translationally (Woeste *et al.*, 1999; Wang *et al.*, 2004). The *eto1* and *eto3* mutants in *Arabidopsis* promoted an increase in ACS activity, without increasing the level of *ACS* mRNA, indicating that they regulate ethylene biosynthesis post-translationally, through interaction of ACS with a ubiquitin-conjugation complex (Wang *et al.*, 2004; Christian *et al.*, 2009) or possibly *via* protein phosphorylation (Woeste *et al.*, 1999).

Post-translational modification of ACS isozymes by protein phosphorylation suggests a tight control of ethylene biosynthesis in plants. In *Arabidopsis* under stress conditions (by wounding and pathogen elicitor), an elevated level of ethylene occurred and it has been reported that ACS is phosphorylated which alters its activity and subsequent ethylene production (Spanu *et al.*, 1994; Felix *et al.*, 2000). The phosphorylation sites for ACS are suggested to occur in the C-terminal region and plant ACS isoforms have been classified into three groups according to their C-terminal sequences (Yoshida *et al.*, 2005; Joo *et al.*, 2008; Christians *et al.*, 2009). These are *Arabidopsis* type-1 (ACS1, ACS2 and ACS6) which have the longest C-terminal region, type-2 (ACS4, ACS5, ACS8, ACS9 and ACS11) which have a longer C-terminal region than the type-3 (ACS7, ACS10 and ACS12) (Liu and Zhang, 2004; Yoshida *et al.*, 2005; Joo *et al.*, 2008) (Figure 1.2). Of these, only type 1 and type 2 are suggested to be regulated by protein phosphorylation.

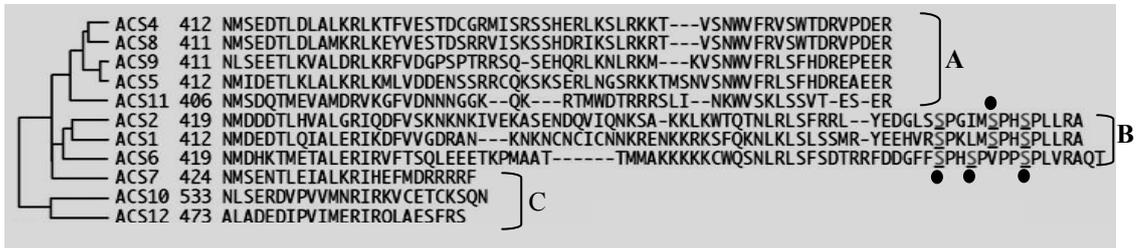


Figure 1.2 Three groups of ACC synthase in *Arabidopsis*, showing different lengths of their amino acid C-terminal end sequences.

One group, type-1 (B), has the longest C-terminal region, with three conserved Serine residues (underlined and denoted by a filled circle) as a putative site for phosphorylation by MAPK. The type 2 (A) has longer C-terminal region than the type 3 (C) which has the shortest C-terminal region (adopted from Liu and Zhang, 2004).

The type-1 and type-2 ACSs have been reported to be stress-associated ACSs in *Arabidopsis* and it has been suggested that the activity of ACS type-1 isoforms is increased during both biotic and abiotic stress (Wang *et al.*, 2003; Liu and Zhang, 2004). It is also suggested that this increased activity is due to increased stability of the ACS protein by phosphorylation (Liu and Zhang, 2004; Wang *et al.*, 2004; Joo *et al.*, 2008). The type-1 ACSs are suggested to be phosphorylated by MAPKs (mitogen-activated protein kinase) (Liu and Zhang, 2004) on the serine residues that are followed by proline (Cohen, 1997; Liu and Zhang, 2004). The MAPK phosphorylation sites are only present on type-1 ACS isoforms (Figure 1.2.), with three phosphorylation sites identified *ie* on serine residues, corresponding to Ser-480, Ser-483 and Ser-488 in ACS6 (Liu and Zhang, 2004). Liu and Zhang (2004) and Joo *et al.* (2008) demonstrated that phosphorylated ACS6 was a more stable protein with a half-life of more than three hours, whilst the non-phosphorylated ACS6 had a half-life of less than 10 minutes and it was rapidly degraded by the ubiquitin-proteasome pathway. The importance of phosphorylation for stability of type-1 ACS proteins was also identified in LeACS2. Following the wounding of

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tomato, an elevated level of ethylene was observed as a result of increased ACS protein activity due to phosphorylation in its C-terminal region.

In common with the type-1 ACSs, the stability of the type-2 ACS protein in *Arabidopsis* has also been reported to be a result of modifications in their C-terminal regions (Chae *et al.*, 2003; Wang *et al.*, 2004). Genetic screening of *Arabidopsis* had identified two ethylene-overproducing mutants (*eto2* and *eto3*), with mutations in the C-terminal regions of ACS5 and ACS9 (type-2 ACS protein), respectively (Kieber *et al.*, 1993; Vogel *et al.*, 1998; Chae *et al.*, 2003; Wang *et al.*, 2004). These two mutants displayed increased ethylene production without an increase in the *ACS5* and *ACS9* transcripts, but by stabilising the ACSs proteins, possibly due to protection of ACS2 and ACS9 from protein degradation via the ubiquitin-proteasome pathway (Kieber *et al.*, 1993; Vogel *et al.*, 1998; Chae *et al.*, 2003; Wang *et al.*, 2004). ETO1 encodes a BTB (Broad-Complex, Tramtrack, Bric-a-brac) domain-containing protein (Wang *et al.*, 2004; Christians *et al.*, 2009) which functions as a substrate adaptor in CUL3-based ubiquitin ligase and interacts with ACS5 to direct degradation by the 26S proteasome pathway (Wang *et al.*, 2004; Yoshida *et al.*, 2005). Mutations in the C-terminal region of ACS5 in *eto2* (or ACS9 in *eto3*) were suggested to reduce the efficiency of ETO1 binding, therefore, resulting in their stabilisation (Wang *et al.*, 2004; Yoshida *et al.*, 2005). Recently, Christians *et al.* (2009) further confirmed that ETO1, EOL1 and EOL2 (ETO1 like protein) specifically targeted only type-2 ACS proteins and ETO1, EOL2 and that EOL3 acted collectively for the efficient degradation of type-2 ACS in *Arabidopsis*. However, the site for phosphorylation of type-2 ACS protein has not yet been identified.

1.3.3. ACC Oxidase

ACC oxidase (ACO; EC 1.14.17.4) is the enzyme that catalyses the final step of ethylene biosynthesis, which is the conversion of ACC into ethylene, and was initially called the ethylene forming enzyme (EFE). Identification and characterisation of ACO has lagged behind ACS, since it was initially proposed that the enzyme was unstable and difficult to purify *in vitro* (Cameron *et al.*, 1979).

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EFE was first identified from cDNA clones of ripening-related tissues in tomato, where one clone was homologous to mRNA which accumulated prior to a wounding-induced ethylene peak in the unripe fruit and leaf tissue of the tomato (Slater *et al.*, 1985). This clone was designated *pTOM13* (now called *LE-ACO1*) and it was shown that it was involved in ethylene production (Slater *et al.*, 1985) and was induced by ethylene in mature green tomato fruit (Maunder *et al.*, 1987). To show this, Hamilton *et al.* (1990) transformed tomato plants with an antisense construct of *pTOM13* and they observed a great reduction in ethylene production in these plants. They suggested that the *pTOM13* gene encoded an enzyme involved in ethylene biosynthesis, but the coded protein is too small to be an ACC synthase (Hamilton *et al.*, 1990). The translated product of *pTOM13* had a molecular mass of *ca.* 35 kDa (Smith *et al.*, 1986), whilst partially purified ACC synthase from zucchini fruit had a molecular mass of *ca.* 53 kDa (Sato *et al.*, 1991). Further confirmation that the *pTOM13* transcript encoded EFE was achieved by comparing the *in vivo* activity of the enzyme obtained from leaf disks of wild type and *pTOM13* transformed plants in the antisense orientation and it was shown that the ethylene-forming activity in the antisense plants was reduced in a dosage-dependent manner (Hamilton *et al.*, 1990). Finally, Hamilton *et al.* (1991) showed that a full length clone of *pTOM13* (*pRC13*) cDNA expressed heterologously in yeast had an EFE activity.

The deduced amino acid sequence of *pTOM13* was shown to have high homology to flavonone-3-hydroxylase (Hamilton *et al.*, 1990), a member of the 2-oxoglutarate-dependant dioxygenases (2-ODDs) (Prescott and John, 1996; Iturriagagoitia-Bueno *et al.*, 1996), which is a non-heme iron enzyme family requiring aerobic conditions during extraction. The majority of its members require Fe^{2+} and 2-oxoglutarate, as co-substrates for activity *in vitro* (Britsch and Griserbach, 1986), but ACO seems to be unique in this family, since it requires ascorbate but not 2-oxoglutarate as co-substrate (Escribano *et al.*, 1996; Schofield and Zhang, 1999). However, when Fe^{2+} and ascorbate were added into a soluble fraction of melon fruit protein, EFE activity could be detected and measured (Ververidis and John, 1991). Subsequently, confirmation that the EFE activity measured *in vitro* was representative of the *in vivo* activity was achieved using pre-climacteric apple fruits treated with ethylene

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(Fernandez-Maculet and Yang, 1992). Following stereospecificity analysis of the reaction, the stoichiometry of the EFE catalysed reaction was determined (Figure 1.3.) (Dong *et al.*, 1992; Fernandez-Macule and Yang, 1992; Kuai and Dilley, 1992; Schofield and Zhang, 1999) and the enzyme was renamed ACC oxidase.

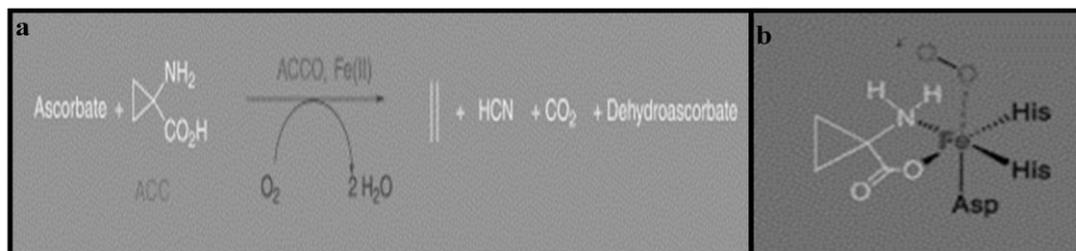


Figure 1.3 Stoichiometry of the reaction catalysed by ACC oxidase.

Reaction catalysed by ACC Oxidase (here named as ACCO) (A), and proposed metal coordination chemistry of ACCO based on spectroscopic studies, showing the Fe, dioxygen binding sites and the amino acid residues, in the active site of the enzyme (B) (Modified from Schofield and Zhang, 1999).

Following the discovery that ACC oxidase activity *in vitro*, with the addition of appropriate cofactors, resembled the *in vivo* activity (Fernandez-Maculet and Yang, 1992), purification and characterisation of the enzyme from many plant species progressed quickly, including that in avocado (McGarvey and Christoferson, 1992), tomato (Zhang *et al.*, 1995), pear (Fonseca *et al.*, 2004) and white clover (Gong and McManus, 2000). Kinetic analysis of ACCO activity demonstrated that the ACCO enzyme exists as more than one isoform, for example, in apple (Binnie and McManus, 2009).

In comparison to ACS, ACCO is encoded by a smaller multi-gene family in many plant species. Four members of ACCO have been identified and isolated from tomato, *LE-ACO1*, *LE-ACO2*, *LE-ACO3* and *LE-ACO4* (Bouzayen *et al.*, 1993; Barry *et al.*, 1996; Nakatsuka *et al.*, 1998), whilst three members were isolated and identified from melon, *CM-ACO1*, *CM-ACO2* and *CM-ACO3* (Tang *et al.*, 1993). Similarly, two members of ACCO have been identified in peach, *PP-ACO1* and *PP-ACO2* (Holdsworth *et al.*, 1987) and three in apple, *MD-ACO1*, *MD-ACO2* and *MD-ACO3*

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(Binnie and McManus, 2009). Members of the ACO gene family share a high nucleotide sequence identity (more than 70%) in their coding regions, both within the same family and between families from different plant species (Lasserre *et al.*, 1996), but these genes have more distinct sequences in the 5' and 3'UTRs.

Initially ACS was thought to have been the key step which limited ethylene biosynthesis, and ACO was considered to be expressed constitutively during plant development which led to the suggestion that ACO did not play a part in the crucial regulation of ethylene biosynthesis (Yang and Hoffmann, 1984; Theologies *et al.*, 1993). However, evidence is now accumulating to show that ACO also contributes towards the fine tuning of ethylene production in higher plants. As with ACS, members of *ACO* gene families have been reported to be regulated at both the transcriptional and post-transcriptional levels.

Studies of *ACO* gene expression and ACO protein accumulation in many plant species have shown these to be developmentally regulated (Barry *et al.*, 1996; Alonso *et al.*, 2003; Shan and Goodwin, 2006; Higgins *et al.*, 2006, Binnie and McManus, 2009). In relation to the four members in the tomato gene family, *LEACO1* and *LEACO3* are expressed in the floral and senescent leaf tissues, *LEACO1* and *LEACO4* are found in the fruit and their expression increases during fruit ripening, while *LEACO2* is expressed in the anther (Barry *et al.*, 1996; Nakatsuka *et al.*, 1998; Anjanasree *et al.*, 2005). Similarly, the lowest level of the *Ca-ACO1* transcript has been found during the early ripening stages of coffee fruit development (Pereira *et al.*, 2005). In apple, three *MD-ACO* genes are also differentially expressed where *MD-ACO1* is only expressed in mature apple fruit, whilst *MD-ACO2* is expressed predominantly in younger fruit tissues, but it is also expressed in young leaf tissues, while *MD-ACO3* is predominantly expressed in young and mature leaf tissues, with less expression in young (pre-ripening) fruit tissues (Binnie and McManus, 2009).

In addition to the developmental regulation of the *ACO* multi-gene family, evidence is now emerging to suggest that *ACO* gene expression is responsive to abiotic or environmental cues, in addition to exogenously applied hormones. For example,

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following wounding (at post-harvest), elevated ethylene production in broccoli has shown to be associated with expression of *BO-ACO1* and *BO-ACO2* (Higgins *et al.*, 2006). In melon, only *CM-ACO1* was responsive to wounding and ethylene treatment, whilst both *CM-ACO2* and *CM-ACO3* were not (Laserre *et al.*, 1996). Similarly, there are two members of *PP-ACO* in peach where expression of *PP-ACO1* is induced by wounding and propylene (ethylene) treatment in young fully-expanded leaves, but expression of *PP-ACO2* transcript is absent in leaves and is not induced by wounding and ethylene treatment (Ruperti *et al.*, 2001). In addition, Shan and Goodwin (2006) reported that *NbACO1* expression is induced by fungal infection in *Nicotiana banthamiana*, whilst expression of *RP-ACO1* in rumex (*Rumex palustris*) is induced by submergence (Rieu *et al.*, 2005).

This evidence supports the view that ACO may also regulate ethylene biosynthesis in plants, both during plant development and in response to environmental cues. However, involvement of ACO has not yet been studied as extensively as ACS, as so less evidence is currently available to support the idea that only a specific ACO isoform is regulated by certain environmental cues and at which levels (transcriptional, post-transcriptional and/or post-translational) this regulation occurs .

1.4. Ethylene Perception and Signal Transduction

A unique response of etiolated dicotyledonous seedlings to ethylene is called the triple response and in *A. thaliana* the triple response is characterised by an inhibition of hypocotyl and root cell elongation, thickening of the hypocotyl and exaggeration of the apical hook (Chen *et al.*, 2005).

In *Arabidopsis*, the ethylene receptor gene family is comprised of five members, *ETR1*, *ETR2*, *ERS1*, *ERS2* and *EIN4*, which are related to the two-component system in bacteria or fungi (Schaller and Bleecker, 1995; Rodriguez *et al.*, 1999; Hwang *et al.*, 2002, Chen *et al.*, 2005). In other plant species that have been fully characterised, the receptor family comprises six members in tomato (Klee and Tieman, 2002) and six in rice (Rzewuski and Sauter, 2008). Analysis of the localisation of ethylene receptor in *Arabidopsis* and other plants suggests that

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proteins have transmembrane domains and they are located on the endoplasmic reticulum membrane (ER) (Chen *et al.*, 2005; Grefen *et al.*, 2008). Finally, a series of genetic studies using loss of function mutations have shown that receptors act as negative regulators of the ethylene response (loss of function mutation resulted in triple response phenotype) (Chang and Schokey, 1999; Chen *et al.*, 2005).

The downstream components of ethylene receptors have also been identified in *Arabidopsis* and they include CTR1 (CONSTITUTIVE TRIPLE RESPONSE1) (Kieber *et al.*, 1993), EIN2 (ETHYLENE INSENSITIVE2) and EIN3 and ERF (ETHYLENE RESPONSIVE FACTOR) (Chang and Shockey, 1999; Ohta *et al.*, 2001; Lorenzo *et al.*, 2003; Chen *et al.*, 2005). In common with *Arabidopsis*, many downstream components of ethylene signalling have also been identified in many plant species including tomato (Klee and Tieman, 2002; Adams-Phillips *et al.*, 2004) and maize (Gallie and Yang, 2004).

A proposed model for ethylene signalling has been suggested based on data from the model species *Arabidopsis* and in rice (Figure 1.4.). According to the model, the receptors stimulate CTR1 in the absence of ethylene which then inhibits downstream components of the pathway (Chen *et al.*, 2005; Rzewuski and Sauter, 2008). On the other hand, in the presence of ethylene, the receptors are inhibited which then also decreases the activity of CTR1, and this then releases downstream components from inhibition by CTR1 resulting in the ethylene response phenotype (Chen *et al.*, 2005; Rzewuski and Sauter, 2008).

Analysis of CTR1 in *Arabidopsis* suggests that the protein has serine/threonine protein kinase activity and it acts as a negative regulator of the ethylene response (Kieber *et al.*, 1993; Huang *et al.*, 2003). Sequence analysis of the CTR1 reveals that the protein has a high similarity with the Raf family of the MAPKKK proteins and (based on sequence homology) it has been suggested that ethylene signalling may involve MAPK as part of the signal transduction mechanism (Phillips *et al.*, 2004; Chen *et al.*, 2005).

1.5. Responses of Higher Plants to Water Deficit

1.5.1. Physiological and Biochemical Responses

The growth responses of plants to water deficit is a complex process, involving the sensing and signalling of a soil water deficit by the root through to the activation of specific genes, which then brings about a change in the physiology and morphology of the plant (Chaves *et al.*, 2003; Kim *e. al.*, 2003, Liu and Zhang, 2004; Boudsocq and Lauriere, 2005). Rodriguez-Uribe & O'Connell (2006) suggested that the adaptive strategies developed by plants during water deficit conditions involve responses that occur at the morphological, physiological, cellular and metabolic levels.

Soil water availability influences plant water status and eventually plant turgor. Water deficit has been shown to decrease leaf water status as indicated by a reduction in relative water content (RWC) in addition to a reduction in leaf water potential and leaf osmotic potential (McManus *et al.*, 2000; Kim *et al.*, 2004; Cechin *et al.*, 2006; Gabrielle and Bendh, 2006). With a decrease in turgor there then ensues stomatal closure resulting in the control of water loss from the plant during water deficit (Tanaka *et al.*, 2005; Gabrielle and Bendh, 2006). Stomatal closure is suggested to be controlled by changes in the turgor of guard cells, metabolic energy (from photosynthesis), membrane permeability and also the level of ABA (Schroeder *et al.*, 2001; Chaves *et al.*, 2003; Kwak *et al.*, 2003; Tanaka *et al.*, 2005). The effect of ABA on stomatal closure is suggested to be mediated by chemical signals, including calcium and phosphatidic acid, originating from the roots which is transported to the leaves and then drives the changes in apoplastic ABA level thus causing stomatal closure (Davis and Zhang; McRobbie, 2000; Chaves *et al.*, 2003).

A reduction in leaf water status, as a result of reduced water availability to plants, is suggested to directly affect the majority of the physiological processes involved in plant growth and development. It is suggested that cell division, cell enlargement and cell differentiation are all affected by water deficit and a decrease in cell expansion is suggested to be an early adaptive response of plants to a water deficit (Schroeder *et*

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al., 2001; Sanchez *et al.*, 2004). Cell expansion is partly a turgor-driven process, but it is also driven by changes in hormonal balance in plant cells as a result of reduced water availability (Schroeder *et al.*, 2001; Sanchez *et al.*, 2004). Reduction in cell expansion and decreases the growth rate result in reduced leaf expansion, leaf area and leaf elongation (Bissuel-Belaygue *et al.*, 1996; Bissuel-Belaygue, 2002). Such adaptations are suggested to be beneficial responses which allow plants to conserve water longer, since the area for transpiration is reduced. In addition, under water deficit conditions, the production of sugars (*via* photosynthesis) and root uptake of mineral ions is likely to be impaired (Chapin, 1991; Neumann, 1995; Achard *et al.*, 2006). Therefore, leaf growth inhibition may then allow diversion of essential solutes from growth requirements to stress-related house-keeping functions, such as osmotic adjustment that improves cell water retention and turgor maintenance. Thus, shoot growth inhibition, in response to a water deficit, may extend the period of soil water availability and plant survival (Chapin, 1991; Neumann, 1995; Achard *et al.*, 2006).

Other responses of plants to water deficit stress are premature necrosis and abscission (Bleecker and Kende, 2000). Water deficit has been shown to increase ethylene concentration, which then drives premature senescence and abscission in the leaf of maize (Young *et al.*, 2004). Drought-induced mature leaf senescence may be beneficial for transferring nutrients from mature leaves to younger organs (or to the growing seeds) thus allowing plants to survive longer (Yang and Zhang, 2006; Foulkes *et al.*, 2007).

Premature leaf senescence processes in vegetative plants exposed to water deficit is suggested to be associated with a decrease in total chlorophyll content within the leaves which impacts on photosynthesis. Prolonged and severe water deficits are factors which are suggested to induce premature necrosis by reducing total chlorophyll accumulation (Abreu and Munne-Bosch, 2008; Javadi *et al.*, 2008; Guerfel *et al.*, 2009). These decreases also decrease the photosynthetic capacity of the mature leaves, which then limits the plant's productivity under water deficit conditions (Abreu and Munne-Bosch, 2008; Javadi *et al.*, 2008; Guerfel *et al.*, 2009). In contrast to its effect on the mature leaves, an increase in the accumulation of

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ethylene by water deficit stress has also been shown to increase the Rubisco content per unit leaf area (Davies *et al.*, 1998) and the capacity of younger leaves to be a source of photosynthates (Ludlow and Ng, 1974).

Although water deficit decreases leaf area and leaf elongation, it is suggested that it promotes root growth and extension in some plants (Lucero *et al.*, 1999; Spollen *et al.*, 2000; Sharp *et al.*, 2000; Sharp and LeNoble 2002) to enhance the capacity of the plant to extract water from deeper soil reserves. However, more severe water deficit is suggested to inhibit root growth. In maize, root growth inhibition is suggested to promote the survival of the apical meristem regions, by increasing the relative availability of water, minerals and sugars for these tissues since these compounds are no longer required for growth. This meristem protection serves as an important method to assist rapid root growth after renewal of irrigation (Fan *et al.*, 2006).

The influence of water deficit on reproductive organs depends on the degree and time of water deficit imposition. For example, a water deficit experiment during the development of early cotton bolls promoted fruit abortion (Guinn, 1976) and it decreased grain weight in wheat (Xu *et al.*, 1995; Beltrano *et al.*, 1999). However, a mild water deficit during grain filling increased the carbon mobilisation from vegetative tissues to the grain, which then increased the grain-filling rate in rice and wheat but severe water stress decreased the rate of grain-filling in these species (Yang *et al.*, 2000; 2001; 2003; 2004; 2006).

There have been extensive studies on biochemical adaptations of plants to water stress, including the accumulation of compatible solutes (Chen and Murata, 2002), osmotic adjustment (Lopez *et al.*, 2009; Ahire *et al.*, 2005), accumulation of protective proteins and sugars (Porcel *et al.*, 2005; Rorat *et al.*, 2006; Saavedra *et al.*, 2006), and alteration or change in the balance of plant hormones (e.g. Chen *et al.*, 2002; Wang *et al.*, 2004; Tanaka *et al.*, 2005). Compatible solutes, which are synthesised by plants, include sugar and sugar alcohols or carbohydrates (including fructan and sucrose), protein and amino acids (such as proline, aspartic acid and glutamic acid) (McManus *et al.*, 2000; Shvaleva *et al.*, 2006), methylated quaternary ammonium compounds (such as glycine betaine and alanine betaine) (Chen and

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Murata, 2002) and cyclitols (for example pinitol, mannitol) (McManus *et al.*, 2000; Anderson and Kohorn, 2001).

Among these compatible amino acids, free proline accumulation is a focus of this thesis. The majority of other studies have suggested that free proline plays an important role in plant adaptation to water deficit in many plant species, including potato (Gabrielle and Bendh, 2006), eucalyptus (Shvaleva *et al.*, 2006), sunflower (Cechin *et al.*, 2006), olive (Ennajeh *et al.*, 2006) and chickpea (Ahire *et al.*, 2005). Proline is an important solute in cytoplasmic tolerance, which alters resistance of the whole plant to water deficit stress and it is proposed that it acts as a hydroxyl radical scavenger (Hong *et al.*, 2000) preventing membrane damage from reactive oxygen species (ROS). It also prevents protein denaturation, preserving enzyme structure and activity (Rajendrakumar *et al.*, 1994, Samuel *et al.*, 2000; Hamilton and Heckathorn, 2001). It also acts as an osmoregulator (Shetty *et al.*, 2002) and it is involved in osmotic adjustment (Ennajeh *et al.*, 2006). Its accumulation is associated with a decrease in leaf water potential and leaf relative water content (Gabrielle and Bendh, 2006). However, other reports have suggested that proline may not play an important role in osmotic adjustment (Delauney and Verma, 1993; Lutts *et al.*, 1999; Satchesz *et al.*, 1998) and its more important role is to stabilise the macromolecules and membrane from the toxic effects of excess N (Kim *et al.*, 2004).

1.5.2. Changes in Hormonal Balance

It has been known that adverse environmental conditions change the level of several plant hormones. Ethylene is one of the plant hormones that have been shown to play an important role during water deficit stress. Ethylene accumulation during water deficit stress accelerates premature necrosis of the mature leaf (Chen *et al.*, 2002) and dry matter partitioning to the younger or storage organs (e.g. Ludlow & Ng, 1974; Mohapatra *et al.*, 2000; Naik and Mohapatra, 2000). These adaptations, together with stomatal closure induced by ABA (e.g. Chaves *et al.*, 2003; Kwak *et al.*, 2003; Tanaka *et al.*, 2005) and the maintenance of a balanced ratio of shoot and root growth, by ABA and ethylene, are useful strategies for plant adaptation to water deficit stress (e.g. Spollen *et al.*, 2000; Tanaka *et al.*, 2005). A more detailed

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progression on how water deficit alters ethylene biosynthesis is discussed in Section 1.8.

Many reports suggest that ethylene interacts with other plant hormones in determining morphological changes in response to water deficit stress. Ethylene and ABA have been shown to interact in the regulation of responses of plants to water deficit stress (Davis and Zhang, 1991; Beltrano *et al.*, 1999; Yang *et al.*, 2000; Wilkins and Davis, 2002; Yang *et al.*, 2004). An increase in the endogenous ABA level due to water deficit stress which limits ethylene production has been reported to maintain the growth ratio between the root and shoot of maize and tomato (Sharp *et al.*, 2000; Spollen *et al.*, 2000; Sharp and LeNoble 2002). In addition, increased ethylene due to water stress inhibits the capacity of ABA to promote stomatal closure in *Arabidopsis* (Tanaka *et al.*, 2005). Decreased ethylene concentration correlated with an increase in ABA concentration during mild water stress increasing the grain filling rate in wheat and rice (Yang *et al.*, 2000; 2001; 2003; 2004; 2006). How such interaction occur (and at what stage of signalling this interaction is regulated) is now being studied at the molecular level, in order to obtain a better understanding of the regulation of plant hormones in adverse environmental conditions. However, such interaction will not be studied here as this thesis will focus on changes in ethylene biosynthesis under water deficit in white clover, in particular changes in ACC oxidase gene expression and protein accumulation.

1.6. Water Deficit in White Clover

White clover (*Trifolium repens* L.) is one of the most important perennial forage legumes in temperate regions including New Zealand (Lucero *et al.*, 1999; Annicchiarico and Piano, 2004). There are four main reasons for the importance of white clover in New Zealand: a) the dependency of the New Zealand economy on agricultural based industries, particularly those related to meat and dairy production, b) the nitrogen-fixing ability of white clover roots, which reduces the need for applied nitrogen fertilisers, c) maximum clover growth in some seasons (late spring and summer), and d) the high nutritive value of white clover which increases milk yield for the dairy industry (Lucero *et al.*, 1999; Annicchiarico and Piano, 2004).

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With these features, white clover is an ideal pasture legume for temperate regions if it is productive in all seasons. However, soil water availability is one of the most important resources for optimum pasture and crop productivity and for yields in many areas of the world including New Zealand (Scotter *et al.*, 1979; Campbell, 1997; Woodward *et al.*, 2001). The optimum growth period for white clover is in late spring and early summer (Hutchinson *et al.*, 1994; Hunt *et al.*, 2002) and it is vulnerable to water stress that reduces its growth rate and productivity especially when drought occurs in late summer and autumn (Hutchinson *et al.*, 1994; Hunt *et al.*, 2002).

White clover is assumed to be sensitive to water deficit since water deficit limits its growth and yield in several cropping zones (Collins *et al.*, 2002; Bissuel-Bellaygue, 2002; Sanderson *et al.*, 2003; Ayres *et al.*, 2007). The response to water deficit depends upon the degree of water deficit experienced. Moderate water deficit (reduction of water potential by 12% or at -0.8 MPa soil water potential) (Bissuel-Bellaygue *et al.*, 1996; Bissuel-Bellaygue, 2002) reduces the vegetative growth of white clover *via* a significant reduction in stolon production and branching (Bissuel-Bellaygue *et al.*, 1996; Sanderson *et al.*, 2003) and leaf expansion and leaf production (Karsten and McAdam, 2001; Bissuel-Bellaygue *et al.*, 2000). In contrast, root responses to water deficit are varied. Lucero *et al.* (1999) and Sanderson *et al.* (2003) suggested that water deficit reduced root dry matter whilst Annicchiarico and Piano (2004) found that water stress increased root dry weight. Differences in leaf size have been suggested to contribute to root system development under water deficit. Large leaf genotypes have been suggested to develop deeper root systems under water stress (Williams *et al.*, 1978; Caradus, 1990). However, other studies found that leaf morphology has no effect on root system development during water deficit in white clover (Brock and Kim, 1994; Brick and Pederson, 1998; Annicchiarico and Piano, 2004). Annicchiarico and Piano (2004) found that stolon thickness and the deepness of the soil horizon contributed to a deeper root system, but not leaf size, in white clover grown under water deficit. Another physiological change observed is stomatal closure, which reduces the photosynthetic activity (Grieu *et al.*, 1995). It was also observed that in the early stages of water deficit (0 to -1.2 MPa), stomatal

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closure strongly reduced leaf gas exchange, thus causing decreased photosynthetic activity (Grieu *et al.*, 1995). However, Grieu *et al.*, 1995 found that a reduction in photosynthetic activity was not due to a change in the biochemistry of the photosynthesis but instead it was due to a reduction of CO₂ allocation to the chloroplast due to stomatal closure.

In contrast to its impact on vegetative growth, a moderate level of water deficit increases the proportion of reproductive organs to vegetative organs which then increase seed set. However a severe water deficit decreases pollen fertility and efficiency of pollination which reduces seed production (Bissuel-Belaygue *et al.*, 2002). In addition, in more severe water deficit conditions the osmotic potential in white clover leaves decreases below the level to maintain sufficient pressure potential thus promoting premature leaf wilting and death (Turner, 1990). Prolonged water deficit can have a dramatic impact on white clover since it can kill the plant (Kim *et al.*, 2004).

Water deficit has also been shown to result in an increase in compatible solute accumulation including proline. Proline has been found to accumulate in higher concentrations in the root, stolon and leaves exposed to water deficit conditions (Turner, 1990; McManus *et al.*, 2000; Singh *et al.*, 2000; Hofmann *et al.*, 2003; Kim *et al.*, 2004). It is suggested that the accumulation of proline in white clover is associated with the tolerance and the ability of white clover to recover after water stress (Singh *et al.*, 2000). Proline is suggested to play a role in osmotic adjustment in white clover since it accumulates in response to a drop in leaf water potential (Turner, 1990). However, Sing *et al.* (2000) found that proline made a relatively small contribution to the osmolarity of leaf sap and therefore these authors suggested that proline may play a role other than that in osmotic adjustment. More recent findings also suggest that the role of proline in water stress tolerance may not be through osmotic adjustment, since it was only contributed to 0.8 to 1.1 % of solute accumulation (Kim *et al.*, 2004). These authors found that proline accumulation in white clover is important due to its ability to detoxify the excess of NH₃⁻ and NH₄⁺ in the cells. This function was supported by findings which suggested that reduced leaf

growth and ammonia accumulation during water deficit stress can be reduced by *de novo* synthesis of protein-amino acids such as proline and arginine (Lovatt, 1990; Slocum and Weinstein, 1990; Lazcanno-Ferrat and Lovatt, 1999). The important role of proline indicates that accumulation of the amino acid during water deficit remains an important indication for white clover tolerance to water deficit.

1.7. Ethylene Biosynthesis in White Clover

Ethylene biosynthesis in white clover has been studied during leaf development, maturation and senescence (Hunter *et al.*, 1999; Gong and McManus, 2000; Murray and McManus, 2005; Chen and McManus, 2006). The typical stoloniferous growth habit, with sequential leaf development, has been used as a model system to study ethylene biosynthesis during growth, leaf maturation and senescence (Hunter *et al.*, 1999). Leaf senescence in white clover has been related to ethylene biosynthesis and an endogenous level of ACC has been found to be related to an increase in ethylene production (Hunter *et al.*, 1999).

Earlier studies on white clover biosynthesis suggested that there were three different ACO genes in the family (Hunter *et al.*, 1999) but recently a fourth member has been identified (Chen and McManus, 2006). During leaf development, *TR-ACO1* is expressed in the apex, axillary buds and leaf petioles in younger tissues, *TR-ACO2* is expressed in the newly initiated and early mature green leaves, *TR-ACO3* is expressed in the senescent tissues. *TR-ACO4* is expressed in similar tissues to *TR-ACO3* including axillary buds and leaf petioles subtending from ontologically older nodes (Chen and McManus, 2006).

Recently, changes in *TR-ACO* transcripts have been studied in white clover roots under phosphate starvation, by both RT-PCR and examination of GUS expression (Roldan, 2008). *TR-ACO1* expression was found in the root meristem and the expression of this gene increased during phosphate starvation (Roldan, 2008). Changes in ACC oxidase by other environmental cues have not yet been studied, but the phosphate starvation study and previous studies on the developmental regulation

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of the *ACO* gene family in other species suggest that expression and accumulation will be altered by other environmental cues.

Similarly, three different *ACS* genes have been cloned from white clover and the transcripts shown to be expressed in the different developmental stages of the leaves (Murray and McManus, 2005). *TR-ACS1* is specifically expressed in the apical structure of the stolon, in mature green leaf tissues and in leaf tissue at the onset of senescence. *TR-ACS2* is expressed in the apical structure of stolon and in newly initiated leaves, whilst *TR-ACS3* is only expressed in the senescent leaf (Murray and McManus, 2005). Attempts to study the induction of these *ACS* genes, by wounding and exogenous application of IAA, have been undertaken, but wounding or IAA treatment did not significantly induce the expression of the three *ACS* genes (Murray and McManus, 2005).

Antibodies against TR-ACO1 and TR-ACO2, and TR-ACS1 have been raised. The anti TR-ACO1 and TR-ACO2 antibodies recognised a protein of *ca* 37 kDa, whilst TR-ACS1 recognises several bands, with a major band at *ca* 57 kDa in leaf tissue (Hunter *et al.*, 1999; Gong and McManus, 2000; Murray and McManus, 2005). The specificity of the anti TR-ACO1 and TR-ACO2 antibodies to recognise only TR-ACO1 and TR-ACO2 proteins has been tested using recombinant TR-ACO1 and TR-ACO2 proteins, which make it possible to separately distinguish the accumulation of these two proteins in white clover. Therefore changes in TR-ACO can be examined at the transcription and post-transcriptional levels.

1.8. Water Deficit and Ethylene

The importance of ethylene in regulating many physiological changes related to plant growth and development has been well-established. Ethylene is also called the stress hormone since it often accumulates as a response of plants to various biotic and abiotic stresses including water deficit (Bleecker and Kende, 2000).

Evidence on the effect of water deficit on ethylene biosynthesis is very limited and available reports are conflicting. Initially, water deficit was suggested to trigger

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ethylene biosynthesis as has been reported in cotton (Jordan *et al.*, 1972) and wheat (Apelbaum and Yang, 1981; McKeon *et al.*, 1982). However, in these reports water deficit was imposed artificially by detaching leaves and it could be argued that this may not resemble the response of a whole plant.

Reports on the effect of water deficit on ethylene biosynthesis in intact plants are varied based on plant species, varieties used, the growing state of plants exposed to water deficit and the degree of water deficit imposed. McMichael *et al.* (1972) suggested water deficit increased ACC oxidase activity (and so ethylene production) in mature intact cotton petioles which led to abscission. Similarly, Van Den Driessche and Langebartels (1994) reported an increase ethylene production (by gas chromatography measurement) when Norway spruce seedlings (four-year-old) were exposed to an early period of water deficit which decreased as the water deficit progressed (although the increase was suggested to be associated more to the ozone added in combination than the water deficit itself). Water deficit treatment alone has been reported to reduce ethylene production in the cambial/xylem region of mature Norway spruce trees (24 years-old) (Eklund *et al.*, 1992) or in bean, miniature rose and cotton plants (Morgan *et al.*, 1990) and in both drought-sensitive and drought-tolerant wheat (Balota *et al.*, 2004). Other authors have suggested that ethylene may not play an important role during water deficit stress since no significant changes had been observed in ethylene accumulation, for example in sorghum (Zhang and Kirkham, 1995), wheat plants (six week-old) (Narayana *et al.* 1991) or rosemary (Munne-Bosch *et al.*, 2002).

In mature wheat plants, it seems that changes in ethylene biosynthesis differed between varieties and the degree of water deficit imposition (Chen *et al.*, 2002). In the drought-tolerant variety, ACC oxidase activity increased over the first 24 hours of a water deficit (following withholding of water) and then decreased, whilst in the drought-sensitive cultivars, ACC oxidase activity decreased continuously during water deficit (48 h period). Although these experiments were conducted over a very short-term water deficit treatment (only 48 h), it gives an indication that alteration in ethylene biosynthesis will differ in drought-sensitive and drought-tolerant varieties

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during a water deficit. Chen *et al.* (2002) found no changes in ACC content throughout water deficit, but ACC oxidase activity was altered as described above. This report supports previous finding of McMichael *et al.* (1972) who also reported increased ACC oxidase activity, but not ACC content, during water deficit in the cotton petiole. Therefore, it seems that water deficit alters ethylene biosynthesis in cotton and wheat *via* alteration of ACC oxidase activity but not by alteration of ACC content. Involvement of ethylene biosynthesis in the plant response to water deficit has also been reported in maize (Young *et al.*, 2004). These authors measured the level of ethylene produced in the wild-type and *Zmaccs6* mutant (loss of function of *ZmACS6*) and compared the occurrence of senescence in those plants exposed to water deficit. They found a 80% reduction in ethylene production in the *Zmaccs6* mutant which also showed reduced leaf senescence under water deficit.

Taken together, all of these findings suggest a connection between water deficit, ethylene biosynthesis and plant response to water deficit which might differ in drought-sensitive and drought-tolerant varieties. Moreover, alteration in ethylene biosynthesis during water deficit may occur at the transcriptional and/or post-transcriptional level. However apart from the report by Young *et al.* (2004), no other paper has reported changes in ethylene biosynthesis genes or/and protein during water deficit.

As outlined previously, genes encoding ACO and ACS have been cloned from many plant species, such as *Arabidopsis*, maize, tobacco, tomato and white clover and it has been shown that both ACS and ACO are encoded by multi-gene families (Hunter *et al.*, 1999; Woeste *et al.*, 1999; Gong and McManus, 2000, Young *et al.*, 2004; Murray and McManus, 2005; Chen and McManus, 2006). Although it is suggested that different member of the *ACS* and *ACO* gene families are differentially expressed in response to different environmental stimuli (Woeste *et al.*, 1999; Wang *et al.*, 2002; Kim *et al.*, 2004; Young *et al.*, 2004), evidence is still needed, however, to further investigate the expression of *ACO* and/or the *ACS* gene during environmental stresses including water deficit. In *Arabidopsis*, wounding inhibited the expression of *ACS1* and *ACS5*, but it enhanced the expression of *ACS2*, *ACS4*, *ACS6*, *ACS7*, *ACS8*,

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and *ACS11* (Tsuchisaka and Theologis, 2004). *ACS2*, *ACS6* and *ACS7* are proposed to be the stress-induced ACS genes that promote stress-ethylene production in *Arabidopsis* because the transcription of these genes is activated by ozone, wounding, salt and pathogen infections (Vahala *et al.*, 1998; Wang *et al.*, 2002; Liu and Zhang, 2004; Joo *et al.*, 2008). Although salt and drought can both induce a water deficit, none of these findings state that certain members the *ACO* or *ACS* gene family are water deficit stress-induced and this is the particular focus of this thesis.

In the case of white clover, amongst the three *ACS* and/or four *ACO* genes, none have been shown to be induced by environmental stimuli, although an attempt to investigate the effect of wounding on *ACS* genes expression has been undertaken (Murray and McManus, 2005). For *ACS*, it is possible that they are all developmentally regulated and therefore there will be other transcripts induced by water deficit. Another possibility is that the same transcripts are both regulated during plant development and in response to water deficit stress. This has been observed in *Arabidopsis*, where some *ACS* isoforms have overlapping expression both during plant development and in response to wounding (Tsuchisaka and Theologis, 2004; Liu and Zang, 2004). Therefore, this thesis will investigate changes in ethylene biosynthesis during water deficit and in particular ACC oxidase gene expression and protein accumulation in two white clover varieties with different sensitivities to water deficit. In addition, this thesis will also attempt to isolate and characterise expression of water-deficit ACC synthase gene in white clover.

1.9. Thesis Aims

The investigation in this thesis is divided into two parts. The aim of part one is to investigate changes in ACC oxidase gene expression and protein accumulation during water deficit and part two aims to isolate and characterise a water-deficit associated ACC synthase in white clover. To achieve these aims, investigation in this thesis will be focused on:

1. Observation of changes in leaf water potential and growth of two white clover varieties, one more drought-tolerant and one more drought-susceptible, during

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water deficit conditions. In addition, changes in leaf chlorophyll content and proline accumulation will be measured,

2. Examination of the expression patterns of three ACC oxidase transcripts and accumulation patterns of two ACC oxydase protein during water deficit in those two white clover varieties,
3. Screening for and isolation of a water-deficit associated ACC synthase gene and characterisation of expression during leaf development and in response to water deficit and hormonal cues.

2. MATERIALS AND METHODS

2.1. Propagation and Growth of White Clover Plants

2.1.1. Establishment of Stock Plants

Two white clover varieties were used in the experiments described in this thesis: the Tienshan ecotype and the Grasslands Challenge Kopu II cultivar (referred as Kopu, henceforward). The stock plants were obtained from cuttings taken from AgResearch Grasslands, Palmerston North, New Zealand. These two varieties were selected based on their previous field performance under water stress (Hofmann *et al.*, 2003). Tienshan is a small-leafed (5 to 7.5 mm²) ecotype originating from high altitude mountainous areas at 2000 m above sea level with an annual precipitation of 600 mm per year. Kopu is an elite New Zealand large-leafed (10 to 15 mm²) cultivar. Kopu maintains a robust growth in well-watered conditions. Differences in growth habit suggest that these two varieties show differing sensitivities to water stress with Tienshan more tolerant to water stress than Kopu (Hofmann *et al.*, 2003).

The cuttings taken from these two varieties were rooted in vermiculite and maintained in the glasshouse at the Plant Growth Unit, Massey University, Palmerston North (See 2.1.2) for two weeks, then potted singly into 10-L-capacity pots to serve as stock plants (See 2.12). The stock plants were placed on glasshouse benches, one variety per bench, labelled and regularly watered (automatic watering system). Flowers were regularly removed to prevent cross pollination between cultivars. When stock plants were mature (three months old), they were re-juvenated by completely trimming of all plant parts above the ground to encourage growth of new juvenile stolons. The stock plants were discarded and replaced by new stock plants every six months. To do this, apical cuttings were undertaken as described in Section 2.1.3.

2.1.2. Plant Growth Conditions

Stock plants (Figure 2.1) were maintained in a temperature-limited glasshouse at Plant Growth Unit, Massey University, Palmerston North (latitude 40° 21'0S, longitude 175° 38' 0E, altitude 29 m), New Zealand. Plants were grown in horticultural grade

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potting mix (Oderings, Palmerston North, NZ) supplemented with nutrients (Table 2.1).



Figure 2.1 Stock plants of the Tienshan (T) ecotype and Grasslands Kopu II (K) cultivar grown in the Plant Growth Unit, Massey University.

Table 2.1 Composition of slow-released fertilizer added to the horticultural grade bark base

Ingredient	Quantity (g/L)	Supplier
Dolomite	3.0	Daltons, Matamata, NZ
Agricultural lime	3.0	Daltons, Matamata, NZ
Iron sulphate	0.5	Hodder Toley, Palmerston North, NZ
Osmocote	50	Carran Agencies, Hamilton, NZ

The glasshouse was maintained (where possible) at a minimum temperature of 14 °C (night time) and 18 °C (day time), and then vented when the internal temperature exceeded 24 °C. The plants were irrigated automatically twice a day for 5 min at 10 am and 5 pm using a time-controlled mist watering system (Automation Service Ltd., Auckland, New Zealand). To control aphids, whitefly and mites, the plants were sprayed regularly, every 2 weeks, with the insecticide Attack® (Crop Care Holdings Ltd., Richmond, Nelson, New Zealand).

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2.1.3. Propagation and Maintenance of Plants for Water Deficit, Ethylene and Auxin Treatments

Routinely, apical cuttings with four nodes were taken from the stock plants. All leaves were excised at the junction of the petiole and stolon, except the youngest unfolded leaf. The cuttings were placed in propagation trays (60 cuttings per tray) with the basal node buried in vermiculite, watered twice a week with Hoagland's solution (half-strength) (Gibeaut *et al.*, 1997) and rooted for two weeks. Rooted stolons were transferred to pots containing potting mix as described in Section 2.1.2. For water deficit experiments, two rooted stolons were transferred into 10-L-capacity pots (experiment 1) or a single rooted stolon was transferred into a 5-L-capacity pot (experiment 2). For ethylene and NAA treatments, single rooted stolons were potted into 2-L-capacity pots. The plants were maintained in the glasshouse for four weeks before being used for each experiment.

2.1.4. Initiation of a Plant Growth Model System

A single stolon growth system was achieved by transferring a selection of the most uniform rooted apical cuttings from vermiculite into trays containing potting mix (Table 2.1). Initially six stolons were established per tray, and were maintained for four weeks before three stolons were discarded leaving the three most identical stolons to be trained out from the tray. Two trays were placed on a bench covered with a dry polythene surface and the stolons trained out over this dry surface to prevent the development of nodal roots.

To maintain single stolons attached to a basal root, all axillary shoots and buds, flowers, and unhealthy leaves were removed routinely (at 3-day intervals). Stolons were allowed to grow for 16 weeks until they provided a consistent pattern of leaf developmental stages, ranging from initiation through to senescence (Figure 2.2).



Figure 2.2 A single stolon of the Tienshan ecotype displaying a series of 19 leaf developmental stages. The leaves are numbered from the apex to the senescent leaves.

2.2. Treatments and Harvesting of White Clovers Leaves

2.2.1 Water Deficit Treatments

Two experiments were undertaken in The New Zealand Control Environment Laboratory (NZCEL), Plant and Food Research, Palmerston North, New Zealand from November to December in 2006 and in 2007. Two sets of plants, composed of Tienshan and Kopu plants (Figure 2.3), were maintained separately in each room, acclimated for 1 week in the NZCEL before being exposed to water deficit treatments. The Climate Room conditions were maintained at a constant temperature of 21 °C (day) and 14 °C (night), constant relative humidity (RH) of 75% and CO₂ at 350 ppm. Each room was equipped with 4 x Metal Halide (1.0 kW) and 4 x Tungsten Halogen (1.0 kW) lights providing 650 $\mu\text{mm}^{-2}\text{s}^{-1}$ PFD over a 14 h photoperiod. The rooms are equipped with built-in mirrors, placed on all of the walls 1.5 m above the floor, to provide uniform illumination to all parts of the room.

The water deficit conditions were obtained by the complete withholding of water. There were two different water deficit treatments imposed on each set of plants. One set of plants was subjected to a water-deficit directly after an acclimation period of 7 days. This treatment was designated the non pre-stressed treatment (NPS). Another set

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of plants were subjected to one week of water-deficit followed by one week of re-watering before the actual-deficit treatment. This treatment was designated as the pre-stressed treatment (PS). Tissues were harvested, as appropriate, as described in Section 2.2.5 with leaves immediately frozen in liquid nitrogen and stored at -80 °C until required.



Figure 2.3 A set of white clover plants maintained in a NZCEL room experiencing water stress treatment.

2.2.2. Ethylene Treatment

White clover plants were placed in a *ca.* 38.5 L capacity glass chamber prior to ethylene treatment. The lid of chamber was sealed with Vaseline and after 5 h in the chamber, the lid cover of the chamber was re-opened, the plants were left to aerate for 30 min before being left in the closed chamber overnight. Following the 20 h incubation, the lid cover was re-opened, the plants were aerated for 30 min, and the first fully-expanded leaves of control plants were harvested. The chamber was re-sealed and filled with ethylene gas (at a concentration of 10 ppm) for half an hour at flow rate of 1 L/min. At completion of this, the exhaust holes were blocked to prevent any escape of ethylene from the chamber. The plants were kept in this condition during treatment and harvested after 1, 2 and 4 h intervals after ethylene treatment. During the harvest, leaf samples (first fully-expanded leaves) were immediately frozen in liquid

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nitrogen and stored at -80 °C until required. After each sampling time, the chamber was refilled with ethylene (10 min) as described earlier.

2.2.3. NAA Treatment

In common with the ethylene treatment, 12 pots of white clover plants were placed in a *ca.* 38.5 L capacity glass chamber prior to the treatment. The lid of chamber was sealed with Vaseline and after 5 h in the chamber, the lid covering the chamber was re-opened and the plants were left to aerate for 30 min before being left in the closed chamber overnight. Following the 20 h incubation, the lid cover was re-opened, the plants aerated for 30 min, after which the first fully-expanded leaves of control plants were then harvested. The remaining nine plants were sprayed with 100 µM NAA (pH 6.0; dissolved in ethanol and containing 0.05 % (v/v) Tween-20) (50 mL per plant). The first-fully expanded leaves were harvested from three plants at 1, 2 and 4 h intervals after treatment and samples were immediately frozen in liquid nitrogen and stored at -80 °C until required.

2.2.4. 1-methylcyclopropene (1-MCP) Treatment

The compound 1-MCP was used in combination with ethylene (1-MCP and ethylene) or NAA (1-MCP and NAA). In the 1-MCP and ethylene treatment, 12 pots of white clover plants were placed in a *ca.* 38.5 L capacity glass chamber prior to the treatment (the same way as described in Sections 2.2.2 and 2.2.3). The plants were aerated for 30 min after 5 h in the chamber before being incubated overnight in the closed-chamber and after this period, the 1-MCP and ethylene treatments were undertaken. For this, the plants were aerated for 30 min and the first fully-expanded leaves of 0 h treated plants were harvested. To treat with 1-MCP and ethylene, the chamber was filled with ethylene (at concentration of 10 ppm) for 30 min at a flow rate of 1 L/min. 1-MCP (25 mg) was dissolved immediately in warm water (40 mL) to provide a concentration of 3000 ppb (inside the chamber), the chamber was immediately closed, and re-gassed with ethylene for another 10 min. The first fully-expanded leaves were harvested from three plants at 1, 2 and 4 h intervals after treatment and samples were immediately frozen in liquid nitrogen and stored at -80 °C until required. At each sampling time, 1-MCP was added and the chamber refilled for 10 min with ethylene at a flow rate of 1 L/min.

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For the 1-MCP and NAA treatments, after the 20 h (overnight) incubation as described, the first fully-expanded leaves of the 0 hr treated plants were harvested. Nine remaining plants were sprayed with 100 μ M NAA (pH 6.0; dissolved in 0.05 % ethanol (v/v) and containing 0.05 % (v/v) Tween-20), placed immediately in the chamber and 1-MCP was applied as described previously. The first fully-expanded leaves were harvested from three plants at 1, 2 and 4 h intervals after treatment and immediately frozen in liquid nitrogen and stored at -80 °C until required. At each sampling time, the 1-MCP was added into the chamber

2.2.5. Control Treatments for Ethylene, NAA and 1-MCP Treatments

The control treatments, 12 pots for ethylene or NAA treatment, were maintained in the *ca.* 38.5 L capacity glass growth chamber as previously described (Section 2.2.3 and 2.2.4). As the control for the ethylene treatment, the samples were harvested (without any ethylene and 1-MCP treatment) at the same time intervals as the ethylene treatments. To act as the control for the NAA treatment, following 20 h incubation in the growth chamber, the plants were sprayed with 0.05 % (v/v) ethanol and 0.05 % (v/v) Tween-20 and incubated and harvested over the same time intervals as previously described (Section 2.2.4). Harvested first fully-expanded leaves were frozen immediately in liquid nitrogen and stored at -80 °C until required.

