ETHYLENE BIOSYNTHESIS DURING LEAF MATURATION AND SENESCENCE IN WHITE CLOVER

A thesis presented in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

at

Massey University

STEPHEN MARK BUTCHER

1997

ABSTRACT

Aspects of leaf senescence in relation to ethylene biosynthesis in the plagiotropic herbaceous plant white clover (*Trifolium repens* L.) have been studied. Two stolons growing from clonally propagated plants were trained over a dry substratum that inhibits nodal root growth, and all axillary stolons and flowers were removed. White clover plants grown using this method produce leaves at all stages of development along a single stolon from initiation at the apex, through expansion, maturity, senescence and then necrosis. The study shows that modification of the stolon growth habit of white clover by the suppression of nodal roots provides a suitable system for the study of leaf development and senescence in relation to ethylene metabolism.

The pattern of leaf development (the number of leaves at each stage of development present on the stolon) and senescence (as measured by changes in leaf chlorophyll content) along the white clover stolon is consistent between plants of the same genotype growing under the same environment, but varied greatly between the different cultivars and genotypes examined. The rate of change between the different stages of leaf development and senescence within the one genotype used in this study, AgResearch Grassland genotype 10F, differed when grown under two different environments.

On mature stolons (stolons with 6 or more nodes with senesced leaves) of genotype 10F grown using the modified stolon system, the number of green leaves was maintained at a constant number as the leaf appearance rate was balanced by the senescence rate. However, the number of leaves maintained on the stolons differed between the two growing environments used in this study, from 9.85 +/- 0.23 for plants grown at Levin, to 14.57 +/- 1.99 for plants grown at Palmerston North.

The total chlorophyll concentration in the leaves from plants grown at Levin increased from leaf one (the youngest opened leaf; 740 µg/g.fw) to a maximum in leaf five (mature green leaf; 2240 µg/g.fw), declined rapidly from leaf five to leaf seven (senescing leaves; 1500 µg/g.fw), and then remained constant from leaf seven to leaf ten. A similar pattern of change in chlorophyll concentration was measured in leaves from plants grown at Palmerston North, but the maximum concentration was found in leaf 4 (1750 µg/g.fw), remained relatively constant to leaf 8, before decreasing in leaf 9 (750 µg/g.fw) and declining to a minimum in leaf 15 (250 µg/g.fw). The chlorophyll *a:b* ratio in mature green leaves from plants grown at Palmerston North (1.46:1 to 2.63:1) was lower than the ratio in leaves from plants grown at Levin (3.72:1 to 4.98:1).

Leaves of white clover produce ethylene. Ethylene evolution from attached

leaves varied from 1 nL/g.fw/h (mature green leaves) to 3 nL/g.fw/h (senescing leaves). Ethylene evolution from detached leaves was initially high (12.6 nL/g.fw/h at 15 min) but declined to 3.8 nL/g.fw/h by 45 min before increasing again.

Detached mature green leaves (leaves four to six) of white clover are sensitive (as measured by chlorophyll loss) to low concentrations (<1 ppm) of exogenous ethylene. The chlorophyll concentration in these leaves after four days of ethylene treatment (1, 10 or 100 ppm ethylene) was significantly lower than the chlorophyll concentration in freshly harvested leaves. However, the chlorophyll concentration in leaves two and three (early mature green) treated with ethylene was not significantly different from the concentration in freshly harvested leaves.

1-aminocyclopropane-1-carboxylic acid (ACC) concentration was low in leaves one to four (<1 nmoles/g.dw), increased to reach a maximum concentration of 11.4 nmoles/g.dw in leaf seven and declined to 2 nmoles/g.dw in leaf ten. 1-aminocyclopropane-1-carboxylic acid (MACC) concentration was highest in leaf one (11.3 nmoles/g.dw), declined to 6 nmoles/g.dw in leaf two, and remained constant for all other leaves.

ACC synthase activity could not be determined in protein extracts from white clover leaf tissue. ACC oxidase activity in protein extracts varied in the different leaves examined. The activity versus substrate concentration curve for leaves one, three, five and six displayed saturation kinetics with respect to the substrate, ACC, whereas the data for leaves eight and ten did not show saturation kinetics over the range of ACC concentrations used. The ACC oxidase activity varied from 0.81 nL/mg.protein/h in extracts from leaf six, to 1.64 nL/mg.protein/h for leaf five. The apparent Km varied from 61 μM for leaf six to 138 μM for leaf five, while the Vmax varied from 0.92 for leaf six to 2.06 for leaf five.

Degenerate oligonucleotide primers corresponding to conserved regions found among diverse ACC synthases were used for reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify DNA fragments from RNA extracted from white clover leaf tissue. A DNA clone, ACS7, showed 88% homology at the nucleotide level to ACC synthase from *Glycine max.* The ACS7 sequence contained the three conserved domains (including the reaction centre, and the three residues known to bind the pyridoxal phosphate coenzyme) identified in published ACC synthase sequences. The derived amino acid sequence for the conserved domains are identical with other published sequences. Southern analysis indicates ACC synthase is represented by a multigene family in white clover. Northern analysis of the expression of ACC synthase using ACS7 as a hybridisation probe was unsucessful.

Preliminary screening of a white clover leaf cDNA library produced a clone with 72.5% homology to a putative cysteine proteinase from *Pisum sativum,* and 63.4% homology to a cysteine proteinase from *Vicia sativa.*

ACKNOWLEDGEMENTS

This thesis would not have been possible without the assistance, advice and encouragement from many people. I would like to thank my supervisors Drs. Mike McManus, David Fountain from the Plant Biotechnology Department at Massey University, and Dr. Derek White from AgResearch Grasslands, for their advice, support and encouragement. In particular, I would like to thank Dr. Mike McManus for his openness, the close working relationship, the excellent advice throughout, and the friendship he has given. I would also like to thank my fellow students in the laboratory for their help, friendship and fun they provided.

I would like to thank Dr. Ross Lill (Crop and Food Research, Levin) and Dr. Mike Hay (AgResearch Grasslands) for their willingness to read the draft manuscript and provide feedback.

I would like to thank AgResearch Grasslands for the opportunity to use the laboratories and other facilities at Palmerston North, and the excellent and friendly support and advice I received from the staff. I would like to thank the British Council and Massey University for the financial support received for travel to the United Kingdom. I would also like to thank the New Zealand Crop and Food Research Institute for the generous financial support received.

For Gail, Nathaniel, Matthew and Hannah

who give me so much love and joy.

For my parents who have supported and

encouraged me in everything.

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ATP	adenosine triphosphate
AdoMet	s-adenosylmethionine
a.i.	active ingredient
AOA	aminooxyacetic acid
AVG	aminoethoxyvinylglycine
BSA	bovine serum albumin
CAP	calf alkaline phosphatase
CTP	cytosine triphosphate
cDNA	complementary DNA
CoA	coenzyme A
DMF	N, N-dimethylformamide
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EFE	ethylene-forming enzyme
HOAc	acetic acid
IAA	indole-3-acetic acid
KOAc	potassium acetate
MACC	1-aminocyclopropane-1-carboxylic acid
PAGs	photosynthesis-associated genes
PCR	polymerase chain reaction
pfu	plaque forming units
ppm	part per million
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SAGs	senescence-associated genes
SA-PMP's	strepdavidin para-magnetic particals
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane

CHAPTER 1

INTRODUCTION

1.1 Ethylene

Ethylene has been used, knowingly and unknowingly, to modify the physiology of plants for hundreds, perhaps thousands of years. In biblical times, people would wound figs (thereby increasing the production of ethylene) in order to ripen them on the tree, and incense smoke containing ethylene has been used to ripen fruit in Asian cultures. More recently, the shedding of leaves exposed to coal gas was described (1864), and the active ingredient in the gas identified in 1901 when Neljubow described the "triple response" of etiolated pea plants in response to ethylene (see Osborne, 1989a). However, the pathway of ethylene biosynthesis in higher plants was not characterised until 1977 (Adams and Yang, 1977, 1979), and the enzymes involved in this biosynthesis, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, have been characterised only very recently (Yu *et al.,* 1979, Ververidis and John, 1991).

Ethylene, a gas under normal physiological conditions, is considered a plant hormone because it is a product of natural plant metabolism, acts in trace amounts in conjunction with, or antagonistic to, other plant hormones, and is neither a substrate or cofactor in reactions associated with major developmental plant processes (Lieberman, 1979). It has been called the ripening hormone (Burg, 1962) as it has a well known role in accelerating ripening and senescence in fruits and flowers (Thimann, 1987).

"Senescence is a process describing those deteriorative changes which lead sooner or later to the natural death of an organism or some part of it. It can be compared with ageing which is defined as all those processes of accruing maturity which occur with time, without reference to death as a consequence"

(Woolhouse, 1980). Senescence therefore refers to those changes that provide for the endogenous regulation of death (Thimann, 1980), and may be induced (for example by wounding) or be considered 'natural' and genetically programmed (Hillman *et al.*, 1994).

Ethylene is produced and liberated by all parts of growing plants and it is now accepted that ethylene synthesis distinguishes plant cells from other eukaryotes (Osborne *et al.*, 1996). However, the pathway of ethylene biosynthesis may be different in lower plants compared with higher plants (see Osborne *et al.*, 1996). This study investigates changes in the biosynthesis of ethylene in relation to leaf maturation and senescence in a plagiotropic herbaceous plant, white clover (*Trifolium repens* L.), and therefore the discussion is limited to ethylene production by higher plants.

1.2 Ethylene biosynthesis

1.2.1 Introduction

Ethylene is produced from two major sources in higher plants; the methionine cycle, and from non-enzymatic oxidative processes resulting from the interaction of free radicals with ACC (Lesham, 1992). Some studies have indicated the possibility of ACC independent pathways for ethylene biosynthesis in higher plants (Mattoo *et al.,* 1986; Chen *et al.,* 1990; Chen and Wellburn, 1991; Mattoo, *et al.,* 1992), but a recent study using inhibitors including aminooxyacetic acid (AOA), cycloheximide, aminoethoxy-vinylglycine (AVG), and selenomethionine (Ivenish and Tillberg, 1995) could not find any specific evidence for an alternative pathway which could be distinguished from side-effects of the inhibitor treatments.

The production of ethylene is regulated by many endogenous and exogenous factors including spatial, development, and temporal factors within the plant. For example, ethylene production is regulated by wounding (section 1.2.4), plant hormones including ethylene (Suttle, 1991), CO_2 (Mathooko, *et al.,* 1995; Mathooko, 1996), and light and carbohydrates (Mattoo and White, 1991).

The regulation of ethylene production by ethylene may be autocatalytic, for example in ripening or senescing tissues (Hoffman and Yang, 1982; Riov and Yang, 1982; Chalutz *et al.,* 1984; Liu, *et al.,* 1985b), or autoinhibitory for example in wounded tissue (Vendrell and McGlasson, 1971; Yang and Hoffman, 1984). A model for the regulation of ethylene biosynthesis has been proposed by McMurchie *et al.* (1972). In this model, system I controls basal ethylene levels and stress-related ethylene production by promoting the activity of the ethylene-forming enzyme (EFE, the former name for ACC oxidase), and ACC malonylation. System II, regulates autocatalysis and operates only in climacteric fruit by stimulating ACC synthase activity.

Ethylene also inhibits its own biosynthesis (autoinhibition). For example, Vendrell and McGlasson (1971) demonstrated that ethylene treatment

inhibited wound ethylene production in banana pulp slices. Similarly, wound ethylene production from green tissues was inhibited by exogenous application of ethylene (Saltveit and Dilley, 1978).

1.2.2 Lipid peroxidation

Lipoxygenase activity increases during fruit tissue senescence in apples (Marcelle, 1991). Lipoxygenase catalyses the stereospecific dioxygenation of polyunsaturated fatty acids producing ethane, pentane, ethylene, jasmonic acid, malondealdehyde, and a series of oxy-free radicals (Lesham, 1992). The ethylene released during lipid peroxidation may have a role in leaf senescence (Bousquet and Thimann, 1984), possibly prior to the autocatalytic production of ethylene (De Pooter and Schamp, 1989).

The production of ethane from free-radical oxidation, mediated by lipoxygenase activity, may represent a marker of membrane damage and is associated with cell death, while ethylene production is associated with stressed living cells. "Increased ethylene production is associated with stressed living cells, while increased ethane production is associated with cell death. In many tissues, ethylene production first increases and then decreases as the extent of the injury increases, while ethane production continues to increase with increasing injury" (Abeles, *et al.,* 1992c). However, the ethylene produced here may not be the result of enzymatic synthesis, but rather the result of interactions with the breakdown products of lipid catabolism.

More recently the role of lipoxygenase in the production of ethylene was supported by Knudsen *et al.* (1994), who propose that the formation of ethylene from the peroxidation of short linear aldehydes represents a second pathway of ethylene formation in plants. The ethylene produced in this case is the product of a specific enzyme reaction, and not just the result of interactions between the breakdown products of lipid catabolism as suggested by Abeles, *et al.,* (1992c).

1.2.3 The methionine cycle

The main pathway of ethylene biosynthesis in higher plants is via the methionine cycle. Ethylene is produced from the number 3 and 4 carbons of methionine (Lieberman *et al.,* 1966), and Adams and Yang (1977, 1979) showed that s-adenosyl methionine (AdoMet) and ACC were intermediates between methionine and ethylene. The production of ethylene via the methionine cycle (Figure 1.1) conserves the CH_3S group in the regeneration of methionine. Thus high levels of ethylene production can be achieved from relatively low concentrations of methionine in plant tissues. The conversion of methionine to ethylene involves the enzymes methionine adenosyltransferase (ATP:methionine s-adenosyltransferase), also known as ACC *N*-malonyltransferase, which converts methionine to AdoMet; ACC synthase (s-adenosyl-L-methionine methylethioadenosine-lyase) which converts AdoMet to ACC; and ACC oxidase (formerly ethylene-forming enzyme, EFE; Yu and Yang, 1979) which converts ACC to ethylene.

The intermediate compound AdoMet is also an intermediate in the biosynthesis of the polyamines, spermine and spermidine (Mattoo and White, 1991), and there is evidence that the two pathways are in competition (Even-Chen *et al.,* 1982). This is supported by the work of Fuhrer *et al.* (1982) who showed that polyamines inhibit the formation and action of ethylene, and by the work of Apelbaum *et al.* (1975) who showed that ethylene inhibits the conversion of arginine to the polyamine putrescine. Further, the inhibition of polyamine synthesis induces ACC synthase activity (Gallardo, *et al.,* 1994). In plants (and animals), polyamine biosynthesis and concentration are highest in regions of active cell division, and diminish progressively with ageing and senescence (Slocum *et al.*, 1984). The application of the polyamine spermine gives complete protection against chlorophyll loss (an indicator of senescence) in excised peeled oat leaves, possibly by inhibiting or reducing ethylene production (Kaur-Sawhney and Galston, 1979).

ACC synthase and ACC oxidase may not always be induced in the same tissues. For example, ACC may be synthesised in one part of the plant and transported to another part (Tudela and Primo-Millo, 1992; O'Neill *et al.,* 1993;

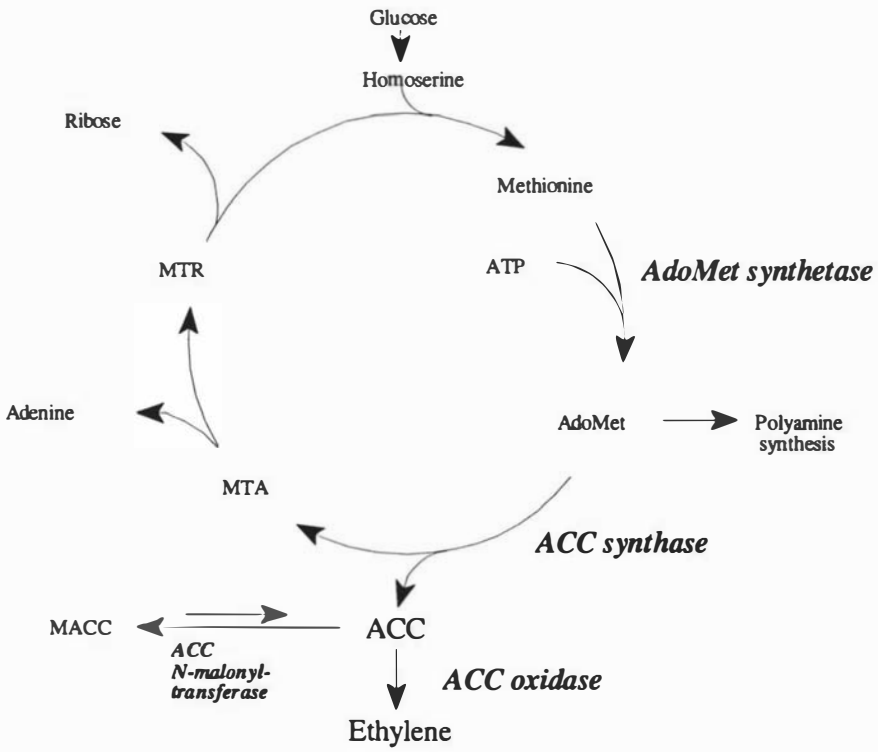


Figure 1.1: Diagram showing the ethylene biosynthesis pathway in relation to the methionine cycle and polyamine synthesis.

ACC:	1-aminocyclopropane-1-carboxylic acid
MACC:	1-(malonylamino)cyclopropane-1-carboxylic acid
AdoMet:	s-adenosylmethionine
MTA:	5'-methylthioadenosine
MTR:	5'-methylthioribose

Jackson, 1994). Similarly, the conjugated form of ACC, 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) may be synthesised in one plant tissue and transported to another before being converted to ACC (Fuhrer and Fuhrer-Fries, 1985; Jiao, *et al.,* 1986).

1.2.3.1 ACC synthase

ACC synthase, present in the cytosolic fraction of plant tissues, uses AdoMet as a substrate, and pyridoxal-5'-phosphate as a cofactor (Imaseki, 1991). This enzyme, like other pyridoxal-5'-phosphate requiring enzymes, is inhibited by AVG and AOA (Imaseki, 1991). The reported Km values for ACC synthase range from 12.1 μM (wounded winter squash fruit, Nakajima and Imaseki, 1986) to 60 μM (etiolated mung bean hypocotyls, Yoshii and Imaseki, 1981), with an optimal pH range of 8.5 to 9.5.

Auxin- or wound-induced ACC synthase is rapidly inactivated within cells, with a half-life estimated to be 25 min (Yoshii and Imaseki, 1982) to 30 min (Kende and Boller, 1981). The nature of the inactivation is at least in part due to "suicide" inactivation of the enzyme by its substrate (Nakajima and Imaseki, 1986; Satoh and Esashi, 1986; Satoh and Yang, 1988; Casas, *et al.*, 1993). The half-life of ACC synthase *in vitro* when extracted from etiolated mung bean hypocotyls was reduced from 23.5 min to 12 min when the AdoMet concentration was increased from 40 μM to 150 μM (Satoh and Esashi, 1986). The ACC synthase protein occurs as both monomeric and dimeric forms when extracted from a range of sources (for example, ACC synthase extracted from tomato fruit tissue occurs as a monomer, whereas, the enzyme extract from the fruit of the winter squash occurs as the dimer), with the monomer having a molecular mass of about 50 kDa (Satoh *et al.*, 1993) These workers showed that the monomeric form was less sensitive to suicide inhibition.

Ripening tomato fruit are a source of relatively high ethylene production (22 to 30 nL/g.fw/h; Rothan and Nicolas, 1989) compared with that considered 'usual' for expanding (0.1 to 0.4 nL/g.fw/h) and fully expanded leaves (<0.1 nL/g.fw/h; Osborne, 1991). However, the concentration of ACC synthase is estimated to be less than 0.0001% of total soluble protein in the pericarp of

ripening tomato fruit (Bleecker, *et al.*, 1986), while the RNA encoding ACC synthase in "normal ripening tomato fruit ...is estimated as low as 0.0001%" of total RNA (Van Der Straeten *et al.,* 1990, 1992b). ACC synthase is believed to be the rate limiting step in ethylene biosynthesis (Yang and Hoffman, 1984; Arteca, 1989; Yang and Dong, 1993). However, Pogson *et al.* (1995) have shown that in broccoli florets, two cDNAs which have a high degree of nucleotide identity to ACC oxidase from tomato fruit were differentially expressed and their appearance was coincident with an increase in ethylene production that subsequently regulates chlorophyll loss in sepals in the absence of an observable increase in ACC synthase transcript level. These cDNAs apparently encode mRNAs from different genes as Southern analysis exhibits a different banding pattern when each cDNA is used as a probe. In this system, an increase in ethylene production was correlated with a rapid increase in the level of one of the ACC oxidase transcripts (*Ox2*). These authors suggest that in this system, ACC oxidase may also be a rate limiting step.

The regulation of ethylene production is achieved at a number of stages in the pathway (Figure 1.2). For example, ACC synthase, and hence the production of ACC, is regulated primarily at the transcriptional level (Mattoo and White, 1991). In addition, ACC synthase activity is enhanced by ethylene in ripening apple fruits (Buffler, 1984), but inhibited by ethylene in etiolated pea segments (Saltveit and Dilley, 1978) and banana fruit (Vendrell and McGlasson, 1971). The level of ACC is further regulated by ACC *N*-malonyltransferase activity, converting ACC to MACC. For example, in experiments investigating the induction of ethylene during mechanical impedance of maize seedling growth, under unimpeded conditions, about 77% of ACC in tissue was found conjugated as MACC, 17% was free ACC, and only 6% was converted to ethylene (Sarquis, *et al.,* 1992). Ethylene production may also be regulated by ACC oxidase activity. For example, in tomato and cantaloupe fruits ACC oxidase activity is enhanced by ethylene (Liu *et al.,* 1985b).

ACC synthase is a member of a multi-gene family, defined as having members that are preferentially expressed in response to an internal or environmental signal (Yang and Dong, 1993). Southern analysis has been

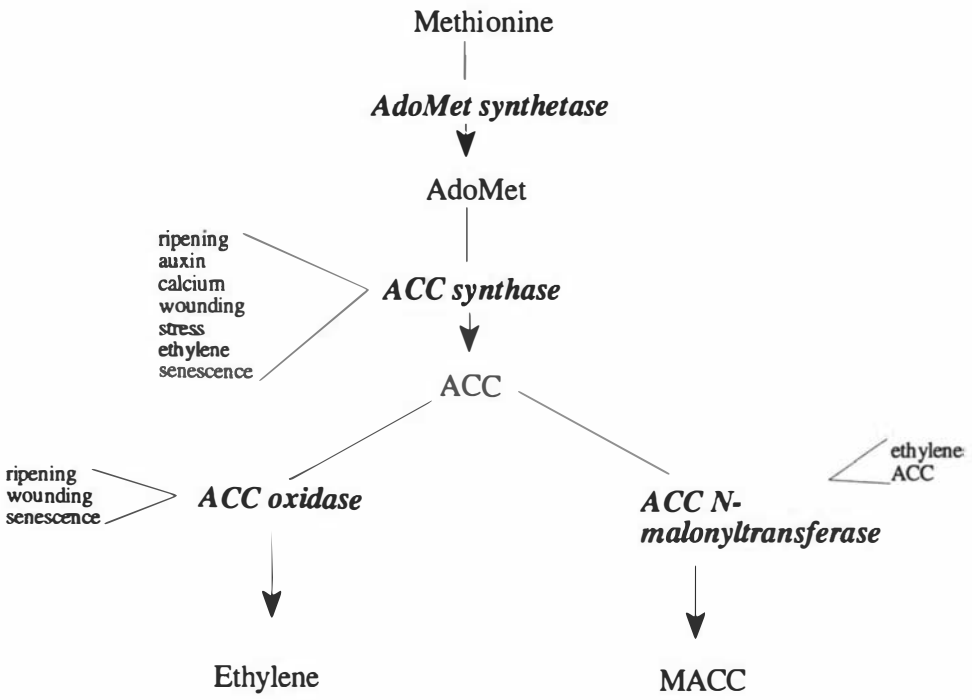


Figure 1.2: Ethylene biosynthesis pathway in higher plants showing some factors which affect the production of ethylene.

used to confirm the existence of six members of the gene family in tomato (Yang and Dong, 1993), five in *Arabidopsis thaliana* (Liang *et al.*, 1992), three in potato (Destèfano-Beltrán *et al.,* 1995), and two in carnation (Park *et al.,* 1992). At least one member of the gene family in *A. thaliana* has been identified as a pseudogene since it has a truncated sequence (Liang *et al.*, 1995). Using RNA hybridisation analysis, individual members of the family have been shown to be differentially expressed in ripening or wounded tomato fruit (Olson, *et al.*, 1991), amongst different tissues in *A. thaliana* (Liang, *et al.,* 1992), or in response to many factors, for example; wounding in winter squash fruit (Nakajima *et al.,* 1990), wounding, anaerobiosis, lithium chloride, and auxin in *A. thaliana* (Liang *et al.,* 1992), or fungal infection in tomato plants (Spanu, *et al.*, 1993).

Liang *et al.,* (1992) showed that various ACC synthase mRNAs were differentially expressed in tissues of *A. thaliana.* For example, *A. thaliana* ACC2 mRNA was expressed at high levels in roots, at moderate levels in leaves and flowers, at low levels in siliques, and at almost undetectable levels in stems. On the other hand, ACC1 and ACC3 mRNAs were expressed only in siliques, ACC4 mRNA was expressed only in roots, leaves and flowers, and ACC5 mRNA was expressed only in flowers and siliques.

A detailed study of differential expression of ACC synthase mRNAs in tissues of *A. thaliana* was also done by Van Der Straeten *et al.,* (1992a). The ACC synthase clones were generated by screening a cosmid library and a flower-specific cDNA library with a conserved sequence oligonucleotide. The expression of the clones was analysed using RT-PCR due to the lack of sensitivity of northern blotting in detecting low abundance mRNAs. One ACC synthase clone, *AT-ACC1*, was expressed prominently in young leaves and flowers, but was not expressed in leaves of mature plants, nor in green or ripe siliques. These workers also found that *AT-ACC1* levels were influenced by ethylene as exposure to ethylene induced an accumulation of the mRNA coding for *AT-ACC1* after 2 h. This gene was however, not wound-inducible, nor induced in senescing leaves.

The members of the ACC synthase gene families show considerable divergence amongst the members within a plant. In contrast, ACC synthase

genes from different plant species under the same regulatory control (for example, auxin) share a high level of similarity (for example, auxin-induced ACC synthase from *A. thaliana*, winter squash, tomato, and rice; Liang *et al.*, 1992). These data suggest that the divergence in ACC synthase under different regulatory control occurred prior to the divergence of monocots and dicots, and suggests a very complex pattern of control of ethylene biosynthesis in plants.

In a further study in *A. thaliana*, Rodrigues-Pousada *et al.*, (1993), investigated the spatial and temporal expression of one member of the ACC synthase gene family using the ACC synthase gene promoter fused to the β-glucuronidase gene. High expression was found in young tissues, but no expression was found in mature tissues. In addition, activity was associated with lateral root formation. These studies provide data on the activity of the gene promoter, but do not provide information on regulation that may occur at the translational or post-translational level.

1.2.3.2 ACC oxidase

In spite of many years research, the biochemical nature of the ACC oxidase could not be determined as activity was completely abolished upon tissue homogenisation (Yang and Hoffman, 1984; Imaseki, 1991). Imaseki (1991) identified two possibilities for the inactivity after homogenisation: "ACC oxidase is an enzyme complex that is active only in a particular conformation delineated by the membrane integrity or the correct topological arrangement with the membrane", or "ACC oxidase reaction may simply require as yet unknown factor(s) that are diluted or inactivated during disruption of cells". Any cell free system that did produce ethylene from ACC was subsequently found to be due to lipoxygenase activity (John, 1991).

In 1991, Hamilton *et al.* reported they had identified a cDNA in tomato (pTOM13) which encoded for the ACC oxidase. The translated amino acid sequence from pTOM13 resembled that of flavonone-3-hydroxylase, an enzyme active in the biosynthesis of anthocyanins. Flavonone-3-hydroxylase is a member of the 2-oxoglutarate-dependent dioxygenases (Prescott, 1993), which are enzymes that require ferrous iron, and a reducing agent (usually

ascorbate) for maximal activity. By incorporating these components in their reaction *in vitro*, Ververidis and John (1991) were able to demonstrate full activity of ACC oxidase from fruits of melon. Subsequently, the enzyme has been identified or characterised from several tissues (for example: apple fruit, Kuai and Dilley, 1992, Fernández-Maculet and Yang, 1992, Dong *et al.*, 1992a; etiolated pea epicotyls, Peck *et al.*, 1993; kiwifruit fruit, MacDiarmid and Gardner, 1993; winter squash fruit, Hyodo, *et al.*, 1993; carnation petals, Nijenhuis-De Vries *et al.*, 1994; and tomato fruit, English *et al.*, 1995). Smith and John (1993) determined the conditions for maximum activity of ACC oxidase *in vitro* in protein extracts from melon fruit. It is now known that the enzyme requires ascorbate and oxygen as co-substrates, and Fe^{2+} and CO_2 as cofactors (Yang and Dong, 1993), and therefore the name ethylene-forming enzyme was changed from EFE to ACC oxidase.

ACC oxidase has been purified to near homogeneity, and the enzyme was found to have a molecular weight of 35 kDa (Dong *et al.*, 1992a) to 40 kDa (Smith, *et al.*, 1994), and a pH optima near 7.5 (Smith *et al.*, 1992; McGarvey and Christoffersen, 1992). The apparent Km (Km determined from impure preparations of the enzyme) for ACC oxidase has been reported to be between 6.4 μM and 175 μM (melon fruit, Ververidis and John, 1991; melon fruit, Smith *et al.*, 1992; apple fruit, Fernández-Maculet and Yang, 1992; Kuai and Dilley, 1992; avocado fruit, McGarvey and Christoffersen, 1992; winter squash fruit, Hyodo *et al.*, 1993). However, the apparent Km depends on the assay conditions used. For example, in winter squash fruit Hyodo *et al.*, (1993) found an apparent Km of 175 μM (with respect to ACC) under atmospheric conditions, but this increased to 435 μM under 4.4% (v/v) CO_2. Similarly, Nijenhuis-De Vries *et al.*, (1994) found the apparent Km for ACC oxidase from carnation petals increased from 30 μM to 425 μM (with respect to ACC) in the presence of increased CO_2 concentration. The activity of the enzyme extracted from melon fruit declines over time due to attack on the enzyme by an intermediate generated during the catalytic turnover (Smith, *et al.*, 1994). Under the conditions for maximal activity, the enzyme has a half-life of 19 min (Smith, *et al.*, 1994).

ACC oxidase is encoded by a multigene family (Bouzayen, *et al.*, 1993; Kim and Yang, 1994; Pogson, *et al.*, 1995), and is thought to be constitutively

expressed in most plant tissues examined so far, as the tissues are able to convert exogenous ACC to ethylene even though the tissues do not produce appreciable amounts of ethylene (Imaseki, 1991). Further, the ethylene production rate from tissues is roughly proportional to the endogenous concentration of ACC (Yoshii and Imaseki, 1981) indicating the rate limiting factor to be the supply of ACC. However, the activity of ACC oxidase may increase during fruit development in tomato and cantaloupe (Liu, *et al.*, 1985b), in cell suspensions of *Acer psuedoplatanus* (Malerba *et al.*, 1995), and during flower development in petunia (Tang *et al.*, 1994) and carnation (Jiang *et al.*, 1994).

The activity of ACC oxidase is regulated temporally in orchid flower tissues (Nadeau, *et al.*, 1993), and spatially in broccoli florets (Pogson, *et al.*, 1995) and sunflower seedlings (Finlayson, *et al.*, 1996). An increase in ethylene production can be achieved by the activation of ACC oxidase in the absence of protein synthesis, or an increase in ACC concentration (Jiang, *et al.*, 1994; Bailly, *et al.*, 1995; Malerba, *et al.*, 1995). While ethylene production is regulated by many factors, ethylene action may also be controlled in some plants by metabolising ethylene to ethylene oxide (Jerie and Hall, 1978), but this does not appear to have a significant effect on the endogenous ethylene concentration (Hall, 1991).

Using immunolocalisation, ACC oxidase has been reported as being located primarily in the cell walls of ripening fruit (Rombaldi, *et al.*, 1994) although these workers also report that sequence analysis of cDNA clones encoding ACC oxidase do not support the targeting of the protein to the cell wall (Balagué, *et al.*, 1993). In contrast, Reinhardt, *et al.*, (1994), using cell fractionation and immunoblot analysis, reported that ACC oxidase was absent from the vacuole, and that most of it was localised in the cytoplasm without being associated with membranes.

1.2.3.3 MACC synthase

1-(malonylamino)cyclopropane-1-carboxylic acid is the only major conjugated form of ACC known in higher plants (Hoffman *et al.*, 1983). Exogenously applied labelled ACC is primarily converted to MACC (Hoffman and Yang,

1982), and although MACC is considered by some workers to be an inactive end-product (Hoffman *et al.*, 1982, 1983), others have shown MACC to be hydrolysed to ACC and then converted to ethylene (Jiao *et al,* 1986). Further, applied MACC induces ethylene production and premature senescence in carnation flower tissues (Hanley *et al.,* 1989). The MACC hydrolase enzyme has a pH optimum of 8.0, and an apparent Km of 450 μM (Jiao, *et al.,* 1986). The physiological importance of the hydrolase reaction is unclear as the endogenous levels of MACC are usually far below the apparent Km for the enzyme (Mattoo and White, 1991). However, Yang *et al.,* (1993) showed that the concentration of MACC did not continue to increase as might be expected of an inactive end-product, but remains in a 'dynamic balance' with ACC. These workers could not determine whether MACC was hydrolysed to ACC, or was transported to other tissues.

The role of MACC may be similar to that seen for conjugates of other plant hormones. For example, conjugates of IAA are thought to be slow release sources of free IAA (Hangartner and Good, 1981). These workers presented evidence that IAA conjugates are metabolised via enzyme-catalysed hydrolysis to free IAA and that their biological activities are related to the rates at which they are hydrolysed. The effect of IAA conjugates was determined by measuring the release of CO_2 from pea stem segments after treatment with various IAA conjugates, and comparing the results with IAA treated controls. These data suggest that the conjugated hormone can act as a slow release source of the free hormone. A similar situation may exist in the control of ethylene, but in this case the ethylene precursor ACC, rather than the hormone itself, may be the controlled entity in the form of MACC.

MACC is formed from ACC by the enzyme ACC *N*-malonyltransferase (methionine adenosyltransferase). The enzyme extracted from tomato fruit has a pH optimum of 8.0, and an apparent Km for ACC of 500 μM (Martin and Saftner, 1995). However, Benichou *et al.,* (1995) reports that at least two different isoforms of ACC *N*-malonyltransferase are present in protein extracts from etiolated mung bean hypocotyls. The smaller of the two enzymes (36 kDa) has an apparent Km of 68 μM with respect to ACC (Benichou *et al.,* 1995), compared with 500 μM for the 55 kDa ACC *N*-malonyltransferase isolated from the same tissues (Guo *et al.,* 1992).

ACC *N*-malonyltransferase activity is increased by ethylene and participates in the autoinhibition control of ethylene synthesis (Liu, *et al.,* 1985a), but may occur in the absence of increased activity of extractable enzyme (Jiao, *et al.*,1987). The activity of the enzyme increased only after the concentration of ACC had reached a relatively high level and may therefore be important in regulating the concentration of free ACC in cells (Sarquis, *et al.,* 1992).

1.3 Ethylene in plant development

Ethylene has observable effects on practically all aspects of plant growth and development including seed germination (Esashi, 1991; Katoh and Esashi, 1975; Ketring and Morgan, 1972; Rudnicki *et al.*, 1978; Sińska and Gladon, 1984), seedling growth (Burg and Burg, 1968), root initiation (Abeles *et al.* 1992a), root growth (Chadwick and Burg, 1970), nodule formation (Abeles *et al.* 1992a), growth of leaves (Van Andel and Verkerke, 1978; Kays and Pallas, 1980; Bleecker *et al.*, 1987; Osborne, 1991), abscission (Osborne, 1989b), fruit ripening (Burg and Burg, 1965; Brady and Speirs, 1991), and flower (Reid and Wu, 1991) and leaf senescence (Roberts and Osborne, 1981). Two of these aspects are considered here; the correlation between ethylene production and leaf development (including senescence), and the role of ethylene in plant stress.

1.3.1 Ethylene production and leaf development

Ethylene has different effects in distinct tissues, and the effect may vary depending on the stage of development of the tissue. Further, the rate of ethylene production (determined by the measurement of evolved ethylene) is modified by different factors, and the effect may also vary depending on the stage of development of the tissue. For example, a peak of ethylene production occurs during the early development of leaves, and a second peak may occur in senescing leaves. Roberts and Osborne (1981), working with detached leaves, showed that during the early development of leaves of *Phaseolus vulgaris*, high ethylene production is correlated with high IAA concentration, while during the later stages of expansion and maturation the concentration of both hormones declines by 75%. As the leaves senesce, however, the level of ethylene production remains constant, while the concentration of auxin continues to decline. In contrast, ethylene production in senescing leaves of *Prunus serrulata* increases many-fold while the IAA concentration remains high during leaf maturation, before falling in senescing leaves. These workers also showed that treating developing leaves of *P. vulgaris* with exogenous IAA (applied to leaf surfaces in methanol) increased ethylene production while treating mature or senescing leaves with

exogenous IAA does not increase ethylene production.

In tomato leaf segments, McGlasson *et al.*, (1975) showed that ethylene production (and respiration) first decreased, then increased in a manner similar to the climacteric in fruit and flowers. A surge of ethylene production is associated with a phase of rapid chlorophyll breakdown in dark-induced senescence in bean, tobacco, and sugar beet leaf discs, while treatment with silver ions, an inhibitor of ethylene action, delays chlorophyll loss in all these tissues (Aharoni *et al.*, 1979a). In the experiments reported by McGlasson, *et al.,* (1975), Aharoni *et al.*, (1979a) and Roberts and Osborne (1981), it is unclear whether the responses would be similar in attached leaves under natural conditions.

1.3.2 Ethylene and plant stress

Ethylene biosynthesis is induced in higher plants in response to a wide range of environmental perturbations (commonly called stress) such as wounding, disease, drought, waterlogging, and chilling (Boller and Kende, 1980; Metraux and Kende, 1983; Roby, *et al.* 1985; Hyodo, 1991) and exposure to chemicals such as sulphur dioxide, ozone, and mercury (Hyodo, 1991). Ethylene production from mechanically wounded tissue has been characterised in many systems. For example, Saltveit and Dilley (1978) identified two peaks of ethylene production in excised epicotyl segments of etiolated pea seedlings. An increase in ethylene production was measured after an initial lag period of 26 min which peaked at 56 min, declined to a minimum at 90 min, and was then followed by a second smaller peak at 131 min.

Wound ethylene is also produced by leaves. Leaf discs from olive produced about twice as much ethylene as controls (Lavee and Martin, 1981), and wound induced ethylene production was detected after a lag of 15 to 20 min in bean leaves (Konze and Kwiatkowski, 1981). However, wounding may also result in the production of ethane. Kimmerer and Kozlowski (1982), found that ethylene production increased to a small peak from the start of the application of a sulphur dioxide stress, and then reached a second higher peak just prior to the appearance of leaf lesions. Ethane production, however, did not

increase until the leaf lesions appeared, and while ethylene production subsequently decreased, ethane production increased linearly with the percentage of necrotic tissue.

Ethylene production in response to stress involves the induction of ACC synthase, and in some cases, ACC oxidase. Yu and Yang (1980) showed that wound ethylene was produced via the methionine cycle, and was inhibited by the application of AVG, an inhibitor of ACC synthase. The application of ACC, however, resulted in an increase in ethylene production in freshly cut discs from albedo tissue of citrus fruits (Hyodo and Nishino, 1981), suggesting to these workers that ACC oxidase activity was present prior to wounding. However, these workers first added ACC 1 h after cutting the discs, and this may have been sufficient time for the induction of ACC oxidase. For example, Jiang *et al.,* (1994) identified an increase in ACC oxidase activity one hour after the exposure of carnation petal tissue to exogenous ethylene, and Olson, *et al.,* 1991, identified an increase in ACC synthase mRNA within 40 min of wounding tomato pericarp tissue.

Ethylene produced after wounding induces changes in the induction of other enzymes. For example, in mesocarp discs of winter squash, a peak in ethylene production was detected 30 h after cutting (Hyodo, *et al.*, 1985) and this was followed by an increase in peroxidase activity (Hyodo, *et al.*, 1991). Both the increase in ethylene and peroxidase activity was inhibited by AVG indicating that the peroxidase activity may have been induced by the increased ethylene production. Similarly, carnation petals treated with exogenous ethylene induced the activity of β-cyanoalanine synthase (Manning, 1986) which metabolises cyanide released from ACC oxidation to β-cyanoalanine and asparagine (Peiser *et al.,* 1984). The release of cyanide may have a role in the induction of cell death under stress conditions (Grossmann, 1996).

1.3.3 Transport and perception of ethylene by plants

The biological activity of ethylene will only be apparent in cells which have the ability to detect the hormone. "For all practical purposes, ethylene is produced by all cells all of the time, and transport is not a concern" (Abeles *et al.*,

1992b). However, not all cells have the same ability to detect ethylene, and the sensitivity or response may change depending on the developmental stage of the cell or tissue. For example, in leaf abscission layers in *Phaseolus vulgaris*, application of 1000 μL/L ethylene will not cause cell separation in the first four days from germination, but subsequent applications of 1 to 10 μL/L ethylene will cause separation after day 5 (Osborne, 1991).

Ethylene perception by plants has been studied in detail following the selection of mutant plants insensitive to ethylene. Bleecker *et al.,* (1988) described a mutant line of *A. thaliana* with a dominant mutation at a locus designated *etr*, which lacks a number of responses to ethylene seen in the wild-type plant. In particular, these plants fail to show an acceleration in leaf senescence. The *ETR1* gene was eventually cloned by chromosome walking (Chang *et al.,* 1993) and it was found that each of the four *etr1* mutant alleles contains a miss-sense mutation in the predicted protein. The protein was found to have similarities with two-component regulatory systems in prokaryotes (hisitidine kinase) suggesting the protein may be a sensor of ethylene. A gene homologous to *ETR1* has also been found in the never-ripe (*Nr)* mutant form of tomato (Yen *et al.,* 1995).

Ethylene may be produced in one part of the plant and transported to another part, or may be released from the plant as a gas. Ethylene released from plant tissues or applied exogenously hastens the ripening of fruit (Theologis, 1992), or hastens the senescence in leafy vegetables in the same environment (Philosoph-Hadas, *et al.,* 1989). Ethylene is soluble in aqueous solutions, reaching 140 ppm in pure water (Osborne, 1989), and is 14 times more soluble in lipids than in water (Abeles, *et al.,*1992d). Ethylene can be transported around plants and act as a mobile factor responsible for inter-organ signalling (Woltering *et al.,* 1995).

Exogenously applied free or conjugated ACC both induce ethylene production (Hyodo and Nishino, 1981) and may also be transported around the plant. ACC is synthesised in the cytoplasm and can be transported into vacuoles (Saftner and Martin, 1993), and from roots to shoots after water-logging in tomato plants (Bradford and Yang, 1980), and after water stress in citrus plants (Tudela and Primo-Millo, 1992). Similarly, MACC is synthesised in the

cytosol and may be transported into the vacuole (Pech *et al.*,1989) or out of the vacuole (Pedreño *et al.*, 1991), or around the plant (Fuhrer and Fuhrer-Fries, 1985; Jiao *et al.*, 1986). The inter-organ regulation of flower senescence in orchids apparently depends on the translocation of ACC between the different flower tissues (Woltering, 1990, O'Neill, *et al.*, 1993). However, Woltering *et al.*, (1995) found that ACC and MACC are probably not mobile between the different flower tissues of carnation.

1.4 Ethylene and leaf senescence

1.4.1 Introduction

In many plants, senescence of the leaves is characterised by yellowing, the result of a loss of chlorophyll unmasking carotenoid and other pigments (Thimann, 1980). While ethylene has effects on practically all aspects of plant growth and development (section 1.3), the role of ethylene in leaf senescence has been less clear. Thimann (1980) states "Ethylene has long been known to promote chlorophyll loss in fruits, but with few exceptions most investigators have found it to have small or negligible effects on leaf senescence", and Saniewski (1995) states "generally speaking, the ethylene production of leaves is not a factor in their senescence". In contrast, Kao and Yang (1983) conclude that ethylene is involved in the regulation of senescence in detached rice leaves for the following reasons: i) maximum ethylene evolution preceded chlorophyll degradation, ii) ACC application promoted chlorophyll degradation, iii) inhibitors of ethylene production and action retarded chlorophyll degradation, and iv) treatments which retarded chlorophyll degradation also inhibited ethylene production. Similarly, Ferguson *et al.*, (1983) conclude that ethylene is "a dominant hormone in the regulation of leaf senescence", and Smart (1994) states that for leaves, "ethylene, like cytokinins, is thought to play a prominent role in the regulation of senescence".

A prominent role for ethylene in leaf senescence is supported by studies using transgenic plants. For example, tomato plants transformed with antisense ACC oxidase gene constructs result in tissues with reduced ACC oxidase activity and ethylene production, and leaf senescence is delayed compared with control plants (Picton, *et al.*, 1993; John *et al.*, 1995) Recently, Grbić and Bleecker (1995) showed that in *A. thaliana*, exogenous ethylene accelerated the induction of senescence-associated genes (SAGs) and decreased the expression of photosynthesis-associated genes (PAGs). Further, mutant *A. thaliana* plants insensitive to ethylene (plants with the dominant *etr1-1* mutation), show a delay in the induction of SAGs, and higher expression levels of PAGs compared with wild-type plants. Leaves of the

etr1-1 plants survive approximately 30% longer than leaves on wild-type plants, but the extended period is associated with low levels of photosynthetic activity, indicating the leaves had functionally senesced even though the apparent life-span of the leaf was prolonged. Ethylene, therefore, may not be the 'trigger' for senescence, but may regulate the timing and rate of some aspects of senescence in plant tissues.

The role of ethylene in leaf senescence may be different to its role in fruit and flower senescence. Roberts *et al.*, (1985) states "in contrast to flowers and fruit the increase in ethylene production during foliar senescence appears to occur after the onset of the senescence programme when a decline in protein and chlorophyll has already commenced". However, at a physiological and biochemical level, there are similarities between fruit ripening and leaf senescence (Davies and Grierson, 1989). Recent studies have provided further evidence for the genetic programming of leaf senescence. Abeles and Dunn (1990) found that DNA remains intact during cotyledon senescence in *Cucumis sativus*. Lohman *et al.*, (1994) showed that a major change in gene expression occurs during leaf senescence in *A. thaliana*, and Smart *et al.*, (1995), demonstrate that the gene expression during leaf senescence in *Zea mays* has many similarities with the pattern of gene expression observed during seed maturation and germination.

Roberts *et al.*, (1985) conclude "the majority of data on leaf senescence comes from experiments using detached leaves which again may not accurately reflect the situation in the whole plant". These authors promote the requirement for studies to be conducted on various organs on intact plants within a single species. These studies may be difficult in plants in which the onset of senescence cannot be accurately predicted and where the early stages of senescence cannot be determined. Some workers have investigated leaf senescence *in situ*, for example on the experimental plant system *A. thaliana* (Lohman *et al.*, 1994) where rosette leaves appear to have an intrinsic life span that is independent of the reproductive status of the plant, and senescence can be predicted from the age of the leaf (Hensel, *et al.*, 1993).

In New Zealand, white clover (*T.repens)* is the most important pasture legume

(Williams, 1987a) and its growth and development in pastures and under controlled conditions has been extensively studied (see Baker and Williams, 1987). White clover has a stoloniferous growth habit which may provide a suitable system for studying ethylene production and leaf ontogeny.

1.5 White clover

In many temperate regions of the world, white clover is the most important pasture legume because it fixes nitrogen, improves sward quality, complements seasonal growth pattern of commonly used grass species, and improves the intake and utilisation rates of protein by animals. In addition, white clover has widespread adaptability and can withstand grazing stress and interspecific competition better than other legumes (Caradus *et al.*, 1996).

1.5.1 Taxonomy

White clover is the most agronomically important of the 200 to 300 species of the genus *Trifolium*. This genus is classified taxonomically in tribe Trifolieae, subfamily Papilionoideae, of the family Leguminosae (Fabaceae; Williams, 1987b). All members of the tribe Trifolieae have the basic chromosome number 8. The somatic chromosome number of *T. repens* is 2n = 32 and it is therefore a polyploid (tetraploid) member of the tribe (Williams, 1987b).

1.5.2 Plant morphology

White clover plants have two distinct mature forms. The first is the mature rosette form with an erect stem and very short internodes resulting from the growth of the seedling plant. The second is the mature stolon which is ultimately derived from the seedling plant. The mature stolon form is the dominant form of the plant in pastures (Thomas, 1987a).

After seed germination, two cotyledons and a unifoliate primary leaf emerge, and all subsequent leaves are trifoliate. After about four weeks under favourable conditions, the white clover seedling plant consists of a central primary axis of stem and root with a rosette of leaves. The stem is erect with very short internodes. This form is the first mature stage of the developing plant. At about four weeks, axillary buds in the axils of older leaves grow to produce secondary stolons.

The stolon, the basic structural component of the second stage of the mature clover plant, consists of a series of internodes separating the nodes which form as a result of growth at the apical bud. Each node produces a single trifoliate leaf with an erect petiole, and two root primordia. In addition, an axillary bud may produce a lateral stolon or an inflorescence, or may remain dormant. The growth of the stolon apex is indeterminate and in theory the apex could be immortal, and hence produce a stolon that is rooted at the nodes, and dying at the base (Thomas, 1987a). The mature clover sward therefore consists of a number of clonally produced independent cohorts which are no longer attached to the 'mother' plant (Figure 3.1). Thus vegetative propagation is the main system of plant maintenance in pastures in moist temperate climates (Turkington *et.al.*, 1979, Chapman, 1987).

Leaves are arranged alternately on the stolon usually orientated so that they grow from the sides of the stolon relative to the surface of the ground. The leaves are usually trifoliate and either uniformly green or patterned with a coloured (usually whitish) V-shaped mark. The leaves are attached by an erect petiole, and each leaflet by a petiolule. The petiolule acts as a pulvinule, allowing movement of the leaflets to 'follow' the sun (Thomas, 1987b). The size of the leaves, and the length of the petiole varies greatly with environmental conditions.

The primary root of the seedling develops into the tap root of the mature plant and may grow to a length of 30 cm or more. The tap root bears several lateral roots which in turn bear smaller laterals. The tap root eventually dies leaving an independent system of stolons nutritionally supplied by adventitious roots. The two root primordia at each node are located one each side of the axillary bud. The upper root primordia usually remains inactive but the lower primordia is closer to the ground and in moist conditions will grow to form a nodal root system. The adventitious roots may be fibrous or form a tap root depending on the genotype and possibly influenced by the environment. The growth of roots is strongly influenced by the presence of foliage, with defoliation limiting the elongation of roots (Evans, 1973). All the roots have the potential to become nodulated when infected with nitrogen-fixing bacteria.

1.5.3 Growth and development of stolons in white clover

The apical bud gives rise to all the major shoot components of the stolon, including the leaves. It is usually comprised of 5 to 8 leaf primordia, all of which except the two youngest normally possess an axillary bud primordium in their axils. The leaf appearance rate varies from 0 to 2 leaves per week under New Zealand environmental conditions, but this varies greatly under other conditions. "Under controlled environment conditions, when the rates of leaf initiation and emergence are constant, the changes from node to node represent changes in time" (Thomas, 1987b), with a constant developmental age difference between them.

Leaf emergence from the stipular sheath is accompanied by leaflet expansion and petiole elongation. The process was described by Carlson (1966) who noted ten stages of development (0.1 to 1.0) which took approximately the same time to pass from each one to the next, and 7 to 10 days to pass through all the stages. The leaves emerge horizontally and then the petiole elongates and grows upright. Petiole elongation is a result of both cell division and cell elongation, and the ultimate length varies depending on the environmental conditions, and is affected by the adjacent tissues. For example, defoliation decreases the petiole length in subsequently developing leaves (Thomas, 1987b). The axillary buds develop within the apical bud, but the effect of apical dominance inhibits their growth at nodes bearing unfolded leaflets. The outgrowth of the axillary buds usually does not occur until several nodes basal to the apical bud, and depends on stolon vigour and environmental conditions.

The internodes elongate in the region immediately basal to the apical bud. This elongation is most rapid in the region of leaf emergence from the bud but has virtually ceased by the second internode basal to the apical bud. The ultimate length of the internode is dependent on the adjacent tissues. For example, defoliation decreases the internode length (Thomas, 1987b) suggesting that photosynthate may be a limiting factor in stolon elongation.

1.5.4 Stolon senescence in white clover

Stolon tissue comprises at least half of the total shoot weight of white clover plants in grazed pastures (Brock *et al.*, 1988). Hay *et al.* (1991) found that under intensive grazing pressure by sheep, 50% of all stolon nodes in a white clover population were located at nodal positions >8 from the stolon apex. These nodes have apparently little input into the growth of new stolon branches as most branches first emerge from their enclosing stipule when at nodal positions <8 (Chapman, 1983; Hay *et al.*, 1991). However, old stolon material is an important source of stored carbohydrate as up to 60% of dry matter is in stolons and their carbohydrate content is high relative to roots. In addition, some well-established roots on the old stolon are linked to the active growing regions by this tissue (Harris, *et al.*, 1983). Chapman and Robson (1992) found that rapid death of old stolon material occurred perhaps as the result of reallocation to the apex of carbon previously supplied to this stolon tissue.

In pastures, the growth of white clover plants is clonal and the size of the individual plants reflects primarily the equilibrium between the rate of stolon formation at the apices, and the rate of senescence of older basal material (Hay *et al.*, 1988). Hay *et al.* (1989a) found that shoot dry weight was at a minimum in spring, and a maximum in summer. Further, the population showed an increase in the proportion of plants of first branching order, and a lower proportion of third order plants during spring-early summer compared with the rest of the year. However, plant density did not increase at this time suggesting that many of the smaller first order plants were not surviving.

White clover stolons can contribute a significant amount of photosynthate to the actively growing regions of the plant, but the older stolon material is usually buried in autumn by earthworm activity and stock treading (Hay *et al.*, 1987), and much of the older stolon material senesces in spring (Hay *et al.*, 1989a). If the stolons are buried and the plants defoliated early in the growing season, significant senescence of the secondary stolons occurs (Grant *et al.*, 1991).

1.5.5 Leaf senescence in white clover

The first visible sign of leaf senescence in white clover is yellowing that begins at the leaf margins. Leaf senescence occurs in the oldest leaves and may be defined as 'progressive or sequential' (Simon, 1967). The growth of white clover plants varies throughout the year and this has an effect on the number of leaves maintained on the stolons (Brock *et al.,* 1988; Hay *et al.,* 1989a). Leaf initiation is affected by environmental factors and under constant conditions, the rate of leaf initiation and emergence is constant (Thomas, 1987b). In a pure undisturbed sward of white clover, the leaf area index is always above the critical value for photosynthesis throughout the year (Brougham, 1962) indicating that light has an effect on the number of leaves maintained on the plant. Brougham (1958) showed that in a mature closed canopy, new leaves are subjected to lower irradiances of different quality during their development, and the rate of leaf senescence increases.

A critical balance in carbohydrate allocation is maintained within the white clover plant (Hay *et al.,* 1989b). Plants grown under low irradiance show a redistribution of photosynthate toward the stolon apex, and have increased flower or inflorescence abortion, and reduced seed set (Parsumarty and Fountain, 1993). The level of photosynthate available appears to be a major factor determining senescence of tissues in white clover. After defoliation of a white clover stand, renewal of the leaf canopy is initiated from reserves translocated from stolons and roots (Harris, 1987). In New Zealand in spring, the white clover sward reached a leaf area index of 5.5 twenty days after defoliation (Brougham, 1958), and leaf senescence began when the leaf area index reached about 4.0. Severe defoliation decreases the carbohydrate available to the plant which results in a reduction in the number of leaves produced and increases the rate of stolon senescence (Chapman and Robson, 1992).

1.6 Summary

Ethylene is produced by all growing tissues of higher plants via a well characterised pathway. Regulation of ethylene production occurs at many points in this pathway similar to the controls observed for other plant hormones.

The action of ethylene has also been well characterised in several aspects of plant growth and development but the role of the hormone in leaf senescence is less clear. However, recent studies using transgenic plants have supported the hypothesis that ethylene has a central role in moderating the rate of leaf senescence in tomato and *A. thaliana*.

The role of ethylene in leaf senescence may not be apparent from studies on ethylene production alone. For ethylene, as with other plant hormones, the sensitivity to, and competence to respond to the hormone are equally important factors in determining its role in affecting physiological change.

In New Zealand, white clover is the most important pasture legume (Williams, 1987a) and its growth, development and senescence in pastures has been extensively studied (see Baker and Williams, 1987). The phenomenology of white clover development, especially leaf initiation and development is well understood. Senescence is a feature of the stoloniferous phase of white clover growth, and is sequential. This gradient in senescence implies a developmental control which possibly involves ethylene.

