Molecular Characterisation and Regulation of Acyl-CoA Oxidase 1 (ACX1) in *Arabidopsis thaliana*

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The true way leads along a tightrope,
which is not stretched aloft but just
above the ground. It seems more designed
to trip one than to be walked upon.

Beyond a certain point there is no return.
This point is to be reached.

-Franz Kafka

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ABBREVIATIONS

ACC Oxidase	Amino-cyclopropane-carboxylic acid oxidase
ACAD	Acyl CoA Dehydrogenase
ACX	Acyl CoA Oxidase
ATG	three base codon signifying methionine
A.tumifaciens	*Agrobacterium tumifaciens*
A.thaliana	*Arabidopsis thaliana*
At-glw	Arabidopsis green photomixotrophic cell culture
bp	base pair
CEP	2-chloroethylsulphonic acid
C2	2 carbon
cDNA	complementary Deoxyribonucleic Acid
CoA	coenzyme A
dNTP	deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic Acid
DEPC	Diethyl Pyrocarbonate
E.coli	*Escherichia coli*
EDTA	diaminoethanetetra acetic acid disodium salt
E.S.T.	Expressed Sequence Tag
EtBr	Ethidium Bromide
FAD	Flavin adenine nucleotide

g	gram
GA20 Oxidase	Gibberellin 20 Oxidase
gL^{-1}	grammes per litre
H_2O	water
H_2O_2	hydrogen peroxide
HR	Hypersensitive Response
ICL	Isocitrate Lyase
IPTG	Isopropyl-β-D-Thiogalactopyranoside
JA	Jasmonic Acid
kb	kilobase
LA	Linolenic Acid
M	molar
μm	micro meter
μg	micro gram
μL	micro litre
μM	micro molar
mM	milli molar
ml	milli litre
MCTE	Medium Chain Thioesterase
MS	Malate Synthase
MFP	Multifunctional Protein
nm	nano meter

ng	nano gram
NAD	nicotinamide adenine dinucleotide
MAR	Matrix Attachment Region
PCR	Polymerase Chain Reaction
PHA	Polyhydroxyalkanoate
psi	pounds per square inch
PTS	Peroxisomal Targeting Signal
5'RACE	5' Rapid Amplification of cDNA Ends
rpm	revolutions per minute
RNA	Ribonucleic Acid
s^{-1}	per second
SDS	Sodium Dodecylsulphate
T-DNA	Transfer Deoxyribonucleic Acid
TAG	Triacyl Glycerol
TCA	Tricarboxylic Acid
TE	Tris EDTA
UTR	Untranslated Region
UV	Ultra Violet
(v/v)	volume to volume ratio
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Summary

The metabolic status of a plant is fundamental to its ability to respond either to internal developmental signals or external environmental signals. The metabolic pathway of fatty acid ß-oxidation is of particular importance during the germination of oil seed plants since the conversion of lipids into carbohydrate is essential for post-germinative growth. The first step of fatty acid ß-oxidation is catalysed by the acyl-CoA oxidase enzyme. In the model oilseed plant *Arabidopsis thaliana*, four genes encoding acyl-CoA oxidase enzymes with different substrate specificities have been functionally characterised. *ACX1* is one member of this gene family which has been shown to be induced during germination (Hooks *et al.*, 1999a). Interestingly, this particular member has also been shown to be induced under stress and wounding conditions. This study aims to further characterise *ACX1*.

The genome sequencing project was completed at the end of the year 2000 (The Arabidopsis Genome Initiative, 2000). An earlier publication detailing sequence from an area of chromosome IV (Bevan *et al.*, 1998) was found to include the genomic sequence for *ACX1*, including the sequence of the putative promoter. Annotation of the published sequence and further sequence analysis revealed that there was a cluster of putative germination-induced genes within 10kb of *ACX1*. To investigate expression patterns of these putative genes, mRNA transcript levels were monitored at day 2 of germination using both gel blot northern analysis and 'electronic' northern analysis of transcript abundance in an Expressed Sequence Tag (E.S.T.) database produced from a cDNA library prepared during the course of this work. Transcript levels of these genes were too low in 2 day old seedlings to allow any

conclusion about co-expression to be drawn. The implications of this level of control are discussed.

From the published genomic sequence it was possible to design oligonucleotide primers to be used in a PCR reaction to amplify the putative *ACX1* promoter. This putative promoter was fused to the luciferase reporter gene which was then transformed into Arabidopsis by *A. tumifaciens*. This system allowed investigation of the developmental and stress induction of *ACX1*. This study shows that the *ACX1* promoter is induced during both germination and dark-induced senescence. Induction is also seen upon wounding of the transgenic plants. It can be concluded that *ACX1* is a gene of particular interest not only due to its important developmental role but also in its suggested role in the plant defence system.

To elucidate the signalling pathways involved in the induction of the *ACX1* transcript, Arabidopsis cell culture was used as an easily manipulatable system. Northern analysis of *ACX1* expression revealed that during the period of rapid growth between subculturing, the metabolic demand for *ACX1* expression was dominant, resulting in constitutive expression from 24 hours. If acid treatment experiments were carried out 18 hours after subculturing it was possible to observe a rapid induction of *ACX1*. This induction was accompanied by an immediate alkalinisation of the cell culture media. Cold treatment of the cells also resulted in alkalinisation of the media possibly suggesting that the respective acidification of the cytosol may be an early stress response which preceeds *ACX1* expression. The cell culture system has proven to be valuable and should be useful in the future elucidation of the signalling pathways.

CHAPTER 1: INTRODUCTION

1.1 Lipid Metabolism and Development

1.1.1 Germination

Germination and post-germinative development constitute a critical stage in the life cycle of plants and are the times at which the basic architecture of the mature plant is established. Lipid and protein reserves are mobilised in the germinating seed to provide carbon and nitrogen for growth prior to the initiation of photosynthesis. As lipids do not migrate from cell to cell, oil stores are converted into soluble sugars, which are then translocated to the growing root and shoot.

The conversion of lipids to soluble sugars involves several consecutive pathways which occur in various cellular compartments:

(i) hydrolysis of triacylglycerols in lipid bodies (Section 1.3.1),

(ii) ß-oxidation in peroxisomes (Section 1.3.2),

(iii) glyoxylate cycle in glyoxysomes (Section 1.3.3),

(iv) partial tricarboxylic acid cycle in mitochondria (Section 1.3.4),

(v) gluconeogenesis in the cytosol (Section 1.3.5)

1.1.2 Photosynthetic Tissue

In photosynthetic tissue enzymes of ß-oxidation are down-regulated to a level at which they maintain the housekeeping function of membrane lipid turnover. Although reduced compared to levels found in post-germinative seedlings these levels are still signifficant as determined by gene transcript and enzyme activity analysis (Hooks *et al.*, 1996).

1.1.3 Flowering and Meristem Development

After establishment of roots, shoots stems and leaves, flowers develop which give rise to seeds thus completing the life cycle. Until recently ß-oxidation was not thought to play a role in flower development. However, Richmond and Bleecker (1999) reported that a ß-oxidation mutant showed abnormal inflorescence and floral development. The abnormal inflorescence meristem (aim1) mutant was subsequently identified by screening T-DNA tagged lines for flowering mutants. The aim1 phenotype is visible as an inability to change from an inflorescence meristem to a flowering meristem, resulting in an undifferentiated mass of cells. Cloning and characterisation of the *AIM1* gene revealed that it was homologous to an enoyl-CoA hydratase (see Section 1.3.2) and was expressed throughout the inflorescence meristem. Biochemical analysis of lipid catabolism showed that the *aim1* mutant had reduced catabolism of long and saturated fatty acids although germination did not appear to be disrupted. These findings led to the hypothesis that the disruption of ß-oxidation results in a build up of lipid metabolites, which disrupt intercellular communication in the reproductive meristem. However, as yet, there is no direct evidence for this.

1.1.4 Seed Development

During embryogenesis the root and shoot apical meristems are defined. Maturation events prepare the seed for germination and subsequent development of the mature plant (West and Harada, 1993). During the maturation process, the developing seed increases dramatically in volume and mass due to significant cell expansion and concomitant accumulation of storage proteins and lipids, which act as nitrogen and carbon sources during germination.

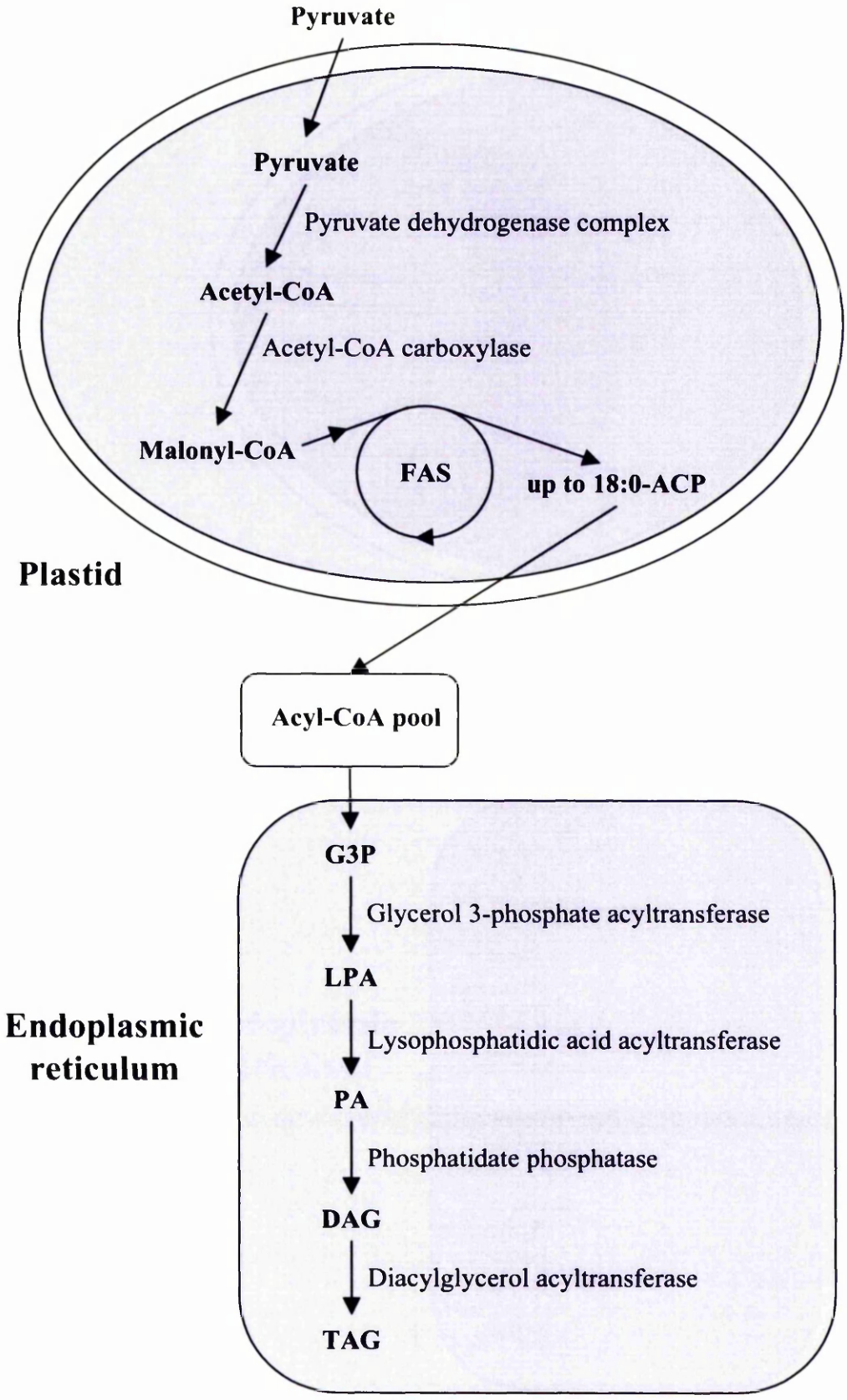
Fatty acid synthesis and subsequent triacylglycerol (TAG) synthesis occurs predominately during seed development in oil seed plants. At this stage the plant stores energy to be used by its progeny upon germination. Plant fatty acid synthesis occurs mainly in the plastid and in most species produces long chain fatty acids such as oleate (18:1) and palmitate (16:0) (Harwood, 1988; Slabas and Fawcett, 1992; Weaire and Kekwick, 1975).

The amount of TAG stored in the seeds of different species varies from as little as 1% to as much as 60% of the total dry weight. Arabidopsis seeds contain approximately 40% TAG which is stored in oil bodies. The pathway by which fatty acids are reported to be synthesised uses acetyl-CoA (2C) as building blocks. The source of the acetyl-CoA is still under debate, although recent research indicated that plastidial pyruvate dehydrogenase complex mRNA is upregulated during seed development. This suggests that during seed development the acetyl-CoA is derived from pyruvate present in the plastid (Ke *et al.*, 2000). The biochemical pathway which results in TAG biosynthesis is shown in Figure 1.1.

Figure 1.1 Triacylglycerol Biosynthesis Pathway

This figure shows a simplified overview of the pathways involved in the synthesis of the plant storage lipid triacylglycerol from pyruvate. Pyruvate is transported into the plastid where its conversion to acetyl-CoA is catalysed by the pyruvate dehydrogenase complex. Acetyl-CoA is in turn used to synthesise malonyl-CoA in a reaction mediated by acetyl-CoA carboxylase. Malonyl-CoA is converted via a malonyl-CoA-acyl carrier protein (ACP) intermediate (reaction catalysed by ketoacyl-ACP synthase 1) to fatty acid-ACP molecules up to 18C in length by the enzymes involved in the fatty acid synthesis (FAS) cycle (ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase and enoyl-ACP reductase).

Fatty acid-ACP molecules are converted to acyl-CoA molecules by acyl-CoA synthetase upon leaving the plastid. Acyl-CoA molecules are transported into the endoplasmic reticulum where they are attached to a glycerol 3-phosphate backbone, enter the Kennedy pathway and are converted in turn to lysophosphatidic acid, phosphatidic acid, diacylglycerol and triacylglycerol by the enzymes glycerol 3-phosphate, lysophosphatidic acid acyltransferase, phosphatidate phosphatase and diacylglycerol acyltransferase respectively.


Pyruvate
Pyruvate
Pyruvate dehydrogenase complex
Acetyl-CoA
Acetyl-CoA carboxylase
Malonyl-CoA
FAS
up to 18:0-ACP
Plastid
Acyl-CoA pool
G3P
Glycerol 3-phosphate acyltransferase
LPA
Lysophosphatidic acid acyltransferase
PA
Phosphatidate phosphatase
DAG
Diacylglycerol acyltransferase
TAG
Endoplasmic reticulum

Fatty acid synthesis commences with the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACCase). Malonyl CoA is then converted to malonyl-ACP by a transacylase. A cycle then starts whereby a malonyl-ACP is added to the fatty acid-ACP, with the loss of CO_2 until the required chain length is synthesised and the reaction is terminated by an acyl-ACP acyltransferase. The enzymes involved in the fatty acid synthesis (FAS) cycle are 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase and enoyl-ACP reductase (Ohlrogge and Browse, 1995).

The conversion of fatty acyl-CoA to TAG continues in the endoplasmic reticulum (ER). The mechanism by which the fatty acyl-CoA is transported to the ER is not well characterised. Glycerol 3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, phosphatidate phosphatase and diacylglycerol acyltransferase are all ER enzymes which constitute the Kennedy pathway. This pathway results in the acylation of glycerol 3-phosphate at the sn-1 and sn-2 positions to form diacylglycerol (DAG). This is the branch point between membrane lipid biosynthesis and storage lipid biosynthesis. The diacylglycerol acyltransferase catalyses the conversion of DAG into TAG which is then transported to oil bodies for storage until germination.

1.1.5 Senescence

Leaf senescence is the sequence of degradative processes leading to the remobilisation of nutrients and eventual leaf death. During senescence, nutrients such as nitrogen, phosphorous and metals which were invested in the leaf are reallocated to younger leaves

and growing seeds (Buchanan-Wollaston, 1997). The senescence process is highly regulated, involving photosynthetic decline, protein degradation, lipid peroxidation and chlorophyll degradation (Smart, 1994). Total RNA levels decline during senescence as RNase activity increases (Blank and McKeon, 1991). The nucleus, which is required for gene transcription, and the mitochondria, which are essential for providing energy, remain intact until the last stages of senescence (Smart, 1994).

While the initiation of leaf senescence depends upon the age of the leaf and the reproductive phase of the plant, external factors such as nutrient deficiency, pathogen attack, drought, light limitation and temperature can induce premature senescence (Smart, 1994). Using differential screening and subtractive hybridisation techniques many genes with increased expression during senescence have been identified (reviewed by Weaver *et al.*, 1997). Amongst the genes identified were RNases, proteases, glyoxylate cycle enzymes and pathogenesis-related proteins. Studies into the role of peroxisomes and H_2O_2 during senescence have shown that there is an enhancement of the activity of active oxygen-producing enzymes in leaf peroxisomes and that catalase activity is reduced (Pastori and del Rio, 1997). Ultrastructural studies of dark-induced senescent leaves showed that peroxisomes preserved their structure and their population in leaf cells was four times greater than in control leaves. The researchers concluded that the H_2O_2 produced by the peroxisomes mediates its effect by leaking out of the peroxisomes into the cytosol (Boveris *et al.*, 1972). The oxidative burst reported to play a role in senescence (Pastori and del Rio, 1997) parallels the role of the oxidative burst in pathogen attack. Interestingly, many defence-related genes have been shown to be induced during senescence even when plants are grown in sterile conditions (Quirino *et al.*, 1999). One explanation for this is based on

the observation that both leaf senescence and the hypersensitive response (HR), involving the oxidative burst, are forms of programmed cell death (PCD). However, leaf senescence occurs over a relatively long period of time with the HR being rapid and involving only a small region of cells around the infection (Mittler and Lam, 1996). This does not exclude the possibility that both processes share certain steps.

The connection between catabolism of membrane lipids and senescence has been demonstrated recently by Hong *et al.* (2000). The researchers isolated and characterised a lipase which is expressed at the beginning of senescence. Further northern analysis revealed that the gene could be induced in leaves treated with ethylene leading the researchers to conclude that this lipase plays a fundamentally central role in mediating the onset of senescence.

A current hypothesis for the mechanism of induction of senescence involves the rapid decline in photosynthesis. When photosynthetic activity falls below a certain threshold, senescence is induced (Bleecker and Paterson, 1997). Support for this hypothesis comes from observations with transgenic plants which over-express hexokinase. Hexokinase has been proposed to play an active role in sugar sensing in plants (Jang *et al.*, 1997). The fact that sugars are primary products in photosynthesis suggests that sugar levels are likely components of the signalling system. In these plants the rate of senescence has been reported to be accelerated, which implies that, as high sugar levels lead to a decrease in expression of photosynthetic genes, the reduction in photosynthetic gene expression somehow leads to accelerated senescence (Dai *et al.*, 1999). However, studies in tobacco plants showed that the glucose and fructose levels increase as leaves progress through senescence (Wingler *et al.*,

1998). This conflicting evidence indicates that further research is required to pinpoint the role of sugar levels in senescence.

1.2 Peroxisomes

1.2.1 Peroxisomal Biogenesis and Structure

Peroxisomes are functionally diverse organelles which are found in nearly all eukaryotic cells. In higher plants, peroxisomes contain enzymes involved in a variety of developmentally-influenced metabolic processes, including lipid mobilisation, photorespiration and nitrogen transport (reviewed by Olsen and Harada, 1995).

Peroxisomes were identified in plants by Frey-Wissling *et al.* (1963) and Mollenhauer *et al.* (1966). Peroxisomes are bound by a single membrane and range in size from 0.2-1.7 μm in diameter. As they lack organeller DNA, ribosomes and internal membrane systems, all peroxisomal proteins must be encoded by the nuclear genome and transported into the organelle (Douglass *et al.* 1973).

The defining characteristic of all peroxisomes is that they are respiratory organelles which contain H_2O_2-producing oxidases, such as acyl-CoA oxidase and catalase to inactivate H_2O_2 (deDuve and Baudhuin, 1966). Different classes of peroxisomes have been reported in plants depending on the tissue or the developmental stage. The four classes of peroxisomes found

in plants are glyoxysomes, leaf-type peroxisomes, root nodule peroxisomes and unspecialised peroxisomes.

Glyoxysomes are specialised peroxisomes found in tissues involved in lipid mobilisation. Glyoxysomes were so named because they contain the enzymes of the glyoxylate cycle, with the exception of aconitase which is found in the cytosol (Courtois-Verniquet and Douce, 1993; Hayashi et al., 1995). As well as supporting the glyoxylate cycle, the ß-oxidation enzymes are also present (Breidenbach *et al.*, 1967; Cooper and Beevers, 1969). Glyoxysomes are found in higher plants during specific developmental stages, such as germination and senescence, when storage lipids are converted to sucrose to be translocated to growing organs in the plant.

Leaf-type peroxisomes are most commonly found in photosynthetically active tissues and can be found associated with both mitochondria and chloroplasts (Frederick and Newcomb, 1969). These peroxisomes are involved in the oxygen and light-dependent evolution of carbon dioxide (CO_2) known as photorespiration. The photorespiratory enzymes which characterise this class of peroxisome are glycolate oxidase, serine-glyoxylate aminotransferase and hydroxypyruvate reductase.

Root nodule peroxisomes have been shown to play a role in nitrogen transport. The peroxisomal enzyme urate oxidase is involved in the final steps of converting the nitrogen in urate (fixed by Rhizobium) to allantoin which can be readily transported to the areas of the plant where it is required for growth (Schulbert, 1986).

Unspecialised peroxisomes contain the marker enzymes catalase and H_2O_2-producing oxidases but are typically smaller and less dense than other peroxisomes. They are found in greatest abundance in tissues which are neither photosynthetically active nor involved in lipid catabolism. Based on the nature of the enzymes found in unspecialised peroxisomes it has been hypothesised that they are involved in protecting cells from oxygen toxicity (Huang *et al.* 1983).

Peroxisome biogenesis is thought to occur through the growth and division of existing peroxisomes (Borst, 1989; Lazarow and Fujiki, 1985). Evidence suggests that organellar growth is mediated by the addition of membrane lipids and post-translational import of proteins (Osumi and Fujiki, 1990). The observation that in cotyledons undergoing the transition from heterotrophic to autotrophic growth, peroxisomes have been found which still contain glyoxylate cycle enzymes but already contain photorespiratory enzymes provides additional evidence for this method of biogenesis (Nishimura *et al.*, 1986). This suggests that individual peroxisomes change class according to developmental stage, each class of peroxisome does not have its own biogenesis pathway and that they differ from each other only in the proteins imported, which is ultimately controlled at the nucleus.

1.2.2 Peroxisomal Membranes and Protein Transport

Proteins destined for peroxisomes are encoded by the nuclear genome and translated by free ribosomes before transportation into the organelles (Subramani, 1993; Borst, 1986). Two protein targeting signals have been identified which are both necessary and sufficient to

direct the peroxisomal matrix proteins from the cytosol into the peroxisome. Peroxisomal Targeting Signal (PTS) 1 is a C-terminal tripeptide with a consensus sequence S-K-L (Ser-Lys-Leu). Conservative substitutions in the tripeptide sequence of PTS1 have been documented and these have shown a high degree of species specificity (Van der Bosch *et al.*, 1992; Purdue and Lazarow, 1994). However the consensus sequence always consists of a small uncharged amino acid in the first position, a positively charged amino acid in the second and a hydrophobic amino acid in the third position. This import signal has been shown to be conserved throughout the eukaryotic kingdom (Gould *et al.*, 1990; Keller *et al.*, 1991).

One other, less common, targeting signal has been identified. PTS2 has a consensus sequence, R L/I-X_5-H/Q L (DeHoop and Ab, 1993). In higher eukaryotes such as plants and mammals the PTS2 is found in a cleavable N-terminal presequence, whereas in lower eukaryotes such as yeast, it is located in the amino-terminus of the mature protein. Some peroxisomal proteins contain neither PTS1 nor PTS2. A probable internal signal has been suggested as the mechanism used by yeast catalase A (Kragler *et al.*, 1993).

The peroxisomal membrane mediates the transport of metabolites and the import of proteins that maintain or modify the identity and function of the organelle. Due to difficulties found in the isolation of pure peroxisomal membranes the identification of peroxisomal membrane proteins has been achieved using yeast mutants defective in peroxisomal function. Nineteen genes have been identified to date (Waterham *et al.*, 1996; Tan *et al.*, 1995; Eitzen *et al.*, 1995; Elgersma *et al.*, 1996 and Elgersma *et al.*, 1997).

The mechanism of peroxisomal membrane import differs greatly from mitochondria and chloroplast in that peroxisomes can translocate oligomeric proteins across their membranes, (Walton *et al.*, 1995; Lee *et al.*, 1997). It has been postulated that pores in the membrane facilitate this transport although there is no direct evidence for this. The pores are thought to be large because 9nm gold particles coated in the S-K-L motif were successfully targeted into peroxisomes (Walton *et al.*, 1995).

With the existence of these two specific targeting signals it was logical to look for the receptors to which they would bind. Genetic studies in yeast and human mutant cells selectively deficient in the PTS1 or PTS2 import pathways have been instrumental in the identification of the PTS receptors. The PTS1 receptor, Pex5p and the PTS2 receptor, Pex7p, have been identified in yeast and humans (Distel *et al.*, 1996 and Subramani, 1997, respectively).

In watermelon the PTS1 receptor has been identified. The protein is found primarily in the cytosol but also in the peroxisomal matrix (Wimmer *et al.*, 1998). This suggests that the receptor binds to a PTS1 containing protein in the cytosol and they are co-transported across the organeller matrix, where they dissociate (Van der Klei and Veenhuis, 1996).

Chaperone involvement in protein transport across many cellular membranes has been documented. Chaperones such as Hsp70 and Hsp90 control protein folding and have been implicated in protein transport into mitochondria, plastids, nuclei and the ER (Miernyk, 1997; Hartl, 1996 ; Boston *et al.*, 1996). It has recently been suggested that chaperones may play a role in peroxisomal protein transport as an Hsp40 and an Hsp70 have been localised

to the glyoxysomal membrane (Preisig-Müller *et al.*, 1994; Corpas and Trelease, 1997) and an Hsp70 has been identified in the glyoxysomal matrix (Wimmer *et al.*, 1997). Evidence supporting this hypothesis comes from research using an established *in vitro* protein import assay (Brickner and Olsen, 1998). The researchers demonstrated that the efficiency of isocitrate lyase import into peroxisomes appeared to be influenced both by chaperones and by the folding state of the protein. Heat shock proteins were shown to improve the efficiency of import by maintaining the targeted protein as a monomer, the preferred substrate for import (Crookes and Olsen, 1998).

1.3 Lipid Catabolism

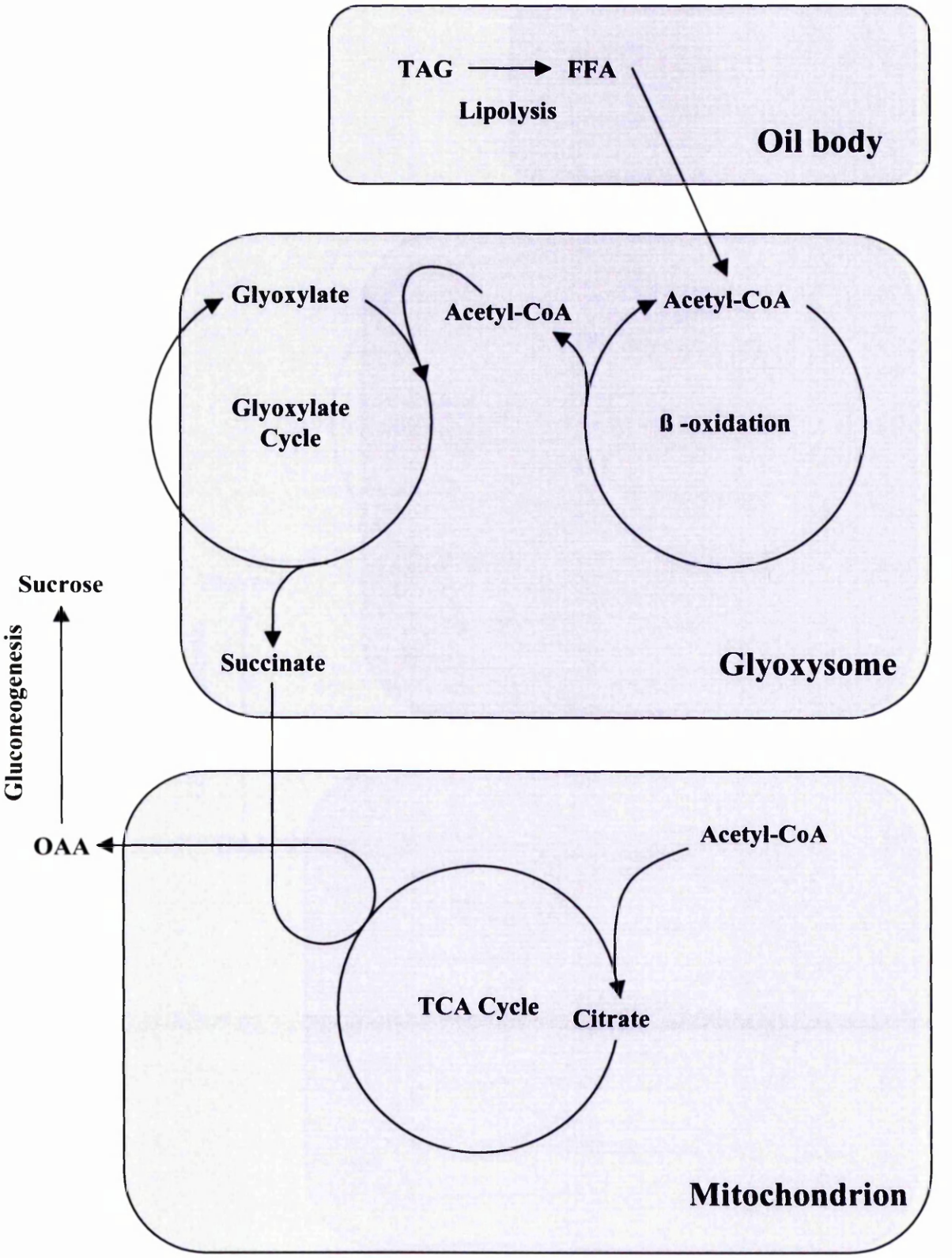
1.3.1 Lipases and Transport to the Glyoxysome

Studies have shown that germination of oil seeds occurs after seeds have been imbibed when other environmental conditions of light and water are suitable (Kornberg and Madsen, 1957; Canvin and Beevers, 1961). The complete pathway of lipid catabolism is shown in Figure 1.2. Huang (1987) showed that the first stage of lipid mobilisation is the hydrolysis of the fatty acids from the glycerol backbone of the triacylglycerols (TAGs) stored in the oil bodies. Lipases have been shown to catalyse this reaction. These enzymes appear to be absent in dormant seeds but expression studies have shown them to be rapidly synthesised at the onset of germination. The mechanism by which the lipases come into contact with the TAGs has not yet been established.

Figure 1.2 Triacylglycerol Catabolism

This figure represents an overview of the pathway of TAG mobilisation during germination resulting in the production of sucrose, which can be easily transported throughout the developing seedling.

TAG molecules are converted to free fatty acids (FFAs) by lipases in oil bodies. The mechanism by which FFAs are transported into the glyoxysome where they are converted to acyl-CoA synthetase is not yet characterised. Each cycle of ß-oxidation yields an acetyl-CoA molecule while shortening the acyl-CoA chain of the substrate by 2 carbons. Acetyl-CoA is then converted to succinate by the glyoxylate cycle. Succinate is transported to the mitochondia where enzymes involved in the tricarboxylic acid (TCA) cycle convert it to oxaloacetic acid (OAA). OAA is the substrate for gluconeogenesis in the cytoplasm which produces sucrose.


TAG
FFA
Lipolysis
Oil body
Glyoxylate
Acetyl-CoA
Acetyl-CoA
Glyoxylate Cycle
ß -oxidation
Sucrose
Gluconeogenesis
Succinate
Glyoxysome
OAA
Acetyl-CoA
TCA Cycle
Citrate
Mitochondrion

Transport of the liberated fatty acids from oil bodies to the glyoxysome, where they are further catabolised, is not well characterised at present. Fatty acids must be linked to a coenzyme-A group by a thioester bond before ß-oxidation can occur. The enzyme which catalyses this reaction is acyl-CoA synthetase. Acyl-CoA synthetase has been shown to be associated with the membrane in peroxisomes. Evidence supporting this hypothesis comes from peroxisomal biogenesis disorders in humans. Patients with X-linked adrenoleukodystrophie (ALD), have a deficiency in peroxisomal very long chain fatty acid acyl-CoA synthetase (Lazo *et al.*, 1988). However the ALD gene encodes a peroxisomal membrane protein and not a synthetase. This transmembrane protein is a member of a family of translocators involved in transport of molecules ranging from ions to large proteins (Higgens, 1992). This suggests that the ALD protein is required for the import or stability of the synthetase.

1.3.2 ß-Oxidation

In 1969, using techniques to separate organelles from castor bean endosperm (*Ricinus communis* L.) on sucrose density gradients, Cooper and Beevers were able to demonstrate that the enzymes which catalyse ß-oxidation are present in peroxisomes (Cooper and Beavers, 1969). Later research by Gerhardt (1983) and Macey and Stumpf (1982) found evidence for ß-oxidation in peroxisomes in photosynthetic tissue, roots and other tissues devoid of storage lipids. The presence of ß-oxidation enzymes in these tissues has led to the hypothesis that their role is one of housekeeping, in the form of membrane lipid turnover.

The ß-oxidation pathway consists of four enzymatic steps which result in production of an acetyl-CoA molecule at the end of each cycle. The pathway is shown in Figure 1.3. The first step of ß-oxidation is catalysed by acyl-CoA oxidase which converts acyl-CoA to trans-2-enoyl-CoA. The reaction is oxygen dependant and releases two electrons which are transferred to an oxygen molecule, via an associated FAD (Flavin Adenine Dinucleotide) group, producing H_2O_2.