2.2.6. Harvesting White Clover Leaves

For other experiments, three different tissues were harvested: apical structures, first fully-expanded and second fully-expanded leaves. All harvests were conducted between three to four hours after dawn for each sampling point. Leaves were excised with sharp scissors or scalpels at the junction between the lamina and petiole, submerged in liquid nitrogen and stored at -80 °C until use. When pooled leaf tissue was to be used in multiple analyses, the tissue was removed from -80 °C, powdered and apportioned into separate pre-chilled 1.5-mL-capacity Nunc™ centrifuge tubes.

In the single stolon model system, each leaf was numbered based on their developmental stage from the apex through to senescence and the appropriate development stage sampled, frozen and treated as described previously.

2.3. Physiological Measurements

2.3.1. Measurement of Soil Water Content

Soil water content (SWC) was estimated using electrical conductivity probes placed at a 15 cm depth connected to a TDR (Time Domain Refractometer) apparatus (Trase Soil Moisture Measuring System, Soilmoisture Equipment Corp, Santa Barbara CA, USA). The measurement was based on the dielectric properties of soil and its moisture content. Trase generates a fast-rise pulse, sent at the speed of light down a transmission line consisting of two parallel waveguides (probes) that are inserted into soil. The velocity of propagation of the high frequency, broad band 3GHz wave in soil, is determined primarily by the water content. The travel time of the wave is used to calculate the dielectric constant of the soil and the correlated volumetric water content then calculated by a microprocessor. Observations on soil water content were recorded every day, at 3 h after dawn, in two pots per treatment (Experiment I, 2006) or four pots per treatment (Experiment II, 2007).

2.3.2. Measurement of Leaf Water Potential

Leaf water potential (LWP) was measured in the first fully-expanded leaves by a Scholander pressure chamber, also called a pressure bomb (Soilmoisture Equipment Corp, Santa Barbara CA, USA). All measurements were performed between three to four h after dawn at each sampling point. To measure the LWP, the leaf was excised with a sharp scalpel blade at the junction between the stolon and petiole, and immediately the petiole was inserted into the chamber lid (Figure 2.4) and secured tightly, with the cut edge of the petiole facing outwards and the leaf blade inside the chamber. The chamber was then slowly pressurized with nitrogen gas while the exposed edge of the petiole was examined for the first appearance of exuded xylem sap. When the pressure exerted on the leaf in the chamber equals the negative pressure inside the leaf, liquid in the leaf blade will be forced out of the cut edge of the leaf petiole. The application of pressure was stopped when the exuded xylem sap was first visible and the pressure required recorded.



Figure 2.4 A Scholander-type pressure bomb showing a white clover petiole (p) inserted into the chamber during the measurement of LWP.

2.3.3. Measurement of Petiole Elongation Rate

The petiole elongation rate (PER) was calculated as the rate of petiole extension per day (as mm/day). The PER was measured in the petiole of the first-fully expanded leaf daily at approximately 3 h after dawn. A day before the measurement was taken, each petiole was measured, marked at the mid-point, then two marks were made from this mid-point: 1 cm up (toward the lamina) and 1 cm down (toward the stem), to provide the initial 2-cm reading in the middle part of the petiole (Figure 2.5.). The distance between the two marks was measured the next day and PER was calculated by subtracting this from the initial reading. The PER was measured daily until PER was ceased.

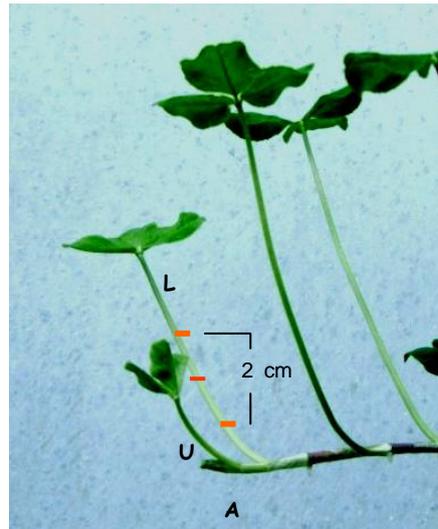


Figure 2.5 A white clover stolon showing the apical structure (A), an unfolded leaf (U) and a first-fully expanded leaf (L).

2.4. Biochemical Methods

2.4.1. Preparation of Commonly Used Reagents

Unless otherwise stated, all general chemical reagents used were of analytical grade and sourced either from BDH Laboratory Supplies (Dorset, England), or Sigma Chemical (St. Louis, MO, USA). Water for making solutions was produced by reverse-osmosis (RO) following by a micro-filtration system containing ion exchange, solvent exchange, organic and inorganic removal cartridges (Milli-Q, Millipore Corp., Bedford, MA, USA). All solutions were prepared using Milli-Q water.

2.4.2. Extraction and Measurement of the Chlorophyll Concentration

Reagent:

- ***N,N*-dimethyl formamide (DMF)**

Measurement of the chlorophyll concentration in white clover leaves was achieved using the method as described by Moran and Porath (1980).

2.4.2.1. Extraction of Leaf Chlorophyll

The first-fully expanded or second-fully expanded leaves were powdered in liquid nitrogen with a mortar and pestle. Approximately 50 to 100 mg of ground leaf material was extracted in 1 mL of chilled (4 °C) DMF by incubating the slurry in darkness at 4 °C for 14 h. The mixture was then vigorously mixed by vortexing, and cellular debris pelleted by centrifugation at 20800 x g for 5 min at room temperature. The supernatant was then transferred into a fresh centrifuge tube.

2.4.2.2. Measurement of Chlorophyll Concentration

A 300 µL aliquot of the supernatant (2.4.2.1.) was transferred into a 3 mL glass cuvette containing 2700 µL of DMF. The solution was mixed by pipetting and the absorbance was read at both 647 and 665 nm against a DMF blank.

The chlorophyll concentrations were calculated using the formula as described by Inskeep and Bloom (1985):

$$\text{Total chlorophyll} = 17.9 A_{647} + 8.08 A_{664.5} \text{ (mg/mL)}$$

The total chlorophyll concentration was then converted to μg^{-1} FW from the known fresh weight of leaf tissues used in each sample.

2.4.3. Extraction and Measurement of Proline Concentration

Reagent

- Proline standard
- 3% (w/v) Sulphosalicylic acid
- Extraction reagent: 1% (w/v) ninhydrin in 60% (v/v) GAA
- Toluene

The method of Magne and Larher (1992) was used to extract and measure the proline concentration in white clover leaves.

2.4.3.1. Extraction of Leaf Proline

Approximately 50 to 80 mg of powdered leaf tissues was typically used and weighed into a 1.5 mL centrifuge tube. The powdered leaf tissues were suspended in 1.2 mL of 3 % (w/v) sulphosalicylic acid, the tubes vigorously mixed by vortexing for approximately 30 sec, and the cellular debris pelleted by centrifugation at 20 800 x g for 7 min at 4 °C. A 500 µL aliquot of supernatant was transferred to a 15 mL Falcon tube and the volume was made up to 1 mL by the addition of 500 µL of water, followed by 2 mL of extraction reagent. The extraction reaction was carried out by incubating the tube in a water bath at 98 °C for 1 h, after which the reaction was stopped by cooling the tube in an ice bath. Proline was extracted from the aqueous fraction by the addition of 3 mL of toluene with vortexing for 30 second. The tube was then left to stand for 5 min to separate the toluene phase from the rest of supernatant. The toluene (upper) phase containing the proline was then transferred into a 3 mL glass cuvette (in a fumehood) and the absorbance was read at 518 nm against a toluene blank.

2.4.3.2. Measurement of Proline Concentration

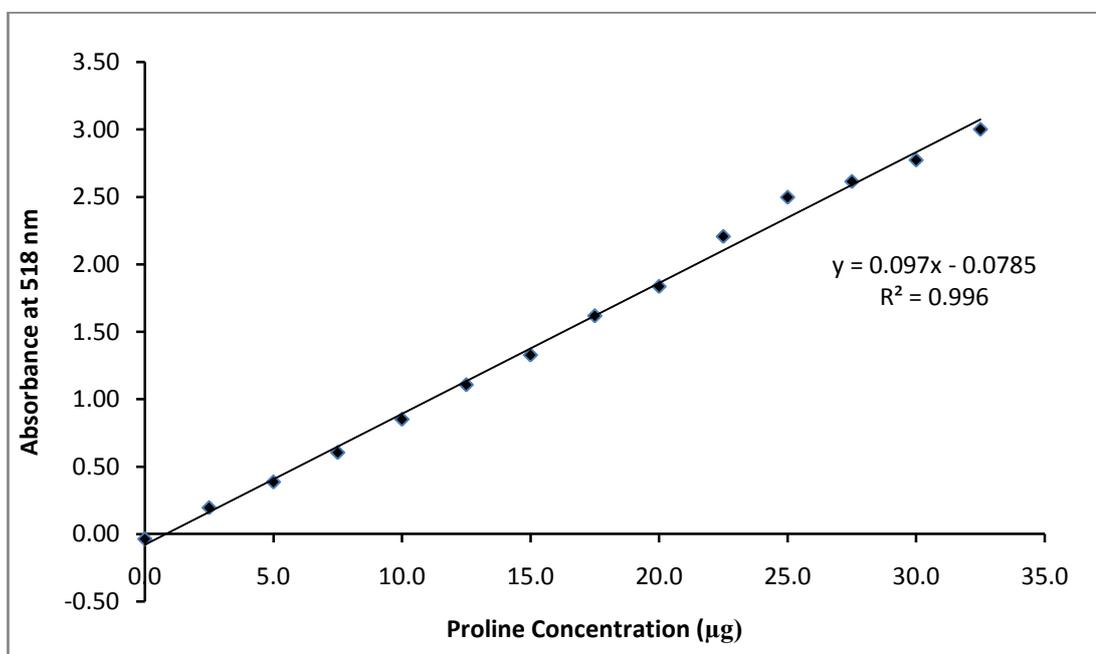


Figure 2.6 A typical standard curve for the proline assay. The points are means of triplicates and the line represents the regression line.

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The concentration of proline was calculated from the formula generated from the regression of a series measurements using a proline standard (Figure 2.6). The standard curve was generated from absorbance readings of a series of proline standards. To do this, a triplicate series of proline standards at concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 $\mu\text{g mL}^{-1}$ were added to a 15 mL Falcon tube containing 2 mL of extraction reagent. Following one hour of incubation at 98 °C, the extraction and measurement was as described for the samples in 2.4.3.1.

2.4.4. Protein and Immunological Analysis

2.4.4.1. Protein Extraction

Reagents:

- Extraction Buffer : (100mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, and 2 mM DTT)

Routinely, frozen (-80 °C) tissues (apical structures, first- and second-fully expanded leaves) were ground to a fine powder stage in liquid nitrogen in a pre-chilled mortar and pestle, and approximately 100 mg (apical structures) and 200 mg (first- and second- fully expanded leaves) were transferred into a new mortar and pestle on ice containing 3 volume of extraction buffer and some acid-washed sand. The powdered tissue was ground to a fine slurry, transferred into a 1.5 mL chilled-microcentrifuge tube and cell debris pelleted by centrifugation at 20 800 for 10 min at 4 °C. The resulting supernatant (crude extract) was used further for protein determinations and western blot analysis.

2.4.4.2. Protein Quantification

Reagents:

- Protein assay reagent (Bio-Rad, Richmond-CA, USA)
- Bovine serum albumin (BSA) (1 mg/10 ml stock)

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The protein concentration of samples was estimated by a microassay version of the Bradford method as described by Bradford (1976) and Zor and Selinger (1996) with BSA as the protein standard. Prior to protein measurement of the samples, a standard curve was generated. To do this, a series of BSA protein standards was prepared to give a concentration range of 0 to 15 mg mL⁻¹. Triplicates aliquots (10 µL) of each protein standard were pipetted into a microplate plate (NuncTM Brand Product, Nulgen International Denmark) and made up to 160 µL with water. Then, 40 µL of protein assay reagent was added and the contents mixed gently. After standing for 10 min, the absorbance at 595 nm was measured using an Anthos HTII plate reader (Anthos Labtech Instruments, Salzburg, Austria). The actual reading was corrected against a blank reading (160 µL water and 40 µL of protein assay reagent) and the protein standard curve was made from these measurements (Figure 2.7).

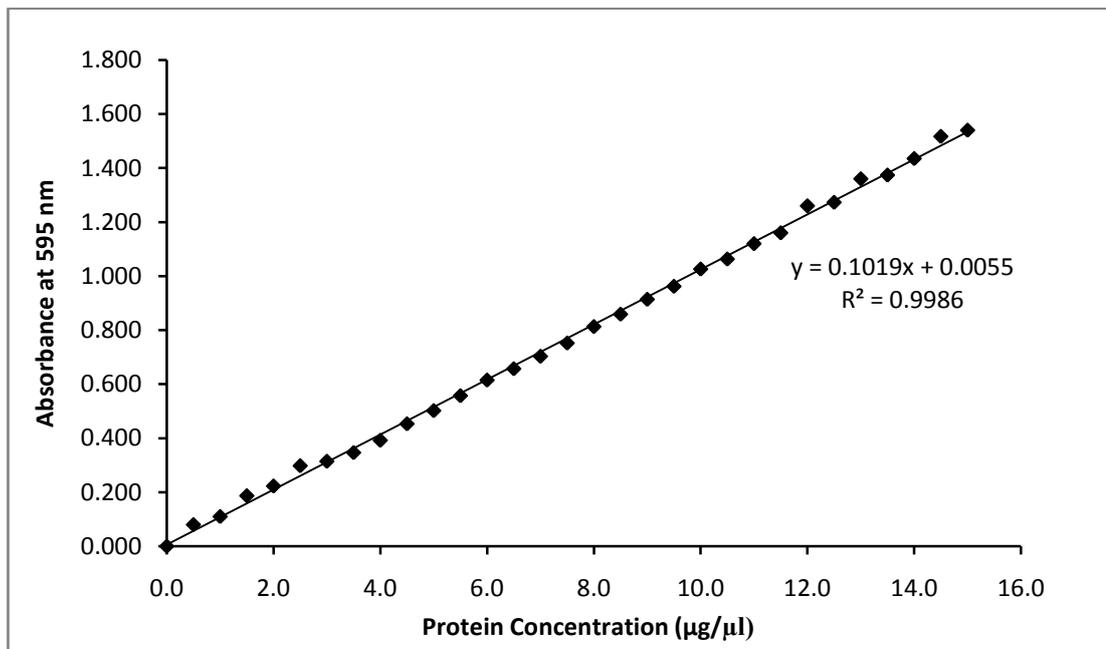


Figure 2.7 A typical BSA standard curve. The points are means of triplicates and the line represents the regression line.

To measure protein concentration, samples were diluted to an appropriate concentration before measurement and 10 µL aliquots were pipetted in triplicate into the wells of the microtitre plate and made up to 160 µL with water and assayed as described above. The protein concentration was then quantified using the equation from the standard curve as shown in Figure 2.7. Only absorbance values within the

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linear region of the standard curve were used to calculate the concentration of the sample.

2.4.4.3. Protein Analysis by SDS-PAGE

Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins on the basis of molecular mass and is based on the method described by Laemmli (1970).

Reagents:

- 40% (w/v) acrylamide stock solution (Bio-Rad)
- 4 x resolving (separating) gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4 % (w/v) SDS)
- 2 x stacking gel buffer (0.25 M Tris-HCl, pH 6.8, 0.2 % (w/v) SDS)
- 2 x SDS gel loading buffer (24 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, 5 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 0.04 % (w/v) bromophenol blue.
- 10 x SDS Running buffer (0.25 M Tris-HCl pH 8.3, 2 M glycine, 1 % (w/v) SDS)
- 10 % (w/v) ammonium persulfate (APS) (Univar, Auburn, NSW, Australia)
- *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Riedel-de Haen Ag Seelze, Hannover, Germany).
- Prestained protein mass standards (Fermentase)
- Water-saturated isopropanol.

A 12% resolving gel was prepared by mixing the components in the order as outlined in Table 2.2. The APS and TEMED were added just prior to pouring the gel, as these reagents promote and catalyse the polymerization of acrylamide. Once the APS and TEMED were added, the gel was immediately poured between two glass plates assembled in a Bio-Rad Mini Protein apparatus (Bio-Rad Laboratories) until the level reached approximately 1 cm below where the bottom of the well-forming comb sits. Water-saturated isopropanol was then over-layered onto the gel surface to minimize atmospheric oxidation, and the gel allowed to polymerise for approximately 20 to 30 min. After the resolving gel was polymerised, a stacking gel (4 %) was prepared as outlined in Table 2.2. The water-saturated isopropanol layer above the resolving gel was discarded and the stacking gel solution was carefully added to the remaining space between the glass plate and well-forming comb. Following polymerisation for about 15 min, the comb was removed, the gel assembly transferred to the electrophoresis apparatus (Bio-Rad) and gel running buffer was added to both the inner and outer compartments prior to sample loading.

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Protein samples (2.4.4.1) were prepared by mixing the protein samples with two volumes of 2 x SDS gel loading buffer in a microcentrifuge tube, followed by incubation in a boiling water bath for 3 min and then cooling for 10 min in room temperature. The tubes were centrifuged at 10 000 x g for 1 min at room temperature, and aliquots of the supernatant (typically 20 µg for apical structures, 5 to 7 µg for first-fully expanded leaves and 10 µg for second-fully expanded leaves) were loaded into the gel wells. A 3 µL aliquot of molecular weight markers was routinely loaded separately as required. Electrophoresis was conducted at 100 V for 130 min.

Table 2.2 Composition of the separating and stacking gels used for SDS-PAGE with the mini-protein apparatus

Order of Addition	Components	Separating gel (mL)	Stacking Gel (mL)
1.	MQ-Water	2.25	1.25
2.	4 x separating gel buffer	1.25	
3.	2 x stacking gel buffer		1.25
4.	40 % (w/v) acrylamide-bis-stock	1.5	0.25
5.	10 % (w/v) APS	0.050	0.025
6.	TEMED	0.005	0.0025

2.4.4.4. CBB Staining of SDS-PAGE Gels

Reagents:

- CBB Stain (0.1 % (w/v) Coomassie Brilliant Blue R-250, 40 % (w/v) methanol, 10 % (w/v) acetic acid)
- Destaining solution (30 % (v/v) ethanol)

After completion of SDS-PAGE, gels used to determine loading controls were immersed in CBB stain for 30 min with gentle agitation and then rinsed with several changes of destaining solution until the background became clear (typically 30 min).

2.4.4.5. Western Analysis Following SDS-PAGE

2.4.4.5.1. Transfer of Protein from the SDS-PAGE gel to a PVDF Membrane

Reagents:

- Transfer Buffer (25 mM Tris-HCl, pH 8.3, 190 mM glycine and 10 % (v/v) methanol)

The wet transfer method was used to transfer separated proteins from the gel (following separation of protein by SDS-PAGE) to a Polyscreen polyvinyl fluoride membrane (PVDF, PerkinElmer™ Life Sciences, Inc., Boston, USA) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). To do this, the gel was removed from the electrophoresis apparatus and equilibrated in transfer buffer for *ca.* 10 min. At the same time, the PVDF membrane was soaked in 100% methanol for *ca.* 15 sec, then equilibrated in transfer buffer for *ca.* 5 min. Pre-cut 3 mm Whatman papers and the Schott pads were simultaneously soaked in transfer buffer during preparation of the transfer sandwich in the cassette holder. The transfer sandwich was then set up as shown in Figure 2.8.

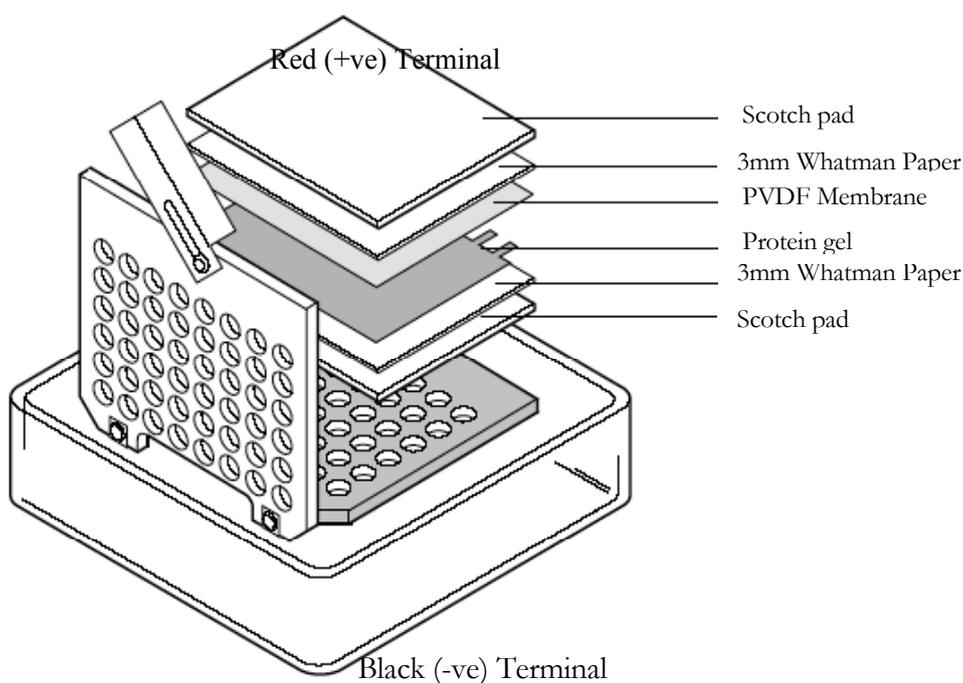


Figure 2.8 A diagrammatic set up for electrophoretic transfer of proteins from an acrylamide gel to PVDF membrane.

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The cassette was set up while immersed in transfer buffer so as to exclude air bubbles which can interfere with the transfer and each layer was rolled gently with a glass tube in order to remove any further bubbles trapped in between the layers. After with assembly, the cassette was placed into the transfer device to which pre-chilled transfer buffer was added and transfer was performed at 100 V for 1 hour. The transfer was either undertaken at room temperature, in which case an ice block was incorporated in the tank, or performed at 4 °C (when the ice block was not included). In either case, the transfer buffer was continuously stirred.

2.4.4.5.2. Staining of the Protein with Ponceau S and Blocking of the Membrane

- Ponceau S Stain (Sigma)
- Blocking Solution (Blocking Solution (12 % (w/v) milk fat in 1x PBST buffer)

Following electrophoretic transfer, the PDVF membrane was placed protein side up and routinely stained with Ponceau S for 5 min, and then destained with several rinses of water prior to the addition of blocking buffer. The blocking was routinely performed at 4 °C and incubated for overnight or at room temperature for 2 h, with gentle shaking.

2.4.4.5.3. Immunodetection of TR-ACO1 and TR-ACO2 Proteins

Reagents:

- Phosphate buffered saline (PBS) (50 mM sodium phosphate, pH 7.4 containing 250 mM NaCl)
- PBST (1x PBS containing 0.05 (v/v) Tween 20)
- Primary antibody: anti-TR-ACO1 and TR-ACO2 antibodies
- Secondary antibody: anti-rabbit IgG horse radish peroxidase (HRP) conjugate
- Blot Rinse (10mM Tris HCl, pH 7.4 containing 150 mM NaCl, 1.0 mM EDTA and 0.1% (w/v) Tween-20)
- Supersignal substrate solution (1:1 ratio of luminol and hydrogen peroxide) (Supersignal West Pico-chemiluminescent substrate, PIERCE, Rockford II, Thermo Fisher Scientific).

Following blocking, the solution was discarded and the membrane was rinsed three times with PBST for 5 min each rinse. The membrane was then incubated with primary antibody, either anti TR-ACO1 or TR-ACO2 (1:5000 dilution in PBST), for 1 h at 37 °C with gentle shaking. After this, the primary antibody was discarded and the

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membrane was rinsed five times in PBST with shaking for 5 min each rinse. The membrane was then incubated with secondary antibody (1:25 000 or 1:15 000 dilutions in PBST for TR-ACO1 and TR-ACO2 respectively) for 1 h at room temperature with gently shaking. The membrane was then washed with PBST five times for 5 min each wash to remove any unbound conjugate prior to detection. Detection of the protein was undertaken using the chemiluminescent method according to the instructions supplied by Thermo Fisher Scientific. The detection substrate was composed of a stable luminol with enhancer and a stable peroxide solution. Oxidation of the chemiluminescent solution (luminol) by the peroxide produces 3-aminophthalate which is an excited state and when it returns to a lower energy state it releases photons of light that are captured by X-ray film. Prior to detection, the membrane was equilibrated in the blot rinse for *ca.* 5 min. Whilst the membrane was still in the equilibration buffer, the working solutions of SuperSignal substrate solution were prepared by mixing equal parts of the luminal/enhancer solution and the stable peroxide solution. The blot was then incubated with a working solution of SuperSignal substrate for 5 minutes, then removed from the working solution, excess liquid removed by tipping the blot onto absorbent tissues, and the blot was then placed in a plastic sheet protector or wrapped in clear plastic (Glad wrap). The membrane was then placed inside the Fuji Cassette with protein side up and, in the dark room, a piece of X-ray film (Kodak) was placed on top of the membrane. The membrane was exposed for *ca.* 10 sec to 5 min and the film was developed using an automatic X-ray film processor (100Plus™, All Pro Image, Hickville, NY, USA).

2.5. Molecular Biology Methods

2.5.1. Chemicals

All general chemical reagents used in this section were molecular biology or analytical grade, obtained from either BDH or Sigma. All solutions and buffers were prepared with Milli-Q water (as described in Section 2.3.1) and sterilized either by autoclaving at 103 kPa or by filtering through a 0.22 µM nitrocellulose filter (Millex®-GS sterilizing filter unit, Millipore).

2.5.2. Growth of Bacteria Culture

2.5.2.1. Preparation of LB Media

Reagents:

- LB (Luria-Bertani) broth [1% (w/v) bacto-tryptone (DIFCO Laboratories, Detroit, MI, USA), 0.5% (w/v) bacto-yeast extract (DIFCO Laboratories), 1% (w/v) NaCl, pH 7.0]
- Amp100 (Ampicillin 100 mg/mL)
- 1.5% (w/v) agar (Life Technologies, Gathersbur, MD, USA)
- Glycerol
- 0.1 M IPTG stock solution (filter-sterilised and aliquots stored at -20 °C)
- X-Gal (50 mg/ml dissolved in *N,N'*-dimethyl-formamide)

E. coli cultures were maintained on LB plates. LB broth growth medium, supplemented with 100 µg mL⁻¹ Ampicillin (LB-Amp¹⁰⁰ broth), was used to grow liquid *E. coli* cultures with vigorous shaking (200 rpm) at 37 °C. To make the LB broth, all components of LB media were mixed by stirring, the pH was adjusted to 7.0 and this solution autoclaved. When required, 100 µg mL⁻¹ of Ampicillin was added to the cooled media following autoclaving. To prepare the LB plate, 1.5% (w/v) agar was added to the LB media prior to autoclaving and when required, the media was supplemented with 100 µg mL⁻¹ Ampicillin (LB-Amp¹⁰⁰ medium) or with 100 µg mL⁻¹ Ampicillin, 0.5 mM IPTG and 80 µg mL⁻¹ X-Gal (LB Amp¹⁰⁰/IPTG/X-Gal) as appropriate. These components were added when the agar medium had cooled down to *ca.* 50 °C, after which the medium was poured into sterile plates in a laminar flow cabinet, left to solidify for 15 min and plates were sealed using parafilm and kept at 4 °C until required.

2.5.2.2. Preparation of SOC Medium

Reagents:

- 2 % (w/v) bacto-tryptone (DIFCO Laboratories, Detroit, MI, USA)
- 0.5% (w/v) bacto-yeast extract (DIFCO Laboratories)
- 1% (w/v) NaCl
- 2.5 mM KCl
- 20 mM filter sterilised-Mg²⁺ (from stock of from 10 mM MgCl² + 10 mM MgSO₄)

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- 20 mM filter sterile-glucose (from stock of 1M glucose)

SOC medium was prepared by adding all components above (except the Mg^{2+} and glucose). Following sterilization by autoclaving, the media was left to cool and filter sterilized Mg^{2+} stock and glucose was added, each to the final concentration of 20 mM. The final pH was 7.0.

2.5.2.3. Preparation of Competent Cells

Reagents:

- 60 mM $CaCl_2$
- Glycerol
- LB broth

E. coli cells used for transformation were prepared from *E. coli* strain DH5 α (GIBCO BRL). Bacterial cells, from a single colony or a glycerol stock, were cultured in 10 mL LB broth at 37 °C overnight with vigorous shaking (200 rpm). An aliquot (0.5 mL) of the overnight culture was removed and used to inoculate 500 mL of fresh LB media, the culture was then incubated at 37 °C until cell growth reached an optical density of 0.6 at 600 nm. The cells were pelleted by centrifugation at 6000 x g for 5 min at 4 °C, the pellet resuspended in 10 mL of ice-cold 60 mM $CaCl_2$ followed by the addition of a further 10 mL of ice-cold 60 mM $CaCl_2$. Following incubation on ice for 30 min, the cell suspension was centrifuged at 2,000 x g for 5 min at 4 °C and the pellet resuspended in 4 mL of 60 mM $CaCl_2$ containing 15% (v/v) glycerol. Aliquots of the cell suspension (300 μ L) were transferred to microfuge tubes, snap frozen in liquid nitrogen and stored at -80 °C until required for transformation.

2.5.2.4. Preparation of Glycerol Stock

Reagents:

- LB Broth
- Glycerol

Single colonies of interest were used to inoculate 10 mL LB broths (see 2.5.2.1) which were cultured at 37 °C with vigorous shaking (200 rpm) overnight. A 850 μ L of

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aliquot of the overnight culture was added into a cry tube containing 150 μ L of glycerol, the suspension mixed, snap frozen and stored at -80 °C.

2.5.3. Isolation and Quantification of RNA

2.5.3.1 Extraction of Total RNA

Reagents:

- Borate Buffer (200mM di-sodium tetraborate decahydrate, 500 mM EDTA, 10 % (w/v) EDTA, 10% (w/v) sodium deoxycholate, 1% (w/v) SDS, 100 mM DTT), pH 9.0
- Extraction Buffer (Borate Buffer, 2% (w/v) PVP-40, 1 % (w/v) IGEPAL CA-630), pH 9.0
- Proteinase-K (20 mg/mL)
- 2 M KCl, 4M LiCl and 3 M Sodium acetate
- Chloroform: isoamyl-alcohol (24:1 (v/v))
- Isopropanol
- 80% (v/v) ethanol
- DEPC-treated water

Total RNA was isolated using the hot borate method (Hunter and Reid, 2001; Moser *et al.*, 2004) with some modifications. To prevent RNA degradation during extraction, all glassware, mortars and pestles, and spatulas used were wrapped with aluminum foil and baked in a dry oven at 180 °C for at least 14 h. All disposable plastic was either new, or treated overnight in 0.3% (v/v) hydrogen peroxide (Andrew Industrial Ltd), then rinsed well with Milli-Q water, wrapped with aluminium foil and sterilised by autoclaving. Chemicals used for RNA work were not used for other purposes and weighed by pouring the chemicals. Clean disposable gloves were always used and regularly changed. All solutions used were made to required concentrations by DEPC-treated water.

To extract total RNA from leaf samples, frozen tissues (100 mg for apical structures and 200 mg for first and second fully-expanded leaves) were ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle before transfer into a microtube containing five volumes (w/v) of warm (85 °C) extraction buffer. The tissues and extraction buffer were mixed by vortexing for 30 sec, and before Proteinase-K (0.75 %; v/v) was added to the slurry. The tubes containing the mixtures were incubated at 42 °C, with shaking at *ca.* 100 rpm, for 90 min. Immediately after

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incubation, 2 M KCl was added to a total concentration of 160 mM [0.08 (v/v)], mixed and incubated in an ice bath and shaken horizontally at *ca.* 100 rpm for 30 min. The extraction mix was then centrifuged at 10 800 x g for 20 min at 4 °C, and the supernatants transferred to a fresh tube and an equal volume of cold 4 M LiCl (to give final concentration of 2 M) added and after mixing well, the RNA was precipitated by overnight incubation at 4 °C. The precipitate was then collected by centrifugation at 20 800 x g for 30 min at 4 °C, the supernatant discarded and the pellet resuspended in 500 µL of DEPC-treated water prior to the addition of 50 µL of 3 M sodium acetate (to give a final concentration 0.3 M) and 550 µL [1: 1 (v/v)] of chloroform/isoamylalcohol. The aqueous and organic phases were shaken vigorously for 30 sec and then separated by centrifugation at 20 800 x g for 5 min at room temperature. The upper aqueous phase was carefully pipetted and transferred into a fresh sterile microfuge tube and then isopropanol (1:1; v/v) was added, the contents mixed well by inverting the tube and then the mixture incubated on ice for 1 h or at 4 °C overnight to precipitate the RNA. The RNA was then pelleted by centrifugation at 20 800 x g for 30 min at 4 °C, the pellet washed with 500 µL of 80 % (v/v) ice-cold ethanol, air dried for 5 min and resuspended in 500 µL of DEPC-water. To remove genomic DNA contamination, the RNA was routinely precipitated with 4 M LiCl (added to a final concentration of 2 M) and incubated either overnight at 4 °C or 1 h on ice. The RNA was pelleted by centrifugation at 20 800 x g for 30 min at 4 °C, then washed with ice-cold 80% (v/v) ethanol, air dried, then resuspended in 30 to 50 µL of DEPC-water. The RNA was quantified (Section 2.5.3.2), and aliquots (5 µL) snap frozen in liquid nitrogen and stored at -80 °C until required.

2.5.3.2. Quantification of RNA in Solution

The total RNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) using an Ultrospec uv/visible spectrophotometer (Pharmacia Biotech). Samples were diluted and aliquots of 100 µL were transferred into a quartz cuvette and measured against a DEPC-water blank. For RNA, an OD_{260} of 0.1 corresponds to approximately 40 µg mL⁻¹ (Sambrook *et al.*, 1989). Therefore, the equation to determine RNA concentration is:

$$\text{RNA concentration } (\mu\text{g mL}^{-1}) = A_{260} \text{ nm} \times \text{dilution factor} \times 40$$

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The purity of RNA was determined by measuring the A_{260}/A_{280} ratio (also measured against a water blank), with relatively pure RNA solutions have an A_{260}/A_{280} ratio of 2.0 (Sambrook *et al.*, 1989).

2.5.4. Reverse Transcription for cDNA Synthesis

The ThermoScript™ RT-PCR system (Invitrogen) was used to synthesise the first single strand of DNA using either an oligo (dT) primer (when β -actin was used as the internal control) or random hexamer primers (when 18s-rRNA was used as the internal loading control).

Reagents:

- Oligo(dt)₂₀ primer, random hexamer primer, 10 mM dNTP mix, 5x cDNA synthesis buffer, 0.1 M DTT, RNaseOUT™ (40 U/ μ L), ThermoScript™ RT (15 units/ μ L), RNase H, DEPC-treated water.

The first strand of complementary DNA (cDNA) was synthesised using reverse transcriptase (ThermoScript™). To do this, total RNA (typically 4 ug) was combined with either 50 μ M Oligo(dt)₂₀ primer or random hexamer primer and a 10 mM dNTP mix (2 μ L) in 0.5 mL tubes and the volume was adjusted to 12 μ L with DEPC-treated water. RNA and primers were denatured at 65° for 5 min and placed on ice for 2 min. Eight μ L of enzyme reaction mixture comprising cDNA synthesis buffer, 0.1 M DTT, RNaseOUT (40 U) and ThermoScript™ RT (15 units) was added to the RNA and primer mixture and after brief mixing, the tube was centrifuged to collect the contents at the bottom of the tube. The tube was placed in the Palm-Cycler (Corbett Life Science, Corbett Research Pty Ltd, Australia) and, typically, cDNA synthesis was carried out at 50 °C for 60 min when using the oligo(dt) primer. When random hexamers were used as the primers, the tube was placed in the Palm-Cycler at 25 °C for 10 min to extend the primer prior to cDNA synthesis which was carried out at 50 °C for 50 min. Upon completion, the synthesis of cDNA was terminated by incubation at 85 °C for 5 min, RNase H (2 U) was then added to digest any remaining RNA by incubation at 37 °C for 20 min. The cDNA was either used immediately or stored at -20 °C until required.

2.5.5. Amplification of cDNA by PCR (Polymerase Chain Reaction)

2.5.5.1. Primers used for amplification of cDNA by PCR

Amplification of putative *TR-ACS* genes and specific *TR-ACS* and *TR-ACO* transcripts was carried out using PCR with degenerate primers or gene-specific primers. The degenerate primers sequences used for amplification of putative *TR-ACS* genes were generously supplied by Professor S.F Yang (UC Davis, USA), and were designed according to the conserved regions of ACS genes from many plants species. The gene-specific primers for amplification of *TR-ACS* genes were designed to distinct regions within the coding frame of each transcript to minimise cross contamination between primer sets. In the case of the *TR-ACO* gene transcripts, the primers were designed to amplify *ca.* 100 bp of the coding frame and *ca.*200 bp of 3'UTR region to allow specific amplification of each *TR-ACO* transcript. The sequences and names of primers used in this thesis are presented in Table 2.3.

Each primer (Sigma) was dissolved in sterile water to give a stock concentration of 100 μ M and stored at -20 °C until required. A working stock was prepared by dilution to a concentration of 10 μ M. PCR was undertaken using the PCR Master Mix Kit (Promega).

Table 2.3 Primers used in PCR for amplification of ACC synthase and oxidase sequences.

Degenerate ACC Synthase Primers	
Primer Name	Sequences (5' to 3')
First-round Forward (ACSR1F)	GCCGAATTCATGGGNYTNGCNGARAA Y
Second- round Forward (ACSR2F)	CTGGATCCGTWYCARGAYTAYCAYGG
Reverse (ACSR6R)	CTCAAGCTTARN SYRAARCTNGACAT

Table 2.3 Continued.

<i>ACC Synthase Gene-specific Primers</i>	
Primer Name	Sequences (5' to 3')
<i>TR-ACS1</i> Forward (ACS1F)	GGCTAAATTCATGTCTAGAACA
<i>TR-ACS2</i> Forward (ACS2F0)	CCCTTCATCAAACAAAGCATTGGTAG
<i>TR-ACS3</i> Forward (ACS3F)	AAAAGTGAGAGGTGGTAGGGTAAG
<i>TR-ACS4</i> Forward (ACS4F)	CTTAAGTCATTTAGAAAAGCAA
<i>Reverse</i> (ACSR6R)	CTCAAGCTTARNZYRAARCTNGACAT
<i>TR-ACS4</i> Reverse	GAGAACTGAACGTTGAATC
<i>ACC oxidase Gene-specific Primers</i>	
<i>TR-ACO1</i> Forward	CACCAGCACCAAACCTTTAT
<i>TR-ACO2</i> Forward	AAGAGAATAATGAAGTTTACC
<i>TR-ACO3</i> Forward	AAAAGAAACAGAGGAAAAAA
<i>TR-ACO1</i> Reverse	TCTAAAATCAAACCTTTAATCAT
<i>TR-ACO2</i> Reverse	CACTCACTATATAGTAAGTAAACA
<i>TR-ACO3</i> Reverse	GATGTTCGAACTCTAATCCC
<i>β-actin Primers</i>	
<i>β-actin</i> Forward	TGAAGTACCCCATCGAGCACG
<i>β-actin</i> Reverse	AGTGATCTCCTTCTGCATCCTGT

2.5.5.2. PCR Conditions

Reagents:

- 2x PCR Master Mix containing *Taq* DNA polymerase, dNTPs, MgCl₂, and reaction buffer (Promega)
- Primers (Forward and Reverse)

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For amplification of putative *TR-ACS* transcripts using degenerate primers, a nested two round PCR was undertaken using similar conditions to those described by Murray (2001). Typically, the first-round PCR comprised 1 µL of ACSR1F and ACSR6R as primers (final concentration of 500nM), 10 µL of 2x PCR Master Mix, 2 µL of cDNA with the volume adjusted to 20 µL with Nuclease-free water. The second round of PCR reaction used 2 µL of ACSR1F and ACSR6R primers, 20 µL of 2x PCR master mix, 5 µL DNA (first round products) and the final volume was adjusted to 40 µL, with an annealing temperature at 42 °C (Murray, 2001).

The conditions for amplification of specific *TR-ACS* and *TR-ACO* transcripts were optimised in a Palm-cycler. Typically, a 20 µL reaction volume was used comprising 10 µL of 2x PCR master mix, 2 µL of cDNA and 1 µL of primers (final concentration of 500 nM). Optimisation was undertaken to find the ideal annealing temperature for the amplification of each transcript and this was achieved through gradient PCR. The length of DNA to be amplified determined the extension time needed for complete synthesis of the PCR product (estimated from approximately 2 min per kb of DNA).

Similar conditions were used for colony PCR screening of clones containing putative *TR-ACS*s genes, but the initial incubation was performed at 95 °C for 5 min to promote cell lysis from the *E. coli* before amplification of the DNA.

2.5.6. Cloning of PCR Products into Plasmid Vector

2.5.6.1. Agarose Gel Electrophoresis of DNA

Reagents:

- UltraPURE™ agarose (Life Technologies)
- 25 x TAE Buffer
- 10 x SUDS (0.1 M EDTA, pH 8.0, 50% (v/v) Glycerol, 1% (w/v) SDS, 0.025% (w/v) bromophenol blue)
- Ethidium bromide (10 mg/ml)
- HyperLader 1 DNA ladder (Bioline)

Horizontal 1% (w/v) agarose gels with 1xTAE buffer were used routinely to separate DNA fragments generated by PCR (Section 2.5.5.2). To make the gel, an appropriate amount of agarose powder was dissolved and heated in a suitable volume of 1 x TAE buffer. After the gel solution was cooled to approximately 55 °C, the solution was

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immediately poured into a gel-forming apparatus with a sample-well-forming comb inserted. When the agarose had polymerised, the comb was removed and running buffer (1xTAE) added to the gel electrophoresis apparatus to cover the gel surface. DNA samples were mixed with 0.1% (w/v) SUDS, loaded into the wells and the DNA separated by electrophoresis at 70 to 100 V. After electrophoresis, the gel was stained with $0.1 \mu\text{g mL}^{-1}$ ethidium bromide for 15 to 20 min, and then destained with water for 15 to 20 min. The DNA fragments were visualized following staining with ethidium bromide.

2.5.6.2. DNA Recovery from Agarose Gels

Reagents:

- QIAquick Gel Extraction Kit (Qiagen, Australia) composed of Buffer QC (pH ≤ 7.5), Buffer PE (ethanol added), Buffer EB (10 mM Tris-HCl, pH 8.5)

DNA fragments for subsequent cloning into the pGEM T-easy vector were either purified by column purification (Section 2.5.6.3.) or recovered from the agarose gel using the QIAquick extraction kit according to the manufacturer's instructions. The separated DNA was visualised quickly, DNA fragments of interest were excised with a sterile, sharp scalpel and then transferred into a labelled microcentrifuge tube, weighed, and three volumes (by weight) of buffer QC was added. The mixture was incubated at $50 \text{ }^\circ\text{C}$ for 10 min and after the gel slice was completely dissolved, the sample was transferred onto a QIAquick column inserted into a 2-mL collection tube. The binding of the DNA onto the column was achieved by centrifugation for 1 min at $20\ 800 \times g$ and the flow through into the collection tube was discarded. To remove all traces of agarose, 0.5 mL of buffer QC was added onto the column and the tube was centrifuged as described above. After discarding the flow through, the column was washed with 0.75 mL of buffer PE by centrifugation at $20\ 800 \times g$ for 1 min, and then the flow-through was discarded and any residual alcohol was removed by a repeat centrifugation, as described. To elute bound DNA, the column was placed into a sterile collection (microcentrifuge) tube and elution buffer (EB) was added and DNA eluted by centrifugation at $20\ 800 \times g$ for 1 min. The DNA was routinely eluted in 30 to 50 μL of EB and quantified by spectrophotometer as described in Section 2.5.7.3 below.

2.5.6.3. In Column Purification of PCR Product

Reagents:

- High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Germany) contains Binding Buffer (10 mM Tris-HCl, pH 6.6, 3 M guanidine-thiocyanate, 5% (v/v) ethanol), Washing Buffer (2 mM Tris-HCl, pH 7.5, 20 mM NaCl), Elution buffer (10 mM Tris-HCl, pH 8.5).

Upon completion of PCR, the total volume of PCR products was adjusted to 100 μ L and then 500 μ L of Binding buffer was added. The solution was mixed well by vortexing and then transferred onto a High Pure filter tube inserted into a collection tube. The DNA was bound to the column by centrifugation at 20 800 x g for 1 min. After discarding the flow-through solution in the collection tube, the column was washed with 500 μ L of washing buffer followed by centrifugation as above. The flow-through was again discarded, 200 μ L of washing buffer was added and the tube was centrifuged at 20 800 x g for 1 min. Following this washing, the column was inserted into a sterile microcentrifuge tube and the DNA eluted with 50 to 100 μ L of elution buffer followed by centrifugation at 20 800 x g for 1 min. The concentration of eluted DNA was estimated as described in Section 2.5.7.3.

2.5.6.4. Ligation of DNA into the pGEM® T Easy vector

Reagents:

- 2X Rapid Ligation Buffer, T4 DNA Ligase
- pGEM®-T Easy Vector (50ng)
- PCR product
- T4 DNA Ligase (3 Weiss units/ μ l)
- Nuclease-free water

DNA sequences were ligated into the pGEM®-T Easy vector (Promega) according to the protocol supplied with the cloning kit. The cloning technique relies on a terminal deoxyadenosine at the 3' end of the PCR product, generated by the terminal transferase activity of the *Taq* DNA polymerase, which is complementary to an overhanging 3'T in the vector (Figures 2.9 and 2.10).

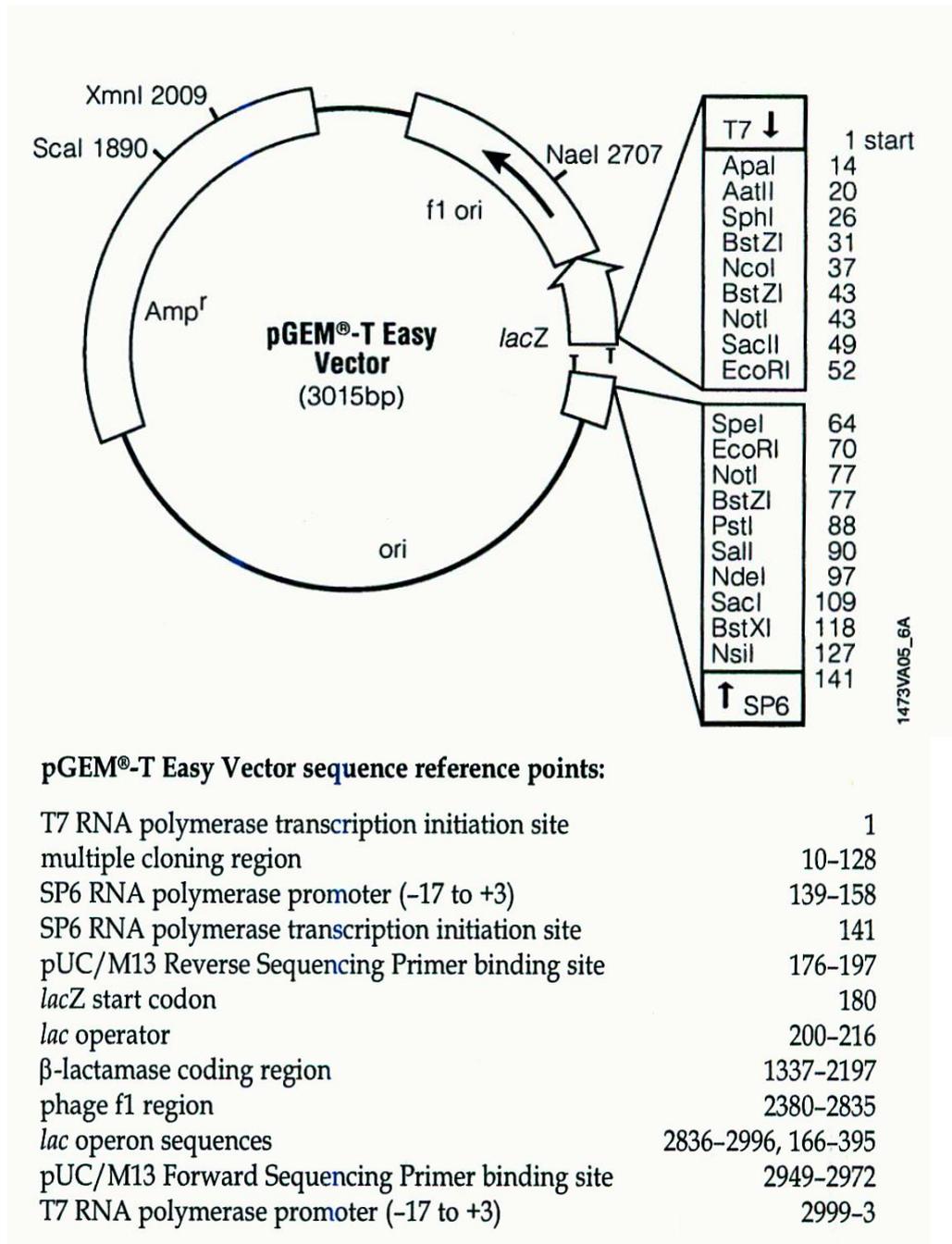


Figure 2.9 Plasmid map of the pGEM®-T Easy Vector with sequence reference points (Adopted from Promega Catalogue, 2007).

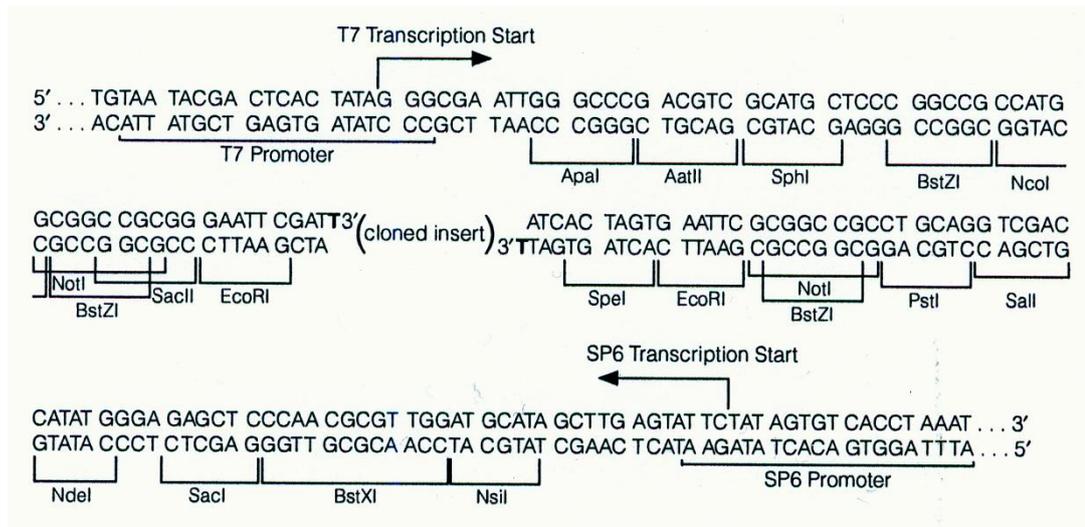


Figure 2.10 Sequence of the promoter and multiple cloning site of the pGEM®-T Easy Vector.

The 5' → 3' sequence corresponds to the RNA synthesised by T7 RNA polymerase while the 3' → 5' corresponds to the RNA synthesised by SP6 RNA polymerase (Adopted from Promega Catalogue, 2007).

Ligation of DNA into the pGEM®-T Easy vector was performed using a 1:3 molar ratio of vector to insert. The quantity of PCR products (insert) to be included in the ligation reaction was calculated according to the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The appropriate amount of PCR product was ligated in a reaction mix that contain 50 ng of pGEM®-T Easy vector, 5 µL of 2x Rapid Ligation Buffer, 3 Weiss units of T4 DNA ligase and sterile water to make the final volume of 10 µL. The ligation reaction was incubated at 25 °C overnight.

2.5.6.5. Transformation of *E. coli* with pGEM®-T Easy Vector

Reagents:

- SOC medium
- LB Amp¹⁰⁰/IPTG/X-Gal

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The putatively ligated pGEM®-T Easy Vector was transformed into competent cells of *E. coli* strain DH5 α using the heat-shock method. To do this, competent DH5 α *E. coli* cells, stored at -80 °C, were placed on an ice bath until just thawed. Half of the ligation mixture (5 μ L) was transferred to a new microcentrifuge tube on ice and 50 μ L the competent *E. coli* cells added and the contents mixed gently. The cell mixture was maintained on an ice bath for 20 min and then heat-shocked at 42 °C for 45 to 50 sec before being placed back immediately on the ice bath for 2 min after which 950 μ L of SOC medium was added. The transformation mixture was incubated at 37 °C for 1.5 h, with shaking at 150 rpm. The cells was then pelleted by centrifugation at 1000 x g for 10 min at room temperature, and the pellet resuspended in 200 μ L of SOC medium. An aliquot (100 μ L) of cells was then plated onto a LB Amp¹⁰⁰/IPTG/X-Gal plate, air dried in the laminar air flow for 5 min, then incubated at 37 °C overnight.

