The role of acyl carrier protein in strawberry fruit ripening

Themis, Matthew

Department of Biological Sciences, Durham University

Doctor of Philosophy

2000

The Role of Acyl Carrier Protein in Strawberry Fruit Ripening

Matthew Themis, B.Sc.

Supervisors:

Mr K. Manning (Horticulture Research International, Wellesebourne)

Professor A.R. Slabas (Durham University, Durham)

Declaration

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

This information is not confidential.

Signature

Mathew Themis

Date 27.9.2001

Durham University

Matthew Themis, B.Sc.

PhD 2000

THE ROLE OF ACYL CARRIER PROTEIN IN STRAWBERRY FRUIT RIPENING

Abstract

Strawberry (*Fragaria ananassa*) is an economically important soft fruit that is highly valued as a fresh product and flavouring. During ripening, strawberry fruits undergo a number of physiological changes affecting colour, texture and flavour. An understanding of these changes at the biochemical and molecular level will be important in developing strategies for enhancing the quality attributes of this fruit. A cDNA encoding a ripening-enhanced acyl carrier protein (RE-ACP) was previously isolated from strawberry fruit. ACP is an essential component of fatty acid synthesis in both plants and animals. The aims of this thesis were to isolate and characterise this and other members of the ACP multigene family expressed in strawberry fruit. Six closely related putative ACP cDNA isoforms were identified from strawberry. Two of these were obtained by screening a cDNA library from ripe fruit and three were obtained by a technique known as candidate fragment length polymorphism (CFLP) that utilised ACP gene-specific primers for AFLP-cDNA display. Northern analysis was not able to differentiate their expression but ACP was highly up-regulated in ripening fruit whereas low levels of expression were detected in other strawberry tissues, including achenes (seeds), expanding leaves and flowers. The RE-ACP was over-expressed in *E. coli* and the recombinant protein partially purified. The over-expressed protein had a M_r of 20kDa on SDS-PAGE and appeared to form a dimer. A genomic library was constructed from *F. ananassa* from which two different genomic clones closely related to RE-ACP were obtained. Promoter analysis indicated the presence of regulatory elements. The characterization of putative ACP cDNA and genomic clones, including the 5' upstream regions, is described and their possible role in strawberry fruit is discussed.

Key words: Strawberry, fruit, ripening, gene expression, genomic, cDNA, fatty acid, acyl carrier protein, aroma, promoter.

ACKNOWLEDGEMENTS

I am grateful to my supervisor Ken Manning (Horticulture Research International, Wellesbourne) for his guidance and support throughout this project. In addition, I would also like to thank Ken for the hours of training he gave me during my time at HRI. I thank my external supervisor Professor Slabas (Durham University) for his constructive criticism and advice during difficult times.

Thank you to Bill Simon and Simon Doig (Durham University) for their collaboration in the expression of ACP in *E. coli.* Thank you Helena Silkowski (Horticulture Research International, Wellesbourne) for the use of your cDNA library constructed from strawberry ripe fruit, and to Mahmut Tor for your protocol for Candidate Fragment Length Polymorphism, plus the excellent tips and advice. To Aram Buchanon and Liz Harrison for their encouragement and advice in matters of radioactivity and other protocols. Thank you Alex Tabrett for showing me how to use the DNA sequencer and to Lynette Shakespeare for helping me with the sequencer when I forgot what Alex taught me. Thank you Antony Yemm for the best competent cells in the world!

I would like to thank Tony Martucci, KarlMorris, Ruth Dadswell, Graham Seymour and Laurence Trueman, and to everyone else I have not mentioned, for their advice and encouragement throughout my time at HRI.

Finally, I would especially like to thank my parents for their encouragement and support.

TABLE OF CONTENTS

CHAPTER 2

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

A	adenine
ACP	acyl carrier protein
[α-^{32}dNTP]	α-^{32} P-labelled deoxynucleotide
ATP	adenosine triphosphate
2-BE	2-butoxyethanol
Bis-acrylamide	Bis(N,N′-methylene-bis-acrylamide)
bp	base pair
°C	degree centigrade
C	cytosine
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CFLP	candidate fragment length polymorphism
Ci	Curie
C-terminal	carboxy terminal
cv	cultivar
d	days
DAA	days after anthesis
dATP	deoxyadenosine-5′-triphosphate
dCTP	deoxycytidine-5′-triphosphate
ddH_2O	double distilled water
DEAE	diethylaminoethyl
dGTP	Deoxyguarnine-5′-triphosphate

DIG	digoxigenin
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide-5′-triphosphate
ds	double stranded
DTT	dithiothreitol
dTTP	deoxythymidine-5′-triphosphate
dUTP	deoxyuridine-5′-triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
ETOH	ethanol
FW	fresh weight
G	guanine
g	grams
GA_3	gibberellic acid
GUS	ß-D-glucuronidase
h	hour
HCl	hydrochloric acid
IAA	indoleacetic acid (auxin)
IPTG	isopropyl-1-thio-ß-D-galactopyranoside
kb	kilobase pairs
KCL	potassium chloride

kDa	kilodalton
KP	potassium phosphate buffer
λ	phage λ
LB	Luria-bertani
LiCl	lithium chloride
M	molar
M13	M13 bacteriophage
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
Min	minute
MOPS	3-[N-morpholino]propanesulphonic
M_r	relative molecular weight
MW	molecular weight
N_2	liquid nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
1-NAA	1-napthaleneacetic acid
NAD	nicotinamide adenine dinucleotide
NADH	reduced NAD
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
N-terminal	amino-terminal
OD	optical density
ORF	open reading frame

PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PEG	polyethylene glycol
%	percentage
pfu	plaque forming unit
pI	isoelectric point
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SSC	saline sodium citrate
T	thymine
T3/T7	T3/T7 bacteriophage
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TE	Tris-EDTA
Temed	N,N,N′N′-tetramethylethylenediamine
TBE	tris/boric acid/EDTA
TCA	Tricarboxylic acid cycle
TF	transcription factor

Tris	Tris (hydroxymethyl) aminomethane
U	unit
UTR	untranslated region
UV	ultraviolet
v/v	volume for volume
w/v	weight to volume
X-gal	5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside

Chapter 1

Literature Review

Introduction

This Ph.D. Research studentship is funded by the BBSRC and arises from a BBSRC-funded program to investigate the biochemical factors affecting maturation, ripening and senescence which was initiated by Mr. K. Manning (HRI Wellesbourne), who has focused on strawberry (*Fragaria* x *ananassa* Duch.), a non-climacteric fruit.

Ripe fruit from a day neutral strawberry (c.v. Brighton) was used to produce a cDNA library. A differential screen of this library isolated approximately 100 ripening-enhanced cDNA clones. Partial sequencing of these clones, and comparisons with sequences in the database, have identified three cDNAs with homology to acyl carrier protein (ACP). This protein is known to be an essential component of fatty acid synthesis in both plants and animals. These three clones may or may not be different isoforms.

The aim of this project is to investigate the role of ACP in the ripening process of strawberry fruit in relation to lipid metabolism.

1.1 Development and Biology of the Strawberry

The modern strawberry (*Fragaria* x *ananassa*), from the family Rosaceae, is a herbaceous plant. It originates from crosses of two *Fragaria* species *F. virginiana* from North America and *F. chiloensis* from Chile, as described by A.W. Duchesne. The fruit itself is described as a soft fruit and has been developed for commercial production in temperate regions across the world. Many cultivars are grown, for instance Elsanta is currently the predominant cultivar of central Europe and the U.K.

The widespread consumption of strawberries makes them an economically important fruit crop. For example, in the U.K the value of the home produced fruit is around £62 million, with 36,000 tones produced annually in 1998 (MAFF). Strawberry fruit quality at the point of sale is of prime importance to consumers and producers. Important quality attributes include fruit size, flavour (sweetness and aroma), colour and texture. Strawberry breeding aims to improve disease resistance, extend picking season and lengthen shelf life. In the U.K there is increasing competition from strawberries imported from the Netherlands, Belgium, Spain and California, and quality of the fruit from home producers will be of major importance.

In addition to classical breeding methods, attempts to modify the strawberry are being made by genetic manipulation. An early example of such a manipulation was that carried out in California in 1990 to transform strawberry plants with the protein sweetener thaumatin and other ripening genes (Righelato and Traill, 1996). The tomato is another fruit widely used as a model system in which genetic modification has been employed to alter ripening. In this case ethylene production by the fruit was reduced allowing the fruit to stay on the vine longer without becoming overripe too rapidly (Evans, 1996).

1.1.1 Strawberry fruit anatomy

A fruit is a ripened ovary of a flower. It protects the seed, assists in dispersal and may be important in germination. Examples of fruits include apple, plum, peach, cherry, orange, beans etc. Fruit can be classified according to certain criteria such as simple and compound forms (Weier *et al.*, 1992). Simple fruits are derived from a single ovary,

being either dry or fleshy, composed of one carpel, and the mature fruits may be dehiscent (open to liberate seeds) or indehiscent. Compound fruits are aggregates or clusters of simple fruits, their complexity depending on the flower number involved.

In the strawberry, as well as the pineapple, mulberry and blackberry, the fruit is derived from the many ovaries of a single flower and these are attached to a common receptacle (Weier *et al.*, 1992). The strawberry has numerous carpels, each one having an ovary in which there is one ovule, and these are attached to a single receptacle, as shown in Figure 1.1.

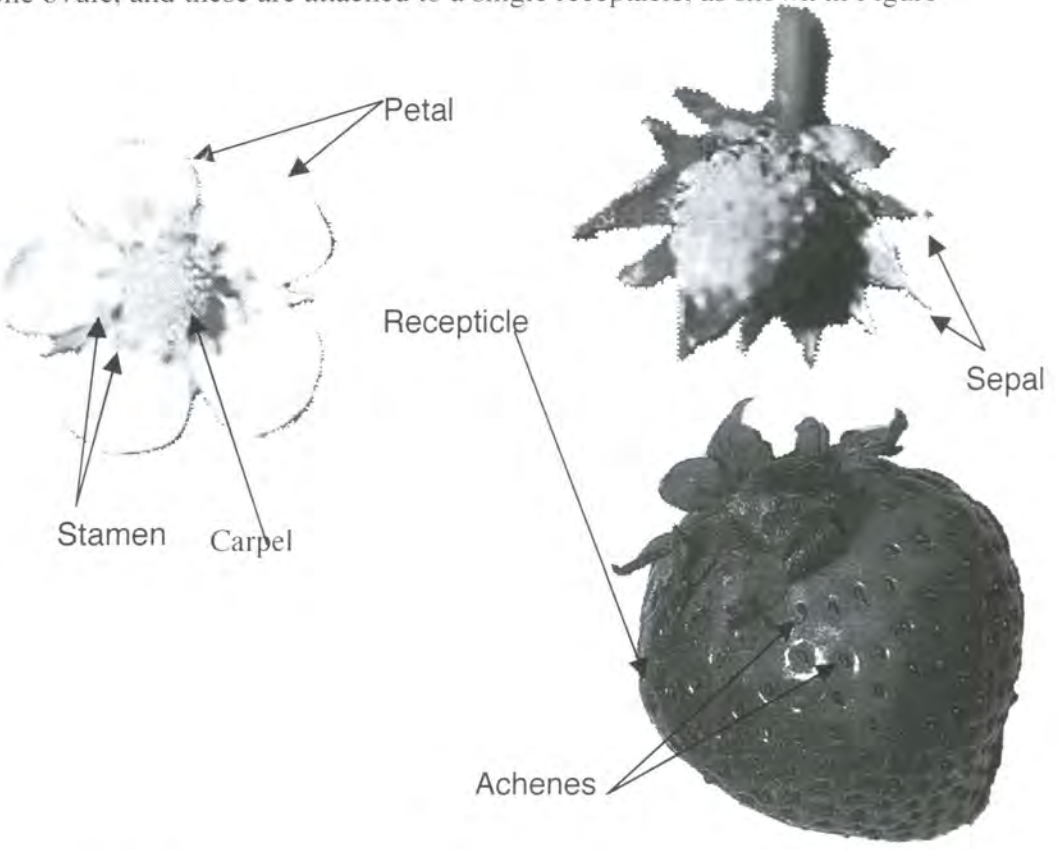

Figure 1.1. Anatomy of the strawberry

The ovary develops into the achene (a one seeded dry fruit) and the receptacle forms the fleshy part to which the fruit is attached. The strawberry is the aggregation of simple fruits, each being an achene. The receptacle consists of stem tissue; a fleshy pith and cortex with a vascular bundle between them. By this former definition the strawberry is not a true fruit as the fleshy receptacle is derived from stem tissue not the ovary (Weier *et al.*, 1992). The fleshy receptacle is attractive to birds and facilitates seed dispersal.

Soft fruits, of which strawberry is the most important in economic terms, are used widely in processed foods including frozen products, yogurts, tinned products, jams etc. As a fresh product, however, it has a short shelf life and bruises easily.

1.1.2 Fruit physiology

Fruits can be categorised into two groups. These are referred to as climacteric and non-climacteric fruits. Climacteric fruits are defined according to a dramatic rise in respiration with the onset of ripening. Ethylene production increases too at this time and can do so gradually, such as in banana, or as a sudden rise in concentration, as in apple. Ethylene production has been shown to be autocatalytic in many climacteric fruits (Giovannoni, 1993). Non-climacteric fruits, such as strawberry, do not undergo a respiratory rise, and increased ethylene production does not occur at the onset of ripening (Manning, 1993).

The developing strawberry fruit exhibits a characteristic set of physio-chemical changes as it grows and ripens (Manning, 1993). These include changes in pigments, texture, flavour compounds (sugars and aromatics) etc., and they occur in overlapping phases of maturation, ripening and senescence. The final size achieved, approximately 30 days after anthesis, is dependent on the fruit's position on the flowering shoot, with the smallest fruits occurring at the most distal positions. Genetic and environmental factors determine fruit size. Factors such as temperature may govern the metabolic rate and the ripening characteristics described above. Receptacle size increases with cell division, which ceases at approximately seven days, and thereafter occurs by expansion. Immature fruit have small vacuoles and a dense cell wall. Starch is present in the plastids and ribosomes and golgi apparatus are abundant. However, during maturation and ripening the cell wall becomes disorganised and starch grains decrease (Knee *et al.*, 1977). The cell wall is important for fruit firmness, and changes within it are believed to be responsible for softening. Therefore this is an important process to understand in regards to the consumer. Although highly important, the cell wall will not be addressed here.

1.2 Fruit Biochemistry

1.2.1 Energy metabolism

Respiration allows for the production of energy, which in turn is used for the synthesis of primary and secondary metabolites, needed by the plant for growth and development, such as ripening. Respiratory substrates include sugars and organic acids, which are found largely in the vacuole of cells. Respiratory pathways used by fruit tissues are common to all living plant cells e.g. glycolysis, the pentose phosphate pathway and the TCA cycle. The concentration of ATP increases during ripening and phosphofructokinase, one of the rate limiting enzymes of glycolysis, has been shown to be insensitive to this, suggesting that glycolysis is unaffected by ATP increases. Pyruvate is known to enter the tricarboxylic acid (TCA) cycle, but how the TCA cycle is regulated during ripening is poorly understood. The TCA cycle is coupled to the electron transport chain but a build up of ATP in climacteric fruit does not inhibit respiration. How this is achieved is also unknown, and the reason why increased respiration occurs in climacteric fruit but not in non-climacteric fruit is also uncertain.

Malate, a metabolite of the TCA cycle, is an important respiratory substrate found in most fruits. Malic enzyme converts malate to pyruvate for its use in the TCA cycle and this enzyme is localised in the cytosol and mitochondria (Tucker, 1993). Its activity has been shown to correspondingly increase with respiration in apples and pears. The content of malic acid is very similar throughout the development of strawberry fruit (Montero *et al.*, 1996).

1.2.2 Flavour changes

The distinctive palatability of a fruit lies in our ability to detect the flavour compounds it produces. Generally, in fruits sugars and organic acids provide the tastes of sweet and sour respectively. The presence of volatile components gives fruits their unique character (Montero *et al.*, 1996). Phenolics and tannins give fruit an astringent character.

1.2.2.1 Sugars and organic acids

Soluble sugars and organic acids, although produced in excess, are used for respiration. The ripe strawberry, for example, accumulates glucose, fructose and sucrose during ripening and declines during senescence. These three sugars represent 99% of the sugars, with glucose and fructose representing 83% in a 1:1 ratio (Montero *et al.* 1996). Also present in minor amounts are sorbitol, xylitol, and xylose. The sugar content is affected by environmental conditions.

Several enzymes may be involved in sucrose metabolism in fruit. The pathways are shown below in Figure 1.2.

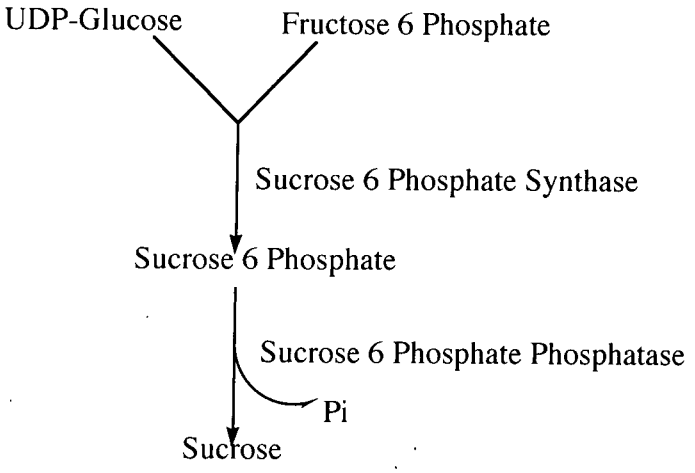

Figure 1.2. Biochemical pathway for sucrose synthesis.

Invertases hydrolyse sucrose to glucose and fructose and may also have a role in sugar transport from one tissue (source) to another (sink). In the strawberry sucrose is known to be the main carbon source transported from the leaves to the apoplastic region of the cell and the enzyme invertase may play a role in converting this into glucose and fructose for cellular uptake. Two invertases have been reported in strawberry, one being a soluble from and the other being a bound form, with the latter thought to be the main contributor to the sucrose sink strength (Poovaiah and Veluthambi, 1985). In most plants soluble invertases are found in the vacuole and have a pH optimum of 5.0, while the bound forms are found in the cell wall and have a pH optimum of 7.5 (Davies and Robinson, 1996). Two main ways are known to transport sugars, one is via the symplast (cell cytoplasm)

and the other is via apoplast (through the cell wall of plants). Whichever route is used sucrose is loaded into the phloem vessels and this is thought to be through active transport which utilises a proton gradient (generated by an ATPase) used by a proton symporter. Invertases at the sink metabolise the sucrose into hexose sugars which are believed to be taken up into the cells by an energy dependant mechanism mediated by transporters (Heineke *et al.*, 1994). Invertases and sucrose synthase may regulate sink strength by maintaining a low concentration of sugars.

Organic Acids

Organic acids vary widely in fruits and in strawberry they increase between the immature and mature green stages then decline at the ripening stage (Montero *et al.*, 1996). In the strawberry 33 organic acids have been identified by Mussinan and Walradt (1975), with citric acid being the most abundant (Montero *et al.*, 1996). The ratio of organic acids to sugars is an index of fruit quality, a high content of acids gives a sour flavour to the fruit while non-volatile acids may affect taste. Volatile compounds contribute to overall flavour.

Carbohydrates

Fruits may accumulate different carbohydrates, e.g. starch in unripe banana, and simple sugars in tomato, prior to ripening (Manning, 1993). Strawberry fruit, however, continue to take up sugars during ripening (Forney and Breen, 1986) and the level of sugar accumulating in the fruit tissue determines optimum harvesting time.

In fruits, such as banana, starch is degraded to sucrose, glucose and fructose during ripening (Tucker, 1993). This breakdown is catalysed by several enzymes including α-amylase (specific for endo α-1,4 glucose linkage), ß-amylase (specific for exo α-1,4 glucose linkages) and α-1,6-glucosidase (specific for non-reducing end of starch to yield glucose-6-phosphate), which have been reported to increase in the ripening fruit. However, amylases do not hydrolyse amylopectins and amylose, which require other enzymes to complete their degradation.

1.2.2.2 Phenolics

Phenolic compounds also determine fruit quality giving fruit its astringent taste when unripe. As the strawberry fruit ripens the phenol content declines (Manning, 1993, Montero *et al.*, 1996). An example of a phenol found in fruits is given below in Figure 1.3.

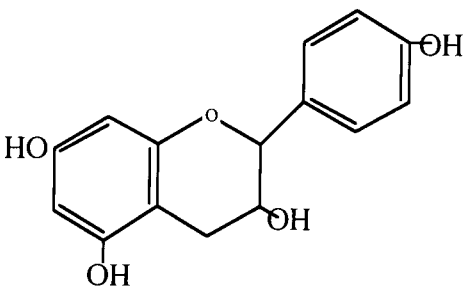

Figure 1.3. Structure of Flavon-3-ol (Catechins).

Phenolics are derived from phenylalanine, via cinnamic and coumaric acids, and consist of different classes of compounds such as hydroxycinnamic esters, flavonoids (anthocyanidins and tannins) and lignin (Ellis, 1997). Phenylalanine ammonia-lyase (PAL) is the first enzyme of phenylpropanoid biosynthesis and its activity increases during strawberry fruit ripening (Given *et al.*, 1988, Martinez *et al.*, 1996).

Phenols comprise a large family of compounds including the tannins and proanthocyanins etc., and thousands of compounds are produced as secondary metabolites, many of which may have therapeutic properties and are of interest to the pharmaceutical industry. The brown colour produced by wounding plant tissues is due to the enzymes catechol oxidase and laccase which oxidise ortho and para phenols (Tucker, 1993).

Phenol derivatives play a vital role in electron transport, but also have important antioxidant and antifungal properties in the plant. Suberin and lignins are also derived from phenols, but are present in dead or dying cells providing structural support to plants. The interaction of tannins and the proteins on the tongue provides an intense astringency

sensation providing a powerful feeding deterrent until the fruit is ripe when such compounds decline (Montero *et al*, 1996).

1.2.2.3 Flavour volatiles

Flavour volatiles are present in minute quantities and are a very diverse group of compounds that give fruits their characteristic aroma. More than 200 volatile compunds have been identified in strawberry fruit, and those having most impact in flavour include Methyl butanoate, ethyl butanoate, methyl hexanoate, ethyl hexanoate, trans-2-hexenyl acetate, trans-2-hexenal, trans-2-hexen-1-ol and 2,5-dimethyl-4-hydroxy-3 (2H)-furanone (DMHF) (Schreier, 1980). The important aroma compound DMHF or FURANEOL is found in many fruits and is a key component of strawberry flavour (Douillard and Guichard, 1990, Pickenhagen *et al.*, 1981, Dirinck *et al.*, 1981). Its aroma has been described as strawberry-like at low concentrations while at higher levels it resembles caramel. Its methyl ester gives a sherry-like aroma (Pickenhagen *et al.*, 1981). Many different pathways may be involved in aroma production. Metabolism of amino acids, keto sugars, the action of lipoxygenase on membrane lipids, and isoprenoid pathways may be involved. For example, esters are made from the reactions between alcohols and acids. However, enzymic pathways involved in these reactions in strawberry have not been fully defined. The large number of compounds produced may result from the low specificity of relatively few enzymes for the substrates involved. An understanding of these pathways may lead to future crop improvements. Certain components give fruits their characteristic flavour (Table 1.1)

Table 1.1. Flavour characteristics of biological compounds.

Flavour Characteristics	Compound
Fruity	Esters, Alcohols, Carbonyls
Strawberry Flavour	Sulphur compounds e g . dimethyl disulphides

The C6 volatile components probably derive from the enzymic oxidation of linoleic and linolenic acids (Schreir, 1980). Most of the aroma compound in strawberry are esters of short chain fatty acids and C6 compounds. Strawberry fruit can convert six and nine carbon aldehydes to their corresponding volatile alcohols and acetate esters (Hamilton-Kemp *et al.*, 1996). For example, hexanal was readily metabolised by the fruit to 1-hexanol and the ester hexylacetate.

Bandyopadhyay and Gholap (1973) have suggested that there is a relationship between the changes in the fatty acid composition of mango pulp and in the development of aroma and flavour. Many of the aroma compounds of strawberry fruit have important flavour and antimicrobial properties during ripening. Although not all aspects of aroma biosynthesis are fully understood, the metabolic pathways involving lipid metabolism are being elucidated, especially in tomato and bean, and these may be similar in strawberry. In tomato, lipoxygenase (LOX) activity increased sharply during early ripening stages and decreased after the fruit had ripened fully (Ealing, 1994), as did LOX from strawberry (Perez *et al.*, 1999). These authors suggested that the products of LOX activity, such as 13-hydroperoxide are metabolised to produce compounds such as cis-3-hexanol and hexenyl esters that are key flavour components of tomato and strawberry fruits. A tomato and strawberry hydroperoxide lyase (HPL) protein was isolated which has highest activity towards the LOX product 13-hydroperoxide derived from linolenic acid (Matsui *et al.*, 2000 and Perez *et al.*, 1999, respectively), as shown in Figure 1.4. Hydroperoxide lyase activity is relatively constant during tomato fruit ripening (Riley *et al.*, 1996) but rises in strawberry (Perez *et al.*, 1999), indicating it may not be the key step in this pathway in tomato. Two enzymes from strawberry, alcohol acyltransferase (SAAT) (Aharoni *et al.*, 2000) and alcohol dehydrogenase (ADH) (Mitchell and Jelenkovic, 1995) may have a role in flavour biosynthesis in this fruit. The activities of both enzymes increase during the period of fruit maturation and ripening.

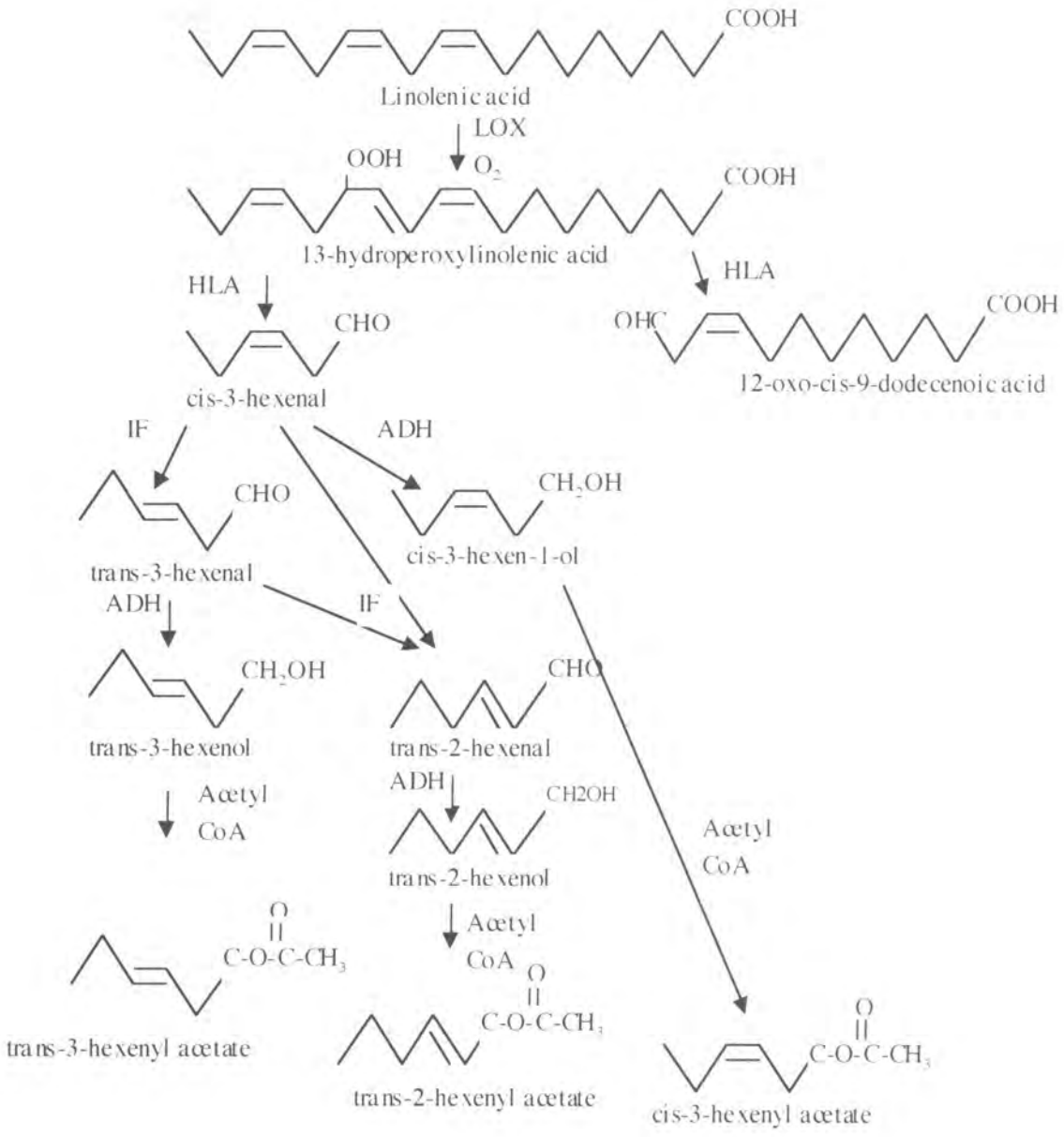

Figure 1.4 Biochemical pathways of the lipoxygenase cascade. LOX, lipoxygenase; IF, isomerization factor; ADH, alcohol dehydrogenase and AAT, alcohol acyltransferase (from Croft *et al.*, 1993).

These enzymes from strawberry were able to metabolise C6 aliphatic alcohols. ADH can convert aldehydes to their corresponding alcohols and these can be esterified by SAAT to form volatile esters, such as trans-3-hexenyl acetate (Fig. 1.4). The expression of a gene encoding the enzyme pyruvate decarboxylase is also enhanced in ripening strawberry

(Manning, 1998, Ahavoni *et al.*, 2000). This enzyme may provide the acetate group for ester formation. The fact that ACP, ADH and SAAT are all up-regulated during fruit ripening strongly suggests that these enzymes are acting together to generate volatile aroma compounds. All the compounds in Figure 1.4 have been identified in strawberry fruit (Hamilton-Kemp *et al.*, 1996; Zabetakis and Holden, 1997), except for trans-3-hexenal, trans-3-hexenol and trans-3-hexenyl acetate, suggesting the biochemical pathway involving these compounds is not active in strawberry. In addition, the compound 12-oxo-cis-9-dodecenoic acid has not been reported. The reasons for this is unclear but it may be due to the compound being rapidly metabolised, or the putative LOX isozymes in strawberry result in other compounds being formed.

The antimicrobial properties of several of the volatile compounds formed in strawberry may be important in retarding the post harvest spoilage of the fruit.

1.2.3 Colour changes

Chlorophylls and carotenoids

Chlorophyll breakdown occurs during the development of many fruits including strawberry. There is no clear explanation as to how this occurs but loss of chlorophyll often coincides with the appearance of other coloured compounds, such as carotenoids. Chlorophyllase and peroxidase are believed to be involved in chlorophyll degradation (Martinez *et al.*, 1996). Both of these enzyme activities decline during strawberry fruit ripening. In tomato fruit, for example, colour changes indicate ripening. The loss of chlorophyll is coordinated with the accumulation of carotenoid pigments, such as ß-carotene and lycopene as the plastids become chromoplasts (Brady, 1987). In this process the highly organised photosynthetic apparatus of the chloroplast becomes disorganised which causes the loss in green colour (Martinez *et al.*, 1996). The carotenoids are less diverse than other pigments, such as anthocyanins, although they may be produced in large quantities such as lycopene in the tomatoes. Formed from acetyl-CoA, carotenoids are produced via the isoprenoid pathway. Little information is available on the control mechanisms in this biochemical pathway. Recently, two pathways were identified that generate isoprenes in tomato fruit. These are known as the

mevalonate pathway, located in the cytosol/endoplasmic reticulum, and the mevalonate-independent pathway, located in the plastids (Lois *et al.*, 2000). There is a strong correlation between the activity of the first enzyme of this plastid biosynthetic pathway and carotenoid synthesis during fruit development. Ripening does not, however, depend on the carotenoids, as demonstrated by tomato mutants that remain green when ripe (Giovannoni, 1993) and that these are known as 'green flesh' mutants. The breakdown products of chlorophyll in ripening fruit may possibly be utilised for the formation of other compounds. Pigments not only indicate ripeness but may be important as vitamin precursors or as antioxidants, such as lycopene.