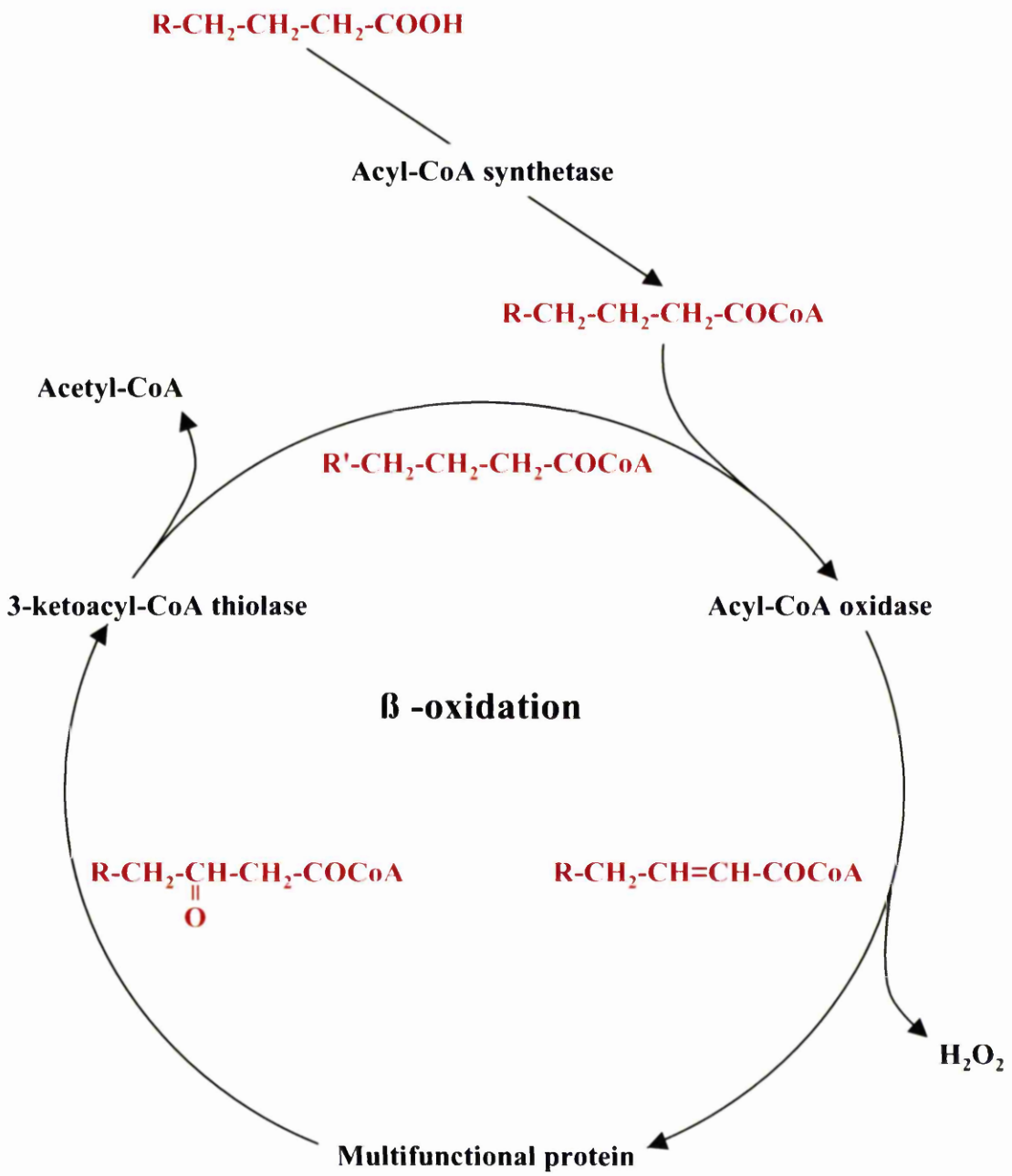
The Arabidopsis acyl-CoA oxidase (ACX1) has been shown to be a homodimer (Hooks *et al.* 1999a), as is the case in rats and yeast (Osumi *et al.* 1980 and Coudron *et al.* 1983 respectively). Hooks *et al.* (1999a) identified two long chain specific acyl-CoA oxidases, ACX1 and ACX2. The researchers identified full length cDNA clones which were then expressed in *E. coli* under the control of the ß-galactosidase promoter. From these experiments, the specific range of fatty acid chain lengths with which the enzymes were active were identified. ACX1 was shown to have a broader substrate range than ACX2 with maximum activity being found with the substrate (14:0-CoA). ACX2 had a narrower substrate range and showed the highest activity with (18:0-CoA).

Anti-sense plants expressing either anti-ACX1 or anti-ACX2 RNA failed to show any phenotypical abnormalities during germination (Hooks *et al.*, 1999a). The ACX1 anti-sense plants did, however, show reduced root growth during post-germinative growth. These observations led the researchers to conclude that loss of either ACX1 or ACX2 during germination had no effect on growth and development as they compensated for each other. Northern analysis confirmed that both *ACX1* and *ACX2* transcript levels increase during germination in wild-type plants (Hooks *et al.*, 1999a).

Figure 1.3 The ß-oxidation Pathway

This figure represents the ß-oxidation pathway which results in the release of 1 acetyl-CoA molecule per cycle and the shortening of the acyl-CoA chain by 2 carbons.

Free fatty acids are converted to acyl-CoA molecules by acyl-CoA synthetase upon entry to the glyoxysome. This activation of the fatty acid allows it to enter the ß-oxidation pathway where acyl-CoA oxidase converts it to enoyl-CoA and H_2O_2. Multifunctional protein then converts the enoyl-CoA to 3-ketoacyl-CoA. The final step in the pathway is catalysed by 3-ketoacyl-CoA thiolase which produces acetyl-CoA and an acyl-CoA 2 carbons shorter than at the start of the cyle. This progressive cycle results in complete degradation of the fatty acid.


R-CH2-CH2-CH2-COOH
Acyl-CoA synthetase
R-CH2-CH2-CH2-COCoA
Acetyl-CoA
R'-CH2-CH2-CH2-COCoA
3-ketoacyl-CoA thiolase
Acyl-CoA oxidase
ß -oxidation
R-CH2-CH-CH2-COCoA
O
R-CH2-CH=CH-COCoA
H2O2
Multifunctional protein

A short chain-specific acyl-CoA oxidase was identified in Arabidopsis by Hayashi *et al.* (1999). ACX4 was shown to be active on butyric acid (4:0-CoA), have maximum activity with hexanoic acid (6:0-CoA) and have decreased activity with octanoic acid (8:0-CoA). A peroxisomal targeting signal (PTS1) was identified in the cDNA sequence of the *ACX4* gene. Hayashi *et al* concluded that ACX4 is active as a homotetramer, a novel observation among acyl-CoA oxidases. ACX4 was further characterised by northern analysis and an assay for acyl-CoA dehydrogenase (mitochondria-specific) activity. These experiments confirmed that ACX4 is expressed during germination and, although it has sequence homologies to the dehydrogenase, it showed no activity. Taken together these observations suggest that *ACX1*, *ACX2* and *ACX4* are part of an acyl-CoA oxidase gene family in Arabidopsis.

Another likely member of the family was reported recently by Froman *et al.* (2000). It is a medium chain acyl-CoA oxidase designated ACX3 which was shown to have maximal activity with myristic acid (14:0-CoA). ACX3 contains a peroxisomal targeting signal (PTS2) and northern analysis showed an induction pattern very similar to other ß-oxidation genes. The characterisation of ACX3 is complemented by Eastmond *et al.*, (2000b). These researchers used promoter trapping experiments, using the ß-glucuronidase (GUS) reporter gene to isolate the same *ACX3* gene from Arabidopsis. *In situ* assays for GUS activity identified constitutive expression in roots and strong expression in cotyledons and seedling hypocotyls. Eastmond *et al.* (2000b) also identified an *acx3* mutant using a reverse genetic approach. Analysis revealed that, even though there was a 95% reduction in enzyme activity from wild-type, seedling development appeared normal. From these observations it was concluded that, due to residual medium chain activity arising as a result of overlapping chain

length specificities of the gene family members, a complete block of ß-oxidation at this step would require more than one of the ACX family members to be knocked out.

With their overlapping specificities, the four acyl-CoA oxidases identified to date in Arabidopsis are sufficient to catalyse the complete degradation of fatty acids in peroxisomes. Indeed, Kleiter and Gerhardt (1998) demonstrated, using ^{14}C-C1 labelled fatty acids and measuring ^{14}C-acetyl-CoA production, complete catabolism of fatty acids in peroxisomes. This landmark observation revealed an important difference in plant and animal fatty acid catabolism, with no requirement for mitochondrial ß-oxidation in Arabidopsis, as has been previously observed in mammalian systems (see Section 1.3.5).

The next stage in peroxisomal ß-oxidation is catalysed by multifunctional protein, which catalyses the conversion of 2-trans-enoyl CoA to L-3-hydroxyacyl-CoA and then to 3-oxoacyl-CoA with the reduction of NAD (Nicotinamide Adenine Dinucleotide). A multifunctional protein isolated from cucumber has been shown to have 2-trans-enoyl CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, D-3-hydroxyacyl-CoA epimerase and Δ^3,Δ^2-enoyl-CoA isomerase activities in distinct domains (Preisig-Müller *et al.*, 1994). In Arabidopsis two multifunctional proteins have been identified, MFP2 (Eastmond and Graham, 2000a) and MFP-a (AIM1), a mutant of which was disrupted in inflorescence meristem development (Richmond and Bleecker, 1999). Northern analysis has shown that MFP2 has the same induction pattern during germination as the other ß-oxidation genes leading to the hypothesis that there is a common signalling pathway which induces these genes (Eastmond and Graham, 2000a).

The final enzyme involved in the ß-oxidation cycle is 3-ketacyl-CoA thiolase, which converts 3-oxoacyl-CoA to fatty acyl-CoA and acetyl-CoA. Expression studies on thiolase genes isolated from cucumber (Kato *et al.*, 1996) and pumpkin (Preisig-Müller and Kindl, 1993) have shown induction during germination. An Arabidopsis thiolase gene has recently been described in a study of mutants which have defects in glyoxysomal fatty acid ß-oxidation (Hayashi *et al.*, 1998). In this study levels of thiolase protein were shown to rise transiently during germination. Analysis of this thiolase mutant, termed *ped1* (Peroxisome Deficient) demonstrated that lipid breakdown is essential for post-germinative seedling growth and this growth defect could be rescued by exogenous sucrose (Hayashi *et al.*, 1998).

1.3.3 Glyoxylate Cycle

The glyoxylate cycle was first described in *Pseudomonas* (Kornberg and Madsen, 1957) where its presence was linked to the organisms ability to use acetate as its sole carbon source. The cycle is an anapleurotic pathway which serves to convert C2 compounds to the C4 acids of the tricarboxylic acid cycle. During rapid lipid mobilisation the acetyl-CoA from ß-oxidation is converted to succinate, which is then used as a substrate for carbohydrate synthesis (Huang *et al.*, 1983). Two enzymes are unique to the glyoxylate cycle, namely malate synthase (MS) and isocitrate lyase (ICL). MS and ICL were found to be co-ordinately synthesised in cucumber cotyledons (Weir *et al.*, 1980). These two enzymes are used as markers for the activity of the glyoxylate cycle and have been reported to be present during pollen development (Zhang *et al.*, 1994), embryogenesis (Turley and Trelease, 1990) and during natural senescence (Graham *et al.*, 1992). Studies in starved cucumber cell cultures

showed that ICL and MS were inducible outwith developmental regulation (Graham *et al.*, 1994). This reported response of the plant to its metabolic status has also been reported to effect photosynthetic gene expression (Sheen, 1990).

Further insight into the role of the glyoxylate cycle during postgerminative growth comes from studies on 2 Arabidopsis mutants of ICL (Eastmond *et al.*, 2001). Germination of these mutants (icl-1 and icl-2) was not affected, leading to the conclusion that the glyoxylate cycle and lipid mobilisation are not essential for germination in Arabidopsis. However, it was reported that the postgerminative growth of these mutants was retarded. The postgerminative growth of the *icl-1* and *icl-2* seedlings was found to be no different from wild type when there was either an alternative carbon source or adequate light source present. It was therefore concluded that the glyoxylate cyle, unlike ß-oxidation, is not essential for seedling establishment in Arabidopsis (Eastmond *et al.*, 2001).

1.3.4 Tricarboxylic Acid Cycle and Gluconeogenesis

The succinate produced by the glyoxylate cycle is transported to the closely associated mitochondria where it is metabolised by a partial tricarboxylic acid cycle (TCA) to produce oxaloacetic acid (OAA). The OAA is then transported to the cytosol where the enzymes of gluconeogenesis convert it to sucrose to be transported to the rapidly growing areas of the plant. As the complete TCA cycle catalyses two decarboxylative reactions, the conversion of lipid to succinate can only occur if certain TCA cycle reactions are by-passed. During gluconeogenesis, only the TCA cycle activities of succinate dehydrogenase, fumarase and

malate dehydrogenase are required. The proposed TCA cycle bypass was first demonstrated by *in vivo* labelling studies in castor bean endosperm (Canvin and Beevers, 1961), when radiolabelled acetate was shown to be converted to carbohydrate. Later work done with germinating castor bean, showed that mitochondria rapidly oxidise succinate, malate and glutamate, whereas the other intermediates in the decarboxylative portion of the TCA cycle were shown to be slowly oxidised (Millhouse *et al.*, 1983). Recent studies into transcriptional and post-transcriptional control of gene expression of the TCA enzymes, have confirmed that during lipid mobilisation in oil seeds, the decarboxylative enzymes of the TCA cycle are strongly repressed (Falk *et al.*, 1998).

1.3.5 Mitochondrial ß-Oxidation

The existence in higher plants of an additional ß-oxidation system in mitochondria, in conjunction with the well characterised peroxisomal system, is often considered controversial. It is well established that the large amount of lipid mobilisation observed during germination of oil seeds proceeds through glyoxysomal ß-oxidation (Cooper and Beevers, 1969). Although certain ß-oxidation genes have been reported to be present in mitochondria (Miernyk *et al.*, 1991), it was not until recently that the mitochondria specific enzyme, acyl-CoA dehydrogenase (ACAD), was reported to be present and active in maize and sunflower (Bode *et al.*, 1999). ACAD catalyses the same reaction as acyl-CoA oxidase in peroxisomes. Bode and co-workers isolated mitochondria from embryonic axes of sunflower and then partially purified and characterised ACAD activity. It was concluded

from these results that mitochondrial ß-oxidation does occur although the physiological significance of this is still unknown.

1.4 Transgenic Oil Crops

1.4.1 Advances in Controlling Lipid Synthesis

One of the main objectives of modern plant biotechnology is to genetically engineer oil seeds to synthesise and store unusual fatty acid which are of agronomic importance. Initial attempts at producing transgenic crop plants to synthesise foreign fatty acids met with limited success.

An important breakthrough and one of the few early successes, was with the tissue-specific medium chain thioesterase (MCTE) from *Umbellularia californica.* The gene encoding this enzyme was expressed in *Brassica napus* under the control of the constitutive cauliflower mosaic virus 35S promoter. Previous studies had shown that this enzyme leads to the premature termination of fatty acid synthesis and the accumulation of medium chain (C_{10}-C_{12}) TAGs in the seeds (Voelker *et al.*, 1992). In the transgenic seeds, oil composition was dramatically altered from virtually undetectable levels of C_{12} lauric acid to 30-40% of total seed oil. Lauric acid is of economic value because of its wide spread use in soaps, detergents and surfactants, due to its excellent solubility properties.

An interesting aspect of this work was that, although the *MCTE* gene was expressed constitutively, no lauric acid was found in the leaves or roots. It was important to confirm that MCTE, which is normally only expressed in developing seed tissues, maintained activity after being incorporated into leaf plastids. To provide this confirmation, Eccleston *et al.* (1996) isolated leaf plastids and demonstrated that lauric acid was indeed synthesised in these organelles. The most likely explanation for this is that ß–oxidation is induced in order to degrade the foreign fatty acid which may interfere with the balance of phospholipids in the cellular membranes. This hypothesis is supported by an observed increase in isocitrate lyase activity (Eccleston *et al.*, 1996). Further evidence comes from work done with *E. coli* (Voelker and Davies, 1994). When wildtype *E. coli* strains are transformed with the *MCTE* gene, no lauric acid can be detected. If, however, ß-oxidation mutants are transformed with *MCTE* gene, large quantities of lauric acid accumulate in the medium in the form of free fatty acids. Interestingly, lauric acid was not detected in the membrane glycerolipids and it was therefore hypothesised that ß-oxidation is involved in the catabolism of foreign fatty acids.

1.4.2 Involvement of Lipid Catabolism

The *E. coli* experiments by Voelker and Davies were the first to provide evidence for the role of ß-oxidation in the futile cycling of foreign fatty acids. Recent research by Eccleston and Ohlrogge (1998), indicated that the medium chain-specific acyl-CoA oxidase was induced in the transgenic MCTE plants. Their experiments showed that in plants strongly expressing MCTE, correspondingly high levels of a medium chain-specific acyl-CoA

oxidase were also induced. However, no induction of long chain-specific acyl-CoA oxidase activity could be detected in these transgenic plants. Interestingly, in the developing seeds of these transgenic plants both ß-oxidation and fatty acid biosynthesis pathways were found to be co-ordinately induced. This can be explained by the observation that lauric acid was found on the sn-2 position of only 5% of TAGs (Voelker *et al.*, 1996). Therefore in those transformants expressing very high levels of MCTE there may be an excess production of lauric acid which cannot be incorporated into TAG. These high levels are hypothesised to induce ß-oxidation for lauric acid catabolism. As lauric acid is rarely found in the sn-2 position replacement fatty acids must be synthesised as the lauric acid is being catabolised. This observation also infers a very close link in the control of both fatty acid synthesis and degradation, as has been reported in yeast (Chirala *et al.*, 1992; Schuller *et al.*, 1992).
The researchers then looked for isocitrate lyase (ICL) and malate synthase (MS) activity as indicators of glyoxylate cycle induction. As with the ß-oxidation enzymes, an induction of the glyoxylate cycle enzymes was also observed during seed development. This increased activity in MS and ICL strongly suggests that a high production of laurate can stimulate peroxisome/glyoxysome interconversion in *Brassica napus*. The presence of the medium chain specific acyl-CoA oxidase only, indicated a different signalling pathway from the other acyl-CoA oxidases, specifically in reponse to the abundance of lauric acid.

In contrast to the conclusions of Eccleston and Ohlrogge (1998), expression of the MCTE gene in Arabidopsis led to no significant induction of ß-oxidation even in high laurate producers (Hooks *et al.*, 1999b). This observation led Hooks and co-workers to conclude that endogenous basal levels of ß-oxidation enzymes were sufficient to cope with the increased flux of fatty acids through the ß-oxidation pathway in the transgenic leaf tissue.

1.4.3 Characterisation of Fatty Acid Flux through ß-Oxidation

Further insight into the role of ß-oxidation in plants expressing genes to synthesise unusual fatty acids has come from studies into the biosynthesis of biodegradable thermoplastics and elastomers. Polyhydroxyalkanoates (PHAs) are polyesters of hydroxy acids naturally synthesised by a wide range of bacteria (Poirier *et al.*, 1995). They have plastic and elastomeric properties as well as being biodegradable, making them an interesting source of renewable and environmentally friendly polymers. Poirier and co-workers demonstrated that expression of bacterial PHA synthase in the peroxisomes of transgenic Arabidopsis leads to the accumulation of PHA inclusions inside the organelle (Mittendorf *et al.*, 1998a, 1998b). In this system, PHA was shown to be synthesised from the polymerisation of the 3-hydroxyacyl-CoA intermediates of ß-oxidation of fatty acids. Synthesis of PHA in peroxisomes appeared to parallel the activity of the ß-oxidation cycle, being high during germination and senescence and low during photosynthetic growth (Mittendorf *et al.*, 1998b).

Considering these results, the researchers investigated the range of fatty acids which could be degraded by ß-oxidation and analysed whether the intermediates could be incorporated into PHAs. Their results showed that changes in plant PHA monomers are directly linked to the fatty acids being catabolised and that feeding experiments with the unusual fatty acid, lauric acid (12:0), produced a larger induction of ß-oxidation and a higher rate of PHA biosynthesis (Mittendorf *et al.*, 1999). An interesting aspect of such feeding experiments was

that these transgenic plants which synthesise PHAs provide an ideal system for indicating the flow of fatty acids through ß-oxidation.

More recent analysis has shown that these plants are indeed very good indicators of ß-oxidation substrates (Poirier *et al.*, 1999). Changes were analysed in the quantity of PHA synthesised in developing seeds and the researchers concluded that plants can respond to the inadequate incorporation of fatty acids into TAG by recycling fatty acids via ß-oxidation. In addition it was suggested that a considerable flow towards ß-oxidation can occur even in a plant tissue primarily devoted to the accumulation of storage lipids. These results support the hypothesis that ß-oxidation is involved in the futile cycling of unusual fatty acids even though induction of ß-oxidation genes is not always apparent.

1.5 The Role of Acyl-CoA Oxidase in Plant Defence

1.5.1 Production of H_2O_2 and the Oxidative Burst

The understanding of plant responses to pathogen attack, be it chemical or physical damage, has advanced rapidly in recent years. Rapid recognition of a potential invader is a prerequisite for the initiation of an efficient defence response. One of the best characterised of these is the oxidative burst (reviewed by Lamb and Dixon, 1997). The oxidative burst describes the rapid production of active oxygen species (H_2O_2, O_2^- and OH^-) which has been described on numerous occasions in response to pathogens and pathogen elicitors (Doke, 1983; Mehdy, 1994; Dixon et al., 1994; Baker and Orlandi, 1995,). These chemicals have

been reported to precede increases in glutathione and ethylene levels, lipoxygenase induction and cell death.

Several roles for active oxygen species during infection have been proposed: as direct anti-microbial agents (Doke *et al.*, 1996), as activators of defence genes (Doke *et al.*, 1996; Low and Merida, 1996), as agents for cross-linking proteins (Brisson *et al.*, 1994) and as inducers of cell death, systemic acquired resistance and salicylic acid production (Doke *et al.*, 1996; Low and Merida, 1996). Maintaining of high levels of reactive oxygen species or production of a second burst hours after the initial attack has been suggested as the switch between a defence response and programmed cell death (Lamb and Dixon, 1997).

1.5.2 Systemin and the Oxidative Burst

Systemin is an 18-amino acid polypeptide which was originally identified as a serine proteinase inhibitor in potato and tomato (Green and Ryan, 1972). The purified peptide was isolated and shown to induce 15 wound and defence-related genes in tomato (Ryan, 1992). The small size of systemin allows its transport between cells possible, leading it to be considered the primary systemic signal upon wounding (Ryan and Pearce, 1998).

The signalling pathway for activation of defence genes by systemin is thought to be mediated by lipid-derived intermediates (Farmer and Ryan, 1992). Systemin activates an intracellular cascade that results in the release of linolenic acid (LA) from the membranes,

with the LA subsequently converted to jasmonic acid (JA) (Vick and Zimmerman, 1984), a powerful inducer of defence genes in plants (Weiler, 1997). The pathway from LA to JA is called the octadecanoid pathway which appears to be a general signalling pathway for many plant processes involved in defence, stress and development (Weiler, 1997). The JA biosynthetic pathhway is shown in Figure 1.4. Evidence linking systemin-induced defence genes to the octadecanoid pathway comes from analysis of a mutant tomato line, deficient in the pathway. This mutant expressed only low levels of defence genes when wounded and was unresponsive to systemin (Howe *et al.*, 1996).

An interesting connection between the oxidative burst and systemin was demonstrated in tomato cell suspension cultures. Addition of a cell wall degradation product, oligogalacturonic acid (OGA), caused an oxidative burst (Legendre *et al.*, 1993).

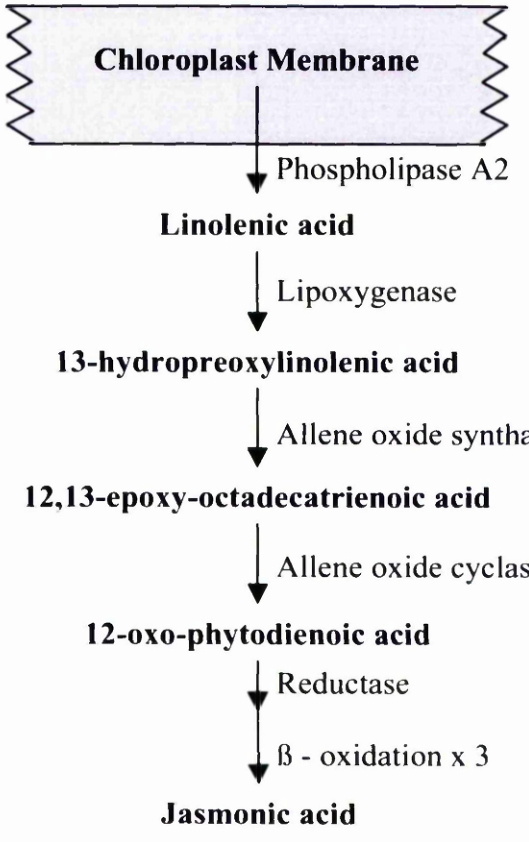
Addition of systemin alone did not cause an oxidative burst (Stennis *et al.*, 1998). However, within 9 hours of the addition of systemin to the cultures, the generation of reactive oxygen species in response to OGA was increased 16-fold over the response in the absence of systemin (Stennis *et al.*, 1998). These observations indicate a mechanism by which the plant can respond more strongly to persistent pathogen attack.

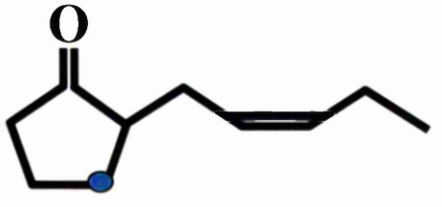
Figure 1.4 The Jasmonic Acid Biosynthesis Pathway

This figure represents an overview of the pathway by which membrane lipids are converted to the fatty acid signalling molecule, JA. Particular attention is paid to the final steps which are thought to be cataysed by the enzymes of ß-oxidation.

The release of linolenic acid from the cellular membranes (particularly chloroplast membranes) is catalysed by phospholipase A2. Linolenic acid is converted to the cyclopentane, 12-oxo-phytodiencoic acid, by the enzymes lipoxygenase, allene oxide synthase and allene oxide cyclase.

Three cycles of ß -oxidation are shown in detail at the bottom of the figure. They have been postulated to convert the cyclopentane to JA by removing 2 carbons from the fatty acid chain attached to the oxopentenyl ring. The blue circle represents the same carbon atom of the cyclopentane. The fatty acid chain is attached to the ring at this position and remains so throughout the ß -oxidation steps.





Cyclopentanes

Oxo(pentenyl)cyclopentane 8 (OPC: 8)

Oxo(pentenyl)cyclopentane 6 (OPC: 6)

Oxo(pentenyl)cyclopentane 4 (OPC: 4)

Jasmonic acid

1.5.3 Jasmonic Acid Biosynthesis and ß-Oxidation

Many organisms use fatty acid derivatives as biological regulators, controlling processes as diverse as reproduction, social behaviour, sleep, metabolism and defence. In plants, fatty acid signals play a crucial role in defence and development, a well characterised member of which is JA. JA is a 12 carbon fatty acid cyclopentanone which has been shown to be essential for completion of the plant's life cycle. Mutant plants which either do not produce or cannot detect JA do not generate viable pollen (Feys *et al.*, 1994), or release viable pollen too late for pollination to occur (Sanders *et al.* 2000) and are susceptible to herbivory (McConn *et al.*, 1997).

JA has been shown to be present during germination, 12 hours after imbibition, at levels which are 5-fold greater than that found in dormant soybean seeds (Creelman and Mullet, 1995). This increase was correlated with lipid mobilisation and was thought to be a consequence rather than a trigger of germination. These higher levels of JA were reported to decline with further seedling development.

The biosynthesis of JA has been reported to occur through the octadecanoid (Figure 1.4) and hexadecanoid pathways. The first step in the biosynthetic pathway is the release of LA from phospholipid membranes. Plant membranes, especially chloroplast membranes, are a rich source of LA esterified in glycerolipids and phospholipids. This led to the hypothesis that JA could result from the activation of phospholipases that release LA from the membranes (Farmer and Ryan, 1992). A plant phospholipase D, which cleaves the head group from the

phospholipids, has been identified and proposed to have a role in plant defence (Wang, 1993). Lipoxygenases (LOX) catalyse the conversion of LA to 13-hydroperoxylinolenic acid and studies in transgenic plants with reduced LOX activity have shown a reduced ability to synthesise JA (Bell *et al.*, 1995). JA arises from 13-hydroperoxylinolenic acid via an allene oxide synthase and an allene oxide cyclase with the final stages of the pathway proposed to be completed by the enzymes of ß-oxidation (Creelman and Mullet, 1997).

Sanders and co-workers (2000) have recently reported that another enzyme involved in JA biosynthesis, 12-oxophytodienoate reductase results in delayed dehiscence when knocked-out. The researchers found that these male sterile plants could have their fertility restored by exogenous application of JA. These experiments confirm the important role that JA plays in the ordinary development of a plant as well as in plants under stress.

1.5.4 Stress Conditions and Cytosolic Acidification

It has been documented that cytosolic acidification is a common early intracellular response of tobacco cells to fungal elicitors (Mathieu *et al.*, 1996). A pH change has also been observed in animal cells in response to growth-factor stimulation (Isofort *et al.*, 1993). This acidification resulted in biochemical changes in the cells including protein synthesis and activation by protein phosphorylation.

Preliminary experiments from Mathieu and co-workers used ^{31}P NMR to show that addition of elicitors to tobacco cell culture resulted in cytosolic acidification (Mathieu *et al.*, 1994).

Further analysis showed that use of different elicitors all resulted in cytosolic acidification followed by induction of plant defence genes e.g. phenylalanine ammonia lyase (PAL) (Mathieu *et al.*, 1996).

Acid treatments of detached Arabidopsis leaves were shown to result in a lowering of cytosolic pH. Induction of *ACX1* was also found in these leaves suggesting that the protons are involved in the signalling pathway inducing transcription from the *ACX1* promoter. (Hooks *et al.*, 1998).

1.5.5 ACX1 Induction during Several Stress Treatments

Evidence supporting the role of ß-oxidation in the wounding response has come from work by Titerenko and co-workers. Using differential display, they reported induction of an acyl-CoA oxidase upon wounding (Titarenko *et al.*, 1997). A second report of stress-induced expression of an acyl-CoA oxidase was reported in barley (Grossi *et al.*, 1995). Acyl-CoA oxidase was induced in response to drought, cold and abscisic acid (ABA).

Further evidence supporting the hypothesis that acyl-CoA oxidase plays a role in the general stress response of Arabidopsis comes from studies using 2-chloroehylphosphonic acid (CEP) commonly known as ethaphon, an ethylene precursor. Northern analysis revealed that ethaphon treatment of detached Arabidopsis leaves led to a rapid induction of ACX1 and no other ß-oxidation genes (Hooks *et al.*, 1998). Control experiments revealed that it was acid breakdown products of ethaphon which actually appeared to be causing the induction.

Further experiments showed that wounding of the detached leaves also led to a rapid induction of *ACX1* (Hooks *et al.*, 1998). These results suggested that ACX1 plays a role in the plants early defence response by contributing to the oxidative burst and being involved in JA biosynthesis.

1.5.6 Nitric Oxide and Plant Defence

Nitric oxide (NO) is a well documented signalling molecule involved in the actions of the mammalian immune system. In particular it has been shown to function with reactive oxygen intermediates (ROI) from the oxidative burst in macrophage killing of bacteria and tumour cells (Schmidt and Walter, 1994; Nathan, 1995).

A role for NO in plants has recently become clear, which parallels the animal system. Delledonne and co-workers (1998) showed with the use of inhibitors that NO was necessary for the oxidative burst to result in programmed cell death. This observation was supported by Durner *et al.* (1998) who reported that NO and ROI are involved in the activation of the disease resistance genes, PAL and pathogen response-1 (PR-1).

A further parallel to animal systems has been reported by Navarre and co-workers (2000) when they showed that tobacco aconitase is sensitive to NO. The reversible inactivation of aconitase during pathogen attack could reduce both mitochondrial metabolism and the activity of the glyoxylate cycle.

NO and ROI not only appears to interact synergistically in programmed cell death signalling but it has been reported recently that NO can increase ROI by reversibly inhibiting two major H_2O_2-scavenging enzymes, catalase and ascorbate peroxidase (Clark *et al.*, 2000). These results are supported by observations using *in vivo* imaging of elicitor-induced NO burst in tobacco which showed that NO production preceeds H_2O_2 accumulation and is therefore one of the very early responses of a plant to pathogen attack (Foissner *et al.*, 2000).

1.6 Conclusions

The characterisation of ß-oxidation genes is relevant not only from the aspect of growth and development of oil seed crops but also for the possible role in the futile cycling of designer oils. During its initial characterisation, *ACX1* was found to be induced during germination (Hooks *et al.*, 1999a) and during stress (Hooks *et al.*, 1998), which could be the link to JA biosynthesis that has long been suggested. Acyl-CoA oxidases have been identified in other plant species during stress suggesting that these fundamental metabolic enzymes have a second biological role in plant defence systems. It is therefore of importance to further characterise *ACX1*, using biochemical and genetic approaches, with a view to investigating the role for this gene not only during development but also under stress conditions.

1.7 Project Aims

The overall aim of this project was to characterise the control of *ACX1* transcription. To achieve this it was planned to use the *ACX1* putative promoter fused to the luciferase reporter gene and to analyse induction from the promoter in transgenic plants after *Agrobacterium*-mediated transformation. The main questions to be addressed using this system were:

(I) Is the reported induction of *ACX1* transcript abundance during germination regulated at the level of transcription?

(II) Is *ACX1* induced during senescence?

(III) Does acid treatment of detached leaves result in activation of the *ACX1* promoter?

(IV) Does the *ACX1* promoter respond to wounding of the leaves?

With the aim of using Arabidopsis cell culture to investigate signalling pathways involving *ACX1*, it would be important first to characterise *ACX1* transcript expression during cell culture growth between subculturing. After establishing this, the cell culture could be used to address the following questions:

(V) Does acid treatment induce ACX1 activity in cell culture?

(VI) Is acidification of the cytosol involved?

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study were obtained from BDH (AnalaR® grade, Poole, UK) unless otherwise indicated. The luciferin used in the experiments described in Chapter 4 was obtained from BIOSYNTH AG, Germany.

2.1.2 Radiochemicals

The radiochemicals used in this study were supplied by Amersham International (Amersham, UK).

2.1.3 Bacterial Strains

E.coli XL-1 Blue (Stratagene, UK) was used as a host for the amplification of plasmids and *A. tumifaciens* strain GV3101(pMP90) (Koncz and Schell, 1986) was used for the transformation of Arabidopsis plants.

2.1.4 Liquid and Solid Bacterial Growth Media

LB Media: 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ tryptone and 10 $g.L^{-1}$ NaCl.

SOC Media: 2%(w/v) tryptone, 0.5%(w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl,

10mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

2YT Media: 16 $g.L^{-1}$ tryptone, 10 $g.L^{-1}$ yeast extract and 10 $g.L^{-1}$ NaCl.

2.1.5 Antibiotics

The antibiotics used were supplied by Sigma, UK. Ampicillin was dissolved in distilled water (100 mg/ml), filter-sterilised and used at a final concentration of 100 μg/ml. Kanamycin was purchased as a sterile solution (50 mg/ml) and used at a final concentration of 50 μg/ml. Gentamycin was purchased as a sterile solution (50 mg/ml) and used at a final concentration of 25 μg/ml. Tetracycline was dissolved in distilled water (50 mg/ml), filter sterilised and used at a final concentration of 50 μg/ml.

2.1.6 DNA Modifying Enzymes

All DNA modifying and restriction enzymes were purchased from Promega (Southampton, UK) together with their reaction buffers which were provided at 10 x concentration unless otherwise indicated.

2.1.7 Expressed Sequence Tags (E.S.T.s)

The following E.S.T.s were used in the study and were verified by sequencing and comparing the results to the genebank database:

Putative ACC Oxidase (AA713273)

Putative GA20 Oxidase (T22434)

Putative Triacylglycerol Lipase (T76589)

Putative Enoyl CoA Hydratase (N97282)

2.1.8 Oligonucleotides

All oligonucleotides used in this study were synthesised by MWG Biotech Ltd., Milton Keynes, UK.

Promoter Amplification:	35Hex3	CCA TGG TTC GTA ATT CAG AAT CTA GTA GC
	35H1kb	AAG CTT TTA ACT ATA CTT TGA AGG AAA CAC
Luciferase Internal	kate156	GTC GCC AGT CAA GTA ACA ACC G
	kate151	CTT CAT AGC CTT ATG CAG TTG CTC
5'RACE	G20BLG	TTC CAT GGT TTG CCT CCA TGA T
	ACCBLG	GAA GTC CTG GAA CTC CAT CAG T
	ACCG20LG	GAT ATA GTC GCC GGC GAG AAT A
	35H3P3	TCA ATG TAG GCA CAT GTC GG
	35H3P5	ACA CCG TCA ACA GAG TTC AG
	35H5P2	GGA TTA TCG AGC TTC GTC

2.1.9 Plant Material

Arabidopsis thaliana ecotype Columbia 0 was obtained from the Nottingham Arabidopsis Stock Centre (NASC), UK.

2.2 General Laboratory Procedures

2.2.1 pH Measurement

All solutions including the cell culture solutions were measured for pH using a Hanna Instruments pH meter HI9321.