2.5.7. Characterisation and Sequencing of Cloned DNA in E. Coli

2.5.7.1. Isolation of Plasmid DNA from *E.coli*

2.5.7.1.1. Isolation of Plasmid DNA using the Alkaline Lysis Method

Reagents:

- LB-Amp¹⁰⁰
- Solution A (25 mM Tris-HCl, pH 8.0, 50mM Glucose, 10 mM Na₂EDTA)
- Solution B (0.2 M NaOH, 1 % (w/v) SDS)
- Solution C (3 M Potassium acetate, 2 M acetic acid, pH 4.8)
- RNase A (10 mg/mL)
- Chloroform/isoamyl alcohol (24:1, v/v)
- Isopropanol
- 80 % (v/v) ethanol
- Nuclease-free water
- 4 M NaCl
- 13 % (w/v) PEG₈₀₀₀
- 10mM Tris-HCL (pH 8.0)

Plasmid DNA was isolated from *E. coli* using the alkaline lysis method and plasmid DNA used for sequencing was purified using the PEG precipitation method. To isolate plasmid DNA (using alkaline lysis), a single *E. coli* colony was inoculated into a 10 mL LB-Amp¹⁰⁰ broth and the culture was incubated at 37 °C overnight with vigorous

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shaking (200 rpm). Cells were then pelleted by centrifugation at 3 000 x g for 10 min at room temperature. The supernatant was discarded and cells were resuspended in 200 μ L of solution A. The cell suspension was then transferred into a microfuge tube and cells were lysed by gently mixing with 300 μ L of solution B followed by incubation at room temperature for 5 min. Three hundred μ L of solution C was then added and the mixture was incubated on ice for 5 min and cell debris was pelleted by centrifugation at 10 800 x g for 10 min at room temperature. The supernatant was transferred into a new microfuge tube, treated with 2.5 μ L of RNase A solution and incubated at 37 °C for 20 min, after which 400 μ L of chloroform/isoamyl alcohol (24:1, v/v) was added and the mixture was vortexed for 30 second. The aqueous layer was separated from the chloroform mixture by centrifugation 20 800 x g for 1 min at room temperature and transferred into a fresh microfuge tube, and an equal volume of isopropanol added, the contents mixed well and the DNA then pelleted by centrifugation at 20 800 x g for 10 min at room temperature. The DNA pellet was washed with 70% (v/v) ethanol, centrifuged at 20 800 x g for 2 min at room temperature, after which the DNA pellet was dried, resuspended with 32 μ L of MQ water, 8 μ L of 4 M NaCl was then added and the plasmid DNA precipitated by the addition of 13 % (w/v) PEG₈₀₀₀ followed by incubation on ice for 20 min. The DNA was pelleted by centrifugation at 20 800 x g for 20 min at 4 °C, the pellet washed with 500 μ L of 70 % (v/v) ethanol, dried for 5 min in a fume hood, resuspended in 25 μ L of 10 mM Tris-HCl (pH 8.0) and stored at -20 °C until required.

2.5.7.1.2. Isolation of Plasmid DNA Using Plasmid Miniprep Kit

As an alternative to the alkaline lysis method, plasmid DNA was also isolated using the ChargeSwitch®-Pro Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions. The purification column in the kit contains a membrane that is positively charged at low pH and neutral at pH 8.5. The DNA is bound to the column at the low pH provided by the binding solution, and then eluted by neutralising the charge of the membrane using the elution solution at pH 8.5. To isolate plasmid DNA, 1 to 5 mL of overnight *E. coli* cultures were harvested by centrifugation at 10 000 x g for 10 min. The cell pellet was resuspended in Resuspension Buffer and then 250 μ L of Lysis Buffer added. The cells were mixed by inversion until the solution becomes clear and further cell lysis is undertaken at room temperature for 5 min. The

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cell debris was then pelleted by centrifugation at 20 800 x g for 10 min at room temperature, and the supernatant transferred onto the ChargeSwitch®-Pro column inserted in a collection tube. The DNA was bound to the column by centrifugation at 20 800 x g for 1 min at room temperature, then 750 µL of wash buffer added to the column and the column was centrifuged at 20 800 x g for 1 min. After discarding the flow-through, the column was again washed with 250 µL of wash buffer and centrifuged at 20 800 x g for 1 min. The column was then placed into a clean microcentrifuge tube and 30 µL of elution buffer added, the column incubated for 1 min at room temperature and then DNA eluted by centrifugation at 20 800 x g for 1 min. The quantity of eluted DNA was estimated either in solution (Section 2.5.7.2) or following separation by agarose gel electrophoresis (Section 2.5.7.3).

2.5.7.2. Quantification of DNA in Solution

Aliquots of DNA (typically 5 µL) were diluted (typically) 100-fold (in 495 µL of 10mM Tris-HCl, pH 8.0) and quantified by comparing the absorbance at 260 nm against a Tris-HCl blank in a 100 µL cuvette. For double-stranded DNA, an OD of 1 corresponds to approximately 50 µg mL⁻¹ (Sambrook *et.al.*, 1989). The concentration of nucleic acid present in the sample was calculated using the following equation:

$$\text{DNA concentration } (\mu\text{g mL}^{-1}) = A_{260} \times \text{dilution factor} \times 50$$

The purity of nucleotide in solutions was determined by calculating the $A_{260} \text{ nm}/A_{280} \text{ nm}$ ratio. Relatively pure DNA has an A_{260}/A_{280} ratio of 1.8 (Sambrook *et.al.*, 1989).

2.5.7.3. In gel Quantification of DNA

Reagents:

- UltraPURE™ agarose (Life Technologies)
- 25 x TAE Buffer
- 10 x SUDS (0.1 M EDTA, pH 8.0, 50% (v/v) Glycerol, 1% (w/v) SDS, 0.025% (w/v) bromophenol blue)
- Ethidium bromide (10 mg/ml)
- HyperLadder 1 DNA ladder (Biolone)

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DNA to be quantified was separated using 1% (w/v) of agarose gel electrophoresis (Section 2.5.6.1). Following electrophoresis, the gel was stained with ethidium bromide and visualised in the Gel Doc System (BioRad). The quantity of DNA was estimated by comparing the relative intensity of the DNA band to the DNA ladder.

2.5.7.3. Preparation for Automatic Sequencing of DNA

Reagents:

- ABI PRISM™ Big Dye Terminator, Sequencing buffer, M13 primer (forward or reverse) or gene specific primers (forward or reverse)
- EDTA (125 mM), sodium acetate (3 M), absolute ethanol

The method used for automatic sequencing of DNA is known as the Big Dye Terminator method or the dideoxy method. For sequencing, DNA (2 ng of PCR product or 300 ng of plasmid DNA per 100 bp) was mixed with 5 µL of sequencing buffer, 3.2 pmol of the appropriate primer and 2 µL of Big Dye Terminator and the volume made up to 20 µL with sterile water. The tube containing the sequencing reaction was placed in the Palm-Cycler programmed for 25 cycles and set at 96 °C for 10s, 50 °C for 5 s and 60 °C for 1 min 30s. Upon completion, the DNA was cleaned and precipitated by the addition of 2 µL EDTA, 2 µL of sodium acetate and 50 µL of ethanol. The DNA was mixed well by inversion and incubated for 15 min at room temperature and then pelleted by centrifugation at 20 800 x g for 30 min at room temperature. The DNA was washed twice with 70% (v/v) ethanol, centrifuged at 20 800 x g for 5 min at room temperature then air dried. Samples were submitted to the DNA Analysis Facility, Allan Wilson Centre, Massey University for sequencing based on the standard protocol of automated ABI PRISM™ 3730 DNA Capillary Sequencer (Applied Biosystems).

2.5.8. DNA Sequence Analysis

A BLAST search based of the nucleotide collection (nt/nr) of the plant genomes in the Genebank database was used to search for nucleotide sequences of interest. The sequences were aligned using ClustalW (EMBL-European Bioinformatic Institute, Cambridge, UK, <http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

2.5.9. Semi-quantitative Reverse Transcript Polymerase Chain Reaction (sqRT-PCR)

2.5.9.1. PCR Amplification of Specific Transcript

Amplification of specific transcripts was performed from cDNA as described in section 2.5.5.2. Typically, PCR reactions were performed in a total volume of 20 μ L and gene-specific primers were added to give a total concentration of 500 nM. The DNA was separated using 1% (w/v) of agarose gel electrophoresis as described in Section 2.5.6.1.

2.5.9.2. Labeling of DNA Probe and Quantification of Probe Yield

2.5.9.2.1. Labelling of DNA Probe

Reagents:

- DIG High Prime Labelling and Detection DNA Kit (Roche Diagnostic GmbH, Germany)

The probes for sqRT-PCR expression analysis were generated by a non radioactive labelling method using DIG labelling kits. The method used an alkali-labile version of digoxigenin (DIG)-dUTP to label the DNA, RNA and oligonucleotides. DNA amplified by RT-PCR was DIG-labelled using the DIG-High Prime Labelling of DNA kit (Roche Diagnostic GmbH, Germany) according to the manufacturer's instructions. During the labelling, the Klenow enzyme will copy the DNA template in the presence of hexameric primers and alkali-labile DIG-dUTP. The enzyme will inset one DIG-moiety in every stretch of 20 – 25 nucleotides.

Amplified cDNA using gene-specific primers was used for probes. The amplified DNA was sequenced to confirm the PCR product, the DNA purified (QIAQuick PCR products Purification Kit) and typically 1 μ g was made up to 50 μ L with sterile water. The DNA was denatured by heating in a boiling water bath for 5 min, quickly placed on an ice bath and on cooling, 4 μ L of DIG-High Prime Labelling mix was added to the denatured DNA, the content mixed thoroughly by vigorous vortexing and centrifuged at 20 800 x g for 30 second at room temperature to collect the contents to the bottom of the tube. The labelling was performed by incubating the tube at 37 °C for 19 to 20 h

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and reaction terminated by the addition of 2 μL of 0.2M EDTA. The probes were then stored at $-20\text{ }^{\circ}\text{C}$ until required or used immediately after removing 1 μL aliquots for the determination of the probe yield.

2.5.9.2.2. Determination of DNA Probe Yield

Reagents:

- DIG High Prime Labelling and Detection DNA Kit (Roche Diagnostic GmbH, Germany) which contains control labelled DNA ($5\text{ }\mu\text{g}/\mu\text{L}$), anti-DIG alkaline phosphatase antibody, blocking solution and BCIP/NBT stock
- Sterile water

The spot-hybridisation method as described by the manufacturer's instructions (Roche Diagnostic GmbH, Germany) was used to determine probe yield. The yield of labelled probe was estimated based on the initial amount of template used and the length of time the labelling reaction was performed. According to the manufacturer, usually 3 μg of labelled probe can be obtained from 1 μg of template when the labelling was performed for 20 h. To verify this, a series of dilutions of the control DNA ($5\text{ }\mu\text{g}/\mu\text{L}$) and labelled probe were prepared by diluting DNA with nuclease-free water to provide final concentrations of $1\text{ }\mu\text{g }\mu\text{L}^{-1}$, $0.3\text{ }\mu\text{g }\mu\text{L}^{-1}$, $0.1\text{ }\mu\text{g }\mu\text{L}^{-1}$, $0.03\text{ }\mu\text{g }\mu\text{L}^{-1}$ and $0.003\text{ }\mu\text{g }\mu\text{L}^{-1}$. One μL of diluted control and labelled DNA were spotted onto the HybondTM-N⁺ (Amersham, GE Healthcare UK Limited, Buckinghamshire UK) membrane, and the DNA fixed by UV crosslinker. The membrane was incubated in 1x blocked solution, with shaking, at room temperature for 30 min. After decanting the blocking solution, the Anti-DIG alkaline phosphatase antibody was added (1 000 times dilution in 1 x blocking buffer) and the blot was incubated with the anti-DIG antibody for 15 min at room temperature with gentle shaking. Following several washes in washing buffer, the blot was developed by adding NBT/BCIP with incubation in the dark until the signal is visible (typically 1 to 5 min). The intensities of the spots obtained from the probe were compared to those of control DNA from which the labelling yield could be estimated.

2.5.9.3. Transfer of DNA onto the Nylon Membrane

Reagents:

- Denaturation solution : (0.5 M NaOH, 1.5 M NaCl)
- Neutralization solution (0.5 M Tris-HCl pH 7.5, 1 mM EDTA, 1.5 M NaOH)
- Transfer solution (20x SSC : 3 M NaCl, 0.3 M Sodium citrate, 1 mM EDTA pH 7.0)
- Washing Buffer (2x SSC : 0.3 M NaCl, 0.03 M sodium citrate, 0.1mM EDTA pH 7.0)

Downward alkaline capillary transfer (Chomeczynski, 1992) was used to transfer DNA fragments onto the positively charged nylon membrane (Roche Diagnostic GmbH, Mannheim, Germany) or Hybond™-N⁺ nylon membrane (Amersham, GE Healthcare UK Limited, Buckinghamshire UK). To do this, following electrophoresis, the DNA fragments were viewed under UV in the gel documentation system and the gel was rinsed in distilled water. The DNA was then denatured in-gel by immersing the gel twice into the denaturation solution with gentle agitation for 15 min. After decanting the solution, the acid was neutralised by placing the gel in neutralization solution for 15 min with gentle agitation. This neutralisation procedure was undertaken twice, after which the gel was transferred and equilibrated in transfer solution for 15 to 30 min. While the gel was in the transfer solution, the membrane was cut (slightly bigger than the gel) and also equilibrated in transfer solution for 15 min. The transfer cassette was then prepared according to Figure 2.11.

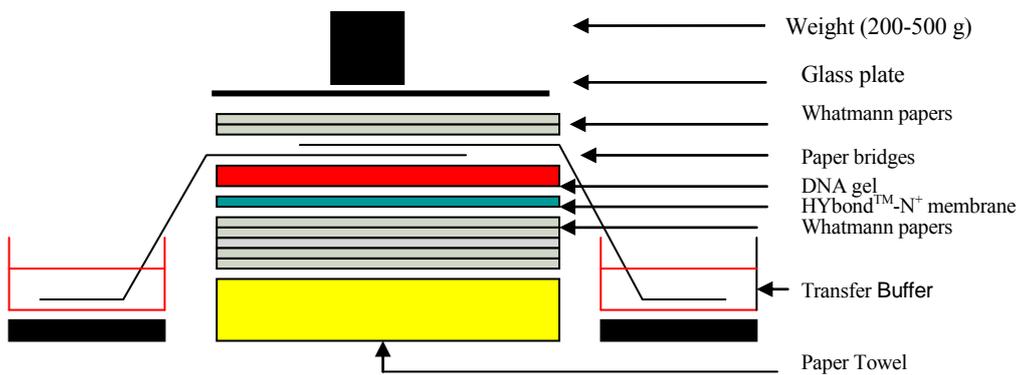


Figure 2.11 Arrangement of the apparatus used to transfer DNA and RNA samples to Hybond™-N⁺ membrane.

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The transfer of DNA was normally undertaken for 6 h to 16 h. After the transfer, the DNA was fixed by UV-crosslinking whilst the membrane was still moist.

2.5.9.4. Hybridisation of DIG-labelled probes to a DNA Blot

Reagents:

- Labelled DNA probes
- Hybridisation buffer (5x SSC contained 36 to 40% (v/v) formamide, 0.02% (w/v) SDS, 0.1% (w/v) N-laurasylsarcosine and 1% (w/v) blocking solution)
- Low stringency buffer (2x SSC containing 0.1% (w/v) SDS)
- High Stringency buffer (0.5x SSC containing 0.1% (w/v) SDS)

The hybridisation temperature for each DNA target was determined prior to hybridisation based on the percentage of the GC content in each target DNA and percentage of salt in the hybridisation buffer (DIG Application Manual, Roche Diagnostic GmbH, Germany). For each hybridisation reaction, 25 ng of labelled probe was used.

The DNA blot was equilibrated in pre-warmed hybridisation buffer (pre-hybridisation) for 30 to 60 min. While the blot was still in pre-hybridisation, the appropriate amount of probe (25 ng) was placed in a sterile microcentrifuge tube containing 50 μ L of sterile water. The probe was denatured by incubating the tube in a boiling water bath for 5 min and then the tube was chilled immediately in an ice bath. The denatured probe was then mixed with 10 μ L of pre-warmed hybridisation buffer, and after decanting the pre-hybridisation solution from the blot, the hybridisation buffer containing probe was added to the DNA blot and the hybridisation bottle was sealed. The blot was incubated, with shaking, in the hybridisation oven set at an appropriate hybridisation temperature overnight (6 to 16 h). Upon completion, the membrane was washed twice in low stringency conditions with incubation at room temperature with gentle shaking for 5 min for each wash. While the blot was in the low stringency washing steps, the high stringency buffer was pre-incubated in a water bath set to 65 °C, the low stringency buffer then poured off and then the pre-warmed high stringency buffer added and the blot was washed twice in the high stringency buffer at 65 °C, with shaking, for 15 min for each wash.

2.5.9.5. Immunodetection of the DNA Blot

Reagents:

- Maleic Acid buffer (0.1M Maleic acid, 0.15 M NaCl, pH 7.5)
- Membrane washing buffer (Maleic acid buffer pH 7.5 contained 0.3 % (v/v) Tween-20))
- 1x DIG blocking buffer (Prepared from dilution of 10x stock in Maleic acid buffer) (Roche Diagnostic GmbH, Germany)
- Anti-DIG-Alkaline Phosphatase antibody (Roche Diagnostic GmbH, Germany)
- Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5).
- Chemiluminescent alkaline phosphatase substrate (CDP star) (Roche Diagnostic GmbH, Germany)

Following hybridisation, the blot was incubated with washing buffer at room temperature, with gently shaking, for 10 min. After the washing buffer was discarded, the blocking solution was added and the blot was incubated for 30 min, with gently shaking, at room temperature. The blocking buffer was then poured off the membrane and the anti-DIG-Alkaline Phosphatase antibody was added to the blot at a ratio of 1:1 000 (antibody: 1x blocking solution) and the blot was incubated for 30 min, with gently shaking, at room temperature. The unbound antibody was removed with two washes in the wash buffer, each for 10 min with shaking at room temperature, then the blot was equilibrated in detection buffer for 10 min. To develop, the blot was placed in a layer of plastic film, chemiluminescent substrate was added and then the blot covered with another layer of plastic film and placed inside the Kodak Cassette. X-ray film (Kodak) was placed on top of the membrane, the assembly exposed for *ca.* 10 sec to 5 min and the film was developed using an automatic X-ray film processor (100Plus™, All Pro Image, Hickville, NY, USA).

2.6 Graphs and Statistical Analysis

All graphs presented in the Chapter three were created using Sigma Plot. Statistical Analysis (Analysis of variance) was undertaken using Minitab for Window's version 11.

3. RESULTS 1:

CHANGES IN WATER RELATIONS AND ACC OXIDASE EXPRESSION AND ABUNDANCE DURING THE IMPOSITION OF WATER DEFICIT IN WHITE CLOVER

3.1. Physiological Analysis of White Clover Growth Responses to a Water Deficit

3.1.1. Time Course and Water Deficit Experiments

Two water deficit experiments were undertaken in the NZCEL, one in 2006 and one in 2007. Both experiments were undertaken over the period from 15 November to 20 December in 2006 and 2007. During the course of the experiments, two sets of white clover plants were maintained in two NZCEL rooms. Each set of plants was composed of one group of the Tienshan ecotype and one of Kopu cultivar and both were subjected to different water-deficit conditions, designated as non-pre-stressed treatment (NPS) and pre-stressed treatments (PS). The actual water withholding was initiated a week after the acclimation in the NZCEL environments (Figure 3.1).

All of the physiological data reported here is from the water deficit experiment performed in 2007. The physiological data for the experiment in 2006 were similar to those of 2007 (data not shown). All gene expression and protein accumulation analysis is also from experiments performed in 2007. The results for 2006 are attached as Appendix.

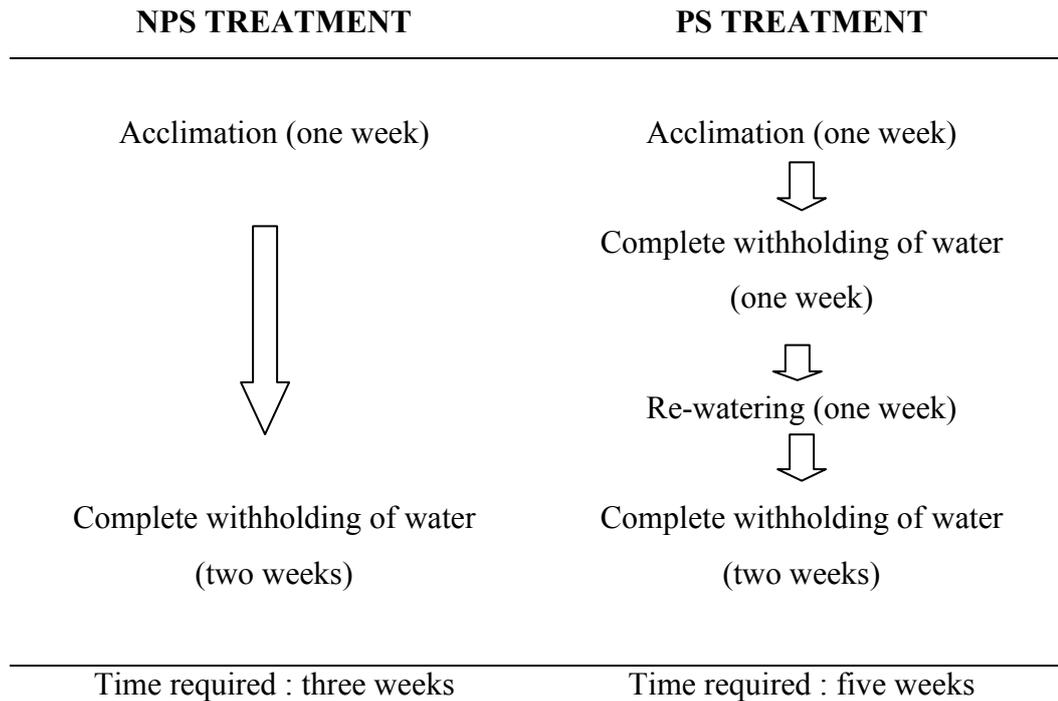


Figure 3.1 A schematic of the two water deficit treatments applied and total time required for each experiment.

3.1.2. Changes in Soil Water Content (SWC)

The SWC in both cultivars exposed to the two different treatments (Section 3.1.1), was monitored on a daily basis using time-domain reflectometry (TDR) (Figure 3.2).

The SWC of full watered soil (before watering ceased), was maintained at approximately 30% for both NPS and PS treatments in the Tienshan ecotype. In the small-leafed Tienshan ecotype, the SWC gradually decreased as plants were exposed to a complete withholding of water. There was no significant difference in the rate of SWC decline between the NPS and PS treatments (Appendix I). In both treatments, similar trends of decrease in SWC were observed (Figure 3.2.A). The SWC of the Tienshan ecotype significantly decreased, from approximately 30% to 26% two days after the imposition of a water deficit. It took eight days to halve the SWC, from 30% to 15% and this was observed in both the NPS and PS treatments. The SWC then decreased to approximately 6% after 13 days of water-

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deficit treatment, and at this SWC, the petiole elongation rate (PER) in the first-fully expanded leaves of NPS and PS Tienshan ceased (Figure 3.4.A).

In common with the Tienshan ecotype, there was a similar but not significant difference in the rate of decrease in SWC of the Kopu cultivar exposed to the NPS and PS treatments (Appendix I). The SWC of both the NPS- and the PS-treated Kopu cultivar was maintained at approximately 30% as the fully-hydrated state, before the imposition of the water deficit treatment. However, in the large-leafed Kopu cultivar, a significant decrease in SWC was observed, after only one day of water deficit imposition, and at this stage, the SWC in both the NPS and PS treatments declined from 30% to 24%. With a faster rate of decline in SWC, the SWC in NPS- and PS-treated Kopu declined to half (from *ca.* 30% to 15%) in only 3.5 days. The SWC in both NPS- and PS-treated Kopu reached approximately 8% at the time when the PER in the first fully-expanded leaves ceased. This occurred after only seven days, a rate that was twice that observed for the Tienshan ecotype (Figure 3.2.B).

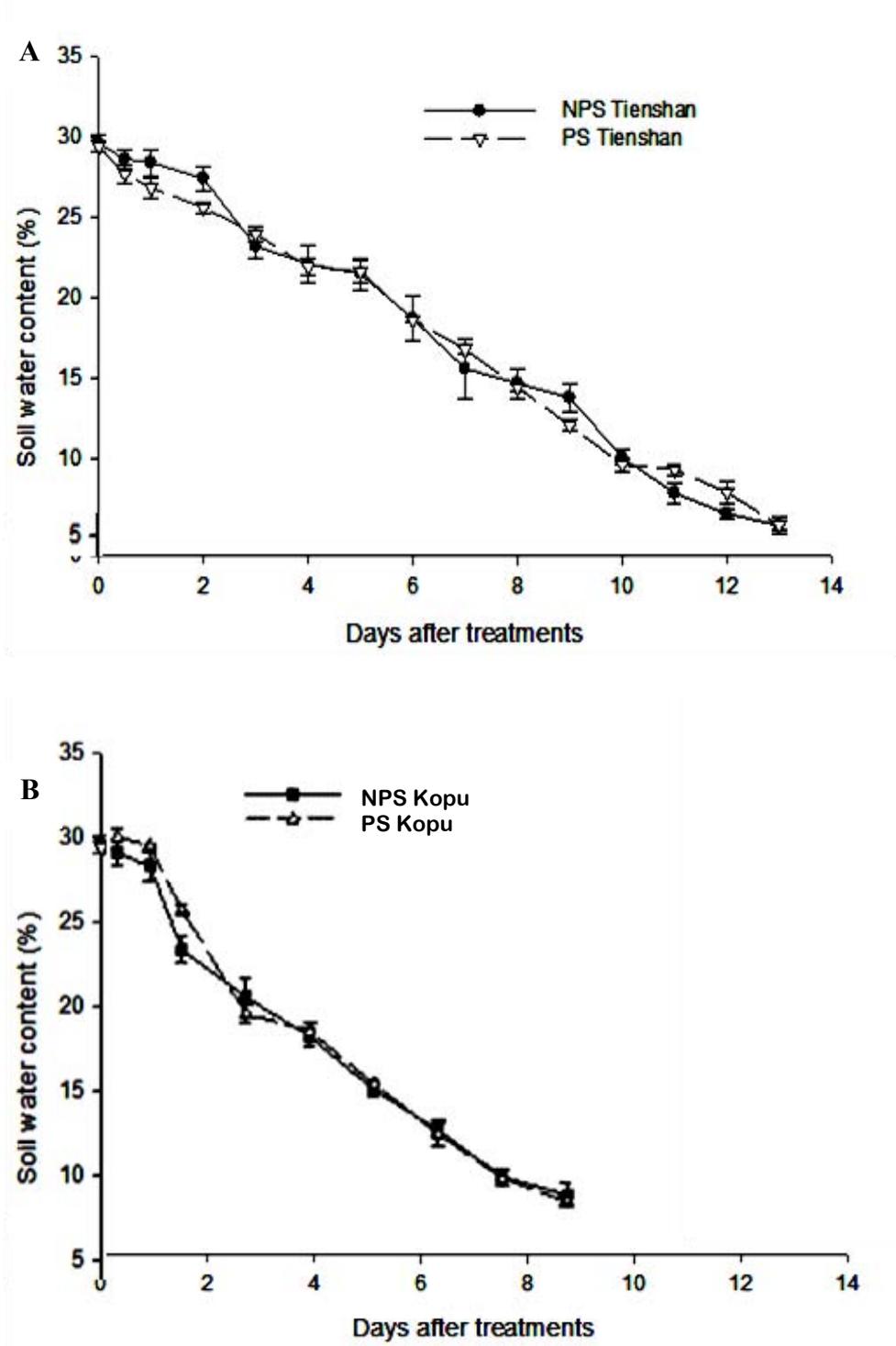


Figure 3.2 Changes in SWC during exposure of the NPS- and PS-treated Tienshan ecotype (A) and the NPS- and PS-treated Kopu cultivar (B), as indicated, to water deficit. Each value is mean of four replicates, \pm SE.

3.1.3. Relationship between SWC and LWP

Changes in water availability will change the leaf water status including relative water content (RWC) and leaf water potential (LWP) (Cechin *et al.*, 2006). In the experiments reported in this thesis, changes in LWP are used as indicators of water status of the plants that are exposed to the two water deficit treatments.

The relationship between LWP and SWC in the Tienshan ecotype and Kopu cultivar that are exposed to the two different water-deficit treatments is shown in Figure 3.3. These changes were measured until petiole elongation rate (PER) in the first-fully expanded leaves of Tienshan and Kopu ceased. The two different water-deficit treatments (NPS and PS) decreased the LWP in both the Tienshan ecotype and the Kopu cultivar. In both experiments reported here, there was no significant difference in the rate of decline in LWP between the NPS and PS treatments for each white clover varieties (Appendix II).

In the Tienshan ecotype (Figure 3.3.A), a similar LWP at *ca.* -0.55 MPa was recorded for the first fully-expanded leaves of fully-turgid NPS- and PS-treated plants maintained. The water-deficit treatment significantly reduced the LWP with the lower the SWC, the lower the LWP. The LWP gradually dropped to an approximately similar level in the NPS and PS treated plants of *ca.* - 0.9 MPa, when the SWC fell to 20%. The LWP then further decreased, following a further decrease of SWC. The LWP fell to *ca.* -1.4 MPa at *ca.* 6 % SWC, by which time the experiments were stopped (the PER in the first fully-expanded leaves ceased). In both treatments, the correlations were significant and the R^2 values were more than 0.90.

Similar trends of decline in the LWP were also observed for the Kopu cultivar exposed to the NPS and PS treatments (Figure 3.3.B). In fully-turgid conditions, the LWP of NPS- and PS-treated Kopu (*ca.* -0.45 MPa) are slightly less negative, compared to the NPS- and PS-treated Tienshan (*ca.* - 0.55 MPa). Interestingly, both the NPS- and PS-treated Kopu plants maintained a similar LWP of *ca.* - 0.9 MPa, when the SWC declined to 20%. These patterns were similar to the LWP in the NPS- and PS-treated Tienshan plants at the same SWC (approximately 20%).

Results 1

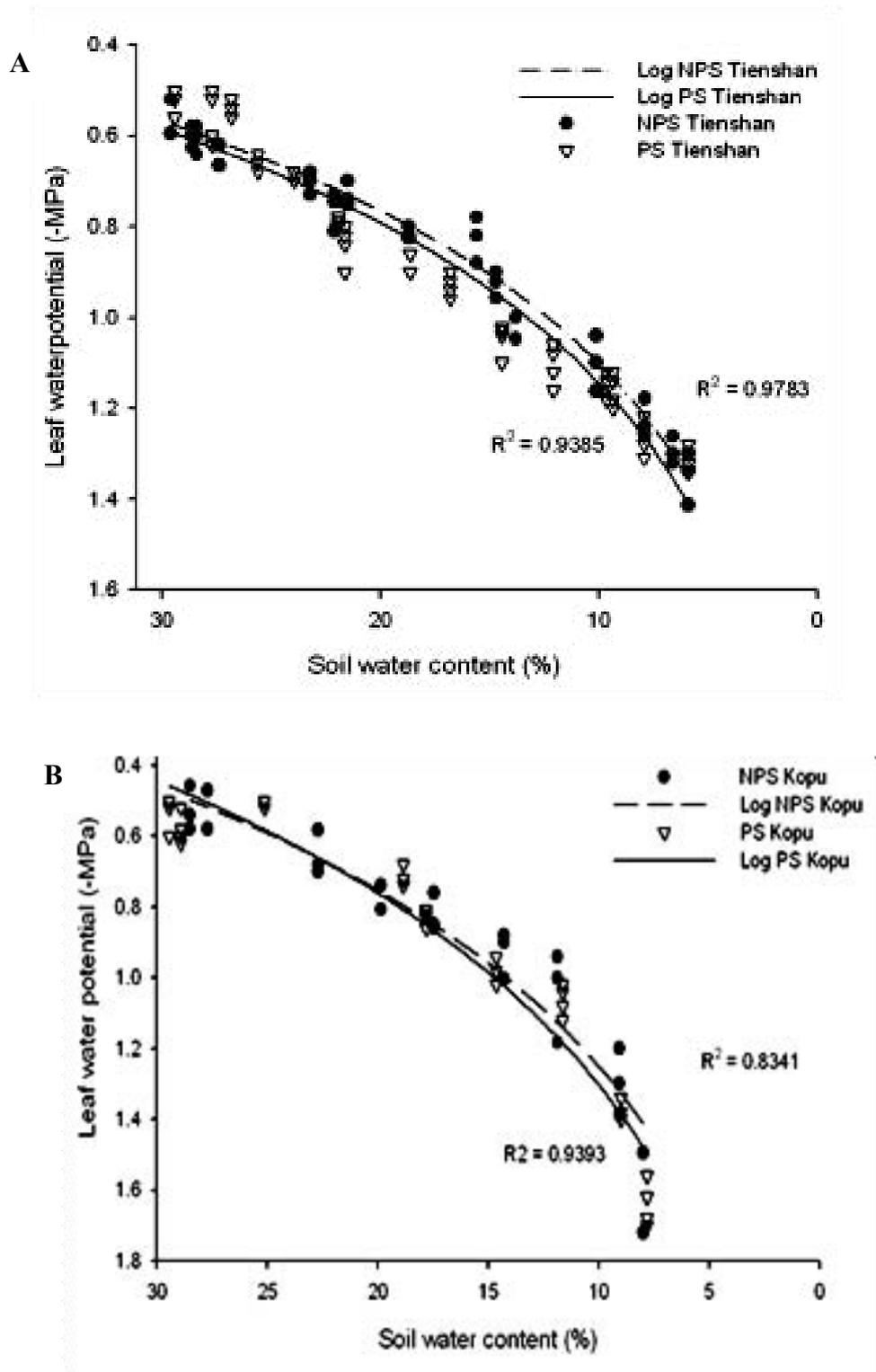


Figure 3.3 Relationship between LWP and SWC during exposure of the NPS- and PS-treated Tienshan ecotype (A) and the NPS- and PS-treated Kopu cultivar (B), as indicated, to a water deficit. The relationship curves are logarithmic regressions of four replicates of LWP.

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However after this point, there was a sharp decline in LWP in the NPS- and PS-treated Kopu, compared to a more gradual decrease in NPS- and PS-treated Tienshan (Figures 3.3.A and 3.3.B). Finally, the LWP in the NPS- and PS-treated Kopu plants fell to -1.8 MPa at the time when the experiments were terminated (the point at which the PER in the first fully-expanded leaves of the NPS- and PS-treated Kopu ceased). At this stage (8% SWC), there was a significantly lower LWP (-1.8 MPa.) in Kopu when compared with Tienshan (-1.4 MPa.). There were also significant correlations between LWP and SWC in the NPS- and PS-treated Kopu plants, although the coefficient correlation value was higher in the PS-treated Kopu (0.943), compared to the NPS-treated Kopu (0.8305).

3.1.4. Changes in PER during Treatments

Changes in the PER during different water-deficit treatments in the Tienshan ecotype and Kopu cultivar are shown in Figure 3.4. Under well-watered conditions, both the NPS- and PS-treated Tienshan plants had a PER of approximately 5.5 mm per day. This PER was maintained in the NPS-treated Tienshan plants which had been exposed to an early decrease of SWC to *ca.* 22%. A significant decline in the PER of the NPS-treated Tienshan was first observed when the SWC further decreased to 18%, at which stage the PER declined from *ca.* 5.5 mm/day to *ca.* 4 mm/day. Following this, the PER declined, as the SWC decreased, and it finally ceased at *ca.* 6 % SWC. Similar trends in the PER were also observed in the PS-treated Tienshan and there were no significant differences between the two treatments (Appendix III). The PER in the PS-treated Tienshan declined earlier than the NPS-treated Tienshan and occurred at *ca.* 22 % SWC after which it declined from *ca.* 5.5 mm/day to *ca.* 3.5 mm/day. Below this SWC, the PER in the PS-treated Tienshan declined significantly and finally it ceased at the same SWC, as the NPS-treated Tienshan, *i.e.* at *ca.* 6 % SWC.

Overall, the water-deficit treatment resulted in a decrease in the PER in the Tienshan plants and similar trends were observed in both the NPS- and PS-treated Tienshan. Therefore the different water-deficit treatments did not modify the rate of decrease in PER in the Tienshan ecotype (Figure 3.4.A).

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In the Kopu cultivar, there was a significant difference between the changes in the PER of the NPS- and PS-treated plants (Appendix III). A PER in the fully-hydrated NPS-treated Kopu of *ca.* 9.5 mm per day (two-fold higher than that of the Tienshan ecotype) was observed (Figure 3.4.B). In the NPS-treated Kopu, the PER was maintained at a higher rate, in common with the fully-turgid plants, when Kopu was exposed to the early stages of NPS treatment. This similar high PER was observed to 20% of SWC. A significant reduction of the PER was observed firstly at *ca.* 18% SWC (at the same SWC as the NPS-treated Tienshan). At this SWC, the PER declined from *ca.* 9.5 mm/day to *ca.* 8.5 mm/day. Below this SWC, the PER in the NPS-treated Kopu declined sharply following the decrease in SWC and it finally ceased at *ca.* 8 % SWC.

The Kopu plants subjected to the PS treatment initially had a lower PER of *ca.* 8 mm/day when fully-hydrated. A significant decrease in PER was observed at *ca.* 19% SWC, by which time the PER had declined from *ca.* 8 to 6 mm/day. However, below this SWC, the decline in the PER of the PS-treated Kopu occurred more gradually and finally it ceased, at the same SWC as the NPS-treated Kopu, *i.e.* at *ca.* 8 % SWC (Figure 3.4.B).

Results 1

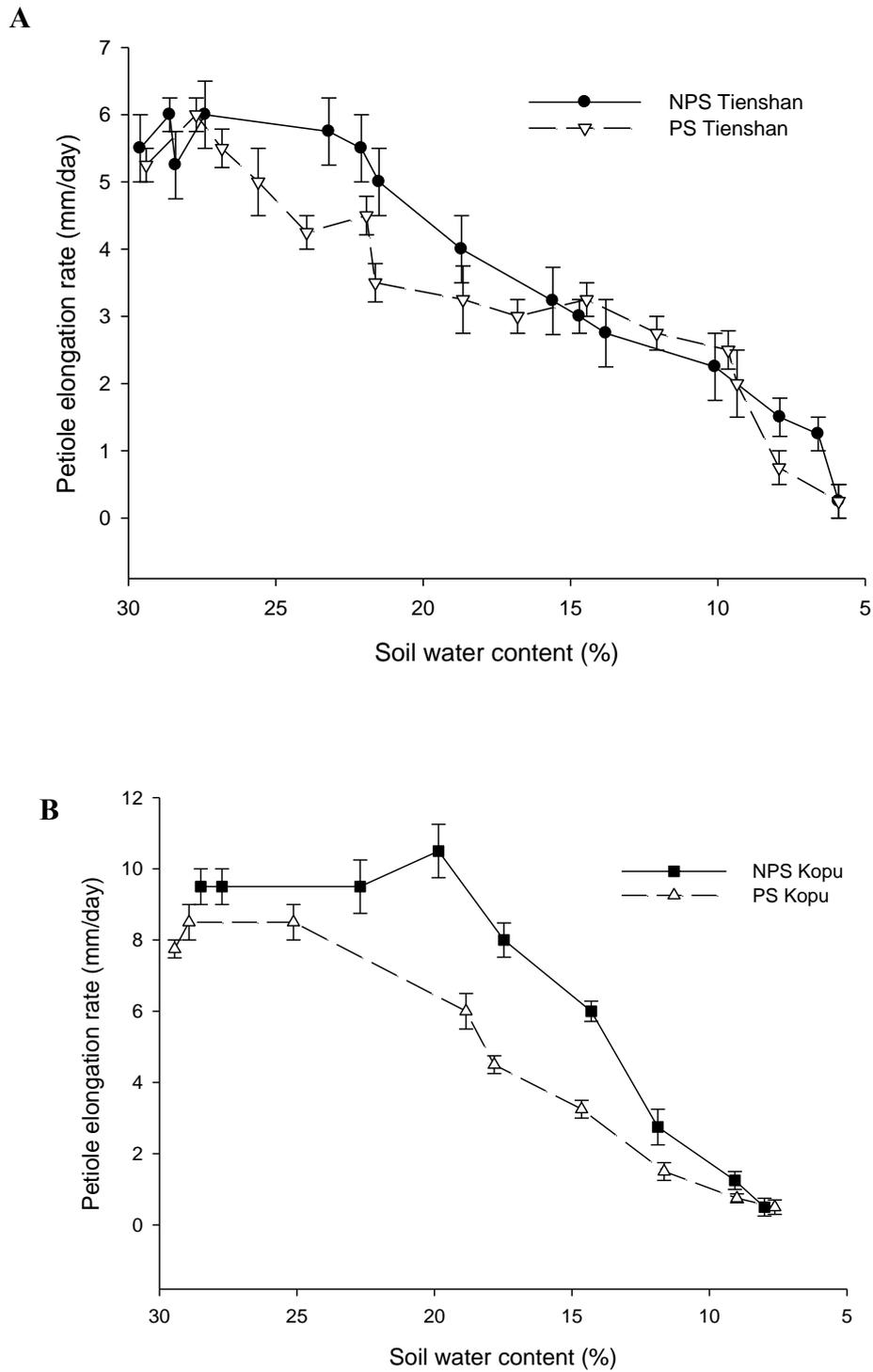


Figure 3.4 Changes in PER during exposure of NPS- and PS-treated Tienshan ecotype (A) and NPS- and PS-treated Kopu cultivar (B), as indicated, to a water deficit. Each value is a mean of four replicates \pm SE.

3.1.5. Accumulation of Proline

Changes in proline concentration in the first fully-expanded leaves of NPS- and PS-treated Tienshan and Kopu was monitored, using the method as described by Magna and Larher (1984).

Well-watered NPS-treated Tienshan plants accumulated *ca.* 100 $\mu\text{g/g}$ FW of proline (Figure 3.5.A). The proline concentration did not significantly change in the NPS-treated Tienshan plants subjected to decreasing SWC to 10%, with the level remaining between 100 and 150 $\mu\text{g/g}$ FW. After this point, at *ca.* 9 % SWC, proline concentration suddenly increased to be *ca.* four-fold higher than that of the well-watered leaves as it increased from 150 $\mu\text{g/g}$ FW to 600 $\mu\text{g/g}$ FW. These higher proline concentrations were maintained when the SWC further decreased to *ca.* 8%, a point before PER in the first-fully expanded leaves ceased. The concentration then declined to *ca.* 550 $\mu\text{g/g}$ FW when the PER ceased (at *ca.* 6 % SWC).

In the PS-treated Tienshan plants, there was a gradual increase in the accumulation of proline as the SWC declined. The fully-hydrated PS-treated Tienshan leaves accumulated approximately 100 $\mu\text{g/g}$ FW proline, and a similar range of proline accumulation (100 to 140 $\mu\text{g/g}$ FW) was maintained in leaves subjected to early stages of water deficit (to *ca.* 23% SWC). After this point, a two-fold increase in proline accumulation was observed as the water deficit progressed and this increase was maintained until the SWC decreased to 9%. A further decrease in SWC below 9% resulted in a *ca.* 3.5 fold increase in proline accumulation (100 to 350 $\mu\text{g/g}$ FW) which was maintained until the PER ceased.

The patterns of proline accumulation in the NPS- and PS-treated Kopu are presented as Figure 3.5.B. The well-watered NPS-treated Kopu leaves accumulated *ca.* 75 $\mu\text{g/g}$ FW proline. There was then no change observed in proline accumulation in the leaves subjected to a water deficit up to 12% SWC. In these leaves, proline concentrations varied between 75 to 90 $\mu\text{g/g}$ FW. When the SWC decreased to 9% (just before the PER ceased) there was a sudden *ca.* four-fold increase in proline concentration from 75 to 330 $\mu\text{g/g}$ FW. After this, the

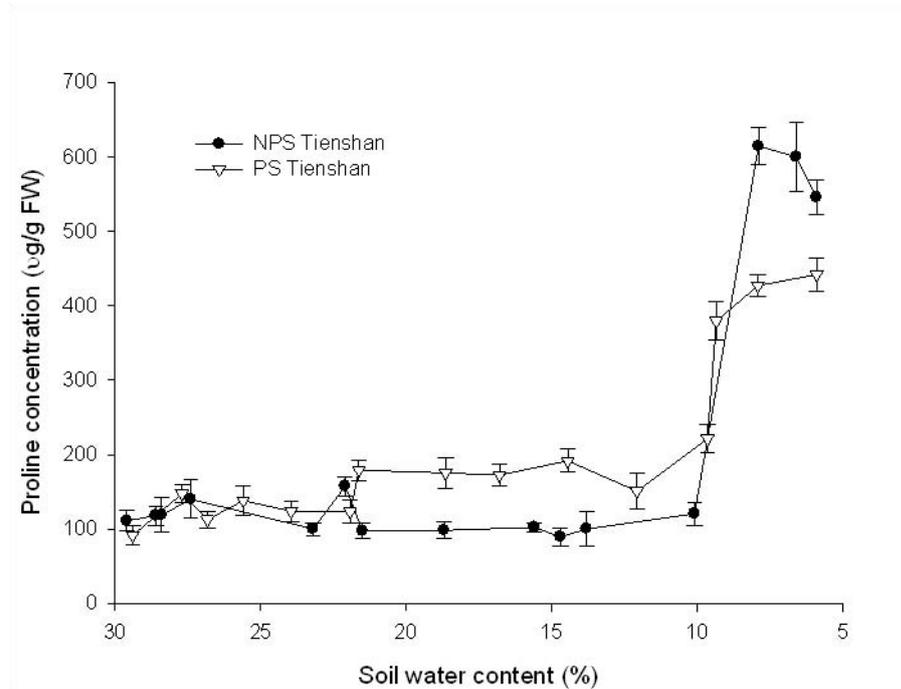
Results 1

proline concentration then decreased to *ca.* 190 $\mu\text{g/g}$ FW, at the point where the PER in the first-fully expanded leaves of the NPS-treated Kopu ceased (at *ca.* 8% SWC). Although this figure was lower than the proline concentration before PER ceases, the proline concentration at the time the PER ceased was *ca.* two-fold higher than those of the well-watered NPS-treated Kopu leaves. In the PS-treated Kopu, a gradual increase in proline accumulation was observed earlier at *ca.* 18% SWC. At this point, proline concentration increased *ca.* two-fold from 50 to 100 $\mu\text{g/g}$ FW. After this, a subsequent increase in proline concentration was observed with a *ca.* four-fold increase observed following a decrease in the SWC to *ca.* 18%, and this proline concentration was then maintained until the PER ceased at *ca.* 8% SWC (Figure 3.5.B).

Overall, there were similar patterns of proline accumulation between the NPS-treated Tienshan and NPS-treated Kopu. Sudden increases in proline concentration in both varieties were observed just before the PER ceased, but the proline concentration then decreased when the PER ceased. Interestingly, alterations in proline accumulation profiles following PS treatment were observed in both varieties. Gradual increases in proline concentration were observed in the PS treatments, as the SWC decreased, and these levels were maintained until the PER ceased in both the PS-treated Tienshan and Kopu.

Results 1

A



B

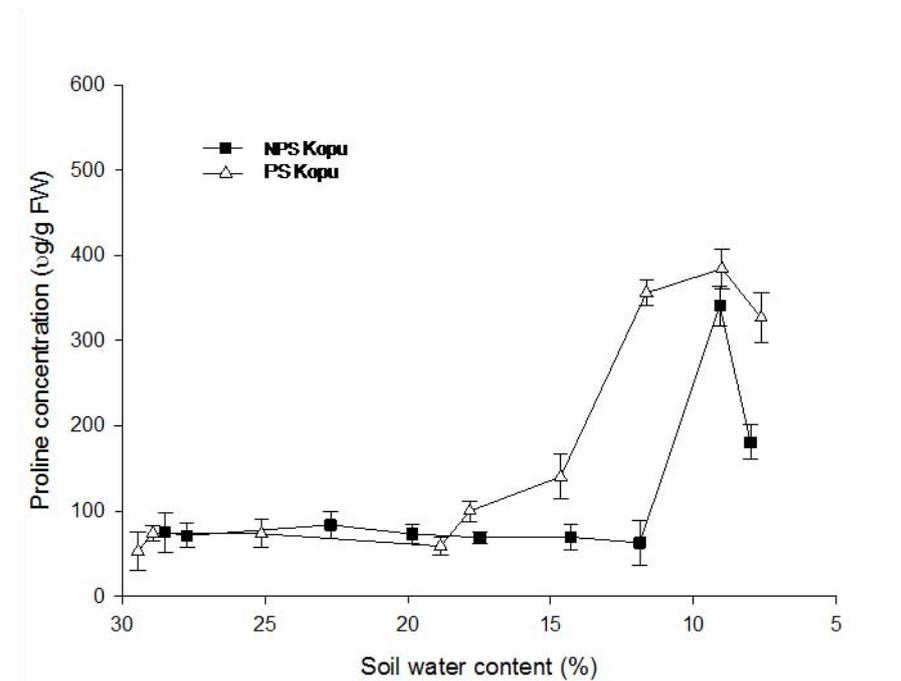


Figure 3.5 Accumulation of proline in the first-fully expanded leaves of the NPS- and PS-treated Tienshan ecotype (A) and NPS- and PS-treated Kopu cultivar (B), as indicated, during a water deficit. Each value is a mean of triplicates \pm SE.

3.2. Characterisation of ACC Oxidase Expression during Water Deficit in White Clover

The characterisation of ACC oxidase expression during water deficit reported in this thesis was carried out from the tissue samples collected from two repeats of water-deficit experiments undertaken in the NZCEL in 2006 and 2007 (Section 2.2.1). All *TR-ACO* gene expression and TR-ACO protein accumulation results described here are from experiments undertaken in 2007. The results from experiments undertaken in 2006 can be found in Appendices VI to VII.

3.2.1. Development of the SqRT-PCR Technique to Study the Expression of TR-ACO1, TR-ACO2 and TR-ACO3

A semi-quantitative Reverse Transcript Polymerase Chain Reaction (sqRT-PCR) technique was used to examine the expression of the *TR-ACO1*, *TR-ACO2* and *TR-ACO3* genes during the water deficit study reported in this thesis. The *TR-ACO1*, *TR-ACO2* and *TR-ACO3* sequences of the *Trifolium repens* genotype 10F of cultivar Grasslands Challenge obtained from GeneBank, were used as the sequence reference. The *TR-ACO* genes share a high homology (more than 80 %) in the coding frame regions but they have more distinct 3'UTR regions (less than 40% homology) (Appendix VIII). Therefore, gene-specific primers, for each of the *TR-ACO* transcripts, were designed to amplify *ca.* 120 bp of the coding regions and *ca.* 200 bp of the 3'UTR regions. The specificity of each primer set was first tested using each of the *TR-ACOs* sequences as PCR templates, with the amplified products separated by 1 % (w/v) agarose gel electrophoresis and ethidium bromide stained (Figure 3.6). Each of the *TR-ACO* primer sets specifically amplified only their target sequence, and the *TR-ACO1*, *TR-ACO2* and *TR-ACO3* primer sets amplified *ca.* 320, 300 and 320 bp PCR products, respectively (Figure 3.6)

Results 1

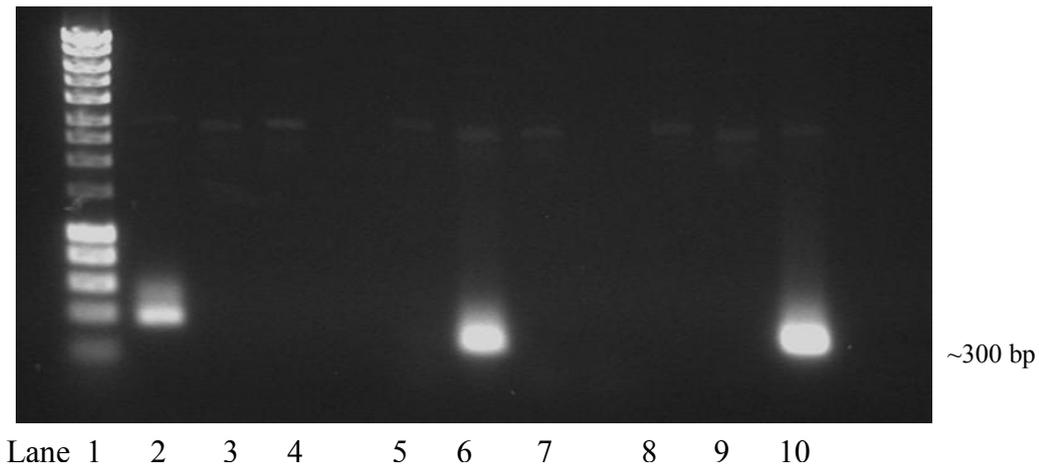


Figure 3.6 Specificity of *TR-ACO1*, *TR-ACO2* and *TR-ACO3* primer sets using RT-PCR.

Lane 1. Ladder.

Lanes 2 to 4. Amplified products as revealed by ethidium bromide staining using *TR-ACO1* primer sets and plasmid harbouring the reading frame and 3'UTR of *TR-ACO1*, *TR-ACO2*, and *TR-ACO3* as templates, respectively.

Lanes 5 to 7. Amplified products as revealed by ethidium bromide staining using *TR-ACO2* primer sets and plasmid harbouring the reading frame and 3'UTR of *TR-ACO1*, *TR-ACO2*, and *TR-ACO3* as templates, respectively.

Lanes 8 to 9. Amplified products as revealed by ethidium bromide staining using *TR-ACO3* primer sets and plasmid harbouring the reading frame and 3'UTR of *TR-ACO1*, *TR-ACO2*, and *TR-ACO3* as templates, respectively.

Following this, the primers were used to amplify *TR-ACO1*, *TR-ACO2* and *TR-ACO3* genes from cDNA pools made to RNA isolated from apical structures, first-fully expanded leaves and second fully-expanded leaves of Tienshan and Kopu, respectively. The RT-PCR products of the *TR-ACO1*, *TR-ACO2* and *TR-ACO3* were confirmed by 1% (w/v) agarose gel electrophoresis and ethidium bromide staining and further confirmed by sequencing (data not shown) before being used to generate specific probes, using the DIG-High Prime method (Section 2.5.9.2). The specificity of *TR-ACO1*, *TR-ACO2* and *TR-ACO3* probes to only hybridise to the respective target sequence was determined prior to sqRT-

Results 1

PCR analysis. Each of the DIG-labelled *TR-ACO1*, *TR-ACO2* or *TR-ACO3* cDNA probes were incubated with amplified *TR-ACO1*, *TR-ACO2*, *TR-ACO3* transcripts and each probe specifically hybridised to the expected target sequences (Figure 3.7)

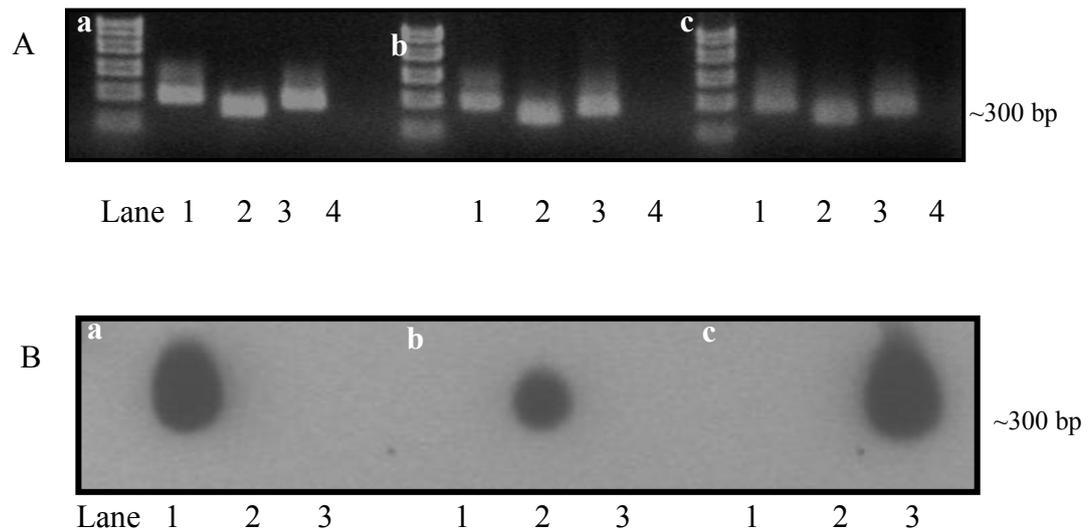


Figure 3.7 Specificity of *TR-ACO1*, *TR-ACO2* and *TR-ACO3* as probes.

