CHARACTERISATION OF ACC SYNTHASE DURING LEAF DEVELOPMENT IN WHITE CLOVER (*Trifolium repens* L.)

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Abstract

ACC synthase catalyses the rate limiting step in the ethylene biosynthetic pathway, and in all plants studied has been shown to be encoded by a highly divergent gene family. These different ACC synthase genes are differentially regulated in response to a variety of developmental and environmental stimuli. In this thesis, ACC synthase gene expression during leaf ontogeny in white clover (*Trifolium repens* L.,) has been studied. This study utilises the stoloniferous growth pattern of white clover, which provides leaf tissue at different developmental stages, ranging from initiation at the apex, through mature green to senescent, and then finally necrotic.

RT-PCR, using degenerate primers to conserved regions of ACC synthase genes in the database, was used to amplify putative ACC synthase sequences from mRNA isolated from white clover leaf tissue. Sequencing and GenBank database alignment of the PCR products revealed that ACC synthase sequences comprising approximately 670 bp of the reading frame were amplified. Sequence alignments indicate that the sequences from white clover represent three distinct ACC synthases, and these were designated *TR-ACS1 (Trifolium repens ACC synthase 1)*, *TR-ACS2* and *TR-ACS3*. *TR-ACS1* is 62 % and 71 % homologous to *TR-ACS2* and *TR-ACS3* respectively, and *TR-ACS2* and *TR-ACS3* are 63 % homologous, in terms of nucleotide sequence. Genomic Southern analysis, using the amplified reading frame of each gene as a probe, confirmed that the sequences are encoded for by distinct genes.

In a GenBank database search, *TR-ACS1* shows highest homology to an ACC synthase sequence from IAA-treated apical hooks of pea, and *TR-ACS2* shows highest homology to an ACC synthase isolated from etiolated hypocotyls of mungbean. An ACC synthase isolated from white lupin, which was found to have increased expression during germination and in response to IAA and wounding, was most similar to *TR-ACS3*. Phylogenetic analysis determined that the three white clover ACC synthase genes are highly divergent. Phylogenetic analysis also determined that *TR-ACS1* groups with ACC synthase sequences isolated from IAA-treated apical hooks of etiolated pea seedlings and IAA-treated mung bean hypocotyls. *TR-ACS2* was found to group with

ACC synthases isolated from etiolated hypocotyls of mung bean and *TR-ACS3* was closest to a cDNA clone isolated from *Citrus parapdisi.*

Northern analysis has shown that two of these genes are expressed differentially during leaf ontogeny. *TR-ACS1* is expressed in mature green leaves and *TR-ACS3* is expressed in senescent leaf tissue. The expression of *TR-ACS2* was unable to be determined by northern analysis, and so the more sensitive method of RT-PCR was used. This procedure determined that *TR-ACS2* is expressed predominantly in the apex, newly initiated and mature green leaves, and again at the onset of senescence.

Sequence analysis of *TR-ACS3* revealed that the coded protein is missing the active site of the enzyme. Using a primer specific for the conserved active site of ACC synthase, a sequence which was similar to *TR-ACS3*, but not completely homologous, was amplified by RT-PCR and designated *TR-ACS3A*. This sequence included the region encoding the active site of the enzyme, but contained an additional four nucleotides in the sequence. Thus the sequence may also encode a non-functional protein, and the possible roles of such non-functional proteins are discussed.

The pattern of *TR-ACS* gene expression observed during leaf ontogeny suggests that these genes are under precise developmental control. Further, an indication as to the nature of the stimuli that may regulate the expression of the ACC synthase genes was provided by the phylogenetic analysis. To learn more of these physiological stimuli, white clover leaves were treated with two of the primary stimuli of ACC synthase gene expression, IAA and wounding; factors that were also shown to regulate the expression of sequences phylogenetically related to each *TR-ACS* gene. While the results of this experiment do not appear to be definitive, *TR-ACS1* was the only gene that hybridised to timepoints in the IAA-treated tissue, and *TR-ACS3* was the only gene to hybridise to the wound-induced tissues. The significance of ACC synthase gene expression during leaf ontogeny in white clover and its regulation is discussed.

Antibodies were raised to the gene product of *TR-ACS1* expressed in *E. coli.* Using western analysis, the antibodies were shown to recognise the gene products of all three *TR-ACS* genes and a protein of 55 KD with highest intensity in mature green leaf extracts. Minor recognition of proteins of 29, 34, 37, 69 and 82 KD was also observed.

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Abbreviations

A_{260} nm	absorbance in a 1 cm light path at 260 nm
ACC	1-aminocyclopropane-1-carboxylic acid
Amp^{100}	ampicillin (100 mg/ml)
APS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EIN	ethylene insensitive
FW	fresh weight
h	hour
IAA	indole-3-acetic acid
Kb	kilo-bases
kD	kilo-dalton
LB	Luria-Bertani media
MACC	1-(malonylamino)cyclopropane-1-carboxylate
1-MCP	1-methylcyclopropane
min	minute
NaOAc	sodium acetate
NBT	p-nitro blue tetrazolium chloride
Ni-NTA	nickel-nitrilotriacetic acid
PAG	photosynthesis-associated gene
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric-point
RNase	ribonuclease
RO	reverse osmosis
RT-PCR	reverse transcriptase-dependent PCR
SAG	senescence associated gene
SAM	S-adenosylmethionine

SA-PMP	streptavidin magne-sphere particles
SDS	sodium dodecyl sulphate
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Tris	tris (hydroxymethyl)aminomethane
U	units
UV	ultra violet
V	volt

1. INTRODUCTION

1.1 The plant hormone ethylene

Ethylene is a gaseous plant hormone involved in the regulation of many physiological responses in higher plants (Mattoo and Suttle, 1991; Kende, 1993; Dolan, 1997). The production of ethylene in plant tissue is normally low. However, as part of the normal life of a plant, an increase in ethylene biosynthesis is induced during certain stages of development. This increased production of ethylene can influence many developmental stages including seed germination, seedling growth, leaf abscission, organ senescence, flowering, diageotropism and fruit ripening (Yang and Hoffman, 1984; Abeles *et al.*, 1992; Fluhr and Mattoo, 1996). For example, a moderate level of ethylene production (0.1 to 0.4 nl g^{-1} F. wt h^{-1}) generally occurs in expanding leaves from seed germination to the early stages of seedling growth, particularly in the apical portions and at the nodes. This production rate of ethylene then decreases to lower levels during later vegetative growth (less than 0.1 nl g^{-1} F. wt h^{-1})(Osborne, 1991; Imaseki, 1999). However, when leaves or flower petals senesce, ethylene production again increases, and just prior to abscission of these organs a transient burst of ethylene production occurs in the abscission zone. In deciduous leaves of *Prunus serrulata*, ethylene production rates as high as 16 nl g^{-1} F. wt h^{-1} have been observed during the yellowing associated with the degeneration of the chloroplasts, while in herbaceous leaves, lower rates (up to 6 nl g^{-1} F. wt h^{-1}) have been detected (Osborne, 1991). In an other example, low amounts of ethylene are produced by immature fruit (between 0.03 and 3 nL g^{-1} h^{-1} in tomato fruit), but when climacteric fruit enter the ripening stage, a large production of ethylene occurs in this organ (e.g. 2 to 8 nL g^{-1} h^{-1} in tomato fruit) while low rates are maintained in other organs (Abeles, 1973; Yang and Hoffman, 1984; Nakatsuka *et al.*, 1998; Imaseki, 1999; Liu *et al.*, 1999).

In addition to the role of ethylene in regulating plant cell development, exposure to other plant hormones such as indole-3-acetic acid (IAA), abscisic acid (ABA), cytokinins and methyl jasmonate, metabolites such as carbohydrates, orthophosphate and polyamines, and various environmental stresses and stimuli such as wounding, flooding, chilling, oxygen deficiency, ozone, pathogen attack, light, touch and gravity,

also influence the ability of plant tissues to produce ethylene (e.g. Mattoo and Suttle, 1991; Abeles *et al.*, 1992; Theologis, 1992; Kende, 1993; Avni *et al.*, 1994; Ecker, 1995; Olson *et al.*, 1995; Fluhr and Mattoo, 1996; Lynch and Brown, 1997; Shiu *et al.*, 1998; Vahala *et al.*, 1998; Woeste *et al.*, 1999; Wong *et al.*, 1999). Due to the diverse physiological effects of ethylene, the organ/ tissue-specific and stage-specific regulation of biosynthesis is important for maintaining regular plant development. In addition, the large increase in ethylene production which occurs in living tissue near cells damaged by wounding, stress, pathogen infection or exposure to toxic chemicals is a response crucial for the plant to regenerate new protective tissues, and to heal the damaged parts (Tudela *et al.*, 1992; Larigaudiere and Vendrell, 1993; Pena-Cortes and Wilmitzer, 1995; Gomez-Cadenas *et al.*, 1996; Brown, 1997; Morgan and Drew, 1997; Shiu *et al.*, 1998; Imaseki, 1999). While many factors are involved in the regulation of the pattern of plant development, ethylene clearly plays a key role in the coordination of these processes (Dolan, 1997).

As a gas, ethylene moves from its site of synthesis by diffusion. Therefore, ethylene synthesised in one part of the plant can affect other tissues as well (Tieman and Klee, 1999). The cyclic amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) (the immediate precursor of ethylene) can be transported and may also account for ethylene effects at a distance from the stimulus (O'Neill *et al.*, 1993; English *et al.*, 1995). Throughout the tissue or organ, the internal distribution of ethylene may not be uniform as tissues produce ethylene at different rates, and have differing resistance to diffusion. The transport of ethylene can occur across dead and living tissues (Abeles *et al.*, 1992).

Ethylene action can be regulated at the level of both hormone synthesis and sensitivity. The effects of ethylene are dependent on cell and tissue type indicating the presence of different processes of recognition and/ or different levels of responsiveness of these cells and tissues (Tieman and Klee, 1999).

1.2 Mode of ethylene action

The mechanism by which plant cells perceive and transduce the ethylene signal has been a long-standing question (Trewavas, 1983; Abeles *et al.*, 1992). Ethylene is presumed to act by binding, most probably in a reversible way, to a receptor protein. The ethylene-receptor complex alters the activity of signal transduction reactions, leading to the transcription of specific genes and the synthesis and/ or activation of enzymes responsible for the physiological effects (Woltering and de Vrije, 1995). Attempts to isolate and purify ethylene receptors or ethylene binding proteins using biochemical methods alone have so far failed (Jiang and Fu, 2000). Recent advances in understanding ethylene signal transduction have been enhanced by analysis of *Arabidopsis* and tomato mutants defective in ethylene-dependent responses (Kende and Zeevaart, 1997; Mita *et al.*, 1998). Thus far, two categories of these mutants have been defined. For the first, dark-grown seedlings do not exhibit the "triple response" (i.e., stunted growth, swelling of the root and hypocotyl, and exaggerated apical hook formation) in the presence of ethylene. These are known as the ethylene insensitive (EIN) mutants as they show reduced or no response to exogenously applied ethylene. Members of this class of mutations include *etr1* (for ethylene triple response), *etr2*, *ein2* (for ethylene insensitive), *ein3*, *ein4*, *ein5*, *ein6* and *ein7* and *ain1* (for ACC insensitive) (Bleecker *et al.*, 1988; Johnson and Ecker, 1998; Hua *et al.*, 1998). The second category of ethylene perception mutants, the constitutive ethylene response (CER) mutants, demonstrate the triple response phenotype even in the absence of ethylene (Ecker, 1995). These are divided into two sub-classes according to their sensitivity to ethylene inhibitors (Johnson and Ecker, 1998). In one, the constitutive triple response appears to be caused by the overproduction of endogenous ethylene. These mutants, whose phenotypes are suppressed by ethylene synthesis inhibitors, produce 10-100-fold more ethylene than wild type seedlings and are called ethylene overproducer (*eto*) mutants. Examples include *eto1*, *eto2*, *eto3* and *eto4* (Vogel *et al.*, 1998). The second class of CER mutants display the triple response regardless of the presence of ethylene or inhibitors of ethylene synthesis, and are termed constitutive triple response (*ctr*) mutants (Johnson and Ecker, 1998).

The cloning and characterisation of several of the genes disrupted in such mutants has led to the identification of proteins involved in ethylene biosynthesis, perception and signal transduction. The first of these was cloned from *etr1*, a mutant that is dominant to the wild-type allele and displays an ethylene insensitive phenotype (Bleecker *et al.*, 1988). The *ETR1* gene was isolated by the map-based cloning technique (Chang *et al.*, 1993). The predicted ETR1 protein shows significant sequence homology to a class of histidine kinase proteins known as two-component regulators (Lelievre *et al.*, 1997). In bacteria, these two-component regulators typically consist of at least two proteins; a sensor histidine kinase (the receptor) that autophosphorylates an internal histidine residue in response to environmental signals, and a response regulator that receives the phosphate group on a conserved aspartate residue (Johnson and Ecker, 1998). The ETR1 protein from *Arabidopsis* exists as a membrane-associated dimer and can be divided into three functional domains. The amino-terminal sensor domain contains three putative transmembrane segments within which all known mutations resulting in loss of ethylene binding are located. This domain binds ethylene when expressed in yeast, and the *etr1* mutation abolishes ethylene binding (Schaller and Bleecker, 1995). Ethylene binding in the N-terminal domain of ETR1 has been demonstrated to require a copper ion and the *etr1-1* mutation abolishes the capacity of the receptor to coordinate this ion (Rodriguez *et al.*, 1999). The second domain has homology to histidine kinases that, in bacterial two-component sensing systems, are autophosphorylated. This portion of the ETR1 protein has been shown to exhibit histidine kinase activity *in vitro* (Gamble *et al.*, 1998). The third domain, the response regulator, may receive the phosphate from the histidine of the histidine kinase domain at an aspartate residue (Tieman and Klee, 1999).

Using an ETR1 cDNA as a probe, Hua *et al.* (1995) isolated the *ERS1* (ethylene response sensor) gene from *Arabidopsis*. Transgenic *Arabidopsis* plants which contain the mutated *ERS1* gene are ethylene-insensitive, indicating that ERS1 may also act as an ethylene binding protein, in addition to ETR1 (Hua *et al.*, 1995). Three additional ethylene receptor genes, *ETR2*, *EIN4* and *ERS2* have recently been isolated from *Arabidopsis* (Hua *et al.*, 1998; Sakai *et al.*, 1998). These results suggest that ethylene sensing in *Arabidopsis* could involve the distinct as well as redundant functions of multiple ethylene receptors (Kim *et al.*, 1999).

The five ethylene receptor family members in *Arabidopsis* share the most extensive sequence identity in an N-terminal domain (or sensor) that is sufficient for ethylene binding. A histidine kinase domain, C-terminal to the membrane-spanning region is easily recognisable in the ETR1 and ERS1 receptor coding regions, which contain all signature motifs found in bacterial histidine kinase homologs. The histidine kinase domains in ETR2, ERS2 and EIN4, however, are more significantly diverged, casting doubt on their ability to participate in phosphorylation cascades. ETR1, ETR2 and EIN4 have an additional receiver domain, which, in other systems is capable of relaying the phosphate group from the histidine kinase domain to the response regulator, usually via another protein. This receiver domain has limited homology to response regulators, suggesting that in the ethylene response pathway a separate response regulator may not be necessary (Clark *et al.*, 1998; Johnson and Ecker, 1998; Mita *et al.*, 1998).

Ethylene has been found to induce a rapid and transient phosphorylation of several proteins in tobacco, which supports the idea that a protein phosphorylation cascade is involved in the ethylene signal transduction pathway (Raz and Fluhr, 1993). Genetic evidence indicates that the ETR1 receptor family signals through the CTR1 protein (Hua *et al.*, 1995). The deduced CTR1 protein sequence is most similar to the Raf family of serine/threonine protein kinases found in eukaryotes, suggesting that CTR1 may act in a mitogen-activated protein (MAP) kinase cascade (Kieber *et al.*, 1993). Loss of function mutations in *CTR1* show a constitutive triple-response phenotype, indicating that CTR1 acts as a negative regulator of ethylene-response pathways (Kieber *et al.*, 1993). Hua and Meyerowitz (1998) demonstrated that combining loss-of-function mutants in three or more members of the *ETR1* family also results in plants with a constitutive ethylene-response phenotype. These results favour a model for receptor signaling in which the ETR1 receptor family acts in conjunction with CTR1 to suppress response pathways in the absence of ethylene. Ethylene binding would convert receptors to a non-signaling state, resulting in derepression of the response pathway (Hall *et al.*, 1999).

The tomato ethylene-receptor homolog gene family consists of at least five members that exhibit overlapping expression in virtually all tissues. However, relative expression levels of the individual genes vary significantly during development. The individual putative receptor proteins also exhibit significant structural divergence, which, for the

functional domains of the proteins, could greatly affect the mechanisms and efficiencies with which they transduce the signal. The differential response to ethylene in response to developmental and environmental stimuli may be a result of several factors working singly or in combination, including the differential regulation of gene expression, the differential binding abilities of the receptors for ethylene, the formation of receptor heterodimers or homodimers from the complement of receptors found in a tissue and the differential interactions with downstream components of the ethylene signal transduction pathway (Tieman and Klee, 1999). If the members of the ethylene receptor family act as negative regulators of the ethylene signal transduction pathway as suggested by Hua and Meyerowitz (1998), high levels of receptors in fruit tissues may act to modulate the fruit response to the high levels of ethylene produced during fruit ripening. Although the actual mechanisms for modulating ethylene sensitivity have yet to be elucidated, it is clear that there is ample opportunity for plants to adjust ethylene responses at the receptor level (Tieman and Klee, 1999).

1.3 Ethylene biosynthesis

1.3.1 Overview of the biosynthetic pathway

Ethylene production is stringently regulated during plant development with the rate of biosynthesis frequently changing depending on the developmental stage and the hormonal status of tissues, and in response to environmental stresses and stimuli (Nakagawa *et al.*, 1991). The biochemical aspects of ethylene biosynthesis have been reviewed extensively (Mattoo and Suttle, 1991; Kende, 1993; Zarembinski and Theologis, 1994; Ecker, 1995; Peck and Kende, 1995; Fluhr and Mattoo, 1996; Imaseki, 1999; Jiang and Fu, 2000).

The ethylene biosynthetic pathway (Figure 1.1) was established by Adams and Yang (1979) and has provided the basis for all subsequent biochemical and molecular genetic analysis of the pathway (Zarembinski and Theologis, 1994).

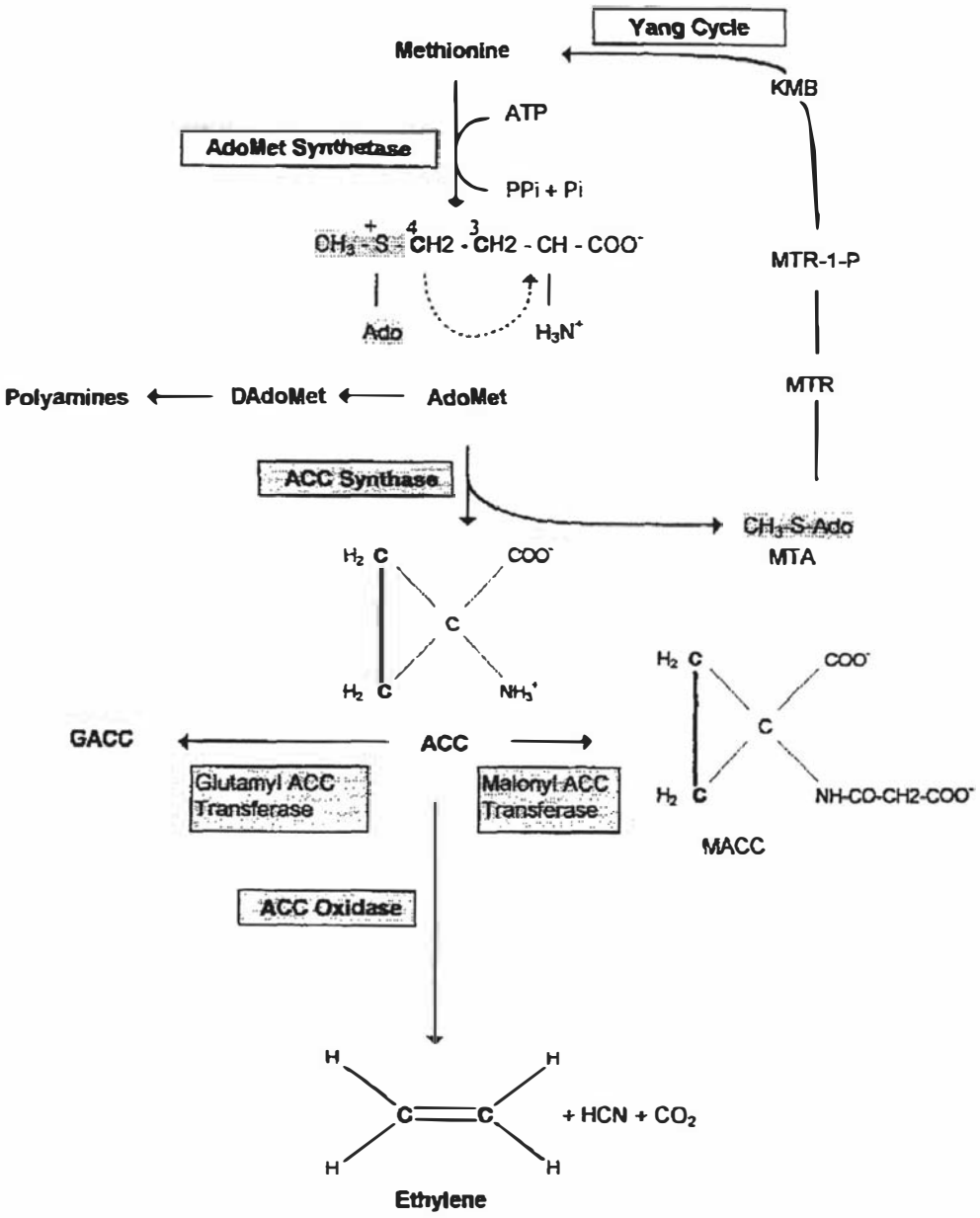


Figure 1.1 The ethylene biosynthetic pathway.

ACC, 1-aminocyclopropane-1-carboxylic acid: **AdoMet**, S-adenosylmethionine; **DadoMet**, decarboxylated AdoMet; **MTA**, 5′-methylthioadenosine; **MTR**, 5′-methylthioribose; **MTR-1-P**, 5′-methylthioribose-1-phosphate; **KMB**, 2-keto-4-methylthiobutyrate; **GACC**, 1-(gamma-L-glutamylamino) cyclopropane-1-carboxylic acid; **MACC**, 1-(malonylamino) cyclopropane-1-carboxylic acid. From Hunter (1998).

The amino acid L-methionine is the biological precursor of ethylene in higher plants (Yang and Hoffman, 1984). Plants, unlike other higher eukaryotes, possess all the necessary enzymatic equipment for *de novo* synthesis of methionine (Ravenel *et al.*, 1998). The biosynthesis of ethylene begins with the conversion of methionine and ATP to S-adenosyl-L-methionine (SAM, or also known as AdoMet), catalysed by the enzyme SAM synthetase (EC 2.5.1.6). SAM is then converted by ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase; EC 4.4.1.14) to ACC and 5′-methylthioadenosine (MTA). MTA is utilised for the synthesis of methionine (a less abundant amino acid) via a modified methionine cycle (Yang and Hoffman, 1984). This salvage pathway results in the ribose moiety of ATP giving rise to the four-carbon skeleton of methionine from which ethylene is derived. The methylthiol group, however, is conserved for continued regeneration of methionine. Thus with a constant pool of the methylthiol group and available ATP, high rates of ethylene biosynthesis can be maintained, even if methionine concentrations are low (Yang and Hoffman, 1984; Miyazaki and Yang, 1987).

ACC is the immediate precursor of ethylene in higher plants. Some lower plants such as the semiaquatic fern *Regnellidium diphyllum* and the liverwort *Riella helicophylla* do not use ACC as a precursor, and studies by Osborne *et al.*, (1996) provide evidence for a non-ACC-dependent pathway.

Oxidation of ACC by the enzyme ACC oxidase (also known as the ethylene-forming-enzyme; EC 1.4.3) results in the production of ethylene, CO_2, and HCN. HCN is detoxified through conversion to β-cyanoalanine, which is further modified to asparagine or γ-glutamyl-β-cyanoalanine. This detoxification pathway prevents the accumulation of HCN, even in plants with a high rate of ethylene biosynthesis (Yip and Yang, 1998).

In addition to being converted to ethylene, ACC can also be conjugated by the enzyme ACC *N*-malonyl transferase in what is thought to be a largely irreversible reaction (Yang and Hoffman, 1984). The conjugated form of ACC has been identified as a malonyl conjugate malonyl-ACC (MACC). Malonylation of ACC occurs largely in the cytosol, and the MACC is then transported to the vacuoles where it is sequestered.

ACC *N*-malonyl transferase has been described as a constitutive enzyme, although, the physiological role of MACC and the possible participation of ACC malonylation in the regulation of ethylene biosynthesis remain an open question (Kende, 1993; Benichou *et al.*, 1995; Martin *et al.*, 1995; Fluhr and Mattoo, 1996; Peiser and Yang, 1998; El Meskaoui *et al.*, 2000). It is known that ACC *N*-malonyltransferase activity increases as a result of stimulation with ethylene (Liu *et al.*, 1985). Firstly, in climacteric fruits such as apple, more than 40 % of the ACC synthesised in the skin, and 5 % in the flesh of immature fruit can be diverted to MACC (Mansour *et al.*, 1986). In another example, young carnation petals have been shown to possess a more active system for the conjugation of ACC compared to senescing petals, indicating that this reaction may be used to limit the availability of free ACC as a substrate for ACC oxidation and thus ethylene production (Peiser, 1986). Finally, ACC malonylation has been suggested to be a contributing factor to the low rate of ethylene production in preclimacteric fruit (Lelievre *et al.*, 1997).

1.3.2 Overview of the regulation of the pathway

It has been shown by many researchers that factors which promote ethylene production, such as fruit ripening, IAA application and various environmental stimuli also cause an increase in ACC synthase activity (Yang and Hoffman, 1984). Because ACC synthase activity increases rapidly from extremely low or undetectable levels when exposed to a stimulus that promotes ethylene production, and because ACC oxidase is usually constitutively present at least at low levels, ACC synthase has been widely regarded as the rate limiting step in ethylene biosynthesis (Yang and Hoffman, 1984). Changes in ACC oxidase levels may contribute to the overall increase in rates of ethylene production (Peck and Kende, 1995; Mita *et al.*, 1999). This has been shown in studies where ACC oxidase levels were increased following wounding of tomato fruit (Holdsworth *et al.*, 1987) and wounding of etiolated mung bean hypocotyls (Kim and Yang *et al.*, 1993). Treatment with 2, 5-norbornadiene (NBD), a competitive inhibitor of ethylene action, blocked this wound-induced accumulation of ACC oxidase. An increase in ACC oxidase transcript levels following pollination in orchid flowers could also be inhibited by NBD (Neill *et al.*, 1993). These results show that a stimulus causing an increase in ethylene production can also cause an increase in ACC oxidase

activity (Peck and Kende, 1995). The ethylene biosynthetic enzymes have been suggested to be sequentially induced by a stimulus promoting ethylene production (Hyodo *et al.*, 1993). A model proposed by Peck and Kende (1995) for the sequential induction of these enzymes after IAA treatment suggests that IAA causes an increase in ACC synthase transcript abundance leading to an increase in ACC synthase activity. The newly formed ACC is converted to ethylene by a low, constitutive level of ACC oxidase. The ethylene produced then causes an increase in the level of ACC oxidase transcript and activity via a positive feedback loop. Both ACC synthase and ACC oxidase are also subject to positive feedback by ethylene in several climacteric ripening fruit and senescing flowers (Lincoln *et al.*, 1993, Lelievre *et al.*, 1997; Mita *et al.*, 1998). However, expression of ACC oxidase is more sensitive to exogenous ethylene than ACC synthase in these plants (Mita *et al.*, 1999). Negative feedback regulation by ethylene has also been recognised in a number of fruit and vegetative tissues (Mattoo and White, 1991; Nakatsuka *et al.*, 1998).

The genes that encode S-adenosylmethionine synthetase, ACC synthase and ACC oxidase have been cloned and all are encoded by multi-gene families. The cloning and characterisation of these gene families, in tandem with biochemical studies, has added significantly to our understanding of the regulatory mechanisms that control the biosynthesis of ethylene (Destephano-Beltran *et al.*, 1995).

1.3.3 SAM synthetase

SAM synthetase is a ubiquitous enzyme involved in the conversion of methionine to SAM (Thomas and Surdin-Kerjan, 1991). In addition to being used for ethylene biosynthesis, the substrate SAM is also used in the synthesis of polyamines and for transmethylation reactions in proteins, carbohydrates, lipids and nucleic acids (Tabor and Tabor, 1984). Only a minor portion of the cellular SAM is thought to be utilised for ACC production, and so it has been suggested by Yu and Yang (1979) that SAM levels do not rate-limit ethylene production. However, studies using inhibitors of either ethylene or polyamine biosynthesis (Roberts *et al.*, 1984) indicated that ethylene production may be regulated by SAM.

SAM synthetase is encoded by a multigene family (Larsen and Woodson, 1991). The gene family members have been found to be differentially expressed in different organs in tomato (Esparetero *et al.*, 1994). Differential accumulation of these transcripts was also seen during ethylene-mediated senescence of carnation petals (Woodson *et al.*, 1992). A tight control in coordinating the relationship between SAM synthetase activity and the transcript level in plant organs has been suggested to be involved in maintaining a stable intracellular level of SAM (Fluhr and Mattoo, 1996).

1.3.4 ACC oxidase

The gene encoding ACC oxidase was first identified when it was shown that transformation of the gene (pTOM13) in an antisense orientation prevented tomato fruit ripening (Hamilton *et al.*, 1990). Subsequent work confirmed that pTOM13 (a cDNA isolated by Davies and Grierson, 1989, by differential cloning techniques) or its homolog pTOM5, conferred ACC oxidase activity when expressed in yeast (Hamilton *et al.*, 1991) or *Xenopus* oocytes (Spanu *et al.*, 1991).

1.3.4.1 Biochemical studies

Sequence comparisons of pTOM13 revealed that the protein product had high identity to flavonone-3-hydroxylase, an enzyme that requires Fe^{2+} and ascorbate for activity *in vivo* (Hamilton *et al.*, 1990). Subsequent biochemical experiments have now confirmed that ACC oxidase requires ascorbate and oxygen as co-substrates, and Fe^{2+} and CO_2 as co-factors (Yang and Dong, 1993). Calcium fluxes and intracellular calcium levels have also been suggested to be involved in regulating the activity of ACC oxidase (Gallardo *et al.*, 1999) and ACC oxidase is activated by one of its products, carbon dioxide.

ACC oxidase has been purified or partially purified and shown to be a monomer of 35 to 41 KD with K_m values for ACC varying from 20 μM for the enzyme from apple, 56 μM for the enzyme from banana and 60 μM for the melon fruit enzyme (Fluhr and Mattoo, 1996). ACC oxidase also undergoes inactivation, which is proposed to occur as two different processes. One occurs during enzyme catalysis, possibly involving an

intermediate during catalytic turnover. The second has been postulated to involve oxidation of the enzyme protein without involving enzyme catalysis (Smith *et al.*, 1994).

1.3.4.2 Molecular studies

Numerous cDNAs for ACC oxidase have been isolated from a variety of plant species and the enzyme is encoded by small multi-gene families (reviewed in Kende, 1993; Barry *et al.*, 1996; Fluhr and Mattoo, 1996; Imaseki, 1999). Multiple gene members have been identified in many species, including four members in *Petunia hybrida* (Tang *et al.*, 1993), three members in mung bean (Kim and Yang, 1994) three members in melon (Lasserre *et al.*, 1996) and four members in tomato (Holdsworth *et al.*, 1987; Hamilton *et al.*, 1991; Barry *et al.*, 1996). Members of these multi-gene families have been shown to be differentially regulated according to the tissue type and the nature of the stimuli, and the regulation of expression of the ACC oxidase gene family has been suggested to constitute an extra tier of control of ethylene biosynthesis (Barry *et al.*, 1996; Hunter *et al.*, 1999). In melon for example, three members have been isolated: *CM-ACO1*, *CM-ACO2* and *CM-ACO3*. *CM-ACO1* is induced in ripe fruit, and in leaves in response to wounding and ethylene treatment. *CM-ACO2* is detectable at low levels in etiolated hypocotyls while *CM-ACO3* is expressed in flowers and is neither induced by wounding nor ethylene treatment (Lasserre *et al.*, 1996). In another example, the use of gene-specific probes for three ACC oxidase genes of tomato, distinct patterns of expression in various organs and at different stages of development have been observed (Barry *et al.*, 1996). One gene (*LE-ACO1*) transcript accumulates during fruit ripening, leaf senescence and in response to wounding. The *LE-ACO3* transcript accumulates in different organs and is similar to the *LE-ACO1* transcript except that it is transiently expressed and differs in abundance. The expression pattern of these tomato genes has been found to differ from that of petunia ACC oxidase genes (Tang *et al.*, 1994). In petunia, two of the four ACC oxidase genes identified are pistil-specific, whereas only the tomato *LE-ACO2* transcript is organ specific (to the anther cone).

The ACC oxidase proteins encoded by these genes are highly identical (overall identity is nearly 80 %), while ACC oxidase genes share identical numbers and positions of introns (Jiang and Fu, 2000). There are clusters of long conserved sequences which

span more than two thirds of the coding regions, and the only significant divergence is observed in the sequence of the N-terminal 20 amino acids and the C-terminal 10-12 amino acids (Tang *et al.*, 1993; Imaseki, 1999).

ACC oxidase, as measured by ethylene production in the presence of a saturated concentration of ACC, is constitutively present in most tissues of higher plants. Although it has been concluded that ACC synthase provides the rate limiting step for ethylene biosynthesis, small changes in ACC oxidase activity may provide the timing of ethylene evolution (Kende, 1993). ACC oxidase accumulation has been demonstrated to increase rapidly in tissues in response to a variety of stress conditions, in response to ethylene, or during certain developmental stages such as fruit ripening and senescence (Yang and Hoffman, 1984; Dilley *et al.*, 1993; Pogson *et al.*, 1995; Kim *et al.*, 1998).

In addition, the positive feed back loop in which treatment of tissue with ethylene often stimulates ethylene production by that tissue (described in section 1.3.2) appears to take place through enhanced expression of ACC synthase and ACC oxidase (Kende and Zeevaart, 1997). Taken together, such observations suggest that ACC oxidase does play an important role in the regulation of ethylene production in vegetative tissues.

1.4 ACC synthase

1.4.1 Characterisation of ACC synthase at the biochemical level

ACC synthase was first identified in a homogenate of ripening tomato pericarp (Boller *et al.*, 1979), and it has been shown subsequently to be a cytostolic enzyme which is highly labile and undergoes suicidal inactivation by its substrate SAM during catalysis (Boller, 1985; Satoh and Esashi, 1986; Kim and Yang, 1992). ACC synthase shows a high substrate specificity for SAM, and affinity to the substrate is also high with Km values ranging from 12 to 60 μM, and the pH optimum is between 8.5 and 9.5. Incubation of a partially purified tomato ACC synthase preparation with SAM was demonstrated by Satoh and Yang (1989) to result in the inactivation of enzyme activity, and that this inactivation is due to the covalent linkage of the aminobutyrate portion of

SAM to the enzyme. Biochemical investigation of ACC synthase has been hampered due to it being present in low abundance with respect to other proteins in tissues, and by its short half-life (Bleecker *et al.*, 1988). The half-life of ACC synthase, as determined by the rapid decay of enzyme activity after treatment of tissues with cycloheximide, varied from 58 minutes in wounded tomato fruit (Kim and Yang, 1992), 25 minutes in mung bean hypocotyls (Yoshii and Imaseki, 1982) to 20 minutes in tomato leaves (Spanu *et al.*, 1990). ACC synthase is dependent on pyridoxal-5'- phosphate for activity (Yang and Hoffman, 1984). The active site of ACC synthase has been identified in apple and tomato fruits by covalent labeling with SAM and pyridoxal phosphate (Yip *et al.*, 1990). Both the substrate and the cofactor bind to a lysine residue contained within the tryptic dodecapeptide SLSKD(M/L)G(L/F/I/M)PG(F/L)R. The consensus active site is conserved in all ACC synthases reported except for three positions (6, 8 and 11) where divergence has been observed (Yip *et al.*, 1990; Botella *et al.*, 1992; Cazzonelli *et al.*, 1998; Imaseki, 1999).

ACC synthase exists in several isoforms. The amino acid sequence identities of the different ACC synthases vary from 48 % to 97 % (Spanu *et al.*, 1994). The proteins are also diverse in their molecular mass. Tissue from wounded tomato fruit contains at least three isoenzymes with pI values of 5.3, 7, and 9 (Mehta *et al.*, 1988). This is consistent with the existence of a highly divergent gene family that encodes ACC synthase (e.g., Theologis, 1992; Kende, 1993; Zarembinski and Theologis, 1993; Fluhr and Mattoo, 1996). *In vitro* translation products fractionated by SDS-PAGE show M_rs of 48 KD for apple (Dong *et al.*, 1991), 55 KD for zucchini (Sato *et al.*, 1991), 56 KD for tomato (Van Der Straeten *et al.*, 1990) and 58 KD for winter squash (Nakajima *et al.*, 1990). The partially purified native ACC synthase has been found to be 8-9 KD smaller than the *in vitro* translated protein in several cases, raising the possibility that the protein is processed (Nakajima *et al.*, 1988; Van Der Straeten *et al.*, 1990). However, Sato *et al.* (1991) found no size difference in the *in vivo* and the *in vitro*-translated zucchini ACC synthase. Northern blotting indicates that the size of the new mRNAs encoding ACC synthase varies from 1.8-2.1 Kb (Rottman *et al.*, 1991), supporting the molecular mass range of the proteins described above.

ACC synthase isoforms fall into four groups of acidic, near neutral, neutral and alkaline, when based on the pIs of the deduced amino acid sequences (Fluhr and Mattoo, 1996).

Each class has different distinct regions or residues. Phylogenetic analysis of ACC synthase genes shows trifurcation into at least three major branches. The polymorphism of these genes is considered to have risen prior to the divergence of moncots and dicots, since monocot sequences exist in two separate branches (Liang *et al.*, 1992; Lincoln *et al.*, 1993). The acidic ACC synthases appear to be progenitors of the alkaline and neutral isoforms. (Fluhr and Mattoo, 1996). Proteins encoded by multigene family members are known to share similar functions but differ in subtle characteristics that may have selective advantage to the cells or tissues in which they are expressed and it has been suggested by Smith (1990) that differential expression of family members may serve as a molecular basis of phenotypic plasticity in plants.

A high degree of conservation encompasses eight distinct regions of ACC synthase (designated as regions 1 to 8), which contain at least eight amino acid residues and show greater than eighty percent identity (Imaseki, 1999). Most notable of these conserved regions is the one at the active site. All ACC synthases contain at comparable positions, eleven of twelve invariant amino acids that are involved in the binding of pyridoxal phosphate and substrate in aminotransferases (Rottman *et al.*, 1991; Mori *et al.*, 1993; Abel *et al.*, 1995). ACC synthase shares the highest sequence identity with subgroup 1 aminotransferases, which include the aspartate aminotransferases (Tarun *et al.*, 1998). There is also extensive conservation between the predicted secondary structure of ACC synthase and that of the aspartate aminotransferase obtained by X-ray crystallography (Zarembinski and Theologis, 1994). The identity between ACC synthases and aminotransferases suggests that these two groups of enzymes may be evolutionary related and raises the possibility that the quaternary structure and co-factor binding sites of these two groups of enzymes may be similar (Rottmann *et al.*, 1991; Tarun and Theologis, 1998). Site-directed mutagenesis of some of these conserved residues in ACC synthase indicate that they are essential for activity and may have similar roles in catalysis as their counterparts in aspartate aminotransferase (Tarun *et al.*, 1998; Zhou *et al.*, 1999).