2.2.2 Autoclaving

Equipment and solutions were sterilised at 15 psi for 20 minutes in a Laboratory Thermal Equipment Autoclave 225E.

2.2.3 Filter Sterilisation

Heat-labile solutions were sterilised by passing them through a Nalgene filter (pore diameter 0.2 μm) into a sterile receptacle.

2.2.4 Solutions and Equipment for RNA Work

Water for RNA work was treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC, Sigma, UK) overnight and then autoclaved. Sterilised glassware and plasticware were used throughout.

2.3 Plant Growth Conditions

2.3.1 Seed Sterilisation

Surface sterilisation of the seeds was carried out using a freshly prepared solution of 10%

chloros (BDH, UK) and 1% triton X-100. The seeds were immersed in the solution for 10 minutes. The bleach solution was then poured off and the seeds rinsed in sterile water six times. The seeds were finally dispersed onto 1/2 MS plates (Section 2.3.2) using a solution of sterile water or sterile 0.15% agar depending on the application. The seeds were then imbibed at 4°C for 2-4 days.

2.3.2 Germination Conditions

250 ml of 1/2 MS media was prepared in the following way: 1 g of MS (Sigma, UK) was dissolved in distilled water and the pH of the solution adjusted using 0.1 M KOH to a value of 5.8. 0.8 g of sucrose and 1.7 g of agar was added before autoclaving and pouring the plates in a sterile flowhood. The plates were covered with circles of sterile filter paper and sterile seedlings suspended in water were spread on top. The plates were then moved to growth rooms with continuous white light (120 $mmol.m^{-2}.s^{-1}$) at 22°C.

2.3.3 Growth of Plants for Transformation

The sterile, imbibed seeds were suspended in 0.1% agar to control the even spread of seeds onto the soil. Plant pots were filled with an autoclaved 1:1 mixture of potting compost (William Sinclair Horticultural Ltd., Lincoln, U.K.) and sharp sand before covering with mesh. The seeds were then evenly spread on the surface and the pot moved to growth conditions as described in 2.3.2. About 7-10 days after the pot was moved to the growth room the seedlings were thinned down to leave 10-12 seedlings per pot before returning to the growth rooms until the plants begin to bolt. The primary bolts were cut back to encourage lateral branching and therefore denser flowering. About a week later as the plants

begin to flower, *A. tumifaciens* mediated transformation was carried out. Before beginning, all flower heads already showing white petals were removed.

2.3.4 Growth of *Arabidopsis* Cell Cultures

The light-grown At-glw *Arabidopsis* cell and callus cultures were supplied by Dr. M. May (Dept. of Plant Sciences, University of Oxford) and have been described previously (May and Leaver, 1993). Cells were grown photomixotrophically in 200 ml culture media containing Murashige-Skoog salts, 0.5 mg/l α-naphatheleneacetic acid, 0.05 mg/l kinetin (all obtained from Sigma, UK), 3% (w/v) sucrose, pH 5.8 in 500 ml conical flasks. Suspension cultures were grown at 20°C in continuous low intensity white light (20 $\mu mol.m^{-2}.s^{-1}$) with constant shaking (110 rpm). The cells were subcultured every week by a 1/10 dilution. Callus cultures were grown in culture media supplemented with 0.8% (w/v) agar (Sigma, UK) and subcultured to fresh growth media every 2 weeks.

2.4 Illumination of Plant Material

2.4.1 Light Sources

Illuminations were carried out in controlled environment rooms at 22°C. The spectra of light qualities were measured with a spectroradiometer (Macam SR9910). Low white light (120 $\mu I.m^{-2}.s^{-1}$) was provided by warm white fluorescent tubes (Osram, Münich, Germany).

2.5 Isolation of Total RNA from Plant Material

Approximately 0.5 g of tissue was ground to a fine powder in liquid nitrogen using a mortar

and pestle and transferred to a 1.5 ml Eppendorf® tube. Total RNA was then extracted using the PUREscript® RNA Isolation Kit (Flowgen, Lichfield, UK) according to the manufacturers instructions. 300 µl of cell lysis solution (containing citric acid, EDTA and SDS) was added to the tube and then vortexed. 100 µl of protein/DNA precipitation solution (containing citric acid and NaCl) was then added and mixed gently. After mixing, samples were incubated on ice for 5 minutes and microcentrifuged for 5 minutes. The supernatant was transferred to clean 1.5 ml Eppendorf® tubes containing 300 µl of *iso*-propanol and mixed by inversion before microcentrifugation for a further 5 minutes. The supernatent was then discarded and the pellet allowed to dry before washing in 70% (v/v) ethanol. Samples were microcentrifuged for 1 min and the resulting pellet dried and resuspended in an appropriate volume of DEPC-treated dH_20.

The purity of the preparation was estimated by measuring the absorbance at 260 nm and 280 nm. A pure preparation was taken as having a A260/280 ratio of 1.8-2.0. The integrity of the RNA was examined on a 1.0% agarose mini-gel. All RNA preparations were stored at –80°C.

2.6 Amplification and Preparation of Plasmid DNA

A single bacterial colony was selected from a plate and 5 ml of LB with antibiotic was innoculated. The culture was grown overnight at 37°C with shaking. The plasmid was isolated using the Wizard™ Minipreps DNA Purification System (Promega) as follows. 1ml of the overnight culture was transferred to a 1.5 ml eppendorf tube and centrifuged at 10,000 x g for 5 minutes. The supernatent was discarded and the bacterial pellet resuspended by vortexing in 200 µl of cell resuspension solution (50 mM Tris-HCl pH 7.5, 10mM EDTA,

100 mg.ml^{-1} RNase A). Once fully resuspended, 200 µl of cell lysis solution (0.2 M NaOH, 1% SDS) was added and mixed by gentle inversion. Next, 200 µl of neutralisation solution (1.32 M KOAc pH 4.8) was added and mixed as before. The tube was centrifuged at 10,000 *g* for 5 min after which the supernatant was removed to a 2.0 ml Eppendorf® tube. 1 ml of the Wizard™ Minipreps DNA purification resin was added and the solution gently mixed. The resin/DNA mixture was then transferred to a 3 ml disposable syringe, attached to a minicolumn and placed on a vacuum manifold (Promega). A vacuum was then applied to pull the slurry into the minicolumn. The vacuum was broken, 2 ml of wash solution (100 mM NaCl, 10 mM Tris-HCl pH7.5, 2.5 mM EDTA, 55% (v/v) ethanol) added and the vacuum reapplied. Once the wash solution had passed through, the column was left to dry for 2 min. The vacuum was broken and the minicolumn removed from the syringe and transferred to a 1.5 ml Eppendorf® tube. This was microcentrifuged for 20 s to further dry the resin. The minicolumn transferred to a clean 1.5 ml Eppendorf® tube, 50 µl of TE (pH 7.6) added and allowed to stand for 1 min at room temperature. Plasmid DNA was eluted by microcentrifugation for 20 s and the minicolumn discarded.

2.7 Quantification of DNA and RNA

An aliquot of the nucleic acid solution to be quantified (usually 5-10 µl) was made up to 1 ml with DEPC-treated dH_2O. The absorbance of the solution was measured between 220 nm and 320 nm against a DEPC-treated dH_2O blank. An absorbance at 260 nm of 1 was taken to indicate the following concentrations:

Form of nucleic acid	Concentration ($\mu g.ml^{-1}$)
Double stranded DNA	50
Single stranded DNA and RNA	40

2.8 Preparation of Electrocompetent Cells

The XL1-Blue strain of *E.coli* was used to make electrocompetent cells. A single colony was picked from a tetracycline plate and grown overnight at 37°C in 2YT media plus tetracycline (see 2.4.1). The overnight culture was used to innoculate 500ml of 2YT containing tetracycline and grown at 37°C until an O.D. (600nm) of 0.5 was reached. The culture was chilled at 4°C for 30 minutes and maintained at 4°C for the duration of the protocol. The cells were centrifuged at 5000 rpm for 15 minutes followed by resuspension in sterile, distilled water. After the first centrifugation the cells were resuspended in an equal volume of water. Cells were centrifuged 3 times, using the same conditions described above, and subsequently resuspended in decreasing volumes: (i) 250ml H_2O, (ii) 10ml H_2O-10% glycerol, (iii) 1ml H_2O-10% glycerol. The final 1ml volume of cells was split into 40 µl aliquots, frozen in liquid nitrogen and stored at -80°C.

2.9 Transformation of Competent Cells

Frozen competent cells (Section 2.6.4) were thawed at room temperature and placed on ice. Approximately 200 ng of plasmid DNA, in a volume of 0.5-1 µl, was added to an aliquot of competent cells and the tube gently mixed and left on ice for 1 minute. The cell and DNA mixture was transferred to a cold 0.2 cm electroporation cuvette (BIORAD, UK). The Gene

Pulse II with Pulse Controller II and Capacitance Extender II from BIORAD was used to perform electroporations, the conditions for electroporation were 25 μF, 2.5 kV and 200 ohms with the time constant being displayed at 4.5-5.0. Immediately after electroporation 1 ml of SOC media (see 2.1.4) was added and the culture transferred to an Eppendorf® and incubated at 37°C for 1 hour (shaking at 225 rpm). 200-500μl of the culture was then plated on selective media. The plates were allowed to dry and placed at 37°C overnight. As a control, transformed cells were plated on agar in the absence of antibiotic selection. A second control was also performed where competent cells were treated as above, in the absence of plasmid DNA and plated on antibiotic-containing plates.

2.10 Gel Electrophoresis

All agarose (ultra PURE™) was supplied by Life Technologies, UK. Acrylamide was supplied by Merck, UK.

2.10.1 Agarose Gel Electrophoresis of DNA

The appropriate concentration of agarose [0.5 - 2.0% (w/v)] was added to the volume of 1 x TAE (0.09 M Tris-acetate, 2 mM EDTA). The agarose suspension was heated in a microwave oven until the agarose had completely dissolved. The solution was allowed to cool to around 60°C at which point 10 $\mu g.ml^{-1}$ EtBr was added to a final concentration of 1 $mg.ml^{-1}$ and the gel solution poured into the electrophoresis apparatus. After allowing the gel to set, enough 1 x TAE running buffer was added to just submerge the gel. The samples to be loaded were mixed with 1/10 volume of loading buffer (20% (v/v) glycerol, 25mM NaOH, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) and loaded with an

automatic pipette. Electrophoresis was carried out at 15 - 80 mA until the bromophenol blue had migrated two-thirds of the way down the gel. The gel was then visualised under UV light (Spectroline® transilluminator, Model TC-312A).

2.10.2 Denaturing Electrophoresis of RNA

This method was used for gels that were to be northern blotted for hybridisation analysis. 1.3 g of agarose was added to 80 ml of DEPC-treated dH_2O. The agarose suspension was then heated in a microwave oven until the agarose had completely dissolved. Once the agarose solution had cooled to 60°C, 10 ml of formaldehyde [37% (v/v), Sigma, UK] and 10 ml 10 x MOPS buffer [0.2 M MOPS, (Sigma, UK), 0.05 M NaOAc, 0.01 M EDTA (pH 7.0)] was added and the gel mixed by swirling prior to pouring into the electrophoresis apparatus. After setting, the gel was placed into the electrophoresis tank and submerged in 1 x MOPS (pH 8.0). 10 µg of RNA was prepared in a solution of 70% (v/v) formamide (Fluka Biochemicals, Gillingham, UK), 2.3 x MOPS, 1 $mg.ml^{-1}$ EtBr, 2.3% formaldehyde to a volume no greater than 50 µl. This solution was heated to 65°C for 5 min and placed on ice. Electrophoresis was carried out at 15 - 80 mA for two hours. The gel was then visualised under UV light.

2.10.3 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of DNA was performed according to the method of Sambrook *et al.* (1989). The gel was made by dissolving 33.6 g urea in 30 ml dH_2O and adding 16 ml 40% acrylamide solution (63.3% (w/v) acrylamide, 3.3% (w/v) *N, N'* - methylenebisacrylamide), 8 ml 10 x TBE buffer (890mM tris-borate, 2mM EDTA), 480 µl

10% (w/v) ammonium persulphate and 60 µl TEMED (*N, N, N', N'* - tetramethylethylenediamine) before pouring in between the plates of the gel apparatus. The gel apparatus was assembled and 1 x TBE buffer was added as a running buffer. Denatured ^{32}P-labelled-DNA samples were loaded and the gel run at approximately 70°C and 85 watts (150 mA) for around 3 hours. The gel was soaked in 10% acetic acid, rinsed with dH_2O and dried at 80°C for 1 hour. The gel was then visualised by autoradiography (Section 2.21.3).

2.11 Isolation of DNA Fragments from Agarose Gels

For this method the Qiaex Gel Extraction System (Qiagen) was used in accordance with the manufacturer's instructions. The DNA of interest was isolated from the agarose gel as described in 2.10.1. The gel slice was transferred to a 1.5 ml Eppendorf® tube, containing 300 ml buffer QX1 (3 M NaI, 4 M $NaClO_4$, 10 mM Tris-HCl (pH 7.0), 10 mM sodium thiosulphate). The sample was incubated at 50°C until the agarose had completely dissolved. 10 ml Qiaex resin was added and the sample incubated at 50°C for 10 min to allow the DNA to bind to the resin. After incubation, the sample was microcentrifuged for 30 s and the supernatant removed. The pellet was resuspended in 0.5 ml of buffer QX2 (8 M $NaClO_4$, 10 mM Tris-HCl (pH 7.0)) and the sample centrifuged once more. This wash step was repeated twice more with an equal volume of buffer QX3 (70% (v/v) ethanol, 100 mM NaCl, 10 mM Tris-HCl (pH 7.5)). The final pellet was air-dried for 10 min and resuspended in 20 ml TE (pH 7.6) then incubated at room temperature for 5 min, vortexing periodically, to elute the DNA from the Qiaex resin. The sample was centrifuged as before and the supernatant transferred to a clean tube.

2.12 Digestion of DNA with Restriction Endonucleases

DNA to be restricted was prepared in a solution of 1 x the appropriate buffer and 1 - 20 units of the restriction enzyme(s) added ensuring that its concentration did not exceed 10% (v/v). Reactions were incubated at 37°C for 1 hour and monitored where sufficient DNA was available by electrophoresis on an agarose gel against uncut and marker DNA.

2.13 DNA Ligations

Purified DNA restriction fragments were ligated based on the method of Sambrook *et al.* (1989). 0.1 µg of vector DNA was transferred to a sterile Eppendorf® tube and an equimolar amount of insert DNA added. To the tube, 1 µl of 10 x T4 DNA ligase buffer (200mM Tris.Cl (pH 7.6), 50mM $MgCl_2$, 50mM dithiothreitol), 1 µl 5mM ATP and 1 unit T4 DNA ligase (Promega, UK) was added. This mixture was diluted to 10 µl with dH_2O. The samples were incubated either at room temperature for 2 hours or overnight at 16°C. Control reactions containing only vector DNA or only insert DNA were also set up. Ligations were directly transformed into competent *E. coli* (Section 2.9) or analysed by agarose gel electrophoresis (Section 2.10.1).

2.14 Colony Hybridisations

This method screens large numbers of colonies or plaques grown on selective plates after transformation / infection. The plates were chilled for one hour before overlaying the surface of the plates with circular, nylon membrane (Hybond-N, Amersham, UK). While the filter was in place orientation marks were made with a needle going through the filter paper and into the agar. When the filter was lifted, it was immediately placed colony side up on layers

of 3MM Whatman paper which had been soaked in 10% SDS. The filters were left for 3 minutes to lyse the cells. Next the filters were placed upon Whatman paper soaked in 0.5M Na OH and 1.5M NaCl for 5 minutes to denature the DNA. The next step was to neutralise the DNA by placing the filters on Whatman paper soaked in 1.5M NaCl and 0.5M Tris-HCl (pH 7.4) for 5 minutes. The final step was to wash the filters on 3MM paper soaked in 2X SSC (3 M NaCl, 0.3mM tri-sodium citrate) before drying at room temperature for 30 minutes. The DNA is finally fixed to the filter using the UV crosslinker (CL-100 UV-crosslinker, UVP, UK) set to deliver 120,000 μJoules.cm^{-2}. Hybridisations were carried out in the same manner as Southern blot hybridisations (see 2.18) using the original ligation fragment as the probe for identifying positive transformants.

2.15 DNA Sequencing

DNA for sequencing was prepared as described in Section 2.6. All sequencing reactions were done commercially by MWG Biotech, Ltd., Milton Keynes, UK. The analysis of DNA sequences was performed using the GCG program package (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, USA).

2.16 Polymerase Chain Reactions (PCR) (Mullis and Faloona, 1987)

All PCR reactions were carried out using Taq Polymerase (Promega) unless otherwise indicated. The reactions were performed on a thermocycler (Perkin Elmer Gene Amp PCR System 2400).

2.16.1 Colony PCR

This protocol is used for carrying out PCR on transformed single colonies to determine if there is an insert and if so which size it is (Gussow and Clackson, 1989). A single colony was scraped off the plate and resuspended in 200 µl of sterile distilled H_2O which was then boiled in a water bath for 5 minutes. The tube was then centrifuged for 2 minutes at 13000 rpm to remove debris then 2 µl of this solution was used as template in the PCR (Mullis and Fuloona, 1987).

2.17 Isolation of Genomic DNA

This was carried out using CTAB buffer [2% (w/v) CTAB (Sigma, UK), 1.4M NaCl, 0.2% (w/v) 2-mercaptoehanol (Sigma, UK), 20mM EDTA, 100mM Tris-HCl (pH 8.0)]. About 1 g of tissue was ground up in liquid nitrogen with a pestle and mortar then put directly into preheated CTAB buffer (60°C). The sample was then incubated at 60°C for 30 minutes with occasional gentle mixing. This was then extracted once with chloroform-isoamyl alcohol (24:1; v:v) after gentle but thorough mixing. The aqueous phase is removed using a wide bore pipette after 10 minutes centrifugation at 1600 x g. The DNA was precipitated by adding 2/3 volume cold isoproponol and then spinning at 500 x g for 2 minutes. To clean up the DNA the pellet was resuspended in wash buffer (76% ethanol (v:v) 10mM ammonium acetate for 10 minutes before centrifuging at 1600 x g for 10 minutes. The pellet was allowed to dry at room temperature before being resuspended in 1ml of TE [10mM Tris-HCl (pH7.4), 1mM EDTA] ready for use.

2.18 Southern Blotting of Genomic DNA

Southern blotting was carried out as described by Sambrook *et al.*, 1989. 10 µg of genomic DNA was digested with different restriction enzymes and run on a 1% agarose gel as described in Section 2.10.1. Before the gel was blotted as described in Section 2.22, it was submerged for 15 minutes in 12M HCl to hydrolyse and fragment the DNA thus making transfer of the molecules possible. The gel was then submerged in denaturing solution (0.5M NaOH and 1.5M NaCl) for 30 minutes followed by renaturation [1.5M NaCl and 0.5M Tris-HCl (pH 7.4)] solution for 30 minutes. The blot was set up as follows: A plastic box containing 20 x SSC (3M NaCl, 0.3M tri-sodium citrate) was used as the base for the blot. Across the top of the box was a glass plate which acted as a bridge over which 3MM (Whatman) paper sat with either end in the 20 x SSC completing a circuit. The gel was placed on top of this and before a gel sized piece of membrane was placed on top the surface of the gel was wetted with the 20x SSC solution. On top of the filter went three layer of Whatman paper also cut to size and it was finished off with many layers of tissues held down by a second glass plate with a weight on top. To stop the blot short circuiting a layer of Nescofilm was placed around the edges of the gel on top of the bridge. The blot was left overnight for total transfer to occur. After successful transfer, the filter was dried at room temperature and the DNA fixed using an UV-crosslinker (CL-100 UV-crosslinker, UVP, UK) to deliver 120,000 µJoules.cm^{-2}.

2.19 Northern Blotting

When the quality of a denaturing RNA gel had been checked under UV for the integrity of the RNA, the gel was blotted onto nylon membrane(zeta probe) for further analysis. The blot

was set up as follows: 500mls of transfer solution (50mM NaOH) was placed in a box at the bottom of the blot. Across the top of the box was a glass plate which acted as a bridge over which 3MM (Whatman) paper sat with either end in the NaOH completing a circuit. The gel was placed on top of this and before a gel sized piece of membrane was placed on top the surface of the gel was wetted with the NaOH solution. On top of the filter went three layer of 3MM paper also cut to size and it was finished off with many layers of tissues held down by a weight onto of a second glass plate. To stop the blot short circuiting a layer of Nescofilm was placed around the edges of the gel on top of the bridge. The blot was left overnight for total transfer to occur. After successful transfer, the filter was dried at room temperature and the RNA fixed using an UV-crosslinker (CL-100 UV-crosslinker, UVP, UK) to deliver 120,000 μJoules.cm^{-2}.

2.20 Radiolabelling of DNA

2.20.1 Preparation of DNA to be Labelled

The DNA sequences to be labelled were amplified using PCR and vector specific primers as described in Section 2.16. The amount of the DNA was estimated by running an aliquot of the probe on an agarose gel as described in 2.10.1 and comparing it to the known amounts of DNA loaded onto the same gel.

2.20.2 Random Priming of dsDNA

DNA was labelled with [α-32P]dCTP using the Decaprime™ DNA labelling kit (Ambion Inc., Austin, USA) in accordance with the manufacturers instructions. Approximately 25 –

50 ng of DNA, prepared as described in section 2.20.1, was added to a tube containing 2.5 µl of decamers in a total volume of 11.5 µl. This solution was incubated at 100°C for 10 minutes before being frozen in liquid nitrogen. As this solution thawed on ice 5µl of 10X buffer, 1µl of Klenow (5U) and sterile H_2O was added to a total volume of 25 µl. After a brief microcentrifugation, 50 µ Ci (3000 Ci.mmol^{-1}) of [α-32P]dCTP was added to the reaction mixture and the tube was incubated at 37°C for 60 minutes. The reaction was stopped by the addition of 5 µl of 0.5M EDTA.

2.20.3 Separation of Labelled DNA from Unincorporated Radionucleotides using Spin Columns

G-50 spin columns were used to separate the unincorporated nucleotides from the radiolabelled DNA probe. The probe was eluted in 150 µl of 1 x TE. Once purified, the probe was denatured at 95°C before adding the total volume to the hybridisation solution.

2.21 Hybridisation Analysis of Northern Blots

2.21.1 Hybridisation Analysis using Homologous Probes

The uv-crosslinked filters were pre-hybridised for 1hour in 0.2M H_2HPO_4, 7% SDS at 55°C. The pre-hybridisation solution was poured away and fresh solution was added prior to addition of the probe (see 2.20.3). The hybridisation was incubated overnight at 55°C.

2.21.2 Washing of Northern Blots

Washing the filters removes the non-specific binding of the radiolabelled DNA to the filter. Washes were carried out as follows: 100mls of 20mM Na_2HPO_4 and 7% SDS wash solution was added to filter in a box. This was incubated for 20 minutes at 55°C, the wash solution was discarded and counts on the filter checked using a hand-held Geiger counter. In some cases the procedure was repeated 2-3 times moving the box to room temperature if a less stringent wash was needed. The filters were then sealed in plastic bags and placed in a cassette for autoradiography.

2.21.3 Autoradiography

Filters to be autoradiographed were sealed in a hybridisation bag and exposed to Fuji X-ray film (type RX) in a film cassette with an intensifying screen at –80°C for the appropriate length of time.

2.21.4 Stripping Filters of Bound Radiolabelled Probes

The filters were stripped of the remaining radioactive label using 0.1% SSC and 0.5% SDS. The filters were incubated at 95°C for 20 minutes. The counts were checked using a hand-held Geiger counter and the procedure repeated if necessary.

2.22 Hybridisation of Southern Blot

2.22.1 Hybridisation Analysis using Homologous Probes

The Southern filters were hybridised in 6 X SSC, 0.5% SDS, 10% Denhardt's solution [10 $g.L^{-1}$ Ficoll (type 400, Pharmacia, UK), 10 g polyvinyl pyrrolidone, 10 g bovine serum

albumin with the volume made up 1 L with sterile H_2O] and 100 µl of salmon sperm DNA (100 $\mu g ml^{-1}$) overnight at 65°C (Sambrook *et al.* 1989). The probe was synthesised as described in Section 2.20.

2.22.2 Washing of Southern Blots

The filters were washed using the protocol detailed by Sambrook *et al.* (1989). Varying the concentrations of SSC and SDS depending on the desired stringency of the washes.

2.22.3 Autoradiography

As described in Section 2.21.3.

2.23 Primer Extension

The primer extension was carried out using the 35Hex3 oligonucleotide described in Section 2.1.8. The first step in primer extension was to use polynucleotide kinase and [gamma-^{32}P] ATP (3 µl of 10 mCi/ml, specific activity 3000 $Ci.mmol^{-1}$) to radioactively label the oligonucleotide primer. This reaction was carried out at 37°C for 10 minutes. 10 µg of total RNA was used with 100 fmol of the labeled primer then incubated at 58°C for 20 minutes to anneal the primer. Buffer (50mM tris-HCl, 50mM KCl, 10mM$MgCl_2$, 10mM DTT, 1mM each dNTP, 0.5mM spermidine) and reverse transcriptase (1 unit) was then added and the primer extension reaction was incubated at 42°C for 30 minutes. To visualise the results of the reaction it was run on a polyacrylamide gel as described in Section 2.10.3.

2.24 5' RACE

The 5'RACE was carried out using the 5'RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Life Technologies Inc.). RNA was prepared from day 2 after imbibition germinating seedlings as described in Section 2.5. First strand cDNA synthesis was carried out using 500ng of total RNA which was denatured at 70°C for 10 minutes before being annealed to a gene specific primer (35H3P5). 200 units of SUPERSCRIPT II reverse transcriptase was added and the reaction incubated at 42°C for 30 minutes. The reaction was terminated by incubation at 70°C for 15 minutes before RNase mix was added and the reaction incubated at 37°C for 30 minutes. The cDNA was purified using the GlassMax spin cartridge provided in the kit. The single stranded cDNA was tailed with dCTP using TdT (terminal deoxynucleotidyl transferase) during a 10 minute incubation at 37°C.

The final PCR was carried out as described in Section 2.16 using an annealing temperature of 55°C as suggested in the kit protocol. The abridged anchor primer (poly-G) in the kit was used along with the second gene specific primer (35H3P3). 5-20 µl of this reaction was run on an agarose gel as described in Section 2.10.1.

2.25 Growth of *Agrobacterium tumifaciens*

For the transformation of *Arabidopsis thaliana* the strain of *A. tumifaciens* used was GV3101, which contains a helper Ti plasmid, pAL4404. A 5 ml overnight culture was innocculated. GV3101 was grown in LB containing 25 µg/ml gentamycin, to which it is resistant and shaken vigorously at 28°C overnight. The 5 ml overnight culture was added to 50 ml of LB and gentamycin (25 µg/ml) in a 250 ml flask and shaken vigorously at 28°C until

the O.D. of the culture reached an absorbance at 600 nm of 0.5 to 1.0. The culture was chilled on ice to stop any further cell divisions before being spun at 3000 x g for 5 minutes at 4°C. The supernatent was discarded and the cells were resuspended in 1 ml of ice-cold 20mM $CaCl_2$. This was then dispensed into 100 µl aliquots and frozen in liquid nitrogen before being stored at -80°C.

2.26 Transformation of *Agrobacterium tumifaciens*

The transformation of GV3101 with the binary vector of interest was carried out by adding 1 µg plasmid DNA to one of the aliquots of frozen cells. The cells were thawed by incubating at 37°C for 5 minutes. 1ml of LB was then added to the tube and it was shaken gently at 28°C for 4 hours to allow expression of the antibiotic resistance gene, in this case neomycin phosphotransferase which confers resistance to kanamycin. The cells were then pelleted by centrifuging in a microcentrifuge for 30 seconds. The supernatent was discarded and the cells were resuspended in 0.1 ml of LB before the cells were spread on LB agar plates containing 25 µg/ml gentamycin and 50 µg/ml kanamycin. The plates were then incubated at 28°C for 3 days after which the transformed colonies were visible and could be tested for the presence of the correct plasmid.

2.27 Transformation of *Arabidopsis thaliana*

The transformation of Arabidopsis plantlets, grown as described in Section 2.3.3, by *A tumefaciens* was carried out using vacuum infiltration. A 25 ml culture of the transformed *A. tumifaciens* in LB with 25 µg/ml Gentomycin and 50 µg/ml kanamycin was started 3 days

before vacuum infiltration. The day before infiltration the 25 ml were added to 400 ml of identical media and grown overnight. The cells were harvested, when the OD (600nm) was > 2.0, by centrifuging them for 10 minutes at 5000 rpm and room temperature before resuspending them in three volumes infiltration medium [1/2 MS salts (Sigma, UK), 1 x B5 vitamins (Sigma, UK), 5% sucrose and 0.044μM Benzylamino Purine (Sigma, UK)], to give an OD (600 nm) of 0.8. The cells in media were added to a dish big enough to hold an inverted plant pot, then the dish containing the inverted plant pot was placed in a bell jar and a vacuum was drawn until bubbles appeared on the leaf and stem surfaces. The vacuum was then released quickly and the saturated plants were stored overnight in plastic bags to maintain humidity and dim light to reduce further stress. The next day the plants were moved to growth rooms to continue their life cycle.

2.28 Selection of Transformed Plants

When the plants that had undergone transformation completed their life cycle, the dried siliques were collected and the seeds within were isolated. These are the T_1 generation and the transformants were selected on agar plates for their resistance to the antibiotic kanamycin. The plates contained 1/2 MS (Sigma, UK), 0.8% Agar, 1 x B5 vitamins and 50 μg/ml kanamycin. After the seeds were sterilised as described in Section 2.3.1, they were resuspended in 0.1% sterile agarose to aid distribution onto plates. The plates were stored in the coldroom (4°C) for 4 days before being moved to the growth rooms as described in Section 2.4.1. After 10 days seedlings which had germinated and whose cotyledons had not bleached were transferred to soil and allowed to continue growing. These were considered putative positive transformants.

2.29 *In vivo* Analysis of Luciferase Expression

The activity of the luciferase reporter gene was monitored by using a Hamamatsu Photonics KK - C2400 (Japan) photon counting camera. The image processor and image intensifier controller (M4314) were from ARGUS as was the ARGUS-50 software. The Luciferin was introduced to the leaves using an airbrush and an airbrush compressor BM310 Mini Micon II from Badger. Previous methods have used detergent for the delivery of luciferin but in this case there was no need as the luciferin-buffer mix was sprayed directly. The pressure successfully delivered the substrate to the reporter enzyme. Seeds germinated for luciferase analysis were grown in 18 hour day, 6 hour night cycles.

At the specific timepoints the seedling or leaves were sprayed with luciferin and left in a darkened chamber for 10 minutes to allow the reaction rate to settle. The photon counting apparatus was then used to obtain a total count of photons over different time points. The ARGUS-50 software was then used to change the quality of the image and superimpose the photon image onto a light image of the samples. Values for the exact number of photons counted were also obtained.

2.30 Cloning of cDNA Library

The cDNA library was cloned using RNA from seedlings that had been grown fo 2 days following imbibition. The Lambda ZAP II kit (Stratagene, UK). At day 2 the radicle is starting to emerge from the seed coat and is taken to represent the stage of maximal lipid mobilisation. PolyA RNA was isolated using the magnetic beads kit (Promega, UK). First

strand synthesis was carried out using 50 units of StrataScript reverse transcriptase, 50 μg of polyA RNA and 2.8 μg of random primer with an XhoI linker (Stratagene). The reaction was incubated at 42°C for 1 hour. The second strand synthesis was carried out using the first reaction as template, 100 units of DNA polymerase I, 3 units of RNase H and 2 μl of [α-^{32}P]dATP (800 Ci/mmol) and incubating at 16°C for 2.5 hours. The cDNA termini were then blunt ended using 5 units of Pfu DNA polymerase and incubating at 72°C for 30 minutes. The cDNA was prepared for packaging by ligating EcoRI linkers and then phosphorylating them using 4 units of T4 polynucleotide kinase, this results in sticky ends at the 5' end of the cDNA. The cDNA was then digested with XhoI to give sticky ends at the 3' end.

Size fractionation was carried out using Sepharose CL-2B gel filtration medium (Stratagene, UK) in a 1 ml pipette. Fractions from the column were collected and samples were loaded onto a polyacrylamide gel (Section 2.10.3) (Figure 3.3). The fractions with the largest cDNA molecules were then ligated into the Uni-ZAP XR vector using 2 units of T4 DNA ligase with an overnight incubation at 12°C. 2 μl of the ligation was added to the packaging reaction (Stratagene, UK) and incubated at 22°C for 90 minutes. The library was then titred using *E. coli* (XL1-Blue MRF' which are RecA$^-$) from Stratagene, UK.

After packaging, plaque PCR (Section 2.14) was used to confirm that each plaque had an insert and that the average insert size was 1 kb (ranging from 0.8 – 1.2 kb). This was done using the T3 and T7 primers compatible with the vector.

2.31 E.S.T. Database from cDNA Library

An aliquot of the cDNA Library in Section 2.30 was sent to BASF Plant Sciences (Germany) where they excised plasmids based on the Stratagene protocol. *E. coli* was transformed and 5000 white colonies were selected and DNA was prepared for 5' end sequencing. Generation of the E.S.T. database was performed as part of an E.U. funded project on the control of fatty acid breakdown (QLRT-1999-00213). The E.S.T. database was produced by Dr. Pawel Hezyck and is only currently available by the E.U. partners. The database was used in the current project to perform 'electronic' northerns and analysis of specific transcripts.

CHAPTER 3: GERMINATION INDUCTION OF GENE EXPRESSION

3.1 Introduction

Peroxisomal ß-oxidation is the predominant pathway of fatty acid catabolism in plants. ß-oxidation is of particular importance during the stages of oil seed germination when there is a rapid mobilisation of lipid reserves to supply carbon skeletons for growth and respiration. Transcript levels of ß-oxidation genes rapidly increase at the onset of germination (Hayashi *et al.*, 1998; Hayashi *et al.*, 1999; Hooks *et al,* 1999a; Froman *et al.*, 2000; Eastmond *et al.*, 2000a). The first step in ß-oxidation is catalysed by a family of acyl-CoA oxidase isozymes with different, but overlapping, fatty acyl-CoA chain length specificities. Assays which measured the activity of the two acyl-CoA oxidases, ACX1 and ACX2, showed that the increase in enzyme activity mirrored the increase in transcript levels, suggesting that expression is regulated predominantly at the level of transcription (Hooks *et al.*, 1999a). More recently the Arabidopsis *ACX3* gene, which encodes a medium chain acyl-CoA oxidase, has been shown to be regulated at the level of transcription during germination (Eastmond *et al.*, 2000b).

Recently the sequence and annotation of the Arabidopsis genome has been published (The Arabidopsis Genome Initiative, 2000). The annotation of the sequences gives preliminary information on a given gene, from which further experiments can be designed. Publication of the first major block of Arabidopsis genome sequence, representing a region from chromosome IV, revealed that it included the sequence of *ACX1* (Bevan et al., 1998). This

created an opportunity to study the *ACX1* gene in the context of the genomic region in which it occurs.

The genomic sequence information along with a wealth of data available from E.S.T. databases has allowed a new electronic approach to the analysis of gene expression. Mekhedov *et al.* (2000) have used this approach, which they call 'electronic' northern analysis, to investigate expression of genes associated with lipid biosynthetic pathways. This analysis involved searching E.S.T. databases for putative transcripts. Large numbers of clones were retrieved, which on further investigation appeared to be chimaeric, containing sequence from different cDNAs. In contrast the identities of other putative clones were confirmed by further sequence alignments. Mekhedov and co-workers concluded that the annotation of the databases is initially useful but that with further sequence alignments and database analysis more information could be found. After this analysis Mekhedov and co-workers were left with 2600 clones thought to be involved in plant glycerolipid biosynthesis. They then plotted these clones in a graph to show abundance relative to each other and suggested that analysis of E.S.T. databases in this way produces an electronic northern reflecting the abundance of a particular transcript.