1.7 Aims

In this thesis I investigate leaf maturation and senescence, and the involvement of ethylene in these processes in the stoloniferous plant system, white clover. There were four main aims for the project:

1. To survey a range of white clover genotypes and identify a genotype suitable for use in this study.

2. To define the major physiological changes associated with maturation and senescence of leaves along stolons of white clover grown under controlled conditions.

3. To identify changes in the biosynthesis of ethylene at a physiological and biochemical level and correlate them with the physiological changes during leaf maturation and senescence.

4. To identify changes in the expression level of the genes coding for the enzymes (ACC synthase and ACC oxidase) involved in the biosynthesis of ethylene during leaf maturation and senescence.

CHAPTER 2

MATERIALS AND METHODS

2.1 Propagation and growth of white clover plants

2.1.1 Propagation and potting mix

White clover plants were propagated and grown in a steam sterilised horticultural potting mix consisting of a 50:50 mixture of granulated bark (Grade No.2, Granulated Bark Supplies Ltd, Putararu, New Zealand) and medium grade pumice (Tuakau Sands Ltd, Tuakau, New Zealand) supplemented with nutrients (Table 2.1).

2.1.2 Growth of white clover plants

Stock plants of white clover were grown in eight litre planter bags in a greenhouse, and hand watered daily. The greenhouses were maintained at a minimum temperature of 18°C (day) and 12°C (night), and vented at 25°C (day) and 18°C (night). Pests were controlled by spraying alternately with Orthene^{R} (a.i. acephate; Monsanto Company, USA), Lannate^{R} (a.i. methomyl; E.I. du Pont de Nemours and Company Ltd, Wilmington, Delaware, USA) and Attack^{R} (a.i. pirimiphos-methyl; I.C.I. Industries Plc, UK) every 7-10 days. Measurol™ (a.i. methiocarb; Bayer AG, Germany) pellets were distributed to control slugs.

Apical cuttings with two or three leaves were taken from the stock plants and all leaves were excised at the junction of the petiole and stolon, except the youngest fully emerged leaf. The cuttings were placed with the basal node buried in trays of potting mix (section 2.1.1). The trays were placed on a heated (21°C) sand propagation bed with a timer controlled mist watering system (Automation Services Ltd., Auckland, New Zealand).

The cuttings were grown for about four weeks after the production of roots (typically after 14 days) and then potted along the centre line of a seedling tray, with five plants per tray. The trays were left on the propagation bed for seven days to establish the plants, then transferred to the growing position in the greenhouse. Two stolons from each plant were trained over an upturned tray (Figure 2.1) and all axillary stolons and flowers were removed using sharp scissors at weekly intervals. The stolons were tied down using plastic coated wire if necessary, to ensure they grew straight and did not intertwine. Plants grown at Levin were watered for six min every five h using an automatic watering system (Automation Services Ltd, Auckland, New Zealand). Plants grown at Palmerston North were watered for 5 min three times a day using an automatic watering system (Nylex Gardena Ltd., Auckland, New Zealand).

2.1.3 Harvesting white clover leaves

The leaf numbering system adopted for this study is a modification of that proposed by Thomas (1987a). Leaves were numbered sequentially from the apex. The first leaf with unfolded expanding leaflets (Carlson scale 0.7 to 0.9, Carlson, 1966) was designated leaf number one, and the first fully expanded leaf was designated leaf number two. The smallest leaf (excluding leaves enclosed within the apical bud) with folded leaflets (Carlson scale 0.2 to 0.6, Carlson 1966) was designated leaf zero when used.

To harvest tissue, stolons were cut from the plants and individual leaves removed at the top of the petiole immediately below the junction of the pulvinules, weighed, and placed individually into a labelled plastic container. Leaves were either used immediately after harvest ('fresh') or stored for use when required. To store tissue, all the leaves from one stolon were harvested and the tissue frozen by filling the containers with liquid nitrogen. The tissue was then stored at -80°C.

Table 2.1 Nutrient additions to the propagation and potting mixes used for the propagation and growth of white clover plants

Nutrient	Quantity (per 100 L of 50:50 bark:pumice potting mix)
Osmocote^{R} Plus (Scotts Europe B.V., Holland)	384 g
Dolomite lime (Golden Bay, Auckland, New Zealand)	480 g
Superphosphate (Ravensdown Fertiliser Company, Hastings, New Zealand)	85 g
Sequestrene^{R} 330 (Ciba-Geigy Ltd, Basle, Switzerland)	17 g
Fritted Trace Elements (Smiths Horticultural Distributors Ltd, Auckland, New Zealand)	10 g

Figure 2.1: Stolons of white clover genotype 10F growing under controlled conditions at Levin.

2.2 Biochemical and physiological methods

2.2.1 Preparation of commonly used reagents

Reagents and chemicals used in this part of the study were analytical grade supplied by BDH Laboratory Supplies (Poole, Dorset, England) or Sigma Chemical Company (St. Louis, Mo., USA) unless otherwise stated. Solutions were prepared using deionised water and stored at room temperature unless otherwise stated.

2.2.2 Measurement of chlorophyll concentration

This method uses the solvent N, N-dimethylformamide (DMF) as described by Moran and Porath (1980). DMF is useful for the extraction and determination of chlorophyll concentration both because it can be used on intact plant tissues (Moran, 1982), and because "chlorophyll is quite stable in DMF" (Inskeep and Bloom, 1985).

Materials

DMF

Methods

Up to 400 mg of freshly harvested leaf material was immersed in 5 mL of cold (4°C) DMF in 15 mL glass test tubes, and incubated in darkness at 4°C for 48 h. The solutions were then mixed by inverting the tubes and the absorbance of 1 mL aliquots (care was taken to avoid any particulate matter) determined at both 664.5 nm and 647 nm.

Calculations

Chlorophyll concentrations were calculated using the formulae described by Inskeep and Bloom (1985) which are corrected for errors incorporated in the original formulae devised by Moran (1982):

Chlorophyll a	=	12.7 $A_{664.5}$	-	2.79 A_{647} (mg/mL)
Chlorophyll b	=	20.7 A_{647}	-	4.62 $A_{664.5}$ (mg/mL)
Total chlorophyll	=	17.9 A_{647}	+	8.08 $A_{664.5}$ (mg/mL)

2.2.3 Treatment of detached leaves with exogenous ethylene

The effect of exogenous ethylene on the rate of de-greening of white clover leaves was determined by placing the leaves (with the petioles attached) into 1 L glass containers with airtight lids. The lids had a hole drilled to accommodate a rubber stopper from a 5 mL Vacutainer^{R} tube (Becton Dickenson and Company, Rutherford, N.J., USA) which enabled ethylene (1000 ppm ethylene in nitrogen gas standard; BOC Gases (NZ) Ltd, Wellington, New Zealand) to be injected into the containers to give nominal final concentrations of 0, 1, 10 or 100 ppm. An additional treatment with 0 ppm added ethylene also contained 10 g of a commercially available ethylene absorbing compound (Purafil^{TM}, Papworth Engineering, Cambridge, New Zealand; Faubion and Kader, 1996) in a small vial to remove endogenously produced ethylene. All the containers had moistened filter paper in the bottom to provide a humidified atmosphere. The containers were vented and the ethylene replaced daily. In this experiment, only fully expanded leaves (leaves 2 to 7) were used.

2.2.4 Determination of ethylene evolution from white clover leaves

2.2.4.1 Measurement of ethylene production

Ethylene production from individual detached (section 2.2.4.2) or attached (section 2.2.4.3) leaves of white clover was measured using a PhotoVac 10S70+ gas chromatograph (PhotoVac, Markham, Canada). All other ethylene measurements (sections 2.2.3, 2.2.5, and 2.2.6) were made using either a Hewlett Packard Model 5890 (Hewlett Packard Company, Avondale, Pa., USA) or a Shimadzu Model GC8A (Shimadzu Corporation, Kyoto, Japan) gas chromatograph (depending on availability) fitted with a flame ionisation

detector. The Hewlett Packard instrument had a 3' x 1/8" (outside diameter) stainless steel column packed with Porapak^{R} N (80/100 mesh; supplied by Alltech Associates Inc., Deerfield, Il., USA), and was run with the oven temperature at 65°C and the injector and detector temperature at 150°C. The Shimadzu instrument had a 6' x 1/8" (outside diameter) stainless steel column packed with Porapak Q (80/100 mesh), and was run with the oven temperature at 50°C, and the injector and detector temperature at 100°C. The Hewlett Packard had a sample loop injection system of 0.5 mL nominal volume, while the Shimadzu was a direct injection system. The instruments were calibrated using alpha standard calibration gas (1 ppm ethylene in nitrogen, BOC Gases (NZ) Ltd.).

2.2.4.2 Measurement of ethylene evolution from detached leaves

Individual leaves were placed into a 10 mL syringe with the plunger inserted so that a 10 mL volume was retained, and the needle blocked by insertion into a rubber bung. The syringe also contained a moistened filter paper disc to provide a humidified atmosphere. After 1h, a 1 mL gas sample was injected into the sample loop of the gas chromatograph directly from the syringe. The plunger was then removed to flush the compartment with air and the plunger reinserted before the next measurement.

2.2.4.3 Measurement of ethylene evolution from attached leaves

Individual leaves were enclosed in 30 mL plastic screw top containers with a 5 mL Vacutainer lid inserted into a hole drilled in the bottom. The lids had a slot cut to accommodate the petiole, and the gap blocked with petroleum jelly. After 1 h, a 1 mL gas sample was withdrawn through the rubber bung and the concentration of ethylene measured using the PhotoVac gas chromatograph (section 2.2.4.1). The PhotoVac instrument was calibrated using an alpha standard calibration gas (0.1 ppm ethylene in nitrogen, BOC Gases (NZ) Ltd).

2.2.5 Measurement of ACC and MACC concentration in white clover leaves

ACC and MACC were extracted from leaf tissue using a method modified

from that described by Whitehead *et al.* (1984). The ACC concentration in the extracts was determined using the method described by Lizada and Yang (1979). The method uses NaOCl which reacts with α-amino acids (for example, ACC) to form N-chloroamine as an intermediate, and this is chemically degraded to ethylene, ammonia and CO_2 with a yield of about 13%. In the presence of heavy metal ions (particularly mercury), the yield of the ethylene increased up to about 80% (Lizada and Yang, 1979). Conjugated ACC (MACC) concentration was measured by first hydrolysing the MACC to ACC using acid as described by Hoffman e*t al.* (1983), and then measuring the ACC concentration as above.

Materials

30 mM $HgCl_2$
80% (v/v) ethanol
6N HCl
Chloroform
NaOH (saturated solution)
6N NaOH
3.15% NaOCl solution (Janola^R; Marketed by Reckitt and Coleman (NZ) Ltd., Auckland, New Zealand)

Method

Individual leaves were harvested and stored at -80°C as described in section 2.1.3. The leaves were placed into liquid nitrogen, lyophilised and powdered with a mortar and pestle. The freeze-dried powder was weighed, and then extracted in 80% ethanol (10 mg of powder/mL ethanol) overnight at 4°C. The extract was centrifuged for 5 min and the supernatant collected in a 150 mL round-bottom flask. The pellet was resuspended in 5 mL of 80% (v/v) ethanol and re-extracted at 80°C for 20 min. This extract was centrifuged for 5 min and the supernatant combined with the first ethanol extract. The combined extracts were dried in a rotary evaporator (Rotovapour; Buchi Laboratoriums Technik Ag., Flawil/Schweiz, Switzerland) at 50°C, the dried product dissolved in 1 mL of chloroform, partitioned once with 2 mL of water, and the chloroform fraction discarded.

ACC concentration was determined directly from the water fraction. Aliquots of $HgCl_2$ (100 μL) were added to 800 μL of the aqueous extract in a 5 mL Vacutainer tube on ice, and the tube stoppered. An ice-cold mixture of 2:1 (v/v) NaOCl:NaOH was prepared, and 100 μL added by syringe to the extract. The tube was vortexed for 5 s, incubated on ice for 2 min, and re-vortexed for 5 s after which a 1 mL gas sample was withdrawn for analysis by gas chromatography (section 2.2.3).

MACC concentration was measured by first hydrolysing the MACC to ACC and measuring the ACC concentration as above. Aliquots of the aqueous extract (1 mL) were placed in a 15 mL glass test tube, 1 mL of HCl added, and the tube stoppered with a glass marble. The extracts were then heated at 100°C for 3 h in a heating block. The hydrolysed extract was neutralised with 1 mL of NaOH, and the total ACC concentration determined as above. The MACC concentration was calculated from the difference between the ACC concentration, and the MACC plus ACC concentration.

2.2.6 Measurement of enzyme activity

2.2.6.1 ACC-synthase

ACC synthase (S-adenosyl-L-methionine: methylthioadenosine lyase; E.C. 4.4.1.14) converts s-adenosyl methionine (AdoMet) to ACC, and is usually considered to be the rate limiting step in the production of ethylene in higher plants (Arteca, 1989). ACC synthase activity was measured indirectly by chemically converting the enzyme product (ACC) to ethylene and measuring the ethylene by gas chromatography. The method was modified from that described by Tsai *et al.* (1991).

Materials

1 M KH_2PO_4/K_2HPO_4 , pH 8.0
200 mM s-adeonsyl methionine sulphate (AdoMet-sulphate)
40 mM DTT
10 mM pyridoxal phosphate
Triton X-100
PVPP
NH_4SO_4

Method

Leaves were harvested and used immediately, or placed in sealed jars with 1 ppm ethylene in air, and stored overnight at 20°C in the dark. The harvested leaf material (about 10 g) was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Apple fruit (cultivar 'Granny Smith') cortical tissue was cut into 5 mm cubes, immediately frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. The powder was added to cold (4°C) extraction buffer (5 mL KH_2PO_4/K_2HPO_4, 5 mL DTT, 25 μL pyridoxal phosphate, 100 μL Triton x-100, and 2.5 g PVPP made to a total volume of 50 mL with water) at a ratio of 1:3 (w/v). The mixture was stirred for 15 min at 4°C, passed through two layers of Miracloth (Calbiochem-NovaBiochem Corporation, San Diego, Ca., USA) and centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was transferred to a clean beaker and solid NH_4SO_4 added to make a 30% (w/v) solution. The extract was stirred for 30 min at 4°C, and centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was transferred to a clean beaker, and solid NH_4SO_4 added to make a 90% (w/v) solution. The extract was stirred for 30 min at 4°C and centrifuged at 10,000 x g for 10 min at 4°C, the supernatant discarded, and the pellet redissolved in a minimal volume of assay buffer (usually 8 to 10 mL of an assay buffer prepared by combining 80 mL of KH_2PO_4/K_2HPO_4, 10 mL of DDT, and 400 μL of pyridoxal phosphate, and making this to a total volume of 4 L with water). The extract was dialysed for 12 h at 4°C against 4 L of the assay buffer, with one change.

Aliquots (600 μL) of the dialysed extract were placed into 1.5 mL microfuge tubes, and the enzyme substrate AdoMet-sulphate added to give a final concentration of 0, 5, 10, 15, 20, 25, or 50 μM. The volume was made up to 800 μL with water, and the reaction incubated for 4 h at 30°C. The assays were done in triplicate. The enzyme activity was measured by converting the enzyme product (ACC) to ethylene (section 2.2.5), and measuring the quantity of ethylene by gas chromatography (section 2.2.4.1).

2.2.6.2 ACC-oxidase

The activity of ACC oxidase was assayed using a method modified from

Ververidis and John (1991), Fernández-Maculet and Yang (1992) and McGarvey and Christoffersen (1992). ACC oxidase resembles 2-oxoglutarate-dependent dioxygenase enzymes in its requirement for Fe^{2+} and ascorbate, and in the sequence homology with flavanone-3-hydroxylase (a 2-oxoglutarate-dependent dioxygenase active in flavonoid biosynthesis; Ververidis *et al.*, 1992). The assay measures ACC oxidase activity directly by measuring the amount of ethylene produced from the enzyme substrate ACC in the presence of both Fe^{2+} and ascorbate.

Materials

1 M Tris-HCl, pH 7.5
1.67 mM $FeSO_4$
4 mM ACC
1 M DTT
$NaHCO_3$
1 M sodium ascorbate (freshly prepared)

Method

Fresh leaf material (about 10 g) was frozen in liquid nitrogen and ground to a fine powder (no granular material visible) with a mortar and pestle. The ground powder was added to cold (4°C) extraction buffer (10 mL Tris-HCl, 10 mL glycerol, 6.6 mL sodium ascorbate, 500 μL DTT, and 100 μL Triton X-100 made to a total volume of 100 mL of water) at a ratio of 1:3 (w/v). The extract was stirred for 1 h at 4°C, passed through two layers of Miracloth and centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was transferred to a clean beaker and solid NH_4SO_4 added to make a 30% (w/v) solution with respect to the aqueous component (i.e. excluding the glycerol in the buffer). The extract was stirred for 1 h at 4°C and centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was then transferred to a clean beaker and solid NH_4SO_4 added to make a 90% (w/v) solution with respect to the aqueous component. The extract was stirred for 1 h at 4°C and centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was discarded and the pellet redissolved in a minimal volume of assay buffer (usually 10 to 12 mL of buffer prepared by combining 5 mL of Tris-HCl, 50 mL of glycerol, and 2.5 mL of DTT, and making this to a total volume of 500 mL with water). Aliquots (1.25 mL) of this

extract were then passed through Sephadex G25 (Pharmacia Biotech, Upsala, Sweden) spin columns (section 2.2.6.3) equilibrated with assay buffer. The eluates were then combined and made up to 15 mL with assay buffer. Aliquots of the eluate were assayed for ACC oxidase activity, protein (section 2.2.6.4) and ACC concentration (section 2.2.5).

To measure ACC oxidase activity, assay tubes (5 mL Vacutainer tubes) were prepared by adding 60 μL sodium ascorbate, 60 μL $NaHCO_3$, 60 μL $FeSO_4$, 750 μL 2 x assay buffer, and ACC (to a final concentration of 0, 50, 100, 150, 200, 300, 400, 500 or 1000 μM), and the volume made up to 1500 μL with water. When the tubes had reached assay temperature (30°C), 500 μL of the eluate was added and the tube sealed and vortexed gently (5 s). The tube was placed in a shaking water bath (200 rpm, 30°C) and incubated for up to 1 h. ACC oxidase activity was determined by withdrawing a 1 mL gas sample from the head space in the assay tube and measuring the concentration of ethylene (section 2.2.4.1).

2.2.6.3 Desalting protein solutions in spin columns

Protein solutions were desalted using a method modified from that described by Neal and Florini (1973).

Materials

Sephadex G25

Method

Spin columns were prepared using 5 mL disposable syringes (Becton-Dickensen, Lincoln Park, N.J., USA). Glass fibre (Whatman International, Maidstone, England) discs were cut using a cork borer and placed at the bottom of the barrel, and the barrel filled with a slurry of Sephadex G25 hydrated in the appropriate buffer. The column was placed into a centrifuge tube, centrifuged at 1000 x g for 2 min and the tube emptied. An aliquot of buffer (1.25 mL) was placed on top of the gel, the column re-centrifuged for 2 min at 1000 x g, and the amount of eluted buffer measured. This was

repeated until 1.25 mL of buffer was recovered in each spin. The tubes were stored dry at 4°C for up to 1 day until required.

Protein extracts were desalted by carefully loading them onto a spin column and centrifuging at 1000 x g for 2 min at 4°C. The desalted sample was recovered from the centrifuge tube. The column was used once and then discarded.

2.2.6.4 Measurement of protein concentration

Protein concentration was measured using the method based on that described by Bradford (1976).

Materials

Bradford Reagent (Bio-Rad, Richmond, Ca., USA)
Bovine serum albumin (BSA)

Method

The protein reagent was diluted 1:4 with deionised water and was equilibrated against a BSA standard curve.

Aliquots of the standard protein or the sample extract (100 µL) were mixed with 5 mL of the diluted reagent, incubated at room temperature for 10 min, and the absorbance of the solutions at 595 nm measured. For dilute samples, 800 µL of the sample was mixed with 200 µL of the undiluted reagent, and incubated at room temperature for 5 min.

2.3 Molecular biology methods

2.3.1 Preparation of commonly used reagents

This section describes the preparation of reagents used in the molecular procedures used in this study. Unless otherwise stated, molecular biology or analytical grade chemicals and reagents supplied by BDH or Sigma were used, and all solutions were made with deionised water. Solutions were sterilised by autoclaving at 103 Kpa for 15 min, or filter sterilised through a sterile 0.22 μm nitrocellulose filter. Reagents for RNA extraction and manipulation were made from chemicals in containers reserved for RNA use, and dispensed by pouring (no spatulas were used). Solutions were made with deionised water dispensed directly into sterile unused Falcon^{R} (Becton-Dickenson) plasticware. All solutions were stored at room temperature unless otherwise stated.

i) Luria broth (L broth; Luria and Burrows, 1957)

a) L broth: 1.0 % (w/v) bacto-tryptone
0.5 % (w/v) bacto-yeast extract
1.0 % (w/v) NaCl
The components were dissolved in water and the pH adjusted to 7.0 before autoclaving.

b) L agar: L broth with 1.5 % (w/v) Bacto-agar

c) L top agarose: L broth with 0.7 % (w/v) agarose
The melted solution was stored at 65°C.

ii) Buffer equilibrated phenol:

Phenol equilibrated with 10 mM Tris, pH 7.5, or 10 mM Tris, pH 8.0. Typically 500 g of phenol crystals were dissolved overnight in 7.5 mL of 2 N NaOH, 130 mL of water, and 6 mL of 1 M Tris-HCl, pH 8.0.

iii) TE buffer: 10 mM Tris-HCl, pH 8.0
0.1 mM EDTA

iv) STE buffer: 10 mM Tris-HCl, pH 8.0
0.1 mM EDTA
150 mM NaCl

v) SM buffer: 0.1 M NaCl
10 mM $MgSO_4$
50 mM Tris-HCl pH 7.5
0.01 % (w/v) gelatin

vi) 20 x SSPE: 17.53 % (w/v) NaCl
2.76 % (w/v) $NaH_2PO_4.H_2O$
0.74 % (w/v) EDTA
The final solution was adjusted to pH 7.4

vii) 20 x SSC: 17.53 % (w/v) NaCl
8.82 % (w/v) sodium citrate
The final solution was adjusted to pH 7.0

viii) Ethidium bromide:
10 mg/mL ethidium bromide in sterile deionised water. Stored in the dark.

ix) SDS: 10 % (w/v) SDS
The pH was adjusted to 7.2.

x) TBE buffer: 45 mM Tris-borate
1 mM EDTA
pH 8.0

xi) TAE buffer: 40 mM Tris-acetate
1 mM EDTA
pH 8.0

xii) KH_2PO_4: 0.75 M KH_2PO_4, pH 3.5

xiii) 10 x MSE: 200 mM MOPS
50 mM NaOAc
10 mM EDTA
The final solution was adjusted to pH 7.0

xiv) Denhardts reagent (Denhardt, 1966):
2% (w/v) BSA
2% (w/v) ficoll
2% (w/v) polyvinylpyrrolidone

xv) X-gal (5-bromo-4-chloro-3-indoyl-b-D-galactoside) stock solution
20 mg/mL X-gal in water

2.3.2 Transformation of *Escherichia coli*

2.3.2.1 Preparation of competent cells

Treatment of *E. coli* cells with ice-cold $CaCl_2$ followed by brief heating induces a transient competence in the cells to take up DNA from a variety of sources (Mandel and Higa, 1970).

Materials

20% (w/v) glucose solution
1 M $MgSO_4$
50 mM $CaCl_2$
Glycerol
L agar (section 2.3.1)
L broth (section 2.3.1)
Ampicillin solution (10 mg/mL)

Method

E. coli strain DH5α (Life Technologies, Galthersburg, MD., USA) was plated onto an L agar plate and cultured overnight at 37°C. A single colony from the

overnight culture was used to inoculate a culture vessel containing 200 mL L broth supplemented with 2 mL $MgSO_4$ and 2 mL glucose solution. The culture vessel was placed on an orbital shaker at 37°C until the optical density at 600 nm had reached 0.4. The culture vessel was then placed on ice for 10 min, and centrifuged at 2000 x g for 10 min at 4°C. The supernatant was carefully poured off and the pellet resuspended in 100 mL ice-cold $CaCl_2$. The culture vessel was centrifuged at 2000 x g for 10 min at 4°C, resuspended in 20 mL ice-cold $CaCl_2$ supplemented with 15 % (w/v) glycerol, and placed on ice for 12-24 hours. The culture was aliquoted into microfuge tubes (200 μL), frozen in liquid nitrogen and stored at -80°C.

2.3.2.2 Plasmid transformation of *E. coli*

DNA (10 ng) was added to an aliquot of the competent cells on ice, mixed gently by pipetting up and down, left on ice for 30 min, and then heated for 2 min at 42°C. L broth (700 μL) was added and the cells incubated at 37°C for 1 h, plated onto L agar containing an antibiotic for the selection of cells containing the plasmid, and incubated overnight at 37°C. Individual colonies were selected for plasmid preparation (section 2.3.3).

2.3.3 Extraction and purification of plasmid DNA from cultures of *E. coli*

The extraction and purification of plasmid DNA involves three steps: the growth of bacterial cultures, the harvesting and lysis of the bacterial cells, and the purification of the plasmid DNA. High copy number plasmids (eg pBluescript^R; Short *et al.*,1988) can be extracted directly, whereas low copy number plasmids often require chloramphenicol amplification to obtain reasonable quantities of DNA.

Materials

L broth (section 2.3.1)	
Antibiotic solution	The antibiotic used depends on the plasmid. For pBluescript^R, ampicillin at 100 μg/mL was used.
Solution A	50 mM glucose 25 mM Tris-HCl

	10 mM EDTA
Solution B (Made fresh)	0.2 N NaOH
	1% (w/v) SDS
Solution C	3 M KOAc
	2 M HOAc

Method

L broth (5 or 10 mL) was inoculated with bacteria containing the plasmid (section 2.3.2.2) by stabbing a single colony with a sterile toothpick and breaking off the lower portion of the toothpick directly into the broth. The cultures were incubated with vigorous shaking (200 rpm) at 37°C overnight. The tubes were centrifuged at 5000 x g for 5 min at 4°C, and the supernatant decanted off. The tubes were left upside down to drain while other tubes were prepared and decanted. The pellet was resuspended in 50 μL of solution A by gently pipetting up and down with a large tip, and the cells lysed by adding 200 μL of solution B and mixing by gently inverting the tubes. Care was taken not to mix vigorously as this can shear the bacterial genomic DNA and cause contamination of the plasmid DNA. When the cells had fully lysed (the solution had cleared), 150 μL of solution C was added and the tubes mixed by pipetting up and down with a large tip. The tubes were then incubated on ice for 15 min, centrifuged for 5 min, and 380 μL of the supernatant carefully removed (avoiding any particulate matter) to a new microfuge tube. Two volumes of isopropanol (section 2.3.4) were added and the solutions mixed by inversion. The tubes were centrifuged at 13,000 x g for 5 min, and the supernatant decanted carefully. The tubes were left upside down to drain for about 5 min before the pellet was washed with 75% (v/v) ethanol. The ethanol was decanted off and the pellet dried briefly under vacuum. The pellet was dissolved in sterile water or TE buffer.

2.3.4 Precipitation of nucleic acids in aqueous solution

In the presence of 0.1 to 0.5 M monovalent cations, ethanol or isopropanol will cause the aggregation and precipitation of nucleic acids from aqueous solution through dehydration. The three major variables are:

i) the temperature for precipitation.

ii) the type and concentration of monovalent cations used.

iii) the time and speed of centrifugation used to collect the precipitating nucleic acids (Sambrook *et al.*, 1989).

i) Although low temperature (-20°C) was thought to be important, it is now known to be unnecessary and that precipitation at 0°C is sufficient (Sambrook *et al.*, 1989).

ii) The choice of monovalent cations is dependent largely on the requirements of subsequent manipulations. To selectively precipitate large oligonucleotides (>30 bp) from solutions containing dNTP s, ammonium acetate (2-2.5 M) was added and the nucleic acid precipitated as described below. This salt should not be used if the nucleic acid is to be phosphorylated as the ammonium ions inhibit T4 polynucleotide kinase. Lithium chloride (0.8 M) is frequently used for precipitating from large volumes of solution. However, this salt should be avoided if RNA is to be used for reverse transcription as chloride ions suppress the activity of RNA dependent DNA polymerases. Lithium chloride can be used to selectively precipitate large RNAs from tRNAs and 5S RNA molecules. Sodium chloride (0.2 M) was used for precipitating DNA in the presence of SDS as the detergent then remains soluble in the ethanol. Sodium acetate (0.3 M, pH 5.2) was used for most routine precipitations of RNA and DNA.

iii) Centrifugation of precipitated DNA was generally done in a microfuge at 13,000 x g for 20 min at room temperature. For RNA samples, centrifugation was done at 13,000 x g for 20 min at 4°C unless otherwise stated.

2.3.4.1 Precipitation of nucleic acids with ethanol

Materials

100% (v/v) ethanol
75% (v/v) ethanol
3 M sodium acetate, pH 5.2 (or other salts as described above)
7.5 M ammonium acetate

The volume of the solution containing the nucleic acid was measured and 0.1 volumes of sodium acetate added (unless salts were already present). Two volumes (calculated after the addition of salts) of cold (-20°C) 100% (v/v) ethanol was then added and the tube inverted several times to mix. If the quantity of nucleic acid in the solution was low (for example, in cDNA library reactions), the tube was held at -20°C for 1 hour before centrifugation at 13000 x g for 20 min at room temperature. If the concentration of nucleic acid was high (for example, plasmid DNA preparations), the tube was centrifuged immediately. The supernatant was carefully removed by decanting, and the pellet washed with 500 μL of cold (-20°C) 75% (v/v) ethanol to remove salts. The tube was centrifuged for 2 min and the supernatant carefully removed (the pellet does not stick well to the microfuge tube after this wash). The pellet was air dried or dried under vacuum for a minimum period (excessive drying under vacuum causes denaturation of small fragments of DNA and greatly reduces recovery of larger fragments of DNA; Svaren *et al.* 1987), and dissolved in either water or TE buffer. Centrifugation was generally carried out at room temperature for DNA samples and 4°C for RNA samples. For RNA samples stored in formamide (section 2.3.12.4), RNA was precipitated using four volumes of 100% (v/v) ethanol, centrifuged at 13,000 x g for 20 min, and then washed as described above (Chomczynski, 1992).

2.3.4.2 Precipitation of DNA with isopropanol

Isopropanol can be used in a similar way to ethanol for precipitation of nucleic acids. Isopropanol is particularly useful when the volume of the solution containing RNA or DNA is large as usually only one volume of isopropanol is required. However, isopropanol is less volatile than ethanol, and the removal of salt from the pellet is often more difficult when this procedure is used.

2.3.5 Restriction endonuclease digestion of DNA

Restriction endonuclease digestion was performed routinely with about 1 to 2 μg of plasmid DNA in 1 to 10 μL water or TE buffer. Two microlitres of the

specific enzyme buffer (10X) supplied by the manufacturer was added, the volume made up to 18 or 19 μL with water and the solution mixed by pipetting up and down. Either 1 or 2 μL of the restriction enzyme was added and the solution again mixed thoroughly by pipetting up and down. If required, 1 μL of RNase was added to the digest prior to adding the restriction enzyme. The digest was incubated for 1 to 2 h at 37°C (or the temperature recommended for the enzyme used). The restriction digest was analysed by separating the fragments using agarose gel electrophoresis (section 2.3.6).

2.3.6 Agarose gel electrophoresis of DNA

DNA migrates through an agarose gel matrix at a rate dependent on its molecular size, the amount of agarose in the gel (Table 2.2) and the voltage applied to the gel. DNA migrates toward the anode at neutral pH due to the negatively charged phosphate backbone. High molecular weight DNA migrates relatively faster as the voltage is increased, hence this DNA is resolved better at low voltages (2-3 V/cm) (Helling *et al.* 1974).

Materials

Gel loading buffer (GLB):

10 mM Tris-HCl
1 mM EDTA, pH 8.0
20 %(w/v) glycerol
0.025 % (w/v) bromophenol blue

Gel loading buffer (SUDS):

50 % (w/v) glycerol
100 mM EDTA, pH 8.0
1 % (w/v) SDS
0.025 % (w/v) bromophenol blue

Method

Agarose gels were prepared by adding the appropriate amount of agarose to 100 mL of TAE or TBE buffer in a glass erlynmeyer flask. The flask and agarose was weighed, the agarose melted by boiling in a microwave oven, and the flask re-weighed and any weight loss made up with water. The gel

was then poured into the gel-forming apparatus. After the gel had set, it was placed into the electrophoresis apparatus and submerged in TAE or TBE buffer containing 0.2 μg/mL ethidium bromide. Excess gel mix was stored at room temperature and re-melted in a microwave oven when required. DNA gel loading buffer was added to the sample in the ratio of 1:9 SUDS:sample or 1:1 GLB:sample depending on the concentration of the sample. Electrophoresis was performed at 5 V/cm. The DNA was visualised by viewing under U.V. light on a transilluminator (UVP Inc., San Gabriel, Ca., USA) and photographed using a Polaroid^{R} camera and Polaroid 667 film (Fabrique au Royanne-Uni., UK., Ltd. Hertfordshire, England).

2.3.7 Recovery and purification of DNA from agarose gels

The method is based on that described by Vogelstein and Gillespie (1979).

Materials

GlassMax^{R} kit (Life Technologies)
TE buffer (section 2.3.1)
TAE (or TBE) buffer (section 2.3.1)
Low melting point agarose (Pharmacia Biotech)

Method

DNA was separated by electrophoresis in an agarose gel using TAE or TBE buffer (TBE can interfere with the binding of DNA to the glass matrix; Sambrook *et al.* 1989). The desired DNA band was identified using UV light, cut from the gel with a scalpel blade, and placed into a microfuge tube with 3 volumes of the supplied NaI solution for each gram of gel. The mixture was heated at 50°C until the agarose was completely melted, and 550 μL added to a spin column and the column capped. The column was centrifuged at 13,000 x g for 20 s at room temperature, the tube emptied, and 400 μL of wash solution added. The column was again centrifuged at 13,000 x g for 20 s at room temperature. This wash step was repeated two more times. The tube was then emptied and the column centrifuged at 13,000 x g for 1 min at room temperature. The column was transferred to a fresh tube and 40 μL of hot

(65°C) TE buffer or water added, and the column centrifuged at 13,000 x g for 20 s to elute the DNA.

2.3.8 Preparation of plasmid cloning vectors for the insertion of DNA fragments

Plasmid "cloning" vectors are plasmids used to amplify DNA fragments of interest. Typically the vector is a high copy number plasmid containing a multiple cloning site (MCS) often bordered by specific promoter regions, an origin of replication, and containing a region coding for antibiotic production. Amplification is usually performed in *E. coli*.

Plasmid cloning vector DNA was prepared for insertion of the DNA fragments by digesting the plasmid with an appropriate restriction enzyme (section 2.3.5) which cuts in the multiple cloning site. If the digestion of the plasmid resulted in compatible termini the digested vector was treated with calf alkaline phosphatase (CAP) to prevent self-ligation. The vector was then treated to remove all traces of the CAP which could dephosphorylate the insert DNA and prevent ligation.

Materials

10 % (w/v) SDS
STE buffer (section 2.3.1)
Phenol, buffer equilibrated, pH 7.5 (section 2.3.1)
3 M sodium acetate, pH 4.8
100 % (v/v) ethanol
Chloroform
CAP (Boehringer Mannheim, GmbH, Mannheim, Germany)

Method

Restriction enzyme digestion of the plasmid cloning vector was completed as described in section 2.3.5. If the digestion resulted in 5' overhangs in the linearised plasmid (for example after digestion with *Eco*R 1), dephosphorylation was achieved by adding 8 μL CAP directly to the completed restriction digest and the mixture incubated for 15 min at 37°C. If

the digestion resulted in 3' overhangs, the vector was treated as for the 5' dephosphorylation followed by the addition of another 8 μL of CAP and incubation for a further 45 min at 55°C.

To remove the CAP, 5 μL of SDS and 10 μL of STE buffer were added and the volume made to 100 μL with water. The preparation was heated at 68°C for 15 min to deactivate the enzyme, the mixture partitioned with phenol/chloroform (1:1), and the organic phase was re-extracted with 50 μL water. The phenol/chloroform extraction was repeated and the aqueous extracts pooled. Sodium acetate (0.1 volumes), and two volumes of 100% (v/v) ethanol were added, and the DNA precipitated overnight at -20°C. The DNA was centrifuged for 10 min, and the supernatant carefully removed. The pellet was washed with 75% (v/v) ethanol, centrifuged for 2 min, the supernatant removed and the pellet dried. The pellet was redissolved in water to give 10 ng/μL plasmid DNA.

2.3.9 Ligation of insert DNA to plasmid vectors

Ligation of insert DNA into plasmid cloning vectors (section 2.3.7) was achieved using T4 DNA ligase.

Materials

T4 DNA ligase (Boehringer Mannheim)
5 x ligation buffer (Boehringer Mannheim)

Method

A ligation mixture was prepared comprising 1 μL (10 to 20 ng) of CAP- treated plasmid cloning vector DNA (section 2.3.7), 3 μL (20 to 50 ng) insert DNA , 4 μL ligation buffer, 2 μL T4 DNA ligase and 10 μL water. The mixture was incubated for 3 h at 26°C or overnight at 8°C. The ligated vector-insert was transformed into competent *E. coli* cells (section 2.3.2.1) for amplification.

2.3.10 Extraction of genomic DNA from leaf tissue of white clover

Materials

Extraction buffer:

50 mM Tris-HCl, pH 8.0
10 mM EDTA
100 mM NaCl
1% (w/v) SDS
10 mM 2-mercaptoethanol

5 M potassium acetate
3 M sodium acetate
TE buffer (section 2.3.1)
100% (v/v) isopropanol
75% (v/v) ethanol

Method

Aliquots (7 mL) of the extraction buffer were placed into 15 mL Falcon tubes. Typically 1 g of fresh or stored (-80°C) leaf tissue was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle, added to the extraction buffer and the tube capped and shaken vigorously until the powder had melted. The mixture was then incubated at 65°C for at least 5 min. Potassium acetate (2.5 mL) was then added and the tubes again capped and shaken vigorously, and incubated on ice for at least 15 min. The extracts were centrifuged at 10,000 x g for 15 min at 4°C, and a 5 mL aliquot of the supernatant passed through a layer of Miracloth into a tube containing 5 mL of isopropanol (section 2.3.4.2). The tube was inverted several times to mix, placed at -20°C for 1 h, centrifuged at 10,000 x g for 15 min at 4°C, and the supernatant carefully removed by pipetting.

The pellet was resuspended in 700 µL of TE buffer, transferred to a microfuge tube, and the DNA precipitated with 75 µL of sodium acetate and 500 µL of isopropanol. The DNA was pelleted by centrifuging at 13,000 x g for 2 min at room temperature, and the supernatant carefully removed. The pellet was washed with 1 mL of ethanol, dried briefly under vacuum, and dissolved in 100 to 200 µL of TE buffer or water.

Table 2.2: Molecular size of DNA fragments efficiently separated by agarose gels (Helling et al., 1974).

% agarose	Range of DNA sizes separated (kb)
0.5	30 to 1
0.7	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

2.3.10.1 Quantitation of DNA samples

The concentration of DNA samples was measured by diluting the sample (typically 5 µL of sample made up to 1000 µL with water) and determining the absorbance at 258 nm. The concentration (in µg/mL) was then calculated using the following formula:

Absorbance at 258 nm X dilution X 50.

2.3.11 Southern blotting

The analysis of DNA for the presence of particular sequences is achieved by the technique described by Southern (1977). DNA is digested with restriction enzymes and the fragments separated according to size by electrophoresis in an agarose gel. The DNA is then denatured *in situ* and transferred and fixed to a solid support (nylon membrane). The fixed DNA is hybridised with a radiolabelled DNA sequence to detect regions with similar sequences.

2.3.11.1 Alkali transfer of DNA to nylon membranes

Materials

0.4 N NaOH transfer buffer
Hybond N^{+R} membrane (Amersham International Plc., Amersham, Buckinghamshire, England)
5 x SSPE buffer (section 2.3.1)

Method

Aliquots of DNA (usually 30 µg) were digested with restriction enzymes (section 2.3.5) and separated by electrophoresis in an agarose gel (section 2.3.6). DNA size markers were included in a separate lane on the gel. The gel was photographed under UV light, then rinsed briefly in deionised water and placed DNA-side up on the 3 MM paper wick in the prepared transfer container (Figure 2.2). Hybond N^+ membrane, cut to the same size as the gel, was carefully laid on top of the gel, and good contact made by rolling a glass

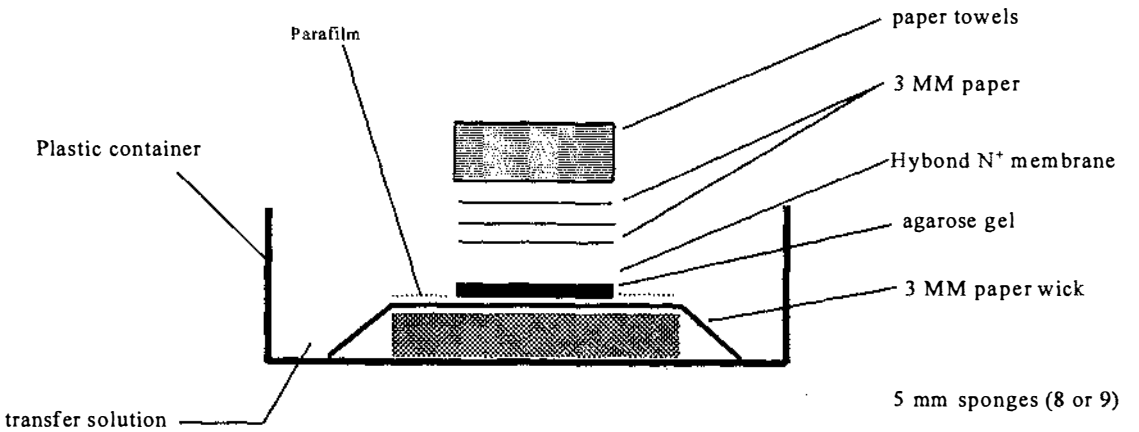


Figure 2.2: Arrangement of apparatus used to transfer DNA or RNA samples to Hybond N$^+$ membrane.

test tube across the membrane. Three pieces of 3 MM paper were cut to the same size as the gel. The first piece was soaked in transfer buffer, laid carefully on top of the membrane and rolled flat with the test tube. This was repeated with a second piece on top of the first. A third piece of 3 MM paper was laid on top of the first two without soaking in the transfer buffer. Paper towels cut to approximately the same size as the gel were placed on top of the 3 MM paper and stacked so that about 1 cm protruded above the top of the container. The transfer buffer was replenished to just below the top of the sponges, and the lid of the box carefully clipped in place. The transfer was allowed to proceed overnight at room temperature. In the morning, the apparatus was dismantled, the membrane rinsed in 5 x SSPE buffer, sealed into a plastic bag, and stored at 4°C until required for hybridisation (section 2.3.11.3 or 2.3.11.4).

2.3.11.2 Preparation of radiolabelled DNA by random priming

Random priming of DNA was achieved using either of the two commercial protocols described below. The main difference between the systems is that the components (including the polymerase) of "Ready-to-go" (Pharmacia Biotech) system are lyophilised, whereas the "Quickprime" (Pharmacia Biotech) components and polymerase are stored separately in solution at -20°C.

2.3.11.2.1 Random priming of DNA using Ready-to-go

Materials

Ready-to-go^{R} labelling kit (Pharmacia Biotech)
0.75 M KH_2PO_4 pH 3.5
α-^{32}P CTP

Method

DNA (25 to 50 ng) dissolved in water or TE buffer was made to a total volume of 45 µL with water, denatured by heating to 95°C for 3 min, and chilled quickly on ice. The contents of the tube were collected by brief centrifugation, and added to a tube of Ready-to-go reagent mix. The lyophilised reagent mix

was dissolved by pipetting up and down briefly, and 5 μL of α-^{32}P CTP added, the solution again mixed by pipetting up and down, and then incubated for 15 to 20 min at 37°C.

After 15 min, 2 μL of the solution was removed and the components separated by thin layer chromatography using KH_2PO_4 as the solvent. Once the solvent front had migrated near the top of the TLC plate (15 to 20 min), the plate was placed into a plastic bag and autoradiographed for 20 to 30 min. DNA was retained at the origin and the level of incorporation can be estimated by comparing the amount of radioactivity detected at the origin with the radioactivity from the unincorporated α-^{32}P CTP near the solvent front. Incubation of the remaining reaction mix was continued at 37°C until the autoradiograph had been developed. After the efficiency of labelling had been assessed, the remaining labelled DNA in the Ready-to-go reaction was denatured by heating at 95°C for 5 min, and then added directly to the vessel containing the membrane (sections 2.3.11.3 or 2.3.11.4).

2.3.11.2.2 Random priming of DNA using Quickprime

Materials

Quickprime^{R} labelling kit (Pharmacia Biotech)
α-^{32}P CTP (3000 Ci/mM) (Amersham)
Scintillation fluid (Amersham)
TE buffer (section 2.3.1)

Method

The DNA (10 ng) to be labelled was dissolved in 34 μL of TE buffer, denatured by heating at 95°C for 5 min, centrifuged briefly, and held on ice until required. Quickprime reagent mix (10 μL) and α-^{32}P CTP (5 μL) was added, mixed by pipetting, and then 1 μL of T7 DNA polymerase added. The complete reaction mixture was then mixed by pipetting, and incubated for 15 to 20 min at 37°C. When the reaction was complete, the volume was made up to 100 μL with water and unincorporated α-^{32}P CTP and labelling reaction components were separated from the radiolabelled DNA using Sephadex

G50 spin columns.

Sephadex columns were prepared using 1 mL disposable syringes (Becton Dickenson). Glass fibre discs were cut using a cork borer, slightly less than the inner diameter of the syringe barrel. The syringe plunger was removed, a glass fibre disc was placed at the bottom, and the barrel filled with a slurry of autoclaved Sephadex G50 in water. The syringe barrel was placed into a microfuge tube (with the cap removed) and this assembly placed into a 15 mL Falcon tube and centrifuged at 1000 x g for 20 s. The microfuge tube was emptied, and 100 μL of water placed on top of the gel. The tubes were centrifuged for 20 s at 1000 x g, and the amount of buffer in the microfuge tube measured. This was repeated until 100 μL of water was recovered in each spin.

A 2 μL aliquot was removed from the Quickprime mixture and spotted onto a 5 mm diameter piece of glass fibre filter paper and this was placed into a scintillation vial. The remaining labelling solution was carefully loaded onto the prepared spin column (above) and the column centrifuged at 1000 rpm for 20 s at room temperature. The labelled DNA was recovered from the microfuge tube, and a 2 μL aliquot spotted onto a second piece of glass fibre filter paper and placed into a labelled scintillation vial. The DNA aliquots on the glass fibre paper were dried for 10 min at 95°C, and 3 mL of scintillation fluid added. The radioactivity was determined in each sample by scintillation counting, and the percentage incorporation of label calculated from the difference in scintillation counts between the two samples. Labelled DNA with scintillation counts above 100,000 (usually 300,000-400,000 counts and >30% incorporation was achieved) were used for hybridisation to DNA or RNA bound to Southern or northern blots, or cDNA library filters.

The labelled DNA was denatured by heating at 95°C for 5 min, and then added directly to the hybridisation solution (sections 2.3.11.3 or 2.3.11.4).

2.3.11.3 Hybridisation of Southern blots with radiolabelled DNA using the 'Amersham' method

Single stranded nucleic acid oligonucleotides will hybridise with DNA or RNA

if the sequences share some homology. The amount of hybridisation will depend on the degree of homology of the sequences and the conditions under which the hybridisation occurs. If the sequences are not very similar, "low stringency" conditions will be required in order to achieve hybridisation. Low stringency is defined as low temperature (for example, 50°C) and high salt (for example, 1 or 2 X SSPE) which stabilises DNA hybrids with a low degree of homology. High stringency conditions (for example, 65°C, 0.1 X SSPE) may be used when the sequences are homologous. The radiolabelling of DNA enables the oligonucleotide to be used as a probe to determine whether similar sequences are present in the target DNA, and to detect the hybridisation by use of autoradiography (Sambrook *et al.*, 1987).

Materials

20 x SSPE (section 2.3.1)
5 mg/mL Herring sperm DNA
10% (w/v) SDS (section 2.3.1)
100 x Denhardts reagent (section 2.3.1)

Method

The Southern blot (section 2.3.11.1) to be hybridised was rinsed in a filter-sterilised pre-hybridisation solution consisting of 6.25 mL 20 x SSPE, 1.25 mL Denhardts reagent, 1.25 mL SDS solution, and 16.25 mL water. Hybridisation was performed in hybridisation bottles (Hybaid Ltd., Teddington, Middlesex, United Kingdom) or 50 mL Falcon tubes containing 25 mL of the pre-hybridisation solution, pre-warmed to 65°C. Herring sperm DNA (1 mL) was denatured by boiling for 5 min and added to the solution. The membrane was pre-hybridised with the herring-sperm DNA for at least 1 h at 65°C.

When the pre-hybridisation incubation was completed, the denatured radiolabelled DNA (section 2.3.11.2) was added to the hybridisation solution, incubated for at least 12 h at 65°C, then washed (section 2.3.11.5) and autoradiographed.

2.3.11.4 Hybridisation of Southern blots with radiolabelled DNA using the Church and Gilbert method

This method uses the hybridisation solutions described by Church and Gilbert (1984), but the hybridised blots or filters were washed using SSPE or SSC as in section 2.3.11.3. This method does not require pre-hybridisation but relies on a higher concentration of SDS to reduce non-specific background hybridisation.

Materials

20 x SSPE (section 2.3.1)
Hybridisation solution:
1 mM EDTA
0.5 M $NaHPO_4$
7% (w/v) SDS

Method

The Southern blot (section 2.3.11.1) or cDNA library filter (section 2.3.17.8) was placed into the hybridisation container with sufficient hybridisation solution to cover it completely (usually 30 to 50 mL). The hybridisation solution and membrane were pre-warmed at 65°C for 10 min, the denatured probe (section 2.3.11.2) added, incubated for at least 12 h at 65°C, then washed (section 2.3.11.5) and autoradiographed.

2.3.11.5 Washing Southern or northern blots, or cDNA library filters hybridised with DNA probes

Washing Southern and northern blots and cDNA library filters was achieved by using increasing levels of 'stringency' until a clear signal free of background radiation counts was observed. At each stage of washing in high stringency (1.0 to 0.1 x SSPE or SSC) the membranes were checked for radiation counts using a hand-held radiation detector (Morgan Mini-Instruments Ltd, Essex, England) and if the counts were low (5 to 20 counts/min), autoradiographed prior to any further washing.

Materials

2 x SSPE (section 2.3.1), 0.1% (w/v) SDS
1 x SSPE (section 2.3.1), 0.1% (w/v) SDS
0.1 x SSPE (section 2.3.1), 0.1% (w/v) SDS

Method

The membrane was rinsed by replacing the hybridising solution (section 2.3.11.3 or 2.3.12.4) with 2 x SSPE containing 0.1% (w/v) SDS (200 to 300 mL). The membrane was then washed in 2 x SSPE containing 0.1% (w/v) SDS (about 200 mL) for 15 min at 65°C on a shaking platform (30 rpm). The wash solution was replaced and the wash repeated with 2 x SSPE containing 0.1% (w/v) SDS, then two further washes in 1 x SSPE containing 0.1% (w/v) SDS, and the membrane checked for localised radiation counts using a hand-held radiation detector. If the labelled DNA used was heterologous to the DNA on the membrane, or if the radioactivity counts were low, the membrane was sealed into a plastic bag and autoradiographed immediately. If the labelled DNA was homologous to the DNA on the membrane and if the radioactivity counts were high (>20 counts/min), the membrane was washed twice in 0.1 x SSPE containing 0.1% (w/v) SDS, and autoradiographed as described previously.

2.3.11.6 Stripping Southern and northern blots, and cDNA library filters

Stripping blots of hybridised DNA allows them to be re-hybridised with labelled DNA. Blots can be stripped provided they have been kept moist. Blots that have been dried are difficult to strip.

Materials

0.5% (w/v) SDS
0.1% (w/v) SDS
5 x SSPE (section 2.3.1)

Method

Southern blots (section 2.3.11.4 or 2.3.11.5) or library filters (section 2.3.17.8) were immersed in a hot (100°C) solution of 0.5% (w/v) SDS and allowed to cool to room temperature. The membranes were rinsed in 5 x SSPE, and stored in sealed plastic bags with a small volume (1 to 2 mL) of 5 x SSPE at 4°C. Northern blots were treated the same except that a 0.1% (w/v) SDS solution was used.

2.3.12 Extraction of RNA from leaf tissue of white clover

The critical component of successful isolation of RNA is minimising endogenous RNase activity during the initial extraction (Chomczynski, 1992). In addition, great care must be taken to avoid the introduction of trace amounts of RNase from external sources. Gloves were used at all times and changed regularly. Wherever possible, the equipment used for RNA extraction was set aside for this purpose only, and new sterile plasticware (Falcon) was used. Other equipment was sterilised by rinsing in 1 N NaOH, or baking for 4 h at 180°C. Pipette tips and microfuge tubes were packed with gloved hands and autoclaved before use. Solutions containing RNA were kept on ice whenever possible.

2.3.12.1 Extraction of total RNA using Trizol^{R} (Life Technologies)

This method is based on the method described by Chomczynski and Saachi (1987). Trizol contains phenol and guanadinium isothiocyanate which are strong RNase denaturants.

Materials

Trizol reagent
Chloroform
100% (v/v) Isopropanol
Formamide

Method

Fresh or stored (-80°C) samples were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Trizol was added to the frozen powder (1 mL to 100 mg fresh weight) and the mixture placed into microfuge tubes. The extracts were incubated for 10 min at room temperature, and 200 μL of chloroform added. The extracts were vortexed and the phases separated by centrifuging at 13,000 x g for 15 min at 4°C. The supernatant was carefully transferred to a clean tube, and 500 μL of cold (-20°C) isopropanol added. The tubes were inverted several times to mix the contents, centrifuged at 13,000 x g for 10 min at 4°C, and the supernatant carefully removed. The pellet was dissolved in a minimum volume of formamide and stored at -20°C.

2.3.12.2 Extraction of total RNA using lithium chloride and phenol

Total RNA was extracted using a method based on that described by Van Slogteren *et al.* (1983).

Materials

1 M LiCl
0.5 M EDTA
4 M LiCl
Phenol, buffer dissolved (section 2.3.1)
1 M Tris-HCl, pH 8
Chloroform: isoamyl alcohol 24:1
10% (w/v) SDS (section 2.3.1)
3 M NaOAc pH 5.2
100% (v/v) ethanol
Water (nuclease free; LifeTechnologies)
75% (v/v) ethanol

Method

An extraction buffer was prepared by mixing 500 μL of 1 M LiCl, 500 μL of Tris-HCl, 500 μL of SDS, 100 μL of EDTA, 3.4 mL of water, and 5 mL of phenol in a new 50 mL Falcon tube, and incubating the mixture in a water bath at 80°C. Fresh or stored (-80°C) leaf material (1.5 to 2 g fresh weight)

was frozen in liquid nitrogen and ground to a fine powder with a pre-cooled mortar and pestle. The powder was added to the hot extraction buffer with a pre-cooled spatula. The slurry was vortexed vigorously for 30 s, 5 mL of chloroform/isoamyl alcohol added, and the mixture vortexed vigorously for a further 30 s. If more than one sample was being prepared, the extracts were stored at room temperature after the addition of the chloroform/isoamyl alcohol until required (usually 10-15 min). The extracts were centrifuged at 4500 x g for 5 min to separate the phases, and the water phase carefully transferred to a new 15 mL Falcon tube on ice. If the RNA was to be used for making a cDNA library, the water phase was re-extracted with a further 5 mL of chloroform and re-centrifuged as before. Following the extraction with chloroform, an equal volume of ice-cold 4 M LiCl was added and the RNA precipitated overnight at 4°C.

Following the precipitation step, the samples were centrifuged at 4500 x g for 10 min at 4°C, and the supernatant carefully poured off. The RNA pellet was dissolved in 250 μL ice-cold water and 0.1 volume of ice-cold NaOAc added. The solution was transferred to a microfuge tube on ice, and precipitated with two volumes of cold (-20°C) 100% (v/v) ethanol for 1 h. The RNA was collected by centrifugation at 13,000 x g for 10 min at 4°C, and the supernatant carefully poured off. The RNA pellet was washed with 500 μL of cold (-20°C) 75% (v/v) ethanol, centrifuged again for 2 min, and the supernatant removed. The pellet was (usually) air dried or dried briefly under vacuum (RNA dried under vacuum was often difficult to re-dissolve). The RNA was stored either as the ethanol precipitate prior to last centrifugation step, or in formamide at -20°C (short-term) or at -80°C (long-term).

2.3.12.3 Isolation of poly $(A)^+$ RNA

The majority of mRNA, in contrast to rRNA and tRNA, carry tracts of poly (A) at their 3' termini. Poly $(A)^+$ RNA can therefore be isolated from other RNA species by annealing the poly (A) tail an oligo (dT) moiety bound to a column or a magnetic bead.