The high level of identity between different ACC synthases is in the interior portion of the polypeptide, while the carboxyl termini are the most divergent (Park *et al.*, 1992; Theologis, 1992). The C-terminal domain is, however, conserved in its net positive charge. The non-conserved C-terminus of ACC synthase has been found to affect its

enzymatic function as well as dimerisation (Li and Mattoo, 1994; Fluhr and Mattoo, 1996). Discrepancies exist in the literature on whether the native enzyme exists as a monomer or a dimer (Fluhr and Mattoo, 1996; Li *et al.*, 1997). However, characterisation of the apple enzyme expressed in *E. coli* (White *et al.*, 1994) and preliminary X-ray analysis of the apple ACC synthase crystal structure (Hohenester *et al.*, 1994) were consistent with the dimeric nature of ACC synthase. In addition, complementation studies with ACC synthase mutants have indicated that, like aspartate aminotransferase, ACC synthase functions as a dimer with shared active sites (Tarun *et al.*, 1998; Tarun and Theologis, 1998)

The presence of distinct domains that influence catalytic and structural aspects of ACC synthase have been revealed by studies on C-terminal truncated enzymes. Two domains separated by the internal sequence VGVEKS affect enzyme catalysis and dimerisation, while one 52 amino acid domain in the C-terminal 52 region is necessary for a relatively inhibited state of the enzyme that is more susceptible to suicidal inactivation by SAM (Li and Mattoo, 1994). Processes that induce ethylene production have been suggested to be associated with limited cleavage of ACC synthase *in vivo*. Endogenous or exogenous stimuli that cause activation of ACC synthase might utilise post-translational cleavage at the C-terminus to form a monomeric enzyme with better catalytic efficiency (Vmax/Km).

Enzyme activity was abolished in engineered ACC synthase expressed in *E. coli* in which the first 20 or more amino acids residues were deleted from the N-terminus. However, nested deletions closer to the N-terminus resulted in expressed protein (deleted in residues 2-12) exhibiting higher activity than the wild-type protein (Li and Mattoo, 1994).

The relative locations of the conserved regions are almost identical in all of the ACC synthase enzymes, i.e., the numbers of amino acids present between two adjacent conserved regions are nearly the same. It is implied that the portion between these regions composes a core of ACC synthase. Thus the different numbers of amino acid residues outside the core portion, upstream of region 1 and downstream of region 8, results in the different molecular masses of different enzymes (Imaseki, 1999).

1.4.2 Characterisation of ACC synthase at the molecular level

Genes that encode ACC synthase have been cloned from many plant species (reviewed in Fluhr and Mattoo, 1996; Johnson and Ecker, 1998; Imaseki, 1999; Mita *et al.*, 1999) and the enzyme is encoded by a multi-gene family (Theologis, 1992; Kende, 1993). The isolation of these cDNA clones was achieved initially using oligonucleotide probes derived from peptide sequences or antibodies for immunoscreening expression libraries. Functional identification of cDNA clones encoding ACC synthase has been achieved by expression studies in *E. coli* and yeast, and by sequence comparisons to peptides purified from ACC synthase protein (Sato and Theologis, 1989; Van Der Straeten *et al.*, 1990; Dong *et al.*, 1991; Li *et al.*, 1992; Hohenester *et al.*, 1994; Abel *et al.*, 1995; Li *et al.*, 1996; Li *et a*l., 1997; Tarun and Theologis, 1998).

Rapid progress is being made in the characterisation of the ACC synthase gene families. Based on the presence of introns, the ACC synthase genes fall into classes of two, three and four intron genes (Lincoln *et al.*, 1993; Destephano Beltran *et al.*, 1995; Fluhr and Mattoo, 1996).

The multigene family of ACC synthase is differentially expressed in various tissues at different stages of development and in response to a variety of environmental and chemical factors (Dong *et al.*, 1991; Van Der Straeten *et al.*, 1992; Kende, 1993; Destephano-Beltran *et al.*, 1995; Wang and Arteca, 1995). For example, in *Arabidopsis*, six different ACC synthase genes have been identified by molecular cloning techniques, with at least four of these expressed and coding for active enzymes (Liang *et al.*, 1992; Van Der Straeten *et al.*, 1992). Wounding, auxin, cycloheximide, LiCl, and anaerobiosis differentially elevate the steady-state level of these *Arabidopsis* genes (Liang *et al.*, 1992; 1996; Arteca and Arteca, 1999). Auxin has been found to specifically induce the transcription of *AT-ACS4*, and several auxin-response elements have been identified upstream of the *AT-ACS4* coding region (Abel *et al.*, 1995). Treatment with lithium ions increased the steady-state level of *AT-ACS5* mRNA (Liang *et al.*, 1996). Vogel *et al.* (1998) demonstrated that *AT-ACS5* is also involved in the response of etiolated seedlings to low doses of cytokinin by a post-transcriptional processing mechanism. Wounding stimulated the expression of *AT-ACS2* and *AT-ACS4*

genes, but suppressed expression of *AT-ACS5* (Liang *et al.*, 1992). *AT-ACS6* was found to be induced rapidly in response to touch (Arteca and Arteca, 1999). *AT-ACS3* was found to be a pseudogene, representing a truncated form of *AT-ACS1*. *AT-ACS1* was missing the tripeptide TNP and could not be functionally expressed in *E. coli* and yeast (Liang *et al.*, 1995). The steady-state level in all four active ACC synthase genes in *Arabidopsis* is increased by treatment with the protein synthesis inhibitor cycloheximide, suggesting that they are under the control of a short-lived negative regulator (Liang *et al.*, 1992). Expression of these genes was not uniform over the different organs. *AT-ACS4* was expressed only in roots, leaves and flowers, but not in stems and siliques. *AT-ACS5* was expressed only in flowers and siliques (Liang *et al.*, 1992). In tomato, ACC synthase is encoded by at least nine divergent genes (Jiang and Fu, 2000). It has been shown that the ACC synthase tomato gene *LE-ACS2* is expressed during fruit ripening (Olsen *et al.*, 1991; Rottman *et al.*, 1991) and is super-induced on wounding of the pericarp tissue (Yip *et al.*, 1992). The *LE-ACS4* gene seems to be equally expressed during these stages, albeit, at a lower level (Olsen, 1991), and the *LE-ACS3* gene is highly induced in auxin-treated vegetative tissue (Yip *et al.*, 1992). Another observation from such expression studies is that ACC synthase genes expressed in response to a particular stimulus (e.g., the application of auxin) are more similar to genes controlled by the same stimulus in other species than they are to other ACC synthase genes in the same species (Liang *et al.*, 1992; Trebitsh *et al.*, 1997; Kende and Zeevaart 1997).

Many of the ACC synthases cloned have been demonstrated to be inducible by protein synthesis inhibitors such as cycloheximide (Huang *et al.*, 1991; Liang *et al.*, 1992; Lincoln *et al.*, 1993; Liu *et al.*, 1993; Zarembinski and Theologis, 1993; Kim *et al.*, 1997; Mathooko *et al.*, 1999; Bekman *et al.*, 2000). Cycloheximide inducibility is the hallmark of primary response genes (Theologis *et al.*, 1985). It has been suggested that the expression of the ACC synthase genes may be under the control of a short-lived repressor protein (Theologis *et al.*, 1985; Liang *et al.*, 1992) or a rapidly-turning-over RNase (Franco *et al.*, 1990) responsible for rapid degradation of some transcripts.

1.4.3 Induction of ACC synthase gene expression by environmental stimuli

Much research has been devoted to the study of ACC synthase gene expression in response to environmental stimuli. In many plant tissues, the level of ACC synthase mRNA has been shown to increase in response to wounding, suggesting that the regulation of wound-induced ethylene biosynthesis may lie at the level of transcription (Nakajima *et al.*, 1990; Olson *et al.*, 1991; Lincoln *et al.*, 1993). Ethylene biosynthesis in response to wounding follows a consistent pattern in most plants. The local rate of ethylene biosynthesis begins to increase rapidly 20-30 minutes after wounding, reaches a peak at 40-60 minutes and then declines (Campbell and Labovitch, 1991). The ethylene that is produced plays an important role as a mediator of the wound signal, inducing the expression of various defensive genes (Watanabe and Sakai, 1998; Leon *et al.*, 2001; Watanabe *et al.*, 2001). Generally this stress-induced ethylene produced via the ACC pathway is primarily due to an increased activity of ACC synthase, although the activity of ACC oxidase may increase as well (Spanu *et al.*, 1993). However, it is now recognised that under certain stress conditions, plants also produce ethylene through a route where ACC is not an intermediate (Mattoo and White, 1991). The potato ACC synthase genes *ST-ACS1A/1B* are transiently down-regulated in response to wounding, while the third gene, *ST-ACS2*, is up-regulated in tubers after wounding that is synergistic in the presence of IAA (Destephano-Beltran *et al.*, 1995).

In addition to wounding, other environmental stimuli also regulate ACC synthase gene expression. In response to ozone, the foliage of potato plants sequentially expressed two ACC synthase genes (*ST-ACS4* and *ST-ACS5*). The expression pattern of the two genes also occurred in response to Cu^{2+} and infection with *Alternaria solani*. *ST-ACS5* expression increases very rapidly reaching a maximum earlier than *ST-ACS4* transcripts, after which *ST-ACS5* expression declines. *ST-ACS4* expression increases at a slower rate (and reaches its maximum after *ST-ACS5*). The sequential nature of expression suggests that the two genes have different signal transduction and gene regulatory mechanisms (Schlagnhaufer *et al.*, 1997; Tatsuki and Mori, 1999). The tomato *LE-ACS2*, *LE-ACS5* and *LE-ACS6* genes were found to be strongly induced in suspension-cultured cells that had been treated with elicitor (Oetiker *et al.*, 1997). *LE-ACS3* and

LE-ACS7 were rapidly induced in flooded roots and wounded leaves within one hour of treatment (Shiu *et al.*, 1998). These results show that one or more ACC synthase genes are responsible for ethylene production in response to one stimulus (Tatsuki and Mori, 1999). The examples cited above are cited to illustrate the induction of ACC synthase gene expression in response to environmental stimuli.

The presence of several genes and proteins for ACC synthase raises questions about their functional significance. However, it is evident that most of these genes are induced in a regulated manner, each gene differentially regulated by a similar group of inducers. Whether cell types with different lineages express different ACC synthase gene(s) and are regulated differentially remains to be determined (Fluhr and Mattoo, 1996). In addition, studies of precise temporal and spatial involvement of individual ACC synthase genes and the use of more sensitive methods of gene expression analysis will be required for better understanding of the regulation of ACC synthase gene expression (Jiang and Fu, 2000).

1.5 Regulation of ethylene biosynthetic gene expression during growth and development

Ethylene production in higher plants is regulated by endogenous and exogenous biotic and abiotic factors and has been implicated in diverse processes such as seed germination, fruit ripening, senescence and leaf abscission (Fluhr and Mattoo, 1996; Imaseki, 1999). The ability of ethylene to promote senescence, causing cells to shift from a program of growth to one of senescence, highlights the intricate controls in plants to regulate ethylene production. That plants have evolved to tightly regulate the production of ethylene is attested by the fact that multiple genes encode key enzymes in ethylene biosynthesis (ACC oxidase and ACC synthase) whose transcripts are differentially regulated (Fluhr and Mattoo, 1996).

Imaseki (1999) has described several characteristics in the regulatory systems of ethylene production. Firstly, the ethylene production rate is determined developmentally, with the basic program of ethylene biosynthesis genetically laid out

for regular development. During the plant life cycle, endogenous ethylene plays various roles to ensure that plants undergo their regular epigenetic development. Ethylene biosynthesis increases or decreases frequently in specified tissues or organs depending upon the stage of development. This implies that ethylene production is a combined outcome of tissue- and stage-specific gene expression according to a preset program. Secondly, changes in environmental factors largely affect the rate of ethylene production. These include regular changes, resulting from revolution and rotation of the earth, including diurnal light/dark cycle, photoperiod and thermoperiod, and irregular changes, such as drought, flooding, extreme temperatures, wound or stress, touch or pressure by other objects and infection by pathogenic microorganisms. The regular environmental changes are necessary for the regular development of the plant, but when plants receive irregular stimuli, ethylene often serves as an effector, which modulates multiple metabolic processes that lead to the acquisition of tolerance to environmental changes or which heal wounds. This characteristic indicates that ethylene synthesis is regulated by a set of genes specific to different physical as well as chemical stimuli. Thirdly, ethylene production is regulated by other plant hormones. Ethylene biosynthesis is induced by auxin in vegetative tissue, and auxin action is synergistically enhanced by cytokinin and antagonised by abscisic acid. Ethylene itself regulates its own synthesis in autocatalytic or inhibitory ways, depending on the tissues or species. Positive feedback regulation of ACC synthase and ACC oxidase genes by ethylene was found in tomato fruit (Rottmann *et al.*, 1991) and carnation petals (Woodson and Lawton, 1988; Woodson *et al.*, 1992) while negative feedback of ACC synthase genes was found in citrus leaf explants (Sisler *et al.*, 1985) and in deepwater rice internodes (Bleecker *et al.*, 1987). Thus any factors affecting endogenous auxin, cytokinin or abscisic acid levels will regulate ethylene biosynthesis (Imaseki, 1999).

Of particular relevance to this thesis, is the regulation of ethylene biosynthesis during organ development and senescence.

1.5.1 Fruit ripening

The ripening of fruit corresponds to a series of biochemical, physiological and structural changes. Although these processes vary from one type of fruit to another, fruits can be

divided into two broad categories, known as climacteric and non-climacteric (Lelievre *et al.*, 1997). Separation into these two groups depends on the presence or absence of a peak in respiration accompanied by a burst of ethylene synthesis at the onset of ripening, and the response of the fruit to exogenous ethylene (Biale and Young, 1981). In ripening climacteric fruits, the sharp increase in climacteric ethylene production at the onset of ripening is considered as controlling the initiation of changes in colour, aromas, texture, flavour and other biochemical and physiological characteristics (Gray *et al.*, 1994). Exogenously applied ethylene induces ripening and endogenous ethylene production (Liu *et al.*, 1999). Both ACC synthase and ACC oxidase are induced in ripening climacteric fruits and the pattern of change in the rate of ethylene biosynthesis is well correlated with the patterns of changes in the levels of ACC synthase and ACC oxidase gene transcripts (Yang and Hoffman, 1984; Liu *et al.*, 1999).

Fruit ripening in the climacteric tomato fruit has been one of the most intensely studied ethylene-mediated developmental processes (Kende, 1989; Rottman *et al.*, 1991; Magne and Lahre, 1995; Harpster *et al.*, 1996). A large number of dramatic changes occur within a short period of time as the tomato fruit ripen, many of which are under ethylene control or initiated by ethylene exposure. One of the first detectable signs of fruit ripening is the dramatic rise in ethylene production associated with the respiratory climacteric. Autocatalysis of ethylene production is a characteristic feature of ripening fruits and other senescing tissues in which a massive increase in ethylene production is triggered by exposure to ethylene (Zarembinski and Theologis, 1994). In climacteric fruit, the transition to autocatalytic ethylene production is due to a series of events in which ACC synthase and ACC oxidase are expressed developmentally (Mason and Botella, 1997; Mita, *et al.*, 1998; Jiang and Fu, 2000). In contrast, the ripening of non-climacteric fruits is generally considered to be an ethylene-independent process and little is known of the regulatory mechanisms underlying the biochemical changes (Lelievre *et al.*, 1997; Shiomi *et al.*, 1998).

Two systems of ethylene regulation have been proposed to operate in higher plants. System I, operating in both climacteric and non-climacteric fruits as well as vegetative tissues, has been proposed to be responsible for basal and wound-induced ethylene production and ethylene is auto-inhibitory. System 2 operates during ripening of climacteric fruit and during petal senescence when ethylene is auto-stimulatory and

requires the induction of both ACC synthase and ACC oxidase (Lelievre *et al.*, 1997; Barry *et al.*, 2000). The signaling pathways operating to result in the induction of these two enzymes by coordinated regulation of ACC synthase and ACC oxidase gene families remain unknown, although much evidence indicates that a combination of both ethylene and developmental factors are required (Barry *et al.*, 2000).

Until recently, there existed a great deal of discussion as to whether ethylene is the trigger for ripening in climacteric fruit, or is simply a by-product of the ripening process. Data using antisense technology in tomato show ethylene is the controlling factor for fruit ripening (Cambell and Labovitch, 1991; Oeller, 1991; Klee and Romano, 1994; Cooper *et al.*, 1998). Transgenic tomatoes have been constructed in which ethylene biosynthesis is blocked by expression of antisense message for ACC synthase (Oeller *et al.*, 1991), or ACC oxidase (Hamilton *et al.*, 1990; Picton *et al.*, 1993), or by overexpression of the ACC deaminase gene from *Pseudomonas syringae* (Klee, 1993). (This enzyme degrades ACC to α-ketobutyric acid thus preventing its conversion to ethylene). In tomato fruit where ACC synthase activity is inhibited with antisense RNA, the antisense phenotype can be reversed with six days of ethylene treatment. Antisense fruits treated for up to two days with ethylene do not develop fully ripe phenotypes compared with control fruits. In addition, removal of ethylene from antisense fruits once they have fully ripened prevents over-ripening (Oeller *et al.*, 1991). This suggests that ethylene-mediated ripening requires continuous transcription of the necessary genes, which may be due to the short half-life of the enzyme (Theologis, 1992). In transgenic tomato constructed with the antisense message for ACC oxidase, ethylene synthesis in ripening fruit was reported to be reduced by 97 % relative to controls (Klee *et al.*, 1994), while overexpression of the ACC deaminase gene in tomato reduced ethylene synthesis in leaves by more than 95 % and in fruit by 90 % (Klee *et al.*, 1991).

The finding that the application of ethylene to transgenic fruit can induce the climacteric respiratory rise in the absence of endogenous ethylene production and reverse the antisense phenotype demonstrates that ethylene controls the climacteric rise in respiration in tomato fruit (Oeller, 1991). Ethylene is therefore a causal agent of fruit ripening and not simply a by-product of the ripening process (Theologis, 1992). The hypothesis that ethylene alone is not the sole determinant of ripening is supported by

other data, since application of ethylene to transgenic ACC oxidase antisense fruit fails to cause complete reversal of the antisense phenotype (Hamilton *et al.*, 1990). The requirement for continued ethylene treatment to overcome the ACC synthase antisense phenotype demonstrates that ethylene acts in the ripening process as a regulator, modulating gene expression, rather than simply a switch that activates the process at the onset of ripening (Fluhr and Mattoo, 1996). Picton *et al.*, (1993), showed that the foliar senescence of ACC oxidase antisense plants was temporarily delayed. In contrast to the altered fruit ripening phenotype, where ripening was initiated at the same time but proceeded at a reduced rate, leaf senescence appeared to be delayed, but once initiated, proceeded at the same rate as wild-type controls. This suggests that both ethylene concentration and/or changes in tissue sensitivity or responsiveness to ethylene may be important in altering gene expression.

1.5.2 Ethylene and leaf senescence

Senescence is the final stage of leaf development. It is a highly regulated process during which new metabolic pathways are activated and others are turned off as cell death occurs in tissues at the end of their life (Gan and Amasino, 1997). Senescence involves the ordered disassembly of cellular components in the senescing tissue and allows for maximum recovery of nutrients, particularly nitrogen and phosphorus, from this tissue for recycling to other parts of the plant such as growing leaves or developing seeds. Senescence is largely characterised by a cessation of photosynthesis, disintegration of organelle structures, intensive losses of chlorophyll and storage proteins and a large increase in lipid peroxidation and membrane leakiness (Becker and Apel, 1993; Hensel *et al.*, 1993; Smart, 1994; Magne and Lahre, 1995; Borochov *et al.*, 1997; Buchanan-Wollaston, 1997; Nooden *et al.*, 1997; Liu *et al.*, 1998). While the initiation of leaf senescence depends upon the age of the leaf and the reproductive phase of the plant, external factors including nutrient deficiency, pathogenic attack, ozone exposure, drought, light limitation and temperature can induce premature senescence (Smart, 1994; Miller *et al.*, 1999).

Ethylene has been implicated as having a significant role in the senescence of many plant tissues (Abeles *et al.*, 1992; Woodson *et al.*, 1992; Becker and Apel, 1993; Smart,

1994; Altworst and Bovy, 1995; Grbic and Bleecker, 1995; John *et al.*, 1995). For example, in naturally senescing carnation flowers, ethylene biosynthesis starts in the ovary as a result of the expression of ACC synthase and ACC oxidase genes. Gaseous ethylene, diffusing to the petals, is presumed to trigger the expression of a petal-specific ACC synthase and ACC oxidase gene leading to increased petal ethylene production and senescence (ten Have and Woltering, 1997). In leaf senescence studies, chlorophyll content has commonly been used as a marker of leaf senescence. In addition, cDNAs that accumulate during both leaf senescence and fruit ripening in tomato have been identified (Davies and Grierson, 1989). Induction of these cDNAs coincided with the peak of ethylene biosynthesis in both leaves and fruits. In addition, an inhibitor of ethylene action, silver thiosulphate, decreased the expression of marker genes. These results have been suggested to imply that ethylene controls gene expression during leaf senescence as it does during tomato fruit ripening (Davies and Grierson, 1989). In tomato, transgenic plants that both over- and under-produce ethylene are altered in some aspects of senescence (Picton *et al.*, 1993; Lanahan *et al.*, 1994). For example, plants that contain the ACC synthase gene under the control of the CaMV 35 S promoter synthesise up to 100 times as much ethylene as controls (Lanahan *et al.*, 1994). These plants are usually infertile due to the abortion of most flowers prior to fertilisation. The abortion is due to the premature induction of the abscission zone in the pedicel. Leaves of these plants, however, do not senesce, and vegetative tissue remains green for up to one year. The signal for vegetative senescence appears to be related more to establishment of a strong sink tissue than to ethylene content as it is only when the plants set fruit that the leaves senesce. The converse experiment has been performed with the *Nr* mutant of tomato (Lanahan *et al.*, 1994). In *Nr*, leaf senescence is slightly delayed, but not prevented, relative to control plants. However, flowers remain viable and abscission zone formation is delayed for at least four weeks regardless of whether fertilisation has occurred. These results indicate that ethylene does promote senescence by affecting the rate at which some senescence associated changes occur (Smart, 1994), but only if the leaf is competent to respond (Fluhr and Mattoo, 1996). Ethylene appears to have a clear role in regulating ripening (senescence) in climacteric fruit but not in non-climacteric fruit, even within the same genus. Grbic and Bleecker (1995) proposed that age-related factors signaled a decrease in the expression of photosynthesis-associated genes (PAGs) and an induction of senescence-associated genes (SAGs).

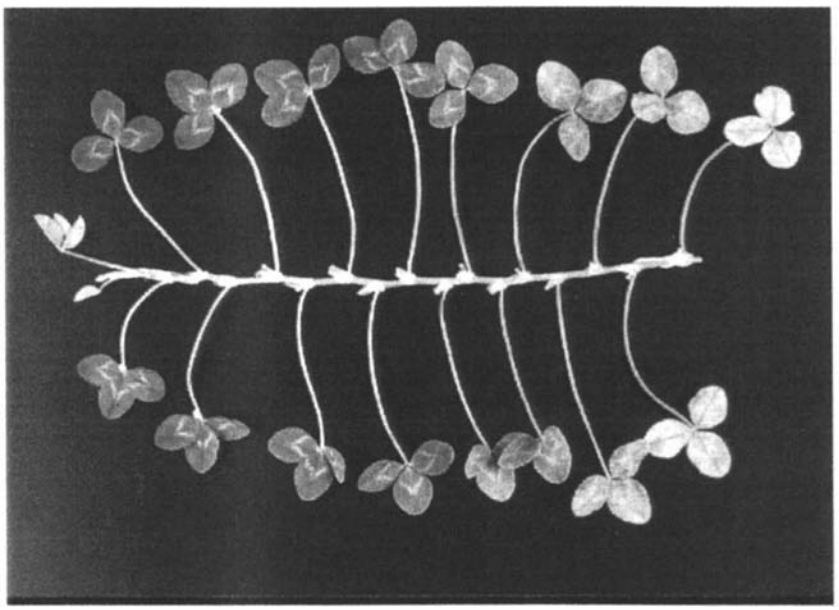
1.6 Leaf ontogeny and the role of ethylene in white clover

White clover (*Trifolium repens* L.) is the plant used in this thesis for the study of ACC synthase gene expression during leaf ontogeny. *T. repens* is the most agronomically important member of the 200 to 300 species comprising the genus *Trifolium* (Baker and Williams, 1987). It is an important component of intensely grazed dairy pasture in New Zealand where nitrogen fertiliser use is low and, through nitrogen fixation, is the main source of nitrogen (Ball *et al.*, 1979).

White clover has a clonal growth form and exists largely through vegetative propagation (Harris, 1994). In pasture, white clover has a stoloniferous growth habit, where the stolon consists of a series of internodes separated by nodes which form as a result of growth at the apical bud (Thomas, 1987). Each node bears a trifoliate leaf with an erect petiole, an axillary bud and two root primordia, the uppermost of which usually remains dormant while the lower primordium gives rise to a fibrous root when in contact with moist stratum. The axillary bud can remain dormant or can develop into a lateral stolon or a compound flower. Production of lateral stolons leads to the vegetative spread of a plant (Thomas, 1987). The growth of stolons is indeterminate and during summer white clover plants can possess up to six orders of branching. The vegetative propagation of white clover stolons involves a constant cycle of growth and branching towards the stolon apices and death and decay of the older basal sections of stolons (Brock *et al.*, 1988). In spring, senescence occurs in many stolons resulting in fragmentation of plants (Hay *et al.*, 1989). Leaf senescence is a highly regulated developmental phase where degraded macromolecules are mobilised to young leaves, developing seeds or storage tissue (Buchanan-Wollaston, 1997). Senescence of leaves and stolons is an important factor in the persistence of white clover in pastures.

The stoloniferous growth pattern of white clover provides a system where leaf tissue at different stages, ranging from initiation at the apex, through maturation and senescence to necrosis, can be studied (Figure 1.2a). To obtain individual stolons exhibiting this range in developmental stage, axillary buds were removed from vegetatively propagated stolons, which were grown over a dry matrix to inhibit root formation at nodes resulting in a large basal root initiating from a single node. Using this growth system, white

A



B

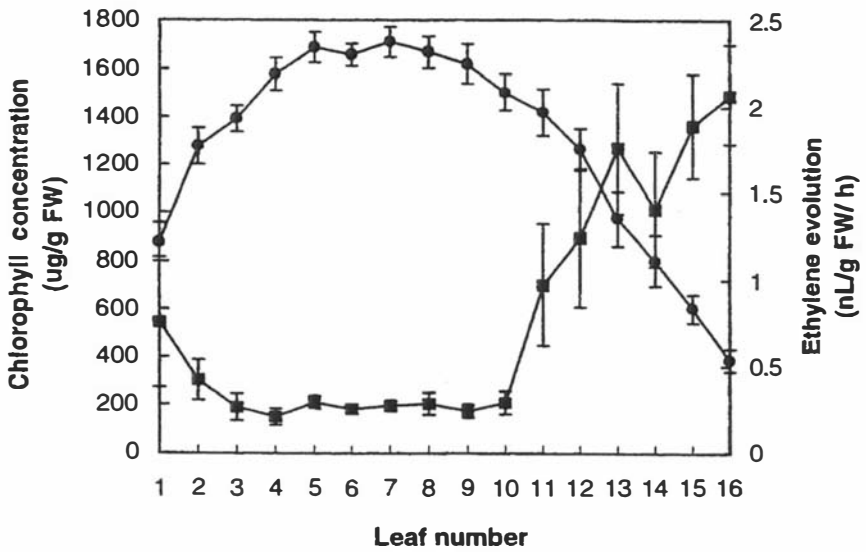


Figure 1.2 A. Stages of leaf development along a single stolon of white clover. **B.** Ethylene evolution (▮) and total chlorophyll (●) determined from leaves excised from single stolons identical to that shown in A. Results are mean values ± SE; n = 5 (From Hunter 1998).

clover stolons produce leaves at a constant rate and exhibit a constant pattern allowing replication of plant material for analysis. The number of leaves attached to the stolon reaches a constant number as the production rate is balanced by the rate of senescence.

This model system has been used to study changes in ethylene biosynthesis associated with leaf ontogeny in several previous studies (Butcher, 1997; Hunter, 1998; Yoo, 1999). Physiological data by Hunter (1988) demonstrated an initial peak in ethylene evolution associated with leaf initiation at the apex (Figure 1.2b). Chlorophyll levels increase at this stage until leaves were fully expanded (leaf 3). From leaf 3 to leaf 8 the chlorophyll content was maintained at this high level while ethylene evolution was low. From leaf 9 onwards, a senescent associated decrease in chlorophyll levels was observed while ethylene levels increased. Using this experimental system, two ACC oxidase genes (*TR-ACO2* and *TR-ACO3*) were cloned by Hunter (1998) and a third ACC oxidase gene (*TR-ACO1*) was cloned by Yoo (1999) and these genes were found to be differentially expressed during white clover leaf ontogeny (Hunter *et al.*, 1999). *TR-ACO1* was expressed in the apex, while *TR-ACO2* was expressed in newly initiated leaves and mature green leaves with highest expression in newly initiated leaves. *TR-ACO3* was expressed in senescent leaves. In terms of ACC synthase genes, Butcher (1997) cloned an ACC synthase gene from mature green leaves of white clover using RT-PCR, but no expression studies were carried out. Several studies in other plant species have examined the expression of ACC synthase in leaf tissue, but these are in response to environmental stimuli. Some examples include the expression of *ST-ACS4* and *ST-ACS5* in potato, which were shown to be induced by ozone in leaves (Schlagnhaufer *et al.*, 1995) and *AT-ACS6* from *Arabidopsis* (Vahala *et al.*, 1998) which was also found to be induced by multiple stimuli including touch in *Arabidopsis* leaves (Arteca and Arteca, 1999). Fewer studies have addressed the concept of differential expression of ACC synthase genes during leaf development. In *Arabidopsis*, *AT-ACS1* was found to be developmentally regulated with highest expression in developing leaves (Van Der Straeten *et al.*, 1992; Rodrigues-Pousada *et al.*, 1993; 1999) and again after the leaf expansion phase (Smalle *et al.*, 1999). In *Pelargonium*, Wang and Arteca (1995) determined the differential expression of two ACC synthase genes (*GAC-1* and *GAC-2*) in leaves. *GAC-2* was expressed in the leaf bud and young leaf tissue, while the second gene, *GAC-1* was expressed in fully expanded leaves but was undetectable in older leaves. Therefore, the focus of this thesis is to determine whether there is

differential expression of ACC synthase genes during leaf maturation and senescence in white clover leaf tissue.

1.7 Thesis aims

- To obtain a full length clone of ACC synthase by screening a cDNA library made to white clover leaf tissue.
- To isolate gene sequences encoding ACC synthase from white clover leaf tissue at different developmental stages.
- To characterise the expression patterns of ACC synthase during leaf ontogeny in white clover
- To examine factors that may induce the expression of these ACC synthase genes
- To produce antibodies against an ACC synthase protein expressed in *E. coli.*

2. Materials and Methods

2.1 Clonal propagation and harvest of white clover plant material

2.1.1 Plant material and growth conditions

White clover (*Trifolium repens* L.) genotype 10F of cultivar Grasslands Challenge (AgResearch Grasslands, Palmerston North, NZ) was used for all experimental analysis in this study. Plants were propagated in horticultural grade bark/ peat/ pumice (50:30:20) (Dalton Nursery Mix, Tauranga, NZ) supplemented with nutrients (Table 2.1) and grown in temperature-controlled greenhouses (Plant Growth Unit, Massey University, Palmerston North, NZ). Minimum greenhouse temperatures of 12 °C (night) and 15 °C (day) with venting at 25 °C were maintained. Plants were watered at 10 am and 5 pm for 5 minutes each using a time-controlled mist watering system (Automation Services Ltd., Auckland, NZ). Aphids and whitefly were controlled by spraying with the insecticide Attack® (Crop Care Holdings Ltd., Richmond, Nelson, NZ). Benlate (Dupont de Nemours and Co., In., Wilmington, Delaware, USA) was used for the irradication of blackspot.

Table 2.1 Composition of potting mix used to propagate white clover

Nutrient	*g/100 L*
Dolomite	300
Agricultural lime	300
Iron sulphate	50
Osmocote	500

2.1.2 Plant propagation

To maintain a continual supply of the single genotype of white clover stock material, apical cuttings comprising the apex and two or three nodes were excised from existing

stock plants. Cuttings were propagated in trays containing bark/ nutrient mix and grown under greenhouse conditions until required.

2.1.3 Model system for growth of white clover stolons

Apical cuttings comprising the apex and two or three nodes were excised from stock plants for the initiation of single stolons. All leaves except those ensheathing the terminal bud and the first leaf were removed by excision of the petiole at the junction with the stolon, and the cuttings placed along a central line of the seedling trays containing bark/ nutrient mix (six per tray). Single stolons were established from each cutting by training out of the tray over a dry polythene surface to inhibit root formation at the nodes. Axillary buds and flowers were removed routinely to maintain single stolons attached to a basal root. Stolons were grown until they provided a consistent range of leaf developmental stages, ranging from initiation at the apex to senescent.

2.1.4 Harvesting of plant material

At least two days prior to harvesting leaf material, stolons were undisturbed by axillary bud and flower removal. At harvest, leaves were excised from a single stolon at the junction of the lamina and petiole (the pulvinule) and placed, either as pooled leaves from the same position (leaf number) of several stolons, or as pooled leaves grouped from the same developmental stage of one or more stolons into 50 ml Nunc™ centrifuge tubes (Nalgen Nunc International, Naperville, USA). Leaf tissue was immediately frozen in liquid nitrogen and stored at –80 °C.

2.1.5 Treatment of plant material

2.1.5.1 Wounding treatment

Mature green leaves (nodes 4-6) of white clover stock plants (approximately eight stolons per tray) were punched with forceps to make small holes (approximately 0.1 mm in diameter), to give approximately 10 % coverage of the trifoliate leaf. Leaves were

harvested (typically 4 g/ sample) at the appropriate time following wounding as described in section 2.1.4 and frozen immediately in liquid nitrogen and stored at –80 °C.

2.1.5.2 IAA treatment

Leaves of white clover stock plants were treated with IAA using the method described by Peck and Kende (1995). Trays containing approximately 8 stolons each were sprayed with 100 μM IAA (pH 6.0, containing 0.05 % (v/v) ethanol and 0.05 % (v/v) Tween 20) or just 0.05 % (v/v) ethanol and 0.05 % (v/v) Tween 20 to act as a control. Leaves (typically 4 g/ sample) were harvested at the appropriate time interval as described in section 2.1.4 and immediately frozen in liquid nitrogen and stored at –80 °C.

2.1.5.3 Treatment of plant tissue with 1-methylcyclopropene (1-MCP)

Whole plants were treated with 1-MCP (EthylBlock™; obtained from Yates NZ Ltd., New Zealand). Trays of white clover stock plants (approximately 8 stolons per tray) were placed in an environmentally-controlled growth chamber (Contherm Scientific Company, Lower Hutt, New Zealand) for 2 hours prior to 1-MCP treatment. Lighting was provided by 8 X TRUE LITE® 40 W fluorescent tubes and the temperature of the chamber was maintained at 25 °C. Following the two hour pre-incubation, 1-MCP was evaporated as a gas from EthylBlock with 1 % (w/v) KOH to achieve a final concentration of 3000 nL L^{-1} of 1-MCP in 0.48 m^3 (the internal volume of the growth cabinet). To achieve this, 1.65 g of EthylBlock was dissolved in 100 ml of 1 % (w/v) KOH, according to the manufacturers' instructions. Plants were treated for 1 h, then aerated, and then treated either by wounding or with IAA, or used as the 1-MCP control treatment for the wounding or IAA treatments. Leaf tissue was harvested before and after 1-MCP treatment, and then at specific time intervals during the subsequent wounding, IAA or control treatments. Leaves were excised as described in section 2.1.4 and immediately frozen in liquid nitrogen and stored at –80 °C.

2.2 Biochemical methods

The biochemical reagents used in experimental work in this thesis, unless specified, were analytical grade, obtained either from BDH Laboratory Supplies (Dorset, England) or Sigma Chemical Company (St. Louis, Mo., USA). Purified water for making solutions in this thesis was produced by reverse-osmosis and microfiltration (Milli-Q, Millipore Corp., Bedford, MA, USA).

2.2.1 Protein extraction with 30 % - 90 % (w/v) saturated ammonium sulphate fractionation

Tissue samples (typically 1 g aliquots) were powdered in liquid nitrogen and mixed with two volumes of extraction buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 25 mM 2-mercaptoethanol). The extracts were then centrifuged at 26 000 x g for 10 min at 4 °C. The supernatant was collected and its volume adjusted to 30 % (w/v) saturated (164 g L^{-1}) ammonium sulphate. The mixture was incubated on ice for 30 min and then centrifuged at 26 000 x g for 10 min at 4 °C to pellet residual nucleic acid and cellular debris. The supernatant was collected and the volume was adjusted to 90 % (w/v) saturated (402 g L^{-1}) ammonium sulphate. Following incubation on ice for 1 h, protein was pelleted by centrifugation at 26 000 x g for 10 min at 4 °C, the supernatant discarded, and the pellet resuspended in 1 volume of resuspension buffer (50 mM Tris-HCl, pH 7.5, containing 10 % (v/v) glycerol and 2 mM DTT). The protein was then desalted by passage through a column containing Sephadex G-25 (section 2.2.2). The column eluate was collected for measurement of protein (section 2.2.3) and for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (section 2.2.4).

2.2.2 Sephadex G-25 column chromatography

Sephadex G-25 (Pharmacia Biotech, Uppsala Sweden) was used to desalt ammonium sulphate-containing protein extracts. Spin columns were prepared by lining the bottom of 10 ml syringe barrels (Becton Dickensen) with three layers of GF-A glass microfilter paper (Whatman International Ltd., Maidstone, England). The tips of the barrels were

sealed with parafilm and the barrels filled with Sephadex G-25 resin pre-equilibrated with column buffer (50 mM Tris-HCl, pH 7.5 containing 10 % (v/v) glycerol and 2 mM DTT). The parafilm was removed from the syringe barrels and these then placed in 50 mL centrifuge tubes and the assembly centrifuged at 178 x g for 1 min at 4 °C. The column was then transferred to a new 50 mL centrifuge tube and a 1 mL aliquot of protein extract for desalting was loaded onto the resin and the assembly centrifuged at 178 x g for 1 min at 4 °C. The eluate was collected from the bottom of the 50 mL centrifuge tube.

2.2.3 Quantification of protein in solution

Aliquots (1-10 μL) of protein extract were pipetted into individual wells of a microtitre plate (Nunc™ Brand Product, Nalgen International, Denmark) in triplicate, and made up to 160 μL with water. Forty μL of Bio-Rad Reagent (Bio-Rad Laboratories, Hercules, CA USA) was added and mixed by pipette. After standing for 5 min, the absorbance at 595 nm was measured using an Anthos HTII platereader (Anthos Labtech Instruments, Salzburg, Austria). A triplicate series of protein standards, prepared to give concentrations of 0, 2, 4, 6, 8 and 10 mg/mL of bovine serum albumin (BSA; Sigma Chemicals) in 160 μL were assayed and measured, as described above, in parallel with protein extracts. A protein standard curve made from measurements of the BSA samples was used to calculate the protein content in each sample.

2.2.4 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins solely on the basis of their molecular mass, and the gel system used is based on the method described by Laemmli (1970). A 10 % resolving gel was prepared by mixing the components in the order outlined in Table 2.2. The APS and TEMED were added just prior to pouring the gel, as these reagents promote and catalyse the polymerisation of the acrylamide. The solution was poured between glass plates assembled in a Bio-Rad Mini-Protein apparatus (Bio-Rad Laboratories) until the level was approximately 1 cm below where the bottom of the well-forming

comb sits. Water was then overlayed onto the gel surface and the gel allowed to polymerise for approximately 30 min.

Table 2.2 Composition of resolving gel and stacking gels used for SDS-PAGE with the Mini-Protein apparatus.

Stock solution composition described below.