In this chapter the expression level of *ACX1* during germination is compared with other genes that occur at the same locus of chromosome IV and which may be functionally related.

3.2 *ACX1* Genomic Locus

The sequencing of the Arabidopsis genome has provideda great deal of information on the close proximity of related genes. From the annotated sequences there appears to be many clusters of functionally related genes close to each other, which suggests that this may not be a coincidence. Functionally related genes may be co-regulated and clustering could allow co-ordinate control of gene expression. There are four members of the ACX gene family that have been functionally characterised and two others that can be identified in the genome sequence based on homology, *ACX5* and *ACX6* (Figure 3.1). *ACX5* is 93% identical to *ACX3* at the amino acid level although E.S.T.s for its specific sequence have been identified in the MIPS database (http://mips.gsf.de/proj/thal/). Similarly *ACX6* is 85% identical to *ACX1* at the amino acid level and gene specific E.S.T.s have been identified.

As outlined in Chapter 1, the ACX gene family is of particular interest during germination of oilseed crops due to their role in ß-oxidation. It was therefore of particular interest when other putative genes of enzymes known to have a role in germination were identified within 100 kb of *ACX1*. A putative GA20 (Gibberellin) oxidase, triacylglycerol lipase, enoyl-CoA hydratase and an ACC (1-Amino-Cyclopropane-1-Carboxylic acid) oxidase were identified. The putative ACC oxidase gene was identified by doing Blastn analysis of the chromosome sequence against the Arabidopsis E.S.T. database (Genbank).

GA20 oxidase is involved in gibberellin biosynthesis and may therefore be expressed during germination and post-germinative growth. ACC oxidase catalyses the final step in ethylene

Figure 3.1 Location of the acyl-CoA oxidases on the Arabidopsis chromosomes

This figure shows the five Arabidopsis chromosomes and the locations of the 4 functionally characterised members of the acyl-CoA oxidase family, ACX1-4. 2 further genes have been identified on the basis of homology using the MIPS database (http://mips.gsf.de/proj/thal/). These genes have been named ACX5 and ACX6.

The MIPS designation for the 6 genes are as follows:

ACX1	At4g16760
ACX2	At5g65110
ACX3	At1g06290 and At1g06300
ACX4	At3g51840
ACX5	At1g06310
ACX6	At2g35690

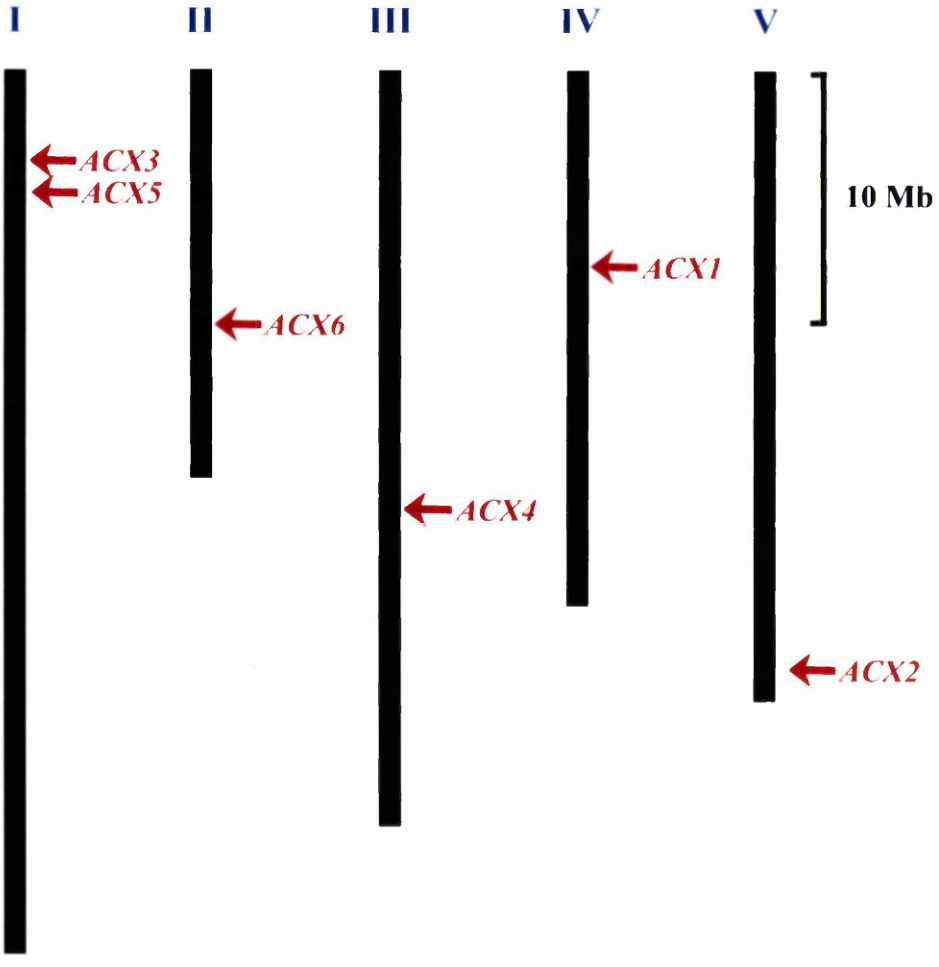
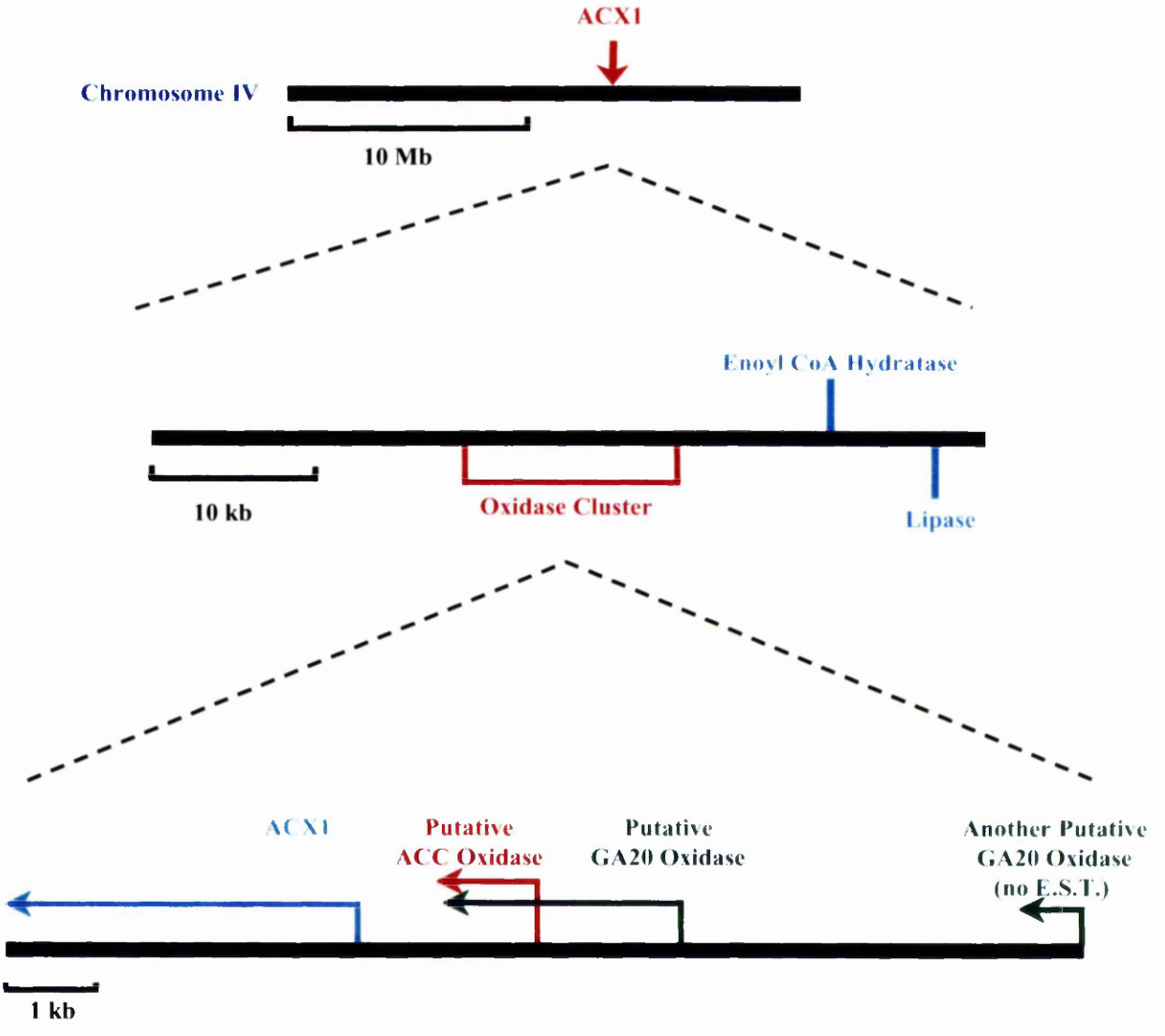

I
II
III
IV
V
ACX3
ACX5
ACX6
ACX4
ACX1
ACX2
10 Mb

Figure 3.2 The putative germination cluster

This figure shows the location of the putative germination cluster on chromosome IV and with increasing detail the presence of the oxidase cluster and the (putative) genes encoded at this location.

The putative lipase and enoyl-CoA hydratase (both shown in blue) are located within 40kb of the oxidase cluster. The oxidase cluster represents three oxidase genes acyl-CoA oxidase 1 (shown in blue), GA20 oxidase (shown in green) and ACC oxidase (shown in red). The coding regions of the GA20 oxidase and the ACC oxidase overlap.


ACX1
Chromosome IV
10 Mb
Enoyl CoA Hydratase
10 kb
Oxidase Cluster
Lipase
ACX1
Putative
ACC Oxidase
Putative
GA20 Oxidase
Another Putative
GA20 Oxidase
(no E.S.T.)
1 kb

biosynthesis and since ethylene also plays an important role in early seed growth it may also be expressed at this stage. Triacylglycerol lipase is involved in the conversion of triacylglycerol to fatty acids and glycerol during the early stages of lipid breakdown in the oil bodies and could therefore also be expressed during germination. Finally the enoyl-CoA hydratase is part of the multifunctional protein involved in ß-oxidation and is thus also implicated in germination. These putative genes and their relative positions can be seen in Figure 3.2.

It can be seen from Figure 3.2 that there is an overlap in the coding sequences of the GA20 oxidase and the ACC oxidase. This was very surprising and as these are putative genes it was important to try and confirm that they produce two different gene products and were not due to some redundant gene duplication or E.S.T. anomaly.

All five sequences are within 100 kb of each other on chromosome IV, which is of particular relevance due to the fact that this is the average size of a chromosome loop with matrix attachment regions (MAR) (Jackson *et al.*, 1990). This suggested that the control of transcription, from genes in this locus during germination could at least in part be at the level of chromatin structure. To test this, expression patterns of these five genes were investigated as co-expression would provide support for this hypothesis.

3.3 Cloning of Day 2 of Germination cDNA Library

An *Arabidopsis thaliana* cDNA library was made with RNA from seedlings at day 2 of germination, with the intention of screening for genes expressed during germination. As mentioned in Section 2.30, the library was made using the λ-ZAPII vector kit from Stratagene and during this process experiments were carried out to monitor the quality of the library.

In order to ensure that the clones in the library were likely to be full length, the cDNA was size fractionated by running it through a sephadex column and collecting fractions. Samples were then run on a 5% polyacrylamide gel to determine which fractions should be taken forward and cloned into the vector. The gel was visualised using a phosphor-imager as the cDNA was synthesised using ^{32}P-dCTP. The results of this analysis are shown in Figure 3.3.

Fractions 7 and 8 clearly contain the larger cDNA products and these were cloned into the λ-ZAPII vector as described in Section 2.30. After the library had been packaged into phage, it was important to ascertain what percentage of the phage contained an insert and what the average size of the insert was. Plaque PCR was carried out using the colony PCR protocol (2.16.1) and standard T3 and T7 primers. The products were run on a 1% agarose gel with the 1kb ladder (Figure 3.4).

The results suggested that almost all of the phage had inserts, which was substantiated by the fact that no blue plaques were found when the library was plated out on IPTG and X-GAL

Figure 3.3 Phosphor-image of cDNA size fractionation

Polyacrylamide gel electrophoresis of the different fractions collected after size of newly synthesised ^{32}P labelled cDNA. The sample loading was as follows:

Lane 1	Fraction 5	No cDNA present
Lane 2	Fraction 6	No cDNA present
Lane 3	Fraction 7	High molecular weight cDNA present
Lane 4	Fraction 8	High molecular weight cDNA present
Lane 5	Fraction 9	Medium molecular weight cDNA present
Lane 6	Fraction 10	Low molecular weight cDNA present
Lane 7	Fraction 11	Low molecular weight cDNA present
Lane 8	Fraction 12	Low molecular weight cDNA present

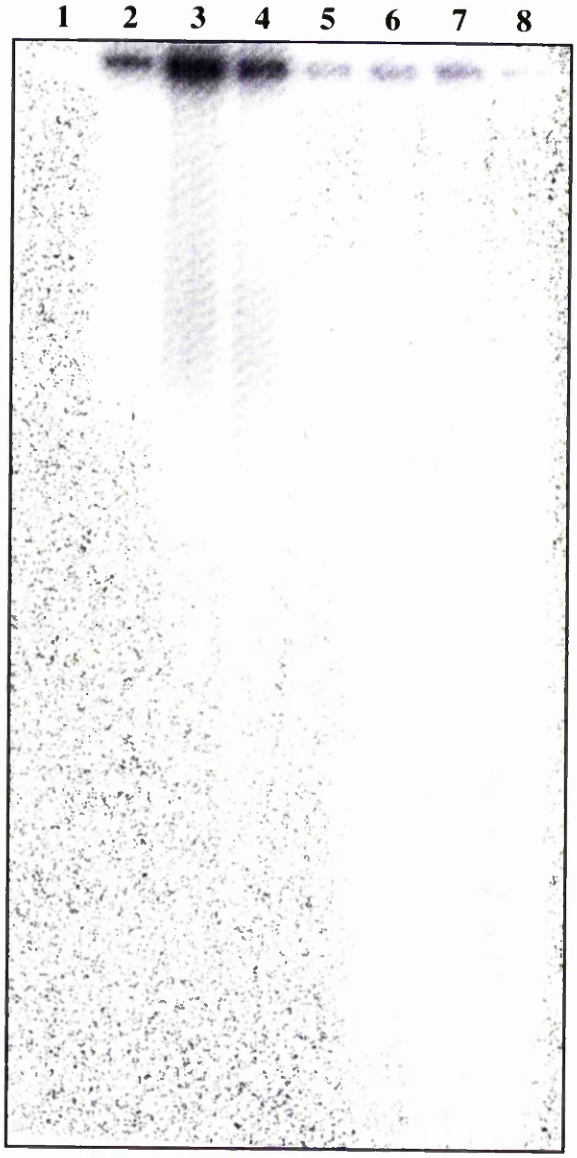
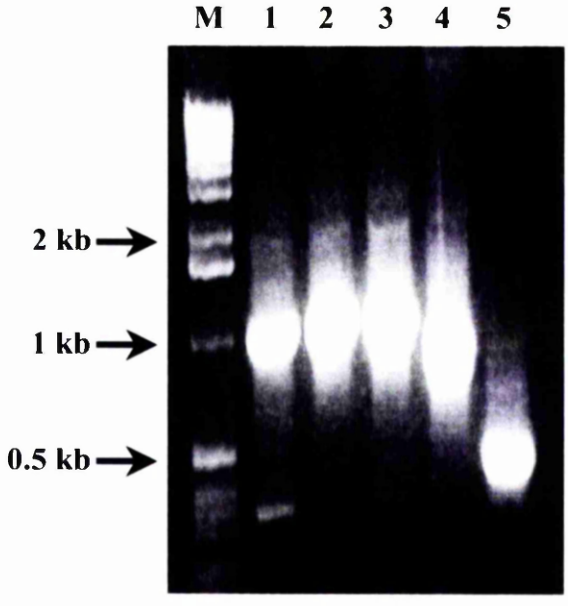
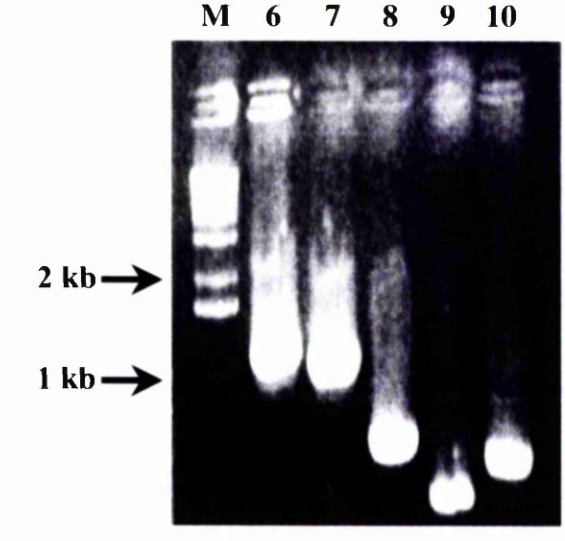

1
2
3
4
5
6
7
8

Figure 3.4 Average size of cDNA insert in the day 2 of germination library

This figure shows the products of a PCR reaction using T3 and T7 primers to amplify the cDNA inserts from random phage picked from the library.

The marker lane contains 1kb ladder. Lanes 1-10 represent randomly picked plaques as template for PCR.


M 1 2 3 4 5
2 kb
1 kb
0.5 kb


M 6 7 8 9 10
2 kb
1 kb

plates. It could also be concluded that the average insert size was around 1kb, which approximates to the average size of Arabidopsis mRNA (1.2kb). The library therefore appeared to be of high quality and was therefore amplified for use in subsequent analysis.

3.4 E.S.T. Library and 'Electronic' Northerns

The cDNA library from day 2 germinating Arabidopsis seedlings was used to synthesise an E.S.T. database (TwoDogs - 2 Days Of Germination), by sequencing the 5'end of the clones present. The sequencing of the cDNA clones was carried out by BASF, Germany. The E.S.T. database represents the results of an annotation of a set of 4007 E.S.T.s carried out by Dr. Pawel Herzyk at the Centre for Novel Agricultural Products (C.N.A.P.), University of York, U.K. The annotation was performed using the Blast2 program against non-redundant (NR) peptide and nucleotide databases available from NCBI (National Centre for Biotechnology Information) as well as Arabidopsis peptide and nucleotide sequences available from TAIR (The Arabidopsis Information Resource). The production of the database was supported by a European Community Grant (no. QLRT-1999-00213) and is currently only available for access by the members named on the grant.

An 'electronic' northern analysis was performed using the known sequences of lipid catabolism genes and also the four available E.S.T.s for the putative genes in the genomic cluster, details of which are in the table in Section 3.5. The results of this analysis are shown in Figure 3.5. As a comparison it was possible to search for E.S.T. sequences specific for these putative genes on the MIPS database (http://mips.gsf.de/proj/thal/).

From this data it can be concluded that although the databases are a valuable resource, more sequences need to be completed and added to the database to ensure finding the low expressers such as *ACX1, ACX3, ACX4*. The 4007 E.S.T.s present at the moment in the TwoDogs database are adequate to detect reasonable numbers of the more highly expressed genes such as *MS, ICL*, thiolase, *ACX2* and *PEPCK*. Failure to detect any of the four putative genes or *ACX1* suggests that a low level of all these transcripts are present in germinating tissue. This means that no conclusion can be drawn with respect to co-regulation of the genes in the putative germination cluster.

It is interesting to note that in the MIPS database *ACX1* is more abundant than *ACX2* which suggests that at other times and under different environmental conditions *ACX1* is expressed at higher levels than during germination and therefore E.S.T.s are detected. Consistant with this observation is that *ACX2* appears to be expressed mainly during germination.
The eleven thiolase clones identified in the TwoDogs database can be aligned to give an indication of the quality of the library. Due to the fact that E.S.T. databases are constructed by sequencing the 5'end of cDNA clones it is possible to observe what percentage of the clones appear to be full length sequences. The results of this sequence alignment are shown in Figure 3.6.

From this sequence alignment it can be concluded that the size fractionation of the cDNA before it was packaged has increased the abundance of full length clones in the library. This is a good indication that the library is of high quality.

Figure 3.5 Relative abundance of lipid catabolism and germination related genes identified by the E.S.T. database

This figure shows a table of the results obtained by quantitative and comparative analysis of the TwoDogs database and the MIPS database (http://mips.gsf.de/proj/thal/). This type of analysis is known as an 'electronic' northern.

ACX1, ACX2, ACX3, ACX4 and thiolase represent ß-oxidation with only ACX1 having no clones identified in the TwoDogs database.

ICL (isocitrate lyase) and MS (malate synthase) represent glyoxysome enzymes, both of which are shown to have clones present at day 2 of germination.

PEPCK (phosphoenol pyruvate carboxylase kinase) is an enzyme involved in gluconeogenesis and appears to be abundant in the TwoDogs library.

ACC oxidase, GA20 oxidase, triacylglycerol lipase and enoyl-CoA hydratase are the putative genes in the germination cluster.

NA - Not Available

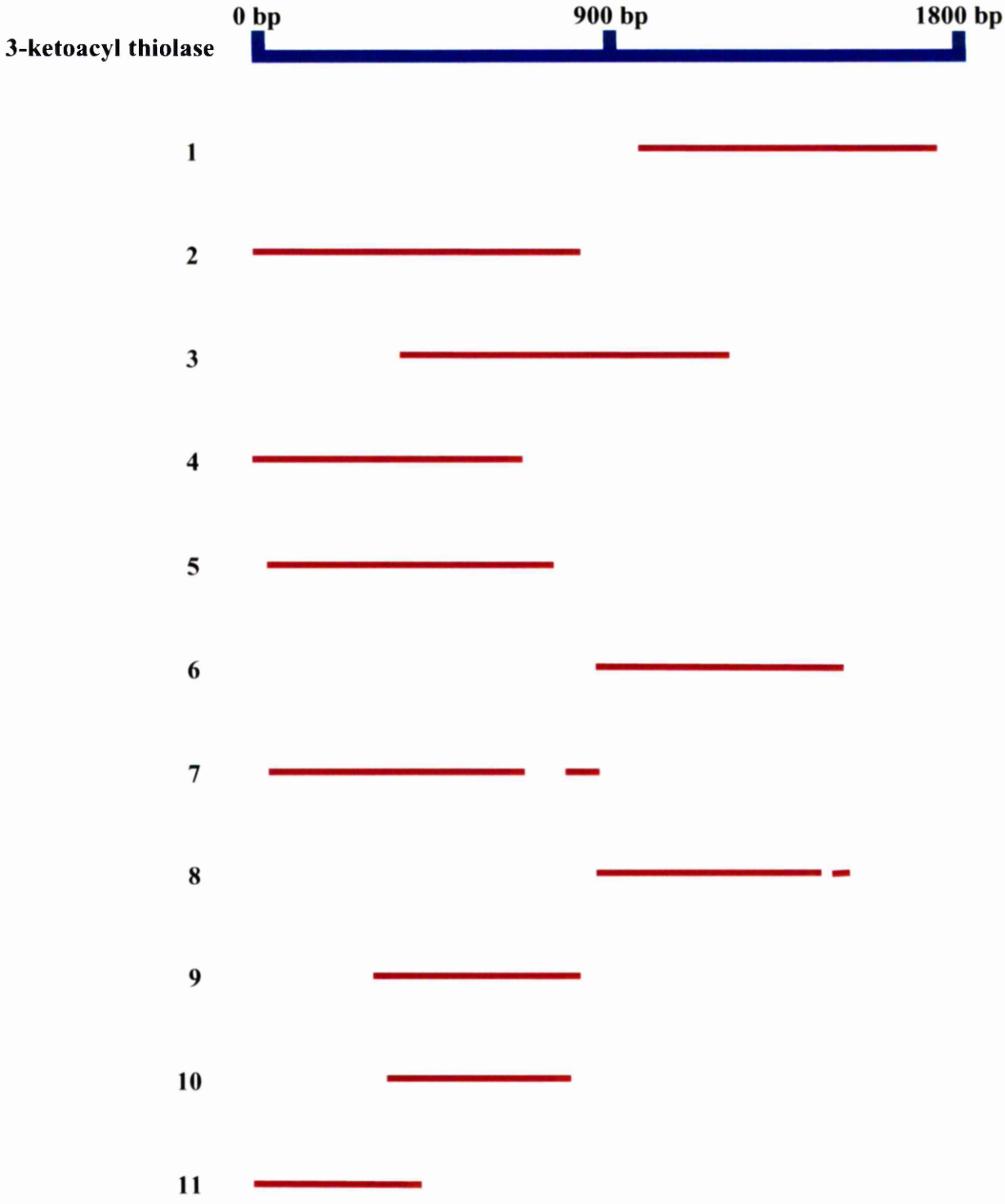
ND - Not Done

Clone Identity	Accession Number	No. of Clones Identified in TwoDogs Database	No. of Clones Identified in MIPS Database
ACX1	AF057044	0	30
ACX2	AF057043	7	17
ACX3	AF207994	2	0
ACX4	AB017643	3	0
ACX5	NA	0	2
ACX6	NA	0	2
Thiolase	AB008855	11	ND
ICL	ACEA_ARATH (swissprot)	39	ND
MS	CAB85506	7	ND
PEPCK	CAB38935	31	ND
ACC Oxidase	AA713273	0	0
GA20 Oxidase	T22434	0	0
Triacylglycerol Lipase	T76589	0	0
Enoyl CoA Hydratase	N97282	0	0

Figure 3.6 Alignment of E.S.T. thiolase clones identified in the library

This figure shows the alignment of the 11 thiolase clones identified in the library and how many of them represent full-length cDNA clones.

The size bar across the top represent the sequence of the known thiolase gene. Clones from the TwoDogs database which start at 0 represent full length clones (i.e. clones 2, 4 and 11). Clones starting further downstream from the 0 have 5' sequence missing (i.e. clones 1, 3, 5, 6, 7, 8, 9, 10).


0 bp
900 bp
1800 bp
3-ketoacyl thiolase
1
2
3
4
5
6
7
8
9
10
11

3.5 Northern Analysis for Co-Expression

The *ACX1* gene is found in the middle of a putative germination cluster as shown in Figure 3.2. As a means of trying to ascertain whether this cluster had genuine significance in terms of control of expression, the E.S.T. databases were searched for clones identical to the genomic region. Genomic sequences were used in BlastN searches of the Arabidopsis E.S.T. database (Genbank), the results of which are shown in Appendix 1.

The E.S.T. database numbers identified are shown below:

Putative Gene	**Corresponding E.S.T. Database Number**
GA 20 Oxidase	T22434
Enoyl CoA Hydratase	N97282
ACC Oxidase	AA713273
Triacylglycerol Lipase	T76589

PCR was used to amplify the inserts from the four E.S.T. clones shown in the table above. The inserts were then used as probes for northern blots. Each blot had RNA from day 0 to day 7 germinating seedlings which would have indicated if all the putative genes were co-expressed. They were first probed with *ACX1* before being probed with each of the other

sequences. After exhaustive attempts positive hybridisations with these probes appeared to be unattainable, possibly because the genes are expressed at very low levels. We were therefore unable to demonstrate co-expression of these genes.

3.6 5' RACE on Overlapping Putative Clones

It was of particular interest to characterise the 5' end of the two overlapping clones, GA20 oxidase and ACC oxidase, described in Section 3.2. Successful cloning of a 5' RACE product for the ACC oxidase and the GA20 oxidase using day 2 of germination RNA, would confirm the presence of these transcripts in germinating tissue and also be interesting with respect to the structure of the cluster.

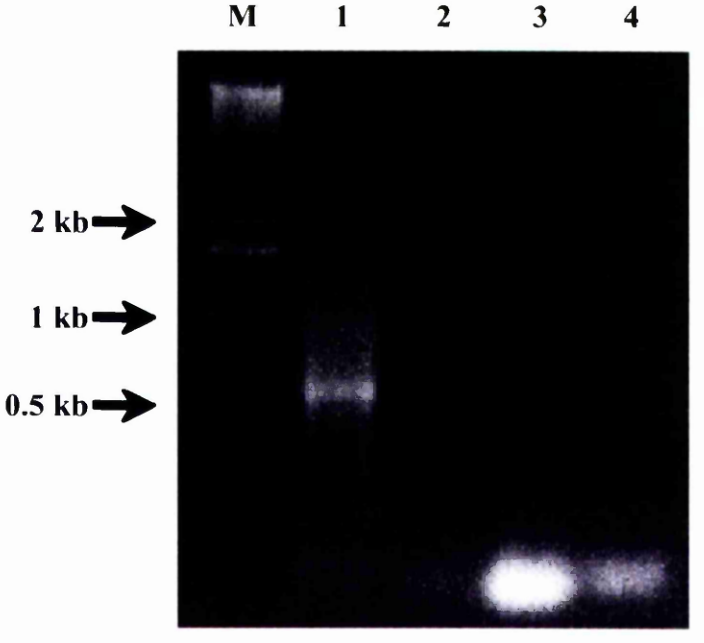
Therefore primers were designed to perform 5'RACE the sequences of which can be found in Section 2.1.8. ACCG20LG was used in the first strand synthesis and is common to both clones. G20BLG and ACCBLG were then used as nested primers in the PCR to specifically amplify the 5' end of each clone. The ACX1 reaction is described in Section 4.2 and is a positive control in this experiment. The products are visualised on a 1% agarose gel. The results of this analysis are shown in Figure 3.7.

It can be concluded from this reaction that no 5'RACE products could be produced even though the positive control of *ACX1* was used and which resulted in a product. It is possible that neither transcript is present in day 2 germinating tissue or that they are below limits of detection. RT-PCR would have been the next approach to try and detect the presence of

Figure 3.7 5' RACE

This figure shows the results of a 5' RACE experiment run on a 1% agarose gel. The lanes were loaded as follows:

Lane M	1kb ladder size marker
Lane 1	5' RACE product of the *ACX1* reaction (550 bp)
Lane 2	product of the ACX2 reaction
Lane 3	product of the ACC oxidase reaction
Lane 4	product of the GA20 oxidase reaction


M
1
2
3
4
2 kb
1 kb
0.5 kb

these transcripts but due to time constraints this was not performed.

3.7 Presence of Matrix Attachment Regions

The fact that all these genes are found in such close proximity suggests that there might be some kind of chromatin or chromosomal control exhibited over their expression. Research completed by van Drunen and co-workers (1997) on the plastocyanin gene in Arabidopsis revealed the presence of MARs in the chromatin domain and confirmed a consensus sequence specific to Arabidopsis. It was therefore possible to check the *ACX1* locus for the presence of MARs.

The consensus sequence is: TAWAWWWNNAWWRTAANNWWG; where W=A or T, R=A or G and N=A, T, C or G. Using the actual sequences used to make the consensus sequence the *ACX1* locus was searched for MARs using GCG. The results of these searches can be seen in Figure 3.8.

Although alignments were found, it can be concluded that the homology scores are not high enough to lead to any convincing conclusions. This level of homology is not surprising as the consensus sequences is very A/T rich and most plant promoters are also A/T rich.

3.8 Discussion

The experiments described in this chapter lead to the conclusion that although the identification of a putative germination gene cluster on chromosome IV is of interest, no co-regulation of the putative genes could be shown. The following observations could be made:

1) The identification of E.S.T. clones for all the putative genes in the cluster means that these genes are expressed under certain conditions.

2) The fact that the 'electronic' northern failed to identify any clones for the four putative genes or ACX1 suggests that there might be very low expression of the cluster genes. This is substantiated by the fact that no E.S.T.s were identified for these clones in the MIPS database. However, with a larger population of E.S.T. clones in the TwoDogs database, they may still be identified.

3) The representation of other lipid catabolism genes in the TwoDogs database suggests that 'electronic' northerns could be a useful tool for analysing gene expression in the future, assuming the original cDNA library is of good quality. However, with the increasing availability of microarray chips, analysis of E.S.T. libraries may not prove to be the best option.

Figure 3.8 Putative matrix attachment regions (MAR)

This figure shows sequence alignments of the *ACX1* promoter (lower strands) to the consensus sequence of Arabidopsis matrix attachment regions (upper strands).

The consensus sequence is TAWAWWWNNAWWRTAANNWWG ; where W=A or T, R=A or G and N= A,T,C or G. marcon, mar 2, mar 3 and mar 4 are examples of this consensus sequence

marcon vs. acoxprom

```
  13 taatcatttt 4
     ||||||||||
4982 TAATCATTTT 4991
```

marcon vs. acoxprom

```
   2 aaaaaatgattataaggaa 20
     ||||  ||  ||| ||| ||
2112 AAAATATTCTTACAAGCAA 2130
```

mar2 vs. acx1prom

```
   5 atttaaaagtaattttg 21
     |||  ||| ||  ||||||
 874 ATTGAAATGTGATTTTG 890
```

mar2 vs. acx1prom

```
  20 aaaattacttttaaattat 2
     |||||  |  |||||| |||
 543 AAAATAAAATTTAAAATAT 561
```

mar3 vs. acx1prom

```
   1 taaattagtaaagtaatgtag 21
     | |||||||  ||  |||||  |
 342 TTAATTAGTCAACGAATGTTG 362
```

mar3 vs. acx1prom

```
  20 tacattactttactaattta 1
     | | |  |||  |||  |||||
 115 TGCCTCACTCTACCGATTTA 134
```

mar4 vs. acx1prom

```
   2 aaataaaaatgataagaaag 21
     ||||||||||  | |   |||
 771 AAATAAAAATAAGATTTAAG 790
```

mar4 vs. acx1prom

```
  21 ctttcttatcatttttattta 1
     ||||  ||  ||  |  |||  |  ||
 218 CTTTTTTTTCTTCTTTCTATA 238
```

Chapter 4: Characterisation of the ACX1 Promoter

4.1 Introduction

It is well established that Arabidopsis makes an excellent model plant system. The small genome size, short life-span and large progeny number make it ideal for genetic characterisation. Since it is a close relative of the major crop, oilseed rape (*Brassica napus*), it is a particularly useful tool for the discovery of genes associated with oil seed metabolism. This is applicable in the case of the acyl-CoA oxidase gene family of which *ACX1* is a member.

During germination and post-germinative growth of oilseed plants there is a large mobilisation of the lipid stores to support seedling establishment (Beevers *et al.* 1958; Weir *et al.* 1980; Eastmond *et al.* 2000a). Targeting the enzymes involved in the mobilisation has become a focus of researchers involved in the transgenic manipulation of oil seed crops. The low percentage yield of designer oil yields achieved when the fatty acid biosynthetic pathway was manipulated led to the hypothesis that ß-oxidation is responsible for the futile cycling of designer oils (Voelker and Davies, 1994). Support for this hypothesis comes from enzyme analysis of transgenic oil crops which show increased acyl-CoA oxidase activity during seed development (Eccleston and Ohlrogge, 1998).

Mobilisation of oilseed lipid reserves involves the pathways of ß-oxidation, the glyoxylate cycle and gluconeogenesis. This results the eventual synthesis of sucrose, which can easily be

transported around the seedling to where it is needed. Acyl-CoA oxidase catalyses the first and possible rate limiting step of ß-oxidation (Hooks *et al.*, 1999a) and is therefore of interest in applied research into controlling fatty acid breakdown and increasing novel oil yields. Preliminary research into the expression of acyl-CoA oxidase genes found two full-length cDNAs (*ACX1* and *ACX2*) which were induced during the first day of germination (Hooks *et al.*, 1999a). ACX1 protein was found to have activity with medium-long chain fatty acyl-CoAs and ACX2 protein showed activity with long chain acyl-CoAs.