Gross (1982) has analysed the pigments in developing strawberry fruits. The chlorophylls and carotenoids in the fruit fell rapidly to low levels during ripening. Anthocyanin production increased rapidly when both the carotenoids and chlorophylls had reached their lowest levels.

Anthocyanins

Anthocyanins are another important class of pigments found in the cells of certain fruits and flowers that occur in a wide range of colours. Differences in colour may result from complex formation between pigments, i.e. anthocyanins with flavones, or metal ions, glycosylation and acylation (Ellis, 1997). Leucoanthocyanins are the intermediate colourless precursors of anthocyanins that when coupled to glycosyl residues give various colours. The primary precursor of anthocyanin biosynthesis is phenylalanine and the key enzyme committing phenylpropanoid synthesis is phenylalanine ammonia lyase (PAL). Anthocyanins are chemically based on the structure of flavone (Figure 1.5).

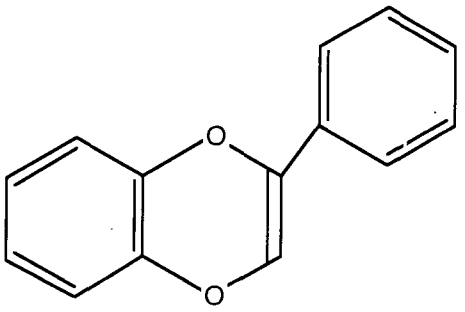

Figure 1.5. The chemical structure of flavone.

In strawberry fruit PAL activity increases during the transition from green to the ripened fruits. This is due to *de novo* synthesis, resulting in pigment (anthocyanins) accumulation (Hyodo, 1971, Martinez *et al.*, 1996). However, anthocyanin levels continue to increase after PAL activity has reached a maximum. Another enzyme that follows a similar pattern of activity is uridine diphosphate-glucose: flavonoid-O-3- transferase (UDPGFT), which produces the major anthocyanin pelargonidin-3-glucoside. This is the terminal step of anthocyanin biosynthesis.

1.2.4 Plant hormones

Auxin is probably essential for the growth of all fruits. Seeds are the source of this hormone and parthenocarpic fruit (ie. lacking seeds) fail to grow unless another source of auxin is provided. Free and conjugated auxins have been found in strawberry fruit and have been reviewed by Manning (1993). The formation of these different forms is not fully understood, but the bound form may act as a reservoir or store. The unbound (active) form is most abundant in the achenes. Studies by Given *et al.* (1988) showed that removal of the achenes causes the receptacle of the strawberry to turn red and ripen early and application of the synthetic auxin, 1-napthylacetic acid (1-NAA) prevented this occurring. Therefore, auxin was implicated to act as a ripening inhibitor but it also acts to promote growth of the receptacle. Furthermore, PAL was induced in deachened fruit, while chlorophyll content declined, and this could be prevented by application of a synthetic auxin.

Ethylene is the main hormone involved in the ripening of climacteric fruits. In non-climacteric fruits only low levels of ethylene are produced, and for strawberry ethylene production declines after the mature green stage. Ethylene production is non-autocatalytic in these fruits, but when applied exogenously does raise respiratory levels (Atta-aly *et al.*, 2000). Ethylene is not thought to be significant in strawberry fruit ripening, but is known to cause abscission in the raspberry, another non-climacteric soft fruit. A recent comparison was made between the effects of ethylene in tomato and strawberry fruit. Exogenous ethylene application to tomato fruit pericarp tissue resulted in an inhibitory effect on ethylene biosynthesis at the immature stage of development (Atta-aly *et al.*, 2000). The effect was reversed at the onset of ripening. In contrast, application of ethylene to strawberry fruit caused a short-term increase in ethylene production levels that fell to control values. Strawberry fruit showed a negative feedback to ethylene at all developmental stages with no increase in CO_2 production levels (Atta-aly *et al.*, 2000). However, strawberry fruit colour development and softening were slightly accelerated by the application of ethylene (Tian *et al.*, 2000). This suggests that strawberry may possess ethylene receptors but they have different actions to those found in climacteric fruit.

Gibberellins (GA_3), cytokinin (CK) and abscisic acid (ABA) have been reported generally in fruit. GA_3 together with a synthetic auxin promoted growth and ripening, but in combination with an synthetic CK was inhibitory. ABA was shown to increase in the achenes and induces ripening when applied exogenously (Kano and Asahira, 1978). Exogenous application of gibberellin to whole strawberry fruit delayed the increased activity of PAL and colour development (Martinez *et al.*, 1996) and lowered chlorophyllase and peroxidase activities.

In summary, auxin appears to be the main elicitor of both growth and ripening in strawberry, with its actions modified by other hormones. The hormonal response by a tissue may depend on its developmental stage.

1.2.5. Genetic controls of fruit ripening

This section mainly relates to the ripening of strawberry. Evidence for ripening being a genetically controlled event has been obtained for a wide range of fruits. Both anabolic

and catabolic processes are involved during ripening, possibly controlled by a range of plant growth regulators including gibberellins (GA_3), cytokinin (CK), auxin (IAA), ethylene, and abscisic acid (ABA). These hormones may interact with each other, and the responses that are brought about may be dependent on the type of tissue and its maturity. Some ripening genes have hormone regulatory sequences. The promoter region of a ripening-related sucrose transporter gene (Vvht1) from grape (Fillon *et al.*, 1999) has several potential regulatory sequences including those for the hormones ethylene, abscisic acid and sugar responsive sequence elements. This indicates that plant growth regulators act on certain genes in concert with other regulatory pathways to control temporal and spatial gene expression. A putative UDPglucose IAA-glucosyltransferase cDNA has been identified from ripening strawberry fruits that may have a role in inactivating free auxin by forming auxin conjugates (Manning, 1998) (see 1.2.4. Plant Hormones). These events may enable a plant to 'fine tune' its response to certain stimuli.

Changes in gene expression during fruit development and ripening in the strawberry have been described by Manning (1994). A complex pattern of mRNA expression was observed in a comparison between ripe and unripe fruit with a set of mRNAs increasing in abundance at the turning stage of ripeness. This group of mRNAs also appeared in de-achened fruit showing enhanced ripening but was suppressed by the application of auxin. A number of workers have now identified several ripening enhanced genes in strawberry that affect a range of metabolic activities. Manning, (1998) has identified genes putatively involved in anthocyanin biosynthesis (Table 1.2), sugar transport (sucrose transporter) and cell wall hydrolysis (cellulase). Three cDNAs encoding ACP were also identified and are the subject of this study.

Table 1.2. Putative identities of some of the ripening-related cDNA clones associated with anthocyanin biosynthesis from strawberry receptacles (Manning, 1998).

Gene	**Putative identity**
FAN R2	Flavonoid-3-hydroxylase
FAN R74	UDPglucose glucosyl transferase
FAN R5	Chalcone synthase
FAN R31c	Chalcone reductase
FAN R1	O-methyl transferase

Of the anthocyanin genes flavonoid-3-hydroxylase (F3H) and chalcone synthase (CHS) are also expressed at the immature stages of fruit development. Another anthocyanin related ripening-enhanced gene isolated from strawberry, encoding dihydroflavonol 4-reductase (DHFR) (Moyano *et al.*, 1998) mirrors the same expression patterns as CHS and F3H. These enzymes may supply substrates for flavonol biosynthesis, responsible for the astringency of the immature fruit.

Using PCR differential display Wilkinson *et al.* (1995) identified five ripening enhanced cDNAs of which three were fruit specific, including a cDNA encoding the Ca2+ dependent phospholipid-binding protein annexin. Although non-climacteric fruit have not been characterised as fully as the climacteric fruits, the patterns of ripening genes emerging indicates that several are common to certain fruits of both types including those encoding a protease inhibitor, heat shock proteins, UDPglucosyl transferase, UDPglucuronosyl transferase, and cell wall degrading enzymes.

Other studies of genes up-regulated in strawberry fruit include Nam *et al.*, (1999) and Medina-Escobar *et al.*, (1997) and most recently Aharoni et al. (2000).

1.3 Plant Lipids and ACP

1.3.1 Major classes of Plant lipids

Major plant lipids, described in a review by Lea and Leegood (1993) have a very diverse structure and distribution and are summerised in Table 1.3.

Table 1.3. Lipid classes found in plants.

Lipids	**Physiological Function**
Phospholipids / glycolipids	Membrane components
Triacylglycerols	Energy storage
Sterols	Structural functions in plant membranes. Possible roles as hormones. May play part in cell division.
Chlorophylls / Carotenoids	Pigments
Waxes	Provides protective role by waterproofing and a barrier against pathogens.

1.3.2 Lipids as pigments

Plant pigments derived from lipids are shown in Table 1.4.

Table 1.4. Pigments identified in plants.

Pigment	**Example**
Tetrapyrrole (chlorophylls)	Chlorophyll a
Tetraterpenoid (carotenoids)	Carotene, Xanthophylls

The chlorophylls (see section 1.2.3)

The chlorophylls consist of the basic structure of four pyrrole molecules in cyclic or linear forms. Chlorophyll is synthesised from the precursor δaminolaevulinic acid (itself produced from glutamate) to form protoporphyrinogen, which feeds into two separate pathways. These pathways may be for chlorophyll production or cytochrome, peroxidase and catalase biosynthesis. By complexing the tetrapyrrole molecule with different ligands molecules, for example Mg or H, and different side chains, e.g. methyl, proprionic acid, phytyl etc., absorption of light at different frequencies can be achieved. Chlorophyll is the most abundant pigment on the planet.

Carotenoids

The carotenoids typically have a yellow, orange or sometimes red colour. These molecules consist of a 40 carbon skeleton built up from five carbon isoprenoid units. Carotenoids are found within plastids and not only act to capture light energy but act as antioxidants against oxygen radicals. In banana fruit the degree of ripening influences the amount of carotenoid fatty esters and free/monoester diester ratio of lutein (Subagio *et al.*, 1996). During ripening, the chloroplast thylakoid membranes are degraded gradually and release fatty acids. It was proposed that oxygenated carotenoids are esterified by the released fatty acids, mainly myristate (C14) and to a lesser extent laurate (C12), palmitate (C16) and caprate (C10) (Subagio *et al.*, 1996). In contrast, carotenoid content decreases in ripening strawberry fruit (Manning, 1993).

1.3.3 Steroyl fatty acyl esters in plants

Steroyl esters are found by the conjugation of sterols to other compounds and include fatty acyl esters (SE), steroyl glycosides (SG), and acylated steroyl glycosides (ASG). An example of an SE is sitosteryl linoleate (Fig. 1.6). SE are found in many plants tissues e.g. roots and shoots, and in a range of species e.g. maize, tobacco, banana and tomato. They may differ in abundance, depending on factors such as maturity, light, tissue type, germination, and senescence, and in the fatty acid steroyl moieties (Dyas and Goad 1993).

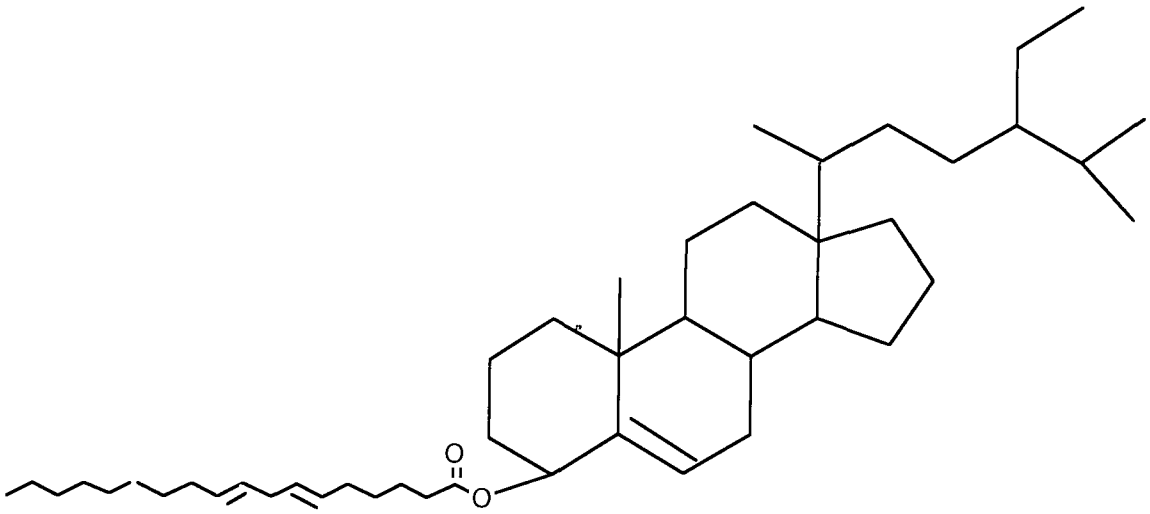

Figure 1.6. The chemical structure of Sitosteryl linoleate

The fatty acid part of SE are typically C12 to C22 in length, with palmitic, stearic, oleic, linoleic, and linolenic acids being most common. These fatty acids may be found in different proportions in different tissues e.g. palmitic acid is predominant in the shoots of maize. Further investigations have found SE located in the mitochondrial and nuclear fractions of the cell. In some plants during germination SE decrease while free sterol (FS) increases, but in others no changes are seen. In germinating tobacco seeds the fatty acid moiety changed from palmitic to linoleic acids (Dyas and Goad 1993) . The sub-cellular, cellular and tissue distribution of steroyl/FA moieties, depend on the stage of plant development and environmental conditions. In tomato fruit, for example, the FS and steroyl moieties differed between the mature green and red ripe stages of development. Some tomato non-ripening mutants did not show this change indicating that it was associated specifically with fruit ripening (Whitaker, 1988).

SE are found in the microsomal fractions of the cell, but the physiological purpose of these compounds is unknown. In animals, cholesterol esters occur in the bile acids, for emulsifying fats and for the transport of fats via lipoproteins etc. In plants FS and SE are inter-convertible and it is speculated that SE could provide FS for the cell during cell division and growth, or for storing sterols when alterations in development occur, e.g. senescence, seed maturation etc. In addition, SE may have a transportational role, or they

may be a store for FA precursors to act in a hormonal capacity. Furthermore, the fluidity of cell membranes and associated biochemical processes and may be affected by SE (Dyas and Goad, 1993).

1.3.4 Waxes

Waxes provide waterproofing and form a barrier to protect plants from environmental stress e.g. dehydration or pathogens. Waxes include long chain fatty acids, hydrocarbons, alcohols, aldehydes, ketones, esters, triterpenes, sterols and flavonoids. The distribution of these compounds varies from species to species, and between different epidermal tissues of the same plant. A large number of genes are thought to be involved in their biosynthesis and deposition, but these have not been fully characterised (Lemieux, 1996).

For wax production fatty acids are elongated, to between 20 to 32 carbons, or 40-60 carbons when esterified to alcohols. These reactions are carried out by elongases (but the acyl chain is not esterified to ACP). The starting points for elongation are the C16 and C18 fatty acids. The pathways for the production of waxes from fatty acid precursors are shown in Figure 1.7.

Fatty acid precursors are required for the synthesis of glycerolipids, waxes, cutin and suberin. The pathway through which a fatty acid is channelled depends on the type of fatty acid precursor. For example, C16: 0 and C18: 0 FA's produce glycerolipids, C18: 0 possibly produce waxes, and C16: 0 and C18: 1 hydroxy fatty acids produce cutin and suberin (Post-Beittenmiller, 1996).

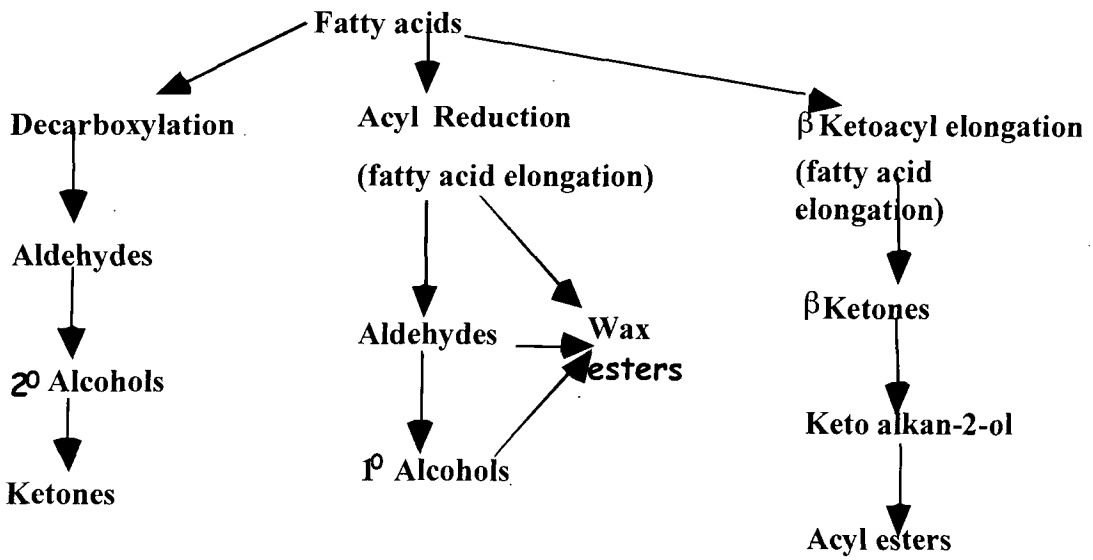

Figure 1.7. A general overview for the production of waxes in plants. (Post-beittenmiller, 1996)

The production of C18: 0, C18: 1 and C16: 0 fatty acids depends upon their esterification to ACP, which is enzymatically removed from the completed fatty acid acyl chain. The enzymes responsible for the further processing of these acyl-ACP thioesters are stearoyl-ACP desaturase, plastidial acyl transferases, and acyl-ACP thioesterases (hydrolases) (Post-Beittenmiller, 1996). Further reactions are carried out extraplastidially by microsomal enzymes known as elongases, with malonoyl-CoA serving as the carbon donor.

1.3.5 Glycolipids and phospholipids

Phospholipids and galactolipids are major components of plant membranes, with neutral lipids being used as an energy source e.g. seed oils. The thylakoid membranes comprise mostly of galactolipid (about 75%). Animals require polyunsaturated fatty acids for their optimal function. However, certain desaturases are absent from animal metabolic processes and therefore are unable to produce these fatty acids. These fatty acids are required in the diet and hence α-linolenic (18:3 Δ9,12,15c) and linoleic acid (18:2 Δ 9,12c) are known as essential fatty acids (Lea and Leegood, 1993).

Fatty acids have important commercial uses including the production of cosmetics, detergents, cooking oil, pharmaceuticals, paints etc. It is their importance in plants, however, that this study is concerned with.

Fatty acids typically occur as 16-18 carbon molecules, except in seeds where a variable 8-24 carbon chain length can occur. They can be esterified to other molecules e.g. glycerol to give glycerolipids (GLs). There are three major groups of GLs: the acylglycerols, the phosphoglycerols, and the glycolipids. Their functions in plants are listed in Table 1.5.

Table 1.5. Glycerolipids of plants and their functions.

Lipid	**Funtion**
Acylglycerols	Seed store oils (energy)
Glycolipids	70-80% lipid in plant thylakoid membranes
Phosphoglycerols	All membranes

1.3.6 The biosynthesis of fatty acids

The biosynthesis of fatty acids occurs in the plastid and their precursor is acetyl-CoA (reviewed by Lea and Leegood, 1993 and Ohlrogge and Browse, 1995). The source of the acetyl-CoA pool is unknown but is present in the plastid at a concentration of 30 to 50mM and remains relatively constant. The rate of FA biosynthesis is higher in the light (Hanapel and Ohlrogge, 1988, Bao *et al.*, 2000).

Acetyl-CoA may be generated in the plastid by several possible routes. One of these may involve plastidial pyruvate dehydrogenase (PDH) acting on pyruvate a substrate derived either from glycolysis or via ribulose bisphosphate carboxylase activity. However, the activity of plastidial PDH is lower than that required for FA biosynthesis (Ohlrogge and Browse, 1995). The activity of another enzyme in the plastid, acetyl-CoA synthetase, is 5 to 15 times greater than that required to supply acetyl-CoA for *in vivo* fatty acid

synthesis. Since acetate concentration in plastids is higher than that of pyruvate, it may be that acetyl-CoA synthetase is more important in maintaining a steady pool of acetyl-CoA for fatty acid synthesis. Because of the number of potential pathways involved, the actual *in vivo* generation of the acetyl-CoA pool in plastids is not understood. In addition, the flux of metabolites from one pathway to another may vary in different species, tissues, environmental conditions and developmental stages.

Bao *et al* (2000) attempted to determine whether fatty acids were generated from C3 compounds or from free acetate using *Arabidopsis* leaf. Radiolabelling and chromatography-mass spectroscopy were used to determine that ^{14}C-labelled CO_2 rapidly appeared in fatty acids. However, only 5% of the label appeared in free acetate, suggesting that fatty acid biosynthesis does not depend on a high concentration of acetate but the direct use of newly fixed carbon in the chloroplast.

A series of enzymatic reactions participate in fatty acid synthesis (FAS), starting with acetyl-CoA carboxylase (220-240kDa), which produces malonyl-CoA. In the next step fatty acid synthetase (FASase) utilises acetyl-CoA and malonyl-CoA to produce fatty acids.

Acetyl-CoA carboxylase

Malonyl-CoA is produced via a two-step reaction utilising ATP. The enzyme responsible for this is acetyl-CoA carboxylase (ACC) which is thought to be made up of a biotin carboxylase (BC), a biotin carboxyl carrier protein (BBCP) and a carboxyltransferase (CT) domain. However, it is now clear that at least two types of ACC exist. The ACC involved in the prokaryotic pathway (p-ACC) has several subunits, which have not been fully isolated, and assembled into a 700kD complex (Ohlrogge and Browse, 1995). The ACC, active in the eukaryotic pathway (c-ACC), occurs as a single polypeptide. Dicots and many monocots have both forms of ACC, the prokaryotic form occurring in the plastid and the eukaryotic form possibly occurring in the cytosol (Konishi *et al*, 1996). However, some plants, e.g. Gramineae (a monocolyledonous family containing the grasses) appear to have another eukaryotic form of ACC in their plastids. The p-ACC is thought to contribute malonyl-CoA for *de novo* fatty acid synthesis, while the c-ACC

may produce malonyl-CoA for extraplastidial elongation reactions (Ohlrogge and Browse (1995).

Fatty acid synthase

Unlike the animal FAS (FAS I), the plant FAS (FAS II), located in the stroma of plastids, comprises individual subunits each of which can be isolated (Ohlrogge *et al.*, 1986). It is unknown whether these enzymes exist as a complex (Ohlrogge and Browse, 1995). The reactions are outlined below.

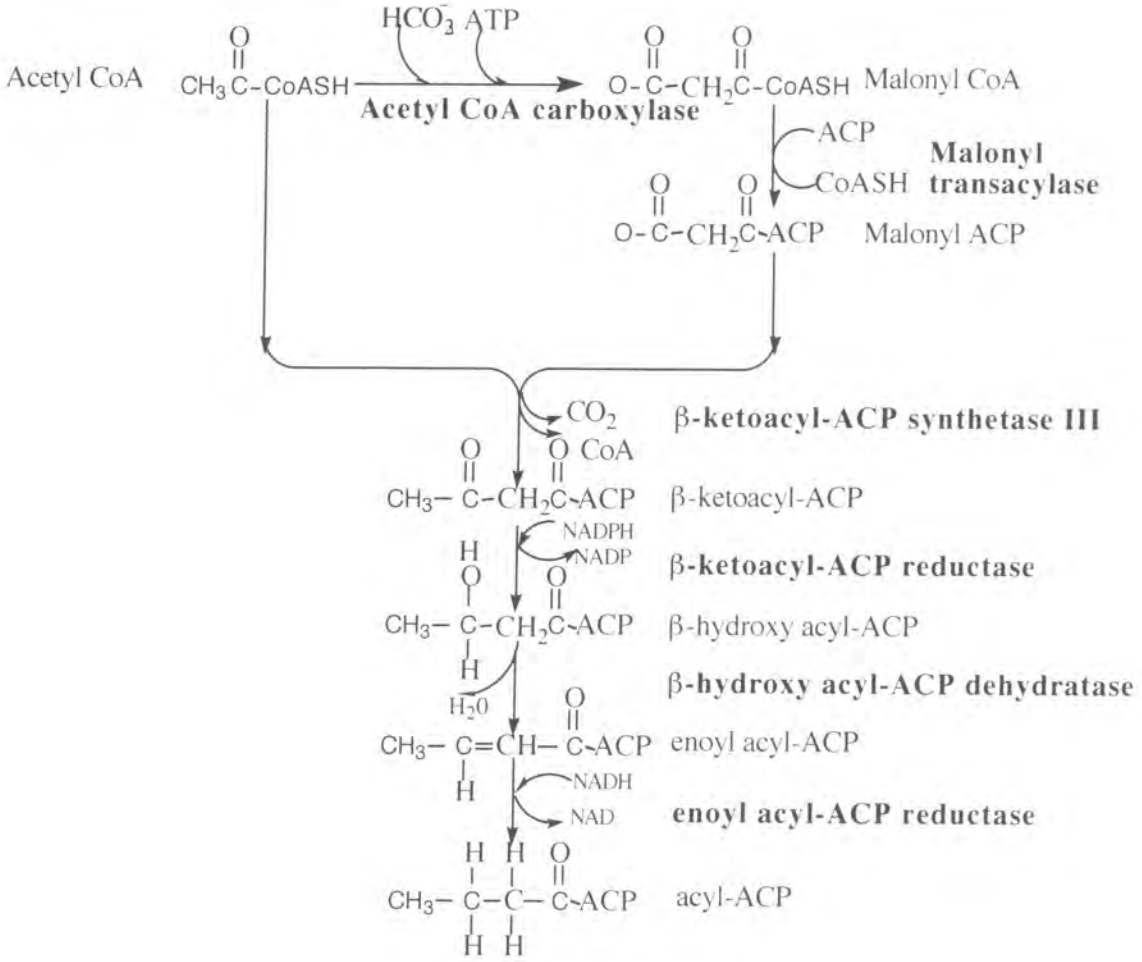

Figure 1.8. The reactions of FASase involved in the first stages of FA synthesis.

The cycle of reactions is initiated when acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase. Malonyl-CoA is transacylated to an acyl carrier protein (ACP) with the removal of CoA (CoASH). The enzyme catalysing this reaction is malonyl-CoA

ACP transacylase. Malonyl-ACP and acetyl-CoA are coupled together by the condensing enzyme ß-keto acyl ACP synthetase III (ß-KAS III) with the release of CO_2 and CoA to form ß-keto acyl ACP. ß-keto acyl ACP is converted to ß-hydroxy acyl ACP by the enzyme ß-keto acyl ACP reductase (ß-KAR), using NADPH to reduce the keto group on the ß-carbon.

ß-hydroxy acyl ACP is converted to enoyl acyl ACP by the enzyme ß-hydroxy acyl ACP dehydratase (ß-KAD). Finally, NADH reduces the double bond of enoyl acyl ACP in a reaction catalysed by the enzyme enoyl acyl-ACP reductase, to give an acyl ACP of four carbons in length.

Further cycles, in which acyl-ACP replaces acetyl-ACP in the reaction, with malonyl-ACP yields a two carbon increase per cycle up to palmitoyl-ACP (16: 0). This is believed to be carried out by the same enzymes, as already mentioned, except for ß-KAS III. This enzyme catalyses the initial reaction which produces a four carbon acyl-ACP. However, it is thought that ß-KAS I is used to carry on further reactions to produce 6 to 16 acyl-ACP, and that ß-KAS II produces acyl chain lengths of between sixteen and eighteen carbons in length. Shorter carbon chain lengths are produced by premature termination at a particular cycle, as occurs in seeds. If further elongation is required palmitoyl elongase carries this out forming stearoyl-ACP (18:0C). Desaturases such as stearoyl ACP desaturase can produce double bonds at a $\Delta 9$ position to form oleoyl ACP (18:1) from stearoyl ACP. The oleoyl ACP is transported outside the plastid as its CoA thioester for further modifications e.g. elongation, desaturation etc., or used in the formation of glycolipids in the plastid.

In plants having the prokaryotic pathway of lipid metabolism these further lipid desaturations occur in the plastids and this is thought to provide lipids for the plastid membranes. In the case of plants having the eukaryotic pathway the lipid modifications take place outside the plastid and the lipids have to be re-imported.

Detailed accounts of other aspects of lipid metabolism are outside the scope of this writing but are summarized schematically below in Figure 1.9.

Acyl-CoA can be esterified at the sn1 or sn2 positions of glycerol-3-phosphate (G3P) to form phosphatidic acid and CoA. This is the precursor of the acyl-glycerols, galactolipids and phospholipids. Triacylglycerols are stored in the oil bodies.

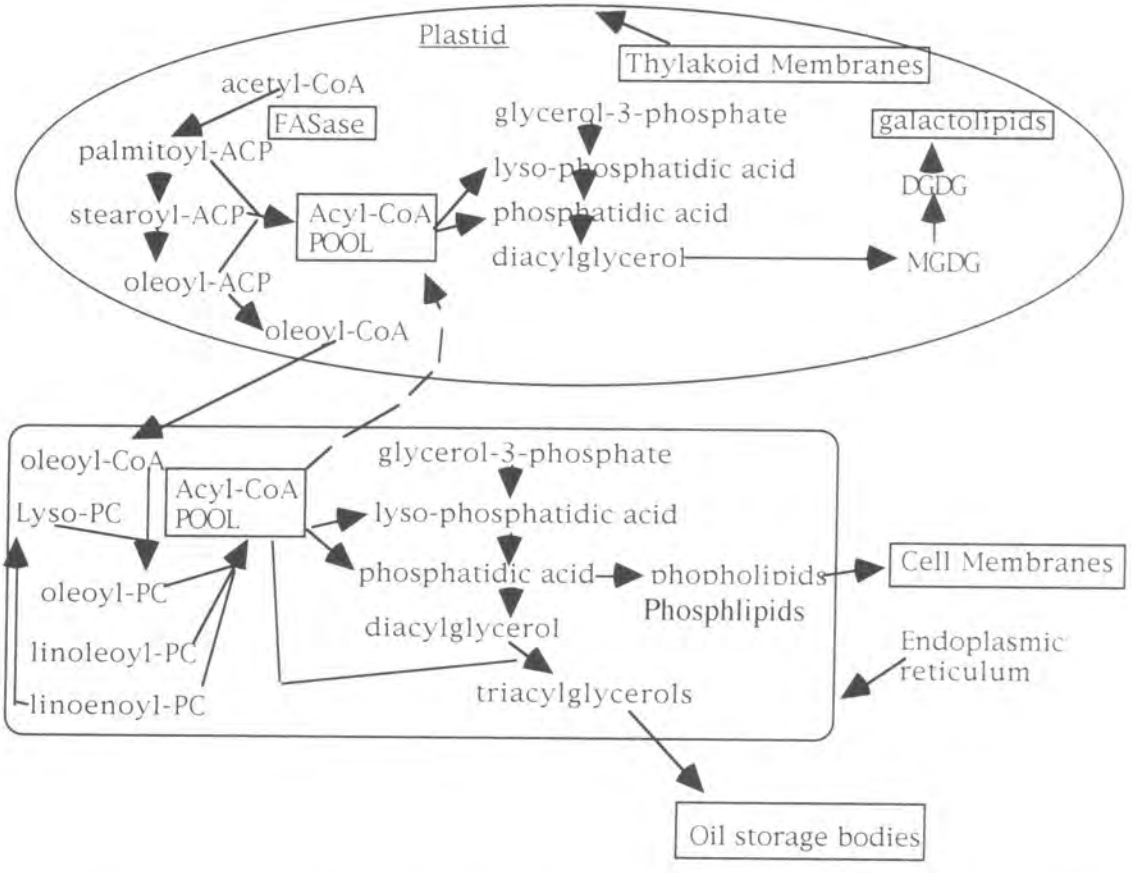

Figure 1.9. A simplified scheme for lipids biosynthesis and the relationship between the endoplasmic reticulum and the plastid. DGDG, Digalactosyldiacylglycerol; MGDG, Monogalactosyldiacylglycerol; FASase, Fatty acid synthetase; PC, Phosphatidylcholine.

