Regulation of Ethylene Biosynthesis in *Festuca novae-zelandiae* (*Hack.) Cockayne* and in *Festuca arundinacea* (*Schreb.)* in Response to a Water Deficit

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Abstract

Changes in ethylene evolution and the associated biosynthetic enzyme ACC oxidase to a water deficit, were examined in intact leaves of *Festuca novae-zelandiae* and *F. arundinacea* cultivar 'Roa' (syn. *Schedonorus phoenix*). The aim was to establish a role, or otherwise, for ACC oxidase as a regulator of ethylene biosynthesis in response to a water deficit. While ACC synthase has long been recognised as the major rate-limiting enzyme in ethylene biosynthesis, there is mounting evidence to suggest that ACC oxidase may also regulate the ethylene biosynthesic pathway in higher plants.

Leaf tissues from the two species were harvested at regular intervals during the experimental dry-down, and dissected into two leaf zones; regions enclosed by the ligule, comprising the meristematic and elongating leaf zone (the enclosed tissue), and exposed regions comprising the mature green leaf zones. Leaf proline content and the rate of leaf elongation (LER) were used as internal and external indicators of physiological changes in response to the water-deficit. Ethylene evolution in response to a water-deficit was found to be tissue-specific in *F.arundinacea*. In the rapidly expanding leaf zones, i.e. enclosed tissue, ethylene was maintained at levels similar to control tissue. In the mature green regions of leaves, ethylene followed changes in the leaf elongation rate (LER) with observed peaks in ethylene evolution occurring approximately 48 hours after a rapid decline in the LER. This burst of ethylene was found to precede any accumulation of proline. Increases in the proline content in both leaf zones, only became significant after the ethylene evolution had subsided to below base levels. This stage-specific ethylene evolution in leaves suggests preferential protection of the rapidly expanding leaf cells, an observation that has been documented by other authors.

ACO specific enzyme activity was greatest at soil water contents of *ca*. 9% in the enclosed and 10% in the exposed leaf tissues of *F.arundinacea*. On further purification of the enzyme, two novel proteins were recognised by polyclonal antibodies in water-stressed leaves of *F.arundinacea*. A 32 kDa protein was identified in the enclosed leaf tissue and a 37 kDa protein was identified in the exposed leaf tissue, by SDS-PAGE. These proteins eluted from a Mono Q column at different points in the separation process, i.e at salt concentrations of 320-340 and 300-320 mM NaCl respectively, indicating that they may represent two distinct isoforms of the ACO enzyme. Both proteins are active at pH 7.5 with saturating substrate (ACC) and co-substrate (Na

ascorbate) concentrations of 1 mM and 30 mM respectively, and co-factor concentrations of 0.02 mM Fe^{2+} and 30 mM $NaHCO_3$. When compared with results from western analyses, maximum specific enzyme activity correlated well with the water-deficit induced protein from partially purified enclosed leaf tissue, but only loosely with the protein identified in the exposed leaf tissue. The presence of high molecular weight proteins in both the crude and the purified (Mono Q) leaf extracts of *F.arundinacea* together with the novel proteins, suggests that the ACO enzyme in this species may exist as a dimer. In *F.novae-zelandiae*, the presence of high molecular weight molecules in the crude and partially purified (Sephadex G-25) extracts also suggests dimerisation of the enzyme in this species.

From this study however, it is not possible to establish a clear regulatory role for the ACO enzyme in ethylene biosynthesis in either *F.arundinacea* or *F.novae-zelandiae*. While two novel water-deficit-induced proteins were associated with increased ACO activity in purified leaf extracts of *F. arundinacea*, there was no obvious correlation between ethylene evolution and enzyme activity.

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List of Abbreviations

A (518nm)	absorbance at a wavelength of 518 nm
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
Adomet	*S*-adenosyl-$_L$-methionine
ANOVA	analysis of variance
APS	ammonium persulphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
°C	degree Celsius
ca.	*circa* (approximately)
CO_2	carbon dioxide
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EFE	ethylene forming enzyme
FPLC	fast protein liquid chromatography
FW	fresh weight
GACC	1-(gamma-$_L$-glutamylamino) cyclopropane-1-carboxylic acid
G	gram
GC	gas chromatography
h	hour
HCN	hydrogen cyanide
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid
HSP	heat shock protein
kDa	kilodaltons
K_m	substrate concentration at half maximum reaction rate
L	litre
LEA	late embryogenesis abundant protein
LER	leaf elongation rate
Log	logarithm
MACC	1-(malonylamino) cyclopropane-1-carboxylic acid
M	Molar, moles per litre
MES	2-(N-Morpholino) ethane-sulfonic acid
Mg	milligram
µg	microgram
µL	microlitre
MilliQ water	water purified by a MillQ ion exchange column
min	minutes
mL	millilitres
Mr	relative molecular mass (g mol^{-1})
MTA	5'-methylthioadenosine
n	number of replicates
N	enclosed leaf tissue
Na HCO_3	sodium bicarbonate
NBT	*p*-nitro blue tetrazolium chloride
2-ODD	2-oxoacid dependent dioxygenase
PA	1, 10-phenanthroline
PAGE	polyacrylamide gel electrophoresis
PBSalt	50mM sodium phosphate, ph 7.4, containing 50 mM NaCl

p.	page
pH	- log [H^+]
pI	isoelectric point
PIPES	piperazine-1, 4-Bis (2-ethanesulfonic acid)
ppm	parts per million
PVDF	polyvinylidene difluoride
resp.	respectively
RO	reverse osmosis
SAM	*S*-adenosyl-$_L$-methionine
SDS	sodium dodecyl sulphate
SE	standard error of mean
SWC	soil water content
TEMED	*N, N, N', N'*-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
V_{max}	maximum rate of reaction
v/v	volume per volume
v/w	volume per weight
w/w	weight per weight
X	exposed leaf tissue

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Chapter One: Introduction

1.1 Overview

Ethylene is an important signal molecule synthesised in both higher plants and in microorganisms. In addition to its role in plants in sustaining a programme of normal growth and development, ethylene regulates plant responses to biological and environmental stresses. It is one of several signalling compounds in plants that are induced following early signalling events such as calcium influx and protein phosphorylation/dephosphorylation. Changes in global gene expression in response to these primary and secondary signals eventually alters the metabolism/physiology of plants and leads to their adaptation to the new environment (Liu and Zhang, 2004).