Each lane contained 100 ng of PCR product amplified from the reading frame and 3'UTR of *TR-ACO1* (lane 1), *TR-ACO2* (lane 2), and *TR-ACO3* (lane 3) using primers as described in Figure 3.6. The PCR products were separated in 1% (w/v) of agarose gel electrophoresis and stained with ethidium bromide (A). The separated DNA, as shown in gel A, was blotted to HybondTM-N⁺ membrane and hybridised with DIG-labelled cDNA probes of *TR-ACO1* (a), *TR-ACO2* (b) or *TR-ACO3* (c). The blots were challenged with anti-DIG IgG alkaline phosphatase conjugated antibody then treated with chemiluminescent substrate (CDP Star) and exposed to X-ray film for visualisation (B).

Following these specificity experiments, a number of cycles for RT-PCR were performed to establish the linear relationship for each gene (Figure 3.8). For *TR-ACO1*, *TR-ACO2* and *TR-ACO3* expression, 21, 16 and 28 RT-PCR cycles, respectively, fitted within the linear range and so these numbers of cycles were then used for the expression analysis reported subsequently in this thesis.

Results 1

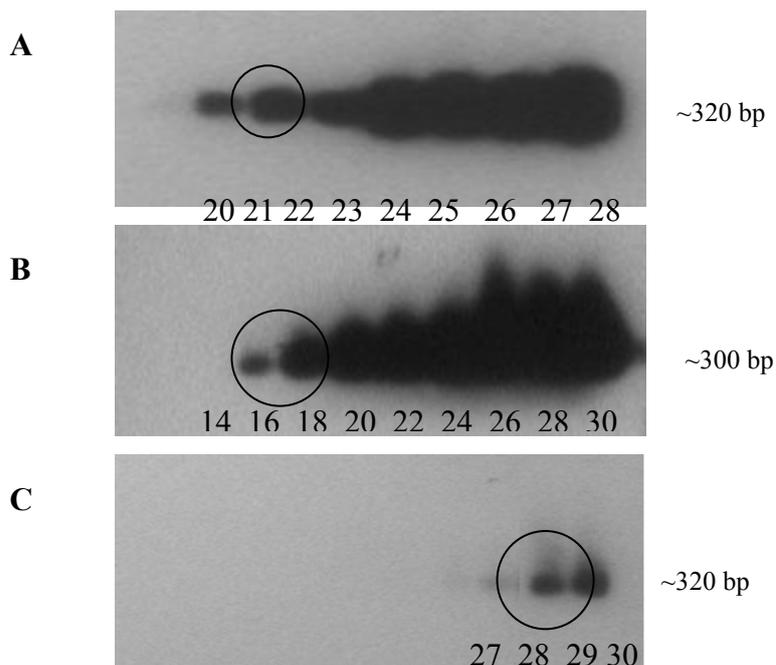


Figure 3.8 Experiments to identify the linear range of PCR cycles used to amplify each *TR-ACO* transcript for subsequent sqRT-PCR analysis.

Each lane, containing the same equivalent of RNA used in the RT reaction, was amplified for a different numbers of PCR cycles (as the numbers indicate), transferred to a HybondTM-N⁺ membrane and hybridised with the *TR-ACO1* (A), *TR-ACO2* (B) or *TR-ACO3* probes (C). The numbers of cycles chosen for the sqRT-PCR analysis are indicated in the circles.

3.2.3. Accumulation of TR-ACO Proteins in the Leaves of White Clover during A Water Deficit

It has been suggested that environmental changes, which alter plant growth and development can also alter the rate of protein synthesis (He *et al.*, 1999). Therefore western analysis, based on an equal amount of protein loading, may not reflect change in the total cellular pool of protein. In order to address this, two sets of western blot analyses were conducted based on a loading as equal fresh weight basis and an equal protein content basis. Examples of one of these are shown in Figure 3.9. Using the SDS-PAGE mini-protein gel system, with either 20 μ g of protein (equal protein loading) or 2 μ l of protein (equal fresh weight loading), and western blot analysis using the anti-TR-ACO1 antibody, a similar pattern of

Results 1

TR-ACO1 protein accumulation was observed. Coomassie brilliant blue (CBB) staining was used to stain the SDS-PAGE protein gels to ensure equal protein loading and Ponceau S staining was used to stain the PDVP membrane, following the electroblotting of protein, to ensure that the protein was equally transferred to the PDVF membranes. Therefore, any changes of TR-ACO1 accumulation are not due to uneven loading or blotting of the protein onto the PDVF membranes.

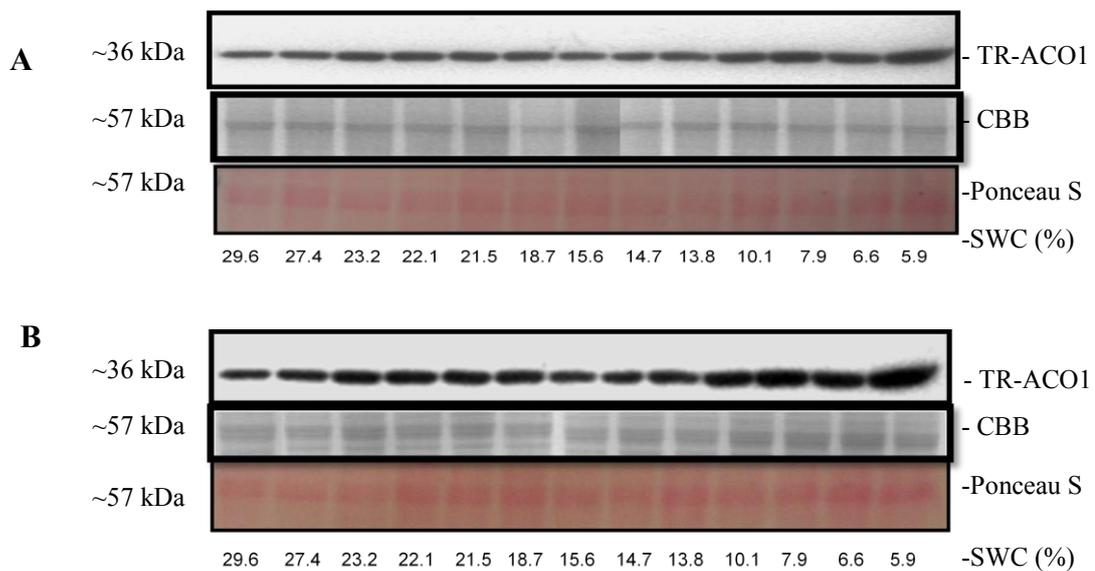


Figure 3.9 Western blot analysis of TR-ACO1 protein accumulation in the apical structures of NPS-treated Tienshan as a function of changes in SWC with samples loaded as a per protein basis (A) or per fresh weight basis (B).

Aliquots of total crude protein extracts of apical structures harvested from plants growing at the SWC, indicated, were separated using SDS-PAGE, electroblotted onto PDVF membrane and challenged with anti-TR-ACO1 antibodies (upper panels). Coomassie staining of each SDS-PAGE gel, corresponding to gel used for western blot is shown in the middle panels and Ponceau S stained PDVF approach is shown in the lower panels.

These results indicate that for western blot analyses in response to a water-deficit to the degree reported in this thesis, both approaches would provide similar

sensitivity to observe changes in the TR-ACO protein accumulation. Thus western blot results subsequently reported in this thesis were based on equal protein loads. The corresponding western blot sets loaded according to equal fresh weight, are attached as Appendices IX to XI.

3.2.4. Expression of TR-ACO1 Transcripts in the Apical Structures of White Clovers during Water Deficit

The expression of *TR-ACO1* during a water deficit in the apical structures of white clover was studied using sqRT-PCR using the probe developed in the specificity and linearity assays (Figure 3.7 and 3.8). Apical structures were harvested daily at different SWCs from the two varieties of white clover plants (Tienshan ecotype and Kopu cultivar) subjected to two different water deficit treatments (see Section 2.2.1).

For the expression of *TR-ACO1* in the NPS- and PS-treated Tienshan, the probe hybridised to a single transcript of *ca.* 320 bp but there were no consistent trends in the patterns of *TR-ACO1* expression in the apical structures (Figure 3.10). In the NPS-treated Tienshan (Figure 3.10.A), a similar pattern of *TR-ACO1* expression was observed in the fully-hydrated apical structures (at *ca.* 29.6% SWC) and apical structures harvested at and above *ca.* 22 % SWC. The expression of *TR-ACO1* then decreased slightly in the apical structures harvested from *ca.* 21.5 and 18.7% SWC, and after this point expression increased again to an approximately similar level as seen in the fully-hydrated state. When SWC fell to below 7%, a slight increase in expression was again observed which was higher than that observed in the fully-hydrated apical structures. Although the trends of expression observed in the apical structures of the NPS-treated Tienshan were not consistent, the overall expression of *TR-ACO1* over the decrease in SWC was quite similar, with a possible induction at *ca.* 6.6 and 5.9 % of SWC. Therefore, these results indicate that there were no major changes in the expression of *TR-ACO1* in the apical structures of NPS Tienshan subjected to water deficit to *ca.* 7.9 % SWC, but there was some induction just before the PER in the first fully-expanded leaves ceases.

Results 1

To ensure loading of cDNA at each time point, the expression of β -actin was also examined and used as an internal standard. The β -actin transcript was amplified by degenerate primers (Section 2.5.5.1) from the same cDNA pool as used for *TR-ACO1* expression analysis, the products were separated by 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide (Figure 3.10). Over the SWC values examined, similar β -actin expression was observed in each lane. Therefore, the differential *TR-ACO1* transcript expression patterns seen were not caused by uneven loading of cDNA.

In the PS-treated Tienshan, a similar level of *TR-ACO1* expression was detected in fully-hydrated apical structures (at *ca.* 29.4 % SWC) and in all subsequent harvest points until *ca.* 14.4% SWC (Figure 3.10.B). The expression of *TR-ACO1* then declined as the SWC decreased to *ca.* 12% and 9.7% SWC, and then increased again to a level similar to that observed in the fully-hydrated plants when the SWC decreased to *ca.* 7.9% and 5.9%. The decrease in *TR-ACO1* expression observed in the apical structures at *ca.* 12% and 9.7% SWC was probably due to uneven loading of cDNA, since the expression of β -actin at these sampling points had also slightly decreased. It can also be noted that expression of β -actin at *ca.* 5.9% SWC was much less than the level of expression at other SWC points. When any changes in β -actin expression are taken into account, the expression pattern of *TR-ACO1* in PS-treated Tienshan was essentially the same, except for the increase at *ca.* 5.9% SWC. Therefore, overall there were similar patterns of *TR-ACO1* expression in the apical structures of NPS and PS Tienshan.

Results 1

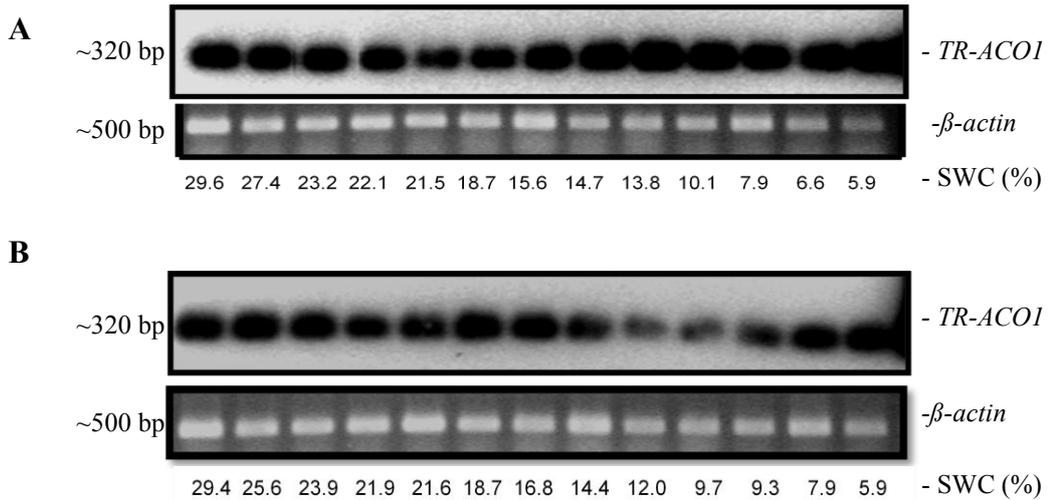


Figure 3.10 Expression of *TR-ACO1* in the apical structures of NPS-treated Tienshan (A) and PS-treated Tienshan (B) revealed using sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene-specific primers for *TR-ACO1* and the products were probed with a DIG-labelled *TR-ACO1* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR, using degenerate primers to amplify β -actin from the same cDNA pool. RT-PCR products were separated by electrophoresis and visualised, following ethidium bromide staining (lower panels).

Expression of *TR-ACO1* was also examined in the NPS- and PS-treated Kopu plants (Figure 3.11). As can be seen in Figure 3.11, there was differences in *TR-ACO1* expression in the apical structures between NPS- and PS-treated Kopu. In the NPS-treated Kopu, similar expression of *TR-ACO1* was observed in the fully-hydrated apical structures (at *ca.* 28.5% SWC) and in the apical structures harvested as the SWC declined to *ca.* 19.8 %. After this point, expression of *TR-ACO1* declined as the SWC decreased. In the PS-treated Kopu, there were no real changes in the expression of *TR-ACO1* after the water-deficit imposition. An approximately similar level of *TR-ACO1* expression was observed, regardless of the SWC. In these experiments, expression of β -actin was also used as an internal standard and a similar level of expression was detected in the NPS- and PS-treated

Results 1

Kopu, thus confirming that the differences in expression of *TR-ACO1* between the NPS- and PS-treated Kopu were not due to uneven loading.

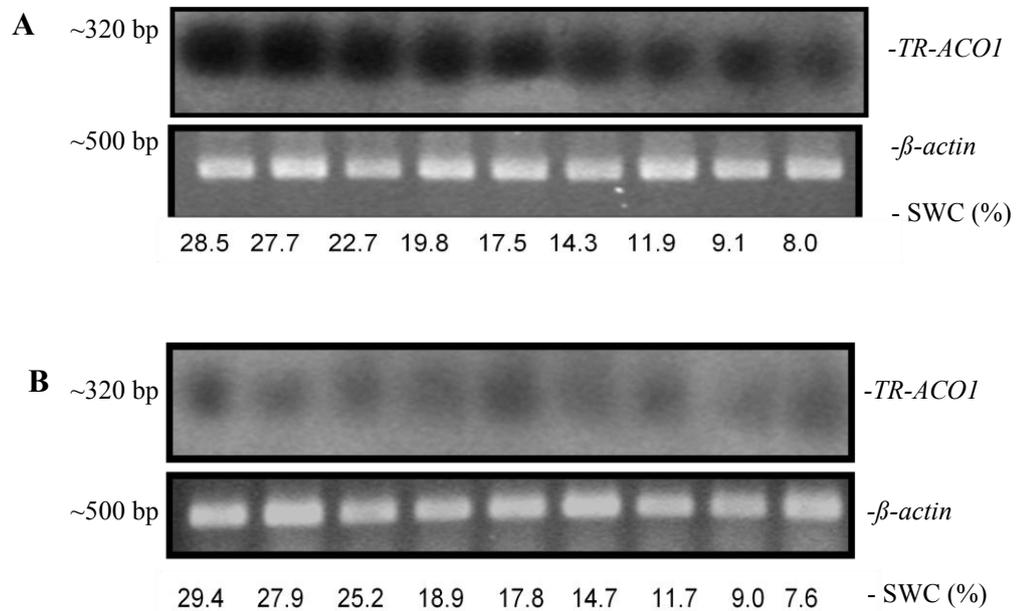


Figure 3.11 Expression of *TR-ACO1* in the apical structures of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed using sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWC, as indicated. One round of PCR was performed using gene-specific primers for *TR-ACO1* and the products probed with a DIG-labelled *TR-ACO1* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate β -actin primers to amplify β -actin from the same cDNA pool. RT-PCR products were separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

3.2.5 Accumulation of TR-ACO1 Protein in the Apical Structures of White Clover Subjected to a Water Deficit

Changes in TR-ACO1 protein accumulation during a water deficit in the NPS- and PS-treated Tienshan ecotype and Kopu cultivar were examined by western blot analysis. The TR-ACO1 protein accumulation was determined using an antibody raised against the recombinant TR-ACO1 protein from white clover genotype 10-F of cultivar Grassland Challenge, as reported by Hunter *et al.* (1999). The anti-TR-ACO1 antibody recognised a protein band of *ca.* 36 kDa in both the Tienshan ecotype and Kopu cultivar, which is in the range reported from other plant species (36 to 41 kDa).

Changes in TR-ACO1 protein accumulation during a water-deficit in NPS- and PS-treated Tienshan are shown as Figure 3.12. A similar level of TR-ACO1 protein accumulation is observed in the apical structure of fully-hydrated NPS-treated Tienshan (at *ca.* 29.6% SWC) and in the apical structures as the SWC decreased to *ca.* 14.7%. After this point, TR-ACO1 accumulation increased as the SWC decreased. Similar patterns in term of changes in the TR-ACO1 protein accumulation were also observed in the apical structures of PS-treated Tienshan (Figure 3.12.A). A similar level of TR-ACO1 protein abundance was observed in the fully-hydrated apical structures (at *ca.* 29.4% SWC) and in extracts from apical structures until *ca.* 12% SWC was reached (Figure 3.12.B). The CBB staining of SDS-PAGE gels from the same samples as those used for western blot analysis showed that approximately the same amount of protein was loaded for each SWC sampling point. These results suggest that there was no major changes in TR-ACO1 protein accumulation in the apical structures of both NPS- and PS-treated Tienshan subjected to a water deficit treatment above *ca.* 12% SWC, but as water-deficit progressed to below 12% SWC, there was an increase in TR-ACO1 protein accumulation.

Results 1

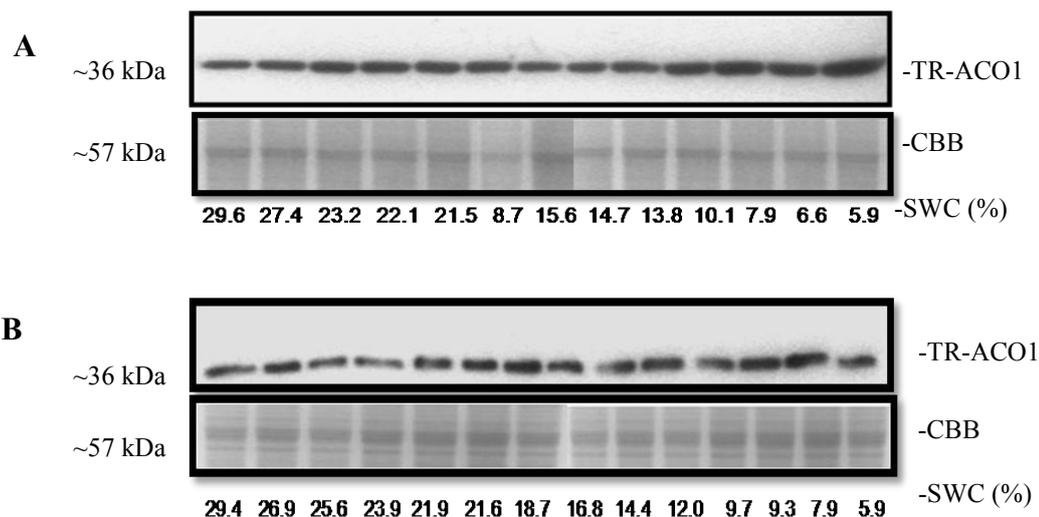


Figure 3.12 TR-ACO1 protein accumulation in the apical structures of NPS-treated Tienshan (A) and PS-treated Tienshan (B) revealed using western analysis.

Aliquots (20 μ g) of total crude protein extract from apical structures harvested at different SWCs, as indicated, were separated using SDS-PAGE, electroblotted onto PDVF membrane and challenged with anti-TR-ACO1 antibodies. Protein-antibody recognition was determined using the chemiluminescent system following incubation of the protein blot in anti-rabbit IgG HRP conjugated secondary antibody and exposure of the blot to an X-ray film (upper panels). CBB staining of the SDS-PAGE gel, corresponding to protein used for the western analysis (lower panels).

In the NPS- and PS-treated Kopu, different TR-ACO1 protein accumulation profiles were observed (Figure 3.2.8). In the NPS-treated Kopu (Figure 3.13.A), a higher level of TR-ACO1 protein accumulation was observed in fully-hydrated apical structures (at *ca.* 28.5% SWC) which decreased as the SWC decreased. This decrease in TR-ACO1 protein accumulation was in contrast to an increase in the TR-ACO1 protein accumulation seen previously in the NPS- and PS-treated Tienshan. However, in PS-treated Kopu, and unlike the NPS-treated Kopu, there was approximately the same level of TR-ACO1 protein accumulation observed regardless of the SWC. Therefore, there was no change in the accumulation of

Results 1

TR-ACO1 in the apical structures of PS-treated Kopu. In both treatments, CBB staining of SDS-PAGE gels confirmed that approximately the same amount of protein was loaded in each lane and therefore the observed changes were not due to a loading artefact.

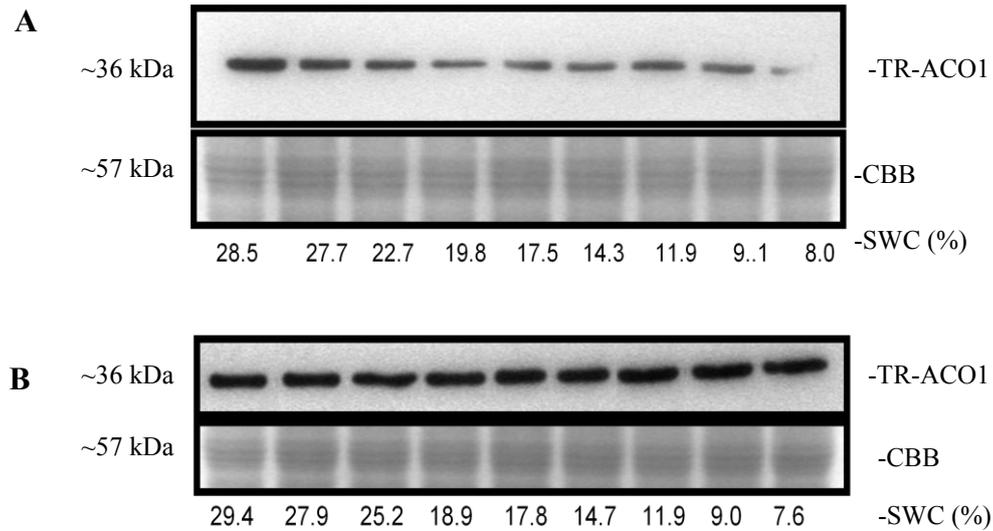


Figure 3.13 TR-ACO1 protein accumulation in the apical structures of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed using western analysis.

Aliquots (20 μ g) of total crude protein extract from apical structures harvested at different SWCs, as indicated, were separated using SDS-PAGE, electroblotted onto PDVF membrane and challenged with anti-TR-ACO1 antibodies. Protein-antibody recognition was determined using the chemiluminescent system following probing of the protein blot with anti-rabbit IgG HRP conjugated secondary antibody and exposure of the blot to an X-ray film (upper panels). CBB staining of the SDS-PAGE gel, corresponding to protein used for the western analysis (lower panels).

3.2.6 Changes in Chlorophyll Concentration in the First-Fully Expanded Leaves of White Clover Stolon Subjected to a Water Deficit

Total chlorophyll was measured by Magna and Larher's (1984) method using first and second fully-expanded leaf extracts harvested at different SWCs. Changes in total chlorophyll concentration, in the first fully-expanded leaves of the NPS- and PS-treated Tienshan ecotype are shown in Figure 3.14.A. In the first fully-expanded leaves of Tienshan exposed to the early stages of the water deficit (to *ca.* 23% SWC) a similar total chlorophyll content was maintained as the fully-hydrated leaves (*ca.* 1420 to 1460 $\mu\text{g/g}$ FW). A significant decrease in chlorophyll concentration was only observed in leaves subjected to *ca.* 21% SWC, at which point the total chlorophyll decreased from *ca.* 1460 to 1370 $\mu\text{g/g}$ FW. After this point, however, the total chlorophyll content increased again as the SWC decreased to reach a similar concentration to that observed in fully-hydrated leaves. This trend was observed until the PER in the first fully-expanded leaves ceased. Therefore, the NPS treatment did not alter the total chlorophyll level in the first fully-expanded leaves of NPS-treated Tienshan. In PS-treated Tienshan, the water deficit treatment also did not significantly alter the total chlorophyll concentration in the first fully-expanded leaves. The total chlorophyll concentration in the first fully-expanded leaves of the PS-treated Tienshan was maintained between *ca.* 1366 and 1534 $\mu\text{g/g}$ FW.

The changes in total chlorophyll concentration in the first fully-expanded leaves of NPS- and PS-treated Kopu are presented as Figure 3.14.B. The first fully-expanded leaves of NPS Kopu had a total chlorophyll content of *ca.* 1426 $\mu\text{g/g}$ FW, and approximately the same concentration of total chlorophyll was also observed in the first fully-expanded leaves of NPS-treated Kopu harvested from plants experiencing the early stages of a water deficit to *ca.* 18% of SWC. When the SWC was further decreased to *ca.* 14% of SWC, there was an increase in the total chlorophyll concentration to *ca.* 1679 $\mu\text{g/g}$ FW and this concentration was maintained until the PER in the first fully-expanded leaves ceased, *i.e.* at *ca.* 6% SWC.

Results 1

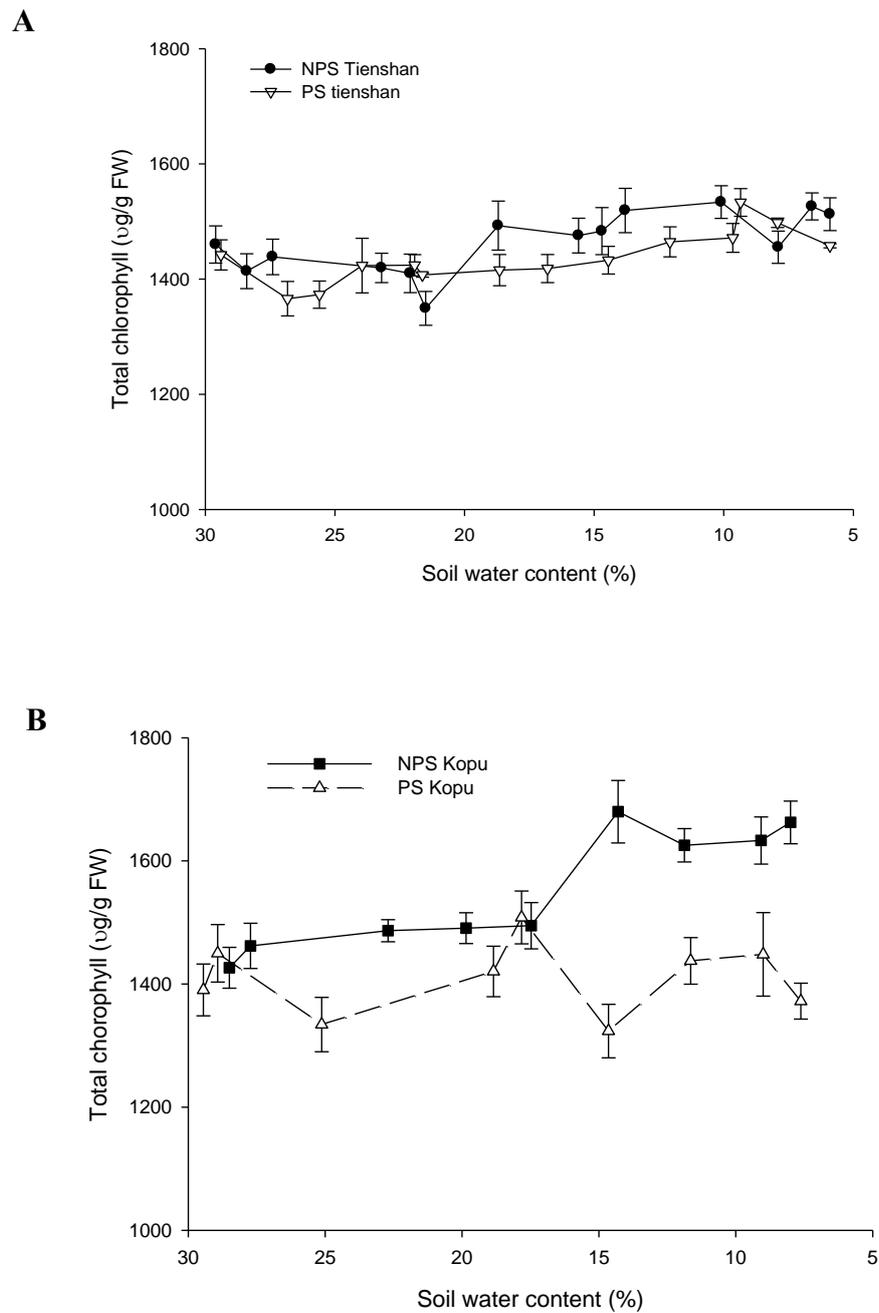


Figure 3.14 Changes in chlorophyll concentration in the first fully-expanded leaves of the NPS- and PS-treated Tienshan ecotype (A) and the NPS- and PS-treated Kopu cultivar (B). Each value is the mean of four replicates \pm SE.

Results 1

The fully-hydrated leaves and subsequent leaves harvested from the PS-treated Kopu plants subjected to an early decrease of SWC (to *ca.* 19%) maintained a similar total chlorophyll concentration of between *ca.* 1390 to 1420 $\mu\text{g/g}$ FW. After this point, there was an increase in the total chlorophyll concentration to *ca.* 1500 $\mu\text{g/g}$ FW in the PS-treated Kopu exposed to *ca.* 18% SWC. The total chlorophyll then decreased again as the SWC decreased to *ca.* 15%, but after this point the total chlorophyll then increased to a similar concentration as that observed in fully-hydrated leaves, and this chlorophyll concentration was maintained until the PER ceased. Therefore, overall the water deficit stress did not significantly alter the total chlorophyll concentration in the first-fully expanded leaves of PS-treated Kopu.

3.2.7. Expression of TR-ACO2 Transcript in the First Fully-Expanded Leaves of White Clovers during Water Deficit

The expression of *TR-ACO2* during water-deficit was studied by sqRT-PCR, as described previously. RT-PCR was performed on total RNA isolated from first fully-expanded leaves harvested daily at different SWCs from the Tienshan ecotype and Kopu cultivar subjected to two different water deficit treatments (Section 3.1.1). The RT reaction was carried out using equal amounts of total RNA as a template from each time point of the treatment. Aliquots of one round of PCR products were separated by electrophoresis on a 1% (w/v) agarose gel, blotted onto HybondTMN⁺ nylon membrane and probed with a DIG-labelled *TR-ACO2* probe.

In NPS-treated Tienshan, expression of *TR-ACO2* increased in the first fully-expanded leaves subjected to early stages of the water deficit (to *ca.* 13.8 % SWC). After this point, the expression of *TR-ACO2* decreased as SWC decreased, and this continued until the PER in the first fully-expanded leaves ceased at *ca.* 5.9% SWC. In this experiment, a similar level of β -*actin* expression was observed at each sampling point. Therefore the changes in expression of *TR-ACO2* observed were not due to uneven loading of cDNA.

Results 1

A similar trend of *TR-ACO2* expression was also observed in PS-treated Tienshan (Figure 3.15.B). *TR-ACO2* expression increased in the first fully-expanded leaves subjected to early stages of water deficit (to *ca.* 18% SWC). After this harvest point, lower levels of *TR-ACO2* expression was observed and that decreased expression continued until PER in the first fully-expanded leaves of PS Tienshan ceased at *ca.* 5.9% SWC. In this experiment, again, similar expression of β -actin was seen at each sampling point. Thus exposure of Tienshan plants to both NPS and PS treatments resulted in an increased of expression of *TR-ACO2* in the early stages of water deficit, but further exposure of Tienshan plants to water deficit (lower than 14% SWC) subsequently resulted in a decrease in expression of *TR-ACO2* in the first-fully expanded leaves (Figure 3.15.B).

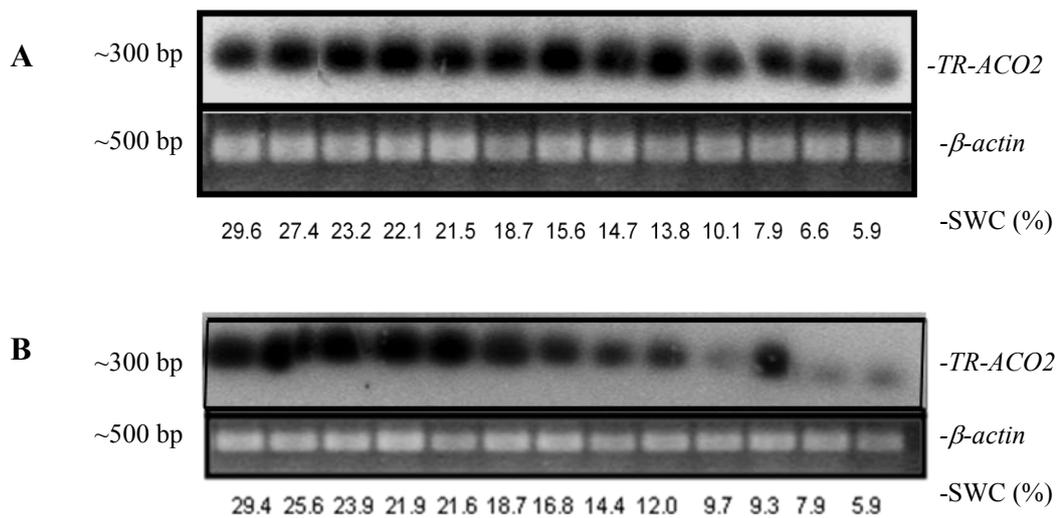


Figure 3.15 Expression of *TR-ACO2* in the first-fully expanded leaves of NPS-treated Tienshan (A) and PS-treated Tienshan (B) revealed using sqRT-PCR. RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO2* and the products probed with a DIG-labelled *TR-ACO2* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate primers to amplify β -actin from the same cDNA pool. RT-PCR products was separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

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Expression of *TR-ACO2* was also observed in the first fully-expanded leaves of NPS-treated and PS-treated Kopu (Figure 3.16). In the NPS-treated Kopu, a similar level of *TR-ACO2* expression was observed regardless of SWC. In these leaves, there was also a similar expression of β -actin seen suggesting that similar level of *TR-ACO2* expression observed as water deficit progressed was not due to cDNA loading artefacts. Therefore, water deficit did not significantly alter *TR-ACO2* expression in the first fully-expanded leaves of NPS-treated Kopu.

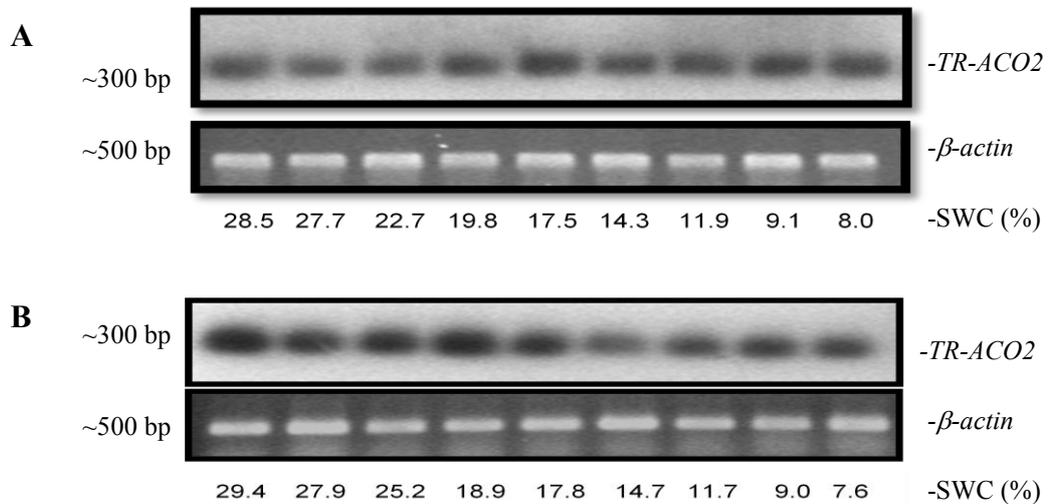


Figure 3.16 Expression of *TR-ACO2* in the first fully-expanded leaves of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed by sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO2* and the products probed with a DIG-labelled *TR-ACO2* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate primers to amplify β -actin from the same cDNA pool. RT-PCR products was separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

In the PS-treated Kopu, a higher level of *TR-ACO2* expression was seen in the well-watered first fully-expanded leaves and leaves subjected to the early stages

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of water deficit (to *ca.* 18% SWC). After this sampling point, expression of *TR-ACO2* decreased as the SWC decreased, and this decreased expression was maintained until PER ceased at *ca.* 7.6% SWC. Expression of *β-actin* at each sampling point of the PS-treated Kopu was similar confirming that these changes were not due to unequal loading of RNA.

3.2.8. Accumulation of TR-ACO2 Protein in the First Fully-Expanded Leaves Subjected to A Water Deficit

Western blot analysis was used to examine the patterns of TR-ACO2 protein accumulation during NPS and PS treatments, in both the Tienshan ecotype and the Kopu cultivar. As an initial attempt at the protein accumulation study, 10 µg aliquots of total crude protein were used in both NPS and PS Kopu and Tienshan. However, both varieties seemed to have a different TR-ACO2 abundance and therefore to be able to observe linear changes the amount of total protein loaded was altered to 5 µg in the Tienshan ecotype and 7 µg in the Kopu cultivar.

Aliquots of total protein, extracted from the first fully-expanded leaves harvested from different SWCs were separated by SDS-PAGE, transferred to PDVF membrane and TR-ACO2 protein detected with the anti-TR-ACO2 antibody. The anti-TR-ACO2 antibody, used in the experiments reported in this thesis, was raised against recombinant TR-ACO2 protein from white clover genotype 10-F of cultivar Grassland Challenge, reported by Hunter *et al.* (1999). In both varieties, the TR-ACO2 antibody recognised a protein band of *ca.* 37 kDa which is in the range reported from the cultivar Grassland Challenge and other plant species (36 to 41 kDa).

Accumulation of TR-ACO2 proteins in the first fully-expanded leaves of NPS- and PS-treated Tienshan are shown as Figure 3.17. In NPS-treated Tienshan, a similar level of TR-ACO2 protein abundance was seen in the well-watered plants and plants subjected to water deficit to *ca.* 27.4 % SWC. As the SWC decreased to *ca.* 18.7% (at which stage the PER declined), there was an increased accumulation of TR-ACO2. However, any additional decrease in SWC did not result in a greater accumulation of TR-ACO2 protein but the TR-ACO2 protein

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accumulated to a lower level than was observed in the well-watered plants (Figure 3.17.A). The CBB staining of the SDS-PAGE gel of the corresponding proteins used in western blot analysis showed that approximately the same amount of proteins was loaded for each sampling point. These results suggest differential accumulation of TR-ACO2 protein seen in the first fully-expanded leaves of NPS-treated Tienshan is not due to protein loading artefact.

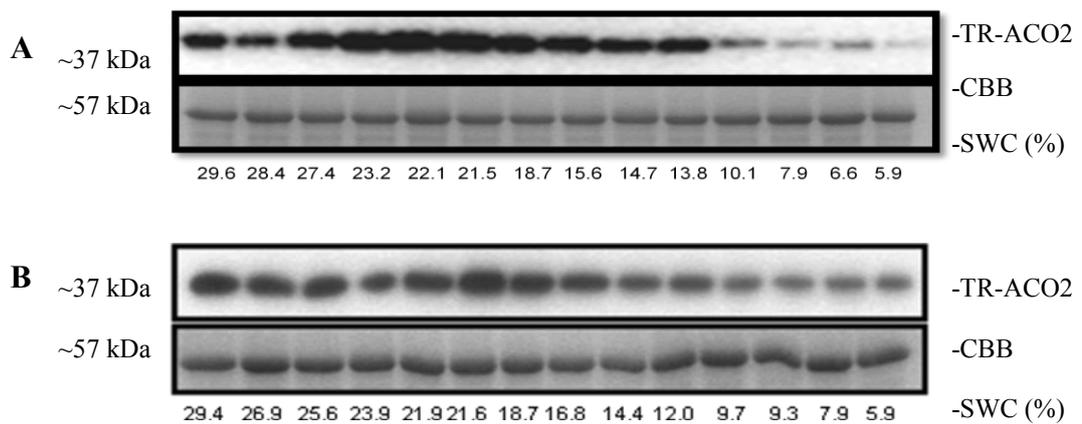


Figure 3.17 TR-ACO2 protein accumulation in the first fully-expanded leaves of NPS-treated Tienshan and PS-treated Tienshan (B) revealed using western analysis.

Aliquots (5 μ g) of total crude protein extract from apical structures harvested at different SWCs, as indicated, were separated using SDS-PAGE, electroblotted onto PDVF membrane and challenged with anti-TR-ACO1 antibodies. Protein-antibody recognition was determined using the chemiluminescent system following probing of the protein blot with anti-rabbit IgG HRP conjugated secondary antibody and exposure of the blot to an X-ray film (upper panels). CBB staining of the SDS-PAGE gel, corresponding to protein used for the western analysis (lower panels).

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A similar trend in TR-ACO2 accumulation, in common with NPS-treated Tienshan, was also observed in PS-treated Tienshan. A similar level of TR-ACO2 protein abundance was detected in the fully-hydrated first fully-expanded leaves of PS-treated Tienshan plants subjected to early stages of water deficit treatment (to *ca.* 23.9 % SWC). After this, the TR-ACO2 protein accumulation increased as SWC decreased to *ca.* 18.7% (at which point the PER also declined). In common with NPS-treated Tienshan, accumulation of TR-ACO2 protein decreased following the further reduction in SWC to *ca.* 5.9% (at which point the PER had ceased) (Figure 3.17.B). The CBB staining of the SDS-PAGE gel of corresponding samples of PS-treated Tienshan showed that approximately the same amounts of proteins were loaded in each sampling point, and therefore alteration of TR-ACO2 protein accumulation was not due to uneven loading of protein.

The changes in TR-ACO2 protein accumulation were also examined in the first fully-expanded leaves of Kopu subjected to NPS and PS treatments (Figure 3.18). In the NPS-treated Kopu, there was a similar level of TR-ACO2 protein accumulation detected in the leaves of well-watered plants and plants subjected to the early stages of water deficit (to *ca.* 17.8 % SWC, at which point the PER declined). After this, TR-ACO2 protein accumulation increased as the SWC decreased to *ca.* 8% (at which point the PER ceased). CBB staining of an SDS-PAGE gel of corresponding samples confirmed that similar amounts of protein were loaded for each sample (Figure 3.18.A). In the PS-treated Kopu, accumulation of TR-ACO2 protein increased in the first-fully expanded leaves harvested from plants subjected to the early stages of water deficit (to *ca.* 17.8%, at which point the PER significantly decreased). After this, a decrease in the TR-ACO2 protein abundance was observed in leaves exposed to a further decrease in SWC (to *ca.* 7.6 % SWC, at which point the PER ceased). Again CBB staining of an SDS-PAGE gel of corresponding samples showed that approximately the same amount of protein was loaded.

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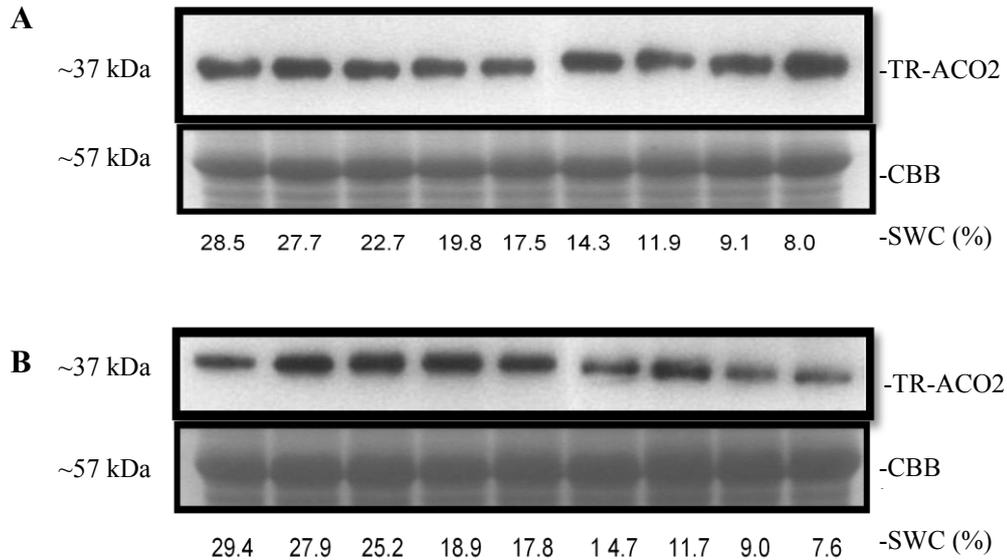


Figure 3.18 TR-ACO2 protein accumulation in the first-fully expanded leaves of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed using western blot.

Aliquots (7 μ g) of total crude protein extract from apical structures harvested at different SWCs, as indicated, were separated using SDS-PAGE, electroblotted onto PDVF membrane and challenged with anti-TR-ACO2 antibodies. Protein-antibody recognition was determined using the chemiluminescent system following probing of the protein blot with anti-rabbit IgG HRP conjugated secondary antibody and exposure of the blot to an X-ray film (upper panels). CBB staining of the SDS-PAGE gel, corresponding to protein used for the western analysis (lower panels).

3.2.9. Expression of TR-ACO3 Transcripts in the First Fully-Expanded Leaves Subjected to a Water Deficit

To investigate if water deficit induced the expression of *TR-ACO3*, sqRT-PCR as described previously (Section 3.2.2.) was used. RT-PCR was performed from total RNA isolated from the first fully-expanded leaves harvested from white clover plants experiencing different SWCs. From previous studies (Hunter *et al.*, 1999), expression of *TR-ACO3* was predicted to be low in mature green tissues and therefore more PCR cycles were used to amplify the *TR-ACO3* gene (Section

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3.2.2.). Aliquots of PCR products were separated by 1% (w/v) agarose gel electrophoresis, blotted onto HybondTM-N⁺ membrane and probed with a DIG-labelled *TR-ACO3* probe, before being exposed to X-ray film for visualisation.

In the first fully-expanded leaves of NPS-treated Tienshan, a blot probed with a DIG-labelled *TR-ACO3* probe showed hybridisation to an approximately 320 bp band. The hybridisation was detectable at all sampling points and similar expression of *TR-ACO3* was seen in leaves harvested from full-hydrated leaves (*ca.* 29.6 % SWC) and leaves exposed to a water deficit to *ca.* 5.9% SWC. Slightly lower expression of *TR-ACO3* was seen in some samples *i.e.* at *ca.* 23.2 %, 21.5 % and 18.7 % SWC, and slightly higher expression was observed in other samples *i.e.* at *ca.* 13.8%, 10.1 and 5.9 % SWC. However, overall these changes did not follow a consistent pattern. They were also not due to uneven loading of cDNA in these samples, as indicated from the expression of β -*actin* of the same samples (Figure 3.19.A). These results suggested that water deficit treatment did not significantly alter the expression of *TR-ACO3* in the first fully-expanded leaves of NPS-treated Tienshan.

A similar pattern of *TR-ACO3* expression was also observed in PS-treated Tienshan (Figure 3.19.B). In the first fully-expanded leaves of PS-treated Tienshan, a blot probed with DIG-labelled *TR-ACO3* also showed hybridisation to an approximately 320 bp band and there was a similar expression of *TR-ACO3* observed in all sampling point regardless of the SWC. In these samples, the expression of β -*actin* was again used as an cDNA loading control and a similar expression of β -*actin* was seen in all sampling points, suggesting that the similar expression is not due to cDNA loading artefacts. Therefore, both NPS and PS treatments did not significantly induce expression of *TR-ACO3* in the first-fully expanded leaves of Tienshan plants when exposed to a water deficit.

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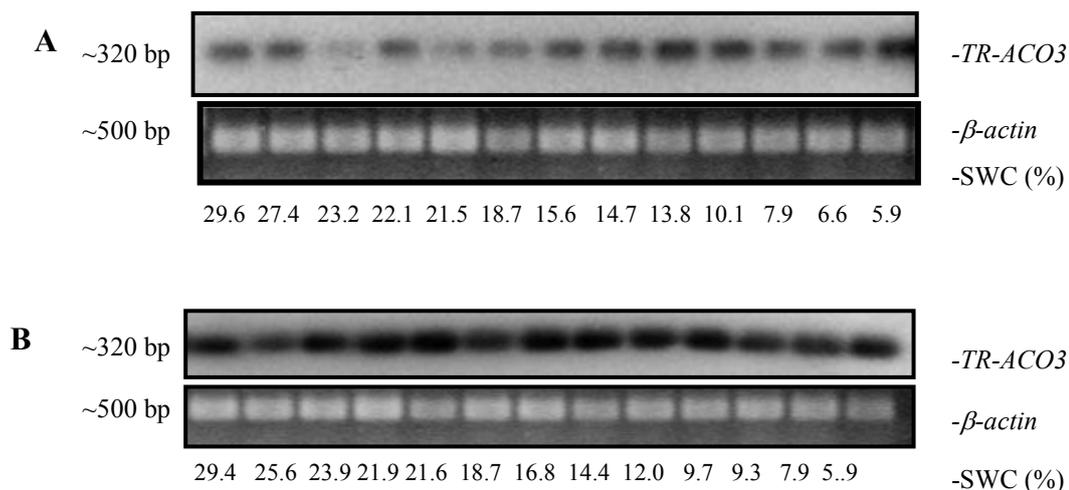


Figure 3.19 Expression of *TR-ACO3* in the first fully-expanded leaves of NPS-treated Tienshan (A) and PS-treated Tienshan (B) revealed by sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWC, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO3* and the products probed with a DIG-labelled *TR-ACO3* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate *β-actin* primers to amplify *β-actin* from the same cDNA pool. RT-PCR products was separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

SqRT-PCR analysis was also used to examine the expression of *TR-ACO3* in the first fully-expanded leaves of NPS- and PS-treated Kopu (Figure 3.20). Blots hybridised with a DIG-labelled *TR-ACO3* probe could be detected in the first fully-expanded leaves and these blots showed hybridisation to a *ca.* 320 bp band. In the first fully-expanded leaves of NPS-treated Kopu plants, a similar level of *TR-ACO3* expression was seen in all sampling point regardless of the SWC (Figure 2.20.A.). In these samples, a similar expression of *β-actin* was also observed suggesting that the similar level of *TR-ACO3* seen was not due to uneven loading of cDNA. Thus water deficit to *ca.* 8% SWC (the point at which the PER ceased) did not significantly alter the expression of *TR-ACO3* in the first fully-expanded leaves of NPS-treated Kopu. In PS-treated Kopu, in common with the NPS-treated Kopu, a similar level of *TR-ACO3* expression was also seen in the

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first fully-expanded leaves harvested from plants grown at all SWCs (Figure 3.20.B). Again, using β -actin as a loading control of RNA, similar expression was also seen in all samples.

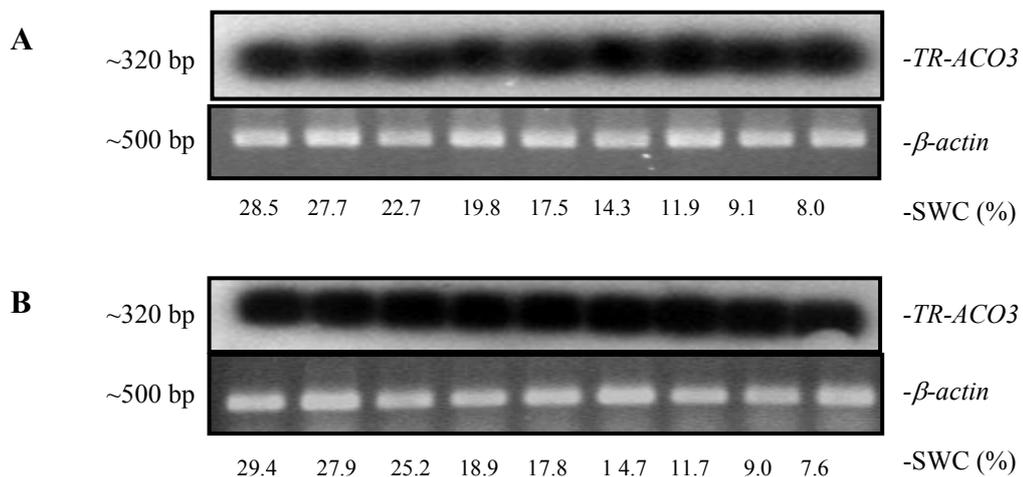


Figure 3.20 Expression of *TR-ACO3* in the first fully-expanded leaves of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed by sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO3* and the products probed with a DIG-labelled *TR-ACO3* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate primers to amplify β -actin from the same cDNA pool. RT-PCR products were separated by electrophoresis and visualised following ethidium bromide staining

Overall, the results of expression analysis of *TR-ACO3* in the first fully-expanded leaves of NPS- and PS-treated Tianshan and Kopu showed that the two water deficit treatments did not significantly induce the expression of *TR-ACO3* in the first fully-expanded leaves in either variety.