1.3.7 Lipids and ripening

Relatively little is known about the involvement of lipids in the ripening process. Izzo *et al.* (1995) analysed lipid content during peach fruit ripening. They found that phospholipids (PL) and glycerolipids (GL) decreased during ripening, with the double bond index (DBI) increasing, while the level of sterols declined towards maturation, before increasing again. During ripening of peach an increase in the free sterol to phospholipid ratio (FS: PL) correlates with a decrease in membrane fluidity, which in

turn may activate desaturases causing an increase in the DBI. The FS limits the loss of structural integrity as the PL declines. These major changes occurred between the climacteric and post-climacteric stages of the peach. Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) content changed during ripening but MGDG was always higher. MGDG levels dropped by 5% during peach development before rising by 14% at the ripe stage. DGDG levels at the same stages increased by 14% before dropping by 40% and a fall in galactolipid may indicate a decrease in chloroplast integrity. Membranes of the peach eventually become disorganised during senescence. MGDG, DGDG and the DBI of PL, decreased in ripening apple, another climacteric fruit, with an increase in free sterol: PL ratio, indicating plastid membrane breakdown. In tomato the PL fraction also declined towards maturation of the fruit (Izzo *et al.*, 1995). In ripening banana fruits the relative proportions of glycolipid and phospholipid were not altered (Wade and Bishop, 1978). However, an increase in unsaturated fatty acid levels occurred in the phospholipid fraction. Wade and Bishop (1978) showed that the membrane fluidity of liposomes prepared from banana phospholipids increased during fruit ripening. It was suggested that change in the fluidity of the cellular membranes influence membrane permeability.

Epicuticular waxes produced in fruit are not well defined. It is unknown if strawberry fruit produce waxes on their surface. However, fruits such as apple are known to produce surface waxes (Belding *et al.*, 1998). These waxes decrease as the fruit develops and mainly consist of ursolic acid (a triterpene), as well as alkanes, primary and secondary alcohols, aldehydes and ketones. The percentage of primary alcohols in the epicuticular wax layers increases with fruit development.

In fruit, the source of acetyl-CoA, generated from photosynthesis in leaf, may not be available due to the lack of chlorophyll. Alternatively, acetate may be produced from malate, by an NADP+ malic enzyme that occur in non-photosynthetic plastids (Emes and Dennis, 1997). Malate can also be generated from citrate, the most abundant acid present in strawberry fruit (see section 1.2.2.1).

1.4 Acyl Carrier Protein

Acyl carrier protein (ACP) is a small acidic protein (Harwood, 1988) of around 9.8 to 10.3kD, that has been isolated from a wide range of organisms including: *E. coli*, *Arthrobacter viscosus*, avocado mesocarp and spinach leaf (Stumpf *et al.*, 1969). It is an essential component in the biosynthesis of fatty acids and in the formation of membrane derived oligosaccharides and polyketides (Shintani and Ohlrogge, 1994). Long chain acyl-ACPs are substrates for desaturases, hydrolases and acyl transferases (Ohlrogge *et al.*, 1991). In plants and bacteria, ACP is an isolatable low molecular weight protein, distinct from that found in animals which forms part of the multicatalytic polypeptide. ACP has a phosphopantetheine group bound to its serine residue, which has a terminal sulphydryl group to which the growing fatty acid chain is esterified during each cycle of fatty acid biosynthesis

Savage and Post-Beittenmiller (1994) examined ACP from spinach. They found that ACP was processed in the plastid into its active form and that the phosphopantetheine group, from coenzyme A (Baerson and Lamppa, 1993), was attached covalently by the action of the enzyme holoACP synthase. It was also demonstrated that the precursor ACP was processed in a similar way in the cytosol. The phosphopantetheine group was attached to the precursor ACP and then imported into the plastid before being processed into its active form. This demonstrated that the enzyme, holoACP synthase, could accept ACP in either its modified or unmodified form. It was also shown that this event was stimulated by CoA and inhibited by adenosine 3', 5'-bisphosphate. The N-terminal transit peptide was cleaved upon plastid import. A 16 amino acid domain in ACP to which phosphopantetheine is attached has been found to be highly conserved in animals and plants (Shintani and Ohlrogge, 1994, Ohlrogge *et al.*, 1991).

ACP has been studied in a range of plant species including *Arabidopsis*, spinach, *Brassica*, barley etc. (Baerson and Lamppa, 1993, Ohlrogge *et al.*, 1991). ACP not only occur in plastids but are also found in mitochondria (Shintani and Ohlrogge, 1994). In *Brassica napus* there is a distinct seed ACP multigene family described with an N-terminal 51 amino acid presequence believed to be required for plastid importation (de

Silva *et al.*, 1990, Safford *et al.*, 1988). In *B. napus* ACP appears prior to lipid biosynthesis and remains high even after lipid biosynthesis declines. The biosynthesis of ACP is coordinated with fatty acid biosynthesis, suggesting a regulatory role (Slabas *et al.*, 1987). de Silva *et al.* (1990) suggests there may be as many as 35 ACP genes per haploid genome in *B.napus*. At least five plastidial and three mitochondrial ACP isoforms have been identified in *Arabidopsis* (Mekhedov *et al.*, 2000).

The promoters of ACP genes have been examined in a number of species. Some isoforms exist which are hypothesized to have 'house-keeping functions', while others have a tissue specific activity, with both groups being under the control of different promoters (Baerson *et al.*, 1994).

In spinach, ACP I was most abundant in the leaf whereas ACP II was found in leaves, roots and developing seeds and appeared to be constitutive. In *Arabidopsis*, five isoforms were identified: ACII.1, ACII.2, ACII.3 etc., with ACII.1, ACII.2 and ACII.3 found in the leaves, roots and seeds. A fourth uncategorised form was present in the leaf, while a fifth form occurred in the seeds (Baerson and Lamppa, 1994). The first three isoforms are thought to have housekeeping functions (Ohlrogge *et al.*, 1991).

Different ACP isoforms may appear in response to variable cellular demands for fatty acid biosynthesis (de Silva *et al.*, 1990). This additional ACP would be needed for increased fatty acid synthesis for oil production in the seeds of *B. napus*. It was hypothesised that these ACP isoforms may provide the substrate specificity neccessary for the synthesis of fatty acids (Safford *et al.*, 1988). For example, in spinach the Km of oleoyl-ACP thioesterase for oleoyl-ACP-II is 10 fold higher than for oleoyl-ACP-I, whereas the Km of acyl-ACP glycerol-3-phosphate acyl transferase is 5-fold higher than for oleoyl-ACP-II (Ohlrogge *et al.*, 1986 and 1991). However, total *de novo* fatty acid synthesis and malonyl-CoA: ACP transacylase do not appear to discriminate between ACP isoforms (Ohlrogge *et al.*, 1986). In a similar experiment, Suh *et al.* (1999) observed that palmitoyl-ACP substrates from coriander, a plant that synthesises C16: 1Δ4 and C16: 1Δ6 fatty acids, were preferred by the coriander Δ4-palmitoyl ACP desaturase and not the *E. coli* and spinach ACP substrates. Stearoyl-ACP substrates from all three

organisms were not selectively desaturated by stearoyl-ACP desaturase, suggesting that ACP from coriander seed determines the fate of its esterified fatty acid to some degree. Furthermore, *in vitro* experiments were carried out to reconstruct the fatty acid synthase reaction in extracts of spinach leaf and *B. napus* seeds to identify the role of two ACP isoforms, ACP 1 and 2, isolated from *C. lanceolata* seeds (Schutt *et al.*, 1998). Both ACP 1 and 2 supported medium chain fatty acid synthesis in spinach, but ACP 2 was more effective in *B. napus* extracts. No preference for either ACP 1 or 2 by a medium chain acyl-ACP-specific thioesterase was observed, indicating that ACP influences medium chain fatty acid biosynthesis. Shintani and Ohlrogge (1994) have characterized a mitochondrial ACP (mtACP) isolated from *A. thaliana*. Its cDNA has a high homology to the mtACP of both *N. crassa* and bovine heart muscle. The *Arabidopsis* cDNA encodes a putative 35 amino acid mitochondrial presequence. However, it is known that mtACP can function as a cofactor in a chloroplast fatty acid synthase system (Ohlrogge *et al.*, 1986). The fatty acids produced by this mtACP were of short to medium chain lengths, indicating that this isoform may be involved in premature fatty acid chain termination.

It must be stressed, however, that the true *in vivo* functions of various plant ACP isoforms are unknown (Shintani and Ohlrogge, 1994). The distribution of ACP isoforms in different tissues may determine if the acyl chain is released or esterified to glycerol-3-phosphate (Ohlrogge *et al.* 1986). The signal mechanisms that govern the regulation and synthesis of lipids are as yet unknown. An important question yet to be resolved is whether a particular ACP isoform directs a specific lipid into a specific biochemical pathway. There is no evidence in the literature that ACP isoforms do this *in vivo*. To this author's knowledge no analysis of the *in vivo* storage products has been made correlating a specific ACP isoform to a particular reaction product.

1.4.1 Identification of tissue specific promoters

When a mRNA species is produced at a particular stage of development or growth, it has been transcribed from the gene as a result of a cis acting element called the promoter.

The corresponding cDNA can be used to isolate the promoter associated with that gene from the genomic library.

The *E. coli* gene ß-glucuronidase (GUS) is used as a reporter gene to study plant promoters, as it is absent or present at very low levels in plants. Therefore, coupling GUS to a tissue specific promoter can help to identify its tissue specificity and developmental phase in which the promoter is most active. Expression of GUS can be monitored directly by using a labelled substrate (X-Glucuronide) that gives an intense blue colour (or by assaying for GUS activity), or indirectly with either labelled GUS mRNA probes or by locating GUS protein using antibodies on histological tissues.

Promoters are located at the 5' position adjacent to the gene. Although promoter sequences are difficult to identify, due to variations in their sequences and their position on different genes, common motifs exist. One of these is the TATAA (TATA box) sequence at -25 to -30 nucleotides upstream of the transcription. Other upstream sequences include CAAT and GC boxes (CCAAT and GGGCG) that are found upstream of the TATA box (Lefebvre and Gellatly, 1997).

In order to identify promoter regions, which are important for a genes activity, sequence mutagenesis and deletions can be performed on promoter sequences which are then transformed, along with GUS, into a transgenic plant. By measuring the levels of GUS the transcriptional power of the promoter can be determined, compared to the control, and therefore the region vital for the promoter activity can be determined (Baerson and Lamppa, 1993).

de Silva *et al.* (1992) isolated a 1.4kb 5' flanking region of the seed expressed *ACP05* from *B. napus* and fused it to the reporter gene GUS. The chimeric gene was expressed in tobacco leaves with a 100-fold increased activity compared to untransformed leaf tissue. Comparisons of the ACP05 5' flanking region with other 5' regions of ACP09, from *B. napus*, and an *Arabidopsis* ACP gene were made. ACP05 and ACP09 had 82% homology in the -184 to -1 region, with ACP05 having 65% homology to *Arabidopsis* ACP. Three particular elements were perfectly conserved within the -184 to -1 region. These elements were:

- Element 1 (-32 to -21), containing the TATA box.
- Element 2 (-72 to -67), containing the sequence GCCCAT, found previously in seed expressed genes.
- Element 3 (-105 to -100), containing the sequence ATGGG which was a perfect inverted repeat of Element 2.

A fourth 10bp sequence was found to be identical between ACP05 and the *Arabidopsis* ACP located at an upstream position -146 to -137. This fourth sequence contained the ABRE core sequences; these sequences have been identified in the upstream region of a number of abscisic acid (ABA) responsive genes (de Silva *et al.*, 1992).

Tissue specific promoters are important in studying the action of ripening related transgenes. Complications and even lethal effects can arise if a transgene is constitutively expressed. The *Arabidopsis* ACP Acl1.2 gene promoter regions that are responsible for controlling gene activity in different tissues are shown in Figure 1.10.

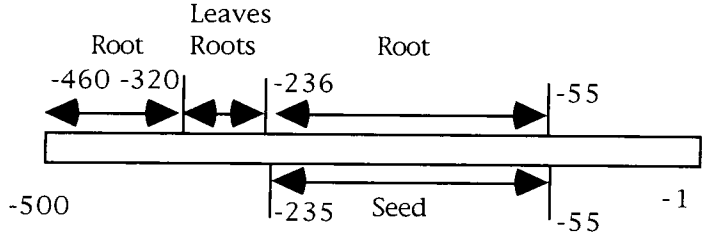

Figure 1.10. The regions involved in *Arabidopsis* ACP *Acl1.2* gene promoter activity in different tissues (Baerson *et al.*, 1994).

In *A. thaliana* the expression patterns of ACP were examined using a 915bp 5' region of the *Acl1.2* promoter coupled to GUS transformed into tobacco (Baerson and Lamppa 1993). GUS expression was found to be high in developing seeds and roots but lower in young leaves declining as the leaf matures. Promoter activity was high in meristematic tissue, stigma. style, the tapetum of anther, developing pollen and ovules. The results indicated a complex pattern of control during organogenesis. Baerson *et al.* (1994)

showed that the -235 to -55 domain (Figure 1.10) contained the GCCAAGCA motif identical to that of yeast FAS 1 and FAS II gene promoters. The GCCAA domain may interact with a protein factor. G-box like elements are recognised by the bZip transcription factor family. A -235 to -55 domain contains TGACG elements, found also in the CaMV 23S and wheat histone promoters, and is thought to bind TGA1 and bZip proteins. The same authors have also described other motifs. Recently, Baerson *et al.* (1998) has investigated the expression patterns of two ACP isoforms (*Acl1.2 and Acl1.3*) that differ by a single amino acid residue in transgenic *Arabidopsis*. Promoter expression studies identified common and distinct patterns of activity. In general, *Acl1.2 and Acl1.3* promoters exhibited meristematic and constitutive activities, respectively. This indicates that the levels of gene expression may be determined by the demand for fatty acid biosynthesis in each tissue, and that Arabidopsis and tobacco contain similar gene controlling mechanisms.

1.5 Aims and Objectives

The aim of this study is to investigate the role of ACP in the ripening process of strawberry fruit in relation to lipid metabolism.

It is of interest to study aspects of strawberry fruit ripening, firstly, to understand how the ripening events are controlled, and secondly, to develop methods for crop improvement. In order to investigate the role of ACP in strawberry a number of objectives had to be fulfilled.

Firstly, the number of ACP isoforms expressed in the fruit will be identified. This is important to establish how many ACPs are involved in fruit developement. The information about each clone will effect the interpretation of northern analysis, influence the general framework of this study and determine future investigations involving antisense technology. The sequence information obtained is vital to prepare primers and probes for specific ACPs.

Secondly, northern analysis will be used to identify the expression pattern of ACP in developing strawberry tissues.

Thirdly, the ripening-enhanced ACP cDNA will be expressed in *E. coli* to purify and partially characterise the protein. The protein will be used in future studies involving antibody production and crystallography.

Lastly, a genomic library will be generated and screened for the homologue of the ripening-enhanced ACP cDNA isoform to isolate its 5' upstream region. The sequence analysis of the isolated gene(s) and its 5' flanking region will be used to determine the coding regions and regulatory sequences, respectively, using information databases.

Chapter 2

Materials and Methods

2.1 Acyl Carrier Protein cDNAs in Unripe and Ripe Strawberry Fruit (chapter 3).

Three partially sequenced ripening enhanced ACP clones (RE-ACP), isolated from a differential screen (Manning 1998), were fully sequenced and the information was used to isolate further ACP clones from a ripe fruit cDNA library and from mRNA by Candidate Fragment Length Polymorphism (CFLP).

2.1.1 Subcloning of ACP inserts from lambda gt_{10} clones

Lambda DNA (1μg) from three ripening-enhanced clones was digested with 5U EcoRI (Boehringer) overnight, at 37 °C, in a volume of 20μl. The reaction was stopped by heat inactivation at 65°C for 10 min and the DNA was separated by electrophoresis through a 1% agarose gel in 1X TAE buffer. The gel was stained with ethidium bromide (0.5μg/ml) for 30 min, then de-stained in ddH_2O for 30 min. After visualising the gel under UV light the DNA insert bands were cut out and isolated using the QiaEx II (Qiagen) gel extraction kit. The DNA was eluted from the silica particles with 100μl ddH_2O and it was assumed a 90% recovery of DNA was obtained.

Ligations were carried out using 25ng gel purified pBK-CMV vector (Stratagene), predigested with EcoRI, and 20ng ACP cDNA insert in a total volume of 10μl. Insert and cut vector were heated at 65°C for five min, cooled on ice and incubated in 1X ligase buffer with 0.5 unit T4 DNA ligase (Boehringer) at 16°C overnight.

Five microlitres of the ligation reaction were added to 50μl competent XL1-Blue MRF' cells (Stratagene), including 0.8μl 2-mercaptoethanol, and incubated on ice for 30 min. The cells were heat shocked at 42°C for 45s, and placed on ice for two min. The addition of 0.45ml SOC medium to the cells was followed by incubation at 37°C on an orbital

shaker for one hour. The cells were then plated onto LB-agar plates (containing 50μg/ml kanamycin, 34μg/ml tetracycline, 80μg/ml X-gal and 20mM IPTG) and incubated at 37°C overnight. White colonies were picked into LB media (containing 50μg/ml kanamycin and 34μg/ml tetracycline) and incubated with shaking at 37°C overnight. Plasmid DNA was isolated using the QIAprep plasmid purification kit (Qiagen).

2.1.2 Sequencing

DNA samples were sequenced using the Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystems). Reactions contained 2μl Dye Terminator, 4 pmol primer, 200ng plasmid (>5Kb) or 100ng (<5kb), and made to a final volume of 10μl. Cycle sequencing was performed on a Perkin Elmer 2400 thermal cycler for 30 cycles at: 95°C for 30s, 50°C for 10 s and 60°C for 4 min. Sequencing products were precipitated with 1μl 3M sodium acetate: acetic acid, (pH 5.2) and 25 μl 100% ethanol (ETOH), at room temperature, for 10 min before centrifugation (13000 x g) for 30 min. The supernatant was carefully removed and the pellet was washed with 150μl 70 % ethanol, and dried at room temperature. The reaction products were sequenced on an ABI 377 DNA Sequencer.

2.2 Screening and Isolation of ACP Isoforms Contained in a Ripe Fruit cDNA Library.

A ripe fruit cDNA library constructed in the Lambda Screen vector (Novogen) was screened for ACP containing clones using the DIG non-radioactive labelling and detection system (Boehringer).

2.2.1 Probe synthesis

DIG-labelled ACP probes were produced using the PCR DIG Probe Synthesis Kit to generate ACP probes, using the putative ripening-enhanced ACP cDNA (RE-ACP) as a template. Primers PPf (5'-CTTGGAGCTGATTCTCTTG-3') and PPr (5'-AAATTCCTCCTCAAGTCCC-3') were designed to generate a PCR product 57bp in length covering the conserved phosphopantetheine region of ACP.

The PCR labelling reaction was performed according to the manufacturer's instructions and included: 0.1μM each of primers PPf and PPr, 50pmol RE-ACP cDNA template, and 2.6 units Expand High Fidelity Enzyme mix in a total volume of 50μl.

Thermal cycling conditions were 1 cycle at 95°C for 2 min, and 10 cycles at 95°C for 10 s, 55°C for 30 s and 72°C for 2 min. Twenty further cycles, at 95°C for 10 s, 55°C for 30 s (increasing by 20s per cycle) and 72°C for 2 min concluded with 1 cycle for 7 min at 72°C

A 5μl aliquot of PCR reaction mixture was analysed on a 1% agarose gel in 1X TAE buffer. Before use, the labelled probes were diluted 1000-fold in DIG Easy Hyb hybridization solution.

2.2.2 Colony blotting

The λScreen cDNA library from ripe fruit was plated at 3 X 10^4 pfu on 10cm square plates containing LB agar. Duplicate positively charged nylon membranes (Boehringer) were used to take plaque lifts on pre-cooled (4°C) plates. The first membrane was left on the plate for one min, and the second membrane for 30s longer. Each blot was

sequentially placed on 3MM paper soaked in denaturing solution (0.5M NaOH, 1.5M NaCl) for five min, neutralisation buffer (1.5M NaCl, 1.0M Tris-HCl, pH 7.4) for 15 min and 2 X SSC for 10 min. The membranes were air-dried and the DNA was fixed by UV light for five min. To lower the background signal each blot was placed in aluminium foil and treated with 9ml proteinase K (2.0 mg/ml) in 2X SSC and incubated at 37°C for one hour prior to hybridization. The blots were removed from the foil and squeezed between two pieces of 3MM paper moistened with ddH_2O to remove cell debris.

2.2.3 Hybridization and probe detection

The membranes were pre-hybridised with 40ml DIG Easy Hyb at 42°C for one hour. This solution was then removed and 4ml of diluted DIG-labelled probe, denatured in boiling water for five minutes, was added. The membranes were incubated at 42°C overnight in roller bottles.

2.2.4 Post hybridisation washes

Each membrane was washed twice, with gentle agitation, at low stringency with 80 ml 2X SSC, 0.1% SDS, for 5 min at room temperature. Two further washes were performed at high stringency using 0.5 X SSC, 0.1% SDS, for 15 min at 65°C.

2.2.5 Immunological detection of bound probes

All steps were carried out at room temperature. The membranes were washed with 80 ml washing buffer (0.1M maleic acid, pH 7.5, 0.15M NaCl), with the inclusion of Tween 20 at 0.3 % (v/v) concentration, for five min. The washing buffer was removed and replaced with buffer 2 (washing buffer including 1% blocking reagent (Boehringer)) for 30 min. Buffer 2 was replaced with a 1:20,000 dilution of Anti-Digoxigenin-AP, Fab fragments (Boehringer) in 20 ml buffer 2 and incubated for 30 min, followed by two 15 min washing steps with 80 ml washing buffer.

To detect bound alkaline phosphatase the nylon membranes were washed in 20 ml buffer 3 (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for 5 min, briefly blotted on 3MM paper, and placed in a 1:500 dilution of CDP-Star (Boehringer) in 5 ml buffer 3. The membranes

were briefly blotted, wrapped and incubated for 30 min in cling film before exposure to X-ray film usually for between 5 min and 1h.

2.2.6 Isolation and sequencing of cDNA clones

2.2.6.1 cDNA clone isolation

Potential ACP clones identified on duplicate X-ray films were used to locate the corresponding plaques which were picked as an agar plug using a sterile pipette tip. The plaque plug was placed into 100µl SM buffer overnight, at 4°C. The plug was centrifuged for five min (4°C) and the supernatant was removed into a clean tube and 50µl/ml chloroform added. After titration the eluted phage were stored at 4°C.

2.2.6.2 Cre-mediated excision of the λBlueSTAR clones.

BM25.8 cells were grown to an $O.D_{600}$ of 1 in LB media containing 0.2% maltose and 10mM $MgCl_2$. Equal volumes (100µl) of BM25.8 cells and phage were combined and incubated at 37°C for 30 min. The infected cells were plated onto LB-agar plates containing carbenicillin (50µg/ml) and incubated at 37°C overnight. A single colony was picked into 5ml LB media (containing 50µg/ml carbenicillin), and grown at 37°C overnight. Plasmid DNA from the culture was isolated by the QIAprep kit and recovered in 50µl TE, pH 8.0. The cre recombinase present in the BM25.8 strain produces plasmid multimers that are unsuitable for analytical purposes. Therefore to obtain normal plasmids the DNA was transformed into DH5α cells.

2.2.6.3 DH5α transformation

Competent cells were thawed on ice and 20µl aliquots were used for transformation. One microlitre of plasmid DNA from the BM25.8 cultures was mixed with the cells and incubated on ice for 30 min. The cells were heat shocked at 42°C for 40s and returned to ice for two min. SOC medium was added to give a final volume of 100µl and the DH5α cells were incubated at 37°C for 1h on a horizontal shaker. All of the cells were plated onto LB-agar plates containing 50µg/ml carbenicillin and incubated as before. Single

colonies were used to inoculate 5ml LB (contâining 50μg/ml carbenicillin) and grown overnight at 37°C.

Plasmid DNA was extracted from the DH5α cells using the QIAprep plasmid extraction kit. Sequencing was carried out, as described in 2.1.2, with the forward and reverse primers ACP S (5'-TCACTCCGCTCTCGCCTTCACC-'3) and ACP A (5'-TGACCACACAAACTCAAATCC-'3), respectively.

2.3 Isolation of ACP cDNA Fragments by CFLP

The technique of candidate fragment length polymorphism (CFLP) was used to identify differentially expressed ACPs in unripe and ripe strawberry fruit.

2.3.1 Isolation of mRNA

Large unripe green fruit and ripe fruit (with achenes removed) were used to isolate mRNA for the synthesis of cDNA. The isolation of mRNA is described in section 2.5.

2.3.2 First and second strand cDNA synthesis

All steps were according to the Amersham protocol. The first strand cDNA synthesis in a volume of 9.5µl contained 0.5µg mRNA and 0.5µl reverse transcriptase, and was incubated at 42°C for 60 min.

Second strand cDNA was synthesised from the first strand reaction in a total volume of 50µl and included 0.4 units ribonuclease H and 11.5 units DNA polymerase. The components were mixed and incubated at 12°C for 60 min then at 22°C for 60 min. The mixture was then incubated at 70°C for 10 min, briefly centrifuged, and placed on ice. One unit of T4 DNA polymerase was added to the second strand cDNA synthesis reaction and incubated at 37°C for 10 min. The reaction was stopped by adding 2µl 0.25M EDTA, pH 8.0.

2.3.3 Purification of double stranded cDNA

The synthesised ds cDNA was vortexed with an equal volume of phenol/chloroform (1:1 v/v) and centrifuged at maximum speed (13,000rpm) in a microcentrifuge for 5 min. The upper phase was re-extracted, as above, and then mixed with an equal volume of chloroform, vortexed and re-centrifuged as before. The upper phase was mixed with one volume of 2M ammonium acetate, pH 5.8, and two volumes of 100% ethanol at -20°C, followed by incubation on ice for 15 min. The mixture was brought to room temperature

and centrifuged at 13,000 rpm for 10 min. The supernatant was removed and the pellet was washed with a mixture of 50µl 2M ammonium acetate and 100µl 100% ethanol, re-centrifuged, and air-dried before resuspending in 25µl ddH2O.

The mass of cDNA obtained from 0.5µg mRNA was calculated according to the Amersham protocol, assuming an average yield of 20-50% first strand cDNA and a 90% yield of double stranded cDNA. Therefore, 0.5µg mRNA is expected to yield between 180-450ng cDNA.

2.3.4 Candidate fragment length polymorphism (CFLP) analysis

2.3.4.1 Digestion of cDNA template

The cDNA (~250ng), prepared from green and ripe fruit tissue, was digested with Mse I (1.25 units) in a volume of 50 µl, for two hours at 37°C and the reaction was stopped by heating to 65°C for 10 min. The cDNA was precipitated by the addition of 0.1M sodium acetate/acetic acid (pH 6.0) and two volumes of 100% ethanol (-20°C), and incubated on ice for one hour. The precipitated cDNA was collected by centrifugation and the pellet washed sequentially with 70% and 100% ethanol, dried in air and resuspended in 25µl ddH_2O on ice for 1h.

2.3.4.2 Ligation of adapter

To amplify each potential ACP cDNA fragment, the ligation of an adapter was required to provide a template for PCR. Adapters were generated by mixing 16µg of the 16-mer 5'-GACGATGAGTCCTGAG-3' with 14µg of the 14-mer 3'-TACTCAGGACTCAT-5' in a total volume of 60µl, resulting in a solution containing 50µM of the following adapter:

Mse I adapter

5'-GACGATGAGTCCTGAG-3'

3'-TACTCAGGACTCAT-5'

The adapter (1µl) was ligated to the 25µl of Mse I digested cDNA (10ng/µl) in a total volume of 50µl, as described above, using 4U/µg T4 DNA ligase (Boehringer). A 1/10 dilution of this mixture was prepared for the pre-amplification step.

2.3.4.3 Preamplification of the ligated cDNA

The Mse I adapter primer 5'-GACGATGAGTCCTGAG-3' and one gene specific primer was used to preamplify the ACP cDNA transcripts derived from ripe and green strawberry fruit. Conserved regions in ACP were identified from database searches using the National Center for Biotechnology Information (NCBI) BLAST program. Sequences for several different plant ACPs were aligned, using the DNASTAR program MEGALIGN, to identify consensus sequences. Gene specific primers, forward PPf and reverse PPr were designed to the phosphopantetheine ACP consensus (see 2.2.1).

Mixtures for PCR were prepared with two primer combinations: 1) MseI primer/PPf primer and 2) MseI primer/PPr primer and either green or ripe fruit cDNA. Preamplification of the adapter ligated cDNAs used PCR beads (Pharmacia), with 0.4 µM of each primer combination and 2.5µl of the diluted cDNA, in a total volume of 25µl. The reactions were incubated for 20 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1min. The PCR products were diluted 50-fold in TE, pH 8.0, and used as the template CFLP PCR.

2.3.4.4 CFLP amplification using labelled ACP-specific primers

The ACP specific primers were phosphorylated at the 5' end with [α-^{33}P] dATP using T4 polynucleotide kinase (Boehringer) in a reaction containing 3.6µl of the ACP specific primer PPf or PPr (27ng/µl), 2.0µl [α -^{33}P] dATP (1000-3000 Ci/mmol-Amersham) and 20units T4 polynucleotide kinase in a total volume of 10µl. The mixture was incubated at 37°C for one hour, followed by inactivation at 70°C for 10 min.

The CFLP reaction contained either the [α-^{33}P] dATP labelled PPf (tube A) or the PPr (tube B) primers, the MseI linker primer and Pharmacia PCR beads in a volume of 15µl. Each mixture was split into two 7.5µl aliquots and placed in tubes 1A, 2A, 1B and 2B. Tubes 1A and 1B received 2.5µl of the diluted ripe fruit template, and tubes 2A and 2B received 2.5µl of the green fruit template. Touchdown PCR was performed on a Perkin-Elmer 2400 thermal cycler, initially for 10 cycles of 94°C for 30s, 65°C (decreasing by 1°C/cycle) for 30s and 72°C for 1 min. Twenty-five further cycles of 94°C for 30s, 56°C for 30s and 72°C for 1min concluded with 1 cycle of 65°C for 30 min.

2.3.4.5 Gel analysis of the labelled PCR product

The labelled PCR products were mixed with 10µl loading dye (98% formamide, 10mM EDTA, 0.1% bromophenol blue (w/v) and 0.25% xylene cyanol (w/v)). A 5% acrylamide gel (37:1, acrylamide: bisacrylamide) solution was prepared containing 8M Urea and 1x TBE buffer. To 50 ml of this solution, 360µl 10% ammonium persulfate and 60µl TEMED were added to initiate polymerisation. The labelled PCR products were heated at 94°C for 30 min, cooled on ice and 3µl were loaded onto the preheated gel (50°C) and electrophoresis begun at 100 Watts constant power, until the dye front was at the bottom of the gel. The gel was then dried directly onto Watman 3MM paper on a slab gel drier and exposed to X-ray film (Kodak X-AR) overnight after marking the position of the gel on the film.

2.3.4.6 Isolation of the selected CFLP bands using the crush and soak method

(Sambrook *et al.*, 1989)

The bands of interest, separated on the polyacrylamide gel, were excised using a clean scalpel blade and placed into 2ml Eppendorf tubes. 500µl of elution buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA, pH 8.0, 0.1% SDS) were added to each tube and a pipette tip was used to crush the polyacrylamide gel. The tubes were placed on a rotary platform at 37°C and after 4hrs were centrifuged at 13,000 rpm for 5 min at 4°C before 100µl of the supernatant was removed into a clean tube. The

eluted DNA fragments were precipitated with 1µl glycogen (Boehringer), 0.1M Sodium acetate/acetic acid (pH 6) and 2.5 volumes of 100% ethanol (-20°C) and centrifuged, as before. The pellets were washed consecutively with 70% and 100% ethanol, dried, and resuspended in 20µl ddH2O. A 1/10 dilution of the extracted DNA was made and 1µl was amplified in a standard PCR reaction using Pharmacia PCR beads with an annealing temperature of 55°C, using the same combination of primers used in the original CFLP PCR (see section 2.3.4.3). Southern analysis was used to confirm the identity of the amplified CFLP PCR products.