In higher plants, the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to the gaseous plant hormone ethylene (C_2H_4) by the enzyme ACC oxidase (ACO) is the final step in ethylene biosynthesis. Together with ACC synthase (ACS), ACO is one of two crucial enzymes (each catalyses an irreversible reaction) in the pathway. ACS has long been recognised to play a major role in the regulation of ethylene biosynthesis, i.e. is regarded as the rate-determining step, while ACO has often been regarded as constitutive in plant tissues. However, there is increasing evidence to suggest that ACO is an additional regulator of ethylene biosynthesis in plants. Initially, support was provided by a multigene family for ACO. The temporal and spatial expression of ACO to a wide range of developmental and physiological signals has been widely observed, and adds further support (Barry *et al.*, 1996; Hunter *et al.*, 1999; Nie *et al.*, 2002; Chen and McManus, 2006). In addition, observed increases in ACO activity correlative with increases in ethylene production and ACC content, suggest a regulatory role (Hunter, 1998; Gong, 1999).

Two distinct ACO isoforms from the same tissue at different developmental stages (mature green and senescent leaves) have been isolated and characterised in white clover (Gong and McManus, 2000). This shows that the widely observed transcriptional regulation of the ACO gene family is also expressed at the protein level by differential regulation of enzyme isoforms (isozymes). While many studies have shown that ethylene evolution is induced by a water deficit, none have examined whether particular ACO isoform/s are induced.

This study sets out to observe changes in ethylene evolution and ACO activity and protein levels in response to a water-deficit in the leaf tissues of two closely related grass species, *Festuca novae-zelandiae* and *F. arundinacea*. Physiological /biochemical measurements such as the leaf elongation rate and proline content of leaves were made, to act as external and internal indicators of physiological responses to a water-deficit. Isoform analysis was attempted in the immature and the mature leaf zones of both species and water-deficit responses in the two tissue types are compared. The aim is to establish a role or otherwise for ACO in regulating the biosynthesis of ethylene.

1.2 The Ethylene Biosynthesis Pathway

The biosynthesis of ethylene is a multi-step enzymatic pathway that converts the amino acid methionine to (gaseous) ethylene. In higher plants, the major biosynthetic pathway, commonly called the ACC-dependent pathway (figure 1.1, p. 3) includes $_{L}$-methionine, *S*-adenosylmethionine and ACC as the intermediates.

Methionine is converted by the enzyme S-adenosyl-$_{L}$-methionine (Adomet) synthetase in an ATP-dependent reaction to S-adenosyl-$_{L}$-methionine (Adomet). Adomet is an important metabolic intermediate in a wide range of metabolic reactions as well as ethylene biosynthesis, and is therefore not regarded as a committed step in ethylene biosynthesis (Imaseki 1999). The cleavage of Adomet to release ACC and 5'–methylthioadenosine (MTA) in a reaction catalysed by 1-amino-cyclopropane-1-carboxylic acid synthase (ACC synthase) is the first committed step in the ethylene biosynthetic pathway (Kende 1993, Imaseki 1999). MTA is recycled via the Yang cycle to regenerate methionine. This recycling is critical because without regeneration, cellular levels of methionine could become limiting for translation (Yang & Hoffman 1984). At this point in the pathway there are at least two possible fates for ACC: malonylation of ACC by ACC-malonyltransferase to form 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC) in an essentially irreversible reaction (Satoh & Esashi 1984), or the generation of ethylene together with HCN and CO_2 from ACC by the enzyme ACC oxidase (Imaseki 1999). The latter reaction is regarded as the second committed step in the ethylene biosynthetic pathway.

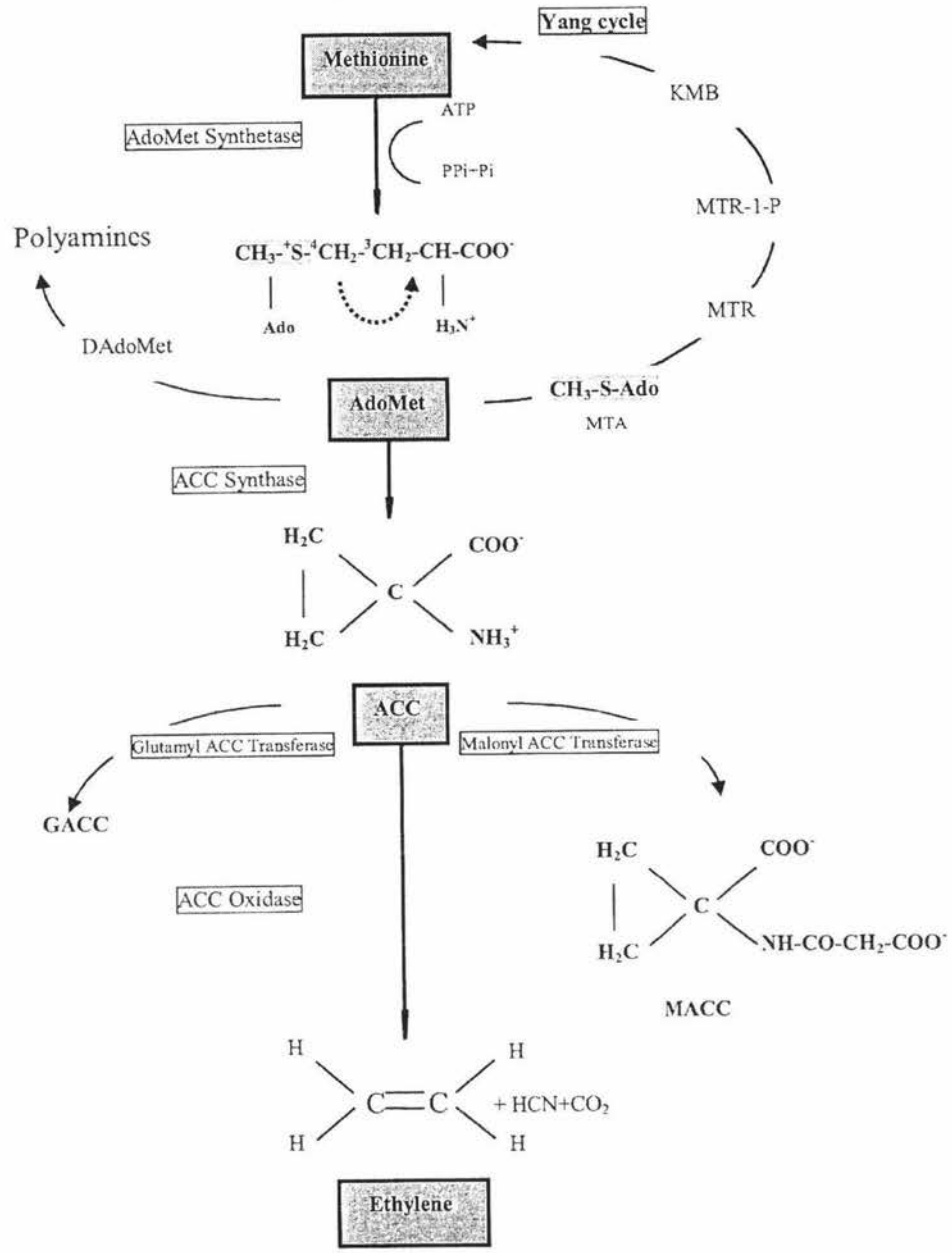

Figure 1.1 The ethylene biosynthetic pathway in higher plants [adapted from Du (2005)]