Reagents	**Resolving gel solution (10 %) (mL)**	**Stacking gel solution (mL)**
Water	10	13
4 x resolving gel buffer	5	
4 x stacking gel buffer		5
Acrylamide-Bis stock	5	2
APS	0.2	0.2
TEMED	0.02	0.02

- 4 x resolving gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4 % (w/v) SDS)
- 4 x stacking gel buffer (0.5 M Tris-HCl, pH 6.8, 0.4 % (w/v) SDS)
- 40 % (w/v) acrylamide stock solution (Bio-Rad)
- 10 % (w/v) APS (ammonium persulphate; Univar, Auburn, NSW, Australia)
- TEMED (N,N,N',N-tetramethylethylenediamine) (Riedel-de haen ag seelze, Hannover, Germany)

During polymerisation of the resolving gel, a stacking gel was prepared as outlined in Table 2.2. The water layer above the resolving gel was then discarded, a well-forming comb was inserted and the stacking gel solution added to the remaining space between the glass plates. Following polymerisation for approximately 30 min, the gel assembly was transferred to the electrophoresis apparatus, and gel running buffer (0.25 M Tris, 0.2 M glycine buffer, pH 8.3, containing 1 % (w/v) SDS) was added to both the inner and outer compartments of the gel apparatus. The combs were removed in preparation for sample loading. Protein samples were prepared by mixing the protein sample with one volume of 2 x gel loading buffer (60 mM Tris-HCl pH 6.8, 25 % (v/v) glycerol, 5 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol, 0.1 % (w/v) bromophenol blue) and boiling for 3 min (except for samples that contained imidazole which were heated at 37 °C for

10 min). Samples were then centrifuged at 20 800 x g for 1 min at room temperature. A 10 µL aliquot of prestained molecular weight markers (Low Range 20.5-103 kD, Bio-Rad Laboratories or High Range 43-205 kD, Pharmacia Biotech) were routinely loaded as required. Electrophoresis was conducted at 200 V for 60 min.

2.2.5 Coomassie Brilliant Blue (CBB) staining of SDS-PAGE gels

After completion of SDS-PAGE, gels were immersed in CBB stain (0.1 % (w/v) Coomassie Brilliant Blue R-250, 40 % (v/v) methanol, 10 % (v/v) acetic acid) for 30 min with gentle agitation and then rinsed with several changes of CBB destain (30 % (v/v) ethanol) until the background became clear.

2.2.6 Drying of gels following SDS-PAGE

Polyacrylamide gels were air dried by placing between sheets of GelAir Cellophane support (BioRad), and standing the assembly at room temperature for (typically) 48 h.

2.3 Molecular methods

Solutions used in this section were sterilised either in a bench pressure cooker or by autoclaving at 103 kPa for various times dependent on the volume of liquid (20 min for 500 mL or less; 30 min for 1 L; 45 min for 2 L), or solutions were filter sterilised through a 0.22 µM nitrocellulose filter (Millex®-GS sterilising filter unit, Millipore).

2.3.1 Culture of bacteria

2.3.1.1 Preparation of LB media

LB (Luria-Bertani) growth medium (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) was used for growth of *E. coli* cultures with vigorous shaking (225 rpm) at 37 °C. *E. coli* cultures were maintained on LB plates which contained 1.5 % (w/v)

agar (GIBCO BRL) added to the LB media. Media was supplemented with 100 μg mL^{-1} (Amp^{100}) when required.

2.3.1.2 Preparation of competent cells

E. coli cells used for transformation were prepared from *E. coli* strain DH5α (GIBCO BRL). From a single *E. coli* colony, bacterial cells were cultured in 10 mL LB broth at 37 °C with vigorous shaking (225 rpm) overnight. Fresh LB medium (40 mL) was then inoculated with 0.4 mL of the overnight culture and incubated at 35 °C until cell growth reached an optical density at 600 nm of 0.4. The broth was centrifuged at 2 000 x g for 5 min at 4 °C and 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 $CaCl_2$. After incubation on ice for 30 min, the cell suspension was centrifuged at 2 000 x g for 5 min at 4 °C and the pellets resuspended in 4 mL 60 mM $CaCl_2$, containing 15 % (v/v) glycerol. Aliquots of the cell suspension (300 μL) were transferred to microfuge tubes and stored at –80 °C until required for transformation.

2.3.2 Characterisation and sequencing of cloned DNA in E. coli

2.3.2.1 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* using the alkaline lysis method (Sambrook *et al.*, 1989). A single colony was used to inoculate 10 mL LB-Amp^{100} broth and the broth incubated at 37 °C overnight with shaking (225 rpm). Cells were then pelleted by centrifugation at 3 000 x g for 5 min at room temperature. The supernatant was drained and cells were resuspended in 200 μL of alkaline lysis solution A (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA, pH 8). The cell suspension was transferred to microfuge tubes and cells were lysed by gently mixing with 400 μL of alkaline lysis solution B (0.2 M NaOH, 1 % (w/v) SDS) followed by incubation on ice for 10 min. Alkaline lysis solution C (3 M potassium acetate, 2 M glacial acetic acid) was then added (300 μL) to precipitate contaminating chromosomal DNA and protein, and the mixture was shaken vigorously and then incubated on ice for 5 min. Cell debris was pelleted by centrifugation at 20 800 x g for 5 min at room temperature, and the

supernatant transferred to new microfuge tube where plasmid DNA was precipitated by the addition of an equal volume of isopropanol. The DNA was pelleted by centrifugation at 20 800 x g for 10 min at room temperature, the pellet rinsed with ice-cold 80 % (v/v) ethanol, dried briefly in a SpeedVac (DNA Speed Vac DNA 110 Savant, Biolab Scientific) and resuspended in sterile water (typically 30 µL) or TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and stored at –20 °C until used.

2.3.2.2 Ethanol precipitation of DNA or RNA

Precipitation of nucleic acids was performed by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes (for DNA) or 3 volumes (for RNA) of ice-cold 100 % (v/v) ethanol followed by incubation either at –80 °C for 1 h, or overnight at 4 °C (when maximal yield was required). The nucleic acid precipitate was then pelleted by centrifugation at 20 800 x g for 20 min at 4 °C, and the pellet rinsed with 80 % (v/v) ethanol, with any residual ethanol removed with a pipette following collection by pulse-centrifugation. The pellet was air dried or dried in a heating block at 40 °C for 3 min and resuspended in sterile water.

2.3.2.3 Quantitation of DNA in solution

An aliquot of DNA (typically 5 µL) diluted in 1 mL of water, was quantified by comparing the absorbance at 260 nm against a water blank in a 1 cm light path quartz cuvette. The concentration of nucleic acid present in the sample was calculated using the following equation:

A_{260} X dilution factor X 50 = DNA concentration (µg/ mL)

An OD of 1 corresponds to approximately 50 µg/ mL of double stranded DNA (Sambrook *et al.*, 1989).

The A_{260}/A_{280} ratio was used to determine the purity of nucleotide solutions. Relatively pure DNA has an A_{260}/A_{280} ratio of 1.8 (Sambrook *et al.*, 1989).

2.3.2.4 Digestion of DNA with restriction endonuclease enzymes

DNA digestion was performed for preparation of plasmid DNA and insert DNA for cloning, to confirm the presence and size of an insert in a plasmid, or to excise an insert from a plasmid. Plasmid DNA (or PCR product for insertion in plasmids) was digested in reaction mixtures containing 40 U restriction enzyme (GIBCO BRL), 0.1 volumes of 10 X restriction buffer (GIBCO BRL), 20 µg RNase A (Sigma) and sterile water to 40 µL. Reaction mixtures were incubated for 2-3 h at 37 °C. Digested DNA (or an aliquot of digestion mixture) was then separated by agarose gel electrophoresis (section 2.3.2.5) to determine whether the enzyme had cut to completion (by the presence of a linearised plasmid) or to confirm the presence or size of an excised insert, or for separation from other components in the reaction mixture prior to gel purification (2.3.2.6). Remaining digested DNA not used for agarose gel electrophoresis could be ethanol precipitated (section 2.3.2.2) prior to digestion with a second enzyme that required different buffer conditions than those in the original digestion.

2.3.2.5 Agarose gel electrophoresis

A 1 % (w/v) agarose gel was prepared by dissolving agarose (UltraPURE™ agarose, GIBCO BRL), with heating, in 1 X TAE buffer (10 X TAE: 0.4 M Tris, 0.2 M glacial acetic acid, 10 mM EDTA pH 8.0). An aliquot of ethidium bromide (stock concentration 10 mg/ mL) was added to the gel solution (1 µL/ 30 mL gel) after the solution had cooled sufficiently, and the gel was then poured into a horizontal mini gel apparatus (Bio-Rad DNA Mini Sub Cell) with a comb inserted for loading wells for samples. No ethidium bromide solution was added to the gel solution used for genomic analysis.

The gel mixture was allowed to cool and then immersed in running buffer (1 X TAE). DNA samples, including molecular size markers (1 kb DNA ladder; GIBCO BRL), were prepared by the addition of 0.1 volumes of 10 X SUDS (0.1 M EDTA, pH 8.0, 50 % (v/v) glycerol, 1 % (w/v) SDS, 0.025 % (w/v) bromophenol blue), loaded and separated by electrophoresis at 5 to 10 V cm^{-1} for plasmid and PCR generated DNA or at 5 V cm^{-1} for genomic DNA. After electrophoresis, the DNA fragments were

visualised on a short wavelength (340 nm) UV Transilluminator (UVP Inc., San Gabriel, CA, USA), and photographed either digitally with an Alpha Imager™ 2000 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA, USA) or using a Polaroid Land Camera with Polaroid 667 film (Fabrique au Royanne-Uni. UK, Ltd., Hertfordshire, England).

For gels used for genomic analysis, the gel was stained after electrophoresis with 0.1 µg/ mL ethidium bromide for 20 min and then destained with water for 20 min.

2.3.2.6 Recovery of DNA from agarose gels

DNA fragments were excised from agarose gels for ligation reactions or labelling reactions. Following separation of DNA by electrophoresis, the desired DNA fragment was identified under long-wave UV, excised from the gel with a sterile scalpel blade and placed in a pre-weighed microfuge tube. Three volumes of 6 M NaI were added per gram of excised gel and the gel heated at 50 °C until the gel was completely melted. Resuspended DNA purification resin (1 mL) provided in the Wizard™ MiniPreps DNA Purification System (Promega Corporation, Madison, WI USA) was then added. After sitting for 2 min at room temperature, the DNA/resin mixture was transferred to a 3 mL syringe barrel (Becton Dickensen) and pushed through an attached minicolumn. Minicolumns were flushed with 2 mL of column wash solution (8.3 mM Tris-HCl, pH 7.5, 80 mM KOAc, 40 µM EDTA, pH 8.0, 55 % (v/v) ethanol) and residual wash solution was removed by centrifugation at 3 800 x g for 1 min. Sterile water (50 µL) preheated to 70 °C was then added to the column and after 2 min at room temperature the DNA was eluted by centrifugation at 18 000 x g for 20 sec.

2.3.2.7 DNA ligation

DNA or cDNA sequences were ligated into the pCR®2.1 linearised T-vector (Invitrogen, Leek, The Netherlands) by following the method provided with the TA-cloning® kit. This cloning technique relies on a deoxyadenosine attachment at the 3'-ends of the PCR product by the terminal transferase activity of *Taq* DNA polymerase, which is a complementary to an overhanging 3'-T in the vector, as shown in Figure 2.1.

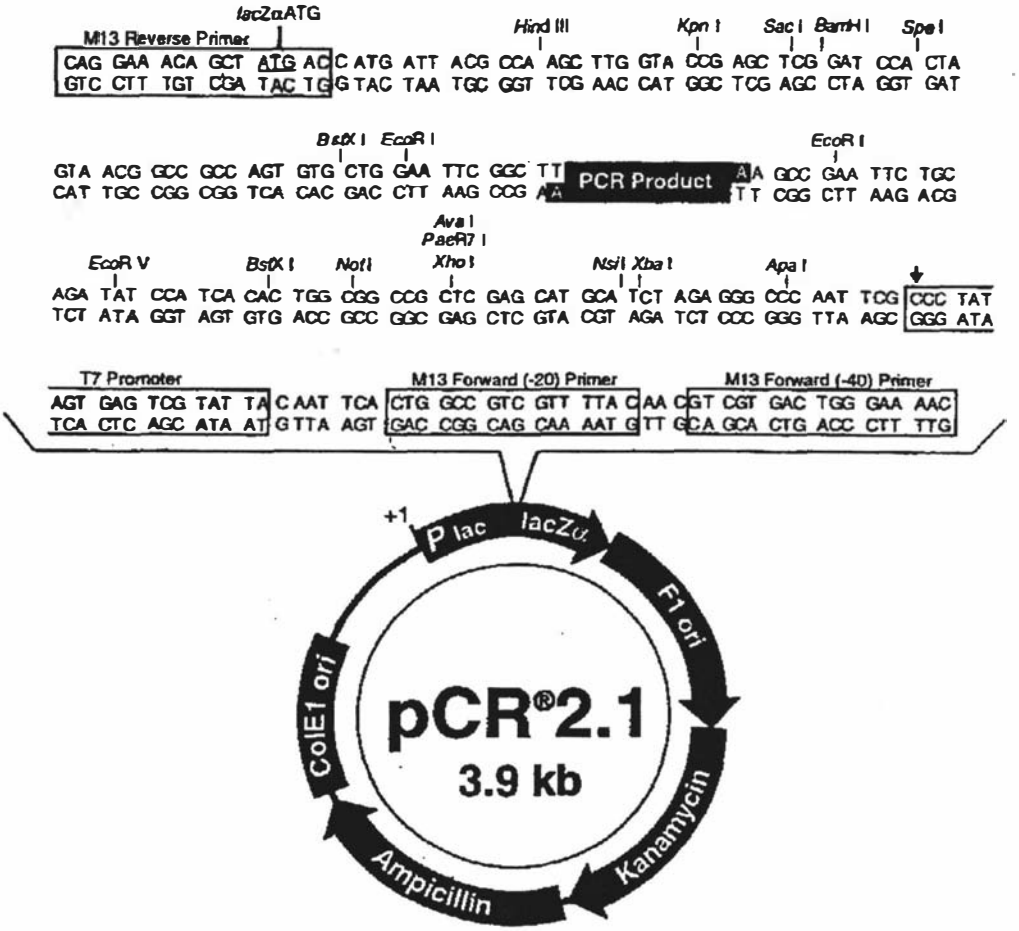


Figure 2.1 Map of the pCR 2.1 plasmid used for TA-cloning PCR generated sequences.

The sequence of the multiple cloning site is shown with a PCR product inserted by TA-cloning. The inserted PCR product is flanked on both sides by *Eco*R 1sites that are used to excise the insert from the plasmid. The M13 Forward and Reverse Primers used in sequencing are indicated. (Reproduced from the TA-cloning® manual).

For ligations into the pPROEX-1 vector (Figure 2.2), an approximately 1:3 molar ratio of vector to insert was used. The quantity of vector and insert DNA was determined visually by resolving an aliquot of vector and PCR product on the same 1 % (w/v) agarose gel and estimating from the ethidium bromide staining intensities. The appropriate amount of vector and PCR product was ligated in a reaction mix that contained 2μL of T4 DNA ligase, 2 μL of 10 X ligation buffer and sterile water to 20 μL. The ligation mix was incubated at 14 °C overnight in a PTC-200 Peltier Thermal Cycler.

2.3.2.8 Heat shock transformation of *E. coli*

The ligated vector and insert was transformed into *E. coli* strain DH5α cells made competent (section 2.3.1.2). A 300 μL aliquot of competent cells was thawed on ice and an aliquot (1 –10 μL) of the ligation mixture was added. Following a 30 minute incubation on ice, the cells were heat-shocked at 42 °C for 2 min, placed back on ice for 2 min, 1 mL LB media was added, and the cells were incubated with shaking at 37 °C for 45 min. Cells were then pelleted by centrifugation at 8 000 x g for 30 sec at room temperature and resuspended with 200 μL of fresh LB media. Aliquots (20 μL and 100 μL) of the cell suspension were spread onto separate LB-AMP^{100} plates and the plates were incubated at 37 °C overnight and stored at 4 °C.

Preparation of glycerol stocks for long term storage

Glycerol stocks of colonies of interest were made for long-term storage. This was done by inoculating a 10 mL LB-Amp^{100} broth with a single colony and incubating overnight at 37 °C with shaking (225 rpm). Cells were then pelleted by centrifugation at 3000 x g for 10 min, resuspended in 200 μL of fresh LB media and 100 μL of the suspension added to a mixture of 200 μL of sterile glycerol and 750 μL of LB media. The cells were mixed well and stored at –80 °C until required.

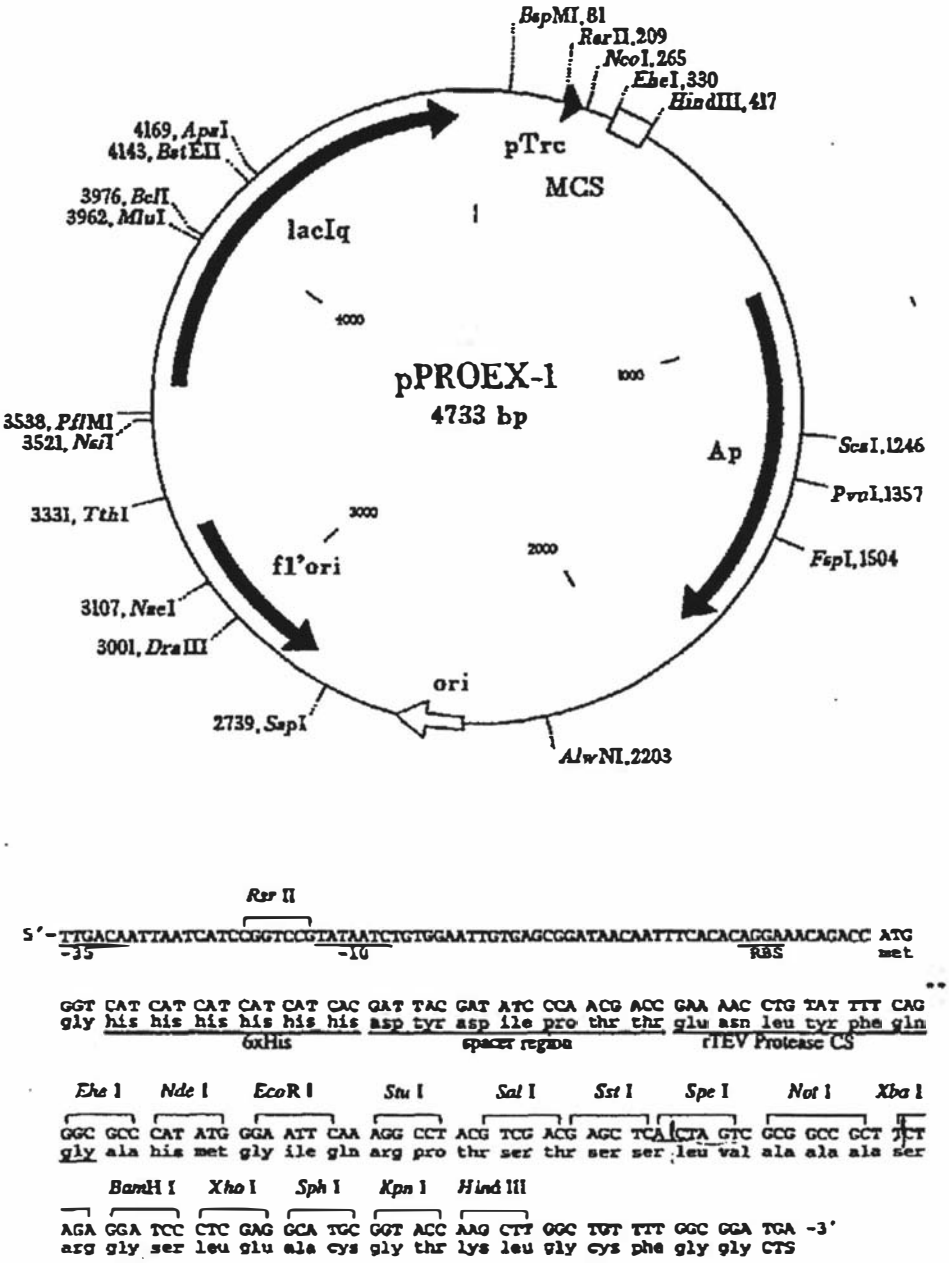


Figure 2.2 Map of the pPROEX-1 plasmid and multiple cloning sites.

The restriction enzyme sites used for cloning *TR-ACS1*, *TR-ACS2* and *TR-ACS3* in-frame into the multiple cloning site are included in the vector sequence. The 6 X His repeats, used for purification of the expressed protein by Ni-NTA column chromatography are also shown in the vector sequence.

2.3.2.9 Purification of DNA for sequencing

Plasmid DNA isolated by alkaline lysis (section 2.3.2.1) was made up to 100 µL with sterile water and 10 µL (50 µg) of RNase was added. Following a 10 min incubation at 37 °C, 1 mL of thoroughly resuspended Wizard™ MiniPreps DNA purification resin was added, and the plasmid column-purified (section 2.3.2.6).

2.3.2.10 Automated sequencing of DNA

Plasmid DNA was sent for automated sequencing to the Massey University DNA Sequencing Centre in the Institute of Molecular BioSciences, Massey University, Palmerston North, NZ. DNA was prepared at a concentration of 200 ng μL^{-1} and was sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpiTaq®DNA polymerase, FS (Perkin Elmer, Foster City, CA, USA), and the products analysed with an automated ABI PRISM™ 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.3.3 DNA sequence analysis

2.3.3.1 Sequence alignment

Alignment analysis of DNA sequences was performed using the Align Plus sequence Alignment Program (version 2, Science and Educational Software, State Line, PA, USA). GenBank database searching was performed using a Basic Alignment Sequence Tool (Altschul *et al.*, 1990).

2.3.3.2 Phylogenetic analysis of sequences

A phylogenetic tree was built (with the aid of Dr Peter Lockhart, Institute of Molecular BioSciences, Massey University, Palmerston North, NZ) using a heuristic search with default parameters of a pre-release β-version of the phylogenetic analysis using parsimony (PAUP version 4.0 od64, Sinaur Assoc., Inc. Publishers, Sunderland Massachussetts©1998 Smithsonian Institute).

2.3.4 Southern analysis

2.3.4.1 Isolation of genomic DNA

Genomic DNA was isolated by a modified method of Junghans and Metzlaff (1990). Approximately 1.6 g of frozen, powdered leaf tissue was thawed in a mixture of 10 mL lysis buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 50 mM EDTA, 0.5 % (w/v) SDS) and 10 mL Tris-buffered phenol (6 mL 1 M Tris-HCl, pH 8.0, 7.5 mL 2M NaOH, 130 mL H_2O, 500 g commercial phenol crystals) and shaken vigorously for 3 min. Five mL of chloroform: isoamyl alcohol (24:1) was added and mixed for a further 3 min. The cellular debris was then pelleted by centrifugation at 26 000 x g for 10 min at 4 °C. The aqueous supernatant was transferred to a new centrifuge tube containing 5 mL of Tris-buffered phenol and shaken for 3 min. A further 5 mL of chloroform: isoamyl alcohol (24:1) was added and the mixture shaken again for 3 min. Centrifugation (26 000 x g for 10 min) was used to separate the aqueous and organic phases, and the aqueous phase was transferred to a new tube containing 10 mL of chloroform: isoamyl alcohol (24:1) and shaken for 3 min. The aqueous phase was again separated and removed to a new tube as before. Isopropanol (0.67 volumes) was used to precipitate the nucleic acid, which was then pelleted by centrifugation at 26 000 x g for 10 min. The pellet was then rinsed with 80 % (v/v) ethanol, air-dried and resuspended in 4 mL TES/RNase solution (made up as 25 mL TES: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, containing 40 µL RNase A (10 mg/ ml). After incubation at 37 °C for 20 min, the nucleic acid was transferred to a fresh centrifuge tube containing 2 mL phenol and shaken for 3 min. Two mL of chloroform: isoamyl alcohol (24:1) was added and the solution shaken for a further 3 min. The aqueous phase was separated by centrifugation as before, transferred to a fresh tube that contained 4 mL chloroform: isoamyl alcohol (24:1), shaken for 3 min, and the centrifugation repeated. The aqueous phase was transferred to a new tube, the nucleic acid was precipitated with ethanol (section 2.3.2.2), and then resuspended in 500 µL of 0.25 M NaCl. While mixing, 0.35 volumes of 100 % (v/v) ethanol were slowly added to precipitate contaminating polysaccharides and the mix incubated on ice for 15 min. Following centrifugation (20 800 x g for 5 min at 4 °C) the supernatant was removed and the DNA precipitated with ethanol,

resuspended in sterile water and quantified (section 2.3.2.3). DNA was stored at –20 °C until required.

2.3.4.2 Digestion of genomic DNA

Genomic DNA was separated in three aliquots and each was digested with either *Eco*R I, *Xba* I or *Hind* III restriction enzymes. Each digestion mixture contained 30 µg DNA, 80 U of restriction enzyme, 20 µL of 10 X restriction buffer and was made up to 200 µL with sterile water. Following an overnight incubation at 37 °C, a further 30 U of enzyme was added and the DNA digested for another 3 h. Ethanol precipitation of the DNA was carried out overnight (section 2.3.2.2), and the DNA was resuspended in 20 µL sterile water for agarose gel electrophoresis.

2.3.4.3 Southern blotting of genomic DNA

Following electrophoresis (section 2.3.2.5), genomic DNA fragments were transferred to a nylon membrane by the alkaline downward capillary transfer method of Chomczynski (1992). The gel was immersed in depurination solution (0.25 M HCl) for 7 min, rinsed twice with RO water and placed in denaturation solution (1.5 M NaCl, 0.4 M NaOH) for 1 h. Following two more rinses with RO water, the gel was immersed in transfer solution (3 M NaCl, 8 mM NaOH; pH 11.45) for 15 min. The positively charged membrane (Hybond-N^+, Amersham, UK) was first wetted in transfer solution and the transfer stack set up as shown in Figure 2.3. Upon completion of transfer, the membrane was post-fixed by UV-crosslinker (UV Stratalinker®2400; STRATAGENE, La Jolla, CA, USA). The membrane was then neutralised in neutralisation solution (0.1 M $Na_2PO_4.2H_20$, pH 7.2) for 10 min, sealed in a plastic bag and stored at 4 °C until required.

2.3.4.4 Southern blotting of cDNA fragments

DNA fragments generated by PCR were transferred to Hybond-N^+ membrane by the alkaline downward capillary transfer method (section 2.3.4.3), except the gel was not depurinated or denatured before transfer.

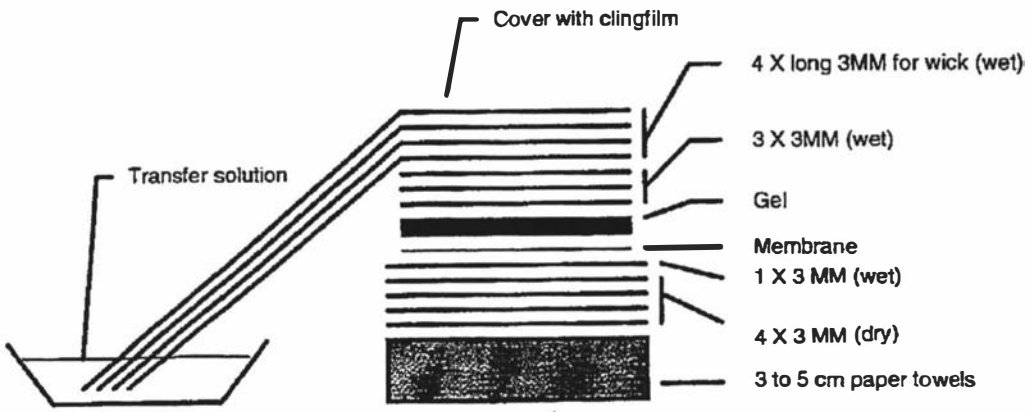


Figure 2.3 Arrangement of the apparatus used to transfer DNA and RNA samples to Hybond-N^+ membrane. From Hunter, 1998

2.3.4.5 Labelling DNA for Southern and northern analysis with [α-^{32}P]-dCTP

An aliquot of DNA (25 ng) was labelled with [α-^{32}P]-dCTP (Amersham) using the Ready-To-Go™ DNA labelling Kit (Pharmacia Biotech). The DNA was diluted with sterile water, denatured in a boiling water bath for 2 min, chilled on ice for 2 min and collected by pulse centrifugation at the bottom of the microfuge tube. The Ready-To-Go bead was resuspended with the denatured DNA and 5μL of [α-^{32}P]-dCTP (3 000 Ci/ mmol) was added and the labelling reaction was incubated at 37 °C for 15 min. The ProbeQuant™ Sephadex G-50 Micro Column (Pharmacia Biotech) was prepared by loosening the lid and snapping off the sealed bottom and centrifugation of the column in a microfuge tube at 735 x g for 1 min to remove the liquid. The column was transferred to a new microfuge tube and the labelled sample was layered onto the Sephadex. Centrifugation at 735 x g for 2 min removed unincorporated nucleotides, dyes and salts and the labelled DNA was collected at the bottom of the microfuge tube. Labelled DNA was denatured in boiling water for 2 min and added to the pre-equilibrated (65 °C) membrane-containing hybridisation solution (section 2.3.4.7).

2.3.4.6 Labelling DNA for Southern analysis with [α-^{32}P]-dATP

The Megaprime™ DNA Labelling Kit (Amersham) was used to label DNA with [α-^{32}P]-dATP (Amersham). Five μL of diluted DNA (approximately 5 ng μL^{-1}) was combined with 5 μL of nonamer primers and denatured in a boiling water bath for 3 min. Following pulse centrifugation to collect the contents, 4 μL each of the unlabelled nucleotides (dGTP, dCTP and dTTP) were added. Reaction buffer (5 μL) was added with 2 μL of 'Klenow' polymerase and 17 μL of sterile water to the DNA. After the addition of 5 μL of [α-^{32}P]-dATP (3 000 Ci/ mmol), the reaction was incubated at 37 °C for 15 min, then stopped by the addition of 5 μL of 0.2 M EDTA, pH 8.0. The radiolabelled DNA was then purified through a ProbeQuant Sephadex G-50 Micro Column, as described in section 2.3.4.5, and denatured for 3 min in a boiling water bath before adding to the pre-equilibrated (65 °C) hybridisation solution in which the membrane was immersed (section 2.3.4.7).

2.3.4.7 Hybridisation and washing of northern and Southern blots

The Church and Gilbert (1984) method was used for labelling and washing of Hybond-N^+ membranes. The membrane was rolled up (nucleic acid facing inwards) and placed in a Hybaid™ glass tube (Hybaid Ltd., Teddington, Middlesex, UK) containing 25 mL of Church hybridisation solution (0.25 M $Na_2PO_4.2H_2O$, pH 7.2, 7 % (w/v) SDS, 1 % (w/v) BSA fraction V, 1 mM EDTA, pH 8.0) and placed in a rotary oven at 65 °C. The denatured labelled probe (sections 2.3.4.5 and 2.3.4.6) was added to the hybridisation solution and circulated over the membrane overnight at 65 °C. A series of washes were then undertaken, each at 65 °C. In the first, 100 mL of hybridisation wash solution (20 mM $Na_2PO_4.2H_2O$, pH 7.2, 5 % (w/v) SDS, 0.5 % (w/v) BSA fraction V, 1 mM EDTA, pH 8.0) was used to wash the membrane for 20 min. This was followed by washes in dilutions of a 20 X SSPE solution (0.2 M Na_2HPO_4, pH 6.5, 3.6 M NaCl, 20 mM EDTA). The first of these washes was in 2 X SSPE (2 X SSPE pH 6.5, 0.1 % (w/v) SDS) for 20 min, followed by 20 min washes in 1 X SSPE (1 X SSPE pH 6.5, 0.1 % (w/v) SDS) and 0.2 X SSPE (0.2 X SSPE pH 6.5, 0.1 % (w/v) SDS). A final 40 min wash was in 0.1 X SSPE (0.1 X SSPE pH 6.5, 0.1 % (w/v) SDS). The membrane was then wrapped in a plastic bag, sealed and placed in a X-OMATIC cassette (Eastman Kodak Company, Rochester, NY, USA) containing a single X-OMATIC intensifying screen and XAR-5 X-ray film (Kodak). After exposure for the appropriate time period at –80 °C, the film was developed using an AUTOMATIC X-RAY FILM PROCESSOR (100Plus™, All-Pro Imaging, Hicksville, NY, USA) using Kodak developer (HC110) and Rapid Fixer Solution A (East Kodak Company).

2.3.5 Northern analysis

2.3.5.1 Extraction of total RNA

Total RNA was isolated using the method described by Van Slogteren *et al.*, (1983) with some modifications. Frozen tissue (1 to 4 g) was powdered in liquid nitrogen and added to five volumes of extraction buffer [50 mM Tris-HCl, pH 8.0, 50 mM LiCl, 5 mM EDTA pH 8.0, 0.5 % (w/v) SDS, 50 % (v/v) phenol (prepared by dissolving 500 g of phenol in 6 mL of 1 M Tris-HCl, pH 8.0, 7.5 mL of 2 M NaOH, 130 mL of H_2O)]

equilibrated at 80 °C. The extraction mix was vortexed for 30 sec and chloroform: isoamyl alcohol (24: 1) was added (in equal volume to the phenol component of the extraction buffer) and the mixture vortexed again for 30 sec. The extraction mix was then incubated at 50 °C for 20 min followed by centrifugation at 4 000 x g for 30 min at 4 °C. The upper aqueous supernatant was transferred to a fresh tube, an equal volume of ice cold 4 M LiCl was added and the RNA precipitated overnight at 4 °C. The precipitate was then pelleted by centrifugation at 4 000 x g for 30 min at 4 °C, the pellet was resuspended in sterile water, an equal volume of chloroform: isoamyl alcohol (24: 1) was added and the mixture was vortexed then separated by centrifugation at 20 800 x g for 3 min at room temperature. The aqueous phase was transferred to a new tube and the RNA precipitated with ethanol (2.3.2.2), resuspended in 50 µL RNase-free water and stored at –80 °C until required. The quantity of RNA was measured as described in section 2.3.5.3.

2.3.5.2 Isolation of poly(A)$^+$ mRNA

Poly(A)$^+$ mRNA was extracted from total RNA using biotinylated oligo(dT) bound to streptavidin MagneSphere™ particles, following the method supplied with the PolyAT tract mRNA isolation system IV kit (Promega, Madison, WI, USA).

Total RNA (100 to 1000 µg) was made up to 500 µL with RNase-free water and incubated at 65 °C for 10 minutes to denature the RNA. A 3 µL aliquot of the biotinylated 17 mer oligo (dT) primer and 13 µL of 20 X SSC (0.3 M sodium citrate, pH 7.0, 3 M NaCl) were added and mixed, and the solution allowed to cool down at room temperature for 10 min. The Streptavidin Magne-Sphere® Particles (SA-PMPs) were resuspended by flicking the bottom of the tube, and following the removal of the preservative solution using the magnetic stand for capturing the beads, the SA-PMPs were washed 3 times with 300 µL aliquots of 0.5 X SSC. The SA-PMPs were then resuspended in 100 µL of 0.5 X SSC and the RNA solution added. The mixture was incubated for 10 min at room temperature to allow binding between the biotin and streptavidin beads, and the beads were then washed 4 times using 300 µL aliquots of 0.1 X SSC. Following the final wash, as much supernatant was removed as possible. The bound poly(A)$^+$ mRNA was recovered by resuspending the beads in 100 µL of RNase-

free water and incubating the mixture at room temperature for 5 min. The SA-PMPs were captured using the magnetic stand and the supernatant was transferred to a fresh microfuge tube. The SA-PMPs were again resuspended in 150 µL of sterile water, incubated for 5 min and any residual poly(A)$^+$ mRNA collected and pooled with the previously collected sample. Poly(A)$^+$ mRNA was quantified (as described in section 2.3.5.3) and stored at –80 °C until required.

2.3.5.3 Quantification of RNA in solution

To measure total RNA concentration, the method used for DNA quantification (section 2.3.2.3) was used. For RNA, an OD_{260} of 0.1 corresponds to approximately 40 µg mL^{-1} (Sambrook *et al.*, 1989).

A_{260} nm X dilution factor X 40 = RNA concentration in µg/ mL

The purity of RNA was determined by measuring the A_{260}/A_{280} ratio. Relatively pure RNA solutions have an A_{260}/A_{280} ratio of 2.0 (Sambrook *et al.*, 1989).

The quantification of poly(A)$^+$ mRNA was in a 250 µL volume, using a 1 cm light path, and a quartz cuvette that had been incubated with 50 mM NaOH to denature RNases and rinsed with large amounts of sterile water. Sterile water was used for rinsing between measuring samples.

2.3.5.4 Electrophoresis of RNA

Total and poly(A)$^+$ mRNA were resolved by formaldehyde gel electrophoresis in a Bio-Rad DNA Sub Cell™ (225 cm^2 gel bed: 100 mL volume). A 1 % (w/v) agarose gel was prepared by adding 95 mL of 1 X MSE buffer (10 X MSE: 200 mM MOPS, 50 mM NaOAc, 10 mM EDTA) to 1 g of agarose. The weight of the flask was noted, the gel mix then heated to dissolve the agarose, the flask reweighed and any water that had evaporated during heating was replaced. After the gel had cooled sufficiently, 5 mL of 37 % (v/v) formaldehyde was added, to give a final concentration of 3 % (v/v), and the

solution poured into the gel forming apparatus. After the gel had set, running buffer (0.22 M formaldehyde in 1 X MSE) was added and the RNA samples loaded.

Aliquots of 10 µL containing 4 to 12 µg of poly(A)$^+$ mRNA were mixed with 20 µL of RNA loading buffer (1 mL RNA gel loading buffer comprises 212.5 µL of 37 % (v/v) formaldehyde, 125 µL MSE (10 X), 5 µL ethidium bromide stock solution (section 2.3.2.5) and 625 µL formamide/BB/XC stock solution (formamide/BB/XC stock solution: 0.01 % (w/v) bromophenol blue, 0.01 % (w/v) xylene cyanol in formamide), and then the RNA was denatured at 65 °C for 15 min, cooled on ice for 2 min and then loaded into wells. A GIBCO BRL 0.24 – 9.5 Kb RNA ladder (Life Technologies) was denatured in the same way and loaded into separate wells.

Electrophoresis was conducted at 80 V for 5 h, after which the gel was visualised using a short-wave (340 nm) UV transilluminator. The migration distance of the molecular weight markers was marked and gels were photographed either digitally with an Alpha Imager 2000 Documentation and Analysis System or with a Polaroid Land Camera using Polaroid 667 film. The RNA was then transferred to a Hybond-N$^+$ membrane.

2.3.5.5 Northern blotting

RNA was transferred to a Hybond-N$^+$ membrane by the method of Chomczynski (1992) as described for Southern blotting (section 2.3.4.3), except the gel was not pre-treated by depurination or denaturation, and the transfer was carried out for only 4 h.

2.3.6 Reverse transcriptase dependent polymerase chain reaction

2.3.6.1 Generation of cDNA using reverse transcriptase

Five µg of total RNA or 1 µg poly(A)$^+$ mRNA in 5 µL of sterile water was denatured with 0.5 µL (0.5 µg) of 17 mer oligo-dT primers at 70 ° C for 10 min. The mixture was then cooled on ice for 2 min and centrifuged briefly. A reaction buffer was then added comprising 4 µL of 5 X first strand buffer (GIBCO BRL, Life Technologies), 2 µL of 0.1 M DTT (GIBCO BRL, Life Technologies), 1 µL of 10 mM dNTP mix (GIBCO

BRL, Life Technologies), 2 μL of ribonuclease inhibitor (GIBCO BRL, Life Technologies) and the reaction mixture made up to 19 μL with sterile water. The mixture was incubated at 42 °C for 2 min and 1 μL of reverse transcriptase (Superscript II; GIBCO BRL) was added. The reaction was mixed by pipette and then incubated at 42 °C for 50 min. The enzyme was then denatured by incubating the reaction at 70 °C for 10 min.

2.3.6.2 Amplification of DNA or cDNA by PCR

Primers (GIBCO BRL, Life Technologies) were dissolved in sterile water at a concentration of 100 μM. A working stock was prepared by diluting one part primer to two parts water, giving a concentration of 33 μM. In a 50 μL volume of PCR mixture, typically 0.5 μL of primer was used, giving a final primer concentration of 330 nM.

Degenerate primers used in RT-PCR

Nested PCR reactions were carried out using two sets of degenerate oligonucleotide primers (Table 2.3). The primers are known to bind to conserved regions of the ACC synthase gene and were provided by Professor S.F. Yang (University of California, Davis, USA).

PCR was performed in 50 μL volumes using the Expand™ High Fidelity system as described by the manufacturer (Boehringer Mannheim, GmbH, Mannheim, Germany). Two master mixtures were prepared on ice. Master mix 1 consisted of 1 μL of 10 mM dNTP mixture (GIBCO BRL), 0.5 μL of 33 μM upstream (5′) primer, 0.5 μL of 33 μM downstream (3′) primer, DNA template (either 3 μL of first strand cDNA template, 1 μL of spin-column purified plasmid (section 2.3.2.6) or 2 μL of first round PCR mixture) and made up to 25 μL with water. Master mixture 2 consisted of 0.5 μL of Expand High Fidelity enzyme mixture, 5 μL of 10 X PCR buffer with 15 mM $MgCl_2$ (GIBCO BRL) and sterile water to 25 μL. The two mixtures were combined, placed into a PTC-200 Peltier Thermal Cycler and the PCR carried out for 30 cycles, with each cycle consisting of 1 min at 92 °C (denaturation), 1 min at 42 °C (annealing) and 1 min 40 sec at 72 °C (extension). After 30 cycles, a final extension time of 10 min at 72 °C

was used. The annealing temperature was raised to 50 °C when amplifying from plasmid templates.