2.3.4.7 Southern analysis of putative ACP bands

DNA samples were mixed with 1/10th volume of 10X sample buffer (50% glycerol (w/v), 1mM EDTA, 0.4% bromophenol blue, pH 8.0) and separated on a 1% agarose gel in 1 X TAE buffer at 5V/cm. The gel was stained with ethidium bromide (1µg/µl) to locate the DNA before setting up the Southern blot apparatus, as described by Sambrook *et al.* (1989). The DNA was transferred overnight to a positively charged Nylon membrane (Boehringer) using 0.4M NaOH. The blot was then treated with neutralisation buffer (0.5M Tris-HCl, pH 7.5; 3M NaCl) for 15 min, followed by 10 min in 2 X SSC (0.3M NaCl, 0.03 M trisodium citrate). The DNA was fixed by UV light for 2 min then detected using a DIG-labelled ACP cDNA probe, as described in sections 2.2.3-2.2.5. Fragments that hybridised with the probe were cloned.

2.3.4.8 Cloning of CFLP re-amplified PCR products

The re-amplified PCR fragments generated from the CFLP techniques were cloned using the ZeroBlunt™ TOPO™ PCR Cloning Kit (Invitrogen), essentially according to the manufacturer's instructions, but using only half of the reaction volumes recommended. Firstly, the PCR products were purified by electrophoresis through a 1% agarose gel and the DNA fragments were recovered using the Qiagen QIAquick Gel Extraction Kit, followed by resuspension in 20µl ddH2O. The PCR products were blunt-ended in a mixture containing 0.5 units Pfu DNA polymerase (Stratagene), 0.2 mM dNTP's, 1 X

PCR buffer and 10 µl PCR product, in a total volume of 13.3µl. The polishing reaction was incubated for 30 min at 72°C.

The PCR products were cloned into the pCR-Blunt II TOPO vector in a reaction mixture containing: 0.5µl polished PCR product, 0.5µl pCR-Blunt II TOPO vector in a total volume of 2.5µl. After five min at room temperature 0.5µl of the 6 X TOPO cloning stop solution was added and incubated for 10s before placing the mixture on ice. The mixture (1µl) was added to 25µl competent cells and incubated on ice for 30 min. The cells were heat shocked at 42 °C for 30s, incubated on ice for two min, before 250µl SOC medium were added to the cells and shaken on a rotary platform at 37°C for one hour. A 50µl aliquot of transformed cells was spread onto LB-kanamycin (50µg/ml) plates and incubated overnight at 37°C. Non-recombinant plasmids contain a functional lethal gene that destroys the host cells, whereas recombinant plasmids, in which the lethal gene is interrupted, allow the host cells to survive (positive selection). A colony was picked from each plate and grown in 5ml LB medium containing 50µg/ml kanamycin. Plasmid DNA was isolated using the QIAprep plasmid isolation kit (Qiagen). Inserts were sequenced using M13 primers.

2.4 The Expression and Partial Characterisation of the Ripening-Enhanced ACP Isoform from Strawberry (Chapter 4).

2.4.1 Primers for expression cloning

The primers 5′-GCGCCATATGGCAGCCAAACCAGAGACAATG-′3 and 5′-GCGCGAATTCCTGCATACATAACCAGTCTAC-′3, designed to the 5′ and 3′sequences bordering the protein coding region of RE-ACP, respectively, were obtained from VH BIO Ltd. The 5' region of both primers contained an adapter sequence with respective restriction enzyme recognition sites for NdeI and EcoRI, respectively.

2.4.2. PCR amplification of ACP cDNA and ligation into the plasmid expression vector pET-24a

The coding region of the RE-ACP mature protein was amplified with the above primers using Pharmacia PCR beads. Thermal cycling conditions were as follows: 1 cycle at 94°C for 5min, then 15 cycles at 94 °C for 2 min, 55 °C for 1min, 72 °C for 1 min and 1 cycle at 72 °C for 10 min. The PCR product was gel purified on 1% agarose and extracted with the QIAquick gel extraction kit. The purified PCR product was digested with 5units of NdeI and EcoRI per μg DNA at 37 °C for two hours and heat inactivated at 65°C for 15 min. The digested DNA was precipitated and washed in 70% and 100% ethanol, as described before, and ligated to NdeI/EcoRI digested and gel purified pET-24a DNA (Novagen). The ligation reaction (1μl) was used in transform (25μl) Xl1-Blue MRF supercompetent cells (Stratagene) which were grown over night on LB-agar plates containing 50mg/ml kanamycin.

A single colony was selected and grown in LB media, including 30μg/ml kanamycin, and plasmid DNA was extracted with Qiagen QIAprep plasmid purification kit. The DNA was sequenced, as described before, with the T7 promoter primer (Novagen) to confirm the correct in-frame sequence.

2.4.3 Protein expression

The recombinant plasmid was transformed into the BL21 (DE3) strain of *E. coli,* as described for the DH5α cells (see section 2.2.6.3), and the cells were grown in 800ml batch culture in LB medium, containing 30μg/ml kanamycin, at 37°C until an OD_{600} of 0.6-1.0 was reached. Recombinant protein over-expression was induced with 0.5mM IPTG and after three hours the cells were collected by centrifugation at 5,000 x g for 10 min at 4°C. The cells were resuspended in 20mM Tris-HCl, pH 8.0, pelleted again by centrifugation and stored as a frozen pellet at –80°C.

2.4.4 Protein extraction by freeze thawing

The cell pellet was resuspended in 10mM KP buffer (0.66M KH_2PO_4, 014M K_2HPO_4; pH 6.2), containing 0.1% ß-mercaptoethanol, and centrifuged at 40,000 x g. The supernatant containing RE-ACP was purified on a porous HQ anion exchange column (Perseptive Biosystems), equilibrated with 10mM KP buffer, pH 6.2, and eluted with the same buffer containing a 0 - 1.0M LiCl gradient. Protein fractions were analysed by SDS-PAGE on a 12% gel and the most enriched fractions for ACP were pooled, dialysed and freeze-dried.

2.5 Analysis of the Expression Patterns of Strawberry ACP Isoforms

(Chapter 5).

2.5.1 Plant tissue sampling

The day-neutral strawberry (*Fragaria x ananassa.* c.v. Brighton) was used for study. The plants were maintained in a glasshouse with a 12°C night and 18°C day temperature. Fruit tissues for RNA extraction were sampled according to developmental time from anthesis and the achenes (seeds) were separated from the receptacles. Fruits from Days 0 (flower), 4, 10, 16, 19, 22 (turning), then at the orange, ripe and overripe stages were washed with ddH_20 before snap freezing in liquid N_2 and stored at -70°C. Other tissues sampled (from plants grown in solution culture) included roots, young and mature leaves, young and mature petioles. These tissues were also stored at -70°C after freezing in liquid N_2.

2.5.2 Isolation of total RNA

Plant tissue was ground in liquid N_2 and mixed with 2.5 ml/gfw^{-1} extraction buffer (0.2M boric acid/Tris, 10mM Na_2EDTA, pH 7.6), with the addition of 1/50 vol. of 25% SDS and 1/50 vol. 2-mercaptoethanol just before use. An equal volume of phenol/chloroform (1:1 v/v) was shaken with the tissue extract and centrifuged at 20,000 x g for 10 min at room temperature. The upper phase was removed, mixed with an equal volume of phenol/chloroform and centrifuged as before. The upper phase was weighed and the volume (ml) calculated as the weight (g) of upper phase multiplied by density (1.024g ml^{-1}). The upper phase was accurately mixed with 1.4 volumes of ddH_2O and 0.1 volumes of 1M sodium acetate/acetic acid (pH 4.5). This diluted phenol phase was mixed with 0.4 vol of 2-butoxyethanol and incubated on ice for 30 min. The mixture was centrifuged at 20,000 g for 10 min at 4°C and the supernatant was transferred to a clean tube before 0.6 vol of 2-butoxyethanol was added. The mixture was incubated and centrifuged, as before, and the supernatant was discarded. The pellet containing total nucleic acids was then washed consecutively with 0.2M boric acid/Tris, 10mM Na_2EDTA, pH 7.6: 2-butoxyethanol (1:1 v/v), 70% ethanol and 100% ethanol, before drying *in vaccuo*.

The nucleic acid pellet was resuspended in 300µl TE, pH 8.0. Total RNA was isolated by the addition of 1/3 volume 12M LiCl (0.2µM sterile filtered) and incubated on ice for one hour, before centrifugation at 20,000g for 10 min at 4°C. The pellet was washed consecutively in 3M LiCl, 70% ethanol, and 100% ethanol and air-dried. The pellet was dissolved in 40µl ddH_20 and 4µl of this was taken for UV determination. The RNA was precipitated with ethanol and stored at -70°C under 100% ethanol until required. Samples for northern blotting were stored at –70°C in the following mixture: 2.0µl RNA in ddH_2O (5µg/µl), 2.0µl X 10 MOPS buffer, pH 7.0, 2.0µl X 10 loading buffer, 3.5µl formaldehyde and 10.0µl deionised formamide. A 0.5µl aliquot of ethidium bromide (10mg/ml) was added before use.

2.5.3 Northern analysis

2.5.3.1 Northern blotting

The RNA (10µg), in storage mixture, was heat denatured at 65°C for 15 min and cooled on ice. The RNA samples were separated at 5V/cm on a 1.4% agarose gel, containing 1 X MOPS buffer (pH 7.0) and 0.22M formaldehyde, overlaid with 1X MOPS buffer containing 0.22M formaldehyde. The gel was viewed under UV light to check that the RNA loading was uniform, and the RNA was transferred by capillary action to a positively charged nylon membrane (Boehringer), using 10 X SSC. The nylon membrane was then viewed under UV light to check for complete transference, photographed and RNA fixed to the membrane by UV crosslinking for five min.

2.5.3.2 Probe radiolabelling and hybridisation

A 550bp probe was prepared from RE-ACP cDNA template, by using primers ACPA and ACPS (see section 2.2.6.3) and PCR beads. The probe was gel purified and quantified by agarose gel electrophoresis against a 100bp DNA ladder (Boehringer). Approximately 25ng probe was labelled using the Pharmacia Ready to Go labelling kit and [α ^{32}P]dCTP (Amersham International, UK), according to manufacturer's protocol.

The reaction was passed through a Sepharose-CL6B packed column to remove unincorporated label. The northern blot was blocked with freshly made prehybridization buffer (0.25M sodium phosphate buffer, pH 7.2, 7% SDS) for four hours at 65°C. Radiolabelled probe was denatured in boiling water for 5 min, mixed with prehybridization buffer and incubated at 65°C, overnight. The membrane was washed twice at low stringency in 2X SSC, 0.1% SDS for 15 min at 65°C. Two further washes were performed at high stringency using 0.5 X SSC, 0.1% SDS at 65°C for 15 min. The membranes were wrapped in saran wrap (Genetic Research Instrumentation Ltd., Cambridge, UK) and exposed to Kodak X-Omat AR Film (IBI Ltd) in an exposure cassette with an intensifying screen at -70°C.

2.6 Characterization of ACP genomic clones from strawberry (Chapter 6)

2.6.1 Isolation of genomic DNA

After examining many methods of isolating high quality restrictable genomic DNA from strawberry the method of Dobbeling *et al.* (1997) was used with modification. Young unexpanded strawberry leaves (*Fragaria x ananassa* c.v. Brighton) were ground to a fine powder in a pestle and mortar in liquid N_2. Extraction buffer (7M Urea, 2% SDS, 5mM EDTA) was added (5mL/gfw^{-1}) and the tissue was macerated until the mixture was homogenous. An equal volume of phenol/chloroform (1:1 v/v) was added to the leaf extract and shaken vigorously for two to three seconds to form an emulsion. The extraction mixture was centrifuged at 12,000 rpm for ten min in an SS-34 rotor (Sorval) at room temperature. The upper phase was transferred to a sterile tube and an equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and mixed as before. The centrifugation step was repeated and the upper phase was mixed with 1 vol of isopropanol, placed on ice for ten min, and the precipitated nucleic acids were spooled out with a sterile glass pipette. Nucleic acid strands were washed in 70% and 100% ethanol and dried in the air for 15 min. The nucleic acid spool was dissolved in TE, pH 8.0, overnight at 4°C. The nucleic acid solution was precipitated and spooled, as before, a further two times before finally dissolving the spooled nucleic acids in TE, pH 8.0. The nucleic acids were approximately quantified by UV absorbance and RNase One (Promega) was added, at 9 units per μg nucleic acid, before incubated at 37°C for two hours. An equal volume of 13% polyethylene glycol 8000 (w/v) in 1.6M NaCl was added, at room temperature, to precipitate DNA. The solution was centrifuged at 15,000 rpm for 10 min in an SS-34 rotor at room temperature, and the DNA pellet was washed in ethanol, as before. The DNA was dissolved in TE, pH 8.0, overnight at 4°C and quantified by UV absorbance.

2.6.2 Construction of *F. ananassa* genomic library in Lambda BlueStar™

2.6.2.1 Partial digestion of DNA

To optimise conditions, a small-scale partial digestion of 1 μg genomic DNA was performed with a range of MboI enzyme concentrations from 0.044 to 0.25 units/μg DNA. The reactions were incubated at 37°C for 15 min, inactivated at 65°C for 10 min and analysed by 0.8% agarose gel electrophoresis at 2.5 Volts/cm. The concentration of enzyme that produced the highest quantity of fragments between 7 and 20kb in size was 0.188 units/μg DNA. However, when this concentration of enzyme was used in the large scale restriction digestion of 100μg genomic DNA the DNA was overdigested. Under the optimum conditions 100μg DNA was digested with two concentrations of enzyme being 0.035 and 0.047 units μg^{-1} MboI for 30 min at 37°C and the DNA digests were combined. Two DNA digests were used to ensure as much of the genomic DNA as possible was represented in the construction of the genomic library. Mbo I is an isoschizomer of Sau3A I and was the preferred choice as it is less sensitive to methylated DNA such as 5′-CpG-′3 methylated DNA.

2.6.2.2 Isolation of the partially digested genomic DNA

Aliquots containing 10 μg digested DNA were loaded into each well of a 0.8% agarose gel and separated at 2.5V/cm. After visualising with ethidium bromide gel slices were excised to include DNA in the size range 7 and 20kb (under low power UV light to avoid photodamage). The DNA was isolated from the gel slices by electroelution in the Strataeluter™ Electroelution Device (Stratagene) at 100V for 2h. The DNA in the collection cup was precipitated by the addition of 0.1M Sodium acetate/acetic acid (pH 6) and 2.5 volumes of 100% ethanol at -20°C. After 30 min on ice, the precipitated DNA was centrifuged at 13,000 rpm for 15 min, at room temperature, in a microcentrifuge. The pellet was washed with 70% and 100% ethanol, dried and resuspended to give a concentration of 0.2μg DNA/μl in ddH_2O.

2.6.2.3 Partial fill-in of restriction overhangs

The genomic DNA fragments were partially filled in to make the end termini compatible with the λBlueSTAR vector arms. The reaction mixture (200µl) comprised 5.2µg genomic DNA, 1 Unit/µg Klenow DNA polymerase (Boehringer), 1x insert fill-in buffer and 1mM dithiothreitol. The mixture was incubated at 30°C for 30 min before inactivating the enzyme at 70°C for 10 min. The partially filled-in DNA was precipitated and washed, as before, and dissolved into 30µl ddH2O.

2.6.2.4 Test ligations of the partially digested DNA into λBlueSTAR vector arms

To determine optimum cloning conditions the partially filled-in DNA was ligated to 0.5µg lambda BlueSTAR Xho I vector arms in vector: insert molar ratios of 0.6, 1.2, 2.1, and 2.9. Each ligation (10µl) contained 4 units T4 DNA ligase (Boehringer), 1 X buffer and 10mM DTT and was incubated overnight at 4°C.

2.6.2.5 Packaging of test ligations and titration

Each ligation (1.7µl) was mixed with 8.3µl of the packaging extract (Novagen's Phagemaker® System) and incubated at 22°C for 2h then stopped by the addition of 73.3µl SM buffer.

A culture of ER1647 cells grown in LB medium (containing 0.2% maltose and 10mM $MgSO_4$) for 5h to an $O.D_{600}$ of 1. The packaged DNA was diluted by 10^{-2}, 10^{-3} and 10^{-4} in SM buffer and mixed with an equal volume of the ER1647 culture then incubated statically at 37°C for 30 min. The infected cells (100µl) were mixed with 3ml top agarose, pre-warmed to 47°C, and poured onto the surface of a pre-warmed (37°C) LB agar plate. The set plates were incubated at 37°C overnight.

2.6.2.6 Large scale packaging of genomic DNA

In the main ligation reaction a vector arms: DNA ratio of 2.9 was used in a total reaction volume of 60µl. Five packaging extracts (50µl) were each mixed with 10µl of the

ligated DNA and treated as described in 2.6.2.5. The packaging reactions were combined to form the primary library and 10µl were removed for titration.

2.6.2.7 Amplification of primary library

Aliquots (16x160µl) of the remaining primary library (5.2 X 10^5 pfu) were each mixed with 320µl ER1647 cells grown in LB-maltose, as described in 2.6.2.5. Each aliquot of cells was mixed with 3ml of top agarose, as before, and poured onto a 10cm square plate. The plates were incubated at 37°C until the plaques became visible (5-6 hours). Each plate was flooded with 8ml SM buffer, incubated at 4°C and the lysates was collected and combined. The plates were washed with a further 2ml SM buffer and the washings combined with the first lysate. After addition of chloroform (0.1ml per 10ml phage suspension) the lysate was centrifuged (3000rpm) for five min at room temperature in a GS-A rotor (Sorvall). An aliquot of the amplified phage was removed for titration, as described in 2.6.2.5, and Dimethylsulphoxide was added to the remainder to give a final concentration of 7%. The phage were stored at -70°C. The final titre of the amplified library was 1.7 X 10^9 pfu/ml.

2.6.3 Screening of genomic library

An aliquot of the amplified genomic library containing 2.5 X 10^5 pfu was plated on a 22cm square plate of LB agar, as described in section 2.6.2.5. Screening was essentially carried out as described in section 2.2.

2.6.3.1 Probe synthesis

The synthesis of the probe used to screen the genomic library was carried out as described in 2.2.1 using primers ACPA and ACPS (2.2.6.3) and the RE-ACP clone as a template to generate a 550bp DIG-labelled product.

2.6.3.2 Hybridization and probe detection.

Duplicate plaque lifts were probed with DIG-labelled RE-ACP as described in 2.2.3-2.2.6.2. Plaques giving a positive signal from the screen were re-plated at a lower density

on 10cm square plates and re-screened. Single, well isolated plaques were excised into pBlueScreen. Putative ACP genomic clones that were isolated were sequenced with ACPA and ACPS (2.2.6.3) primers and analysed with DNASTAR. A single genomic clone was further sequenced with the following primers: PPf and PPr (2.2.1), ACP3 (5'-CTCAAGCCACTATCAGGT-3') and ACPEX1 (5'-CTGTAACCCGAAAGAAAGAGAAC-3'). The 5′ upstream region of the putative genomic ACP DNA was also sequenced with ACP 61/78 (5'-GAAGGCGAGAGCGGAGTG-3') primer.

Chapter 3

Acyl Carrier Protein Isoforms in Unripe and Ripe Strawberry Fruit

3.1. Introduction

Three ripening enhanced acyl carrier protein (ACP) cDNA clones were identified from a differential screen of a cDNA library from ripe strawberry fruit (Manning, 1998). ACP is a small acidic protein involved as an essential co-factor in *de novo* biosynthesis of fatty acids which are essential precursers for membrane synthesis and triglyceride storage reserves (Harwood, 1996), but the function of ACP in ripe fruit is unknown. Multiple ACP isoforms are found in plants, but their role in lipid metabolism has not been examined in edible fruits and it is not known how these isoforms are specifically utilised by the plant. Some ACP isoforms are constitutively expressed, and others are developmentally and tissue-specifically regulated (de Silva *et al.*, 1990). Because ACP has a central role in lipid metabolism, it is likely to be involved in 'house-keeping' functions, including the synthesis and maintenance of membranes in developing fruit. However, a differential screen on ripe blackcurrant fruit, another soft fruit, did not identify ACP from the isolated cDNA clones (Woodhead *et al.*, 1998). In banana, a climacteric fruit, 59 differentially expressed clones were sequenced (Medina-Suárez, 1997) with no ACP cDNA identified. This may indicate that the up-regulated form of ACP in strawberry has a special function in ripening. To address the possibility that other ACPs are expressed in strawberry fruit, further research is required.

Differential screening, as was used for strawberry, is likely to isolate the more abundant cDNAs that are expressed in the fruit. To identify ACP cDNAs, representing lowly expressed genes, alternative strategies are needed. Two approaches will be used to investigate if other ACP genes are expressed in strawberry fruit. In the first, a cDNA library from ripe strawberry fruit will be screened with a probe derived from the highly

conserved phosphopantetheine recognition sequence present in all ACPs. This probe is expected to hybridise to all ACP cDNAs present in the library but may not detect very low abundance clones. The number of clones obtained by this method, however, will depend on the quality of the library, the number of clones screened and the relative abundance of ACP cDNAs. The second approach involves the use of a novel technique known as Candidate Fragment Length Polymorphism (CFLP). The CFLP method is a PCR-based technique that produces amplified fragment length polymorphisms (AFLPs) specific for the family of genes being analysed. To isolate ACP fragments a specific primer derived from the conserved phosphopantetheine sequence will be used. This method, which is theoretically capable of detecting relatively low abundance gene products, displays the amplified cDNA fragments on a polyacrylamide gel. Bands of interest will be re-amplified, cloned and sequenced.

The main aims of this chapter are to fully sequence the three ACP cDNA clones previously isolated from strawberry and compare their sequences. Other ACP cDNAs, putatively expressed in strawberry fruit, will be isolated and the sequences obtained will be compared with the ripening enhanced ACP cDNA(s) and with ACPs from other species.

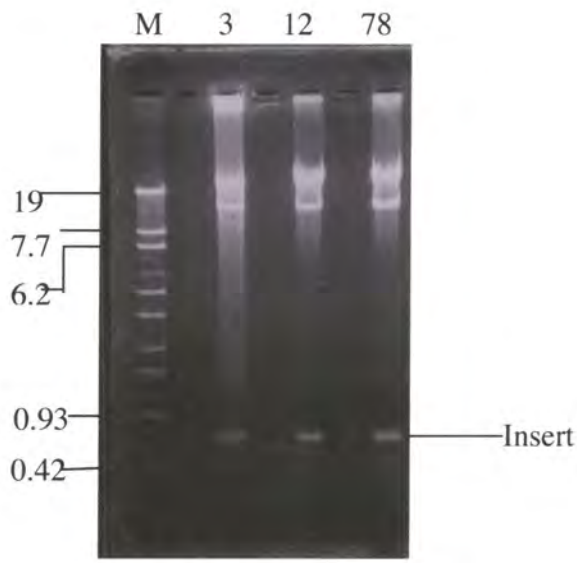

Figure 3.1. Separation of ACP cDNA inserts by agarose gel electrophoesis after digestion of λgt10 clones with EcoRI. ACP clones 3, 12, 78 and λ/StyI markers (M) were separated on a 1% agarose gel.

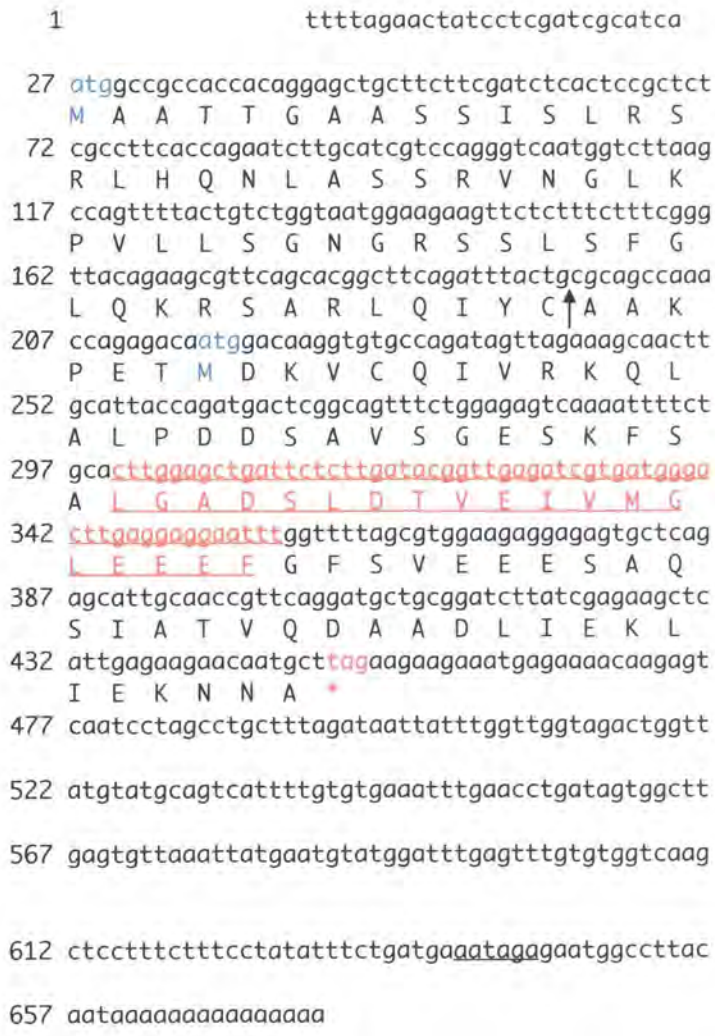

Figure 3.2. Nucleotide and predicted amino acid sequence of the *F. ananassa* ripening enhanced ACP cDNA. An arrow marks the precursor cleavage site. The putative polyadenylation signal sequence is underlined in black. The putative phosphopantetheine-attachment site is underlined in red.

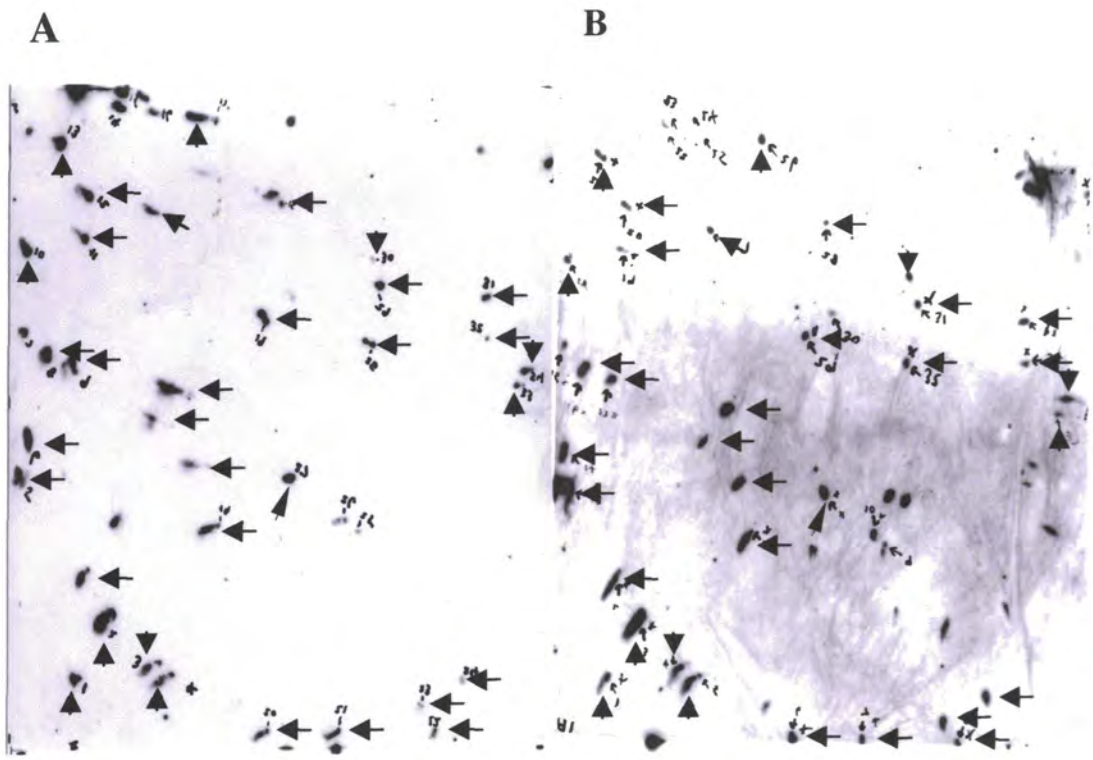

Figure 3.3. Primary screening of plaques from a ripe strawberry fruit cDNA library in λScreen. The arrows represent positive clones identified in duplicate blots A and B.

3.2 RESULTS

3.2.1 Sequencing of the ripening enhanced ACP cDNAs

The cDNA inserts (Fig.3.1) from three independently isolated putative ACP clones (3, 12 and 78) in the λgt10 vector were sub-cloned into pBK-CMV and fully sequenced using M13 forward and reverse primers and internal ACP primers. The sequences obtained from the three clones were found to be identical (Fig. 3.2).

3.2.2 Screening of a ripe fruit cDNA library in λscreen vector

For the primary screen $3x10^4$ pfu were plated and probed with a DIG-labelled DNA comprising the phosphopantetheine recognition sequence. From the duplicate plaque lifts (Fig. 3.3 A and B) 35 hybridising plaques were identified. From the secondary screen, nine plaque pure clones (5, 8, 13, 14, 15,17, 20, 21 and 24) were obtained and *in vivo* excised into the pScreen plasmid vector for sequencing. Clones 5, 8, 13, 15, 17 and 21 possessed homology to ACP sequences (see section 3.2.4 for comparisons with other strawberry ACPs).

3.2.3. Identification of ACP cDNAs by CFLP

3.2.3.1. Expression profiles of ACP genes

cDNA fragments were amplified from cDNAs isolated from ripe (R) and white (W) strawberry fruit by PCR, using either the forward (PPf) or reverse (PPr) radiolabelled primers designed to the conserved phosphopantetheine binding region of ACP and an Mse I linker primer. Bands of interest, displayed on a polyacrylamide gel and numbered as shown (Fig. 3.4 A and B), were cut out from the gel and designated Rr, Rf and Wr, Wf for ripe and white fruit, respectively

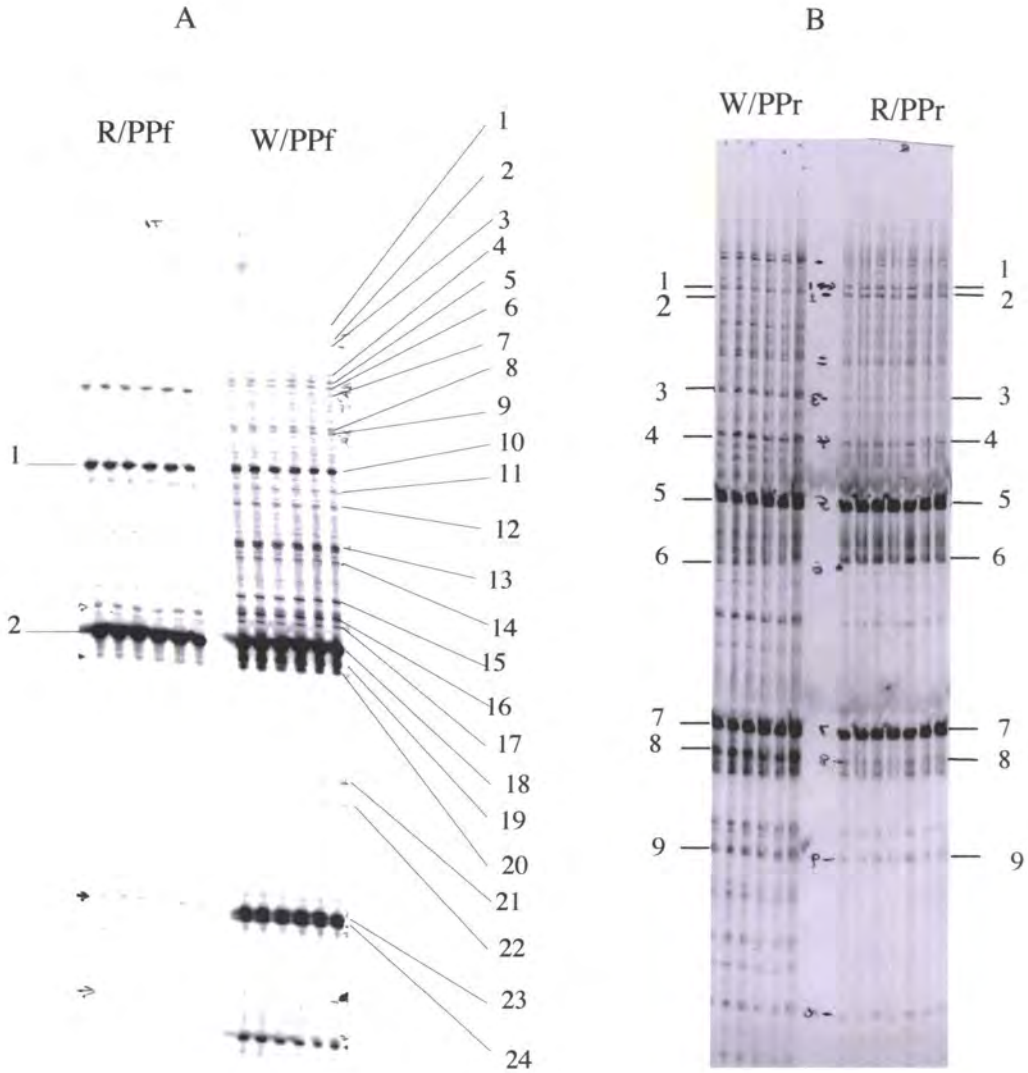

Figure 3.4. Candidate Fragment Length Polymorphism (CFLP) analysis of ACP cDNAs in ripe and unripe strawberry fruit. Autoradiograph images of ^{33}P-labelled amplification products, from Ripe (R) and White (W) strawberry fruit cDNA, were obtained with MseI/PPf primers (A) and MseI/PPr primers (B). PPr (reverse) and PPf (forward) are ACP-specific primers. Bands numbered as shown were excised for Southern analysis.