Poly $(A)^+$ RNA was isolated using the PolyATtract system IV (Promega, Madison, Wi., USA) according to the manufacturers protocol. This system

utilises a biotinylated oligo (dT) which is annealed to the poly (A) tail of the mRNA. This complex is then captured by binding the biotin moiety to MagneSphere^{R} (Promega) streptdavidin paramagnetic particles (SA-PMP). The SA-PMPs are then captured by a magnet during washing, and the poly $(A)^{+}$ RNA released by incubating the particles in water.

Materials

PolyATtract mRNA isolation system IV kit (Promega)
3 M sodium acetate
100% (v/v) isopropanol
75% (v/v) ethanol

Method

Total RNA (100 to 1000 μg; section 2.3.12.1) was made to a total volume of 500 μL with nuclease-free water in a microfuge tube. The tube was placed in a water bath at 65°C for 10 min to denature the RNA. An aliquot of the biotinylated oligo (dT) oligonucleotide (3 μL) and 13 μL of 20 x SSC added and mixed, and the solution incubated at room temperature for 10 min. The SA-PMPs were resuspended by flicking the bottom of the tube, and then captured on a magnetic stand. The preservative solution was carefully removed, and the SA-PMPs washed three times with 300 μL of 0.5 x SSC by first resuspending the particles and then capturing them on the magnetic stand. Following the third wash, the SA-PMPs were resuspended in 100 μL 0.5 x SSC, and the entire contents of the RNA/oligo (dT) annealing reaction added. The reaction was incubated at room temperature for 10 min to allow binding between the biotin and streptdavidin components, the SA-PMPs captured on the magnetic stand and the supernatant removed. The SA-PMPs were washed four times by resuspending them in 300 μL of 0.1 x SSC, capturing the particles on the magnetic stand, and removing the supernatant On the last wash, care was taken to remove as much of the supernatant as possible.

The poly $(A)^{+}$ RNA was recovered by resuspending the SA-PMPs in 100 μL of nuclease-free water, incubating for 5 min at room temperature, and capturing

the particles with the magnetic stand. The supernatant was carefully transferred to a clean microfuge tube. The SA-PMPs were again resuspended in 150 μL nuclease-free water, incubated for 5 min and the particles captured with the magnetic stand. The supernatant was removed and added to the first supernatant, and the poly $(A)^+$ RNA precipitated by adding 0.1 volumes of sodium acetate and 1 volume of cold (-20°C) isopropanol at -20°C overnight. The poly $(A)^+$ RNA was pelleted by centrifugation at 13,000 x g for 10 min at room temperature, the pellet washed in cold (-20°C) ethanol, re-centrifuged briefly, and air dried or dried briefly under vacuum. The pellet was resuspended in water if used immediately, or stored in formamide (section 2.3.12.4) until required.

2.3.12.4 Storage of RNA in formamide

Degradation by RNase enzymes is a major concern when handling or storing RNA samples. These enzymes are heat stable and very difficult to remove. Every effort is made to remove endogenous RNases during extraction but the accidental introduction of RNases from a range of sources can quickly degrade samples. The storage of RNA in formamide protects RNA from degradation even in the presence of high concentrations of RNases (Chomczynski, 1992).

Materials

Formamide

Method

RNA was dissolved in small volumes of formamide in place of water or TE buffer, and stored at -20°C for short periods (up to one month), or -80°C for longer periods. The RNA dissolved in formamide was used directly in denaturing agarose electrophoresis, or precipitated out of formamide using four volumes of 100% (v/v) ethanol (section 2.3.4.1).

2.3.12.5 Quantitation of RNA

The concentration of total RNA samples was measured by diluting the sample

(typically 5 µL of sample made up to 1000 µL with water) and determining the absorbance at 258 nm. The concentration (in µg/mL) was then calculated using the following formula:

Absorbance at 258 nm X dilution X 40

The concentration of poly $(A)^+$ RNA samples were measured determining the absorbance on undiluted samples using a 50 µL curvette, and then calculating the concentration as described above. In each case, the curvettes used for RNA were pre-washed in 0.05 N NaOH to remove any RNases.

2.3.13 Formaldehyde agarose gel electrophoresis of RNA

RNA may be electrophoresed directly from samples dissolved in water, TE buffer or formamide.

Materials

10X MSE buffer (section 2.3.1)
Formaldehyde 37% (v/v)
Ethidium bromide solution (section 2.3.1)
Loading dye: 0.01 % (w/v) bromophenol blue
0.01 % (w/v) xylene cyanol
in formamide

Methods

The RNA denaturing gel was prepared by adding 0.9 g agarose and 65 mL water to an erlynmeyer flask. The agarose was dissolved by boiling the solution in a microwave oven. The solution was transferred to a fume hood and 7.5 mL of MSE buffer added. When the gel had cooled sufficiently (to about 60ºC), 2.25 mL of formaldehyde was added. The agarose mixture was immediately poured into the prepared gel apparatus used only for RNA. When set, the gel was covered with a denaturing gel running buffer (75 mL 10X MSE and 13.4 mL formaldehyde made to a total volume of 750 mL with water).

RNA samples in water or formamide were heated at 65°C for at least 5 min then chilled on ice before loading onto the gel. Typically 10 μg of RNA in 10 μL of formamide was added to 10 μL of formamide-sample loading buffer (700 μL loading dye, 425 μL formaldehyde, 250 μL 10X MSE, and 10 μL EtBr made up to a total volume of 550 μL with water), or 10 μg RNA in 2 μL water was added to 7 μL water-sample loading buffer (1250 μL loading dye, 425 μL formaldehyde, 250 μL 10X MSE and 10 μL EtBr) was used in each lane. Electrophoresis was performed at 40 V per cm of gel.

2.3.14 Northern blotting of RNA

Northern blotting was accomplished by alkali transfer using the same arrangement as described for Southern blotting (section 2.3.11.1) except that the transfer buffer used was 0.05 N NaOH.

2.3.15 *In vitro* amplification of DNA by the polymerase chain reaction (PCR)

2.3.15.1 First strand cDNA synthesis by reverse transcription

Materials

Superscript 2 Reverse Transcriptase (Life Technologies)
4 x RT buffer (Life Technologies)
10 mM dNTP mix
RNasin (20-40 U/μL; Life Technologies)

Method

Typically 5μg of total or poly $(A)^+$ RNA was placed into a microfuge tube, 0.5 μL of oligo(dT) primer (500 ng) added, and the volume made up to 5.5 μL with water. The mixture was heated at 80°C for 5 min to denature the RNA, centrifuged for 20 s, and then 4 μL of reverse transcriptase buffer, 2 μL dNTP mix, 1 μL RNasin, 1 μL reverse transcriptase and 6.5 μL water added. The mixture was incubated at 37°C for 1 hour. The tube was heated to 70°C for 10 min to stop the reaction, and the contents of the tube collected by brief

centrifugation.

2.3.15.2 PCR reactions

The PCR technique amplifies a specific segment of DNA that is bordered by sequences homologous to the primers used in the reaction. These primers are homologous to sequences on opposite strands of the template DNA so that the major product of the amplification process is a double stranded DNA segment that lies between, and includes, the primers. The reaction is accomplished using temperature cycling that first denatures the template DNA in the presence of excess oligonucleotide primer and dNTP s. The temperature is then lowered to allow the primers to anneal to their target DNA sequence, followed by a period for polymerisation. This cycle is repeated many times and potentially doubles the number of copies of the target DNA in each round, hence very high amplification of the target DNA sequences can be achieved after 30 cycles.

Materials

Taq DNA polymerase or 'Expand' DNA polymerase (Boehringer Mannheim)
10 X PCR buffer (Boehringer Mannheim)
10 mM dNTP mix (Boehringer Mannheim)
50 mM $MgCl_2$
Paraffin oil (Life Technologies)

Method

PCR amplification was achieved in either 20 or 100 μL reactions by adding the reaction components to 650 μL volume capacity thin-walled PCR tubes (Table 2.3). Water was added to make the total volume up to 20 μL or 100 μL. The primers used are specific to the DNA to be amplified. For ACC synthase, primers ACSR1F, ACSR2F and ACSR6R (Table 2.4) were used. Magnesium chloride was added if it was not in the PCR buffer supplied by the manufacturer.

When reactions were performed in 20 μL, one drop of paraffin oil was added

to reduce evaporation. The tubes were centrifuged briefly at low speed (6,000 x g) and placed into the thermal cycler (Perkin Elmer Cetus, Norwalk, Co. USA). When DNA from the reverse transcriptase reaction (section 2.3.15.1) was used as a template, 2 μL of the reaction was diluted with 8 μL of water and used as described above. PCR was performed at 92°C for denaturation, 50°C for annealing, and 72°C for extension, and repeated for 30 cycles.

2.3.16 Dideoxy chain termination sequencing of DNA

Dideoxy chain termination sequencing was first described by Sanger *et al.* (1977). The method relies on the random incorporation of a dideoxy nucleotide into a DNA strand being synthesised by a DNA polymerase. The incorporation of the dideoxy residue stops further polymerisation due to the absence of a hydroxyl residue at the 3' position of deoxyribose. Therefore, discrete lengths of DNA are synthesised which are terminated at specific residues dependent on the dideoxy residue added to the mixture. When all four dideoxy residues are incubated in separate reactions along with α-^{35}S ATP, the resulting DNA fragments can be separated by electrophoresis in a polyacrylamide gel and the sequence of bases read directly from the autoradiograph.

2.3.16.1 Sequencing with Sequenase version 2 T7 DNA polymerase

Sequenase version 2 (United States Biochemicals, Cleveland, Ohio, USA; Amersham) is a modified bacteriophage T7 DNA polymerase. The enzyme has been genetically modified to remove the 3' → 5' exonuclease repair activity, is reported to be very stable and have high specific activity.

Materials

2 N NaOH
0.5 M EDTA
2 M NH_4SO_4
100% (v/v) ethanol
75% (v/v) ethanol
α-^{35}S ATP (Amersham)
Sequenase version 2 sequencing kit

Table 2.3: Reaction components used to amplify specific DNA sequences homologous to ACC synthase and ACC oxidase from white clover by PCR.

Component	20 μL	100 μL
10 x PCR buffer	2 μL	10 μL
10 mM dNTP mix	0.5 μL	2 μL
amplification primer 1	0.5 μL	1 μL
amplification primer 2	0.5 μL	1 μL
template DNA	0.5 to 1.0 μL	1 to 2 μL
DNA polymerase	0.5 μL	1 μL

Table 2.4: Primer sequences used to generate DNA fragments homologous to ACC synthase and ACC oxidase from white clover by PCR

Name	Probe	Sequence
ACSR1F	ACC synthase forward	GCC GAA TTC ATG GGN C(T)TN GCN GAA(G) AAC(T)
ACSR2F	ACC synthase forward	CTG GAT CCG TA(T)C(T) CAA(G) GAC(T) TAC(T) CAC(T) GG
ACSR6R	ACC synthase reverse	CTC AAG CTT AA(G)N C(G)C(T)A(G) AAA(G) CTN GAC AT
ACSF-1	internal ACC synthase forward primer	GTT ATC TGC GAA GCG CGA AT
ACSR-1	internal ACC synthase reverse primer	ACG GTC ACA TTC GAT GTC TG
ACOF1	ACC oxidase forward	GTG AAT TCG AC(G)G CNT GC(G)G ANA AC(G)T GTG G
ACOR1	ACC oxidase reverse	TCG TCT AGA TCA AAN CG(T)N GGC(G) T

Method

Approximately 4 μg of DNA was used as a template for the sequencing reactions. The template DNA was added to a microfuge tube, the volume made up to 18 μL with sterile water, 2 μL of NaOH and 2 μL of EDTA added, and the tube vortexed briefly to mix. The reaction mixture was incubated for 5 min at room temperature to denature the DNA. The solution was neutralised by the addition of 2 μL of NH_4SO_4 and then vortexed briefly to mix. Cold (-20°C) 100% (v/v) ethanol (75 μL) was added and the tube placed for 10 min at -80°C. The DNA was pelleted in a microfuge at full speed for 10 min, the supernatant decanted, and the pellet washed with cold (-20°C) 75% (v/v) ethanol. The contents of the tube were collected by brief centrifugation and the pellet dried at 37°C.

The denatured DNA was redissolved in 6 μL water, then 2 μL of sequencing primer and 2 μL T7 sequencing buffer added. The sample was heated for 5 min at 65°C in a heating block to melt the strands. The heating block with the tubes was then placed into ice to rapidly bring the temperature down to 37°C. The temperature was maintained at 37°C for 5 min to finish the primer annealing reaction. The T7 DNA polymerase was diluted 1:7 with Sequenase buffer, and placed on ice until required. The labelling mix was diluted 1:4 with water and placed on ice until required. A reaction cocktail was prepared containing:

1 μL DTT
2 μL diluted labelling mix
2 μL diluted Sequenase version 2 T7 DNA polymerase
0.5 μL α-^{35}S ATP.

When the annealing reaction was completed, the reaction cocktail was added to the tube and mixed by pipetting up and down. The labelling mixture was incubated for 5 min at room temperature to initiate polymerisation, and label the sequencing product a short distance from the primer. The tube was then placed on ice until the termination reaction tubes were prepared.

The dideoxy-nucleotide reagents were aliquoted (2.5 μL) separately into

microfuge tubes held on ice. When the labelling mixture was ready, the dideoxy-nucleotide reagent was pre-heated to 37°C for 2 min, and 3.5 μL of the labelling mixture added to each tube. The tubes were incubated for 5 min at 37°C, the reactions stopped by the addition of 4 μL of stop buffer, and the tubes stored on ice until required.

2.3.16.2 Separation of sequencing products by electrophoresis

The electrophoresis buffer used in this study is a modified TBE buffer (section 2.3.1) that gave better resolution of bands after extended electrophoresis times.

Materials

Polyacrylamide: 40 % (w/v) acrylamide
2 % (w/v) methylene-bis-acrylamide
Modified 10 x TBE: 1.3 M Tris base
450 mM boric acid
25 mM EDTA
10% (w/v) ammonium persulphate (freshly prepared)
TEMED

Method

Sequencing glass plates were thoroughly cleaned with detergent (Decon 90; Decon Laboratories Ltd., Hove, East Sussex, England), rinsed with deionised water, wiped with ethanol and allowed to air dry. A 6% (w/v) acrylamide gel mix was prepared containing 15 mL of polyacrylamide solution, 10 mL of modified TBE buffer, 50 g of urea and 35 mL of water. When the urea was completely dissolved, 1 mL of ammonium persulphate and 50 μL of TEMED were added. The mixture was stirred quickly and drawn into a 60 mL syringe for loading into the assembled glass plates. When the gel had been poured, the gel combs were carefully placed onto the gel upside-down and the top was completely covered with excess acrylamide solution to exclude air. The gel was allowed to set for at least 2 h (usually overnight) before use. When the gel had completely set, the combs were removed, the plates placed onto the electrophoresis apparatus, and the combs placed on top of the gel in the

upright position.

The labelled sequencing products were heated to 70°C for 3 min and loaded onto a polyacrylamide sequencing gel. The sequencing products were separated by electrophoresis at 1650 V, 40 mA and 65 W for 3 h. A second loading from each reaction was made in separate wells, and electrophoresis continued for another two hours. At the completion of electrophoresis, the gel was removed from the electrophoresis apparatus, one glass plate removed, and the gel fixed for 10 min in a solution of 10% (v/v) glacial acetic acid and 10% (v/v) methanol. The gel was then transferred onto 3 MM paper, covered with polyethylene cling film, and dried in a vacuum drier. The radiolabelled DNA fragments were visualised by autoradiography. The sequence data was entered into a computer via a digitiser (DNA Star Inc., Madison, Wi., USA).

2.3.17 Construction of a cDNA library in the excision vector λZipLox

A cDNA library is an array of DNA copies of an mRNA population that are ligated into a cloning vector (for example, λ or plasmid) and amplified in *E. coli*. To produce a cDNA library with a high titre [10^6 plaque-forming-units (pfu) or higher], the poly $(A)^+$ RNA preparation must be free of contaminants and the RNA not degraded. The priming of the first strand cDNA can be performed with either oligo(dT) or random hexaprimers. Oligo(dT) priming is less efficient than random hexamer primers, but produces libraries rich in the 3' sequence. Random hexamer priming will copy all RNA including poly $(A)^-$ mRNA, and tends to produce libraries with shorter sequences but richer in 5' sequences. Libraries can also be produced as directional or non-directional depending on the adapters used for ligation.

2.3.17.1 First strand cDNA synthesis

First strand cDNA synthesis utilises a modified Moloney murine leukemia virus reverse transcriptase (Superscript 2 RT; Life Technologies). The enzyme has been modified to remove the RNase H activity which is an integral component of native reverse transcriptase enzymes.

Materials

Superscript 2 RT (Life Technologies)
5X first strand buffer (Life Technologies)
10 mM dNTP mix (Life Technologies)
Oligo (dT) and random hexaprimers (Life Technologies)
0.1 M DTT

Method

The cDNA yield was maximised by using a mixture of oligo(dT) and random hexamer primers. Two microlitres of oligo (dT) $_{12\text{-}18}$ primer and 1 μL (50 ng) of random hexamers were added to a sterile 1.5 mL microfuge tube. Poly $(A)^+$ RNA was prepared as described in section 2.3.12.2, and 5 μg in 7 μL of water added to the tube. The mixture was heated to 70°C for 10 min and quick-chilled on ice. The contents of the tube were collected by brief centrifugation and the following added:

4 μL 5X first strand buffer
2 μL 0.1 M DTT
1 μL 10 mM dNTP mix
1 μL [α^{32}P]dCTP (1 μCi/μL)

The contents of the tube were mixed by gently vortexing, the reaction collected by brief centrifugation, and the tube placed at 37°C for 2 min to equilibrate to temperature. The polymerisation reaction was started by adding 5 μL Superscript^R 2 RT, mixing gently, and incubating at 37°C for 1 h. The tube was then placed on ice to terminate the reaction.

2.3.17.2 Second strand cDNA synthesis

The second strand synthesis uses DNA polymerase I from *E. coli*. The addition of RNase H in this reaction removes RNA from the RNA/DNA hybrid allowing the polymerase access to the single stranded DNA template.

Materials

DEPC-treated water (Life Technologies)

10 mM dNTP mix (Life Technologies)
E. coli DNA ligase (Life Technologies)
E. coli DNA polymerase I (Life Technologies)
E. coli RNase H (Life Technologies)
5 X second strand buffer (Life Technologies)
0.5 M EDTA, pH 8.0
Chloroform/isoamyl alcohol (24:1)
100 % (v/v) ethanol
75% (v/v) ethanol
7.5 M NH_4OAc
Buffer equilibrated phenol, pH 8.0 (section 2.3.1)

Method

The following reagents were added in the order shown to the completed first strand reaction mixture on ice (section 2.3.17.1):

91 μL DEPC-treated water
30 μL 5X second strand buffer
3 μL 10 mM dNTP mix
1 μL *E. coli* DNA ligase
4 μL *E. coli* DNA polymerase I
1 μL *E. coli* RNase H

The tube was vortexed gently, collected by brief centrifugation, and the reaction incubated for 2 h at 16°C. When this reaction was completed, 2 μL (10 units) of T4 DNA polymerase was added and reaction incubated at 16°C for a further 5 min. The reaction was then placed on ice and 10 μL of 0.5 M EDTA added.

The cDNA was isolated from the reaction components by adding 150 μL of phenol:chloroform:isoamyl alcohol (25:24:1), vortexing, and centrifuging at 14,000 x g for 5 min at room temperature to separate the phases. The upper layer (140 μL) was carefully transferred into a fresh 1.5 mL microfuge tube and the cDNA precipitated by adding 70 μL of 7.5 M NH_4OAc, followed by 500 μL of cold (-20°C) 100% (v/v) ethanol. The mixture was vortexed thoroughly and immediately centrifuged at 14,000 x g for 20 min at room temperature. The supernatant was carefully removed and the pellet washed

with 500 μL of cold (-20°C) 70% (v/v) ethanol. The tube was centrifuged at 14,000 x g for 2 min at room temperature and the supernatant removed carefully. The cDNA was dried at 37°C for 10 min to evaporate any residual ethanol.

2.3.17.3 Preparing the cDNA for ligation to the vector

Adapters with ends compatible with the cut vector arms must be ligated onto the cDNA before the cDNA can be ligated to the λ vector. To stop self-ligation, the cDNA with the ligated adapters were CAP-treated to remove the terminal phosphate residues.

Materials

CAP-treated *Eco*R I (*Not* I) adapters (Life Technologies)
DEPC-treated water
5X adapter buffer
0.1 M DTT
T4 DNA ligase

Method

The dried cDNA prepared as described in section 2.3.17.2 was placed on ice, and the following reagents added, in the order shown:

18 μL DEPC-treated water
10 μL 5X adapter buffer
10 μL *Eco*R I (*Not* I) adapters
10 μL 0.1 M DTT
5 μL T4 DNA ligase

The reaction components were mixed gently and incubated at 16°C overnight. The reaction was heated at 70°C for 10 min to inactivate the ligase, placed on ice, and 3 μL of T4 polynucleotide kinase added, mixed gently and incubated for 30 min at 37°C. When the phosphorylation was completed, the reaction was heated at 70°C for 10 min to inactivate the kinase.

2.3.17.4 Column chromatography

This procedure removes short cDNA fragments and residual adapters from the reaction making cloning of larger inserts more probable.

Materials

SizeSep^R 400 spun columns (Pharmacia Biotech)
7.5 M NH_4OAc
100 % (v/v) ethanol
75% (v/v) ethanol

Method

A SizeSep 400 column was prepared by suspending the Sepharose in the column buffer, removing the caps and allowing the column to drain. The Sepharose was then resuspended in 2 mL of TE buffer and allowed to drain. This procedure was repeated twice more, and the column was capped until required. The column was placed into a microfuge tube (with the cap removed), and this assembly placed into a 15 mL Falcon tube and centrifuged at 400 x g for 2 min. The microfuge tube and contents were discarded. The cDNA sample (section 2.3.17.3) was made up to 100 μL with TE buffer and carefully loaded onto the top of the Sepharose gel in the prepared SizeSep column. The column was placed into the Falcon tube with a clean microfuge tube in the bottom and centrifuged at 400 x g for 2 min. The eluted cDNA sample was transferred into a clean microfuge tube and precipitated by adding 0.1 volumes of NH_4OAc and two volumes of cold (-20°C) 100 % (v/v) ethanol. The cDNA was pelleted by centrifugation at 13,000 x g for 10 min, the supernatant was removed and the pellet washed with 500 μL of cold (-20°C) 75% (v/v) ethanol. The contents of the tube were collected by brief centrifugation, the supernatant carefully removed, and the pellet dried at 37°C for 10 min.

2.3.17.5 Ligating the cDNA fragments to the lambda vector

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 cloned and screened in *E. coli* strain Y1090/ZL (Young and Davies, 1983). The selected cDNA can then be recovered in the autonomously replicating plasmid pZL1 by *in vivo* excision in *E. coli* strain DH10B(ZIP) utilising the *Cre-LoxP* recombination process (D'Alessio, et.al.,1992).

Materials

λZipLox arms digested with *Eco*R 1 (Life Technologies)
5x T4 DNA ligase buffer (Life Technologies)
T4 DNA ligase (Life Technologies)
DEPC-treated water

Method

Ligated cDNA was prepared as described in section 2.3.17.4 and the following components added at room temperature:

1 μL 5XT4 DNA ligase buffer
2 μL λZipLox, *Eco*R I Arms (250 ng/μL)
1 μL DEPC-treated water.

The reaction components were mixed by pipetting up and down. When the cDNA was completely dissolved, 1 μL of T4 DNA ligase was added and mixed by pipetting. The reaction was incubated for 3 h at room temperature

2.3.17.6 Packaging ligated cDNA

The ligated cDNA were packaged into phage particles for infection into *E coli*

Materials

MaxPlax packaging extract (Epicentre Technologies, Madison, Wi., USA)
SM phage buffer (section 2.3.1)
Chloroform
L agar plates (9 cm; section 2.3.1)

L top agar (section 2.3.1)
1 M $MgSO_4$
E. coli strain Y1090 (ZL)

Method

Ligated DNA was prepared as described in section 2.3.17.5. Two packaging extracts were removed from the -80°C freezer and allowed to thaw at room temperature. Immediately the extracts had thawed, 2.5 μL of the cDNA solution were added and mixed by pipetting up and down several times. The reactions were incubated at room temperature for 2 h, and 500 μL of SM phage buffer added and mixed by gentle vortexing. An aliquot of chloroform (25 μL) was then added and mixed by gentle vortexing.

The libraries were titred on *E. coli* strain Y1090 (ZL) plating bacteria. An aliquot of the packaged phage was diluted as follows:

10 μL phage in 990 μL SM (to make 10^{-2} phage)
10 μL of 10^{-2} phage in 990 μL SM (to make 10^{-4} phage)
100 μL of 10^{-4} phage in 900 μL of SM (to make 10^{-5} phage)
10 μL of 10^{-4} phage in 990 μL SM (to make 10^{-6} phage)

A 100 μL aliquot of each of the diluted phage solutions were added to 100 μL of the prepared plating bacteria (section 2.3.17.7) and incubated for 15 min at 37°C. The bacteria were then added to 3 mL of L top agarose supplemented with 30 μL $MgSO_4$, and poured onto pre-warmed L agar plates. The top agarose was allowed to solidify, the plates inverted and then incubated overnight at 37°C. The plaques were counted to determine the titre in pfu.

2.3.17.7 Preparing *E. coli* stocks and plating library

Materials

E. coli Y1090 (ZL)
9 cm L agar plate (section 2.3.1)
15 cm L agar plates (section 2.3.1)
L broth (section 2.3.1)
1 M $MgSO_4$

Maltose
L top agarose (section 2.3.1)

Method

An aliquot of *E. coli* Y1090 (ZL) was streaked onto L agar and grown overnight at 37°C. A single colony was recovered from the agar plate and grown overnight with vigorous shaking (200 rpm) at 37°C in L broth containing 100 μL $MgSO_4$ and 0.2 % (w/v) maltose. The cells were stored at 4°C for up to two weeks.

The *E. coli* cells were dispersed in the L broth, and 100 μL aliquots were transferred to microfuge tubes and incubated for 15 min at 37°C with sufficient phage solution to give approximately 20,000 plaques per plate (section 2.3.17.6). While these were incubating, 6.5 mL aliquots of melted L top agarose 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 L top agarose and then poured onto the pre-warmed (37°C) L agar in 15 cm plates. The plates were swirled to spread the agarose evenly, and when set, inverted and incubated at 37°C overnight.

2.3.17.8 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. This membrane ('filter') is then treated in a similar way to a Southern blot (section 2.3.11) and probed for homologous sequences as described previously.

Materials

2 x SSC (section 2.3.1)
Hybond N^+ membranes (Amersham)
Denaturation buffer:
0.5 N NaOH
0.5 M NaCl
Neutralisation buffer:
2.0 M NaCl

0.5 M Tris HCl pH 7.4

Method

Following overnight incubation, the plates containing the plated library (section 2.3.17.7) were placed at 4°C for a minimum of two hours. In a laminar flow hood a Hybond N^+ membrane filter was placed carefully onto the surface of the agarose avoiding any movement or slipping across the surface. Orientation holes were punched into the edge of the filter with syringe needle, and their positions were marked on the bottom of the plate with a felt pen. The filter was transferred, DNA side up, to 3 MM paper soaked in denaturation buffer, and incubated for 5 min. The filter was transferred to 3 MM paper soaked in neutralisation buffer and incubated for a further 5 min. The filter was then transferred to 3 MM paper soaked in SSC, incubated for a further 5 min, and dried on fresh 3MM paper in the laminar flow hood. The filters were hybridised with labelled DNA and autoradiographed as described in section 2.3.11.5.

2.3.17.9 Plaque purification

Areas or plaques which gave positive signals on the autoradiograph were identified on the cDNA library plates. In the primary screen, these areas were removed from the plate using the wide opening of a sterile pasteur pipette In secondary and subsequent screens, 1 mL pipette tips with the tip cut so that the opening approximated the size of the plaque were used. The pipette was pushed into the top agarose to isolate a small region, and this agarose was transferred to a microfuge tube containing 500 µL SM buffer, vortexed briefly, and held at room temperature for at least 1 h. The isolated plaque solutions were titred and re-screened as described previously, until single isolated positive plaques could be identified on the plate.

2.3.17.10 Amplifying and storing the lambda library

Each isolated plaque identified on the screening plates (ignoring contamination from overlapping phage particles) arose from a single lambda particle produced from a single isolated cDNA fragment. However, the plaque

was formed from the amplification of the phage particles in the *E. coli* cells, and the subsequent bursting of those cells. The plaque therefore contains millions of copies of the original lambda particle and the inserted cDNA fragment. Collecting these particles therefore provides an amplified collection of cDNA inserts from the original library.

Materials

SM buffer (section 2.3.1)
Chloroform

Method

Amplified phage were collected from the primary screening plates (section 2.3.17.3) by adding 3.5 mL (7 mL for 15 cm plates) of SM buffer and incubating the dishes at 4°C overnight. The SM buffer containing the phage was transferred to sterile 15 mL Falcon tubes. The plates were washed with a further 2 mL (or 5 mL) of SM buffer and the wash added to the 15 mL tube. Chloroform was added to give a final volume of 0.3%, the tube vortexed briefly, and then centrifuged at 4000 x g for 10 min at 4°C. The supernatant was carefully transferred to a fresh 15 mL tube, chloroform added to give a final concentration of 0.3%, and the amplified library or sub-library stored at 4°C.

2.3.17.11 Excising pZL1 from positive λZipLox clones

λZipLox is a modified λgt11 containing the plasmid cloning vector pSPORT 1 flanked by *lox*P sequences (Abremski, *et al.*, 1983; Hoess and Abremski, 1985). When *E. coli* containing the plasmid pZIP (containing a gene coding for the recombination protein 'Cre', for example *E. coli* DH10B(ZIP), Life Technologies) are infected with λZipLox, the Cre protein acts on the *lox*P sequences and excises and circularises the pZL1 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.

Materials

E. coli Y1090 (ZL) cells (section 2.3.17.7)
E. coli DH10B(ZIP) cells
Kanamycin (10 mg/mL)
Ampicillin (10 mg/mL)
1 M $MgCl_2$
SM buffer (section 2.3.1)
L broth (section 2.3.1)
L agar (section 2.3.1)

Method

E. coli DH10B(ZIP) cells were streaked onto L agar plates containing 10 μg/mL kanamycin. The cells were prepared for infection by inoculating a single colony into 10 mL L broth containing 10 μg/mL kanamycin, and incubating at 37°C overnight with shaking (200 rpm).

Purified plaques (section 2.3.17.9) were plated onto *E. coli* Y1090 (ZL) and a single isolated plaque selected and removed using a 1 mL pipette tip cut to the size of the plaque. The plaque was transferred to a microfuge tube containing 250 μL of SM buffer, vortexed vigorously for 10 s, and incubated at room temperature for 5 min. The contents of the tube were collected by brief 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 an L agar plate containing 10 mM $MgCl_2$ and 100 μg/mL ampicillin, and the plate inverted and incubated overnight at 37°C. An isolated colony was selected from the plate and used to inoculate 10 mL of L broth. The culture was incubated overnight with vigorous shaking (200 rpm) at 37°C, and the plasmids extracted and purified as described in section 2.3.3, then further purified using a phenol-chloroform partition. The plasmids were then sent to the University of Otago for automatic sequencing, using the universal forward primer and dye termination sequencing.

CHAPTER 3

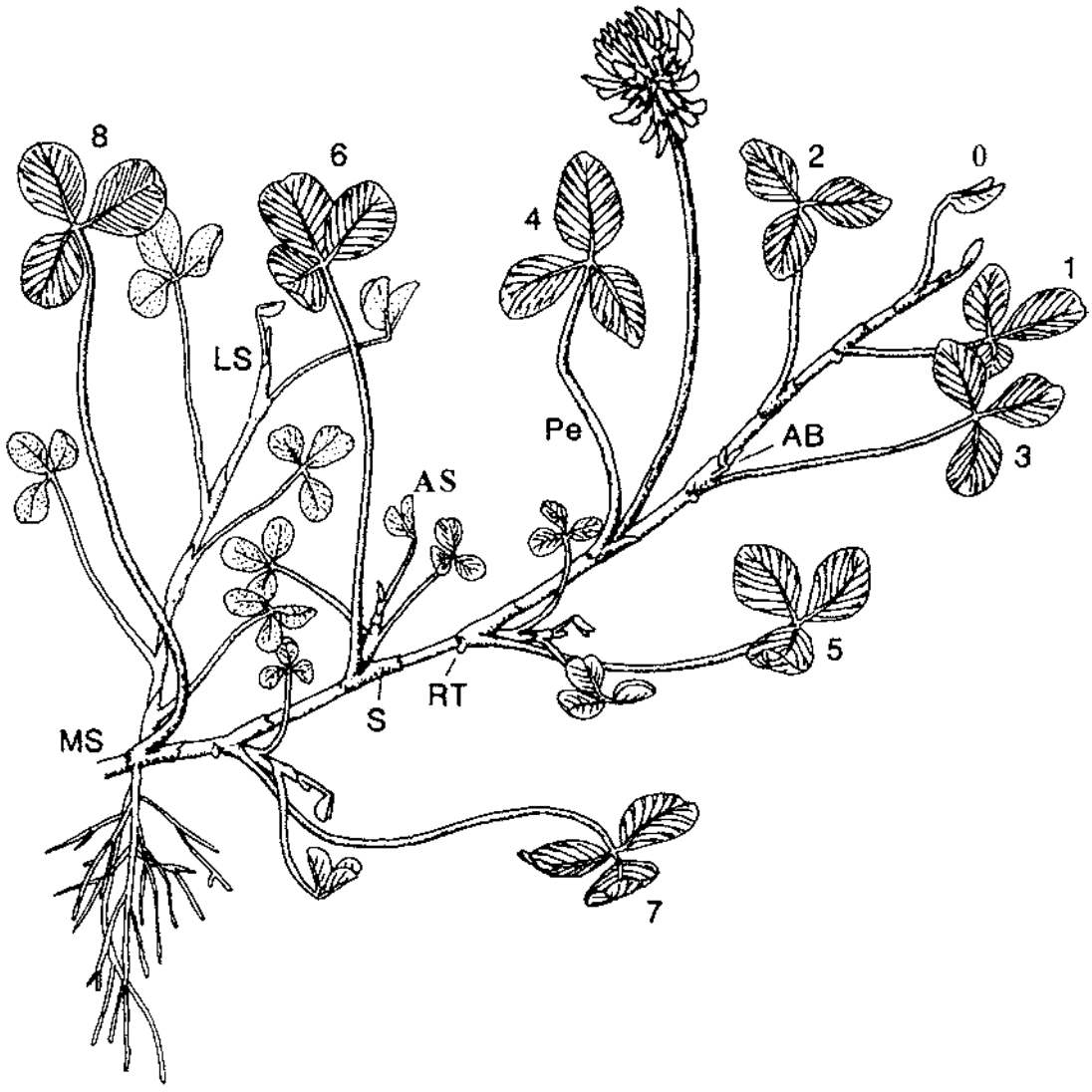
PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISATION OF ETHYLENE BIOSYNTHESIS IN LEAVES OF WHITE CLOVER

3.1 Stolon growth and plant selection

3.1.1 Introduction

Stolon growth in white clover is indeterminate (Figure 3.1). In pastures, white clover can grow into large complex plants with up to 6 orders of branching and this complexity changes in an annual cycle (Brock *et al*, 1988). For example, Hay *et al.* (1989b) found that shoot dry weight per area of pasture was at a minimum in spring (the result of accelerated death of older buried stolon material), and a maximum in summer. In addition, Hay *et al.* (1989a), showed that within the white clover population the proportion of plants of first branching order increased, and that of third order plants decreased during spring-early summer compared with the rest of the year.

Stolon growth and maintenance is dependent to a large extent on the root systems. Thomas (1987b) observed that when "the root primordium at a node is in contact with a moist substratum it grows out into a nodal root". Roots have a large impact on the development of the white clover plant. For example, branching and nodal rooting have been shown to be strongly correlated (Chapman, 1983). Further, the natural decay (senescence) of old sections of the stolon proceeds in waves which are interrupted by the presence of strongly rooted nodes (Sackville Hamilton *et al.*, 1989), and in white clover growing in pasture, persistence was improved by a healthy stolon



MS= Main Stolon. RT=Root. Pe=Petiole. S=Sheath.
LS=Lateral Stolon. AS=Axillary Shoot. AB=Axillary Bud.

Figure 3.1: Drawing of a clover plant modified from Thomas (1987).

Leaves are numbered as descibed in section 2.1.3

adventitious root system (Gibson and Trautner, 1965; James, *et al.*, 1980). The vascular traces from the nodal roots are also associated with leaves (Thomas, 1987b) hence the presence of nodal roots may affect the development and senescence pattern of leaves.

The cultural methods used in this study were selected so as to provide plants of a simplified plant structure suitable for the study of ethylene production in leaves at different stages of development. This section describes the cultivation methods, and the selection and characteristics of a genotype of white clover suitable for the experiments in this study.

3.1.2 Stolon growth of white clover

Two stolons with all axillary stolons and flowers removed growing from white clover plants in trays (section 2.1.2) were trained across upturned trays which provide a dry substratum that inhibits nodal root growth (Figure 2.1). White clover plants grown using the method described here produce leaves at all stages of development along a single stolon from initiation at the apex, through expansion, maturity, senescence and then necrosis (Figure 3.2).

3.1.3 Genotype evaluation

White clover has a well studied gametophytic self-incompatibility system and can generally be described as self-sterile (a small proportion of plants may set some seed after self-pollination). Hence, white clover cultivars are usually synthetic cultivars based on polycrosses of three or more parent plants, or bulks of three or more seed lines (Williams, 1987b). Individual seeds of a cultivar are therefore individual genotypes. In this study, ten individual plants from each of eleven cultivars of white clover were grown at Levin using the method described in section 2.1.2. Cultivars were numbered 1 to 11, and each genotype within a cultivar labelled alphabetically (Table 3.1).

Cuttings were taken on 6th July 1992 (day 0) and potted into trays on day 28. Data on stolon length, leaf (node) appearance, internode length, and the number of leaves maintained on each stolon were collected. The first of the weekly measurements was taken on day 42 and the last was taken on day

Figure 3.2: Pattern of leaf development obtained when growing white clover stolons of genotype 10F using the method described in section 2.1.2.

Table 3.1: White clover cultivars assessed at Levin

Cultivar Number	Name	Country of Origin
1	Gwenda	United Kingdom
2	Trifo	Denmark
3	Ladino Giganteum Lodigiano	Italy
4	Viglasska	Czechoslovakia
5	Huia	New Zealand
6	Isolation V	New Zealand
7	Dusi	South Africa
8	Crau	France
9	El Lucero Inta	Argentina
10	Grasslands Challenge	New Zealand
11	Luclair	France

98. The variation in growth measurements (stolon length, internode length, and the number of nodes produced) within cultivars was greater than the differences between cultivars (Tables 3.2, 3.3 and 3.4 respectively). These data show that individual genotypes within the white clover cultivars examined exhibit wide variation when grown under the same environmental conditions.

As no significant differences were observed between cultivars, data were pooled for all genotypes and cultivars. The mean stolon length increase was nearly linear with time after day 14, and increased about 50 mm every seven days (Figure 3.3). After day 56, individual genotypes varied in stolon length from a minimum of 110 mm (genotype 7E) to a maximum of 1035 mm (genotype 6B). Data on stolon growth was complicated by differences in timing of initiation of stolon growth between individual genotypes. Stolons appeared within a few days from the appearance of roots on cuttings of some genotypes, whereas the appearance of stolons in other genotypes was delayed for some weeks.

The mean length of internodes increased from about 11 mm to about 20 mm in the first 35 days, and then increased more slowly to about 24 mm by day 56 (Figure 3.4). Genotype 6B had the longest mean internode length (54.5 mm) and genotype 7E had the shortest (7.9 mm) after 56 days. The mean number of nodes increased linearly over the assessment period from about 4 nodes at day 0 to about 16 nodes at day 56 (Figure 3.5), giving a mean node appearance rate of 1.5 nodes per week. The number of nodes ranged from 11 (genotype 2H) to 23 (genotype 3A). The node appearance rate is equivalent to the leaf appearance rate as one leaf is produced per node (Thomas, 1987b). The mean leaf (node) appearance rate therefore varied between the genotypes from 1.4 to 2.9 leaves/week.

In "young" stolons (stolons with up to eight nodes), a single green leaf (green leaves are defined as those with 50% or more green area on the leaflets using a visual assessment) was attached to each node. In older stolons, the number of green leaves was maintained at a constant number as the leaf appearance rate was balanced by the leaf senescence rate. The average number of green leaves maintained on "mature" stolons (stolons with 6 or more nodes with senesced leaves) was 8.8 (Table 3.5), but the number

Table 3.2: Average stolon lengths for cultivars grown at Levin

Cultivar	Day								
	0	7	14	21	28	35	42	49	56
1	55* +/-5	72 +/-7	109 +/-11	153 +/-17	200 +/-22	251 +/-26	301 +/-30	353 +/-34	402 +/-38
2	50 +/-10	67 +/-10	92 +/-11	134 +/-14	183 +/-17	242 +/-22	295 +/-28	332 +/-35	356 +/-41
3	46 +/-6	71 +/-8	107 +/-13	149 +/-20	196 +/-25	251 +/-28	318 +/-33	370 +/-43	422 +/-45
4	52 +/-14	86 +/-16	128 +/-22	163 +/-35	221 +/-41	315 +/-57	399 +/-73	470 +/-81	623 +/-91
5	44 +/-6	64 +/-8	102 +/-10	148 +/-11	205 +/-12	263 +/-13	313 +/-17	357 +/-24	405 +/-35
6	77 +/-13	97 +/-13	139 +/-15	186 +/-20	237 +/-27	294 +/-37	348 +/-47	411 +/-56	487 +/-78
7	40 +/-7	58 +/-8	79 +/-12	111 +/-17	150 +/-25	189 +/-31	228 +/-36	266 +/-49	324 +/-57
8	48 +/-5	64 +/-6	91 +/-11	131 +/-15	169 +/-19	212 +/-23	268 +/-29	311 +/-32	364 +/-34
9	42 +/-14	74 +/-18	115 +/-29	116 +/-40	216 +/-48	245 +/-59	299 +/-60	375 +/-111	435 +/-104
10	43 +/-11	43 +/-13	52 +/-15	66 +/-20	74 +/-23	79 +/-25	87 +/-28	145 +/-35	459 +/-36
11	45 +/-9	56 +/-10	74 +/-11	102 +/-13	133 +/-17	171 +/-20	214 +/-23	269 +/-25	316 +/-28

* Measurements shown in mm plus and minus standard error of the sample mean.

Table 3.3: Average internode lengths for cultivars grown at Levin

Cultivar	Day								
	0	7	14	21	28	35	42	49	56
1	10.1 +/-0.7*	12.0 +/-0.6	14.6 +/-1.2	17.5 +/-1.7	19.5 +/-1.8	21.4 +/-1.8	21.7 +/-1.8	22.9 +/-2.0	23.0 +/-1.9
2	11.0 +/-1.6	12.4 +/-1.4	13.3 +/-1.3	16.7 +/-2.3	20.1 +/-2.9	22.6 +/-3.2	23.9 +/-3.5	23.4 +/-3.4	23.4 +/-4.8
3	10.7 +/-0.8	12.2 +/-0.7	14.4 +/-1.2	16.5 +/-1.2	18.5 +/-1.5	20.4 +/-1.4	22.1 +/-1.2	23.1 +/-1.2	23.2 +/-1.2
4	13.7 +/-2.3	16.5 +/-2.7	19.1 +/-3.2	19.1 +/-3.7	22.5 +/-3.5	26.9 +/-4.2	29.5 +/-4.3	30.0 +/-4.6	34.6 +/-5.1
5	9.4 +/-0.8	11.6 +/-0.7	14.7 +/-1.1	17.4 +/-1.1	20.4 +/-1.6	22.8 +/-1.2	23.9 +/-1.1	23.9 +/-1.3	24.2 +/-1.8
6	14.6 +/-1.9	15.6 +/-1.7	17.5 +/-1.8	20.2 +/-2.1	22.9 +/-2.7	25.1 +/-3.1	24.8 +/-3.3	25.8 +/-3.1	28.2 +/-3.9
7	10.2 +/-1.4	11.6 +/-1.4	12.3 +/-1.6	14.3 +/-1.8	15.9 +/-2.3	19.1 +/-3.1	19.8 +/-2.9	19.8 +/-3.2	21.6 +/-3.8
8	11.4 +/-1.3	11.8 +/-1.2	13.3 +/-1.6	16.0 +/-1.8	19.1 +/-2.3	19.7 +/-2.1	22.4 +/-1.6	22.2 +/-2.2	24.0 +/-2.1
9	10.5 +/-2.6	13.7 +/-2.1	17.7 +/-4.1	20.9 +/-4.1	23.8 +/-5.2	22.3 +/-4.8	24.9 +/-4.3	26.8 +/-7.9	27.7 +/-6.6
10	10.7 +/-1.6	11.4 +/-1.2	13.8 +/-1.2	17.1 +/-1.8	21.4 +/-2.3	24.0 +/-2.6	24.4 +/-2.4	26.4 +/-2.7	26.5 +/-1.9
11	10.3 +/-1.6	9.2 +/-1.2	10.8 +/-1.2	11.1 +/-1.1	13.2 +/-1.2	15.7 +/-1.4	16.6 +/-1.4	18.5 +/-1.6	17.7 +/-1.4

* Measurements shown in mm plus and minus one standard error of the sample mean.

Table 3.4: Average number of nodes for cultivars grown at Levin

Cultivar	Day								
	0	7	14	21	28	35	42	49	56
1	5.4 +/-0.3*	6.0 +/-0.4	7.4 +/-0.4	8.7 +/-0.4	10.2 +/-0.5	11.7 +/-0.5	13.8 +/-0.5	15.4 +/-0.6	17.4 +/-0.6
2	4.5 +/-0.6	5.5 +/-0.8	6.9 +/-0.6	8.4 +/-0.6	9.8 +/-0.8	11.3 +/-0.7	13.1 +/-0.8	14.8 +/-0.8	16.6 +/-1.3
3	4.2 +/-0.3	5.8 +/-0.4	7.5 +/-0.7	8.9 +/-0.6	10.4 +/-0.5	12.1 +/-0.6	14.3 +/-0.9	15.8 +/-1.0	18.2 +/-1.4
4	4.0 +/-1.0	4.8 +/-0.6	6.5 +/-0.4	8.0 +/-0.6	9.2 +/-0.6	11.0 +/-0.6	12.8 +/-0.8	15.0 +/-0.6	17.3 +/-0.7
5	4.3 +/-0.4	5.3 +/-0.5	6.7 +/-0.3	8.1 +/-0.5	9.6 +/-0.4	11.1 +/-0.4	12.8 +/-0.4	14.4 +/-0.5	16.3 +/-0.7
6	5.7 +/-0.9	6.5 +/-0.8	8.1 +/-0.8	9.4 +/-0.8	10.5 +/-0.6	12.0 +/-0.9	14.1 +/-0.7	15.9 +/-0.8	17.1 +/-0.8
7	3.9 +/-0.3	5.3 +/-0.6	6.5 +/-0.5	7.8 +/-0.5	9.3 +/-0.7	9.9 +/-0.6	11.4 +/-0.8	13.2 +/-1.0	15.0 +/-1.1
8	4.4 +/-0.4	5.7 +/-0.4	7.0 +/-0.4	8.4 +/-0.4	9.2 +/-0.4	11.0 +/-0.3	12.4 +/-1.1	14.5 +/-0.7	15.6 +/-0.8
9	3.5 +/-0.4	5.0 +/-0.6	6.3 +/-0.5	7.5 +/-0.7	8.8 +/-0.5	10.6 +/-0.5	11.8 +/-0.6	14.0 +/-0.0	15.2 +/-0.7
10	3.6 +/-0.5	4.7 +/-0.5	6.2 +/-0.5	7.4 +/-0.7	8.4 +/-0.7	10.1 +/-0.7	12.0 +/-0.7	13.0 +/-0.9	14.9 +/-1.0
11	4.4 +/-0.5	6.0 +/-0.5	6.8 +/-0.5	9.1 +/-0.7	10.0 +/-0.7	10.8 +/-0.7	13.0 +/-0.9	14.9 +/-1.2	18.0 +/-1.0

*Measurements shown plus and minus one standard error of the sample mean.

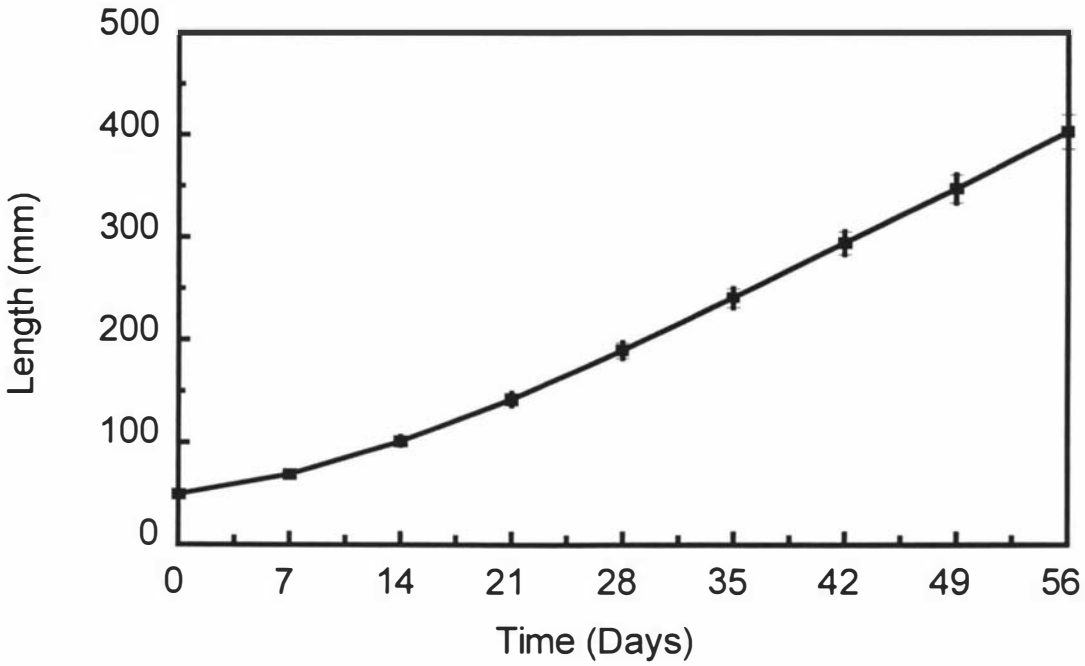


Figure 3.3: Mean stolon length for all genotypes grown at Levin.

Bars represent two standard errors of the sample mean.

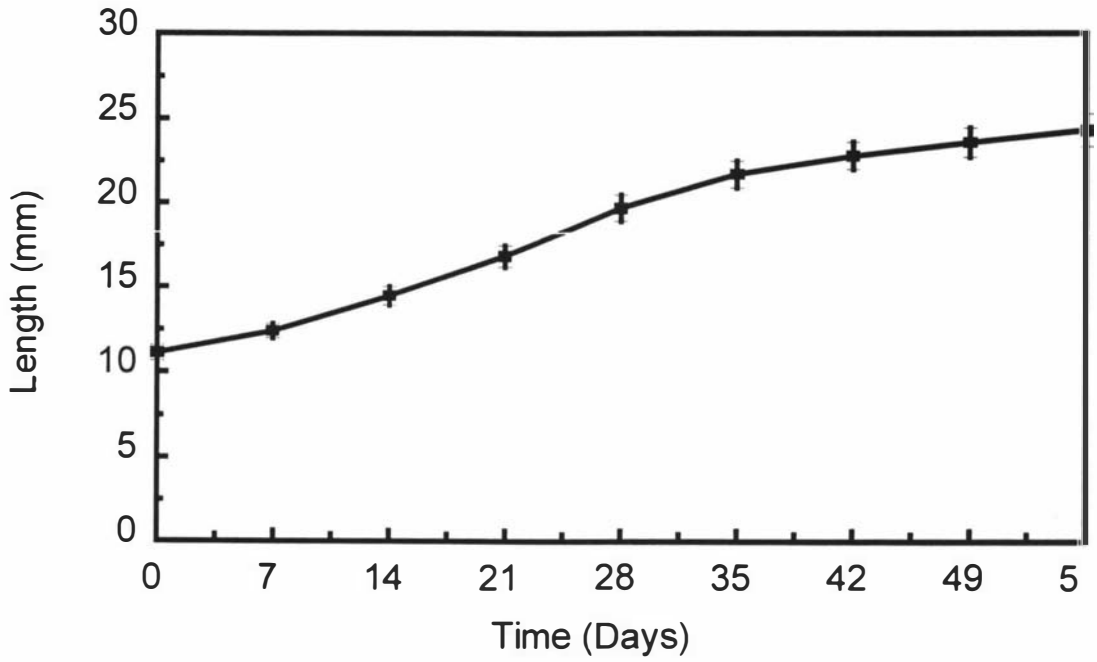


Figure 3.4: Mean internode length for all genotypes grown at Levin.

Bars represent two standard errors of the sample mean.

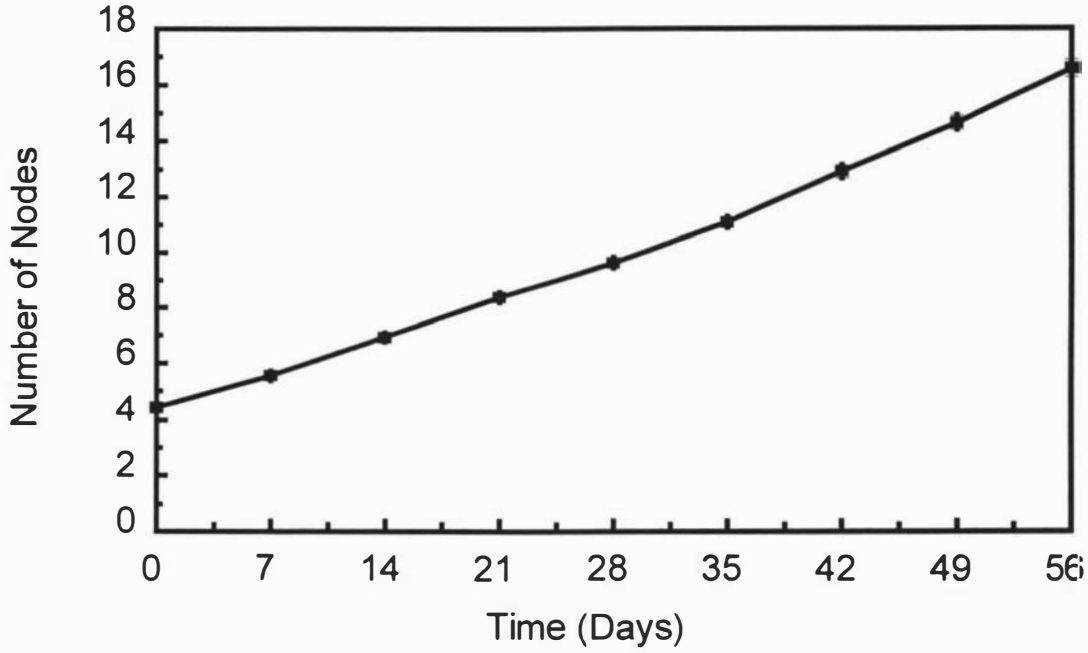


Figure 3.5: Mean number of nodes for all genotypes grown at Levin.

Bars represent two standard errors of the sample mean.

Table 3.5: Average growth measurements for genotype 10F compared with the average growth measurements for all genotypes at harvest (plants grown at Levin)

Genotype	Stolon length (mm)	Internode length (mm)	Number of nodes	Number of green leaves
Average of all genotypes	402.4 +/- 16.8*	24.4 +/- 1.0	16.6 +/- 0.3	8.8 +/- 0.26
10F	367.2 +/- 12.1	27.3 +/- 0.9	13.5 +/- 0.3	9.85 +/- 0.11

* values shown plus and minus one standard error of the sample mean.

ranged from 4 (genotypes 9A, 10D and 5D) to 15 (genotype 4C) leaves. There was no significant difference in the number of green leaves maintained on the stolons between the cultivars.

3.1.4 Genotype selection

In addition to the measurements of growth (section 3.1.2), the genotypes were visually assessed for factors considered important for subsequent studies. The genotypes were assessed for relative leaf size (larger leaves giving more material for analysis) and stolon diameter, and anthocyanin production in the stolon (high concentrations of anthocyanin can interfere with some extraction procedures). Genotype 10F was selected as suitable for physiological and molecular investigations as it has an above average growth rate, maintains 8 green (> 50% green) and 2 green-yellow (10-50% green) leaves on the mature stolon (Table 3.6), and has relatively large leaves and thick stolons with little obvious anthocyanin production. This genotype was initially grown in greenhouses at Levin.