Gene-specific primers used in RT-PCR

Primers (Table 2.3) were designed to amplify regions of specific *TR-ACS* genes either from plasmid DNA, where one round of PCR was used, or from first strand cDNA template, where either one or two rounds of PCR were required. PCR conditions were carried out as above, however, the annealing temperature was increased to 55 °C.

Primers for in-frame cloning into the pPROEX™-1 vector

The GIBCO BRL pPROEX™-1 protein expression system was used to translate foreign gene sequences in *E. coli* (section 2.4.1). The insertion in the correct orientation of the cDNA template is required for in-frame cloning into the pPROEX plasmid vector. Primers were designed to accomplish this (Table 2.3) and used in PCR with plasmid cDNA template to generate sequences containing different restriction sites at their 5′ and 3′ends. These two restriction sites could be used to directionally clone the sequence, in-frame, into the multiple cloning site of the pPROEX vector. Where necessary, spacer nucleotides were added between the coding frame section of the primer and the restriction site for in-frame cloning. Additional nucleotides were added to cap the restriction site because restriction enzymes cut poorly at the ends of PCR fragments (New England Biolabs Catalogue, 1993).

Table 2.3 Primer sequences used in PCR for amplification of ACC synthase sequences (refer Figure 3.10)

	Primers for degenerate ACC synthase sequences
First round Forward (ACSR1F)	GCCGCCTTCATGGGNYTNGCNGARGAAY
Second round Forward (ACSR2F)	CTGGATCCGTWYCARGAYTAYCAYGG
Reverse (ACSR6R)	CTCAAGCTTARNSYTRAARCTNGACAT
	Primers for gene-specific sequences
TR-ACS1 Forward (ACS1F)	GGCTAAATTCATGTCTAGAACA
TR-ACS2 Forward (ACS2F)	CCCTTCATTCAAACAAGCATTGGTAG
TR-ACS3 Forward (ACS3F)	AAAAGTGAGAGGTGGTAGGGTAAG
Reverse (ACSR6R)	CTCAAGCTTARNSYTRAARCTNGACAT
Active site reverse (ACS3AR)	GCGTCCCAKATCTTTTGAAAGACT
	Primers for in-frame cloning into pPROEX
TR-ACS1 Forward (ACSF1)	GCGGGAATTCAATWYCARGAYTAYCAYGGT
TR-ACS2 and *TR-ACS3* Forward (ACSF2)	GCGGGATCCTWYCARGAYTAYCAYGGT
Reverse (ACSR1)	GCGCTCGAGNSYRAARCTNGACAT

Primers for amplification of β-actin

To compare the expression level of a specific mRNA during different developmental stages or during induction treatments an internal control was required to normalise each sample for variation in the amount of starting RNA. β-actin primers were used to amplify a fragment of the β-actin gene (Table 2.4). PCR conditions were as described above except the annealing temperature was increased to 62 °C.

Table 2.4 Primer sequences used for amplification of β-actin

β-actin Forward	TGAAGTACCCCATCGAGCACG
β-actin Reverse	AGTGATCTCCTTCTGCATCCTGT

2.3.6.3 Relative RT-PCR using QuantumRNA™ 18S Internal Standards

The invariant expression of 18S ribosomal RNA makes it an ideal internal control for RNA analysis. By using QuantumRNA™ 18S Internal Standard (Ambion), with competimers, which are specially modified primers that cannot be extended, the signal for 18S rRNA can be attenuated to the level of a rare message by modulating the efficiency of the amplification of the 18S primers.

RT-PCR was carried out using total RNA (5 μg) in 5 μL water. Random primers (GeneWorks) were added (50 μM in 2 μL) to the RNA and the solution heated at 65 °C for 10 min, chilled on ice for 2 min and collected by centrifugation. Sterile water (3 μL), 4 μL of 5 X first strand buffer (Boehringer Mannheim, GmbH, Mannheim, Germany), 2 μL of 0.1 M DTT (Boehringer Mannheim), 1 μL of 10 mM dNTP mix (GIBCO BRL), 2 μL of RNase inhibitor (GIBCO BRL) and 1 μL of Expand Polymerase (Boehringer Mannheim) was then added and the mixture was heated at 30 °C for 10 min, followed by a 45 min incubation at 42 °C.

PCR was carried out using the same primer concentration and PCR parameters as described in section 2.3.6.2. However, for these PCR reactions a supermix (GIBCO BRL) was used which contained the premixed components for PCR. To a 45 μL aliquot of supermix, a 0.5 μL aliquot each of forward and reverse primer to amplify the required ACC synthase sequence, 2 μL of cDNA template and 2 μL of 18S rRNA primers: competimers mix (1:9) was added so that the two primer sets (18S RNA; ACC synthase) are in the one PCR reaction.

2.4 Heterologous protein expression in *E. coli*

2.4.1 Protein expression using pPROEX –1 vector

The pPROEX vector system (GIBCO BRL) (Figure 2.2) was used in *E. coli* strains DH5α and TB1. In-frame PCR products were amplified using the appropriate plasmid DNA as template with the appropriate primers (Table 2.3). PCR was performed as

described in section 2.3.6.2 at an annealing temperature of 50 °C. The amplification product was checked by sizing on a 1 % (w/v) agarose gel (section 2.3.2.5) and the PCR products digested (as described in section 2.3.2.4) with either *Eco*R I and *Xho* I, or *Bam*H 1 and *Xho* 1. The pPROEX vector was also digested with these enzymes in preparation for ligation (described in section 2.3.2.7). Ligation mixes were then used for transformation of *E. coli* (section 2.3.2.8) strains DH5α and TB1. Bacterial cells transformed with the pPROEX vector were selected on LB-Amp^{100} plates and positive colonies were inoculated into a 10 mL LB-Amp^{100} broth and incubated overnight at 37 °C with shaking (225 rpm). Plasmid DNA was prepared by alkaline lysis (section 2.3.2.1) and the presence of the correct sized insert following digestion of plasmid DNA with the appropriate enzymes was confirmed by electrophoresis on a 1 % (w/v) agarose gel (2.3.2.5).

2.4.1.1 Preliminary induction of His-tagged fusion proteins in *E. coli*

A single colony of *E. coli* transformed with recombinant pPROEX was incubated overnight in 10 mL LB-Amp^{100} broths at 37 °C with shaking (225 rpm). A 500 µL aliquot of this overnight culture was then transferred into a fresh 10 mL LB-Amp^{100} broth and incubated using the same growth conditions for 3 h. One mL of the culture was removed to serve as an uninduced control. To induce the expression of foreign proteins, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cell culture to a final concentration of 0.6 mM, and the cell cultures were grown at 37 °C for a further 3 h, during which 1 mL samples were removed each hour. At the conclusion of the sampling period, the bacteria were pelleted by centrifugation at 20 800 x g for 30 sec, resuspended in 60 µL of 2 X SDS-PAGE gel loading buffer (section 2.2.4), boiled for 4 min, centifuged again for 3 min and loaded into a well on a SDS-PAGE gel (section 2.2.4). Generally, between 3 to 8 µL of sample supernatant was loaded. After completion of electrophoresis, the separated proteins in the gel were stained with CBB (section 2.2.5) and the protein profile at each time point was examined to determine the time at which protein induction was maximal, and to determine the induction of protein of the predicted size.

2.4.1.2 Large scale induction of His-tagged fusion proteins in *E. coli*

The previous procedure was scaled up after determination of the time, following IPTG induction, for maximum induction of protein of the predicted size. Broths (10 mL) were inoculated with *E. coli* and grown overnight as described above (section 2.4.1.1). An aliquot of culture (20 mL) was then used to inoculate 2 L of fresh LB-Amp^{100} media (one part overnight culture to 100 parts fresh media) and incubation continued until the bacteria culture reached an optical density at 590 nm of between 0.5 and 1.0. One mL of culture was removed to serve as an uninduced control, and IPTG was added to a final concentration of 0.6 mM to the remaining culture. Incubation was then continued until the time of maximal induction was completed (typically 3 h, based on the result of the small scale induction). The cultures were then transferred to pre-weighed centrifuge tubes and the bacteria pelleted by centrifugation at 10 000 x g for 10 min at 4 °C, the supernatant decanted, the weight of the bacteria determined, and the cells stored at –80 °C until required.

To prepare a crude protein extract containing the induced protein for subsequent purification, the pelleted bacteria were resuspended in four volumes of cell lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol), apportioned into 4 mL volumes and the protein released by sonication with a MSE Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments, Manor Royal, Sussex, England). The sonicator was fitted with a 3 mm diameter probe and used at an amplitude of 14 microns (15 sec on, and 30 sec off, repeated 6 times) with the bacteria/ protein solution kept cool on ice. Following sonication, the cellular debris were pelleted by centrifugation at 10 000 x g for 10 min at 4 °C, the supernatant removed and dialysed against 100 volumes of 50 mM Tris pH 8.0 at room temperature for 7 h to remove SDS (buffer was changed every 2 h).

2.4.1.3 Purification of His-tagged fusion protein by metal chelate affinity chromatography

The induced His-tagged fusion protein prepared in section 2.4.1.2 was purified from bacterial protein contaminants by metal-chelate affinity chromatography according to

the instructions in the pPROEX™-1 Protein Expression System kit. The affinity column (1 mL) was contained in a 10 mL disposable syringe barrel (Becton Dickensen) fitted with 2 layers of GF-A glass microfibre filter paper. The column was equilibrated with 5 volumes of buffer A (20 mM Tris-HCl pH 8.5, 100 mM KCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, 10 % (v/v) glycerol) at a flow rate of 0.5 mL/ min. The sample was then loaded on top of the column, allowed to run in, and the column washed with 10 volumes of buffer A, two volumes of buffer B (20 mM Tris-HCl pH 8.5, 1 M KCl, 10 mM 2-mercaptoethanol, 10 % (v/v) glycerol), two volumes of buffer A, and the fusion protein eluted off the column with 10 volumes of buffer C (20 mM Tris-HCl pH 8.5, 100 mM KCl, 100 mM imidazole, 10 mM 2-mercaptoethanol, 10 % (v/v) glycerol). The eluate was collected in 0.5 to 1.0 mL fractions and 5 µL aliquots of each fraction analysed by SDS-PAGE (section 2.2.4) and the protein quantified (section 2.2.3) to determine which fractions contained the fusion protein. These fractions were then pooled, and stored at 4 °C.

2.4.1.4 Amino acid sequencing

The putative 28 KD protein was purified by metal-chelate affinity chromatography (section 2.4.1.3) and then separated using a 10 % SDS-PAGE gel (section 2.2.4). The gel was then Coomassie stained and the putative TR-ACS1 band excised, placed in a microfuge tube, covered with water and sent to Ms Catriona Knight, SES, University of Auckland, for gas-phase sequencing.

2.5 Immunological analysis

2.5.1 Production of polyclonal antibodies (PAb)

The affinity purified fusion proteins (section 2.4.1.3) were used as the antigen for production of polyclonal antibodies in rabbits at the Small Animal Unit (SAPU), Massey University, Palmerston North, NZ. The primary inoculum consisted of fusion protein, PBSalt (50 mM sodium phosphate, pH 7.4, 250 mM NaCl) and complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA) in a ratio (v/v) of 1: 1:

2. The inoculum was vortexed vigorously and rabbits were inoculated with 500 µg fusion protein in a final volume of 1 mL. The rabbits were boosted three times at 4 to 5 week intervals by injecting the mixture prepared as above, except incomplete Freund's adjuvant replaced the complete adjuvant for the boost inoculations.

The rabbit blood was collected before the first injection as a pre-immune serum, and collected after the third boost as the polyclonal antibody (PAb) serum. Collected blood was allowed to clot at room temperature for 1 h, and then stored overnight at 4 °C. The antisera containing the antibodies was then removed from around the clot using a glass pipette. Any remaining cells were pelleted by centrifugation at 10 000 x g for 10 min and the serum (supernatant) stored at –20 °C.

2.5.2 Western blotting

Proteins separated by SDS-PAGE gel (section 2.2.4) were transferred to a polyvinyl fluoride membrane (PVDF; Immobilin-P, Millipore Corporation, Bedford, MA, USA) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The transfer cassette holder with gel and membrane was set up as shown in Figure 2.4.

(+ve/red terminal)

Scotch pad

3MM paper

PVDF membrane

Protein gel

3MM paper

Scotch pad

(-ve/black terminal)

Figure 2.4 Protein transfer sandwich for electro-transfer of protein onto PVDF membrane

The cassette was set up while partially immersed in transfer buffer (25 mM Tris, 10 % (v/v) methanol, 190 mM glycine) so as to exclude air bubbles, which can interfere with transfer. The PVDF membrane was soaked briefly in 100 % methanol prior to placing on top of the gel. Transfer was performed at 30 V for 1 h at room temperature.

After transfer, membranes were blocked in PBST (50 mM sodium phosphate buffer, pH 7.2, 250 mM NaCl, 0.05 % (v/v) Tween 20) containing 0.5 % (w/v) I-block (Tropix, Bedford, MA, USA) at room temperature for 1 h, or at 4 °C overnight. The membrane was washed briefly with PBST, incubated with the primary antibody at a 1:1000 dilution in PBST for 1 h at room temperature with gentle shaking, and then washed three times in PBST, each for 10 min. The membrane was incubated with the secondary antibody, goat anti-rabbit alkaline phosphatase at a dilution of 1:10 000 dilution in PBST for 1 h at room temperature, washed three times with PBST then washed twice with 150 mM Tris-HCl (pH 9.7), each for 5 min. The substrate (150 mM Tris-HCl, pH 9.7 containing 0.01 % (w/v) 5-bromo-4-chloro-3-indoyl phosphate (BCIP), 0.02 % (w/v) p-nitro blue tetrazolium chloride (NBT), 1 % (v/v) dimethyl sulphoxide (DMSO); 8 mM $MgCl_2$) was added and the reaction allowed to continue in the dark. When sufficient colour had developed, the reaction was stopped by rinsing the membrane several times in RO water.

2.5.3 Size estimation of proteins

Protein size was estimated by comparison of the electrophoretic mobility of the unknown protein with the mobility of marker proteins of known molecular weight. The distance between the origin and the centre of each of the marker proteins was used to plot a calibration curve of distance migrated against the $\log_{10}$ of molecular weight, and the size of the protein of interest determined from the regression curve.

2.6 Screening of a cDNA library

λZiplox™ (Life Technologies) is a lambda expression vector that combines the efficient cDNA cloning by lambda infection, with the convenience of plasmid manipulation. The vector containing the inserted cDNA is first screened in *E. coli* Y1090/ZL. The selected cDNA can then be recovered in the autonomously replicating plasmid pZL 1 (Figure 2.5) by excision *in vivo* in *E. coli* DH10B(ZIP) utilising the *Cre-Lox*P recombination process (D'Alessio *et al.*, 1992).

2.6.1 Preparation of E. coli stocks and plating of cDNA library

An aliquot of *E. coli* Y1090 (ZL) was streaked onto LB agar (section 2.3.1.1) and grown overnight at 37 °C. A single colony was isolated and grown overnight with vigorous shaking (225 rpm) at 37 °C in 10 mL LB media containing 100 µL $MgSO_4$ and 0.2 % (w/v) maltose. The cells were stored at 4 °C for up to 2 weeks.

The *E. coli* Y1090(ZL) cells were dispersed in the LB broth and 100 µL aliquots were transferred to microfuge tubes and incubated for 15 min at 37 °C with sufficient phage solution containing the putative ACC synthase sequence to give approximately 20 000 plaques per plate. While these were incubating, 6.5 mL aliquots of LB top agarose (LB media, section 2.3.1.1, containing 0.7 % (w/v) bacto-agar) were pipetted into 15 mL tubes containing 65 µL $MgSO_4$, and held in a water bath at 48 °C. The incubated cells were pipetted directly into the LB top agarose and then poured onto pre-warmed (37 °C) LB agar (2.3.1.1) plates. The plates were swirled to spread the top agarose evenly, and when set, inverted and incubated at 37 °C overnight.

2.6.2 Screening the cDNA library by plaque lifting

Plaque lifting involves the transfer and fixing of the cDNA contained within the phage particles to Hybond N^+ membrane (Amersham). This membrane is then probed for homologous sequences.

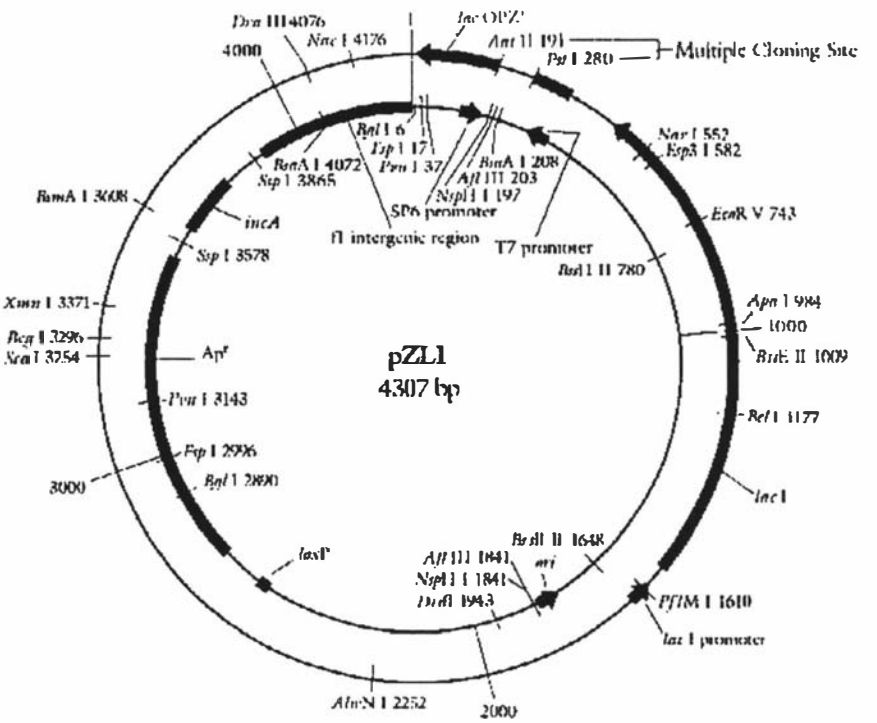


Figure 2.5 Map of the plasmid pZL 1 (Life Technologies) used for the *in vivo* excision of DNA inserts from the λ-cDNA library prepared from leaves of white clover.

Following overnight incubation, the plates containing the plated library were placed at 4 °C for a minimum of 2 h. In a laminar flow hood, a Hybond N^+ membrane was carefully placed onto the surface of the agarose avoiding any movement across the surface. Orientation holes were punched into the edge of the membrane with a syringe needle, and their positions marked on the bottom of the plate. The membrane was then transferred, DNA side up, to 3 MM paper soaked in denaturation buffer (0.5 M NaOH, 0.5 M NaCl), and incubated for 5 min. The membrane was transferred to 3 MM paper soaked in neutralisation solution (2.0 M NaCl, 0.5 M Tris-HCl pH 7.4) and incubated for a further 5 min. The membrane was then transferred to 3 MM paper soaked in 2 X SSC (20 X SSC: 17.53 % (w/v) NaCl, 8.82 % (w/v) sodium citrate; pH 7.0), incubated for 5 min, and dried on fresh 3 MM paper in a laminar flow hood. The membrane was hybridised with labelled DNA and autoradiographed as described in section 2.3.4.5.

2.6.3 Purification of plaques putatively containing cDNA of interest

Areas or plaques which gave positive signals on the autoradiograph were identified on the cDNA library plates. Pipette tips (1 mL) with the tips cut so that the opening approximated the size of the plaque were pushed into the top agarose to isolate a small region, and this agarose was transferred to a microfuge tube containing 500 µL of SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01 % (w/v) gelatin), vortexed briefly, and held at room temperature for at least 1 h. The isolated plaque solutions were re-screened (section 2.6.2) until single isolated positive plaques could be identified on the plate.

2.3.6.4 Excising pZL 1 from positive λZipLox clones

λZipLox is a modified λgt11 containing the plasmid cloning vector pSPORT 1 flanked by *lox*P sequences. When *E. coli* DH10B(ZIP), (Life Technologies) containing the plasmid pZIP (containing a gene coding for the recombination protein 'Cre'), are infected with λZipLox, the Cre protein acts on the *lox*P and excises and circularises the pZL 1 plasmid. The process also yields an intact λ phage, but a resident λ pro-phage in DH10B(ZIP) represses expression of the phage functions and prevents initiation of lytic replication.

E. coli DH10B(ZIP) cells were streaked onto LB agar plates containing 10 µg/ mL kanamycin, and incubated at 37 °C overnight with shaking (225 rpm). Purified plaques (section 2.6.3) were plated onto *E. coli* Y1090 (ZL) and a single isolated plaque selected and removed using a 1 mL pipette tip. The plaque was transferred to a microfuge tube containing 250 µL of SM buffer, vortexed for 10 sec, and incubated at room temperature for 5 min. The contents of the tube were collected by centrifugation and 25 µL of the supernatant added to 100 µL of the prepared *E. coli* DH10B(ZIP) cells as described above. The cells were incubated for 5 min at room temperature, spread onto a LB-Amp^{100} plate containing 10 mM $MgCl_2$, the plate inverted and incubated overnight at 37 °C. An isolated colony was selected from the plate and used to inoculate a 10 mL LB broth. The broth was incubated overnight at 37 °C with shaking (225 rpm), and the plasmids extracted and purified as described in section 2.3.2.1.

3. RESULTS

3.1 Screening and analysis of a cDNA library

3.1.1 Preparation and initial screening

A cDNA library, constructed by S. Butcher (Butcher, 1997), using poly(A)$^+$ mRNA isolated from white clover leaf tissue at an early stage of senescence, was screened to isolate full-length cDNAs which are homologous to ACC synthase genes. Butcher used leaf tissue subtending from node 6 (leaf 6) on stolons grown at Crop & Food Research, Levin, as a source of poly(A)$^+$ mRNA for cDNA library construction. Developmentally, this leaf tissue was just at the onset of significant chlorophyll loss associated with leaf senescence, and is similar to leaves subtending from nodes seven and eight on stolons grown as part of this thesis at the Plant Growth Unit, Massey University, Palmerston North (as determined by changes in chlorophyll levels of leaves) (data not shown). The library, containing 400 000 clones, was produced by cloning double-stranded cDNAs into the lambda expression vector λZipLox™ (Life Technologies). λZipLox is a modified λgt11 lambda expression vector that contains a modified plasmid cloning vector pSport 1 (designated pZL1) flanked by *loxP* sequences. In this system, this λZipLox (containing the inserted cDNA) is first screened in the *E. coli* strain Y1090/ZL. Any cDNA of interest can then be removed as an insert into the autonomously replicating plasmid pZL 1 by *in vivo* excision in the *E. coli* strain DH10B(ZIP), since this strain harbours a gene encoding the 'cre' recombinase (D' Alessio *et al.*, 1992). The library had previously undergone two screens for ACC synthase using a ^{32}P-labelled 670 bp PCR-generated fragment of the ACC synthase reading frame (designated ACS7) (Butcher, 1997). The PCR product was amplified using primers homologous to conserved regions of the gene (designated ACSR1F, ACSR2F and ACSR6R; Table 2.3) using two rounds of PCR with cDNA templates synthesised from RNA extracted from leaf six as template (Butcher, 1997). In the primary screen, two plaques corresponding to positive signals were identified and designated sub-library 6/1 and 10/1. A second screen resulted in seven regions of

hybridisation from sub-library 6/1 (designated 6/1/1 - 6/1/7) and 10 regions of hybridisation from sub-library 10/1 (designated 10/1/1 – 10/1/10). Aliquots of each sub-library (6/1/1 to 6/1/7 and 10/1/1 to 10/1/10) were used to infect *E. coli* DH10B(ZIP), so that any inserts of interest could be sequenced after recombination into the pZL 1 plasmid. One of these colonies, designated 10/1/10, was isolated and sequenced and shown to share homology (63%) with a soybean cysteine proteinase (Becker *et al.*, 1994; cited in Butcher, 1997). The cDNA sublibrary (10/1/1 to 10/1/10) was provided at this stage by S. Butcher for further screening and analysis in this study.

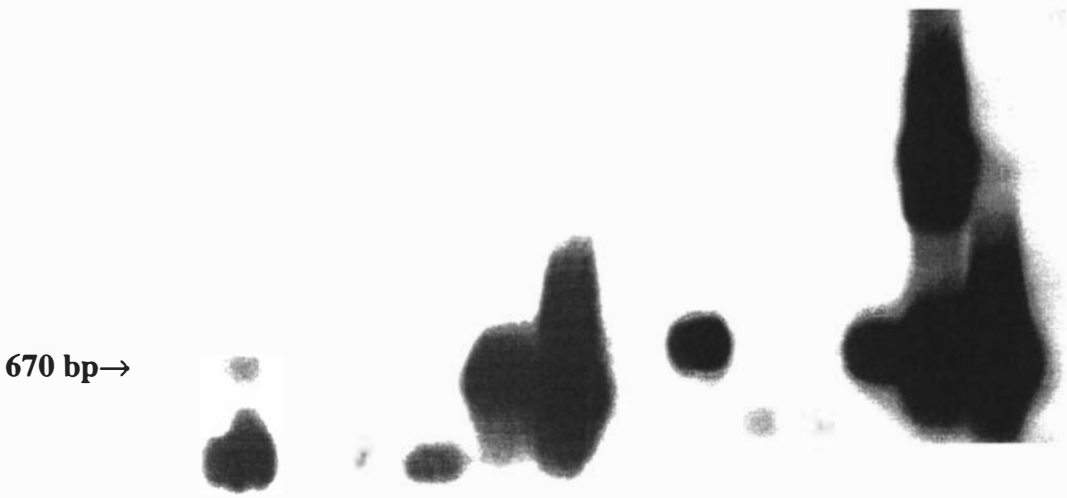
3.1.2 Further screening of cDNA library

Plaques (dispersed in SM media; section 2.6.3) corresponding to the ten regions of hybridisation (positive signals) in the second screen of sub-library 10/1 (designated 10/1/1 to 10/1/10) were used, in aliquots, as templates for two rounds of PCR amplification (30 cycles). The degenerate primers ACSR1F and ACSR6R (Table 2.3) were used with an annealing temperature of 42 °C for round one. Products from the first round of PCR amplification were used as templates for a second round of PCR amplification using the nested degenerate primer ACSR2F and ACSR6R, also with an annealing temperature of 42 °C. PCR products were separated by electrophoresis on a 1 % (w/v) agarose gel and stained with ethidium bromide. No bands were visible after one round of PCR (data not shown). However, bands corresponding to the expected 670 bp fragment of ACC synthase were detected in sub-libraries 10/1/2, 10/1/6, 10/1/7, 10/1/9, and 10/1/10 after a second round of PCR (data not shown). Following electrophoresis, second round PCR products were blotted onto Hybond N^+ membrane and hybridised with ^{32}P-labelled ACS7 (at high stringency; 0.1 x SSPE, 0.1 % (w/v) SDS, at 65 °C). ACS7 hybridised to a 670 bp fragment in all sub-libraries in which a 670 bp band was visualised by ethidium bromide staining (Figure 3.1). In two sublibraries, 10/1/2 and 10/1/5, a lower molecular weight band was also recognised by the ACS7 probe. More intense hybridisation by ^{32}P-labelled ACS7 to a 670 bp PCR product in sub-libraries 10/1/6, 10/1/7 and 10/1/10 was observed. These sub-libraries were, therefore, the focus of further screening. Hybridisation was also

Figure 3.1 Southern analysis of second round PCR products of sub-libraries 10/1/1 - 10/1/10 probed with ^{32}P-labelled ACS7.

PCR-generated DNA fragments were prepared using aliquots of the amplified cDNA from sub-libraries 10/1/1 – 10/1/10 as templates for two rounds of PCR amplification. PCR products were separated by electrophoresis on a 1 % (w/v) agarose gel and probed with ^{32}P-labelled ACS7 at a high stringency (0.1 x SSPE, 0.1 % (w/v) SDS, at 65 °C). The position of the amplified 670 bp cDNA fragment is indicated by the arrow.

Lane 1 & 9	Gibco BRL 1 Kb DNA ladder
Lane 2	Two rounds of PCR amplification of sub-library 10/1/1
Lane 3	Two rounds of PCR amplification of sub-library 10/1/2
Lane 4	Two rounds of PCR amplification of sub-library 10/1/3
Lane 5	Two rounds of PCR amplification of sub-library 10/1/4
Lane 6	Two rounds of PCR amplification of sub-library 10/1/5
Lane 7	Two rounds of PCR amplification of sub-library 10/1/6
Lane 8 & 15	PCR amplification of ACS7 (cloned into pCR 2.1 plasmid) and gel purified
Lane 10	Two rounds of PCR amplification of sub-library 10/1/7
Lane 11	Two rounds of PCR amplification of sub-library 10/1/8
Lane 12	Two rounds of PCR amplification of sub-library 10/1/9
Lane 13	Two rounds of PCR amplification of sub-library 10/1/10
Lane 14	PCR amplification of ACS7 (cloned into pCR 2.1 plasmid)


670 bp→
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

detected with all products from PCR amplification of ACS7, which served as a control (Figure 3.1; lanes 8, 14 and 15).

Aliquots of sub-libraries 10/1/6, 10/1/7 and 10/1/10 were diluted, plated onto 90 mm agar plates, lifted onto nylon filters, and screened using ^{32}P-labelled ACS7 (1 x SSPE, 0.1 % (w/v) SDS, at 65°C). Although all plaques in this third screen from the three sub-libraries resulted in regions of hybridisation, plaques from sub-library 10/1/7 showed the most intense hybridisation signal (Figure 3.2). Ten plaques from sub-library 10/1/7 were picked as agarose plugs and dispersed in SM media and plated out on 90 mm agar plates for a fourth screen (designated 10/1/7/1 to 10/1/7/10). Lifts from six of these plates were hybridised with ACS7 and a representative three of these lifts (10/1/7/1, 10/1/7/7 and 10/1/7/9) are shown as Figure 3.3. All plaques in each plate were recognised by ACS7.

3.1.3 Excision of cDNA clones to pZL 1 from λZipLox phage

Three plaques from each of the fourth screened sub-libraries were picked (18 in total), dispersed in SM media and aliquots were used to infect *E. coli* DH10B(ZIP). Cells were then plated onto LB agar containing 100 µg/ml ampicillin. The plasmid pZL 1, containing putative inserts from the cDNA library, is *in vivo* excised by Cre-*lox* recombination in the *E. coli* DH10B(ZIP) cells. Twenty isolated colonies were selected and grown overnight in 10 ml LB broth containing 100 µg/ml ampicillin. Plasmid DNA (pZL 1, containing putative ACC synthase inserts) was extracted and purified, an aliquot digested with *Eco*R 1, and the products were separated by electrophoresis on a 1 % (w/v) agarose gel. No excised DNA fragments were apparent following the DNA digest (Figure 3.4) and *Eco*R 1 digestion of a further forty colonies also did not yield any inserts (data not shown). The remaining colonies which had not been checked by *Eco*R 1 digestion were then replica plated onto LB agar (12 plates) and grown overnight under ampicillin selection. Lifts onto nylon filters were made from half the number of replica plates (six) and probing with ^{32}P-labelled ACS7 (0.1 x SSPE, 0.1 % (w/v) SDS, at 65°C) revealed regions of hybridisation for all colonies plated (data not shown). However, when a selection of these plasmids were digested with

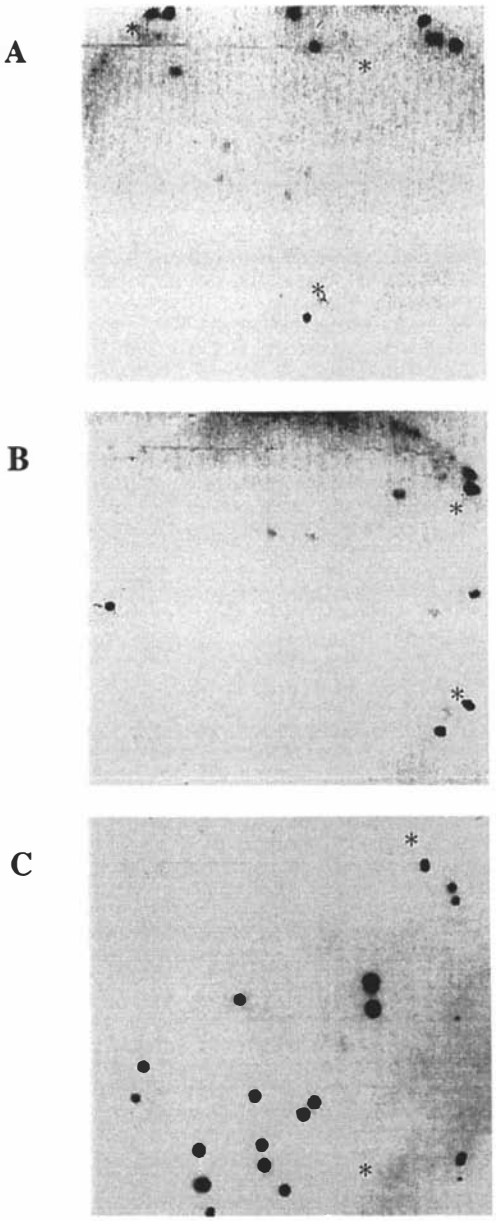


Figure 3.2 Screening of sub-libraries 10/1/6, 10/1/7 and 10/1/10.

A. Filter lifted from sub-library 10/1/6 was probed with ^{32}P-labelled ACS7

B. Filter lifted from sub-library 10/1/10 was probed with ^{32}P-labelled ACS7

C. Filter lifted from sub-library 10/1/7 was probed with ^{32}P-labelled ACS7

Filters were washed at a stringency of 1 x SSPE, 0.1 % (w/v) SDS at 65 °C. The asterisks denote dots that are orientation marks.

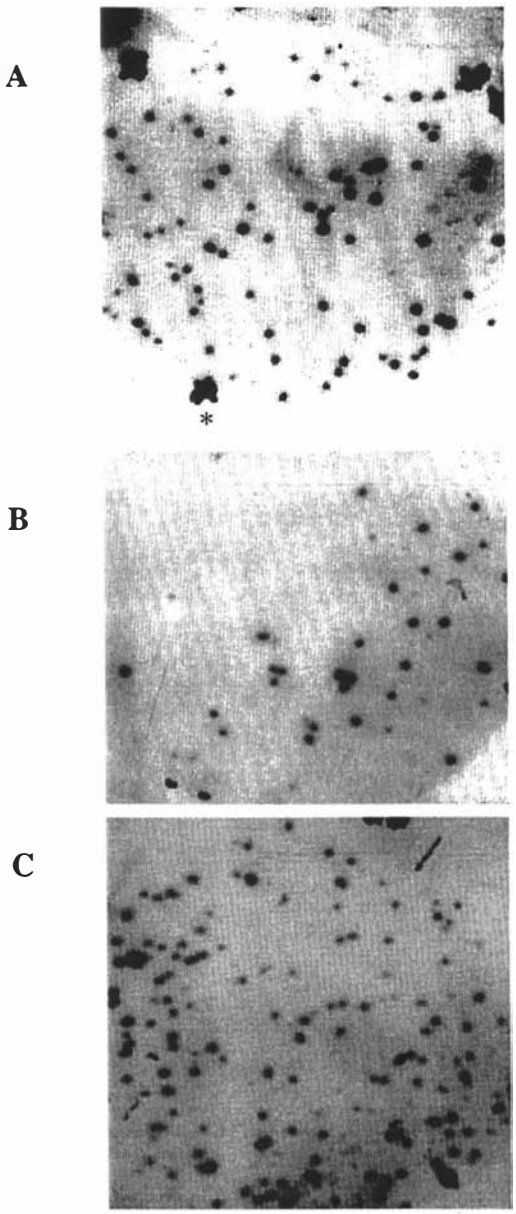


Figure 3.3 Screening of sub-libraries 10/1/7/1, 10/1/7/7 and 10/1/7/9

A. Filter lifted from sub-library 10/1/7/7 was probed with ^{32}P-labelled ACS7

B. Filter lifted from sub-library 10/1/7/1 was probed with ^{32}P-labelled ACS7

C. Filter lifted from sub-library 10/1/7/9 was probed with ^{32}P-labelled ACS7

Filters were washed at a stringency of 1 x SSPE, 0.1 % (w/v) SDS at 65 °C. The asterisks denote dots that are orientation marks.

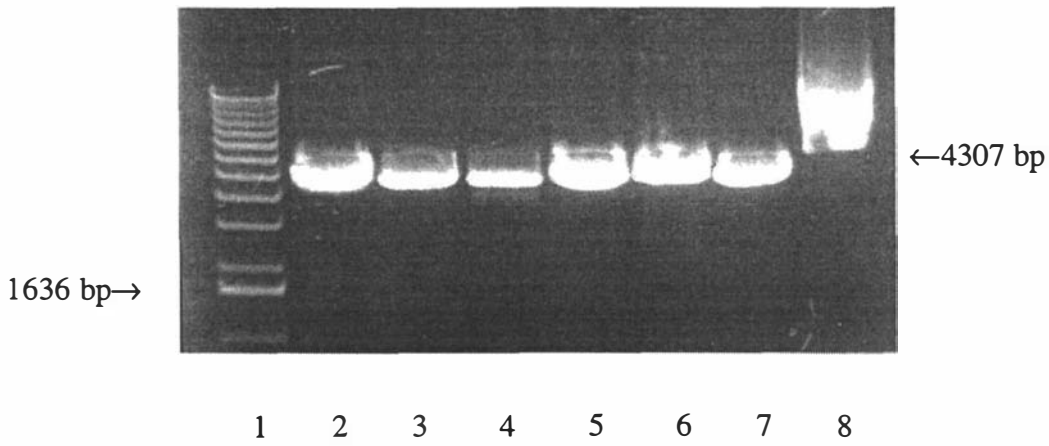


Figure 3.4 Restriction enzyme digests of pZL 1 plasmids excised *in vivo* in *E. coli* DH10B(ZIP).

Products from *Eco*R 1 digests were separated on a 1 % (w/v) agarose gel and visualised with ethidium bromide. The molecular weights of one of the standards is indicated on the left. The size of the vector is indicated on the right.

Lane 1 Gibco BRL 1 Kb DNA ladder

Lanes 2–7 *Eco*R 1 digestion of six representative pZL 1 colonies to excise putative ACC synthase cDNA inserts

Lane 8 Undigested pZL 1

*Eco*R 1, no other bands, apart from the linear plasmid DNA, were detected following gel electrophoresis (data not shown).

3.1.4 Hybridisation specificity of ACS5 and ACS7

In the study by S.D. Yoo (1999), RT-PCR was used to clone a 685 bp ACC synthase fragment (designated ACS5) isolated from leaf two of white clover stolons grown in the same way as described in this study. Sequencing of ACS5 determined that it was 92% homologous with ACS7 at the nucleotide level (data not shown). To determine whether there were differences in hybridisation specificity of ACS7 and ACS5 to the 670 bp PCR product from screened sub-libraries (a putative ACC synthase), sub-libraries 10/1/1 to 10/1/10 were used again as templates for two rounds of PCR. Following electrophoresis, second round PCR products blotted onto Hybond N^+ membrane were hybridised with ^{32}P-labelled ACS5 (Figure 3.5). ACS5 and ACS7 did not appear to have different specificities when used as ^{32}P-labelled probes since PCR products (670 bp) from the same sub-libraries that had shown hybridisation to ^{32}P-labelled ACS7 also hybridised to ^{32}P-labelled ACS5. Strong hybridisation by ACS5 was again observed to PCR products amplified from sub-libraries 10/1/6, 10/1/7 and 10/1/10, in common with that observed with ACS7 (section 3.1.2). ACS5, in common with ACS7, also hybridised to a lower molecular weight band in 10/1/2, but not in 10/1/5.