3.2.10. Changes in Chlorophyll Concentration in the Second Fully-expanded Leaves during Water Deficit

Total chlorophyll content was measured by Magna & Larher (1984) method (Section 2.4.1). The second fully-expanded leaves of NPS-treated Tienshan had *ca.* 1345 $\mu\text{g/g}$ FW of total chlorophyll when fully-hydrated (at *ca.* 29.7 % SWC) with a higher total chlorophyll observed in the leaves harvested from plants exposed to *ca.* 28% SWC. After this increase, the total chlorophyll concentration decreased to the same concentration as the fully-hydrated plants as the SWC subsequently decreased to *ca.* 13%. A significant increase in the total chlorophyll was then observed following further decreases of SWC to *ca.* 6% at which stage the total chlorophyll increased from *ca.* 1345 $\mu\text{g/g}$ FW to approximately *ca.* 1466 $\mu\text{g/g}$ FW (Figure 3.21.A). In the PS-treated Tienshan plants, there was no consistent trend in the changes of total chlorophyll, in the second fully-expanded leaves. The well-watered and water-deficit treated leaves maintained a level between *ca.* 1400 to 1500 $\mu\text{g/g}$ FW of total chlorophyll. Only the leaves of PS-treated Tienshan harvested from *ca.* 28%, 26% and 12% SWC had less chlorophyll of *ca.* 1450 $\mu\text{g g/FW}$. Overall, therefore, the results suggested that there were no real changes in the total chlorophyll concentration of second fully-expanded leaves of PS-treated Tienshan.

Changes in the total chlorophyll level of the second fully-expanded leaves of NPS- and PS-treated Kopu are presented in Figure 3.21.B. The fully-hydrated NPS-treated Kopu plants and plants subjected to the early stages of water-deficit (to *ca.* 21% SWC) maintained a similar chlorophyll concentration of between *ca.* 1580 and 1650 $\mu\text{g/g}$ FW. After this, subsequent decreases in SWC (to *ca.* 6%) reduced the total chlorophyll of NPS-treated Kopu leaves to *ca.* 1470 $\mu\text{g/g}$ FW. In the PS-treated Kopu, however, no significant decrease in total chlorophyll was observed in the plants subjected to water deficit to *ca.* 9 % SWC. The well-watered and water deficit treated leaves of PS-treated Kopu (to 9% SWC) maintained a similar total chlorophyll level of between *ca.* 1513 $\mu\text{g/g}$ FW and 1679 $\mu\text{g/g}$ FW. After this, the total chlorophyll content decreased to *ca.* 1415 $\mu\text{g/g}$ FW in leaves of PS-treated Kopu subjected to *ca.* 7.6% SWC.

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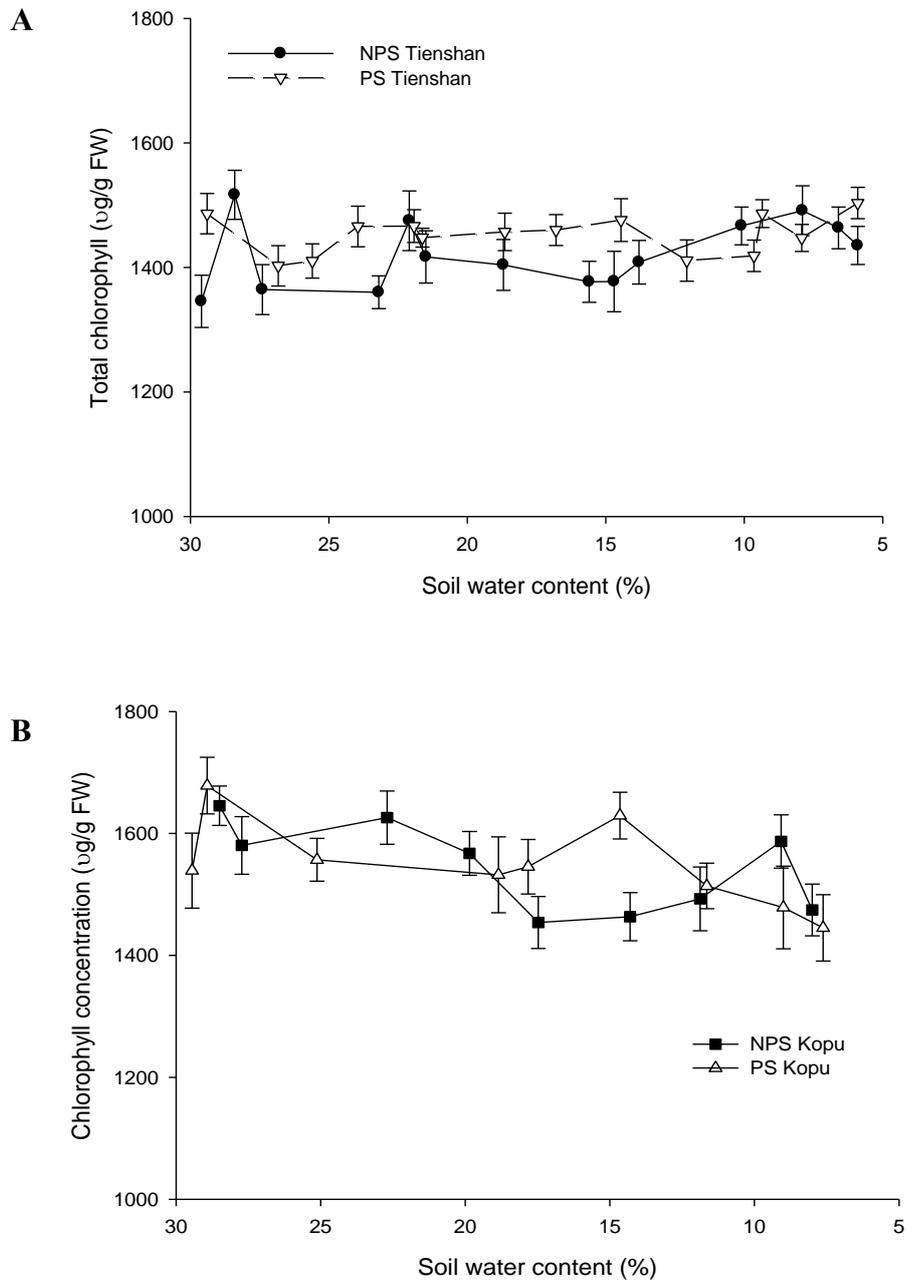


Figure 3.21 Changes in total chlorophyll in the second fully-expanded leaves of NPS- and PS-treated Tienshan (A) and NPS- and PS-treated Kopu (B), as indicated. Each value is the mean of four replicates \pm SE.

3.2.11. Expression of TR-ACO2 in the Second Fully-expanded Leaves of White Clovers during Water Deficit

Expression of *TR-ACO2* was observed in the second fully-expanded leaves of NPS-treated and PS-treated Tienshan and Kopu, using sqRT-PCR as previously described (Section 3.2.2). One round of RT-PCR was performed from total RNA isolated from leaves exposed to different SWCs. Aliquots of PCR products were separated by 1% (w/v) agarose gel electrophoresis, blotted onto HybondTM-N⁺ membrane and hybridised with a DIG-labelled *TR-ACO2* probe.

Hybridisation of the *TR-ACO2* to a *ca.* 300 bp band was detected in the second fully-expanded leaves of NPS-treated Tienshan grown in all SWC (Figure 3.22). A higher level of *TR-ACO2* expression was detected in well-watered leaves (at *ca.* 29.6% SWC) and leaves harvested from *ca.* 27.4% SWC. After this SWC, expression of *TR-ACO2* decreased following a subsequent decrease in SWC, and this decrease in expression was observed in plants grown to *ca.* 5.9% SWC. Slightly higher expression of *TR-ACO2* was observed in leaves exposed to *ca.* 13.8% and 10.1% SWC, but these levels were lower than *TR-ACO2* expression observed in the fully-hydrated leaves. In addition, β -*actin* expression was also slightly higher in the samples harvested from *ca.* 13.8% and 10.1% SWCs.

In common with NPS-treated Tienshan, higher expression of *TR-ACO2* was also seen in the fully-hydrated second fully-expanded leaves of PS-treated Tienshan and in the leaves samples taken from plants exposed to decreasing SWC (to *ca.* 25.6%). After this, expression of *TR-ACO2* decreased as the SWC decreased to *ca.* 5.9% SWC. Expression of β -*actin* was again used as internal control of cDNA loading and a similar level of expression was seen at each SWC sampled. Therefore, the decrease in expression of *TR-ACO2* observed was not due to uneven loading of cDNA.

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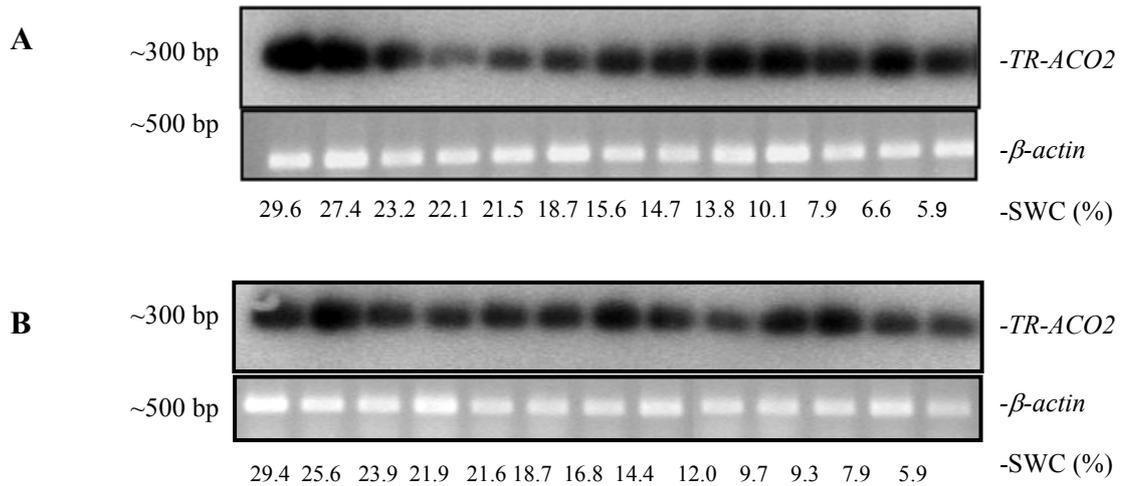


Figure 3.22 Expression of *TR-ACO2* in the second-fully expanded leaves of NPS-treated Tienshan (A) and PS-treated Tienshan (B) revealed by sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO2* and the products probed with a DIG-labelled *TR-ACO2* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate primers to amplify *β-actin* from the same cDNA pool. RT-PCR products were separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

Expression of *TR-ACO2* was also observed in the second fully-expanded leaves of NPS- and PS-treated Kopu (Figure 3.23). Higher expression of *TR-ACO2* was seen in the leaves of NPS-treated Kopu harvested from well-watered plants (at ca. 28.5 % SWC) and subsequent leaves experiencing the early stages of water deficit (to ca. 22.7% SWC). After this, expression of *TR-ACO2* decreased following the subsequent decrease in SWC to ca. 8% (Figure 3.23.A). Expression of *β-actin* was similar at each sampling point, and so the decrease in *TR-ACO2* expression observed in leaves subjected to water deficit of less than ca. 22% SWC was not due to the cDNA loading artefacts.

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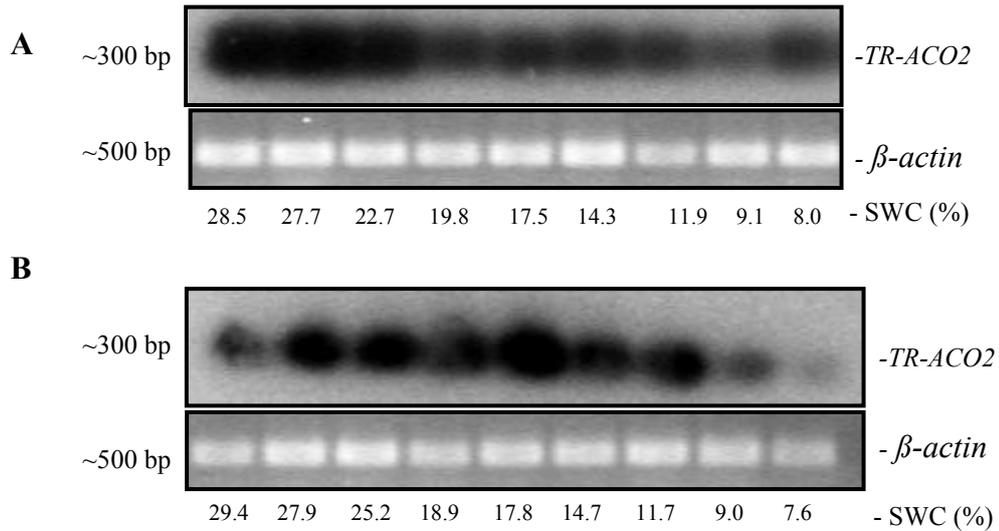


Figure 3.23 Expression of *TR-ACO2* in the second-fully expanded leaves of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed by sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO2* and the products probed with a DIG-labelled *TR-ACO2* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate primers to amplify *β-actin* from the same cDNA pool. RT-PCR products were separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

In the PS-treated Kopu, increased *TR-ACO2* expression was observed in the second fully-expanded leaves subjected to an early decrease of SWC to *ca.* 18.9%. However, expression of *β-actin* was also lower in these leaves harvested from fully-hydrated plants (at *ca.* 29.4% SWC). Considering this therefore, there was a similar level of *TR-ACO2* expression in the second fully-expanded leaves of PS-treated Tianshan subjected to an early decrease of soil water (to *ca.* 18.9% SWC). After this, there was a decrease in *TR-ACO2* expression as the SWC subsequently decreased to *ca.* 7.6%. In each of these sampling points, there was a similar expression of *β-actin* confirming that the decrease in *TR-ACO2* expression in

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leaves subjected to water deficit of less than *ca.* 18.9% was not due to uneven loading of cDNA.

3.2.12. TR-ACO2 Protein Accumulation in the Second Fully-expanded Leaves of White Clovers during Water Deficit

Accumulation of TR-ACO2 protein in the second fully-expanded leaves of both NPS- and PS-treated Tienshan and Kopu were, again, studied by western blot analysis using the anti-TR-ACO2 antibody (Hunter *et al.*, 1999).

In the initial analysis, aliquots of 10 µg of total protein were used. However, only very faint bands were detected indicating that lower TR-ACO2 protein accumulates in more mature leaves. Therefore, the amount of total protein used for TR-ACO2 accumulation analysis was increased to 20 µg. Aliquots of the protein extracted from different SWC were separated by SDS-PAGE, transferred to PDVF membrane and challenged with anti-TR-ACO2 antibody. A single band of approximately 37 kDa was seen following incubation of the protein blot in the secondary antibody (anti-rabbit IgG HRP conjugate secondary antibody) and exposure of the blot to X-ray film.

TR-ACO2 protein accumulation could be detected in the second fully-expanded leaves of NPS- and PS-treated Tienshan harvested from plants grown under well-watered and all water-deficit conditions (Figure 3.24). Exposure of Tienshan plants to an early decrease of soil water (to *ca.* 21.5%) in the NPS treatment resulted in an increase in TR-ACO2 protein abundance. Further decreases of SWC, after this sampling point, altered this trend. A lower TR-ACO2 protein abundance was observed when the SWC decreased below 21% and these lower levels were detected until the SWC decreased to *ca.* 5.9%. The CBB stain of the SDS-PAGE gel of the corresponding samples used for western analysis showed that approximately a similar amount of protein was loaded for each sampling point.

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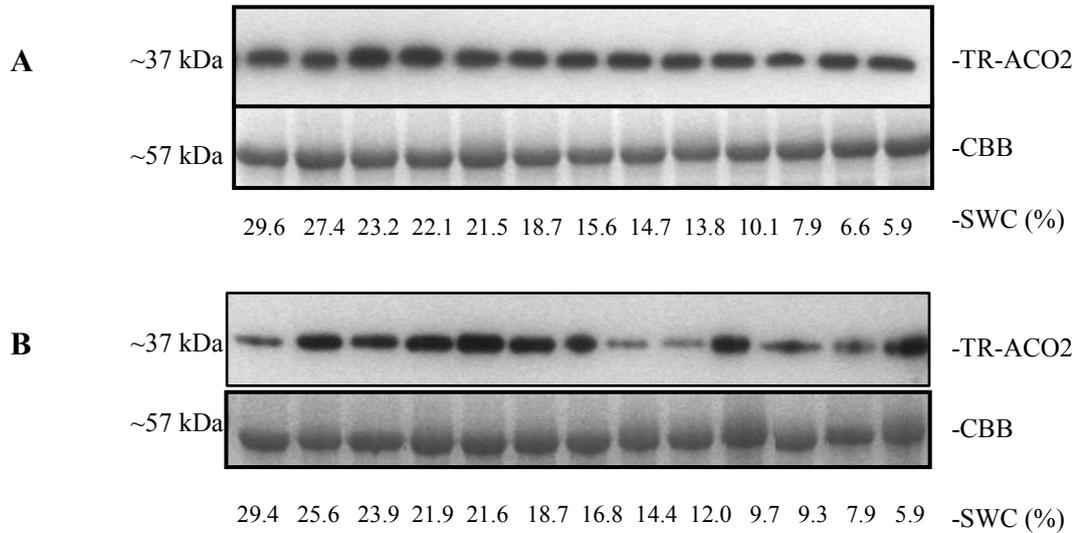


Figure 3.24 TR-ACO2 protein accumulation in the second fully-expanded leaves of NPS-treated Tienshan (A) and PS-treated Tienshan (B) revealed using western analysis.

Aliquots (20 μ g) of total crude protein extract from apical structures harvested at different SWCs, as indicated, were separated using SDS-PAGE, electroblotted onto PDVF membrane and challenged with anti-TR-ACO2 antibodies. Protein-antibody recognition was determined using the chemiluminescent system following probing of the protein blot with anti-rabbit IgG HRP conjugated secondary antibody and exposure of the blot to an X-ray film (upper panels). CBB staining of the SDS-PAGE gel, corresponding to protein used for the western analysis (lower panels).

Similar patterns of TR-ACO2 accumulation were also observed in PS-treated Tienshan (Figure 3.24.B). Increased accumulation of TR-ACO2 protein was seen in the second fully-expanded leaves as the SWC decreased to *ca.* 16.8% SWC. The CBB stain of the SDS-PAGE gel of the corresponding samples showed that approximately the same amount of protein was loaded, and so this increase in TR-ACO2 protein accumulation was not due to uneven loading of protein. After this point, and in common with the NPS-treated Tienshan, accumulation of TR-ACO2 protein decreased following the decrease in SWC to *ca.* 5.9%. The exception was two sampling points (*i.e.* *ca.* 9.7% and 5.9% SWC) but the CBB

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stain of the SDS-PAGE gel separation of those same samples showed that more protein was loaded. Considering this CBB staining gel, therefore, it could be suggested that prolonged imposition of PS-treated Tienshan to a water deficit of lower than *ca.* 16.8% SWC decreased the accumulation of TR-ACO2 protein in the second fully-expanded leaves.

The changes in TR-ACO2 protein accumulation in the second fully-expanded leaves of NPS- and PS-treated Kopu were also examined. A single TR-ACO2 protein band of approximately 37 kDa was detected in all samples collected from different SWCs, of both NPS- and PS-treated Kopu (Figure 3.25). In the NPS-treated Kopu, accumulation of TR-ACO2 protein decreased as the SWC decreased, while the CBB stain of the protein gel of the corresponding samples showed that an approximately similar amount of protein was loaded. Therefore, the observed decrease in accumulation of TR-ACO2 protein as the water deficit progressed was not due to a protein loading artefact.

Western blot analysis of TR-ACO2 protein accumulation in the second fully-expanded leaves of PS Kopu (Figure 3.25.B) showed that a single TR-ACO2 protein band of approximately 37 kDa can be detected in all leaves subjected to different SWCs. In these samples, water deficit treatment below *ca.* 25.2% SWC subsequently decreased the TR-ACO2 protein abundance and these decreases were observed until the SWC decreased to *ca.* 7.6%. The CBB stain of the SDS-PAGE gel of the corresponding samples showed that there was approximately a similar amount of protein loaded for each sampling point

Western blot analysis of TR-ACO2 protein accumulation in the second fully-expanded leaves of NPS- and PS-treated Tienshan and Kopu suggested that both water deficit treatments decreased accumulation of TR-ACO2 protein in the second fully-expanded leaves of both varieties. Moreover, there was a similar pattern of decrease in TR-ACO2 protein accumulation in the NPS- and PS-treated treatments in both Tienshan and Kopu. Higher TR-ACO2 protein was accumulated in the fully-hydrated leaves which then decreased as the water deficit progressed.

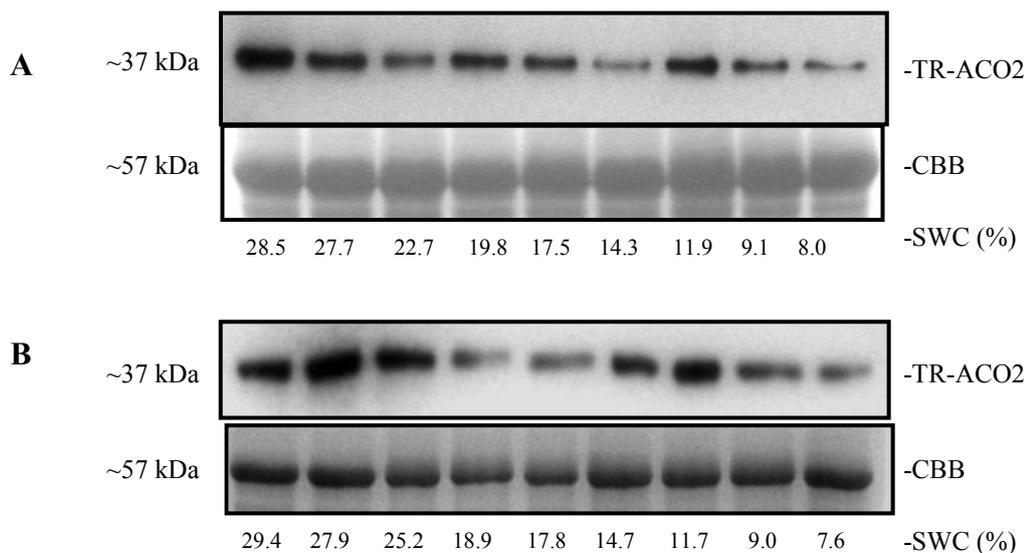


Figure 3.25 TR-ACO2 protein accumulation in the second fully-expanded leaves of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed using western analysis.

Aliquots (20 μ g) of total crude protein extract from apical structures harvested at different SWCs, as indicated, were separated using SDS-PAGE, electroblotted onto PDVF membrane and challenged with anti-TR-ACO2 antibodies. Protein-antibody recognition was determined using the chemiluminescent system following probing of the protein blot with anti-rabbit IgG HRP conjugated secondary antibody and exposure of the blot to an X-ray film (upper panels). CBB staining of the SDS-PAGE gel, corresponding to protein used for the western analysis (lower panels).

3.2.13. Expression of TR-ACO3 in the Second Fully-expanded Leaves of White Clovers during Water Deficit.

Changes in expression of *TR-ACO3* in NPS- and PS-treated Tienshan and Kopu under water-deficit in the second-fully expanded leaves was also undertaken. In this study, again, sqRT-PCR was used from total RNA isolated from leaves harvested from different SWCs. Aliquots of RT-PCR products were separated by a 1% (w/v) agarose gel electrophoresis, blotted into HybondTM-N⁺ membrane and hybridised with a DIG-labelled *TR-ACO3* probe (Section 3.2.2).

Results 1

The hybridised *TR-ACO3* band can be detected in the second fully-expanded leaves of NPS-treated Tienshan subjected to all water-deficit conditions (Figure 3.26). However, there was no consistent trends of *TR-ACO3* expression observed in the leaves harvested from different SWC (to 10.1 %). Some higher expression of *TR-ACO3* was observed at 23.2% SWC and 18.7% SWC, but less expression of *TR-ACO3* was observed at 21.5% SWC and 13.8% SWC. However, below 10.1% SWC, there was a consistent increase in expression of *TR-ACO3*. These patterns of *TR-ACO3* expression above 13.8% SWC were consistent with the pattern of β -*actin* expression. Therefore, water deficit treatment to *ca.* 10.1% SWC did not alter *TR-ACO3* expression, but prolonged exposure of Tienshan plants to a water deficit lower than 10.1% SWC did result in the increase of expression of *TR-ACO3*.

Exposure of PS-treated Tienshan plants to an early decrease of SWC to 18.7% did not significantly alter the expression of *TR-ACO3* in the second fully-expanded leaves. After this point, a decrease in expression of *TR-ACO3* was observed as the SWC further declined to *ca.* 9.3%. When the SWC decreased below *ca.* 9.3%, there was an increase in the *TR-ACO3* detected in leaves of PS-treated Tienshan which was in common with NPS-treated Tienshan. In these samples, expression of β -*actin* was also used as internal loading control and approximately similar expression of β -*actin* was seen in each sampling point.

Overall these results show that exposure of Tienshan plants to the NPS and the PS treatment to SWCs less than *ca.* 9.3% resulted in an increase in expression of *TR-ACO3* in the second fully-expanded leaves.

Results 1

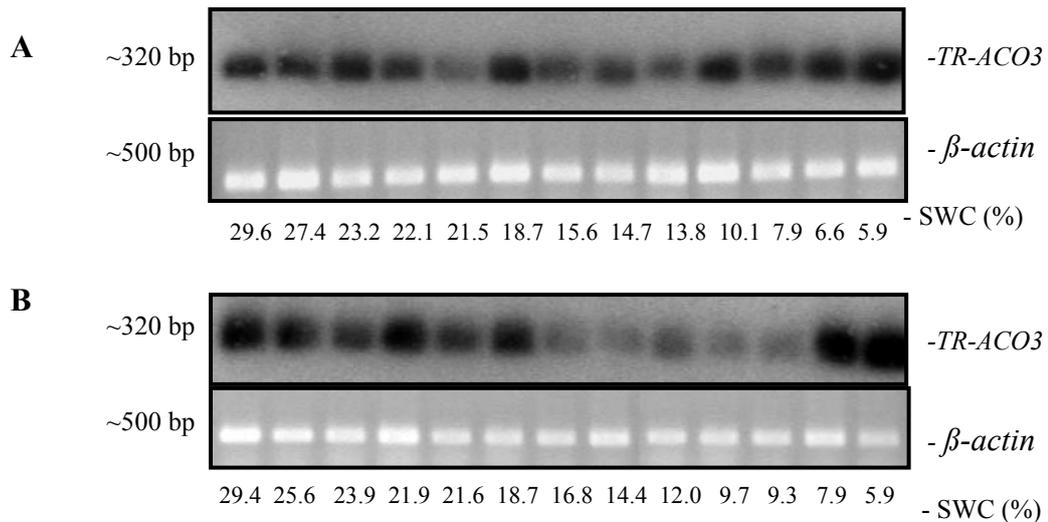


Figure 3.26 Expression of *TR-ACO3* in the second fully-expanded leaves of NPS-treated Tienshan (A) and PS-treated Tienshan (B) revealed by sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO3* and the products probed with a DIG-labelled *TR-ACO3* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate primers to amplify *β-actin* from the same cDNA pool. RT-PCR products were separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

TR-ACO3 expression, using the sqRT-PCR approach as described previously, was also observed in the second fully-expanded leaves of Kopu exposed to NPS and PS treatments (Figure 3.27). In the NPS-treated Kopu, a similar level of *TR-ACO3* expression was observed in leaves harvested from all sampling points regardless of the SWC. In these samples, approximately the same expression of *β-actin* was seen in most samples although slightly less expression was seen in leaves from plants exposed to *ca.* 8% SWC indicating that probably less mRNA was used for the RT-PCR from leaves at *ca.* 8% SWC. These results suggested that there was no change in the expression of *TR-ACO3* in the second fully-expanded leaves of NPS-treated Kopu exposed water deficit above 8% SWC. However a water deficit

Results 1

at 8% SWC induced expression of *TR-ACO3*. In common with NPS-treated Kopu, exposure of Kopu plants to PS treatments to SWC above *ca.* 7.6% did not alter the expression of *TR-ACO3*. However, exposure of Kopu plants to a water deficit to *ca.* 7.6% resulted in increase expression of *TR-ACO3*. Using β -actin expression as an internal loading control, a similar expression of β -actin was seen in most of these sampling point, except for less β -actin expression at *ca.* 7.6% SWC.

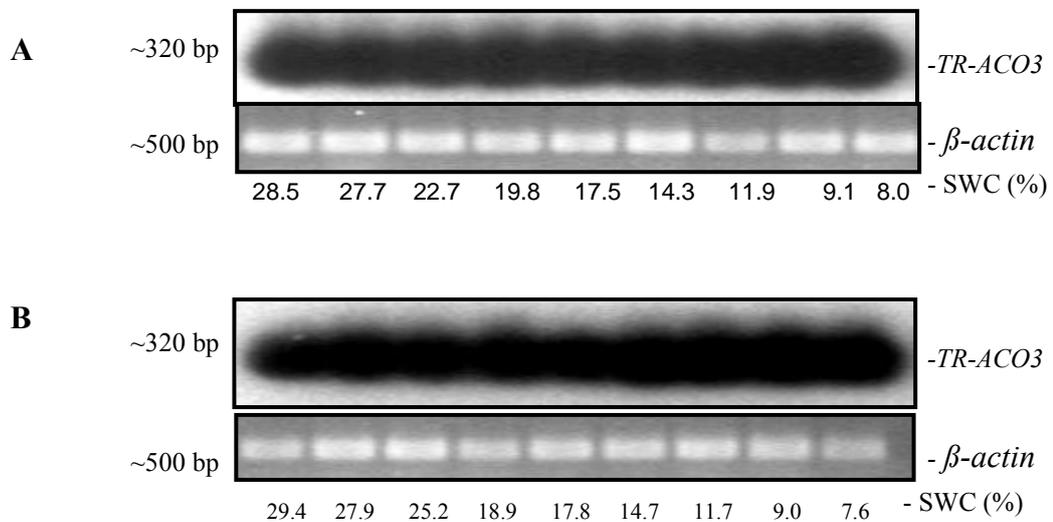


Figure 3.27 Expression of *TR-ACO3* in the second-fully expanded leaves of NPS-treated Kopu (A) and PS-treated Kopu (B) revealed using sqRT-PCR.

RT-PCR was performed using RT-generated cDNA templates from total RNA isolated from the apical structures of white clover harvested at different SWCs, as indicated. One round of PCR was performed using gene specific primers for *TR-ACO3* and the products probed with a DIG-labelled *TR-ACO3* probe (upper panels). Equal loading of cDNA was assessed by RT-PCR using degenerate primers to amplify β -actin from the same cDNA pool. RT-PCR products was separated by electrophoresis and visualised following ethidium bromide staining (lower panels).

Results of expression analysis of *TR-ACO3* in the second fully-expanded leaves of NPS and PS Tienshan and Kopu suggested that exposure of both Tienshan and Kopu plants to water deficit of less than 9% SWC did not alter the expression of

Results 1

TR-ACO3 in the second fully-expanded leaves, but prolonged water deficit treatments to less than 9% SWC induced the expression of *TR-ACO3* in the second fully-expanded leaves of Tienshan and Kopu plants.

4. RESULTS 2:

ISOLATION AND CHARACTERISATION OF ACC SYNTHASE GENES OF WHITE CLOVER

4.1. Isolation of TR-ACS Genes

4.1.1. Sampling Points for Isolation of ACC Synthase Gene Transcripts

Due to low the abundance of ACC synthase transcripts (Yang and Hoffmann, 1984), water-deficit treatment was used to induce the expression of the *TR-ACS* genes in the two white clover varieties used in this thesis. In each variety, isolation of RNA to clone putative water-deficit-associated *ACS* genes was undertaken from five sampling points (based on physiological data, Figures 4.1 and 4.2.).

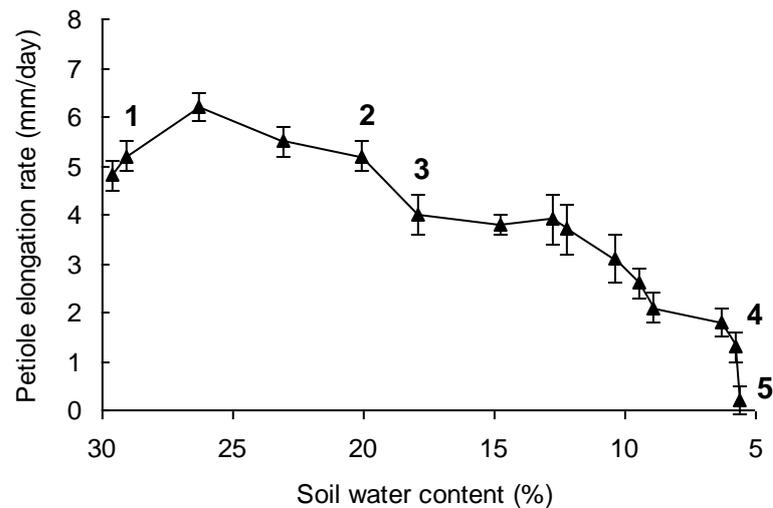


Figure 4.1 Changes in PER of the first-fully expanded leaves of the Tienshan ecotype during the water-deficit treatment.

Each number indicates the sampling points used to isolate RNA to clone putative ACS genes: 1; just after water-deficit imposition, 2; one day before the PER declines, 3: at the time when PER declines, 4: one day before PER ceases, and 5: at the time when PER ceases. $N = 4 \pm SE$.

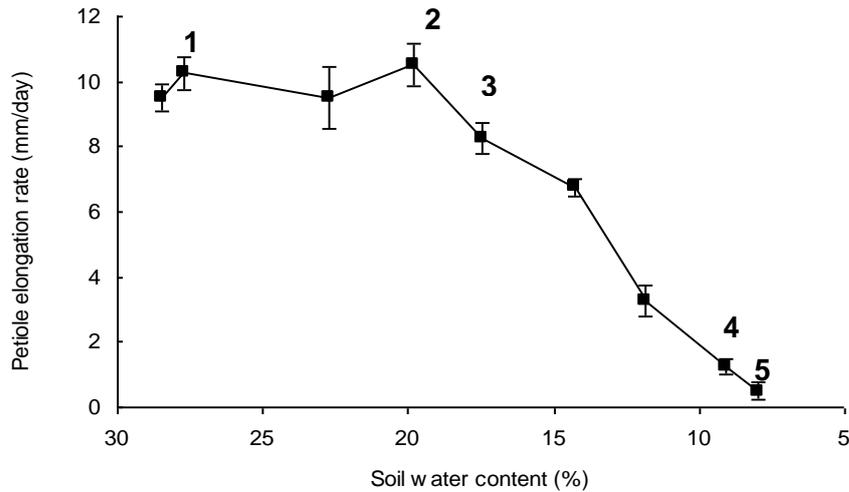


Figure 4.2 Changes in PER of the first-fully expanded leaves of cv. Kopu during water-deficit treatment.

Each number indicates the sampling points used to isolate RNA to clone putative ACS genes: 1; just after water-deficit imposition, 2; one day before the PER declines, 3: at the time when PER declines, 4: one day before PER ceases, and 5: at the time when PER ceases. $N = 4 \pm SE$.

4.1.2. RT-PCR Amplification of Putative ACC Synthase Gene Transcripts

Previously, RT-PCR had been used to isolate and clone three *TR-ACS* genes from the white clover genotype 10F of cultivar Grasslands Challenge (Murray, 2001). Similar procedures were then used in this thesis to isolate orthologues and novel *TR-ACS* genes from the white clover ecotype Tienshan and cultivar Kopu.

Initially, ACC synthase cDNAs were amplified by RT-PCR using nested degenerate oligonucleotide primers (Section 2.5.5.1.) corresponding to conserved sequences within the *ACS* genes (boxes I to VI, Figure 4.3.). The first round primers (ACSR1F and ACSR6R) generated cDNA transcripts of approximately 780 bp from the total cDNA pool, which represented *ACS* transcripts within the conserved boxes I to VI. An aliquot of the first round PCR products were then used as templates for the second round of PCR amplification using ACSR2F and ACSR6R primers. This second round PCR amplified products that corresponded to sequences between conserved boxes II to VI.

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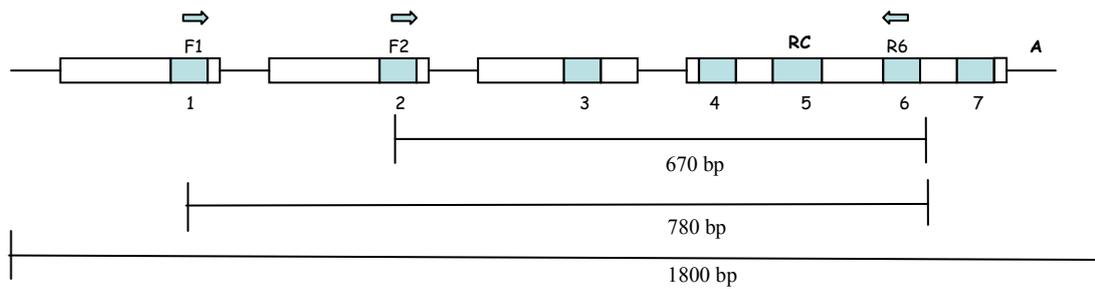


Figure 4.3 Diagrammatic representation of an ACC synthase gene from *Arabidopsis thaliana* (modified from Murray, 2001).

The boxed regions denote the exons and lines denote the introns; conserved regions are denoted by the dark boxes; RC, the reactive centre; A, polyadenylation signal. The position of degenerate primers for the amplification of ACC synthase genes in white clover are shown as: first round forward primer (F1), second round forward primer (F2) and reverse primer (R6).

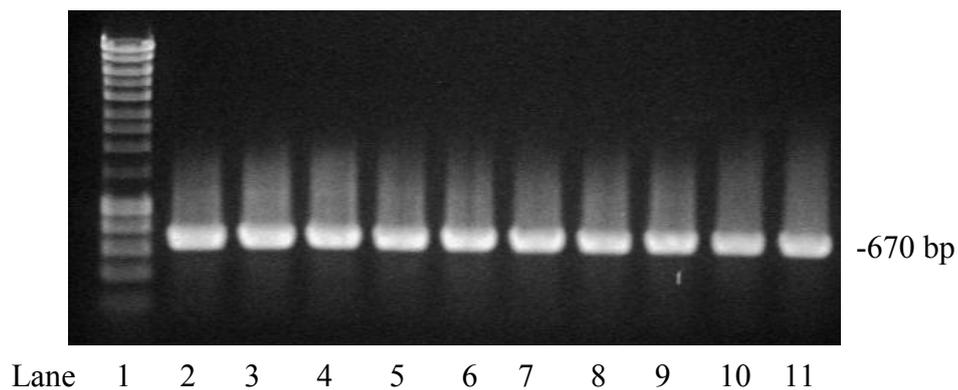


Figure 4.4 RT-PCR using RNA isolated from apical structures and first-fully expanded leaves of the Tienshan ecotype harvested at different soil water contents.

Nested degenerate primers were used for two rounds of PCR amplification. The approximate size of the amplified cDNA is indicated.

Lane 1. DNA Ladder

Lanes 2 to 6. RT-PCR products amplified from RNA isolated from apical structures at *ca.* 28%, 20%, 18%, 8% and 6% SWC respectively.

Lanes 7 to 11. RT-PCR products amplified from RNA isolated from first-fully expanded leaves at *ca.* 28%, 20%, 18 %, 8% and 6% SWC.

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The amplified first round PCR products (*ca.* 780 bp) were unable to be detected after electrophoresis on a 1% (w/v) agarose gel and ethidium bromide staining (data not shown). However, amplified products from the second round PCR (*ca.* 670 bp) were detected and examples are shown as Figure 4.4.

The second round PCR products (Figure 4.4.) were TA-cloned into the pGEM T-easy vector and transformed into the *E. coli* strain DH5 α , and putative inserts detected by blue/white screening (Figure 4.5).

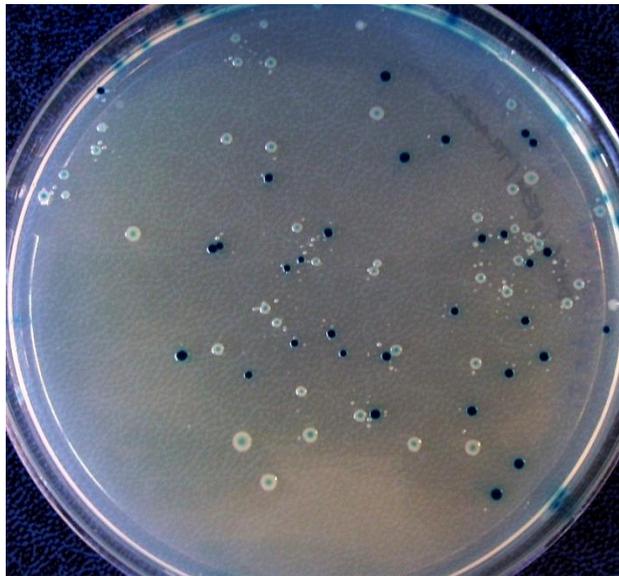


Figure 4.5 White-blue colony selection of sub-libraries generated from the apical structures of the Tienshan ecotype harvested at 8% SWC. Colonies were grown in LB media supplemented with IPTG and X-Gal.

Initially, only white colonies were selected and cultured in LB Amp¹⁰⁰ broth, plasmids isolated (Section 2.5.7.2) and the presence of inserts confirmed by PCR, using M13 primers, and the DNA sequences then obtained. All of the DNA sequences obtained from this protocol belonged to two genes, which have high sequence identity to the *TR-ACS1* and *TR-ACS2* sequences of white clover genotype 10F, Cultivar Grasslands Challenge (Murray, 2001). These results suggested that *TR-ACS3* and any novel *TR-ACS* genes may not occur frequently (or at all) in apical tissues, or they might have been present in the blue or pale blue colonies. Since blue colonies could also contain the insert, as a result of in-frame cloning into the *LacZ*

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gene or the introduction of a mutation during the amplification process, no further blue/white colony selection was carried out.

For subsequent clones, the presence of inserts of approximately 670 bp was determined by PCR from all colonies without selection. To perform this, PCR was carried out directly from the colonies (without prior plasmid isolation) using the second round *ACS* degenerate primer sets (ACSR2F and ACSR6R), prior to separation by 1% (w/v) agarose gel electrophoresis and visualization following ethidium bromide staining (Figure 4.6.A). For further identification and screening, the clones were rescued by sub-culturing onto a new LB Amp¹⁰⁰ plate and numbered. Clones containing inserts were further screened for the presence of *TR-ACS1* or *TR-ACS2*, using gene-specific primers (Section 2.5.5.1.).

Positive clones which were not amplified by the gene-specific *TR-ACS1* or *TR-ACS2* primers could therefore contain either the *TR-ACS3* gene or a novel *TR-ACS* gene not previously identified by Murray (2001). Selections of these are shown in Figure 4.6, and the frequency of the genes isolated is summarised in Tables 4.1 to 4.4.

TR-ACS1 was the predominant clone found in the apical structures and first fully expanded leaves of both the Tienshan ecotype (546 and 641 clones in the apical structures and first-fully expanded leaves, respectively) and the Kopu cultivar (430 and 351 clones in the apical structures and first-fully expanded leaves, respectively). Next was *TR-ACS2* (67 and 133 clones in the apical structures and first-fully expanded leaves of Tienshan, respectively), and 56 and 88 clones in the apical structures and first-fully expanded leaves of Kopu cultivar, respectively. Further, *TR-ACS1* and *TR-ACS2* were found in clones amplified from RNA isolated from both varieties in all SWC conditions (fully-hydrated and water-deficit conditions) (Table 4.1., 4.2., 4.3. and 4.4.).

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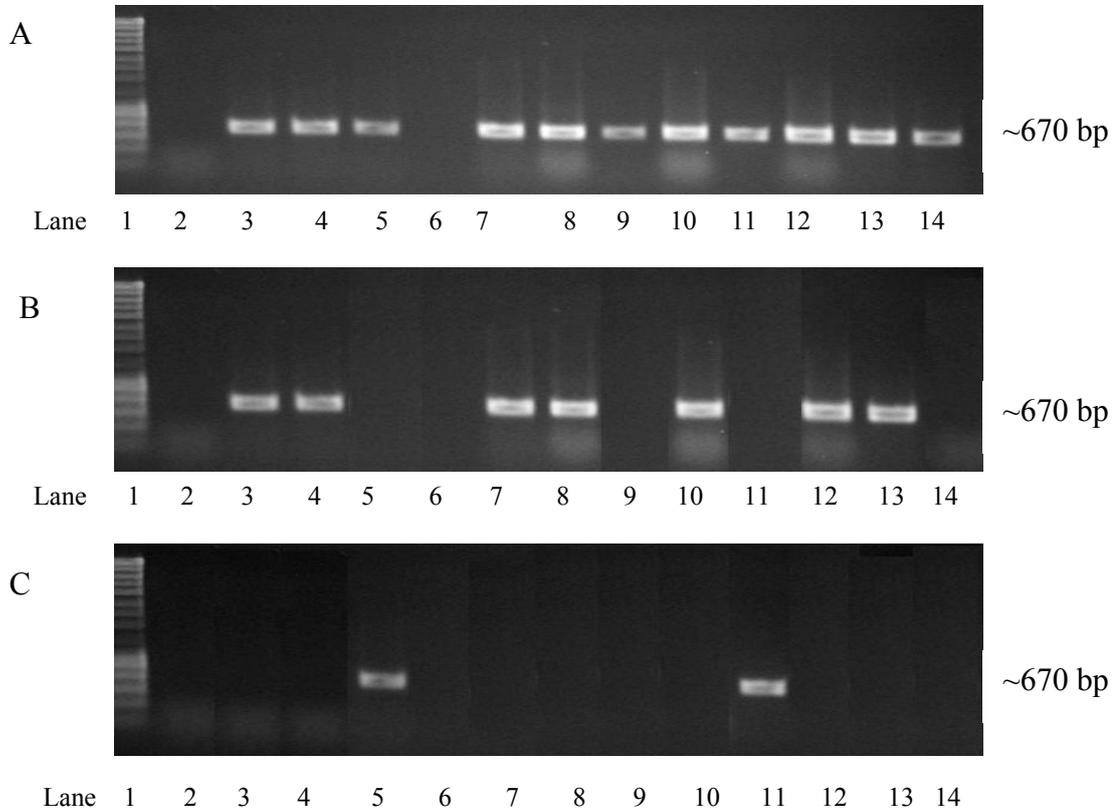


Figure 4.6 PCR selection of putative ACC synthase gene fragments.

A. Amplification of ACC synthase fragments using ACSR2F and ACSR6R as primers from sub-libraries generated from RNA isolated from apical structures of Tienshan ecotype at *ca.* 8% SWC. B. Amplification of *TR-ACS1* from clones positively amplified in plate A using ACS1F and ACSR6R primers. C. Amplification of *TR-ACS2* from clones in plate A using ACS2F and ACSR6R as primers. PCR products were separated on a 1% (w/v) agarose gel and visualised with ethidium bromide. The molecular weights of standards are indicated on the left. The size of PCR products is indicated on the right.

Lane 1. DNA Ladder

Lanes 2 to 14 are amplified products from colonies number 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 respectively.

Table 4.1 Summary of the ACC synthase clones identified by PCR and sequence analysis in the apical structures from plants of the Tienshan ecotype harvested at different soil water contents (SWCs)

% SWC	<i>TR-ACS1</i>	<i>TR-ACS2</i>	<i>TR-ACS3</i>	<i>TR-ACS4</i>	Total clones
28	82	7	-	-	89
20	79	4	-	-	83
18	114	9	-	-	123
8	131	27	1	2	161
6	140	20	-	-	160
Total clones	546	67	1	2	616

Table 4.2 Summary of the ACC synthase clones identified by PCR and sequence analysis in the first-fully-expanded leaves from plants of the Tienshan ecotype harvested at different soil water contents (SWCs)

% SWC	<i>TR-ACS1</i>	<i>TR-ACS2</i>	<i>TR-ACS3</i>	<i>TR-ACS4</i>	Total clones
28	48	22	-	-	70
20	113	31	1	-	145
18	142	22	6	-	170
8	178	19	5	-	201
6	160	39	10	-	209
Total clones	641	133	22	-	796

Table 4.3 Summary of the ACC synthase clones identified by PCR and sequence analysis in the apical structures from plants of cv. Kopu harvested at different soil water contents (SWCs)

% SWC	<i>TR-ACS1</i>	<i>TR-ACS2</i>	<i>TR-ACS3</i>	<i>TR-ACS4</i>	Total clones
28	24	10	-	-	34
22	79	4	-	-	83
18	169	12	-	-	181
11	98	22	-	-	120
8	60	8	-	-	68
Total clones	430	56	-	-	486

Table 4.4 Summary of the ACC synthase clones identified by PCR and sequence analysis in the first-fully expanded leaves from plants of cv. Kopu harvested at different soil water contents (SWCs)

% SWC	<i>TR-ACSI</i>	<i>TR-ACS2</i>	<i>TR-ACS3</i>	<i>TR-ACS4</i>	Total clones
28	24	10	-	-	34
22	57	20	1	-	78
18	92	14	2	-	108
11	62	9	2	-	73
8	80	22	4	-	106
Total clones	351	88	9	-	448

TR-ACS3 was represented by thirty two clones: twenty three were amplified from the Tienshan ecotype and nine from the Kopu cultivar. In both varieties, all *TR-ACS3* genes were amplified from RNA isolated from water-deficit treatments, and most of them were isolated from first-fully expanded leaves (twenty two and nine clones from Tienshan ecotype and Kopu cultivar, respectively). Only one clone was amplified from the apical structures of Tienshan from RNA isolated at *ca.* 8% SWC (just before the PER ceased).

Out of 2346 clones isolated, only two were different with respect to the known *TR-ACSI*, *TR-ACS2* and *TR-ACS3* genes. Both clones were amplified from RNA isolated from apical structures of the Tienshan ecotype harvested at *ca.* 8% of SWC (Table 3.1). After alignment of the sequences (Section 4.1.3), it was confirmed that these two clones represent a novel *TR-ACS* gene, which is designated *TR-ACS4*.

4.1.3. Confirmation of Putative ACC Synthase Gene Transcripts by Sequence Analysis

A total of sixty clones were then subjected to DNA sequencing: twenty that were amplified using *TR-ACSI* and *TR-ACS2* primers, and forty clones that were not amplified with the *TR-ACSI* and *TR-ACS2* primers (and so they could be *TR-ACS3* or *TR-ACS4*). The BLAST analysis in GeneBank indicated that all of these sequences displayed high sequence homology to ACC synthase gene sequences. The sequences obtained were aligned using ClustalW, which revealed that all of the sequences could

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be separated into four groups with each of the sequences within a group showing greater than 98% homology.

The consensus sequences between these groups were generated and then compared for homology at the nucleotide level, and identity at the amino acid level (Table 4.5). The four cDNA sequences share between 60% to 70% nucleotide homology. The low homology between these four cDNA sequences suggested that the four groups represented four distinct ACC synthase cDNAs, and so the genes from Tienshan ecotype were designated, for the purposes of this thesis, as *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T* and *TR-ACS4-T*.

TR-ACS1-T shared the highest nucleotide homology and amino acid identity with *TR-ACS3-T* (70% and 65%, respectively), while *TR-ACS2-T* shared the highest nucleotide homology (64%) and amino acid identity (59 %) with *TR-ACS4-T*. The nucleotide homology and amino acid identity among all others *TR-ACS* genes ranged between 60 to 64% and 50 to 56%, respectively (Table 4.5).