Characterisation of the transcript levels of these genes showed that they were expressed in most tissues and at high levels in young seedlings. As discussed in Chapter 3 the promoter sequence became available and oligonucleotide primers were designed to amplify the putative promoter region.

4.2 Mapping of the 5' End of the *ACX1* Transcript

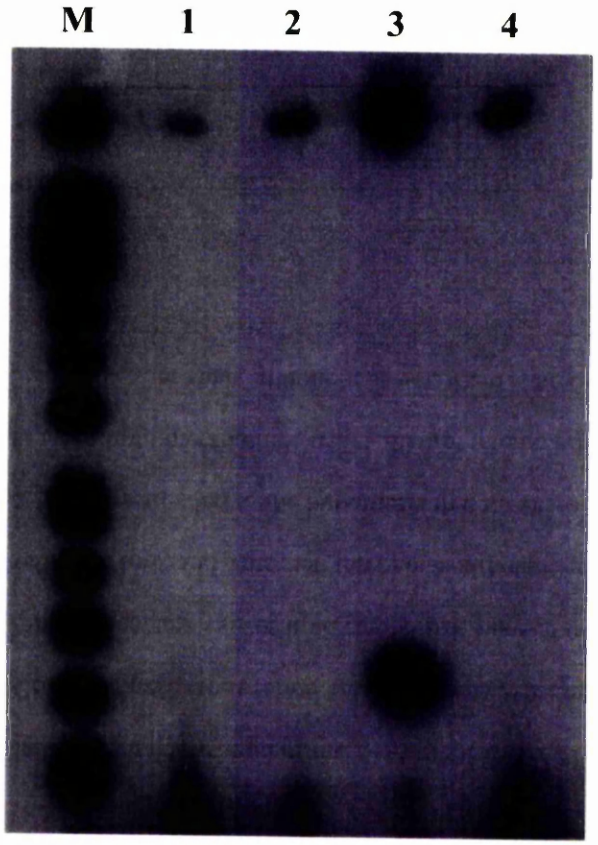
Initially, primer extension analysis was performed on polyA RNA isolated from 2-day-old seedlings and cell culture. A standard protocol as detailed in Chapter 2 was followed and modified to establish conditions that would allow a product to be synthesised. Despite extensive variations in the experimental conditions including extension time, reaction temperature, amounts of radioactive isotope and 3 different extension oligonucleotides (varied length and G-C content) the protocol did not result in a primer extension product being detected. The extension oligonucleotides were used in conjunction with upstream primers in the promoter region to successfully amplify a product therefore confirming that

Figure 4.1 Primer extension analysis of *ACX1*

This figure shows the autoradiograph produced from the polyacrylamide gel analysis of the primer extension experiment. Lanes were loaded as follows:

Lane M	RNA ladder
Lane 1	Primer extension reaction for *ACX1*
Lane 2	Primer extension reaction for *ACX2*
Lane 3	Positive control reaction provided by manufacturer
Lane 4	Empty

No products were seen for the primer extension reactions for *ACX1* and *ACX2*. A band was detected (45 bases) in the positive control reaction.


M
1
2
3
4

the specificity of the oligonucleotides was high. A commercially available primer extension kit (Promega, UK) was also used and again no product was detected. As shown in Figure 4.1 the positive control, which was supplied with the kit, yielded a detectable product.

It is possible that secondary structure existed in the RNA which interfered with the synthesis. Although a 5'RACE approach could also be affected by 3-D RNA structure, it was still a valid method to use as a cDNA template is more stable than an RNA template.

The 5'RACE was carried out as described in Section 2.24. A gene specific primer, 35H3P3, was designed against a region 1 kb downstream from the ATG codon of *ACX1*. This oligonucleotide was used to prime first strand synthesis and the RNA template was then degraded. A poly-C tail was added to the 3' end of the single-stranded cDNA to allow use of a kit primer in the PCR (Section 2.24). The kit primer contained a poly-G sequence (Abridged Anchor Primer) and was used with a second internal gene specific primer, 35H3P5 to amplify the 5' end of *ACX1*. 35H3P5 was complementary to a region 470 bases downstream from the ATG codon. This PCR product was re-amplified according to the kit protocol, with appropriate controls and is shown in Figure 4.2.

This analysis indicates that the 5' end of the *ACX1* transcript has been amplified and was able to be isolated as a product which could be cloned and sequenced to help predict a transcription start site. The control PCR reactions confirm that it is *ACX1* that was amplified and that there is cDNA sequence upstream of the ATG. The 550 bp fragment in lane one (Figure 4.2) was cloned as described in Section 2.13. After cloning, the fragment was

Figure 4.2 5'RACE analysis of the *ACX1* transcript

This figure shows the results of the 5'RACE analysis of *ACX1* amplified by PCR. The products were visualised by running on a 1% agarose gel. The 1kb ladder was run in lane M. In the reactions in lanes 1, 2 and 3, RNA from day 2 germinating seedlings was used, in lanes 4, 5 and 6 acid treated cell culture RNA was used. Lanes 1 and 4 show the results of the 5'RACE amplified using 35H3P5 and the abridged anchor primer from the kit. Lanes 2 and 5 show PCR reactions carried out using 35Hex3, which covers the ATG of *ACX1*, and the abridged anchor primer. The PCR reactions in lanes 4 and 6 were carried out using internal primers from *ACX1* which are 200 bp apart and would confirm that the 5'RACE product is indeed from *ACX1*.

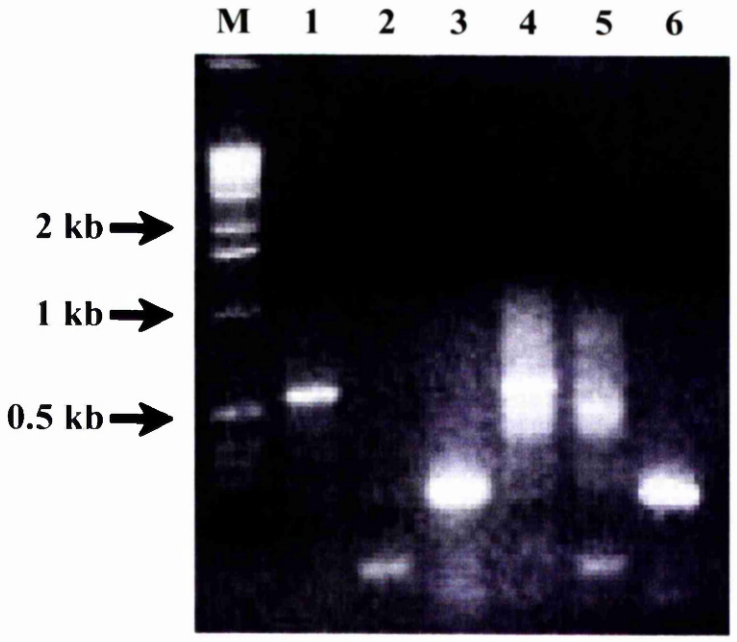

M
1
2
3
4
5
6
2 kb
1 kb
0.5 kb

Figure 4.3 Sequence of the 5'RACE product

This figure shows the nucleotide sequence of the 5'RACE product seen in lane 2 of the gel shown in figure 4.2. The nucleotides shown in black indicate the sequence of the 5'RACE product and those in red indicate vector sequence.

```
1     GATTCTGATT ACGAATCATG GAAGGAATTG ATCACCTCGC CGATGAGAGA

51    AACAAAGCAG AGTTCGACGT TGAGGATATG AAGATCGTCT GGGCTGGTTC

101   CCGCCACGCT TTTGAGGTTT CCGATCGAAT TGCCCGCCTT GTCGCCAGCG

151   ATCCGGTGTT TGAGAAAAGC AATCGAGCTC GGTTGAGTAG GAAGGAGCTG

201   TTTAAGAGTA CGTTGAGAAA ATGTGCCCAT GCGTTTAAAA GGATTATCGA

251   GCTTCGTCTC AATGAGGAAG AAGCAGGAAG ATTGAGGCAC TTTATCGACC

301   AGCCTGCCTA TGTGGATCTG CACTGGGGAA TGTTTGTGCC TGCTATTAAG

351   GGGCAGGGTA CAGAGGAGCA GCAGAAGAAG TGGTTGTCGC TGGCCAATAA

401   GATGCAGATT ATTGGGTGTT ATGCACAGAC TGAGCTTGGT CATGGCTCAA

451   ATGTTCAAGG ACTTGAGACA ACTGCCACAC A
```

sequenced (Figure 4.3).

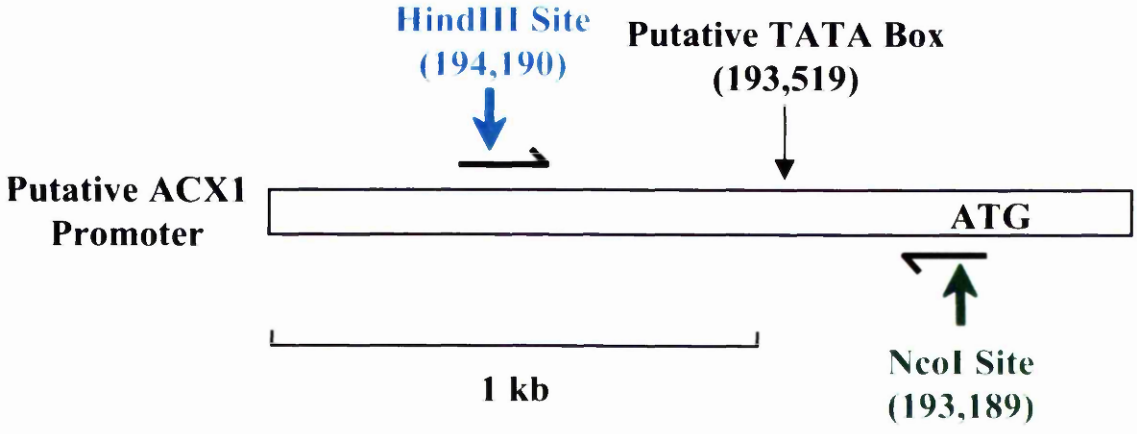
This sequence suggested a transcription start site 17 bp upstream of the ATG giving a untranslated region (UTR) of only 17 bp. At the same time this work was being performed, Dr. Hooks in the Graham lab screened cDNA libraries using a 5'prime sequence probe for the *ACX1* gene. He successfully isolated a cDNA clone with 76 bp of UTR. The new sequence was submitted to Genbank as accession number AF057044. In conclusion, 5'RACE, although it is a valuable tool, has limitations which have been clearly demonstrated in this analysis. Secondary structure can interfere with the synthesis resulting in a product that is truncated. Termination of the first strand synthesis is a limitation and does not necessarily indicate the true end of the transcript.

4.3 Identification and Amplification of ACX1 Promoter

The genome sequencing project for Arabidopsis was completed at the end of the year 2000 (The Arabidopsis Genome Initiative, 2000). The first sequence from this project was published in 1998 by Bevan *et al.* (1998) which contained sequence from a section of chromosome IV. The genomic region containing the *ACX1* gene sequence and putative promoter was annotated in this region, leading to the opportunity to clone the putative promoter without screening a library. From the genomic sequence it was possible to design oligonucleotide primers which would amplify the promoter from the ATG of *ACX1* to 1kb upstream. Primers 35Hex3 and 35H1KB that are described in Section 2.1.8 were used to amplify the 1kb region. These primers also contained restriction enzyme site sequences

Figure 4.4 *ACX1* Promoter Sequence

This figure shows the positions of the primers relative to the *ACX1* putative promoter sequence. The primers were used to amplify the putative promoter sequence from genomic DNA. The Hind III site (shown in blue) and the NcoI site (shown in green) engineered into the primers are indicated. The putative ATG and TATA box sequences are given. The numbers shown indicate the position on the BAC ATFCA6.


HindIII Site
(194,190)
Putative TATA Box
(193,519)
Putative ACX1
Promoter
ATG
1 kb
NcoI Site
(193,189)

engineered into them to allow easier cloning of the fragment into an intermediate vector. A schematic diagram of the promoter region and the primers used are shown in Figure 4.4.

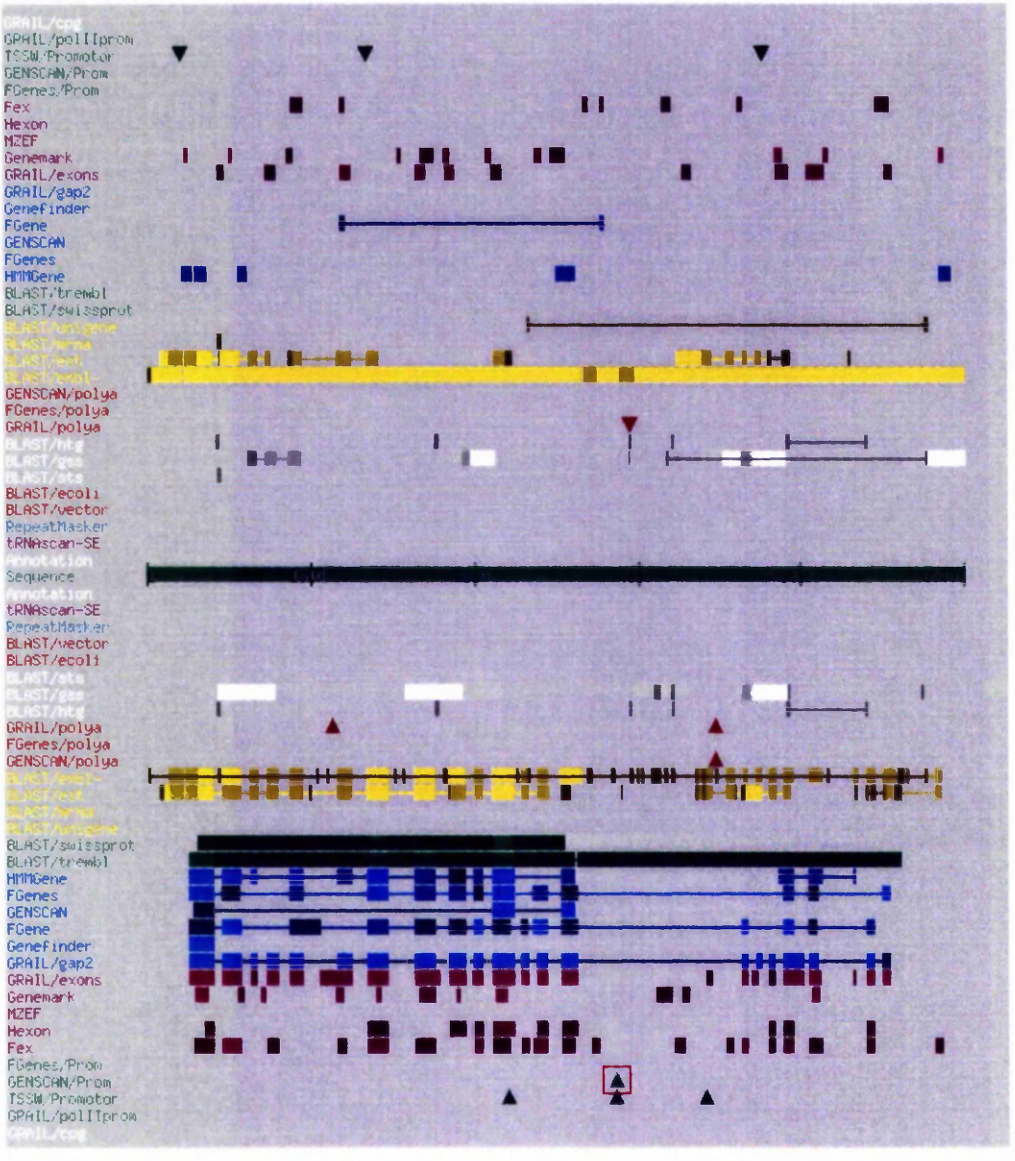
Sequence analysis of the 1kb putative *ACX1* promoter, using NIX from the UK HGMP Resource Centre, identified a possible TATA box at 330bp upstream from the ATG. The actual software analysis and sequence element found by the software is highlighted in Figure 4.5. Further sequence comparison searches revealed no other common promoter motif at that time.

PCR reactions were performed with both Taq and the high fidelity polymerase Turbo Pfu (Promega, UK). Since it was important to isolate a promoter that contained no errors it was desirable to use the high fidelity polymerase. PCR with Taq polymerase generated the appropriate size fragment from two different samples of genomic DNA (Figure 4.6). However, despite changing every parameter in the PCR reaction Turbo Pfu did not amplify the promoter fragment using the same genomic DNA. Turbo Pfu was active under these conditions since it amplified products from other primers on the same DNA samples. A possible explanation is that Turbo Pfu is a lot larger than Taq because it has a proof-reading domain and perhaps there is secondary DNA structure present which interferes with Pfu access to the DNA sequence. After a near exhaustive attempt to establish products that would yield a product it was decided to use digested genomic DNA to overcome the potential problem of DNA secondary structure. EcoRI and BamHI restriction enzymes were used and based on the genomic sequence were expected to give a 1.4kb fragment containing the *ACX1* promoter. This approach was successful in generating a PCR product of 1kb using

Figure 4.5 Analysis of the *ACX1* Putative Promoter

This figure shows the results of a NIX analysis with the aim of identifying putative promoter regions in the putative oxidase cluster 8.04 kb genomic sequence. Further details of NIX analysis can be found at the website of the UK HGMP Resource Centre.

GENSCAN identified one putative TATA box (represented as a green triangle highlighted by a red box) at position 4588 bp (forward sense). The nucleotide sequence identified in this way is also shown.



(Genscan promoter) Score=4.42

Start: **4588**, End: **4627** (of 8040) (counting from the forward sense positions)

Start: **3414**, End: **3453** (counting from the reverse sense positions)

Strand: **negative**

Quality of prediction: **good**

TGGAATAATGTGATTTTTTCCCCCTCATATATATATTTGA

Figure 4.6 Taq Polymerase Amplification of the ACX1 Putative Promoter

This figure shows the products of a single set of PCR reactions to amplify the putative ACX1 promoter. The reactions run in lanes 1-6 used Turbo Pfu as the polymerase and those in lanes 7-12 used Taq polymerase. Lanes 1, 2, 3, 7, 8 and 9 had Arabidopsis Col 0 genomic DNA (preparation 1) in the reaction. Lanes 4, 5, 6, 10, 11 and 12 had Arabidopsis Col 0 genomic DNA (preparation 2) in the reaction.

Lanes 1, 2, 4, 5, 7, 8, 10 and 11 contained the primers for *ACX1* promoter amplification in the reaction.

Lanes 3, 6, 9 and 12 contained control primers complementary to sequences 800 bp apart, to confirm the integrity of the DNA. Each of these reactions produced a 800 bp fragment.

Lanes 7, 8 and 11 contained a 1 kb fragment which had been amplified using Taq. No 1 kb fragments were amplified by Turbo Pfu.

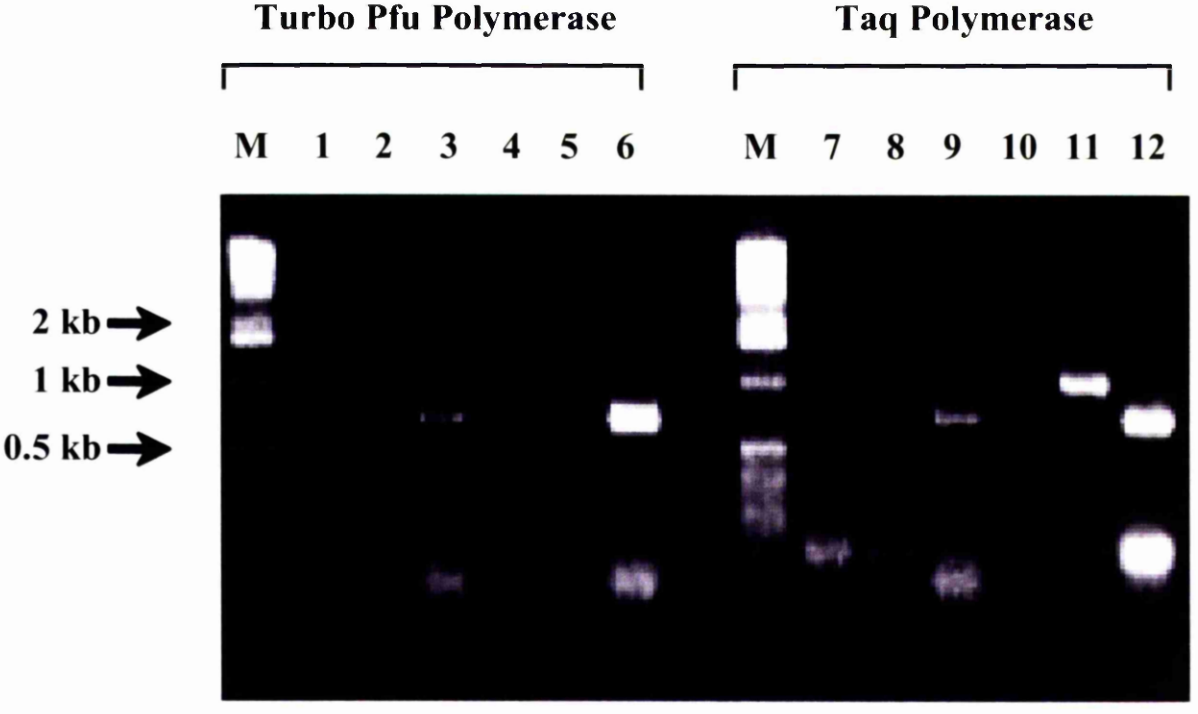
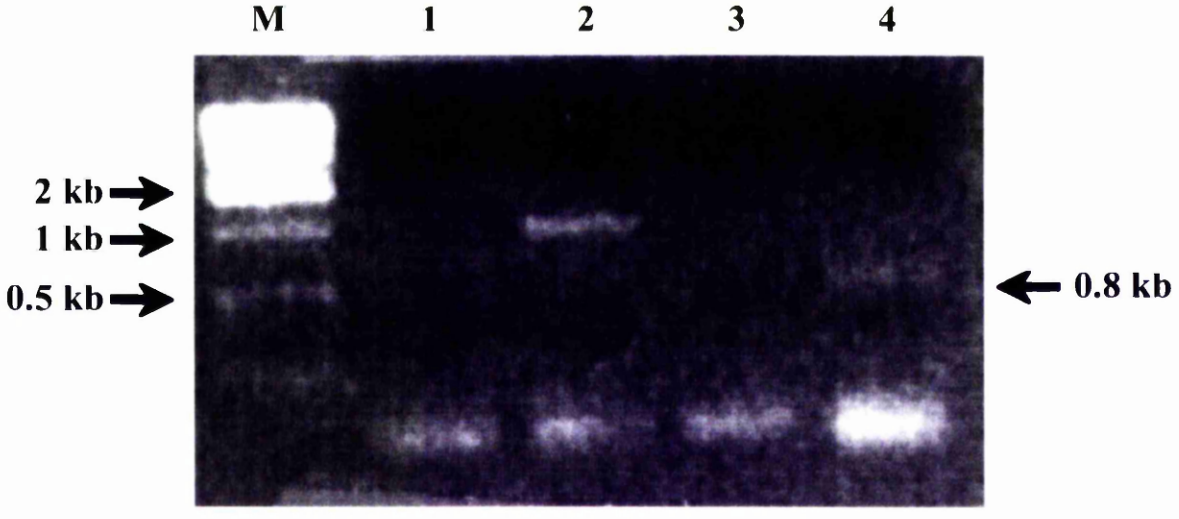

Turbo Pfu Polymerase
Taq Polymerase
M 1 2 3 4 5 6
M 7 8 9 10 11 12
2 kb
1 kb
0.5 kb

Figure 4.7 Turbo Pfu Amplification of the *ACX1* Putative Promoter

This figure shows the results of PCR amplification (of restriction digested genomic DNA) of the putative *ACX1* promoter and the product of a positive control reaction.

Lanes 1, 2 and 3 contained the primers for *ACX1* promoter amplification (35Hex3 and 35H1kb) in the reaction. A 1 kb product was amplified in lane 2 only.

Lane 4 contains the products of the control reaction control primers (complementary to sequences 800 bp apart) to confirm the integrity of the DNA. This positive control has produced a product of the expected size.


M
1
2
3
4
2 kb
1 kb
0.5 kb
0.8 kb

Turbo Pfu polymerase.The results of this experiment are shown in Figure 4.7.

4.4 Cloning into Intermediate Vectors and Confirmation of Sequence

In order to ensure that a promoter fragment with no PCR-generated errors was used for further studies it was decided to clone 3 products from 3 separate PCR reactions, sequence these and confirm their sequence against each other and the genomic sequence. Three clones were generated in PCRscript (Stratagene, UK) and commercially sequenced. The alignment of these sequences with the genomic sequence is shown in Figure 4.8.

Differences between the published genomic sequence and the amplified fragments which were consistent in all three fragments, were taken to be a true representation of the sequence. Both fragments 1 and 2 contained errors where they had a base difference compared to the other three sequences. Fragment 3 did not contain any errors based on the criterion that at a given position the authentic sequences will be present in three out of the four sequences. It should be noted that there are a few differences between the genomic sequence and the 3 PCR clones. As the 3 PCR clones were in agreement with each other we concluded that the error was in the genomic sequence. It is interesting to note that fragment 2 contains a deletion in the region where the putative TATA box is located. The fact that the sequence continues perfectly after this deletion suggests that there may have been a loop structure formed which could prevent the access of proteins, although the Taq polymerase was able to pass by it.

Figure 4.8 Sequence alignment of the three promoter PCR products

This figure shows the alignment of the three promoter fragments with the published genomic sequence. The putative 3AF1 box (transcription factor binding site) is highlighted by a magenta box in all four sequences. Nucleotide substitutions, insertions and deletions are shown in red. Consensus sequences are shown in blue. Sequences highlighted in green represent a putative AGTA repeat. Multiple nucleotide deletions are represented by / symbols. The transcriptional start site (ATG) is shown in bold.

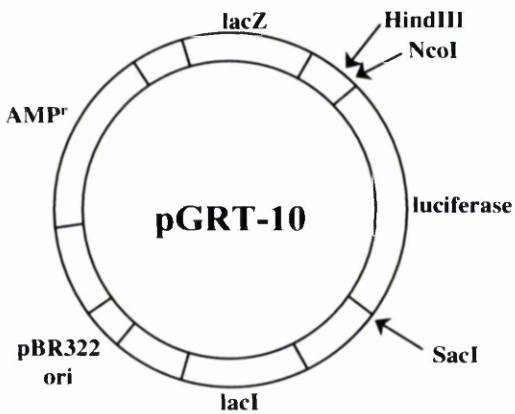

Genomic CAC
frag 3 AAGCTTTTAA CTATACTTTG AAGGAAACAC
frag 2 ACAC
frag 1 AAAACAC
Genomic AAATATTAAT CAAGTTTTAC CATACAGTTG ACAAGCAAAT GCATTTGACA
frag 3 AAATATTAAT CAAGTTTTAC CATACAGTTG ACAAGCAAAT GCATTTGACA
frag 2 AAATATTAAT CAAGTTTTAC CATACAGTTG ACAAGCAAAT GCATTTGACA
frag 1 AAATATTAAT CAAGTTTTAC CATACAGTTG ACAAGCAAAT GCATTTGACA
Genomic GACACAAAAA CACAAATGAA TGTTGACATA GACCATACCC AAAATCACAT
frag 3 GACACAAAAA CACAAATGAA TGTTGACATA GACCATACCC AAAATCACAT
frag 2 GACACAAAAA CACAAATGAA TGTTGACATA GACCATACCC AAAATCACAT
frag 1 GACACAAAAA CACAAATGAA TGTTGACATA GACCATACCC AAAATCACAT
Genomic TTCAATATCA AAATCTATAT ATATATATAT ATATATTTAT GTATTTTCGT
frag 3 TTCAATATCA AAATCTATAT ATATATATAT ATATATTTAT GTATTTTCGT
frag 2 TTCAATATCA AAATCTATAT ATATATATAT ATATATTTAT GTATTTTCGT
frag 1 TTCAATATCA AAATCTATAT ATATATATAT ATATATTTAT GTATTTTCGT
Genomic TATTATTTGA ACCTGCAAAA CGCAGTCACC ATTCGGGCCC TTAAATCTTA
frag 3 TATTATTTGA ACCTGCAAAA CGCAGTCACC ATTCGGGCCC TTAAATCTTA
frag 2 TATTATTTGA ACCTGCAAAA CGCAGTCACC ATTCGGGCCC TTAAATCTTA
frag 1 TATTATTTGA ACCTGCAAAA CGCAGTCACC ATTAGGGCCC TTAAATCTTA
Genomic TTTTTATTTT CTTCGAAATG TGCATAACGC TACTATACCT GCAAAGTTGG
frag 3 TTTTTATTTT CTTCGAAATG TGCATAACGC TACTATACCT GCAAAGTTGG
frag 2 TTTTTATTTT CTTCGAAATG TGCATAACGC TACTATACCT GCAAAGTTGG
frag 1 TTTTTATTTT CTTCGAAATG TGCATAACGC TACTATACCT GCAAAGTTGG
Genomic AACAAAATAC TATTTTAAAC ACAAGATAAC AAAACATTAT CTTATCTTCA
frag 3 AACAAAATAC TATTTTAAAC ACAAGATAAC AAAACATTAT CTTATCTTCA
frag 2 AACAAAATAC TATTTTAAAC ACAAGATAAC AAAACATTAT CTTATCTTCA
frag 1 AACAAAATAC TATTTTAAAC ACAAGATAAC AAAACATTAT CTTATCTTCA
Genomic ATCTAACTAT TTTATCTTCT TTTTATCAAC ATGTTCTTCA GATGCATGCA
frag 3 ATCTAACTAT TTTATCTTCT TTTTATCAAC ATGTTCTTCA GATGCATGCA
frag 2 ATCTAACTAT TTTATCTTCT TTTTATCAAC ATGTTCTTCA GATGCATGCA
frag 1 ATCTAACTAT TTTATCTTCT TTTTATCAAC ATGTTCTTCA GATGCATGCA
Genomic TTTACTAATT CATATGGAAA CACATATGGG AAAAAGAAAA AAAAATTATCC
frag 3 TTTACTAATT CATATGGAAA CACATATGGG AAAAAGAAAA AAAAATTATC
frag 2 TTTACTAATT CATATGGAAA CACATATGGG AAAAAGAAAA AAAAATTATC
frag 1 TTTACTAATT CATATGGAAA CACATATGGG AAAAAGAAAA AAAAATTATC
Genomic ACACATCTGA TTTAACGATA TTTTAAATTT TATTTTAGTA CACAAATACT
frag 3 ACACATCTGA TTTAACGATA TTTTAAATTT TATTTTAGTA CACAAATACT
frag 2 ACACATCTGA TTTAACGATA TTTTAAATTT TATTTTAGTA CACAAATACT
frag 1 ACACATCTGA TTTAACGATA TTTTAAATTT TATTTTAGTA CACAAATACT
Genomic GAAATACAAC CACTTTTTGT CTGTCAATTA TCCTTTGTTA TACAGCATAT
frag 3 GAAATACAAC CACTTTTTGT CTGTCAAATA TCCTTTGTTA TACAGCATAT
frag 2 GAAATACAAC TACTTTTTGT CTGTCAAATA TCCTTTGTTA TACAGCATAT
frag 1 GAAATACAAC CACTTTTTGT CTGTCAAATA TCCTTTGTTA TACAGCATAT
Genomic AAAATTTTCT ATACGTAGAT AT CCTTTTT TTCTTAGTCA AATATATATA
frag 3 AAAATTTTCT ATACGTAGAT ATTCCTTTTT TTCTTAGTCA AATATATATA
frag 2 AAAATTTTCT ATACGTAGAT ATTCCTTTTT TTCTTAGTCA AATATATATA
frag 1 AAAATTTTCT ATACGTAGAT ATTCCTTTTT TTCTTAGTCA AATATATATA
Genomic TGAGGGGGAA AAAATCACAT TATTCCATAC TTGCGAGGTT TTATTT GCA
frag 3 TGAGGGGGAA AAAATCACAT TATTCCATAC TTGCGAGGTT TTATTTTGCA
frag 2 CGAGGGGGAA AAAATCACAT TATTCCATAC TTGCGATGTT TTATTTTGCA
frag 1 TGAGGGGGAA AAAATCACAT TATTCCATAC TTGCGAGGTT TTATTTTGCA

```
Genomic AACAACTTAA TTTGTTTTCA ACATTCGTTG ACTAATTAAC TTATATTAGG
frag 3  AACAACTTAA TTTGTTTTCA ACATTCGTTG ACTAATTAAC TTATATTAGG
frag 2  AACAACGTAA TTTGTTTTCA ACATTCGTTG ACTAA///// //////////
frag 1  AACAACTTAA TTTGTTTTCA ACATTCGTTG ACTAATTAAC TTATATTAGG

Genomic AATATATAAT CCAAAATTTA TTATTTTCCA CGTGGGTTTT ATCTATGTCG
frag 3  AATATATAAT CCAAAATTTA TTATTTTCCA CGTGGGTTTT ATCTATGTCG
frag 2  ////////// //AAAATTTA TTATTTTCCA CGTGGGTTTT ATCTATGTCG
frag 1  AATATATAAT CCAAAATTTA TTATTTTCCA CGTGGGTTTT ATCTATGTCG

Genomic GAGAGTGGAT CTGAGAAAAA GATAAACCCA AAATATATTA ATTATAGAAA
frag 3  GAGAGTGGAT CTGAGAAAAA GATAAACCCA AAATATATTA ATTATAGAAA
frag 2  GAGAGTGGAT CTGAGAAAAA GATAAACCCA AAATATATTA ATTATAGAAA
frag 1  GAGAGTGGAT CTGAGAAAAA GATAAACCCA AAATATATTA ATTATAGAAA

Genomic GAAGAAAAAA AA GAGTCTA TATTTAAATC AATTTGTCTT TCTCTTCTTT
frag 3  GAAGAAAAAA AAAGAGTCTA TATTTAAATC AATTTGTCTT TCTCTTCTTT
frag 2  GAAGAAAAAA A  GAGTCTA TATTTAAATC AATTTGTCTT TCTCTTCTTT
frag 1  GAAGAAAAAA AA GAGTCTA TATTTAAATC AATTTGTCTT TCTCTTCTTT

Genomic TATCTCCGAA ATAAAAAGCT TAATTGTTCT TCCAACTACT CTGCAATTAA
frag 3  TATCTCCGAA ATAAAAAGCT TAATTGTTCT TCCAACTACT CTGCAATTAA
frag 2  TATCTCCGAA ATAAAAAGCT TAATTGTTCT TCCAACTACT CTGCAATTAA
frag 1  TATCTCCAAA ATAAAAAGCT TAATTGTTCT TCCAACTACT CTGCAATTAA

Genomic ATCGGTAGAG TGAGGCATTG TCCTTCTTTT TCTCCCTCTG TTTTTTTTTTTTT
frag 3  ATCGGTAGAG TGAGGCATTG TCCTTCTTTT TCTCCCTCTG TTTTTTTTTT
frag 2  ATCGGTAGAG TGAGGCATTG TCCTTCTTTT TCTCCCTCTG TTTTTTTTTTT
frag 1  ATCGGTAGAG TGAGGCATTG TCCTTCTTTT TCTCCCTCTG TTTTTTTTTT

Genomic TTTTTCCTAT CATCTCTGAG AGTTTTCTCG AGAAACTTTT GAGTGTTTAG
frag 3  TTTTTCCTAT CATCTCTGAG AGTTTTCTCG AGAAACTTTT GAGTGTTTAG
frag 2  TTTTTCCTAT CATCTCTGAG AGTTTTCTCG AGAAGCTTTT GAGTGTTTAG
frag 1  TTTTTCCTAT CATCTCTGAG AGTTTTCTCG AGAAACTTTT GAGTGTTTAG

Genomic CTACTAGATT CTGAATTACG AACCATGG
frag 3  CTACTAGATT CTGAATTACG AACCATGG
frag 2  CTACTAGATT CTGAATTACG AACCATGG
frag 1  CTACTAGATT CTGAATTACG AACCATGG
```

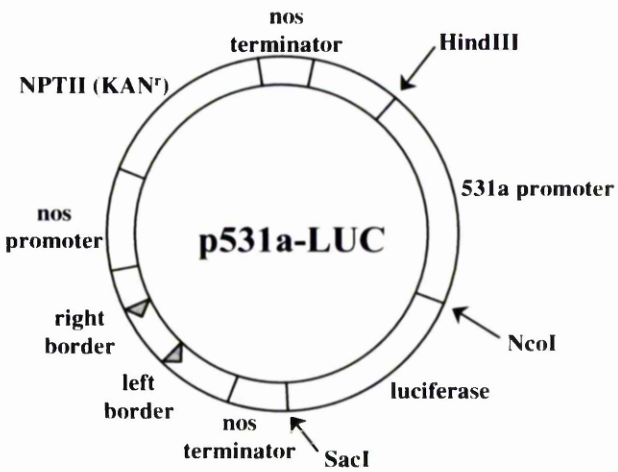
Figure 4.9 Intermediate and binary vectors used in the study

This figure shows a map of the plasmids pGRT-10, p531a-LUC and pALB1, the final vector used to transform plants. All the relevant restriction enzyme cutting sites are shown along with antibiotic resistance genes, promoters and terminators.