3.1.5 Determination of non specific hybridisation

To identify whether the regions of hybridisation observed in Figures 3.2 and 3.3 (library plate screens) were due to hybridisation of ^{32}P-labelled ACS7 to the plasmid pZL 1, and not to putative ACC synthase cDNA fragments, a hybridisation intensity comparison was undertaken. The plasmid pZL 1 digested with *Eco*R 1, the cysteine proteinase excised from pZL 1, and ASC5 (obtained by PCR amplification of the insert in pCR 2.1; Yoo, 1999) were blotted onto Hybond N^+ membrane following gel electrophoresis and hybridised with ^{32}P-labelled ACS5 (Figure 3.6). Although hybridisation of ^{32}P-labelled ACS5 to pZL 1 was observed as a weak signal (lanes 2, 3; Figure 3.6B) the hybridisation to PCR amplified

Figure 3.5 Southern analysis of second round PCR products of sub-libraries 10/1/1 – 10/1/10 probed with ^{32}P-labelled ACS5.

PCR-generated DNA fragments were prepared using aliquots of the amplified cDNA from sub-libraries 10/1/1 - 10/1/10 as templates for two rounds of PCR amplification. PCR products were separated on a 1 % (w/v) agarose gel and probed with ^{32}P-labelled ACS5. The membrane was washed at a high stringency (0.1 x SSPE, 0.1 % (w/v) SDS, at 65 °C. The position of the amplified 670 bp cDNA fragment is indicated by the arrow.

Lanel & 10	Gibco BRL 1 Kb DNA ladder
Lane 2	Two rounds of PCR amplification of sub-library 10/1/1
Lane 3	Two rounds of PCR amplification of sub-library 10/1/2
Lane 4	Two rounds of PCR amplification of sub-library 10/1/3
Lane 5	Two rounds of PCR amplification of sub-library 10/1/4
Lane 6	Two rounds of PCR amplification of sub-library 10/1/5
Lane 7 & 14	ACS5 (amplified from pCR 2.1 plasmid harbouring the insert using one round of PCR)
Lane 8	Two rounds of PCR amplification o f sub-library 10/1/6
Lane 9	Two rounds of PCR amplification of sub-library 10/1/7
Lane 11	Two rounds of PCR amplification of sub-library 10/1/8
Lane 12	Two rounds of PCR amplification of sub-library 10/1/9
Lane 13	Two rounds of PCR amplification of sub-library 10/1/10

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

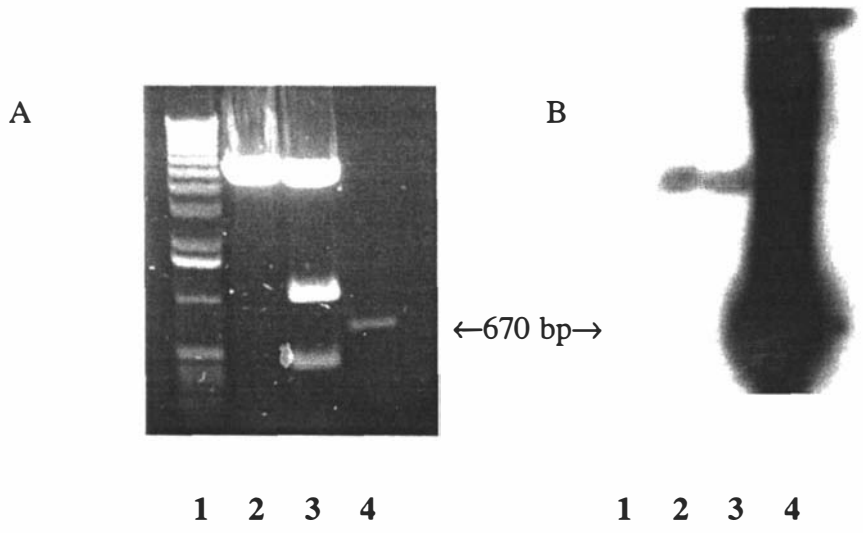


Figure 3.6 Hybridisation intensity comparison of ACS5, cysteine proteinase and pZL 1 vector probed with ^{32}P-labelled ACS5.

Lane 1. Gibco 1 Kb DNA ladder

Lane 2. Restriction enzyme digest of pZL 1 with *Eco*R 1

Lane 3. Restriction enzyme digest of pZL 1 containing cysteine proteinase with *Eco*R 1

Lane 4. PCR amplification of pCR 2.1 containing ACS5 (330 nM ACSR2F, 330 nM ACSR6R, annealing temperature of 50 °C)

A. Products from *Eco*R 1 digests or PCR amplification were separated on a 1 % (w/v) agarose gel and visualised with ethidium bromide. The position of the amplified 670 bp cDNA is indicated on the right.

B. Southern analysis of (A) using ^{32}P-labelled ACS5 (Figure 3.6A, lane 4) as probe. The membrane was washed at high stringency (0.1 x SSPE, 0.1 % (w/v) SDS at 65 °C). The position of the amplified 670 bp cDNA fragment is indicated on the left.

ACS5 produced a comparatively stronger signal. The cysteine proteinase cDNA (previously isolated from the cDNA library) did not hybridise to the ^{32}P-labelled ACS5. Duplicate membranes washed at different stringencies (1 x SSPE and 0.1 x SSPE, both with 0.1 % (w/v) SDS at 65°C) showed no difference in signal following hybridisation with ^{32}P-labelled ACS5 (data not shown).

Due to the hybridisation of pZL 1 to ^{32}P-labelled ACS5, it was necessary to verify that plaques previously selected for third and fourth screens (section 3.1.2), based on hybridisation signals, contained the putative ACC synthase (as amplified from sub-libraries 10/1/6, 10/1/7, and 10/1/10). In previous screening, no insert DNA of the expected size (670 bp) could be excised from *Eco*R 1-digested pZL 1 plasmid (Figure 3.4). Therefore, aliquots of ten sub-libraries from the fourth screen (10/1/7/1 to 10/1/7/10) were used as templates for PCR (two rounds) amplification, and the products separated by electrophoresis (Figure 3.7A). Nine of the ten sub-libraries produced a 670 bp band, which when blotted onto Hybond N^+ membrane hybridised to ^{32}P-labelled ACS5 (Figure 3.7B). Eight bands of 670 bp showed strong hybridisation signals when compared with the PCR amplified ACS5 (control).

Although putative ACC synthase inserts were evident in sub-libraries after a fourth screen, as determined by PCR amplification and probing with ACS5, there were apparent difficulties in excising cDNA clones in pZL 1 from the λZipLox phage. A new pellet of DH10B(ZIP) cells was resuspended for infection of the λZipLox phage. However, when plasmid DNA extracted from colonies arising from this infection were again digested with *Eco*R 1, no inserts were detected following gel electrophoresis and ethidium bromide staining (data not shown). Attempts to amplify the initial cDNA library failed as no products from PCR amplification could be visualised by ethidium bromide following electrophoresis on a 1 % (w/v) agarose gel. This was possibly due to inadequate storage during and following the transfer of the cDNA library between research sites. Later attempts to amplify the previously observed 670 bp PCR products from sub-libraries (e.g. Figure 3.1) were also unsuccessful (i.e. no PCR product was detected). Subsequent information from the manufacturer determined that this was possibly as a result of

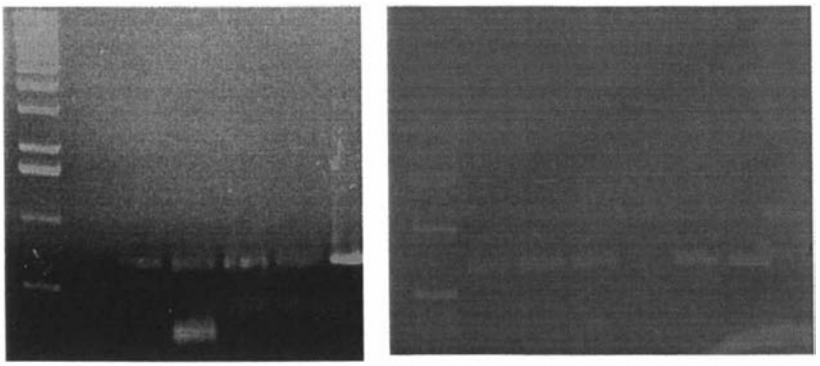
Figure 3.7 PCR amplification of putative ACC synthase fragments.

Lane 1 & 8	Gibco 1 Kb DNA ladder
Lane 2	Two rounds of PCR amplification of sub-library 10/1/7/1/1
Lane 3	Two rounds of PCR amplification of sub-library 10/1/7/1/2
Lane 4	Two rounds of PCR amplification of sub-library 10/1/7/1/3
Lane 5	Two rounds of PCR amplification of sub-library 10/1/7/1/4
Lane 6	Two rounds of PCR amplification of sub-library 10/1/7/2/1
Lane 7 & 14	ACS5 (amplified from pCR 2.1 plasmid harbouring the insert using one round of PCR)
Lane 9	Two rounds of PCR amplification o f sub-library 10/1/7/2/2
Lane 10	Two rounds of PCR amplification of sub-library 10/1/7/4/1
Lane 11	Two rounds of PCR amplification of sub-library 10/1/7/4/2
Lane 12	Two rounds of PCR amplification of sub-library 10/1/7/5/1
Lane 13	Two rounds of PCR amplification of sub-library 10/1/7/5/2

A. PCR-generated DNA fragments, using ACSR1F and ACSR6R for round one and ACSR2F and ACSR6R for round two to amplify putative ACC synthase fragments, amplified from each sub-library. PCR products were separated by electrophoresis on a 1 % (w/v) agarose gel and visualised with ethidium bromide. The position of the amplified 670 bp cDNA is indicated on the left.

B. Southern analysis of (A). Membranes were probed with ^{32}P-labelled ACS5 at a high stringency (0.1 x SSPE, 0.1 % (w/v) SDS at 65 °C. The size of the amplified cDNA is indicated to the left.

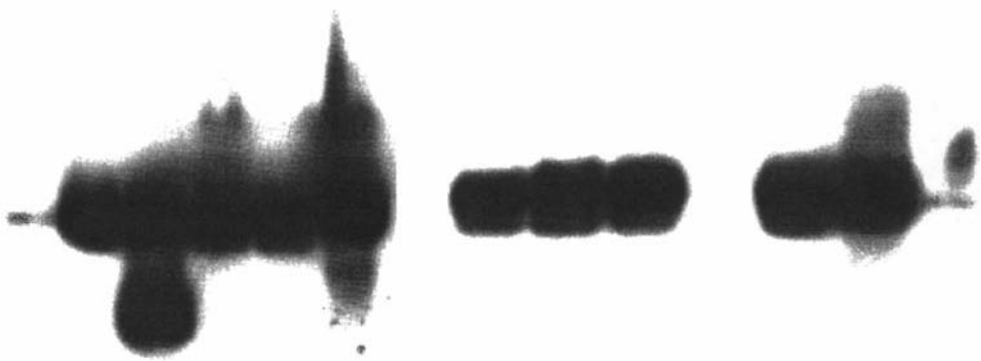
A



670 bp→

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13

B



670 bp→

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13

prolonged storage at 4 °C, as inserts in λZipLox. No further attempts to amplify putative ACC synthase cDNAs from the library were made as part of this thesis. Instead, isolation of developmentally-regulated ACC synthase genes during leaf ontogeny in white clover was attempted using RT-PCR. This technique was shown to be successfully applied to the isolation of ACC oxidase genes (Hunter *et al.*, 1999) and in some preliminary isolation of ACC synthase genes by Sang Dong Yoo (Yoo, 1999).

3.2 Gene expression of ACC synthase during leaf ontogeny in white clover

3.2.1 White clover stolon growth

White clover was grown to obtain stolons sub-tending leaf tissue representative of all developmental stages ranging from leaf initiation to senescence and necrosis (Figure 1.2A). Measurements by Hunter (1998) of changes in ethylene evolution and chlorophyll content during white clover leaf ontogeny within this growth system were used to determine the different developmental stages along the stolon (Figure 1.2B). Leaves at nodes 1 to 16 were used in these studies and were representative of typical stolon growth. The first leaf to have emerged completely from the apex was designated leaf 1, with leaf numbers progressing through the stages of maturation and senescence to leaf 16.

3.2.2 RT-PCR amplification of putative ACC synthase gene transcripts

The reverse transcriptase-dependant polymerase chain reaction (RT-PCR) (section 2.3.6) was used to isolate ACC synthase genes. Due to the low abundance of the ACC synthase transcripts (Butcher, 1997), leaf tissue at similar developmental stages was pooled to comprise three stages of development. The first developmental stage (nodes 4-7; designated as presenescent) comprised leaves that had reached full maturity in terms of maximum chlorophyll content and minimum levels of ethylene evolution. An initial yellowing at leaf margins was observed in leaves comprising the second developmental

stage (nodes 9-12; designated onset of senescence) as chlorophyll concentration of leaf tissue decreased and ethylene levels increased significantly. The third developmental stage (senescence; nodes 13-16) was characterised by a further decline in chlorophyll concentration and a major increase in the rate of ethylene production.

To generate putative ACC synthase sequences, nested degenerate primers (Table 2.3), made to highly conserved regions of ACC synthase and known to amplify a second round PCR product of approximately 670 bp (Butcher, 1997), were used. Eight distinct regions of the gene are considered to be conserved (Imaseki, 1999), including the putative active site (Yip *et al.*, 1990; Van Der Straeten *et al.*, 1992). The eight conserved domains and the positions of primers used for amplification are shown in Figure 3.8. As shown, the products of the first round of PCR amplification were produced using primers homologous to conserved domains one and seven, and primers homologous to domains two and seven were used for second round PCR amplification. The amplified products, therefore, will represent 670 bp of the protein coding sequence of the ACC synthase gene.

The ACSR1F and ACSR6R primers were used for a first round of PCR amplification using RT-generated cDNA transcripts from poly(A)$^+$ mRNA. Aliquots of the first round PCR mixes were used as templates for a further round of PCR amplification, using ACSR2F and ACSR6R as primers. The amplified products from the first round of PCR (with an expected size of 780 bp) were unable to be detected following electrophoresis on a 1 % (w/v) agarose gel and staining with ethidium bromide. However, PCR products of approximately 670 bp were observed following the second round of PCR amplification (Figure 3.9A). The second round PCR products were TA-cloned into the pCR 2.1 vector (Figure 2.1) and transformed into the *E. coli* strain DH5α. A selection of the resulting colonies were grown overnight for plasmid isolation (section 2.3.2.1) and purification (section 2.3.2.2), and the presence of an insert of approximately 670 bp was confirmed by restriction digestion and agarose gel electrophoresis. A selection of these are shown in Figure 3.9B. Thirty four cloned inserts of approximately 670 bp, resulting from PCR amplification of cDNA from the three developmental stages, were then sequenced using an automated sequencer.

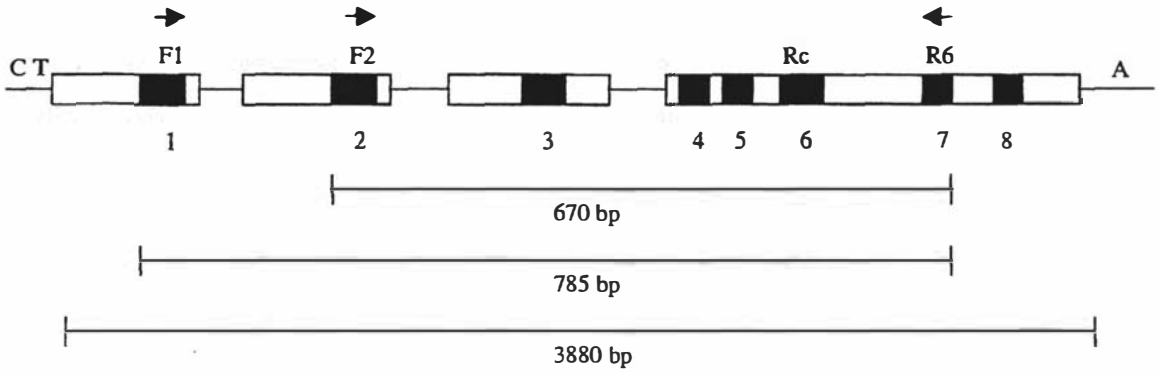


Figure 3.8 Diagrammatic representation of an ACC synthase gene from *Arabidopsis thaliana* (modified from Butcher, 1997).

Exons (boxes), introns (lines), conserved domains (dark boxes), the reactive centre (Rc), putative CAAT box (C), TATA box (T), polyadenylation signal (A), and the positions of the primers (arrows) used for PCR amplification in white clover are shown.

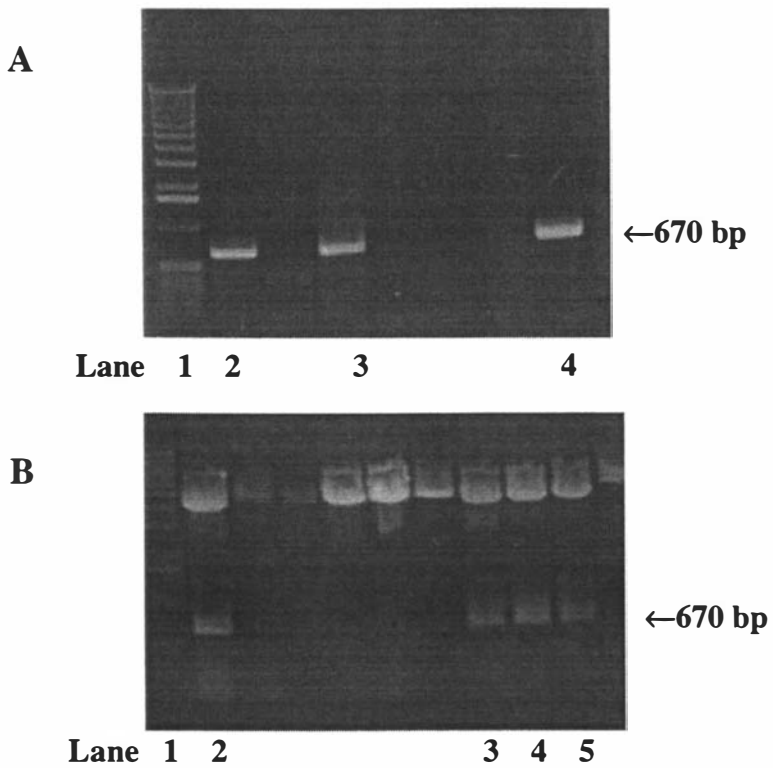


Figure 3.9 A. **RT-PCR on poly(A)$^+$ mRNA pooled from leaf tissue from different developmental stages.** Nested degenerate primers were used for two rounds of PCR amplification. The approximate size of the amplifed cDNA is indicated.

Lane 1. GIBCO BRL 1 Kb DNA ladder

Lane 2. RT-PCR products synthesised and amplifed from RNA isolated from presenescing leaf tissue (nodes 4-7)

Lane 3. RT-PCR products synthesised and amplifed from RNA isolated from leaf tissue at the onset of senescence (nodes 9-12)

Lane 4. RT-PCR products synthesised and amplifed from RNA isolated from senescent leaf tissue (nodes 13-16).

B. Restriction digestion of plasmids to confirm cloning of the putative ACC synthase cDNA fragment amplified in (A). The approximate size of the amplified cDNA is indicated.

Lane 1. GIBCO BRL 1 Kb ladder

Lanes 2-5. Restriction digestion of pCR 2.1 to confirm the cloning of cDNA fragments

3.2.3 ACC synthase sequence alignment

A sequence alignment comparison, using the align+ program, indicated that all 34 sequences displayed high sequence similarity to ACC synthase sequences in the GenBank database. Within these sequences, three distinct groupings of putative ACC synthase cDNA sequence emerged, based largely on sequence differences that occurred in degenerate regions. A consensus sequence was then generated to represent these three groups and these were then compared for homology at the nucleotide level, and identity at the amino acid level (Table 3.1). The low homology between the three consensus cDNA sequences, ranging from 62-71 % and the high sequence homology within the groups resulted in the three groups being proposed to represent three distinct ACC synthase cDNAs (designated *TR-ACS1*, *TR-ACS2* and *TR-ACS3*).

Table 3.1 Comparison of the percentage of nucleotide homology and percentage of amino acid identity (in parentheses) between the three ACC synthase consensus sequences amplified from white clover leaf tissue by RT-PCR.

	TR-ACS1	*TR-ACS2*	*TR-ACS3*
TR-ACS1	-	62 (56)	71 (69)
TR-ACS2	62 (56)	-	63 (55)
TR-ACS3	71 (69)	63 (55)	-

TR-ACS1 shares the highest amino acid identity (71 %) with *TR-ACS3*, whereas the amino acid identity between *TR-ACS2* and *TR-ACS3* is only 55 %, which is similar to the amino acid identity between *TR-ACS1* and *TR-ACS2* (56 %) (Table 3.1).

The consensus sequences of the three ACC synthases isolated from white clover leaf tissue and the derived amino acid sequences were aligned (Figures 3.10 and 3.11 respectively). *TR-ACS1* and *TR-ACS2* sequences were found to be highly similar at the conserved domains three, four, five and six. *TR-ACS3*, however, was missing the nucleotide sequence encoding domain six (the active site of the enzyme).

Eleven of twelve invariant amino acid residues involved in binding of substrate and pyridoxal 5′ phosphate are conserved between aminotransferases and ACC synthase from various plant species (e.g. Nakajima *et al.*, 1990; Van Der Straeten *et al.*, 1990; Dong *et al.*, 1991; Huang *et al.*, 1991; Nakagawa *et al.*, 1991; Olson *et al.*, 1991; Park *et al.*, 1991; Sato *et al.*, 1991). All nine of the conserved amino acid residues that are located within the regions of ACC synthase gene amplified in this thesis are present in *TR-ACS1* and *TR ACS2* (Figure 3.11). Three of the nine conserved amino acid residues are missing from *TR-ACS3*, as they are typically located in the active site of ACC synthase.

The predominantly amplified sequence, *TR-ACS1* (present as 19 out of a total of 34 sequences), was amplified from poly(A)$^+$ mRNA isolated from leaves from all three developmental stages (Table 3.2). Seven sequences were grouped as *TR-ACS2*, and these were all amplified from poly(A)$^+$ mRNA isolated from the presenescing leaves (nodes 4-7). *TR-ACS3* was represented by eight sequences and these were amplified from poly(A)$^+$ mRNA isolated from senescing leaves only (nodes 13-16).

Reference molecule:	*TR-ACS1* (629 bp)	Homology
Sequence 2:	*TR-ACS2* (649 bp)	62 %
Sequence 3:	*TR-ACS3* (562 bp)	63 %

```
ACSR2F CTGGATCCGTWYCARGAYTAYCAYGG
TR-ACS1 TCTACCAGAGTTCAGAAATGCTGTGGCTAAATTCATGTCTAGAACAAGAGGAAACAGAGTTACATTTGATCCTGATC
TR-ACS2 ...CCCTTCATTCAAACAAGCATTGGTAG.T..T...G.CGAG.TC.....C.....T.C.....AA.C.
TR-ACS3 AT.G.....A.....A..T.....AAAAGTGAGAGGTGGTAGGGTAAG.....C.

TR-ACS1 GTATTGTCATGAGTGGTGGAGCAACTGGAGCACATGAGGTTACTGCCTTTTGTTTGGCAGATCCTGGTGATGCTTTTT
TR-ACS2 A...A TC.C.C..CC..CT.T...TCC...A.C...AC.CT.ATG.....C.T..T..GAAA..A..A..A...C
TR-ACS3 ....AT.G.....A..G...A....AT.A.TCATG..C.....T.....C....

TR-ACS1 TGGTACCTACTCCTTACTATCCAGGTTTCGATCGAGATTTGAGATGGAGAACAGGAGTTAAACTTGTTCCGGTTATAT
TR-ACS2 .CC.T.....A..T...A.....C.T.A.....T..T...G.GA.....AA.ACA..
TR-ACS3 ....T....GC.....T.....CA..T.T...T.....T.T.....C..T..GC....AA....T..CCA..

TR-ACS1 GCGAAAGCGCGAACAATTTCAAATTAACAAGACAAGCTTTGGAAGAAGCATATGAAAAAGCCAAAATTGATAACATCA
TR-ACS2 ..A.T...T.T.....C..TC..A....TGA.....A...C..C.....CA..G.T..AC..GACCGC...C.T.
TR-ACS3 .TC.T...T.A.....GA.....G....AC.T.....T...ATG.....AC..GAAAGA.....

TR-ACS1 GAATAAAAGGTTTACTCATAACAAATCCTTCCAATCCATTAGGCACAGTTATGGACACAACCACATTAAAAACCGTTG
TR-ACS2 A.G.C.....AG..A.GG.T.....C..A..A..C..G.....CACAT..TCA.GG.GTGA.....TCTTC.C.
TR-ACS3 ATG.G.....G...A.....A..A..C..TC....A...ACA..A..A.A.GAA...C....G.G.A.A.

TR-ACS1 TAAATTTCATCAACGAAAAGCGTA---TTCATCTTATAAGCGATGAAATTTACGCTGCAACGGTTTTTAGCCACCCAA
TR-ACS2 .TG.C..T..TG.A.....CAAA.ACA.G...T.G.....C..G.....T.C.GG..T.....TC.TCT....
TR-ACS3 .T.G.....A..T.....CAAC.---.....T.AG.GT.T.....C..TT.C.GC..A.....CGA.ACT..G.

TR-ACS1 GTTTCATAAGTATAGCTGAGATCATAGAACAAGAAACAGACATTGAATGTGACCGTAACCTCG----------
TR-ACS2 ....T..C...G.TATG..A...C.TA.GG..AG..AT...C..C.GGA.TT.AAAT..ACT.ATAATATTTGCGAGA
TR-ACS3 AA.A.G.....G.C..C..AG.T..---.....TG..---A.....CA.AAAAG.....A----------

TR-ACS1 ---TTCACATAGTTTACAGTCTTTCAAAAGATATGGGATTCCCCGGTTTTAGAGTCGGTATAATTT-ACTCATACAAT
TR-ACS2 GAG....TG.T..C..T.....C.....CT....T..G..A.....CC.C..T...GC.C...T....CG.A..C
TR-ACS3 ---...-----.....----------

TR-ACS1 GATACCGTTGTTAACTGCGCGCGCAAAC ACSR6R CTCAAGCTTARNSYTRAARCTNGACAT
TR-ACS2 ...--GAA.TG.CG.A..TGCAA.C..G ACS3AR GCGTCCCAKATCTTTTGAAAGACT
TR-ACS3 ...GAA.....G..T....GT..-...A
```

Figure 3.10 Alignment of coding frame regions of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* consensus sequences (.) represents identical sequence (-) represents no sequence. Degenerate primer sequences are underlined and gene specific primer sequences are shaded.

Reference molecule:	*TR-ACS1* (209 aa)	Homology
Sequence 2:	*TR-ACS2* (208 aa)	56 %
Sequence 3:	*TR-ACS3* (187 aa)	60 %

```
                                                                3          *
TR-ACS1 LPEFRNAVAKFMSRTRGNRVTFDPDRIVMSGGATGAHEVTMFCFADPGDAVLVPTPAYPGFDRDL
TR-ACS2 ..S.KQ.LVD..AEI.....S...NH..LTA.S.S.N.TLM....EK.E...L...........
TR-ACS3 .........N...KV..G..R......L........N.LIM.............S....A.V...

                                                                4 **     *
TR-ACS1 RWRLTGVKLVPVICESANNFKLTRQALEEAYEKAKIDNIRIKGLLITNPSNPLGTVMDTTTLKTVV
TR-ACS2 K.....EI..IQ.N.S...QI.E...QQ..KD.QER.LKV..VMV.........TLSRSE.NLL.
TR-ACS3 C.....Q.I..Q.H.S....I..E......M..QER..NV...I..........TIEKE...SI.

                      5  *  *                                              6 *
TR-ACS1 NFINE-KRIHLISDEIYAATVFSHPSFISIAEIIEQE--------TDIECDRNLVHIVYSLSKDM
TR-ACS2 D..E.N.NM........SG....S.....VM..LKERNDLQDFK ..NI.E.--..V.......L
TR-ACS3 S....-NN...VC....SG...DT.KYV.V..V.QEM--------EECKK--------------

              * *
TR-ACS1 GFPGFRVGIIYSYNDTVVNCARK
TR-ACS2 .L.................ALLLRKR
TR-ACS3 --------L.HM...E....G..
```

Figure 3.11 Alignment of deduced amino acid sequences of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* consensus sequences (.) represents identical sequence (-) represents no sequence and * represents conserved residues found in aminotransferase and other ACC synthase sequences. Shaded, numbered sequence represents the conserved regions of ACC synthase, shaded bordered sequence represents the active site of the enzyme.

Table 3.2 Isolation of ACC synthase transcripts from white clover leaf tissue.

RT-PCR was used to isolate ACC synthase genes from white clover leaf tissue at different developmental stages, and the occurrence of each proposed ACC synthase gene transcript at each developmental stage is shown.

Gene	*Tissue source of RNA*
TR-ACS1	Presenescence (nodes 4-7) Onset of senescence (nodes 9-12) Senescence (nodes 13-16)
TR-ACS2	Presenescence (nodes 4-7)
TR-ACS3	Senescence (nodes 13-16)

The GenBank database was also searched using the consensus sequences of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* (Table 3.3). The database sequences with the highest homology to *TR-ACS1* were ACC synthase sequences from IAA-treated tissue from apical hooks of pea (GI 2360989), mature leaves of soybean (GI 18557), and IAA-treated hypocotyls of mungbean (GI 22069). The highest scoring database sequence homologies to *TR-ACS2* were of ACC synthases isolated from etiolated hypocotyls of mung bean (GI 1006805 and GI 1006807) and IAA-treated pea hypocotyls (GI 2360987). An ACC synthase isolated from a white lupin genomic library (GI 6650975) had the highest sequence homology to *TR-ACS3*. The expression of the lupin ACC synthase (*LA-ACS1*) was found to increase during germination and in response to IAA and wounding (Bekman *et al.*, 2000). Other neighbouring GenBank sequences to *TR-ACS3* included ACC synthases isolated from ripening mango fruit (GI 1143811) and immature tomato fruit (GI 3986116).

Table 3.3 GenBank comparison of *TR-ACS* sequences

	Plant	*Tissue type (Accession number)*
TR-ACS1	Pea	IAA-treated apical hook (GI 2360989)
	Soybean	Mature leaf (GI 18557)
	Mungbean	IAA-treated hypocotyl (GI 22069)
TR-ACS2	Mungbean	Etiolated hypocotyl (GI 1006805)
	Mungbean	Etiolated hypocotyl (GI 1006807)
	Pea	IAA-treated hypocotyl (GI 2360987)
TR-ACS3	White lupin	Germination, IAA, wound-induced (GI 6650975)
	Mango	Ripening fruit (GI 1143811)
	Tomato	Immature fruit (GI 3986116)

- Database search on 13/9/2001

3.2.4 Confirmation of an ACC synthase multi-gene family in white clover by Southern analysis

The specificity of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* to hybridise to their own sequence only was determined prior to Southern and northern analysis to determine if these coding sequences can be used as gene-specific probes. Probing of triplicate blots containing DNA of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* with each of the three cDNAs labelled with ^{32}P, and washing at high stringency (0.1 x SSPE, 0.1 % (w/v) SDS at 65 °C) revealed that each sequence only hybridised to the complementary cDNA. No cross-hybridisation was detected between any of the three ACC synthase sequences (Figure 3.12).

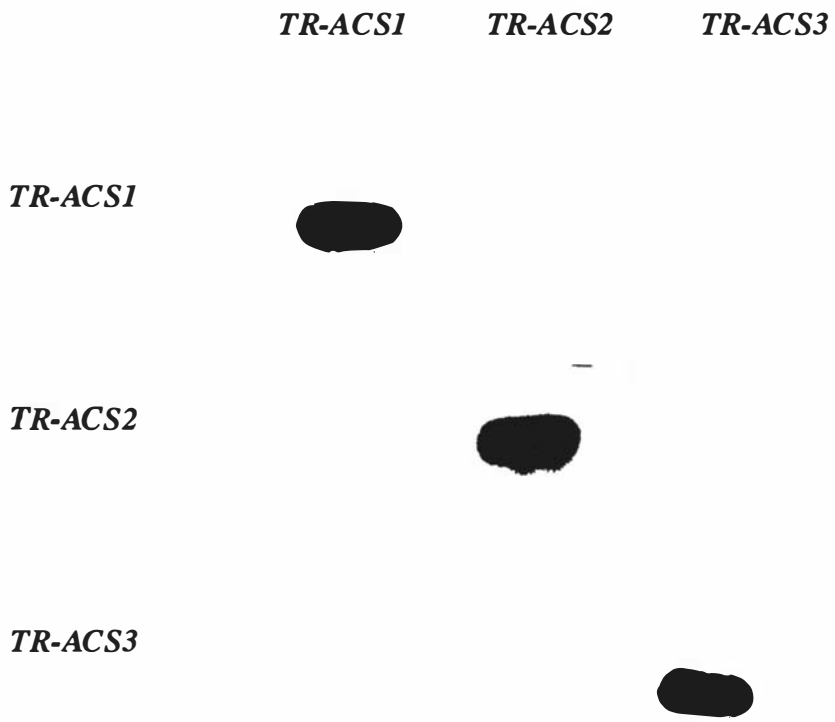


Figure 3.12 Specificity of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* as probes in Southern analysis.

Each lane contained 50 ng of DNA corresponding to a 670 bp cDNA of *TR-ACS1*, *TR-ACS2* and *TR-ACS3*. The blots were hybridised with 32P-labelled cDNA coding-frame probes *TR-ACS1*, *TR-ACS2* and *TR-ACS3* as indicated, and washed at high stringency (0.1 X SSPE, 0.1 % (w/v) SDS at 65 °C.

To perform Southern analysis, white clover genomic DNA was digested in separate aliquots with *Eco*R I, *Hind* III and *Xba* I (section 2.3.4.2) and then separated through a 1 % (w/v) agarose gel (Figure 3.13A). An even smearing pattern of ethidium bromide in each lane and little high molecular weight DNA (higher than 12 000 Kb) indicated that genomic DNA was completely digested. After transfer to Hybond N^+ membrane, ^{32}P-labelled gene-specific probes representing *TR-ACS1*, *TR-ACS2* and *TR-ACS3* were added. Following washing of blots at a high stringency (0.1 x SSPE, 0.1 % (w/v) SDS at 65 °C) hybridisation of ^{32}P-labelled *TR-ACS1*, *TR-ACS2* and *TR-ACS3* to different sequences of genomic DNA on the Southern blot confirmed that distinct genes encoded the three ACC synthases. Each cDNA probe hybridised with multiple bands for each restriction enzyme (Figure 3.13B). This might be expected, however, due to the tetraploid nature of the white clover genome. The potential presence of pseudogenes or closely related genes or the presence of restriction sites for the enzymes used for DNA digestion within putative introns may also contribute towards the multiple sequences observed. These possibilities are discussed later.

3.2.5 Phylogenetic analysis of TR-ACS1, TR-ACS2 and TR-ACS3

A phylogenetic tree, constructed from the alignment of derived amino acid sequences of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* with 116 ACC synthase sequences (from a GenBank search), identified high phylogenetic divergence among the ACC synthase genes cloned from white clover (Figure 3.14).

The three ACC synthases isolated from white clover are closer to other ACC synthase sequences on the phylogenetic tree than they are to each other. TR-ACS1 is most closely related to a pea ACC synthase isolated from IAA-treated apical hooks of etiolated seedlings (GI: 2360989). Other neighbouring sequences included those of ACC synthases isolated from IAA-treated mung bean hypocotyls (GI: 232930 and 398956). TR-ACS2 was found to group with ACC synthases isolated from etiolated hypocotyls of mung bean (GI: 1006805, 1006807 and 1813331). The TR-ACS3 sequence was closest to a cDNA clone isolated from *Citrus paradisi* (GI: 2952297). It is not stated at what developmental stage

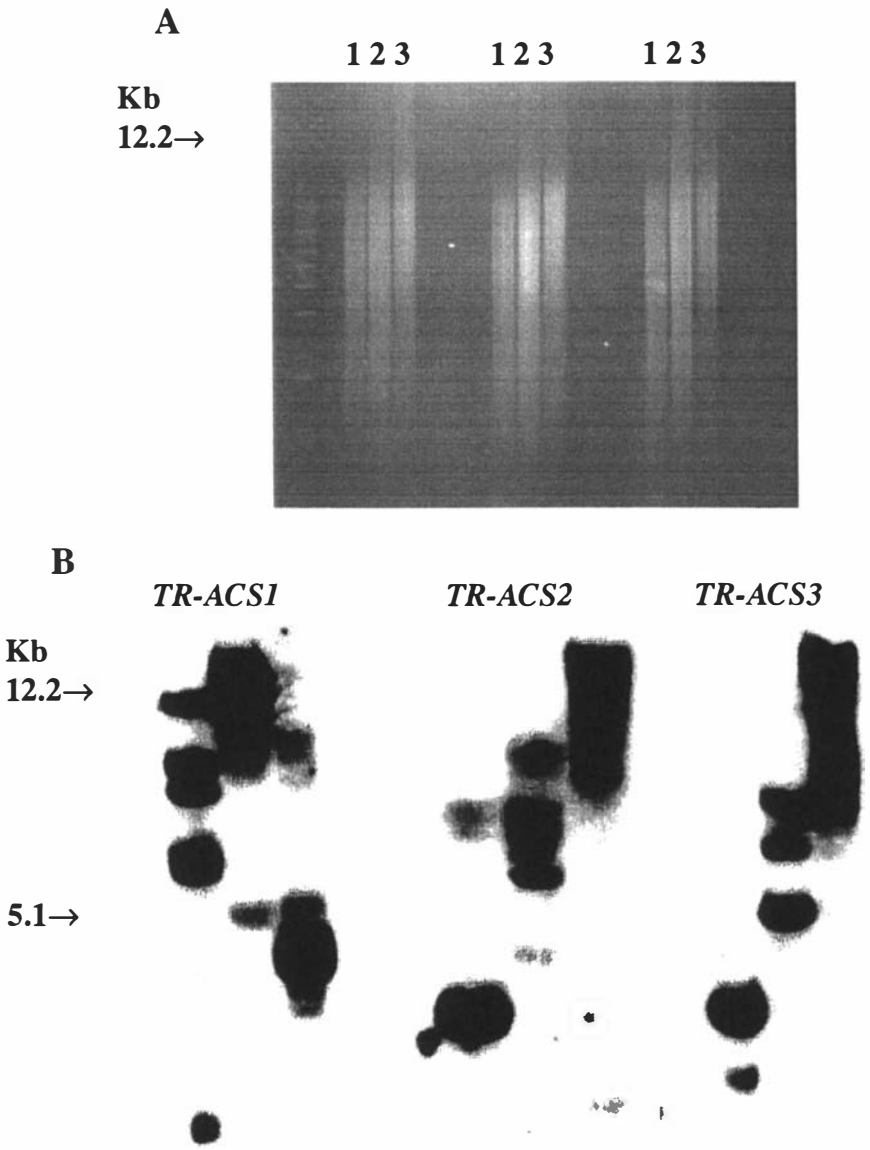


Figure 3.13 Southern analysis of white clover genomic DNA.

A. Genomic DNA following restriction enzyme digestion, visualised by ethidium bromide staining. Lane 1 was digested with *Hind* III, Lane 2 was digested with *Eco*R I, Lane 3 was digested with *Xba* I.