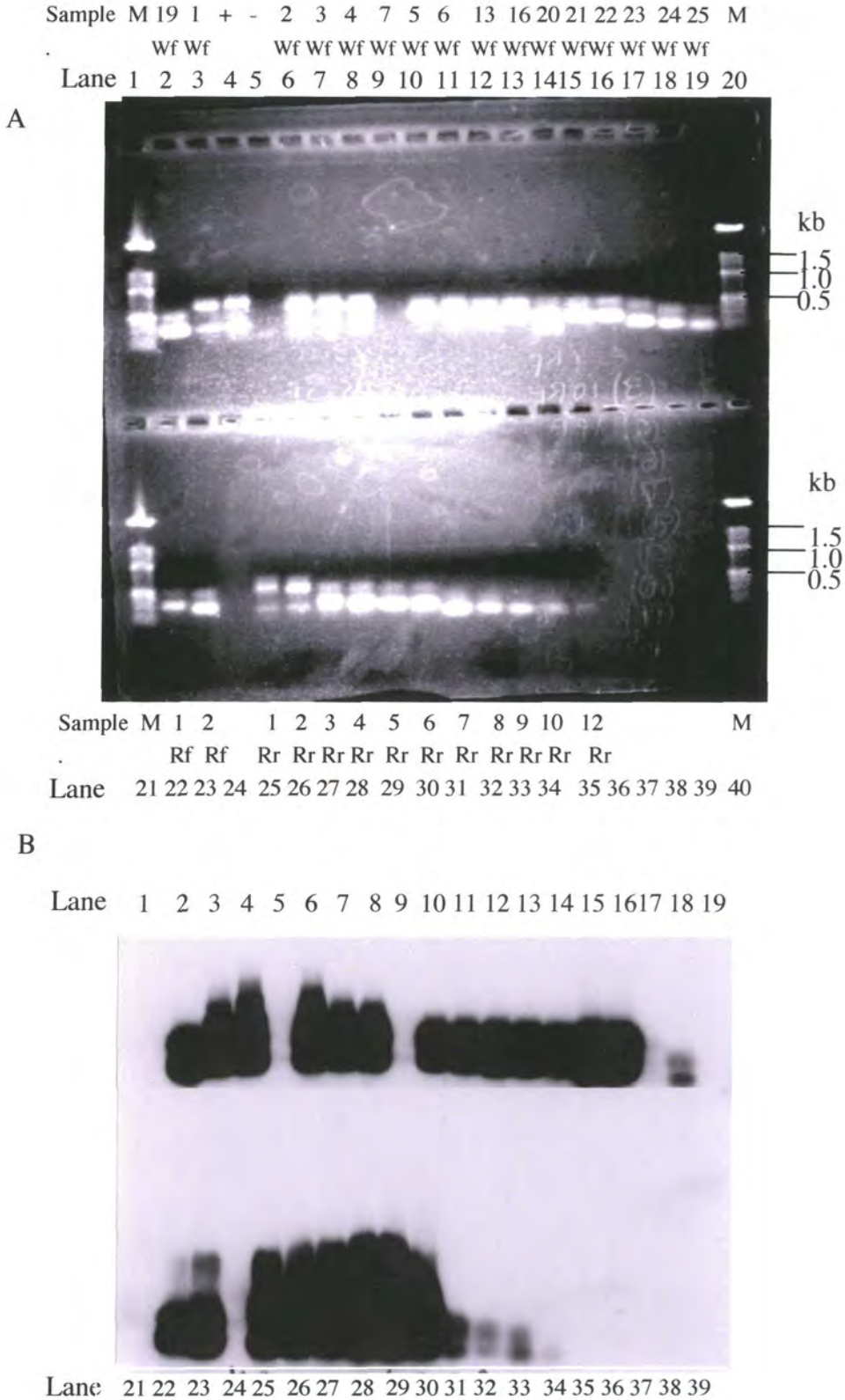

Figure 3.5. PCR re-amplification and Southern blot analysis of bands extracted from the CFLP PAGE. Selected PCR products were re-amplified (A) from the DNA fragments from the CFLP PAGE (see Fig 4), Southern blotted onto a nylon membrane, and probed with a DIG-labelled phosphopantetheine sequence (B). Positive and negative controls are shown in lanes 4 and 5, respectively. Markers (M) are a 100bp ladder (XIV, Boehringer). Lane numbers in (B) correspond to those in (A).

3.2.3.2. Southern analysis

The cDNA fragments, selected according to signal intensity (Fig. 3.4 A and B), were re-amplified, separated on an agarose gel (Fig. 3.5A) and transferred to a nylon membrane for Southern blot analysis (Fig. 3.5B). No staining of PCR product 7Wf (lane 9) was obtained with ethidium bromide.

The DIG-labelled phosphopantetheine probe hybridized to 22 of the 29 ethidium bromide stained fragments (Fig. 3.5B) (including the positive control). cDNA fragments 23Wf, 24Wf, 25Wf, 8Rr, 9Rr, 10Rr and 12Rr showed little or no hybridization with the probe and were not investigated further. Fragments that hybridised strongly to the probe were cloned into the pBlunt II TOPO vector and sequenced. Sequences of clones 16Wf, 19Wf, 2Rf and 5Rr encoded ACP. Sequences showing no database homology or non-ACP identity were not investigated further. Sequence analyses are described below.

3.2.4 Sequence analysis of the ripening enhanced ACP isoform

The full-length sequence of 674bp (Fig. 3.2) has high homology with a number of ACPs. The sequence from *F.vesca,* the wild strawberry, has the highest homology, followed by *C. glauca*, showing identities of 96% and 85%, respectively. The *F. vesca* ACP sequence has the greatest length of identical sequence (639bp) followed by *C. glauca* (159bp). The ripening enhanced ACP cDNA from the cultivated strawberry contains an open reading frame (ORF), initiating at nucleotide 27 and terminating at nucleotide 449, encoding a full-length 141 amino acid polypeptide with a predicted molecular mass of 14986 Daltons. An ACP cDNA isolated from *B. napus* seed encodes a protein of 134 amino acids (M_r 14700) (Safford *et al.*, 1989). Further comparisons with the *B. napus* ACP revealed that the strawberry ACP has the same transit/mature peptide sequence boundary of AAKPET. This enables the transit and mature peptides for strawberry ACP to be defined as 57 (M_r 6031) and 84 (M_r 8972) amino acids in length, respectively, similar to

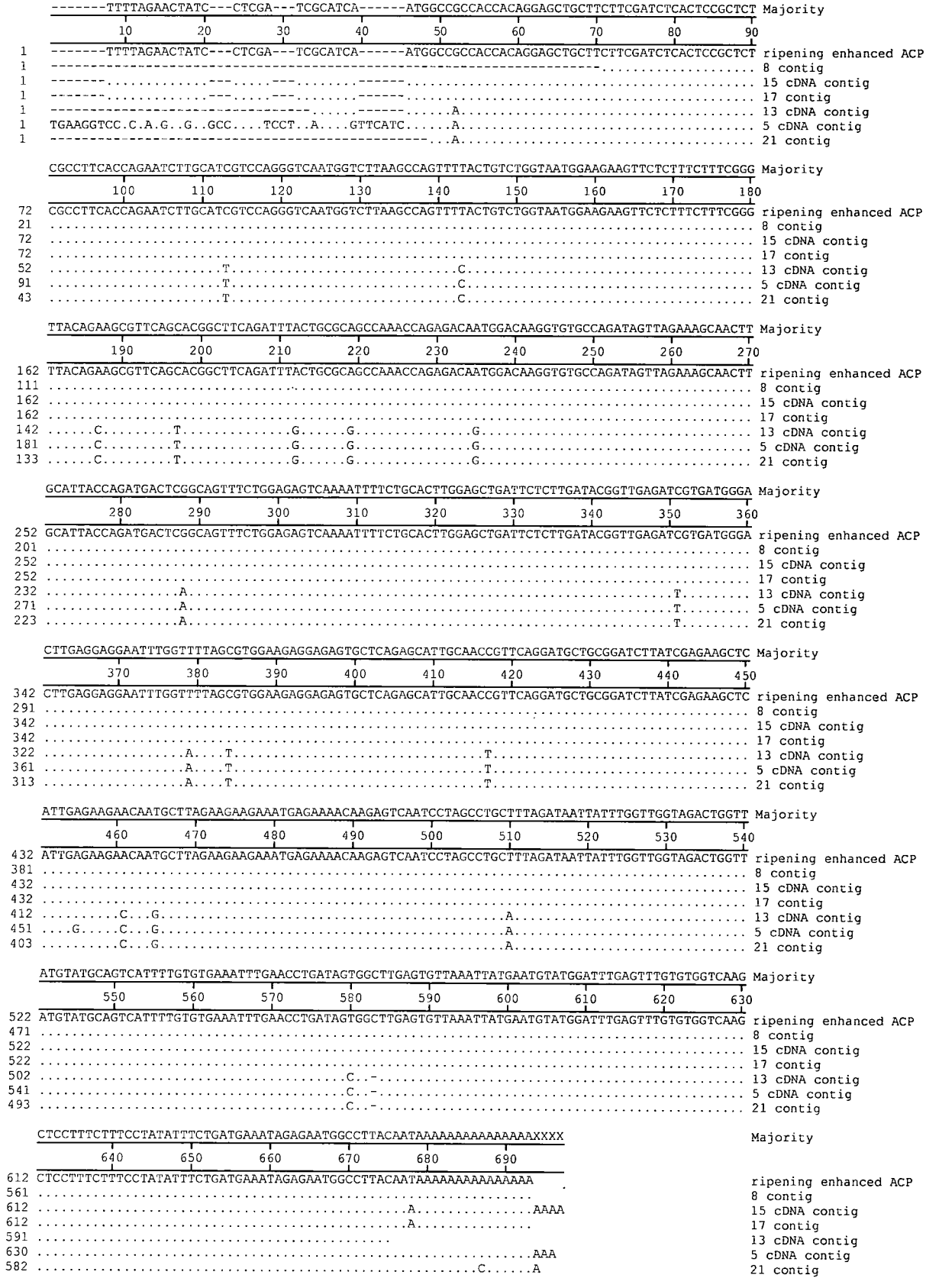

Figure 3.6. Comparison of the nucleotide sequences of seven strawberry fruit ACP cDNAs. The cDNAs from the library screen are compared with the ripening enhanced ACP cDNA. Dots (.) and hyphens (-) represent identical and missing bases, respectively. Only base differences are shown.

those predicted for the *B. napus* ACP (Safford *et al.*, 1989). The transit peptide indicates that ACP is directed to an organelle. Transit sequence analysis using PSORT (Nakai and Horton, 1999) predicts the destination for the protein to be the chloroplast stroma (data not shown), which is known to be the site of *de novo* fatty acid biosynthesis in plants (Safford *et al.*, 1989; Töpfer and Martini, 1994). The translated sequence of the strawberry ACP contains a highly conserved pantetheinylation site between residues 92 (nucleotide 300) and 110 (nucleotide 354) (Fig. 3.2).

3.2.5. An ACP multigene family is expressed in strawberry fruit

Comparison of cDNA nucleotide sequences, isolated by screening the ripe strawberry fruit cDNA library and by the CFLP method, indicates six putative ACP isoforms are expressed in strawberry fruit and therefore that ACP is a multigene family in strawberry.

3.2.5.1. cDNAs from the library screen

The six cDNA clones (5, 8, 13, 15, 17 and 21) isolated from the ripe fruit cDNA library can be grouped into three families (Fig. 3.6). Clones 8, 15 and 17 show 100% identity to the ripening enhanced form. Clones 13 and 21 are identical and differ from clone 5 at nucleotide position 455. A total of 18 nucleotide substitutions and one base deletion occur between clones 5/13 and the ripening enhanced isoform. The differences located at the poly (A)-tail were ignored as these substituted bases could be created by errors during polyadenylation.

The differences in nucleotide sequences between the three groups of ACPs are distributed along the cDNA sequence (Fig. 3.6), with 15 of the 18 base substitutions occurring in the ORF. A single base substitution occurs in the pantetheinylation recognition sequence. Four of the cDNAs (21, 8, 13 and 5) were found to be incomplete at the 5' untranslated region, which makes definitive comparisons impossible. Clone 5 has no homology with the other ACP sequences at the 5' end.

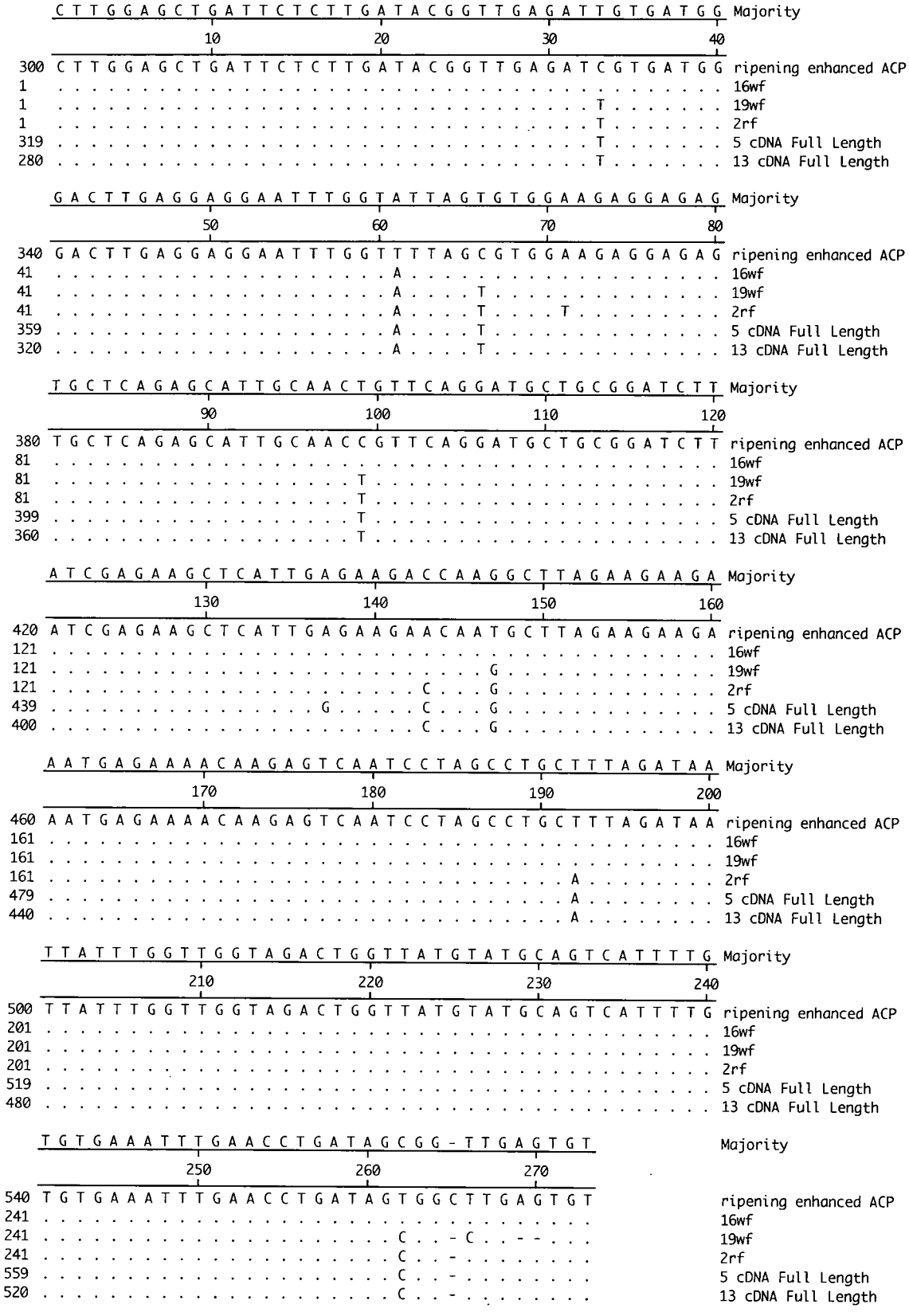

Figure 3.7. Comparison of the partial cDNA sequences from all ACP clones isolated from strawberry fruit. Nucleotide replacements are shown. Dots represent identical bases and hyphens indicate inserted gaps.

3.2.5.2. cDNAs from CFLP analysis

The partial sequences isolated by CFLP also show heterogeneity with the sequences described above (Fig. 3.7) with the exception of clone 5Rr which has 100 % identity with the ripening enhanced clone (data not shown). Similarity comparisons between the strawberry ACPs are summarised in Table 3.1.

Table 3.1. Percentage similarity/divergence of the ACP cDNA isoforms isolated from strawberry fruit.

Percent Similarity

	1	2	3	4	5	6		
1		99.6	96.3	94.9	94.9	95.2	1	ripening enhanced ACP
2	0.4		96.7	95.2	95.2	95.6	2	16Wf
3	2.6	2.3		98.1	98.1	98.5	3	19Wf
4	3.4	3.0	1.5		99.3	99.6	4	2Rf
5	3.4	3.0	1.5	0.7		99.6	5	5
6	3.0	2.6	1.1	0.4	0.4		6	13
	1	2	3	4	5	6		

Percentage Divergence

3.2.5.3. Comparisons of all strawberry ACP isoforms

The isolated putative cDNA ACP clones form two main groups, the first containing the original ripening enhanced cDNA and clone 16Wf, and the second containing the clones 19Wf, 2Rf, 5 and 13 (Fig. 3.8). The second set of clones can be sub-divided further into two groups with clone 19Wf separated from the remainder.

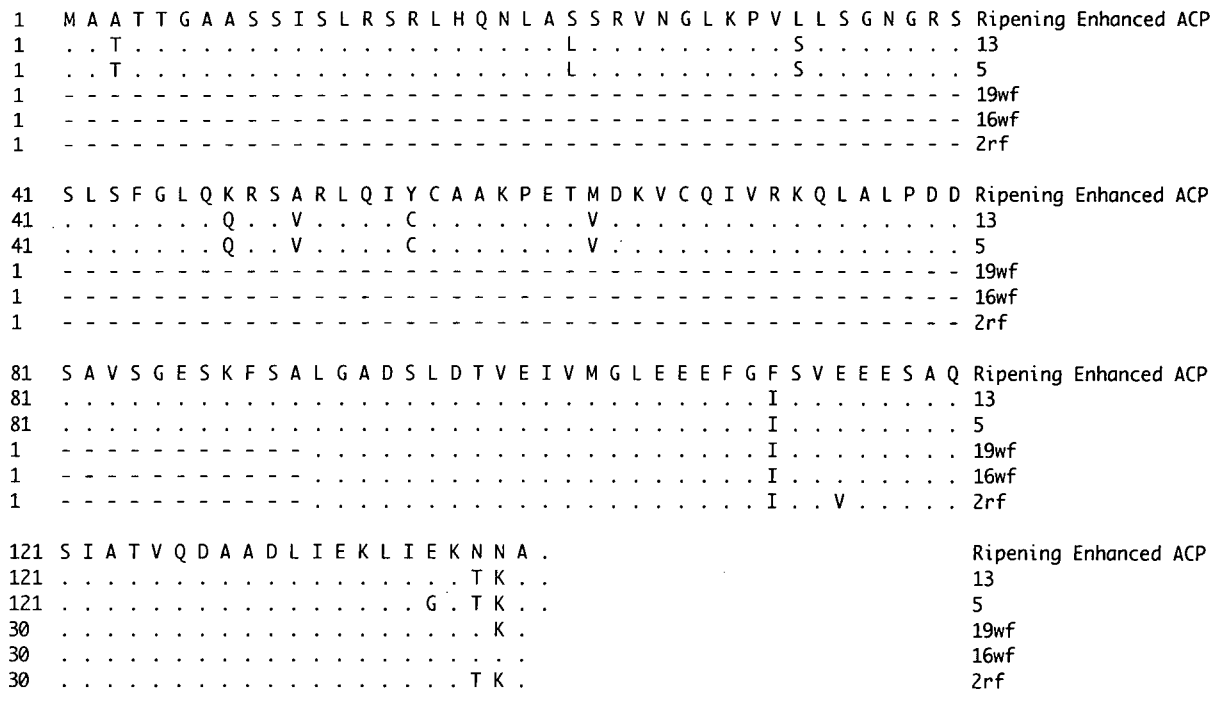

Figure 3.9. Comparison of the predicted amino acid sequences from the ORFs of the ACP clones isolated from strawberry fruit. Dots represent identical amino acids and hyphens indicate inserted gaps.

However, these comparisons are between incomplete sequences and may differ from the full-length homologues.

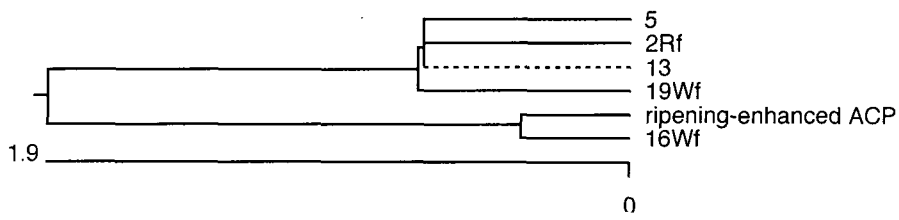

Figure 3.8. Phylogenetic comparison of all the ACP cDNAs isolated from strawberry fruit

Clones 2Rf, 5, and 19Wf have base substitutions that are unique, while other substitutions are shared with at least three other clones (Fig. 3.7). Only one base substitution was observed in the pantetheinylation sequence. The cDNA clones 2Rf, 5 and 13 had a single base deletion at the 3' end, while three were present in clone 19Wf. These deletions lie outside the ORF.

None of the six ACP isoforms were identical at the amino acid level (Fig. 3.9) the nucleotide substitutions in the full-length cDNAs translated into 10 (clone 13) or 11 (clone 5) differences, relative to the ripening enhanced clone. Six of these amino acid changes are in the transit peptide region (1-57). The partial cDNA clones derived from the CFLP method have amino acid identities to the other strawberry ACPs, except for 2Rf which has a unique residue substitution (E/V). Between amino acid 92 to 141 (Fig. 3.9) two substitutions maintain some charge conservation (F/I, N/T) but three (E/V, E/G, N/K) did not and these may be functionally significant. However, the single nucleotide difference in the pantetheinylation recognition site, described above, did not change the amino acid at this position. Three of the seven further substitutions between the full-length ACP clones (Fig. 3.9) also showed conservation (A/V, Y/C, M/V) but the others did not (A/T, S/L, L/S, K/Q).

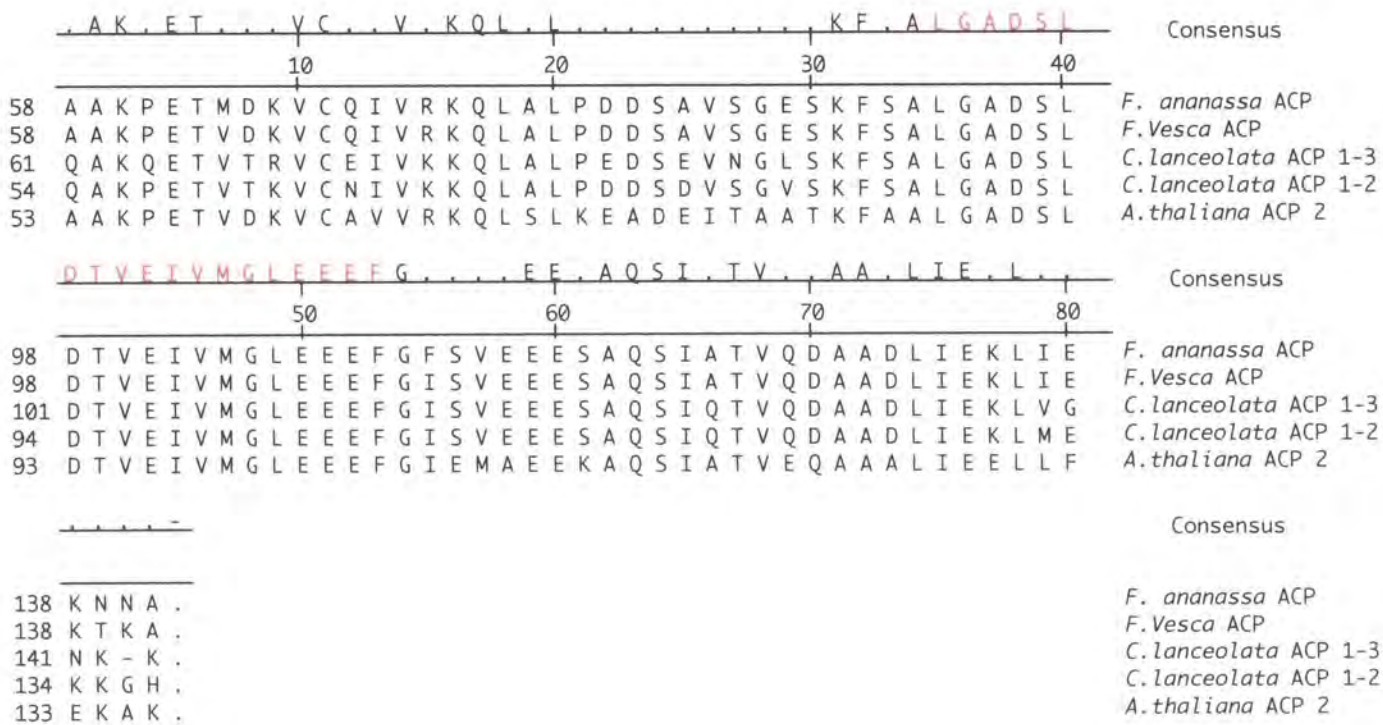

Figure 3.10. Comparison of mature ACP amino acid sequences. The deduced ACP sequences from *Arabidopsis*, *Cuphea* and wild strawberry (*Fragaria vesca*) are compared with the translated product from the ripening enhanced ACP of *Fragaria ananassa*. Conserved regions are shown in the consensus sequence and the phosphopantetheine recognition sequence is in red. Hyphens represent inserted gaps.

3.2.6. Comparison of the strawberry putative ACPs with heterologous ACPs

Comparison of the mature amino acid sequence of the ripening enhanced ACP from strawberry with ACP sequences obtained from *F. vesca* (wild strawberry), *Arabidopsis* and *Cuphea* ACP 1-2 and 1-3, reveal respective homologies of 94.1%, 63.5%, 83.5% and 77.4% (Fig. 3.10). The amino acid sequences within the phosphopantetheine region is identical to that found in ACPs from seed and leaf tissues from different species (Safford *et al.*, 1989; Shintani and Ohlrogge, 1994; Suh *et al.*, 1999).

3.2.6.1. ACP transit peptide amino acid sequence heterogeneity

The greatest variation in the coding region between ACPs from different species occurs in the transit sequences and includes amino acid substitutions and deletions (not shown). The transit sequences of the strawberry ACPs have an identical length, but the transit sequence in clones 5 and 13 which is identical, only has 89.5% homology to the ripening enhanced isoform. No SIP/NHG tripeptide sequences, as described by Safford *et al.* (1989), are present in the strawberry ACPs, but clones 13 and 5 contained the MATT residues (Ohlrogge *et al.*, 1991) at the beginning of the N terminal region (Fig. 3.9), as did the ACP from *F. vesca* (not shown). However, there was no SV/ISC sequence (Ohlrogge *et al.*, 1991) at the C-terminal end of the transit peptide. The sequence KVS(X)IVK is highly conserved in different species (Slabas *et al.*, 1987) and a similar sequence KVC(X)IVR was present in the ACPs from both the cultivated and wild strawberry

3.2.6.2. ACP amino acid charge analysis

The transit peptide is characterised by high levels of serine and leucine (19.3% and 15.7%, respectively), neutral (polar and hydrophobic) and basic amino acids (71.9% and

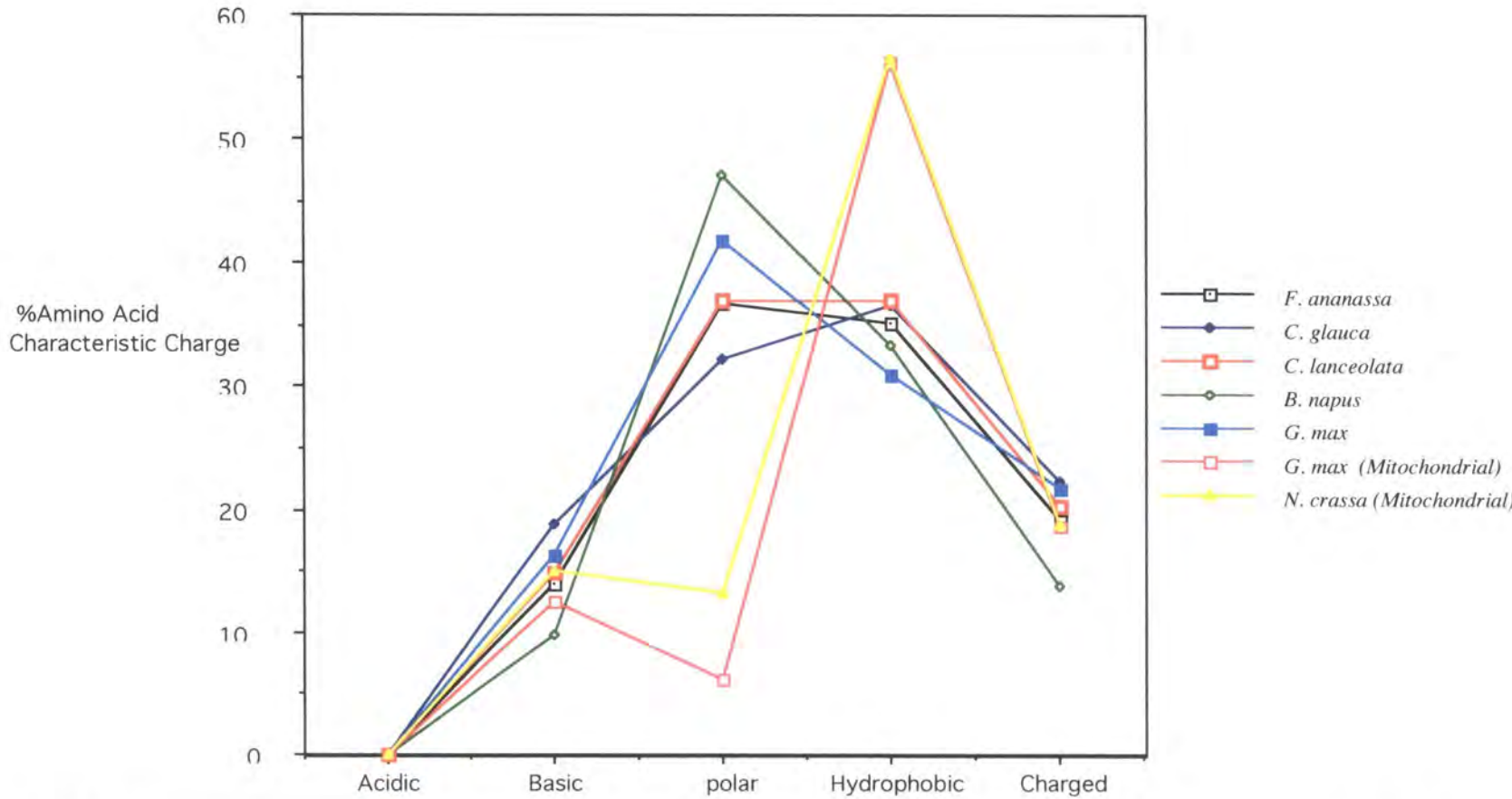

Figure 3.11. Comparison of charge distributions between transit peptides of mitochondrial and chloroplast targeted ACP proteins.