3.1.5 Growth of 10F at Levin

Cuttings of genotype 10F were prepared on the 14th of September, 1992 (day 0), potted into trays, and placed into the growing house on day 42. The stolons were trained over upturned trays as described previously (section 3.1.2; Figure 2.1) and leaves harvested on days 85 and 86. The leaf colour, fresh weights and dry weights are shown in Table 3.6. Leaf fresh weight increases up to leaf six and then decreases slightly, and this pattern is reflected in the dry weight changes. Both leaf fresh weight and dry weight showed increasing variation with leaf age, while the weights tended to decrease or remain constant after leaf six. The mean length of the stolons at day 56 was 367.2 mm, and the number of leaves with 10% or more green area maintained on the stolon was 9.85 (Table 3.5). The mean internode length increased from node one to node two, and then showed little or no increase from node two to node ten (Figure 3.6). The average length of the petioles increased rapidly from node one to node three, increased more slowly from node three to node seven, and then remained constant from node seven to node ten (Figure 3.7). The variation in the length of the petiole

Table 3.6: Leaf colour, fresh weight and dry weight for genotype 10F grown at Levin

Leaf number	Leaf colour	Fresh weight (mg)	Dry weight (mg)
1	100% green	83.3 +/- 7.9*	15.7 +/- 1.4
2	100% green	172.5 +/- 11.4	35.5 +/- 2.4
3	100% green	217.8 +/- 13.4	47.1 +/- 2.6
4	100% green	228.3 +/- 12.7	50.5 +/- 2.7
5	100% green	258.1 +/- 15.6	58.3 +/- 3.3
6	50-100% green	275.4 +/- 15.6	63.0 +/- 3.5
7	50-100% green	266.6 +/- 18.1	60.0 +/- 3.9
8	50-100% green	236.5 +/- 21.8	57.9 +/- 4.8
9	10-50% green	229.7 +/- 23.2	53.0 +/- 4.8
10	10-50% green	230.3 +/- 24.5	54.7 +/- 5.2

*Measurements shown plus and minus one standard error of the sample mean

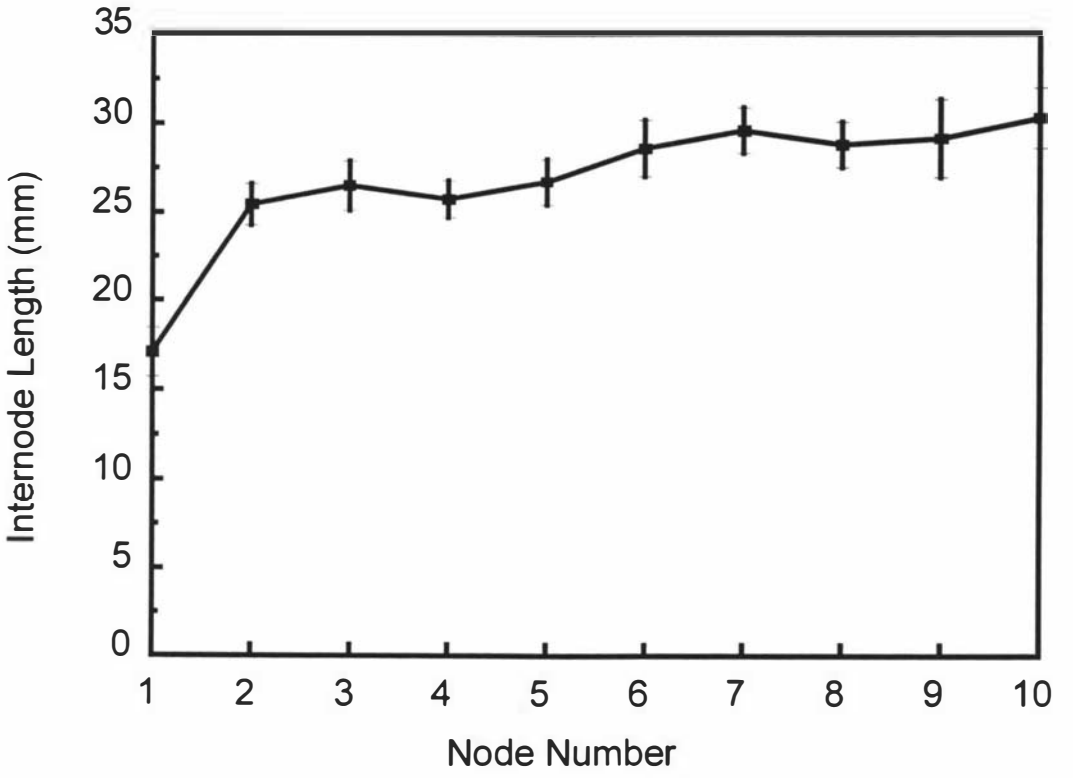


Figure 3.6: Mean internode length for genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

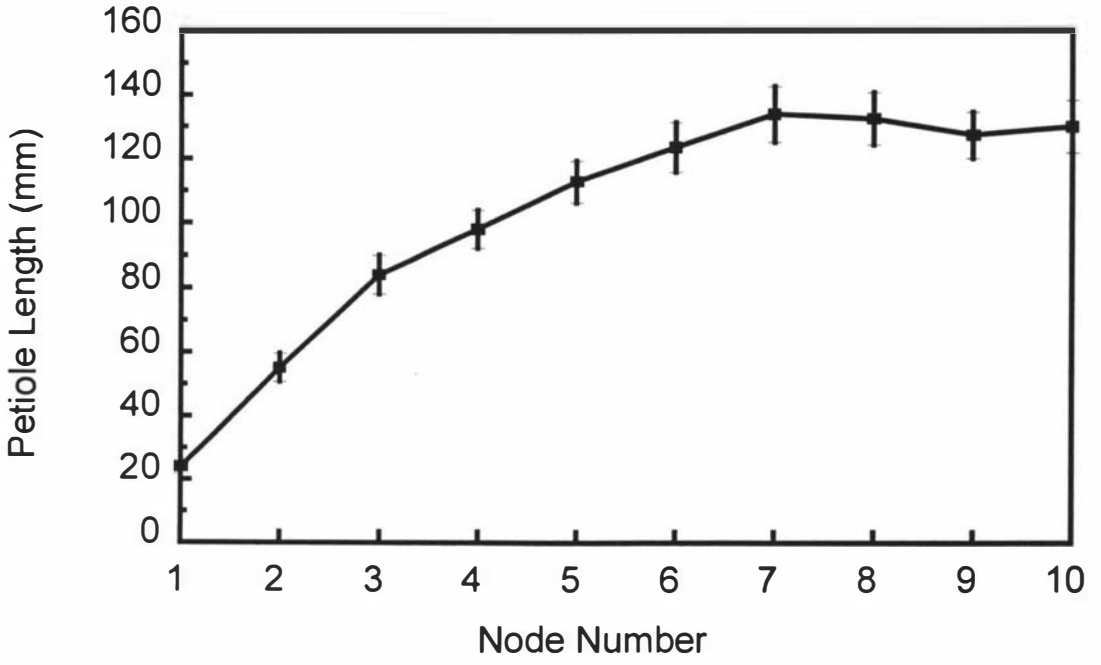


Figure 3.7: Mean petiole length for genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

increased with the increasing length of the petiole.

For genotype 10F, the cultural method used resulted in a pattern of leaf development from leaf initiation at the apex, to green leaves at node two to node five, and leaves from node six to node ten exhibiting progressively more de-greening (Figure 3.2). The total chlorophyll, and chlorophyll *a* and *b* concentration in the leaves follows the pattern of de-greening visible in Figure 3.2 (Figures 3.8, 3.9 and 3.10 respectively). The total chlorophyll concentration increased from leaf one to leaf five, declined rapidly from leaf five to leaf seven, and remained relatively constant from leaf seven to leaf ten.

The variation in chlorophyll concentration was high for leaf one, low for leaves two, three, and four, and rose sharply for leaves five to ten. The change in total chlorophyll concentration results from changes in both chlorophyll *a* and *b* concentration. The chlorophyll *b* concentration represents about 20% of the total chlorophyll present in the leaves, and was consistent in all leaves except for leaves one and two, where the ratio is lower (Table 3.7). The lowest concentration of chlorophyll *b* (12.6%) was measured in leaf number one, and the highest concentration (21.2%) was measured in leaf number five.

3.1.6 Growth of 10F at Palmerston North

Characteristics of individual genotypes within each of the white clover cultivars exhibited wide variation when grown under common environmental conditions at Levin (section 3.1.2). In contrast, clonally grown plants of genotype 10F exhibited little variation in these characteristics (section 3.1.4). Plants of genotype 10F were also grown in greenhouses at Palmerston North, 50 kilometres north-east of Levin. Greenhouse heating and venting settings, and the cultural procedures, were similar to those at Levin (Table 2.1). Potting media was prepared at Levin as described in section 2.1.1, and transported to Palmerston North for use. The major identifiable difference in the growing conditions (apart from factors associated with the location) was the watering system (section 2.1.2). The method of watering container grown plants has a significant effect on plant growth and dry weight, and nutrient availability (Hicklenton and McRae, 1989; Hicklenton and Cairns, 1996).

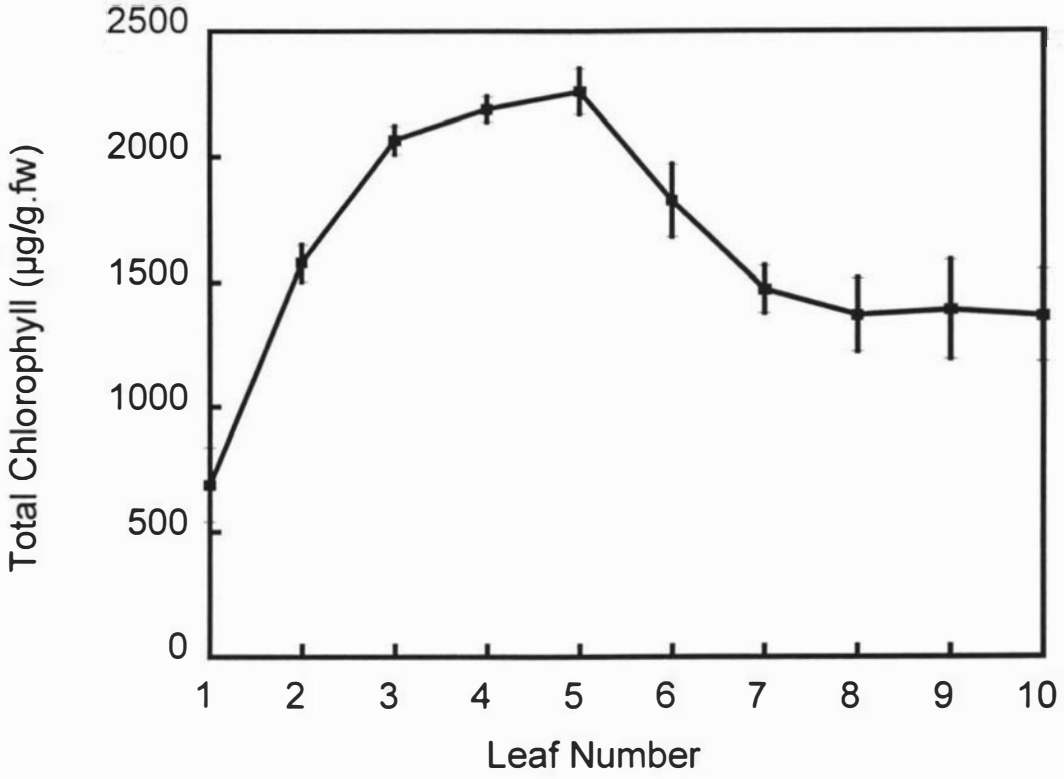


Figure 3.8: Mean total chlorophyll concentration in leaves of genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

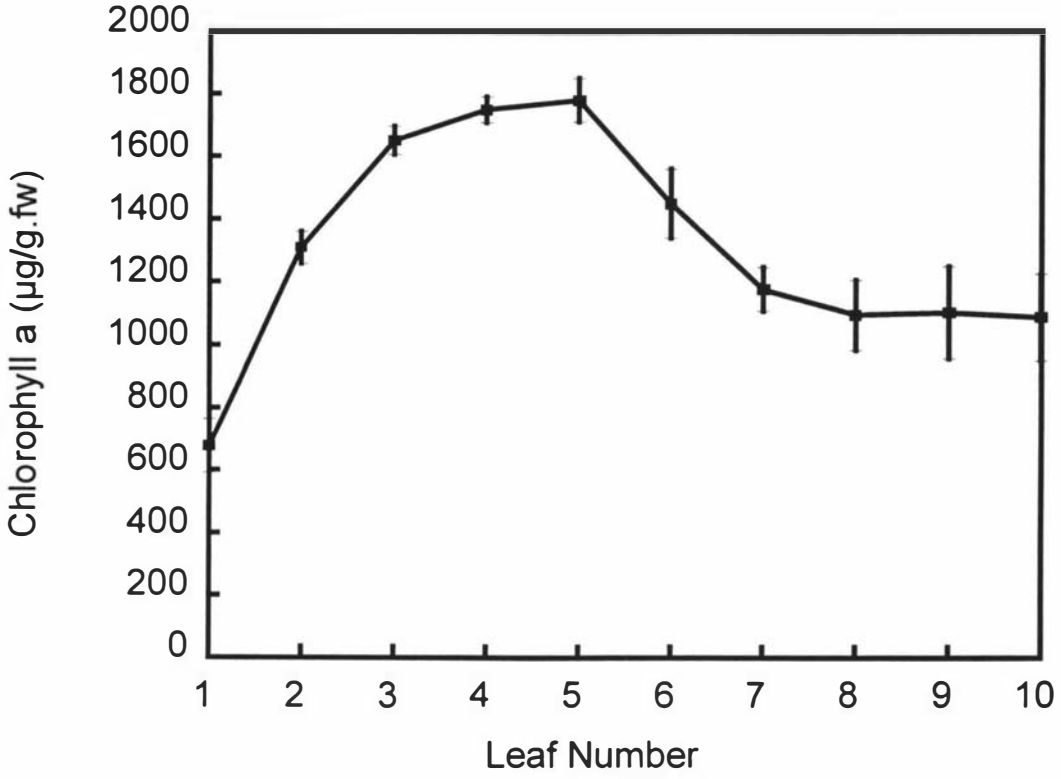


Figure 3.9: Mean chlorophyll *a* concentration in leaves of genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

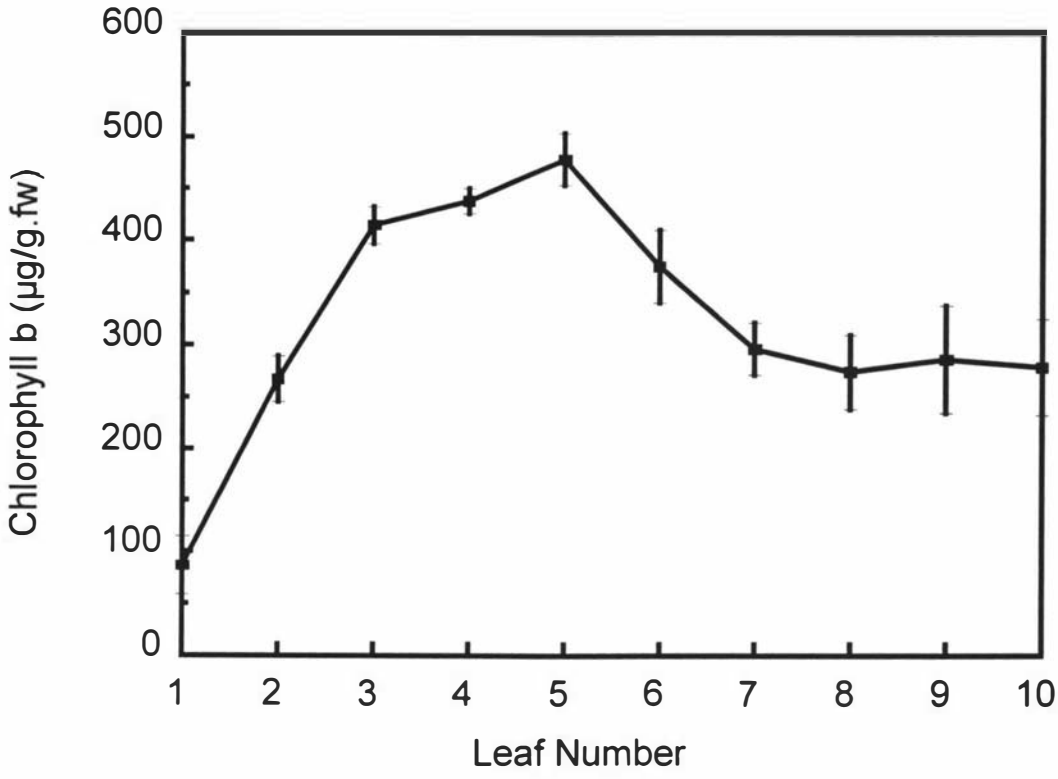


Figure 3.10: Mean chlorophyll *b* concentration in leaves of genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

Table 3.7: Ratio of chlorophyll *b* to total chlorophylls, and the chlorophyll *a*:*b* ratios in leaves of white clover from plants grown at Levin and Palmerston North.

Leaf Number	Chlorophyll *b*: total chlorophylls (percent)		Chlorophyll *a*:*b* ratios	
	Levin	Palmerston North	Levin	Palmerston North
1	12.6	22.6	7.83	3.43
2	17.0	24.6	4.89	3.07
3	20.1	27.5	3.98	2.63
4	20.0	32.9	4.00	2.04
5	21.2	32.6	3.72	2.07
6	20.6	36.2	3.87	1.76
7	20.2	38.7	3.96	1.58
8	20.1	40.7	3.99	1.46
9	20.6	38.5	3.85	1.60
10	20.4	35.2	3.90	1.84
11		33.7		1.97
12		29.0		2.44
13		28.3		2.54
14		27.3		2.67
15		26.9		2.72

The leaf colour and leaf fresh weights for plants grown at Palmerston North are shown in Table 3.8. More green leaves (10% or more area green) were maintained on stolons grown at Palmerston North (14.57 +/- 1.99) compared with plants grown at Levin (9.85 +/- 0.11). The fresh weight of leaves harvested at Palmerston North increased rapidly from leaf one to leaf four, increased more slowly from leaf four to leaf eleven, and then declined from leaf eleven to leaf fifteen. The maximum average leaf fresh weight was greater for plants grown at Palmerston North (approximately 700 mg) compared with those grown at Levin (approximately 275 mg) but the pattern of change in the leaf weight is similar. The variation in leaf fresh weight was small, with the largest variation occurring at leaf fifteen.

The total chlorophyll, and chlorophyll *a* and *b* concentration in leaves harvested at Palmerston North are shown in Figures 3.11, 3.12, and 3.13 respectively. The total chlorophyll concentration increased rapidly from leaf one to leaf two, remained constant from leaf two to leaf eight, declined rapidly from leaf eight to leaf nine, remained constant from leaf nine to leaf eleven, and then declined slowly from leaf 11 to leaf 15. The chlorophyll changes are due to changes in both chlorophyll *a* and *b* as the changes in chlorophyll *a* and *b* follows the same pattern as the changes in total chlorophyll.

The maximum average total chlorophyll concentration for leaves from plants grown in Palmerston North was about 1750 μg/g.fw (Figure 3.11), compared with about 2250 μg/g.fw for leaves from plants grown at Levin (Figure 3.8). The major difference in chlorophyll concentration between plants grown at Levin and plants grown at Palmerston North was the concentration of chlorophyll *a*. The higher concentration of chlorophyll *a* in leaves from plants grown at Levin is reflected in the ratio of chlorophyll *b* to total chlorophyll concentration (Table 3.7). The ratio of chlorophyll *b* to total chlorophylls in leaves from plants grown at Palmerston North was higher than the ratio in leaves from plants grown in Levin. In fully developed leaves (i.e. excluding leaf one), the ratio of chlorophyll *b* was more variable in leaves from plants grown at Palmerston North (24.6% for leaf two to 40.7% for leaf eight) compared the ratio in leaves from plants grown at Levin (17% for leaf two to 21.2% for leaf five). The difference in the concentration of chlorophyll *a* between plants grown at Levin compared with plants grown at Palmerston

Table 3.8: Leaf colour and fresh weight for genotype 10F grown at Palmerston North

LEAF NUMBER	LEAF COLOUR	FRESH WEIGHT (MG)
1	100% green	52.5 +/- 2.6*
2	100% green	169.1 +/- 5.5
3	100% green	291.0 +/- 6.4
4	100% green	365.1 +/- 8.3
5	100% green	401.2 +/- 8.9
6	100% green	439.7 +/- 8.6
7	100% green	481.6 +/- 9.0
8	100% green	527.9 +/- 9.4
9	50-100% green	578.6 +/- 10.0
10	50-100% green	650.7 +/- 12.8
11	50-100% green	691.0 +/- 15.8
12	50-100% green	680.7 +/- 14.9
13	50-100% green	641.3 +/- 17.8
14	10-50% green	623.5 +/- 18.1
15	10-50% green	624.1 +/- 24.7

*Measurements shown plus and minus one standard error of the sample mean

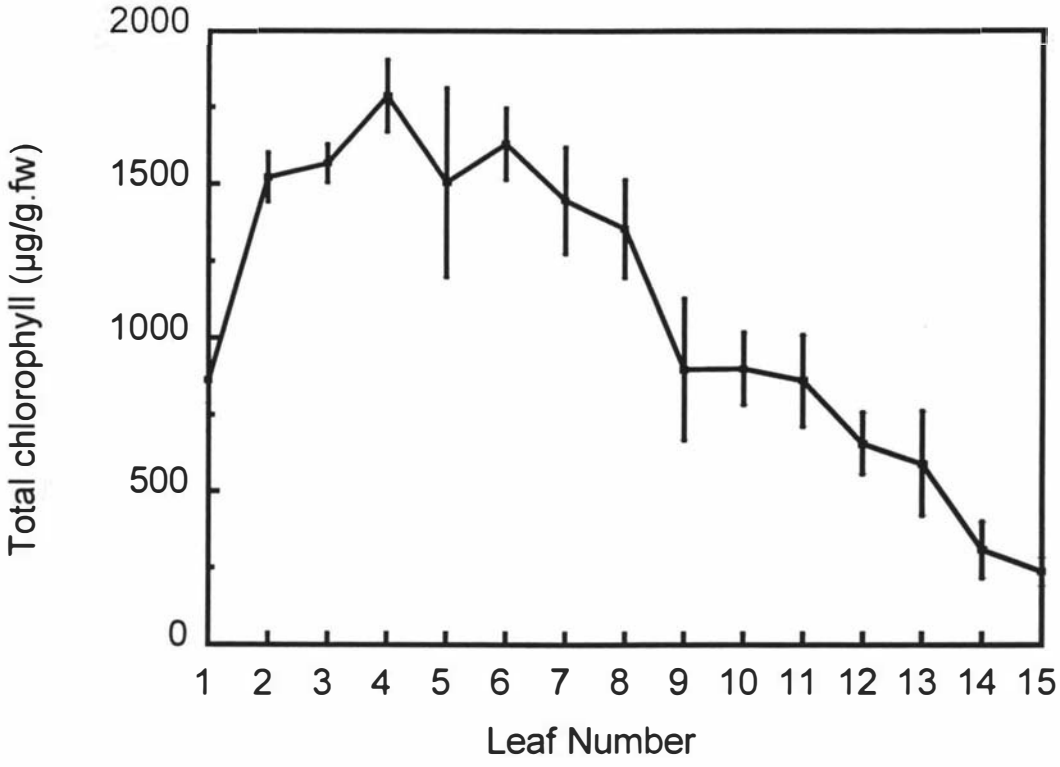


Figure 3.11: Mean total chlorophyll concentration in leaves of genotype 10F grown at Palmerston North.

Bars represent two standard errors of the sample mean.

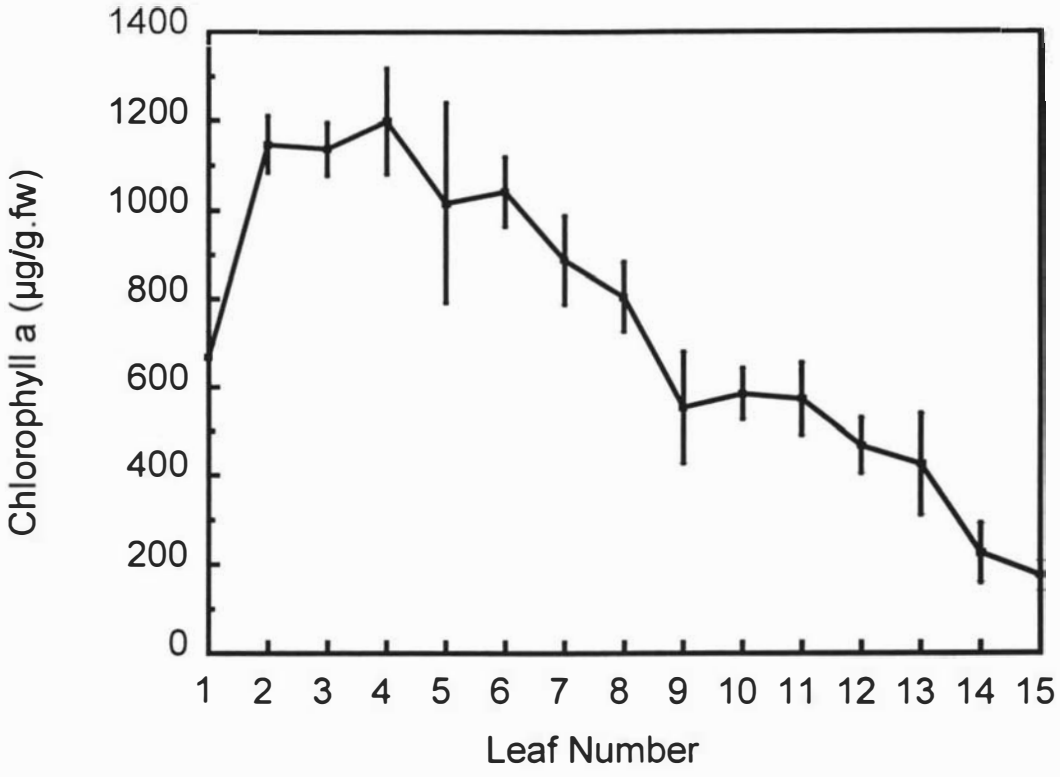


Figure 3.12: Mean chlorophyll *a* concentration in leaves of genotype 10F grown at Palmerston North.

Bars represent two standard errors of the sample mean.

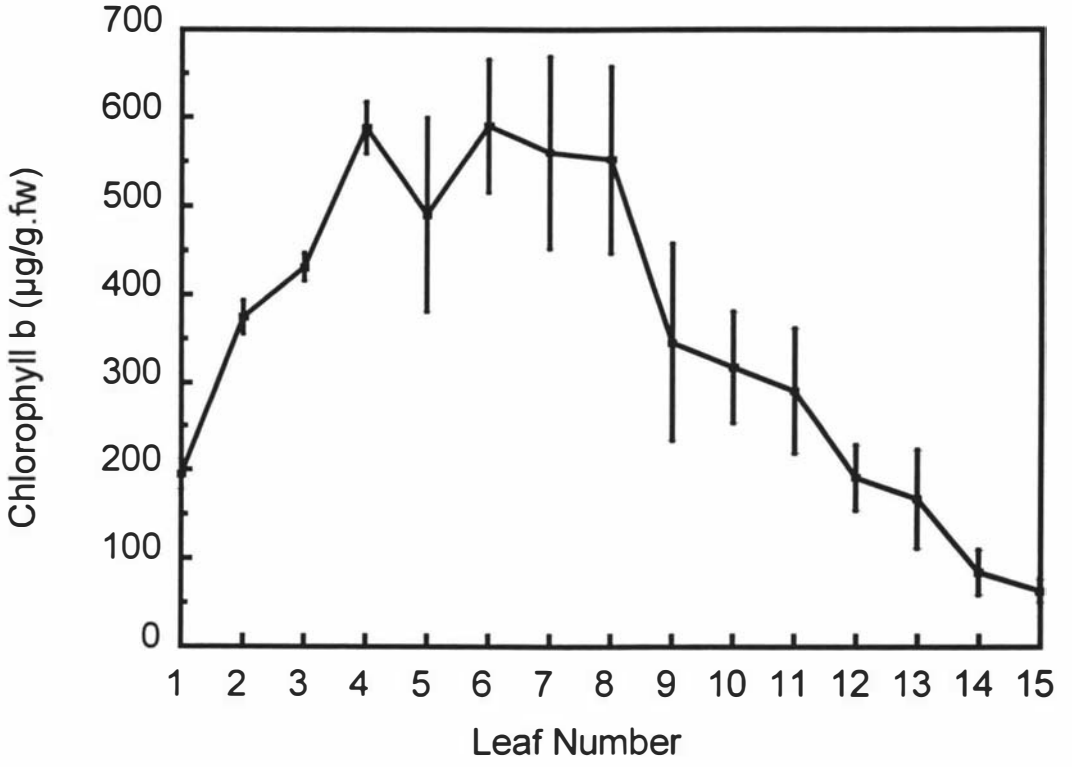


Figure 3.13: Mean chlorophyll *b* concentration in leaves of genotype 10F grown at Palmerston North.

Bars represent two standard errors of the sample mean.

North may reflect differences in light level or quality between the two growing environments.

A preferential loss of chlorophyll *a* from senescing (yellow) leaves has been reported in a study of 25 deciduous plants mature green and yellow leaves collected from the same plants (Wolf, 1956). More recently, chlorophyll loss from leaves of the wild type and a non yellowing mutant of *Festuca pratensis* were measured by Gut *et al.,* (1987). These workers found that the chlorophyll *a*:*b* ratio remained nearly constant in leaves of the mutant plant whereas in leaves of the wild-type genotype, a preferential retention of chlorophyll *b* was observed towards the end of the senescence period. Further, Young *et al.,* (1990) report that chlorophyll *a* was "lost at a slightly faster rate than chlorophyll *b*" in detached primary leaves of *Horedeum vulgare* and *Avena sativum.* However, Adams *et al.,* (1990) reported that the chlorophyll *a*:*b* ratio in leaves of *Platanus occidentalis* exhibited no decline except in leaves with extremely low chlorophyll contents. In contrast, the chlorophyll *a*:*b* ratio in leaves of white clover declined in mature green leaves compared with young leaves (Table 3.7) but remained relatively constant in senescing leaves. In leaves from plants grown at Palmerston North, the ratio in older (senescing) leaves increased compared with mature green leaves although the chlorophyll *a* concentration was lower than in plants grown at Levin.

The variation in chlorophyll data for plants grown at Palmerston North is greater than for the data collected from plants grown at Levin. However, this may reflect the greater number of replicates used for the plants grown at Levin. The measurement of chlorophyll is destructive, hence fewer replicates for plants grown at Palmerston North were used.

The pattern of change in chlorophyll concentration is similar to the data collected from plants grown at Levin (Figures 3.8, 3.9, and 3.10) but the rate of change of chlorophyll concentration is more gradual in plants grown in Palmerston North. These data demonstrate that a consistent pattern of leaf development (for example fresh weight, chlorophyll concentration) is produced when white clover plants are grown using the standard cultural procedure. However, the absolute values of these parameters differed in the

two environments suggesting that the rate of change of leaf development along the stolon is affected by environmental growing conditions.

3.2 Measurement of ethylene evolution from leaves of white clover

3.2.1 Introduction

Ethylene production has been associated with bud break, ageing and de-greening in other plant systems studied (Abeles *et al.*, 1989; Aharoni and Lieberman, 1979; Osborne, 1991), but no data is available on ethylene production from leaves of white clover, or the effect ethylene may have on leaf development and senescence in this plant. This section details experiments aimed at determining the timing and extent of ethylene production in relation to leaf development in white clover.

3.2.2 Ethylene evolution from detached leaves of white clover

Ethylene evolution from individual leaves detached at the base of the pulvinule was measured as described in section 2.2.4.2. The mean ethylene evolution from fully expanded green leaves from plants grown at Levin is shown in Figure 3.14. Ethylene evolution was initially high (at 15 min) but had declined to a basal level by 45 min. This is consistent with ethylene production as a result of mechanical 'stress'. For example, stress ethylene production can be measured 20 min after the excision of tomato petiole segments (Jackson and Campbell, 1976), and by 90 min and 10 min after cutting tomato fruit and leaves respectively (Smith *et al.*, 1986). Elevated levels of ACC synthase activity have also been measured 10 min after wounding bean leaves (Konze and Kwiatkowski, 1981).

The ethylene evolution tended to increase to reach a maximum between 120 and 150 min after detachment, but the variation also increased. The timing of this increase is similar to ethylene peaks observed in wounded green tissue from *Cercis canadensis* (104 min), *Forsythia viridissima* (133 min), and *Loncera flexuosa* (129 min; Saltveit and Dilley, 1978). This 'wound' ethylene production has been associated with increases in ACC synthase activity and ACC concentration (Hyodo *et al.*, 1983), and ACC oxidase activity

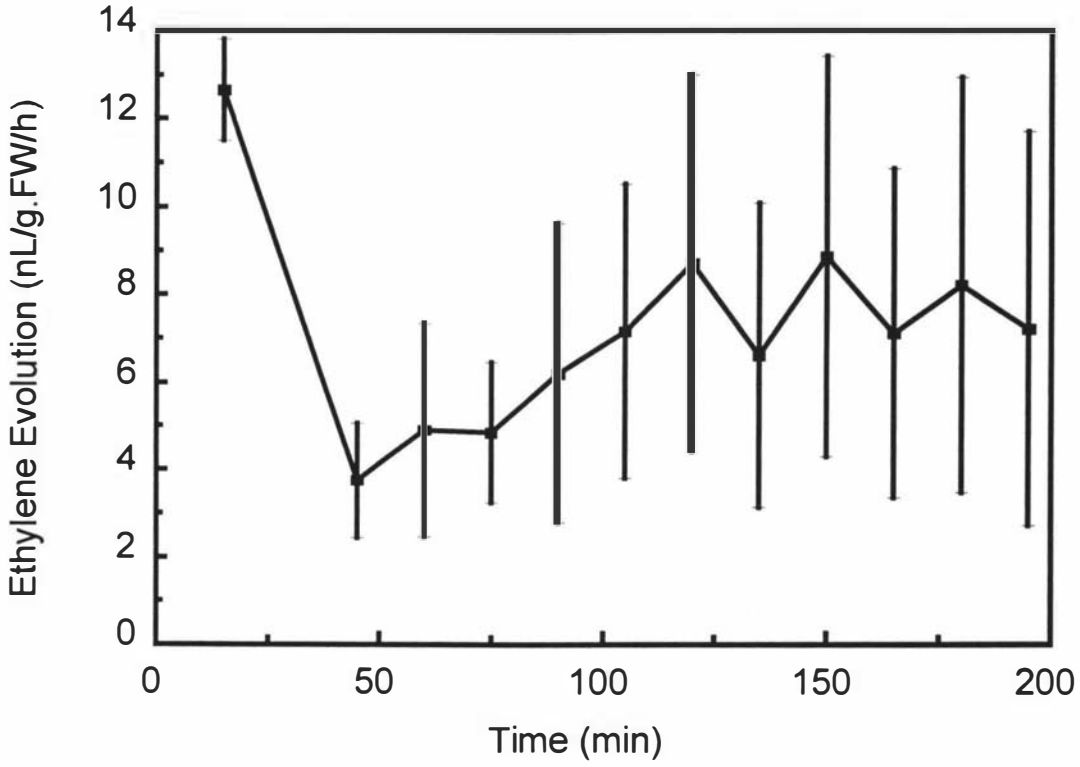


Figure 3.14: Ethylene evolution immediately after harvest from mature green leaves of white clover genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

(Shimokawa, 1983). Ethylene production again peaked at about 31 hours after detachment from the stolon (Figure 3.15), consistent with wound ethylene peaks measured in aged citrus albedo discs 30 h after cutting (Hyodo, 1977), and 32 h after cutting mesocarp discs from winter squash (H*yodo, et al.*, 1985). The ethylene measurements after 25 h were less variable than the data collected immediately after detachment. However, the number of replicates was increased in the second experiment (8 compared with 5) to reduce the variation.

3.2.3 Ethylene evolution from attached leaves of white clover

3.2.3.1 Introduction

Ethylene evolution was measured from intact leaves as leaf detachment often results in marked changes in the senescence pattern of the leaves (Simon, 1967) and the magnitude of ethylene evolution changes rapidly after detachment (section 3.2.2). This section describes experiments conducted with the aim of identifying the magnitude of ethylene production *in vivo* from intact leaves, and to compare the timing of production with changes in physiological parameters along the stolon (for example fresh weight, chlorophyll).

3.2.3.2 Ethylene evolution from attached leaves

Ethylene evolution was measured from individually enclosed intact leaves as described in section 2.2.4.3 (Figure 3.16). Mean ethylene evolution was relatively constant for leaves one to six although leaf two tended to be higher. Ethylene evolution increased from leaf seven to leaf eight, and then remained constant from leaf nine to leaf ten. The magnitude of ethylene evolution from the leaves of white clover (1 to 3 nL/g.fw/h) was greater than that considered 'usual' for expanding leaves (0.1 to 0.4 nL/g.fw/h) and fully expanded leaves (<0.1 nL/g.fw/h; Osborne, 1991).

Ethylene evolution was also measured from leaf 0 (leaves with unfolded leaflets; Carlson scale 0.4 to 0.6, Carlson, 1966). The mean ethylene evolution was significantly higher from this tissue (12.3 nL/g.fw/h) compared

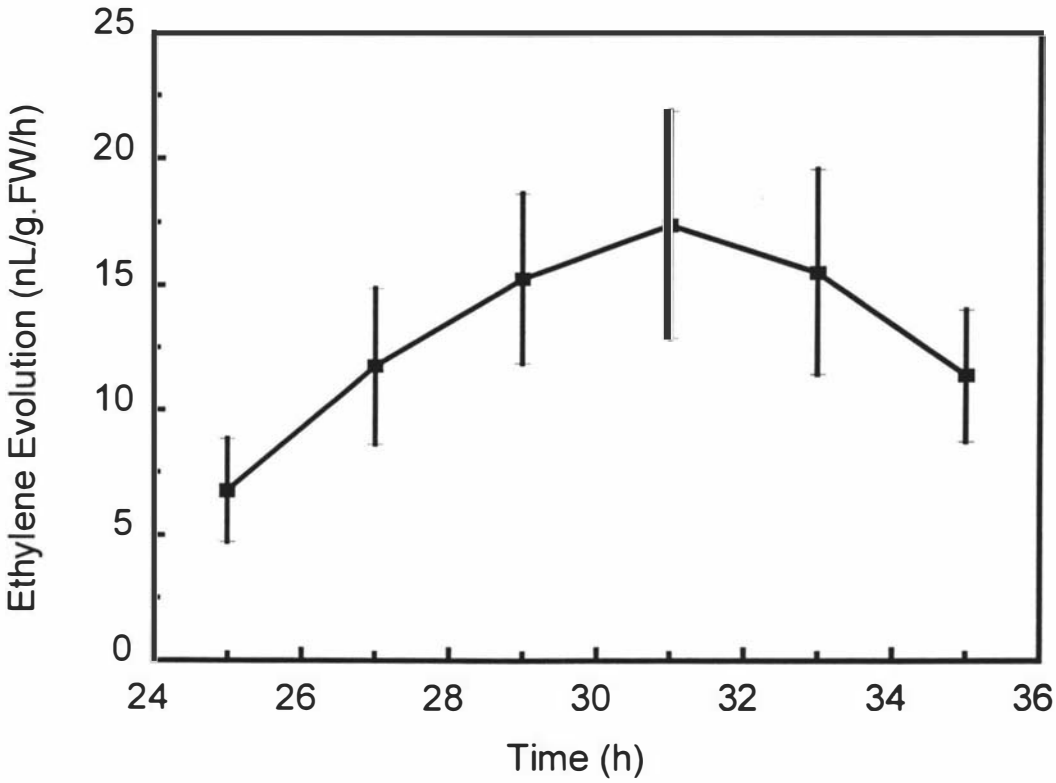


Figure 3.15: Ethylene evolution from detached leaves of white clover, versus time from detachment.

Bars represent two standard errors of the sample mean.

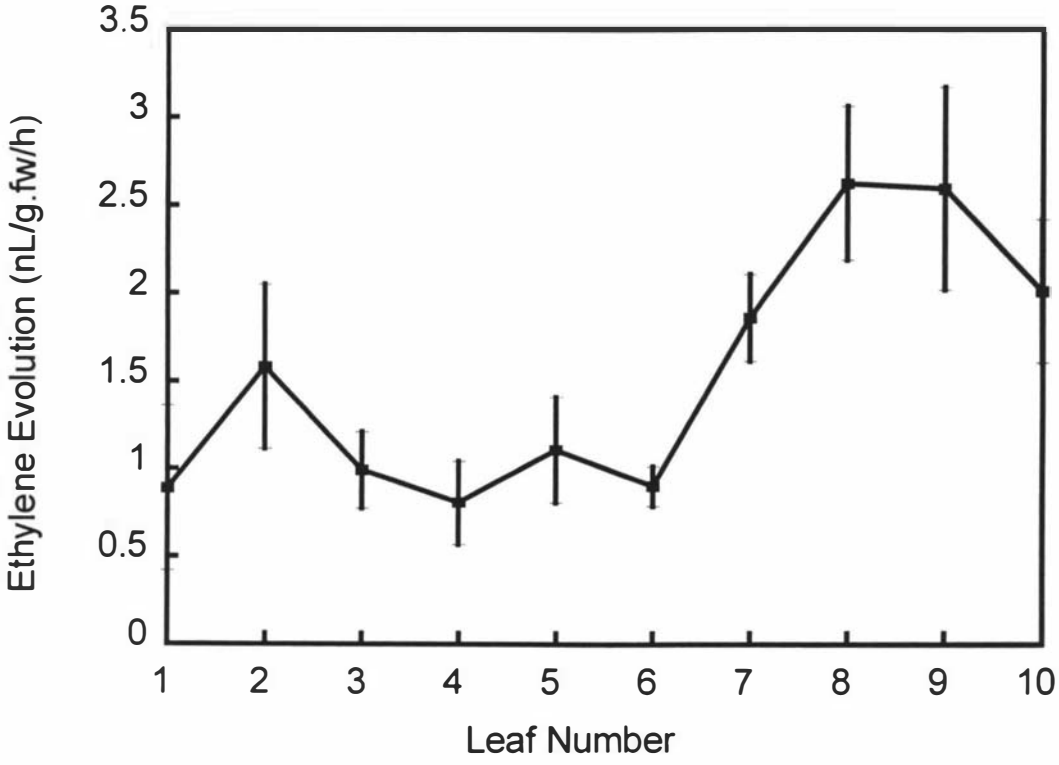


Figure 3.16: Mean ethylene evolution from attached leaves of genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

with all other leaves. High ethylene evolution has also been measured during bud break (very young expanding leaves) but this was in deciduous species (Osborne, 1991) where the bud structure is different to that in white clover.

The pattern for ethylene evolution generally mirrors that seen for total chlorophyll concentration (Figure 3.8). However, the chlorophyll concentration declines from leaf five to leaf six, but an increase in ethylene evolution was not detected until leaf seven.

Ethylene evolution was also measured on plants grown at Palmerston North (Figure 3.17) but older leaves (leaves 11-15) showed higher variation, and a lesser relative increase than ethylene evolution from older leaves grown at Levin. The higher variation in data from plants grown at Palmerston North reflects the fewer replicates used in these measurements. However, consistent with the data collected for fresh weight and chlorophyll concentration (section 3.1.4), the pattern of change in ethylene evolution was similar to the data collected at Levin.

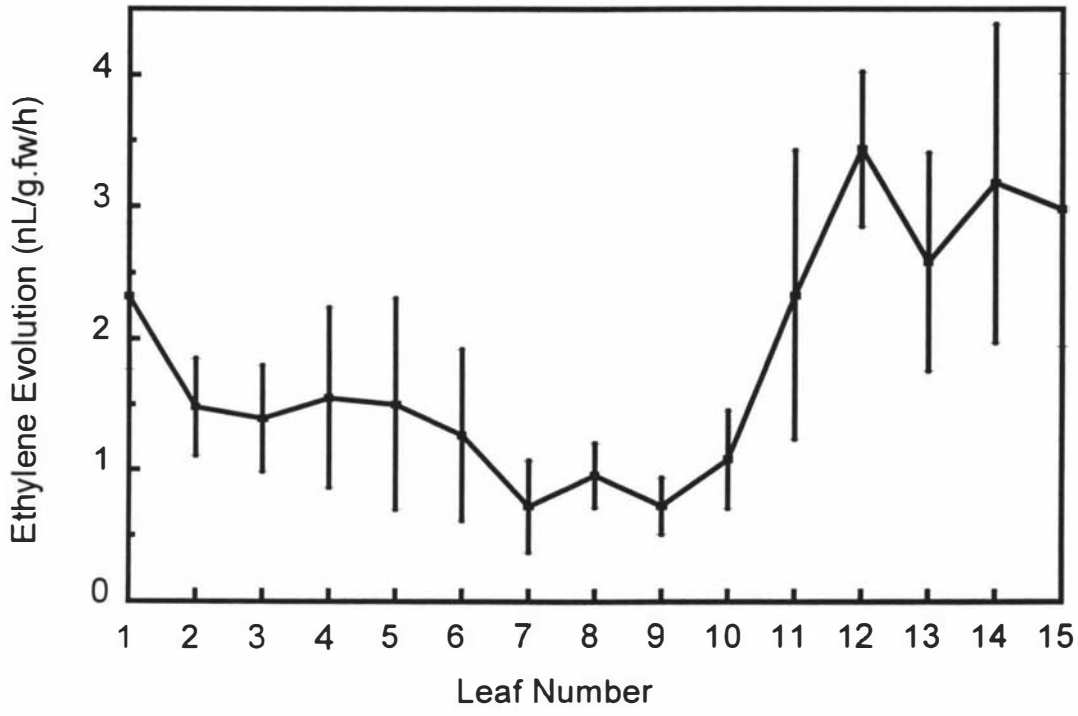


Figure 3.17: Mean ethylene evolution from attached leaves of genotype 10F grown at Palmerston North.

Bars represent two standard errors of the sample mean

3.3 ACC and MACC concentration in leaves of white clover

3.3.1 Introduction

Ethylene evolution has often been used to determine the rate of ethylene production by plant tissues. However, "ethylene gas diffusing from or extracted from, plants is predominantly the ethylene of the intercellular spaces and NOT (sic) that present within plant cells. The ethylene that is functional in growth control is the ethylene that IS (sic) within the cytosol" (Osborne, 1989a). Measuring the concentration of ethylene precursors within the cytosol may provide more precise data on the timing of ethylene production.

ACC is the immediate precursor molecule to ethylene in the biosynthesis of ethylene in plants. ACC is formed from AdoMet by ACC synthase, and in turn is converted to ethylene by ACC oxidase (Figure 1.1). ACC is formed as the free acid in the cytoplasm (Guy and Kende, 1984) and is reported to be translocated into the vacuole (Saftner and Martin, 1993), and around the plant (Finlayson, *et al.*, 1991). In addition, the malonyl conjugated form of ACC, MACC, can be transported into (Bouzayen et al., 1989) and out of the vacuole (Pedreno *et al.*, 1991). Changes in ACC and MACC concentrations may precede changes that can be measured in ethylene evolution and provide more precise data on the timing of induction of enzymes involved in ethylene biosynthesis.

3.3.2 Measurement of ACC and MACC concentration in leaves of white clover

The mean ACC concentration in leaves from plants grown at Levin is shown in Figure 3.18. Low concentrations (less than 2 nmol/g.dw) were detected in leaves one to four and the concentration increased in leaves five to seven before declining in leaves eight to ten. The rise in the concentration of ACC could be detected in leaf five, whereas a rise in the level of evolved ethylene could not be detected until leaf seven (Figure 3.16). The mean concentration

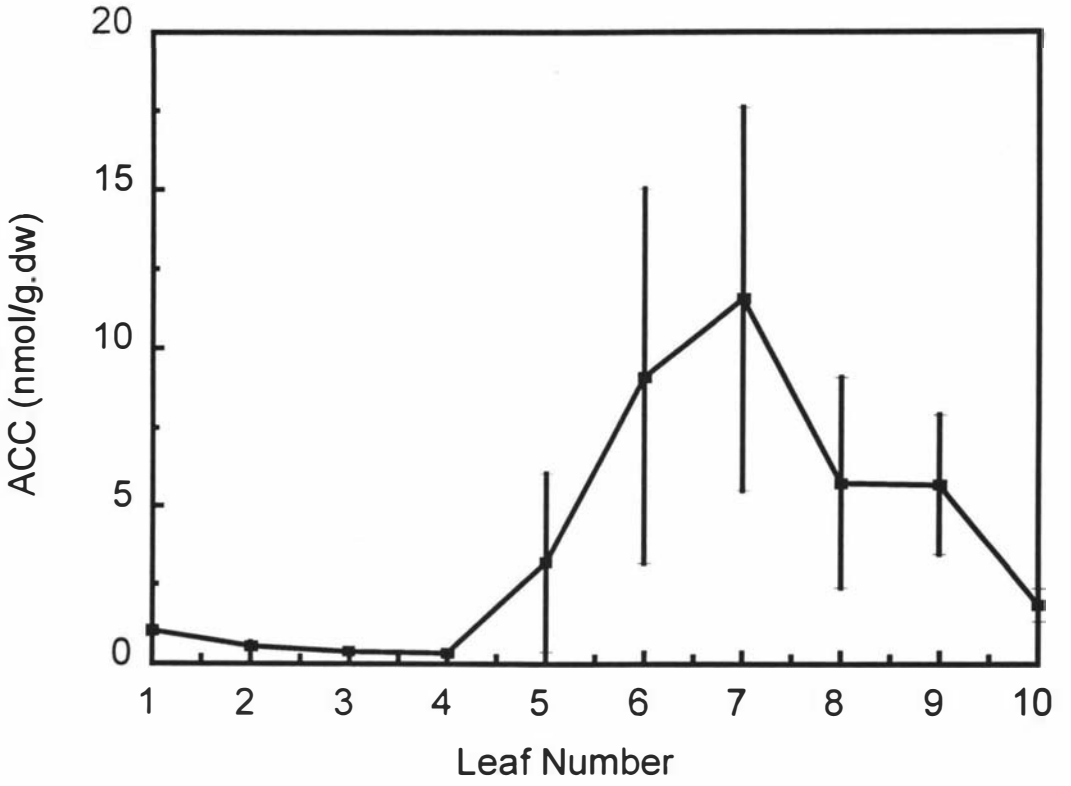


Figure 3.18: Mean ACC concentration in leaves of genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

of MACC was highest in leaf one (Figure 3.19) and remained relatively constant for all other leaves, although leaves four, five and ten tended to be lower.

In some systems MACC is reported to be an inactive end product of ACC synthesis and not oxidised to ethylene (Hoffman *et al.*, 1982). However, other workers have shown that MACC can be converted to ACC and provide a source of ACC for ethylene production (Jiao, *et al.*, 1986). The data for ACC and MACC concentration shows higher levels of variability between replicates than the other physiological and biochemical measurements made in this study and may be a result of inherent reproducibility inaccuracies in the methods.

The methods used to measure ACC and MACC concentration in this study rely on the chemical conversion of ACC to ethylene (Lizada and Yang, 1979). However, the use of this method in plant tissues has "shortcomings that include a lack of internal standard to correct for losses during purification, and poor reproducibility in many tissues arising from interference by phenolic compounds and extraction-solvent residues" (Hall *et al.*, 1989). In particular, the accuracy of the method is reduced by ethanol (Bufler and Mor, 1980) and also protein, sulfhydryl reagents, and amines (Coleman and Hodges, 1991).

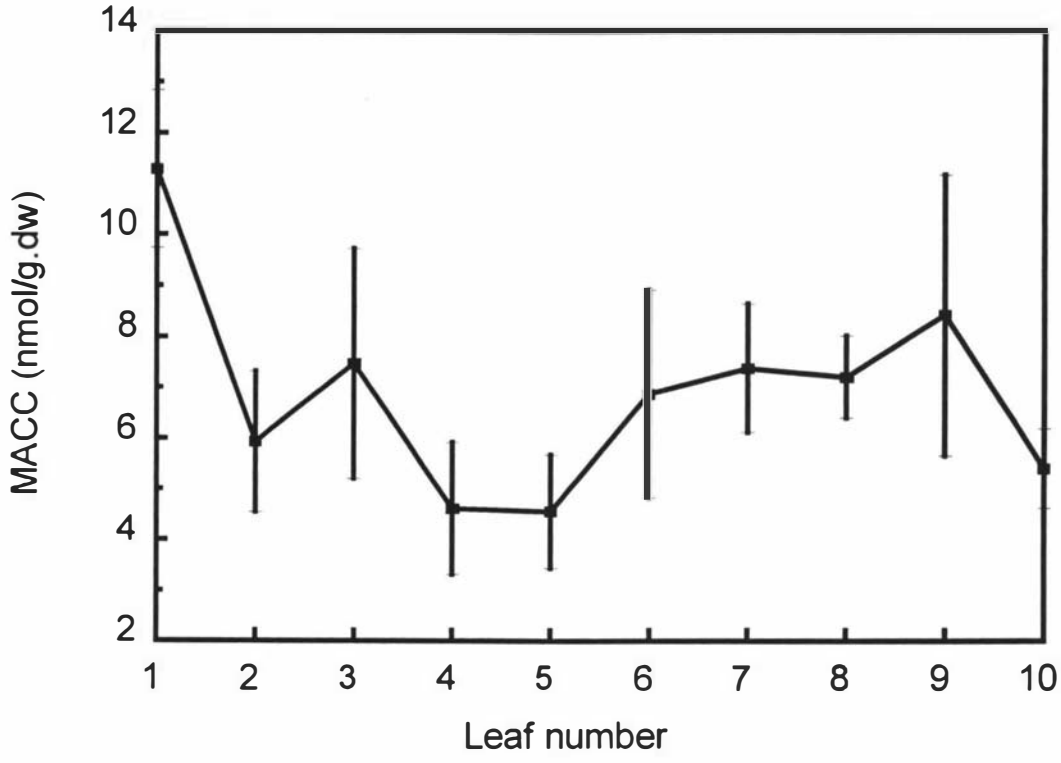


Figure 3.19: Mean MACC concentration in leaves of genotype 10F grown at Levin.

Bars represent two standard errors of the sample mean.

3.4 Measurement of enzyme activity in leaves of white clover

3.4.1 Introduction

Ethylene is formed via the Yang cycle from AdoMet and ACC (Figure 1.1) involving the enzymes ACC synthase and ACC oxidase (Imaseki, 1991). ACC synthase is believed to be the rate limiting step in ethylene biosynthesis in plants (Arteca, 1989).

3.4.2 Measurement of ACC synthase activity

Attempts were made to measure ACC synthase activity in individual leaves, and in bulked leaf samples. Extracts were tested from fresh or frozen tissue, and dialysed or passed through Sephadex G-25 spin columns. ACC synthase activity in attached leaves was compared with activity in detached leaves exposed to ethylene. The activity in leaf extracts was also compared with activity in extracts from stolon and root tissue. However, no reliable activity (activity proportional to the substrate concentration, section 2.2.6.1) above control levels (boiled extract) could be detected in any extracts from white clover tissue (data not shown).

ACC synthase activity responsive to increases in AdoMet concentration could be detected in extracts of apple fruit cortical tissue. However, when the apple extracts were combined with white clover leaf extracts, detectable activity diminished to the control level. When white clover leaf extracts were boiled for five minutes and again combined with apple extract, activity was restored (Table 3.9). The restored activity was higher than the activity expected from diluting the apple extract with an 'inert' component i.e. diluting the extract by 50% should reduce the activity by 50%. However, the combination of the apple extract with the boiled white clover extract resulted in a reduction to 66.8% of the undiluted activity.

Table 3.9: ACC synthase activity in white clover leaf protein extracts, and apple cortical tissue protein extracts.

Extract	Mean relative ethylene production at 50 μM AdoMet (nL/h)
White clover leaf extract (600 μL)	0.664 +/- 0.009
Apple fruit extract (600 μL)	1.785 +/- 0.016
White clover leaf (300 μL) + apple fruit extract (300 μL)	0.568 +/- 0.07
Boiled white clover leaf (300 μL) + apple fruit extract (300 μL)	1.193 +/- 0.044

3.4.3 Measurement of ACC oxidase activity

3.4.3.1 Introduction

A method for determining ACC oxidase activity *in vitro* was first described by Ververidis and John (1991). The method was developed following recognition of the similarity between a putative ACC oxidase cDNA and 3'-hydroxylase enzymes cDNA using sequence data. These hydroxylase enzymes have a requirement for Fe^{2+}, ascorbate and CO_2 for activity. ACC oxidase activity was measured using a method modified from Ververidis and John (1991), Fernández-Maculet and Yang (1992), and McGarvey and Christoffersen (1992).

3.4.3.2 Measurement of ACC oxidase activity

Protein was extracted from fresh (attached) leaf material as described in section 2.2.6.2. Preliminary experiments had shown that in this system, extracting under a stream of nitrogen did not increase the enzyme activity (data not shown). To confirm the assay measured enzyme activity, the assay was first tested on protein extracts from mature green leaves (leaves three to six) from plants grown at Palmerston North. Enzyme activity (ethylene production) decreased approximately in proportion to the dilution of extract (1, 0.5, and 0.25 times dilution with assay buffer) added to the assay at 1 mM added ACC (Figure 3.20A). Further, ethylene was produced approximately in proportion to the added ACC concentration (Figure 3.20B). However, the graph is not linear and the ethylene production is relatively lower at increasing concentrations of ACC.