ACC, 1-aminocyclopropane-1-carboxylic acid; **Adomet**, *S*-adenosyl-$_L$-methionine; **DAdoMet,** decarboxylated AdoMet; **MTA,** 5'-methylthioadenosine; **MTR,** 5'-methylthioribose; **MTR-1-P**, 5'-methylthioribose-1-phosphate; **KMB**, 2-keto-4-methylthiobutyrate; **GACC,** 1-(gamma-L-glutamylamino) cyclopropane-1-carboxylic acid; **MACC,** 1-(malonylamino) cyclopropane-1-carboxylic acid.

As MACC is unable to act as a precursor of ACC, the malonylation reaction can limit ethylene production by limiting endogenous levels of ACC, as ACC levels are regarded as being the primary determinant of the rate of ethylene production (Satoh & Esashi 1984). A second conjugation reaction occurs in a limited number of species such as tomato (*Lycopersicon esculentum*) and involves the gamma-glutamyltranspeptidase catalysed conversion of ACC into 1-(gamma-L- glutamylamino) cyclopropane-1-carboxylic acid (GACC). However, it appears that GACC constitutes only a minor portion of the conjugated ACC in tomato fruit discs. Therefore, this form of conjugation may be relatively unimportant with regard to the regulation of endogenous ACC levels (Peiser & Yang, 1998).

1.3 Regulation of the Ethylene Biosynthetic Pathway

Ethylene acts as a promoter of ageing/senescence in cells and can cause a shift from a programme of growth to one of senescence. Hence, intricate controls are needed in plants to regulate the production of C_2H_4. That plants have evolved to tightly regulate the production of ethylene is supported by the fact that multiple genes encode key enzymes in the C_2H_4 biosynthesis pathway, for example, ACC synthase (ACS) and ACC oxidase (ACO) (Fluhr & Mattoo 1996).

Ethylene production may be regulated developmentally, environmentally and/or by other plant hormones, i.e. by both endogenous and exogenous factors. The basic programme of ethylene biosynthesis during regular development is laid out genetically and C_2H_4 biosynthesis increases and decreases in specified tissues or organs, depending on developmental stage i.e. the transient production of C_2H_4 that soon dissipates according to an epigenetic programme (Imaseki, 1999). For example, during seed germination, fruit ripening, leaf abscission and flower senescence, endogenous C_2H_4 production is induced. Because the physiological effects of ethylene are diverse, this tissue-specific and stage-specific regulation of ethylene biosynthesis is important in sustaining normal plant development.

Ethylene production can also be induced by a variety of external factors such photoperiod, thermo period, drought, flooding, high and low temperatures, wounding (Kim and Yang, 1994; Barry *et al.*, 1996), wind, insects and animals, touch, or infection by pathogens. In many cases, ethylene is an effector that modulates multiple metabolic processes that lead to the acquisition of tolerance to harsh environmental changes or

the healing of wounds(). Ethylene biosynthesis is induced by the hormone auxin in vegetative tissues (Zimmerman and Wicoxon, 1935). Auxin action is in turn synerginistically enhanced by cytokinin, and antagonized by ABA. Therefore, any factors that affect endogenous levels of auxin, cytokinin or ABA will also regulate the synthesis of C_2H_4. Ethylene itself has both a positive and a negative effect on its own biosynthesis. It stimulates a tissue's ability to convert ACC to ethylene as well as to sequester ACC as MACC (Fluhr and Mattoo, 1996). Ethylene also stimulates the development of ACC oxidase activity in a number of vegetative (Kim and Yang, 1994) and reproductive tissues (Liu *et al.*, 1985). This stimulation is correlated with the accumulation of transcripts of ACC oxidase (Kim and Yang, 1994; Tang *et al.*, 1994; Barry *et al.,* 1996) suggesting transcriptional regulation (Fluhr & Mattoo, 1996).

Initially, ACO was thought to be constitutive because exogenously applied ACC led to a rapid increase in C_2H_4 production in tissues that normally produced ethylene at very low rates (Yang & Hoffman 1984), indicating that the ethylene forming enzyme (EFE, later re-named ACO) is induced earlier than ACC synthase (ACS). That is, ACS is the rate-limiting enzyme of the biosynthesis of C_2H_4 (Imaseki 1999). Regulation by ACO then, was not considered a major control point in the ethylene biosynthetic pathway. It is now recognised that C_2H_4 evolution increases correlatively with any increase in ACO activity and is not constitutive (Hyodo *et al.* 1993; Tang *et al.* 1994; Kato *et al.* 2000, Upreti *et al.* 2000; Calvo *et al.*, 2004), and a role for ACO in regulating C_2H_4 biosynthesis has developed in recent years. It is suggested that ACO expression also increases when C_2H_4 production is maximised, such as during fruit ripening (Kende, 1993; Owino *et al.*, 2002). There is increased evidence to suggest that ACO now constitutes an extra tier of control for C_2H_4 biosynthesis in higher plants. Further evidence for the support of ACO as an important regulatory step in ethylene biosynthesis is provided by the discovery that ACO is a multi-gene family (Hamilton *et al.* 1990) and that ACC oxidase has been demonstrated to be rate limiting in the ethylene biosynthesis of *Rumex palustris* during submergence (Vriezen *et al.* 1999).