14.0%, respectively), with a complete absence of acidic residues. In contrast, the mature peptide has a higher level of acidic residues (21%) giving a predicted charge of -11.1 (at pH 7.0), which is a general feature of ACPs (Schmid *et al.*, 1997). The characteristics from the ripening enhanced ACP *F. ananassa* are similar to those of other ACPs described by Ohlrogge *et al.* (1991) and are thought to be indicative of chloroplast targeted proteins. Mitochondrial transit sequences have fewer polar but more hydrophobic residues (Fig. 3.11). The amino acid substitutions in the other ACP cDNAs from strawberry have the effect of decreasing the predicted negative charge in the mature peptide to give values of –9.1 (clone 5) and –10.1 (clone 13), at pH 7.0. The significance of the decreased negative charges is unknown.

3.2.6.3. 5' and 3' non-coding regions

The 5' and 3' non-coding regions (UTR) of the ripening enhanced ACP clone (Figs 3.2 and 3.6) from strawberry are 26 and 210 nucleotides in length, respectively (excluding the poly A tail). A comparison between the ripening enhanced cDNA and the *F. vesca* homologue (not shown) suggests the 5' UTR region of the ripening enhanced cDNA may have been truncated in the cloning process. In addition, a G is present at position +4, relative to the translation start site, but no purine is present at position –3, postulated to be important for the optimal initiation of translation (Lefebvre and Gellatly, 1997).

The partial 3' UTR of clones 5, 13, 2Rf, 16Wf and 19Wf (Fig. 3.7) have few differences and the extreme 3' ends of the full-length clones 5 and 13 were identical (Fig. 3.6). This is contrary to the observation made by Safford *et al.* (1989), in that considerable variations occur in the nucleotide length of ACP clones isolated from *B. napus*. The putative polyadenylation site in the ripening enhanced ACP mRNA (AAUAGA) differs from the consensus AAUAAA. In *B. napus*, the polyA recognition sequence (AAUGAA) also differs from the consensus (Safford *et al.*, 1989). However, plant polyA signals are known to deviate more than those of mammalian genes (Lefebvre and Gellatly, 1997).

3.2.7. ACP structure

ACPs have been studied from different organisms to identify structural features that explain how these proteins function. ACPs have been isolated from organisms and tissues that are involved in the synthesis of medium chain or long chain fatty acids. Long chain fatty acids are present in *E. coli*, spinach leaf and *B. napus* seeds. Unusual and medium chain fatty acids are produced in seeds of coriander and *C. lanceolata*, respectively. The ACPs from *C.lanceolata* and coriander are the most similar (Fig. 3.12) with an homology of 84.3%. The RE-ACP from strawberry had 83.5% and 81.9% homology to *C. lanceolata* and coriander ACPs, respectively. As expected, *E. coli* had the least homology with the other ACPs examined. *B. napus* and spinach ACPs are more distantly related to the other plant ACPs.

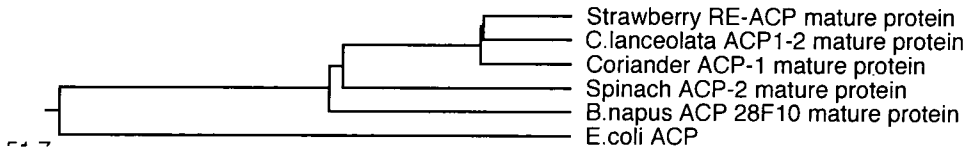

Figure 3.12. Phylogenetic tree of ACP mature protein sequences.

Computer analysis of the structure of mature ripening-enhanced ACP, using Protean (DNASTAR), predicts a mainly alpha secondary structure that is also shared with ACPs from *E. coli, B. napus*, spinach and coriander (Fig. 3.13). NMR studies of *E. coli* ACP (Kim and Prestegard, 1990) have established that ACP forms four α-helices involving amino acids 4-14, 36-50, 56-59 and 66-75 (Fig. 3.14), although this model is not absolute. The serine residue at position 36, which is found within the phosphopantetheine recognition site, is located at the beginning of helix 2 (Fig. 3.14B). A comparison between the structure predicted for *E. coli* ACP by NMR and by the Chan-Fusman method is shown in figure 3.13. The α-helix regions predicted by these methods were generally in good agreement. The Chan-Fasman method predicts that all five ACPs have α-helices beginning immediately after the serine residue located within the phosphopantetheine recognition site and extending towards the C-terminus end. Unlike

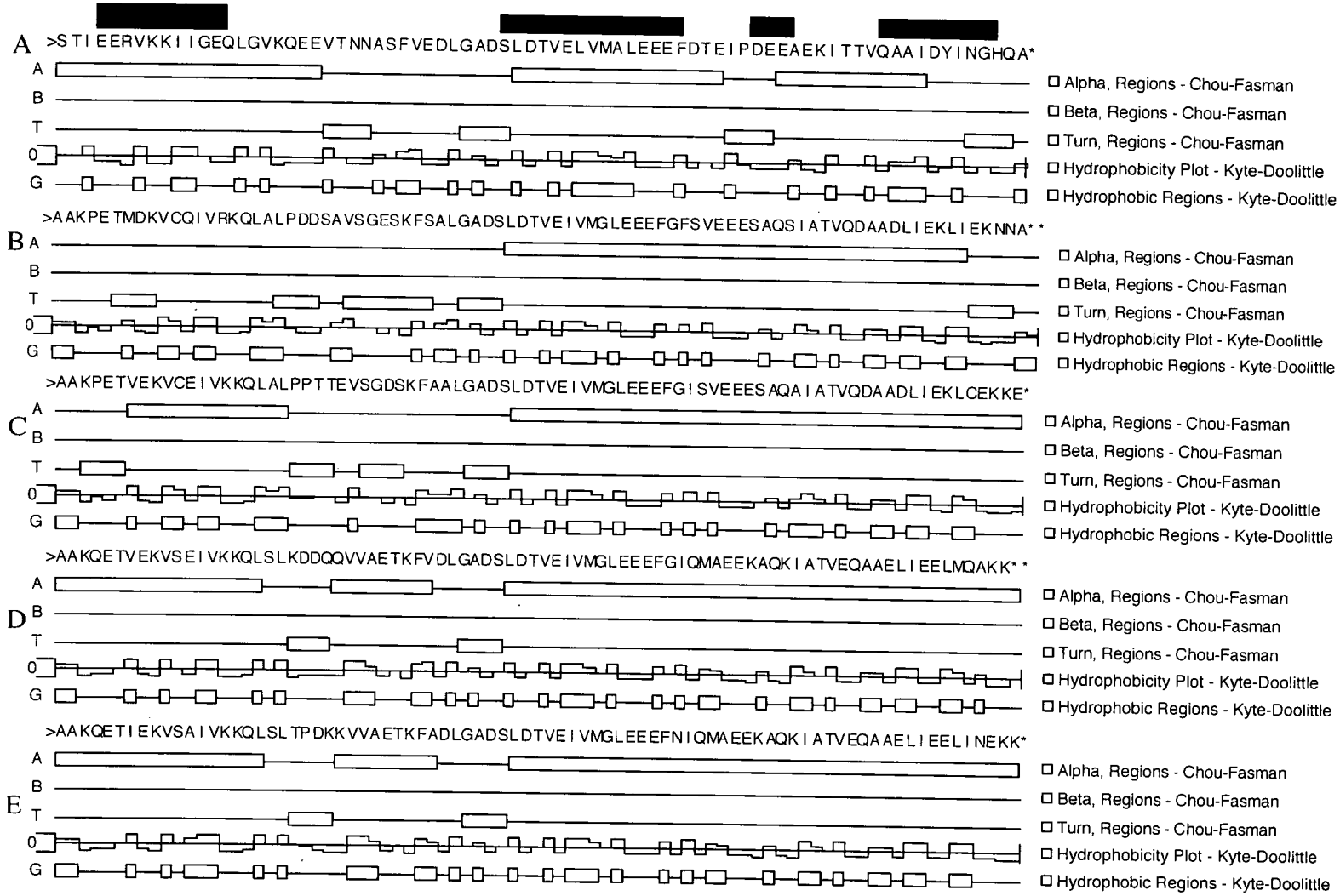

Figure 3.13. Predicted secondary structure of ACPs from *E. coli* (A), strawberry (RE-ACP; B), coriander (ACP-1; C), *B.napus* ACP (28f10; D) and *Arabidopsis* (ACP-1; E). Three structures, alpha, beta and turn (ß bends) regions were determined by the Chou-fasman method (DNASTAR). Black bars indicate α-helices of *E. coli* ACP determined by NMR. Hydrophobicity was measured using the Kyte and Doolittle method.

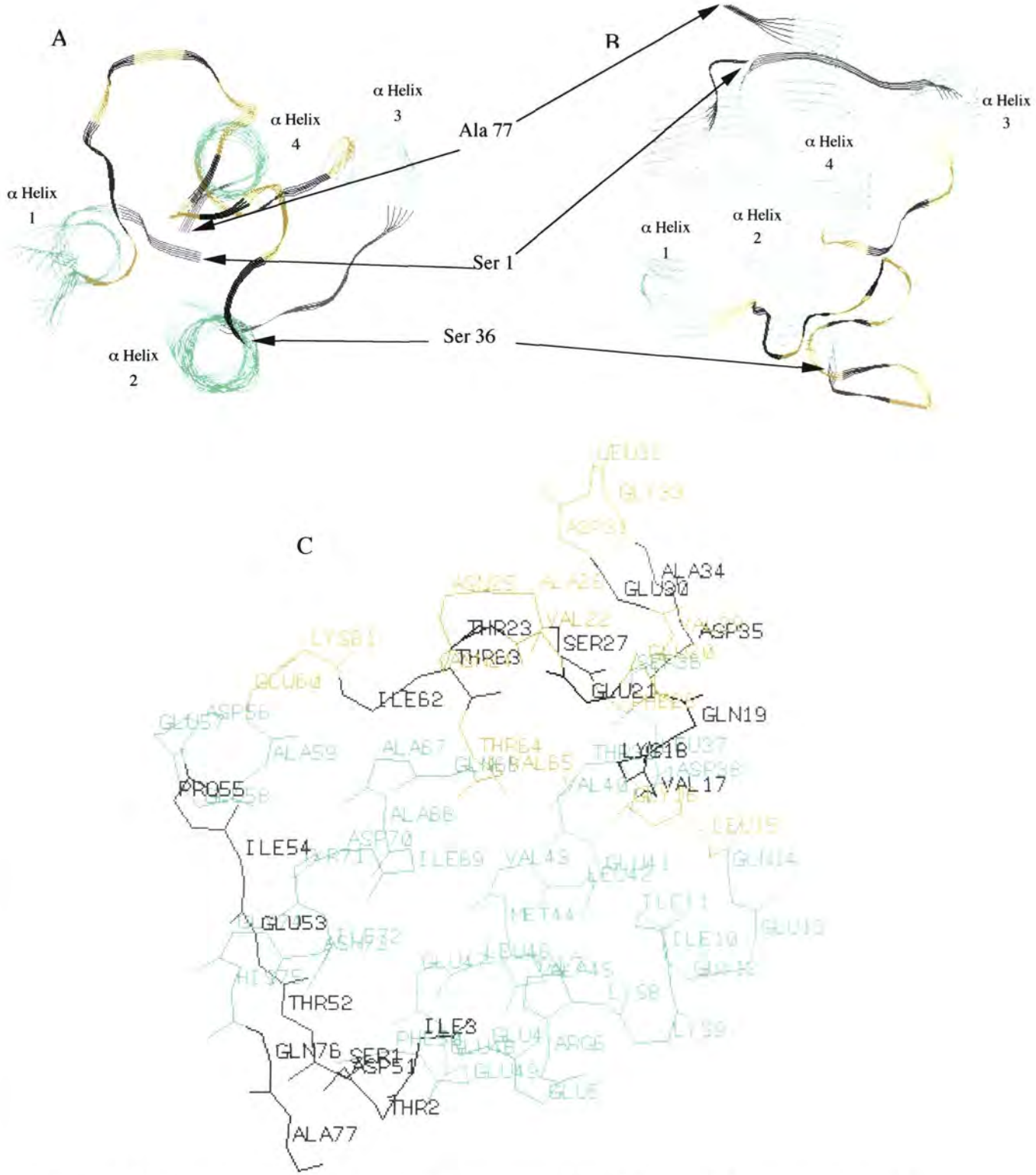

Figure 3.14. The structure of *E. coli* ACP determined by NMR. α–Helices are coloured turquoise. A and B are end and side views of the *E. coli* ACP. The relative positions of amino acids in the protein structure are shown (C). Arrows indicate the serine residue, to which the prosthetic group is attached. Diagrams were obtained by SCOP (**Structural Classification Of Proteins**) at NCBI.

the *E.coli* ACP, the α-helices of the plant proteins in this region is not interrupted. Interestingly, the ACP from ripening strawberry is the only one without an α-helices located towards the N-terminus of the protein (Fig. 3.13).

To examine the organisation of the ACPs in figure 3.13 a hydropathy plot, using the Kyte-Doolittle index, was determined from their primary amino acid sequences. In all ACPs examined the hydrophobic amino acids occurred approximately every three to four residues, particularly in the α-helices. The arrangement of amino acids within the ACPs described is shown in figure 3.15. Two faces of the helix can be seen with the negative residues (red) unevenly distributed on one side, with the opposing side containing hydrophobic residues M, I, L and V (black). The serine residue that is specifically bound to the phosphopantetheine moiety at position 39 (36 in *E.coli*), is located in the hydrophobic portion of the helix (Fig. 3.15; position 15). General features common to all ACPs are the four negatively charged residues that occur at every 18th position within the α-helix that places them directly over each other (position 7). However, *E. coli* (Fig 3.15A) and coriander (Fig 3.15D) ACPs contain a substituted hydroxyl amino acid at the first and second positions, respectively. With respect to the strawberry RE-ACP, positions 13 of all five helices are homologous. In addition, below the amino acid Q (position 13) is a hydrophobic residue (black) that is repeated every 11 amino acids, which results in a spiral occurring on the opposite face of the helix with respect to the negative amino acid residues. All eukaryotic ACPs, including strawberry RE-ACP, have amino acids with similar charge at positions 11 and 16.

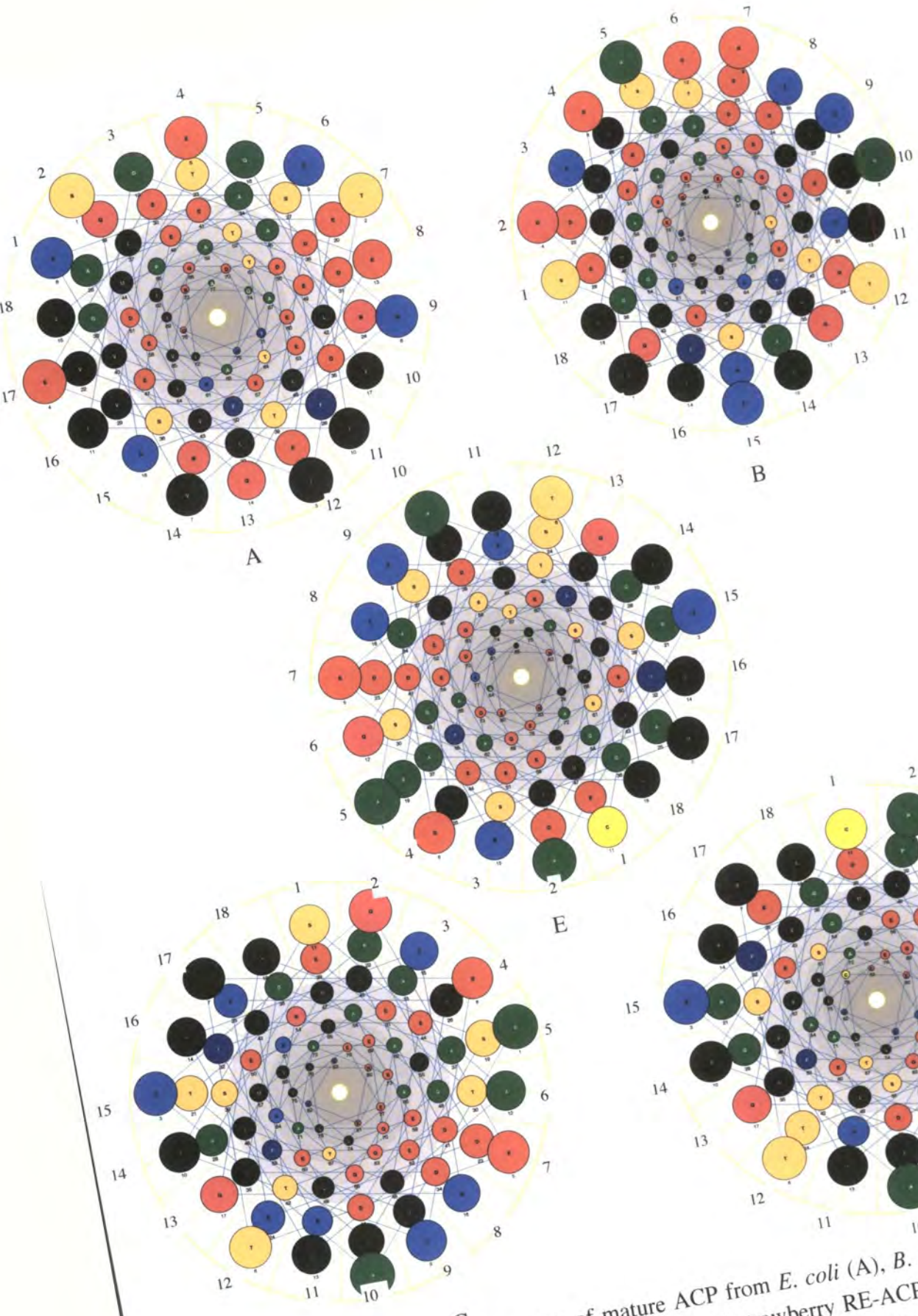

Figure 3.15. Helical wheel structure of mature ACP from *E. coli* (A), *B. napus* 28F10 (B), *A. thaliana* ACP-1 (C), coriander ACP-1 (D) and strawberry RE-ACP (E). Colours represent amino acid charge. Amino acids are represented by black (M, I, L, V), dark blue (F, Y, M), green (G, A, P), red (D, E), pink (N, Q), light blue (K, H, R), orange (S T) and yellow(C).

3.3. DISCUSSION

Two techniques were used to isolate ACP cDNAs from strawberry fruit. The library screen isolated two ACP cDNA isoforms expressed in fruit that are distinct from the previously isolated ripening enhanced clone. The CFLP technique enabled three further ACP variants to be identified from the fruit. The ability to display cDNAs semi-quantitatively from several different tissues or developmental stages is an important advantage of the CFLP method. However, the choice of restriction site used in this technique will determine which cDNAs can be cloned. PCR artefacts in the CFLP technique were found, as the majority of the cDNA fragments isolated were not from ACPs. For example, cDNAs with homology to aconitase and caffeic O-methyltransferase were isolated, the latter was reported in *F. vesca* (Nam *et al.*, 1999), in addition to cDNAs with no database identity.

Although no direct evidence defines the general features of the putative ACP sequences from strawberry fruit, the sizes of the cDNAs and their translated protein sequences (transit and mature peptides) correspond with observations made in other plants (Safford *et al.*, 1989; Suh *et al.*, 1999), and importantly include the conserved phosphopantetheine site. Ohlrogge *et al.* (1994) has described three ACP cDNA sequences from *Arabidopsis* with lengths of 688-810bp. Mature and transit peptide lengths for these clones were 83-84bp and 48-59bp amino acids, respectively. This evidence strongly supports the identity of the clones isolated from strawberry fruit to be ACP.

Strawberry ACP was found to be encoded by a multigene family. Six unique cDNAs, encoding different mature ACP polypeptides, were actively transcribed in fruit tissue. The six cDNA isoforms appear to fall into two groups distinguished by 18 nucleotide substitutions. Similarly, Safford *et al.* (1989) had characterised ten cDNA ACP clones encoding six different polypeptides from *B. napus*. These *B. napus* clones comprised two subfamilies and had 7 to 41 substitutions among them, indicating variations in ACP sequence can be tolerated without destroying its ability to function in fatty acid

biosynthesis. Likewise, strawberry ACP cDNAs contain shared base substitutions between two or more clones suggesting this is not an artefact of cloning. However, the unique base substitutions of some clones will have to be confirmed with further study.

From the 11 amino acid variations found between the strawberry cDNAs five residues appear to maintain a similar charge. Four of these amino acids are located near the conserved regions, such as the transit cleavage site or the phosphopantetheine attachment region. However, ACP families from *B. napus* (Safford *et al.*, 1989) have a greater degree of variation at these same locations. This information suggests that the sequence surrounding the invariant conserved sequences may accommodate limited variation without causing a detrimental effect to the function of ACP. Sequence variation may also indicate a specialised function specific to the respective host species, as indicated by SIP sequences in some isoforms of ACP in *B. napus* (Safford *et al.*, 1989). The significance of these and other conserved sequences, such as the KVS(X)IVK residues, is unknown (Slabas *et al.*, 1987; Ohlrogge *et al.*, 1991). Differences in the strawberry cDNA isoforms were found but the significance of this unknown. Different amino acid charge in the ACP protein may alter its functional characteristics. Other ACP isoforms in species such as in *B. napus* (Safford *et al.*, 1989) and *Arabidopsis* (Hlousek-Radojcic *et al.*, 1992), have a high degree of variation that suggests that further isoforms remain to be identified in strawberry.

The presence of multiple ACP isoforms in the fruit may enable this tissue to respond to different cellular demands for fatty acids during development. It is possible that different ACPs may have particular functions in the fruit tissue. The isoforms of ACP may play a role in determining the end products of fatty acid biosynthesis (Suh *et al.*, 1999), since mitochondrial isoforms are thought to be involved in fatty acid chain termination (Shintani and Ohlrogge, 1994). The strawberry is the only soft fruit in which ACP cDNAs have been reported (Manning, 1998; Nam *et al.*, 1999). One form of ACP appears to be particularly highly expressed in the ripe fruit. However, there is no

information about the relative abundance of other ACP isoforms in the fruit. The ripening-enhanced form may be related to the extraordinary array of aromatic compounds produced by the fruit (Mussinan and Walradt, 1975; Schreier, 1980), many of which are likely to be products of the lipid biosynthetic pathway (Schreier, 1980; Hamilton-Kemp *et al.*, 1996).

Comparisons with a transit peptide database suggest that the subcellular location for the strawberry fruit ACPs is the plastid. This information provides few further clues to their function except that it agrees with the current view that the plastid is the site of *de novo* fatty acid synthesis (Schmid *et al.*, 1997). It is possible that the ACPs in strawberry fruit are functionally indistinguishable and that their roles in fruit development are specified by the promoters of the corresponding genes. The expression patterns of these genes remain to be determined. The sequence data obtained from the ACP isoforms should enable gene specific primers to be designed to differentiate their expression during fruit development and in other tissues.

Homologies of ACPs between different species increase if the amino acid charge is taken into account. *E. coli* ACP forms an α-helical structure indicated by NMR studies. Computer modelling indicates all ACPs examined possess a secondary structure of α-helices with an α-helix extending from the phosphopantetheine group towards the carboxyl terminus. It is possible that ACPs in plants have α-helices with hydrophobic and hydrophilic faces. Amphipathic helices are important in water soluble proteins as their hydrophobic face is turned toward the interior of the protein and the hydrophilic surface faces the hydrophilic environment. This suggests that ACP may form a stable tertiary structure with the possibility that two ACP molecules could interact to form a dimer, as described above. The serine residue that is covalently attached to the prosthetic phosphopantetheine group would be placed at the end of the helix on its hydrophobic side. ACP from *E. coli* exists as at least two distinct conformers in solution (Keating and Cronan, 1996, and references therein). Two models have been proposed for the structure of *E. coli* ACP. In one model the second α-helix of ACP is unstable and the second

model shows a perfect helix (Fig. 3.14). The helices of ACP interact to form a hydrophobic cavity that can accommodate the phosphopantetheine group and up to six carbons of the growing acyl chain. This interaction between the acyl chain and the hydrophobic face of the helix stabilises ACP (Keating and Cronan, 1996).

Certain charged residues are maintained in all ACPs examined indicating that they have an important function. A base substitution from valine to isoleucine altered the compact nature of *E. coli* ACP increasing its electrophoretic mobility at high pH (pH 9.0) (Keating and Cronan, 1996). The stability of this V43I ACP was higher than that of the wild type ACP and this is thought to be the result of the increased stabilisation of helix two (Fig.3.14). In all ACPs examined the valine residue is conserved (Figs. 3.13 and 3.15). Amino acids at key positions may alter the stability of ACP and this in turn could explain the abilities of certain ACPs to influence the length of acyl moieties. It is uncertain how far the molecular structure of ACP from *E. coli* can be extrapolated to strawberry ACP.

Conclusion

Six ACP cDNAs expressed in strawberry fruit have been identified. These cDNAs have nucleotide and translated sequences with features similar to ACPs from other species. They possess a highly conserved phosphopantetheine sequence and the encoded mature protein appears to be targeted to the plastid.

Chapter 4

The Expression and Partial Characterization of Recombinant ACP from Ripening Strawberry

4.1. Introduction

The ACP cDNA clones isolated from strawberry fruit (chapter 3) belong to a multigene family. One of these, a ripening-enhanced isoform known as RE-ACP, encodes a putative protein 141 amino acids in length. The expression pattern and biochemical activities of this protein *in vivo* are unknown. Essentially, ACP is covalently linked to its phosphopantetheine prosthetic group which is bound to extending acyl moieties as a thioester during each cycle of fatty acid biosynthesis (Ohlrogge *et al*, 1991).

Enzymes involved in this pathway may exhibit different specificities towards isoforms of ACP. Guerra *et al.* (1986) had showed that spinach oleoyl-ACP thioesterase had a higher preference for acyl-ACP-II than for acyl-ACP-I. However, no preference for either ACP-I or ACP-II was observed for a thioesterase isolated from *C. lanceolata* seed that supported medium chain fatty acid synthesis in reconstituted fatty acid synthase (FAS) reactions (Schütt *et al.*, 1998). A mitochondrial ACP isoform from *A. thaliana* enabled predominantly medium chain fatty acids to be synthesised in spinach chloroplast FAS reactions (Shintani and Ohlrogge, 1994). Suh *et al.* (1999) has observed that palmitoyl-ACP from coriander seed, a plant producing monoenoic fatty acids, synthesised more Δ^4-hexadecenoic acid than did palmitoyl-ACP derived from spinach or *E. coli* using coriander seed Δ^4- palmitoyl-ACP desaturase. This same experiment, repeated with a coriander seed stearoyl-ACP desaturase, did not strongly discriminate between stearoyl-ACPs from coriander, spinach and *E. coli*. These experimental data suggest that ACPs could determine the end products of fatty acid synthesis.

ACP, a nuclear encoded protein with an N-terminal transit peptide (Ohlrogge *et al.*, 1991), is induced prior to the onset of lipid biosynthesis (Slabas *et al.*, 1987), indicating it has a regulatory role. Slabas *et al.* (1987) have purified ACP from maturing *B. napus*

seeds and shown the acylated protein to have a molecular mass of 15kDa. The mature ACP (Cs-ACP-1) from coriander, expressed in *E. coli* using the pET vector system, was found to co-migrate on SDS-PAGE with the most abundant ACP from endosperm tissues (Suh *et al.*, 1999) indicating the recombinant and native proteins were identical. Western blot analysis of *Arabidopsis* leaf, root and seed tissues demonstrated the presence of at least four ACP isoforms having either tissue specific or constitutive patterns of expression (Hlousek-Radojcic *et al*, 1992). As a first step to understanding the expression and functional characteristics of strawberry RE-ACP, recombinant protein will be expressed in *E. coli* and purified.

Figure 4.1. Nucleotide sequence of *F. ananassa* RE-ACP cDNA cloned into the multiple cloning site of the pET-24a expression vector. The predicted amino acid sequence is shown below the nucleotide sequence. The putative phosphopantetheine attachment site is shown in red. A methionine residue, internal to the translation start site (ATG) on the pET-24a vector, is highlighted in blue.

4.2 Results

4.2.1 Expression of ACP in *E. coli*

Primers were designed to sequences bordering the mature protein coding region of the RE-ACP isoform and used in a PCR to amplify the protein coding sequence. The 5′ regions of these primers incorperate an NdeI or EcoRI restriction site allowing the PCR product to be ligated into the pET-24a expression vector (Novogen) in the correct reading frame. The pET-24a vector exerts tight control over basal expression levels through the LacI/operator, minimising possible toxicity of the recombinant protein to the host cell before induction by IPTG.

The plasmid was initially transformed into DH5α cells but the plasmid DNA obtained from this strain failed to sequence properly with either T7 promoter or terminator primers. The plasmid was re-transformed into XL1-Blue MRF' Cells and this yielded a sequence (Fig. 4.1).

To express the strawberry ACP the recombinant plasmid was transformed into the BL21 [DE3] cell line and the cells were induced by IPTG. Protein was released by freezing and thawing (Johnson and Hecht, 1994) and ACP was purified by chromatography on an HQ anion exchange column using a continuous gradient of 0 - 1.0M LiCl. The proteins in the crude bacterial extract, and in the fractions eluted from the anion exchange column, were analysed by SDS-PAGE on a 12% polyacrylamide gel (Fig. 4.2). Three major bands, with mobilities of approximately 18, 21 and 45 kDa were observed in the crude extract. The dominant polypeptide in this fraction, corresponding to an apparent molecular mass (M_r) of 20kDa, was further enriched by anion exchange chromatography. It is approximately twice as large as the M_r (8.9) predicted for strawberry ACP (chapter three). Anomalous mobility has been observed similarly for a partially purified mature ACP of coriander (Cs-ACP-1) expressed in *E. coli.* This polypeptide migrated with a M_r of 15 kDa (Suh *et al.*, 1999) although its predicted Mr was 8.8 kDa. Slabas *et al.* (1988) and Safford *et al.* (1988) have also described a similar anomaly with the migration of ACP from *B.napus* on SDS-PAGE. Fractions 7-11 were the most enriched for the

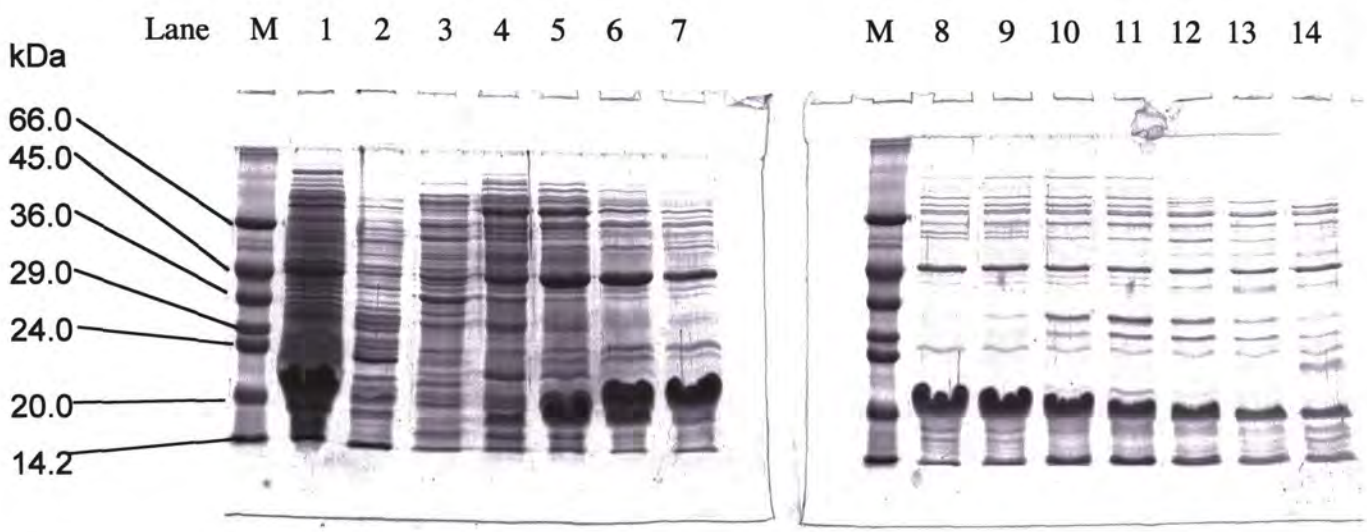

Figure 4.2. SDS-PAGE analysis of strawberry ACP expressed in *E. coli*. Proteins were separated on 12% polyacrylamide and stained with coomassie Blue R250. M= Mark VII-L® SDS markers. Lane 1 is crude bacterial protein, lanes 2 to 14 are protein fractions eluted from the anion exchange column between 300mM and 400mM LiCl.