The effect of ACC concentration was investigated further using higher concentrations of ACC, and in time course studies. Three separate protein extracts, each comprising the protein extracted from leaf one plus the mature green leaves from a single stolon on a plant grown at Palmerston North, were extracted using the method described in section 2.2.6.2. ACC oxidase activity in each of the extracts was measured after different incubation times, and at 1 mM or 10 mM ACC. Ethylene production from the assay was linear up to 60 min at 1 mM ACC, but at 10 mM ACC linearity was lost after 20 min (Figure

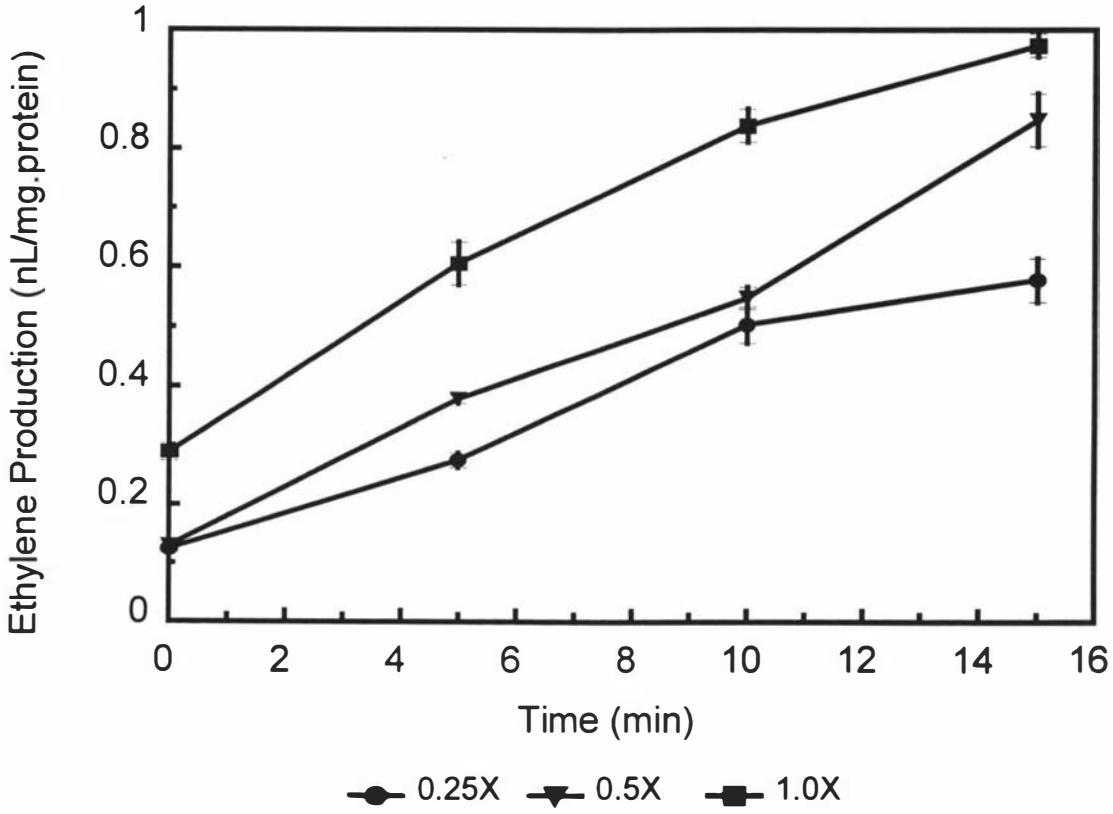


Figure 3.20A: ACC oxidase activity at 1mM ACC for different concentrations of leaf protein extracts.

Bars represent two standard errors of the sample mean.

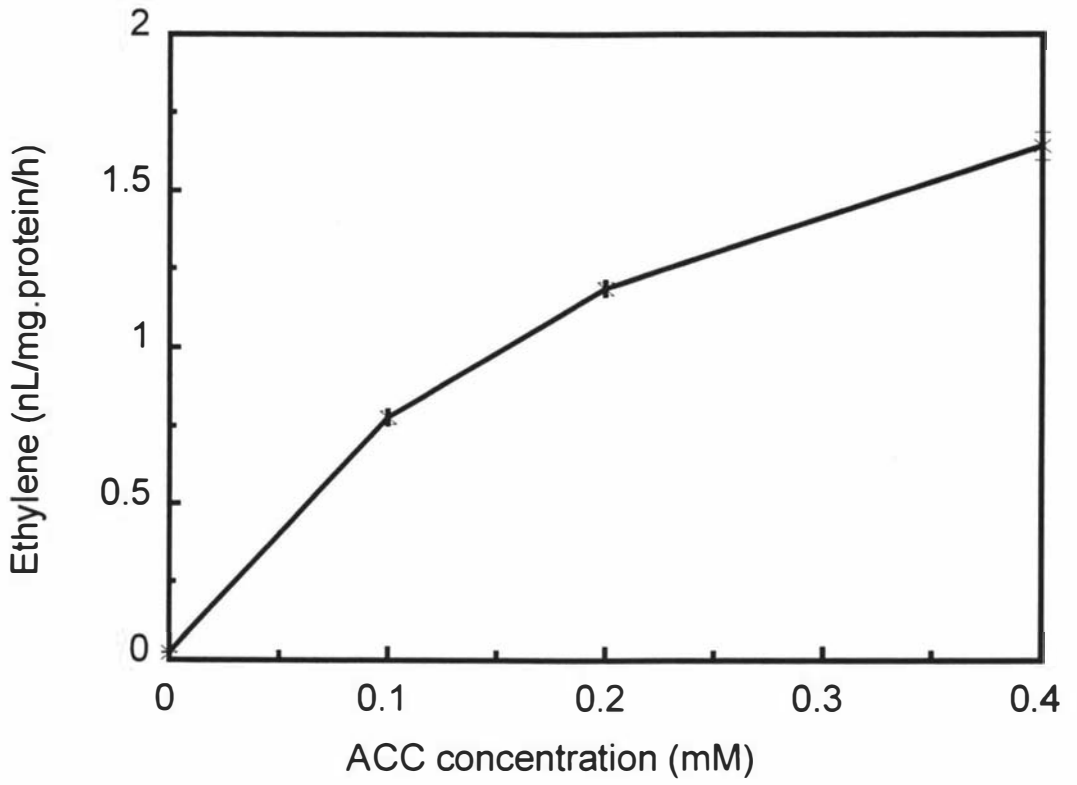


Figure 3.20B: ACC oxidase activity at different added ACC concentrations in protein extracts from mature green leaves from genotype 10F plants grown at Palmerston North.

Bars represent two standard errors of the sample mean.

3.21). This may be due to increased deactivation of the enzyme at the higher concentration of substrate by an intermediate generated during the catalytic turnover. Smith *et al.,* (1994) found that linearity was lost after 14 min for a purified recombinant tomato ACC oxidase, and after 19 min in assays *in vivo* in the presence of ascorbate, Fe^{2+}, and 1 mM ACC.

ACC oxidase activity was measured in leaves one, three, five, six, eight and ten with a minimum of three sample replicates and three internal replicates per sample. The ACC oxidase activity versus substrate concentration curve for leaves one, three, five and six display saturation kinetics with respect to the substrate (Figures 3.22A to 3.25A). However, the data for leaf six indicate that at the highest concentration of ACC used, linearity was at least partly lost, as shown by the outlying point in the Lineweaver-Burk plot in Figure 3.25B (this point was not included in the regression). The data for leaves eight and ten do not show saturation kinetics over the range of ACC concentrations used (Figures 3.26A and 3.27A). These data are shown in Lineweaver-Burk plots in Figures 3.22B to 3.27B. Data for leaves one, three, five and six are approximately linear as expected from the velocity graphs. The transformed data for leaves eight and ten however, do not plot linearly further indicating these data do not follow saturation kinetics at the concentrations of ACC used in these experiments.

Previous workers in other systems have shown apparently high ACC oxidase activity but this has subsequently been attributed to an artifactual, non-enzymatic oxidative breakdown of ACC catalysed by free radicals (Yang and Hoffman, 1984). The apparent Km (Km data obtained from partially purified enzyme preparations) for ACC oxidase is known to be about 100 μM or less with respect to ACC, "while artifactual systems either do not show saturation kinetics or have apparent Km values of *ca.*10 mM or higher" (Ververidis and John, 1991). Apparent Km and Vmax, and activity for ACC oxidase extracts from leaves one, three, five, six, eight and ten are shown in Table 3.10. The apparent Km of about 104 μM (average for leaves one, three, five, and six) is higher than that reported for ACC oxidase by Ververidis and John (1991) (85μM), McGarvey and Christoffersen (1992) (32 μM), Fernández-Maculet and Yang (1992) (17 μM) and Smith *et al.* (1992) (60 μM), while the apparent Km for leaf six (61 μM) was similar to the values reported from these species.

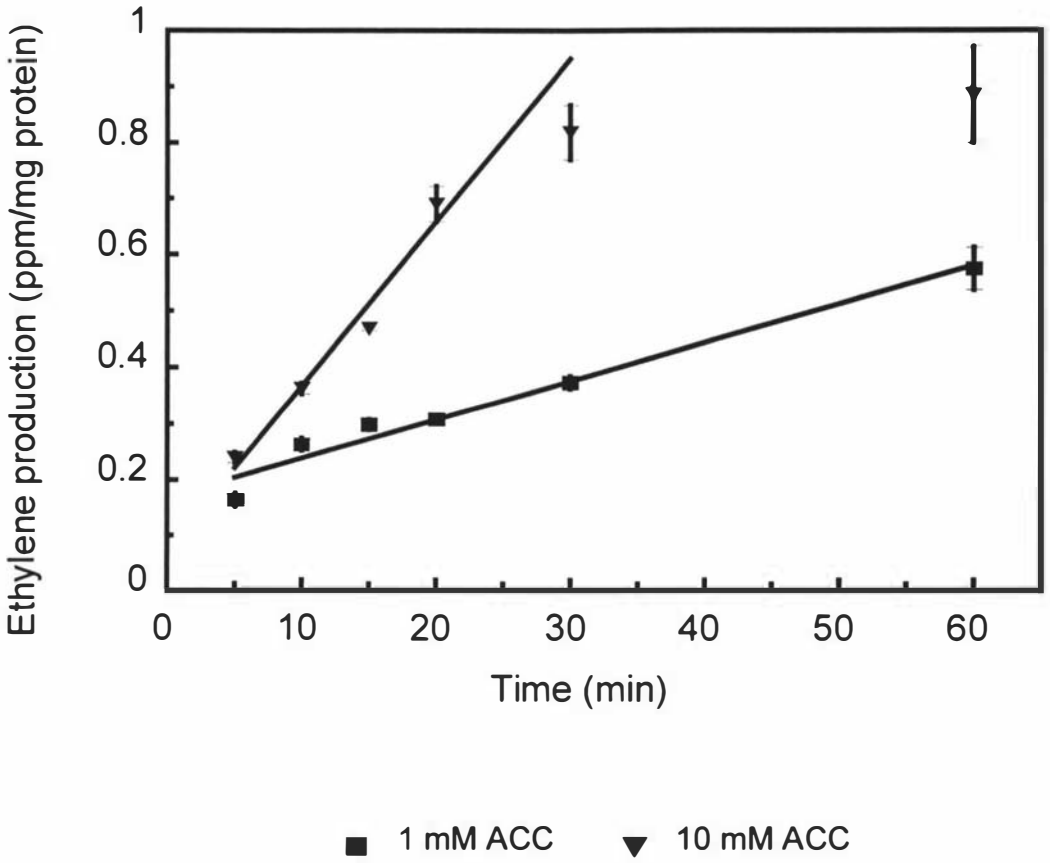


Figure 3.21: ACC oxidase activity in protein extracts from leaf one and mature green leaves from white clover genotype 10F plants grown at Palmerston North.

Bars represent two standard errors of the sample mean.

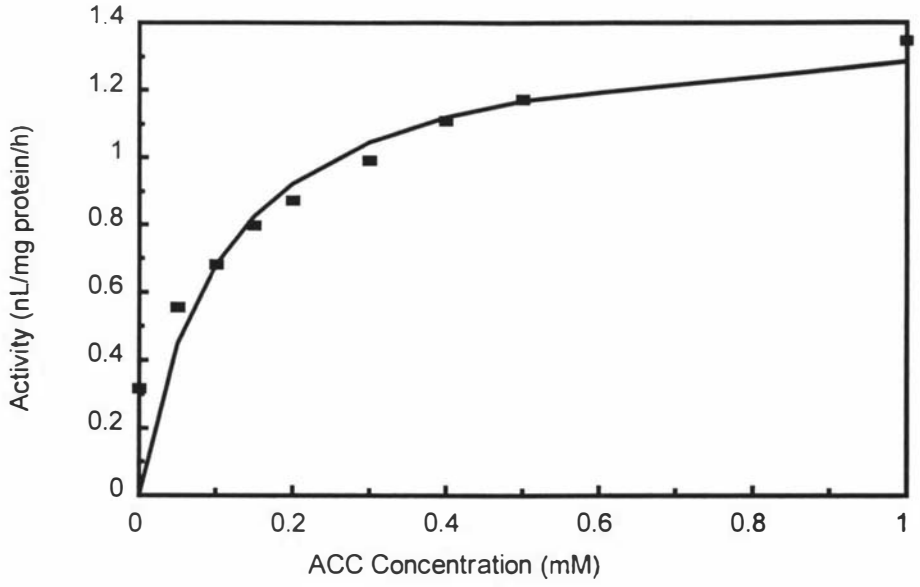


Figure 3.22A: ACC oxidase activity in protein extracts from leaf one of genotype 10F plants grown at Levin.

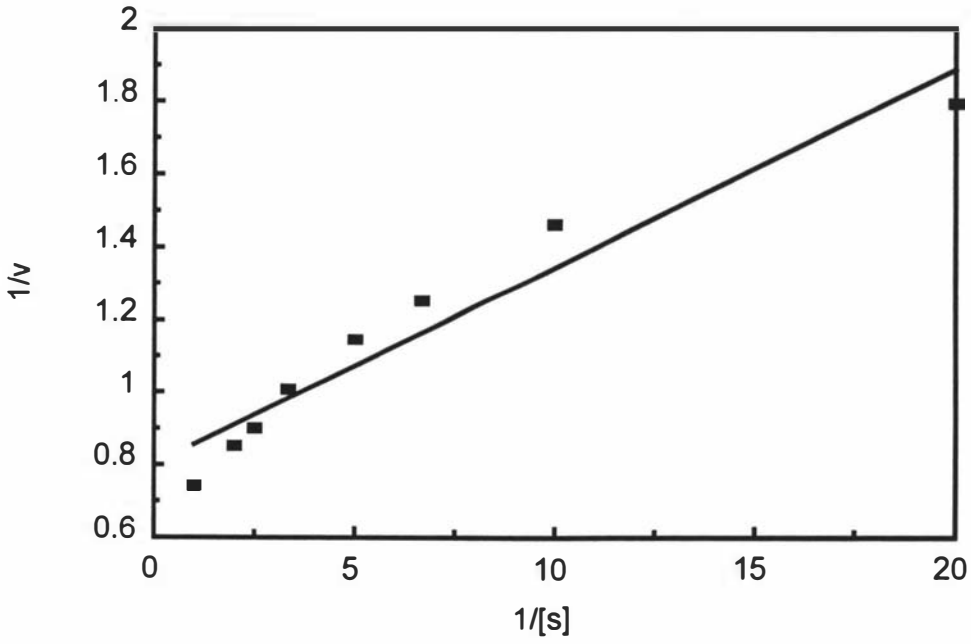


Figure 3.22B: Lineweaver-Burk plot for ACC oxidase in protein extracts from leaf one of genotype 10F plants grown at Levin.

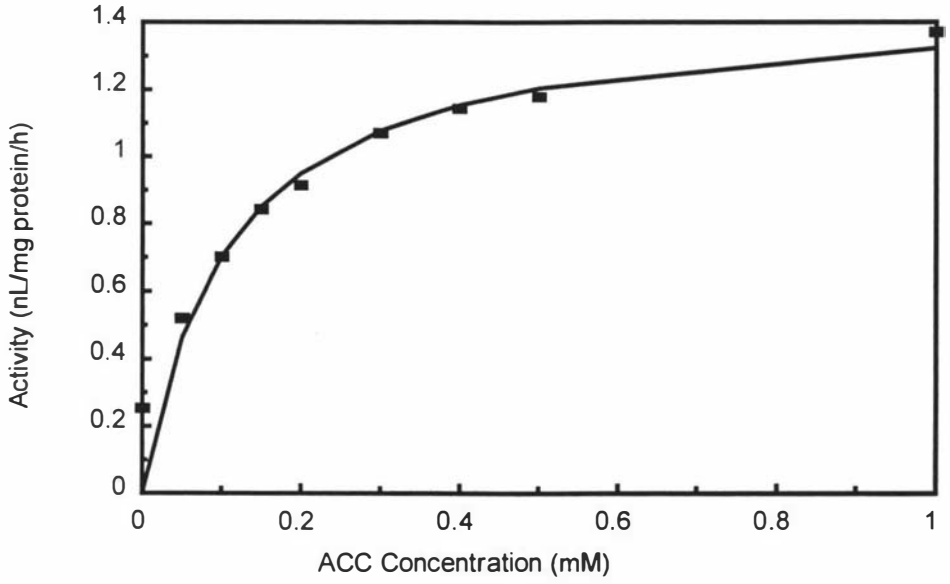


Figure 3.23A: ACC oxidase activity in protein extracts from leaf three of genotype 10F plants grown at Levin.

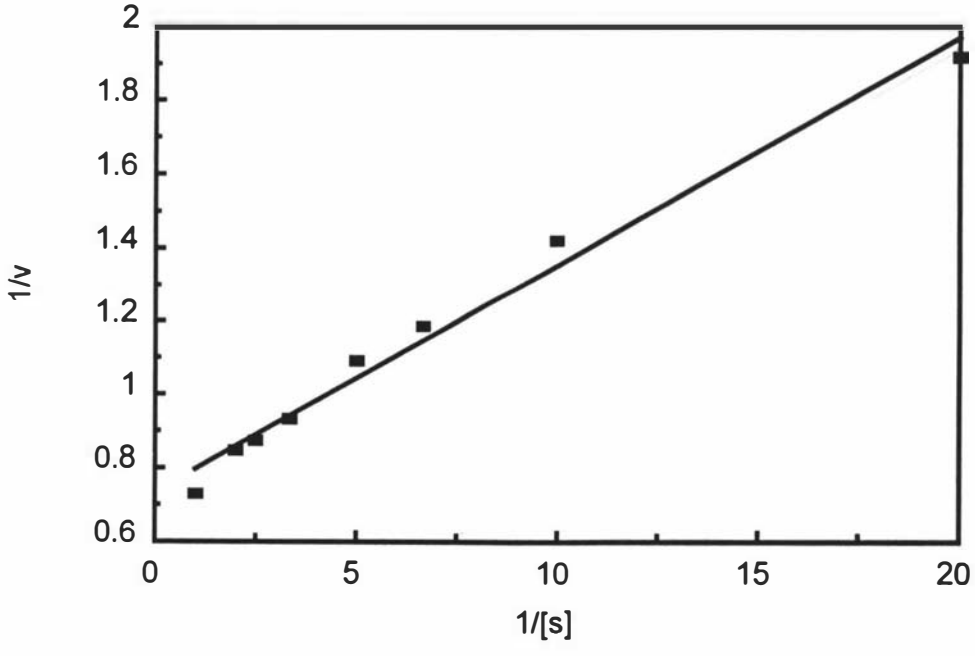


Figure 3.23B: Lineweaver-Burk plot for ACC oxidase in protein extracts from leaf three of genotype 10F plants grown at Levin.

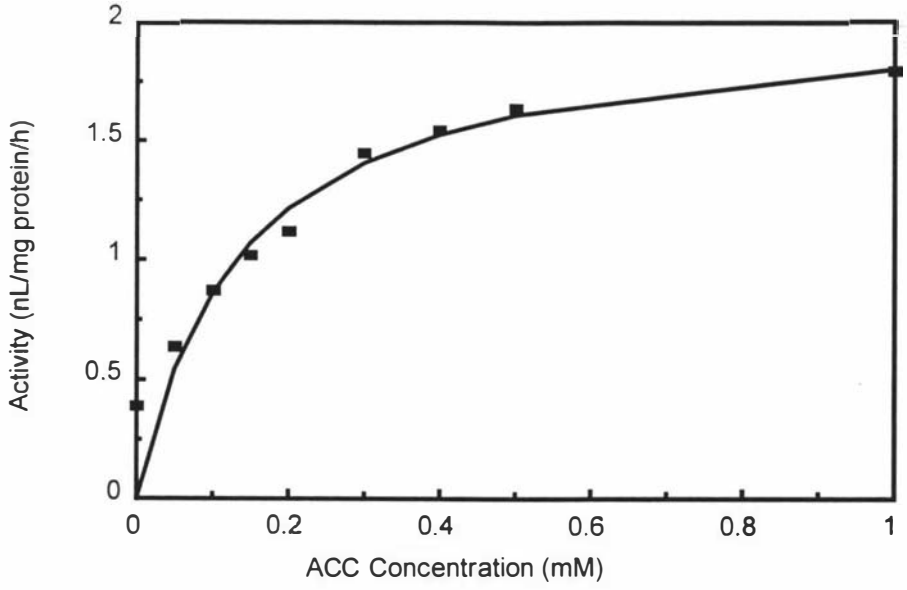


Figure 3.24A: ACC oxidase activity in protein extracts from leaf five of genotype 10F plants grown at Levin.

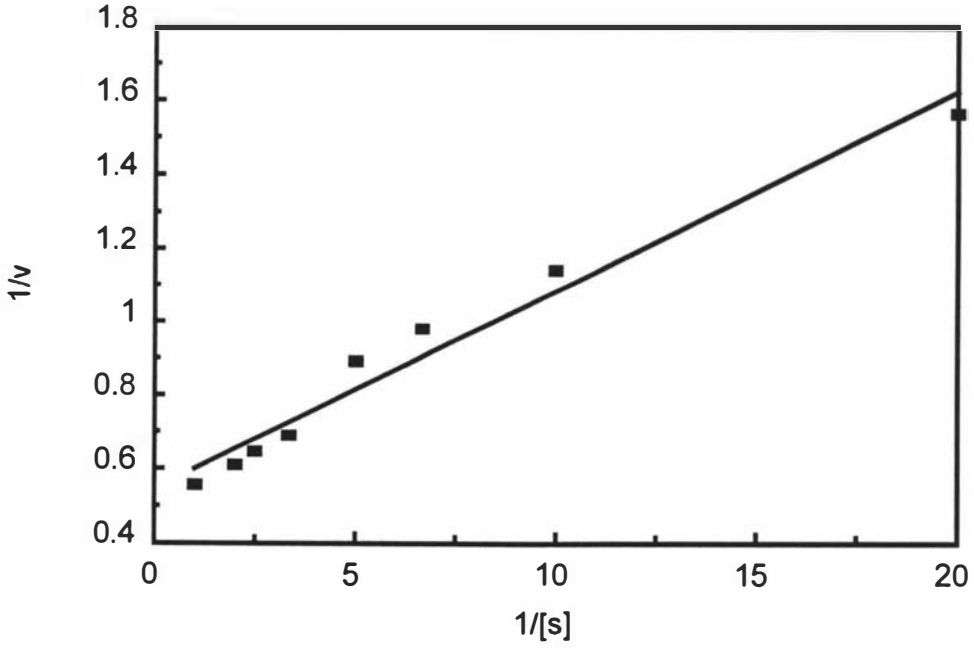


Figure 3.24B: Lineweaver-Burk plot for ACC oxidase in protein extracts from leaf five of genotype 10F plants grown at Levin.

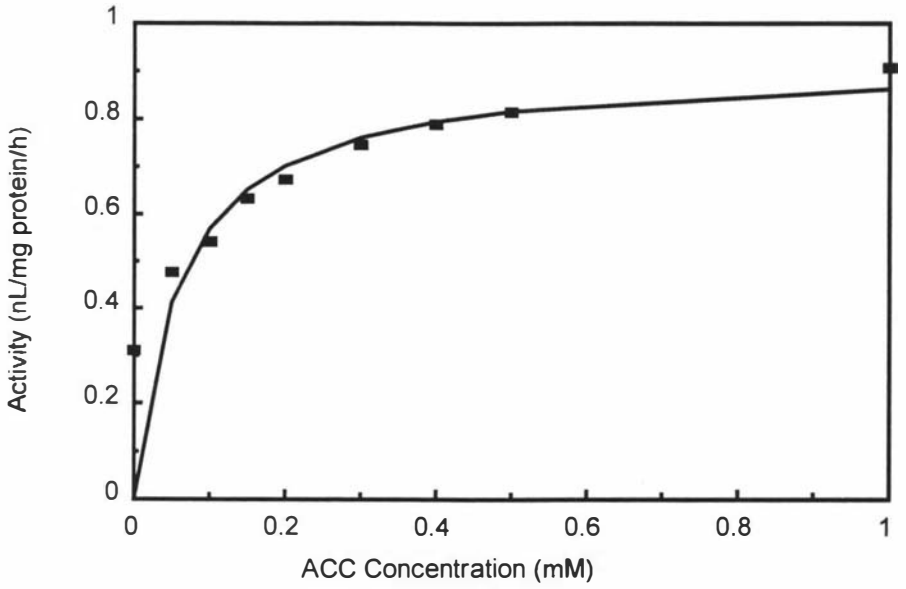


Figure 3.25A: ACC oxidase activity in protein extracts from leaf six of genotype 10F plants grown at Levin.

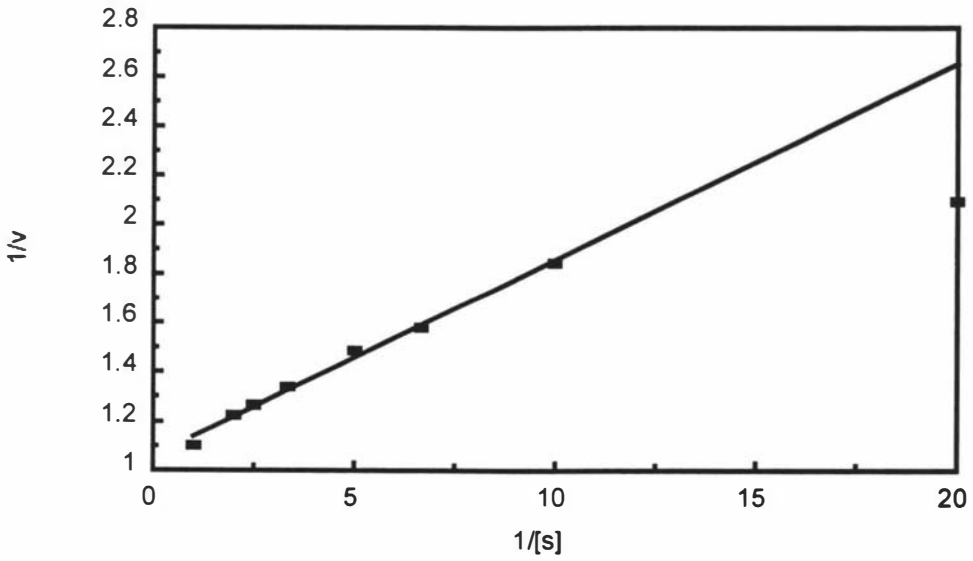


Figure 3.25B: Lineweaver-Burk plot for ACC oxidase in protein extracts from leaf six of genotype 10F plants grown at Levin.

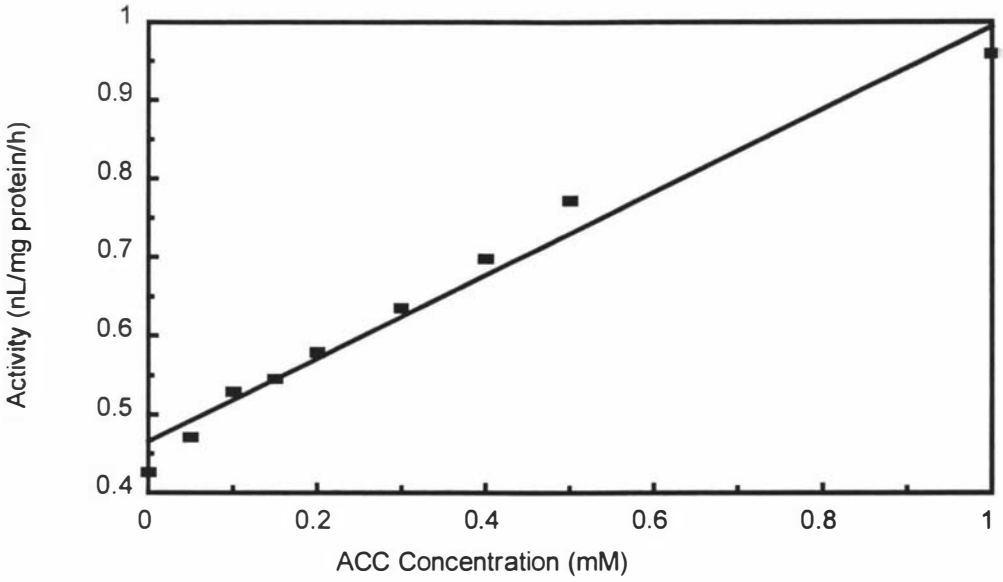


Figure 3.26A: ACC oxidase activity in protein extracts from leaf eight of genotype 10F plants grown at Levin.

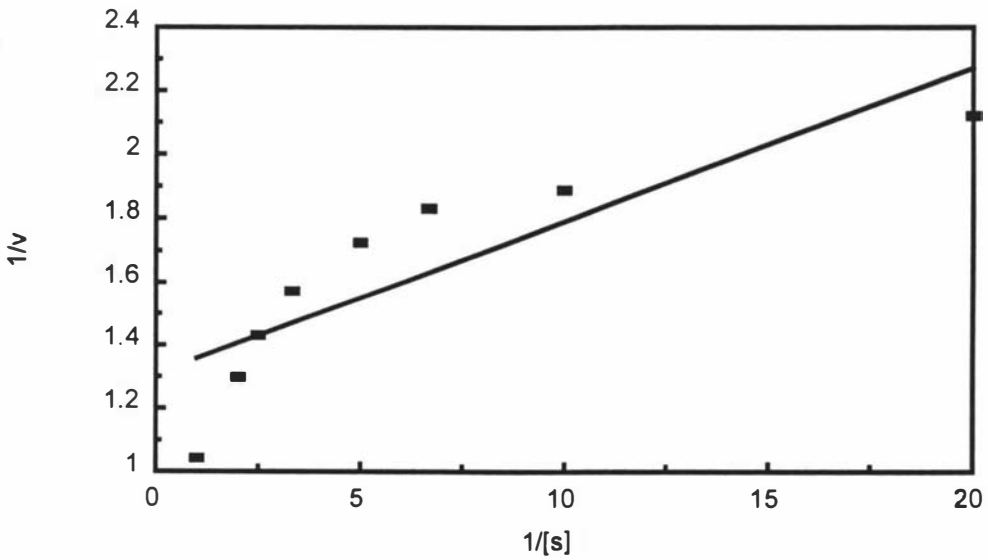


Figure 3.26B: Lineweaver-Burk plot for ACC oxidase in protein extracts from leaf eight of genotype 10F plants grown at Levin.

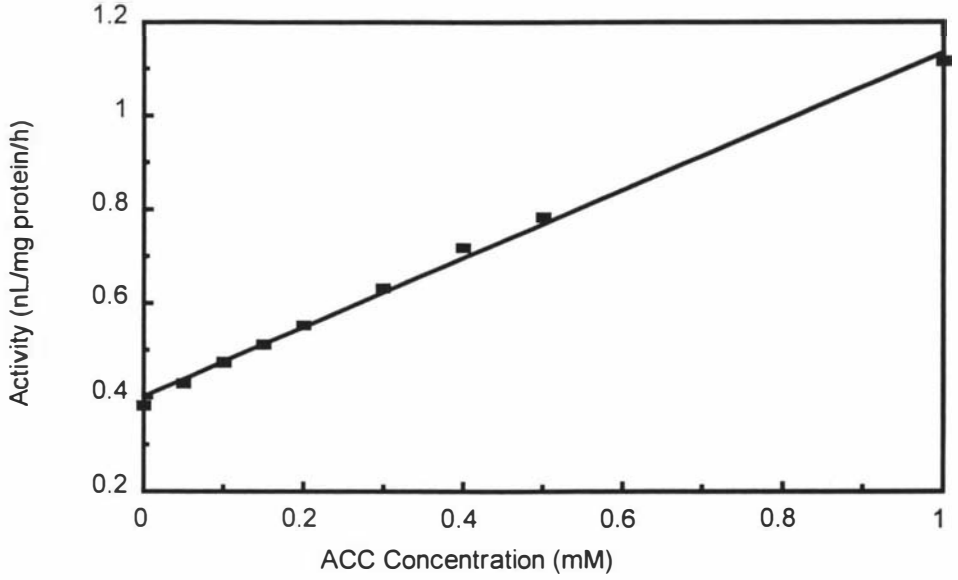


Figure 3.27A: ACC oxidase activity in protein extracts from leaf ten of genotype 10F plants grown at Levin.

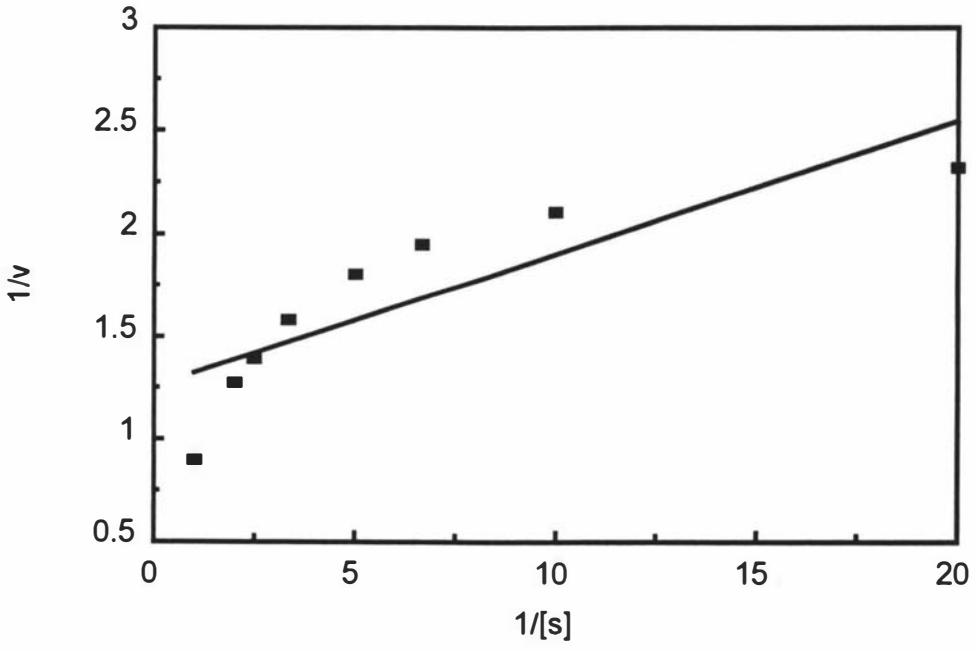


Figure 3.27B: Lineweaver-Burk plot for ACC oxidase in protein extracts from leaf ten of genotype 10F plants grown at Levin.

Table 3.10: Apparent Km and Vmax for ACC oxidase

Leaf Number	Apparent Vmax	Apparent Km (μM)	Activity at 500 μM ACC (nL/mg.protein/h)
1	1.42 ± 0.06*	109 ± 17*	1.17
3	1.48 ± 0.04	108 ± 9	1.18
5	2.06 ± 0.08	138 ±16	1.64
6	0.92 ± 0.31	61 ± 9	0.81
8	ND**	ND**	ND**
10	ND**	ND**	ND**

*values plus and minus one standard error of the sample mean

ND** No data. These data do not show saturation kinetics

3.5 Induction of senescence in leaves of white clover by exogenously applied ethylene

3.5.1 Introduction

An increase in ethylene production has been measured when white clover leaves were detached (section 3.2.2), and attached older (de-greening) leaves had higher levels of ethylene evolution compared with mature green leaves (section 3.2.3). Ethylene accelerates ripening and senescence in many fruits (for example cantaloupe, McGlasson and Pratt, 1964; banana, Brady *et al.* 1970), and increases the rate of de-greening from green tissues (Abeles *et al.*, 1967; Abeles *et al.*, 1989; Aharoni, 1989; Makhlouf, *et al.*, 1989). This section details experiments aimed at determining whether the application of exogenous ethylene affects the rate of de-greening in leaves of white clover.

3.5.2 Ethylene application to detached leaves

Ethylene was applied to detached leaves in gas tight containers (section 2.2.3) to make final concentrations of 0, 1, 10 and 100 ppm. The actual ethylene concentration (Table 3.11A) in control containers (without leaves) after adding the ethylene was determined by gas chromatography using the Shimadzu instrument (section 2.2.4.1). The concentrations were prepared by injecting 100 % (v/v) ethylene into the containers (section 2.2.3). The final concentrations (Table 3.11A) were less accurate (compared with the calculated concentrations) and more variable at the higher concentrations indicating that either the dilution volumes were incorrect or, more likely, the gas seals were imperfect, and relatively more ethylene escaped at the higher concentrations.

Carbon dioxide and oxygen concentration in the containers with the leaves was measured immediately prior to venting after days one, two and four. The concentration of CO_2 in all containers was below the level of detection of the gas chromatograph. The concentration of O_2 varied between 20% and 21.5%. However, a peak of unknown origin with a retention time slightly less than that

Table 3.11A: Ethylene concentration in control containers

Treatment	Nominal concentration (ppm)	Measured concentration (ppm)
A	0	0*
B	0 (+ purafil)	ND
C	1	1.05 +/- 0.04
D	10	8.58 +/- 0.16
E	100	90.71 +/- 0.42

* concentration below detection limit

ND. No data collected.

Table 3.11B: Ethylene concentration measured in containers with leaves after day one

Treatment / Leaf Number	A	B	C	D	E
1	0*	0	1.4	11.5	93
2	0	0	1.2	10.7	109.8
3	0	0	1.2	10.2	103.4
4	0	0	1.1	7.8	57.9#
5	0	0	0.9	11.1	57.9#
6	0	0	0.9	**	93.4
7	0	0	1.7	8.2	83.5

* concentration below detection limit.

** Treatment lost.

Seals leaked

for ethylene was detected for some samples in treatments without added ethylene after day one and two. These peaks were greatest in samples from leaf seven (0 ppm ethylene), and leaves three to eight (0 ppm ethylene plus Purafil) after day two. This peak was also detected in samples from leaves four and six treated with 1 ppm ethylene for one day, and leaves seven and eight treated with 1 ppm ethylene for two days.

The ethylene concentration in the containers was measured immediately prior to venting on day one, two, and four. After day one (Table 3.11B), no ethylene was detected in treatments A or B. The concentration of ethylene measured in containers with treatments C and D were generally above the concentration of the added ethylene, indicating the leaves were producing ethylene. This results contradicts with data from treatment A where no ethylene evolution could be detected. Ethylene was measured at the minimum attenuation that gave a stable baseline on the gas chromatograph. To detect low concentrations of ethylene as might be found in treatments A or B, a lower attenuation may have been required, however, this was precluded by baseline 'noise'.

The ethylene concentrations in the containers after two days are shown in Table 3.11C. An increase in ethylene concentration above the added concentration (Table 3.11A) was detected for most leaves in treatments C and D, again indicating that the leaves were producing ethylene. However, no ethylene could be detected in treatments A or B, and no increase above the added concentration could be detected in treatment E. After four days exposure (Table 3.11D) no consistent increase in ethylene concentration above the added levels could be detected in treatments C, D, or E, suggesting that ethylene production had diminished at this time. In contrast, ethylene was detected for leaves two and three in treatment A, and for leaf three in treatment B indicating that leaves in these treatments were producing ethylene at detectable levels. The presence of ethylene in treatment B indicates that either the leaves were producing more ethylene than the Purafil could absorb, or, more likely, that the sample of Purafil in this treatment had become exhausted. This is supported by the total chlorophyll data from the same experiment (Figure 3.29A) where the concentration for leaf three in treatment B (Purafil) had declined more than the chlorophyll concentration in

Table 3.11C: Ethylene concentration measured in containers with leaves after day two

Treatment / Leaf Number	A	B	C	D	E
1	0*	0	1.4	9.8	75.3
2	0	0	1.6	10.4	92.7
3	0	0	1.8	10.4	80.4
4	0	0	1.7	10.4	48.7#
5	0	0	1.3	10.9	82.5
6	0	0	1.1	8.9	87.2
7	0	0	1.5	6.3	90.2

* concentration below detection limit.

seal replaced

Table 3.11D: Ethylene concentration measured in containers with leaves after day four

Treatment / Leaf Number	A	B	C	D	E
1	0.7	0	1.0	3.2	65
2	1.0	1.3	1.02	9.0	80.7
3	0*	0	0.9	8.9	90.4
4	0	0	1.01	8.8	81.5
5	0	0	0.6	9.2	85.2
6	0	0	1.3	9.5	86.7
7	0	0	1.1	9.8	77.9

* concentration below detection limit.

treatments A or C (0 and 1 ppm ethylene respectively).

These data suggest that leaves exposed to exogenous ethylene (treatments C, D, and E) were responding differently to leaves in treatments A and B. Ethylene production was only detected from leaves in treatments A and B after four days. In contrast, ethylene evolution was detected from leaves in treatments C, D, and E after days one and two, but not after day four. In addition, no increase in ethylene concentration was detected from leaves in treatment E after two days. These data suggest that exogenous ethylene may be decreasing the *in vivo* ethylene production, and the higher concentration of exogenous ethylene (treatment E) is inhibiting the production earlier. This is consistent with autoinhibition of ethylene production in wounded green tissues (Vendrell and McGlasson, 1971; Yang and Hoffman, 1984).

3.5.3 Chlorophyll concentration in leaves exposed to ethylene

The chlorophyll concentration in leaves exposed to exogenous ethylene (section 3.5.2) was measured after day four and day ten. The total chlorophyll concentration in leaves exposed to exogenous ethylene for four days was compared with total chlorophyll concentration in fresh ('control') leaves (Figure 3.28A, error bars omitted for clarity). After four days, leaves four to eight in treatments A, C, D, or E had a significantly lower total chlorophyll concentration than the control leaves. Leaves six to eight in treatment B also had significantly lower chlorophyll concentrations compared with control leaves. Data from leaves in treatments C, D, and E were not significantly different from each other indicating that leaves of white clover are sensitive to 1 ppm ethylene (treatment C), and increasing the concentration of ethylene did not increase the response over this time period. Leaves two and three in treatment A tended to have a higher concentration of chlorophyll compared with leaves in treatments C, D, or E, but the differences were not significant. Leaves two to five in treatment B had significantly higher concentrations of chlorophyll than leaves in all other treatments.

The chlorophyll *a* and *b* concentrations in leaves exposed to exogenous ethylene for four days was compared with chlorophyll *a* or *b* concentrations in

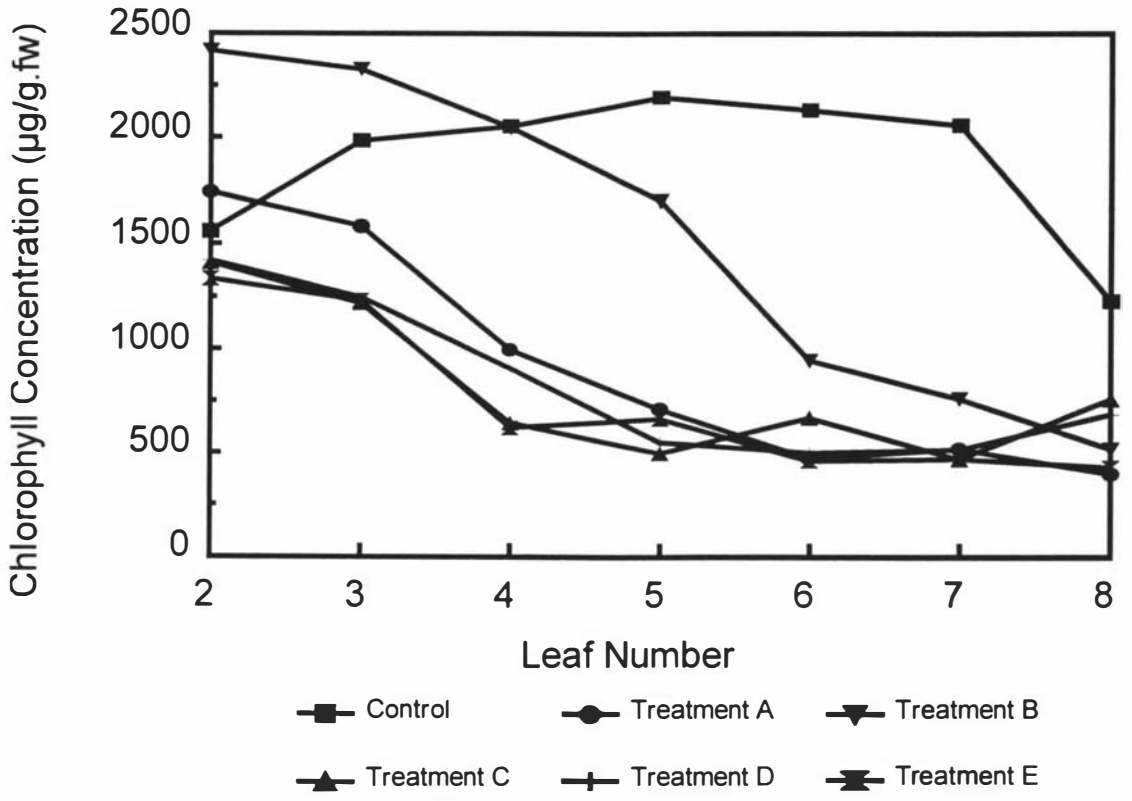


Figure 3.28A: Mean total chlorophyll concentration in detached leaves exposed to ethylene for four days.

See text for treatment details.

fresh ('control') leaves at day 0 (Figures 3.28B and C respectively). These data indicate that the decrease in total chlorophyll concentration results from a decrease in both chlorophyll *a* and *b,* although chlorophyll *a* concentration tended to decrease more than chlorophyll *b* concentration. These data are similar to the data for chlorophyll changes in attached leaves along the stolon (section 3.1.6).

The total chlorophyll, and chlorophyll *a* and *b* concentrations in leaves exposed to ethylene for ten days was compared with total chlorophyll, or chlorophyll *a* or *b* concentrations in fresh ('control') leaves (Figures 3.29A, B, and C respectively). No significant difference in chlorophyll concentration (total, *a* or *b*) was measured between leaves in treatments C, D, or E. The total chlorophyll, and chlorophyll *b* concentration in leaves one and two were significantly higher in treatments without ethylene compared with treatments with exogenous ethylene. Leaf three in treatment B also had a significantly higher total and chlorophyll *b* concentration compared with other treatments. These data suggest that chlorophyll *a* may be preferentially catabolized in detached leaves treated with ethylene.

As no significant differences in chlorophyll concentration were observed between the treatments with exogenous ethylene (treatments C, D, and E), the data were combined and the results from day four compared with results from day ten (Figures 3.30A, B and C). Leaves four to seven showed the greatest decrease in total chlorophyll concentration after four days compared with leaves two and three. Leaf eight showed a lesser decrease in chlorophyll concentration than leaves four to seven, but the concentration of chlorophyll in the control leaf eight was also significantly lower than leaves four to seven in the control leaves. After day four, the total chlorophyll concentration in leaf two was not significantly lower than the concentration in fresh leaves. However, after ten days treatment, all leaves showed significant differences in total chlorophyll concentration compared with the control treatment. The chlorophyll concentration in leaves seven and eight after day ten was not significantly different from the concentration on day four. The decrease in total chlorophyll concentration was generally reflected in the decreases in chlorophyll *a* and *b*.

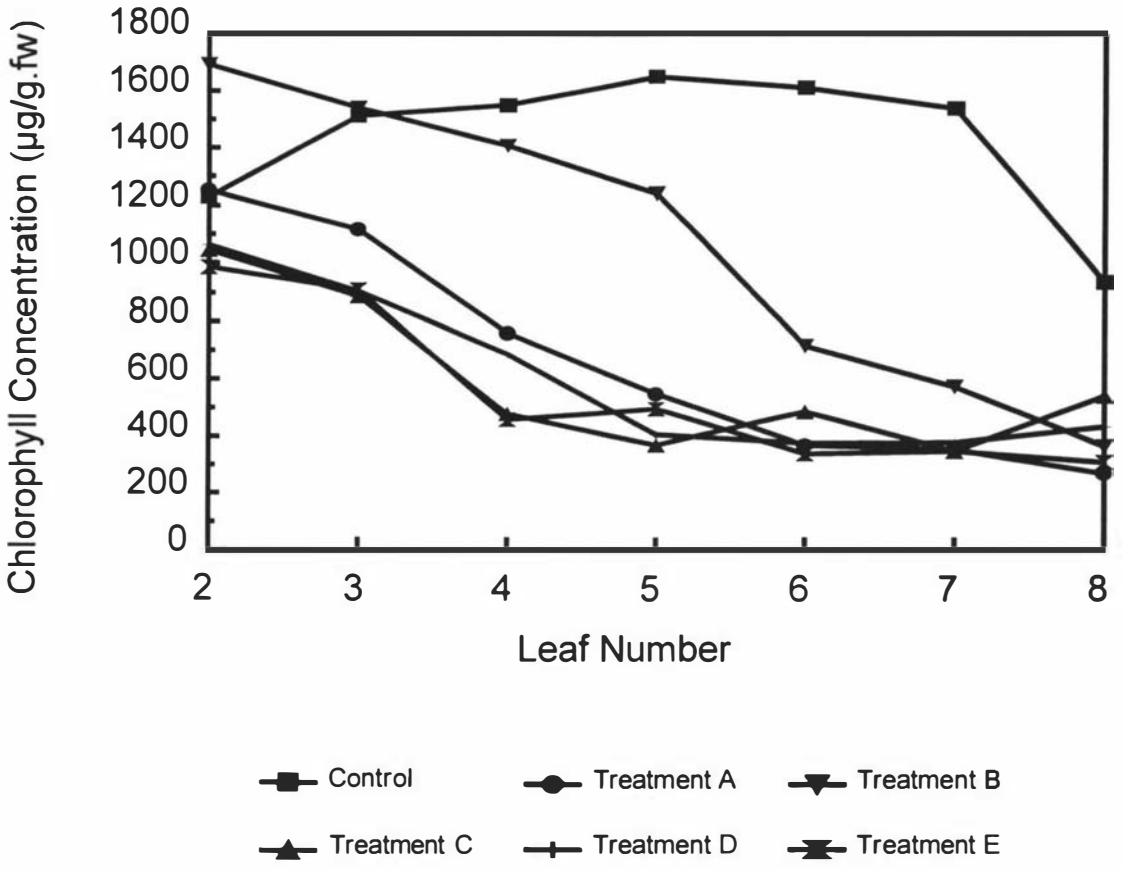


Figure 3.28B: Mean chlorophyll *a* concentration in detached leaves exposed to ethylene for four days.

See text for treatment details.

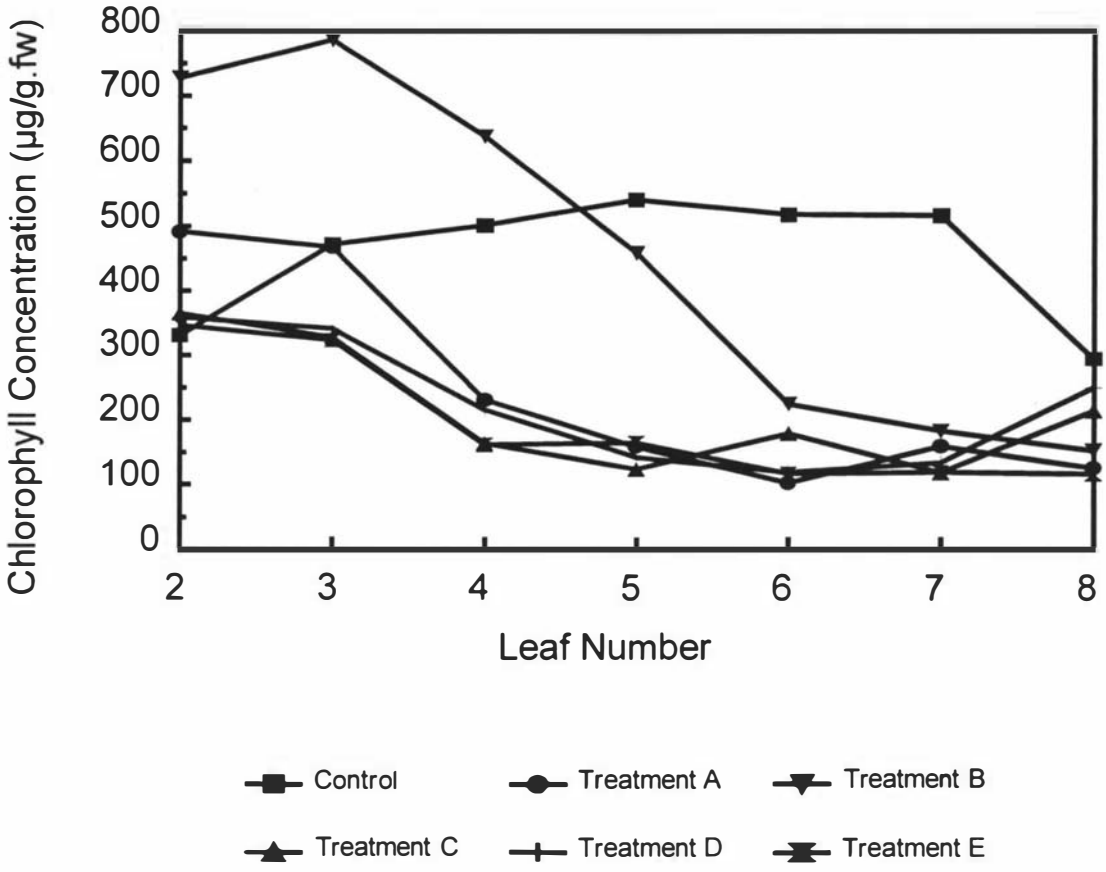


Figure 3.28C: Mean chlorophyll *b* concentration in detached leaves exposed to ethylene for four days.

See text for treatment details.

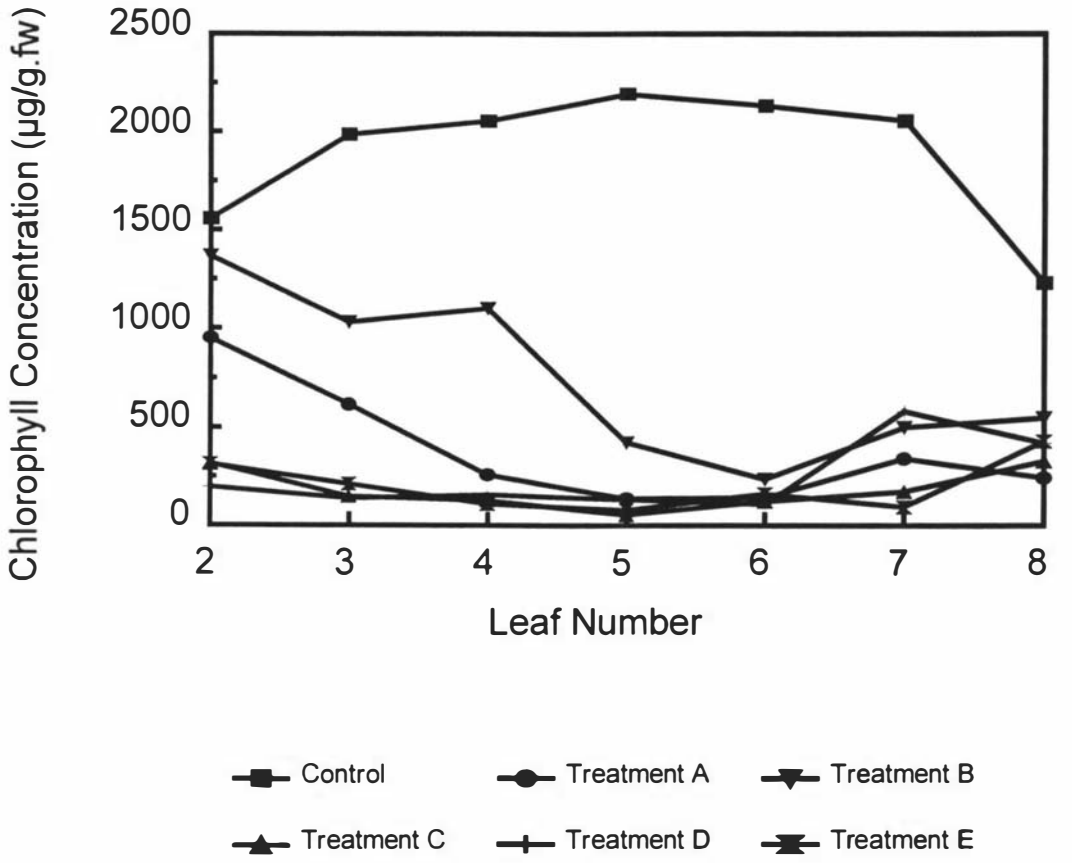


Figure 3.29A: Mean total chlorophyll concentration in detached leaves exposed to ethylene for ten days.