1.3.1 ACO Belongs to a Multigene Family

The first indication that multi-gene families for ACO existed in plants came from the discovery that there were three related genomic clones to the pTOM 13 cDNA in tomato (Hamilton *et al.* 1990). Since then, ACO multi-gene families have been characterised for many plant species. For example, there are five gene members in tomato (Barry *et al.,* 1996; Nakatsuka *et al.*, 1998; Sell and Hehl, 2005); four in *petunia*

hybrida flowers (Tang *et al.* 1993); three in tobacco (*Nicotiana glutinosa)* (Kim *et al.* 1998); and three in white clover leaves (Hunter *et al.* 1999), melon fruits (*Cucumis melo* L.) (Lasserre *et al.*1996), broccoli floral tissue (*Brassica oleracea* L.) (Pogson *et al.* 1995; Yang *et al.* 2003), cucumber floral buds (*Cucumis sativus* L.) (Kahana et al. 1999), rice seedlings (*Oryza sativa* L.) (Chae *et al.* 2000), sunflower seedlings (*Helianthus annus*) (Liu *et al.*1997), potato (Nie *et al.* 2002; Zanetti *et al.* 2002), and papaya (Chen *et al.,* 2003; Lopez-Gomez *et al.,* 2004). There are two gene members in: mung bean (*Vigna radiata*) hypocotyls (Kim & Yang 1994, Jin *et al.* 1999), banana fruits (Huang and Do, 1998), apricot fruits (Mbeguie *et al.,*1999), peach fruits (*Prunus persica* L.) (Ruperti *et al.* 2001), and carnation (Wang and Woodson, 1991), and one in beech seeds (*fagus sylvatica* L.) (Calvo *et al.* 2004).

1.3.2 Differential Expression of the ACO Gene Family

Spatial and temporal differential expression of ACO genes has been demonstrated in a wide range of plant tissues, for example, in tobacco leaves (Kim & Yang 1994), broccoli florets (Pogson *et al.* 1995), tomato (Barry *et al.* 1996), melon fruits (Lasserre *et al.* 1997), sunflower seedlings (Liu *et al.* 1997), passionfruit (Mita *et al.* 1998), white clover leaves and mung bean hypocotyls (Yu *et al.* 1998; Song *et al.,* 2005).

The isogenes in tomato, the most widely studied fruit model, have been found to be developmentally regulated. For example, *LE-ACO1* and *LE-ACO3* transcripts accumulate during fruit ripening; leaf, flower and fruit senescence (Barry *et al.* 1996). During flower development, *LE-ACO1* is expressed predominantly in petals, stigma and style. *LE-ACO3* is expressed in all floral organs except the sepals and *LE-ACO2* expression is confined to the anther cone. Similarly, in white clover, the four distinct isogenes, *TR-ACO1, TR-ACO2, TR-ACO3* (Hunter *et al.*, 1999) and TR-ACO4 (Chen and McManus, 2006) are expressed in leaves at different stages of development. For example, TR-*ACO1* is expressed specifically in the apex, *TR-ACO2* is expressed in the apex and in the developing and mature green leaves, and *TR-ACO3* and TR-*ACO4* are expressed in senescent leaf tissue (Hunter *et al.* 1999; Chen and McManus, 2006).

Differential expression of members of the ACO multigene family also occurs in response to physiological stimuli. For example, in tomato, only *LE-ACO1* is inducible in wounded leaves (Barry *et al.,* 1996). In mature green white clover leaves, *TR-ACO3* transcripts increase following wound treatment (Hunter, 1998; Gong, 1999). In potato, ACO1 is induced markedly in leaves by wounding, soil flooding, heat (40°C) and cold

(0°C) treatments. Induction of ACO2 by wounding and soil flooding is much lower (Nie *et al.*, 2002). *ST-ACO1* but not *ST-ACO2 or 3* transcripts were induced in leaves, stems and tubers by graft inoculating a Potato Virus A (PVA) resistant cultivar with PVA (Nie *et al.*, 2002).

This differential expression of the ACC oxidase multi-gene family in response to a wide range of developmental and physiological signals provides strong support for the recognition of ACO as a critical regulatory enzyme in the ACC-dependent ethylene biosynthetic pathway in vegetative tissues, ripening fruit and flowers.

1.4 Isolation and Characterisation of the Ethylene-Forming Enzyme, ACC Oxidase (ACO)

Once ACC was established as an immediate precursor of ethylene, the next step was to develop an assay *in vitro* for the ethylene-forming enzyme (EFE) (Adams & Yang 1979). In converting ACC to ethylene *in vivo,* this enzyme showed stereo specificity towards different isomers of 1-amino-2-ethyl-cyclopropane-1-carboxylic acid (AEC) (Fluhr & Mattoo 1996). None of the initial *in vitro* systems was able to discriminate between isomers, and EFE activity proved difficult to demonstrate. In most plant organs activity of the EFE *in vivo* was high (Yang and Hoffman, 1984) compared with the activity of the enzyme responsible for converting ACC to ethylene *(in vitro).* Originally then, a membrane-associated system, i.e. a cell wall/cell membrane association, was implicated because membrane integrity was found to be important for activity *in vitro.* Before the absolute requirement of the ACO enzyme for the addition of ascorbate and Fe^{2+} in extraction media and assay reaction mix was known, (Ververidis and John, 1991), membrane integrity appeared to be needed to retain authentic EFE activity in isolated cell-free preparations (refer to section 1.3), suggesting that ACO was membrane bound in the apoplast (Rombaldi *et al.* 1994; Ramassamy *et al.* 1998). Physiological evidence showed that membrane perturbations affected ethylene production, largely due to an effect on the conversion of ACC to ethylene (Mattoo and White, 1991). Indeed, authentic ethylene-forming activity was retained *in vitro,* in vacuoles from pea (*Pisum sativum* L.) and bean (*Vicia faba* L.) (Guy and Kende, 1984), and in membrane vesicles from kiwifruit (Mitchell *et al.*, 1988). However it was noted that none of the ACO cDNAs predicted a signal sequence encoding a peptide that would facilitate transport to the apoplast. It was also suggested that more efficient regeneration of ascorbate would occur in the cytoplasm compared with the apoplast

and this could explain the apparent membrane requirement for ACO activity *in vitro* (Prescot and John, 1996). Hence there were two possible sub-cellular locations for ACO. It wasn't until recently with much improved techniques, e.g. highly specific antibodies raised against ACO and enzyme recovery methods, a cytosolic location for ACO has been shown. Chung *et al.* (2002) re-examined the sub-cellular location of ACO in apple fruits. It was demonstrated that apple ACO, previously thought to be located extracellularly (Rombaldi *et al.* 1994; Ramassamy *et al.* 1998), is located mainly, if not solely, in the cytosol of apple fruit mesocarp cells.