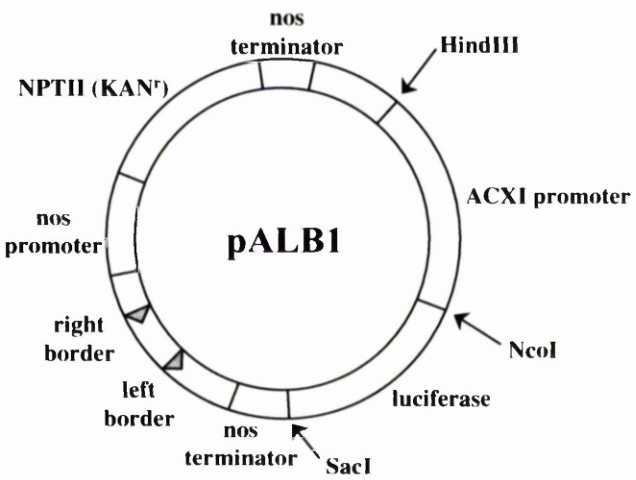
pALB1 is the final construct as it was designed. In this case pALB1 had pUC18 vector sequence inserted at the SacI site which was confirmed by pALB1 being resistant to ampicillin.



Intermediate Vector
(pUC18-derived)



Binary Vector
(pBI101-derived)



Final Construct

Fragment 3 was chosen for further studies and cloned into the intermediate pGRT-10 vector and finally into the binary vector (p531a-LUC) for transformation of *A. tumifaciens.*

The cloning strategy which we used in collaboration with Dr. Alison Baker's laboratory in Leeds involved an intermediate vector, pGRT-10, which contained the luciferase coding region in a pUC based vector (Figure 4.9). From this, the entire *ACX1*-luciferase fragment was cut out and ligated into the binary vector, p531a-luc.

The promoter fragment was cut out of the pCRscript vector using NcoI and HindIII.restriction enzymes The restriction sites were present on the oligonucleotide primers used to amplify the promoter fragment. The digest was run on a 1% agarose gel and the resulting 1kb fragment was then excised from the gel and cleaned up using the Quiagen kit as described in Section 2.11. The clean fragment was then ligated into pGRT that had been prepared in the same manner. XL1-Blue cells were transformed as described in Section 2.9 before being plated on ampicillin plates. Putative clones were identified by colony hybridisations, as described in Section 2.14, using the original amplified promoter fragment as the probe. After aligning positive spots on the film to the original transformation plates, putative clones were picked and streaked out on new ampicillin plates. Restriction digests were used to confirm that these colonies were genuine transformants. The results of one of these digests (clone AL-2) is shown in Figure 4.11.

From this analysis it can be seen that the promoter had been successfully cloned into the intermediate vector, clone number AL2 (ACOX-luciferase 2) was chosen for further cloning

Figure 4.10 Confirmation of the cloning of the *ACX1* promoter into the vector containing luciferase

This figure shows the nucleotide sequence of plasmid construct pAL-2 determined using primer 35H500. The luciferase coding sequence is shown in red. The ATG sequence is presented in bold and the *ACX1* promoter sequence is shown in black. This sequence provides confirmation that the *ACX1* promoter was successfully cloned in frame with the luciferase reporter sequence.

The blue star indicates the NcoI site which was used in the cloning of the putative promoter to the luciferase reporter.

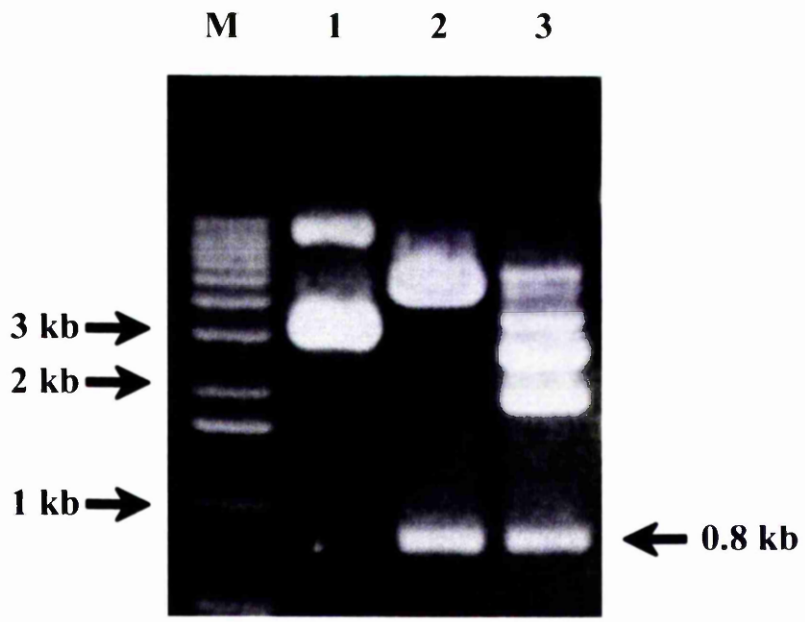
```
  1  TCTAGAGGAT AGAATGGCGC CGGGCCTTTC TTTATGTTTT TGGCGTCTTC
     AGATCTCCTA TCTTACCGCG GCCCGGAAAG AAATACAAAA ACCGCTGAAG

        *
 51  CATGGTTCGT AATTCAGAAT CTAGTAGCTA AACACTCAAA AGTTTCTCGA
     GTACCAAGCA TTAAGTCTTA GATCATCGAT TTGTGAGTTT TCAAAGAGCT

101  GAAAACTCTC CGAGATGATA GGAAAAAAAA AAAAAAACAG AGGGAGAAAA
     CTTTTGAGAG GCTCTACTAT CCTTTTTTTT TTTTTTTGTC TCCCTCTTTT

151  AGAAGGACAA TAGCCTCACT CTACCGATTT AATTGCAGAG TAGTTGGAAG
     TCTTCCTGTT ATCGGAGTGA GATGGCTAAA TTAACGTCTC ATCAACCTTC

201  AACAATTAAG CTTTTTATTT CGGAGATAAA AGAAGAGAAA GACAAATTGA
     TTGTTAATTC GAAAAATAAA GCCTCTATTT TCTTCTCTTT CTGTTTAACA

251  TTTAAATATA GACTCTTTTT TTTCTTCTTT CTATAATTAA TATATTTTGG
     AAATTTATAT CTGAGAAAAA AAAGAAGAAA GATATTAATT ATATAAAACC

301  GTTTATCTTT TTCTCAGATC CACTCTCCGA CATAGATAAA ACCCACGTGG
     CAAATAGAAA AAGAGTCTAG GTGAGAGGCT GTATCTATTT TGGGTGCACC

351  AAAATAATAA ATTTTGGATT ATATATTCCT AATATAAGTT AATTAGTCAA
     TTTTATTATT TAAAACCTAA TATATAAGGA TTATATTCAA TTAATCAGTT

401  CGAATGTTGA AAACAAATTA AGTTGTTTGC AAAATAAAAC CTCGCAAGTA
     GCTTACAACT TTTGTTTAAT TCAACAAACG TTTTATTTTG GAGCGTTCAT

451  TGGAATAATG TGATTTTTTC CCCCTCATAT ATATATTTGA
     ACCTTATTAC ACTAAAAAAG GGGGAGTATA TATATAAACT
```

Figure 4.11 Digest of the plasmid AL-2 containing *ACX1* fused to luciferase

This figure shows a 1% agarose gel loaded as follows:

Lane M	1 kb DNA ladder
Lane 1	pAL-2 digested with SacI
Lane 2	pAL-2 digested with HindIII
Lane 3	pAL-2 digested with SacI and HindIII

The 0.8 kb band was an unexpected product of the digests run in lanes 2 and 3. Subsequenct analysis showed that this product was generated due to an additional HindIII site in the *ACX1* promoter sequence.


M
1
2
3
3 kb
2 kb
1 kb
0.8 kb

reactions. To confirm that the promoter and the ATG of the luciferase had fused without inserting or deleting bases, the ligation site was sequenced using the 35H500 primer. The sequence was shown in Figure 4.10.

The sequence analysis confirmed that the NcoI site ATG translational fusion contained no errors therefore it can be concluded that the authentic open reading frame of luciferase is intact.

4.5 Cloning of *ACX1* Promoter-Luciferase Cassette Into Binary Vector

The vector diagrams in Figure 4.9 show that the *ACX1* promoter-luciferase fusion could easily be excised using the restriction enzymes HindIII and SacI to give a fragment of 2.8kb in size. This DNA fragment could then be ligated into the binary vector (p351a-Luc) which had been digested with the same restriction enzymes. When the AL-2 plasmid was digested there were bands at 800 bp and 2kb (Figure 4.11).

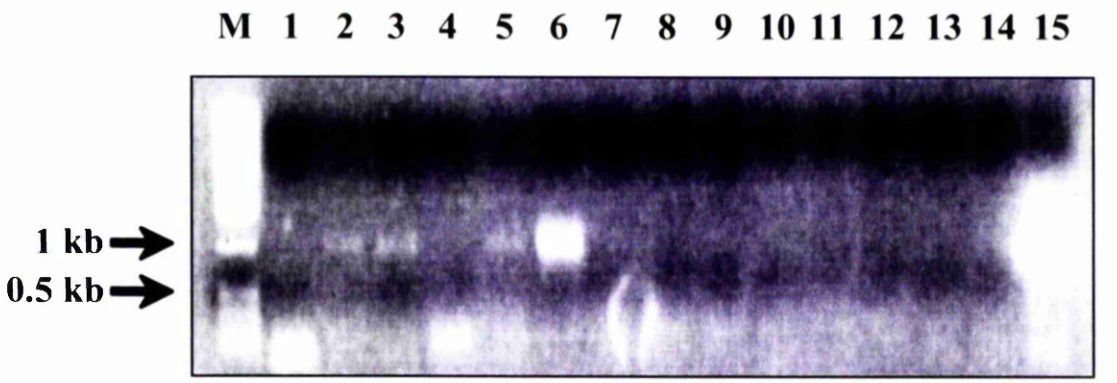
Inspection of the putative promoter sequence revealed a second HindIII site which accounts for the 800 bp fragment and the 2kb fragment seen in Figure 4.11. Due to this, further cloning was not as straightforward as planned. In order to clone the full 2.8kb fragment it was decided to try partial digests of the AL-2 plasmid. This proved to be impossible as even when HindIII was added for only the last five minutes of the digest the promoter fragments were digested to completion.

Figure 4.12 PCR showing the presence of the *ACX1* promoter in the binary vector

This figure shows a 1% agarose gel on which the products of PCR reactions run to identify which clones contained the binary vector with the ACX1 promoter-luciferase cassette. All PCR reactions shown were performed using the 35Hex3 and 35H1kb primers which originally amplified the putative promoter fragment. The lanes were loaded as follows:

Lane M	1 kb DNA ladder
Lanes 1-15	PCR products from 15 different randomly selected colonies

Lanes 2, 3, 5 and 6 contained PCR products of 1 kb in size, indicating that the plasmid containing the ACX1 promoter-luciferase cassette was present in these colonies.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 kb
0.5 kb

After many attempts at this cloning strategy it was decided to set up the digest and take an aliquot from it to put directly into a ligation reaction with the binary vector. The ligated binary vector was first used to transform XL1-Blue cells as subsequent analysis of the vector would be easier in *E. coli* than in *A.tumifaciens.* It was considered important to demonstrate that any kanamycin resistant colonies contained the whole 1kb of putative promoter, this was achieved by performing colony PCR using the 35Hex3 and 35H1kb oligonucleotide primers. The results of colony PCR carried out on ten random kanamycin resistant colonies are shown in Figure 4.12.

It can be seen in this figure that 4 out of the 10 colonies contained the full 1kb of putative promoter. In this case all four were digested with HindIII, NcoI and SacI to confirm that the entire *ACX1*-Luciferase element was present.

4.6 Transformation of Agrobacterium and Subsequent Transformation of Arabidopsis

Once it was confirmed that the binary vector contained the ACX1-Luciferase fusion, the clone from lane 2 (Figure 4.12) was named pALB-2. pALB-2 was transformed into *Agrobacterium tumifaciens* strain GV3101 using the direct transformation method (Bechtold *et al.,* 1998) described in Section 2.26. After 3 days putative positive transformants could be seen on the Kanamycin-Gentamycin selective plates. These colonies were picked and cultured in LB media for plasmid purification as described in Section 2.6. Restriction digest analysis was performed to determine whether the transformed *A. tumefaciens* clones

contained the intact vector. The results of this analysis are shown in Figure 4.13.

From this analysis it was clear the vector was intact in clone pALB-2, although there was an extra band of 2.8kb. This was suspected to be the pGRT-10 vector attached to the 3'end of the luciferase sequence. To confirm that the band was pGRT-10, the transformed *Agrobacterium* was plated on ampicillin media. Only if pGRT-10 was present would the clones be ampicillin resistant. The colonies grew, confirming that the additional 2.8kb fragment carries the gene conferring amp^{res} and this is likely to be the 2.8kb pUC plasmid. This was not likely to cause expression problems as adding sequence to the 3' end of the luciferase gene in the past had not interfered with activity (personal communication, Dr. P. Gilmartin, University of Leeds).

Arabidopsis thaliana, ecotype Col 0, plants were grown as described in Section 2.3.3 before being transformed with clone pALB2 using vacuum infiltration as described in Section 2.27. These plants were incubated overnight in low light and high humidity before being transferred back to growth rooms to be grown for seed collection.

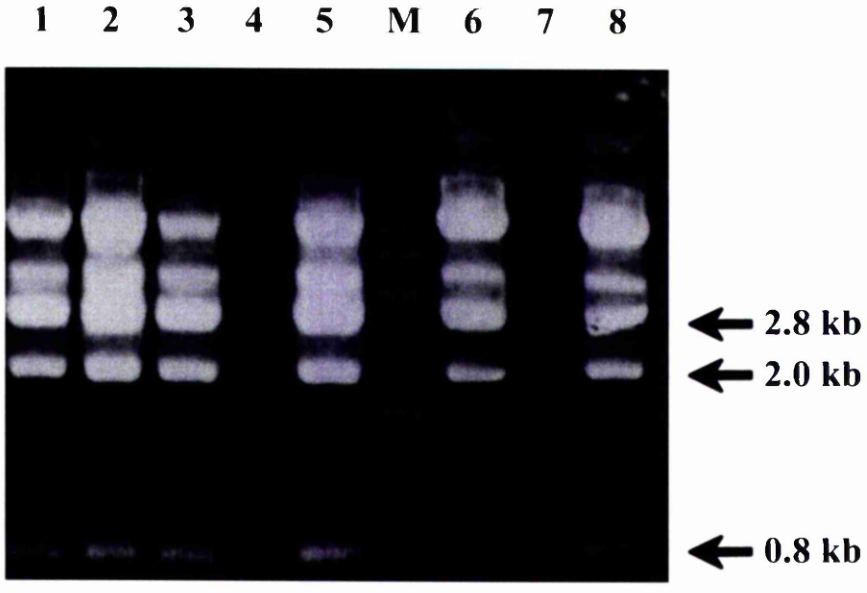
4.7 Analysis of Primary Transformants

Six separate seed batches were collected from the six pots of vacuum infiltrated plants. Seeds were then surface sterilised as described in Section 2.3.1 and imbibed (dark and 4°C) for 2 days in 0.1% agarose. The putative transgenic seeds were then spread evenly on large 1/2 MS agar plates containing the selection antibiotic, Kanamycin (50µg/ml) and germinated under the

Figure 4.13 Digest of binary vector prepared from *A. tumefaciens*

This figure shows a 1% agarose gel loaded with the products of restriction digests of plasmid preparations of the pALB1 construct from colonies 2, 3, 5 and 6 (analysis shown in Figure 4.12). All restriction digests were performed using the SacI and HindIII enzymes. Lanes 1 and 2 show digests of the plasmids isolated from colony 2. Lanes 3 and 4 show digests of the plasmids isolated from colony 3. Lanes 5 and 6 show digests of the plasmids isolated from colony 5. Lanes 7 and 8 show digests of the plasmids isolated from colony 6.

The 2.8 kb band was unexpected and was shown to be pUC18 sequence from the intermediate vector. The 2.0 kb and the 0.8 kb bands represent the *ACX1* promoter-luciferase cassette.


1 2 3 4 5 M 6 7 8
2.8 kb
2.0 kb
0.8 kb

conditions described in Section 2.3.2. After five or six days it was clear that all the kanamycin sensitive seedlings were bleaching and dying. In the first screen for transformants in which 40,000 seeds were plated only 4 seedlings were resistant to kanamycin. This is a very low rate of plant transformation but it was still possible that one of the four seedlings that we identified would have integrated T-DNA from which luciferase expression could be induced. A second *Agrobacterium* mediated transformation of Arabidopsis was carried out but no positive transformants were isolated.

These four seedlings (ALP1, ALP2, ALP3 and ALP4) were transferred from the selective plates to soil, allowing them to continue the normal life cycle. After two weeks, two leaves were detached from each plant and genomic DNA was extracted as described in Section 2.17. Genomic Southern analysis was performed as described in Section 2.18 using a fragment of the T-DNA from the left border to the SacI site of the luciferase gene as a probe. The results of this analysis can be seen in Figure 4.14.

From the bands present on the genomic Southern, it can be concluded that all four plants contain T-DNA. Due to the fact that 10 μg of genomic DNA was digested and then loaded into each lane, the higher intensity of hybridisation in lanes 1 and 3 suggests that there are multiple T-DNA insertions in these plants. Although all four plants have the insert, the position of integration can greatly affect the level of transgene expression. It was therefore important to check that luciferase could be detected in all four plants. Another two leaves of similar size were detached from all four plants to be tested for luciferase activity. These leaves were left wrapped in aluminium foil for 4 days to induce senescence before the assays

Figure 4.14 Genomic Southern analysis of the transgenic Arabidopsis

This figure shows the results of genomic Southern analysis on the four kanamycin resistant transformed Arabidopsis plant. The lanes are as follows:

M- 1kb marker

1- ALP1

2- ALP2

3- ALP3

4- ALP4

10μg of DNA was digested with SacI which would cut at the3' end of the transgene.

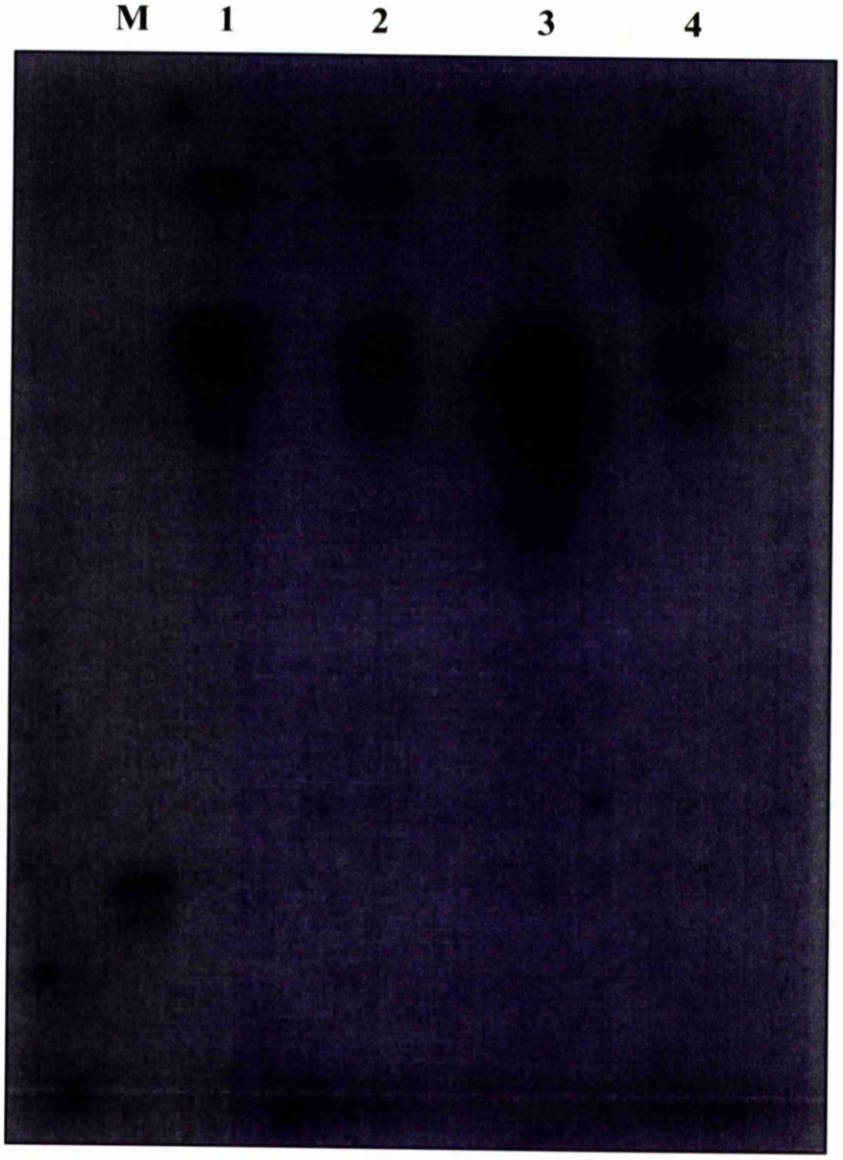

M
1
2
3
4

Figure 4.15 Luciferase analysis of senescing leaves

This figure show an image of leaves from the 4 transformed plants as visualised by a photon counting camera. 2 leaves from each plant were excised and dark-incubated to artificially induce senescence. Luciferase activity results in the release of energy as photons of light which are measured by the camera and visualised as colour.

ALP1

ALP2

ALP3

ALP4

were completed. The results of this analysis are shown in Figure 4.15.

It could be seen from this preliminary experiment that in transformants ALP3 and ALP4 luciferase was being expressed. This confirmed that the presence of the pGRT-10 vector at the 3'end of the luciferase sequence did not completely abolish enzyme activity. It can also be concluded from these experiments that the *ACX1* promoter appears to be active during dark-induced senescence, which is a novel observation. The lack of expression in ALP1 and ALP2 demonstrates that not all leaves that are induced to senesce produce luminescence. These leaves can be considered as a negative control in the absence of a wild type control which was not available in this experiment.

4.8 Analysis of Germination Induction of Luciferase in the T2 Generation

Luminescence detection for luciferase was carried out for all four plant lines in the T2 generation. Seedlings were germinated on 1/2 MS and luciferase activity measured. The results of this analysis are shown in Figure 4.16.

From this figure it is clear that luciferase induction during germination occurs only in the ALP4 transformant. It appears that the luciferase expression is induced at day 2 of germination before decreasing at day 4. The luciferase expression increases again at day 7 which is consistent with the published northern analysis.

A detailed time course of bioluminescence during germination and early seedling establishment was performed on the ALP4 line along with appropriate controls obtained from Dr. Wayne Charlton in Dr. Alison Baker's lab in Leeds. The seedlings were germinated on 1/2 MS agar in a 24-well plate. At each time point over a one week period the tray was sprayed with fresh luciferin and the photon counting camera gave both an image and a photon counts value for luciferase expression. As the different plants were set up in either duplicate or triplicate the average value was calculated for each plant after the background values (wells X and Y which contained no seeds) were subtracted at each time point to take into account the differences in luciferin distribution, as it was applied under pressure by a spray gun. The promoterless control had 100 seeds per well and the ALP4 plants had 20 seeds per well. The photon count values were then expressed on a per seed basis. These calculations can be seen in Figure 4.17. The values from this experiment are presented graphically in Figure 4.18.

This graph shows that *ACX1* expression is induced over a similar time course as has been reported in germinating seedling. This suggests that at least the basic elements for transcriptional induction are present in the putative promoter. Interestingly, the time of induction appears to be delayed by a day, showing a peak on day 2 and not on day 1 as previously reported in northern analysis (Hooks *et al.*, 1999a). Although this is difficult to explain it can be noted that, the seedling were physiologically only as developed as a normal 1 day old seedling when they were in fact 2 days old. This could be due to the fact that the seedlings were being removed from the growth rooms and sprayed with luciferin at two hour intervals, therefore disrupting normal development. The physiological difference could also be due to the fact that the seedlings were grown in 16 hour day/ 8 hour night conditions for

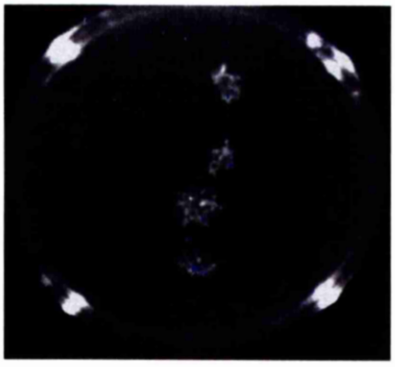
the luciferase experiments. The luminescing seedlings are shown in Figure 4.19.

It can be seen that *ACX1* promoter induction is switched on and turned off as was observed for the published northern analysis of *ACX1* induction. These results suggest that the putative promoter contains enough information for germination induction of the *ACX1* gene. Having established that this promoter contains the sequence elements necessary for *ACX1* induction during germination, it was important to establish whether it also contained the sequence elements required for stress induction.

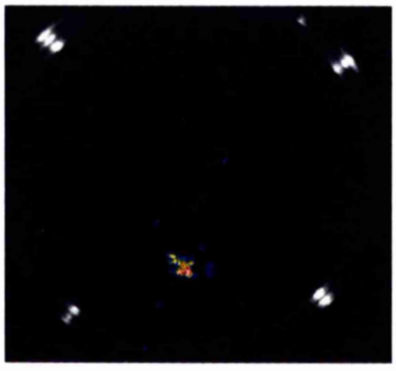
Figure 4.16 Luciferase analysis of all four transgenic lines during germination

This figure shows a petri dish with piles of seeds from the 4 transformed plants being germinated on 1/2 MS agar. ALP1 is at the top with ALP2, ALP3 and ALP4 in order underneath. The luciferase activity was visualised using a photon counting camera. It can be seen that ALP4 is the only plant with luciferase activity during germination.

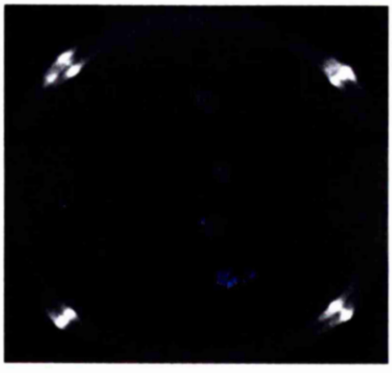
Day 0



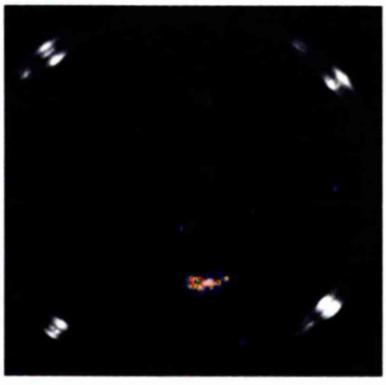
Day 2



Day 4



Day 7



Day 11

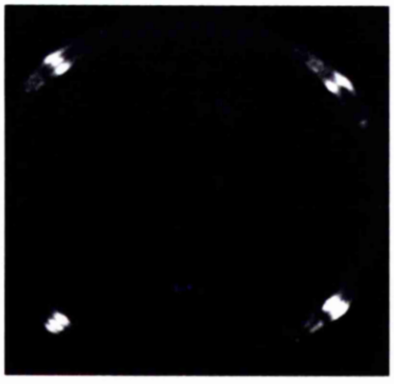


Figure 4.17 Luciferase calculations

This figure shows a sample calculation carried out to compare the luciferase activity of ALP4 (20 seeds) against the promoter-less control (100 seeds). It should be noted that this is a non-segregating population and so the variability in photon counts may be affected by this.

The calculated values were then plotted in Figure 4.18.

Sample calculation using photon counting values from 46 hours of germination (counts in ten minutes)

Promoter-less	11712	10992	13888	11648	24000	9680
ALP4	17728	17648	14464	13200		
no seeds	13728	9664				

The average for the four ALP4 wells was calculated to be 15760

The average for the six promoterless wells was 13653

The average for the wells with no seeds was 11692

To account for the background light emission, the no seed control value was subtracted from the other two.

ALP4 = 4068 and the promoterless control = 1961

The value were then calculated on a per seed basis,

20 seeds of the ALP4 plants = 203/seedling

100 seeds for the promoterless control = 20/seedling

Therefore, for the 46 hour time point values of 203 and 20 were plotted (Fig.4.18).

Figure 4.18 Graph of luciferase activity during germination

This graph depicts the difference in luciferase activity when the ALP4 promoter controls expression in comparison to a promoter-less control. The measurements were taken at 2 hour intervals for the first 3 days and then at less frequent intervals there after. The values are expressed as photon counts on a per seed basis.

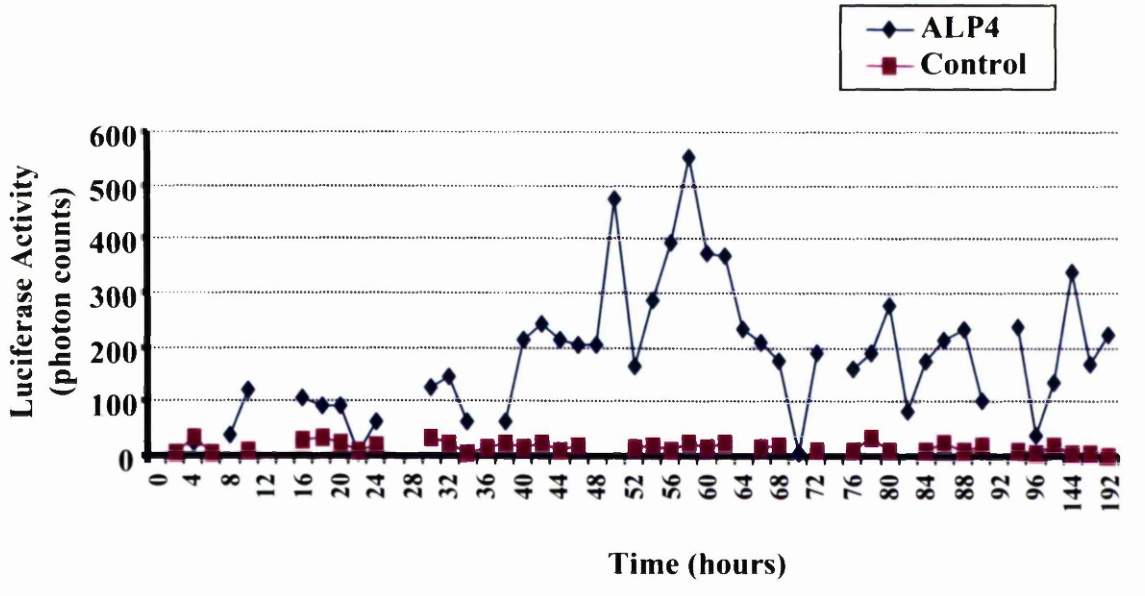

ALP4
Control
Luciferase Activity
(photon counts)
600
500
400
300
200
100
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88
92
96
144
192
Time (hours)

Figure 4.19 Luciferase analysis of ALP4 during germination

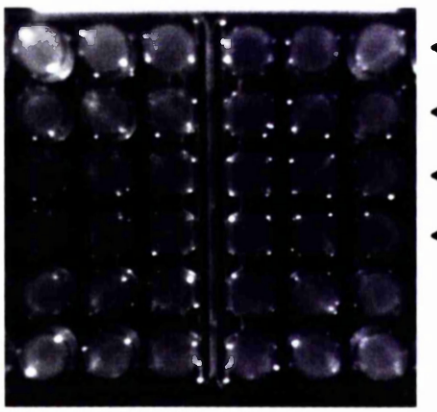
This figure shows the results of luciferase analysis while the gene is under the control of different metabolic putative promoters. The upper figure shows which transgenic seedlings are in which wells. The other figures show this plate visualised by the photon counting camera at different time points.

The other promoters used in this study were cloned by Dr. Wayne Charlton and were as follows:

Thiolase is an enzyme of ß-oxidation.

PEPCK is an enzyme involved in gluconeogenesis.

Malate Synthase is an enzyme specific for the glyoxylate cycle



PEPCK

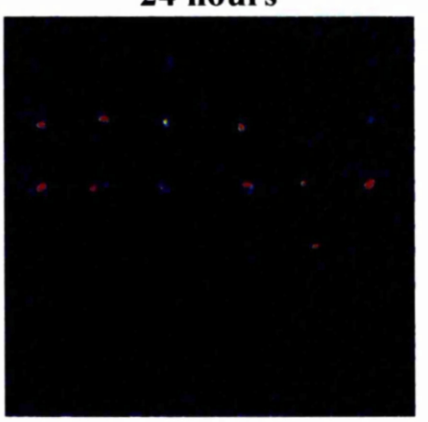
Thiolase

Malate Synthase

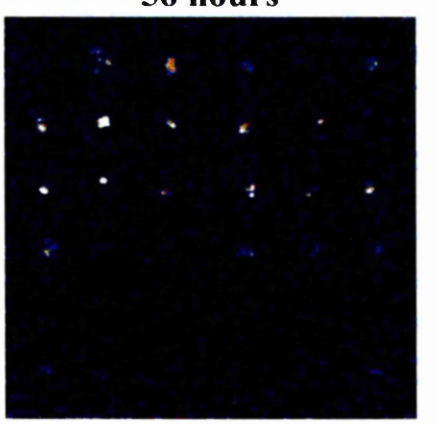
Promoterless Transgenics

ALP4 X Y ALP4

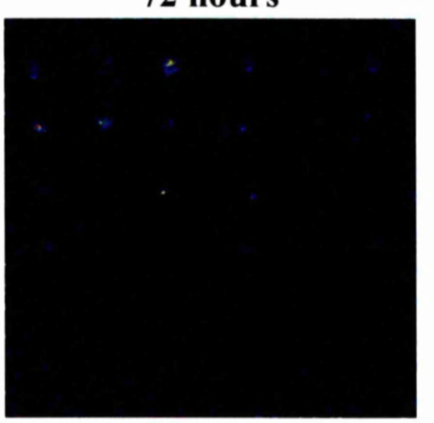
24 hours



56 hours



72 hours



4.9 Analysis of Stress and Wounding Induction of Luciferase

Recently published northern analysis suggests that wounding and certain chemical treatments of detached Arabidopsis leaves, leads to a rapid induction of *ACX1* (Hooks *et al.*, 1998). Based on these experiments, it was decided to investigate the effect of acid treatment on transgene expression. Two primary leaves were detached from the ALP4 transformant and were floated on 1/2 MS to which either HCl (1mM) or H_3PO_3 (1mM) was added. As a control for these acid treatments parallel treatments were set up which contained 10mM MES buffer pH 5.7. Treatments were arranged in a 24-well micro-titre plate and the images from the photon counting camera after 1, 2, 4 and 6 hour incubations are shown in Figure 4.20.