~20kDa polypeptide and these were pooled together. Most of the contaminating polypeptides were eluted off the column at low salt concentrations (lanes 2- 6, Fig 4.2).

4.3 Discussion

In order to understand the molecular characteristics, regulation and structure of strawberry RE-ACP the recombinant mature protein was expressed in *E. coli* and purified.

Sequencing verified that the clone was correctly constructed and free from amplification errors (Taq polymerase has an error rate of 1 per ~9000bp and frameshifts of approximately 1 in every 40,000bp were expected (Kocher and Wilson, 1991)). To ensure the correct in-frame insertion of RE-ACP into the expression vector, primers giving the appropriate restriction sites were used to amplify the insert by PCR.

Purification of recombinant proteins from *E. coli* can often be problematic. The technique of freezing and thawing separates highly expressed recombinant proteins away from the majority of endogenous bacterial contaminants (Johnson and Hecht, 1994). Ion exchange chromatography using DEAE Sephadex was sufficiently specific to enable ACP to be purified largely free of contaminating proteins, which were eluted off the column at a lower salt concentration than the putative ACP. The mild extraction and purification procedures avoid disrupting secondary structures required for antibody recognition. This purified fraction has been used for the production of an antiserum to strawberry ACP with a high titre (K. Manning, personal communication). Further purification of strawberry will be required for RE-ACP protein crystallography studies. The activity of ACP extracted from *B. napus* seeds has been examined by Slabas *et al.* (1987) and the native protein was shown to have limited stability of biological activity during its purification. It has yet to be determined whether strawberry ACP expressed in *E.coli* will be of value in *in vitro* assays.

The anomalous migration of ACPs in SDS-PAGE appears to be due to their acidic character (Safford *et al.*, 1988) and because the acylation is known to alter their electrophoretic mobility (Slabas *et al.*, 1987). Most proteins bind SDS in a ratio of ~1.4g of SDS per gram of protein (Voet and Voet, 1990). Because of the high negative charge on ACP, the SDS-ACP complex may be unusually large due to the repulsive nature

between the SO_4 groups of SDS and ACP, or due to less molecules of SDS binding, resulting in lower gel mobility. The two less intensely staining polypeptide bands of 18 and 45kDa during the purification of the 20kDa polypeptide may represent truncated and dimeric forms of ACP, respectively. If codons, specified by the plant nucleotide sequences, are uncommon to the bacterial host premature termination may result releasing a smaller size product. Dimerisation of ACP may theoretically occur between the cysteine residues located at position 11 of the mature protein and at the end of the prothetic group (Fig. 3.13). SDS disrupts protein subunits linked by non-covalent bonds to give the true M_r of each protein (Voet and Voet, 1990). The presence of 2-mercaptoethanol in the sample buffer used for SDS-PAGE reduces the disulphide bonds. ACP dimers have been observed in the absence of reducing agents in the seed extracts of pisa (*Actinodaphne hookeri*) and ground nut (*Arachis hypogaea*) (Sreenivas and Sastry, 1995). Bienkiewicz and Woody (1997) found that an *E. coli* ACP dimer was converted to the monomeric form by reduction, oxidation, and removal of the prosthetic group. The "20-kDa" band associated with monomeric ACP depends on the specific conditions used in gel electrophoresis and could represent a dimeric ACP species that accounts for the low mobility. In spinach a single ACP isoform was resolved into two bands on SDS-PAGE due to conjugation between glutathione and the phosphopantetheine group via a disulfide bridge (Butt and Ohlrogge, 1991). This suggests ACP can be modified to produce multiple bands in SDS-PAGE. However, it is unknown whether the glutathione conjugation occurs in strawberry ACP.

To understand how ACP functions X-ray crystallography will be an important tool to study the 3-dimentional structure of this protein. A very useful feature of the strawberry mature ACP is the presence of methionine residues at positions 7 and 47. They can be replaced by selenomethionine to map the crystal structure. However, protein crystallography is outside the scope of this discussion.

To further characterise ACP in strawberry studies such as biochemical, immunolocalization, and organelle localisation will be of interest.

Chapter 5

Analysis of the General Expression of Strawberry ACPs

5.1 Introduction

Analysis of cDNAs from strawberry fruit has shown at least six ACP isoforms are expressed (see Chapter three). The roles of these multiple ACP isoforms in fruit metabolism in relation to plant fatty acid biosynthesis are unknown. The methods used to identify these ACPs do not indicate the relative expression levels of their corresponding genes in strawberry fruit. Because of the high homology shared between these ACP clones it is difficult to discriminate their individual expression.

Four independent studies on differentially expressed strawberry fruit ripening genes have been reported (Nam *et al.*, 1999; Manning, 1998; Medina-Escobar *et al.*, 1997; *Wilkinson et al.*, 1995). Of these studies, Manning (1998) and Nam (1999) have isolated ACP cDNA from *F. ananassa* and *F. vesca*, respectively, which were shown by northern analysis to be up-regulated during fruit ripening. ACP was also found to be expressed at very low levels in stem mRNA and did not increase in wounded leaf (Nam *et al.*, 1999).

The expression of ACP genes has been examined in diverse plant species including spinach (Hannapel and Ohlrogge, 1988), *Arabidopsis* (Baerson and Lamppa, 1993), coriander (Suh *et al.*, 1999) and *B. napus* (Safford *et al.*, 1988). *B. napus* shows considerable divergence between seed and leaf isoforms, as northern studies have demonstrated (Safford *et al.*, 1988). Safford *et al.* (1988) has isolated an ACP isoform from rape that was preferentially expressed in embryo tissue. Northern analysis has shown the isoform ACP-2 in *Arabidopsis*, or a closely related isoform cross-reacting with the probe (Hlousek-Radojcic *et al.*, 1992), to be expressed in leaf, root and seeds. However, western blots indicated that ACP-2 is a less abundant isoform in leaf tissue. Similar expression patterns for Cs-ACP-1 was found in coriander, where transcript levels of Cs-ACP-1 were highest in leaf (Suh *et al.*, 1999). Therefore, northern analysis can

indicate the overall expression patterns of closely related genes, but may not distinguish the expression of specific isoforms.

Northern analysis will be used to characterise the spatial and temporal expression of mRNAs from strawberry (*F. ananassa* c.v. Brighton) tissues employing a 550bp general probe derived from the ripening-enhanced ACP isoform. This probe is expected to bind to closely related ACP isoforms of the fruit.

The main aim of this chapter is to determine the overall expression of highly homologous ACP isoforms in different strawberry tissues.

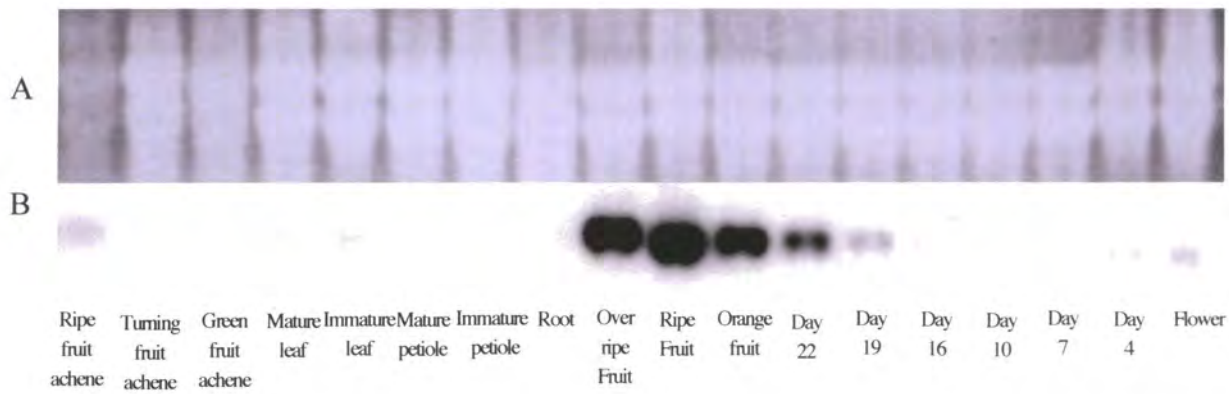

Figure 5.1. Northern analysis of RNA isolated from deachened fruit and other tissues. A. To determine equal loading of total RNA the blot was photographed under UV light. B. Blot probed with ^{32}P-labelled 550bp fragment from ripening-enhanced ACP. Fruit development was defined relative to days after anthesis, then by colour.

5.2 Results

Spatial and temporal expression of the ripening-enhanced ACP (and any cross-reacting isoforms) was examined in a northern blot of strawberry root, petiole, leaf, flower, achene and fruit at different stages of development (Fig. 5.1). ACP transcript was detected at very low levels in flower tissue and in fruit up to16 days after anthesis (DAA). At 19 DAA ACP expression increased in the fruit and continued to rise sharply reading a maximum in ripe fruit before declining slightly in overripe fruit. ACP expression was barely detectable in the achenes from ripe fruit and in young and mature leaf tissues. Little or no detectable ACP expression occurred in the petiole and root and in achenes from green and turning fruit.

5.3 Discussion

In this study the highest strawberry ACP mRNA expression level occurred during fruit ripening, maximising at the ripe fruit stage. Expression in tissues other than fruit was barely detectable. In other species such as *B. napus* an increase in ACP levels precedes an increase in fatty acid biosynthesis (Slabas *et al.*, 1988). Extrapolation to strawberry predicts that rate of flux through the fatty acid biosynthesis pathway increases in strawberry receptacle. As strawberry fruit tissues do not accumulate oils (Couture *et al.*, 1988), it is unknown why such an increase in ACP expression occurs in the ripening fruit and not in immature expanding tissues, such as leaf or fruit, where the requirement for fatty acid for membrane biosynthesis during growth is expected to be higher. Many seeds accumulate fatty acids but even the seeds (achenes) of strawberry have much lower ACP expression levels than the fleshy receptacle. Therefore, the predicted increase in fatty acid biosynthesis in strawberry fruit must be delivered to aspects specific to ripening. A major part of the fruit metabolism is coupled to flavour productions. During ripening, the fruit accumulates sugars and produces volatile compounds to attract animals to disperse the seeds. As levels of lipids do not increase significantly in strawberry one possibility is that fatty acids are used as precursors for the synthesis of volatile and anti-fungal compounds. Mango peroxisomal thiolase, the last step of the ß-oxidation pathway, is another fruit ripening-related gene involved in fatty acid metabolism (Bojorquez and Gómez-Lim, 1995). Changes in fatty acids have been associated with flavour and volatiles and mango thiolase may play a role in the aroma biosynthesis of this fruit (Bojorquez and Gómez-Lim, 1995). Another possible explanation for increased ACP expression in strawberry is that there are changes in membrane fatty acids during fruit ripening. In ripening banana fruits, for example, total lipid content remains constant but an increase in unsaturated fatty acids occurs in the phospholipid fraction (Wade and Bishop, 1978). As a result, an increase in membrane fluidity and in the passive permeability to small molecules may occur as the fruit ripens (Wade and Bishop, 1978). However, Couture *et al.* (1988) has reported a slight increase in saturated fatty acids between unripe and ripe strawberry fruit. Research into the fatty acid metabolism of

fruits is sparse, as apart from a few exceptions such as avocado, these organs do not accumulate lipids.

ACP expression levels have been analysed in both the commercial strawberry *F. ananassa* (Manning, 1998; Nam *et al.*, 1999) and the wild strawberry *F. vesca* (Nam *et al.*, 1999). In both studies ACP levels increase during fruit ripening. However, ACP levels of flower and different developmental stages of achene, leaf and petiole were not examined. Nam *et al.* (1999) used northern analysis to compare ACP expression levels in *F. ananassa* and *F. vesca* fruit tissues and showed that the highest hybridisation signals occurred at the turning stage and ripe stage, respectively. These authors found that ACP was also highly expressed in immature green fruit of *F. ananassa,* in contrast to the findings of Manning (1998) and the results of this study. The *F. vesca* cDNA has 96.6% similarity over 676 nucleotides to the ripening-enhanced clone isolated from *F. ananassa* (Chapter 3) and is expected to cross-hybridise strongly. The reason for this discrepancy may be that different cultivars were used that exhibit biochemical differences during fruit development. However, Nam *et al.* (1999) did not indicate which *F. ananassa* cultivar was used in their studies.

One method that can be used to distinguish transcripts from a closely related gene family is reverse transcription-PCR (RT-PCR), providing significant nucleotide differences exist. In this technique primers are designed to a region of the gene containing base substitutions unique to the isoform to be analysed. Total RNAs from green and ripe strawberry fruit could be used as a template in the RT-PCR to amplify each isoform of ACP examined in chapter 3. This method is theoretically capable of amplifying specific isoforms, and with adequate controls and internal standards, can be quantitative. However, The PCR will depend on the primers ability to distinguish between each clone. Alternatively, a technique such as Single Nucleotide Primer Extension (SNuPE) can enable transcripts differing by a single nucleotide to be quantitatively differentiated (Singer-Sam, 1994). The isolation of the RE-ACP gene promoter will allow the construction of a promoter-GUS gene. Once the gene is introduced into strawberry the tissue specific activity of the promoter can be monitored.

In summary, ACP expression in strawberry is found in most tissues but is highly up-regulated during fruit ripening. Other ACP isoforms isolated from *F. ananassa* are not sufficiently different to distinguish individual expression patterns by northern analysis. Therefore an alternative approach is required.

Chapter 6

Isolation and Sequence Analysis of ACP genes from Strawberry

6.1 Introduction

Six ACP cDNA clones were isolated from strawberry fruit (see chapter 3). One of these isoforms, known as the ripening-enhanced ACP (RE-ACP), was found to be the major form in ripening strawberry fruit, but the expression levels of its corresponding gene were low in all other tissues examined (see chapter 5). Therefore, the RE-ACP isoform is under temporal and spatial transcriptional controls. Regulation of gene expression is controlled at the transcriptional level by cis-regulatory sequence elements located in the 5' upstream proximal region (the promoter) and up to several thousand nucleotides upstream, downstream, or within the introns (the enhancer) of the respective gene (Latchman, 1995).

Genes encoding ACP have been isolated from plants such as *Arabidopsis* (Lamppa and Jacks, 1991) and *Brassica* (de Silva *et al.*, 1990), but not from fruit. Two ACP genes that were identified from *B. napus* were found to be homologous to cDNA isoforms that were isolated from the seeds. A GUS reporter construct fused with 1.4kb 5' flanking region of one of these genes (ACP05) was found to be preferably expressed in transgenic tobacco seed, which correlated with lipid biosynthesis in the seed (de Silva *et al.*, 1992). Comparisons of the *ACP05* gene promoter with the 5' flanking region of an *Arabidopsis* ACP gene had identified four sequence elements that were perfectly conserved, indicating the presence of regulatory sequences. For example, studies using the 1159bp fragment of the promoter from the ripening related ACC oxidase gene from apple was shown to confer both fruit and ripening specificity (Atkinson *et al.*, 1998). Although a 450bp region of this promoter was sufficient to drive gene expression in the fruit, it did not show ripening specificity. However, the -450bp to-1159bp region of the promoter contained elements that directed ripening-specific expression in transgenic tomato fruit. Sequence analysis of the 5' upstream regions from two ripening expressed genes,

tomloxA and *tomloxB*, did not reveal any categorised regulatory elements except for the TATA and CAAT boxes (Beaudoin and Rothstein, 1997). This and other evidence in the literature in plants suggests that at least 1kb of 5' upstream flanking region may be required to control gene expression temporally and spatially. With the expanding genome sequencing projects, including the nearly complete *Arabidopsis* genome, it may be possible to identify potential sequence motifs in promoters that are involved in the specific and in the general mechanism of gene transcription.

Promoters that direct temporal and spatial gene expression can be of use in antisense technology (Cannon *et al.*, 1990) to target the down-regulation of gene activity to a particular tissue, or at a specific stage of development, so as to reduce any adverse effects on the plant as a whole. To down-regulate the strawberry ripening-enhanced ACP isoform by antisense technology it may be necessary to use a fruit ripening and tissue specific promoter to avoid altering the expression of ACPs having similar sequences in other tissues.

To investigate ACP genes from strawberry, a genomic library was produced in lambda phage containing genomic DNA inserts of 7 to 20kb which can be propagated by lytic growth in a suitable bacterial host (Frischauf, 1991). *F. ananassa* contains 1.22pg DNA/nucleus (Nehra *et al.*, 1991) with a calculated length of 1177Mbp, assuming 1pg = 965Mbp (Arumuganathan and Earle, 1991). The octaploid strawberry genome is similar in size to that of the cherry (1370Mbp) and is 2-fold larger than the genome of raspberry (560Mbp), but 4-fold that of Arabidopsis (290Mbp) (Arumuganathan and Earle, 1991). The probability (P) of the presence of any given sequence in a library of N clones with an insert size of f (as a fraction of the genome) is given by the equation (Frischauf, 1991):

$$N=\ln (1-P)/\ln(1-f)$$

Therefore a genomic library of 3.6×10^5 clones from *F.ananassa* would be required for a 99% probability of isolating an insert with an average size of 15kb.

The genomic library will be screened with a 550bp probe derived from the RE-ACP cDNA. This probe is expected to hybridize to the RE-ACP genomic homologue and to genes having highly homologous sequences.

The main aims of this chapter are to isolate, sequence and identify genes encoding ACPs from strawberry, including their respective proximal 5' upstream regions (the promoter). The gene sequences will be compared to the strawberry ACP cDNAs previously described (see chapter three) and the promoter regions will be compared to other gene promoters in the databases, in order to identify potential transcription factor binding sites.

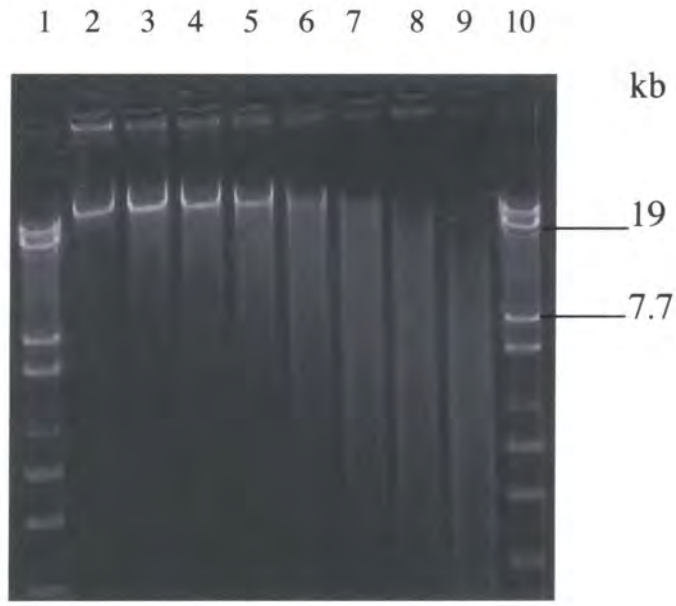

Figure 6.1. Fractionation of genomic DNA isolated from *Fragaria x ananassa* (c.v. Brighton) after partial digestion with MboI. Lanes 1 and 10 are λ/StyI markers. Lane 2 is undigested DNA. Lanes 3 to 9 were DNA digested with increasing concentrations of MboI from 0.044, 0.059, 0.079, 0.105, 0.141, 0.188 and 0.25 units/μg DNA respectively.

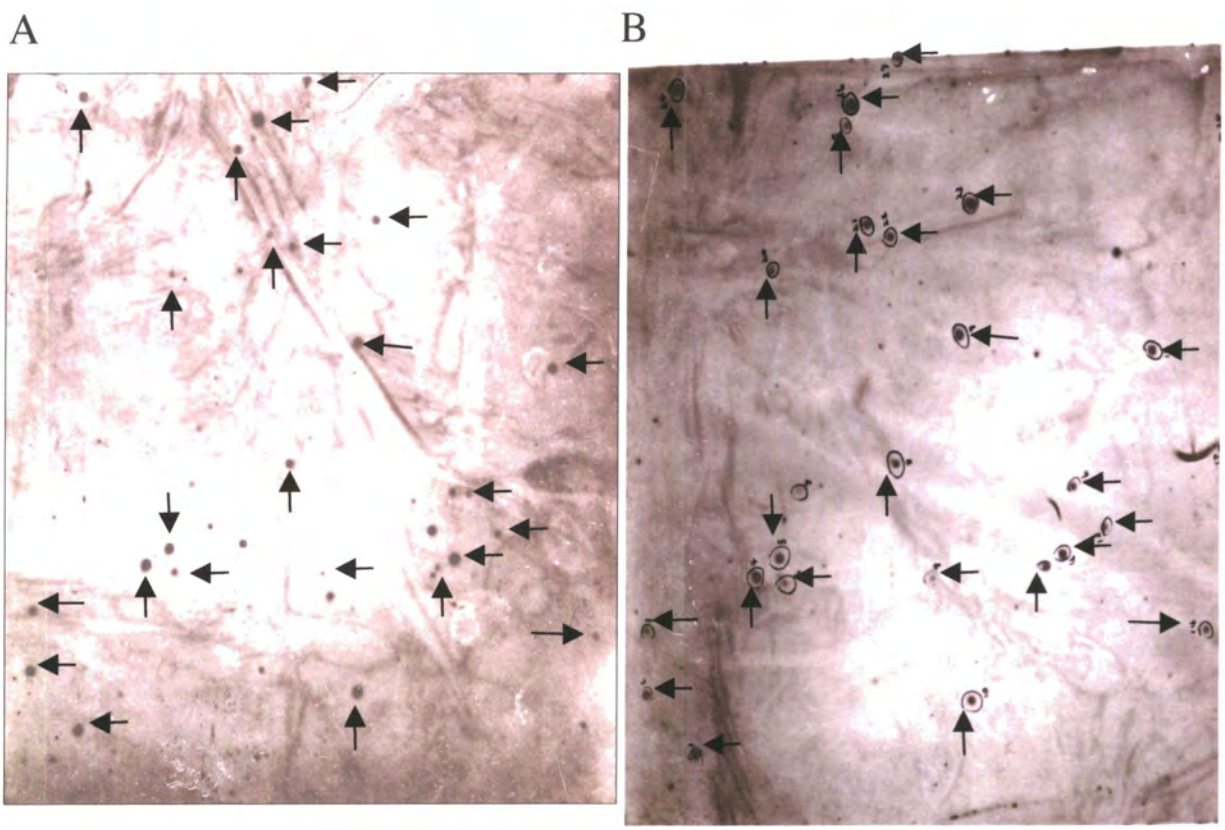

Figure 6.2. Primary screening of plaques from a strawberry genomic DNA library in λBlueStar. The arrows represent positive clones identified in duplicate blots **A** and **B**.

6.2 Results

6.2.1 Construction and screening of *F. ananassa* genomic library

Genomic DNA, isolated from immature strawberry leaves, was partially digested with Mbo I and analysed by agarose gel electrophoresis (Fig. 6.1). Restriction enzyme concentrations of between 0.141 and 0.188 units/μg DNA were optimum for DNA in the size range of 7kbp to 20kbp. A large-scale digestion was set up and DNA fractionating between approximately 7 to 20Kbp was pooled and fractionated, as before, and DNA extracted from the gel was used to construct a primary library of 5.2 $x10^5$ pfu in lambda BlueStar.

For the primary screen $5x10^5$ pfu were plated and probed with a 550 bp DIG-labelled DNA sequence from the RE-ACP cDNA clone. From the duplicate plaque lifts (Figs. 6.2 A and B) 24 hybridizing plaques were identified. Nine plaque pure clones (1, 3, 6, 7, 8, 9, 11, 19 and 24), obtained from a secondary screen, were *in vivo* excised into the pBlueStar plasmid vector for partial sequencing. Clone ACP-G24 was sequenced, including its 5' upstream region. All nine clones possessed homology to ACP sequences.

6.2.2 Sequence analysis of strawberry ACP genomic DNA

6.2.2.1 Sequence analysis of the ACP clone ACP-G24

The nucleotide sequence of the ACP-G24 gene contig is shown in Fig. 6.3. The gene is 1733 nucleotides, beginning from the base corresponding to the full-length ACP cDNA 5' region. The structure of the gene was determined by the comparison with the RE-ACP cDNA isoform, and from the two eukaryotic consensus left (5'-GU-3') and right (5'-AG-3') intron junctions (Lefebvre and Gellatly, 1997). The ACP gene from strawberry comprises three introns and four exons (corresponding to nucleotides 684-772, 1117-1227, 1557-1678 and 2081-2416). The sequence contains a predicted 659bp cDNA, identical in length to the other ACP cDNAs discussed in chapter 3. Intron I (344 nucleotides) interrupts the sequence coding for the transit peptide, at amino acid 21.

```
   1  TTTATATAGC GGAAATTGGT TGGAATATGA TTCATTCCAG TTTGTATGGG
  51  TAGTATGGTA TACATAGCTC AATAGAAGGC ATAGCCGGTT AGCTTCTTGA
 101  ATTAGAATAT GAGCACAAAA GTTAGCTGTT AGAGGGGTGA CCTGATAAAC
 151  TCGATCAGCA TAACATAATA TAGGCATGAC TATTGAGCAT AACATAAATC
 201  TATTTATGTG TATGGCTAAA ACTTGAAAAT AGAAAACCAT ATTATCACAT
 251  AAAGCACGTA CGTAAACCCT AATAAGTTAT ACAAAAAGTT GATTTGTATA
 301  AAATAGATTA GGTATGTATG TAACTTTTAA TCTGACATCA ATTACTCGTT
 351  ATTTCATCAT GTAAATTAGT CGGTTGACCA TAATAAAATT GTTTGATGTG
 401  AAATTTCAAT TTGAAAATGC GACTTAACTC ATTTCGTTCA GATTTTGAGT
 451  CGTGTTTATC TCTACTCCGA ATCCATATTC TATAACCTAG ACGCCCAAAC
 501  AATTTTTAAC CGGAGCAGTA ATTTTTCCGA TTTTACTTTG TTTCACTCAG
 551  ATGGTACACG TGTCGAATAG TGAGCTCCTC TTTGCCTGCA GTCAGTATCA
 601  GAAACCAAAC AGCCTACCTA TAAATACCAA CAAACCAACG ACCTCTCTAT
 651  TTCACTTGTC CTCGTTCTCG CCGTCTCTCT GTGTTTTAGA GCTCTCCTCG
 701  ATCGCATCAA TGGCCGCCAC CACAGGAGCT GCTTCTTCGA TCTCACTCGG
 751  CTCTCGCCTT CACCAGAATC TTgtaatccc atatcacttt gttttcattt
 801  tttctgggag attcttatgt ctaagtttga ttttagtttt atttaattgc
 851  ttgtagtttt ttttagtagt agtgagattt ggttgaatct aatattgatt
 901  tatcaattca tttttgggtt atattagttc tacttgtcat tgggttgatt
 951  ataagaatac tgttgaattg tacaagattt tggatccatt ttttcaatag
1001  tataatgtct atggagattt tttgctattt tgttattgat ttgagatatt
1051  ggattggacc ctgttgatgt gatatgattg atttgagtgt ttactgaagc
1101  agtcatggtt taacaggcat TGTCCAGGGT CAATGGTCTT AAGCTAGTTT
1151  CACTGTCTGG TAATGGAAGA AGTTCTCTTT CTTTCGGGTT ACAGCAGCGT
1201  TCAGCACGGC TTCAGATTTA CTGCGCGgta tgttttgatt tgttatgaag
1251  tgtttttttaa cactcatgtg tccgaaccat cagaaatagt ttcaatcttc
1301  aaagtttaga tcttgaactg gattgatggt aagttactac gttagtgaac
1351  tttctatgca gagaggtaca aaattatgta tttatgtcct gcacctggat
1401  tcatccaagt gactgcctga agtaagtaga agatagtaaa aagtaaaaat
1451  ccaaaacatc atcgatgatc atcatggttg aatcttgata gttgataatg
1501  ttacattgat gatcatttta tttgaatctt gatatcatta cctgtctttg
1551  agcagGCCAA ACCAGAGACA GTGGACAAGG TGTGCCAGAT AGTTAGAAAG
1601  CAACTTGCAT TACCAGATAA CTCGGCAGTT TCTGGAGAGT CAAAATTTTC
1651  TGCACTTGGA GCTGATTCTC TTGATACGgt acatttgatt ttgctatatg
1701  ctcttgttat attcctgttc cctgaattat ctgccttttc atgagcctgt
1751  tacctttttt tttcctttta ctgttcagta aagatctttt tttctgtctt
1801  tttcttgtgc tttgtctgca agcttgttga atcagattct ttcctctgat
1851  ggatttttta tctaccataa acagcagaat agtgtgtcta ggggttgaat
1901  agtgaaaagt aaactacaat ttgaataatg tccaatattt ttctcattaa
1951  ggacattctt tctatttaga acccttttggg agcaacagaa gtagaattgt
2001  attaagtttt gtccatcgat ctggcacttg ctaaatttat gcttgtgcat
2051  ttgaatttac ttaattgatg ccaatttcag GTTGAGATCG TGATGGGACT
2101  TGAGGAGGAA TTTGGTATTA GTGTGGAAGA GGAGAGTGCT CAGAGCATTG
2151  CAACCGTTCA GGATGCTGCG GATCTTATCG AGAAGCTCAT TGAGAAGAAC
2201  AAGGCTTAGA AGAAGAAATG AGAAAACAAG TGTCAATCCT AGCCTGCTTT
2251  AGATAATTAT TTGGTTGGTA GACTGGTTAT GTACGCAGTC ATTTTGTGTG
2301  AAATTTGAAC CTGATAGCGG TTGAGCGTTA AATTATGAAT GTATGGATTT
2351  GAGTTTGTGT GGTCTAGCTC CTTTCTTTCC TATATTTCTG ATGAAATACA
2401  GAATGGCCTT ACAATA
```

Figure 6.3. Nucleotide sequence of the ACP-G24 genomic clone from strawberry. The 5'upstream region of the gene is coloured green. The 5' and 3' UTR are in uppercase black and the intron sequences are lowercase black. The region coding for the transit, mature and the conserved phosphopantetheine recognition site are highlighted light blue, dark blue and red, respectively.

Intron II (328 nucleotides) occurs after the first codon (amino acid 58) of the mature peptide sequence. Intron III (402 nucleotides) interrupts the middle of the region coding for the conserved phosphopantetheine sequence (amino acid 99), three amino acids downstream from the serine residue that binds the phosphopantetheine group.

The intron/exon structure of the strawberry ACP gene is shared with other ACP genes from different species, such as *Arabidopsis A2* (Lamppa and Jacks, 1991), *B. napus BNACP05* (de Silva, 1990), *C. lanceolata Acl1;cl-g1* (Voetz *et al.*, 1994) and *B. campestris ACP-SF-2* (Das and Ghosh, 1995). The introns of the ACP genes from the above species vary in their respective length while the exon regions show less variation (Table 6.1).

Table 6.1. Nucleotide lengths of the introns and exons regions of ACP genes from different plant species.

Species	Exon I	Intron I	Exon II	Intron II	Exon III	Intron III	Exon IV
Strawberry	89	344	111	328	123	402	336
B. napus 05	48	270	108	76	123	402	336
C. lanceolata	60	490	108	164	123	77	129
A. thaliana A1	105	300	108	115	123	324	126
B. campestris	48	266	108	77	123	85	126

In all ACPs examined, the length of introns II and III and exons I and IV appear more variable than intron I and exons II and III.