Table 4.5 Comparison of the percentage of nucleotide homology and percentage of amino acid identity (in parenthesis) between the four ACC synthase consensus sequences amplified from tissues of the Tienshan ecotype by RT-PCR

	<i>TR-ACS1-T</i>	<i>TR-ACS2-T</i>	<i>TR-ACS3-T</i>	<i>TR-ACS4-T</i>
<i>TR-ACS1-T</i>	-	61 (50)	70 (65)	64 (56)
<i>TR-ACS2-T</i>	61 (50)	-	60 (55)	64 (59)
<i>TR-ACS3-T</i>	70 (65)	60 (55)	-	63 (55)
<i>TR-ACS4-T</i>	64 (56)	64 (59)	63 (55)	-

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ACSR2F CTGGATCCGTWYCARGAYTAYCAYGG

```

TR-ACS2-T TCTCCTTCATTCAAACAAGCATTTGGTAGATTTTCATGGCCGAGATCAGAGGAAACCGAGT 60
TR-ACS4-T ACTTAAGTCATTTAGAAAAGCAATGGCAAGTTTCATGGAAAAATAAGAGGAAATAAAGC 60
TR-ACS1-T TCTACCAGAGTTTCAGAAATGCTGTGGCTAAATCCATGTCTAGAACAAGAGGAAACAGAGT 60
TR-ACS3-T ATTGCCAGAATTCAGAAATGCTGTGGCAAATTTTCATGTCAAAAGTGAGAGGTGGTAGGGT 60
          *      ** * * * ** ***      * ****          *****      *

TR-ACS2-T TTCCTTTGATC-CCAACCATATGTTCTCACCGC-GGT-CTACTTCCGCAAACGAGACTCT 117
TR-ACS4-T AAAATTTGATTATGAAAGAATTGTTGTAAGTCTGTTGCTACTGCTGCAATGAACCTCT 120
TR-ACS1-T TACCTTTGATCCTGATCGTATTGTCATGAGTGGTGGAGCAACTGGAGCACATGAGGTTAC 120
TR-ACS3-T AAGATTTGATCCTGACCGTATATTGATGAGTGGTGGAGCAACAGGGGCAAATGAATTAAT 120
          *****      *      * * * * * ** * **      ** * **

TR-ACS2-T TATGTTTTGTCTCGCTGAGAAAGGAGAAGCATTTCCTTCTACTCCTTACTATCCAGG 177
TR-ACS4-T AACCTTCATTCTTGCAAATCCAGGAGATGCTTTACTTGTTC AACACCATACTATCCAGG 180
TR-ACS1-T TGCCTTTTGTGGCAGATCCTGGTGTGCTTTTTTGGTACCTACTCCTTACTATCCAGG 180
TR-ACS3-T CATGTTCTGTTGGCTGGTCTGGTGTGCTTTTTTGGTTCCTAGCCCTTATTATCCAGC 180
          **      * * * *      ** * * * * * * * * * * ** * * *****

TR-ACS2-T GATTTGTAGAGATCTTAAATGGAGAACCGGTGTTGAGATTGTACCAATACAATGCAATAG 237
TR-ACS4-T ATTTGATAGAGATTTAAGATGGAGAACCGGAGTAAACATAGTTCCGATCCATTGCGACAG 240
TR-ACS1-T TTTGATCGAGATTTGAGATGGAGAACAGTAGTTAAACTTGTTCGGTTATATGCGAAAG 240
TR-ACS3-T ATTTGTTGTTGATTTGTGTTGGATAACCGGTGTGCAACTAATTCCTGTCCAATGTCATAG 240
          *      * * * * * *      ***** * * * * * * * * * * ** * * *

TR-ACS2-T GTCCAACAACCTTTCAAATAACTGAACAAGCACTGCAACAAGCATAACAAGATGCACAAGA 297
TR-ACS4-T CTCRAACAATTTCAAATCACACTTGAAGCATTAGAACTGCATACAAAAATGCAGAATC 300
TR-ACS1-T CGCGAACAATTTCAAATTAACAAGACAAGCTTTGGAAGAAGCATATGAAAAAGCCAAAAT 300
TR-ACS3-T CTCAAACAATTTCAAGATAACAAGAGAAGCACTTGAAGAAGYTTATWTGAAAGCACAAGA 300
          * ***** ** * * * *      ***** * * * * * * * * * *

TR-ACS2-T CCGCAACCTTAAAGTCAAAGGAGTAATGGTTACAAACCCGTCAAACCCGTTAGGCACCAC 357
TR-ACS4-T AATGAACATGAAAGTAAAAGCAGTACTTATAACCAACCCATCGAACCCGTTAGGCATATC 360
TR-ACS1-T TGATAACATCAGAATAAAAGGTTTACTCATAACAAATCCTTCAAATCCATTAGGCACAGT 360
TR-ACS3-T AAGAAACATCAATGTGAAAGGGTTAATCATAACAAATCCATCAAACCCCTCTAGGAACAAC 360
          *** * *      * ***** ** * * * * * * * * * * ***** *

TR-ACS2-T ATTGTCAAGGAGTGAATTAATCTTCTCGTTGACTTTATTGAAGAAAACAAAACATGCA 417
TR-ACS4-T GATTC AACGTT CAGTTCTCGAGGACCTTCTCGACTTTGT---GACTCRCAAGAACATACA 417
TR-ACS1-T TATGGACAGAACCAAATTA AAAACCGTTGTAAATTTTCAT---CAACGAAAAGCGTATTCA 417
TR-ACS3-T AATAGAAAAGAAACACTAAAGAGCATAGTTAGTTTCAT---AATGAAAACAACATTCA 417
          *      * *      * *      ** *      ** ** * *

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TR-ACS2-T   TTTG-ATAAGCGACGAGATTTACTCCGGGACTGTTTTTTTCTCTCCAAGTTTATCAGTG 476
TR-ACS4-T   TCTT-GTCTCGGACGAAATCTACTCAGGCTCGGTTTTTCTCTCACACGAATTCATAAGCG 476
TR-ACS1-T   TCTATGGACACAACCACAT-TACCGTTGTCCGGTTTTTAGCCAACCAATTTTCATAAGTA 476
TR-ACS3-T   TTTA-GTGTGTGACGAAATCTATTCGGGCACAGTTTTCAACACTCCGAAATACGTAAGTG 476
          * *           ** * ** ** * * * * * * * * * * * * * *
          * *           ** * ** ** * * * * * * * * * * * * * *

TR-ACS2-T   TTATGGAAATCCTTAACGAAAGAAATGACCTTCAGGATTTCAAATACACTGATAATATTT 536
TR-ACS4-T   TGGCGGAGATTCTG-----GAGTCTCGTCAATACAAAGACGCGGA----- 516
TR-ACS1-T   TAGCTGAGATCATAGAACAAGAAACAGACATTGAATGTGACCGTAACCTCG----- 527
TR-ACS3-T   TCGCCGAAGTTATA---CAAGAAATGGAA---GAATGCAAAAAAGACCTCA----- 521
          *   ** * *           **           **
          *   ** * *           **           **

TR-ACS2-T   GCGAGAGAGTTCATGTGTCTATAGTCTTTCCAAAGACTTGGGTTTGCCAGGTTTCCGCG 596
TR-ACS4-T   ----AAGAGTTCACATTTGTTTATAGTCTTTCAAAGATCTCGGTCTACCTGGTTTTAGAG 572
TR-ACS1-T   -----TTCACATAGTTTACAGTCTTTCAAAGATATGGGATTTCCCGGTTTTAGAG 578
TR-ACS3-T   -----TTCATATCATATATAGTTTATCAAAGACATGGGACTTCCGGGTTTCAGAG 572
          **** * * ** ** * ** * * * * * * * * * * * * * *

TR-ACS2-T   TTGGTGCACTTTACTCCGAAAACGATGAAGTTGT--CGAGCAACCAAC--- 642
TR-ACS4-T   TCGGTACAATTTATTCGTACAACGATAAAGTTGTTACAACCGCTCGAAGA- 622
TR-ACS1-T   TCGGTATAATTTACTCATAACAATGATACCGTTGTTAACTGCGCGCGCAAAC 629
TR-ACS3-T   TCGGTTTAGTTTATTCGTACAATGATGAAGTTGTGAATTGCGGTGCAAAA- 622
          * ** * * * * * * * * * * * * * * * * * * * * * * * *

ACSR6R CTCAAGCTTARNZYRAARCTNGACAT

```

Figure 4.7 Alignment of coding frame region of *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T*, and *TR-ACS4-T* consensus sequences isolated from the Tianshan ecotype.

(*) represents identical base (-) represents no sequence. Degenerate primer sequences are underlined and gene specific primer sequences are shaded.

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Identification of ACS protein family using the NCBI reference protein sequences

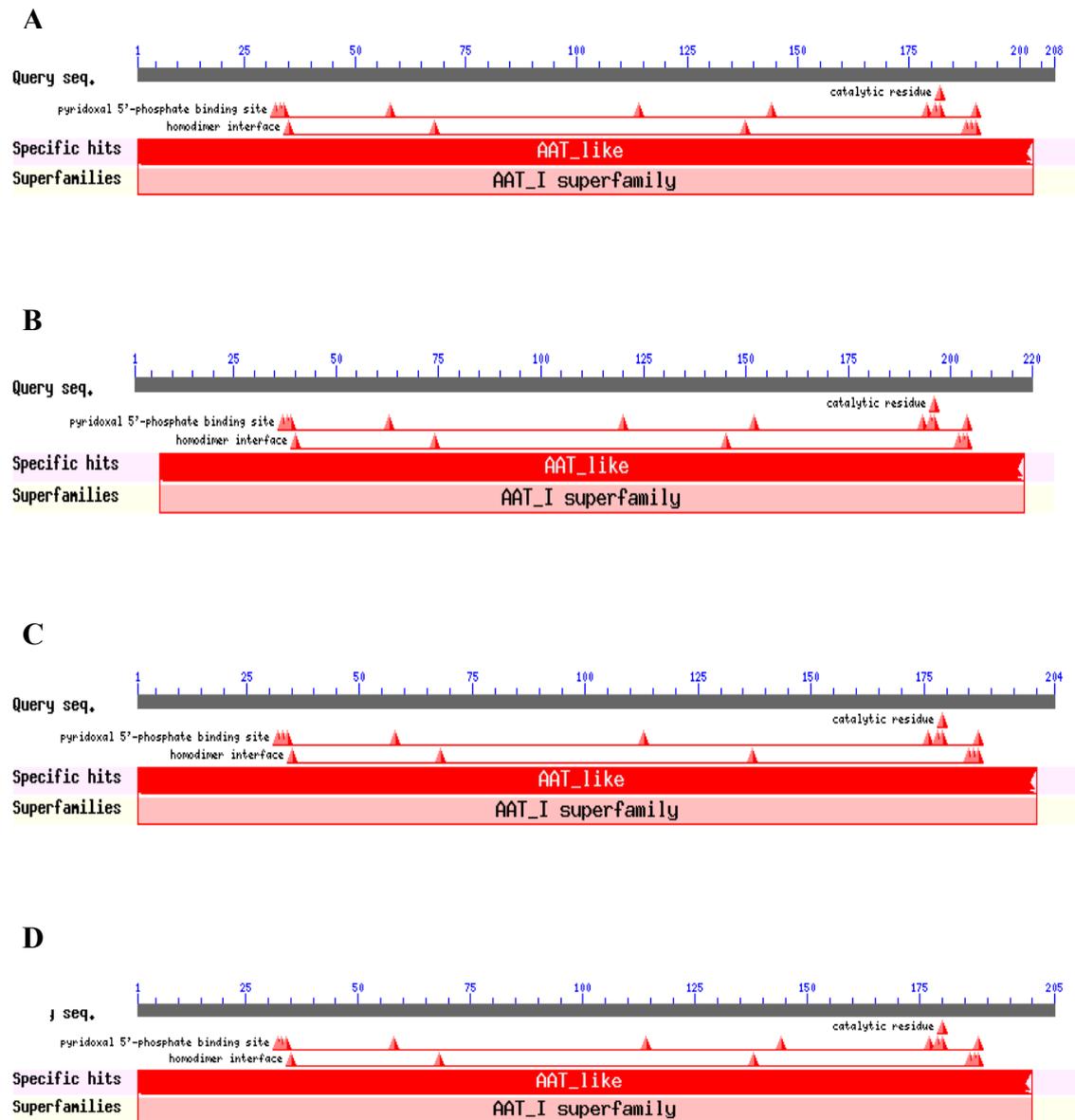


Figure 4.8 Conserved amino acid residues detected in the TR-ACS1-T (A), TR-ACS2-T (B), TR-ACS3-T (C) and TR-ACS4-T (D) when compared to an aminotransferase-I enzyme super family in the NCBI database.

Ten of the 11 amino acid residues required for the binding of the substrate to pyridoxal-5'-phosphate (PLP) are present in the TR-ACS1-T, TR-ACS2-T and TR-ACS4-T and 9 of these are present in the TR-ACS3-T.

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The consensus sequences of these four ACC synthase gene and their derived amino acid sequences were aligned (Figures 4.7 and 4.9). The partial coding regions consist of between 622 to 642 bp, and sequence comparison of their derived amino acid sequences reveal that the four ACC synthase proteins are similar in the conserved domain three, four, five and six. BLAST analysis of the NCBI reference protein sequences (Figure 4.8.) suggested that all these sequences contain the conserved catalytic amino acid residue in the active site (lysine correspond to position 182 or L¹⁸² in TR-ACS1-T). ACS belongs to the Aspartate aminotransferase enzyme family and this enzyme requires pyridoxal-5'-phosphate (PLP) as a cofactor (Metha *et. al*, 1999). The four ACS proteins isolated from Tienshan ecotype contained 8 or 9 (out of 11 known) conserved amino acid residues involved in the binding of substrate to pyridoxal 5'phosphate (Figure 4.8).

As can be seen from Figure 4.9, TR-ACS1-T, TR-ACS2-T and TR-ACS4-T contain ten of these amino acid residues while TR-ACS3-T contains nine of these residues. In the TR-ACS1-T, these include four conserved amino acid residues in conserved domain III (A⁴⁵, N⁴⁶, E⁴⁷ and R⁶²), P in position 111 (P¹¹¹) in conserved domain IV, one conserved amino acid residue in conserved domain V (D¹⁴³), and four conserved residues in domain VI which are in the reactive site of the gene (H¹⁷⁷, Y¹⁸⁰, L¹⁸² and G¹⁹⁰). In addition, the 20 amino acid residues in conserved domain VI, which serve as the active site of the enzyme, are all present and highly conserved in the TR-ACS1-T, TR-ACS2-T, TR-ACS3-T and TR-ACS4-T sequences. This suggests that the four ACC synthase genes all encode for functional enzymes.

The GeneBank database was searched using the consensus sequences of *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T* and *TR-ACS4-T* (Table 4.6.). The database sequences with the highest identity to *TR-ACS1-T* were *MtACS* isolated from *Medicago truncatula* (accession number: AY062022.1), an *ACS* isolated from *Cicer arietinum* seeds (accession number: DQ372685.1), *Ps-ACS2* isolated from IAA-treated apical hooks of *Pisum sativum* (accession number: AF016459.1), and an *ACS* isolated from leaves of *Glycine max* (accession number: DQ273841.1).

TR-ACS2-T shares highest identity to *VRACS6*, isolated from etiolated mungbean (*Vigna radiata*) seedlings treated with auxin (accession numbers: U34986.1, AB000679.1 and AB018355.1). The next three sequences from the GeneBank

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database that share highest identity to *TR-ACS2-T* are: *ACS5* isolated from etiolated seedlings of *Arabidopsis thaliana* (accession numbers AK229087.1, NM_125977.2 and AF334729.1); an *ACS* isolated from young leaves of *Brassica rapa* (accession number: AC189266.1) and *ACS4* isolated from *Sinapsis arvensis* (accession number: AF074930.1). *TR-ACS3-T* shares highest identity to an *ACS* from stored pea pods (accession number AB049725.1), an *ACS* isolated from barrel medic (accession numbers: AC157377.6 and AC186677.3) and an *ACS* isolated from white lupin seedlings treated with IAA and wounding.

The novel ACC synthase gene, *TR-ACS4-T*, has a wide range of identity to other *ACS* genes in the GeneBank database, including those isolated from many different tissues, ranging from the seed, hypocotyl, leaf, stem, flower to the fruits. It has also high identity to wounding, auxin and ethylene associated *ACS* genes. For example, *TR-ACS4-T* has the highest identity to an *ACS1* isolated from developing seeds of Damson plum (*Prunus domestica*; accession number AJ890088.1). It also shares high identity to *ACS* isolated from auxin-treated flower stem tissue of snapdragon (*Anthirrhium majus*; accession number AF083815.2), *pMACS4* and *pMACS5* isolated from mungbean hypocotyl (*Vigna radiate*: accession numbers Z12134 and Z12135.1), the auxin- and ethylene-induced *ACS3* from the petals of carnation (*Dianthus caryophyllus*: accession number AF049137.1), the wounding- and auxin-responsive *CS-ACS3* isolated from female flower apices of cucumber (*Cucumis sativus*: accession numbers AB003683 and AB006805), *CMe-ACS3* isolated from melon seedlings (*Cucumis melo*: accession number D86241) and a wounding-induced *ACS* from *Cucurbita maxima* (accession number: D01033). Together these identities suggest that the *TR-ACS4-T* gene is expressed in a developmental manner but could also be a stress- and hormone-related *ACS* in white clover.

Table 4.6 GeneBank Comparison of *TR-ACS-T* Sequences

Genes	Plants	Tissue type (Accession number)
<i>TR-ACS1-T</i>	Barrel medic (<i>Medicago truncatula</i>)	No information (AY062022.1)
	Chickpea (<i>Cicer arietinum</i>)	Seeds (DQ372685.1)
	Pea (<i>Pisum sativum</i>)	IAA-treated apical hooks (AF016459.1)
	Soybean (<i>Glycin max</i>)	Leaves (DQ273841.1)
<i>TR-ACS2-T</i>	Mungbean (<i>Vigna radiata</i>)	Etiolated hypocotyl (U34986.1, AB0006791.1)
	Arabidopsis (<i>Arabidopsis thaliana</i>)	Etiolated seedling (AK2290807.1, NM_125977.2 and AF334729.1)
	(<i>Brassica rapa</i>)	Young leaves (AC189266.1)
	Sinapsis arvensis	No information (AF074930.1)
<i>TR-ACS3-T</i>	Pea (<i>Pisum sativum</i>)	Stored pods (AB049725.1)
	Barrel medic (<i>Medicago trunculata</i>)	No information (AC157377.6 and AC186677.3)
	White lupin (<i>Lupinus albus</i>)	IAA and wound-treated seedling (AF119411.1)
<i>TR-ACS4-T</i>	Barrel medic (<i>Medicago trunculata</i>)	No information (AC150703.43)
	Damson plum (<i>Prunus domestica</i>)	Developing seeds (AJ890088.1)
	Snapdragon (<i>Anthirrhium majus</i>)	Auxin treated flowers stems (AF083815.2)
	Mungbean (<i>Vigna radiata</i>)	Hypocotyl (Z12134.1, M94863.1 and Z12135.1)

Table 4.6 GeneBank Comparison of *TR-ACS-T* Sequences (continued)

<i>Genes</i>	<i>Plants</i>	<i>Tissue type (Accession number)</i>
	Carnation (<i>Dianthus caryophyllus</i>)	Auxin and ethylene treated petals (AF049137.1)
	Cucumber (<i>Cucumis sativus</i>)	Wounding and auxin treated female flower apices (accession numbers AB003683 and AB006805)
	Melon (<i>Cucumis melo</i>)	Etiolated seedlings (D86241.1)
	Winter squash (<i>Cucurbita maxima</i>)	Stem (D01033.1)

4.1.4. Comparison of ACC Synthase Genes Obtained from the Tienshan Ecotype and from Genotype 10-F of Grasslands Challenge Cultivar

The sequences of the three *TR-ACS* genes; *TR-ACS1*, *TR-ACS2*, and *TR-ACS3*; isolated from the Tienshan ecotype (designated *TR-ACS-T*), as reported in this thesis, and those previously isolated from genotype 10-F (designated *TR-ACS-10F*) were compared for identity at the nucleotide and amino acid level. The *TR-ACS4-T* sequence was not compared as it is the novel *TR-ACS* gene and its sequence has not yet been reported previously from other white clover varieties.

The alignment comparisons between nucleotide and amino acid sequences of *TR-ACS1-T* and *TR-ACS1-10F* and *TR-ACS2-T* and *TR-ACS2-10F* are shown in Appendices XII and XIII. The partial sequences of the two *TR-ACS1* genes consist of 629 bp or 210 amino acids in the reading frame, and they share 96% identity at nucleotide level and 93% amino acid identity. The two *TR-ACS1* genes share high identity in the conserved regions and both have the conserved amino acid residues (eight in *TR-ACS1-T* and nine in *TR-ACS1-10F*) required for the binding of the substrate to PLP. For the nucleotide and deduced amino acid sequences of the *TR-ACS2* genes, the partial sequence comparison shows that *TR-ACS2-T* and *TR-ACS2-10F* consist of a 642 and 645bp coding region, respectively, and they share 98% homology at the nucleotide level and 92% amino acid identity (Appendices XIV and XV).

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In common with the *TR-ACS1* and *TR-ACS2* genes, both *TR-ACS3* sequences comprise the partial coding frame region within box I to VI. The coding frame sequence of *TR-ACS3-T* comprises 622 bp nucleotides which is approximately the same length as *TR-ACS1-T*, *TR-ACS2-T* and *TR-ACS4-T*. However the *TR-ACS3-10F* sequence only comprises 562 bp which is 60 bp smaller and translates into 20 missing amino acids (Figures 4.10 and 4.11). The aligned nucleotide and amino acid sequence of these two *TR-ACS3* show that the *TR-ACS3-10F* sequence is missing 20 of the amino acids that occur in conserved domain six (the reactive site of the enzyme). This reactive site is present in *TR-ACS3-T* and in the three other *TR-ACS-T* proteins (see Section 4.1.2.). Deletion of these 20 aa from *TR-ACS3-10F* means that only 6 out of 11 (3 of these, including the catalytic lysine residue, are in domain VI) of the conserved amino acid residues that are required for the binding of pyridoxal 5'phosphate are present (Metha *et. al.*, 1989). With the exception of the deleted 20 aa, identity values of the two *TR-ACS3* genes are very high (more than 90%). Therefore, *TR-ACS3-T* and *TR-ACS3-10F* represent the same isoform, but only *TR-ACS3-T* occurs as an active ACC synthase.

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TR-ACS3-T   ATTGCCAGAATTCAGAAATGCTGTGGCAAATTTTCATGTCAAAAAGTGAGAGGTGGTAGGGT 60
TR-ACS-10F  ATTGCCAGAATTCAGAAATGCTGTGGCAAATTTTCATGTCAAAAAGTGAGAGGTGGTAGGGT 60
*****

TR-ACS3-T   AAGATTTGATCCTGACCGTATATTGATGAGTGGTGGAGCAACAGGGGCAAATGAATTAAT 120
TR-ACS-10F  AAGATTTGATCCTGACCGTATATTGATGAGTGGTGGAGCAACAGGGGCAAATGAATTAAT 120
*****

TR-ACS3-T   CATGTTCTGTTTGGCTGGTCCTGGTGTGCCTTTTTGGTTCCTAGCCCTTATTATCCAGC 180
TR-ACS-10F  CATGTTCTGTTTGGCTGTATCCTGGTGTGCCTTTTTGGTTCCTAGCCCTTATTATCCAGC 180
*****

TR-ACS3-T   ATTTGTTTCGTGATTTGTGTTGGARAACCGGTGTGCAACTAATTCCTGTCCAATGTCATAG 240
TR-ACS-10F  ATTTGTTTCGTGATTTGTGTTGGAGAACCGGTGTGCAACTAATTCCTGTCCAATGTCATAG 240
*****

TR-ACS3-T   CTCAAACAATTTCAAGATAACAAGAGAAGCACTTGAAGAAGYTTATWTGAAAGCACAAGA 300
TR-ACS-10F  CTCAAACAATTTCAAGATAACAAGAGAAGCACTTGAAGAAGCTTATATGAAAGCACAAGA 300
*****

TR-ACS3-T   AAGAAACATCAATGTGAAAGGGTTAATCATAACAAATCCATCAAACCCTCTAGGAACAAC 360
TR-ACS-10F  AAGAAACATCAATGTGAAAGGGTTAATCATAACAAATCCATCAAACCCTCTAGGAACAAC 360
*****

TR-ACS3-T   AATAGAAAAAGAAACACTAAAGAGCATAGTTAGTTTCATAAATGAAAACAACATTCATTT 420
TR-ACS-10F  AATAGAAAAAGAAACACTAAAGAGCATAGATAGTTTCATAAATGAAAACAACATTCATTT 420
*****

TR-ACS3-T   AGTGTGTGACGAAATCTATTCCGGCACAGTTTTCAACACTCCGAAATACGTAAGTGTGCG 480
TR-ACS-10F  AGTGTGTGATGAAATCTATTCCGGCACAGTTTTCGACACTCCGAAATACGTAAGTGTGCG 480
*****

TR-ACS3-T   CGAAGTTATAACAAGAAATGGAAGAATGCAAAAAAGACCTCATTTCATATCATATATAGTTT 540
TR-ACS-10F  CGAAGTTATAACAAGAAATGGAAGAATGCAAAAAAGACCTCATTTCATAT----- 528
*****