A major development of historical significance relating to the ethylene-forming enzyme was the sequencing of the pTOM 13 clone from tomato, together with the proposal that this cDNA might represent the gene encoding EFE (Hamilton *et al.*, 1990). The deduced amino acid sequence was similar to that for flavanone 3-hydroxylase. Transgenic tomato plants with a pTOM 13 cDNA insert under the control of the CaMV 35S promoter and terminator, in an antisense orientation, showed much reduced ethylene production, indicating that its gene product is essential for ethylene biosynthesis (Fluhr and Mattoo, 1996). Using conditions similar to those used for isolating and assaying flavanone 3-hydroxylase activity, Ververidis and John (1991) successfully isolated and assayed *in vitro* authentic soluble EFE activity from melon fruits. EFE extraction required anaerobic conditions and the addition of Fe^{2+} and Na-ascorbate to the assay reaction mixture. Similar activity was then quickly recovered from avocado fruit, apple, winter squash and pear (Fluhr & Mattoo 1996). The enzyme was shown to require CO_2 for activation (Imaseki 1999). These studies made it possible to obtain homogeneous ACC oxidase from a wide range of fruit tissues (see Section 1.3.1) as well as to characterise the recombinant enzyme from tomato cDNA's expressed in *E. coli* (Fluhr & Mattoo 1996).

From these early characterization studies, it soon became clear that EFE catalysed the oxidation of ACC and its co-substrate ascorbate in the following reaction in the presence of $Fe^{2+}$and CO_2:

$$ACC + O_2 + \text{ascorbate} \rightarrow C_2H_4 + CO_2 + HCN + \text{dehydroascorbate} + H_2O$$

The nature of this reaction called for renaming EFE to ACC oxidase, the most obvious choice of name since the enzyme is a soluble oxidase (Imaseki 1999). Enzyme activity *in vitro* for ACO has been demonstrated in a large number of plant tissues.

1.4.1 Enzymatics (Biochemical Charaterisation) of ACC Oxidase

The ACO protein has now been isolated and characterised from a number of plant species, mostly in fruit tissues but also in some vegetative tissues. The first successful extraction was from melon fruits (Ververidis and John, 1991). Since then the ACO protein has been isolated and partially purified from fruits of apple (Dong *et al.*, 1992; Kuai and Dilley, 1992; Dupille *et al.*, 1993; Pirrung et al., 1993), avocado (McGarvey and Christoffersen, 1992), kiwifruit (MacDiarmid and Gardner, 1993), pear (Vioque and Castellano, 1994; Kato and Hyodo, 1999), banana (Moya-Leon and John, 1995), tomato (English *et al.,* 1995), citrus peel (Dupille and Zacarias, 1996), cherimoya, (Escribano *et al.,* 1996), papaya (Dunkley and Golden, 1998), and breadfruit (Williams and Golden, 2002). The protein has also been isolated and characterised from senescing carnation floral tissue (Kosugi *et al.*, 1997), white clover leaves (Butcher, 1997; Hunter *et al.,* 1999), barley leaves (Kruzmane and levinsh, 1999), pine needles (*Pinus sylvestris* L.) (Kruzmane and levinsh, 1999) and in seedlings belonging to two orders of the Gymnospermae (the Coniferales and Gnetales): *Pinus nigra, Pinus radiata, Pseudotsuga menziesii, Cupressocyparis leylandii, Ephedra major and E. navadensis* (Reynolds and John, 2000). The protein has been purified to homogeneity or near homogeneity in apple, banana, papaya, pear and breadfruit (references as above). However, fewer ACOs have been partially purified to homogeneity from vegetative tissues, for example only in white clover (Gong and McManus, 2000).

ACO enzyme preparations in varying degrees of purification, show that the enzyme is a 27.5 kDa (in papaya fruits, Dunkley and Golden, 1998) to 42.3 kDa (in in breadfruit, Williams and Golden, 2002) monomer with K_m values for ACC varying from 20 μM for the enzyme from apple, to 56 μM for banana enzyme and 60 μM for melon enzyme (Fluhr & Mattoo 1996). In pine needles (*Pinus sylvestris* L.) and in barley leaves, K_m for ACC is 61 μM and 77 μM respectively (Kruzmane and levinsh, 1999). Gong and McManus (2000) reported K_m s for ACC of 34.7 μM and 110 μM for the white clover isoforms MGI and SEII respectively. Optimum PH for maximum enzyme activity ranges from 7.0-7.2 in barley leaves and pine needles (Kruzmane and levinsh, 1999), and 7.5-8.5 in white clover leaf tissues (Gong and McManus 2000). In all of the plant species studied, ACO is active as a monomer with one exception. In cherimoya fruits, Escribano *et al.* (1996) provided evidence that the ACO enzyme may be active as a

dimer, as indicated by the different molecular masses obtained for native (62, 66 kDa) and denatured (35 kDa) ACO protein.

1.4.2 Evidence for the Occurrence of ACC Oxidase Isoforms in Plants

The presence of a multigene family, together with biochemical characterisation of ACO in many plant tissues, supports the notion that the protein may exist as isoforms in plants. Initial evidence for isoforms came from a study in avocado, using differential ammonium sulphate precipitation at extraction. Two authentic ACO proteins with differences in K_m for ACC were isolated and designated EFE1 and EFE2. Optimal temperature differences in pear fruits (Vioque and Castello, 1994), chickpea (Derueda *et al.,* 1995) and citrus peel (Dupille and Zacarias, 1996) implied the presence of more than one ACO in these plant tissues.

Further evidence for ACO isoforms has been found in leaf and root tissues of corn (*Zea mays*) and sunflower (*Helianthus annus*) (Finlayson *et al.,* 1997), in which tissue-specific ACO activity was demonstrated. Observed differences in pH dependence, K_m for ACC, dioxygen, ascorbate and CO_2 for root and leaf ACO in both species, suggests that ACO isoforms may have evolved to adapt to the environment in which each organ of the plant is exposed (Du, 2004). Three isoforms of tomato ACO when expressed in yeast showed differences in K_m for ACC, pI and specific activity (Bidonde *et al.*, 1998). Gong and McManus (2000) purified two ACO isoforms (MGI and SEII) to homogeneity from white clover leaf tissue at different developmental stages. MGI was isolated from mature green leaf tissues and SEII from senescent leaf tissues of white clover. Differences in molecular mass, the pH optimum, iso-eletric point (pI), K_m for ACC and optimal requirements for the co-substrate ascorbate shows that these two ACOs are distinct isoforms.

1.5 Ethylene as a "Stress" Indicator in Plants

Ethylene influences a diverse range of plant processes including seed germination, diageotropism, flowering, abscission, senescence, fruit ripening and pathenogenesis responses (Imaseki 1999). In addition to its role in growth and development, ethylene regulates plant responses to biotic (biological) and abiotic (environmental) stresses. Early studies found that when plants were subjected to chemical defoliants, microbial diseases or mechanical injury, they showed symptoms usually associated with

exposure to ethylene (Hall, 1952; Williamson, 1950). Increased (endogenous) ethylene production in response to stresses represented by drought, mechanical wounding and radiation has been observed in plant tissues that normally produce very little ethylene (Apelbaum & Yang 1981).