See text for treatment details.

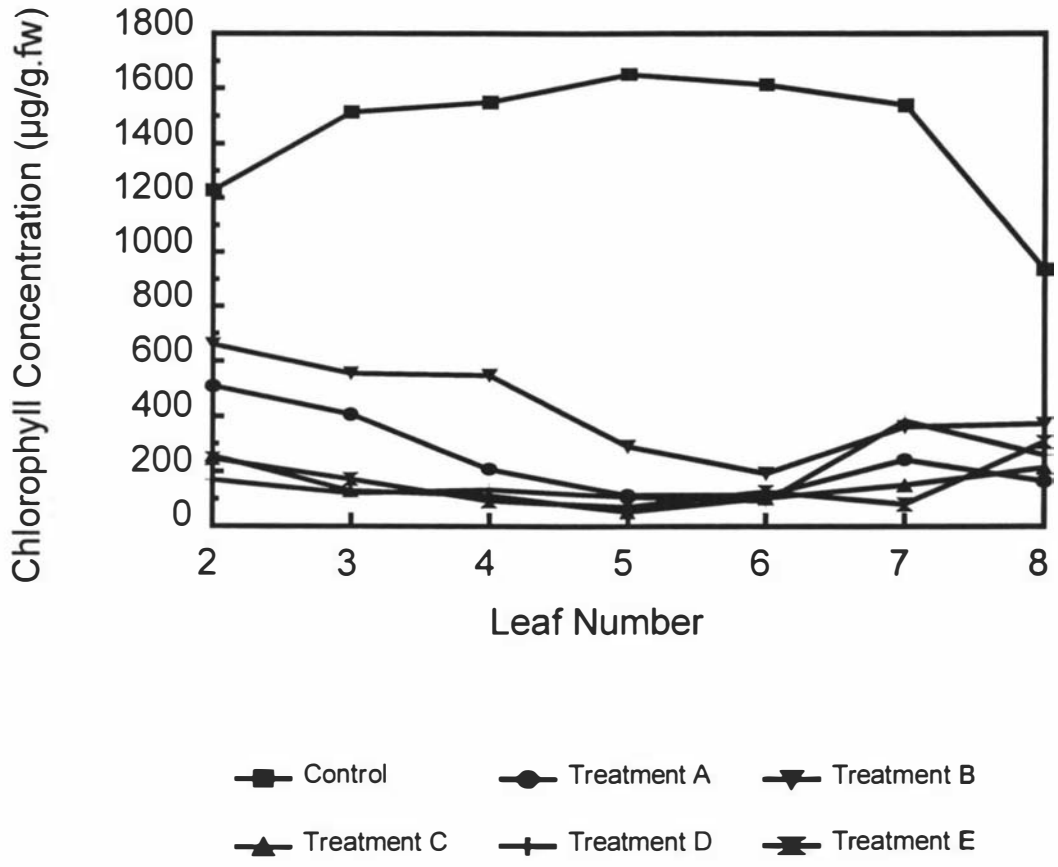


Figure 3.29B: Mean chlorophyll *a* concentration in detached leaves exposed to ethylene for ten days.

See text for treatment details.

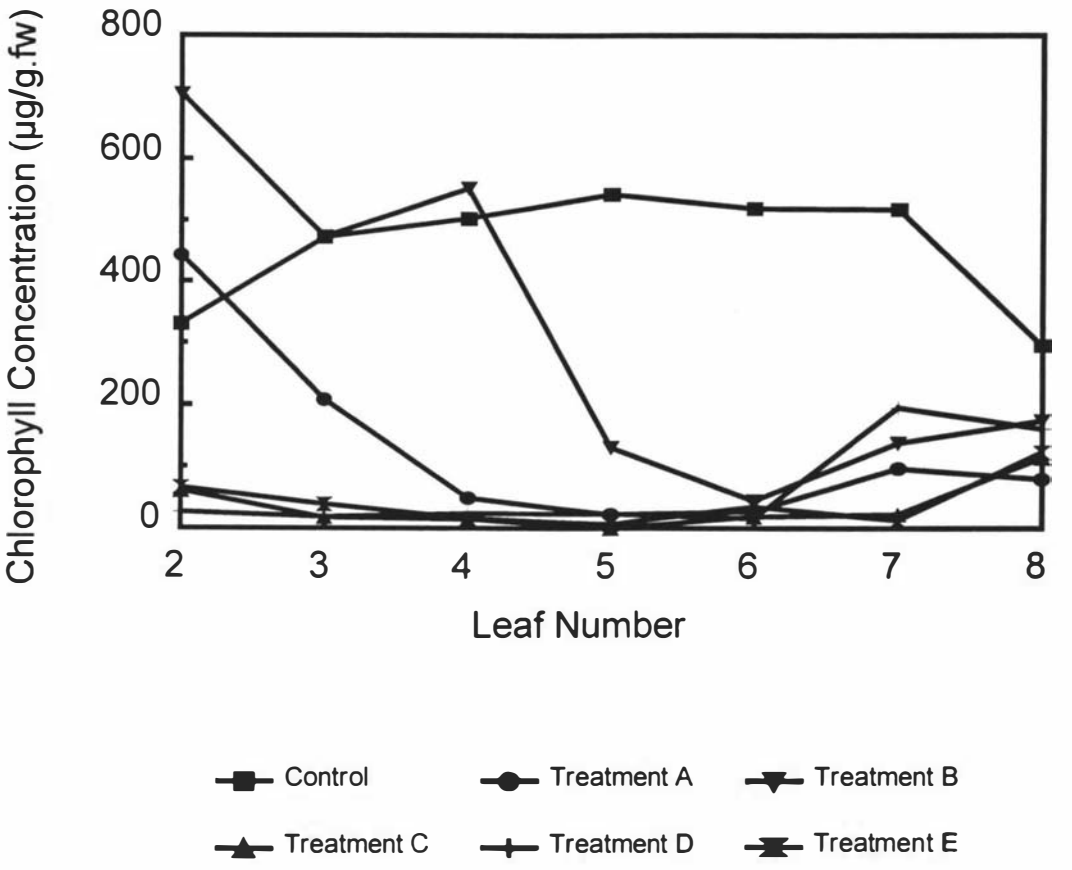


Figure 3.29C: Mean chlorophyll *b* concentration in detached leaves exposed to ethylene for ten days.

See text for treatment details.

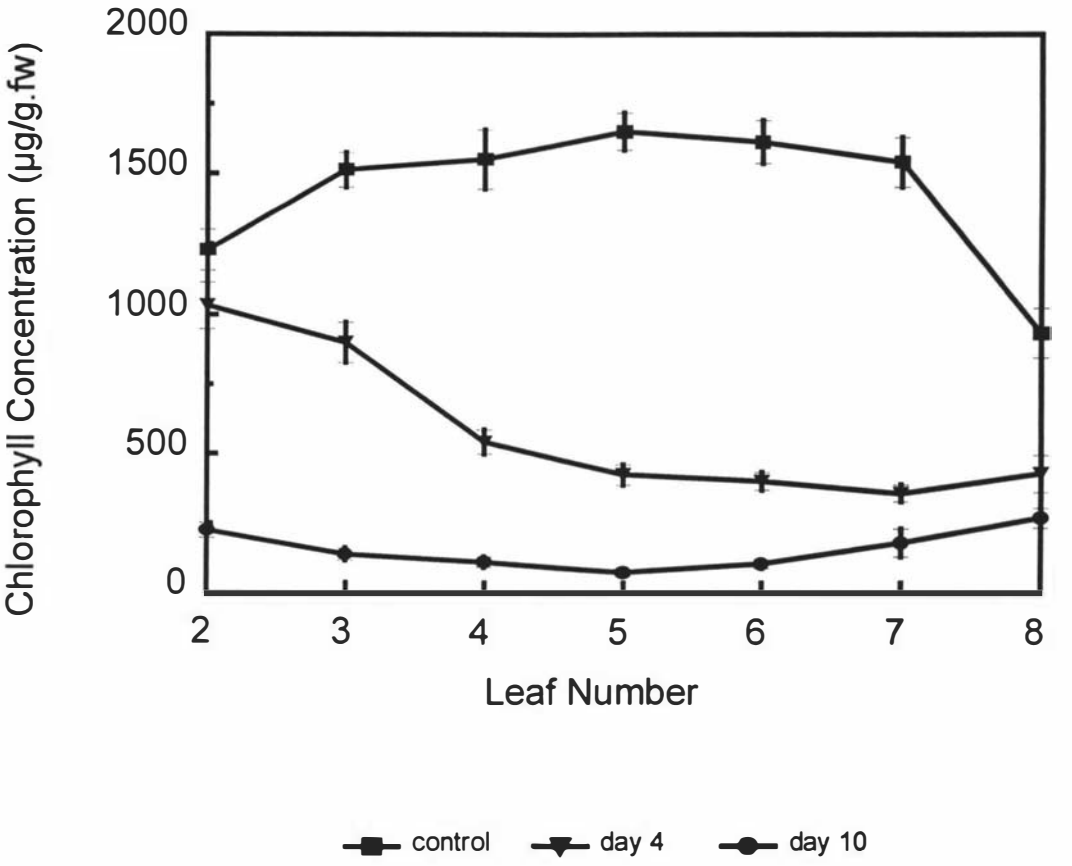


Figure 3.30B: Mean chlorophyll *a* concentration in detached leaves of white clover exposed to ethylene for all treatments combined.

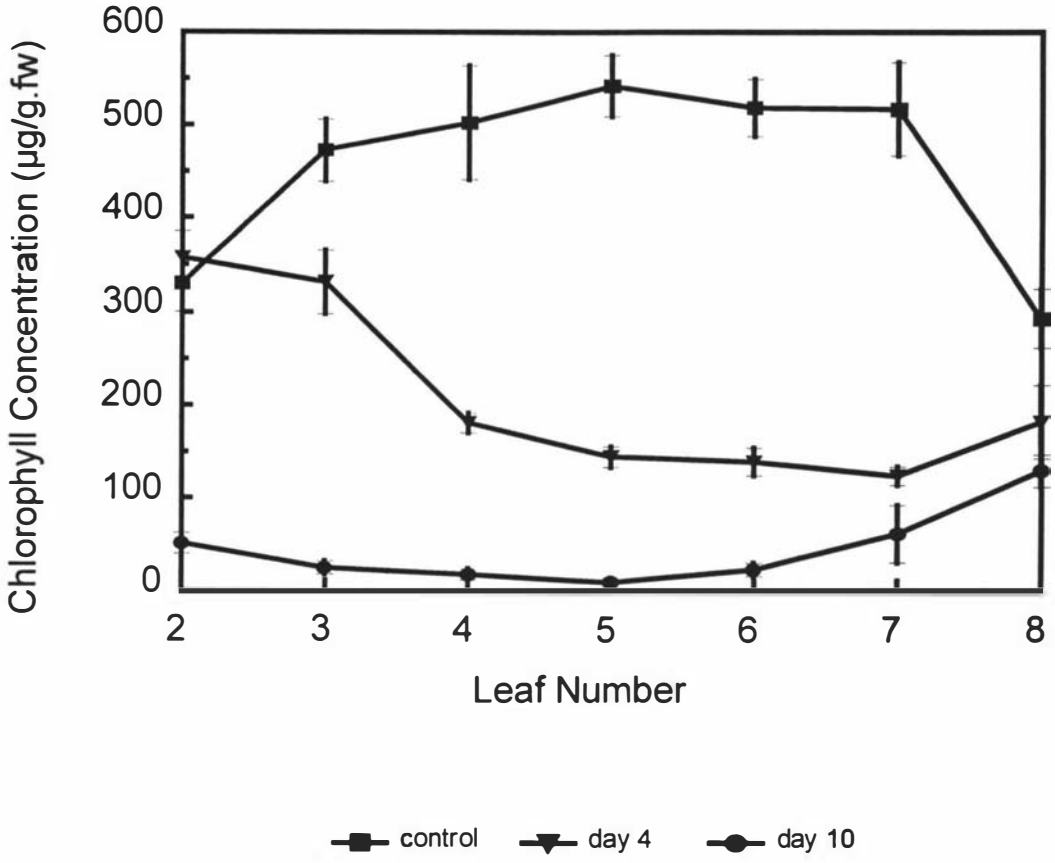


Figure 3.30C: Mean chlorophyll *b* concentration in detached leaves of white clover exposed to ethylene for all treatments combined.

Leaves four to seven showed the greatest reduction in chlorophyll concentration after four days (Figure 3.30A). Leaf three showed a lesser decrease while leaf two was not significantly different from the controls. By day ten, however, all leaves had significantly lower chlorophyll concentrations. These data indicate that the leaves are differentially sensitive to exogenous ethylene, with leaf two relatively insensitive up to day four, leaf three moderately sensitive, and all other leaves sensitive. Leaf eight had the lowest concentration of chlorophyll in the control leaves indicating that processes leading to chlorophyll loss was underway. However, after ten days of ethylene treatment, leaf eight tended to have as high or higher chlorophyll concentration than leaves two to seven. This suggests that the chlorophyll loss processes may already be partly completed and these leaves are no longer as sensitive to ethylene compared with leaves two to seven.

No significant differences could be detected in the sensitivity of the mature leaves (leaves four to seven). This may be because the minimum concentration of added ethylene (1 ppm) was higher than the level required to detect differences in sensitivity of the mature leaves. This is supported by data from the control treatment without Purafil (where a low concentration of ethylene would result from *in vivo* production; section 3.2.2), as no significant differences in chlorophyll concentration could be detected in these treatments compared with treatments with added ethylene.

CHAPTER 4

MOLECULAR CHARACTERISATION OF ETHYLENE BIOSYNTHESIS DURING LEAF MATURATION AND SENESCENCE IN WHITE CLOVER

4.1 Introduction

Ethylene is synthesised in higher plant tissues via the following pathway:

methionine →s-adenosylmethionine→ACC→ethylene

(Adams and Yang, 1979).

The conversion of s-adenosyl methionine to ACC is catalysed by ACC synthase, while ACC is converted to ethylene by ACC oxidase. The activity of ACC oxidase in leaves of white clover has been determined (section 3.4.3), and changes in the concentration of the ethylene precursor molecule (ACC) measured (section 3.3). However, attempts to measure the activity of ACC synthase were not successful (section 3.4.2).

Recently, the timing of induction of ethylene biosynthesis has been studied using molecular analysis of ACC oxidase or ACC synthase. For example, the temporal regulation of ACC oxidase was investigated in orchid flowers by Nadeau *et al.* (1993), and in tomato leaves by John *et al.* (1995). These workers showed that the accumulation of mRNA coding for ACC oxidase occurs prior to post-pollination induced senescence in orchid flowers, and in pre-senescent leaves of tomato respectively. Changes in the abundance of mRNA encoding ACC synthase has also been studied by several workers.

For example, Dong *et al.* (1991) showed that ACC synthase mRNA was undetectable in un-ripe apple fruit, but accumulated "massively during the ripening process". Park *et al.*, (1992) found that ACC synthase mRNA accumulates during senescence of carnation flower petals "concomitant" with an increase in ethylene production and ACC synthase activity.

Smart (1994) concluded that particularly for certain dicotyledonous species, "ethylene, like cytokinins, is thought to play a prominent role in the regulation of senescence. It has the opposite effect to cytokinin as it accelerates many of the physiological changes normally associated with leaf senescence". Ethylene is produced by leaves of white clover and the amount of ethylene evolved increases in senescing (de-greening) leaves (section 3.2). Further, the application of exogenous ethylene accelerates the rate of de-greening of detached white clover leaves (section 3.5).

Ethylene may have a role in the initiation of senescence, or in modifying the rate at which senescence proceeds. "The evidence supporting ethylene as an initiator of senescence might be strengthened if there was a close correlation between the timing of the rise in ethylene production and the onset of leaf senescence." (Roberts *et al.*, 1985). The rise in ethylene evolution was detected subsequent to chlorophyll loss in this study (section 3.2.3.2) and this has also been found by other workers (Even-Chen *et al.*, 1978; Aharoni *et al.*, 1979b). However, Gepstein and Thimann (1981) found that an increase in ethylene production preceded chlorophyll degradation in oat leaf segments

This section describes experiments aimed at identifying molecular markers which can be used to detect changes in abundance of ethylene biosynthesis mRNA species (ACC oxidase or ACC synthase), and relate these changes to the stage of leaf maturation and senescence in white clover.

4.2 Southern hybridisation of white clover genomic DNA with heterologous ACC oxidase DNA sequences

4.2.1 Introduction

ACC oxidase catalyses the conversion of ACC to ethylene in higher plants. A cDNA, isolated by differential cloning techniques in tomato fruit and leaves from mRNA that accumulates in response to ethylene (pTOM13; Davies and Grierson, 1989) was subsequently shown to be ACC oxidase (Hamilton *et al.* 1991; Köck *et al.*, 1991). The use of pTOM13 as a molecular probe for ACC oxidase was investigated for use in white clover. A copy of pTOM13 inserted into pBR322 (Slater *et al.,* 1985) was gifted by Dr. K Davies, Crop and Food, Levin (Figure 4.1) and used to probe a genomic Southern blot of white clover DNA.

4.2.2 Preparation of pTOM13 for use as a molecular probe

The plasmid pBR322/pTOM13 was transformed into competent *E. coli* (strain DH5α) cells and these were grown on L agar containing 2.5 μg/mL tetracyclin. Individual colonies were selected from the plate and grown overnight in 10 mL of L broth containing 2.5 μg/mL tetracyclin. *E. coli* containing the plasmid pBluescript were also grown overnight in 10 mL of L broth containing 100 μg/mL ampicillin. Plasmid DNA was extracted from these broth cultures, digested with *Pst* 1, and the digests electrophoresed in a 1% (w/v) agarose gel (Figure 4.2). Regions of the gel containing the 2.96 kb pBluescript or the 1.3 kb pTOM13 fragments were both removed and the DNA extracted. Aliquots of the fragments were re-electrophoresed to confirm the fragment size and purity. The pBluescript vector was treated with CAP and the pTOM13 fragment ligated into it. Competent cells of *E. coli* (strain DH5α) were then transformed with the pBluescript/pTOM13 construct and the bacteria grown on L agar containing 100 μg/mL ampicillin with 40 μL of 5-bromo-4-chloro-3-indoyl-b-D-galactoside (x-gal) stock solution spread on the surface of the agar. DNA inserted into the multiple cloning site (MCS) of pBluescript disrupt the *lac Z* gene (coding for β-galactosidase) and hence colonies are white

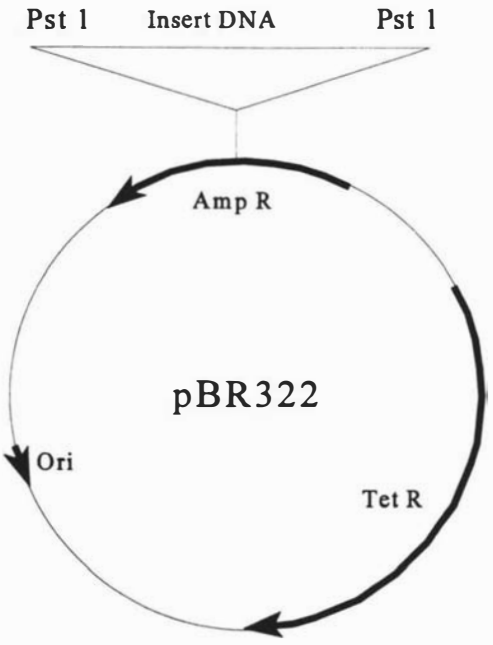


Figure 4.1: Plasmid pBR322 showing the position of the inserted DNA from tomato to make the plasmid construct pTOM13 (Slater *et al.*, 1985).

Amp R:	Ampicillin resistance gene
Tet R:	Tetracyclin resistance gene
Ori:	Plasmid origin of replication

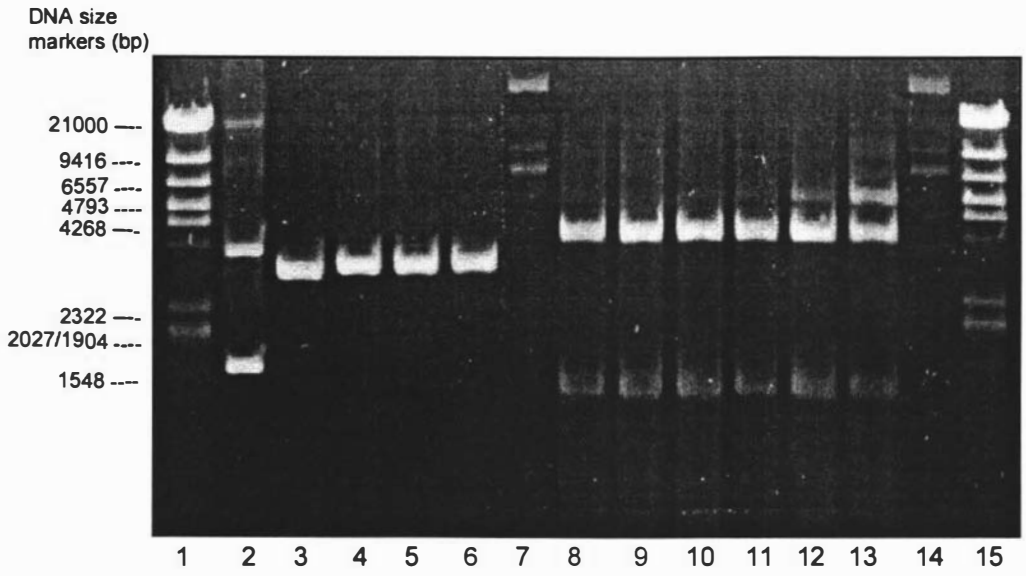


Figure 4.2: Vectors pBR322 and pBluescript digested with *Pst* 1 and separated by electrophoresis in a 1% (w/v) agarose gel.

Lanes 1 and 15:	λDNA size markers
Lane 2:	undigested pBluescript
Lanes 3 to 6:	pBluescript digested with *Pst* 1
Lanes 7 and 14:	undigested pBR322
Lanes 8 to 13:	pBR322 digested with *Pst* 1

(positive) when grown on media containing x-gal, while negative (no insert) colonies are blue.

One blue (pSB6) and five white (pSB1 to pSB5) colonies were picked from the plates and grown overnight in 10 mL of L broth containing 100 μg/mL ampicillin. Plasmid DNA was extracted from the cells, digested with *Pst* 1, and small aliquots electrophoresed in a 1% (w/v) agarose gel (Figure 4.3). Plasmid DNA extracted from the positive clones contained the expected 1.3 kb insert whereas plasmid DNA extracted from the negative clone did not contain any bands other than the digested plasmid band (2.96 kb). These data suggest that CAP treating the digested pBluescript (see above) did not completely inhibit the re-ligation of the plasmid. Aliquots (5 μL) of the DNA extracted from the positive clones were digested with *Pst* 1, the products separated by electrophoresis in a 1% (w/v) agarose gel, and the DNA band corresponding to a molecular weight of 1.3 kb was extracted and stored in TE buffer at -20°C until required.

4.2.3 Extraction and Southern blotting of white clover genomic DNA

DNA was extracted from duplicate samples of fresh leaf tissue of white clover (1.5 g) and dissolved in a minimal volume of TE buffer. DNA extracted from leaves of tomato (1.8 g) was used as a control on the Southern blots. The concentration of the dissolved DNA and 260/280 nm ratio of each preparation is shown in Table 4.1. Aliquots (10 μg) of the DNA from white clover were digested with *Bam*H 1, *Eco*R 1, *Hin*d III, *Pst* 1, *Xba* 1 or *Kpn* 1. The products were separated by electrophoresis in a 1% (w/v) agarose gel and the efficiency of the digestion visually assessed under UV light. DNA digested with *Eco*R 1, *Hin*d III and *Xba* 1 resulted in a even smear of DNA fragments in the gel, whereas DNA digested with *Bam*H 1, *Pst* 1 and *Kpn* 1 appeared to be less well digested with several bands appearing in the gel (data not shown).

Aliquots of DNA from white clover (30 μg) were then digested with *Eco*R 1, *Hin*d III or *Xba* 1, and the products separated by electrophoresis in a 1% (w/v) agarose gel (Figure 4.4). These digests resulted in an even smear of DNA without obvious banding, suggesting the DNA had been efficiently cut.

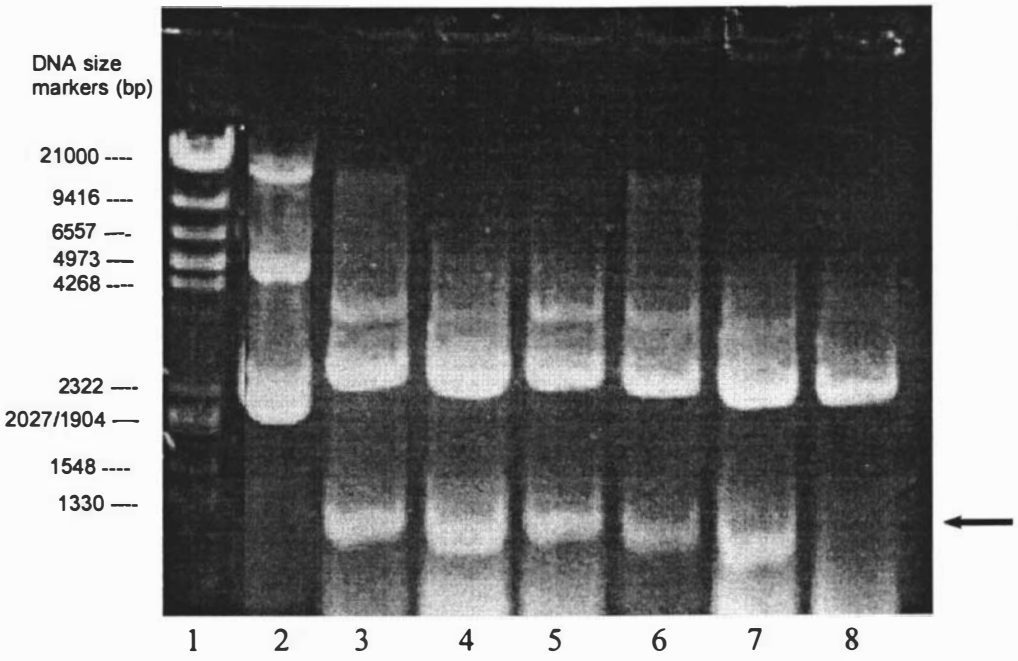


Figure 4.3: Plasmids pSB(1-6) digested with *Pst* 1 and separated by electrophoresis in a 1% (w/v) agarose gel. The 1.3 kb insert DNA band is arrowed.

Lane 1:	λDNA size markers
Lane 2:	undigested plasmid
Lane 3:	pSB1 digested with *Pst* 1
Lane 4:	pSB2 digested with *Pst* 1
Lane 5:	pSB3 digested with *Pst* 1
Lane 6:	pSB4 digested with *Pst* 1
Lane 7:	pSB5 digested with *Pst* 1
Lane 8:	pSB6 digested with *Pst* 1

Table 4.1: Absorbance ratio and quantity of DNA extracted from leaves of white clover and tomato for Southern analysis.

Extract	260/280 nm ratio	DNA (mg/g.fw)
White clover #1*	1.581	245
White clover #2*	1.778	353
Tomato	1.492	200

*duplicate extracts.

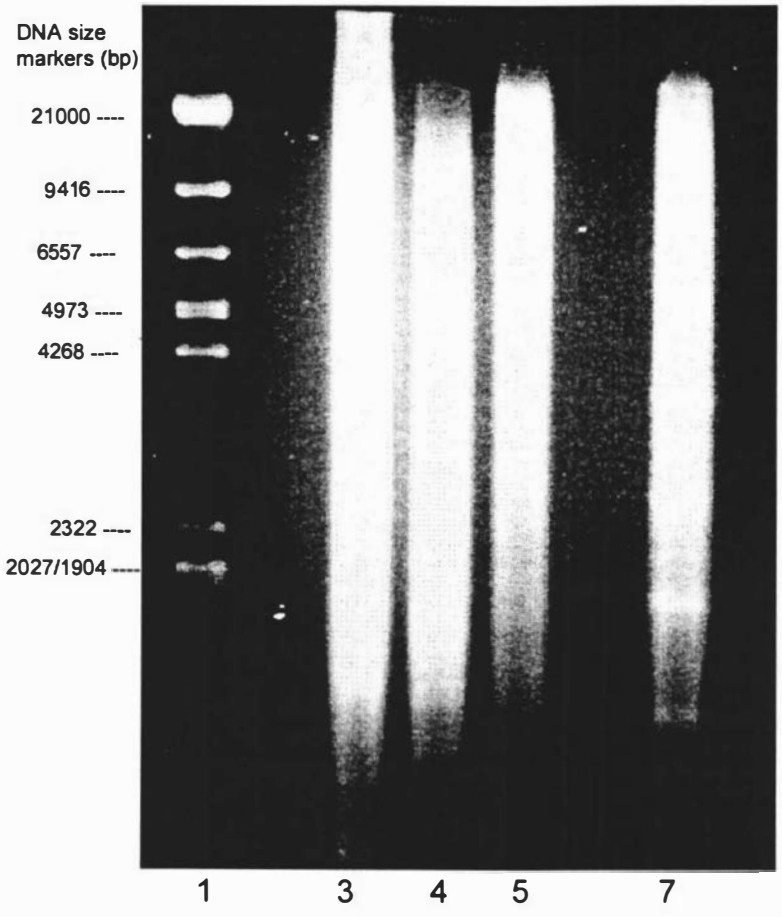


Figure 4.4: White clover genomic DNA restriction enzyme digests separated by electrophoresis in a 1% (w/v) agarose gel.

Lane 1:	λDNA size markers
Lane 3:	white clover DNA digested with *EcoR* 1
Lane 4:	white clover DNA digested with *Hind* III
Lane 5:	white clover DNA digested with *Xba* 1
Lane 7:	tomato DNA digested with *EcoR* 1

Tomato DNA (30 μg) was also digested with *Eco*R 1 and electrophoresed on the same gels. The DNA was blotted onto Hybond N^+ membrane and the membrane washed in 5 x SSC and stored in a sealed plastic bag at 4°C until required.

4.2.4 Hybridisation of genomic DNA with ^{32}P-labelled pTOM13 DNA

A ^{32}P-labelled DNA probe was prepared using the pTOM13 fragment (section 4.2.1) as a template. The ^{32}P-labelled DNA probe was allowed to hybridise overnight with white clover and tomato DNA fixed to the membrane as described in section 4.2.2, and the blot washed at low stringency (1 X SSPE, 65°C) and autoradiographed. The results of this experiment were inconclusive as only non-specific hybridisation (no specific bands detected) of the ^{32}P-labelled DNA could be detected with both the white clover and tomato DNA control (data not shown). The inability to detect the control DNA may have been due to several factors including;

1. A corrupted copy of pTOM13,
2. An ineffective Southern blot, or
3. Incorrect hybridisation conditions.

A second copy of pTOM13 (gifted by, University of Nottingham, Sutton Bonnington) was tested using the same Southern blot and hybridisation conditions as described previously. A second tomato clone, pTOM75 (a membrane channel protein; Dr. J. Roberts, pers.com.) was used as a control on a second Southern blot. Both the second pTOM13 (see Figure 4.5) and pTOM75 (data not shown) showed hybridisation with the tomato DNA controls, but no binding to specific bands in the white clover DNA could be detected. These results suggest that the use of tomato probes to detect ACC oxidase in white clover was not successful and that heterologous probes from other plant species, or homologous DNA sequences would be required to identify the ethylene biosynthetic genes in white clover.

Figure 4.5: Southern analysis of white clover and tomato DNA probed with [^{32}P]-labelled DNA prepared using pTOM13 (J.A.Roberts, University of Nottingham, Sutton Bonnington) as a template. The blot was washed at low stringency (1 x SSPE, 65°C).

Lane 1:	white clover DNA digested with *Eco*R1
Lane 2:	white clover DNA digested with *Hin*dIII
Lane 3:	white clover DNA digested with *Xba* 1
Lane 4:	tomato DNA digested with *Eco*R1

4.3 PCR amplification of ACC synthase and ACC oxidase DNA sequences

4.3.1 Introduction

Southern analysis of white clover genomic DNA using a heterologous DNA probe from tomato was inconclusive and no specific bands could be identified. Reverse transcriptase-dependent polymerase chain reaction (RT-PCR) was investigated as a means of obtaining DNA sequences homologous to ACC oxidase and ACC synthase from white clover. RT-PCR uses RNA as a template for the production of single-stranded cDNA and this is then used as a template for PCR. This section details experiments aimed at producing PCR-generated DNA fragments homologous to ACC oxidase and ACC synthase from the leaves of white clover.

4.3.2 Extraction of total RNA from leaves of white clover using Trizol

Total RNA was extracted using Trizol (section 2.3.21.1) from leaves two to ten from plants grown at Levin. The fresh weight of the leaf material, the amount of RNA extracted per gram of leaf tissue, and the 260/280 nm absorbance ratios are shown in Table 4.2. Aliquots of the RNA extract were electrophoresed under denaturing conditions in a 1% (w/v) agarose gel (Figure 4.6). Six sharp, clear bands were detected against a background smear of RNA in extracts from leaves two and three. However, RNA extracted from leaves four to seven showed a decrease in the size of these bands relative to RNA extracted from leaves two and three. In extracts from leaves eight, nine and ten, fewer bands were visible and the high molecular weight RNA bands were absent when compared with the other extracts. An increased amount of smearing of these bands was visible in extracts from older leaves, and this was particularly noticeable for the extract from leaf ten.

Table 4.2: Total RNA extracted using Trizol from leaves of white clover grown at Levin

Leaf number	Fresh weight (mg)	260/280 nm ratio	Total RNA (mg/g.fw)
2	188	2.046	723.4
3	159	2.242	754.7
4	201	2.115	364.2
5	158	1.980	597.5
6	206	1.977	374.8
7	159	2.243	478.0
8	182	2.093	455.0
9	181	1.785	435.4
10	240	2.090	433.3

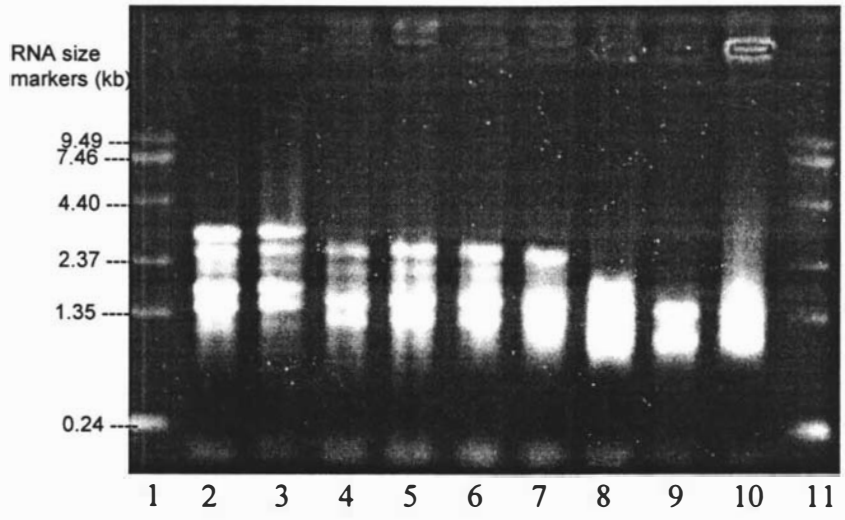


Figure 4.6: Total RNA extracted from leaves of white clover using Trizol and separated by electrophoresis in a 1% (w/v) agarose gel under denaturing conditions.

Lanes 1 and 11:	RNA size markers
Lanes 2 to 10:	RNA extracted from leaf numbers 2 to 10 respectively

4.3.3 Extraction of total RNA from leaves of white clover using hot phenol and lithium chloride

Total RNA was extracted from leaves one to twelve from plants grown at Palmerston North using the method described in section 2.3.12.2. The fresh weight of the leaves, and the amount of RNA extracted per gram of leaf tissue is shown in Table 4.3. The ratio of RNA to leaf fresh weight was usually lower than that obtained using the Trizol method (section 4.3.2). The highest extraction (1290 μg/g.fw) was achieved from leaf one.

The 260/280 nm absorbance ratios for the total RNA extracts are shown in Table 4.3. The ratios are close to the theoretical ratio for pure RNA (1.8, Sambrook *et al.*, 1989) indicating the extracts have a low concentration of impurities. Aliquots (10 μg) of total RNA were electrophoresed under denaturing conditions in a 1% (w/v) agarose gel (Figure 4.7). The ribosomal RNA bands appear clear and sharp for all leaves, and a smear of non-ribosomal RNA appears as a background. RNA extracted using this protocol did not show the shift in the size of the rRNA bands evident in RNA extracted using Trizol (section 4.3.2) suggesting that less degradation of the RNA has occurred using this extraction method.

4.3.4 Amplifying ACC synthase and ACC oxidase DNA fragments using PCR

Plants grown at Levin show a decrease in the concentration of chlorophyll in leaf six (section 3.1.5), and an increase in the level of ethylene evolution compared with leaf five, and similar changes occur at leaf nine for plants grown at Palmerston North (section 3.1.6). The concentration of ACC increased in leaves five to seven (compared with leaves three and four) while the concentration of MACC tended to be higher in leaf six (compared with leaves four and five) in plants grown at Levin (section 3.3.2). ACC is produced from two known sources in plants; from AdoMet by ACC synthase (Adams and Yang, 1979), and from MACC (Jiao *et al.*, 1986). The enzyme which converts MACC to ACC, ACC malonyltransferase (Yang *et al.*, 1990), has a reported Km of 0.45 mM, and "high MACC levels in the plant tissues can

Table 4.3: Total RNA extracted as described in section 4.3.3 from leaves of white clover grown at Palmerston North.

Leaf number	Fresh weight (mg)	260/280 nm ratio	Total RNA (mg/g.fw)
1	279	1.922	1290
2	919	1.989	470
3	1422	2.013	215
4	2209	1.960	318
5	2455	1.903	207
6	2713	1.991	128
7	2849	1.908	237
8	3193	1.907	295
9	3409	1.843	176
10	3910	1.848	66
11	4232	1.839	159
12	4142	1.842	235

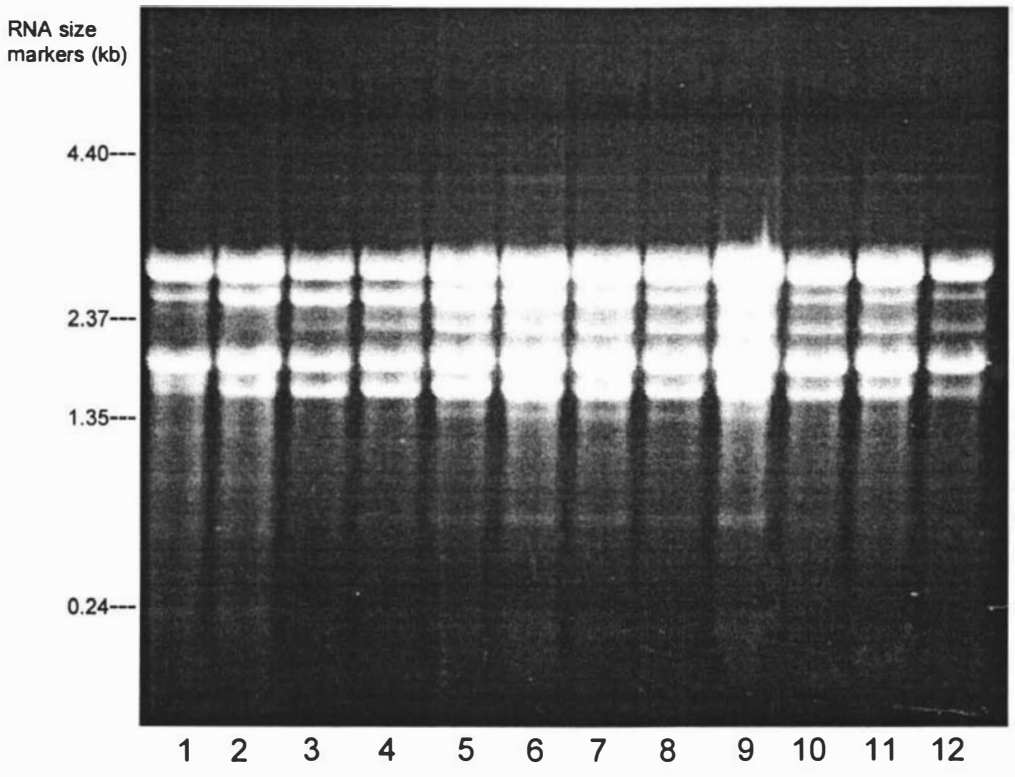


Figure 4.7: Total RNA extracted from white clover leaf tissue and separated by electrophoresis in a 1% (w/v) agarose gel under denaturing conditions.

Lanes 1-12: Total RNA extracted from leaves 1 to 12 respectively.

induce to some extent the capability to convert MACC to ACC" (0.2 to 5 millimolar MACC; Jiao *et al.*, 1986). However, the MACC concentration in leaves of white clover was less than 12 nmoles/g.fw (Figure 3.17) suggesting that MACC is unlikely to be a major source of ACC for ethylene production. These data suggest that ACC is most likely being produced by the action of ACC synthase and hence the enzyme, and the mRNA encoding it may be at a relatively high concentration in this leaf. Leaf six may therefore be a good candidate for the extraction of RNA and analysis by PCR for the presence of ACC synthase.

Total RNA was extracted from leaf six from plants grown at Levin (section 4.3.3) and the pelleted RNA dissolved in formamide. The RNA was given to Dr. M McManus for use as a template in RT-PCR amplification. RT-PCR was performed using primers specific for the conserved domains in ACC oxidase and ACC synthase (Table 2.4; primers gifted by Professor S.F.Yang, University of California, Davis). The PCR generated DNA fragments were inserted into PCR-II plasmid by A/T cloning (Figure 4.8), transformed into *E. coli* (strain DH5α), and the bacteria screened on L agar containing 150 μg/mL ampicillin. Twelve colonies from each set (ACC oxidase and ACC synthase) were picked and subcultured onto replica plates. Overnight broth cultures were prepared from each of the colonies and the plasmid DNA extracted and digested with *Eco*R 1. The digests were then separated by electrophoresis in an agarose gel and the size of the inserted DNA assessed by comparison with DNA size markers. Plates containing each of the twelve clones were gifted for use in the rest of this study. PCR amplified ACC oxidase and ACC synthase cDNA fragments generated using the primers listed in Table 2.4 have expected sizes of 850 and 670 kb respectively.

4.3.5 Southern hybridisation of white clover genomic DNA with PCR generated DNA fragments

Plasmid DNA extracts which showed a high concentration of DNA on the electrophoresis gel, and a band of the correct size, (plasmid preparations ACO4 and ACS7) were selected for use in Southern analysis (data from Dr. M.McManus). Genomic DNA blots were hybridised with ^{32}P-labelled DNA prepared using PCR generated fragments of the putative ACC oxidase and

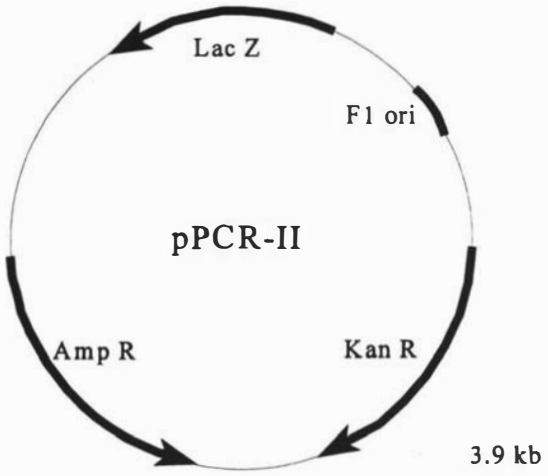


Figure 4.8: Plasmid pPCR-II used for A/T cloning of PCR products generated from white clover leaf RNA extracts.

Amp R:	Ampicillin resistance gene
Kan R:	Kanamycin resistance gene
F1 ori:	Plasmid F1 origin of replication
Lac Z:	β-galactosidase gene from *E coli*

ACC synthase DNA as templates, and the blots were washed at high stringency (0.1 X SSPE, 65°C). Autoradiographs of the blots hybridised with putative ACC oxidase (ACO4) and ACC synthase (ACS7) PCR clones are shown in Figures 4.9 and 4.10 respectively. Strong hybridisation was detected for both the putative ACC oxidase and ACC synthase probes to the white clover DNA. Faint hybridisation was detected between the putative ACC oxidase probe and tomato DNA but no hybridisation could be detected between the putative ACC synthase probe and the tomato DNA. These data indicate the sequences were homologous to genomic DNA sequences of white clover.

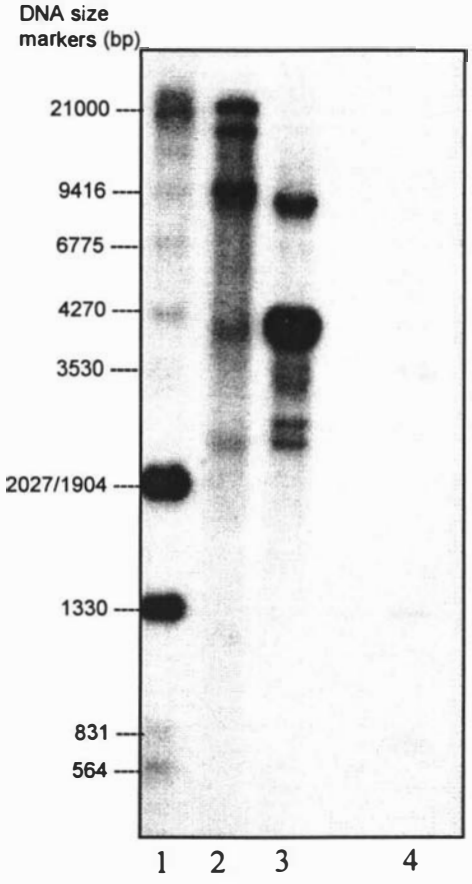


Figure 4.9: Southern analysis of white clover DNA probed with a [^{32}P]-labelled DNA fragment generated by PCR using primers specific for ACC oxidase (see section 4.3.5).

Lane 1:	white clover genomic DNA digested with *EcoR* 1.
Lane 2:	white clover genomic DNA digested with *Hind* III
Lane 3:	white clover genomic DNA digested with *Xba* 1.
Lane 4:	tomato genomic DNA digested with *EcoR* 1.

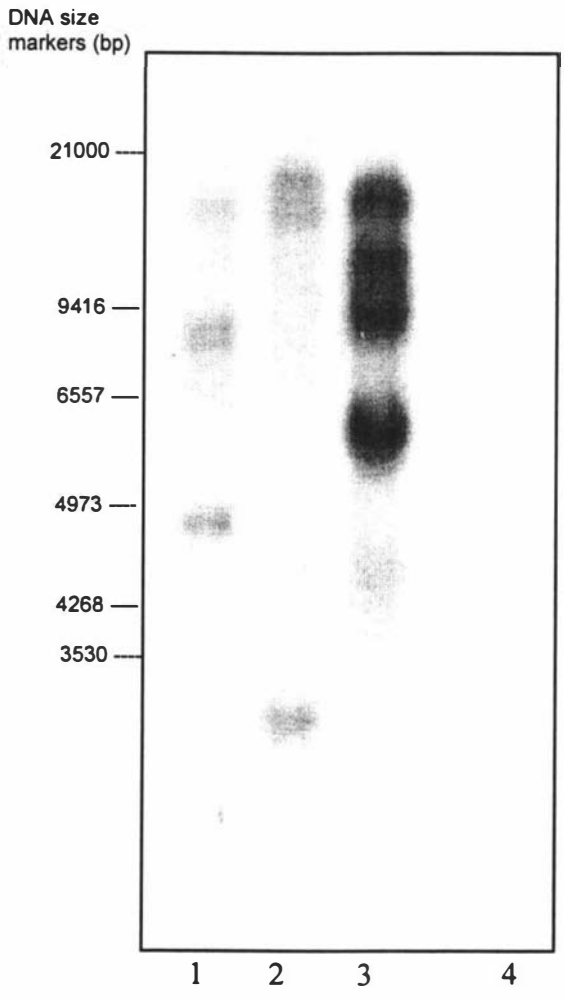


Figure 4.10: Southern analysis of white clover DNA probed with a [^{32}P]-labelled DNA fragment generated by PCR using primers specific for ACC synthase (see section 4.3.5).

Lane 1: white clover genomic DNA digested with Eco R1.
Lane 2: white clover genomic DNA digested with Hind III
Lane 3: white clover genomic DNA digested with Xba1.
Lane 4: tomato genomic DNA digested with Eco R1.

4.4 Molecular characterisation of ACC synthase

4.4.1 Introduction

Attempts to measure the activity of ACC synthase in protein extracts from leaves of white clover grown at Levin were unsuccessful (section 3.4.2) although the activity of ACC oxidase could be measured in similar leaf extracts (section 3.4.3). An increase in the product of ACC synthase, ACC, was measured in leaves 5 and 6, and an increase in ethylene evolution was measured in leaf 7, indicating that ACC synthase activity was present in these leaves. RT-PCR amplification of RNA extracted from leaf six using primers specific to the conserved domains of ACC synthase (section 4.3.4) produced DNA fragments which hybridised strongly with genomic DNA from white clover (section 4.3.5). These data suggest PCR amplification could be a successful method for obtaining DNA sequences homologous to ACC oxidase and ACC synthase from white clover.

ACC synthase is believed to be the rate limiting step in the ethylene biosynthetic pathway (Yang and Hoffman, 1984; Arteca, 1989; Yang and Dong, 1993), and, as would be expected, the protein and mRNA is found at low concentration in ripening tomato fruit (Bleecker, *et al.*, 1986; Van Der Straeten *et al.,* 1990, 1992a). The induction of ACC synthase may therefore be an important indicator in identifying early stages of senescence in white clover leaves. In addition, kinetic data on ACC oxidase had confirmed the activity of the enzyme in leaf extracts, but no data on the activity of ACC synthase had been obtained to date. All subsequent investigations in this project were aimed, therefore, at the molecular characterisation of ACC synthase. This section describes experiments aimed at obtaining the sequence of the putative ACC synthase clones generated by RT-PCR from leaves of white clover.

4.4.2 Preparation of PCR amplified ACS clones for sequencing

PCR amplified ACC synthase cDNA fragments generated using the primers

listed in Table 2.4 have the expected sizes of approximately 780 bp after amplification using the primers ACSR1F and ACSR6R, and 670 kb after amplification using the primers ACSR2F and ACSR6R (based on the fragment sizes obtained from PCR amplification of ACC synthase from *Arabidopsis;* see Figure 4.12b). The plasmids were classified as belonging to one of five classes according to their apparent size in this gel (Table 4.4). Representative plasmids from each class were sequenced in the forward direction to obtain preliminary sequences. The plasmids falling into class 1 were represented by seven of the twelve plasmids and the preliminary sequencing, and *Eco*R 1 fragment size, suggested these were good candidates for fragments of DNA homologous to ACC synthase in white clover. DNA isolated from plasmid pACS7 was shown to hybridise strongly with white clover DNA (section 4.3.5). This plasmid and plasmid pACS8, both shown to contain a DNA fragment of approximately 670 bp (section 4.3.4), were selected for full sequence analysis. *E.coli* containing these plasmids were amplified overnight in 10 mL of L broth cultures containing 150 μg/mL ampicillin, and the plasmid DNA extracted. The purified plasmid DNA was digested with *Eco*R 1 and then separated by electrophoresis in a 1% (w/v) agarose gel to confirm the insert size (Figure 4.11).

4.4.3 Sequencing ACS7 and ACS8

The PCR generated inserts in plasmids pACS7 and pACS8 were sequenced using universal forward and reverse primers. Internal primers (ACSF-1 and ACSR-1) were used (Table 2.4) to obtain internal sequences and a 'contig' sequence generated (Figure 4.12a). The positions of the conserved domains in ACC synthase genes (Harpster *et al.,* 1996), and the positions of the primers used in the PCR amplification of ACC synthase from white clover is shown in Figure 4.12b. The PCR product was produced using primers homologous to conserved domains one and six in the first round of amplification, and domains two and six in the second round of amplification. The three conserved domains between primer sites two and six (domains three, four and five), and the derived amino acid sequences for these regions are shown in bold in Figure 4.12a, and compared with published sequences in Table 4.5. The derived amino acid sequence for the conserved domains are identical with other published sequences. In the reaction centre (domain five),

Table 4.4: Classification of putative ACC synthase clones generated by PCR from cDNA's prepared from leaves of white clover (see section 4.4.2)

Classification	Plasmids
Class I	pACS 2,3,7,8,9,10,12
Class II	pACS 6,11
Class III	pACS 4
Class IV	pACS 5
Class V	pACS 1

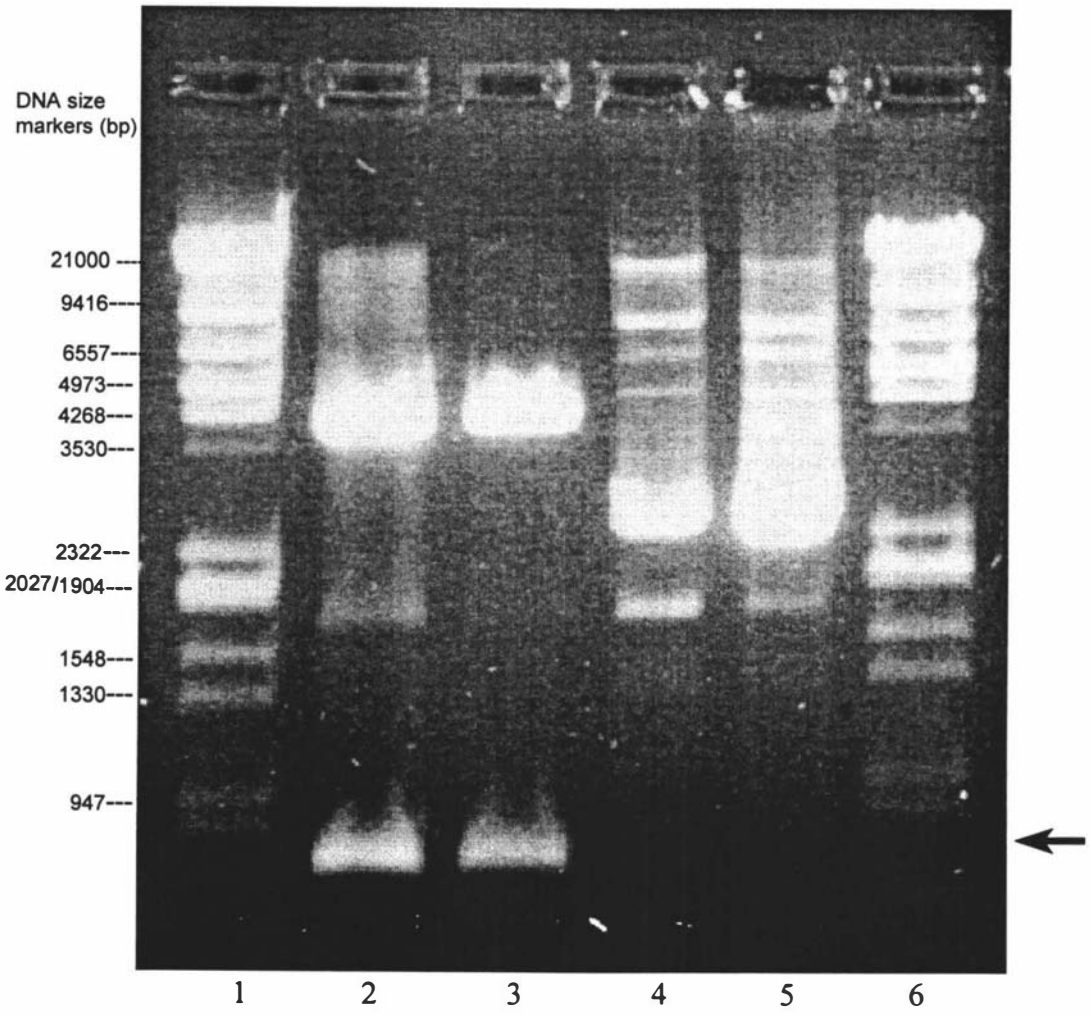


Figure 4.11: Restriction enzyme digests of pPCR-II plasmid DNA containing putative ACC synthase DNA fragments (arrowed) generated by PCR from cDNA s prepared from leaves of white clover. The digests were separated by electrophoresis in a 1% (w/v) agarose gel

Lanes 1 and 6: λ DNA size markers
Lane 2: pASC-7 digested with *Eco*R 1
Lane 3: pACS-8 digested with *Eco*R 1
Lane 4: pACS-7 undigested
Lane 5: pACS-8 undigested

Figure 4.12a: Consensus DNA sequence for ACS-7 and ACS-8. Primer sites are underlined. Conserved domains and the reaction centre are shown in bold, and the predicted amino acids are shown below.