TR-ACS3-T   ATCAAAGACATGGGACTTCCGGGTTTCAGAGTCGGTTTAGTTTATTCGTACAATGATGA 600
TR-ACS-10F  -----GTACAATGATGA 540
*****

TR-ACS3-T   AGTTGTGAATTGCGGTCGAAAA 622
TR-ACS-10F  AGTTGTGAATTGCGGTCGAAAA 562
*****

```

Figure 4.10 Alignment of nucleotide sequences of the *TR-ACS3* genes isolated from the Tienshan ecotype (*TR-ACS3-T*) (this thesis) and that previously isolated from genotype 10-F of the cultivar Grasslands Challenge (*TR-ACS3-10F*, as reported by Murray and McManus, 2005). (*) represents identical sequence, (-) represents no sequence.

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Figure 4.11 Alignment of deduced amino acid sequences of the TR-ACS3 gene isolated from the Tianshan ecotype (TR-ACS3-T) and the TR-ACS3 sequence previously identified from genotype 10-F of the cultivar Grasslands Challenge (TR-ACS3-10F, as reported by Murray and McManus, 2005).

(*) represents identical residue, (.) represents semi-conserved substitution sequence; (:) represents conserved substitution sequence (-) represents no sequence; and (•) represents conserved residues found in aminotransferase and other ACC synthases while boxed-*italics* represent the catalytic residue of the enzyme. Shaded, numbered sequences represent the conserved regions of ACC synthase and shaded underlined sequences represent the active site of the enzyme.

The high identity at the nucleotide and amino acid levels confirmed that the RT-PCR based gene cloning successfully generated ACC synthase genes from the Tianshan ecotype. The identity values of these six *TR-ACS* genes are summarized in Table 4.7.

Table 4.7 Nucleotide homology and amino acid identity values of six ACC synthase genes identified in the Tienshan ecotype and genotype 10F of the cultivar Grasslands Challenge

Nucleotide level	<i>TR-ACS1-T</i>	<i>TR-ACS2-T</i>	<i>TR-ACS3-T</i>
<i>TR-ACS1-10F</i>	96		
<i>TR-AC2-10F</i>		96	
<i>TR-ACS3-10F</i>			92
Amino Acid Level	TR-ACS1-T	TR-ACS2-T	TR-ACS3-T
TR-ACS1-10F	93		
TR-AC2-10F		82	
TR-ACS3-10F			97

4.1.5. Phylogenetic Analysis of *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T* and *TR-ACS4-T*

A phylogenetic tree was constructed, using ClustalW, from the alignment of derived amino acid sequences of TR-ACS1-T, TR-ACS2-T, TR-ACS3-T and TR-ACS4-T with twenty other ACC synthase sequences obtained from GenBank database that have high identity with the ACC synthase genes from white clover (Figure 4.12).

The four ACC synthase genes isolated from white clover were more closely related to other ACC synthase sequences in the phylogenetic tree rather than to each other. The TR-ACS1-T was most closely related to ACC synthases isolated from barrel medic (accession number AAL35745.1), chickpea seeds (accession number ABD16181) and an ACC synthase isolated from IAA-treated etiolated pea seedlings (AAD04199).

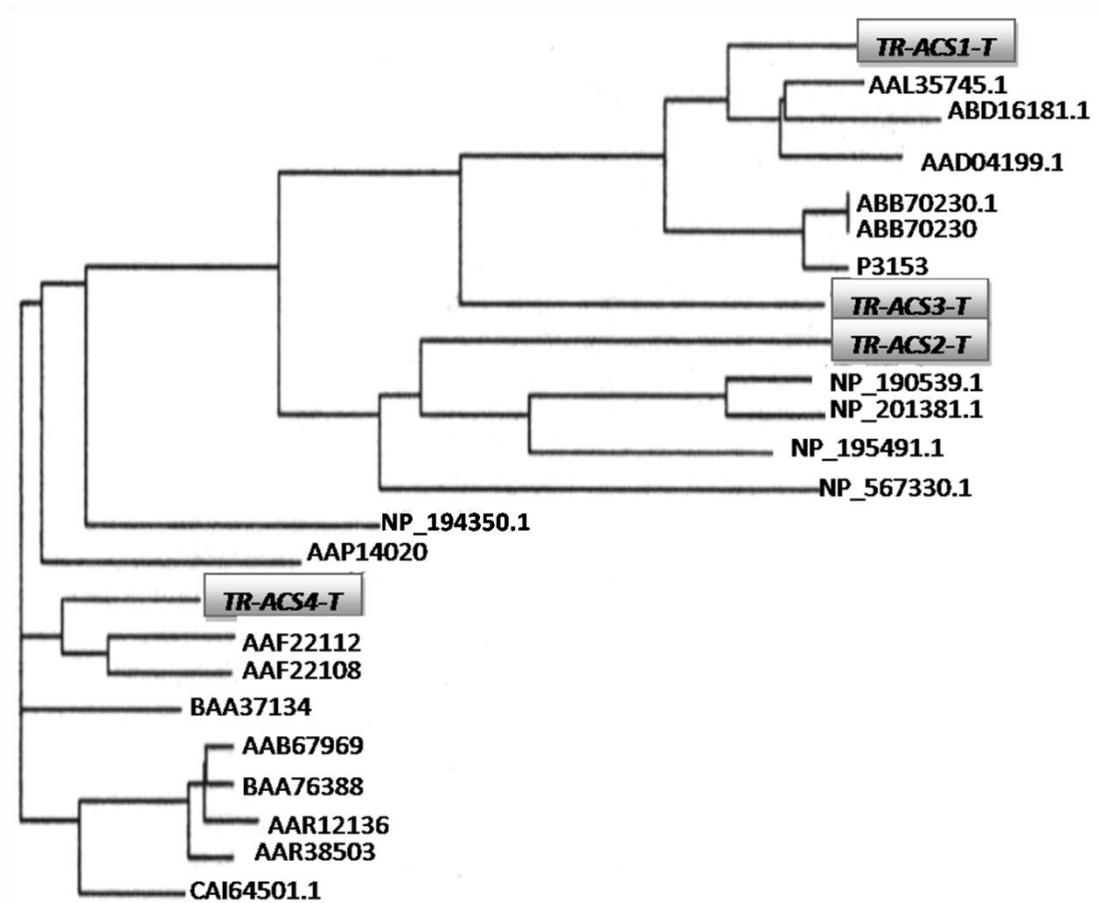


Figure 4.12 Amino acid sequence based phylogeny of the four ACC synthase family.

The phylogenetic tree was generated using ClustalW and constructed from alignment of deduced amino acid sequences of TR-ACS1-T, TR-ACS2-T, TR-ACS3-T and TR-ACS4-T with amino acid sequences of other ACC synthases from the GenBank database. The accession number of each sequence is displayed and the details of the genes related to these accession numbers can be found in Appendix XIV.

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The neighbouring sequences of TR-ACS2-T were the four of type-2 ACC synthase proteins from Arabidopsis including the ETO3 (ETHYLENE OVERPRODUCING) ACC synthase (accession number NP_190539.1) or ACS9, ACS5 (accession number NP_201381.1), ACS8 (accession number NP_195491), ACS11 (accession number NP_567330.1) and ACS7 (accession number NP_194350.1). Interestingly, there was no neighboring sequence to TR-ACS3-T seen in the phylogenetic tree. The TR-ACS4-T was most closely related to ACS5 and ACS4 isolated from IAA- and wound-treated white lupin seedlings (accession numbers AAF221112 and AAF22108) and an ACC synthase protein from ripening fruit of *Passiflora edulis* (accession number BAA37134).

4.2. Expression analysis of the *TR-ACS4-T* gene

4.2.1. Development of sqRT-PCR to Study Expression of the TR-ACS4-T gene

The sqRT-PCR technique was used to study the expression of *TR-ACS4-T* during leaf development. Specific oligonucleotides were designed (Section 2.5.5.1.) as unique forward and reverse primers for *TR-ACS4-T*, and these were made to amplify *TR-ACS4-T* generated from a total cDNA pool made from RNA isolated from white clover leaves. Prior to the expression study, the specificity of the *TR-ACS4-T* primer set was first tested, using plasmids containing *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T* and *TR-ACS4-T* cDNA as PCR templates. Aliquots of the first round PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide, which revealed that only the target gene sequence of approximately 400 bp (*TR-ACS4-T*) was amplified indicating the specificity of *TR-ACS-T* primer sets (Figure 4.13).

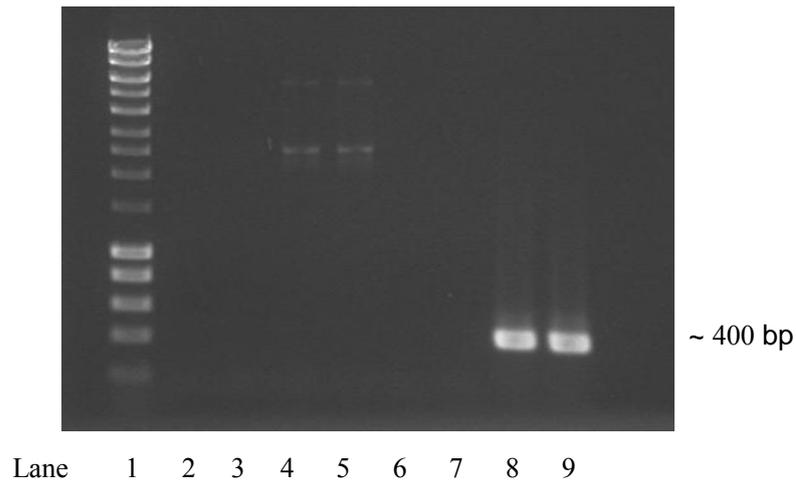
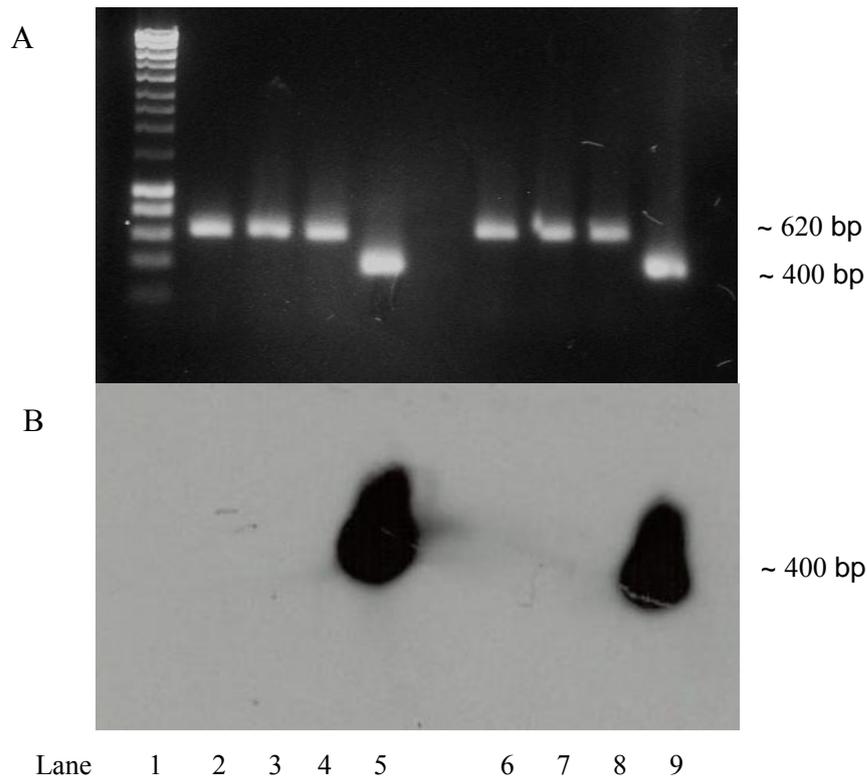


Figure 4.13 Specificity of the *TR-ACS4-T* primer set used to amplify *TR-ACS4-T* in sqRT-PCR. Each lane represents the amplified products using the *TR-ACS4-T* primers with different *TR-ACS-T* clones as templates (see legend).

Lane 1 is the DNA Ladder while lanes 2 and 3 are amplified products with *TR-ACS1-T* as template. Lanes 4 and 5 are amplified products using e *TR-ACS2-T* as template while lanes 6 and 7 are amplified products using *TR-ACS3-T* as template. Lanes 8 and 9 are amplified products of DNA using *TR-ACS4-T* as template.

To increase the sensitivity of the RT-PCR method, the separated PCR products were transferred to a positively charged nylon membrane and probed with a DIG-labeled *TR-ACS4-T* DNA probe. The specificity of the *TR-ACS4-T* probe to hybridise its own target sequence was firstly tested using separated *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T* and *TR-ACS4-T* cDNA. The DIG-labeled *TR-ACS4-T* only hybridized to its own target sequence suggesting that this method could be used to distinguish expression of *TR-ACS4-T* from the other three ACC synthase genes (Figure 4.14).

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Figures 4.14 Specificity of *TR-ACS4-T* probe to hybridise its own sequence.

Each lane contained DNA of either *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T* and *TR-ACS4-T* as indicated in the legend below. One round of RT-PCR products were separated by 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide for visualization (A). The DNA separated in (A) was electroblotted onto HybondN⁺ membrane and probed with a DIG-labeled *TR-ACS4-T* probe (B).

Lane 1. DNA Ladder

Lanes 2 and 6 are amplified products from *TR-ACS1-T*, lanes 3 and 7 are amplified products from *TR-ACS2-T*, lanes 4 and 8 are amplified products from *TR-ACS3-T*, while lane 5 and 9 are amplified products from *TR-ACS4-T*.

4.2.2. Expression of the *TR-ACS4* Gene during Leaf Development

Previously, Murray and McManus (2005) characterized the expression of *TR-ACS1-10F*, *TR-ACS2-10F* and *TR-ACS3-10F* during leaf development. These authors found that the *TR-ACS* genes were developmentally regulated during leaf development. *TR-ACS1-10F* and *TR-ACS2-10F* were expressed in the green leaves whilst the expression of *TR-ACS3-10F* was found in the yellowing and senescent leaves. In the current thesis, therefore, only the expression of *TR-ACS4-T* was studied during leaf development in the Tienshan stolon. To do this, a single stolon growth system was used and leaves were numbered from the apical structures subsequently down to the senescent leaves, giving eighteen different developmental stages (Section 2.1.4.).

The *TR-ACS4-T* probe hybridized to cDNA transcribed from RNA isolated from leaves at most developmental stages but different hybridisation intensities were observed (Figure 4.15). Higher hybridisation intensities were detected in the apical structures and unfolded leaves, after which the intensity of hybridisation decreased (Leaves 3 to 8), and then increased again in the more mature leaves and leaves at the onset of senescence (Leaves 9 to 13). Interestingly the hybridization intensities of *TR-ACS4-T* then decreased in the yellowing and senescent leaves (Leaves 17 and 18). In this experiment, expression of β -actin was used as an internal loading control but expression seemed to also change during leaf development (Figure 4.15). Higher intensity of β -actin expression was detected in the earlier leaf developmental stages and the β -actin bands decreased in the more mature and senescent leaves. Therefore, the expression study was repeated using 18-sRNA as the internal standard.

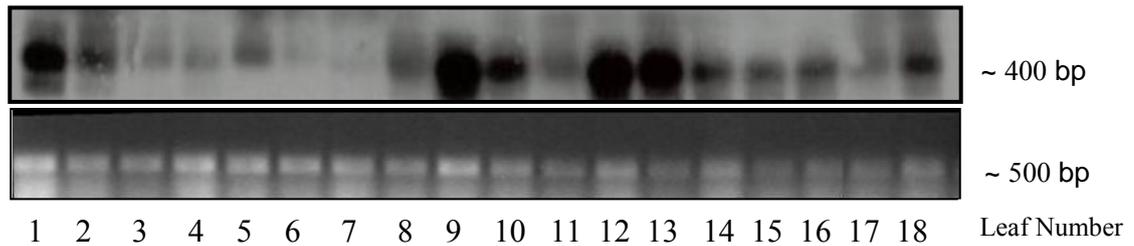


Figure 4.15 Expression of *TR-ACS4-T* during leaf development of the Tienshan ecotype revealed using sqRT-PCR with β -actin as the loading control.

Each lane contained cDNA representing different developmental stages of the leaves (as indicated). One round of RT-PCR was performed and aliquots of PCR products were separated by agarose gel electrophoresis, blotted onto HybondN+ membrane and hybridized with DIG-labeled *TR-ACS4-T* (upper panel). Expression of β -actin, from the same DNA as used for *TR-ACS4-T* amplification, was used as an internal control and visualized using ethidium bromide staining (lower panel).

In the repeat experiment, the *TR-ACS4-T* probe hybridized to a 400 bp product in Leaves 1 and 2, was undetectable in Leaves 3 and 4, very faintly detectable in Leaves 5 and then undetectable again in Leaves 6, 7, and 8 (Figure 4.16). An increase in hybridization intensity was observed in Leaves 9 to 16 (the late mature leaves and the leaves in the onset of senescence). However, *TR-ACS4-T* expression could not be detected in the senescent leaves (Leaves 17 and 18). The 18-sRNA bands in these samples were approximately the same, except for a less intense band in the Leaf 1. Overall, a similar pattern of *TR-ACS4-T* expression was observed during development in the Tienshan ecotype in Figures 4.15 and 4.16, and these results suggest that *TR-ACS4-T* expression is developmentally regulated. It is predominantly expressed in the very young leaves (apical structures and newly initiated leaves) and again in the mature leaves progressing through to the onset of senescence.

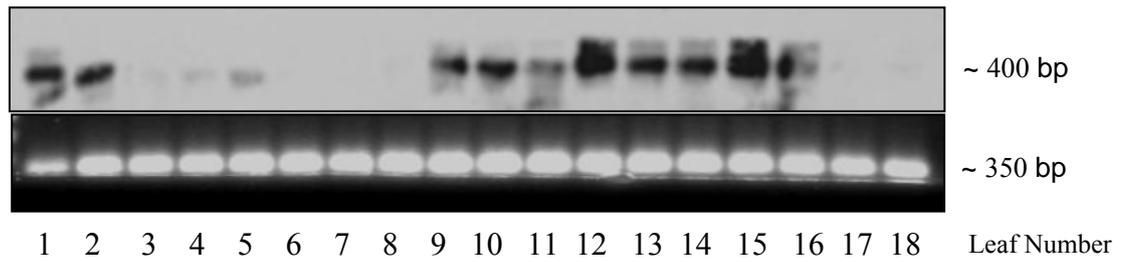


Figure 4.16 Expression of *TR-ACS4-T* during leaf development in the Tienshan ecotype revealed using sqRT-PCR with *18S-rRNA* as the loading control.

Each lane contained cDNA representing different developmental stages of the leaves (as indicated). One round RT-PCR was performed and aliquots of PCR products were separated by agarose gel electrophoresis, blotted into HybondN+ membrane and hybridized with DIG-labeled *TR-ACS4-T* (upper panel). Expression of *18S-rRNA*, from the same DNA as used for *TR-ACS4-T* amplification, was used as an internal control and visualized using ethidium bromide staining (lower panel).

4.2.3. Induction of *TR-ACS4* in White Clover Leaf Tissues

The expression of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* transcripts has been well characterized (Murray and McManus, 2005), and these authors showed that none of these three transcripts were induced by wounding or by auxin treatment. Although these characterizations were undertaken in the white clover genotype 10F from the cultivar Grasslands Challenge, no characterization has been undertaken in the Tienshan ecotype. However, in this thesis, characterization was undertaken for the novel *TR-ACS4-T* only. The *TR-ACS4-T* gene was isolated from apical structures of Tienshan, exposed to 10% SWC. Moreover, the GeneBank comparison of *TR-ACS4-T* indicated that it is closely related to hormone-induced and stress-associated *ACS* genes from many plant species (see Table 4.7). Therefore, *TR-ACS4-T* was further characterized to examine if its expression is altered by ethylene and/or auxin treatment.

The ethylene and auxin induction experiments were undertaken in the first-fully expanded leaves, a developmental stage at which expression of *TR-ACS4* was low in non-treated leaves (see Section 4.2.2.), and the expression study was undertaken

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using sqRT-PCR. The treatment was undertaken in a growth cabinet to which an ethylene action inhibitor (1-MCP) was added and used to act as a control to determine the effect of added ethylene and NAA-induced ethylene, as summarised in Table 4.8.

Table 4.8 Treatment of plant tissues to determine the induction of ACC synthase transcripts

Induction treatment	Control treatment
1. a. Ethylene b. 1-MCP pre-treatment and ethylene	No ethylene and 1-MCP
2. a. Auxin (NAA dissolved in ethanol/Tween 20) b. 1-MCP pre-treatment and NAA	Ethanol and Tween-20 only

In ethylene-treated tissue, the DIG-labeled *TR-ACS4-T* probe hybridized to a *ca.* 400 bp band and hybridisation of *TR-ACS4-T* could be detected in all samples (Figure 4.17). There was equal hybridisation detected in the control treatments before incubation in the growth chamber (0-h) and after 1, 2 and 4 h incubation in the growth chambers. In the ethylene treated leaves, increased hybridisation intensity of *TR-ACS4-T* was observed after 1 h, but after this, the hybridisation intensity (for the 2 and 4 h treatments) decreased to the same intensity as 0 h. Interestingly, the hybridisation intensity of *TR-ACS4-T* was not altered significantly by 1-MCP and ethylene treatment.

To ensure that equal quantities of RNA were used for the RT-PCR reactions, one round of PCR was carried out for the amplification of *β -actin*, the reaction products separated on a 1% (w/v) agarose gel, and visualized following staining by ethidium bromide (Figure 4.17). It can be seen that approximately the same intensity of *β -actin* expression was observed in each lane. Therefore, the differential transcript expression patterns observed for *TR-ACS4-T* were not caused by uneven loading of cDNA.

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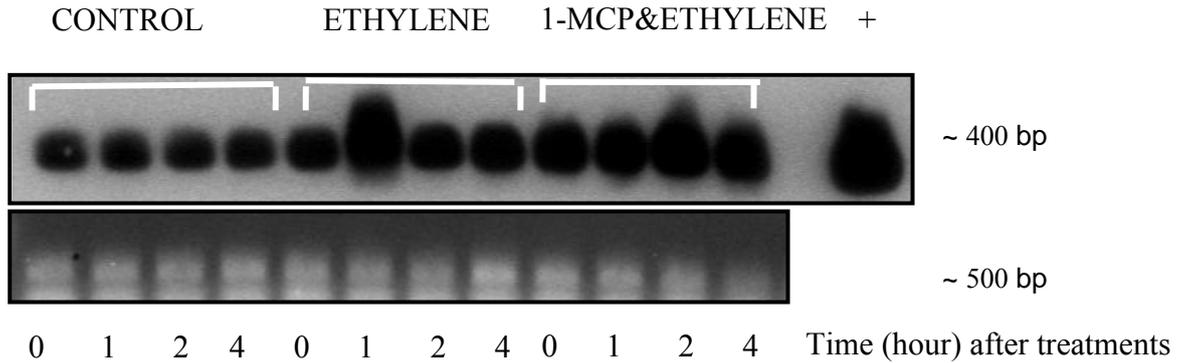


Figure 4.17 Analysis of *TR-ACS4-T* expression in ethylene-treated leaf tissues using sqRT-PCR.

RT-PCR was performed using cDNA representative of RNA isolated from the first fully-expanded leaves of white clover harvested at different times (as indicated) after being incubated in a chamber without treatment (control), with ethylene treatment (ethylene) or treated with both 1-MCP and ethylene (1-MCP/ethylene) (upper panel), as indicated. One round of PCR was performed using gene-specific primers for the *TR-ACS4-T* gene and the products were probed with DIG-labelled *TR-ACS4-T*. An equal loading of cDNA was assessed using degenerate *β-actin* primers from the same cDNA and RT-PCR products (lower panel). + indicates positive control.

In NAA-treated tissue, the *TR-ACS4-T* probe hybridized to a *ca.* 400 bp product which could be detected in all of the time points and treatments (Figure 4.18). In the control treatment (plants sprayed with ethanol and Tween-20), a slight increase in *TR-ACS4-T* hybridisation intensities could be observed at samples collected after 1 h and 4 h incubation in the growth chamber. However, the intensities of *β-actin* bands from the same 1 and 4 h samples were also higher. Therefore, incubation of plants in the chamber did not significantly alter the hybridisation intensities of *TR-ACS4-T*. In the NAA-treated plants, a slightly higher hybridisation intensity was detected in 0-h samples which then decreased slightly at 1 h and 2 h, and then significantly increased after 4 h. The intensities of *β-actin* bands from the same samples indicated that a higher intensity of *TR-ACS4-T* at 0 h sample of NAA treatment was due to higher loading of cDNA in this sample. Therefore, the only real increase in the hybridisation intensity of *TR-ACS4-T* was after 4 h. For the combined 1-MCP and NAA treatments, a higher hybridisation intensity of *TR-ACS4-T* was

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seen in samples collected after 2 h treatment, but it decreased again to a similar intensity as the control after 4 h treatment. These results indicate that spraying the plants with ethanol and Tween-20 and incubating them for 4 h in the growth chambers did not induce any significant expression of the *TR-ACS4-T* gene. However, NAA did induce the expression of *TR-ACS4-T* after 4 h. In addition, the combined 1-MCP and NAA treatment did not significantly alter the gene expression suggesting that NAA-induced expression of *TR-ACS4-T* was indirect and mediated by ethylene.

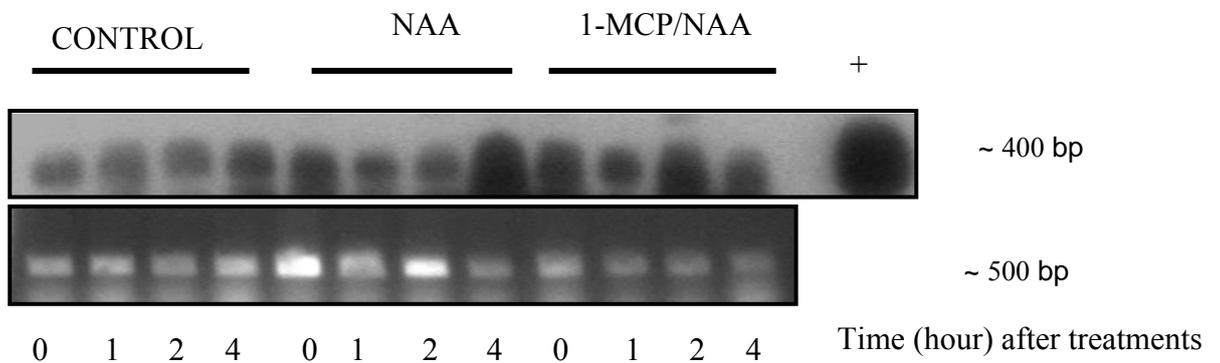


Figure 4.18 Analysis of *TR-ACS4* expression in NAA-treated leaf tissues using sqRT-PCR.

RT-PCR was performed using cDNA representative of RNA isolated from the first fully-expanded leaves of white clover harvested at different time (as indicated) after being sprayed with ethanol and Tween-20 and incubated in a growth chamber (control), or treated with NAA (NAA), or treated with both 1-MCP and NAA (1-MCP/NAA) (upper panel), as indicated. One round of PCR was performed using gene-specific primers for *TR-ACS4-T* and the products were probed with DIG-labelled *TR-ACS4-T*. Equal loading of cDNA was assessed using degenerate β -actin primers from the same cDNA and RT-PCR products (lower panel). + indicates positive control.

5. DISCUSSION

5.1. Overview

Many reports suggest that ethylene is an important mediator of plant responses to different environmental cues (Bleecker and Kende, 2000). The biosynthesis of the hormone involves the two key enzymes, ACC synthase (ACS) and ACC oxidase (ACO) and while many studies on the involvement of ethylene in plant responses to environmental cues have explored the importance of ACS (Liu and Zhang, 2004; Joo *et al.*, 2008), fewer reports have focused on ACO despite the fact that the enzyme catalyses the final step of ethylene biosynthesis in plants (Bleecker and Kende, 2000). The reports on the role of ACS in ethylene biosynthesis suggest that this is regulated at the transcriptional, post-transcriptional as well as the post-translational level. Of the few reports on the involvement of ACO in ethylene biosynthesis, it has been shown that ACO is encoded by a small multigene family and its regulation is controlled (at least) at the transcriptional level. Using water deficit as an environmental stimulus, the first part of this thesis investigated changes in *ACO* gene expression and ACO protein accumulation in white clover leaves during an imposed water deficit. The thesis has extended the study of Hunter *et al.* (1999) and Chen and McManus (2006) who showed that each member of the *TR-ACO* multigene family is differentially expressed during leaf ontogeny of white clover when grown under optimal environmental conditions.

The first part of this thesis examined changes in *TR-ACO* gene expression and *TR-ACO* protein accumulation in response to a water deficit, and was studied by comparing responses of two white clover varieties with differing sensitivities to water deficit. One variety was the ecotype Tienshan which is considered to be drought-tolerant, while the second is the cultivar Kopu which is considered to be more drought-susceptible. The first step of the study was to evaluate the differential sensitivity and growth responses of these varieties to a water deficit by examining their leaf water status and physiological responses to water stress. The second step was to study these alterations and their relation to changes in the patterns of *TR-ACO*

gene expression and TR-ACO protein accumulation in white clover leaves at different developmental stages.

The second part of this thesis extended the study of ACS gene regulation in white clover by screening for a water-stress-associated ACS gene. ACC synthase (ACS) is suggested to comprise a larger gene family in plants and three *ACS* genes have been isolated and characterised from Genotype 10F of the Grasslands Challenge cultivar of white clover (Murray and McManus, 2005). These genes were isolated from different developmental stages of white clover leaves. In this thesis, orthologues of each ACS gene was isolated from the Tienshan ecotype as well as a novel ACC synthase. The expression of this novel ACS gene during leaf development and in response to ethylene and auxin treatments was examined.

5.2. Part I: Changes in Water Relations, Expression of the *TR-ACO* Gene Family and TR-ACO Protein Accumulation during Water Deficit in White Clover

5.2.1. Changes in Water Relations as an Indicator of Differential Sensitivity of the White Clover Varieties to a Water Deficit

This study compared the responses of two white clover varieties to a water deficit, the Tienshan ecotype and the Grasslands Challenge Kopu II cultivar (referred as Kopu hereafter). The Tienshan ecotype is a small-leafed (5 to 7.5 mm²) ecotype originated from the semi arid, high altitude areas of the Tien Shan Mountains in China, areas with an average annual precipitation of 500 mm/year. The Kopu is a large-leafed (10 to 15 mm²) New Zealand elite variety with very robust growth in well-watered conditions. Hofmann *et al* (2003) suggested that these two varieties have a different sensitivity to water deficit, where the Tienshan ecotype is more tolerant than the Kopu cultivar.

Both varieties were treated with two different water deficit treatments: without a previous water deficit treatment (non pre-stressed; NPS) and with one week previous

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water deficit treatment (pre-stressed; PS) (see details in Chapter II). The SWC was monitored on a daily basis and used as an indicator that a soil water deficit was imposed. In both treatments, the SWC declined progressively from the time that the water was completely withheld and had declined to *ca.* 6% in the Tienshan ecotype and *ca.* 8% in the Kopu cultivar at the point where PER ceased and experiments were terminated. Similar trends of decrease in the SWC were observed between the NPS and the PS treatments in both varieties. However, the rate of decrease of SWC in the Kopu cultivar was twice as fast as that observed for the Tienshan ecotype. With the assumption that the soil evaporation rates were similar for all pots, and that the vegetation coverage was similar in all pots, the absorption of water from the soil was faster for the large-leaf phenotype (Masinde *et al.*, 2005) suggesting this variety had a faster transpiration rate. However, the transpiration rate, or the total leaf area was not measured.

Water deficit has been shown previously to decrease leaf water status as indicated by a reduction in the relative water content of the leaf as well as leaf water potential (LWP) and leaf osmotic potential (McManus *et al.*, 2000; Kim *et al.*, 2004; Cechin *et al.*, 2006; Gabrielle and Bendh, 2006). LWP is a common criterion used to determine the intensity of stress experienced by plants (Abraham *et al.*, 2004). In the experiments in this thesis, therefore, LWP was monitored and used as an indicator of water status of the Tienshan and the Kopu plants under the water deficit treatments. The LWP in both the NPS and the PS Kopu and Tienshan decreased as the SWC decreased (Figure 3.3). Again these results indicate that the treatments used were able to provide a water deficit for both the Tienshan and the Kopu plants. The LWP of the NPS and the PS treatments (within the same variety) were not significantly different, suggesting that the PS treatments (exposure to previous water) may not alter the water requirement of the plants during a water deficit.

However, the LWP of the Tienshan plants did differ from the Kopu plants. In well-watered conditions, the LWP in the Tienshan ecotype was less than the Kopu cultivar, but as the water stress progressed, the LWP in the Kopu cultivar became much less (at the same SWC). After this, the decrease in the LWP in the Kopu plants was more

rapid and become more negative than that recorded for the Tienshan plants. At the point at which the PER ceased, the LWP in the Kopu plants was significantly lower (-1.7 MPa) than that observed for the Tienshan plants (-1.4 MPa). Many studies suggest that water status can also reflect the osmotic/elastic property of plant tissues. Plants with a higher water status are better adapted to a water deficit which in part is due to the elastic property of the plant tissues (Van den Boogaard *et al.*, 1997; Zhang *et al.*, 1997; Khurana and Singh, 2004; Monclus *et al.*, 2006; Dias *et al.*, 2007). The Tienshan ecotype, therefore, displayed a better response to water deficit than the Kopu, suggesting that this ecotype has a better control of water loss. This supports the view that the Tienshan ecotype is more tolerant to a water deficit than the Kopu, as suggested by Hofmann *et al.* (2003).

5.2.2. Responses of the Two White Clover Varieties to a Water Deficit

Cell turgor pressure is an important factor required for maintaining plant growth (Proseus and Bryer, 2005). Water deficit decreases cell turgor pressure and reduces plant growth through a reduction in cell elongation (Abe and Nakai, 1999; Bissuel-Belaygue *et al.*, 2002). A decrease in cell turgor occurs at the early stages of water deficit, and this has been suggested to be an adaptive response of plants to a water deficit (Abe and Nakai, 1999; Schroeder *et al.*, 2001; Sanchez *et al.*, 2004). The decrease in the SWC and the LWP resulted in a reduced PER of both the NPS-treated Tienshan and NPS-treated Kopu plants (Figure 3.4). In well-watered soil, and in soil subjected to an early decrease of water content (above 18% SWC), both varieties maintained a high PER. The first significant decrease in the PER in the NPS-treated Tienshan and NPS-treated Kopu was observed at a similar SWC (*ca.* 18%) and at a similar LWP (-0.8 MPa). These early significant decreases in the PER reflected a turgor-driven decrease in cell elongation responses as they occurred at the early stages of water deficit (Abe and Nakai, 1999; Schroeder *et al.*, 2001) and at the same plant water status in both varieties. However, after this, the decrease in the PER in the NPS-treated Kopu was more rapid. When a water deficit persists, the further decline in cell turgor affects the whole plant physiology and hormonal balances in plant cells which then directly affect plant growth (Arend and Framm, 2007). One of

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the hormones that proposed to be involved in regulating these responses is ethylene (Munne-Bosch *et al.*, 2000; Potters *et al.*, 2009). Therefore, this further decrease in cell elongation in both the Tienshan and the Kopu plants after -0.8 MPa of LWP is probably driven by changes in the hormonal balance and the involvement of ethylene in this will be discussed later (Section 5.2.3). Thus further decreases in the PER was used as an indicator that alterations in ethylene biosynthesis may have occurred, and therefore this study was undertaken until the PER in the fastest growing leaves (first fully-expanded leaves) ceased.

In association with the decrease in cell turgor, a series of morphological and biochemical changes occur which are related to both the control of water loss from plant tissues during a water deficit and maintenance of the cell function in a water deficit. Stomatal closure and the accumulation of compatible solutes, such as proline, are amongst these changes (Tanaka *et al.*, 2005; Gabrielle and Bendh, 2006). ABA (Schroeder *et al.*, 2001; Sanchez *et al.*, 2004) and other factors such as changes in the turgor of guard cells, changes in metabolic energy (from photosynthesis) and changes in membrane permeability (Chaves *et al.*, 2003; Kwak *et al.*, 2003; Tanaka *et al.*, 2005) have been suggested to be involved in the regulation of stomatal closure during water deficit. The importance of ABA as a regulator of the response of plants to a water deficit has been extensively studied, but it was not the focus of study in this thesis. However, changes in proline content were examined.

Proline accumulation linked to water deficit has been observed in many species including legumes (Iannucci *et al.* 2002) and it has been also reported in white clover previously (Barker *et al.*, 1993; McManus *et al.*, 2000). The level of proline accumulation is reported to vary between species and also depends on the degree of water deficit imposed. Much evidence indicates that the more tolerant varieties accumulate a higher concentration of free proline than the more sensitive varieties in many plant species including bent grass (Dacosta and Huang, 2006), olive (Ennajeh *et al.*, 2006), Chinese poplar (Xiao *et al.*, 2008) and mulberry (Chaitanya *et al.*, 2009). A similar difference was also observed in white clover in the current study. Proline accumulation in the leaves of the NPS-treated Tienshan ecotype and NPS-

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treated Kopu cultivar were not significantly different in well-watered conditions. In both varieties, accumulation of free proline increased in the leaves of plants exposed to 10% SWC and below (Figure 3.5). However, it increased six-fold in the NPS-treated Tienshan ecotype but only four-fold in the NPS-treated Kopu, and these differences were significantly different. The results support the suggestion that a higher proline concentration accumulates in the tolerant variety and may indicate that these plants show a better adjustment to low water availability (Dacosta and Huang, 2006; Ennajeh *et al.*, 2006; Bouchabke-Coussa *et al.*, 2008; Xiao *et al.*, 2008). The results further suggested that the Tienshan ecotype has the capacity to respond more efficiently to water loss and so display more tolerance to a water deficit when compared with the Kopu cultivar.

In this thesis, both varieties were also subjected to a pre-stress (PS) treatment. Both Tienshan and Kopu demonstrated similar responses in terms of water relations and there was a similar trend in terms of changes to the PER in PS-treated Tienshan as observed in the NPS-treated Tienshan. However, the PER of the NPS- and PS-treated Kopu did show some difference, with a more gradual decrease observed in the PS-treated Kopu which was similar to that observed for both NPS- and PS-treated Tienshan, in contrast to a sharp decline in the PER of NPS Kopu exposed to a water deficit below *ca.* 18% SWC. As well, the proline accumulation profile differed between the NPS-treated and PS-treated Kopu. A more gradual proline accumulation which started earlier (at *ca.* 18% SWC) was observed in PS-treated Kopu when compared with the more sudden accumulation in the NPS-treated Kopu which occurred only after *ca.* 9% of SWC. These results suggest that an acclimation to the water deficit may have occurred in Kopu cultivar following the previous water deficit experience (*ie.* the pre-stress treatment).

As with a reduction in growth rate, premature senescence and/or abscission of mature organs have also been suggested to be involved in the response of plants to a water deficit (McMichael *et al.* 1972; Ludmilla *et al.*, 2000; Young *et al.*, 2004). In this thesis, total chlorophyll in the first and second fully-expanded leaves of both the NPS- and PS-treated Tienshan and Kopu plants was measured and used as an

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indicator of ageing (premature necrosis) during the water deficit treatment. Previous studies in white clover (*T. repens*) and *T. occidentale* (suggested to be one of the progenitor of white clover) reported that the total chlorophyll content of the leaves of clovers grown under optimal conditions was associated with the physiological age of the leaves where the first fully-expanded leaves contained less total chlorophyll than the more developed mature green leaves (Hunter *et al.*, 1999; Du, 2004). The maximum level of chlorophyll occurred in the more mature green leaves, but then it decreased as leaves developed toward the onset of senescence and in the senescent leaves (Hunter *et al.*, 1999; Du, 2004). In response to the water deficit imposition in this thesis, no changes in total chlorophyll were observed in the first fully-expanded leaves of the NPS- and PS-treated Tienshan as well as the PS-treated Kopu. This may indicate that these leaves were maintained at a similar physiological state as the fully-hydrated first fully expanded leaves. However, in the first fully-expanded leaves of NPS-treated Kopu, total chlorophyll increased as the SWC decreased to below 18%. This may suggest that an earlier onset of leaf maturity may have occurred in these leaves of NPS-treated Kopu. In both the NPS- and PS-treated Tienshan ecotype, an increase in total chlorophyll was observed in the second fully-expanded leaves harvested from plants subjected to low SWC (below 10%) again suggesting premature leaf maturation. These trends were in contrast to a decrease in total chlorophyll observed in the second fully-expanded leaves of both the NPS- and PS-treated Kopu grown under water deficit conditions of below 12% SWC. Thus, a sign of premature necrosis or ageing was only observed in the second fully-expanded leaves (an older tissue) of both NPS- and PS-treated Kopu subjected to a water deficit below 12% SWC.

Reports on the effects of water deficit on total chlorophyll accumulation are mainly associated with more severe water deficit and senescence. These reports vary with some suggesting that water deficit decreased total chlorophyll (Javadi *et al.*, 2006; Abrau and Munne-Bosh, 2008; Guerfel *et al.*, 2008), some suggest it increased (Zaefyzadeh *et al.*, 2009) or some reporting that it did not alter total chlorophyll level (Gu *et al.*, 2007). These differences could be due to many factors such as differences

in developmental stages of leaves observed, species or variety differences (Javadi *et al.*, 2006; Abrau and Munne-Bosh, 2008; Guerfel *et al.*, 2008; Zaefyzadeh *et al.*, 2009). These authors suggest further that differences in chlorophyll can also indicate the different sensitivity or different adaptation of plants to water stress. In this thesis, some indication of premature ageing only occurred in the (drought-susceptible) Kopu cultivar which supports this suggestion.

5.2.3. ACO is Involved in Regulating Ethylene Biosynthesis during a Water Deficit

Although much evidence suggests that ethylene is associated with the response of the plant to a water deficit, evidence on how water deficit alters ethylene production is conflicting. Earlier reports suggest that water deficit enhances ethylene production, for example in cotton (Jordan *et al.*, 1972) and wheat (Apelbaum and Yang, 1981). However, in these reports water deficit was imposed artificially by detaching leaves and this may not resemble the actual water deficit conditions experienced by whole plants. When ethylene production was measured in intact plants, levels varied based on plant species, varieties, the growing state of plants exposed to water deficit and the degree of water deficit imposed. For example, in the seedlings of Norway spruce (four year old), Van Den Driessche and Langebartels (1994) observed an accumulation of ethylene in the early period of the water deficit which decreased as the water deficit progressed. However, the increased ethylene production reported was not a direct effect of water deficit but related to ozone treatment. Water deficit treatment alone resulted in reduced ethylene production in the cambial/xylem region of mature Norway spruce trees (24 years-old) (Eklund *et al.*, 1992) or in bean, miniature rose and cotton plants (Morgan *et al.*, 1990). Similarly, water deficit resulted in a decrease in ethylene production by mature wheat plants (six week-old) (Narayana *et al.* 1991). Indeed, exposure of wheat seedlings to up to 48 hours of water deficit decreased ethylene production by both drought-sensitive and drought-tolerant cultivars (Balota *et al.*, 2004). However, Chen *et al.* (2002) found that ethylene production in mature wheat cultivars differed between varieties and the degree of water deficit imposed. In the drought-tolerant variety, ethylene production increased in the first 24 hours of a water deficit and then decreased, whilst in the

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drought-sensitive cultivars, ethylene production decreased continuously during water deficit. In most of these reports, total ethylene production in plants exposed to a water deficit was compared to well-watered plants, or the plant tissue is either exposed to very short period of water deficit (up to 48 h) or very severe water deficit conditions (below -2.0 MPa). These studies did not provide any time scale information as to how ethylene biosynthesis is altered in different developmental stages of the leaves which could indicate a link between biosynthesis of the hormone and the response of the plant to a water deficit. In addition, these reports measured total ethylene production (as ethylene evolved and detected using a gas chromatography), ACC content and/or ACO activity which provided hints of the mechanism of alterations in ethylene biosynthesis, but did not provide evidence at the molecular level as to which members of the *ACS* and/or *ACO* gene families or their gene products are specifically altered by the water deficit.

The availability of earlier information on differential regulation of the *ACO* multigene family during leaf development in white clover (*TR-ACO*) (Hunter *et al.* 1999, Chen, 2005; Chen and McManus. 2006) therefore provides some fundamental background with which to study further regulation of each member of the *TR-ACO* gene family in response to a water deficit. By characterising how each member of the *TR-ACO* gene family, which are expressed in different developmental stages of fully-hydrated leaves, is regulated by water deficit, some clues as to how white clover responds in term of regulation of ethylene biosynthesis may be provided. To meet these goals, changes in the expression of each member of the *TR-ACO* gene family was examined by sqRT-PCR and the changes in the accumulation two protein isoforms was also examined by western blot analysis.

Initially, the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed using total RNA isolated from three different leaf developmental stages to confirm that the expression of each member of the *TR-ACO* gene family in the Tianshan ecotype and Kopu cultivar occurs at the same stage of leaf development in which they all normally expressed in Genotype 10F: *TR-ACO1* in the apical structures, *TR-ACO2* in the first and second fully-expanded leaves, and *TR-ACO3* in

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the first and second fully-expanded leaves. The *TR-ACO1*, *TR-ACO2* and *TR-ACO3* sequences of *T. repens* Genotype 10F, cultivar Grasslands Challenge obtained from the GeneBank, were used as the sequence reference. Gene-specific primers for amplification of each member of the *TR-ACO* gene family were designed. The *TR-ACO* genes share a high homology (more than 80%) in the coding frame regions but have more distinct 3'UTR regions (less than 40% homology) (Appendix VIII). Thus primers were designed to amplify *ca.* 100 bp of the coding region and *ca.* 200 bp of the 3'UTR region of each transcript. Each of these primer sets were successfully used to specifically amplify the *TR-ACO1*, *TR-ACO2* and *TR-ACO3* genes from both the Tienshan ecotype and the Kopu cultivar. Moreover, probes made from each of these amplified products specifically hybridised to their target sequences and hybridisation occurred in the cDNA made to RNA isolated both from the Tienshan ecotype and the Kopu cultivar.

Changes in ACO during water deficit in these two white clover varieties were also examined by observing the accumulation of TR-ACO isoforms corresponding to two members (*TR-ACO1* and *TR-ACO2*) of the gene family by western analysis. These anti-*TR-ACO1* and anti-*TR-ACO2* antibodies were raised against recombinant *TR-ACO1* and *TR-ACO2* proteins from genes cloned from the white clover Genotype 10-F (Hunter *et al.*, 1999). Each of the antibodies recognised a protein band of the expected size (*ca.* 36 to 37 kDa) confirming that these two antibodies can be used to detect *TR-ACO1* and *TR-ACO2* protein in the Tienshan and the Kopu varieties. However, the abundance of the TR-ACO isoforms seemed to differ between these two varieties. Less total protein was required to detect *TR-ACO2* accumulation in the leaves of the Tienshan when compared with Kopu, indicating that the small-leaved Tienshan ecotype may accumulate more *TR-ACO2* enzyme than the large-leaved Kopu on the per leaf protein basis.

The studies on changes of each member of *TR-ACO* gene family and two members of the TR-ACO protein family reported in this thesis have revealed that ACO gene expression and protein accumulation is influenced in plants exposed to a water deficit. These changes occur at both the transcriptional and translational levels. There

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are four major lines of evidence in this thesis to support these findings, and each of these will be discussed in more detail later:

1. Analysis of *TR-ACO1* gene expression in the apical structures, *TR-ACO2* gene expression in the first- and second-fully expanded leaves and *TR-ACO3* gene expression in the first- and second-fully expanded leaves showed that the expression of each transcript was altered in plants exposed to a water deficit. Moreover, expression patterns of *TR-ACO1* and *TR-ACO2* differed between the more drought-tolerant Tienshan ecotype and more drought-sensitive Kopu cultivar (Figures 3.10, 3.11, 3.15, 3.16, 3.19, 3.20, 3.22, 3.23, 3.26 and 3.27).
2. Analysis of TR-ACO1 protein accumulation in the apical structures and TR-ACO2 protein accumulation in the first- and second-fully expanded leaves showed that exposure of plants to a water deficit altered the accumulation of TR-ACO1 and TR-ACO2 proteins. In addition, the pattern of TR-ACO1 and TR-ACO2 protein accumulation following water deficit treatments also differed between the more drought-tolerant Tienshan ecotype and the more drought-sensitive Kopu cultivar (Figures 3.12, 3.13, 3.17, 3.18, 3.24 and 3.25)
3. The two different water deficit treatments used: one and two-cycles of water deficit (hence NPS and PS treatments) also differentially altered the pattern of both the *TR-ACO* gene expression (Figures 3.10, 3.11, 3.15, 3.16, 3.19, 3.20, 3.22, 3.23, 3.26 and 3.27) and TR-ACO protein accumulation (Figures 3.12, 3.13, 3.17, 3.18, 3.24 and 3.25) and these alterations differed in the more drought-sensitive Kopu cultivar, but not in the more drought-tolerant Tienshan ecotype. More interestingly, the expression of *TR-ACO* genes and accumulation of TR-ACO proteins in the apical structures and first fully-expanded leaves of PS Kopu followed similar trends as observed in the more drought-tolerant Tienshan ecotype (Figures 3.12, 3.13, 3.15, 3.16, 3.17 and 3.18).
4. Analysis of *TR-ACO3* gene expression in the first- and second- fully expanded leaves showed that expression of *TR-ACO3* did not alter in the first-fully expanded leaves of plants exposed to water deficit, but did increase in the second

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fully-expanded leaves of both Tienshan and Kopu plants exposed to less than 10% SWC.

Initially, when discussing the evidence that supports the differential expression of the *TR-ACO* gene family and accumulation of TR-ACO1 and TR-ACO2, and further differences between the more drought-tolerant Tienshan ecotype and the more drought-susceptible Kopu cultivar in response to an imposed water deficit, comparison is made between the NPS-treated plants. A further aspect of this thesis, the acclimation of the Kopu cultivar after PS, is then discussed separately particularly with respect to associated changes in ACO gene expression and protein accumulation.

5.2.3.1. *TR-ACO1* Expression and TR-ACO1 Protein Accumulation in the Apical Structures of White Clover during Water Deficit

This study has demonstrated that water deficit differently regulated the expression of *TR-ACO1* in the Tienshan and the Kopu plants. The expression analysis showed that there were few changes in *TR-ACO1* expression in the apical structures of the Tienshan plants exposed to water deficit, except for possibly some induction as the SWC decreased to *ca.* 5.9 %, by which time the PER in the first fully-expanded leaves ceased. However, the expression of the *TR-ACO1* in the apical structures of the NPS-treated Kopu plants decreased when the SWC values decreased to *ca.* 17%.

A common adaptation of plants, including the legumes, to water deficit is to preferentially protect meristematic tissues (Elston and Bunting, 1980; Rossie *et al.*, 2009). Such a mechanism allows the plants to survive until water becomes available again. In white clover plants observed in this study, meristem protection during the water deficit may have occurred in the more tolerant Tienshan ecotype, but not in the more sensitive Kopu cultivar, as indicated by the relatively unchanged expression of *TR-ACO1* and accumulation of TR-ACO1. Only when the water deficit became more severe (at *ca.* 5.9% SWC) did some indication of a change in expression occur.

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Previously, Hunter *et al.* (1999), using northern analysis, demonstrated that *TR-ACOI* was predominantly expressed in the apex or meristematic tissues of white clover and expression of the *TR-ACOI* decreased as the tissues developed into a more organised/leaf organ. As well, Chen (2005) showed that the promoter region of *TR-ACOI* mostly contained Transcription Factor Binding Domains (TFBDs) that were associated with development such as MADS, AGAMOUS (AG) and AGAMOUS-LIKE3 (AGL-3). Further, histochemical analysis of white clover transformed with a *TR-ACOI* promoter-GUS reporter gene fusion (*TR-ACOI*_p::*GUS*) showed that the highest GUS staining was in the ground meristem, which then decreased in the newly initiated leaves (Chen and McManus, 2006). Together these findings indicate a strong connection between *TR-ACOI* expression and tissues undergoing cell division and differentiation (Hunter *et al.*, 1999; Chen and McManus, 2006). Rapidly dividing and expanding tissues exhibit relatively high rates of ethylene production which is proposed to interact with auxin to define cell shape (Osborne, 1991). These very strong relationships therefore suggest that the expression of *TR-ACOI* indicates a state of actively dividing tissues, such as observed in the apex.

In this thesis, only a small change in *TR-ACOI* expression was observed in the apical structures of NPS-treated Tienshan suggesting that the apical structure of the NPS Tienshan plants was maintained in the meristematic stage. In the NPS-treated Kopu plants, expression of the *TR-ACOI* decreased as the water stress progressed, suggesting that a decreased rate of cell division may be occurring. Unlike the Tienshan ecotype, the meristem may not be as well protected in the Kopu such that the normal rate of activity cannot be maintained, but no histological analysis of the cells in the apical structures was performed to examine this further.

5.2.3.2. *TR-ACO2* and *TR-ACO3* Expression and TR-ACO2 Protein Accumulation in the First-Fully Expanded Leaves of White Clover in Response to Water Deficit

In the first fully-expanded leaves, the expression of *TR-ACO2*, but not *TR-ACO3*, was altered in NPS Tienshan and NPS Kopu plants in response to a water deficit. In both NPS Tienshan and NPS Kopu plants, the expression of *TR-ACO2* increased in the very early stages of the water deficit. However, expression then decreased as the water deficit progressed in the NPS Tienshan, but continued to increase in the NPS Kopu plants. This pattern of expression was similar to the pattern of TR-ACO2 protein accumulation. In both the NPS Tienshan and NPS Kopu plants, exposure to the water deficit did not significantly alter the expression of the *TR-ACO3* suggesting that in the first fully-expanded leaves, TR-ACO2 is the major ACO enzyme operating and *TR-ACO2* is the major gene that is influenced by exposure to a water deficit.

Significantly, the expression of *TR-ACO2* and accumulation of TR-ACO2 in the first fully-expanded leaves of the NPS Tienshan ecotype during the water deficit correlated with the pattern of changes in the PER. In the early stages of the water deficit, the high PER measured was consistent with higher expression of *TR-ACO2* and accumulation of TR-ACO2 until the time when the PER first decreased (at *ca.* 18% SWC). After this, the expression of *TR-ACO2* and accumulation of TR-ACO2 decreased as the PER declined further.

As has been discussed earlier (Section 5.2.2.), a decrease in the PER indicates a reduction in cell elongation which is a turgor-driven process (Abe and Nakai, 1999; Schroeder *et al.*, 2001; Sanchez *et al.*, 2004). The first decrease in the PER observed (without corresponding to a decrease in *TR-ACO2* expression and TR-ACO2 accumulation) was probably a direct effect of the decreasing cell turgor through a decrease in water pressure. This occurred in the NPS Tienshan plants at the early stages of the water deficit and has been observed in other plant species previously (Abe and Nakai, 1999; Schroeder *et al.*, 2001; Sanchez *et al.*, 2004). Further

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exposure of plants to a water deficit is then proposed to alter hormonal balances which reduce growth (Arend and Framm, 2007). The observed decreases in the PER following further exposure of the NPS Tienshan plants to the water deficit correlated with a decrease in *TR-ACO2* expression and TR-ACO2 accumulation, thus supporting previous evidence that ethylene may be involved in regulating this decrease in cell elongation (Munne-Bosch *et al.*, 2000; Potters *et al.*, 2009) (although ethylene production was not measured directly).

In contrast, in the NPS Kopu plants, reduction in growth was not consistent with the pattern of *TR-ACO2* expression and TR-ACO2 accumulation. The PER decreased rapidly in the NPS Kopu plants but the accumulation of TR-ACO2 and *TR-ACO2* expression increased. Increased accumulation of TR-ACO2 in leaves of the Kopu cultivar exposed to a water deficit probably indicates an injury response. Plants have been well documented to accumulate reactive oxygen species (ROS) as a universal response to various stresses (Kaminska-Rozek and Pukacki, 2005). Under normal growth conditions, the production and removal of ROS is controlled by a homeostatic mechanism which is compromised when plants are exposed to stresses (Bartoli *et al.*, 2004). Accumulation of ROS leads to cell damage if it is not in balance with scavenging antioxidants (Shen *et al.*, 2008). Further, it has been proposed that either ethylene enhances ROS production or ROS can induce ethylene production (Song *et al.*, 2007; Shen *et al.*, 2008). ROS accumulation was not measured in this thesis, but the rapid decrease in the PER of the Kopu leaves as well as the decrease in free proline accumulation at the time when the PER ceased in Kopu supports the possibility that cell injury might have occurred in the NPS Kopu plants.

In summary then, the decrease in *TR-ACO2* expression and TR-ACO2 accumulation that correlated with a decrease in PER was seen only in the more drought-tolerant Tienshan plants, where the decrease in PER progressed gradually. Therefore, findings reported here support the perspective that a controlled reduction in growth is a key adaptation of a tolerant plant to water deficit (Schroeder *et al.*, 2000; Sanchez

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et al., 2004). Together with other adaptations, a decrease in growth allows plants to conserve water for longer period during a water deficit.

Analysis of the promoter region of the *TR-ACO2* gene has shown that sequence contains the same number of class I, class II and class III TFBDs (as designated by Chen, 2005). The class I TFBD's are associated with hormonal or environmental cues, the class II TFBDs are associated with transcription factors involved in regulating plant growth and development, while class III comprise transcription factors involved in ethylene and wound responses. Therefore, the *TR-ACO2* promoter sequence could be regulated by a broad range of stimuli, including ABA, GA, auxin and ethylene, and these interactions could occur at different developmental stages, in different tissues and in response to a range of environmental cues (Chen, 2005).

Ethylene-mediated cell expansion has been proposed to be the result of the interaction with other plant hormones, such as abscisic acid (ABA), gibberellin (GA) and auxin (Var der Straten *et al.*, 2001; Kurepin *et al.*, 2006, Fukao and Bailey-Serres and Julia, 2008). Further, the induction of ABA by water deficit has been well documented (Davies *et al.*, 2005; Christmann *at al.*, 2007; Okamoto *et al.*, 2009; Ikegami *et al.*, 2009) and it has been suggested that interaction between ABA and ethylene will determine how plants respond to various stresses including water deficit (Cheng and Lur 1996; Davies, 2004). Different degrees of water deficit have been suggested to differentially alter the ratio of ABA to ethylene (Yang *et al.*, 2004), or differences in the balance of ABA to ethylene during a water deficit have also been observed between species and varieties (Cheng and Lur 1996; Davies, 2004) which then specifies adaptation of different varieties to water deficit. Thus, there are many potential mechanisms of regulation, including hormones, that may interact to decrease *TR-ACO2* expression in Tienshan in a controlled way and result in the reduction of PER, while other factors may interact with the gene in Kopu as part of the injury response.

5.2.3.3. *TR-ACO2* and *TR-ACO3* Expression and TR-ACO2 Protein Accumulation in the Second-Fully Expanded Leaves of White Clover in Response to Water Deficit.

The second fully-expanded leaves represent more mature organs (in comparison to the first fully-expanded leaf), and therefore may be more sensitive to water deficit and so more pronounced alterations in *TR-ACO2* and *TR-ACO3* expression and TR-ACO2 accumulation may be expected (No TR-ACO3 protein accumulation was assessed as there is no anti-TR-ACO3 antibody available). Also, in the second fully-expanded leaves, a larger amount of total protein (twice as much as used in the first fully-expanded leaves) was needed to be loaded using SDS-PAGE to detect linear changes in TR-ACO2 protein accumulation, thus confirming that the TR-ACO2 accumulation decreases in more mature leaves (Hunter *et al.*, 1999).

In these experiments, water deficit treatment resulted in a significant decrease in expression of *TR-ACO2* and TR-ACO2 protein accumulation in the second fully-expanded leaves of both the NPS-treated Tienshan and the NPS-treated Kopu plants. These changes support the view that a response to water deficit in vegetative tissue involves a cessation of growth and is accompanied by a decrease in ACO gene expression and protein accumulation (although the PER was not specifically measured in this leaf developmental stage, there was no evidence that growth continued in this leaf when it ceased in the younger, first fully-expanded leaf). In contrast to *TR-ACO2*, the expression of *TR-ACO3* increased in the second fully expanded leaves of both Tienshan and Kopu when exposed to less than 10% SWC. In fully hydrated leaves, expression of *TR-ACO3* is closely related to a decrease in chlorophyll content in the leaves (ageing) (Hunter *et al.*, 1999; Du, 2004). However, in this thesis, decrease in total chlorophyll was only seen in the second fully-expanded leaves of Kopu, but not the Tienshan. Therefore, during water deficit, the increase in *TR-ACO3* expression may have increased as a response to stress which may have occurred (at least in Tienshan) even before ageing visibly occurred (as determined by a decrease in chlorophyll).

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Of further significance is that there were similar trends of *TR-ACO2* and *TR-ACO3* expression observed in the second fully-expanded leaves of both the Tienshan and Kopu plants. These results suggest that no significant differences exist in the responses of more mature leaves between the tolerant and sensitive white clover plants to a water deficit. Thus the differences in responses in the younger tissue (first fully-expanded leaf) and apical structures maybe the critical determinant for the tolerance (or otherwise) of white clover plants to water deficit.

5.2.3.4. Evidence for an Acclimation Response of the Sensitive White Clover Variety to Water Deficit

Examination of changes in water relations, PER, proline accumulation as well as changes in both *TR-ACO1* and *TR-ACO2* gene expression as well as TR-ACO1 and TR-ACO2 protein accumulation in the leaves of the NPS Tienshan and the NPS Kopu plants have all shown differences in the responses between the more drought tolerant-Tienshan and the more drought sensitive-Kopu plants to water deficit (Section 5.2.3.1 and 5.2.3.2.). Moreover, changes in *TR-ACO1* and *TR-ACO2* gene expression as well as TR-ACO1 and TR-ACO2 protein accumulation in the younger tissues could also be used as an indicator of differences in the responses of white clover plants to water deficit. Therefore, TR-ACO1 and TR-ACO2 abundance may be used as a marker for the adaptation responses of white clover to water deficit.

These differences were observed in both the Tienshan ecotype and the Kopu cultivar subjected to one cycle of water deficit treatment (NPS treatments). However, in this thesis, responses of these two varieties to two cycles of water deficit were also investigated. The two cycles of water deficit treatment (the PS treatments) were introduced following initial observations that only one cycle of water deficit showed that the PER in both varieties decreased at a similar SWC value (*ca.* 18%) and the PER also ceased at a similar SWC (at *ca* 6 to 8 %) in both varieties. It was suspected initially, therefore, that the Tienshan ecotype probably would not show its tolerant capability since the stock plants have been maintained in well-watered conditions and not in the conditions that mimic the original habitat of the ecotype before the

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water deficit experiments were undertaken. Therefore, some pre-exposure to a water deficit may be necessary for the Tienshan ecotype to display its tolerant properties. However, following prior exposure to a water deficit (PS treatment), all data obtained in this study indicated little change to the responses of Tienshan plants to the water deficit. However, the pre-stress treatment did alter the responses of the more sensitive Kopu cultivar to water deficit, as shown by alteration of the PER, *TR-ACO1* and *TR-ACO2* gene expression as well as TR-ACO1 and TR-ACO2 protein accumulation. Unlike the decrease in *TR-ACO1* expression and TR-ACO1 abundance observed in the apical structure of NPS Kopu, the expression of *TR-ACO1* and accumulation of TR-ACO1 in the apical structure of PS Kopu remained unchanged such that this response was now similar to those seen in both the NPS and the PS Tienshan (Figures 3.15 and 3.17). This suggests that following the pre-stress, meristem protection occurred in the PS Kopu. More interestingly, the expression of *TR-ACO2* and accumulation of TR-ACO2 in the first fully-expanded leaves of PS Kopu was also altered, and again followed the trends observed in the NPS and the PS Tienshan (Figure 3.17 and 3.18). When the pattern of the PER in the PS Kopu was compared, the decline in the PER also occurred more progressively after the pre-stress treatment (Figure 3.4). All of this evidence suggests that an acclimation to water deficit did occur in the more drought-sensitive Kopu cultivar after the pre-stress treatment which resulted in responses that were more similar to the drought-tolerant Tienshan ecotype.

There is now much evidence that shows the importance of acclimation of plants to environmental cues, including water deficit (Cameron *et al.*, 2008; Chaves *et al.*, 2009; Nunez *et al.*, 2009). Previous water deficit experiences have been reported to promote subsequent resistance of several tree species to a subsequent water deficit by preventing water loss through osmotic adjustment as reported in apricot (Ruiz-Sanchez *et al.*, 2000), oak (Villar-Salvador *et al.*, 2004) and two species of woody ornamental species: *Forsythia x Intermedia* cv. Lynwood and *Cotinus coggygria* (Cameron *et al.*, 2008). In these two ornamental woody species, acclimatised plants showed better control of water loss (higher leaf relative water content and a more

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positive LWP) as well as a higher concentration of leaf ABA (Cameron *et al.*, 2008). In addition, Chaves *et al.*, (2009) suggested that acclimation to a previous water deficit may change gene expression profiles resulting in the modification of plant physiology and morphology. Thus in this thesis, acclimatised Kopu plants demonstrated altered PER, *TR-ACO1* and *TR-ACO2* gene expression as well as altered TR-ACO1 and TR-ACO2 protein accumulation to give a more similar patterns to the more drought-tolerant Tienshan ecotype. These results suggest that either the plant hormone ethylene plays a direct role in the physiological alterations required for the acclimation process, or ethylene biosynthesis is altered as consequence of these changes.

Although ethylene evolution was not measured in this thesis, as ACO catalyses conversion of ACC into ethylene, it is likely that alteration in *ACO* gene expression and ACO protein accumulation could be used as an indicator of alteration in ethylene biosynthesis. Therefore, this evidence indicates that ACO, at least in part, plays some degree of regulation on ethylene biosynthesis during water deficit in white clover. Much evidence on regulation of ethylene biosynthesis by environmental cues has focused on ACS, as it is considered as the rate limiting enzyme for ethylene biosynthesis (for example Bleecker and Kende, 2000; Wang *et al.*, 2002). Therefore, ACS involvement in regulating ethylene biosynthesis in plants exposed to stress has been intensively studied and reviewed (Wang *et al.*, 2002; Tsuchisaka and Theologis, 2004). Studies on *Arabidopsis* suggested that only certain members of the *ACS* gene families are responsive to stress stimuli (Wang *et al.*, 2002; Kim *et al.*, 2003; Tsuchisaka and Theologis, 2004), and ACS activity is regulated at different levels including at the transcriptional, posttranscriptional and posttranslational levels (Wang *et al.*, 2002; Kim *et al.*, 2003; Tsuchisaka and Theologis, 2004).

Although the involvement of ACO has not yet been studied as extensively as ACS, evidence is now accumulating to show that ACO also plays a regulatory role in ethylene biosynthesis both during plant development and in response to hormonal and environmental cues. For example, *MaACO1* in banana is expressed during fruit ripening (Kesari *et al.*, 2007) while four members of the *ZmACO* gene family in

maize are differentially expressed in the root (Gallie *et al.*, 2009). Similarly, three members of *MD-ACO* gene family are differentially expressed in leaves and fruits of apple (Binnie and McManus, 2009). In addition, the *MaACO1* transcript and MaACO1 protein in banana was shown to be upregulated by exogenously applied ethylene, but not responsive to auxin treatment and it was downregulated by wounding and cold treatments (Choudhury *et al.*, 2008). Similar to this is the differential expression of two *ACO* genes in Chinese pear where expression of *PbACO1* is induced by exogenously applied ethylene while *PbACO2* is induced by mechanical wounding (Yamane *et al.*, 2007). In addition, three *NaACOs* transcripts in *Nicotiana attenuata* respond differentially to wound-induced ethylene production (von Dahl *et al.*, 2007). Evidence shown in this thesis supports these previous findings of differential expression of *ACO* transcripts and *ACO* proteins in response to environmental cues, including water deficit.

Although it has not yet been studied at the level of gene expression and protein accumulation, it was previously suggested that ACC oxidase activity is altered during water deficit in cotton petiole (McMichael *et al.*, 1972) and wheat (Chen *et al.*, 2002) with no significant changes in ACC levels. Together, these results indicate that *ACO* may be involved in fine tuning the control of ethylene biosynthesis during a water deficit. But how such environmental cues signal to effect changes of *ACO* transcripts and *ACO* protein abundance remains to be elucidated.

5.3. Part II. Isolation and Characterisation of ACC Synthase

In white clover, three ACS genes (*TR-ACS*) have been isolated previously from different developmental stages of leaves (Murray and McManus, 2005). These genes were isolated from the white clover Genotype 10F of the Grasslands Challenge cultivar, and each of these genes is expressed differentially during leaf development. However, none of these ACS members were induced by wounding or by IAA treatment (Murray and McManus, 2005). Therefore, it is speculated that more members of the *TR-ACS* gene family are present in white clover and these should include members whose expression will be regulated by abiotic and biotic stress

stimuli. As discussed previously, the water deficit treatments altered the expression of the *TR-ACO* gene family in both the apical structures and first-fully expanded leaves, and these changes differed between the drought-tolerant and drought-sensitive varieties (Section 5.2.). Therefore, water deficit was again used to induce expression of *TR-ACS* genes in order to isolate any novel, stress-related *TR-ACS* genes from the Tienshan and the Kopu varieties.

For these experiments, changes in the PER was used as an indicator to determine the critical stages of water deficit at which the *TR-ACS* genes were isolated from both the apical structures and first fully-expanded leaves (Figures 4.1). As ACS is the enzyme converting AdoMet into ACC which is then oxidised by ACO to ethylene (Yang and Hoffmann, 1984; Zarembinski and Theologis, 1994; Bleecker and Kende, 2000), it is then reasonable to predict that alteration in ACS expression might have occurred before any alteration in *ACO* production or before growth ceased. Therefore, five growth stages of white clover at different SWCs were selected to increase the chances of isolating stress-associated *TR-ACS* genes.

5.3.1. RT-PCR Isolation of Orthologous and Novel *TR-ACS* Genes

Nested RT-PCR, using degenerate primers designed to specifically amplify conserved ACS regions within many plant species, were used to isolate orthologous and novel *TR-ACS* genes from the Tienshan ecotype and Kopu cultivar. The partial reading frames of the *TR-ACS* genes were successfully amplified and the sequences obtained from the Tienshan ecotype showed high homology to *ACS* sequences from the Genebank databases. Based on nucleotide sequence identity, the *ACS* sequences obtained were categorised into four groups. For the purposes of this thesis, three groups were designated *TR-ACS1-T*, *TR-ACS2-T*, *TR-ACS3-T* (Figures 4.6.) because they have high identity with the *TR-ACS1*, *TR-ACS2* and *TR-ACS3* genes isolated from the 10F Genotype of Grasslands Challenge (Murray and McManus, 2005). The fourth group represented a novel ACS sequence, designated *TR-ACS4*.

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Colony PCR, using a second round of degenerate primer sets (ACSR2F and ACSR6R) successfully amplified ACS sequences and identified 2346 clones harbouring ACS transcripts (Figure 4.5). Further screening of these clones using gene-specific primers based on the sequence from the genotype 10-F of the cultivar Grasslands Challenge and designed to amplify *TR-ACS1*, *TR-ACS2* and *TR-ACS3* showed more than 98% of the total colonies harboured either the *TR-ACS1* or the *TR-ACS2* genes (Tables 4.1, 4.2, 4.3 and 4.4). The rest could not be amplified by *TR-ACS1* or *TR-ACS2* gene-specific primers. These results indicate that the *TR-ACS1* and the *TR-ACS2*-like transcripts, amplified from the Tienshan and the Kopu, have an identical nucleotide sequence in the primer regions to those from Genotype 10F. Moreover, it further suggested that *TR-ACS1* and *TR-ACS2* may be the most abundant *TR-ACS* transcripts in white clover leaves, and that *TR-ACS3* and *TR-ACS4* genes may be present at lower levels in the apical structures and first fully-expanded leaves, although the plants have been exposed to a water deficit. Further, the percentage of *TR-ACS1-T* clones obtained decreased as the SWC decreased, while the percentage of *TR-ACS2-T* clones obtained increased as the SWC decreased (Tables 4.1, 4.2, 4.3 and 4.4). This may reveal further that the abundance of these transcripts may be altered during a water deficit treatment.

Analysis of the sequences of the two other groups identified in this thesis revealed that one group shared 92% nucleotide homology to *TR-ACS3*, but this clone had an extra 60 nucleotides (20 amino acids). This group was then designated as *TR-ACS3-T*. Another group had low nucleotide homology and amino acid identity to the three *TR-ACS* or *TR-ACS-T* transcripts so may represent a novel *TR-ACS* gene and was, designated *TR-ACS4-T*. The *TR-ACS3-T* transcript comprised only 1% of the clones, and most of these were obtained from RNA isolated from the first fully-expanded leaves exposed to a water deficit and the number of clones obtained increased as the SWC decreased. Another feature of *TR-ACS3-T* is the 20 amino acids corresponding to the active site of the ACC synthase enzyme including three conserved amino acid residues (one of which is the catalytic lysine residue where binding to the pyridoxal phosphate is proposed to occur) (Yip *et al.*, 1990; Capitani *et al.*, 1999) are present.

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These do not occur in TR-ACS3 suggesting that TR-ACS3-T is a functional enzyme. Such differences may be due to variability within species and may indicate the biological adaptation for Tienshan such that it may produce more ethylene than the Genotype 10F, although such relationships remain to be explored further.

TR-ACS4-T was amplified only from RNA isolated from the apical structures of Tienshan grown at less than 10% SWC. Only two *TR-ACS4-T* clones were obtained suggesting a low abundance of this transcript in the total RNA pool. Thus, *TR-ACS4-T* could be a water deficit associated ACC synthase in white clover. The expression of *TR-ACS4-T* in response to a water deficit was examined in the Tienshan ecotype using first fully-expanded leaf material collected from the first NCEL trial conducted in 2006 (Appendix XV). However, no evidence for a consistent change in expression was observed and this was not repeated for the 2007 material.

Differential expression of the *ACS* gene family in different tissues, growth stages and in response to environmental cues is common for many plant species. In *Arabidopsis*, for example, eight of nine functional *ACS* genes are expressed in the seedlings while one, *ACS9*, is not (Tsuchisaka and Theologies, 2004). In flower, these authors found that only *ACS11* was expressed in the trichomes of the sepals, whilst *ACS1* was specifically expressed in the replum. A *PnACS* isolated from the cotyledon of *Pharbitis nil* is expressed constitutively in cotyledons, petioles, hypocotyls, roots and shoot apices of both light- and dark-grown seedlings with the highest expression level in the roots (Franskowski *et al.*, 2009). In addition, only certain members of the *ACS* gene family are reported to be induced by environmental cues. For example, *ACS2*, *ACS4*, *ACS5*, *ACS6* and *ACS7* in *Arabidopsis* are induced by various biotic and abiotic stresses (Liu and Zhang, 2004; Wang *et al.*, 2005), *ACS1* and *ACS2* expression is induced by the pathogen *Penicillium digitatum* in citrus fruit (Marcos *et al.*, 2005) and *NT-ACS2* in tobacco is induced by cold and light but not by wounding (Ge *et al.*, 2000).

Comparison of each of the *TR-ACS-T* sequences with ACS nucleotide sequences in the GeneBank determined that both *TR-ACS1-T* and *TR-ACS2-T* share highest

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homology with ACC synthases isolated from leaves, etiolated or IAA-treated seedlings, while *TR-ACS3-T* shares highest homology to *ACS* genes induced by wounding or ageing (Table 4.7). Thus these results suggest that the *TR-ACS-T* gene family is developmentally regulated. Many papers have described differential regulation of ACC synthase during plant development. Nine functional *AtACS* transcripts in *Arabidopsis* has been isolated and their expression has been reported to be either tissues-specific or some overlap in different tissues (Yamaguchi *et al.*, 2003; Tsuchisaka and Theologis, 2004). Similar to this, three *ZmACS* genes were also found to be specifically expressed in different tissues, with some overlapping or some expressed in specific tissues. Of those, *ZmACS6* was specifically expressed in the root cap and *ZmACS6* expression was found to be important for root development in impeded soil (Gallie *et al.*, 2009). These results suggest that the overlapping expression of *ACS* genes is not unusual.

Sequence comparison of *TR-ACS4-T* with the GeneBank database identified a wide range of similarities with other *ACS* genes isolated from many different developmental stages (Table 4.7). However, generally the results showed that *TR-ACS4-T* was closely related to other *ACS* genes that are induced by a variety of hormonal cues. It has been well documented for many plant species that not all member of ACS gene family is responsive to hormonal cues. In *Arabidopsis* roots, expression of 7 functional *ACS* genes (*ACS2*, 4, 5, 6, 7, 8, and 11) are induced by IAA (Yamagami *et al.*, 2003) while *ACS4*, *ACS5* and *ACS7* expression is induced by exogenously applied ethylene (Wang *et al.*, 2005). However, only *ACS7* is also responsive to gibberellic acid (GA₃), abscisic acid (ABA), salicylic acid (SA) and brassinosteroid (BR) application (Tang *et al.*, 2008). Therefore, it seems that *Arabidopsis ACS7* is responsive to a variety of hormonal cues, and *TR-ACS4-T* may be those responsive to hormonal cues in white clover.

5.3.2. Significance of *TR-ACS4* expression during Leaf Development

To examine *TR-ACS4-T* expression during leaf development, Tienshan stolons were trained out as a single stolon to display each stage of leaf development (Hunter *et al.*, 1999).

In the initial analysis, the expression of β -*actin* was also examined and used as an indicator for equal loading of RNA. However, β -*actin* expression seemed to be developmentally regulated as well (Figure 4.18) as less expression was observed in total RNA isolated from yellowing/senescent leaves (leaf no 15 to 18). Many reports suggest that the mRNA level of β -*actin* varies with tissues/organs at different developmental stages and with the physiological state of the plant (Sturzenbaum and Kille, 2001; Schmittgen and Zakrajsek, 2004; Nolan *et al.*, 2006). Thus using β -*actin*, expression may not be suitable as an internal RNA loading control (Sturzenbaum and Kille, 2001; Schmittgen and Zakrajsek, 2004, Jain *et al.*, 2006; Nolan *et al.*, 2006). Therefore, RT reactions were performed again using random hexamer primers, and prior to expression analysis the expression of *18S rRNA* was also examined and used as the second reference gene for the internal RNA loading control (Figure 4.19).

Expression analysis of *TR-ACS4-T* during leaf development, using both reference genes set as controls, showed two major peaks of expression in the Tienshan stolon. Expression was highest in the apical structures which decreased toward the younger mature green leaves and then increased again at the late mature leaf stage and in leaves at the onset of senescence. Expression decreased again in senescent leaves (Figures 4.18 and 4.19). The apical structures of white clover comprises meristematic tissues (Chen and McManus, 2006) and such rapidly dividing and expanding tissues exhibit relatively high rates of ethylene production (Osborne, 1999) as well as auxin synthesis (Aloni *et al.*, 2003; DeMason and Polawick, 2009). In white clover, Hunter *et al* (1999) identified two peaks of ethylene evolution that occur during leaf development in white clover: in the apical structures and in late mature leaves. Although in this study, ethylene evolution was not measured and a different ecotype of white clover used, the pattern of *TR-ACS4-T* expression appears to be correlated with ethylene evolution as observed by Hunter *et al* (1999). This further confirmed

the bioinformatic analysis (Table 4.7) which showed that *TR-ACS4-T* was closely related to *ACS* genes isolated from seedlings as well as *ACS* genes induced by wounding, auxin and ethylene. So, further exploration as to whether *TR-ACS4-T* is induced by auxin and ethylene was then undertaken (Section 5.3.3).

5.3.3. Ethylene and Auxin Induced the Expression of *TR-ACS4-T*

Using sqRT-PCR as previously described, expression of *TR-ACS4-T* was studied following treatment of plants with ethylene or NAA in a controlled environment growth chamber. The analysis was performed on RNA isolated from the first fully-expanded leaves, a tissue in which expression of *TR-ACS4-T* is low during normal plant development (Figures 4.18 and 4.19). The specific ethylene action inhibitor 1-MCP was used as a control treatment to determine the effect of positive feedback of ethylene-induced *TR-ACS4-T* and NAA-induced ethylene. Control treatments were also established by incubating white clover plants in the growth chamber and harvesting at the same sampling time as the NAA and ethylene treatments to exclude the possibility of diurnal control of *TR-ACS4-T* expression. Another set of control plants were sprayed with ethanol and tween-20 to determine if ethanol and tween-20 (used to dilute the NAA) will induce ethylene in their own right (independent of the NAA treatment).

Expression of *β-actin* was examined and in the 1-MCP-, NAA- and ethylene-treated plants, a similar expression of *β-actin* was observed (Figures 4.20 and 4.21), thus indicating that expression remained constant over the 4 hour period of treatments. Therefore *β-actin* expression can be used in these short-time induction treatments.

In the ethylene-treated plants, expression of *TR-ACS4-T* was induced by ethylene soon after the plants were exposed to the hormone as seen by an increased abundance of the *TR-ACS4-T* transcripts after 1 hour of treatment. However, prolonged ethylene treatment (more than 1 hour) resulted in the expression of *TR-ACS4-T* returning to a similar level as observed in the untreated plants (0 h). Treatments with 1-MCP blocked this ethylene-induced *TR-ACS4-T* expression at 1 h. To determine if this increased expression of *TR-ACS4-T* was due to ethylene induction following

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incubation of plants in the chamber or due to differences in the time of each sampling point (diurnal effect), the expression of *TR-ACS4-T* was also observed in untreated plants. No induction of *TR-ACS4-T* was seen in these plants harvested at the four time intervals, suggesting that the induction observed was due to exogenous ethylene (or 1-MCP) treatment.

Ethylene production has been reported to be regulated by two systems: the auto-inhibitory (negative feedback) and the autocatalytic (positive feedback) system (Tang *et al.*, 2008), and positive or negative feedback regulation of ethylene biosynthesis by ethylene itself has been well documented (Kende, 1993). Ethylene can, in turn, positively regulate endogenous ethylene biosynthesis by increasing the expression of SAM synthetase or ACC synthesis (Riov and Yang, 1982; Inaba and Nakamura, 1996; Nakatsuka *et al.*, 1998). Indeed, induction of many *ACS* genes by exogenous ethylene application has been reported in many plant species including *Arabidopsis* (Tang *et al.*, 2008), tomato (Nakatsuka *et al.*, 1998), banana (Choudhury *et al.*, 2008), plum (El-Sharkawy *et al.*, 2008). In many systems, not all of the *ACS* genes are positively induced by ethylene, with only the expression of three out of the 9 *ACS* genes in *Arabidopsis* positively induced by ethylene (Tang *et al.*, 2003). The present study reports that *TR-ACS4-T* is the member of *ACS* gene family in white clover which is positively regulated by exogenous application of ethylene. Thus this result further supports the view that feedback regulation of ethylene biosynthesis occurs for only certain members of the *ACS* gene family. The process by which the auto-regulation of ethylene biosynthesis by certain members of the *ACS* gene family is regulated and its significance during plant growth and development are still unclear and remains to be investigated.

TR-ACS4-T expression was also induced by NAA treatment (Figure 4.21) confirming the previous indication as shown by the bioinformatics data of the *TR-ACS4-T* sequence homology. However, induction of *TR-ACS4-T* by NAA occurred only after 4 h of treatment and exposure to 1-MCP abolished this induction. Thus suggests that the induction of *TR-ACS4-T* by NAA occurred indirectly and was mediated by ethylene. To confirm that this induction was not due to the effect of spraying with

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ethanol and Tween-20 or diurnal effects, one set of plants were treated with ethanol and Tween-20 only. No significant induction of the *TR-ACS4-T* was observed in this control treatment (Figure 4.21), thus suggesting that induction of *TR-ACS4-T* was due to NAA and was not due to the chemicals used to dissolve the NAA or a diurnal expression effect.

Auxin is reported to be one of the important hormones that interacts with ethylene and this interaction normally results in specific auxin-ethylene phenotypes (Abeles *et al.*, 1992; El-Sharkawy *et al.*, 2008; Tang *et al.*, 2008; Chaabouni *et al.*, 2009). Induction of different *ACS* genes expression by auxin have been reported in many plants species including *Arabidopsis* (Yamaguchi *et al.*, 2003; Tsuchisaka and Theologis, 2004), mungbean (Yoon *et al.*, 1997); rice (Chae *et al.*, 2000); banana (Choudhury *et al.*, 2008), apple (Kondo *et al.*, 2009). However, auxin only induces the expression of specific *ACS* genes. Of the nine *ACS* transcripts in *Arabidopsis*, eight were induced by auxin (Yamaguchi *et al.*, 2003; Tsuchisaka and Theologis, 2004) whilst in apple only the expression of *MdACS4* was induced by auxin, but not *MdACS1* and *MdACO1* (Kondo *et al.*, 2009). Previous studies in white clover suggested that the expression of *TR-ACS1*, *TR-ACS2* or *TR-ACS3* was not induced by auxin (Murray and McManus, 2006). However, *TR-ACS-4-T* in the present study was induced by NAA-treatment. This further suggests that different stimuli affect different members of *ACS* multigene families. Auxin-induced expression of ACC synthase genes has been reported in many plant species including cucumber (Siomi *et al.*, 1999), mungbean (Kim *et al.*, 2001), watermelon (Salman-Minkov *et al.*, 2008) and apple (Kondo *et al.*, 2009). Of these, Kim *et al.* (2001) reported the IAA-induced expression of *VR-ACS1* may be mediated by cross-talk between IAA and ethylene since exogenous application of ethylene decreased expression of *VR-ACS1*. In this thesis, both ethylene and NAA treatments increased the expression of *TR-ACS4* and blocking of ethylene action by 1-MCP resulted in an earlier increase in NAA-induced *TR-ACS4-T* expression (Figure 4.21), indicating that NAA-induced *TR-ACS-4-T* expression was indirect and was mediated by ethylene too.

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Treatment with 1-MCP has been well documented to act as an inhibitor of ethylene action, and is reported to act by binding to ethylene receptors thus preventing the ethylene binding to its receptor (Nakatsuka *et al.*, 1998). Application of 1-MCP to avocado and pears had been reported to significantly delay the expression of *PA-ACSI* and *PP-ACSI* transcripts, respectively. In this thesis, 1-MCP did not alter the expression of the ethylene-induced *TR-ACS4-T* gene, suggesting that 1-MCP partially blocked the positive feedback of ethylene induction of *TR-ACS4-T*.

Taken together, the sequence analysis and the bioinformatic investigation of other *ACS* sequences with high identity to *TR-ACS4-T* and the induction study all suggest that the *TR-ACS4-T* expression is not only regulated in a developmental manner but is also induced by exogenous application of ethylene and auxin. *TR-ACS4-T* is expressed in the apical structures and late mature green leaves where the balance between ethylene and auxin will be expected to be important in controlling white clover stolon growth. This interesting phenomenon of *TR-ACS4-T* expression indicates the biological importance of *TR-ACS4-T* in the regulation of ethylene biosynthesis, which is possibly altered by various biotic and abiotic signals which alter auxin and ethylene biosynthesis. Such interactions, and their roles in regulating white clover adaptation or responses to various abiotic and biotic stimuli will be interesting aspects to be studied further.

5.4. Conclusions

Two varieties of white clover previously suggested to have differing sensitivities to water deficit, were exposed to two water deficit treatments. Selected physiological responses of these two white clover varieties were compared as well as changes in ACO gene expression and protein abundance were observed in selected developmental stages of the leaves.

Plant water relations, changes in the PER and proline accumulation were employed to investigate differences in the sensitivity of the two varieties to water deficit. The drought tolerant Tienshan ecotype showed a less negative LWP when compared with the Kopu cultivar thus indicating better control of water loss which was probably due to a higher proline accumulation, and translated to a more gradual decrease of the PER.

Examination of changes in *TR-ACO* gene expression and TR-ACO protein accumulation revealed that ACO at both the transcriptional and posttranscriptional levels may be part of a response to a water deficit. In the apical structures and first-fully expanded leaves, different patterns of *TR-ACO1* and *TR-ACO2* gene expression as well as TR-ACO1 and TR-ACO2 protein accumulation were observed between the more drought-tolerant Tienshan ecotype and more drought-sensitive Kopu cultivar. In addition, *TR-ACO2* is the main transcript altered by water deficit in the first-fully expanded leaves.

Observation of one cycle of water deficit (the NPS Treatment) showed little change in the expression of the *TR-ACO1* gene and accumulation of TR-ACO1 protein in the Tienshan ecotype suggesting that apical structures of Tienshan are protected during the water deficit. In contrast, the *TR-ACO1* gene and TR-ACO1 protein abundance in the apical structures of Kopu decreased suggesting that the meristem of the Kopu was not protected during water deficit, may indicate an injury response. Differences in the responses between the Tienshan and the Kopu were also observed in the first fully-expanded leaves where TR-ACO2 is the main ACO regulating ethylene biosynthesis in this tissue. In Tienshan, both the expression of *TR-ACO2* and

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accumulation TR-ACO2 decreased as water deficit progressed and this was associated with a decrease in the PER. In Kopu however, expression of *TR-ACO2* gene and accumulation of TR-ACO2 protein increased and may again reflect an injury response to the water deficit. Therefore, changes in expression of *TR-ACO1* and *TR-ACO2* could be used as a marker for responses of white clover to a water deficit, and may suggest further that changes in ACO gene expression and protein accumulation may contribute to the response shown in both varieties.

Following exposure to a previous water deficit, growth responses and the pattern of *TR-ACO1* and *TR-ACO2* gene expression as well as TR-ACO1 and TR-ACO2 protein abundance did not change in the Tienshan ecotype, but did change in the Kopu cultivar. Kopu plants that had been pre-stressed (PS Kopu) displayed a more gradual decrease in the PER during the second cycle of water deficit. The apical structures of PS-treated Kopu plants exhibited no significant change in the expression of *TR-ACO1* and accumulation of TR-ACO1 during the water deficit. This pattern was now in common with that observed in the NPS and PS Tienshan plants and suggests some meristem protection. Moreover, the pattern of *TR-ACO2* expression and TR-ACO2 accumulation in the first fully-expanded leaves of acclimatised Kopu plants was also altered to display a similar pattern as those shown by the NPS and PS Tienshan plants. Thus the PS-treated Kopu plants responded to a water deficit in a similar way as the drought tolerant-Tienshan plants, suggesting some degree of acclimation of the drought-susceptible Kopu variety and again that ACO gene expression and protein accumulation may be associated with the response of plants to a water deficit.

However, expression of *TR-ACO3* observed in the first- and second-fully expanded leaves suggest that there is no significant changes in the expression of *TR-ACO3* in the first fully-expanded leaves of both NPS- and PS-treated Tienshan ecotype and Kopu. This suggest that the responses of younger tissues maybe the critical determinant for the tolerance (or otherwise) of white clover plants to water deficit. Further, expression did increase in the more mature, second fully-expanded leaves, of both NPS- and PS-treated Kopu subjected to less than 10% SWC, suggesting that

older tissues are more prone to premature ageing (the chlorophyll levels in this tissue also decreased) and again that younger tissues may be the critical determinant for the tolerance (or otherwise) of white clover plants to water deficit.

Three *TR-ACS-like* transcripts and a novel *TR-ACS* gene, designated *TR-ACS4-T*, were isolated from the Tienshan ecotype following a water deficit treatment. All of the *TR-ACS* genes isolated from the Tienshan ecotype encoded functional enzymes and contained the conserved amino acid residues in the active site which are required for binding of the substrate to the PLP co-factor.

The novel *TR-ACS4-T* gene was isolated from the apical structures of Tienshan grown at less than 10% SWC, but expression analysis did not indicate that expression of the gene was influenced by water deficit. Bioinformatic analysis indicated a wide range of similarities of *TR-ACS4-T* with other *ACS* genes isolated from various developmental stages of plants, and also those induced by hormonal cues. In the stolon of Tienshan, *TR-ACS4-T* expression peaked in the apical structures then decreased in the younger mature leaves and then increased again as the mature green leaves developed toward the onset of senescence. Expression decreased again as the leaves senesced. In addition, *TR-ACS4-T* expression was induced by both ethylene and auxin, but the auxin-induced *TR-ACS4-T* was mediated by ethylene. All of this evidence suggests that the *TR-ACS4-T* expression is not only developmentally regulated, but is also responsive to hormonal cues.

5.5. Future Directions

The work in this thesis comprised two major parts. The first part was the characterisation of ACO, the enzyme that catalyses the last step of ethylene biosynthesis during water deficit in two white clover varieties that show a differential sensitivity to water deficit: the Tienshan ecotype and the Kopu cultivar. The second part of this thesis isolated and characterised *TR-ACS-like* genes and a novel *TR-ACS4-T* from the Tienshan ecotype following a water deficit.

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Demonstration that ACO gene expression and protein accumulation is influenced by a water deficit indicated that ACO may serve as a fine tuning regulator of ethylene biosynthesis in response to plants to environmental cues. However, in this thesis, ethylene evolution was not measured. Therefore it is still unclear as to whether changes in ACO gene expression and accumulation observed during a water deficit correlated with any alteration in ethylene production. Measurement of ethylene production in parallel with changes in any ACO gene expression and protein accumulation under water deficit will therefore need to be explored. In addition, differential expression of the *ACO* gene family observed between the drought-tolerant and drought sensitive varieties indicate that the level of *ACO* expression and probably ethylene production may be crucial for any response to a water deficit. Further studies, using ACO knock outs (perhaps using RNAi technology) during water deficit will be useful tool to gain more understanding in how directly *ACO* regulates plant responses during a water deficit.

Investigation on selected physiological changes and TR-ACO gene and TR-ACO protein accumulation during water deficit in these two varieties revealed that *TR-ACOs* gene and TR-ACOs proteins could be used as markers for differential responses of white clover plants to water deficit. Although the physiological data supported these indications, more evidence is needed to confirm that, and these include;

- Histological analysis will be a useful tool to observe differences in the meristem of the two varieties during water deficit.
- The capability of Tienshan plants to maintain a higher leaf water status may have been a result of better control of stomatal conductance in the Tienshan leaves. As this was suggested to be mediated by both ethylene and ABA, the relationship of these two hormones in controlling water loss in the drought-tolerant and sensitive white clover will be an interesting aspect to be studied. In addition, changes in the PER is also driven by the balance of ethylene and other plant hormones, and again an understanding of how ethylene biosynthesis alters the biosynthesis of other hormones, such as ABA, during the water deficit in these two varieties will

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be useful to understand how the adaptive responses of the tolerant variety are controlled.

In the second part of study, the water deficit associated *TR-ACS4* gene has been isolated and the biological significant of *TR-ACO4* expression during leaf development and ethylene and auxin treatments investigated. Further studies will be necessary as described below.

- Isolation of full length sequences of the four *TR-ACS* genes will be useful to identify more clues as to their biological function. Analysis of the promoter regions will provide clues as to what type of transcription binding domains are possibly involved in the regulation of expression.
- Investigation of different environmental cues on the expression of *TR-ACS4-T* will be useful to investigate further if the *TR-ACS4-T* gene is the stress-associated ACS in white clover.
- Investigation of the changes of TR-ACS accumulation will be useful to further characterise if regulation of ethylene biosynthesis during development and by environmental cues occurred at both the transcriptional and post-translational level. However, the ACS enzyme is naturally present in very low abundance making it difficult to be detected. Additional approaches such as immunoprecipitation of the protein as well as tagging methods to increase the possibility of identification and detection of TR-ACS protein will to be an important aspect to be developed.

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APPENDICES

Analysis of Variance for Experiment II (2007) (Appendices I to V)

Appendix I: Analysis of variance for changes in soil water content (SWC)

1. NPS Tienshan vs PS Tienshan

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	1.20	1.20	1.20	0.66	0.420	NS
Day	14	7370.62	7370.62	526.47	288.22	0.000	S
Water-Deficit*Day	14	46.73	46.73	3.34	1.83	0.046	NS
Error	90	164.39	164.39	1.83			
Total	119	7582.94					

NS: Non significantly different S : Significantly different

2. NPS Kopu and PS Kopu

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	2.80	2.80	2.80	2.31	0.135	NS
Day	8	3999.21	3999.21	499.90	411.76	0.000	S
Water-Deficit*Day	8	16.34	16.34	2.04	1.68	0.124	NS
Error	54	65.56	65.56	1.21			
Total	71	4083.92					

NS: Non significantly different S : Significantly different

Appendix II: Analysis of variance for changes in leaf water potential (LWP)

1. NPS Tienshan vs PS Tienshan

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	0.00085	0.00085	0.00085	0.58	0.450	NS
Day	14	7.65751	7.65751	0.54697	369.16	0.000	S
Water-Deficit*Day	14	0.15867	0.15867	0.01133	7.65	0.000	S
Error	90	0.13335	0.13335	0.00148			
Total	119	7.95039					

NS: Non significantly different S : Significantly different

2. NPS Kopu and PS Kopu

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	0.00517	0.00517	0.00517	2.09	0.154	NS
Day	8	8.03517	8.03517	1.00440	405.29	0.000	S
Water-Deficit*Day	8	0.21327	0.21327	0.02666	10.76	0.000	S
Error	54	0.13382	0.13382	0.00248			
Total	71	8.38743					

NS: Non significantly different S : Significantly different

Appendix III: Analysis of variance for changes in petiole elongation rate (PER)

1. NPS Tienshan vs PS Tienshan

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	2.852	2.852	2.852	5.35	0.023	NS
Day	14	342.929	342.929	24.495	45.99	0.000	S
Water-Deficit*Day	14	14.929	14.929	1.066	2.00	0.026	S
Error	90	47.938	47.938	0.533			
Total	119	408.648					

NS: Non significantly different S : Significantly different

2. NPS Kopu vs PS Kopu

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	67.087	67.087	67.087	87.43	0.000	S
Day	8	784.778	784.778	98.097	127.84	0.000	S
Water-Deficit*Day	8	38.944	38.944	4.868	6.34	0.000	S
Error	54	41.438	41.438	0.767			
Total	71	932.247					

NS: Non significantly different S : Significantly different

Appendix IV: Analysis of variance for changes in chlorophyll concentration

1. NPS Tienshan vs PS Tienshan

a. First fully-expanded leaves

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	28152	28152	28152	8.87	0.004	S
Day	14	219788	219788	15699	4.95	0.000	S
Water-Deficit*Day	14	74139	74139	5296	1.67	0.076	S
Error	90	285588	285588	3173			
Total	119	607666					
Total	71	932.247					

NS: Non significantly different S : Significantly different

b. Second fully-expanded leaves

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	279.1	279.1	279.1	0.68	0.411	NS
Day	14	70416.1	70416.1	5029.7	12.27	0.000	S
Water-Deficit*Day	14	89938.1	89938.1	6424.1	15.67	0.000	S
Error	90	36892.8	36892.8	409.9			
Total	119	197526.0					

NS: Non significantly different S : Significantly different

2. NPS Kopu vs PS Kopu

a. First fully-expanded leaves

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	363662	363662	363662	1228.92	0.000	S
Day	8	156335	156335	19542	66.04	0.000	
Water-Deficit*Day	8	258801	258801	32350	109.32	0.000	
Error	54	15980	15980	296			
Total	71	794778					

NS: Non significantly different S : Significantly different

Appendices

b. Second fully-expanded leaves

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Water-Deficit	1	52	52	52	0.19	0.662	NS
Day	8	202089	202089	25261	94.66	0.000	S
Water-Deficit*Day	8	174086	174086	21761	81.54	0.000	S
Error	54	14411	14411	267			
Total	71	390638					

NS: Non significantly different S : Significantly different

Appendix V: Analysis of variance for proline accumulation

1. NPS Tienshan vs PS Tienshan

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Water-Deficit	1	190	190	190	0.42	0.517 NS
Day	14	2643409	2643409	188815	419.79	0.000 S
Water-Deficit*Day	14	279460	279460	19961	44.38	0.000 S
Error	90	40480	40480	450		
Total	119	2963540				

NS: Non significantly different S : Significantly different

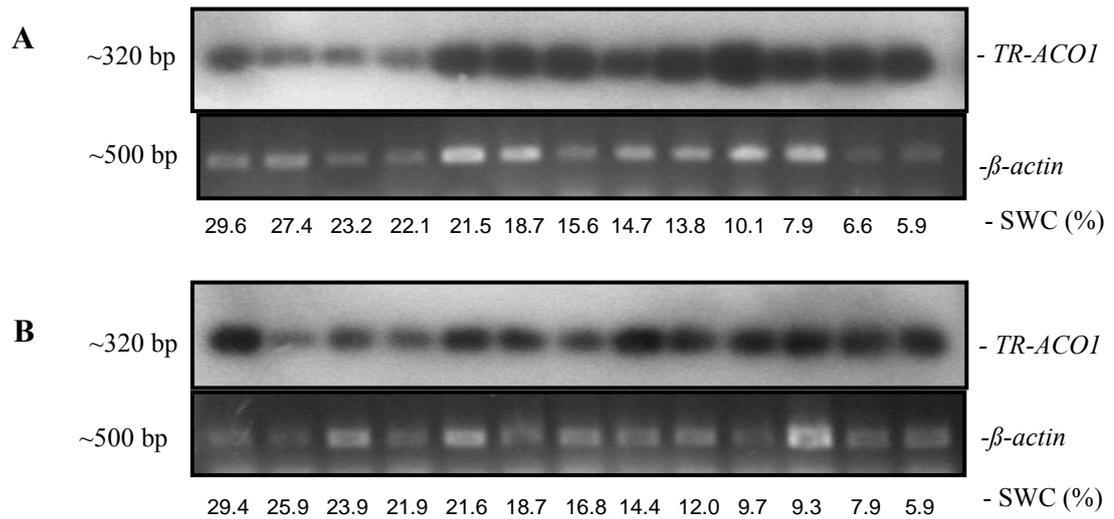
2. NPS and PS Kopu

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Water-Deficit	1	64916	64916	64916	217.14	0.000 S
Day	8	729108	729108	91139	304.85	0.000 S
Water-Deficit*Day	8	166885	166885	20861	69.78	0.000 S
Error	54	16144	16144	299		
Total	71	977054				

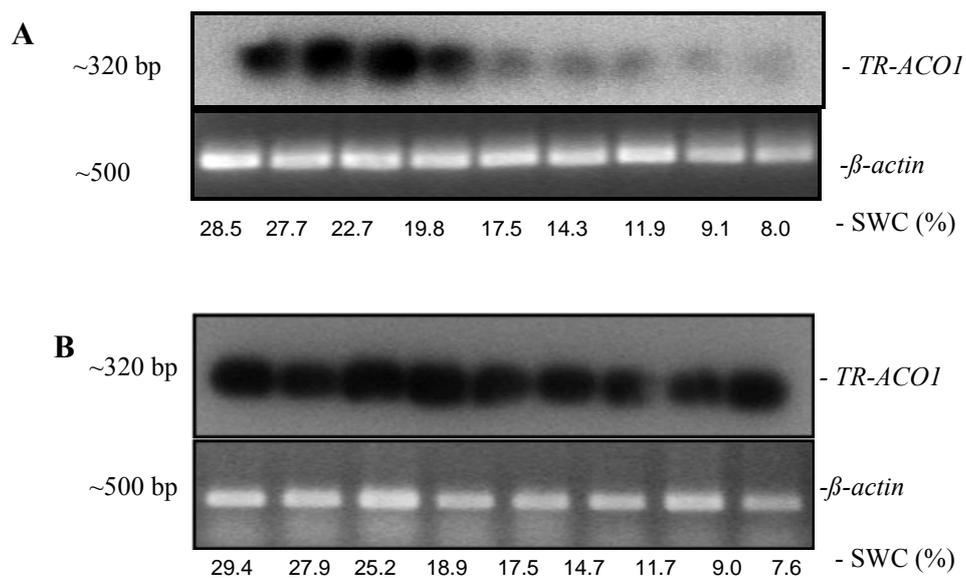
NS: Non significantly different S : Significantly different

Appendix VI: Changes in *TR-ACO1* expression in the apical structures of NPS and PS Tienshan and NPS and PS Kopu in experiments conducted in 2006

1. Expression of *TR-ACO1* in the apical structures of NPS Tienshan (A) and PS Tienshan (B) at different SWCs (as indicated)

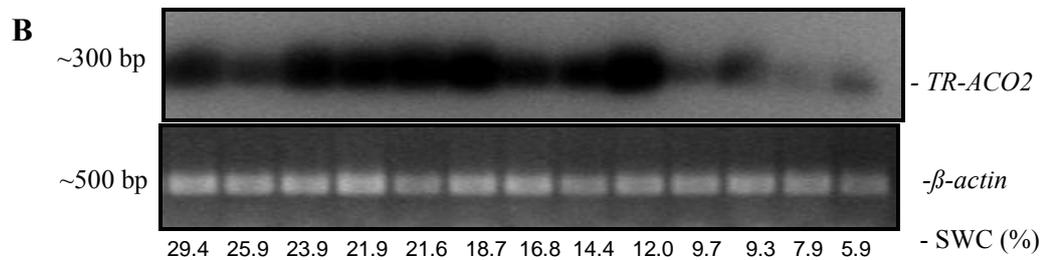
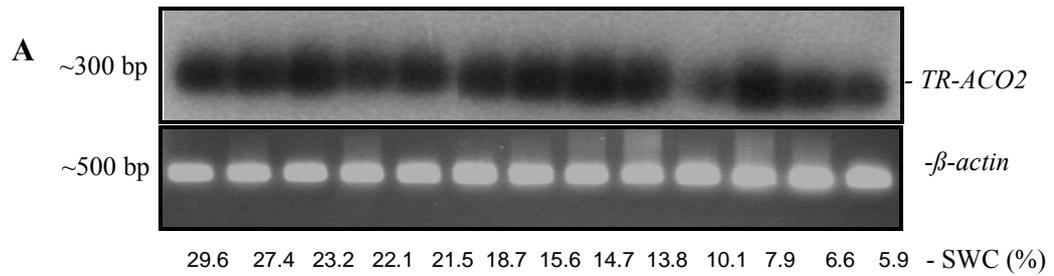


2. Expression of *TR-ACO1* in the apical structures of NPS Kopu (A) and PS Kopu (B) at different SWCs (as indicated)

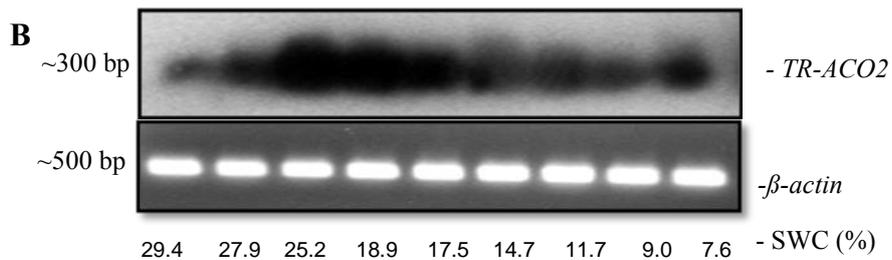
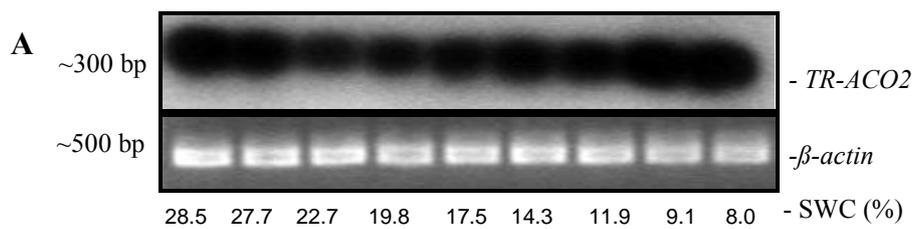


Appendix VII: Changes in *TR-ACO2* expression in the apical structures of NPS and PS Tienshan and NPS and PS Kopu in experiments conducted in 2006

1. *TR-ACO2* expression in the first fully-expanded leaves of NPS Tienshan (A) and PS Tienshan (B) at different SWCs (as indicated)



2. *TR-ACO2* expression in the first fully-expanded leaves of NPS Kopu (A) and PS Kopu (B) at different SWCs (as indicated)



Appendices

Appendix VIII: Alignment of *TR-ACO1*, *TR-ACO2* and *TR-ACO3*