In general, stress increases ethylene production. The term "stress ethylene" was coined by Abeles (1973) to refer to the accelerated biosynthesis of ethylene associated with environmental or biological stresses experienced by plants. The reactions and relationships connecting stress with ethylene-mediated responses are complex. A number of stress responses involve interactions with other hormones, for example auxin and abscisic acid (Gomez-Cadenas *et al.* 1996). "Stress ethylene syndrome" has been used by Morgan & Drew (1997) to describe the variable ethylene-mediated stress responses brought about by stress effects on ethylene perception and the signal transduction pathway. Variable responses to stress may include: an increase, a decrease, or no effect on ethylene biosynthesis (Morgan and Drew, 1997). Stress can alter many steps in the ethylene biosynthesis pathway. For example, the enzymes Adomet synthetase, ACC synthase and enzymes that conjugate ACC, including ACC oxidase, are all regulated by stress (Morgan and Drew, 1997).

Ethylene is an early indicator of stress in plants and as a mediator of the stress-response pathway. It functions as an important signal molecule upstream of any receptors, kinases or transcription factors. Stress-induced activation of the mitogen-activated protein kinases (MAPKs), SIPK, a tobacco (*Nicotiana tabacum*) MAPK and its orthologues, MPK6 in *Arabidopis thaliana,* SIMK (alfalfa, *Medicago sativa),* leSIPK in tomato (*Lycopersicon esculentum*) and PcMPK6 in parsley (*Petroselinum crispum)* have been shown to occur within several minutes and represent one of the earliest responses in plants to stress. It has been known for some time that protein kinases are involved in stress responses, however, it is only very recently that the MAPK cascade (figure 1.2 on the following page) has been linked to the induction of ethylene biosynthesis in plants under stress (Liu and Zhang, 2004).

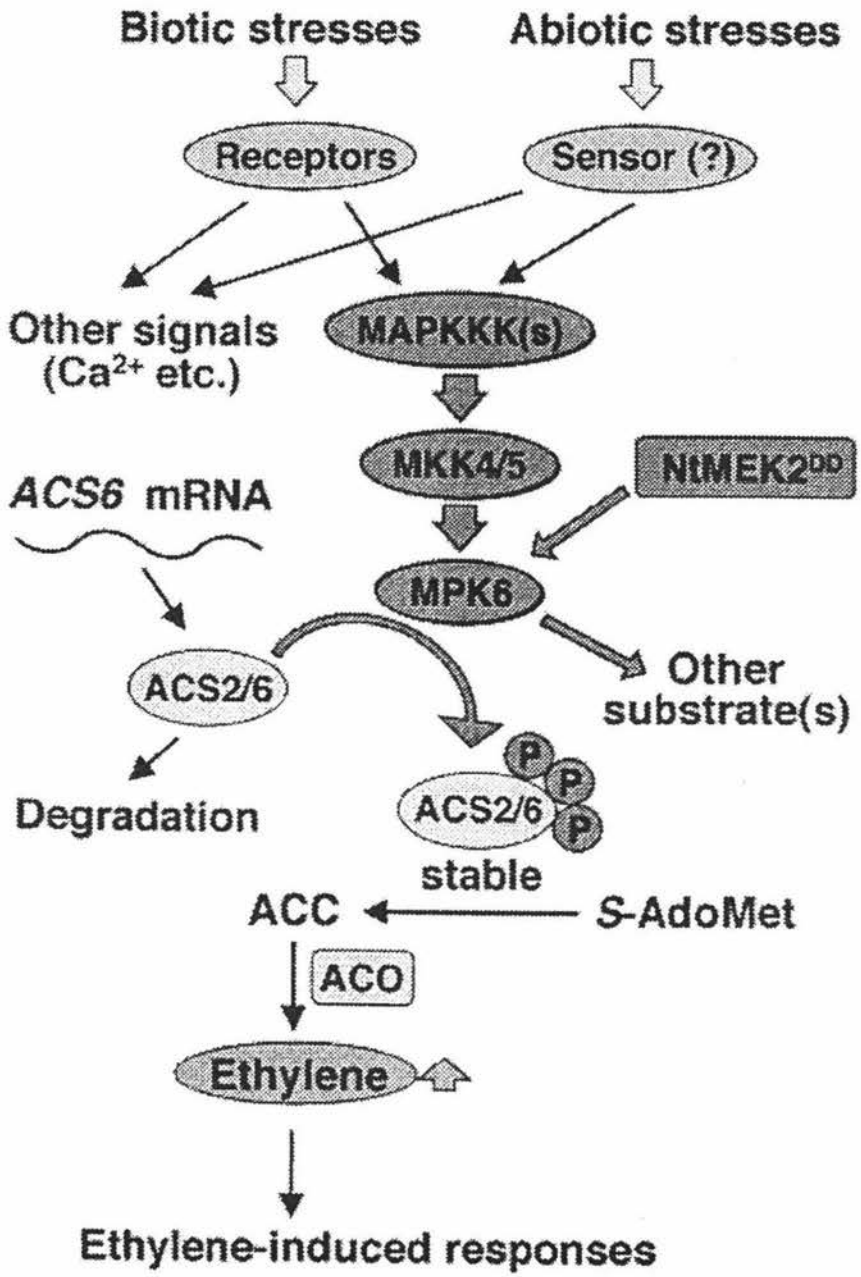

Figure 1.2 MAPK cascade showing responses upstream of ethylene evolution [adapted from Liu and Zhang (2004)]

MAPKs are mitogen–activated protein kinases; **NtMEK2^{DD}** is the active mutant (gain of function) of a MEK2 gene knockout in tobacco; **ACS** is ACC synthase; **ACC** is 1-aminocyclopropane-1carboxylic acid; ***S*-Adomet** is *S*-adenosyl-$_L$-methionine ;**ACO** is ACC oxidase

1.6 Water Deficit Stress in Plants

"Stressed" plants are described as those prevented from expressing their full genetic potential (Boyer 1982). Reductions in plant growth due to stress can be considerable, and are measured as losses in fresh weight. Of all the environmental factors that limit plant growth, for example, drought, cold, salinity, alkalinity, flood, wind, anoxia, and pest organisms, drought or water-deficit stress is the most limiting on plant performance (Boyer 1982).