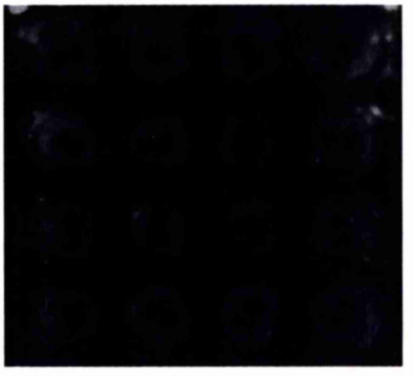
It can be seen from these figures that no luciferase activity was detected. This suggests that the 1kb of promoter does not contain the sequence elements necessary for induction of the *ACX1* gene. However, it is also possible that the presence of the acid on the leaves affected the luciferin so that it was no longer an active substrate for luciferase. This could be verified by decreasing the pH of the luciferin buffer and testing on luciferase positive tissue for luminescence e.g. the constitutive 35S promoter-luciferase. To determine whether the promoter was simply not responding to the stress stimuli, wounding experiments were carried out.

In the wounding experiment 6 plantlets were grown on germination media as described in Section 2.3.2. The bottom 2 plantlets in the dish were injured by stabbing a few of the leaves

Figure 4.20 Acid treatment of detached ALP4 leaves

This figure shows the results of the acid induction experiment. The top figure shows the treatments which the 2 primary leaves from each plant received. No luciferase activity was detected in this experiment.

1 hour after acid added



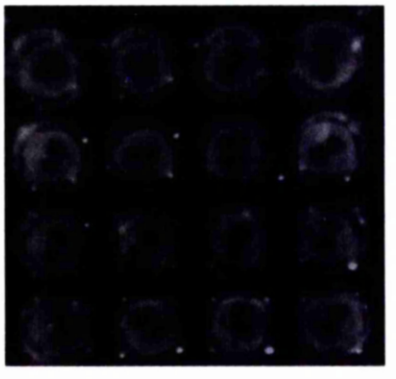
← 1mM HCl

← 1mM HCl + 10mM MES

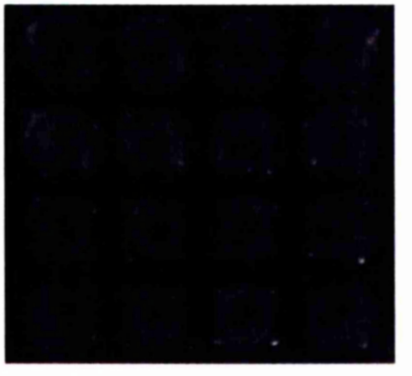
← 1mM H_3PO_3

← 1mM H_3PO_3 + 10 mM MES

2 hours after acid added



4 hours after acid added



6 hours after acid added

with a yellow pipette tip. The middle 2 plantlets were wounded with a razor blade by cutting off sections of the leaf. The 2 remaining plantlets were untreated. The leaves were assayed for luciferase activity at different time points and the resulting luminescence is shown in Figure 4.21. The photon count values during the experiment were plotted against time in a graph shown in Figure 4.22.

In Figure 4.21 it can be seen that luciferase activity is detectable, indicating that the 1kb promoter does indeed encode the information necessary for wound induction. The plants which were treated by detaching part of the leaves with a razor, did not show such a rapid induction, although there was detectable luciferase activity after eight hours. However it can be concluded from this experiment that the 1kb of the *ACX1* promoter contains sequence elements which mediate the induction of transcription upon wounding. The graph in Figure 4.22 shows the time course for this induction.

4.10 Putative Promoter Elements

The putative TATA box was found using NIX at the UK HGMP Resource Centre website funded by the MRC. The putative TATA box is 330bp upstream from the ATG and has been described in Section 4.3. No other sequence homologies had been identified at the time of construct production. Since then two putative promoter sequence elements have been identified by a FASTA search of a Japanese promoter element database at the address fasta@dna.affrc.go.jp. The results of the alignment analysis can be seen in Figure 4.23.

Figure 4.21 Wound induction of luciferase in the ALP4 plants

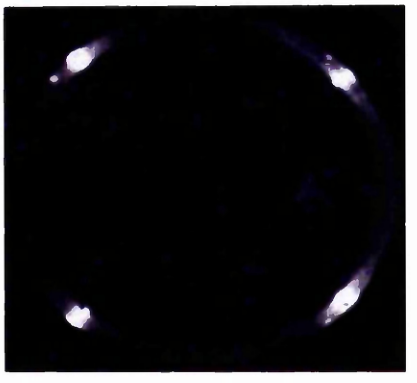
This figure shows the results of wounding treatments to ALP4 leaves. The luciferase activity is visualised using a photon counting camera.

The upper picture shows the positions of each plant whose treatments were as follows:

Plants 1 and 2:	untreated controls
Plants 3 and 4	leaves cut with a razor
Plants 5 and 6	leaves stabbed with a yellow tip

The activity was visualised at the times indicated after treatment.

1 hour after wounding

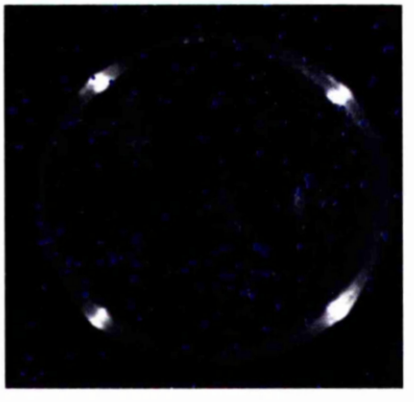


← Plants 1 and 2

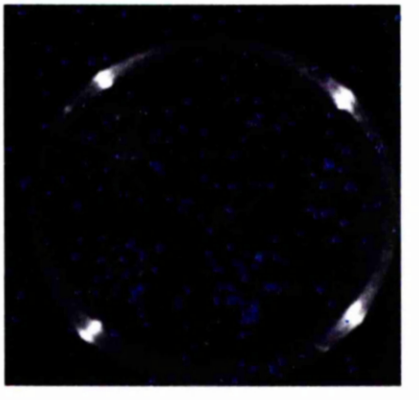
← Plants 3 and 4

← Plants 5 and 6

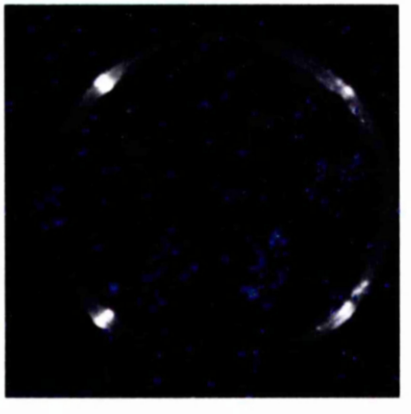
2 hours after wounding



4 hours after wounding



6 hours after wounding



8 hours after wounding

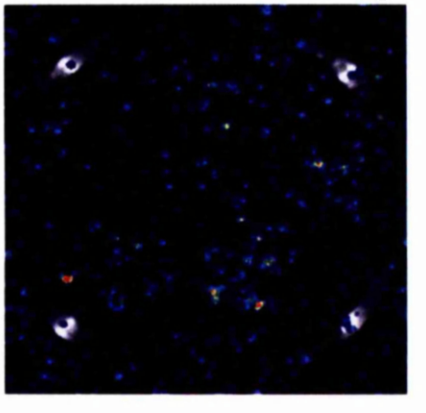


Figure 4.22 Graph of wound induction

This graph shows the average photon counting values for the 2 stabbed plants (visualised in Figure 4.21) plotted against time after treatment..

The standard deviations were calculated using the 2 repetitions. The error bars are large and so it is difficult to conclude that the increase is significant

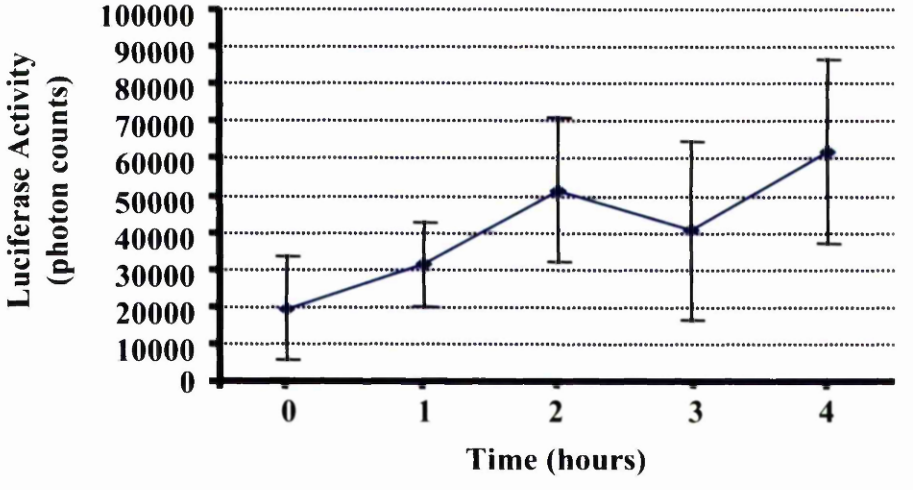

Luciferase Activity
(photon counts)
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0
1
2
3
4
Time (hours)

Figure 4.23 Alignment of putative promoter elements

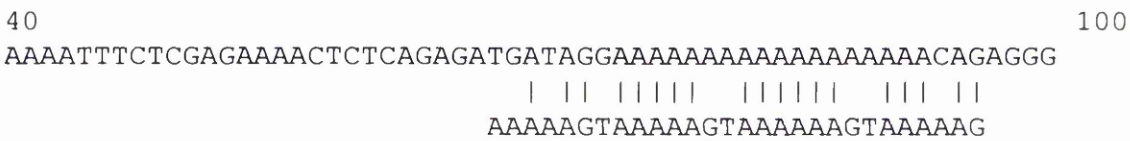
This figure shows the sequence alignment of putative cis-acting elements in the *ACX1* promoter. They were both identified using a Japanese promoter element database (fasta@dna.affrc.go.jp).

The AGTA repeat was characterised in the ascorbate oxidase promoter and is associated with pathogen attack.

The 3AF1 element was characterised in the rbcS-3A promoter which is light responsive.

The AGTA Repeat

73% IDENTITY



The 3AF1 Box

80% IDENTITY

The first putative element identified was AGTAC, which is an AGTA repeat from the pumpkin ascorbate oxidase promoter. The homology was 73% and the element is located 69bp upstream from the ATG. The ascorbate oxidase gene is of particular interest as it has been implicated in the oxidative burst in response to pathogen attack (Smirnoff, 1996). If ascorbate oxidase is involved in the stress response then this represents a functional connection with *ACX1*.

The second putative element which has been identified in the promoter sequence is a 3AF1 binding site which has a homology of 80% and is located 834bp from the ATG of *ACX1*. The 3AF1 transcription factor was originally described as binding to the light-responsive promoter of rbcS-3A (Lam *et al.*, 1990). It has since been shown to bind to the UV-light responsive promoter of the tryptophan decarboxylase gene from Madagascar periwinkle. UV light is a well documented stress for plants (Ouwerkerk *et al.*, 1999) suggesting that this sequence may well be a stress response element.

Although these putative elements are interesting, closer inspection of the homologies revealed that the bases involved were mostly As and Ts. Plant promoters are generally A-T rich so the significance of these homologies is probably less than the percentage scores would suggest. More detailed promoter analysis will be necessary to confirm any real role for these putative elements in the control of *ACX1* gene expression.

4.11 Discussion

Investigating the control of transcript expression of *ACX1* through promoter analysis has confirmed that induction is found during germination as reported in the original northern analysis by Hooks *et al.* (1999a).

1) The analysis of luciferase expression in transgenic Arabidopsis was, although preliminary and only on one transgenic line, conclusive in showing that the 1kb of the *ACX1* promoter is enough to confer germination-inducible and wound-inducible expression. Given the short half-life of luciferase, the results are likely to accurately reflect changes at the level of transcription.

2) The graph of luciferase expression during germination quite clearly shows a rapid induction of expression at 40 hours which then tails off to a basel level by 64 hours. This basel level is thought to be due to the cells need for ß-oxidation enzymes, which are involved in general housekeeping such as the turnover of membrane lipids. This expression pattern is in line with the other promoter-luciferase lines used by Charlton and Baker (unpublished) which are used in this study as controls for germination induction.

3) Treating the transgenic leaves with acid as means of stress did not show any induction of luciferase. This could be due to the acids interfering with luciferin and blocking luciferase activity. It is also possible that the 1kb of promoter does not contain the

elements responsible for this type of stress induction. Further control studies are required to establish which of these possibilities is correct.

4) Physically wounding transgenic leaves induced luciferase expression, although this was only observed with surface wounds and not with a razor. This confirms that the 1kb of promoter contains cis-acting elements with which certain stress signalling molecules can interact and transcription can be activated.

5) Homology searches of databases containing known promoter elements identified putative cis-acting elements present in the *ACX1* promoter. There is a putative TATA box 330bp upstream from the ATG. There are two other putative elements which have been identified, one is a 3AF1 binding site with 80% identity and the other is an AGTA repeat with 73% identity which is Tassociated with ascorbate oxidase expression. Although the homologies are not 100% identical it is interesting to note that both of these sequence elements have been associated with the stress response in plants.

Chapter 5: Characterisation of ACX1 in *Arabidopsis* Cell Culture Including Response to Stress Stimuli

5.1 Introduction

Previous studies on metabolic enzymes and their role during the continual growth and development of plants have demonstrated a correlation between germination of oil seeds and induction of glyoxylate cycle genes (Weir *et al.*, 1980; Smith and Leaver 1986; Harada *et al.*, 1988; Graham *et al.*, 1994). Other studies have shown that when these plants start to senesce at the end of their life cycle, the glyoxlate genes are induced again (Gut and Matile, 1989; Graham *et al.*, 1992). Subsequent analysis of genes encoding ß-oxidation enzymes showed that they followed a similar pattern of induction to glyoxylate cycle genes (malate synthase and isocitrate lyase) (Hayashi *et al.* 1998 ; Eastmond *et al.* 2000a ; Hooks *et al.* 1999a). The glyoxylate cycle converts acetyl-CoA, which is derived from ß-oxidation, into succinate in the glyoxysome. The succinate is then transferred to the mitochondrion where it enters the TCA cycle and is converted to oxaloacetic acid (OAA). The OAA is then converted by the enzymes of gluconeogenesis into sucrose.

Hooks *et al.* (1999a) succeeded in cloning two full-length cDNA clones representing the genes *ACX1* and *ACX2* which encode the medium-long and long chain acyl-CoA oxidase of Arabidopsis. It was of interest to characterise the expression of these genes during senescence since ß-oxidation is thought to play an important role in sequestering carbon derived from the breakdown of lipids during this stage in development. In order to synchronise the senescence of the leaves, an ethylene precursor, 2-chloroethylsulphonic acid

(CEP), was used (Hooks *et al.*, 1998). These initial CEP experiments led to an interesting observation of a rapid induction of *ACX1* at the RNA level. Neither *ACX2* nor any of the other ß-oxidation genes were induced at this point. This was surprising as lipid mobilisation would require all of the enzymes in ß-oxidation. As a control in the CEP treatments the leaves were treated with hydrochloric acid (HCl) and phosphonic acid (H_3PO_3), as they are by-products of the CEP breakdown to ethylene. These control experiments indicated that the acids in fact induced transcription from the *ACX1* locus, an induction which could be prevented by adding buffer to the incubation media. These observations suggest that *ACX1* was being induced due to stress rather than senescence. This was strengthened by the fact that the CEP-treated leaves became chlorotic 2-4 hours after addition of CEP and were completely bleached in 10-12 hours (Hooks *et al.*, 1998). The progression to senescence in detached leaves occurs over a much longer time scale. These results imply that *ACX1* was induced due to stress and may have a role in other stress responses in the plant. It was also found that *ACX1* was induced when the leaves were wounded, indicating that ACX1 could play a role in stress response (Titorenko *et al.*, 1997).

It was not surprising to find that an acyl-CoA oxidase was induced during stress as it had been thought to have a role in biosynthesis of JA, a stress signalling compound in higher plants (Farmer *et al.*, 1998). Since the acyl-CoA oxidase reaction generates H_2O_2, the other important role that ACX1 could perform is in the production of H_2O_2 and therefore maintenance of the oxidative burst. The oxidative burst describes the rapid production of active oxygen species, which has been reported in response to pathogens and pathogen elicitors (Doke *et al.*, 1996). The maintenance of reactive oxygen species levels or production

of a second burst hours after the initial attack may be the switch between a defense response and programmed cell death (Lamb and Dixon, 1997).

Intact leaf or leaf disc experiments, although informative about induction of the *ACX1* transcript, had limitations in terms of the chemical treatments penetrating the leaf. It was therefore desirable to develop a cell culture system which would allow identification of individual components of the signal transduction pathway leading to *ACX1* expression. Previous studies had shown that with different plant cell cultures and different stress signals the common reaction of the cells was to import protons resulting in a measurable alkalinisation of the media (Mathieu *et al*, 1996). If acid treatments of the Arabidopsis cell culture resulted in alkalinisation of the media and induction of *ACX1* transcripts, then it would be a valuable tool for further investigation of the signalling cascade and investigation of whether an increase in JA could be detected.

The light grown At-glw Arabidopsis cell culture was supplied by Dr. M. May (Dept. of Plant Sciences, University of Oxford) and had been described previously (May and Leaver, 1993). The characterisation of the Arabidopsis cell culture with respect to *ACX1* transcript induction and pH changes is addressed in this chapter.

5.2 Acid Treatment of Arabidopsis Cell Culture

The object of these initial cell culture experiments was to investigate whether the acid induction of *ACX1* could be shown using the Arabidopsis cell culture. The cell culture was

incubated with either 1mM HCl or 1mM H_3PO_3 plus or minus 10mM MES buffer (pH5.7). Samples were taken at 15 minute intervals for the first hour and then at hourly intervals up to 4 hours. RNA was isolated from the samples and subjected to northern blot analysis using *ACX1* as a probe (Figure 5.1).

This analysis revealed that steady state levels of *ACX1* mRNA remain at constitutively high levels in all treatments tested including the controls with buffer added. Possible explanations for the constitutivly high levels of *ACX1* mRNA at this point were that the demand for ß-oxidation may be constantly high because the cells are in continual cell cycle replications, as is known to be the case in dividing maize root tips (Hooks *et al.*, 1996). Alternatively, the constant shaking of the cell suspension culture could be acting as a wound stimulus and thus the cells are in a constant state of stress.

5.3 Cell Cycle and *ACX1* Expression in *Arabidopsis* Cell Culture

The maize root tip experiments by Hooks *et al.* (1996) showed that tissue composed of rapidly dividing cells had a constitutively high level of *ACX1* expression. Based on this work, the most likely explanation for the constitutive expression of *ACX1* in the cell culture is metabolic demand for ß-oxidation. To investigate whether there was a stage in the cell cycle where expression was lower, 2ml samples of the cell culture were taken at different time points over 1 week for RNA isolation. The results of this analysis are shown in Figure 5.2.

Figure 5.1 ***ACX1*** **transcript levels in** ***Arabidopsis thaliana*** **cell culture after treatment with acid**

This figure shows the results of northern analysis of the Arabidopsis cell culture treated with either HCl or H_3PO_3. MES buffer has been added along with the acid in three of the samples. The filter has been analysed using a radiolabelled *ACX1* probe. The lanes were loaded as follows:

Lane 1	0 minutes after addition of 1mM HCl
Lane 2	15 minutes after addition of 1mM HCl
Lane 3	30 minutes after addition of 1mM HCl
Lane 4	45 minutes after addition of 1mM HCl
Lane 5	60 minutes after addition of 1mM HCl
Lane 6	2 hours after addition of 1mM HCl
Lane 7	3 hours after addition of 1mM HCl
Lane 8	4 hours after addition of 1mM HCl
Lane 9	0 minutes after addition of 1mM HCl + 10mM MES
Lane 10	2 hours after addition of 1mM HCl + 10mM MES
Lane 11	0 minutes after addition of 1mM H_3PO_3 + 10mM MES
Lane 12	0 minutes after addition of 1mM H_3PO_3
Lane 13	15 minutes after addition of 1mM H_3PO_3
Lane 14	30 minutes after addition of 1mM H_3PO_3
Lane 15	45 minutes after addition of 1mM H_3PO_3
Lane 16	60 minutes after addition of 1mM H_3PO_3
Lane 17	2 hours after addition of 1mM H_3PO_3
Lane 18	3 hours after addition of 1mM H_3PO_3

The blot has been analysed using a radiolabelled *ACX1* probe (A) and the loading checked by visualisation of ribosomal RNA using ethidium bromide (B).

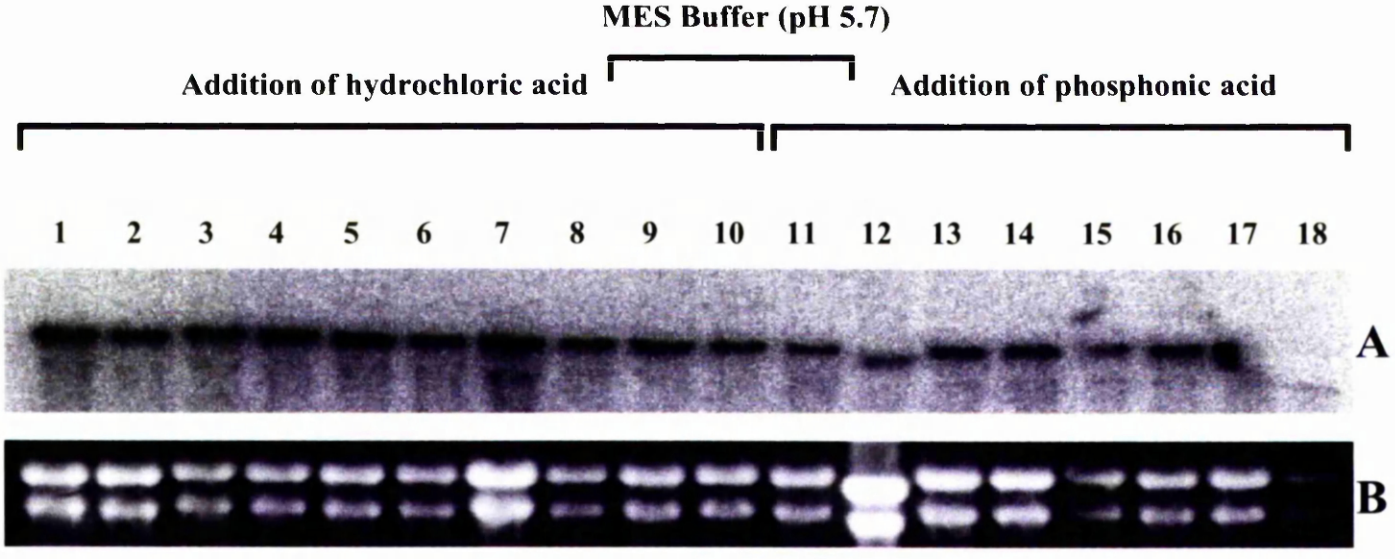
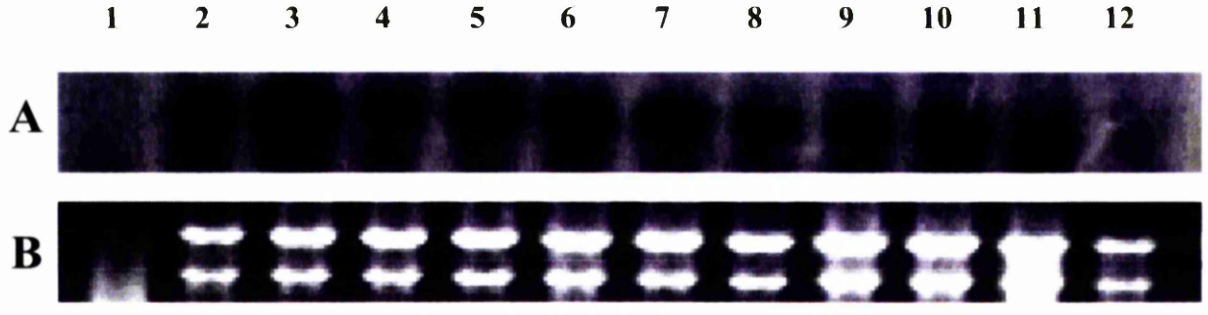

MES Buffer (pH 5.7)
Addition of hydrochloric acid
Addition of phosphonic acid
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A
B

Figure 5.2 ACX1 transcript levels during the one week of growth between subculturing

This figure shows northern analysis of the cell culture with samples being taken for analysis at successive time points between subculturing. The filter has been analysed using a radiolabelled *ACX1* probe. The lanes were loaded as follows:

Lane 1 -	1 week old cell culture
Lane 2	0 hours after subculturing
Lane 3	6 hours after subculturing
Lane 4	18 hours after subculturing
Lane 5	24 hours after subculturing
Lane 6	30 hours after subculturing
Lane 7	42 hours after subculturing
Lane 8	48 hours after subculturing
Lane 9	54 hours after subculturing
Lane 10	66 hours after subculturing
Lane 11	90 hours after subculturing
Lane 12	120 hours after subculturing

The blot has been analysed using a radiolabelled *ACX1* probe (A) and the loading checked by visualisation of ribosomal RNA using ethidium bromide (B).


1 2 3 4 5 6 7 8 9 10 11 12
A
B

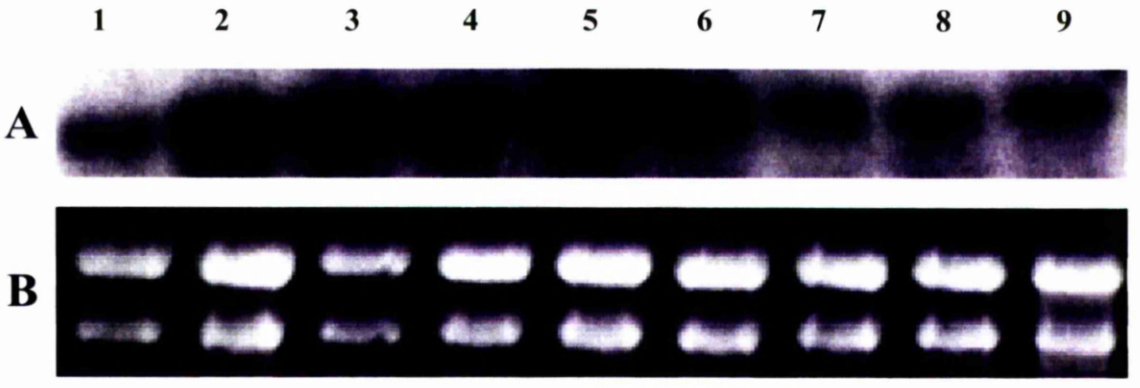
These results suggest that gene expression is low enough to allow any induction to be visible 18hrs after cell subculturing. Interestingly a large induction of ACX1 can be seen within 6 hours of subculturing. This suggests that the subculturing process produces induction of the *ACX1* gene, possibly due to stress caused by the motion of pipetting or changes in environment e.g. media pH, nutrient availability and presence of growth hormones. This is followed by a decrease in *ACX1* expression at 18 hours, probably because the cells were still in the lag phase and not metabolically demanding. The growth curve of this cell culture was characterised by Oswald and co-workers (2001). It can then be noted that there is an increase in *ACX1* mRNA levels which are maintained during the growth period, as determined by Oswald and co-workers (2001). This constant high level explains why an induction of *ACX1* expression was not observed in the original acid experiments. Using the knowledge gained from this experiment it was possible to design further experiments to establish if acid induction occurs in the cell culture.

Figure 5.3 ACX1 transcript levels in the *Arabidopsis thaliana* cell culture, 18 hours after subculturing when treated with phosphonic acid

This figure shows the result of northern analysis of the Arabidopsis cell culture with samples treated with 1mM H_3PO_3 for different periods of time. The filter has been analysed using a radiolabelled *ACX1* probe. The lanes were loaded as follows:

Lane 1	0 minutes after addition of acid
Lane 2	15 minutes after addition of acid
Lane 3	30 minutes after addition of acid
Lane 4	45 minutes after addition of acid
Lane 5	60 minutes after addition of acid
Lane 6	2 hours after addition of acid
Lane 7	3 hours after addition of acid
Lane 8	4 hours after addition of acid
Lane 9	RNA from day 2 germinating seedlings (control)

The blot has been analysed using a radiolabelled *ACX1* probe (A) and the loading checked by visualisation of ribosomal RNA using ethidium bromide (B).


1
2
3
4
5
6
7
8
9
A
B

5.4 Stress Treatments of *Arabidopsis* Cell Culture

In order to determine whether the *Arabidopsis* cell culture would be a useful system to study the mechanism regulating *ACX1* gene expression, the initial acid treatment experiments were repeated on cell culture 18 hour after subculture. The results of an RNA gel blot analysis of phosphonic acid treated cells are shown in Figure 5.3.

This analysis shows that *ACX1* transcript abundance increases within 15 minutes of phosphonic acid treatment to levels significantly higher than those which occur during germination. This rapid induction is similar to the levels observed in the leaf experiments (Hooks *et al.*, 1998). It can also be noted that the increase of ACX1 transcript levels appears to be reversible as the levels decrease 3 hours after treatment.

5.5 Cell Viability Study in *Arabidopsis* Cell Culture

Having demonstrated that acid induction of *ACX1* gene expression can be observed in cell culture 18 hours after subculture, it was important to show that this acid treatment was not killing the cells and that the cells response was a reproducible and reversible one.

Fluorescein diacetate (FDA) (Sigma, UK) was used as a vital stain to establish whether the cells were still alive after the various treatments. Living cells take up the dye and esterase activity cleaves the acetate moieties from the FDA leaving fluorescein which fluoresces in U.V. light. Live cells can be visualised as green using a fluorescent microscope, dead cells

appear red. The acid experiment was repeated, at the standard time points cell samples were taken and washed in 1/2 MS to remove the acid before adding the dye. The images of the cells at the these different time points are shown in figure 5.4.

This experiment demonstrates that the cell culture is still alive and viable even after 4 hours of the acid treatment. This suggests that changes observed after treatment, are due to an active process in the cells and not as some side effect to the death of the cells.

5.6 pH Measurements of *Arabidopsis* Cell Culture

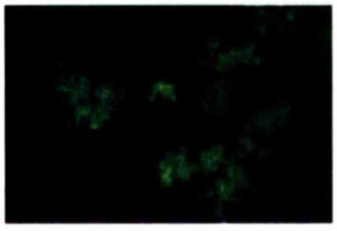
As mentioned in the introduction, previous experiments by other labs into stress signal transduction had observed that acidification of the cytosol and therefore alkalinisation of the media was a physical indication of stress. In order to establish if the Arabidopsis cell culture was responding to acid treatment in a similar manor, the pH of the external medium was monitored and the results are presented in Figure 5.5. Observation of changing pH levels in the cell culture during the treatment could indicate whether *ACX1* was induced as part of a stress response.

The graph in Figure 5.5 shows that there is alkalinisation of the media and that it occurs immediately on addition of the acid. The buffer control in this experiment indicates, as expected, that no pH changes occurred in the absence of cell culture. This suggests that the cells recognise the acid as a stress and respond by importing protons or exporting hydroxyl radicals which would in turn result in acidification of the cytosol.

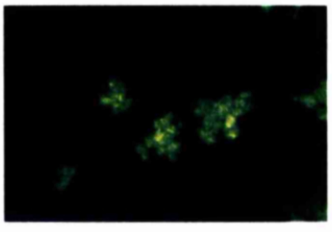
Figure 5.4 Photographs of *Arabidopsis thaliana* cell culture stained with FDA

The photographs show the cell culture stained for viability with FDA and viewed under a fluorescent microscope. Live cells stain green and dead cells stain red. Incubation times and acid used are indicated for each sample. Green cells are viable and red cells are dead. Red cells were only detected in the2 hour HCl treatment.

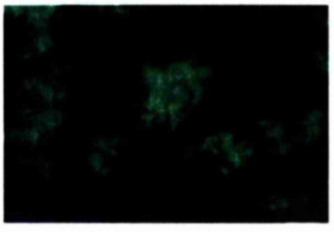
Control (0 hours)



HCl (1 hour)

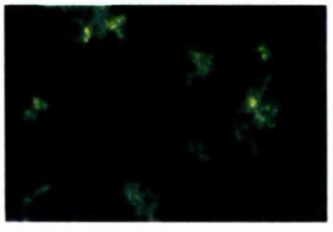


H_3PO_3 (1 hour)

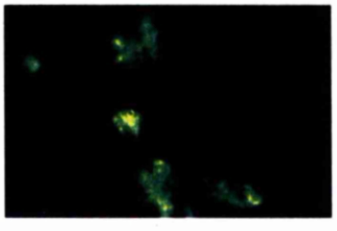


HCl (2 hours)

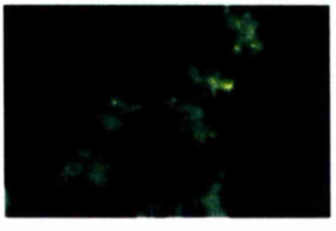
H_3PO_3 (2 hours)



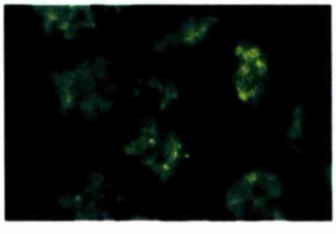
HCl (3 hours)



H_3PO_3 (3 hours)



HCl (4 hours)



H_3PO_3 (4 hours)

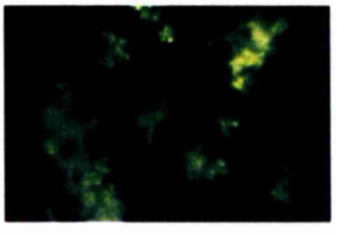
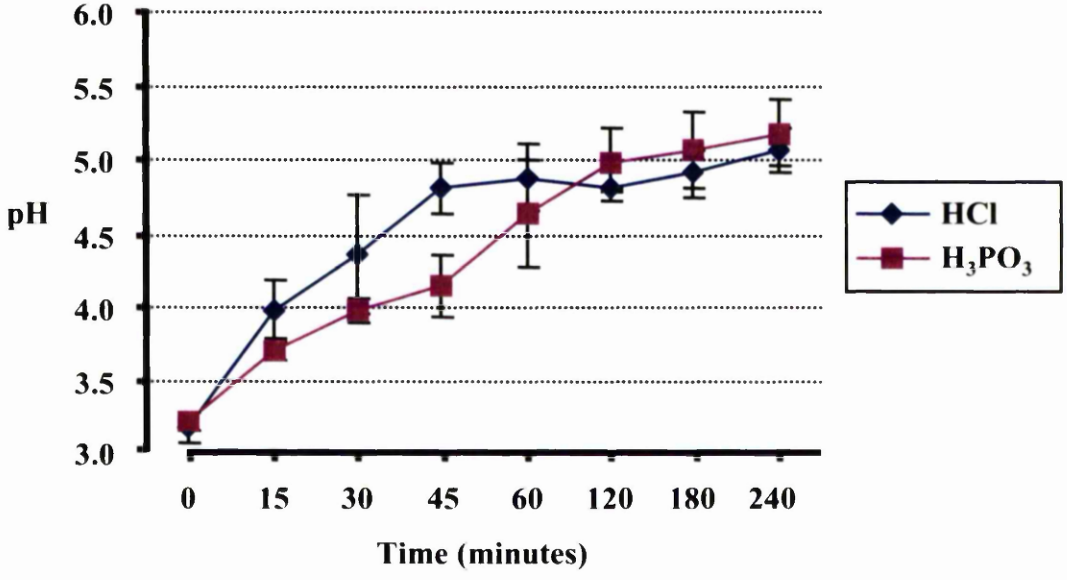


Figure 5.5 Graph of pH versus time during an acid experiment

This graph shows the pH changes that occur in the cell culture after addition of 1mM acid. Measurements were taken at 15 minute intervals for the first hour and every hour thereafter. The pH changes that occurred were measured in three separate experiments and the average has been plotted with error bars indicating the standard deviation. The pH of the media increased after addition of both HCl and H_3PO_3 over time.