CTGGATCCGTWYCARGATTATCACGGTCTACCAGAGTTCAGAAATGCTGT 50

GGCTAAATTCATGTCTAGAACAAGAGGAAACAGAGTTACATTTGATCCTG 100

ATCGTATTGTCATGAGTGGTGGAGCAACTGGAGCACATGAGGTTACT**GC** 150
A

CTTTTGTTTGGCAGATCCTGGTGATGCTTTTTGGTACCTACTCCTTACTA 200
F C L A D P G
(domain 3)
TCCAGGTTTCGATCGAGATTTGAGGTGGAGAACGGGAGTTAAACTGTCGT 250

TATCTGCGAAGCGCGAATAATTTCAAATTAACAAAAACAAGCTTTAGAA 300

GAAGCATATGAAAAAGCCAAAATTTGATAACATCAGAATAAAAGGTTTAC 350

TCATAACA**AATCCTTCAAATCCATTAGGCACA**GTTATGGACAGAACCACA 400
N P S N P L G T
(domain 4)
TTAAAACGTTGTAAATTTCATCAAYGAAAAGCGTATTCATCTTATAAGCG 450

ATGAAATTTACGTGCACGTTTTTAGCCACCCAAGTTTCATAAGCATAGCT 500

GAGATCATAGAAMAGAAACAGACATCGAATGTGACCGTAACCTTGTTCAC 550

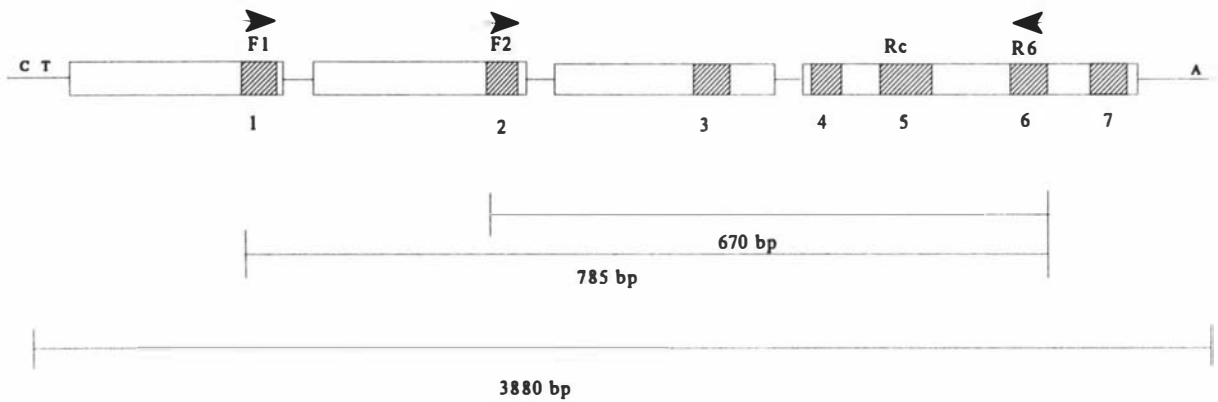
ATAGTTTAC**AGTCTTTCAAAAGATATGGGATTCCCCGGTTTTAGA**GTCGG 600
S L S K D M G F P G F R
(domain 5)
GTATAATTTACTCTTRCAATGATACCGTGTGTTAAYTGCGCGCGCAAAAT 650

GTCWAGTTTYGVHYTAAGCTTGAGA

Nucleotide codes:

A:	adenosine	G:	guanine	T:	thymine	C:	cytosine
W:	A or T	Y:	C or T	H:	A or C or T	V:	A or C or G
R:	A or G	M:	A or C	N:	A or T or C or G		

Figure 4.12b: Diagram of an ACC synthase gene from *Arabidopsis thaliana* (from Van Der Straeten *et al.*, 1992) showing exons (boxes), introns (lines), conserved domains (hatched 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.



the three residues known to bind the pyridoxal phosphate coenzyme are marked (Yang and Dong, 1993).

The EMBL + GenBank database was interrogated with the consensus sequence in Figure 4.12a. The database sequence producing the highest homology score was an ACC synthase sequence from another legume, *Glycine max* (88% homology at the nucleotide level). High homology was also found for ACC synthase sequences from a range of plants including *Vigna radiata* (85%), *Brassica juncea* (75%), *B. oleracea* (75%), *Lycopersicon esculentum* (74%), *Daucus caryophyllus* (73%), and *Nicotiana tobacum* (72%). The sequence for ACC synthase from *Glycine max* (Liu, D., Li, N., and Mattoo, A.K., unpublished sequence submitted on 29 June, 1992) was retrieved from the database, and a sequence alignment with the putative ACC synthase from white clover performed (Figure 4.13). Nucleotides in the clover sequence identical to nucleotides in the soybean sequence are underlined. The sequence alignment has a homology of 88% at the nucleotide level. This high level of homology, and the presence of the expected conserved regions, suggest clones pACS7 and pACS8 are DNA fragments homologous to ACC synthase from white clover.

The DNA fragment ACS7 was used to prepare ^{32}P-labelled DNA for use as a probe on genomic Southern blots (section 4.3.5). The genomic DNA sequence encoding ACC synthase in *A. thaliana* is approximately 3.8 kb and includes three introns (Figure 4.12b; Van Der Straeten *et al.*,1992a). However, ACS7 hybridised with several large DNA fragments on the genomic DNA Southern blot (Figure 4.10) suggesting more than one copy of sequences homologous to ACS7 is present in the genome. This is similar to the findings of other workers who have shown that ACC synthase is represented by a small gene family (Botella *et al.*, 1992; Liang *et al.*, 1992; Park *et al.*; 1992; Yang and Dong, 1993; Destèfano-Beltrán *et al.*, 1995).

Table 4.5: Amino acid sequence of the conserved domains of ACC synthase (Harpster *et al.*, 1996) compared with the derived amino acid sequences from the consensus DNA sequence of pACS7 and pACS8.

Domain number	Conserved sequence	pACS7/pACS8 sequence
one	MGLAENQL	primer ACSR1F
two	FQDYHGLP	primer ACSR2F
three	AFCLADPG	AFCLADPG
four	NPSNPLGT	NPSNPLGT
five (reaction centre)	SLSKDL*GF*PGFR	SLSKDMGFPGFR
six	MSSFGLVS	primer ACSR6R
seven	EPGWFRVC	outside fragment**

• amino acids with known variations between species (Yang and Dong, 1993)

** Domain outside the region amplified by the primers used in PCR

Figure 4.13: Alignment of the DNA sequence of ACS-7 with an ACC synthase DNA sequence from soybean located on the GenBank database (see Figure 4.12 for nucleotide codes).

Reference molecule: SOYINTR.ONS 1 - 682 (682 bps)

Sequence 2: ACC-CONTIG.SEQ 1 - 667 (667 bps)

Parameters set: Mismatch = 2; Open Gap = 4; Extend Gap = 1

Identical nucleotides are underlined in the white clover sequence.

Soybean = SOY; Clover = CLO

SOY ATAGCTAACTTTCAGGATTATCATGGTCTGCCCGAGTTCAGAAATGCTGT

CLO CTXGATCCGTWYCARGATTATCACGGTCTACCAGAGTTCAGAAATGCTGT

SOY GGCTAAATTCATGGGTAGAACAAGAGGAAACAGAGTCACGTTTGATCCTG

CLO GGCTAAATTCATGTCTAGAACAAGAGGAAACAGAGTTACATTTGATCCTG

SOY ACCGTATTGTCATGAGCGGTGGAGCAACTGGAGCACACGAAGTCACTACC

CLO ATCGTATTGTCATGAGTGGTGGAGCAACTGGAGCACATGAGGTTACTGCC

SOY TTTTGTTTGGCAGACCCTGGTGACGCATTTTTGGTGCCCATTCCTTATTA

CLO TTTTGTTTGGCAGATCCTGGTGATGCTTTTTTGGTACCTACTCCTTACTA

SOY TCCAGGTTTTGACCGGGATTTGAGGTGGAGAACAGGAATTAAACTTGTTC

CLO TCCAGGTTTCGATCGAGATTTGAGGTGGAGAACGGGAGTTAAAC--TG-TC

SOY CAGTTATGTGCGATAGCTCAAACAATTTCAAGTTGACAAAGCAAGCATTG

CLO -----GTTATCTGCGA-AGCGCGAATAATTTCAAATACAAACAGC----------TTA

SOY GAAGATGCGTATGAGAAGGCCAAAGAGGATAATATAAGAGTAAAGGGCTT

CLO GAAGA-GCATATGAAAAAGCCAAAATTG--ATAACATCAGAATAAAAGGTTT

SOY GCTCATCACCAATCCATCAAACCCATTAGGCACAGTCATGGACAGAAACA

CLO ACTCATAACAAATCCTTCAAATCCATTAGGCACAGTTATGGACAGAACCA

SOY CACTAAGAACCGTGATGAGCTTCATCAACGAGAAGCGTATCCACCTTGTA

CLO CATTAA--AACCGTTGTAAATTTCATCAAYGAAAAGCGTATTCATCTTATA

SOY TCTGATGAAATATACTCTGCAACAGTTTTTAGCCACCCCAGTTTCATAAG

CLO AGCGATGAAATTTACGCTGCA----CGTTTTTAGCCACCCAAGTTTCATAAG

SOY CATTGCTGAGATATTAGAGGAAGACACAGACATCGAATGTGACCGCAACC

CLO CATAGCTGAGATCATAGAAAMAGAAACAGACATCGAATGTGACCGTAACC

SOY TCGTTCACATTGTTTATAGTCTTTCAAAGGATATGGGGTTCCCTGGCTTC

CLO TTGTTCACATAGTTTACAGTCTTTCAAAAGATATGGGATTCCCCGGTTTT

SOY AGAGTTGGCATCATATACTCTTACAATGATGCTGTGGTCCATTGTGCACG

CLO AGAGTCGGTATAATWTACTCTTRCAATGATACSGTTGTTAAYTGCGCGCG

SOY CAAAATGTCAAGCTTTGGATTGGTGTCAACAC

CLO CAAAATGTCWAG-TTTYGSMYTAAGCTTGAGA-

4.5 Northern analysis of white clover RNA for sequences homologous to ACC synthase

4.5.1 Introduction

If ACC synthase is the rate limiting step in ethylene biosynthesis in leaves of white clover (see section 4.4.1), induction of the enzyme may be a major factor in the control of ethylene biosynthesis. Northern analysis has been used to detect changes in the amount of ACC synthase mRNA in different tissues; for example in different flower organs in orchids (O'Neill *et al.,* 1993), and to determine the timing of induction after various treatments; for example, wounding in winter squash (Nakajima *et al.,* 1990), pathogen infection in tomato cell suspensions (Spanu *et al.,* 1993), and mechanical stress in mung bean (Botella *et al.,*1995). This section details experiments aimed at determining the timing of induction of ACC synthase in relation to the stage of development in leaves of white clover using northern analysis.

4.5.2 Preparation of total RNA northern blot

Total RNA (5 μg), separated by electrophoresis in a 1% (w/v) agarose gel (section 4.3.3) was blotted onto Hybond N^+ membrane, and the northern blot hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template. A low level of hybridisation, possibly to rRNA, was detected at low stringency (2 x SSPE at 65°C; Figure 4.14) but no hybridisation was detected at high stringency (0.1 x SSPE at 65°C, data not shown).

4.5.3 Extraction of poly $(A)^+$ RNA

The results from northern analysis using total RNA (section 4.5.2) were inconclusive but suggested that the amount of poly $(A)^+$ RNA required to detect ACC synthase mRNA was greater than that used here. Data from PCR analysis here (section 4.3.4), and expression studies in other systems (Van Der Straeten *et al.,* 1990, 1992a) suggest that the concentration of mRNA coding for ACC synthase is expected to be low in abundance. To improve the ratio of message for ACC synthase compared with non-homologous RNA,

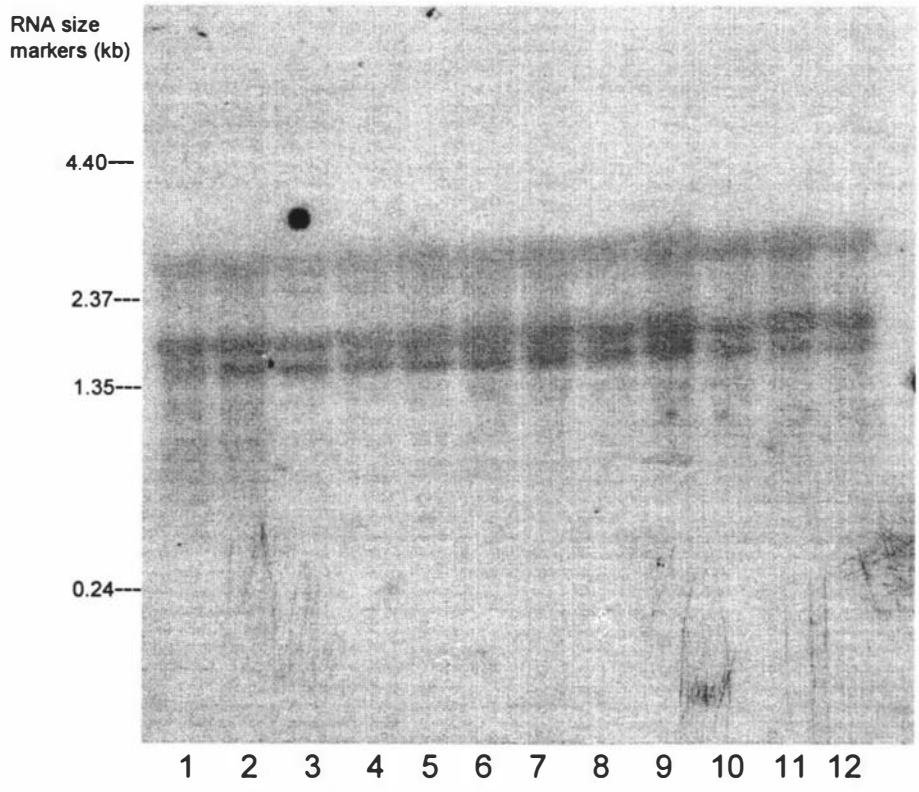


Figure 4.14: Northern analysis of total RNA probed with ^{32}P-labelled DNA prepared using ACS-7 as a template

Lanes 1-12: Total RNA extracted from leaves 1 to 12 respectively

poly $(A)^+$ RNA extracts were made for blotting. Poly $(A)^+$ RNA was isolated from about 300 μg of the total RNA extracted as described in section 4.3.3. The amount of total RNA used to isolate the poly $(A)^+$ RNA, the amount of poly $(A)^+$ RNA isolated, and the percentage of poly $(A)^+$ RNA compared with the total RNA is shown in Table 4.6.

4.5.4 Analysis of poly $(A)^+$ RNA by PCR

Aliquots of the poly $(A)^+$ RNA (0.05 μg) from leaves one to ten were used as a template for reverse transcriptase (section 2.3.15.1). The products from this reaction were then used as a template for two rounds of PCR (30 cycles per round) using ACSR1F and ACSR6R primers in the first round, then ACSR2F and ACSR6R primers in the second round (Table 2.4).The plasmid pACS7 was used as a PCR control. Equal volumes of the products were separated by electrophoresis in a 1% (w/v) agarose gel (Figure 4.15). In all the leaves tested, a band of similar size (670 bp) to that produced from pACS7 was detected. In addition to this band, at least one other band smaller than the 670 bp fragment expected from ACC synthase was visible as a product. Amplification using PCR will be achieved whenever sufficient homology exists between the primers and the template DNA. These data suggest that sufficient homology exists between the ACS primers used in this study that at the annealing temperature used (50°C), some annealing occurred with DNA sequences other than ACC synthase.

The amount of DNA product present in the band corresponding to the band produced from pACS7 was assessed visually. Leaves 7 to 10 produced the most product, followed by leaf 4, then leaves 5 and 6. For plants grown at Palmerston North, leaves 7, 8 and 9 show decreasing concentrations of chlorophyll compared with leaves 4, 5, and 6 (Figure 3.11). The decrease in chlorophyll occurs in leaves 6 and 7 for plants grown at Levin (Figure 3.8).

PCR is not completely accurate in producing DNA product with the same sequence as the template. Frequently, errors of up to 1% of the bases are inaccurately coded. In addition, false-priming may occur resulting in extra DNA sequences being amplified. The amount of PCR amplified product is not always a good predictor of the amount of homologous sequences present in

Table 4.6: Poly $(A)^+$ RNA extracted from leaves of white clover grown at Palmerston North

Leaf number	260/280 nm ratio	RNA (mg)	Poly (A) /total RNA (%)
1	1.293	0.82	0.27
2	2.336	5.37	1.79
3	2.301	5.23	1.88
4	2.302	4.28	1.43
5	2.260	4.35	1.45
6	2.292	6.33	2.11
7	2.321	7.02	1.40
8	2.239	2.80	0.93
9	2.263	3.48	1.16
10	2.286	7.43	3.19
11	2.223	3.28	1.09
12	2.207	2.87	0.96

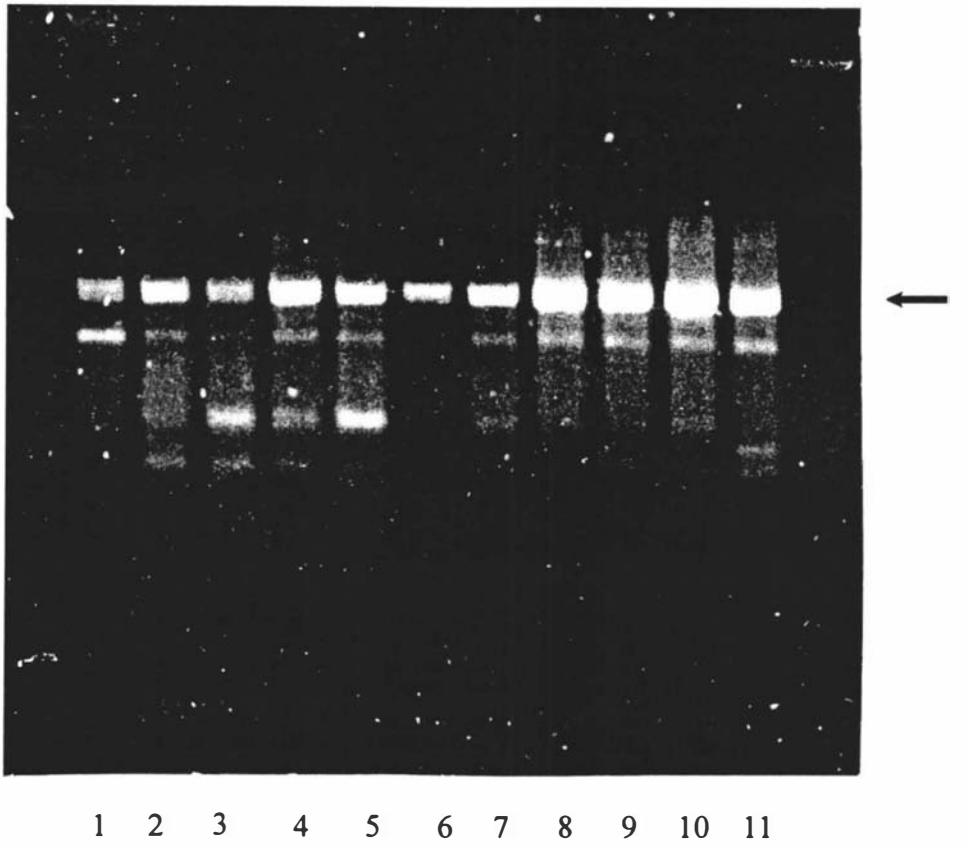


Figure 4.15: Detection of ACC synthase DNA sequence in leaf tissue of white clover by RT-PCR using poly $(A)^+$ RNA as a template. The position of the DNA band the same size as ACS7 (670 bp) is arrowed.

Lanes 1 to 5	leaf numbers 1 to 5
Lane 6	ACS7
Lanes 7 to 11	leaf numbers 6 to 10

the original sample (for example, the number of copies of mRNA). Inaccuracies can occur in the efficiency of reverse transcription, and also in the initial priming when the sequence is in low abundance, hence caution is required when interpreting quantitation data using this technique. However, some quantitative comparisons can be made if the number of cycles of PCR is kept low (30 to 35 cycles; Van Der Straeten, *et al.,* 1992a). In white clover, usually two rounds of PCR (30 cycles each) were required to obtain sufficient quantities of product for analysis (but see section 4.6.5).

4.5.5 Preparation of poly $(A)^+$ RNA northern blot

The ratio of absorbance at 260 nm and 280 nm for the poly $(A)^+$ RNA preparation after one isolation treatment is shown in Table 4.6. The ratio is generally higher than that measured for the total RNA preparations (Table 4.3). These data show that using the extraction methods described here, poly $(A)^+$ RNA represents 0.27% to 3.19% of the total RNA extracted. The lowest percentage was isolated from leaf one, the leaf which gave the highest ratio of total RNA extracted per gram fresh weight. Overall, the poly $(A)^+$ RNA represented about 1.5% of the total RNA extracted from the leaf tissue. The poly $(A)^+$ RNA (1 μg per lane) was electrophoresed on a 1% (w/v) denaturing agarose gel (Figure 4.16). A good smear of RNA was seen as a background although some ribosomal bands were still apparent.

The poly $(A)^+$ RNA was blotted onto Hybond N^+ membrane and hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template (section 2.3.11.2.2). A low level of hybridisation was detected at low stringency (2 X SSPE, 65°C) (Figure 4.17) but no hybridisation could be detected at higher stringency (1 x SSPE, 65°C, data not shown). Although the poly $(A)^+$ RNA northern gave about 20 times the concentration of message compared with the total RNA northern, no signal could be detected.

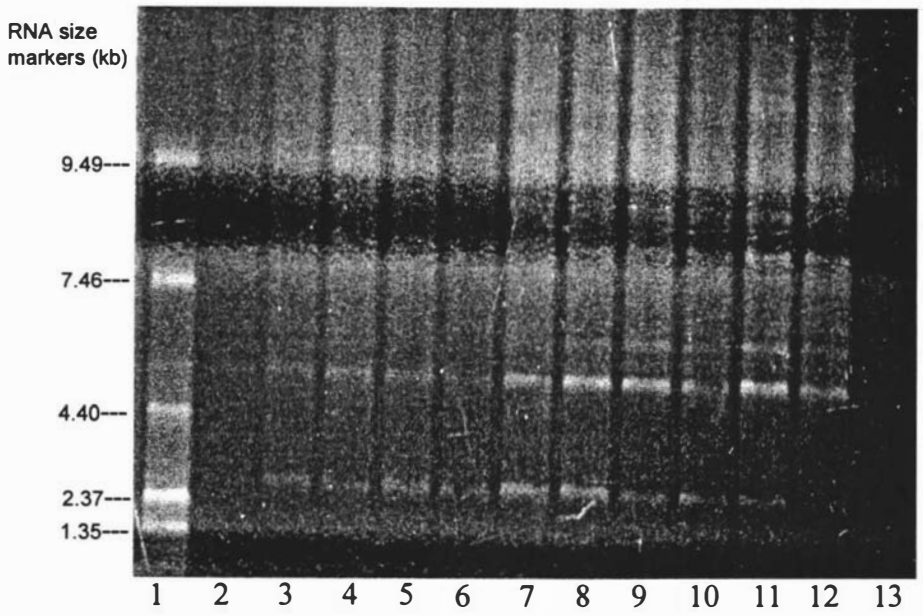


Figure 4.16: Poly (A)$^+$ RNA extracted from leaves of white clover grown at Palmerston North, separated by electrophoresis under denaturing conditions in a 1% (w/v) agarose gel.

Lane 1: RNA size markers
Lanes 2-13: RNA extracted from leaves 1 to 12 respectively

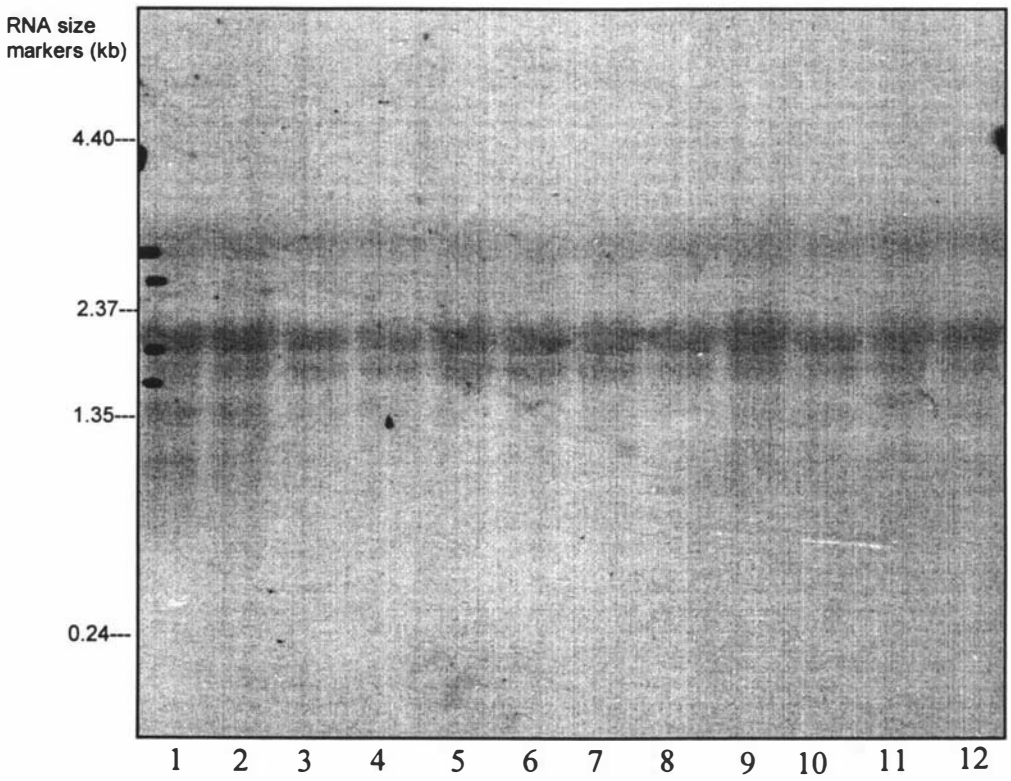


Figure 4.17: Northern analysis of poly $(A)^+$ RNA probed with ^{32}P-labelled DNA prepared using ACS-7 as a template.

Lanes 1-12: Total RNA extracted from leaves 1 to 12 respectively

4.6 Preparation and screening of a cDNA library prepared from senescing white clover leaves

4.6.1 Introduction

Attempts to characterise ACC synthase activity from leaves of white clover using enzyme assays (section 3.4.2), or northern analysis using PCR generated fragments of ACC synthase (section 4.5), were unsuccessful. Increasing the amount of poly $(A)^+$ RNA used to prepare the northern blot may improve the detection of ACC synthase mRNA. However, the ACS7 fragment represents only a portion of the ACC synthase gene from white clover. Hybridisation with a full-length copy of the cDNA will improve the detection and the discrimination between this gene and other ACC synthase sequences present. It was therefore decided to pursue the option of obtaining a full length clone of ACC synthase before repeating the northern analysis.

To determine the complete sequence of ACC synthase from white clover, a full length cDNA clone was required. This section details experiments aimed at obtaining a full length clone of ACC synthase from leaves of white clover using a PCR generated fragment of the gene from white clover to probe a cDNA library.

4.6.2 Total RNA extraction

PCR analysis had demonstrated that ACC synthase mRNA was present in RNA extracts from leaf six on plants grown at Levin (section 4.3.4) and RT-PCR analysis of leaves 7 and 8 from plants grown at Palmerston North resulted in the highest concentration of product using primers specific for ACC synthase (section 4.5.4). Physiological data indicated that leaves 7 and 8 (Palmerston North) and leaf 6 (Levin) were at a similar developmental stage as determined by changes in the chlorophyll content of adjacent leaves. Leaf six was therefore used to prepare a cDNA library from leaves of white clover at an early stage of senescence. The preparation of the library was monitored for the presence of DNA sequences homologous to the ACC synthase clone ACS7 (section 4.3.7) using PCR. The library was screened for ACC synthase

using ^{32}P-labelled DNA prepared using ACS7 as a template.

Total RNA was extracted from fresh leaf tissue from plants grown at Levin. The extracts were partitioned twice with chloroform, and each partition precipitated separately. Extracting twice with chloroform increased the amount of RNA obtained compared with a single partition. The amount of RNA extracted, and the 260/280 nm ratio is shown in Table 4.7. Aliquots (4 μL) of each extract were separated by electrophoresis in a 1% (w/v) agarose denaturing gel (Figure 4.18). Although lanes 5 to 8 are overloaded, the extracts show little evidence of degradation and all the rRNA bands appear sharp. The extracts were combined to give a total of 1229 μg in 728 μL of water. An aliquot of the total RNA extract was analysed for the presence of ACC synthase using RT-PCR. First strand cDNA was made from the RNA and this was used as a template for two rounds of PCR (30 cycles each) using ACSR1F and ACSR6R primers in the first round, then ACSR2F and ACSR6R primers in the second round (Table 2.4).

Aliquots of the product of each PCR round were separated by electrophoresis in a 1% (w/v) agarose gel (lanes 3 and 9, Figure 4.19). No bands were visible after one round of PCR (lane 3) but three bands were visible after the second PCR amplification (lane 9). The smaller of these bands corresponded to the size of the expected 670 bp fragment of ACC synthase (the DNA fragments in the control lanes 7 and 14 are visible on the original photograph). The other bands may be PCR-derived artifacts generated from regions with some homology to the primers used, or may be the result of amplification from a contaminating genomic DNA fragment and therefore contain intron DNA. The products of this PCR analysis were blotted onto Hybond N$^+$ membrane and hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template (section 4.6.5).

4.6.3 Poly (A)$^+$ RNA extraction

Extraction of poly (A)$^+$ RNA from total RNA from white clover using the Promega PolyATract system results in a yield of approximately 1.5% (Table 4.6). Sufficient total RNA extract was used to give an estimated 5 μg poly (A)$^+$ RNA for use in the preparation of a cDNA library. An aliquot of the poly (A)$^+$

Table 4.7: Total RNA extracted from leaf six from white clover grown at Levin

Extract number	Chloroform partition	260/280 nm ratio	RNA concentration (mg/mL)
1	First	1.7381	968
2	Second	1.7289	840
3	First	1.8934	504
4	Second	1.8736	504
5	First	1.8019	2344
6	Second	1.7845	1776
7	First	1.8487	3296
8	Second	1.8810	3272

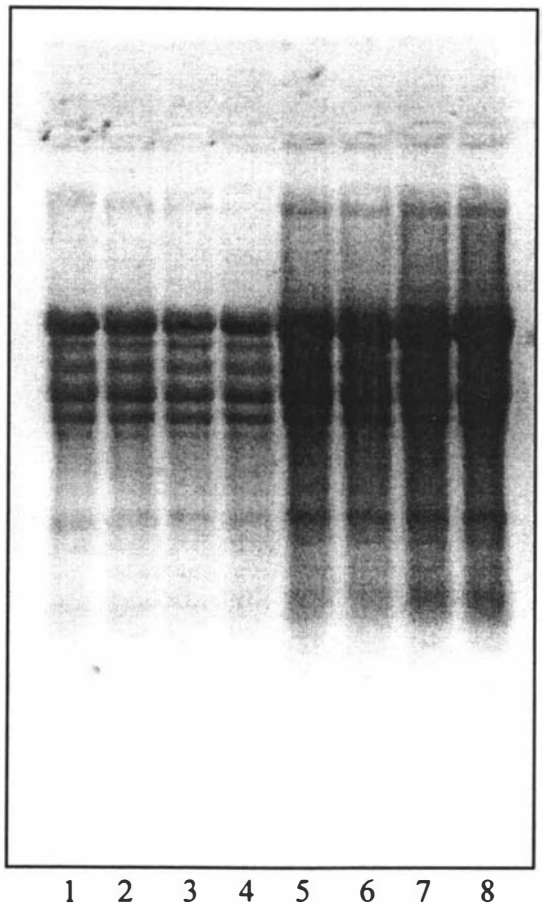


Figure 4.18: Total RNA extracted from leaves of white clover plants grown at Levin and separated by electrophoresis under denaturing conditions in a 1% (w/v) agarose gel.

Lanes 1 to 4: RNA extracted after one chloroform partition
Lanes 5 to 8: RNA extracted after two chloroform partitions

RNA extract was analysed for the presence of ACC synthase using RT-PCR. First strand cDNA was made from the RNA and this was used as a template for two rounds of PCR (30 cycles each) using ACSR1F and ACSR6R primers in the first round, then ACSR2F and ACSR6R primers in the second round (Table 2.4). Aliquots of the product of each PCR round were separated by electrophoresis in a 1% (w/v) agarose gel (lanes 4 and 10, Figure 4.19). No bands were visible after one round of PCR (lane 4) but two bands were visible after the second PCR amplification (lane 10). The smaller of these bands was more abundant and corresponded to the size of the expected 670 bp fragment of ACC synthase. The products of this PCR analysis were blotted onto Hybond N^+ membrane and hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template (section 4.6.5).

4.6.4 cDNA synthesis

The poly $(A)^+$ RNA extract was used immediately for the preparation of cDNA required for the library. No attempt was made to measure the amount of poly $(A)^+$ RNA used, and the number of manipulations was minimised to reduce the chance of degradation. The second strand cDNA synthesis was completed and precipitated after the phenol-chloroform partition, and the product stored frozen (-20°C). An aliquot of the cDNA extract was analysed for the presence of ACC synthase using PCR. The cDNA was used directly as a template for two rounds of PCR(30 cycles) using ACSR1F and ACSR6R primers in the first round, followed by ACSR2F and ACSR6R primers in the second round (Table 2.4).

Aliquots of the product of each PCR round were separated by electrophoresis in a 1% (w/v) agarose gel (lanes 5 and 12, Figure 4.19). No bands were visible after one round of PCR (lane 5) but two bands were visible after the second PCR amplification (lane 12). The smaller of these bands was more abundant and corresponded to the size of the expected 670 bp fragment of ACC synthase. The products of this PCR analysis were blotted onto Hybond N^+ membrane and hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template (section 4.6.5).

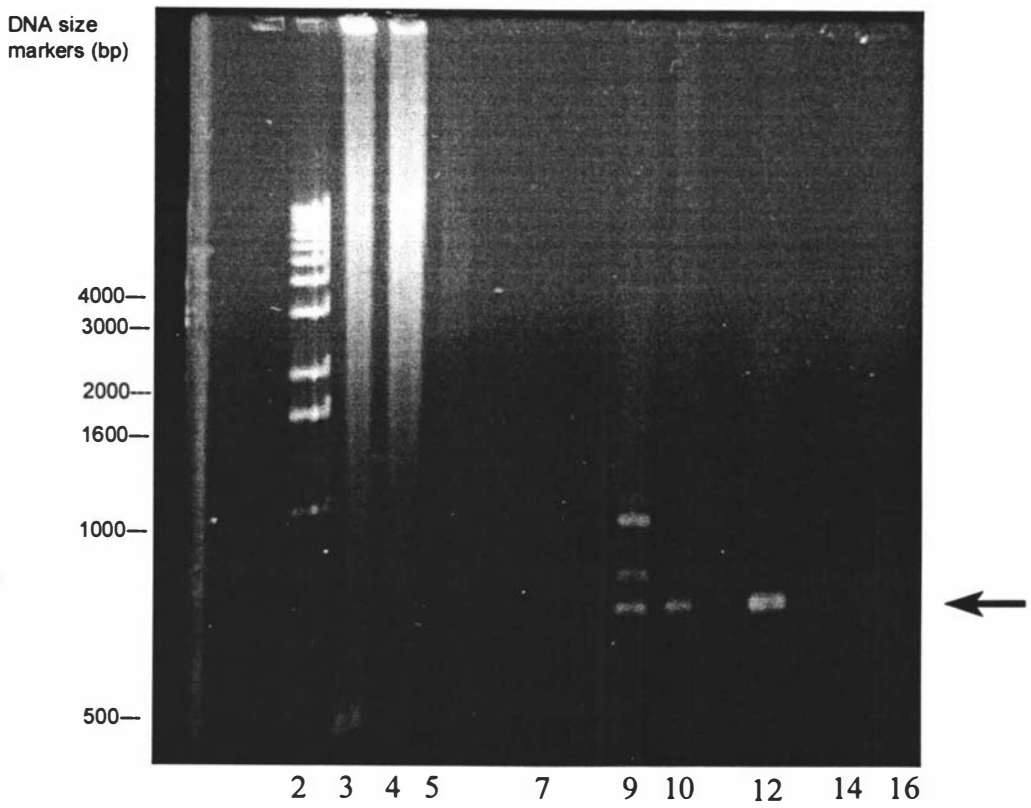


Figure 4.19: DNA fragments prepared by RT-PCR, PCR, and cDNA synthesis from RNA extracted from leaves of white clover, separated by electrophoresis in a 1% (w/v) agarose gel. The position of the 670 bp band is arrowed.

Lane 2:	DNA standard
Lane 3:	one round of RT-PCR from total RNA
Lane 4:	one round of RT-PCR from poly (A)+ RNA
Lane 5:	one round of PCR amplification from cDNA preparation
Lane 7:	pACS7 control
Lane 9:	two rounds of RT-PCR from total RNA
Lane 10:	two rounds of RT-PCR from poly (A)+ RNA
Lane 12:	two rounds of PCR amplification from cDNA preparation
Lane 14:	PCR amplification of pACS7
Lane 16:	cDNA sample from library preparation

4.6.5 Southern analysis of the PCR products

The PCR-generated DNA products separated by electrophoresis (section 4.7.2 to 4.7.4, Figure 4.19) were blotted onto Hybond N^+ membrane and hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template (Figure 4.20). Hybridisation (at high stringency; 0.1% SSPE, 65°C) of ACS7 was detected with products from one round of RT-PCR amplification (expected size 780 bp) using total RNA as the template, and less hybridisation detected with products from poly $(A)^+$ RNA and cDNA after one round of PCR amplification. Two bands were detected after PCR amplification using poly $(A)^+$ RNA and cDNA as the template. The major band corresponds to the expected fragment size of 780 bp in lanes 3 and 4 (RT-PCR amplification from total and poly $(A)^+$ RNA respectively). However, lane 5 (PCR amplification from cDNA preparation) produced a major band bigger than the expected 780 bp, and corresponding to the minor band present in lanes 3 and 4.

Hybridisation was also detected with all products from PCR amplification of ACS7 (control), and the second round of PCR from total and poly $(A)^+$ RNA, and cDNA. Although other bands were present, the major bands corresponds to a fragment size of 670 bp. An aliquot (1 μL) of the prepared cDNA (without amplification) for the library was also loaded onto the gel (Figure 4.19, lane 16), and hybridisation of ACS7 to a band of higher molecular weight was detected (Figure 4.20, lane 16). This band was estimated to be >1000 bp by comparison with the molecular size markers.

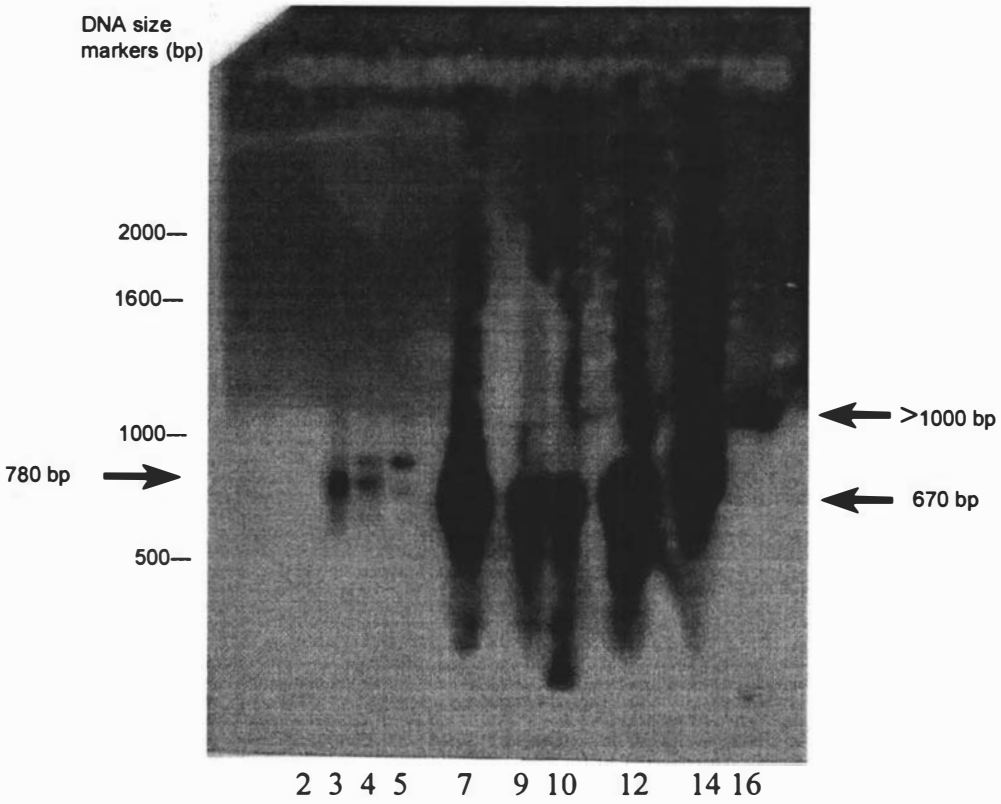


Figure 4.20: Southern analysis of products from RT-PCR, PCR, and cDNA synthesis (see Figure 4.19) probed with ^{32}P-labelled DNA prepared using ACS7 as a template. The position of the 780 bp, 670 bp, and cDNA (>1000 bp) bands are arrowed.

Lane 2	DNA standard
Lane 3	one round of RT-PCR from total RNA
Lane 4	one round of RT-PCR from poly (A)+ RNA
Lane 5	one round of PCR amplification from cDNA preparation
Lane 7	ACS7 control
Lane 9	two rounds of RT-PCR from total RNA
Lane 10	two rounds of RT-PCR from poly (A)+ RNA
Lane 12	two rounds of PCR amplification from cDNA preparation
Lane 14	PCR amplification of ACS7
Lane 16	cDNA sample from library preparation

4.7 Preparation, screening and analysis of the cDNA library

4.7.1 Preparation and screening

The prepared cDNA (section 4.7.4) was sub-aliquoted into two and packaged separately. Library aliquot one had a titre of approximately 800,000 pfu/mL, while aliquot two had 200,000 pfu/mL. The total number of clones in library one was therefore about 400,000, and these were plated onto twenty 150 mm plates (about 20,000 per plate). The plaques were lifted onto nylon filters and the primary screen probed with ACS7, and washed using 1 x SSPE at 65°C. Two positive signals were obtained from these plates. An agarose plug containing the plaques corresponding to the positive signals were 'picked' using a pipette tip, and the plug was dispersed into SM media (sub-library 6/1 and 10/1). The remaining library was then amplified in separate aliquots and stored at 4°C.

Aliquots from these sub-libraries were used as templates for a single round of PCR (30 cycles) amplification using the primers ACSR1F and ACSR6R (Table 2.4), and the products separated by electrophoresis (Figure 4.21). Both sub-libraries produced a single band (lanes 2 and 3) slightly larger than ACS7 (lane 5). This is expected from PCR amplification using the primers ACSR1F and ACSR6R, as these produce an expected band size of 780 bp (section 4.42). Lambda DNA was used as a PCR control template and separated on the same gel (lane 7). A smear of high molecular weight DNA was produced from the lambda DNA but no DNA fragments of a size corresponding to 670 bp were produced indicating the band produced from the sub-library preparations were not amplified from contaminating λ DNA.

Aliquots of the sub-libraries were diluted, plated onto 90 mm agar plates, lifted onto nylon filters, and screened using ^{32}P-labelled ACS7 DNA using 1 x SSPE at 65°C. This second screen resulted in 7 regions of hybridisation from sub-library 6/1, and 10 regions of hybridisation from sub-library 10/1. An agarose plug containing the plaques corresponding to the positive signals were 'picked' using a pipette tip, and the plugs dispersed into SM media (sub-

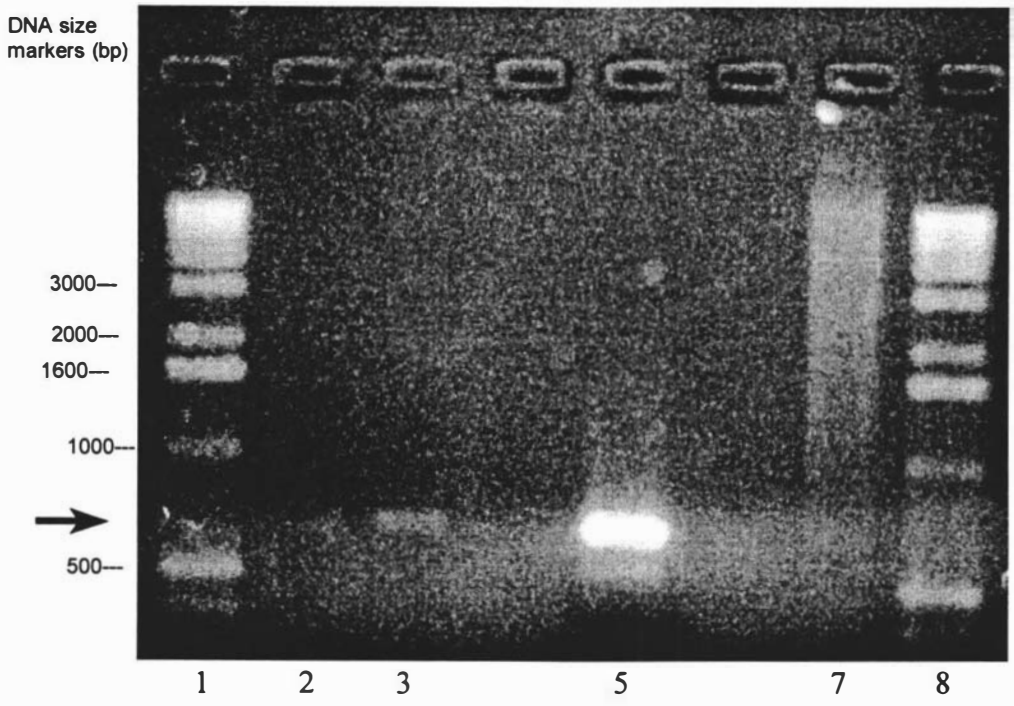


Figure 4.21: PCR generated DNA fragments prepared using aliquots of the amplified cDNA sub-libraries 6/1 and 10/1 as templates, separated by electrophoresis in a 1% (w/v) agarose gel. The position of the 670 bp band is arrowed.

Lanes 1 and 8:	DNA standard
Lane 2:	sub-library 6/1
Lane 3:	sub-library 10/1
Lane 5:	ACS7
Lane 7:	lambda DNA control

libraries 6/1/1 to 6/1/7 and 10/1/1 to 10/1/10 respectively).

4.7.2 Sequencing cDNA library clones

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) grown under kanamycin selection, and the cells plated onto L agar containing 100 μg/mL ampicillin. The plasmid pZL 1 (Figure 4.22) containing inserts from the library are *in vivo* excised by Cre-*lox* recombination in the *E.coli* DH10B(zip) cells. An Individual colony from each the sub-libraries was removed from the agar and grown overnight in 10 mL L broth containing 100 μg/mL ampicillin. Plasmid DNA was extracted and purified, an aliquot digested with *Eco*R 1, and the products separated by electrophoresis on a 1% (w/v) agarose gel (Figure 4.23). The digestion generally showed only a faint band or bands indicating that the digestion may not have been completed. However, the plasmids show a range of insert sizes which may represent partial clones of ACC synthase. The digestion of plasmid 10-1-10 resulted in three distinct bands with a total size of approximately 1.9 kb. This plasmid also gave the most product from the digestion as determined by a visual assessment of the stain density.

The products of the *Eco*R 1 digestion were then blotted onto Hybond N^+ membrane, hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template and washed using 1 X SSPE at 65°C (Figure 4.24). Strong hybridisation was detected with high molecular weight DNA (plasmid DNA) again indicating that the digestion had only been partially completed. Faint hybridisation was detected with lower molecular weight DNA corresponding to the inserts. The strongest hybridisation was detected with insert DNA in plasmids from sub-library 10-1-10. While hybridisation was detected with the two larger bands visible in Figure 4.23, no hybridisation was detected with the smallest band. This may indicate that this portion of the insert is outside the fragment amplified by PCR and represented in ACS7. The insert DNA in plasmid 10-1-10 was selected for sequence analysis because of the large insert size, and the relatively strong hybridisation with ACS7 DNA.

Plasmid DNA extracted from one colony prepared from sub-library 10-1-10 was sequenced using an automatic sequencer. The EMBL+GenBank

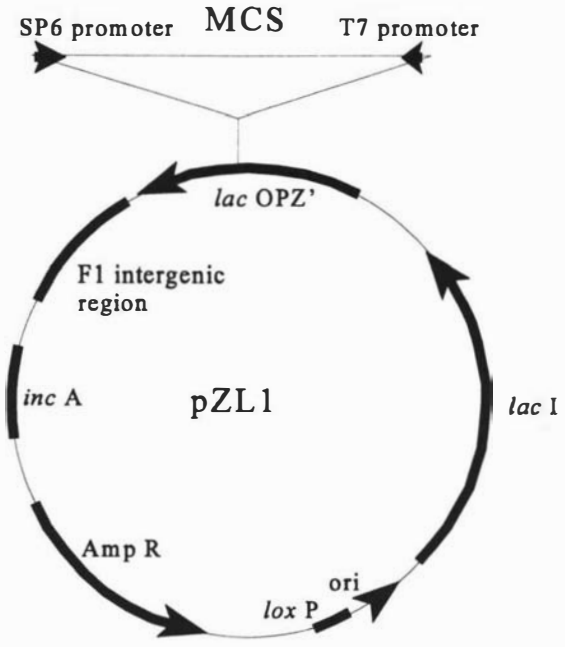


Figure 4.22: Diagram of the plasmid pZL1 (Life Technologies) used for the *in vivo* excision of DNA inserts from the λ-cDNA library prepared from leaves of white clover

inc A:	phage P1 incompatibility locus
lac I:	lac repressor gene
ori:	origin of replication
Amp R:	Ampicillin resistance gene
lox P:	Cre recombination locus
lac OPZ':	lactose operon genes

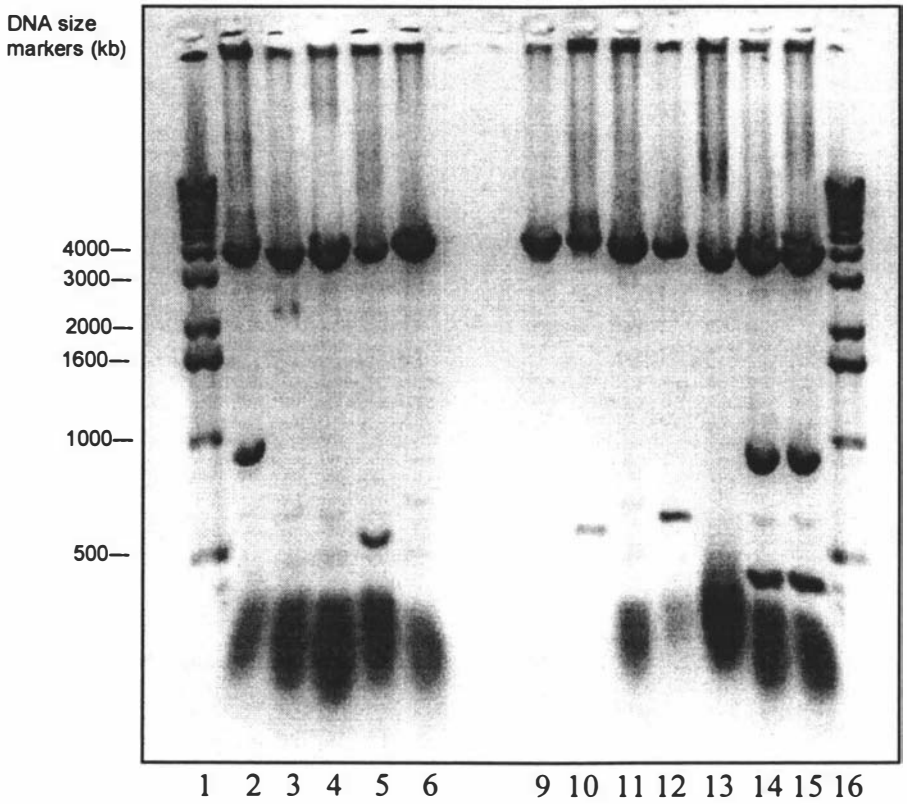


Figure 4.23: Restriction enzyme digests of pZL 1 plasmids excised *in vivo* in *E.coli* DH10B(ZIP), produced from a cDNA library prepared from leaves of white clover, and separated by electrophoresis in a 1% (w/v) agarose gel

Lanes 1 and 16	DNA standards
Lane 2	sub-library 6/1/1
Lane 3	sub-library 6/1/2
Lane 4	sub-library 6/1/3
Lane 5	sub-library 6/1/4
Lane 6	sub-library 6/1/5
Lane 9	sub-library 10/1/3
Lane 10	sub-library 10/1/5
Lane 11	sub-library 10/1/7
Lane 12	sub-library 10/1/8
Lane 13	sub-library 10/1/9
Lane 14	sub-library 10/1/10
Lane 15	sub-library 10/1/10

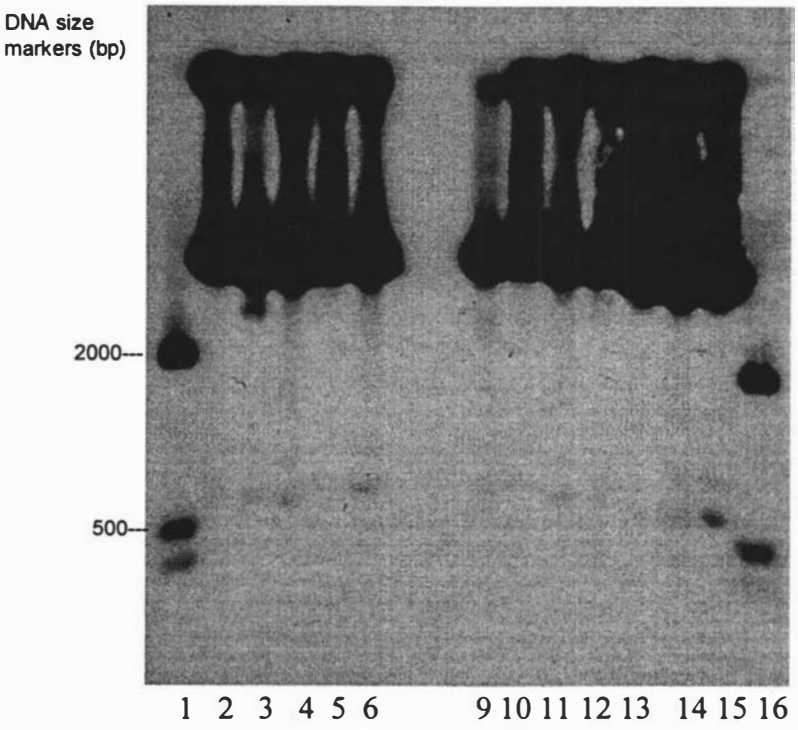


Figure 4.24: Southern analysis of restriction enzyme digests of pZL 1 plasmids *in vivo* excised in *E.coli* DH10B(ZIP), obtained from a cDNA library prepared from leaves of white clover, and hybridised with ^{32}P-labelled DNA prepared using ACS7 as a template

Lanes 1 and 16	DNA standards
Lane 2	sub-library 6/1/1
Lane 3	sub-library 6/1/2
Lane 4	sub-library 6/1/3
Lane 5	sub-library 6/1/4
Lane 6	sub-library 6/1/5
Lane 9	sub-library 10/1/3
Lane 10	sub-library 10/1/5
Lane 11	sub-library 10/1/7
Lane 12	sub-library 10/1/8
Lane 13	sub-library 10/1/9
Lane 14	sub-library 10/1/10
Lane 15	sub-library 10/1/10

database was interrogated with the sequence obtained from this plasmid (Figure 4.25). The database sequence producing the highest homology score was a cDNA clone labelled 15a from *Pisum sativum* (induced in shoots by wilting) which was found to have high homology with a cysteine protease found in *Dictyostelium* (Guerrero, *et al.*, 1990). Clone 10-1-10 also had high homology with a cysteine proteinase sequence from germinating seeds of *Vicia sativa* (Becker *et al..*, 1994). The sequence for clone 15a from *P. sativum* and the cysteine proteinase sequence from *V.sativa* were retrieved from the database, and alignments with the sequence for clone 10-1-10 performed (Figure 4.26, 4.27 respectively). Nucleotides in the clover sequence which were identical to the clone 15a or *V.sativa* sequences are underlined. The high level of homology suggests the clone is a fragment homologous to cysteine proteinase from white clover.

Figure 4.25: Sequence of cDNA 10-1-10.