```
TR-ACO2 ATGGAGAACTTCCCAATCATCAACTTAGAAAACCTCAATGGTGAGGAGAGAAAAGCTACC 60
TR-ACO3 ATGATGAACTCCCCAATTATCAGCTTGGAGAGACTCAATGGTGTGGAGAGAAAAGATACC 60
TR-ACO1 ATGGAAAACCTTCCCAATGTTGACATGGGGAAGCTTAACACAGAAGAGAGAAAATCAACC 60
***      ****      ***** *   * * * * *   * *   *   *   *   *   *   *   *   *   *

TR-ACO2 ATGGAAAAAATCAAGGATGCATGCGAGAATTGGGGCTTCTTTGAGCTGGTGAATCATGGC 120
TR-ACO3 ATGGAGAAAAATAAGGATGCATGCCAGAATTGGGGATTCTTTGAGCTGGTGAATCATGGC 120
TR-ACO1 ATGGAGAAGATCAAAGATGCTTGCAGAGAATTGGGGCTTCTTTGAGTGGTGAACCATGGA 120
***** * * * * *   ***** * *   ***** *   ***** *   ***** *   *****

TR-ACO2 ATATCTCATGACTTAATGGACACTGTGGAAAGGTTGACAAAAGAACACTACAGGATATGC 180
TR-ACO3 ATACCTCATGACCTTATGGACACATTGGAGAGATTGACCAAAGAGCACTACAGGAAATGC 180
TR-ACO1 ATATCTATTGAGATGATGGACAAAGTGGAGAAGCTCACAAAAGATCACTACAAGAAGTGT 180
*** * *   *** *   ***** *   * *   *   *   *   *   *   *   *   *   *

TR-ACO2 ATGGAACAAAGATTC AAGGATTTGGTGGCCAACAAAGGACTAGAGGCTGTTCAAACTGAG 240
TR-ACO3 ATGGAGCAGAGGTTT AAGGAATTGGTATCAAGCAAAGGCTTAGATGCTGTCCAAACTGAG 240
TR-ACO1 ATGGAACAAAGGTTCA AAGAAATGGTTTCAAGCAAAGGTTTGGAGTGTGTTCAGTCAGAA 240
***** * * * * * * * * *   ***** * *   ***** * *   * *   * *   * *

TR-ACO2 GTCAAAGACATGGACTGGGAGAGTACCTTCCACTTGCCTCACCTACCTGAGTCAAACATT 300
TR-ACO3 GTCAAAGATATGGATTGGGAAAGTACCTTCCATGTTGACATCTCCCTGAATCAAACATT 300
TR-ACO1 ATAAATGACTTAGATTGGGAAAGCACTTCTTTTTGCGCCATCTTCCATTTTCTAATATT 300
* * * * * * * *   ***** * * * * *   * * * * *   * * * * *   * * * * *

TR-ACO2 TCAGAGGTCCTGATCTCACTGATGAATACAGGAAAGCAATGAAGGAATTTGCTTTGAAG 360
TR-ACO3 TCAGAGCTCCCTGATCTCAGTGAATACAGGAAGGTGATGAAGGAATTTCTTTGAGG 360
TR-ACO1 TCAGAGATCCAGATCTTGATGATGATTACAGGAAGATAATGAAGGAATTTGCACAAAAA 360
***** ***** ***** ***** ***** ***** ***** ***** * *

TR-ACO2 CTAGAGAACTAGCAGAGGAGCTGCTAGACTTATTATGTGAGAATCTTGGACTAGAAAAG 420
TR-ACO3 TTAGAGAAGCTAGCAGAAGAGCTTTTGGACTTGTATGTGAGAATCTTGGACTTGAAAAA 420
TR-ACO1 TTAGAGAATCTGGCTGAGGAACCTTCTGACTTATTATGTGAGAATCTTGGCTTGAAAAA 420
***** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

TR-ACO2 GGATACCTCAAAAAAGCCTTTTATGGATCAAAGGGACCAACTTTTGGCACCAAGGTTGCA 480
TR-ACO3 GGTACCTCAAAAAAGCCTTCTATGGATCAAGAGGACCAACTTTCCGGCACCAAGGTAGCC 480
TR-ACO1 GGGTATTTGAAGAAGGTGTTTATGGTTCAAAGGTCCAAACCTTTGGTACAAAAGTTAGT 480
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

TR-ACO2 AACTACCCTCCATGCCAAAAACCAGACCTTGTAAGGCTCCGAGCACACACCGATGCC 540
TR-ACO3 AACTACCCTCAATGCCAATCCAGAGCTGGTGAAGGGTCTCCGTGCTCACACCGATGCC 540
TR-ACO1 AACTATCCTCCTTGCTCCTAAGCCTGACCTTATTAAGGGACTTAGAGCCCATACAGATGCT 540
***** ***** * * * * * * * * * * * * * * * * * * * * * * * * * *

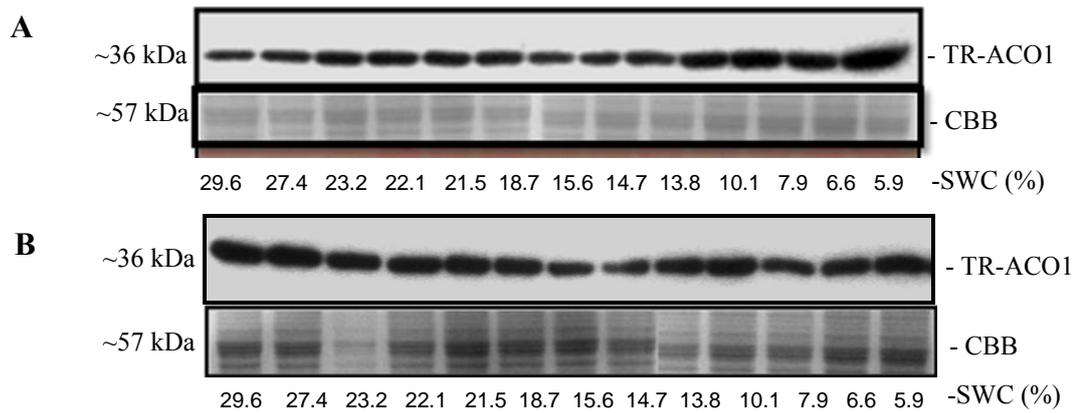
TR-ACO2 GGTGGAATAATCCTCCTTTTCCAAGATGACAAAGTCAGTGGCCTTCCAGCTTCTCAAAGAT 600
TR-ACO3 GGTGGGATCATCCTTCTCTTCCAGGATGACAAAGTCAGCGGCCTTCCAGTACTCAAAGAC 600
TR-ACO1 GGTGGCATCATCCTTCTCTTCCAAGATGACAAAGTCAGTGGACTTCCAGTCTCTCAAAGAT 600
***** * *   ***** * *   ***** ***** ***** * *   ***** *****

TR-ACO2 GGTAATGGGTAGATGTTCTCCCATGCATCATTCCATTGTCATCAACCTTGGTGACCAA 660
TR-ACO3 GACGAGTGGATCGATGTTCCCCAATGCGTCACTCCATTGTTGTCAACCTTGGTGACCAG 660
TR-ACO1 GACCAATGGATTGATGTCCCTCCAATGCGTCACTCTATTGTCATCAACCTTGGTGATCAA 660
*   * * * * *   ***** * *   * * * * *   * *   * * * * *   ***** * *

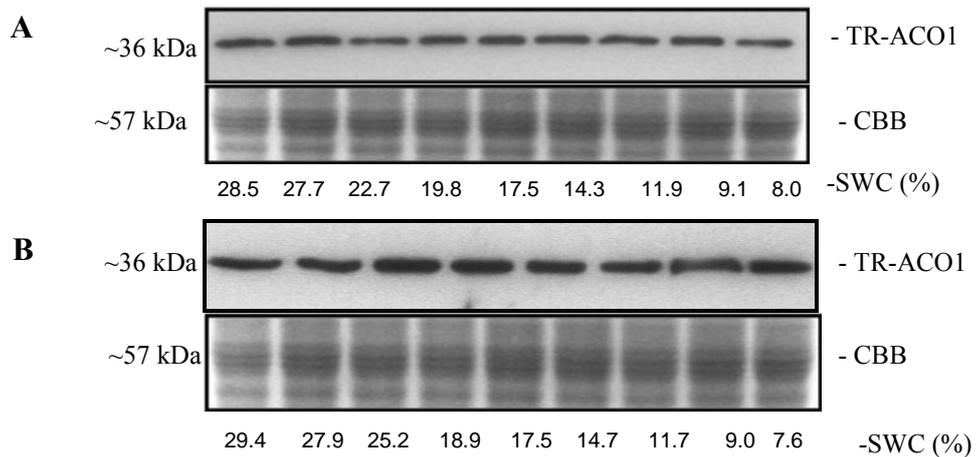
TR-ACO2 CTCGAGGTAATAACAAATGGTAAGTACAAGAGTGTGGAACATCGTGTGATAGCACAAAGT 720
TR-ACO3 CTCGAGGTAATAACAAATGGTAAATATAAGAGTGTGGAGCACCGTGTGATAGCACAAACA 720
TR-ACO1 CTTGAAGTCATAACAAATGGGAAGTACAAGAGTGTGATGCATAGAGTAATTGCTCAAACA 720
* * * * *   ***** ***** * * * *   ***** * *   * * * * *   * * * * *
```


Appendix IX: Western analysis, based on equal fresh weight loadings, of accumulation of TR-ACO1 protein in the apical structures of NPS Tienshan and NPS Kopu (for Experiment II, 2007).

1. Accumulation of TR-ACO1 in the apical structures of NPS Tienshan (A) and PS Tienshan (B) at different SWCs (as indicated)

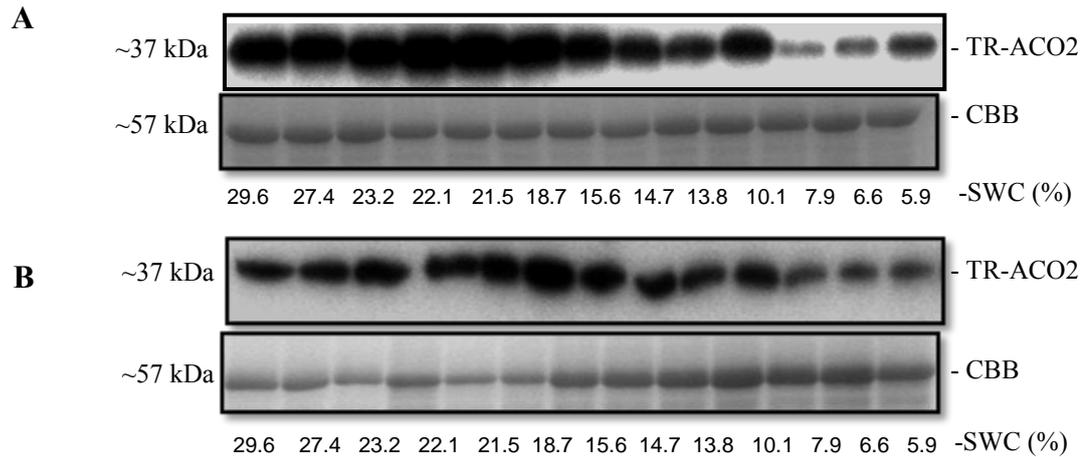


2. Accumulation of TR-ACO1 in the apical structures of NPS Kopu (A) and PS Kopu (B) at different SWCs (as indicated)

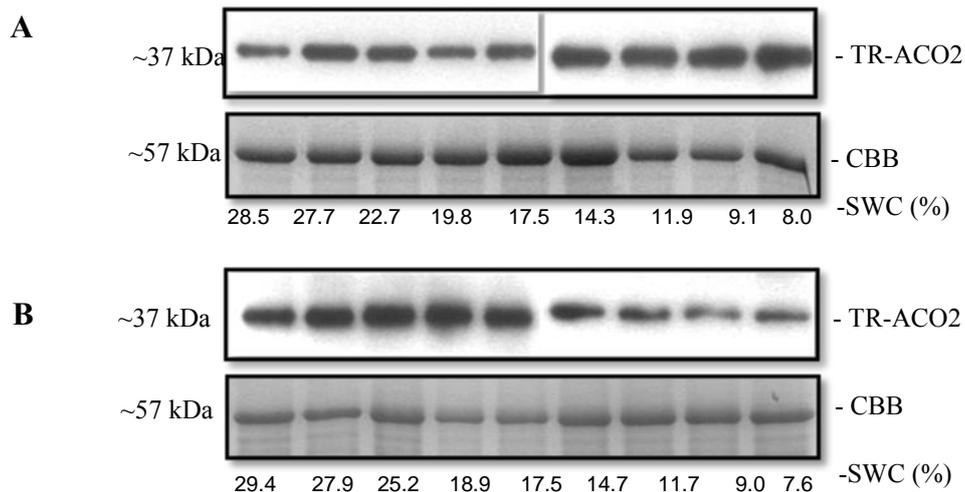


Appendix X: Western analysis, based on equal fresh weight loadings, of accumulation of TR-ACO2 protein in the first-fully expanded leaves of NPS Tienshan and NPS Kopu (for Experiment II, 2007).

1. Accumulation of TR-ACO2 in the first fully-expanded leaves of NPS Tienshan (A) and PS Tienshan (B) at different SCWs (as indicated)

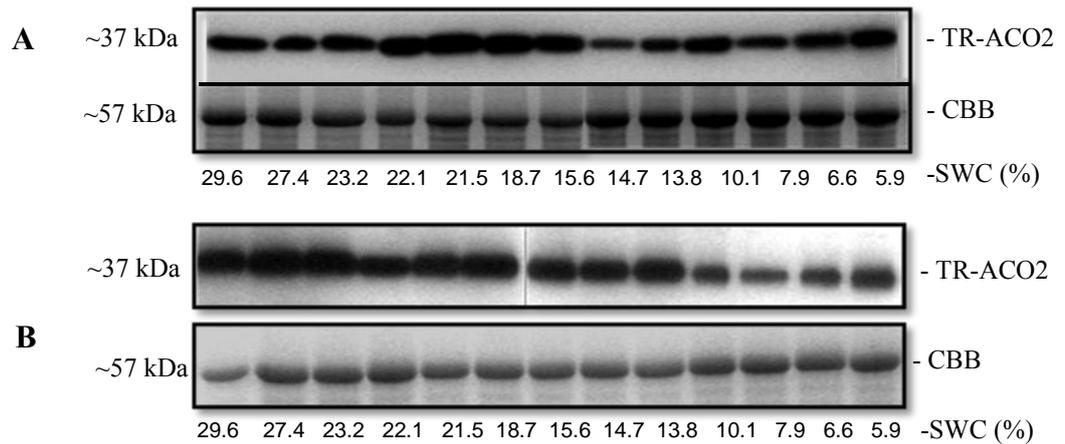


2. Accumulation of TR-ACO2 in the first fully-expanded leaves of NPS Kopu (A) and PS Kopu (B) at different SWCs (as indicated)

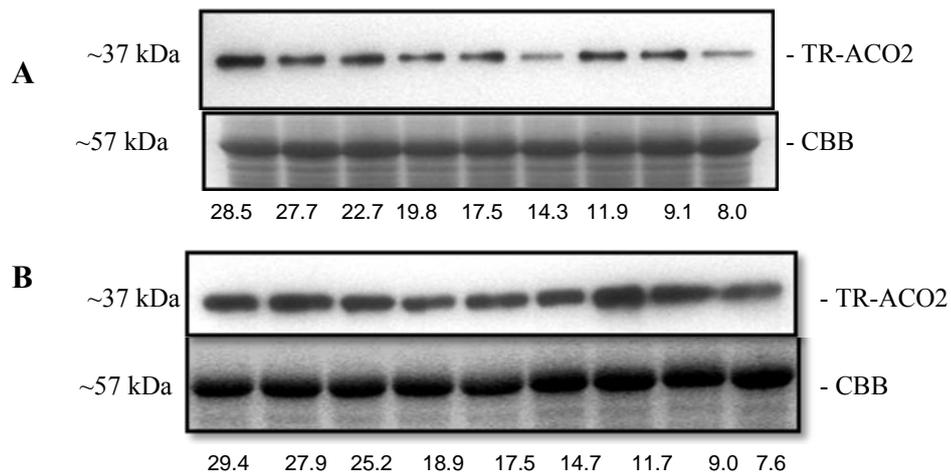


Appendix XI: Western analysis, based on equal fresh weight loadings, of accumulation of TR-ACO2 protein in the second fully-expanded leaves of NPS Tienshan and NPS Kopu (for Experiment II, 2007).

1. Accumulation of TR-ACO2 in the second fully-expanded leaves of NPS Tienshan (A) and PS Tienshan (B) at different SWCs (as indicated).



2. Accumulation of TR-ACO2 in the second fully-expanded leaves of NPS Kopu (A) and PS Kopu (B) at different SWCs (as indicated)



Appendices

2. Amino acid sequence alignment of TR-ACS1-T and TR-ACS1-10F

```

                2                                     3
TR-ACS1-T      LPEFRNAVAKSMSRTRGNRVTFDPDRIVMSGGATGAHEVTAFCLADPGDAFLVPTPYYPG 60
TR-ACS1-10F   LPEFRNAVAKFMSRTRGNRVTFDPDRIVMSGGATGAHEVTAFCLADPGDAFLVPTPYYPG 60
*****

                4
TR-ACS1-T      FDEDLRWFTVVKLVVICESANNFKLTRQALEEAYEKAKIDNIRIKGLLITNPSNPLGTV 120
TR-ACS1-10F   FDEDLRWFTGVKLVVICESANNFKLTRQALEEAYEKAKIDNIRIKGLLITNPSNPLGTV 120
*****

                5
TR-ACS1-T      MDRTKLKTVVNFINEKRIHLWTQPHYFCPVFSQPIFISIAEIIIEQETDIECDRNLVHIVY 180
TR-ACS1-10F   MDTTLLKTVVNFINEKRIHLISDEIIYAATVFSHPSFISIAEIIIEQETDIECDRNLVHIVY 180
** * . ***** : : * . . * : * *****

                6
TR-ACS1-T      SLSKDMGFFGFVGIIYSNDTVVNCARKQ 210
TR-ACS1-10F   SLSKDMGFFGFVGIIYSNDTVVNCARKQ 210
*****

```

(*) represents identical sequence, (.) represents semi-conserved substitution sequence, (:) represents conserved substitution sequence (-) represents no sequence, and (boxes) represent conserved residues found in aminotransferase and other ACC synthase. Shaded, numbered sequences represent the conserved regions of ACC synthase and shaded underlined sequences represent the active site of the enzyme.

Appendices

Appendix XIII: Alignment of the TR-ACS2 sequence isolated from the Tienshan ecotype in this thesis (TR-ACS2-T) with a previously isolated sequences from the 10-F genotype (TR-ACS2-10F)

1. Nucleotide sequences alignment of *TR-ACS2-T* and *TR-ACS2-10F*

```
TR-ACS2-T      TCTCCCTTCATTCAAACAAGCATTGGTAGATTTTCATGGCCGAGATCAGAGGAAACCGAGT 60
TR-ACS2-10F    TCTCCCTTCATTCAAACAAGCATTGGTAGATTTTATGGCCGAGATCAGAGGAAACCGAGT 60
*****
TR-ACS2-T      TTCCTTTGATCCCAACCATATGTTCTCACCGCGG--TCTACTTCCGCAAACGAGACTCTT 118
TR-ACS2-10F    TTCCTTTGATCCAAACCATATAT-CTCACTGCCGGCTCTACTTCCGCAAACGAGACTCTT 119
***** * * * * *
TR-ACS2-T      ATGTTTGTCTCGCTGAGAAAGGAGAAGCATTTCTCCTTCTACTCCTTACTATCCAGGG 178
TR-ACS2-10F    ATGTTTGTCTTGTCTGAGAAAGGAGAAGCATTTCTCCTTCTACTCCTTACTATCCAGG- 178
*****
TR-ACS2-T      ATTTGTAGAGATCTTAAATGGAGAACCGGTGTTGAGATTGTACCAATACAATGCAATAGG 238
TR-ACS2-10F    ATTGATAGAGATCTTAAATGGAGAAGTGGTGTGAGATTGTTCCAATACAATGCAATAGC 238
*** *****
TR-ACS2-T      TCCAACAACCTTCAAATAACTGAACAAGCCTGCAACAAGCATACAAAGATGCACAAGAC 298
TR-ACS2-10F    TCTAACAACCTTCAAATAACTGAACAAGCATTTGCAACAAGCATACAAAGATGCACAAGAC 298
** *****
TR-ACS2-T      CGCAACCTTAAAGTCAAAGGAGTAATGGTTACAACCCGTCAAACCCGTTAGGCACCACA 358
TR-ACS2-10F    CGCAACCTTAAAGTCAAAGGAGTAATGGTTACAACCCATCAAACCCGTTAGGCACCACA 358
*****
TR-ACS2-T      TTGTCAAGGAGTGAATTAATCTTCTCGTTGACTTTATTGAAGAAAACAAAACATGCAT 418
TR-ACS2-10F    TTGTCAAGGAGTGAATTAATCTTCTCGTTGACTTTATTGAAGAAAACAAAACATGCAT 418
*****
TR-ACS2-T      TTGATAAGCGACGAGATTTACTCCGGGACTGTTTTTTCCTCTCCAAGTTTATCAGTGTT 478
TR-ACS2-10F    TTGATAAGCGACGAGATTTACTCCGGGACTGTTTTTTCCTCTCCAAGTTTATCAGTGTT 478
*****
TR-ACS2-T      ATGGAATCCTTAACGAAAGAAATGACCTTCAGGATTTCAAATACACTGATAATATTTGC 538
TR-ACS2-10F    ATGGAATCCTTAAGGAAAGAAATGACCTTCAGGATTTCAAATACACTGATAATATTTGC 538
*****
TR-ACS2-T      GAGAGAGTTCATGTTGTCTATAGTCTTTCCAAAGACTTGGGTTTGCCAGGTTTCCGCGTT 598
TR-ACS2-10F    GAGAGAGTTCATGTTGTCTATAGTCTTTCCAAAGACTTGGGTTTGCCAGGTTTCCGCGTT 598
*****
TR-ACS2-T      GGTGCACTTTACTCCGAAAACGATGAAGTTGTCTGA---GCAACCAAC 642
TR-ACS2-10F    GGTGCACTTT-CTCCGAAAACGATGAAGTTGTCTGCAGCTGCAACCAAG 645
***** * * * * *
```

(*) represents identical sequence, (-) represents no sequence.

Appendices

2. Amino acid sequences alignment of TR-ACS2-T and TR-ACS2-10F

```

                2                               3
TR-ACS2-T      LPSFKQALVDFMAEIRGNRVSFDPNHMFSPRSTSANETLMFCLAEKGEAFLLPTPYYPGI 60
TR-ACS2-10F    LPSFKQALVDFMAEIRGNRVSFDPNHISHCR-LYFRKRDSYVLSCERFSISPSYSLLSRI 59
*****: * .: : *: : .: . . . *

                4
TR-ACS2-T      CDLKWRTGVEIVPIQCNRSNNFQITEQALQQAYKDAQDRNLKVKGVMVTNPSNPLGTTI 120
TR-ACS2-10F    DDLKWRTGVEIVPIQCNSSNNFQITEQALQQAYKDAQDRNLKVKGVMVTNPSNPLGTTI 119
*****

                5
TR-ACS2T       SRSELNLLVDFIEENKNMHLISDEIYSGTVFSSPSFISVMEILNERNDLQDFKYTDNICE 180
TR-ACS2-10F    SRSELNLLVDFIEENKNMHLISDEIYSGTVFSSPSFISVMEILKERNDLQDFKYTDNICE 179
*****:*****

                6
TR-ACS2-T      RVHVVYSLSKDLGLPGFRVGALYSENDVVEQPT- 214
TR-ACS2-10F    RVHVVYSLSKDLGLPGFRVGALYSENDVVAAATK 214
*****

```

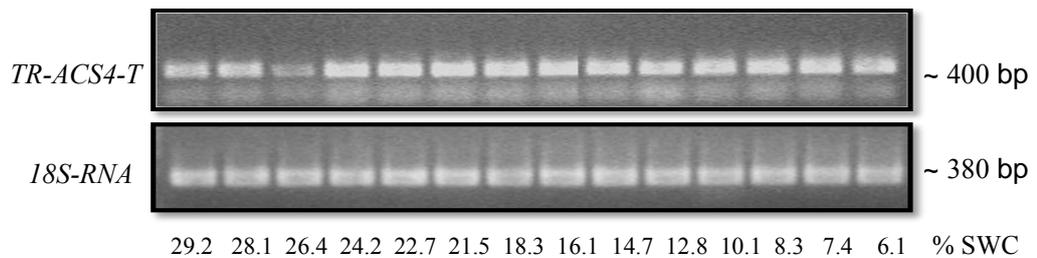
(*) represents identical sequence, (.) represents semi-conserved substitution sequence, (:) represents conserved substitution sequence (-) represents no sequence, and (●) represents conserved residues found in aminotransferase and other ACC synthase while boxed-*italics* represent the catalytic residue of the enzyme. Shaded, numbered sequences represent the conserved regions of ACC synthase, shaded underlined sequences represent the active site of the enzyme.

Appendix XIV. The accession number and gene sequences used to generate phylogenetic tree of the four TR-ACS sequences obtained from white clover of Tienshan ecotype (Figure 4.12).

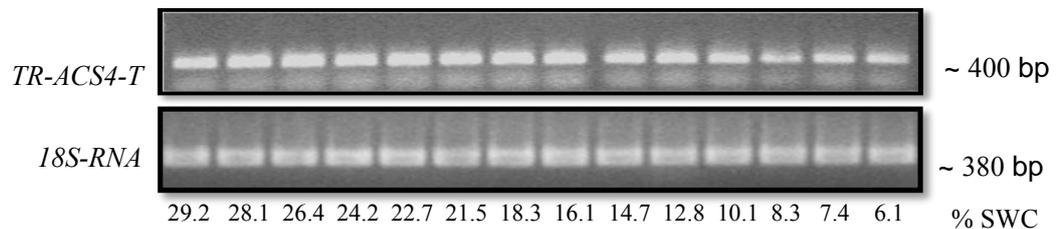
Gene Name	Amino acids	Plant species	Accession number
<i>MtACS</i>	487	<i>Medicago truncatula</i>	AAL35745.1
<i>ACS</i>	436	<i>Cicer arietinum</i>	ABD16181.1
<i>PsACS</i>	487	<i>Pisum sativum</i>	AAD04199.1
<i>ACS</i>	484	<i>Glycine max</i>	ABB70230.1
<i>ACS</i>	484	No information	P31531.1
<i>ACS9</i>	470	<i>Arabidopsis thaliana</i>	NP_190539.1
<i>ACS5</i>	470	<i>Arabidopsis thaliana</i>	NP_201381.1
<i>ACS8</i>	369	<i>Arabidopsis thaliana</i>	NP_195491.1
<i>ACS11</i>	460	<i>Arabidopsis thaliana</i>	NP_567330.1
<i>ACS7</i>	447	<i>Arabidopsis thaliana</i>	NP_194350.1
<i>ACS2</i>	445	<i>Lactuca sativa</i>	AAP14020.1
<i>ACS5</i>	448	<i>Lupinus albus</i>	AAF22112.1
<i>ACS4</i>	446	<i>Lupinus albus</i>	AAF22108.1
<i>ACS</i>	351	<i>Passiflora edulis</i>	BAA37134.1
<i>ACS</i>	446	<i>Malus x domestica</i>	AAB67989.1
<i>ACS</i>	446	<i>Pyrus pyrifolia</i>	BAA76388.1
<i>ACS3a</i>		<i>Pyrus communis</i>	AAR12136.1
<i>ACS2b</i>	446	<i>Pyrus communis</i>	AAR38503.1
<i>ACS</i>	356	<i>Prunus domestica</i> subsp. <i>initia</i>	CAI64501.1

Appendix XV. Expression of *TR-ACS4-T* in tissues (as indicated) of the Tienshan ecotype subjected to a water deficit (from Experiment I, 2006).

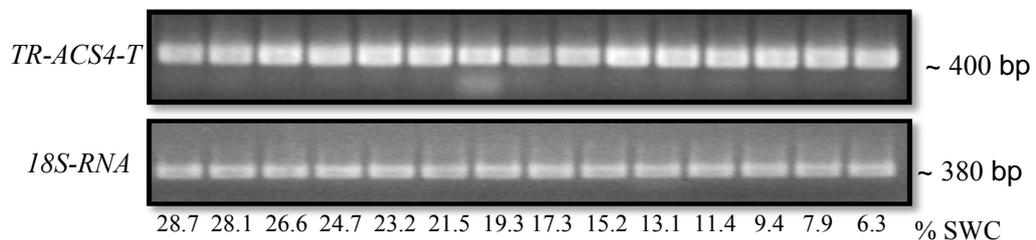
1. Expression of *TR-ACS4-T* in the apical structures of NPS Tienshan subjected to different SWCs (as indicated)



2. Expression of *TR-ACS4-T* in the first fully-expanded leaves of NPS Tienshan subjected to different SWCs (as indicated)

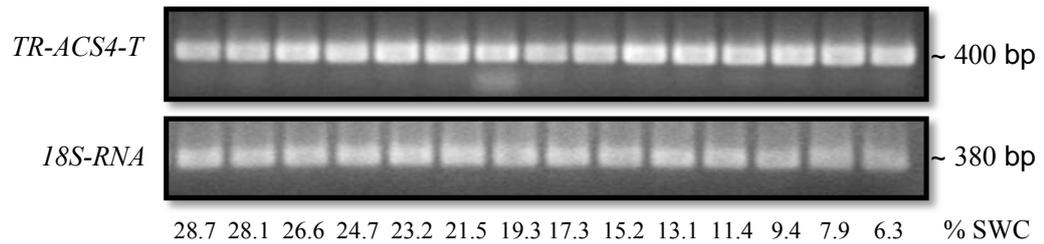


3. Expression of *TR-ACS4-T* in the apical structures of PS Tienshan subjected to different SWCs (as indicated)

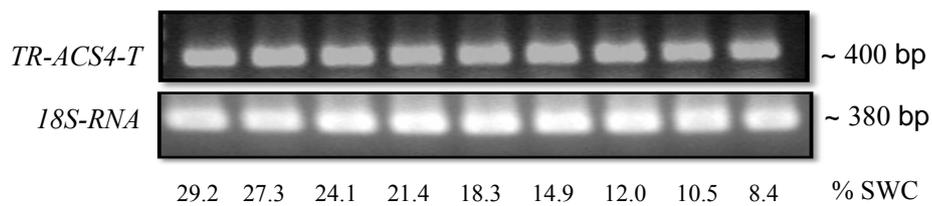


Appendices

4. Expression of *TR-ACS4-T* in the first fully-expanded leaves of PS Tienshan subjected to different SWCs (as indicated)



5. Expression of *TR-ACS4-T* in the apical structures of NPS Kopu subjected to different SWCs (as indicated)



6. Expression of *TR-ACS4-T* in the apical structures of NPS Kopu subjected to different SWCs (as indicated)