6.0
5.5
5.0
4.5
4.0
3.5
3.0
pH
0
15
30
45
60
120
180
240
Time (minutes)
HCl
H3PO3

It was important to establish if factors other than active proton exchange could be responsible for the observed pH changes. For example, cell wall proteins could absorb the protons and effectively act as a buffer. Alternatively, the protons could have been moving from the media into the cytosol based purely on a concentration gradient rather than being actively transported.

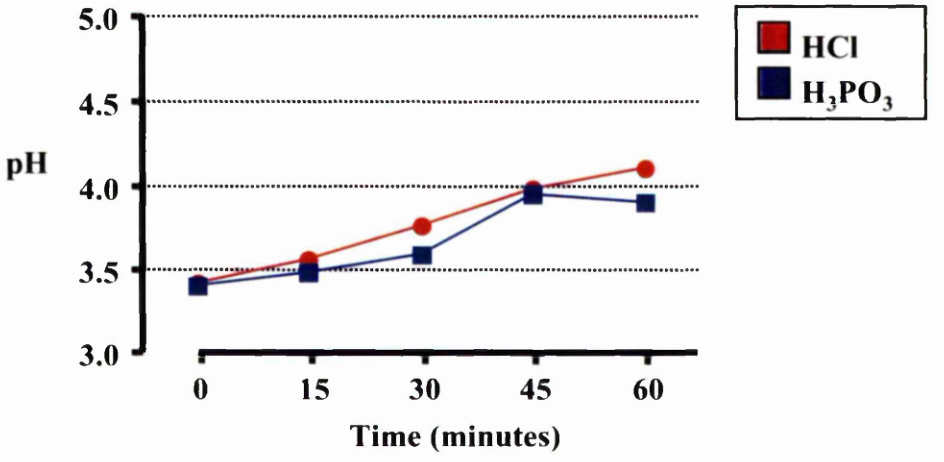
5.7 Cold Treatment of *Arabidopsis* Cell Culture

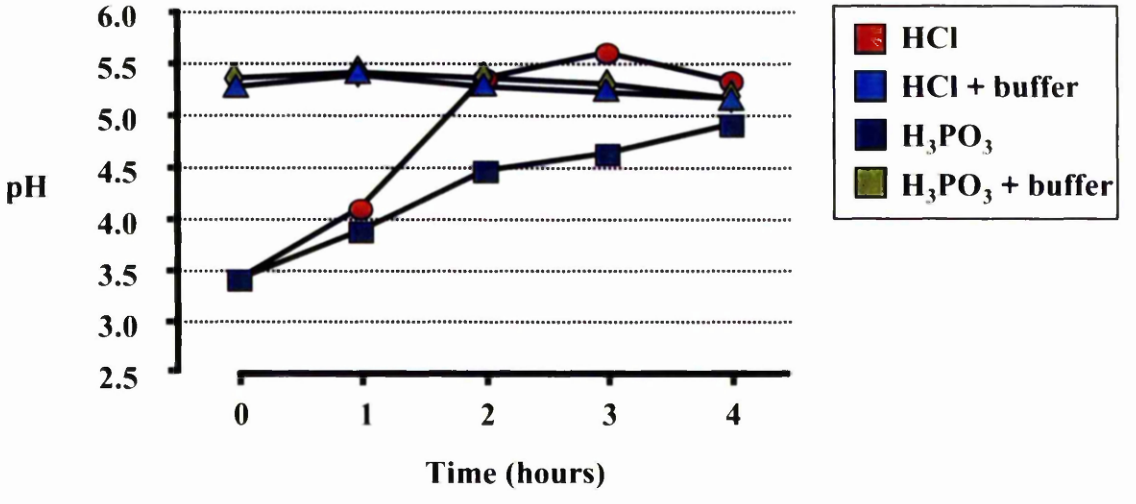
In an attempt to ascertain whether the pH changes occur as a result of an energy demanding process or are due to physical changes external to plant cells, the acid treated experiment was repeated at 4°C. Before adding the acids, the cell culture was left at 4°C for 1 hour to equilibrate to the lower temperature. The results of the pH measurements are shown in Figure 5.6.

The graph shows that the changes in pH at 4°C are similar to that which occurs at room temperature. However there are two important differences: The cell culture pH at the start of the experiment is higher than at room temperature. This suggests that cooling the cells at 4°C for 1 hour had a stressful effect on the cell culture. Another difference is that the media becomes more alkaline 4 hours after addition of acid in the cold treatment before settling back at the original value of 5.2. This could be due to sustained acidification of the cytosol because of the double stress or perhaps just an over compensation. To confirm that the initial change in pH was due to the cells and not a physical change in the media, pH was measured during the acclimatisation period (1 hour period after the transfer of the culture from 20°C to 4°C)

Figure 5.6 Graph of pH versus time during an acid experiment at 4°C

These graphs show the changes in pH during an acid treatment experiment carried out at 4°C. The cell culture had been moved to 4°C one hour before the experiment was started by the addition of acid. pH was measured at 15 minute intervals for the first hour and then every hour for the duration of the experiment. The graphs represents results from a single representative experiment. The top graph shows the pH changes during the first hours after addition of the acids. The lower graph shows all the time points taken and includes the buffered controls. It is clear that the presence of buffer abolishes any effect of the acid. Without buffer the pH of the media increases with time after addition of acid.


5.0
4.5
4.0
3.5
3.0
pH
0
15
30
45
60
Time (minutes)
HCl
H_3PO_3


6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
pH
0
1
2
3
4
Time (hours)
HCl
HCl + buffer
H_3PO_3
H_3PO_3 + buffer

with both cell-free media and the cell culture. These measurements are shown in Figure 5.7.

It is clear that although both flasks show an increase in pH, the change in the flask with cells is significantly greater. This supports the hypothesis that acidification of the cytosol arises as a consequence of general stress, of which cold treatment is one. In order to establish whether the acid induction of *ACX1* could also be observed at low temperature, RNA was isolated from the acid treated cell cultures maintained at 4°C and used for northern blot analysis. The results of this experiment are shown in Figure 5.8.

This analysis showed that there was no induction of *ACX1* at 4°C. The levels remained constant throughout the time course. However, these cells had been subcultured 48 hours before transfer to 4°C therefore any slight induction may have been masked by the elevated levels of *ACX1* transcript prior to the transfer.

Figure 5.7 Graph showing changes in pH during the one-hour acclimatisation to 4°C

This graph shows the changes in pH versus time during a 1 hour incubation of the Arabidopsis cell culture after transfer from 20°C to 4°C. Measurements were taken at 15 minute intervals. This graph shows data from a single representative experiment. These data indicate that alkalinisation of the media only occurs in the presence of cell culture.

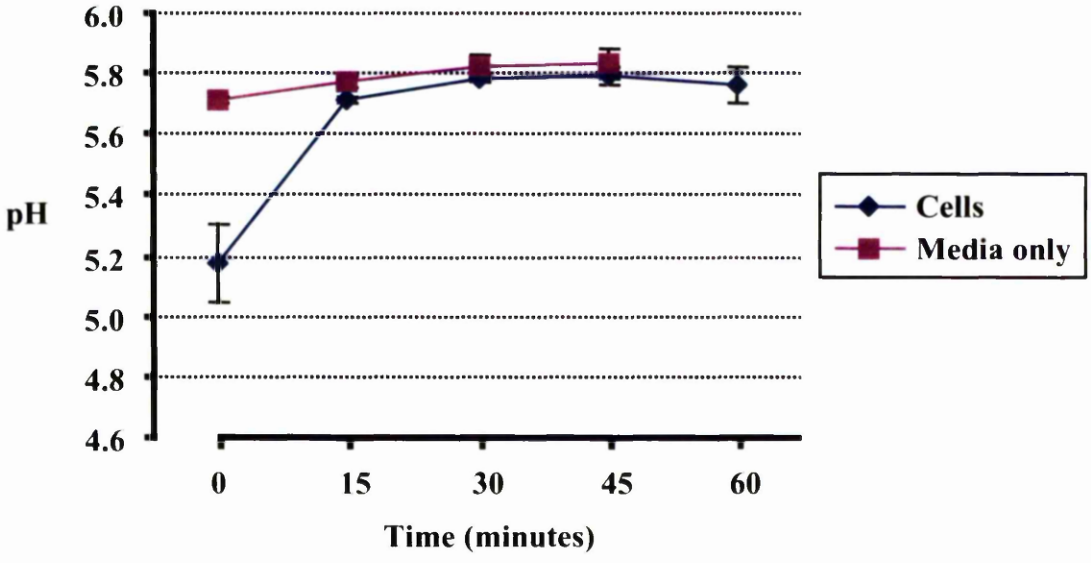
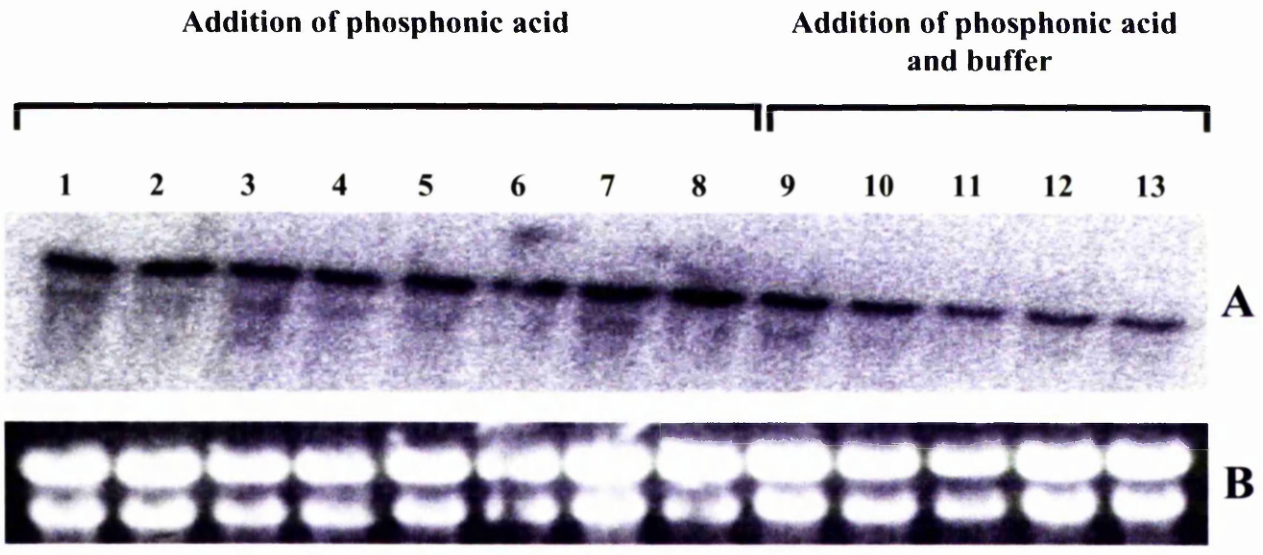

6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
pH
0
15
30
45
60
Time (minutes)
Cells
Media only

Figure 5.8 ACX1 transcript levels in *Arabidopsis thaliana* cell culture when treated with acid at 4°C

This figure shows the results of northern blot analysis of the Arabidopsis cell culture grown for 48 hours and then transferred to 4°C and acclimatised for 1 hour, before being treated with HCl or H_3PO_3, with and without MES. The blot has been analysed using a radiolabelled *ACX1* probe (A) and the loading checked by visualisation of ribosomal RNA using ethidium bromide (B).

The lanes were loaded as follows:

Lane 1	0 minutes after addition of acid
Lane 2	15 minutes after addition of acid
Lane 3	30 minutes after addition of acid
Lane 4	45 minutes after addition of acid
Lane 5	60 minutes after addition of acid
Lane 6	2 hours after addition of acid
Lane 7	3 hours after addition of acid
Lane 8	4 hours after addition of acid
Lane 9	0 minutes after addition of acid and MES
Lane 10	1 hour after addition of acid and MES
Lane 11	2 hours after addition of acid and MES
Lane 12	3 hours after addition of acid and MES
Lane 13	4 hours after addition of acid and MES


Addition of phosphonic acid
Addition of phosphonic acid and buffer
1 2 3 4 5 6 7 8 9 10 11 12 13
A
B

5.8 Discussion

The aim of the work on the Arabidopsis cell culture was to investigate its suitability as a model system to study the signal transduction mechanisms underlying the regulation of the *ACX1* gene in response to stress. This work has demonstrated that the cell culture responds to various stresses by induction of the *ACX1* gene and could be used to study the underlying signalling mechanism and the role *ACX1* plays in the stress response. However, the work also indicates that the metabolic role of the *ACX1* gene product in rapidly growing cell culture is such that gene expression is already high. Cells need to be sampled before the cell culture is in the log phase and ACX1 expression is at a relatively low level in order to allow treatments that cause the induction of the *ACX1* gene to be studied.

Positive conclusions from this work include:

1) The pH measurements on the cell culture suggests that cytosolic acidification may occur in response to the acid treatments. This could be confirmed by use of cytosolic pH indicators.

2) The pH changes observed in the 4°C control experiments suggest that an active uptake of protons by the cells occurs in response to the presence of acid in the media.

3) Cold treatment also induces stress and the consequent change in pH of

the media appears to be an active process.

4) Induction of *ACX1* immediately after subculturing can be observed. This may be a stress response to changes in the environment with respect to media pH, nutrient availability and growth hormones.

5) The reduced RNA expression levels found at 18 hours may be due to the initial stress response subsiding and the metabolic demand during the exponential growth phase not yet having commenced.

6) Acid induction of *ACX1* can be observed in cell culture 18 hour after subculturing, similar to that observed in leaves.

7) Arabidopsis cell culture is a promising system for the identification of the role of *ACX1* in stress and its possible relationship to JA biosynthesis.

CHAPTER 6: DISCUSSION

6.1 Introduction

In this study, an Arabidopsis cell culture system and transgenic Arabidopsis plants were utilised to characterise the expression of an acyl-CoA oxidase gene, *ACX1*. Reporter gene expression studies were undertaken to characterise the activity of the putative 1kb *ACX1* promoter fragment during germination, senescence and in response to wounding and acid treatment in transgenic plants. This analysis was complemented by experiments using the cell culture to look for stress induction of the *ACX1* transcript and examine concurrent physiological changes in the cells. Germination of Arabidopsis is the stage in the plant's life-cycle during which there is the largest conversion of lipids to a soluble carbon source and as such is a major physiological stage at which to study lipid catabolism. This study also involved experiments carried out to investigate co-expression of metabolic genes (e.g. enoyl-CoA hydratase) and other developmentally important genes (e.g. GA20 oxidase). The aim of this chapter is to discuss and draw together the main conclusions of this research and summarise the prospects for future work.

6.2 *ACX1* - A Putative Germination Gene Cluster

Publication of the ACX1 genomic locus on chromosome IV by Bevan *et al.* (1998) revealed not only the sequence of the putative ACX1 promoter but allowed identification of the surrounding putative genes. It was interesting to note two other putative open-reading frames

encoding a GA20 oxidase and an ACC oxidase that could also be involved during germination. The GA20 oxidase could play a role in germination through gibberellin biosynthesis. The ACC oxidase was not annotated in the published sequence but was identified during the course of the analysis using sequence comparisons. ACC oxidase has been shown to catalyse the final step in the biosynthesis of ethylene which is known to be produced during germination.

Within 100kb of the *ACX1* locus, two other genes with a possible role in germination were identified. One of the putative genes encodes a lipase which could be involved in the breakdown of TAG in the oil bodies. The second putative gene encodes an enoyl-CoA hydratase which could be part of the multifunctional protein and therefore involved in ß-oxidation. The proximity of these putative genes may be significant given that it has been shown in mammals that the average chromosome loop is about 90kb, although varying in size from as little as a few hundred bases to 100kb (Jackson *et al.*, 1990). If these putative genes were on the same chromosome loop, their induction could be controlled at the level of chromatin structure as well as by transcription factors.

E.S.T. clones for all four of these genes were obtained from the Nottingham Arabidopsis Stock Centre. Both classical northern analysis and 'electronic' northern analysis using an E.S.T. database prepared from a cDNA library of 2-day-old seedlings, failed to demonstrate any co-regulation of these genes. A possible conclusion from this work was that the transcript abundance for these genes was below the levels of detection of the techniques employed. More abundant transcripts such as those of isocitrate lyase and thiolase

demonstrated the utility of the E.S.T. database and 'electronic' analysis, and showed that the cDNA library produced in the current study was of high quality.

6.3 Developmental Induction of *ACX1* Visualised Using the Luciferase Reporter

Previous studies into the germination induction and stress induction of *ACX1* showed this particular acyl-CoA oxidase to be of importance in metabolism and also during stress responses. Cloning of 1kb of the *ACX1* putative promoter was performed and transcriptional activity of the promoter was monitored by fusing it to the luciferase reporter gene.

Luciferase activity was observed after four days of dark-induced senescence in primary transformed Arabidopsis lines. This was the first time *ACX1* had been shown to be expressed during dark-induced senescence. This implicates ACX1 in the mobilisation of membrane lipids in older tissue. Expression from the *ACX1*-luciferase transgene was monitored over an extensive time period during germination and post-germinative growth. The *ACX1* promoter became active 2 days after germination which was a day later than the northern data suggested. Expression was relatively short lived compared to the other promoter-luciferase transgenic plants analysed, which is in agreement with the northern analysis comparing *ACX1* and *ACX2* (Hooks *et al.*, 1999a). Although this may suggest that *ACX1* is redundant in terms of lipid mobilisation during germination, the *ACX1* and *ACX2* anti-sense plants described by Hooks and co-workers showed no retardation of germination, suggesting that these two enzymes may compensate for each other. It is possible that the role of *ACX1*

during germination is primarily in the biosynthesis of JA, which has been reported to be present in germinating seedlings and developing tissues throughout the life cycle of the plant.

Irrespective of the physiological role of *ACX1* this analysis indicates that the 1kb of *ACX1* promoter encodes information sufficient for the developmental induction of gene expression during both germination and senescence.

6.4 The *ACX1* Promoter is Activated during Wounding

The ethaphon treatment of detached Arabidopsis leaves suggested that *ACX1* is rapidly induced upon exposure to acid conditions. No other ß-oxidation genes have been reported to be induced which suggests that *ACX1* has a role during stress in addition to its metabolic role (Hooks *et al.*, 1998). To confirm these observations and to attempt to quantify the induction, the acid experiments were repeated using leaves from the transgenic ALP4 plants. Unfortunately, no luciferase activity was detected indicating that either the 1kb of promoter which had been cloned did not contain the stress response elements or the acid with which the leaves were treated in some way affected the luciferase measurements, perhaps inactivating the luciferin.

In an attempt to determine whether the promoter could induce luciferase expression in response to a non-chemical stress stimulus, leaves were wounded then assayed for luciferase activity. Wound induction of luciferase was found within 2 hours, suggesting that *ACX1*

does indeed have a role to play in the Arabidopsis defence response and that the sequence elements required to mediate this response are present in the 1kb promoter fragment analysed. The most likely role for ACX1 would be in the biosynthesis of JA. An important experiment in future analysis would be to measure the rate of JA production in parallel with luciferase assays in these transgenic plants to determine whether there is a correlation between the two.

An interesting observation regarding the possible role of ACX1 in JA biosynthesis is the nature of *ACX1* induction during germination. From the published northern analysis it can be seen that after the short initial induction of expression, there is a comparatively small peak in expression at day 6 which lasts for 24 hours (Hooks *et al.*, 1999a). This may initially appear to be an artefact but a germination study with ALP4 plants showed the same second peak in activity (Figure 4.16), suggesting that it is a genuine expression pattern. Such a pattern is indicative of a negative feedback mechanism which has recently been suppressed. If the initial peak of *ACX1* expression is responsible for JA biosynthesis and then JA turns off *ACX1* expression by a feedback mechanism, it could be that as JA levels fall the repression of *ACX1* is lifted, resulting in the second small peak of expression. A role for JA during germination has been reported by Ranjan and Lewack (1992). The researchers demonstrated that JA promotes germination and lipase activity in apple embryos. These results are supported by the experiments of Creelman and Mullet (1995) who showed that JA levels increase in correlation with lipid mobilisation. They reported that JA levels were highest on day 1 of germination and that levels declined with further seedling development. The second peak of *ACX1* expression would only be transient as the plant would become photosynthetically competent and lipid catabolism genes would be down-regulated.

Further support for the connection between ACX1 and JA during development comes from research published by Berger *et al.* (1996). These researchers showed that application of JA to Arabidopsis roots inhibited growth by a mechanism which did not involve ethylene. This observation is in agreement with Hooks *et al.* (1999a) who showed that the only phenotype of *ACX1* anti-sense plants was reduced root growth. Taken together these two results strongly suggest the presence of a negative feedback pathway from JA to one of its biosynthetic enzymes, *ACX1*.

6.5 ACX1 is Inducible Under Stress in Arabidopsis Cell Culture and Coincides with Cytosolic Acidification

The rapid induction of *ACX1* upon treatment of detached Arabidopsis leaves with ethaphon and the proposed involvement of cytosolic acidification (Hooks *et al.*, unpublished) led to the decision to characterise *ACX1* expression in Arabidopsis cell culture. The first important aspect of the study was to characterise the levels of *ACX1* transcript levels during the growth cycle of the cells. This analysis showed a stress induction of *ACX1* in the cells within 6 hours after subculturing which disappeared by 12 hours. From 24 hours after subculturing transcript levels indicated constitutive high level expression of the *ACX1* gene, most probably due to the metabolic demand in the rapidly growing and dividing cells. This confirmed that metabolic status of the cell culture played an important role in regulating *ACX1* expression. In order to use the cell culture as a model system to study acid induction

it was necessary to establish conditions where expression due to metabolic activity was minimised.

Using the knowledge gained from the cell culture growth experiments (Fig. 5.2), experiments were designed for acid induction of *ACX1*. It was possible to see the same rapid induction as had been reported in the detached leaves. By measuring the pH of the cell culture media it was possible to note that an alkalinisation occurred immediately upon addition of the acid. Interestingly, transfer of the cell culture to 4 °C also caused an alkalinisation of the media. This pH change suggests that either protons are moving into the cells or hydroxyl radicals are moving out, either way causing acidification of the cytosol. The observation of pH changes using two different treatments suggests that it is not an artefact and has a physiological significance, perhaps in stress signalling.

6.6 The Role of ACX1 in the Plant Stress Response

The reaction catalysed by ACX1 oxidises a fatty acyl CoA which can then continue in the ß-oxidation pathway, H_2O_2 is a by-product of this reaction. What role could this enzyme play in the plants stress response? It has already been stated that ACX1 may play an important role in the biosynthesis of JA (Section 1.5.3) which is known to mediate an effect both in developing tissue and in response to wounding or pathogen attack. It is important to note that H_2O_2 is a by-product of the ACX1 reaction and could contribute to the oxidative burst (Section 1.5.2). As acyl-CoA oxidase is a peroxisomal enzyme, the H_2O_2 produced is normally degraded by the H_2O_2-scavenging enzymes catalase and ascorbate peroxidase. This

would make the H_2O_2 unlikely to contribute to the oxidative burst. A recent publication by Clark *et al.*, (2000) showed that the stress signal, NO, reversibly inhibited both catalase and ascorbate peroxidase very quickly preceeding the oxidative burst. This could result in an increase in intracellular H_2O_2. These results support the hypothesis that the H_2O_2 produced by ACX1 may have a role in the oxidative burst during plant stress.

6.7 Further Work

This study has provided valuable information about the control of *ACX1* gene expression in Arabidopsis. Both the promoter analysis and the cell culture analysis were preliminary investigations and involved the setting up of useful systems. This means that there is a great deal of scope for future work.

With respect to the germination cluster on chromosome IV, the best method currently available for investigating the possibility of co-expression is RT-PCR. This is a highly sensitive technique which can confirm the presence of very low abundance transcripts in a quantitative manner. Further analysis of E.S.T. sequences when they are added to the TwoDogs database may also identify the presence of the clones. The fact that no E.S.T.s for *ACX1* were present in the 2 DOGs database demonstrates that this approach is not detecting low abundance transcripts.

The 1kb of *ACX1* promoter has been shown to contain the sequence elements required for both developmental and stress induction of the gene. It is important in future studies to

identify these elements. This could be done by making promoter-deletion constructs and assaying, in a transient system, for loss of induction. Once putative elements have been identified, specific base mutations in the constructs would provide confirmation of their function. Band shift assays could be used to confirm that nuclear proteins interact with the promoter region. Additional band shift assays using mutated promoter elements could be performed to investigate the sequence specificity of such an interaction.

With the ALP4 transgenic line, signalling pathways could be investigated by crossing it with known stress signalling mutants, such as JA mutants. This could supply information about upstream factors and the possibility of a negative feedback mechanism. Obtaining knockouts in the *ACX1* gene would allow the role of *ACX1* in germination, wounding and JA to be addressed directly. It is possible to measure JA levels using gas chromatography. Using this method to analyse JA levels in wild-type and ACX1 mutant lines could answer important questions about the role of ACX1 in JA biosynthesis and provide valuable information on the role of JA in developing tissues.

The cell culture system may prove to be valuable in understanding the signalling to the pathways involved in ACX1 expression. This study has shown that *ACX1* can be induced in cell culture under stress. Discovering other stimuli could be straightforward given that chemical signalling molecules can be easily applied to the cell culture. For example, would exogenous addition of JA feedback on the *ACX1* promoter and affect constitutive expression observed in the growing cell culture? Another possible approach with the cell culture would be to transform it with promoter-luciferase constructs using *A. tumifaciens*, as described by Fourreiter *et al.* (1997). This would allow quantitative analysis of *ACX1* promoter induction

by chemical signalling molecules. A lot information about the signalling pathways involved could be elucidated using this approach.

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Appendix 1

T22434

The putative GA20 oxidase aligned with chromosome IV

```
>emb|Z97341.2|ATFCA6 Arabidopsis thaliana DNA chromosome 4, ESSA I FCA contig fragment
No. 6
          Length = 213788

 Score =  196 bits (99), Expect = 4e-48
 Identities = 159/174 (91%), Gaps = 6/174 (3%)
 Strand = Plus / Minus


Query: 66     gtcttgaacatggattctnctatgtaaagaatcatggaatctcggaagaattaat-ggaa 124
              |||||||||||||||||| |||||| | || |||||  ||| ||||||||||||| ||||
Sbjct: 196398 gtcttgaacatggattcttctatgtcagga-tcatg--atc-cggaagaattaattggaa 196343


Query: 125    ggggttttnaaggagagcaaaggcttctttaatctcccattagagggaaagatggcttta 184
              |||||||| ||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 196342 ggggttttaaggagagcaaaggcttctttaatctcccattagaggaaaagatggcttta 196283


Query: 185    ctccgccgtgattttctcggttatannccgttngtatgctgaggaacttgaccc 238
              |||||||||||||| |||||||||| ||||| |||||||||| ||||||||||
Sbjct: 196282 ctccgccgtgatttgctcggttatactccgtt-gtatgctgagaaacttgaccc 196230

 Score = 75.8 bits (38), Expect = 1e-11
 Identities = 55/61 (90%)
 Strand = Plus / Minus


Query: 2      cttccgatcatcgacctttcttcgccgggaaaaatttccactgctaaattnattcgtcag 61
              |||||||||||||| |||||||||||||| |||||| |||||| || |||| |||||||||
Sbjct: 196797 cttccgatcatcgatctttcttcgccggaaaaaatctccactactcaattgattcgtcag 196738


Query: 62     g 62
              |
Sbjct: 196737 g 196737

 Score = 50.1 bits (25), Expect = 6e-04
 Identities = 28/29 (96%)
 Strand = Plus / Minus


Query: 1      acttccgatcatcgacctttcttcgccgg 29
              ||||||||||||||| ||||||||||||
Sbjct: 201619 acttccgatcatcgatctttcttcgccgg 201591
```

N97282

This is the alignment of the putative enoyl CoA hydratase to chomosome IV

```
>emb|Z97340.2|ATFCA5 Arabidopsis thaliana DNA chromosome 4, ESSA I FCA contig fragment
No. 5
          Length = 209164

 Score =  731 bits (369), Expect = 0.0
 Identities = 416/427 (97%), Gaps = 4/427 (0%)
 Strand = Plus / Minus


Query: 1      gaatcgtcgaaaaatggatcaaacagtatcggaaaatctcattcaggtgaagaaagaatc 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 144649 gaatcgtcgaaaaatggatcaaacagtatcggaaaatctcattcaggtgaagaaagaatc 144590


Query: 61     aggcgggatcgc-gtcatcacaatcaaccgtccgaaatctctcaattcactcacgagagc 119
              ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 144589 aggcgggatcgccgtcatcacaatcaaccgtccgaaatctctcaattcactcacgagagc 144530


Query: 120    gatgatggttgatctcgccaaggcattcaaggatatggactccgacgaatcggtccaggt 179
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 144529 gatgatggttgatctcgccaaggcattcaaggatatggactccgacgaatcggtccaggt 144470


Query: 180    cgtgattttnaccggatcaggtcgatctttctgctctggcgttgatttgactgcggcgga 239
              ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 144469 cgtgattttcaccggatcaggtcgatctttctgctctggcgttgatttgactgcggcgga 144410


Query: 240    gtctgttttcaaaggagacgtgaaggatccggaaaccgacccggttgtgcagatggagcg 299
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 144409 gtctgttttcaaaggagacgtgaaggatccggaaaccgacccggttgtgcagatggagcg 144350


Query: 300    gttacgtaaaccgatcatcggagctattaacggttttgccatcaccggccgggnttgaac 359
              |||||||||||||||||||||||||||||||||||||||||||||| |||||| ||||||
Sbjct: 144349 gttacgtaaaccgatcatcggagctattaacggttttgccatcacc-gccgggtttgaac 144291


Query: 360    tcgccttggcctgtaatattttgggtcgctttctagaggagctaaggtcaatggtactca 419
              ||||||||||||| ||||||| |||||| |||||||||||||||| |||  | ||||||
Sbjct: 144290 tcgccttggcctgtgatatttt-ggtcgc-ttctagaggagctaagttcatggatactca 144233
```

```
Query: 420    cgccagg 426
              |||||||
Sbjct: 144232 cgccagg 144226
```

AA713273

This is the alignment of the putative ACC Oxidase to chromosome IV

```
>emb|Z97341.2|ATFCA6 Arabidopsis thaliana DNA chromosome 4, ESSA I FCA contig fragment
No. 6
          Length = 213788

 Score =  317 bits (160), Expect = 2e-84
 Identities = 167/170 (98%)
 Strand = Plus / Minus


Query: 128    gggcgtttatagtcaacattggtgatatgatggagagatggaccaatggattgtttcggt 187
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 195028 gggcgtttatagtcaacattggtgatatgatggagagatggaccaatggattgtttcggt 194969


Query: 188    aaacttaaaccagtttacctagtcgtgagtnoggttttnatgcgttcttcacatttcgta 247
              ||||||||||||||||||||||||||||| ||||||| ||||||||||||||||||||
Sbjct: 194968 aaacttaaaccagtttacctagtcgtgagtgcggtttttatgcgttcttcacatttcgta 194909


Query: 248    tttccttgtaatgggtttaggtctacattgcacagagtgatgccggtggg 297
              ||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 194908 ttttcttgtaatgggtttaggtctacattgcacagagtgatgccggtggg 194859

 Score =  129 bits (65), Expect = 1e-27
 Identities = 69/71 (97%)
 Strand = Plus / Minus


Query: 1      ggtgcctcanctcactcagattatggaatggtcactctnctttgactgatggagttcca 60
              ||||||||| |||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 195312 ggtgcctcagctcactcagattatggaatggtcactcttctttgactgatggagttcca 195253


Query: 61     ggacttcaggt 71
              ||||||||||||
Sbjct: 195252 ggacttcaggt 195242

 Score =  123 bits (62), Expect = 7e-26
 Identities = 62/62 (100%)
 Strand = Plus / Minus
```

```
Query: 68     aggtttgtagagacaaatcgaaacaaccacacatttgggaagatgtccctggaattaaag 127
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 195165 aggtttgtagagacaaatcgaaacaaccacacatttgggaagatgtccctggaattaaag 195106


Query: 128    gg 129
               ||
Sbjct: 195105 gg 195104

 Score = 46.1 bits (23), Expect = 0.012
 Identities = 53/63 (84%)
 Strand = Plus / Minus


Query: 68     aggtttgtagagacaaatcgaaacaaccacacatttgggaagatgtccctggaattaaag 127
               |||||||||||||| |||   ||| ||||| |  |||||||||||||| |||| |||||||
Sbjct: 199824 aggtttgtagagataaagataaagaaccaaaagtttgggaagatgtcgctggtattaaag 199765


Query: 128    ggg 130
               |||
Sbjct: 199764 ggg 199762

 Score = 44.1 bits (22), Expect = 0.049
 Identities = 56/68 (82%)
 Strand = Plus / Minus


Query: 1      ggtgcctcanctcactcagattatggaatggtcactctnctttgactgatggagttcca 60
               ||||||||  |||| ||||||| ||||||| | ||||| |||   ||||||||||| ||
Sbjct: 199979 ggtgcctctgctcattcagattttggaatgataactcttcttgcaactgatggagttgca 199920


Query: 61     ggacttca 68
               || |||||
Sbjct: 199919 gggcttca 199912
```

T76589

This is the alignment of the putative Lipase with chromosome IV

```
>emb|Z97342.2|ATFCA7 Arabidopsis thaliana DNA chromosome 4, ESSA I FCA contig fragment
No. 7
          Length = 201471

 Score =  529 bits (267), Expect = e-148
 Identities = 308/321 (95%), Gaps = 2/321 (0%)
 Strand = Plus / Plus


Query: 1     atcatttnttttctctcaccaaccaacaagttctcctttctacccatttngtctccttcat 60
              ||||||| ||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct: 33301 atcatttctttctctcaccaaccaacaagttctcctttctacccatttcgtctccttcat 33360


Query: 61    tttnacgttttctttagatctgtnaataatcc-atccatggcttggtctagtcactagtg 119
              ||| |||||||||||||||||| |||||||| ||||||||||||||||||||||||||
Sbjct: 33361 tttgacgttttctttagatctgttaataatcccatccatggcttggtctagtcactagtg 33420


Query: 120   gtgttatctctctgtgtggaagaaaaagcaaggtttttnatcacttggattgttttaag 179
              |||||||||||||||||||| |||||||||||||||| ||||||||||||||||||
Sbjct: 33421 gtgttatctctctgtgtggaataaaaagcaaggtttttaatcacttggattgttttaag 33480


Query: 180   atatggcaactttgaaccctttaatttgttggatgatgatgctgaggatccaagccagc 239
              |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct: 33481 atatggcaactttgaaccctttgatttgttggatgatgatgctgaggatccaagccagc 33540


Query: 240   tcgctgtttccatcgagaagattgataagtccaagaaatctggaccgggtttcgagcttg 299
              |||||||| |||||||||||||||||||||||||||||| | ||||||||||||||
```

```
Sbjct: 33541 tcgctgttgccatcgagaagattgataagtccaagaaatctgga-caggtttcgagcttg 33599


Query: 300   cttgctaagtnagctcctaag 320
              | |||||||| ||||||||||
Sbjct: 33600 cctgctaagtcagctcctaag 33620
```