B. Hybridisation pattern of ^{32}P-labelled *TR-ACS1*, ^{32}P-labelled *TR-ACS2* and ^{32}P-labelled *TR-ACS3*

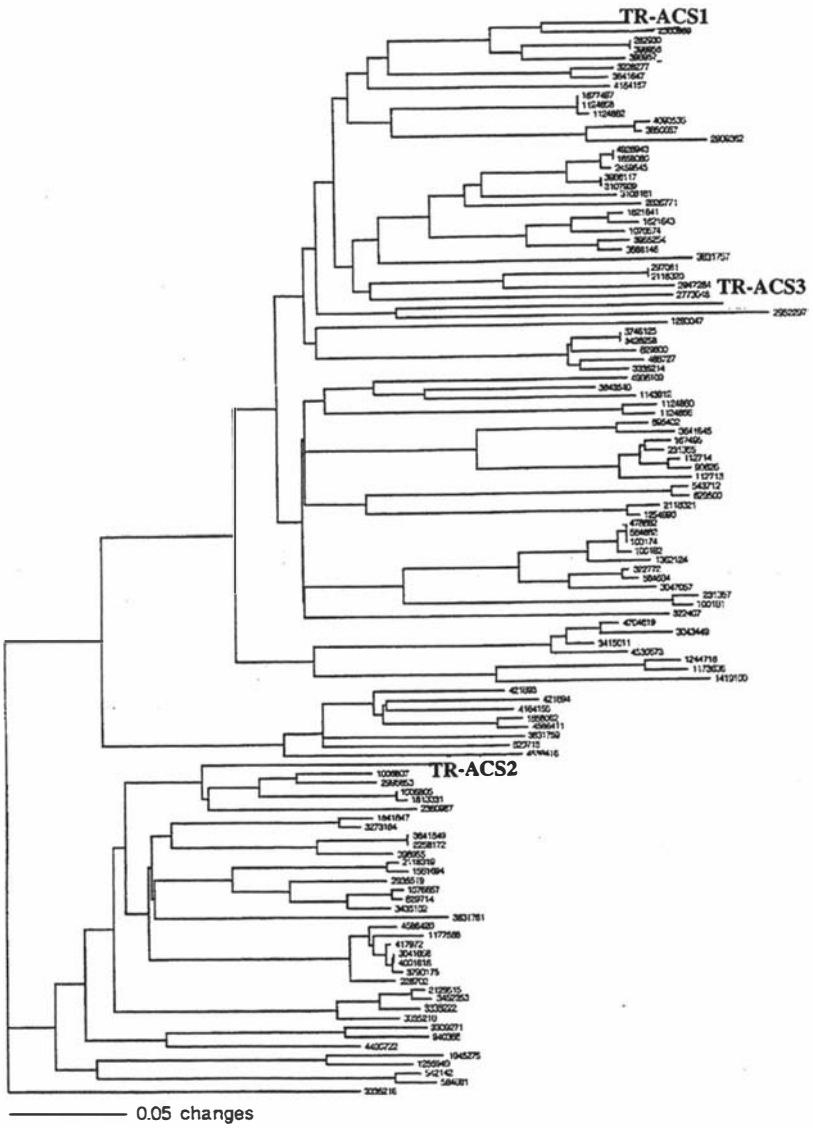


Figure 3.14 Amino acid sequence based phylogeny of the ACC synthase family.

The phylogenetic tree (generated using parsimony) was constructed from alignment of GenBank sequences with deduced amino acid sequences for *TR-ACS1*, *TR-ACS2* and *TR-ACS3*. Each sequence is depicted by its accession number.

the RNA was isolated from. There are no other sequences close to TR-ACS3 on the phylogenetic tree as the branch for TR-ACS3 is very long.

3.2.6 Expression of ACC synthase during white clover leaf ontogeny by northern analysis

The expression of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* during both leaf maturation and senescence was studied using northern analysis. White clover leaf tissue was grouped into four developmental stages for these studies as determined from the chlorophyll content and ethylene evolution data of Hunter (1998) and described in section 3.2.2. The four stages used were a developing stage (represented by leaf tissue pooled from nodes 1-3), a mature green stage (nodes 4,5,6) an onset of senescence stage (nodes 7,8,9), and a senescent stage (nodes 13,14,15). As much care as possible was taken to ensure that the plant material was grown under optimal conditions to avoid insect damage, water-deficit stress and other stresses that have been shown to induce ACC synthase activity.

The expression of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* was studied by northern analysis using equal quantities of polyA$^+$ RNA extracted from leaf tissue pooled from these four stages of development. *TR-ACS1* hybridised to a 2.1 Kb transcript which accumulated predominantly in mature green leaves, and was less abundant in leaves at the onset of senescence (Figure 3.15A). *TR-ACS3* was expressed predominantly in senescent leaves (Figure 3.15B. No expression of *TR-ACS2* was detected by northern analysis (data not shown), and further northern analysis determined that no expression could be determined using up to 12 µg of poly A$^+$ RNA.

3.2.7 Expression of ACC synthase genes during white clover leaf ontogeny by RT-PCR

The lack of detectable expression of *TR-ACS2* using northern analysis may be due to the lack of sensitivity of this technique to detect mRNAs of very low abundance. Therefore, ACC synthase RNA levels were also analysed by RT-PCR. Specific oligonucleotides were

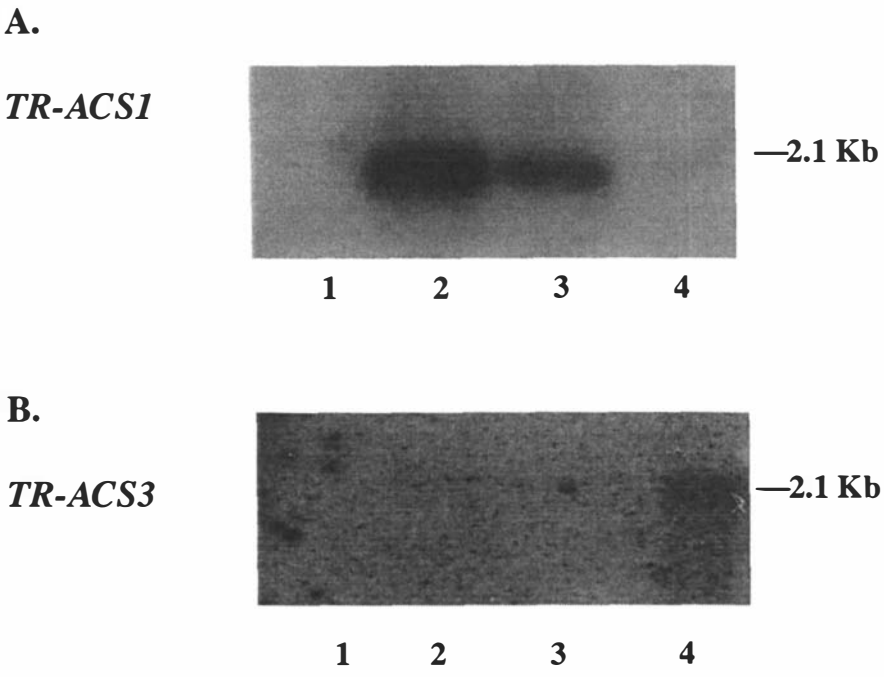


Figure 3.15 Northern analysis of ACC synthase gene expression during leaf ontogeny in white clover. Eight μg of poly(A)$^+$ mRNA was separated on a 1.2 % (w/v) agarose-formaldehyde gel, blotted onto Hybond-N$^+$ membrane with alkaline transfer buffer and probed with ^{32}P-labelled *TR-ACS1* (A) and *TR-ACS3* (B). Membranes were washed at high stringency (0.1 X SSPE at 65 °C) and exposed to X-ray film for 5 days.

Lane 1. Developing leaf tissue (nodes 1-3)

Lane 2. Mature/ green leaf tissue (nodes 4-6)

Lane 3. Green/ yellow leaf tissue (nodes 7-9)

Lane 4. Senescing leaf tissue (nodes 13-15)

designed (Table 2.3) as unique forward primers for each of the three ACC synthase genes. These, with the degenerate 3′ ACC synthase primer ACSR6R, were used to amplify cDNA made to mRNA isolated from white clover leaves at different developmental stages.

The specificity of these primers was first tested using the cDNA clones of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* (in pCR 2.1 vectors) as templates with the corresponding gene-specific primers. For each primer set, only the target sequence was amplified (Figure 3.16) indicating that this method may provide an approach to distinguish between the three ACC synthases using RT-PCR.

To amplify ACC synthase sequences from RNA isolated from plant tissue, two rounds of PCR were again required for visualisation following electrophoresis and ethidium bromide staining on 1 % (w/v) agarose gels. Using the gene-specific primer for *TR-ACS2* (ACS2F) and the reverse primer ACSR6R (Table 2.3), it appeared that the *TR-ACS2* transcript was expressed predominantly in the developing and mature green tissue (in the apex and leaves from nodes 1 and 4) and also with less abundance in the tissue at the onset of senescence (leaves from nodes 9 and 10; Figure 3.17A). To ensure that equal quantities of RNA were used for the RT-PCR reactions, primers specific for β-actin (Table 2.4) were used with the same RT samples as used for ACC synthase amplification. After one round of amplification, transcripts of β-actin appeared to accumulate at consistent levels in the leaves from different developmental stages along the stolon (Figure 3.17B).

The β-actin control was visualised after one round, but the expression of *TR-ACS2* was determined using two rounds of PCR. To confirm the expression of *TR-ACS2*, a single round of PCR was performed using the gene-specific forward primer (ACS2F), the degenerate primer (ACSR6R), and the amplified products probed with ^{32}P-labelled *TR-ACS2* (Figure 3.18). This expression pattern of *TR-ACS2* was consistent with the expression observed in Figure 3.17, with *TR-ACS2* expression being higher in developing and mature green tissue (apex and nodes 2, 3 and 5) and also present in tissue at the onset of senescence (node 8). The plant material used in this experiment was harvested at a different time than that used in the first analysis of *TR-ACS2* expression (Figure 3.17), so

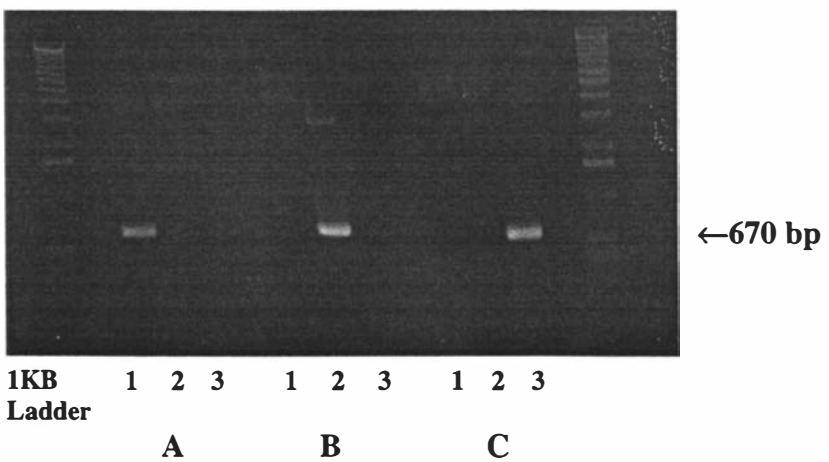
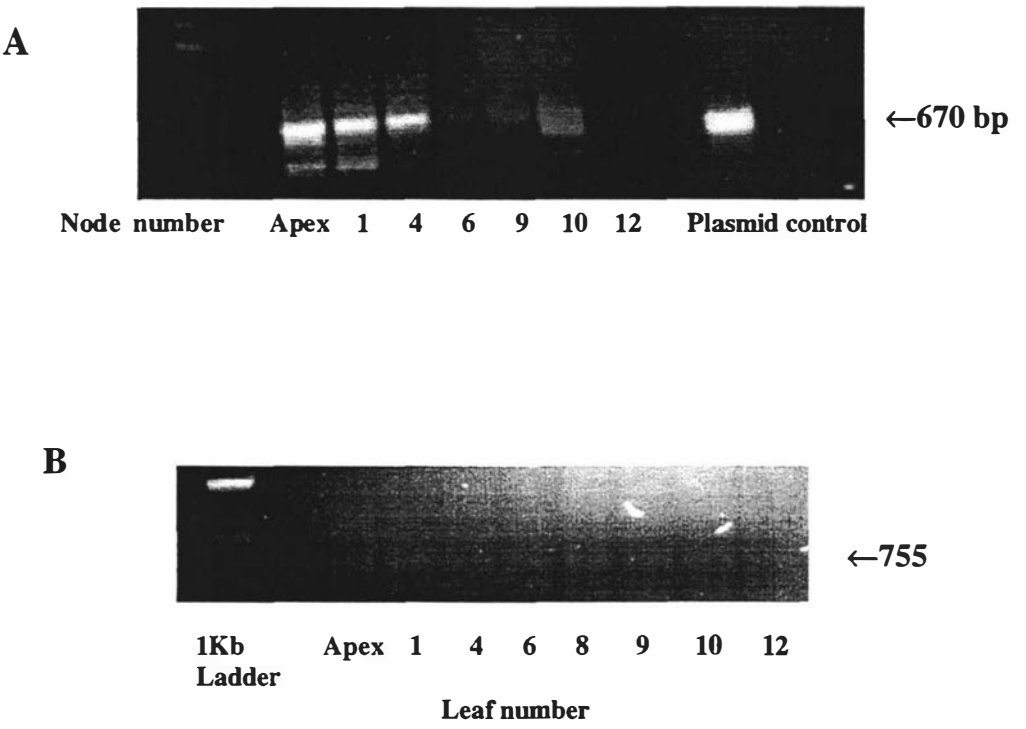


Figure 3.16 Specificity of ACS gene-specific primers. The specificity of primer sets to specifically amplify a single ACS gene was tested using cDNA clones of *TR-ACS1*(Lane 1), *TR-ACS2* (Lane 2) and *TR-ACS3* (Lane 3), in pCR 2.1 vectors, as templates with the gene-specific primers for *TR-ACS1* (A), *TR-ACS2* (B) and *TR-ACS3* (C).



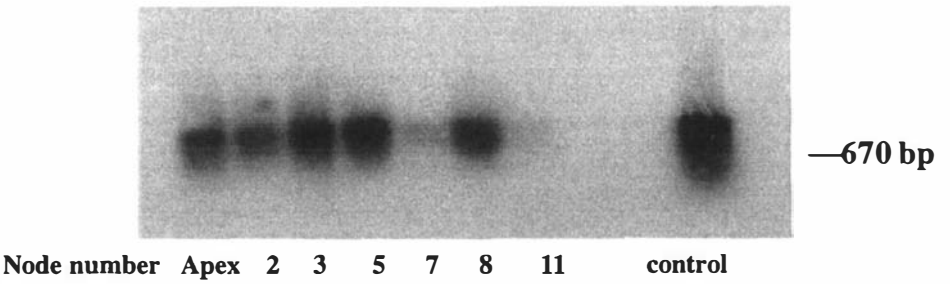
3.17 A) RT-PCR analysis of expression of *TR-ACS2*. Poly(A)$^+$ mRNA, isolated from white clover leaves representative of different developmental stages, was used as substrate for reverse-transcriptase reactions and the cDNA transcripts generated used as template for PCR with primers specific for *TR-ACS2*. Two rounds of PCR amplification were required for visualisation on an ethidium bromide stained gel. Leaves used for RT-PCR are marked below the lanes. *TR-ACS2*, cloned into pCR 2.1, was used as a control and amplified by PCR (one round).

B) The expression of β-actin in white clover leaf tissue at different developmental stages was determined to ensure that equal quantities of RNA was used as template for RT-PCR reactions. One round of PCR amplification on the cDNA transcripts generated in (A) was performed using primers specific for β-actin.

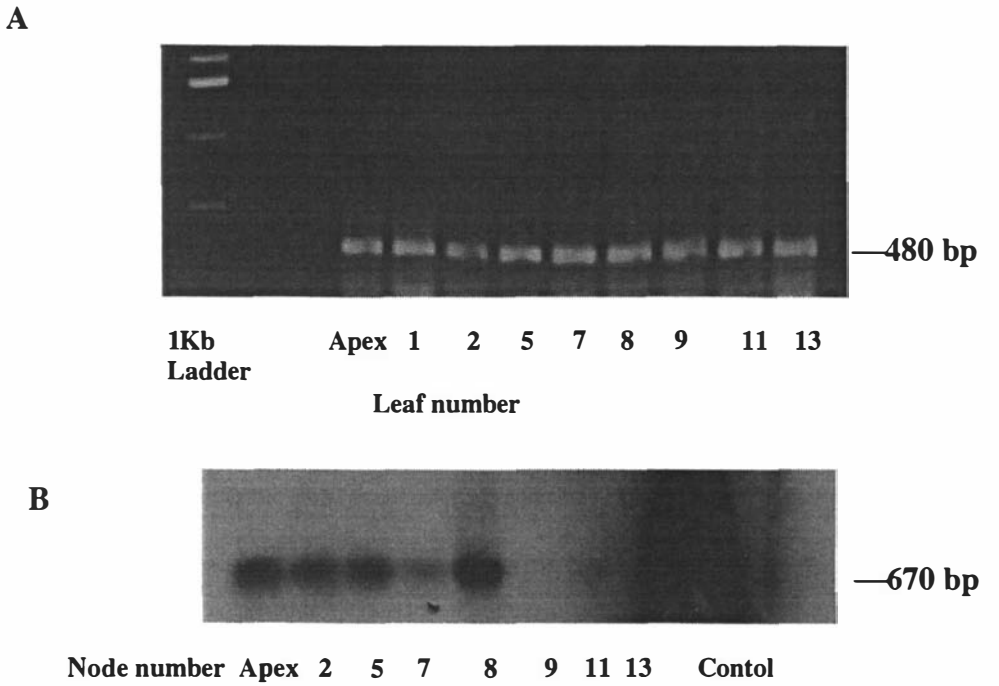
confirming that the expression pattern of *TR-ACS2* is not influenced by external stimuli generated by growing the plants in the glasshouse.

In later studies with *TR-ACS2*, the induction of gene expression was examined in response to auxin (IAA) and wounding (see section 3.3). As part of these induction experiments, 18S RNA was used as the template control for RT-PCR, and so this was also used as a template control to determine the expression of *TR-ACS2* during leaf ontogeny. The purpose was to confirm the apparent biphasic pattern of expression in the apex/ developing tissue and again at the onset of senescence was observed (see Figures 3.17 and 3.18). No change in the intensity of the 18S RNA amplification products (as determined by ethidium bromide staining) was observed in the leaves sampled (Figure 3.19A). In these experiments, degenerate primers (Table 2.3) were used to amplify ACC synthase transcripts and gene-specific probes (Figure 3.12) used to detect the expression of *TR-ACS2*. Using these conditions, the biphasic pattern of expression was confirmed. This expression was highest in the apex, nodes 2, 3 and again in node 8 (Figure 3.19B).

PCR amplification of *TR-ACS1* was attempted using the gene-specific primer for *TR-ACS1* (ACS1F) with ACSR6R using white clover leaf tissue from leaves representative of the different developmental stages. However, following two rounds of PCR, a broad smear at approximately the expected 670 bp region was visualised by ethidium bromide staining following gel electrophoresis on a 1 % (w/v) agarose gel (data not shown). Although not a single band, these smear patterns were in leaves from nodes 4, 6, 9, 10 and 12 which was consistent with the expression pattern determined by northern analysis (Figure 3.15A). This smearing is probably due to the limited gene-specific sequence available for design of gene-specific primers, which lead to different required annealing temperatures for the two primers used for PCR. However, the expression of *TR-ACS1* had been determined by northern analysis with strong hybridisation signals observed.



3.18 RT-PCR analysis of expression of *TR-ACS2* using gene-specific primers. Poly(A)$^+$ mRNA, isolated from white clover leaves representative of different developmental stages, was used as substrate for reverse-transcriptase reactions and the cDNA transcripts generated using primers specific for *TR-ACS2*. One round of PCR amplification was performed and the amplified products probed with ^{32}P-labelled *TR-ACS2*. Leaves used for RT-PCR are marked below the lanes. *TR-ACS2,* cloned into pCR 2.1, was used as a control and amplified by PCR (one round).



3.19 RT-PCR analysis of expression of *TR-ACS2* using degenerate primers. **A.** Primers for 18S rRNA were used as a template control for RT-PCR using total RNA isolated from white clover leaves from the developmental stages indicated.

B. Total RNA, isolated from white clover leaves representative of different developmental stages, was used with degenerate primers for ACC synthase. One round of PCR amplification was performed and the amplified products probed with ^{32}P-labelled *TR-ACS2*. Leaves used for RT-PCR are marked below the lanes. *TR-ACS2,* cloned into pCR 2.1, was used as a control and amplified by PCR (one round).

This method was also used to amplify *TR-ACS3* sequences (Figure 3.20) using the gene-specific primer for *TR-ACS3* (ACS3F) and ACSR6R. This expression pattern appeared consistent with northern expression studies for *TR-ACS3* (c.f. figure 3.15B and Figure 3.20) in which maximum expression of the gene is observed in senescent tissue. However, in addition to *TR-ACS3* expression observed in senescing leaf tissue, the more sensitive method of RT-PCR analysis determined *TR-ACS3* to also be expressed in leaf tissue from all developmental stages assayed. Again, the plant material used in this study was harvested at a different time when compared to that used in northern analysis (Figure 3.15B) so confirming that any external stimuli were not having an effect.

3.2.8 Identification of TR-ACS3-like genes in white clover

Sequence analysis of *TR-ACS3* revealed that the coded protein is missing the active site of the enzyme. However, Southern analysis of *TR-ACS3* determined that it was a distinct gene (Figure 3.13), and northern analysis and RT-PCR expression studies revealed that expression of the gene was developmentally-regulated in white clover leaf tissue (Figure 3.15B; Figure 3.20). To determine if a form of *TR-ACS3* containing a sequence encoding an active site is expressed in white clover leaf tissue, further RT-PCR was carried out using total RNA extracted from white clover leaf tissue from node 13 (within the same developmental stage used for the isolation of *TR-ACS3*). A degenerate reverse primer homologous to the active site (ACS3AR) (Table 2.3) was used with a forward primer specific for *TR-ACS3* (ACS3F) for two rounds of PCR amplification. The second round PCR products of the expected size of approximately 550 bp and also a smaller product of approximately 400 bp were TA-cloned into the pCR 2.1 vector and transformed into *E. coli* strain DH5∝. The inserts were confirmed to be approximately 550 bp and 400 bp by restriction digestion. Sequencing of the cloned inserts found the 550 bp sequence to be highly similar, but not completely homologous, to *TR-ACS3* (88 % homology) within the primer sites used for PCR amplification (Figure 3.21). However, the smaller product (approximately 400 bp) of RT-PCR was not found to have a high homology to any sequences on the GenBank database. The 550 bp sequence, designated *TR-ACS3A*, includes the DNA sequence for domain six (the active site of the ACC synthase enzyme).

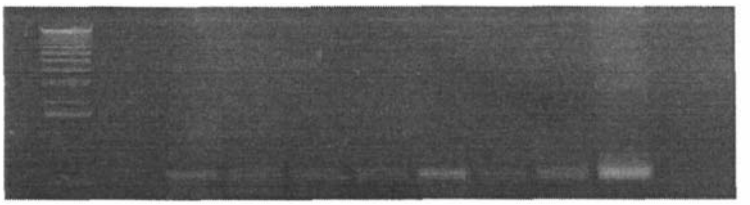


Figure 3.20 RT-PCR analysis of expression of *TR-ACS3*.

Poly(A)$^+$ mRNA isolated from white clover leaves representative of different developmental stages was used as substrate for reverse transcriptase reactions and the cDNA generated used for PCR reactions with primers specific for *TR-ACS3*. Two rounds of PCR amplification were required for visualisation on an ethidium bromide stained gel. Leaves used for RT-PCR are marked below the lanes. A control of *TR-ACS3* cloned into pCR 2.1 and amplified by PCR (one round) is also shown.

Reference molecule:	*TR-ACS3* (523 bp)	Homology
Sequence 2:	*TR-ACS3A* (526 bp)	88 %

```
TR-ACS3   AAAAGTGAGAGGTGGTAGGGTAAGATTTGATCCTGACCGTATATTGATGAGTGGTGGAGCAACA
TR-ACS3A  ............................................................

TR-ACS3   GGGGCAAATGAATTAATCATGTTCTGTTTGGCTGATCCTGGTGATGCCTTTTTGGTTCCTAGCC
TR-ACS3A  ..A.....................................................T.............

TR-ACS3   CTTATTATCCAGC----ATTTGTTCGTGATTTGTGTTGGAGAACCGGTGTCAACTAATTCCTGT
TR-ACS3A  ............CAAA........................................................

TR-ACS3   CCAATGTCATAGCTCAAACAATTTCAAGATAACAAGAGAAGCACTTGAAGAAGCTTATATGAAA
TR-ACS3A  ............................................................

TR-ACS3   GCACAAGAAAGAAACATCAATGTGAAAGGGTTAATCATAACAAATCCATCAAACCCTCTAGGAA
TR-ACS3A  .........G..................................................

TR-ACS3   CAACAATAGAAAAAGAAACACTAAAGAGCATAGTTAGTTTCATAAATGAAAACAACATTCATTT
TR-ACS3A  ...................T.....A.................C.....................

TR-ACS3   AGTGTGTGATGAAATCTATTCCGGCACAGTTTTCGACACTCCGAAATACGTAAGTGTCGCCGAA
TR-ACS3A  ...........................................................G...

TR-ACS3   GTTATACAAGAAATGGAAGAATGCAAAAAAGACCTCATTCATATGTACAATGATGAAGT-TGTG
TR-ACS3A  .............................................-----CAT..AT...C.T.C

TR-ACS3   AATTGCGGTCGAA---AA
TR-ACS3A  ..AA.ATC.G.G.CGC..
```

Figure 3.21 Alignment of *TR-ACS3* and *TR-ACS3A* sequences (.) represents identical sequence (-) represents no sequence. The additional 4 nucleotides are highlighted.

Although *TR-ACS3A* contains the DNA sequence which encodes the active site of the ACC synthase gene, an additional four nucleotides occur in *TR-ACS3A* when compared with *TR-ACS3*. Translation of the *TR-ACS3A* sequence reveals identity for the first forty-seven amino acids of the two sequences, and then complete non-identity for the next four amino acids before translation stops (Figure 3.22). However, if all four additional nucleotides are deleted, then the translated proteins have complete identity. Omission of any one nucleotide of the four creates the additional amino acids valine, aspartic acid, and tyrosine respectively. However, none of these residues occur adjacent to the active site in other ACC synthases.

3.3 Induction of ACC synthases in white clover

To characterise the expression of the three ACC synthase genes in response to wounding and IAA, RT-PCR was performed on total RNA isolated from white clover leaf tissue, which had been harvested from stolons following treatment. The specific ethylene action inhibitor (1-MCP) was used in a growth cabinet to act as a control to determine the effect of IAA- or wound-induced ethylene (Table 3.4). Whole white clover plants were used for these experiments and fully expanded, mature green leaves only were harvested (nodes 4-6).

Table 3.4 Treatment of plant material for induction of ACC synthase transcripts.
Leaf tissue was harvested at 0 h, 0.5 h, 1 h and 2 h following wounding or IAA or their control treatment. Leaf tissue pre-treated with 1-MCP followed by wounding or IAA or a control treatment was harvested at 0 h, 0.5 h and 2 h following treatment.

Induction treatment	*Control treatment*
Wounding	No wounding
1-MCP pre-treatment/ wounding	1-MCP pre-treatment/ no wounding
IAA (dissolved in ethanol/ Tween 20)	Ethanol and Tween 20 only
1-MCP pre-treatment/ IAA	1-MCP pre-treatment/Ethanol and Tween 20

Reference molecule: *TR-ACS3* (159 aa)
Sequence 2: *TR-ACS3A* (51 aa)
Sequence 3: *TR-ACS3A*[1] (126 aa)

```
                                                        3
TR-ACS3    KVRGGRVRFDPDRILMSGGATGANELIMFCLADPGDAFLVPSPYYPAFVRDLCWRTGVQLIPV
TR-ACS3A   ..............................................AYICS------------
TR-ACS3A1                                                  IFVRD.............
                                                    4
TR-ACS3    QCHSSNNFKITREALEEAYMKAQERNINVKGLIITNPSNPLGTTIEKETLKSIVSFINENNIH
TR-ACS3A   ------------------------------------------------------------
TR-ACS3A1  ........................G.....................................
               5                                    6
TR-ACS3    LVCDEIYSGTVFDTPKYVSVAEVIQEMEECKKD---------------
TR-ACS3A   ----------------------------------------------
TR-ACS3A1  ........................--Q.MEECKKDLIHIIVSLSKDLG
```

Figure 3.22 Alignment of deduced amino acid sequences of *TR-ACS3* and *TR-ACS3A* consensus sequences. The TR-ACS3A[1] sequence is the deduced translation of TR-ACS3A following the removal of the four additional nucleotides present in Figure 3.21. (.) represents identical sequence (-) represents no sequence. Shaded, numbered sequence represents the conserved regions of ACC synthase, shaded bordered sequence represents the part of the active site amplified within the primer site for TR-ACS3A.

RT reactions were carried out using equal amounts of total RNA as template from each time point of each treatment. One round of PCR was carried out on each sample, and triplicate aliquots of PCR products were separated by electrophoresis on a 1 % (w/v) agarose gel, blotted onto Hybond N^+ membrane and probed with either ^{32}P-labelled *TR-ACS1*, *TR-ACS2* or *TR-ACS3*. Initially, primers for β-actin were used on cDNA from these RT reactions. However, β-actin expression was found to be developmentally-regulated. In response to wounding, β-actin appeared to be expressed with more abundance one hour following wounding, and then expression decreased two hours following wounding (Figure 3.23A). This pattern of expression following wounding was not considered to be due to unequal template used for RT-PCR reactions, as ethidium bromide staining of first round PCR products showed a different pattern of expression of ACC synthase. In the example shown, ACC synthase expression increased 30 minutes after wounding, followed by a decrease in expression 60 minutes after wounding, when actin expression was highest (Figure 3.23B). It should be noted that the degenerate primers ACSR1F and ACSR6R were used suggesting that the induction may be one or more ACC synthase genes. Nevertheless, the results cast sufficient doubt on the use of β-actin, and so these sequences were not used as endogenous controls to show equal initial template for RT-PCR.

Therefore, 18S rRNA primers were used as an endogenous control, with both the 18S rRNA primers and primers for ACC synthase used in each PCR reaction. Due to the difference in abundance of rRNA and ACC synthase, a linear range of amplification was achieved by adding 18S competimers (primers modified at their 5′ ends to block extension by DNA polymerase) at a ratio to reduce the amplification efficiency of 18S cDNA when compared with that of ACC synthase, and without the primers becoming limiting, or without loss of relative quantification. In the induction experiments, PCR products were separated by electrophoresis on a 1 % (w/v) agarose gel and transferred to Hybond N^+ membrane for probing with either *TR-ACS1*, *TR-ACS2* or *TR-ACS3*. Prior to transfer of cDNA to the membrane, one agarose gel representing each treatment was visualised by ethidium bromide staining to test the constitutive nature of 18S RNA expression. In the example shown, the abundance of 18S RNA is consistent over increasing times (up to 2 h) following IAA or IAA/1-MCP treatments and in the control treatments (Figure 3.24).

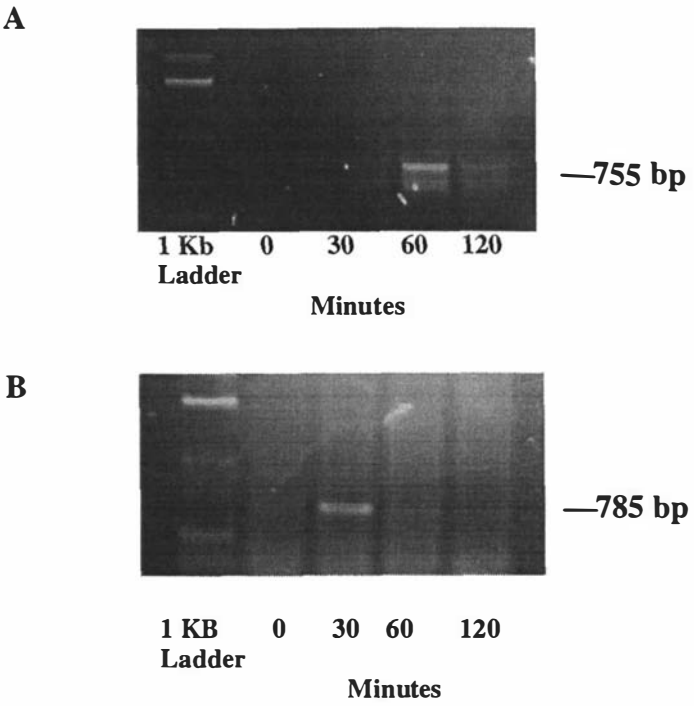


Figure 3.23 RT-PCR analysis of expression of ACC synthase following wounding.

A. Primers for β-actin were used with cDNA from poly(A)$^+$ mRNA isolated from white clover leaf tissue following wounding. One round of PCR amplification was performed and the products visualised using ethidium bromide staining after separation through a 1 % (w/v) agarose gel. Time points for harvest following the wounding treatment are indicated.

B. Poly(A)$^+$ mRNA isolated from white clover leaf tissue following wounding was used for RT-PCR with degenerate primers for ACC synthase. One round of PCR amplification was performed and the products visualised using ethidium bromide staining after separation through a 1 % (w/v) agarose gel. Time points for harvest following the wounding treatment are marked below the lanes.

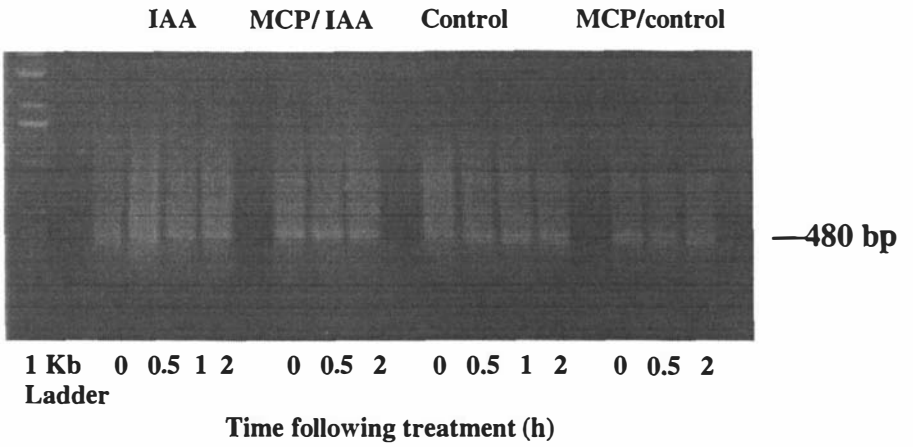


Figure 3.24 Assessment of the use of 18S rRNA as an internal control for RT-PCR. Primers for 18S rRNA were used to amplify 18S rRNA with total RNA as a template isolated from white clover leaf tissue following treatment with IAA (IAA), IAA with a pre-treatment of 1-MCP (MCP/IAA), an IAA control treatment of 0.05 % (v/v) ethanol/Tween 20 (control) and this control which had been pre-treated with 1–MCP (MCP/control). Products were visualised with ethidium bromide following separation through a 1 % (w/v) agarose gel. The expected size of the amplified rRNA product (480 bp) is indicated.

In IAA treated tissue, blots probed with ^{32}P-labelled *TR-ACS1* showed hybridisation to an approximately 785 bp band, consistent with a first round amplification PCR product using degenerate primers (Figure 3.25). In IAA-treated tissues, hybridisation is detectable at 0 h, is undetectable at 0.5 h, then is detectable at 1 h and 2 h, although no apparent increase in hybridisation signal from 0 h to 2 h has occurred. A similar pattern is observed for IAA-treated tissues that had been pre-treated with 3000 nL L^{-1} of 1-MCP. Hybridisation is observed at 0 h, is absent 0.5 h and then is detectable again at 2 h. Again, there is no apparent increase in hybridisation intensity observed from 0 h to 2 h. For these experiments, two control treatments were used. For the first control, plants were sprayed with 0.05 % (v/v) ethylene (the solvent used for the solubilisation of IAA and 0.05 % (v/v) Tween 20). Hybridisation of *TR-ACS1* is observed at 0 h, is undetectable at 0.5 h and 1 h and then is detectable again at 2 h but with no significant difference in intensity between 0 h and 2 h. The second control was to treat tissues that had been pre-treated with 3000 nL L^{-1} of 1-MCP with 0.05 % (v/v) ethanol and to sample tissues at 0 h, 0.5 h and 2 h. No hybridisation of *TR-ACS1* was observed at any of the time points assayed in this treated tissue.

No hybridisation of ^{32}P-labelled *TR-ACS2* was detected in IAA-treated, IAA + 1-MCP, or the appropriate control treated tissues (data not shown) although RT-PCR did amplify *TR-ACS2* from mature green leaf tissue in previous experiments (Figure 3.18). Hybridisation of ^{32}P-labelled *TR-ACS3* was observed to a band of approximately 785 bp in the 0 h IAA treatment, but then no hybridisation was detected in any of the further time points (3.26A). Hybridisation of *TR-ACS3* was also observed in 0.05 % (v/v) ethanol-treated tissue at 0 h, but again no detection of hybridisation signal was observed at any further time points.

In wounded mature-green leaves, no hybridisation of ^{32}P-labelled *TR-ACS1* and *TR-ACS2* was observed in any of the wounded treatments (data not shown). However, ^{32}P-labelled *TR-ACS3* was found to hybridise to two bands both 1-MCP and non-1-MCP treated plant tissues (Figure 3.26B). One band was approximately of the expected 785 bp while the second band was of approximately 500 bp (Figure 3.26B).

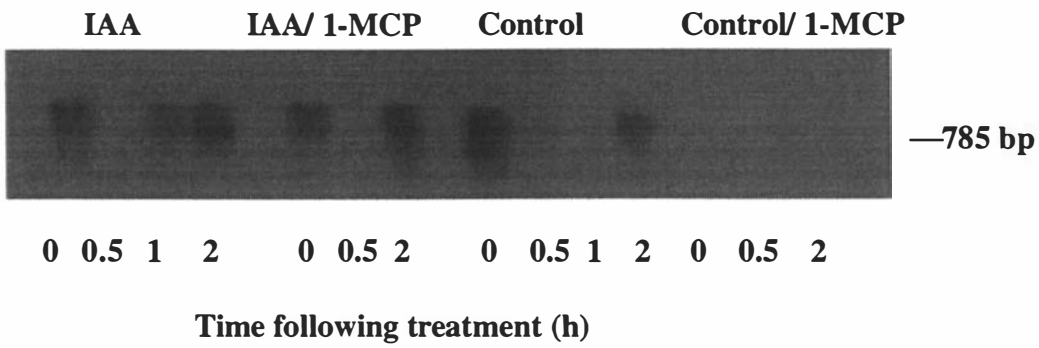


Figure 3.25 RT-PCR analysis of *TR-ACS1* expression in IAA-treated leaf tissue. RT-PCR was performed using reverse-transcriptase generated cDNA as template from total RNA isolated from white clover leaf tissue following treatment with IAA (IAA), IAA with a pre-treatment if 1-MCP (IAA/1-MCP), a control treatment of 0.05 % (v/v) ethanol/Tween 20 (control) and this control which had been pre-treated with 1–MCP (control/1mcp). One round of PCR was performed using degenerate primers for ACC synthase and the products probed with ^{32}P-labelled *TR-ACS1*. The position of the 785 bp cDNA fragment is indicated.

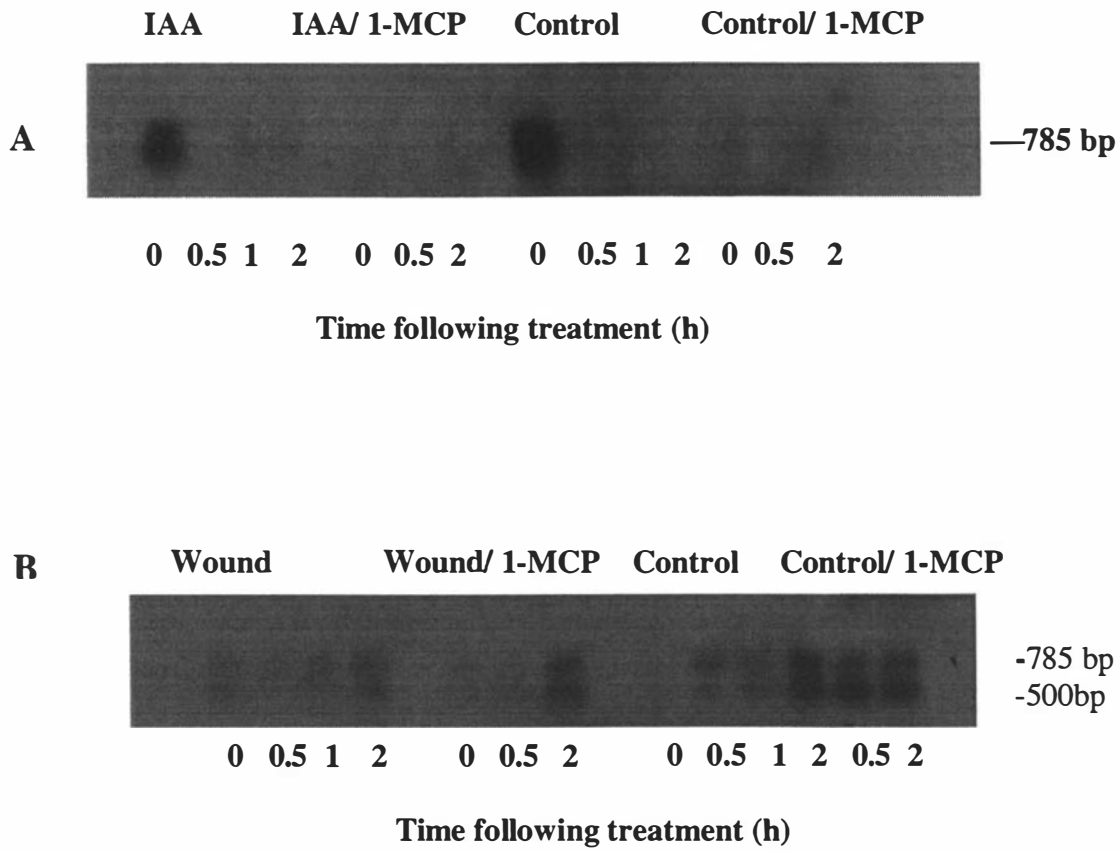


Figure 3.26 RT-PCR analysis of *TR-ACS3* expression in IAA-treated and wounded leaf tissue. RT-PCR was performed using reverse-transcriptase-generated cDNA as template from total RNA isolated from white clover leaf tissue. A) Leaf tissue was treated with IAA (IAA), IAA with a pre-treatment if 1-MCP (IAA/1-MCP), a control treatment of 0.05 % (v/v) ethanol/Tween 20 (control) and this control which had been pre-treated with 1–MCP (control/1-MCP). B) Leaf tissue was treated by wounding (wound), by wounding leaves pre-treated with 1-MCP (wound/1-MCP), no wounding (control) and no wounding of 1-MCP pre-treated tissue (control/1-MCP). One round of PCR was performed using degenerate primers for ACC synthase and the products separated through a 1 % (w/v) agarose gel and then probed with ^{32}P-labelled *TR-ACS3*. The position of the 785 bp cDNA fragment is indicated.