Loss of turgor is the earliest significant biophysical effect of water stress in plants (Taiz and Zeiger, 1998). Therefore, plant turgor-dependent processes are the most sensitive to water deficit. Reductions in turgor cause decreases in growth rates as described by the following relationship:

$$GR=m\ (\Psi_p-Y)$$

where GR is growth rate, m is wall extensibility (responsiveness of the wall to pressure), Ψ_p is turgor and Y is the yield threshold (the pressure below which the cell resists plastic or non-reversible deformation). As the plant water content decreases, cell shrinkage leads to a relaxation of the cell wall. A subsequent decrease in cell volume results in lowered hydrostatic pressure or turgor. Cell expansion is turgor-dependent and therefore extremely sensitive to water deficit. Since leaf expansion depends mostly on cell expansion, a decrease in leaf area is one of the earliest (adaptive) responses by plants to water deficit. Smaller leaf areas transpire less water and allow conservation of a limited water supply in soil for use over a longer period. Water stress not only affects leaf area but may also affect plant size i.e. a decrease in the number of leaves/shoots on an indeterminate plant.

Drought tolerance has been associated with the presence of considerable quantities of non-reducing di- and oligosaccharides, e.g. mannitol, fructan and trehalose, compatible solutes, e.g. praline, and specific proteins, e.g. the late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs). Proline accumulation to very high levels in plant tissues has been shown to correlate with tolerance to drought and salt stress (Kavi Kishor *et al.,* 1995). Deamination during water-stress-induced proteolysis results in characteristic changes in the levels of free amino acids and amides. Proline increases proportionately faster than other amino acids in plants under stress (Magne and Larher, 1992). Increases can be dramatic. For example, in Bermuda grass (*Cynodon dactylon* L.) shoots under water stress, increases of up to 100-fold have been reported (Barnett and Naylor, 1966). This phenomenon was first observed in

wilted ryegrass by Kemble and McPherson (1954). Proline is believed to be involved primarily in osmotic adjustment, as an osmoprotectant, and, as a hydroxyl radical scavenger but has also been reported to play a role in protecting enzymes from denaturation, stabilising machinery of protein synthesis, regulating cytosolic acidity, increasing water-binding capacity and acting as a reservoir for carbon and nitrogen (Kavi Kishor *et al.,* 1995). Proline also has an effect on ubiquitination of damaged proteins for degradation by the proteasome. In this study, proline is used as a biochemical marker of physiological changes associated with a water-deficit.

Current models in plants for sensing osmotic stress, propose that osmotic stress is firstly sensed by plant cells as perturbations in plasmalemmae caused by a loss of turgor pressure (Skriver and Mundy, 1990). This is followed by changes in cell pH, and then an increase in cytosolic and apoplastic ABA because of de *novo* synthesis and/or release of ABA from organelles (Abernethy, 1996). ABA induces changes in gene expression including expression of the LEA proteins during seed maturation and the RAB (responsive to ABA) group of genes (Abernethy, 1996). LEA proteins are heat soluble and highly hydrophilic, and are located primarily in the cytoplasm and nuclei of cells. Some LEAs are thought to coat intracellular macromolecules with a cohesive layer of water and this is based on high numbers of polar residues within their structures. Small HSPs have also been associated with tolerance to desiccation. These proteins are induced by the same stresses as LEAs and are synthesized with the acquisition of stress tolerance. They have been assigned a role in stabilizing enzymes.

1.6.1 Drought Resistance Mechanisms in Plants

Plants use a variety of strategies to resist drought, including desiccation postponement (the ability to maintain tissue in a hydrated state), desiccation tolerance (the ability to function while dehydrated) or escape. Accordingly, plants may be divided into two basic categories: drought avoiders and those tolerant of drought conditions. Escape plants complete their lifecycle during the wet season before the onsets of drought and are the only true "drought avoiders". Drought resistance (avoidance or tolerance) mechanisms may be ecological, developmental, morphological, physiological and/or biochemical.

Desiccation postponement strategies are adaptations that have evolved in plants, by a process of genetic selection in response to prevailing environmental conditions. However, they may be insufficient to prevent water deficit stress from occurring during unusually dry periods. Such traits may be developmental or morphological and occur

typically in mesophytes which are species of temperate (climatic) zones. Desiccation tolerance strategies may be physiological or biochemical, for example, osmolyte accumulation. Such strategies are employed by species of arid or semi-arid places, for example the xerophytes. Traits at the developmental level are more complex organisationally, than morphological or physiological or biochemical traits, and in that order (M^{c}Cue and Hanson (1990). The smaller number of gene products involved in biochemical traits than in developmental traits makes them more suited for practical reasons to applications of genetic manipulation.

1.6.2 Water Deficit Stress and Ethylene

The imposition of water-deficit stress leads to marked effects on plant growth and development (Abou Hadid *et al.* 1986). M^{c}Michael *et al.* (1972) suggested that increased ethylene production under drought conditions causes leaf abscission, and thereby reduces water loss. This in turn aids plant survival.

There is a complexity of documented responses to "drought" involving intact leaves, detached leaves, short and long water stress duration (Upreti *et al.* 2000) and young and mature leaves (Abou Hadid *et al.*, 1986). It is generally accepted that ethylene is induced by water-deficit stress, and many studies in both leaves of intact plants and detached leaves have shown this. For example, in intact cotton petioles (Jordan *et al.*, 1972, McMichael *et al.*, 1972), in detached leaves of Valencia orange, *Citrus sinensis* 'Osbeck', (Ben Yehoshua & Aloni, 1974); and in excised wheat leaves (Apelbaum & Yang, 1981). Thirty-fold increases in ethylene production were observed in intact wheat leaves, within 4 hours of being subjected to a water-deficit (Apelbaum & Yang, 1981). Under normal conditions, wheat leaves produce very little ethylene. Increases in ethylene have been attributed to increased ACC content and ACO activity. In other studies, however, there is no evidence for a water stress induced increase in ethylene production for intact plants, for example, in *Vicia faba* (El-Beltagy & Hall, 1974), in aeroponically grown sunflower, (Hubick *et al,.* 1986; and in wheat seedlings, (Narayana *et al.*, 1991).

When Narayana *et al.* (1991) compared ethylene production rates in dried, excised leaf segments of eight day old and six week old wheat seedlings with intact six week old water stressed seedlings, they concluded that the commonly reported surge in ethylene production by excised leaves subjected to rapid drying represented an artefact, and has little relevance to water stress response of intact wheat plants. Morgan *et al.*,

(1990) also compared the response to dessication of detached leaves with that of leaves from intact plants.It was found that with regard to ethylene production, detached leaves react differently to rapid drying when compared with leaves attached to intact plants drying out more slowly in soil. In the three species examined (bean, *Pheasoelus vulgaris*; cotton, *Gossypium hirsutum* L. cv. 'Stoneville'; and the miniature rose, *Rosa hybrida* L. cv Bluesette), no evidence for a significant increase in ethylene production with water deficit stress, imposed in a way that resembled a natural drought situation (slowly over several days), was found. In most cases, water deficit stress decreased ethylene production.