CTGGGGGGTCNCTTTGAATCNACTTCTTTTNTCCCNTTTAAAAAGGG

CCCCCACNGGACCGGATNANTNTACAACGTTCGCCGACTACTGAGC

TCGTCGACCCNGCCGNGAATTCCCGGCCNCGTCAACNCAAANTTTC

ACANCATACCCGATNATGTCNTTGAAGGTTATATCCAAATNTNAACAC

NTGCCNACATCTTCCTGGGCNCCCTTANTATCATGCNANTCTCTGGT

GAACGTCCCACTTTTGATGCCNACTCCGAAACAANCNTTGCCTACAC

ACTCTCCTANTTTAACATACCNTNCGATGCTNCACNNGTGTNTNCTG

GTTATTNTTTTTTTTTTNACTACNTGGTTTCACTCTANTCCCANTTTCA

TCATTGATTNTAACNTCTNATNCNNACTCTAANGTANACCANTNTTCA

ACTTCCTCACCCCTNGTANACTTTGCTGAATTTTNCGGGTGNANAATC

TCACTTCNAGCNTCTTCTNNANCCNCCNANCNATNATCCCACCCTTGA

ACTCCCACAANATTNCNACANCTTCCATNNANTTTTAAANCTAATTTTT

TTCTCTCCTTATTNACCATTCTCTCTCCCCCCCTTTCCCTCCTACTCTT

TNTNCTNNNNAANTCANACTTTTCTNTAATTNNTANNCNCATATNTTCC

TCTTCTCNTATANTNTTTCNNTAAATANCTCACNAATAATNTCTTCCTTC

TCNTTTTTTAATANTACCCCGNTCCCNCCCTANATNTTCTTCCCCCTCT

GAANTNNCCGTTNTTATTCTCCTCCTTANTNNGCCTCTTTTTCTCTNCT

CCC

Figure 4.26: White clover cDNA 10-1-10 sequence aligned with cDNA 15a sequence from *Pisum sativum* (Guerrero *et al.*, 1990).

Sequence 1: cDNA 10-1-10 (*CLO*)
Sequence 2: cDNA 15a from *Pisum sativum* (*PEA*)

Similarity Index: 72.5%
Gap Number: 16
Gap Length: 44
Consensus Length: 501
Wilbur-Lipman DNA Alignment
Parameters set: Ktuple:3; Gap Penalty: 3; Window: 20

Identical nucleotides are underlined in the *PEA* sequence.

CLO AATCA - - - - NGGG - - - -GGTTTTCNTTTNTAAGTTGGTTNTTTGGG

PEA AATCGCGCCTGGATCATGGTGTTCTTCT - - -AGTTGGTTTTGG- - - -

CLO AAAAAGGCCGGGTTTATGCTTCCCCATTCGGATAGAAGGGGAAAA

PEA AAAAGGTGC - - - - TTATGCT- - CCCATTC -GATTGAAGG - - AAAA

CLO CCCTTAACTGGGTCATAAA GAAATTCATGGGGGCGAGAATTGGGG

PEA GCCTTA - CTGGATCATTAA AACTCCT - - GGGGGC -AGAATTGGGG

CLO AGAGGAGGGATATTACAAGANCTGCAGAGGCCGGGAATGTATGTG

PEA A GAGCAGGGATATTACAAGATCTGCAGA GGTA - GAAATGTATGTG

CLO GAGTGGATTCGATGGTCTCAACTGTAGCTGCAGCTCAAATCAACCA

PEA GAGTCGAT TCAATGGTTTCAACTGTAGCTGCAGCTCAATCCAACCA

CLO TTAAATACTGCGATGCTGATTCTGGTTGGGGTCTCATCTAATGCAATA

PEA TTAAATACAGGGATGCTGATTCTAGTCTGTC T- TCATCTCATGTGACT

CLO -TTAAGCTAGTTGAATTTGTGTAAATATATTATCATGAAGTTGCAAATNNA

PEA CTTAAGTTAGTTGAATTTGTGTAAATATATTATCATGAAGTTGTGAATGTA

CLO TAANAAAGGTTCTGGCAAAAACTATTTCCACGTAGTATCAGGAAATTGC

PEA TCTTCT- GGTTATGGCAAAAACTATCTCTAAGTAGGATCAAGAGACTGC

CLO CTCAGCCATGTTACTATTTATGTAGTTAGTAGTAATTATGTATGCTTGTT

PEA CACGATCATGTTA - - -TTTAAGTAGTTGG - - - - - - TTATGTCTTGCTGTT

CLO ATTCCGGTGATGTTTAT CCATAGCCATTGCAACATGGCAGATACATGAA

PEA ACTGTGGTGATGTTTATCCTTAGCCATGGCAGCATAGCAGGTAAATGAA

CLO TTGGAAAATAATCAGTAGTATAATCTTT

PEA CTGGAAAATAAT - - - - - - TATAAACTTT

Figure 4.27: White clover cDNA 10-1-10 aligned with cysteine proteinase from *Vicia sativa*.

Sequence 1: cDNA 10-1-10 (*CLO*)
Sequence 2: cysteine proteinase from *Vicia sativa* (*VIC*)

Similarity Index 63.4%
Gap Number: 9
Gap Length: 23
Consensus Length: 322 nucleotides
Wilbur-Lipman DNA Alignment
Parameters set: Ktuple: 3; Gap Penalty: 3; Window: 20

Identical nucleotides are underlined in the *VIC* sequence.

CLO GGAAAACCCTTAACTGGGTCATAAAGAAATTCATGGGGGCGAGAATTGG

VIC GGAAAAGCCTTA -CTGGA -CATTAAGAACTCT TGGGGGC- - AGAATTGG

CLO GGAGAGGAGGGATATTACAAGANCTGCAGAGGCCGGAATGTATGTGGA

VIC GGAGAGCAGGGATATTACAAGATCTGCAGAGGTAGA AATGTATGTGGT

CLO GTG GATTCGATGGTCTCAACTGTAGCTGCAGCTCAAATCAACCATTAAAT

VIC GTT GACTCAATGGTTTCAACTGTAGCTGCAGCTCAATCCAACAATTAAAT

CLO ACTG CG -ATGCTGATTCT-GTTGGGGTCTCATCTAATGCAATATTAAGCT

VIC ACAT CTCATGTGACACTAAGTTAGTTGAA TTTGTGTAAATATATTATCA -

CLO AGTTGAAT TTGTGTAAATATATTATCATGAAGTTGCAAATNNATAANAAA

VIC - - - TGAAG TTGTG - -AATGTATCTTCTGGTTATGGCAAAACTATCTCTAA

CLO GGTTCTGGCAAA AACTATTTCCACGTAGTATCAGGAAATT GCCTCAGCC

VIC GTAGTA TCAAGA GACTG- - -CCACCCACGATCATGTTATT - - - TCAGTA

CLO ATGTTA CTATTTATGTA - - - - - GTTA

VIC - GT TGCTAATTATGTA TTCCTGTTA

CHAPTER 5

DISCUSSION

5.1 Stolon growth of white clover

Stolons of white clover genotype 10F grown using the method described in section 2.1.2 produce leaves at a constant rate and exhibit a consistent pattern of leaf development allowing replication of plant material for analyses (sections 3.15, 3.16). The number of leaves attached to the stolon reaches a constant number as the production rate is balanced by the senescence rate. This is consistent with the observation of Thomas (1987b) that "under controlled environment conditions, when the rates of leaf initiation and emergence are constant, the changes from node to node represent changes in time".

At a single harvest time, leaf tissue replicated across individual stolons can be collected representing all stages of development from leaf initiation to maturation, senescence and necrosis. The overall pattern of development exhibited by the leaves was consistent between plants grown at different times and under different environmental conditions (light, temperature, watering), while the rate of change between the developmental stages varied. "The changes taking place in senescence form a genetically programmed sequence, with close co-ordination at the cell and tissue levels" (Smart, 1994). The data presented here suggest that the consistent pattern of development in white clover genotype 10F reflects an underlying genetically programmed sequence but the rate of development is influenced by the growing environment.

The method of plant culture of stolons of white clover allows the collection of

non-senescing and senescing tissues at the same time. Leaf senescence is often studied by comparing physiological and biochemical analyses or gene expression in non-senescent and senescent leaves where, by necessity, the tissues are collected at different times (for example, Kang, *et al.,* 1982; Bate *et al.,*1991; Kamachi, *et al.,* 1992), or the plants are incubated in darkness (for example, Hörtensteiner, *et al.,* 1995), or the leaves are detached with or without dark incubation to induce senescence (for example, Hilditch, *et al.,*1989; Cuello, *et al.,* 1989,1990; Davies, *et al.*, 1990; Thomas, *et al.,* 1992; Ferguson, *et al.,* 1993; Bachmann, *et al.,* 1994). While these methods have some advantages (for example, excision from the plant accelerates senescence, and removes interactions with the rest of the plant), there are dangers in assuming these artificial systems represent a true picture of "natural" senescence in the plant (Smart, 1994). For example, the induction of endoproteinases in detached barley leaves was different to that seen in attached tissues (Miller and Huffaker, 1985). These workers found that during senescence in detached leaves, the activity of two major endoproteinases present in green leaves increased and four new species of endoproteinases 'appeared'. In contrast, during senescence in attached leaves, the activity of the two major endoproteinases did not change, and none of the 'new' endoproteinases appeared.

The different types of senescence have been defined by Hillman *et al.,* (1994): *overall* senescence occurs in monocarpic plants, *selective* or *partial* or *top* senescence happens when there is die-back to a perennating organ such as a rhizome or root, *synchronous* senescence is observed every autumn when the leaves and fine roots of deciduous trees are shed, and *sequential* senescence is the senescence of older organs, tissues and cells as new structures appear. These workers also state that "natural senescence can be regarded as being genetically programmed which assumes built-in obsolescence..", and this can be compared with induced senescence which may be caused "by mechanical damage, xenobiotics, environmental perturbations commonly referred to as stress, and by pests and diseases which could be regarded as combinations of the first two factors".

The senescence observed in white clover using the artificial system in this study can therefore be defined as *natural* and *sequential* because it has not

been induced by external factors, and because it appears in older leaves as new leaves appear. Other workers have also recognised the possible differences between natural and induced senescence. For example, King *et al.*, (1995) compared gene expression in postharvest-induced and natural foliar senescence in asparagus. In this system the accumulation of three harvest-induced cDNA clones also accumulated during 'natural' leaf senescence indicating to the authors a similar underlying regulatory mechanism in both developmental situations.

In contrast to the findings of King *et al.*, (1995), Becker and Apel (1993) compared changes in gene expression between natural and artificially induced leaf senescence in barley and concluded that only a minor part of the mRNA changes observed during dark incubation of detached leaves is connected with senescence while the major changes are associated with stress responses. These workers isolated six cDNA clones that accumulated during dark incubation of detached primary leaf segments of barley. Four of the six clones probably represent the same transcript (based on sequence data) which may be highly abundant in the leaf segments during dark incubation. Five of the six clones were induced by abscisic acid and inhibited by kinetin. They were also induced by wounding and osmotic stress, but could not be detected in naturally senescing leaves of barley. The expression of one cDNA clone however, was not significantly affected by hormone application, wounding or drought stress, but accumulated during natural senescence of barley flag leaves. The changes in gene expression analysed by these workers probably represents a very small proportion of the total changes in gene expression, hence their conclusion is drawn from a very small sample population.

A feature of the cultural system used in this study is the inhibition of nodal root growth. The presence of roots has an effect on the senescence of clover tissues. Stolon senescence was found to be strongly influenced by the presence of nodal roots where the death of nodes and internodes 'progressed in waves' interrupted by the presence of roots (Sackville Hamilton and Harper, 1989). While the specific mechanism for the effect nodal roots have on persistence and senescence in white clover has not been fully determined, roots are generally believed to be a site of cytokinin production in plants

(Kende, 1965; Short and Torrey, 1972; Torrey, 1976) and while cytokinin production has been observed in young leaves in tobacco, mature and senescing leaves appear not able to produce the hormone (Dyer and Osborne, 1971; Venkatarayappa *et al.*, 1984). Leaf senescence has been correlated with decreased endogenous cytokinin activity or concentration in the leaves (Lindoo and Noodén, 1976, 1978; Ambler, *et al.,* 1987; Van Staden *et al.*, 1988). Further, the application of exogenous cytokinin has been shown to delay leaf senescence (Thimann, 1980; Aharoni, 1989; Cuello, *et al.,* 1990), and may cause re-greening of yellow leaves in some species (Smart, 1994).

The controlling influence of cytokinins in leaf senescence is supported by the work of Smart *et al.,* (1991) who transformed *A. thaliana* with a gene which encodes isopentenyl transferase (which catalyses the first step in cytokinin biosynthesis in *Agrobacterium tumefaciens*) under the control of a heat shock promoter (HS6871 from soybean). The transformed plants exhibited increased levels of cytokinin following a heat shock (42°C for 2 h), and had delayed leaf senescence. However, the non-heat shocked transformed plants (maintained at a constant 20°C) also had elevated levels of cytokinin compared with control plants, and also some delay in leaf senescence indicating that the heat shock promoter has some activity at lower temperatures.

In a very elegant study, Gan and Amasino (1995) transformed *N. tobaccum* with a chimeric gene construct linking an isopentyl transferase gene with a senescence-specific promoter from the SAG12 gene isolated from *A thaliana.* The promoter, P_{SAG12}, is activated in early senescence, and the chimeric gene presumably increases cytokinin production in the transgenic plants therefore preventing the leaves from senescing. It should be noted however, that no evidence of raised cytokinin levels could be found by these workers. The prevention of senescence would in turn attenuate expression from the promoter to prevent over expression of the cytokinin. These workers found that leaves of the transformed plant showed no sign of senescence 40 days after detachment, whereas wild-type leaves began senescing 10 days after detachment.

The induction of leaf senescence may therefore be related to the concentration of cytokinin in the leaves derived from both biosynthesis by the leaves, and import from other sources (for example, the roots). This is supported by Singh *et al.,* (1992) who, using radio-tracer studies in tobacco, provided evidence that it may be the difference in the cytokinin biosynthetic capacity between young and old leaves that contributes to the differing cytokinin levels and may participate in the sequential control of leaf senescence in this species. In contrast, Noodén *et al.,* (1990) showed that pod removal during early pod development in soybean caused an increase in xylem cytokinin levels, while removing pods at late podfill did not increase the cytokinin levels. These workers suggest that the control of cytokinin production in the roots (by the developing pods) is important in determining whether the plants undergo monocarpic senescence. Taken together, data from these studies indicate that the concentration of cytokinin in the leaves may be an important determining factor in the control of leaf senescence.

Based on studies with other plant species, young leaves and roots of white clover are likely to be sites of biosynthesis of cytokinin. In white clover plants grown using the system described in this study, a consistent number of leaves was maintained on the stolon after the stolon system matured (defined as having 6 or more nodes with senesced leaves, section 3.1.3). In this situation, the "mature" state exhibited by the leaves may at least in part be determined by the 'distance' from the roots and the site of cytokinin production. In this situation, the mature leaves may senesce when the cytokinin biosynthesis in the leaves reduces below some critical amount. It is interesting to note that during the early growth of the stolons when all nodes are 'close' to the roots, more green leaves are maintained on the young stolon (>10) than on the mature stolon. These data suggest that inhibition of nodal root growth may improve the consistency of leaf development in the cultural system used here by decreasing the number of sites (roots tips) for cytokinin production adjacent to the leaves, that is, there may be a greatly decreased effect of root derived cytokinin, and senescence may simply reflect the leaf cytokinin content.

This role of cytokinin in determining the pattern of leaf senescence in white clover may be tested using several methods including applying cytokinin at

various points on the stolon to plants grown using the system described in this study, and comparing cytokinin concentrations in leaves at different stages of development and senescence on stolons grown with or without roots.

A successful method for the genetic transformation of white clover plants was reported recently (Voisey *et al.*, 1994). The ability to transform white clover allows very specific investigations to be made on the role of various factors in regulating leaf development and senescence. For example, white clover plants transformed with a functional isopentenyl transferase gene linked to a senescence-specific promoter (for example, an equivalent to SAG_{12}) may provide information on the role of cytokinin during leaf development and senescence in a similar way to the work reported by Gan and Amasino (1995). Alternatively, the same gene linked to an inducible promoter would allow an investigation of the effect of cytokinin on development and senescence to be studied at various developmental stages.

The onset of leaf senescence in white clover plants grown using the system described in this study is apparently determined by leaf age and does not require an external factor for initiation. Lohman *et al.*, (1994), state that "the initiation of leaf senescence can be induced by a variety of external factors such as shading, mineral deficiency, drought and pathogen infection and developmental processes such as seed development. In the absence of such factors leaf senescence occurs in an age-dependent manner in many species". White clover plants grown at two different locations (Levin and Palmerston North) exhibit the same pattern of leaf senescence suggesting that senescence in these tissues is apparently not induced by external treatments but rather develop in an age-related manner the rate of which is modified by the environmental conditions. Lohman *et al.*, (1994) also comments that the predictability of the senescence programme in *A. thaliana* facilitated a study of changes in mRNA, chlorophyll, protein and gene expression. The data presented here demonstrate that due to the predictability of leaf development along the stolon of white clover, this is also an appropriate system for the study of leaf senescence.

5.2 Ethylene evolution from leaves of white clover

Attached and unwounded leaves of white clover produce ethylene; a peak of ethylene evolution is associated with young expanding leaves while mature green leaves produce a detectable but low level of ethylene, and senescing leaves produce more ethylene in a second peak. This is similar to the ethylene evolution measured in other systems. For example, ethylene evolution from leaves of the herbaceous plant *P. vulgaris* was high during the rapid expansion phase, declined by 75% as the tissue matured, and remained constant during senescence, while ethylene evolution from leaves of the deciduous plant *Prunus serrulata* increased markedly in senescing leaves (Roberts and Osborne, 1981).

The pattern of ethylene evolution in white clover, generally inversely mirrors the changes in chlorophyll concentration down the stolon, but an increase in ethylene evolution was only detected in leaves which already had shown a decline in chlorophyll concentration. This is similar to the findings of Roberts *et al.*, (1985) who state "In contrast to flowers and fruit the increase in ethylene production during foliar senescence appears to occur after the onset of the senescence programme when a decline in protein and chlorophyll has already commenced". However, this finding may reflect the inability to accurately measure changes in ethylene concentration at the site of action of the hormone.

The measurement of evolved ethylene is detecting predominantly the ethylene present in the intercellular spaces and not that within the cells, and may not reflect physiologically important concentrations. "The ethylene that is functional in growth control is the ethylene that is within the cytosol" (Osborne, 1989a). Therefore, an increase in physiologically-important ethylene production may not be detected in the early stages by measuring evolved ethylene. This is supported by data presented here on the measurement of ACC concentration. The concentration of ACC was low in expanding and mature green leaves, but increased in de-greening leaves prior to the measurement of an increase in ethylene evolution. For plants grown at Levin, the ACC concentration was very low in leaf four (mature

green) but increased in leaves five, six and seven. The total chlorophyll concentration decreased abruptly in leaf six (compared with leaf five) while an increase in ethylene evolution was not detected until leaf seven. The sharp decline in total chlorophyll concentration seen in leaf six is similar to the rapid chlorophyll decline measured in rosette leaves of *A. thaliana* six days after full expansion (Hensel *et al.*, 1993).

5.3 Ethylene biosynthesis

Ethylene evolution, ACC concentration, and ACC oxidase activity were measured in the experimental stolon system in white clover. However, no reliable ACC synthase activity (activity proportional to the concentration of the substrate) could be detected in leaf protein extracts tested with a range of AdoMet-sulphate concentrations (0, 5, 10, 15, 20, 25, 50 μM; section 3.4.2). This may be due to low concentrations of the enzyme in the protein extracts resulting in the production of ACC below the detection limits of the methods used, or may be due to the presence of some (unknown) inhibiting factor(s) in the extracts. Combining extracts from apple with clover leaf extracts decreased the activity of ACC synthase from the apple tissue more than would be expected from the dilution, while boiling the clover extract overcame this effect. The restoration of activity in extracts from apple tissue when boiled white clover protein extracts were added implies a "proteinaceous" inhibitor because boiling should denature the proteins present. Alternatively, boiling may be removing a volatile component from the extract, hence the combined extracts would have a lower concentration of the volatile component (50% of the total extract has been boiled).

The ACC synthase protein is found at very low concentrations even in fruit tissues exhibiting relatively high ethylene production. For example, Bleecker, *et al.*, (1986) purified ACC synthase from tomato pericarp tissue and estimated the protein to represent 0.0001% of total protein. The concentration of ACC synthase present in leaf tissue of white clover is presumably even lower as the ethylene production is approximately 1% of that considered 'normal' for ripening tomato fruit. The possibility of a low concentration of ACC synthase present in the extracts being responsible for the inability to detect reliable activity could be tested by further extraction and purification of the extracts. Further purification and concentration of ACC synthase has been found necessary in order to study the properties of the enzyme in fruit tissues where relatively high levels are expected to be present (for example, tomato fruit, Bleecker *et al.*, 1986; Kim and Yang, 1992; apple fruit, Dong *et al.,* 1991; *Cucurbita* fruit, Sato *et al.,* 1991; carnation petals, Satoh *et al.,* 1994). However, it is unlikely that the low concentration is the only factor in the low

activity as combining the white clover extracts with the apple extracts should reduce activity in proportion only to the dilution.

This low concentration may also mean that the enzyme is very unstable due to the effect of "suicide" inhibition by its substrate. For example, the half-life of ACC synthase extracted from mung bean hypocotyls was reduced from 23.5 min to 12 min when the AdoMet concentration was raised from 40 μM to 150 μM AdoMet (Casas, *et al.,* 1993). Similarly, Satoh *et al.,* (1994) demonstrated a decrease in the half-life of ACC synthase in extracts from senescing carnation flower petals reduced from 3.5 h in the absence of AdoMet, to about 11 min in the presence of 200 μM AdoMet. In this study, the maximum concentration of AdoMet used for extracts from leaves of white clover was 50 μM, suggesting that a reduction in the half-life due to suicide inhibition should not be the main cause of the inability to measure activity of the enzyme.

These data suggest several further avenues to attempt to demonstrate ACC synthase activity. First, the protein extracts should be further concentrated as mentioned in the second paragraph on page 227. Second, the extraction time should be reduced as much as possible because Satoh *et al.,* (1994) showed that the half-life was only 3.5 h in the absence of AdoMet although Tsai *et al.,*(1991) showed that there was no reduction in ACC synthase activity in protein extracts from mung bean hypocotyls when the extracts were stored at 4°C for at least 6 days. Thirdly, the presence of volatile components in the extraction mixture should be investigated. For example, lyophilising the protein extracts may remove any volatile components without denaturing the proteins (including ACC synthase), or further purification using, for example, gel filtration may remove small molecular weight interfering components.

The data for ACC oxidase activity indicates a change in the characteristics of the enzyme in different leaves on the stolon. In leaves one, three and five (expanding and mature green leaves), ACC oxidase activity, Vmax and apparent Km were relatively constant. In leaves six, eight and ten, however, the ACC oxidase activity decreased to approximately half that measured in the younger leaves, and the apparent Km in leaf six decreased to approximately half the apparent Km values determined in these leaves. The kinetic data for ACC oxidase activity in protein extracts from leaves eight and

ten, however, did not show saturation kinetics with respect to the ACC concentrations used.

Several hypotheses may explain the inability to show saturation kinetics in extracts from leaves eight and ten. For example, a change in the chemical environment of the cell may affect the kinetics of the ACC oxidase enzyme. A change in the *in vitro* kinetics of ACC oxidase has been demonstrated by several workers in response to CO_2 concentration (Hyodo *et al.,* 1993; Nijenhuis-De Vries *et al.,* 1994) and a change in the *in vivo* kinetics with respect to O_2 was reported by Bailly, *et al.,* (1995). A change in the catalytic efficiency of the enzyme caused by the application of ferricyanide to sycamore cell suspensions was also reported by Malerba *et al.,* (1995). In the present study, protein extracts from white clover leaves using a similar extraction method for the ACC oxidase assays reduce the activity of ACC synthase extracted from apple tissue and hence may also be affecting the efficiency of the ACC oxidase extracts.

The higher average Km reported here compared with the apparent Km reported for other species (see section 3.4) may also be due to differences in kinetic parameters between the ACC oxidase enzymes(s) in white clover leaves compared with that in other species used by previous workers. For example, ACC oxidase extracted from ripe, or ethylene-treated or propylene-treated fruit tissues has significantly higher *in vivo* and *in vitro* ACC oxidase activity [for example melon: about 100 nL/g.fw/h, (Ververidis and John, 1991); 60 nL/mg protein/h, (Smith *et al.,* 1992)] than measured in white clover leaves (4 nL/g fw/h; 2 nL/mg protein/h). While the Km studies reported by these workers were not done on purified enzymes (and may also be subject to variation from other ACC oxidase isozymes present in the extracts), ripening fruit may contain a significantly higher concentration of one isozyme of ACC oxidase ('ripening-related') compared with other isozymes present in the fruit. The relatively small increase in ethylene production in senescing leaves of white clover indicates that ethylene biosynthesis is not increased to the same extent as it is in fruit tissues, and hence the concentration of a senescence-related ACC oxidase (if present) does not increase to the same relative concentration as a specific ACC oxidase in fruit tissues.

A wide variability in the apparent Km values for ACC oxidase activity in extracts from different tissues and under different assay conditions (especially the concentration of the head-space gas above the extract solution) has been reported. The values vary from 17 μM in apple fruit tissue under atmospheric concentrations of CO_2 (Fernández-Maculet and Yang, 1992) to 435 μM under 4.4% (v/v) CO_2 for extracts from winter squash fruit (Hyodo *et al.,* 1993).

An alternative explanation for the lack of saturation kinetics observed in leaves eight and ten may be the presence of a second isozyme of ACC oxidase in the senescing tissues. The observed change in the enzyme kinetics presented in this study support this hypothesis, and mRNAs encoding two different ACC oxidases have been found in leaves of white clover, one present in young and mature green leaves, and a second present in senescing leaves (D. Hunter, Department of Plant Biology and Biotechnology, Massey University, pers. comm.).

The differential expression of different ACC oxidase genes has been demonstrated recently in other systems (for example, in tomato plants, Bouzayen, *et al.,* 1993 and broccoli florets, Pogson, *et al.,* 1995). More recently, Barry *et al.,* (1996) used the cloned sequence-divergent 3' regions of members of the ACC oxidase gene family from tomato and showed temporal and differential expression amongst the three members examined. For example, two of the members, *ACO1* and *ACO3* accumulate during the senescence of leaves fruit and flowers, with *ACO1* apparently also wound-inducible in leaves. All three members (*ACO1, ACO2, ACO3)* examined were expressed during flower development but in a temporal and tissue-specific manner.

The induction of ACC oxidase in senescing tissues has also been demonstrated in several plant species (Nadeau, *et al.,* 1993; Picton, *et al.*, 1993; John *et al.,* 1995; Pogson, *et al.,* 1995). The ACC oxidase activity present in pre-senescent leaves (leaves three, five, and six) in white clover may represent activity from a non-senescence related isozyme, while the increased activity in senescent tissue (leaves eight and ten) may represent a senescence-associated isozyme, and hence have different kinetic characteristics.

A third possibility is that the ethylene is produced *via* non-enzymatic processes, for example, lipid peroxidation. An increase in lipid peroxidation has been measured in carnation flower petals (the leaves were not analysed for ACC oxidase activity) prior to wilting and senescence (Paulin and Droillard, 1989). However, while this possibility cannot be definitely ruled out, the relatively high level of ethylene production observed in this study was from protein extracts which were responsive to ACC concentration, suggesting the added ACC was the major source of the ethylene. In addition, ACC oxidase is believed to be active in senescent leaves in other species as the yellow leaves of many deciduous and herbaceous plants will readily convert ACC to ethylene, suggesting that ACC oxidase activity "still functions late in senescence" (Osborne, 1991).

The concentration of ACC is maintained in a balance between synthesis (by ACC synthase), conjugation (to MACC by ACC malonyltransferase, Yang *et al.*, 1990), deconjugation (from MACC, Jiao *et al.*, 1986), and oxidation to ethylene (ACC oxidase). In leaf six, ethylene evolution was relatively low (1 nL/mg.protein/h), and while the MACC concentration tended to be higher than that measured in leaves four and five, the changes were not significant. There was no evidence of a significant decrease in MACC concentration, hence it is unlikely MACC is a major source of ACC in this leaf tissue. The ACC concentration was about 3 to 15 nmoles/g.dw suggesting the limiting factor in ethylene biosynthesis was the synthesis of ACC by ACC synthase. As already discussed, however, control may also be accomplished by the activation of ACC oxidase, or the induction of different isoenzymes of ACC oxidase in senescing tissues.

ACC synthase is generally believed to be the rate limiting step in the biosynthesis of ethylene (Yang and Hoffman, 1984; Arteca, 1989; Yang and Dong, 1993). This is supported by data presented here. All leaves tested in the experimental stolon system produced ethylene and had detectable ACC oxidase activity. Further, concentrations of ACC and MACC were measured in the leaves of white clover suggesting that both ACC synthase and ACC *N*-malonyltransferase enzymes were also active in young and mature green leaves (although the possibility of transport of ACC and MACC from other

tissues into the leaves cannot be ruled out). These data suggest that the concentration of ACC limits the production of ethylene by these tissues. This could be tested by adding ACC *in vivo* to white clover leaves and measuring any increase in ethylene evolution.

5.4 The timing of ethylene biosynthesis and perception

Recently, the use of ethylene reception or response mutants, or transgenic plants with modifications in the ethylene biosynthesis pathway has demonstrated an early role for ethylene biosynthesis in leaf senescence. For example, Picton, *et al.* (1993) found that tomato plants transformed with an antisense gene construct to ACC oxidase delayed the onset of leaf senescence (chlorophyll loss), but once started, proceeded at a rate similar to control plants. Leaf senescence in these transformed plants was investigated further by John *et al.,* (1995) who also demonstrated that inhibiting the action of ACC oxidase delays leaf senescence, but once initiated, senescence progressed as normal. These workers concluded that ethylene is implicated in the control of the timing of onset of senescence.

This conclusion is in agreement with the recent findings of other workers. For example, Hensel *et al.,* (1993) conclude that leaf senescence is triggered by age-related declines in photosynthetic processes, and Grbić and Bleecker (1995) suggest that age-dependent factors are necessary and sufficient for senescence-associated gene induction, and that ethylene plays a subsidiary role in regulating the timing of the senescence-associated gene expression.

An alternative explanation for the data presented by Picton *et al.*, (1993) and John *et al.*, (1995) may be that the level of ethylene biosynthesis in the transgenic plants is below that required to accelerate chlorophyll degradation in the older leaves, that is, it may be insufficient to induce a response at that level of production. This is supported by Cuello *et al.*, (1990) who showed that in detached barley leaves the degree of acceleration of chlorophyll loss caused by ethylene application depends on the age of the leaves. The data presented by Picton *et al.,* (1993) show that chlorophyll loss in the young

leaves was the same in both wild-type and transgenic plants suggesting that the wild-type plant is also not responding to the ethylene being produced by the leaves.

The data presented by John, *et al.,* (1995) however, show that ACC oxidase activity in the antisense plants was not completely inhibited and an increase in ethylene evolution and accumulation of ACC oxidase mRNA was detected in leaves of ACC oxidase antisense plants at the onset of senescence. The low level of ethylene produced (0.087 nL/g/h) may be insufficient to initiate a response in accelerating chlorophyll loss in these plants. No data was presented on the effect of low concentrations of ethylene (in the range produced by the transgenic and wild-type plants) on accelerating chlorophyll loss leaves of either the wild-type or transgenic plants in the transgenic studies. However, John *et al.,* (1995) state that when control and transgenic plants were grown together, the differences between them were less apparent, indicating that the ethylene evolved by the control plants was sufficient to overcome the lower biosynthesis in the transgenic plants.

The data presented in this study support the hypothesis that mature leaves perceive ethylene at low concentrations (less than 1 ppm) and respond by increasing the rate of chlorophyll loss. In control treatments with detached leaves of white clover, leaves three, four and five in the presence of the ethylene absorbing material, Purafil, de-greened more slowly than control leaves without Purafil. These data show that removing some of the ethylene produced by these leaves reduced the rate of de-greening, suggesting the ethylene concentrations were below that required for maximum response. After four days treatment, only one container with Purafil had a concentration of ethylene detectable using a flame ionisation gas chromatography detector. There was no significant difference in the rate of de-greening between any of the applied ethylene treatments indicating that the leaves were sensitive to, and responded at a maximum rate to, the lowest concentration used (1 ppm).

The application of ethylene increased the rate of de-greening only in leaves one and two when compared with the control treatment, but the rate was still lower than that measured for mature green leaves. However, the rate of de-greening of detached leaves in these control treatments was also more rapid

than the rate measured in treatments with Purafil. These data suggest that the level of response is maximum at the lowest added ethylene concentration used, and that by comparison with treatments containing Purafil, the control plants are responding to low concentrations of endogenously produced ethylene.

The response of white clover leaves to very low concentrations of ethylene (up to 1 ppm) could be tested by measuring the rate of chlorophyll loss in a flow-through system rather than the static system used in this study. In a flow-through system, air with controlled low concentrations of ethylene can be passed over the leaves so that endogenously produced ethylene can be immediately removed. The level of endogenous production can be determined by measuring the ethylene concentration in the outflow using a gas chromatograph connected to a photoionisation detector. However, as discussed previously (section 3.3.1), the ethylene removed from the leaves is not that which is functional in growth control (Osborne, 1989a).

Leaves respond differently to ethylene depending on the age of the leaf. Cuello, *et al.*, (1989) working with detached barley leaves, showed that ethylene (applied as 'ethrel') accelerated senescence (chlorophyll and protein loss) more in old than in young segments of the leaves. These findings contrast with the conclusions of some previous workers using an induced senescence system. For example, Thimann (1980), reported that the application of ethrel has not had any detectable effect on senescence in darkness, and citing workers who have added very high concentrations of ethylene (for example, 25, 50, or 100 ppm) concluded that "one really has to deduce that the ethylene production of the leaves is not a factor in their senescence". A similar conclusion was reached by Saniewski (1995), who, citing studies that attempted to relate ethylene production to the degradation of membrane phospholipids, states "Generally speaking, the ethylene production by leaves is not a factor in their senescence". The data presented here indicate that mature green leaves of white clover are sensitive to very low concentrations of ethylene and suggest that the response range is within the level of ethylene production by the leaves. This is consistent with the results of experiments with transgenic plants where "in contrast to expectations from ethylene application experiments" by other workers,

transgenic plants with constitutive overproduction of ethylene did not show premature leaf senescence (Lanahan, *et al.*, 1994; Guzman and Ecker, 1990), suggesting that the response may already have been swamped at the lower production level.

The induction of senescence may not require an increase in the production of ethylene, but may involve an increase in the sensitivity of the tissue to ethylene. In particular, the ethylene response pathway linked to senescence may be the important factor. Grbić and Bleecker (1995) found that in ethylene insensitive *A. thaliana* plants, the *etr1-1* (ethylene insensitivity) mutation affected only the timing of expression of SAG genes, but had little or no effect on maximum SAG mRNA levels. These workers conclude that ethylene acts in conjunction with age-related factors in *A. thaliana* but that these factors did not determine sensitivity *per se*, since an ethylene-responsive reporter gene unrelated to senescence, was highly induced in younger leaves (Chen and Bleecker, 1995). In other words, the young leaves of *A. thaliana* can detect ethylene but do not respond by undergoing senescence.

This is similar to the effect of ethylene on abscission zone tissues in *P. vulgaris* where the sensitivity to ethylene changes with tissue age. Here, the application of high concentrations of ethylene (1000 μL/L) will not cause cell separation in the first four days from germination, but subsequent applications of low concentrations of ethylene (1 to 10 μL/L) will cause separation after day 5 (Osborne, 1991). In a different system, an increase in ethylene sensitivity following pollination is the initial event triggering an increase in ethylene production and enhanced senescence of *Phalaenopsis* orchid flowers (Porat *et al*,. 1995). This is consistent with the view that ethylene does not initiate senescence but rather acts on tissues after the initiation of the senescence programme, and is consistent with the data presented in this study.

The data presented for the biosynthesis and perception of ethylene in white clover are consistent with the role of ethylene as a plant hormone. At least three separate components of ethylene metabolism must be taken into account when considering the action of the hormone; the ability and extent of the tissue under study to produce ethylene; the ability of the tissue to

perceive the hormone at the concentration it is being subjected to (i.e. ethylene concentration arising from endogenous production by the tissue, and production or exposure from other sources including other tissues on the same plant); and the ability of the tissue to respond in the manner being studied (for example, by undergoing senescence). The data presented here demonstrate that all three aspects change in leaves of white clover at different developmental stages.

5.5 PCR amplification of ACC oxidase and ACC synthase

The physiological data presented in chapter 3 provides some quantified information on the timing and extent of ethylene biosynthesis, perception and response in leaves of white clover. However, the physiological data relies on the measurement of ethylene released from the plant tissues, or substrate concentration or enzyme activity in tissue extracts. These methods may not be providing precise information due to errors inherent in the methods (for example, see the discussion on ACC and MACC concentration in section 3.3.2), or are limited by the level of sensitivity such that changes can only be detected once a certain level of measurable component is reached.

The inability to detect small changes or low concentrations will affect the determination of the timing of changes, generally delaying the detection until the process has already been underway for some time. In white clover using the growing system described in this study, this delay will result in the ascribing of changes to leaves at a later stage of development than may be the case if more sensitive techniques were used. Molecular techniques offer increased sensitivity compared with physiological techniques. The molecular techniques used in this study were aimed at providing further information on ethylene biosynthesis in white clover which had been unattainable using the physiological methods employed, and also used to determine changes in the in the expression level of the genes coding for ACC synthase and ACC oxidase during leaf maturation and senescence. The changes in the expression level of genes can be detected before changes at the physiological level can be measured and therefore provides more precise data on the timing of these changes.

The use of heterologous sequences to detect ACC oxidase in white clover in this study was unsuccessful. Other workers have used pTOM13 to isolate ACC oxidase homologues from tissues in other plant systems. For example, Ross *et al.* (1992) were successful in isolating an ACC oxidase homologue from ripening apple cortical tissue by screening a cDNA library with pTOM13. Similarly, Kim and Yang (1994) isolated ACC oxidase homologues from mung

bean hypocotyls using a combination of apple and tomato ACC oxidase cDNAs as probes. In this case, the isolated clones were shown to share 83% identity at the nucleotide level. However, other workers have used PCR amplification to identify ACC oxidase homologues from cDNAs (for example, Dong *et al.,* 1992b), or used PCR-generated fragments to screen cDNA libraries (for example, Peck *et al.,* 1993).

In this study, primers homologous to conserved domains in ACC oxidase and ACC synthase were used to produce DNA fragments by RT-PCR. The putative ACC oxidase and ACC synthase fragments hybridised strongly on a white clover genomic DNA Southern blot, indicating they were homologous to DNA from this source. As ACC synthase is likely to be the rate limiting step in the biosynthesis of ethylene in leaves of white clover, it was decided to concentrate the rest of this study on the characterisation of this enzyme. However, the putative ACC oxidase clone (section 4.3.4) was subsequently sequenced by another worker, and shown to have high homology to ACC oxidase sequences published in the GenBank database (D. Hunter, Plant Biology and Biotechnology Department, Massey University, pers. comm.).

The pattern produced in Southern hybridisation of genomic DNA from white clover with the putative ACC oxidase fragment indicated that it was represented by more than one copy in the genome. This is in agreement with other studies where ACC oxidase has been shown to be encoded by a multigene family in other species including tomato (Bouzayen, *et al.,* 1993), mung bean (Kim and Yang, 1994), and broccoli (Pogson, *et al.,* 1995). The homologues of ACC oxidase within and between species share significant homology with each other. For example, two ACC oxidases isolated from broccoli share 83% homology at the nucleotide level in their putative translated regions but only 48% homology in the 3' untranslated region, and there was 99% identity between one of these clones and ACC oxidase from mung bean. The ACC oxidase clones also shared 70 and 71% homology with ACC oxidase from tomato (Pogson, *et al.,* 1995).

The pattern produced in the Southern hybridisation of genomic DNA from white clover with the putative ACC synthase fragment indicated that it was represented by more than one copy in the genome. ACC synthase has also

been shown to be encoded by a multi-gene family in other species including tomato (Yang and Dong, 1993), *A. thaliana* (Liang *et al.*, 1992), potato (Destèfano-Beltrán *et al.,* 1995), and carnation (Park *et al.,* 1992).

Two of the PCR generated clones using primers homologous to conserved domains in ACC synthase were sequenced and found to have high homology to ACC synthase from *Glycine max, Vigna radiata, Brassica juncea, B. oleracea, Lycopersicon esculentum, Daucus caryophyllus* and *N. tobaccum* (section 4.4). The fragment was also found to contain sequences homologous to the expected conserved domains internal to the primer sites. One of the conserved domains, domain 5, is believed to be the active site domain (Yip *et al.,* 1990; Yang and Dong, 1993). In all 15 of the ACC synthase sequences reported by Yang and Dong (1993), the amino acid sequence in the reaction centre differs in only two residues as follows: S-L-S-K-D-M(L)-G-F(M,L)-P-G-F-R. The reaction centre sequence determined in white clover is identical to an ACC synthase sequence from mung bean (Botella, *et al.,* 1992). In spite of the high level of homology between the putative white clover ACC synthase and ACC synthase from *L. esculentum* (74%), no hybridisation was detected between the tomato ACC synthase probe (pTOM13) and DNA from white clover in Southern analysis at the high stringency level used.

Previous workers studying ACC synthase have generally used fruit or flower tissue with high ethylene production as a source of mRNA for amplification by PCR (for example, tomato Van Der Straeten, *et al.,* 1990; Dong *et al.,* 1991; Olson *et al.*, 1991; carnation flower petals, Park, *et al.,* 1992; *Phalaenopsis* flower tissue, O'Neill, *et al.,* 1993), or wounded tissues with induced ethylene production (for example, etiolated mung bean hypocotyls, Nakajima, *et al*, 1990, Botella, *et al.,* 1992; infected or elicitor-treated tomato cell suspensions, Spanu, *et al.,* 1993). The tissue used for PCR amplification of ACC synthase in white clover produces significantly less ethylene than those tissues used in the studies by other workers. Therefore, if ACC synthase is the rate limiting step in the biosynthesis of ethylene in leaves of white clover, the concentration of the enzyme and its mRNA will also be significantly lower than in tissues used in other studies.

In white clover, the isolation of ACC synthase DNA fragments from total RNA

by PCR required two rounds of amplification in order to generate sufficient DNA to visualise in an agarose gel. Maximum yield was achieved using ACSR1F and ACSR6R primers in the first round, followed by amplification using a primer internal to ACSR1F (ACSR2F), and ACSR6R in the second round. If the products after one round of amplification were separated by electrophoresis, blotted onto nylon membrane, and probed with ^{32}P-labelled ACS7, a product of the expected size (780 bp) could be detected. The requirement for two rounds of amplification provides further evidence that ACC synthase mRNA may be present at very low concentration in leaves of white clover. However, as discussed previously (section 4.5.4) PCR is at best semi-quantitative so that caution must be used in interpreting this data.

Two rounds of PCR amplification of ACC synthase sequences have been used in other studies. Liang *et al.,* 1992, used two rounds of PCR amplification using the same set of primers each time, to enrich the ACC synthase products from *A. thaliana* for subcloning, and Park, *et al.,* (1992), used random hexaprimers to amplify carnation petal cDNA by PCR, followed by a second PCR amplification using primers specific for ACC synthase.

The northern analysis of expression of ACS7 in the experimental stolon system using total or poly $(A)^+$ RNA did not produce a reliable pattern of hybridisation above background levels. This result may be improved by using higher concentrations of RNA. Previous workers studying ACC synthase in green tissues in other systems have used higher concentrations of poly $(A)^+$ RNA on RNA or northern blots to improve detection levels (5.0 μg in apple tissue, Dong *et al.,* 1991; 3.0 μg in tomato leaves, Spanu, *et al.,* 1993, 2.5 to 5.0 μg in orchid flower tissue, O'Neill, *et al.,* 1993).

Recently, a study of ACC synthase expression in developing leaves of white clover was successful using 4.0 μg of poly $(A)^+$ RNA (Sang Dong Yoo, Department of Plant Biology and Biotechnology, Massey University, pers. comm.). Here, a PCR-generated fragment (using the same primer sets as used in this study) with high homology to ACC synthase genes in the GenBank database (but only 60% homology to ACS7) was generated from developing leaf tissue and used as the hybridisation probe. However, using the same northern blot, no hybridisation was detected when ACS7 was used

as the hybridisation probe. This finding is similar to that found by Nakagawa *et al.,* (1991): two ACC synthase clones, one induced by IAA and the other induced by wounding, were extracted from winter squash but the cDNAs representing these clones did not cross-react. Taken together, these data show that ACC synthase mRNA can be detected in white clover leaf tissues but the detection by northern analysis is gene-dependent. It also shows that amounts in excess of 4.0 μg of poly $(A)^+$ RNA may be required to detect hybridisation of ACS7.

A low concentration of ACC synthase in leaves of *A. thaliana* is also implied by Van Der Straeten *et al.,* (1992a) who used RT-PCR analysis ACC synthase mRNA in leaves "due to the lack of sensitivity of northern (RNA) blotting in detecting very low-abundance mRNAs". Similarly, Destèfano-Beltrán *et al.,* (1995) used RT-PCR in expression studies of ACC synthase in leaves and petioles of *Solanum tuberosum* as "transcripts were expected in low abundance". The concentration of ACC synthase mRNA in tomato fruit has been estimated to be as low as 0.0001% of total RNA (Van Der Straeten *et al.,* 1990, 1992b). If the same relative concentration is also true for white clover, the approximate loadings of ACC synthase mRNA used in the northern analysis in this study would be 5×10^{-12} g on the total RNA northern, and this may be below the level of detection using radio-labelled probes.

The data presented in this study together with the data from S.D.Yoo discussed above suggest that different ACC synthase genes with only a low degree of homology may be present in white clover. This is in agreement with other studies where the degree of homology for different ACC synthase cDNAs within a species was found to vary from being moderately high to very low. For example, three ACC synthase cDNAs in mung bean share about 80% homology at the nucleotide level (Botella, *et al.,* 1992), while two clones from tomato share 75% homology (Olson, *et al.,* 1991), but the homology falls from 75% to 31% between some ACC synthase clones from *A. thaliana* (Liang, *et al.,*1992), and an IAA-induced ACC synthase cDNA did not hybridise with wound-induced ACC synthase cDNA from winter squash (Nakagawa *et al.,* 1991).

The expression of the ACC synthase gene represented by ACS7 may be

examined by increasing the concentration of poly $(A)^+$ RNA used in northern analysis. However, the isolation of greater quantities of poly $(A)^+$ RNA requires considerable amounts of leaf tissue. However, as ACC synthase is represented by a multigene family in other species, and Southern analysis in this study suggests this may also be true for white clover, it was unknown whether ACS7 represents a fragment from an ACC synthase associated with senescence or with some other physiological function (for example, wounding). In order to gain the maximum information about the ACC synthase represented by ACS7, and to determine the physiological function most likely associated with it, a full length clone is required. Therefore, the poly $(A)^+$ RNA available was used to produce a cDNA library, and ACS7 was used as a genetic probe with the aim of isolating a full-length ACC synthase clone.

5.6 Screening a cDNA library for ACC synthase

The data reported in this study presents strong evidence for ACS7 being homologous to an ACC synthase from white clover. A cDNA lambda library was prepared from de-greening white clover leaf tissue, and the putative ACC synthase sequence ACS7 used as a hybridisation probe. Only two hybridising plaques were detected in the primary screen. The library filters were washed at low stringency (1 x SSPE at 65°C) to increase the probability of isolating any ACC synthase clones present in the library.

A clone rescued after the second screen at low stringency was sequenced and found to share high homology with a cysteine proteinase. Cysteine proteinase homologues have also been isolated using a specific 5' oligonucleotide primer designed to amplify an ACC synthase cDNA in combination with a non-specific oligo dT-based 3' primer (Jones *et al.*, 1995). These workers found that the cysteine proteinase sequence shared significant homology to other reported cysteine proteinase sequences but no significant homology was found to ACC synthase sequences. When the clone was sequenced, it was found that the nucleotides 2 to 29 were identical to the last 28 nucleotides of the 5' primer. In the data presented here, only a low level of homology was found between the ACC synthase clone from clover (ACS7) and the putative cysteine proteinase clone 10-1-10 (40% at the nucleotide level).

Cysteine proteinases are expected to be active in senescing leaf tissues. Leaf senescence involves degradation of proteins, nucleic acids, and membranes, and the subsequent transport of these nutrients to other regions of the plant (Huffaker, 1990; Lohman, *et al.,* 1994). These workers identified and characterised several senescence-associated genes (SAGs), and two of these (SAG2 and SAG12) were identified as cysteine proteinases. Cysteine proteinase (also known as thiol proteases), as well as other proteinase (serine, aspartic acid and metalloproteinases; Jones *et al.,* 1995) activity has been demonstrated in senescing tissues of a range of plants and is thought to have a role in the mobilisation of nitrogen and carbon from proteins (Thayer, *et al.,*1987).

Thiol proteases have been detected in senescing tissues of pea (Granell *et al.,* 1992), barley (Rogers *et al.,* 1985), and *A. thaliana* leaves (Hensel, *et al.,*1993), and in tissues suffering various stress (Schaffer and Fischer, 1988, 1990; Koizumi, *et al.,* 1993; Linthorst, *et al.*, 1993). A senescence-enhanced cDNA with homology to a cysteine protease was also found in maize leaves indicating a similarity in gene expression during seed maturation and germination, and leaf senescence (Smart, *et al.,* 1995), and the putative cysteine proteinase from white clover shares significant homology to that found in germinating seed of *Vicia sativa* (Becker, *et al.,*1994).

Attempts at using tomato ACC2 as a hybridisation probe to detect a cDNA clone of ACC synthase from a cDNA library prepared from *Capsicum annuum* were also unsuccessful (Harpster *et al.,* 1996). These workers concluded that ACC synthase expression is sufficiently low to exclude its representation in screenings of 200,000 to 300,000 plaques. This is similar to the level of screening used in this study (400,000). These workers were successful in obtaining a DNA fragment homologous to ACC synthase by using PCR amplification of cDNA's synthesised from poly $(A)^+$ RNA extracted from *C. annuum*. However, using a similar technique to that presented in this study, Lay-Yee and Knighton (1995) isolated a full-length cDNA encoding ACC synthase from a cDNA library prepared from ripe apple fruit. This clone was isolated by screening the library with a partial ACC synthase clone (pAAS2; Dong *et al.,* 1991) which was previously isolated from a separate apple fruit cDNA library.

It is suggested by the data presented in this study that ACC synthase is represented in low concentrations in the white clover leaf cDNA library, and that sufficient homology exists between the ACS7 and the putative cysteine proteinase to detect some hybridisation at the low stringency used. However, it is also possible that the cysteine proteinase fragment was picked up as a contaminant from the library plate, and that sequences with higher homology to ACC synthase were also present. Further screening of the sub-libraries should result in the identification of plaques containing a single cDNA clone which hybridise with ACS7.

5.7 Conclusions

The data presented in this study show that the stoloniferous growth habit of white clover provides a good system for the study of leaf development and senescence in relation to ethylene metabolism. The pattern of change in senescence in leaves of white clover (as measured by changes in leaf chlorophyll content) is consistent between plants of the same genotype, but the rate of change between the different stages of de-greening is affected by the environment.

The data also indicate that leaves of white clover produce ethylene and the rate of ethylene evolution varies with the stage of development. In addition, an increase in the rate of ethylene evolution is associated with leaves undergoing senescence in a manner similar to other plant systems studied, but this increase was detected after a decrease in chlorophyll concentration was measured. Data is also presented indicating a change in the activity and kinetic parameters of ACC oxidase in protein extracts from leaves at different stages of development. Taken together, these data support the hypothesis that ethylene modifies the rate of senescence but does not in itself act as the trigger of senescence.

The physical and biochemical data were used to determine an appropriate stage of leaf development to use as source material for molecular analysis of ethylene biosynthesis. The developmental stage selected was a 'late mature-green' stage, prior to a decrease in chlorophyll concentration (leaf six for plants grown at Levin). A PCR-generated fragment of ACC synthase and ACC oxidase from white clover leaves (leaf six) have been sequenced and were used to probe Southern blots and a cDNA library prepared from leaves of white clover. The Southern analysis shows that consistent with other systems studied, both ACC oxidase and ACC synthase is represented by a multigene family in white clover.

PCR analysis of the cDNA library suggests that cDNA clones homologous to the PCR-generated ACC synthase fragment are present but the initial screen did not result in the isolation of an ACC synthase homologue. However, one

cDNA clone isolated from the library has a high sequence homology to a cysteine proteinase from isolated from *Pisum sativum.*

5.8 Future directions

The use of this experimental stolon system for the study of leaf senescence opens up many exciting possibilities for future research, and these fall into three main groupings:

1). isolation, sequencing and expression analysis of ACC synthase(s),

2). isolation, sequencing and expression analysis of ACC oxidase(s), and the further characterisation of the enzyme kinetics, and

3). an investigation on the changes in sensitivity of the tissues to ethylene.

5.8.1 Further investigations on ACC synthase

The complete screening of the white clover leaf cDNA library should enable an ACC synthase clone to be isolated and sequenced. This clone can then be used to analyse the expression of ACC synthase in leaf tissue by using higher concentrations of poly $(A)^+$ RNA preparations in northern or RNA dot blot analyses. In addition, the further use of PCR techniques (for example, quantitative RT-PCR, 5' RACE) may allow a more sensitive analysis of expression, and be useful in isolating more members of the ACC synthase gene family in white clover.

5.8.2 Further investigations on ACC oxidase

The kinetic data suggest that more than one ACC oxidase may be present in leaves of white clover. Recently two different putative ACC oxidase clones have been identified in young and older leaves of white clover (D. Hunter, Department of Plant Biology and Biotechnology, Massey University, pers. comm.). The proteins should be purified and the physical characteristics (size, isoelectric point etc.) determined, and the kinetic characteristics of these

isozymes should be further analysed in the different developmental stages in the leaves (expanding, mature green and senescing).

5.8.3 Sensitivity of white clover leaves to ethylene

White clover leaves are differentially sensitive to ethylene depending on the stage of development. A further investigation of this sensitivity will provide information on the regulation of senescence by ethylene. Changes in the ethylene-receptor protein (for example, in abundance or conformation) coded by the *etr1* gene in *A. thaliana* (Bleecker, *et al.,* 1988; Chang, *et al.,* 1993) may provide some evidence for the mechanism of the change in sensitivity. However, an ethylene-responsive reporter gene in *A.thaliana* unrelated to senescence was highly induced in younger leaves indicating these leaves were responding to ethylene but not undergoing senescence (Chen and Bleecker, 1995). Further, Zhou *et al.,* (1996) found that three *etr1* homologues exist in tomato, and the mRNA coded by one homologue was constitutively expressed in all tissues examined, and its accumulation was unaffected by ethylene or silver ions (an inhibitor of ethylene action), or auxin. However, it is unknown whether this homologue is responsible for the perception of ethylene by tomato plants. Together, these data testify that the mechanism of sensitivity to ethylene in plants is not yet understood. These data also suggest that more than one ethylene receptor gene is likely to be present in white clover and that the relationship between the receptor proteins and senescence will be complex.

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Erratum

P(iii)L16 "1-aminocyclopropane-" should read "1-(malonylamino)cyclopropane-"
P(iii)L41 "unsucessful" should read "unsuccessful"
P(xix)L20 "1-aminocyclopropane-" should read "1-(malonylamino)cyclopropane-"
P3L12 "development" should read "developmental"
P5L10 "ACC *N*-malonyltransferase" should read "AdoMet synthase"
P12L8 "tomato fruit" should read "tomato leaves and petioles"
P13L6 "ACC oxidase may increase" should read "ACC oxidase does increase"
P70L7 "curvettes" should read "cuvettes"
P94L11 "stolon growth was complicated" should read "stolon growth were complicated"
P110L2 "leaves of white clover" should read "leaves of genotype 10F"
P116L4 "plants mature green and yellow leaves collected" should read "plants where mature green and yellow leaves were collected"
P118L6 "data is available" should read "data are available"
P14527 "leaves two and three" should read "leaves one and two"
P145L28 "three" should read "two"
P148L32 "four days was compared" should read "four days were compared"
P159L9 "chlorophyll loss was underway" should read "chlorophyll loss were underway"
P159L11 "concentration than leaves" should read "concentration as leaves"
P183L25 "white clover is shown" should read "white clover are shown"
P233L9 "no data was presented" should read "no data were presented"
P245L13 "Data is also" should read "Data are also"
P251L12 insert "Barry, C.S. Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J. and Grierson, D. 1996.
Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *The Plant Journal* 9:525-535."

Table 3.2		n = 10
Tables 3.3-3.4	cultivars 1-3,5-6,8,9	n = 10
	cultivars 7,11	n = 9
	cultivar 4	n = 6
	cultivar 9	n = 7
Table 3.5	all genotypes	n = 101
	10F	n = 120
Table 3.6		n = 20
Table 3.8		n = 96
Table 3.9		n = 3
Table 3.10		n = 3 with 3 internal replicates
Tables 3.11A-3.11D		n = 4
Figures 3.3-3.5		n = 101
Figures 3.6-3.7		n = 120
Figures 3.8-3.13, 3.16		n = 10
Figure 3.14		n = 5
Figure 3.15		n = 8
Figures 3.17-3.19		n = 4
Figures 3.20A-3.27B		n = 3 with 3 internal replicates
Figures 3.30 A-3.30C		n = 12