For *TR-ACS3*, some hybridisation to both bands was observed at 0 h and at 0.5 h, 1 h and 2 h post-wounding, with some indication of an increase in expression after 2 h. In wounded tissues that had been pre-treated with 3000 nL L^{-1} 1-MCP, hybridisation to both bands was observed in 0 h tissues and at 0.5 h and 2 h post-wounding, with the intensity of recognition highest at 2 h. In the first of two controls, tissues were not wounded and sampled at 0 h, 0.5 h and 1 h intervals. Hybridisation was observed to both bands in all sampled tissues, with a faint signal detectable at 0 h and the most intense signal from the 2 h treatment. In the second control, tissues were pre-treated with 1-MCP then sampled at 0.5 h and 2 h. For these tissues, ^{32}P-labelled *TR-ACS3* hybridised to both bands at both time points with equal intensity.

3.4.1 Identification of TR-ACS proteins in *E. coli*

The *TR-ACS1*, *TR-ACS2* and *TR-ACS3* genes were translated in *E. coli* using the pPROEX™-1 protein expression system (Figure 2.2). This system enables the sequence, which is cloned into the vector, to be induced for translation by IPTG when transformed into *E. coli*. The translated protein is fused to six histidine residues, which allows for purification using a nickel affinity column. Purified proteins can then be used for inoculation into rabbits to raise antibodies for identification and quantification of ACC synthase proteins by western analysis. Initially, the translated protein from *TR-ACS1* was used for the production of polyclonal antibodies, as it was necessary to determine that the antibodies raised to the translated products of one gene did not cross react with the translated products of the other genes. As well, northern analysis suggested that *TR-ACS1* was the most abundantly expressed transcript of the three ACC synthase genes identified in this study (the discovery of TR-ACS3A was made after the initiation of these translation studies).

TR-ACS1, *TR-ACS2* and *TR-ACS3* were amplified by PCR from pCR 2.1 vectors using degenerate primers (Table 2.3). ACSF1 and ACSR1 were used to amplify *TR-ACS1* and ACSF2 and ACSR1 were used to amplify *TR-ACS2* and *TR-ACS3*. These primers

contained restriction sites for digestion (*Eco*R 1 for ACSF1, *Bam*H 1 for ACSF2 and *Xho* 1 for ACSR1) so enabling in-frame cloning of the restriction digested PCR products into the pPROEX-1 vector (section 2.3.6.1). Following ligation of the digested PCR products into the polycloning sites of the pPROEX-1 vector, the recombinant vectors were transformed into the *E. coli* strain DH5∝ and restriction digestion confirmed that the vectors contained inserts of the expected size of approximately 670 bp (data not shown). These inserts were sequenced in the pPROEX-1 vector to confirm that they had been cloned in-frame for expression of the correct protein. The recombinant plasmids were also transformed into *E. coli* strain TB-1 as a comparison with DH5∝ for the production of fusion protein.

To produce antibodies to *TR-ACS1*, IPTG was used for the induction of expression in both bacterial strains. Crude protein was extracted from cell lysates and the proteins purified using a Ni-NTA column. SDS-PAGE and staining with Coomassie Brilliant Blue was used to visualise proteins from crude extracts of both uninduced and IPTG-induced bacterial strains containing *TR-ACS1* recombinant pPROEX vectors (Figure 3.27). Both bacterial strains could be induced to produce fusion proteins of approximately 28 kD. This is similar to the size of the predicted TR-ACS1 His-tagged fusion protein from translation of the *TR-ACS1* sequence (27.4 kD). IPTG-induced protein accumulation appeared to be higher in TB-1 than in DH5∝, and so TB-1 was used for all further protein expression studies.

TR-ACS1 fusion protein accumulation was up-scaled to gain the required amount of protein for the production of antibodies in rabbits, and for amino acid sequencing to confirm that the fusion protein was TR-ACS1. Using a Ni-NTA column, TR-ACS1 His-tagged fusion protein was purified from the crude extract. Column fractions of TR-ACS1 fusion protein were collected and the protein content of each fraction determined. The putative TR-ACS1 fusion protein eluted in column fractions 5-20 (with the highest accumulation in fraction 12). SDS-PAGE, followed by Coomassie Brilliant Blue staining (Figure 3.28), was used to confirm that the eluted protein was of the predicted size of the translated His-tagged TR-ACS1 (27.4 KD), and to determine that the fractions at the beginning and end of the elution profile did not contain degraded TR-ACS1 proteins. In addition to a band of approximately 28 KD, which corresponded to the induced band in the

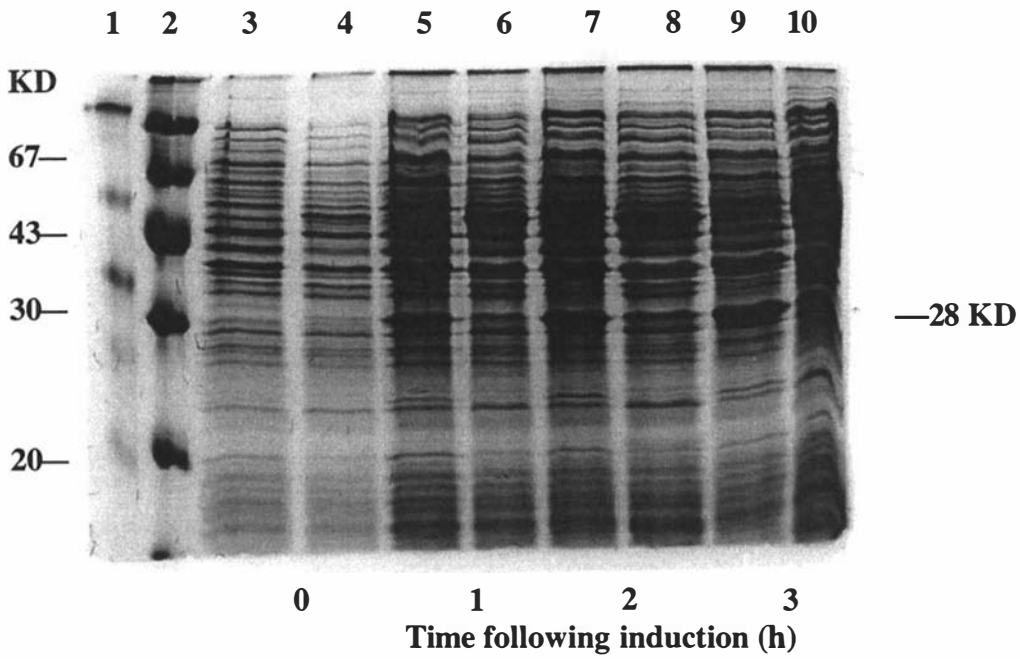


Figure 3.27 SDS-PAGE analysis of induction of TR-ACS1 fusion protein in *E. coli* strains DH5α and TB-1.

Total protein extracts from *E. coli* strain DH5α (lanes 3, 5, 7 and 9) and TB-1 (lanes 4, 6, 8 and 10) were separated on 10 % (w/v) SDS-PAGE gel and proteins visualised with Coomassie Blue staining. Lanes 3 and 4 are protein extracts from un-induced *E. coli* cells and lanes 5-10 are protein extracts from *E. coli* cells induced by IPTG for increasing time periods. Lanes 1 and 2 are two different molecular weight markers from BioRad and Pharmacia with some molecular weights indicated. The position of the induced TR-ACS1 fusion protein is marked on the right.

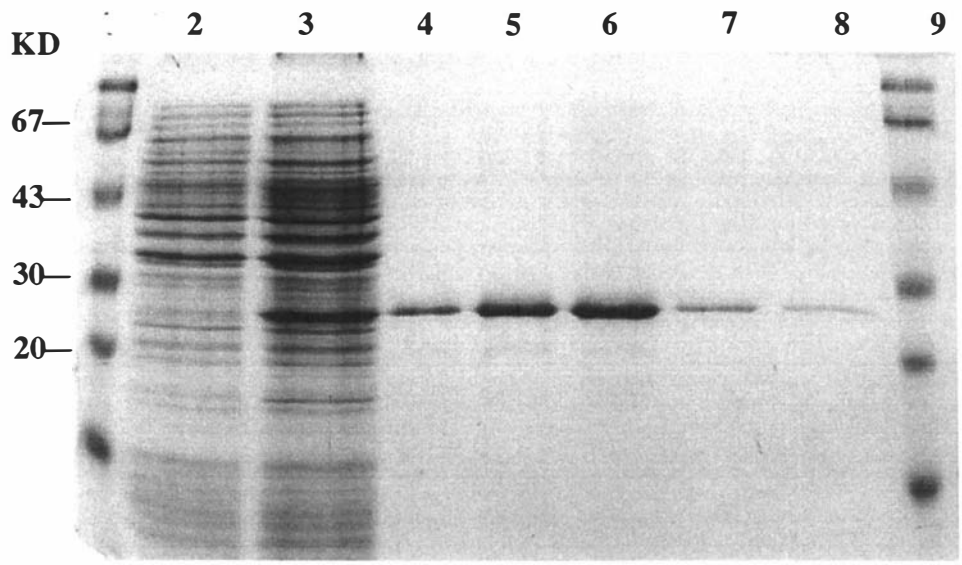


Figure 3.28 Identification of column fractions containing the induced TR-ACS1 fusion protein.

Column fractions containing TR-ACS1 fusion protein following Ni-NTA column purification were separated on a 10 % (w/v) SDS-PAGE gel and proteins visualised by Coomassie Blue staining.

Lane 1 and 9. Pharmacia markers

Lane 2. Total protein extracts from un-induced *E. coli* strain TB-1.

Lane 3. Total protein extracts from IPTG-induced *E. coli* strain TB-1.

Lane 4. Fraction 5

Lane 5. Fraction 8

Lane 6. Fraction 12

Lane 7. Fraction 16

Lane 8. Fraction 20

crude extract, a smaller band of approximately 23 KD was also detected. This low molecular weight band had been previously identified as a nickel binding protein common to strains of *E. coli* (M. T. McManus, *personal communication*). Eluted fractions 5 to 20, containing high concentrations of protein, were pooled and concentrated in preparation for antibody production in rabbits.

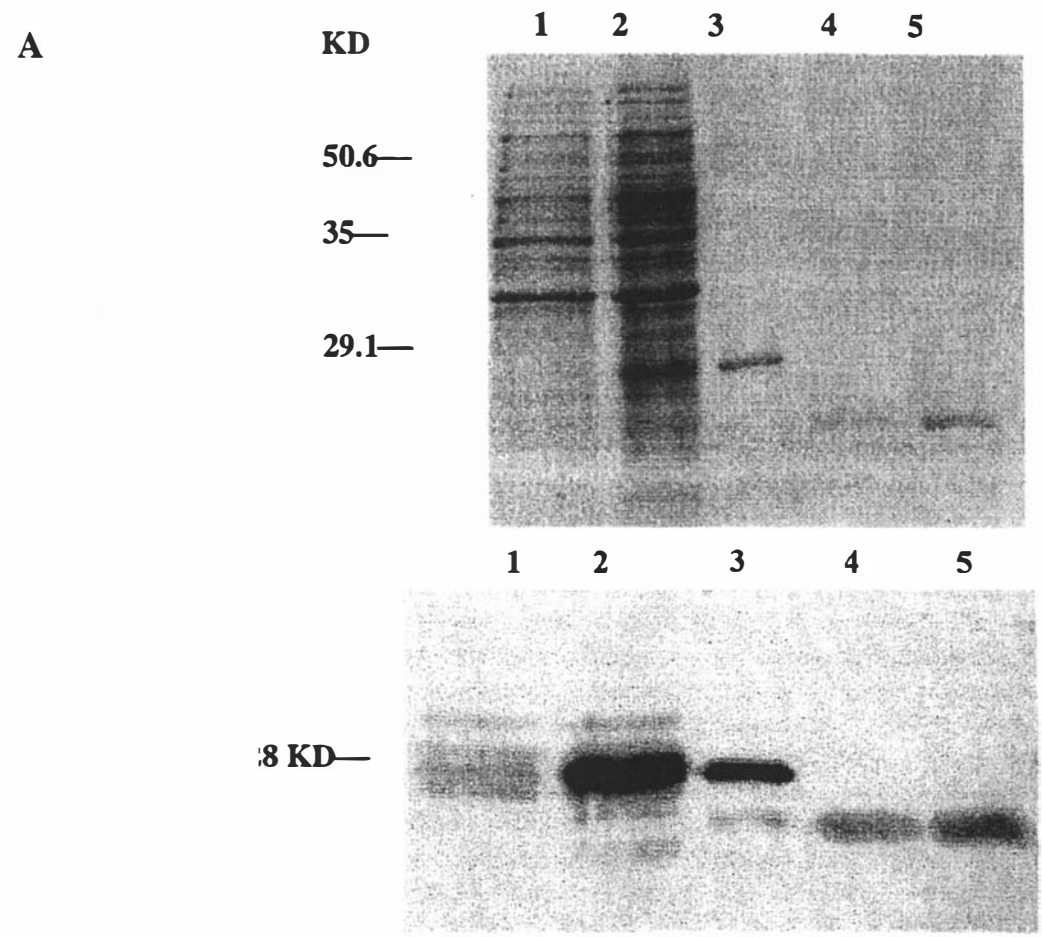
Prior to the production of antibodies it was necessary to confirm that the expressed protein was the translated product of *TR-ACS1*. To do this, the 28 KD protein band was purified by Ni-NTA column chromatography, separated on a 10 % (w/v) SDS-PAGE gel and excised. The excised protein band was trypsin digested and selected fragments were subjected to amino acid sequencing. Amino acid sequencing of the trypsin digested protein identified a sequence of six amino acids (Gln-Ala-Leu-Glu-Glu-Ala), which was also present in the deduced translated sequence of *TR-ACS1* (Figure 3.29). This confirmed the in-frame translation of the *TR-ACS1* sequence (Figure 3.10). Polyclonal antibodies to the TR-ACS1 fusion proteins were then raised in rabbits. The His-tag was not excised prior to the protein being injected into rabbits, as it was shown previously to be a poor antigenic determinant (Hunter, 1998).

3.4.2 Western analysis using anti-TR-ACS1 antibodies

Polyclonal antibodies to the *TR-ACS1* gene product were used for western analysis (section 2.5.2) to determine specificity and cross reactivity to the translated products of *TR-ACS2* and *TR-ACS3*. To do this, *TR-ACS2* and *TR-ACS3* were expressed using the pPROEX-1 vector in *E. coli* strain TB1 and column purified with a Ni-NTA column. Protein was loaded in equal amounts on duplicate gels and separated by SDS-PAGE. One gel was stained with Coomassie Brilliant Blue to visually confirm the equal loading of purified fusion protein, and also to identify the size of the TR-ACS2 and TR-ACS3 proteins (Figure 3.30A). The purified putative TR-ACS2 and TR-ACS3 fusion proteins (both approximately 26 KD) were similar in size to the predicted translated gene products fused to a His-tag, with TR-ACS2 predicted to be 25.9 KD and TR-ACS3 predicted to be 24.7 KD. From western analysis, the antibody raised against the TR-ACS1 fusion protein

LPEFRNAVAKFMSRTRGNRVTFDPDRIVMSGGATGAHEVTAFCLADPGDAFLVPT
PYYPGFDRDLRWRTGVKLVPVICESANNFKLTRQALEEAYEKAKIDNIRIKGLLITN
PSNPLGTVMDTTTLKTVVNFINEKRIHLISDEIYAATVFSHPSFISIAEIIEQETDIECD
RNLVHIVYSLSKDMGFPGFRVGIIYSYNDTVVNCARK

Figure 3.29 Deduced amino acid sequence of *TR-ACS1*. Shaded area corresponds to sequence obtained by amino acid sequencing of the *TR-ACS1* gene expressed in *E. coli.*



rn analysis of expressed TR-ACS1, TR-ACS2 and TR-usion-proteins using the antibody raised to TR-ACS1

ACS protein purified from *E. coli* was separated using duplicate 10 % s. Gel A was stained with Coomassie Blue for protein visualisation blotted onto PVDF membrane. The membrane was developed for 1

xtracts from uninduced *E. coli* strain TB-1
xtracts from IPTG induced *E. coli* strain TB-1
fied TR-ACS1 fusion protein

recognised a band of approximately 28 KD in extracts of the *E. coli* strain TB1 harbouring the pPROEX1 vector containing the *TR-ACS1* gene (Figure 3.30B; lane 2). The antibody also recognised a 28 KD protein in the column-purified TR-ACS1 protein preparation (lane 3) which corresponds to the major band visualised by Coomassie Blue staining in the column-purified TR-ACS1 protein preparation (Figure 3.30A; lane 3). Bands corresponding to the putative TR-ACS2 and TR-ACS3 Ni-NTA column purified fusion proteins were also recognised by the polyclonal antibody to the TR-ACS1 gene product (Figure 3.30B; lanes 4, 5) although with less intensity. A band below the TR-ACS1 protein band (approximately 23 KD), possibly corresponding to the nickel binding protein visualised on many of the Coomassie Brilliant Blue stained SDS-PAGE gels of Ni-NTA column purified proteins, was also recognised.

Western analysis was performed using the polyclonal antibody to the TR-ACS1 gene product using protein extracted, following 30 % to 90 % (w/v) ammonium sulphate fractionation, from mature green white clover leaf tissue (nodes 4-6) (Figure 3.31). Multiple protein bands were recognised at different intensities by the polyclonal antibody. A 55 KD band was recognised, as were fainter bands at 29 KD, 34 KD, 37 KD, 82 KD and a faint broad band centred at 69 KD. The protein band of 55 KD was recognised with the most intensity, and this is also close to the range of molecular masses (48-52 KD) reported for other ACC synthase proteins. However, without further detailed characterisation, the relationship of this putative ACC synthase band to the other protein bands recognised is uncertain. Therefore, attempts to characterise TR-ACS1 protein expression during white clover leaf ontogeny was not pursued further in the time available.

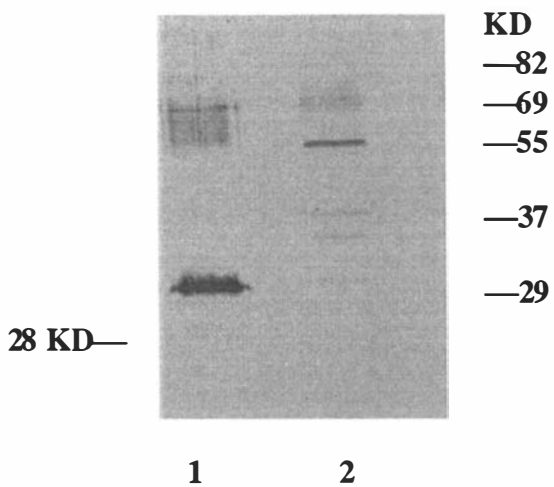


Figure 3.31 Western analysis of ACC synthase protein expression in white clover leaf tissue, using the antibody raised to TR-ACS1 fusion-protein.

One μg of expressed TR-ACS protein from *E. coli*, and 1 μg protein from mature green leaves (nodes 4-6) was separated through a 10 % (w/v) SDS-PAGE gel and blotted onto a PVDF membrane. The separated proteins were challenged with anti-TR-ACS1 antibodies and antibody recognition determined using alkaline-phosphatase-linked secondary antibodies.

Lane 1. TR-ACS1 fusion protein purified by Ni-NTA column chromatography

Lane 2. Protein extracted from white clover leaf tissue

4. Discussion

ACC synthase, the key regulatory enzyme of the ethylene biosynthetic pathway, is encoded by a multi-gene family in many plant species, with many of these genes cloned from a range of different tissues (Fluhr and Mattoo, 1996). In most cases, enhanced ethylene production rates appear to correlate with enhanced ACC synthase transcription (Kende, 1993; Zarembinski and Theologis, 1994; Mason and Botella, 1997) and the enzyme has been shown to be responsive to many developmental and environmental stimuli (Abeles, 1973; Yang and Hoffman, 1984; Yu *et al.*, 1998).

The physiological role of ethylene and the changes in ACC synthase gene expression in late development (ripening and senescence) have been well documented in fruit and floral tissue (e.g. Roberts *et al.*, 1985; Broglie and Broglie, 1991; Van Der Straeten and Van Montagu, 1991; Woodson *et al.*, 1992; Lay-Yee and Knighton, 1995; Van Altworst and Bovy, 1995; O'Neill, 1997; Ten Have and Woltering, 1997; Whittaker *et al.*, 1997; Mita *et al.*, 1998; Nakatsuka *et al.*, 1998; Liu *et al.*, 1999; Mita *et al.*, 1999; Shiomi *et al.*, 1999; Barry *et al.*, 2000; Jiang and Fu, 2000; Llop-Tous *et al.*, 2000). However, even though ethylene is considered to be involved in the regulation of leaf ontogeny in higher plants (Osborne, 1991; Abeles *et al.*, 1992), comparatively fewer studies have been undertaken on ACC synthase gene expression during leaf development (Van Der Straeten *et al.*, 1992; Rodrigues-Pousada *et al.*, 1993; Schlagnhaufer *et al.*, 1995; Wang and Arteca, 1995; Schlagnhaufer *et al.*, 1997; Vahala *et al.*, 1998; Jones and Woodson, 1999; Rodrigues-Pousada *et al.*, 1999; Smalle *et al.*, 1999).

Leaf senescence (culminating in necrosis) is the final stage of leaf development (Gan and Amasino, 1997). It is not simply a degenerative process, but is a highly regulated, developmental phase that results in the co-ordinated degradation of macromolecules and the subsequent mobilisation of compounds to young leaves, developing seeds or storage tissue (Buchanan-Wollaston, 1997; Gan and Amasino, 1997). One of the most conspicuous features of senescence is the rapid loss of chlorophyll associated with the degeneration of the internal structure of the chloroplasts (Hensel *et al.*, 1993). White clover plants have a stoloniferous growth habit, where plant size is dictated by the balance between growth at the apices, and gaining a supply of nutrients from the older

basal leaves and stolon, and the rate of leaf and stolon senescence markedly influences the carbon economy of the plant (Hay *et al.*, 1988). A high level of ethylene production is also associated with leaf senescence in white clover (Butcher, 1997; Hunter, 1998) and so the clonal growth form of white clover, therefore, provides a model system to study ACC synthase gene expression during leaf maturation and senescence.

White clover stolons have a sequential growth pattern with subtending leaves displaying the full range of development from initiation at the apex through maturation, senescence and necrosis. Stolons grown as part of this study exhibit consistent patterns of leaf development, with the production rate of leaves being balanced by the rate of senescence (Butcher, 1997). This thesis parallels studies by Hunter (1998) and Yoo (1999) who, using this growth system, found ACC oxidase to be differentially expressed during white clover leaf ontogeny. The focus of this study, therefore, is to determine the level of regulation of ACC synthase during leaf maturation and senescence in white clover leaves.

Previously determined physiological data characterising changes in ethylene evolution and chlorophyll levels during leaf ontogeny (Hunter, 1998) were used to target specific developmental regions of the stolon. Leaves at individual nodes along the stolon were categorised into four developmental stages (developing, mature green, onset of senescence and senescent). During white clover leaf ontogeny, an initial peak in ethylene evolution consistently coincided with leaf initiation at the apex. Ethylene production typically declined to reach a minimum value by leaf three in the white clover leaves and was maintained at this level through the mature green stage of leaf development (Hunter, 1998). An increase in ethylene production in senescing leaves coincided with chlorophyll loss. To identify ACC synthase genes expressed during leaf ontogeny two approaches were used, a cDNA library approach and an RT-PCR approach to generate ACC synthase cDNA sequences.

4.1 Screening and analysis of a cDNA library

The initial strategy for cloning an ACC synthase gene from white clover leaf tissue involved the screening of a cDNA lambda library, constructed by S. Butcher, (1997) using poly(A)$^+$ mRNA isolated from white clover leaf tissue at an early stage of senescence. The cDNA library was screened with ^{32}P-labelled ACS7, a 670 bp PCR-generated fragment of the ACC synthase reading frame. Two initial screens of the cDNA library were carried out by Butcher (1997). Two plaques corresponding to positive signals were identified in the first screen and were designated sub-library 6/1 and 10/1. The second screen resulted in seven regions of hybridisation from sub-library 6/1 (designated 6/1/1 to 6/1/7) and ten regions of hybridisation from sub-library 10/1 (designated 10/1/1 to 10/1/10). Aliquots of each sub-library from the second screen were used to infect *E. coli* DH10B(ZIP), to enable the sequencing of inserts of interest following recombination into the pZL 1 plasmid. The isolation and sequencing of one colony found it to share 63 % homology with a soybean cysteine proteinase (Bleecker *et al.*, 1991; cited in Butcher, 1997). Further screening and analysis of the cDNA sub-library (10/1/1 to 10/1/10) was then carried out in this study. Initially, two rounds of PCR amplification were carried out on templates from aliquots of plaques corresponding to regions of hybridisation from the second screen. Primers made to conserved regions of the ACC synthase gene were used for PCR amplification. PCR products of the expected size of 670 bp were detected by ethidium bromide staining following electrophoresis on a 1 % (w/v) agarose gel in five of these sub-libraries. ^{32}P-labelled ACS7 hybridised to the 670 bp fragment in these same five sub-libraries and also to a lower molecular weight band in two of the sub-libraries (Figure 3.1). More intense hybridisation by ^{32}P-labelled ACS7 to three of the 670 bp PCR products from these sub-libraries was observed, resulting in these three sub-libraries being the focus of further screening. Although sequencing of the lower molecular weight band could have been of interest, the expected size of an ACC synthase PCR product was 670 bp, therefore, this smaller PCR product was not pursued further. All plaques in lifts from the third and fourth screens were recognised by ^{32}P-labelled ACS7. Aliquots of plaques, picked and dispersed in media, from the fourth screened sub-libraries, were then used to infect *E. coli* DH10B(ZIP) cells, for the excision of putative ACC synthase

inserts. Attempts at rescuing putative ACC synthase cDNA clones from plaques corresponding to regions of hybridisation to ^{32}P-labelled ACS7 following four screens of the cDNA library were unsuccessful. No excised DNA fragments were detected following *Eco*R1 digestion of pZL 1 (Figure 3.4).

A further ACC synthase cDNA fragment (designated ACS5), supplied by S. D. Yoo (1999) and with 92 % identity at the nucleotide level to ACS7, was used to repeat probing of PCR products amplified from plaques picked from the second screen. No differences in hybridisation specificities were observed between ACS5 and ACS7, with both ACC synthase fragments hybridising to the same bands in the same sub-libraries (cf. Figure 3.1 with 3.5). A hybridisation intensity comparison was then carried out and determined that, although non-specific hybridisation of ^{32}P-labelled ACS5 to the plasmid pZL 1 occurred, this was observed as a weak signal in comparison to hybridisation to ACS5. Due to this occurrence of non-specific hybridisation and also the lack of an excised fragment following *Eco*R 1 digestion of pZL 1 plasmids from the fourth screen, PCR amplification was used to confirm that plaques selected from the fourth screen, based on hybridisation signals, contained the putative ACC synthase insert. A 670 bp band, which hybridised to ^{32}P-labelled ACS5, was amplified from nine of ten sub-libraries checked by PCR from the fourth screen (Figure 3.7). Therefore, although the putative ACC synthase cDNA clones were evident in sub-libraries following a fourth screen, a problem with the excision process of cDNA clones in pZL 1 from the λZipLox phage appeared to result in the inability to rescue the cDNA clones. However, it should be noted that none of these PCR products from the fourth screen have been sequenced to confirm unequivocally that these PCR products are ACC synthase clones.

PCR amplification of the initial cDNA library and later attempts to amplify the previously observed 670 bp band from sub-libraries were unsuccessful, and may be due to inadequate storage during and following the transfer of the library between research sites. Although no PCR product was detected from these later attempts to amplify the 670 bp PCR product, subsequent experimental work has found that cDNA separated on a 1 % (w/v) agarose gel with yields too low to be detected by ethidium bromide staining, still hybridised strongly when probed with ^{32}P-labelled probes made to regions of the ACC synthase reading frame. Therefore, although the library and sub-libraries

appeared unstable during long term storage, it is possible that the yield of cDNA was just diminished and not non-existent. This could have been confirmed by probing second round PCR products with ^{32}P-labelled ACS7 to determine the presence of ACC synthase cDNA in the sub-libraries, although the ability to recover the cDNA as an insert by *in vivo* excision in *E. coli* strain DH10B(ZIP) would still have needed resolving. Possible future methods could involve using an alternative system such as Genetrapper (GIBCO BRL), which is compatible with this cDNA library, to rescue the putative ACC synthase fragments, or by using primers to the SP6 and T7 promoter sites on either side of the multiple cloning site of the pZL 1 vector, to amplify the inserted fragment between these sites.

Data presented in other studies suggests that the expression of ACC synthase is low enough to exclude its representation in screening of a cDNA library. For example, Harpster *et al.*, (1996) were unsuccessful in the detection of an ACC synthase cDNA clone from a cDNA library of 200 000 to 300 000 plaques prepared from *Capsicum annum*. Whittaker *et al.*, (1997) screened approximately 75 000 clones from a ripening *Actinidia chinensis* cDNA library for homology to a kiwifruit ACC synthase fragment obtained by PCR. No hybridising colonies were detected indicating low abundance of the message even though ripening of these climacteric fruit is typically associated with a high level of ethylene production along with an increased respiration rate (Abeles *et al.* 1992; Henskens *et al.*, 1994). In normal ripening tomato fruit, which also produce a large amount of ethylene, the ACC synthase mRNA is estimated to be as low as 0.0001 % of total mRNA (Van Der Straeten *et al.*, 1990). During vegetative growth where very low levels of ethylene are evolved (Abeles, 1992; Imaseki, 1999), such as in white clover leaf tissue where maximum evolved ethylene levels reach 1600 nL g^{-1} FW h^{-1} in senescing leaves (Hunter *et al.*, 1999), ACC synthase gene expression would be expected to be extremely low. For example, *AT-ACS1* is the most abundant ACC synthase transcript in *Arabidopsis* leaves (Liang *et al.*, 1992), but its expression level is still too low to be analysed using RNA gel blot analysis.

To overcome the limitations of screening a cDNA library for a poorly represented message, Harpster *et al.*, (1996) used cDNA synthesised from poly(A)$^+$ mRNA isolated at different stages of fruit development as templates in PCR reactions. Degenerate olionucleotides were used which corresponded to conserved domains found among

diverse ACC synthases. This approach had been successful in the isolation of ACC oxidase genes within the white clover model growth system (Hunter *et al.*, 1999) and was used in this study in an attempt to isolate developmentally-regulated ACC synthase genes during leaf ontogeny in white clover.

4.2 Identification of ACC synthase genes using RT-PCR

Using poly(A)$^+$ mRNA isolated from white clover leaf tissue at different developmental stages, RT-PCR was used to generate ACC synthase sequences. In ACC synthase, there are eight regions of the primary sequence which are highly conserved (Figure 3.8). A characteristic feature of the structure of ACC synthase is the active site which is encoded by the conserved sequence of region six (Nakajima *et al.*, 1990; Van Der Straeten *et al.*, 1990; Yip *et al.*, 1990; Mori *et al.*, 1993; Cazzonelli *et al.*, 1998). The relative location of the conserved regions are almost identical and are considered to compose a structural core of ACC synthase (Imaseki, 1999). Nested degenerate primers made to three of these regions were used to amplify cDNA sequences from white clover leaf tissue. A previous study by Yoo (1999) determined that two rounds of PCR (of 30 cycles each) were required for visualisation of the PCR products on an ethidium bromide stained gel. This is probably due to the low expression level of ACC synthase in white clover leaf tissue (Butcher, 1997). Sequencing of second round PCR products (approximately 670 bp) determined there to be three distinct ACC synthase genes. These genes, designated *TR-ACS1*, *TR-ACS2* and *TR-ACS3*, had low homology (62 % to71 %) (Table 3.1) which is typical of the diversity of ACC synthases, with nucleotide and deduced amino acid sequences usually ranging from 55 % to 75 % (Imaseki, 1999). *TR-ACS1* and *TR-ACS2* sequences contained six of the eight conserved regions commonly found in all ACC synthases (Figure 3.10). These six regions were all of the conserved domains within, and including, the second round PCR primer sites (Figure 3.8). *TR-ACS3*, however, did not contain the nucleotide sequence encoding domain six (the active site of the enzyme).

Eleven of twelve invariant amino acid residues involved in binding of substrate and pyridoxal phosphate are conserved between aminotransferases and ACC synthase from

various plant species (Nakajima *et al.*, 1990; Van Der Straeten *et al.*, 1990; Dong *et al.*, 1991; Huang *et al.*, 1991; Nakagawa *et al.*, 1991; Olson *et al.*, 1991; Park *et al.*, 19991; Sato *et al.*, 1991). All nine of the conserved amino acid residues that are located within the regions of the ACC synthase gene amplified in this thesis are present in *TR-ACS1* and *TR-ACS2*. Three of these nine conserved amino acid residues are missing from *TR-ACS3* as they are typically located in the active site of ACC synthase. In addition, the lysine residue in the active site, which has been shown by Yip *et al.* (1990) to be the site of pyridoxal phosphate attachment, is conserved in *TR-ACS1* and *TR-ACS2* but is absent from *TR-ACS3*.

Because ACC synthase has been shown to be a large multi-gene family (e.g. Theologis, 1992; Kende, 1993; Zarembinski and Theologis, 1993; Destephano-Beltran *et al.*, 1995; Kim *et al.*, 1997; Oetiker *et al.*, 1997; Bui and O'Neill, 1998; Yu *et al.*, 1998; Arteca and Arteca, 1999; Woeste *et al.*, 1999; Barry *et al.*, 2000) in other plant species, it is reasonable to assume that not all of the white clover ACC synthase genes were necessarily identified using the approach of RT-PCR with degenerate primers made to conserved regions of the gene. The ACC synthase transcript is present in extremely low abundance, as indicated by the necessity for two rounds of PCR to amplify cDNA sequences for visualisation by ethidium bromide staining. The three ACC synthase transcripts identified were the result of sequencing a large number of cloned PCR products, with *TR-ACS1* being sequenced predominantly. The extremely low level of variation within the three groupings of sequences, together with ACC synthase sequences commonly having low homology to other members of the gene family within a species, indicated that from the large number of clones sequenced, there were only the three distinct ACC synthase transcripts isolated. Sequencing of more clones may well identify further members of the ACC synthase gene family in white clover, particularly those that may be present in even lower abundance in leaf tissue of white clover. However, this study was based on a developmental system, whereas many of the ACC synthase genes isolated in other studies have been from systems where tissue has been induced by factors that are known to increase ethylene biosynthesis and the steady-state level of ACC synthase mRNA. Treatments that have previously been found to induce ACC synthase expression include wounding, flooding, treating tissue with heavy metals such as lithium and copper, or plant signalling molecules including auxin, cytokinins, brassinosteroids, jasmonic acid and ethylene itself (reviewed in; Theologis, 1992;

Kende, 1993; Yang and Dong, 1993; Fluhr and Mattoo, 1996; Kende and Zeevart, 1997; Imaseki, 1999). It may be that treating white clover leaves with an inducer prior to RNA isolation may lead to the identification of other ACC synthase transcripts.

Genomic Southern analysis, using the gene-specific coding regions of *TR-ACS1*, *TR-ACS2* and *TR-ACS3*, confirmed that these three genes represented three different members of the white clover ACC synthase gene family (Figure 3.13B), supporting the existence of a multi-gene family (Kende, 1993; Zarembinski and Theologis, 1993). Multiple banding patterns were observed which is possibly due to the allotetraploid nature of the white clover genome (Williams, 1987). The white clover genome consists of two independently segregating genomes, therefore, polymorphisms can occur in either or both genomes, with the potential of up to four polymorphic alleles for each gene. The potato genome, which is also tetraploid, exhibits multiple bands with Southern analysis (Destephano-Beltran *et al.*, 1995). It is also possible that two ACC synthase genes may be highly homologous genes that have resulted from a gene duplication event, such as ACC synthase genes of potato and zucchini (Trebitsch *et al.*, 1992). In tomato and zucchini, the similar genes (*LE-ACC1A* and *LE-ACC1B* in tomato and *CP-ACC1A* and *CP-ACC1B* in zucchini) are clustered in the same locus and convergently transcribed (Huang *et al.*, 1991; Botella *et al.*, 1993). However, the banding patterns for the three white clover ACC synthases are not similar to each other and are, therefore, not likely to be clustered. Alternatively, the presence of multiple bands observed in this thesis could be explained by the presence of introns in each gene, which contain restriction sites for the enzymes used. Bands of weaker intensity are apparent in the *Xba* I digested lanes of *TR-ACS1* and *TR-ACS2* and the *Eco*R I digested lane of *TR-ACS2*. These may correspond to divergent ACC synthase sequences that are either unexpressed or not isolated using the degenerate oligonucleotides for PCR amplification.

A comparison of the consensus sequences of each of the three ACC synthase genes isolated from white clover leaf tissue with sequences in the GenBank identified a different set of genes with homology to each white clover ACC synthase sequence aligned with *TR-ACS1*, *TR-ACS2* and *TR-ACS3* (Table 3.3). All of the 116 sequences present in the GenBank are annotated as ACC synthases providing further confirmation that these three white clover genes encode ACC synthase. Interestingly, GenBank

sequences with the highest homology to *TR-ACS1*, which had been amplified from cDNA isolated from the three developmental stages initially used for RT-PCR (presenescing, onset of senescence and senescent), showed highest homology to ACC synthase sequences isolated from IAA-induced apical hooks of pea (GI 2360989) and mung bean (GI 22069) and from mature soybean leaf tissue (GI 18557). *TR-ACS2*, isolated from presenescing leaf tissue, showed highest homology to ACC synthase sequences isolated from etiolated mung bean hypocotyls (GI 1006805 and 1006807) and IAA-treated pea hypocotyls (2360987). GenBank sequences from white lupin (GI 6650975; where the expression of *LA-ACS1* was found to increase during germination and in response to IAA and wounding), ripening mango fruit (GI 1143811) and immature tomato fruit (3986116) were most similar in homology to *TR-ACS3*, which had been isolated from senescing white clover leaves. These GenBank alignments may provide the first indication of the physiological factors that regulate the expression of the three ACC synthases, and although the range of inducers for the expression of each gene may be diverse, *TR-ACS1* and *TR-ACS2* may be induced by IAA, and *TR-ACS3* may be senescence-associated or wound induced.

In other species, ACC synthase genes appear to be highly phylogenetically divergent. Phylogenetic analysis has previously revealed trifurcation of the ACC synthase genes into at least three branches of the phylogenetic tree, indicating the existence of three major classes of ACC synthase polypeptide (Lincoln *et al.*, 1993). The extraordinary degree of divergence among ACC synthase isoenzymes within each species has been suggested to have arisen early in plant evolution and before the divergence of monocotyledonous and dicotyledonous plants (Zarembinski and Theologis, 1993).