Morgan & Drew (1997) have suggested the term "water-deficit stress" be used to describe plants (or plant parts) subjected to rapid drying conditions, such as experienced by excised leaves (or very small volumes of soil) such that a severe deficit develops in one day or less, and "drought" to describe slow drying in pots with volumes of soil large enough so that the water supply declines more slowly over several days. Other workers consider that a drought is where water deficit is influenced by other environmental factors (eg wind, temperature, humidity) and approximates a field situation (Abernethy, 1996). The terms used here are those used by the relevant authors. In glasshouse conditions, it is possible to simply remove water from the plant and keep all other environmental factors identical, and this is usually considered a water deficit. In both situations, when the growth of the plant is affected they are considered to be under drought-stress or water-deficit stress.

In this study, intact plants are used. It is known that the excision of leaves induces ethylene production without the need for drought stress (i.e. wound stress). Therefore, to determine any role for ethylene in plants experiencing a water-deficit, leaves will be excised from whole plants at the appropriate sampling points. The measurement of evolved ethylene as the sole indicator of response to a water-deficit or water-deficit stress is also of limited value. It is possible that responses to a water-deficit may be mediated by changes in the ethylene biosynthetic pathway, and it is these changes that will be examined in this study. Thus far in the literature, there have been no studies on the control of ACC oxidase activity in response to a water deficit or water deficit stress.

1.7 The Plants: *Festuca novae-zelandiae* and *Festuca arundinacea*

Many of the traits associated with water-deficit stress have been discovered and researched in crop and pasture species, especially cereals, and in a few unusual plants, for example, *Craterostigma plantagineum,* the dessication tolerant plant; *Mesembryanthemum crystallinum*, a facultative halophyte; the desert plant Anastatica hierochuntica; and the extremely xerophytic liverwort, *Exormotheca holstii* (Abernethy, 1996). In this study, tissue-specific responses to a water-deficit stress will be examined in the two grass species: *Festuca novae-zelandiae* and *Festuca arundinacea* cv.'Roa' (syn. *Schedonorus phoenix*). Both are members of the Gramineae (grasses) family, however there are important differences. While *F. arundinacea,* or "tall fescue", is an improved forage species of pasture and a mesophyte, that is, it inhabits temperate climatic zones; *F. novae-zelandiae* is a native (unimproved) xerophyte i.e. an inhabitant of harsh, semi-arid environments. *Festuca novae-zelandiae,* commonly known as "hard tussock", is a dominant member of the short tussock grasslands that extend east of the main divide from Marlborough to Southland, in areas of low rainfall to elevations of 1450m, and in isolated regions of the volcanic plateau in the central North Island (Abernethy, 1996). *F. novae-zelandiae* conforms to the accepted definition of a xerophytic plant species in terms of anatomy, morphology, habit and distribution (Connor, 1960). For example, morphological adaptations include rolled leaves that form a tight cylinder enclosing a void which is densely packed with trichomes and a continuous leaf margin gap whose aperture is controlled by the turgidity of bulliform cells located between the vascular ribs (Abernethy *et al.,* 1998).

Studies in several species of the Gramineae show tissue-specific responses to a water deficit. The differential accumulation of metabolites, for example proline, ABA and glycine-betaine (selected species only), suggests preferential protection of growing tissues in response to a water deficit. For example, in wheat (Munns *et al.*, 1979), barley (Matsuda and Riazi, 1981), maize (Michelena and Boyer, 1982) and in *F. arundinacea* (West *at al.,* 1990; Barlow, 1986), the leaf base regions show a greater resistance to water-deficit stress effects than the mature leaf regions. Similarly, in the xerophytic grasses, *Agropyron dasystachyam* and *A. smithii*, maximum osmotic adjustment was observed in the meristematic regions of leaves (Abernethy *et al.*, 1998).

In previous studies in *F. novae-zelandiae,* proline was shown to accumulate preferentially in the leaf bases compared with the mature leaf tissue when subjected to a water deficit (Abernethy *et al.,* 1998). Together with the patterns of protein ubiquitination in leaf bases, immature and mature leaf regions of *F. novae-zelandiae* in response to water deficit (Abernethy and McManus, 1999), this supports the concept of preferential protection of rapidly expanding cells (in the meristematic and elongating leaf regions) in xerophytes during a water deficit (Maxwell and Redmann, 1978). By protecting growing tissue in this way, it is proposed that growth can resume once conditions become favourable (Redmann, 1976).

Biochemical characterisation of the ethylene biosynthetic enzyme, ACC oxidase, will be attempted firstly in the xerophytic native species, *Festuca novae-zelandiae* and secondly in the mesophytic pasture species, *F.arundinacea*. The question central to this project is: Does *Festuca novae-zelandiae*, a xerophytic plant species, produce "ethylene" in response to water-deficit? Previous studies of the plant's response to a water deficit reveal a series of specific cellular responses that result in targeted protection of meristematic tissue (Abernethy *et al.,* 1998) and there may be no induced stress response in the meristem. That is, the plant already exists in a "prepared" state, or signals do not permeate to the protected meristem.

This study will seek to answer this question by examining the existence or otherwise of a pronounced response to a water-deficit in meristematic leaf tissue through the induction of specific ACC oxidase isoforms. Mature, immature and meristematic leaf tissue types will be examined for the presence of such a response. A major aim therefore, will be to establish a role, particularly in a tissue specific context, for ACO as a regulator of ethylene biosynthesis to a water deficit. The results from this study will add to our knowledge of drought response strategies used by plants that grow in arid environments and, unlike crop species have not undergone plant-breeding improvements. Further, any information gained might be used to aid the improvement of commercial crop species.

1.8 Project Objectives

- To compare some responses to a water deficit in the xerophyte *F. nove-zelandiae* with those of the mesophyte *F. arundinacea*.

- To examine ethylene production in meristematic, elongating and mature leaf tissues of *Festuca novae-zelandiae* and *Festuca arundinacea* subjected to a water deficit.

- To examine the activity of the ethylene biosynthetic enzyme ACC oxidase in meristematic, elongating, and mature leaf tissues in response to a water deficit.

- To identify any specific isoforms of ACC oxidase in meristematic, elongating and mature leaf tissues specifically involved in response to a water deficit.