Phylogenetic analysis in this study demonstrates that the three ACC synthase genes isolated from white clover leaf tissue are also extremely divergent (Figure 3.14). As indicated by GenBank alignment, *TR-ACS1*, *TR-ACS2* and *TR-ACS3* group with other ACC synthase genes which demonstrate similar environmental/hormonal stimuli. The results indicate that *TR-ACS1* is most closely related to a pea ACC synthase (*PS-ACS1*), isolated from IAA-treated apical hooks of etiolated seedlings (GI 2360989). Together with other neighbouring sequences from IAA-treated hypocotyls of mung bean (GI 232930 and 398956), these ACC synthase isogenes comprise a major lineage in the ACS phylogenetic tree (Figure 3.14). The *PS-ACS1* sequence, which is closest to *TR-*

ACS1 on the phylogenetic tree was also determined to have the highest homology to *TR-ACS1* in a GenBank comparison (Table 3.3). As was also found by Liang *et al.*, 1992 and Abel *et al.*, 1995, most of the genes in this lineage are auxin regulated in vegetative tissues. *TR-ACS2*, which had been isolated from presenescent white clover leaves (nodes 4-7), was found to group with ACC synthases isolated from etiolated hypocotyls of mung bean (GI 1006805, 1006807 and 1813331). Two of these sequences were included in Table 3.3 as having high homology to *TR-ACS2* in a GenBank alignment. The sequences with which *TR-ACS2* grouped also comprised a major lineage that was separate to that of *TR-ACS1*. The use of phylogenetic analysis has placed the *TR-ACS3* sequence, isolated from senescing leaves (nodes 13-16), closest to an ACC synthase isolated from *Citrus paradisi* (2952297). However, it was not reported in this study whether this sequence was expressed specifically in ripening tissues as in GenBank sequences with the highest homology to *TR-ACS3*. The branch for *TR-ACS3* is very long and, therefore, the relative position to other sequences is less stable on the tree. These results support the suggestion by Lincoln *et al.* (1993) that a striking correlation exists between the phylogenetic relationship and the pattern of expression among the ACC synthase family from various plant species and also reinforces the previous indication determined by GenBank comparison, of the physiological factors that may regulate the expression of the three white clover ACC synthases. Therefore, it was of interest to examine the expression and induction of the three white clover ACC synthases during leaf ontogeny.

4.3 Gene expression of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* during leaf ontogeny

Initially, northern analysis was used to study the expression of all three ACC synthase genes isolated from white clover leaf tissue as maturation and senescence proceeded along the stolon. Although the translated sequence of *TR-ACS3* has the active site of the enzyme missing, suggesting that the gene product is a non-functional protein, the PCR product was amplified from poly(A)$^+$ mRNA suggesting that *TR-ACS3* is expressed in leaf tissue. Therefore, examination of the expression of this gene during leaf ontogeny may provide important information as to the function of this apparently aberrant

transcript. Due to the low abundance of the ACC synthase transcripts in white clover leaf tissue (as determined by the requirement for two rounds of PCR to visualise an RT-PCR product by ethidium bromide staining) leaves from different developmental stages were pooled. Leaves from nodes 1-3 represented the developing stage, the mature green stage was represented by leaves from nodes 4-6, the onset of senescence stage contained leaves from nodes 7-9 while leaves from nodes 13-15 represented a senescing stage. Equal quantities of poly(A)$^+$ mRNA extracted from pooled leaves from each developmental stage were used for northern analysis. Differing amounts of leaf tissue were required from the different developmental stages to get equal quantities of mRNA. Studies by Butcher (1997) and Yoo (1999), on white clover leaf tissue, also found that leaves at different developmental stages contain different proportions of poly(A)$^+$ mRNA. Bleecker *et al.* (1998) reported that total RNA declines significantly during leaf senescence. Together, these studies conclude that it is mainly the ribosomal RNA being measured as consistent when quantifying total RNA, and as total RNA levels change during leaf senescence, gene expression studies using total RNA may not be an accurate reflection of changes in gene expression. The use of equal loadings of poly(A)$^+$ mRNA, therefore, provides the opportunity to compare the abundance of ACC synthase transcripts as a proportion of other mRNA transcripts in tissues at specific developmental states.

In northern experiments, *TR-ACS1* hybridised to a 2.1 Kb transcript which accumulated predominantly in mature green leaves, and was less abundant in leaves at the onset of senescence (Figure 3.15A). At these developmental stages, ethylene levels were low following a peak in ethylene evolution associated with leaf initiation at the apex and prior to an increase in ethylene evolution associated with senescence (Hunter, 1998). Although it is expressed in tissues at the onset of senescence, its abundance is decreasing. *TR-ACS3* is expressed predominantly in senescing leaves (Figure 3.15B), where ethylene levels were determined previously to be increasing (Hunter, 1998). *TR-ACS3* expression in these leaves is probably not responsible for the regulation at the onset of senescence but may be induced by factors initiated by the senescence process.

No expression of *TR-ACS2* was detected by northern analysis even when 12 µg of poly(A)$^+$ mRNA was used. In a study by Shiomi *et al.* (1998), 8 µg poly(A)$^+$ mRNA was used to determine ACC synthase gene expression in wound- or IAA-induced leaf

tissue. However, in this thesis induced leaf tissue (ie. wounded or treated with IAA; conditions known to induce ACC synthase gene expression) were not used. As another comparison, ACC synthase gene expression was determined in ripening tomato fruit using 25 μg of total RNA (Barry *et al.*, 2000).

In common with *TR-ACS2* in non-treated *Arabidopsis* leaves, Liang *et al.* (1992, found the expression level of *AT-ACS1* to be too low to analyse using northern analysis and used a semi-quantitative RT-PCR method to determine the expression of this transcript. Likewise, Van Der Straeten *et al.*, (1992) used a method of semi-quantitative RT-PCR to study the expression *AT-ACS1*. Therefore, to analyse the expression of *TR-ACS2* during leaf ontogeny, RT-PCR was also used, as a putatively more sensitive method than northern analysis. Gene-specific primers were used for two rounds of PCR to amplify *TR-ACS2* transcripts from leaves representative of different developmental stages.

Using two rounds of PCR with gene-specific primers, the expression of *TR-ACS2* was shown by ethidium bromide staining to be predominantly in the developing tissue (Figure 3.16), coinciding with a peak in ethylene evolution associated with the apex. Of the three ACC synthases isolated from white clover leaf tissue, *TR-ACS2* mRNA was the only ACC synthase transcript isolated from developing tissue, suggesting that *TR-ACS2* may play a key role in the control of ethylene production during leaf initiation and expansion. However, it is not necessarily the only ACC synthase in the apex, but the only one picked up by the methods used in this study. RT-PCR showed that *TR-ACS2* is also expressed, but with less abundance in leaves at the onset of senescence (nodes 9 and 10). To confirm this biphasic pattern of expression (particularly expression of the gene at the onset of senescence), gene-specific primers for *TR-ACS2* were used with total RNA for a single round of RT-PCR, which was blotted and probed with ^{32}P-labelled *TR-ACS2* (Figure 3.18). The use of ^{32}P-labelled *TR-ACS2* to hybridise to PCR products provided enough sensitivity to detect products not visible by ethidium bromide staining. This approach enabled a comparative single round of PCR, using primers specific for β-actin, to be used to demonstrate equal amounts of RNA template used in the RT-PCR reactions. The pattern of expression observed was identical to that observed using gene-specific primers and two rounds of PCR (Figure 3.17A). These two expression studies were conducted using RNA extracted from leaves of different

harvests. This was to rule out any of the observed expression patterns of *TR-ACS2* being due to induction by wounding of leaf tissue during harvest or due to any environmental stimuli which could have been affecting the white clover leaves used for the initial RT-PCR. In later studies that examined the induction of ACC synthase expression by auxin and wounding, degenerate primers were used to amplify ACC synthase sequences from total RNA and the products probed with ^{32}P-labelled *TR-ACS2* (Figure 19B). Again, the biphasic expression pattern was confirmed with expression in the apex and newly initiated leaves and then again at the onset of senescence.

RT-PCR, using gene-specific primers for two rounds of PCR, followed by ethidium bromide staining of agarose gels, was also used to confirm the expression pattern of *TR-ACS3* (Figure 3.20) previously determined by northern analysis. The observed pattern of expression was broadly consistent with the northern analysis for *TR-ACS3* with maximum expression of the gene in senescing tissues. However, *TR-ACS3* was also determined to be expressed in leaf tissue representative of all developmental stages by the more sensitive method of RT-PCR. The use of RT-PCR was also used to verify *TR-ACS1* expression as determined by northern analysis. However, following two rounds of PCR, only broad smears were visible by ethidium bromide staining following agarose gel electrophoresis. This may be due to the limited gene-specific sequence available for gene-specific primers, which lead to different required annealing temperatures for the two primers used for PCR.

4.3.1 Significance of TR-ACS gene expression during leaf ontogeny

Three ACC synthase genes have been identified during leaf ontogeny in white clover that display a progression of expression such that *TR-ACS2* is expressed predominantly in the apex, newly initiated and mature leaves. *TR-ACS1* is expressed in mature green leaves and this decreased at the onset of senescence, and then *TR-ACS3* is expressed predominantly in senescent leaf tissue. These genes were cloned from tissues that, as far as possible, had not been perturbed (e.g. wounded or water stressed) on the days before harvest and so may represent developmentally-regulated genes.

In common with *TR-ACS2* expression in white clover leaves, Smalle *et al.* (1999) found that transcript levels of an *Arabidopsis* ACC synthase gene (*AT-ACS1*) were high in emerging unexpanded leaves, and subsequently decreased sharply, to rise again as leaves matured and gradually stopped expanding. The higher *AT-ACS1* levels in older, full-grown leaves, proceeded visible signs of senescence (e.g. reduction in chlorophyll content).

Analysis of a transgenic line containing *AT-ACS1* promoter-β-glucuronidase (gus) fusion reporter construct indicated that this gene is strongly developmentally regulated and associated with tissues that are undergoing differentiation and tissues that are an auxin source or where auxins are known to act (Rodrigues-Pousada *et al.*, 1993).

In view of the strong correlation of *AT-ACS1* expression and immature tissue, Rodrigues-Pousada *et al.* (1993) suggest that *AT-ACS1* is possibly involved in limiting cell wall expansion and consequent determination of size and shape, where ethylene is considered to have a role (Osborne, 1991; Abeles *et al.*, 1992; Kieber *et al.*, 1993; Lee *et al.*, 1996). Rapidly dividing and expanding tissues exhibit relatively high rates of ethylene production (Osborne, 1991). *Arabidopsis* seedlings grown in the presence of ethylene display a reduction in rosette diameter, resulting from an inhibition of cell enlargement (Kieber *et al.*, 1993). In addition, the characterisation of ethylene signal transduction mutants has demonstrated that ethylene produced by *Arabidopsis* seedlings is a major factor that determines leaf size (Bleecker *et al.*, 1988; Kieber *et al.*, 1993). Therefore, ethylene-insensitive mutants, such as *etr1*, *ein2* and *ain1* develop larger rosettes because of increased cell expansion (Bleecker *et al.*, 1988; Van Der Straeten *et al.*, 1993). However, ethylene biosynthesis is known to be under a feedback inhibition control in vegetative tissues (Yang and Hoffman, 1984; Rodrigues-Pousada *et al.*, 1999) and, therefore, the *AT-ACS1* (and *TR-ACS2*) expression pattern may be a reflection of the level and regulation of ethylene sensitivity in these developing tissues (Smalle *et al.*, 1999).

TR-ACS1, which was isolated by RT-PCR from leaf tissue of white clover from all three developmental stages (mature green, onset of senescence and senescent) is most similar in a GenBank comparison and by phylogenetic analysis to *PS-ACS1* (an ACC synthase isolated from IAA-treated apical hooks of pea seedlings) and other ACC synthases

isolated from IAA-treated vegetative tissue. Young leaves are typically associated with IAA (Rodigues-Pousada *et al.*, 1993). The expression of *TR-ACS1*, however, is in mature green leaves and in leaves at the onset of senescence (Figure 3.15A). It may be due to the development of axillary buds at the base of leaves at these developmental stages, which is a feature of the clonal growth of the white clover stolon (Brock *et al.*, 1988; Newton and Hay, 1996), but care was taken to excise these tissues during the maintenance of the clonal growth system. Alternatively, the expression of *TR-ACS1* during leaf ontogeny may be involved in the ethylene regulation system 1, which has been proposed to be responsible for basal and wound-induced ethylene production in vegetative tissues as well as climacteric and non-climacteric fruit (Lelievre *et al.*, 1997; Barry *et al.*, 2000). This system is ethylene auto-inhibitory and the decreasing level of expression of *TR-ACS1* in leaves at the onset of senescence may be due to ethylene levels (previously determined by Hunter, 1998) to be increasing at this stage.

Phylogenetic analysis and a GenBank comparison indicate that *TR-ACS3* may be induced by senescence or wounding. The expression of *TR-ACS3* in senescing leaves of white clover is consistent with this. It is possible that *TR-ACS3* expression is associated with senescence where ethylene may be operating in conjunction with age-related factors in regulating senescence. Grbic and Bleecker (1995) postulate that age-dependent factors are necessary and sufficient for senescence-associated gene (SAG) induction, and that ethylene plays a subsidiary role in regulating the timing of SAG expression. However, as *TR-ACS3* expression is not predominant until senescence is established (Figure 3.15B) it may be that the expression of *TR-ACS2* (Figure 3.17A, nodes 9 and 10) in tissues at the onset of senescence is involved in regulating the timing of senescence. In contrast *TR-ACS3* expression may be associated with wounding, as senescence is accompanied by cell degradation which would induce the wound-like signals (Imaseki, 1999).

Using the white clover clonal growth system used in this study, three ACC oxidase genes were previously cloned and found to be differentially expressed during leaf ontogeny (Hunter, 1998, Yoo, 1999). *TR-ACO1* is expressed in the apex with less abundance in leaf 1. *TR-ACO2* is detectable in the apex, shows maximal expression in leaves 1 and 2 and then gradually decreases in intensity until undetectable in leaf 11. The expression of *TR-ACO3* is clearly detectable first in leaf 8 with maximum

expression reached in leaves 13 to 16. The expression of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* may fore-shadow the expression of these three ACC oxidase genes during leaf maturation and senescence white clover with *TR-ACS2* being expressed in developing and mature green leaf tissue, *TR-ACS1* expression being highest in mature green leaf tissue and decreasing in leaves at the onset of senescence and *TR-ACS3* expression being at maximum intensity in senescing leaves. However, further research is needed to define the relationship and significance of this apparent parallel in gene expression between ACC oxidase and ACC synthase genes during leaf ontogeny.

A review of the literature suggests that there are few studies on ACC synthase gene expression during leaf development (Van Der Straeten *et al.*, 1992; Rodrigues-Pousada *et al.*, 1993; Wang and Arteca 1995; Rodrigues-Pousada *et al.*, 1999; Smalle *et al.*, 1999) and of these studies, only one study (Wang and Arteca, 1995) focuses on the differential expression of ACC synthase during leaf ontogeny. There are interesting parallels with the differential expression of ACC synthase genes observed during leaf development in *Pelargonium* (Wang and Arteca, 1995) and the results from this study in white clover. In *Pelargonium*, *GAC-2* is expressed in leaf buds and in young leaves and in terms of developmental expression most closely parallels *TR-ACS2*. The second ACC synthase from *Pelargonium*, *GAC-1*, is expressed in mature leaves and the expression diminishes in senescent leaf tissue. This pattern of expression most closely parallels *TR-ACS1*. To learn more about the possibility of developmentally-regulated ACC synthase genes expressed during leaf ontogeny, some of the physiological factors that may induce ACC synthase gene expression were examined.

4.4 Induction of *TR-ACS* genes in white clover leaf tissue

In this study, the *TR-ACS* genes were cloned from a developmental system and the genes isolated may be induced in a developmentally regulated manner. However, in several plant species it has been shown that different members of the ACC synthase gene family are expressed more strongly in response to a variety of chemical and environmental factors, such as environmental stress, physical wounding, noxious chemicals, auxins, ripening and senescence (e.g. Yang and Hoffman, 1984; Nakajima *et al.*, 1990; Van Der Straeten *et al.*, 1990; Dong *et al.*, 1991; Olson *et al.*, 1991; Rottmann

et al., 1991; Yip *et al.*, 1992; Lelievre *et al.*, 1997). When data from several extensively studied plant species were compiled, the genes were functionally classified in terms of the primary stimuli; wound-, auxin- and ripening signal-inducible genes (Imaseki, 1999). The primary stimulus-induced expression of these genes is further positively or negatively modulated by secondary stimuli. For example, wound-induced expression is modulated positively by auxin and ABA, but negatively by ethylene. Cytokinin positively modulates auxin-induced expression, while ABA and ethylene have a negative effect. Therefore, it may be that different stimuli induce the expression of the three ACC synthase genes in white clover leaves, or a combination of stimuli and developmental stage acting together may be involved in the induction.

In this study of the identification of ACC synthase genes during leaf ontogeny of white clover, phylogenetic analysis has provided some clues as to the nature of stimuli that may induce the expression of these ACC synthase genes. Sequence comparison has revealed that *TR-ACS2* is homologous with *AT-ACS1* gene expression in *Arabidopsis*. It is known that young leaves are a good source of auxin, which is a known mediator of various ethylene effects, and so it is possible that the expression of *AT-ACS1* in immature tissues of *Arabidopsis* and *TR-ACS2* in developing leaves of white clover is related to the presence of auxin. Similar analysis has shown that *TR-ACS3* aligns with transcripts induced by wounding and in senescing tissue.

Therefore, white clover leaves were treated with two of the primary stimuli of ACC synthase gene expression, IAA and wounding, to characterise the differential regulation of the three members of the ACC synthase gene family that have been identified in white clover, and to initiate some understanding of the nature of the internal cues that may regulate the developmental expression of these genes.

RT-PCR was used to study the pattern of expression of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* in response to wounding and IAA using total RNA isolated from white clover leaf tissue, which had been harvested following treatment. The specific ethylene action inhibitor 1-MCP, was used as a control to determine the effect of IAA- or wound-induced ethylene. 1-MCP has been used successfully in inhibiting ethylene-induced effects in studies including inhibiting wilting in cut carnations (Serek *et al.*, 1995a) and

petunia (Serek *et al.*, 1995b), and inhibiting ACC synthase transcript accumulation in ripening tomato (Nakatsuka *et al.*, 1998) and melon fruit (Shiomi *et al.*, 1999).

Equal amounts of template mRNA were used for each time point of each treatment for RT reactions. A single round of PCR (30 cycles) was carried out. As described in section 3.2.7, gene-specific primers were unable to be used to amplify the *TR-ACS1* PCR product, and so degenerate primers were used in this experiment to amplify the three white clover ACC synthase genes. Gene-specific probes were then used to discriminate between the three genes. To confirm that equal template was used for PCR reactions, β-actin primers were used initially with the cDNA from each RT reaction. However, β-actin expression was found to be developmentally regulated, with expression increasing one hour following wounding and decreasing after two hours (Figure 3.23). This expression pattern was not considered to be due to unequal template in PCR reactions, as ethidium bromide staining of first round ACC synthase PCR products (using degenerate primers) showed a different expression pattern. ACC synthase expression increased 30 minutes after wounding and decreased 60 minutes following wounding, when β-actin expression was highest. In other studies, β-actin expression was found to be developmentally regulated (Schitten and Zakrajsek, 2000; Goidin *et al.*, 2001) and was, therefore, not deemed suitable as an endogenous control to show equal initial template for RT-PCR. Instead, 18S rRNA primers were used in a method where both the 18S primers and the primers for ACC synthase were used in a single PCR reaction, thus eliminating differences between amounts of initial template between the sample and the control. Due to the difference in abundance of rRNA and ACC synthase transcripts, a linear range of amplification was achieved by adding 18S competimers (primers modified at their 5′ ends to block extension by DNA polymerase) at a ratio to reduce the amplification efficiency of 18S cDNA when compared with that of ACC synthase. Using this method, the abundance of 18S RNA was found to be consistent (using ethidium bromide staining of single round PCR products) following wounding and other tissue treatments (Figure 3.24).

In the *TR-ACS* expression studies (section 3.2.6), poly(A)$^+$ mRNA was used and different amounts of leaf tissue was required, depending on the developmental stage, to obtain equal quantities of mRNA. In contrast, in the induction experiments, as

ribosomal RNA was needed for the use of 18S primers to show consistency in initial template for RT-PCR reactions, total RNA was used. Using total RNA is not ideal to study changes in gene expression, as it is mainly the ribosomal RNA that is measured as consistent when quantifying total RNA. Leaves at different developmental stages contain different proportions of poly(A)$^+$ mRNA and different rRNA profiles (Yoo, 1999) and so to negate this phenomenon, all induction studies were carried out on leaf tissue of the same developmental stage (leaf tissue subtending from nodes 3-5).

In IAA-treated tissues (sprayed with 100 μM IAA, pH 6.0, containing 0.05 % (v/v) ethanol and 0.05 % (v/v) Tween 20), ^{32}P-labelled *TR-ACS1* hybridised to a band of approximately 785 bp, which is consistent with a first round PCR product using degenerate primers (Figure 3.25). However, apart from hybridisation to the RT-PCR products, no obvious effect of IAA is observed. Hybridisation is detectable at 0 h, is undetectable at 30 min, and is detectable again at 60 min and increasing at 120 min to reach similar levels as at 0 h. In IAA-treated tissue, which had been pre-treated with 3000 nL L^{-1} 1-MCP, a similar pattern was observed. A control treatment in which the plant tissue is sprayed with the treatment solution without IAA, also showed hybridisation at 0 h with no detectable hybridisation at 30 min and 60 min and hybridisation again at 120 min, although not at the intensity of 0 h. A second control where plant tissue treated with ethanol/ Tween 20 was pretreated with 1-MCP showed no hybridisation at any time points. The expression of *TR-ACS1* at the 0 h time point in all samples may be due to *TR-ACS1* being expressed at this developmental stage during normal leaf development (Figure 3.15A). Therefore, *TR-ACS1* expression may be inhibited by the spray solution both with and without added IAA by the 30 min time point and this inhibition decreases over the next 90 min (such that expression is observed again). It should be noted, however, that all 0 h time points in this experiment were from pooled leaves harvested on a different day to the other time points, and this may contribute to a difference in expression between 0 h and 30 min. It was necessary to use separate trays of plants for each time point to minimise any ACC synthase induction by touching the leaves and to avoid exposure of unharvested tissue to wound-induced ethylene produced by plants following the harvest of leaf tissue. Therefore, any induction in these treatments cannot be due to wounding effects.

The pattern of induction of *TR-ACS1*, which is similar in all treatments apart from the 1-MCP control treatment, shows an increase in *TR-ACS1* expression following treatment with the spray solution. This indicates that it may be the solution itself, regardless of whether IAA is added which is inducing the expression of *TR-ACS1*. Although leaf material was pooled for RNA extraction for the 0 h time points, individual RT reactions were performed and there may be variation in these reactions, since in the 1-MCP control (no IAA) treatment, no expression at 0 h is observed.

No hybridisation of ^{32}P-labelled *TR-ACS2* was detected in IAA-treated tissues, although *TR-ACS2* was found to be expressed in these leaves during normal leaf ontogeny in the white clover stolon (Figure 3.17A). Whole white clover plants were used in these experiments and only fully expanded, mature green leaves were harvested (nodes 3-5).

In the wound-treated plants, no hybridisation of ^{32}P-labelled *TR-ACS1* or *TR-ACS2* was observed in any of the wounded treatments. For *TR-ACS2*, this is consistent with observations in *Arabidopsis*. The *AT-ACS1* promoter, which is expressed in tissue at the same developmental stage as *TR-ACS2*, was not affected by wounding of young or old vegetative light-grown tissue (Rodrigues-Pousada *et al.*, 1993).

For *TR-ACS3*, hybridisation to two bands was apparent at 0 h and at 30 min, 60 min and 120 min after wounding, with one band approximately the expected 785 bp size and a smaller 500 bp band (Figure 3.26B). There was some evidence of an increase in expression over these time points. In wounded tissue that had been pre-treated with 3000 nL L^{-1} 1-MCP, hybridisation to the two bands was observed at all time points of 0 h and 30 min and more intense hybridisation detected at 120 min following wounding. As a control, a tray of plant tissue was sampled without a wounding treatment at 0 h, 30 min, 60 min and 120 min. In all samples, hybridisation to two bands was observed, with faint hybridisation detected at 0 h and with the intensity of hybridisation increasing during the sampling times. A second control of non-wounded plant tissue, which had been pre-treated with 1-MCP, was sampled at 30 min and 120 min and showed hybridisation to two bands with equal intensity at both time points. The observed expression pattern of *TR-ACS3* in this experiment is difficult to interpret. The induction of *TR-ACS3* appeared to be independent of 1-MCP, with the hybridisation intensity being higher in these samples than in non 1-MCP treated tissues. This could, again, be

due to variation amongst trays or 1-MCP could be inducing the expression of *TR-ACS3*. In ripening tomato fruit, Nakatsuka *et al.* (1998) determined that although treatment with 1-MCP prevented an increase in the transcript abundance of *LE-ACS2* and *LE-ACS4* in untreated fruit, the *LE-ACS6* transcript, which was not detected in untreated ripening fruit, was induced by 1-MCP treatment.

The presence of two hybridising bands is not unusual. Gene-specific probes have detected two transcripts in other ACC synthase expression studies in several plant species (Ten Have and Woltering, 1997; Peck and Kende, 1998; Bekman *et al.*, 2000). Peck and Kende (1998) demonstrated with *PS-ACS1* from pea, that the smaller mRNA (*PS-ACS1b*) arises from an alternative promoter, giving rise to a transcript where translation starts on the conservative methionine residue located in the second exon. Since the deletion of the first 27 amino acid residues completely abolishes ACC synthase activity (Li *et al.*, 1996), this truncated protein has been suggested to play a regulatory role (Bekman *et al.*, 2000).

TR-ACS3 expression was also observed in IAA-treated tissue and control spray-treated tissue at 0 h, but not at later time points (Figure 3.26A). There was no observed expression of *TR-ACS3* at any time points of 1-MCP pre-treated tissues followed by IAA- or control-treatment. During white clover leaf ontogeny, *TR-ACS3* is expressed predominantly in senescing leaves (Figure 3.15B) and would not be expected to be observed in mature green leaves. Using the more sensitive method of RT-PCR, *TR-ACS3* expression could be detected at low levels in leaves from all developmental stages (Figure 3.20). The expression of *TR-ACS3* at only the 0 h time point could, therefore, indicate that the leaves used in the 0 h time point were induced in some way and that leaves in other time points were not, or the variation between trays was having an effect. Although plants were handled carefully and leaves were harvested directly into liquid nitrogen, leaf tissue is very sensitive to being touched. Similarities between touch response and wounding in *Arabidopsis AT-ACS6* expression indicates that they may have overlapping signal transduction pathways or that the response is the same but the level of induction and decline is due to the severity of the treatment (Arteca and Arteca, 1999). *AT-ACS6* expression had decreased to undetectable levels 60 minutes following touching, however, expression of *AT-ACS6* was detectable up to 2 hours after wounding (Arteca and Arteca, 1999). In tomato fruits and seedlings touch stimulation and

wounding also led to a large, transient increase in two transcripts, but a third transcript was induced by wounding but was not detected in touched tissues (Tatsuki and Mori, 1999).

In addition to these possible effects due to touching, trays of plants were removed from the glasshouses where they were grown, and then equilibrated over night in the laboratory. This was to enable 1-MCP treated plants, which required incubation in growth cabinets, to be treated as similarly as possible to the non 1-MCP pre-treated plants. These plants, which were not incubated in growth cabinets, were kept in a laboratory at the same temperature as the growth cabinets. However, the transition to the laboratory from the glasshouse environment may have had stress effects on the plants and affected their diurnal rhythm with change in day length and light intensity due to laboratory lights. This disruption may influence the pattern of expression observed for both wounding and IAA treatments.

At the initial level of inspection, the IAA- and wounding of plant tissues in this thesis has not apparently been definitive in terms of the induction of ACC synthase gene expression. Nevertheless, these induction experiments have identified some potentially interesting trends. It may be significant that *TR-ACS1* is the only gene that showed widespread recognition to time points in the IAA-treated tissue, and that *TR-ACS3* is the only gene that displays widespread hybridisation to the wound-induced tissues. It is intriguing that the senescence (ageing) –associated gene (*TR-ACS3*) does display this homology as there may be some connection between the signals associated with ageing and wound-induced expression (Imaseki, 1999; Mita *et al.*, 1999). However, such signals may not be operating in the comparatively young tissue used in these induction experiments.

In terms of the auxin-induced experiments, in addition to the eight highly conserved regions found in all ACC synthases studied (Imaseki, 1999), all auxin-induced ACC synthases have been suggested to contain four more regions of homology which are at least ten amino acid residues and show greater than 90% identity (Kim *et al.*, 1997). *TR-ACS1*, the putative IAA-associated ACC synthase identified in this thesis, and the nearest neighbouring sequences determined by phylogenetic analysis and GenBank alignment do exhibit high homology to these regions (Figure 4.1) and some of these

	BOX 2	BOX 3
VR-ACS7	PTPYYPGFDRDLKWRTG	HLISDEIYSGT
TR-ACS1	.....R....	.....AA.
TR-ACS2	.....	.....
TR-ACS3	.....A.V...C....	..VC.....

Figure 4.1 Alignment of conserved regions of deduced amino acid sequences of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* with the regions of amino acid sequence of VR-ACS7 considered to be conserved within auxin-induced ACC synthases (Kim *et al.*, 1997). Box 1 and 4 of VR-ACS7 are not within the sequence of *TR-ACS1*, *TR-ACS2* and *TR-ACS3*. (.) represents identical sequence

have been shown to be IAA-induced (eg. apical hooks of pea (GI 2360989); IAA-treated hypocotyls of mungbean (GI 22069). However, the amino acid sequence of *TR-ACS2* and the neighbouring sequences grouped with *TR-ACS2* contained regions *identical* to these (IAA) conserved regions. *TR-ACS2* and the sequences with highest homology to *TR-ACS2* were isolated from developing tissue, which is typically associated with IAA, but in the induction experiments performed here, mature-green leaves were used (when *TR-ACS1* expression predominates). It may be, therefore, that it is necessary to replicate developmental cues as well as hormonal cues in order to recreate the regulatory conditions *in vivo* in induction experiments performed *in vitro*. For example, IAA-treatment of developing tissues may induce the expression of *TR-ACS2*. Therefore, any of the three *TR-ACS* genes may be responsive to IAA or wounding, but these cues may need to be transduced in association with developmental cues.

Finally, for some members of the ACC synthase multi-gene family in other plant species, no chemical inducers or treatments have been found to activate or modulate the expression (Huang *et al.*, 1991; Rottmann *et al.*, 1991; Botella *et al.*, 1993). Therefore, although there is some homology with these IAA regions, the three ACC synthase genes identified as being constitutively expressed during leaf ontogeny in white clover may simply not be induced by wounding or IAA.

4.5 Identification of *TR-ACS3*-like genes in white clover

The *TR-ACS3* sequence amplified by RT-PCR encodes a protein with a deleted active site. Northern (and RT-PCR) analysis suggested that the *TR-ACS3* gene was being transcribed in white clover leaf tissue, and that this transcript appeared to be developmentally-regulated. In addition, Southern analysis confirmed that this sequence was distinct from *TR-ACS1* and *TR-ACS2*. However, the translated protein is lacking the active site of the enzyme.

Initially, to discount any technical reasons (for example, deletion of the sequence during plasmid replication in the *E. coli* host), the RT-PCR of *TR-ACS3* was repeated. To

determine directly that *TR-ACS3* did exist in another form, which contained the active site, a degenerate reverse primer homologous to the active site sequence was used with a forward primer specific for *TR-ACS3* for two rounds of PCR. Sequencing of second round PCR products confirmed that a sequence (designated *TR-ACS3A*) did exist with high homology to *TR-ACS3* and contained the nucleotide sequence encoding the previously non-existent active site. However, in addition to *TR-ACS3* and *TR-ACS3A* differing in the presence of an active site region, the *TR-ACS3A* sequence also contained four additional nucleotides in domain three, which are not present in any of the other *TR-ACS* genes. The presence of these four nucleotides means that *TR-ACS3A* encodes a non-functional protein. Resequencing confirmed this DNA sequence, although, the same RT-PCR reactions were used. Using fresh RNA, further RT-PCR and sequencing would be required to confirm that this is a transcribed sequence and not a result of technical error, and also to determine whether a further sequence exists encoding a functional *TR-ACS3*.

If *TR-ACS3* and *TR-ACS3A* are shown not to be PCR artifacts, then these genes may be pseudogenes arising during the evolutionary process. In the white clover genome, four pseudogenes to alcohol dehydrogenase have previously been found to be present (Nick Ellison, AgResearch, Palmerston North, NZ *pers. comm.*). Southern analysis would not distinguish between pseudogenes if using *TR-ACS3* as a probe in this study. Alternatively, studies by Peck and Kende (1998) have shown that a single ACC synthase gene of pea (*PS-ACS1*) produces two transcripts, one of which does not encode a functional enzyme. In *Arabidopsis*, two of six ACC synthase genes have been identified and characterised, and have been found to be encoded by highly homologous sequences. It has been demonstrated that *AT-ACS3* is a pseudogene representing a truncated version of *AT-ACS1* (Liang *et al.*, 1995), and that the *AT-ACS1* polypeptide does not display ACC synthase activity when expressed in *E. coli.* The predicted amino acid sequence of *AT-ACS1* is missing the highly conserved tripeptide, Thr-Asn-Pro (TNP), between Ile^{204} and Ser^{205}. Liang *et al.* (1992) suggested that since *AT-ACS1* is transcriptionally active in *Arabidopsis,* the possibility exists that the AT-ACS1 polypeptide may be post-translationally modified and be active in the intact plant. In addition, the possibility that it may also play a role as a dominant negative regulator of other ACC synthases by forming heterodimers has also been suggested (Herskowitz, 1987). In a model favoured by Peck and Kende (1998), the smaller transcript (*PS-*

ACS1b) accumulates prior to the transcription of a full-size ACC synthase mRNA and "titrates away" a putative repressor protein. This assumption is based partly on the possible occurrence of a strong loop structure in the last exon of the *PS-ACS1* gene.

4.6 Identification of TR-ACS proteins in protein extracts of white clover leaf tissue

In an attempt to characterise TR-ACS protein expression during white clover leaf ontogeny as a complement to the gene expression studies, *TR-ACS1*, *TR-ACS2* and *TR-ACS3* were translated in pPROEX-1 expression vectors and purified using nickel affinity columns. The aim was to produce antibodies to these proteins to examine their accumulation during leaf ontogeny. A putative TR-ACS1 fusion protein of approximately 28 kD was detected by SDS-PAGE and staining with Coomassie Brilliant Blue and was similar to the predicted size of the His-tagged fusion protein from translation of the *TR-ACS1* sequence (27.4 KD). Purified putative TR-ACS2 and TR-ACS3 fusion proteins (both approximately 26 KD) were also similar in size to the predicted translated gene products fused to a His-tag with TR-ACS2 predicted to be 25.9 kD and TR-ACS3 predicted to be 24.7 kD. Only the translated protein from *TR-ACS1* was used initially for the production of polyclonal antibodies as it was necessary to determine that the antibodies raised to the translated products of one gene did not cross react with the translated products of the other genes. *TR-ACS1* was selected as northern analysis indicated that it was the most abundantly expressed transcript of the ACC synthase genes identified in this study. Amino acid sequencing of the 28 kD protein band, following trypsin digestion, was performed to confirm that the expressed protein was the translated product of *TR-ACS1*. A six amino acid sequence, which was also present in the deduced translated sequence of *TR-ACS1,* confirmed the in-frame translation of the TR-ACS1 sequence. Polyclonal antibodies to the TR-ACS1 fusion proteins were raised in rabbits and used for western analysis to determine specificity and cross reactivity to the translated products of *TR-ACS1*, *TR-ACS2* and *TR-ACS3*. The antibody recognised a band of approximately 28 KD in extracts of the *E. coli* strain TB1 harbouring the pPROEX-1 vector containing the *TR-ACS1* gene (Figure 3.30B, lane 2). A 28 KD band in the column-purified preparation (lane 3) which corresponds

to the major band visualised by Coomassie Brilliant Blue, was also recognised by the antibody. Bands corresponding to the putative TR-ACS2 and TR-ACS3 purified fusion protein (Figure 3.30B; lanes 4 and 5), loaded in equal amounts to TR-ACS1, were also recognised by the polyclonal antibody to the TR-ACS1 gene product, although strongest recognition corresponding to the 28 kD band of TR-ACS1 (lane 3). A band below the TR-ACS1 protein band (approximately 23 kD), possibly corresponding to a nickel binding protein common to strains of *E. coli* (M. T. McManus, *personal communication*), was also recognised, although faintly.

Western analysis was performed using the polyclonal antibody to the TR-ACS1 gene product using protein extracted, following 30 % to 90 % (w/v) ammonium sulphate fractionation, from white clover leaf tissue (Figure 3.31). The antibody recognised a band of 55 KD with highest intensity, and bands of 29, 34, 37, 69 and 82 KD with a lower intensity. It is unknown whether these bands correspond to ACC synthase or unrelated proteins which share a common epitope with the enzyme. In other plant species, the multiple ACC synthase genes encode proteins that show considerable diversity in molecular mass. Translation products *in vitro* fractionated by SDS-PAGE, show M_rs ranging from 48 kD to 58 kD (Van Der Straeten *et al.*, 1990; Dong *et al.*, 1991; Sato *et al.*, 1991). Several studies report that the in vitro-translated ACC synthase is larger by 8 to 9 kD, when compared to the partially purified native protein (Sato *et al.*, 1991; Yip *et al.*, 1991). This size difference has been suggested to be a result of proteolytic processing which may have occurred *in vitro* during the isolation of the native ACC synthase from plant tissues (Li *et al.*, 1996). However, Sato *et al.* (1991) reported that the *in vivo* and *in vitro* translated zucchini ACC synthase are the same size (55 kD), the same size as the major staining band in white clover leaves (Figure 3.31). Discrepancies also exist in the literature on whether the native enzyme exists as a monomer or a dimer. For example, a wound-induced tomato ACC synthase has been suggested to exist as a monmer (Bleecker *et al.*, 1986), while the corresponding squash ACC synthase may exist as a homodimer (Sato *et al.*, 1991). The TR-ACS1 antibody did not recognise a single protein band in mature green leaf tissue of white clover, but the protein recognised with the highest intensity was 55 KD. However, without further detailed characterisation, the relationship of this protein to ACC synthase, and to the other proteins recognised is uncertain.

4.7 Future studies

In terms of future studies, the expression of *TR-ACS1*, *TR-ACS2* and *TR-ACS3* during leaf ontogeny provides a model system for the study of transcriptional activation of a gene by various inducers which may or may not be in association with developmental cues. This system also provides the opportunity to investigate the molecular mechanisms underlying developmentally regulated gene expression. Therefore, some suggestions for further study are:

- Further investigation is required on the induction of the three ACC synthases in white clover leaf tissue. In future, induction experiments would be better conducted in the glasshouse, where the environmental conditions for the plants are maintained. Using silver thiosulphate instead of 1-MCP would stop any requirement for placing plants in the growth cabinet, which, being a small volume of space and containing a limited air supply, is not an ideal environment for non-stressed plant growth. In addition, IAA could be dissolved in sodium salt to eliminate any possible effect of ethanol on the plant tissue. An alternative method for studying the induction of the three white clover ACC synthases would be to treat leaf tissue by wounding or with IAA, both with and without pre-treatment with another inhibitor (to replace 1-MCP) silver thiosulphate and then perform RT-PCR on extracted poly(A)$^+$ mRNA using degenerate primers for two rounds of PCR, and sequence the products and compare the sequences between control and induced samples.

- The induction experiments should also be repeated with a view to comparing the age of tissue that is treated. For example, IAA may be effective at inducing ACC synthase gene expression in young tissues, and wounding may induce ACC synthase gene expression in older tissues. Further, RT-PCR analysis should be performed on poly(A)$^+$ mRNA isolated from these treated tissues to determine if the expression of distinct ACC synthase genes is being induced.

- As part of a longer term study, the promoters of these genes could be isolated and sequenced. Ethylene, auxin and wound-response elements have now been identified in a wide range of plant genes.

- To determine if the 55 KD protein recognised by the TR-ACS1 antibody is ACC synthase, western blots could be performed on leaf tissue extracts from different developmental stages. An affinity column could be made with the antibodies, the recognised proteins trapped on the column and then eluted, and any proteins that do elute (and so recognised by the TR-ACS1 antibody) can be sequenced.

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