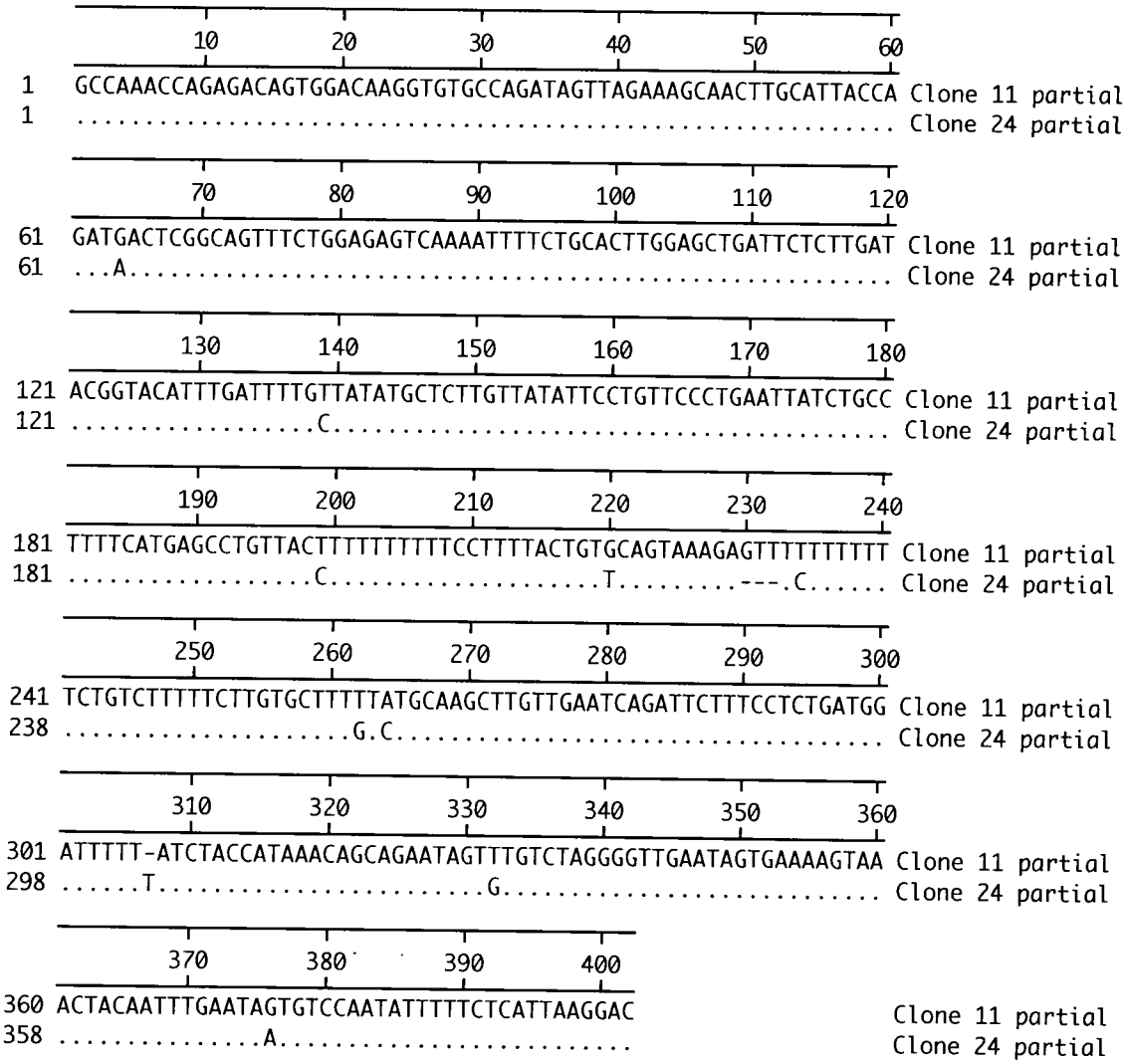

Figure 6.4. Comparison of the partial nucleotide sequences obtained from nine strawberry genomic clones encoding ACP. Dots (.) and hyhens (-) represent identical and missing bases, repectively. Only base differences are shown,

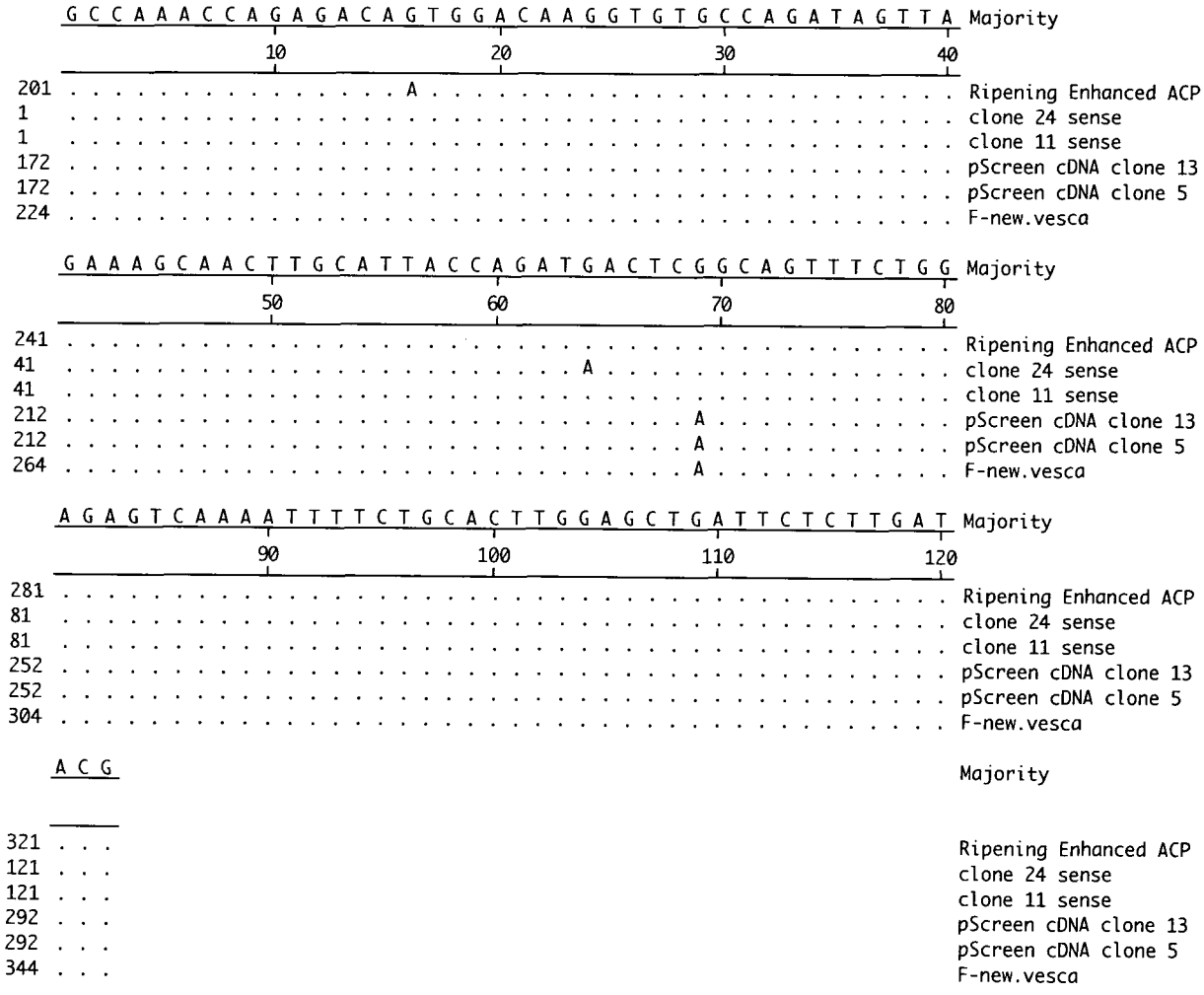

Figure 6.5. Comparison of the nucleotide sequences of the ACP gene exon region three with ACP cDNA clones isolated from strawberry. Dots (.) and hyphens (-) represent identical or missing bases, respectively. Only substituted bases are shown.

6.2.2.2 Sequence comparisons between genomic ACP clones

The partial sequences of the nine genomic clones isolated from strawberry show two isoforms over a 402bp region (Fig. 6.4). The partial sequences cover 123bp of exon 3 (Fig. 6.3) which exhibits one base substitution (G/A) in this region. A total of 10 base substitutions and three base deletions occur between the two isoforms. The unassigned bases (N) in clones ACP-G6 and ACP-G7 make definitive comparisons impossible (Not shown).

6.2.2.3 Sequence comparisons between ACP genomic and cDNA clones

Comparisons of the partial nucleotide sequences of the two genomic isoforms and the cDNA clones isolated from ripe strawberry fruit (chapter 3) indicate that neither gene is homologous to any of the cDNA clones including RE-ACP (Fig. 6.5). The ACP-G11 and ACP-G24 genomic clones each had respective homologies of 99.2% and 98.4% with cDNA clones 5/13 and the RE-ACP isoform. The partial sequence of ACP-G11 did not overlap the sequence of the ACP fragments obtained by CFLP therefore a definitive comparison was not possible. The putative cDNA sequence, derived from the full-length genomic clone ACP-G24, was compared to all strawberry ACP cDNA clones (Fig. 6.6). Nucleotide differences from ACP-G24 were found in all cDNA families (see chapter 3 for a description), but three differences were unique, between nucleotides 300 to 570, indicating that seven ACP isoforms have been identified. The partial nucleotide sequence homologies with ACP-G24 range between 97.4% for ACP cDNA 13 and 95.6% for the RE-ACP cDNA. ACP cDNAs identified from CFLP clones 16Wf, 19Wf and 2Rf had nucleotide homologies of 96%, 97% and 97.1% respectively to ACP-G24. Nucleotide substitutions in clone ACP-G24 that is common to the other ACP clones are discussed in chapter three.

6.2.2.4 Sequence comparison of translated ACP cDNA regions

The translated partial nucleotide sequences of ACP-G11 and ACP-G24 are 99.2% and 98.4% identical, respectively, to that of the ripening-enhanced isoform (Fig. 6.7). The

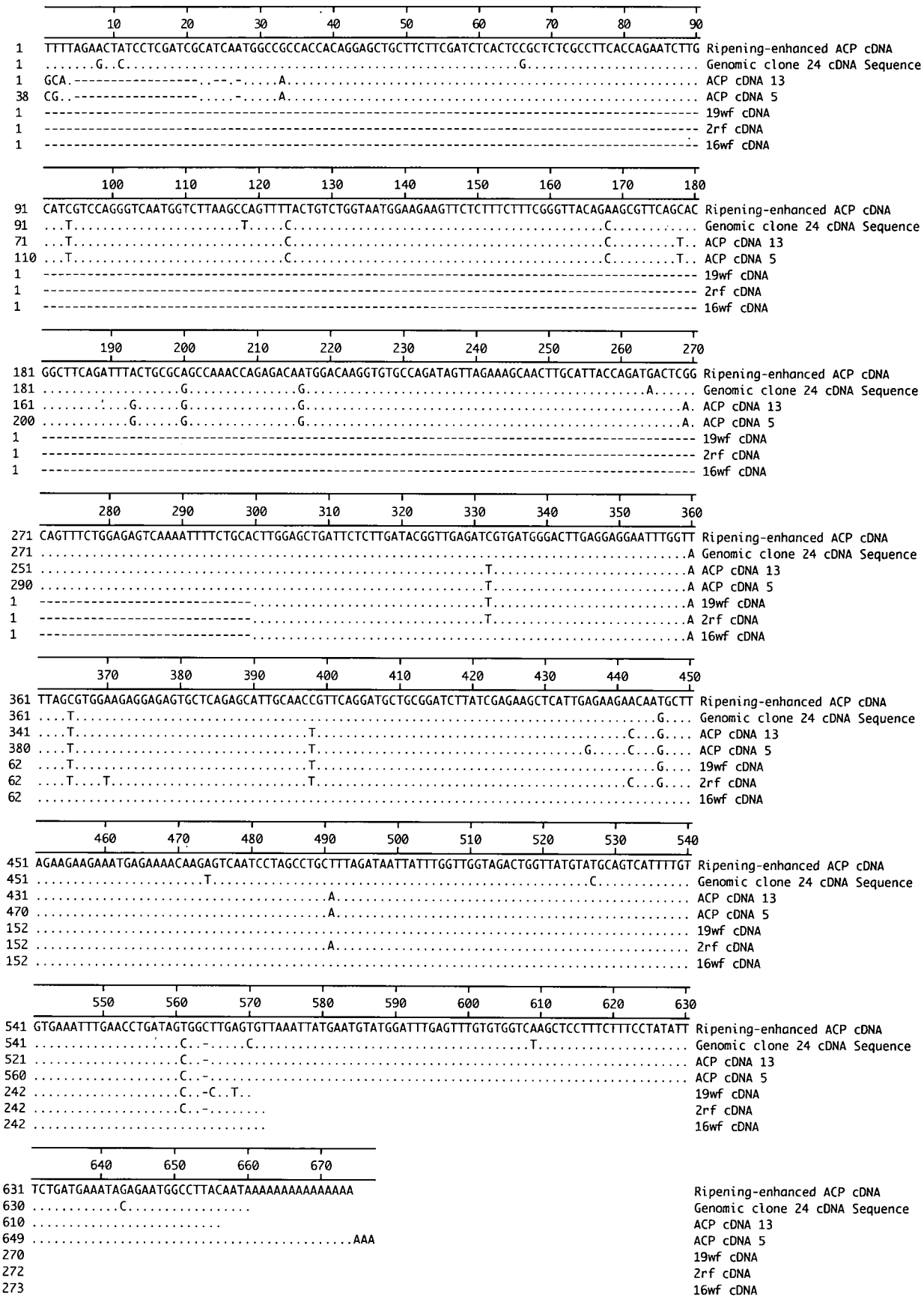

Figure 6.6. Comparison of the nucleotide sequences of strawberry ACP cDNAs with the predicted cDNA sequence from the genomic clone ACP-G24. Dots (.) and hyphens (-) represent homologous or missing bases. Only substituted bases are shown

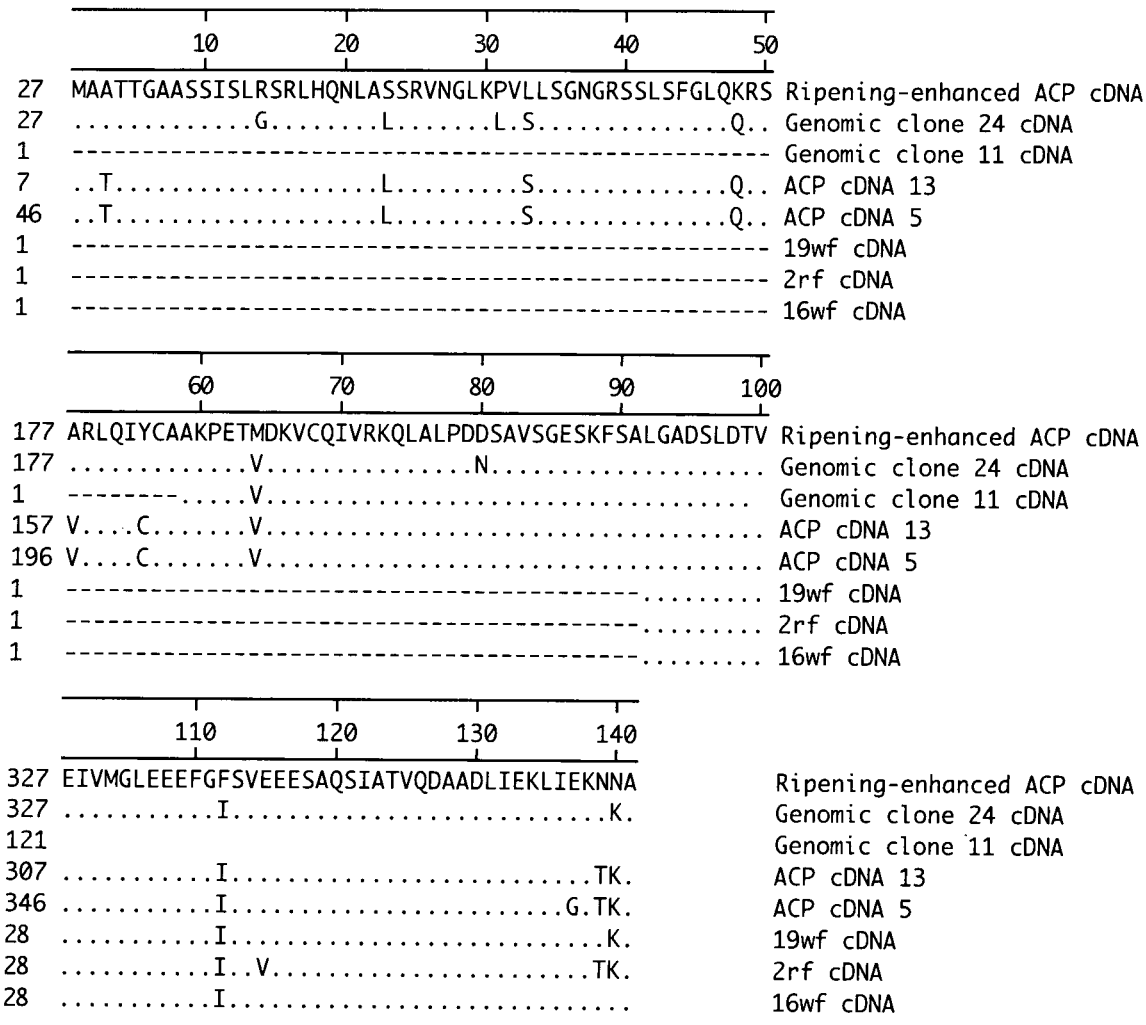

Figure 6.7. Comparison of the predicted amino acid sequences from the ORFs of the ACP clones isolated from strawberry fruit. Dots (.) and hyphens (-) represent homologous or missing bases. Only substituted bases are shown

polypeptide encoded byACP-G11 shares identity with the translated (partial) sequences of clones 5 and 13 but not that of the RE-ACP cDNA, which has a amino acid substitution (M /V) that contributes to its the hydrophobic character (Fig. 6.7). Again, the limited nucleotide sequence of clone ACP-G11 precludes definitive comparisons. The protein sequence encoded byACP-G24 has 141 amino acids and 99.8% and 97.4% identity to the sequences predicted from cDNAs 13 and RE-ACP clones, respectively. The ACP-G24 gene product has nine, eight and seven amino acid differences with respect to the translated sequences of RE-ACP and clones 5 and 13 cDNAs, respectively. Three unique amino acid substitutions can be identified in the protein encoded by genomic clone ACP-G24 (R/G, P/L, D/N) when compared with all the other ACP isoforms. The charges predicted from the transit and mature peptide fragments from ACP-G24 are 6.04 and -9.1, at pH 7.0, respectively, similar to the values from the other ACP cDNAs described in chapter 3. The ACP-G24 gene product has amino acid substitutions common to the predicted partial sequences from the CFLP cDNAs 19wf, 16wf and 2rf, described in chapter 3 (Fig. 6.7), with corresponding identities of 100%, 98% and 96.0%.

6.2.3 Promoter analysis of genomic clone ACP-G24

6.2.3.1 Identification of the transcription start site

The 5' upstream region of the ACP-G24 gene was sequenced with the 61/78 primer designed from the 5' region of the ripening-enhanced cDNA clone. The 5' upstream region of clone ACP-G24 (Fig. 6.3) was compared with promoter sequences in the databases and analysed for repeats and transcription factor (TF) binding sites using plant TF databases. The putative transcription start site was determined by comparison to the ACP A2 gene from *Arabidopsis* (Lamppa and Jacks, 1991). A sequence in the *Arabidopsis* A2 gene is similar to the strawberry sequence and **T** indicates the putative transcription start site:

5'-TTCAC**T**-GTGTCTC-3' Arabidopsis A2
5'-TTCAC**T**TGTC-CTC-3' Strawberry ACP-G24

However, these sequences were not present in other ACP genes including A1 of *Arabdopsis* (Lamppa and Jacks, 1991) and ACP05 to 09 of *B. napus* (de Silva *et al.*,

1990). The transcription start site of the strawberry gene indicates that the putative exon I has a length of 117bp, which is comparable to the corresponding region of the *B. napus* ACP 09 and ACP 05 genes which are 115nt and 117nt in length, respectively. A TATA box is located 30 nucleotides upstream of the putative transcription start site of ACP-G24, a feature that is conserved in eukaryotic promoter regions (Lefebvre and Gellatly, 1997).

6.2.3.2 Base composition

The base composition of the 5' upstream region of strawberry ACP-G24 gene was compared with other promoter sequences (Table 6.2). The genes used in the comparison were chosen by the following criteria: a) Apple and strawberry share the same family (Rosaceae), b) tomato lipoxygenase and apple ACC oxidase genes are ripening-related and c) the *B. napus* ACP05 (de Silva *et al.*, 1990) gene encodes an ACP that is tissue specifically expressed in seed.

Table 6.2. Base composition of plant promoter regions. Analysis was carried out using 696 nucleotides of each sequence.

Promoter	Species	Accession No.	Base composition of promoter region (%)			
			A	C	G	T
ACP-G24	Strawberry		31.6	19.7	16.4	32.3
ascorbate peroxidaseAPX26	Strawberry	AF158654	32.9	24.6	16.2	26.3
ACP05	*B. napus*	A26254	24.7	16.8	23.0	35.5
lipoxygenase *tomloxB*	Tomato	U63118	34.1	13.4	12.2	40.4
ACC oxidase	Apple	AF030859	32.6	16.8	14.7	35.9
RE-ACP cDNA	Strawberry		25.9	19.0	25.0	30.0

In general, all the promoter regions had a high A+T base percentage. The ACP-G24 gene promoter had >60% A+T residues a value that was similar for the apple and tomato gene

Strawberry *ACP-G24* gene promoter (0.696 kbp)

Direct Repeats:

Len	Sequence	Sites	
12	AGCATAACATAA	133	162

Short Dyads:

Len	Site	Sequence
11	511	ACTTTGTTTCA
11	476	CAATTTTTAAC

Palindromes:

Len	Site	Sequence
10	375	TGAAATTTCA

Inverted Repeats:

Stem	Loop	Site	End	Sequence	End
	Site				
11	100	361	5'	AAAATTGTTTG 3'	371
		482	3'	TTTTAACAAAC 5'	472

Strawberry *APX26* ascorbate peroxidase gene promoter (0.635kbp)

Direct Repeats:

Len	Sequence	Sites	
10	CTCTCCTCAC	588	614

Short Dyads:

Len	Site	Sequence
11	290	GAAAAGAAAAG

Inverted Repeats:

Stem	Loop	Site	End	Sequence	End
	Site				
10	238	36	5'	TTTCATTTTT 3'	45
		293	3'	AAAGTAAAAA 5'	284

Tomato lipoxygenase promoter (2.031kbp)

Direct Repeats:

Len	Sequence	Sites			
57	GTATAATTTTCTTCATTTTTTTAGACTCTTAATTATAATTT TGTCTCTTTATATTAT	8	118		
31	TTTAAAATTCTCTGACATTTACATTATGATG	74	184		
14	TTTTTTTTTTTTTT	1570	1571		
13	TAATGTAATTCTT	233	675		
13	TTTAATTTCATTT	1012	1680		
13	TTTTAAATTCTTT	244	692		
13	TTTTTTTTTTTTT	1570	1572		
12	AGTAATTACACT	566	1499		
12	ATTCTTTTAACT	1063	1898		
12	ATTTTTAATTTT	1154	1333		
12	GAATATATGTAA	106	216		
12	TAATTCTTTTAA	238	1896		
12	TATTCAATTTAT	62	1287		
12	TATTTTTAATTT	1008	1153		
12	TTTAATTTTTAT	326	1157		
12	TTTGTATTTTTA	650	1149		
12	TTTTAATTTTTA	855	1156		
12	TTTTTTTTTTTT	1570	1573		
14	TTTTTTTTTTTTTT	1571			
13	ATTTTTATTTTTA	1354			
13	TCTTTTCTTTTCT	1968			
13	TTTTGTGTGTTTT	1786			
13	TTTTTTTTTTTTT	1570			
13	TTTTTTTTTTTTT	1572			
12	TAAAAAAAAAAT	1985			
12	TTTACTTCATTT	786			
12	TTTTTTTTTTTT	1570			
12	TTTTTTTTTTTT	1573			

Short Dyads:

Len	Site	Sequence
18	1152	GTATTTTTAATTTTTATG
15	1570	TTTTTTTTTTTTTTT

Dyad Repeats:

Len	Site	Sequence	Site	Sequence
16	854	ATTTTAATTTTTAAAT TAAATTTTTAATTTTA	1330	
13	921	TTTTAAATATATT	1325	TTATATAAATTTT
12	1008	TATTTTTAATTT	1157	TTTAATTTTTAT
12	326	TTTAATTTTTAT	1008	TATTTTTAATTT
12	326	TTTAATTTTTAT	1153	TATTTTTAATTT
12	855	TTTTAATTTTTA	1154	ATTTTTAATTTT
12	1156	TTTTAATTTTTA	1333	ATTTTTAATTTT

Palindromes:

Len	Site	Sequence
16	871	AAAATGAATTCATTTT
14	1721	AAATTTATAAATTT
12	1235	AAACATATGTTT
12	924	TAAATATATTTA
12	1246	TATTAATTAATA

Inverted Repeats:

Stem	Loop	Site	End	Sequence	End
	Site				
12	36	1832	5'	GACAAATTTATA	3'
	1843				
		1891	3'	CTGTTTAAATAT	5'
	1880				
12	492	715	5'	TTATTATTATAA	3'
	726				
		1230	3'	AATAATAATATT	5'
	1219				

B.napus ACP05 gene promoter (1.0 kbp)

Direct Repeats:

Len	Sequence	Sites	
13	ATTTTAATTCGGC	518	713
11	TGATATGTTTA	132	792
10	GAAGGATAAA	16	320

Short Dyads:

Len	Site	Sequence
11	397	GTTTTTTTTTG
10	437	AGAAGGAAGA
10	818	TAGTAATGAT

Figure 6.8. Sequence repeats located within the 5' upstream region of various plant genomes.

sequences. The three introns of strawberry ACP-G24 comprise an average 68.8% A+T a value similar to that within of the introns of the *B. napus ACP 05* gene (de Silva, 1990).

6.2.3.3 Sequence repeats

The 5' flanking region of the ACP-G24 gene was analysed for sequence repeats that may have functional significance. The promoter region contained three direct, 15 dyad, four palindromes and 14 inverted repeats between 8bp and 12bp. Examples of these repeats are shown in Figure 6.8. In comparison, the ripening-enhanced cDNA clone had 26 repeats that were all dyad symmetry ranging from between 8bp and 13bp.

A comparison was made of the repeats in a similar length of promoter sequence of the genes chosen for the analysis of base composition. The promoter of the apple ACC oxidase gene had the largest number of repeats, followed by tomato lipoxygenase, strawberry ACP-G24, strawberry ascorbate peroxidase apx26 and *B. napus* ACP05 (Table 6.3).

Table 6.3. Sequence repeats identified in plant promoters.

Promoter	Direct repeats	Dyad repeats	Palindromes	Inverted repeats	Total No.
Strawberry ACP-G24	3	15	4	14	36
Strawberry ascorbate peroxidase APX26	8	18	2	3	31
B. napus ACP05	10	17	1	1	29
tomato lipoxygenase		38	8		46
apple ACC oxidase	12	22	5	10	49
RE-ACP cDNA		26			26

Most plant promoter elements contain all four types of repeat described above. The tomato lipoxygenase promoter differed in having dyad symmetry and palindromes. Further examination of the 2.031kbp tomato promoter revealed more repeat elements, including a direct repeat of 57bp in length (Fig. 6.8). Dyad repeats were the most

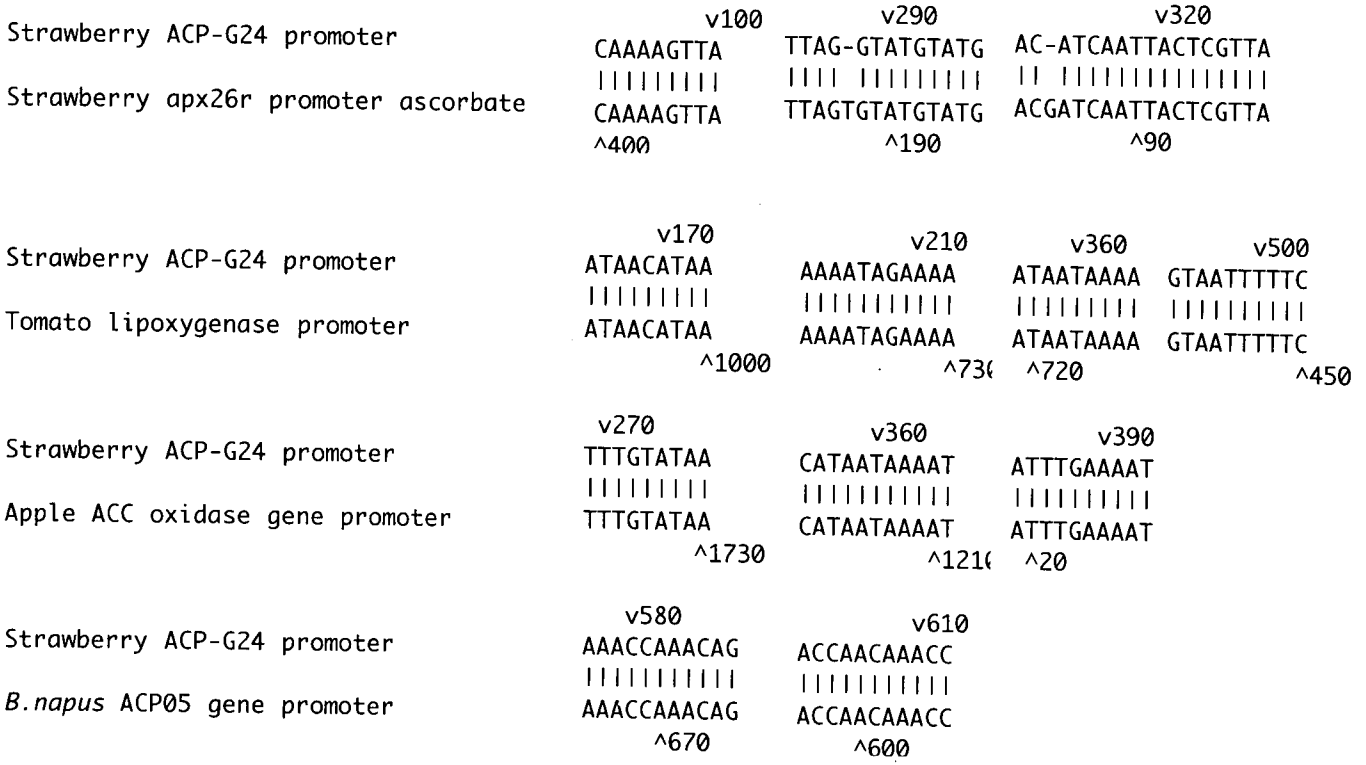

Figure 6.9. Sequence comparison of ACP 5' flanking regions. The –696 to –1 region of *F.ananassa* ACP-G24 gene is compared to other gene promoter sequences.

numerous feature of the promoters examined. To observe features that are unique to promoters a comparison was made to cDNA sequences. The RE-ACP cDNA clone contained dyads but none of the other types of repeat. An ACP cDNA sequence, isolated from *C.lanceolata* (data not shown), contained all of the above repeats in its 800bp sequence.

6.2.3.4 Promoter sequence alignments

To identify sequence elements that might be of functional significance in the 5' flanking regions a sequence alignment was carried out between ACP-G24 and the *B. napus* ACP05, the tomato tomloxB and the strawberry ascorbate peroxidase APX26 genes (Fig. 6.9). The promoter of ACP-G24 shares small regions of homology, from 9 to 17 nucleotides, with all of these promoters. Four, three and two sequence elements were common to the tomato, strawberry apx26/apple and *B. napus* promoters, respectively. The strawberry apx26 promoter elements shared the largest homologies of 13 and 17 bases. An element, identified at nucleotide 360, overlapped the ACP-G24, apple and tomato promoters and formed part of an inverted repeat with nucleotide 472 (Fig. 6.10).

6.2.3.5 Transcription factor binding sites analysis

The ACP-G24 gene promoter sequence was compared against databases of plant transcription factor binding sites. Three independent searches were performed using Matinspector (http://www.gsf.de/cgi-bin/matsearch.pl), NCBI BLASTN epd (http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=0) and the Plant Cis-acting Regulatory DNA Elements (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) or PLACE databases.

A large number of potential transcription binding factor motifs were identified with all three database searches in both 5'-3' (+) and 3'-5' (-) DNA strands. Table 6.4 shows 20 different potential sequence motifs in the 696bp 5' upstream region. Several of these are present in multiple copies.

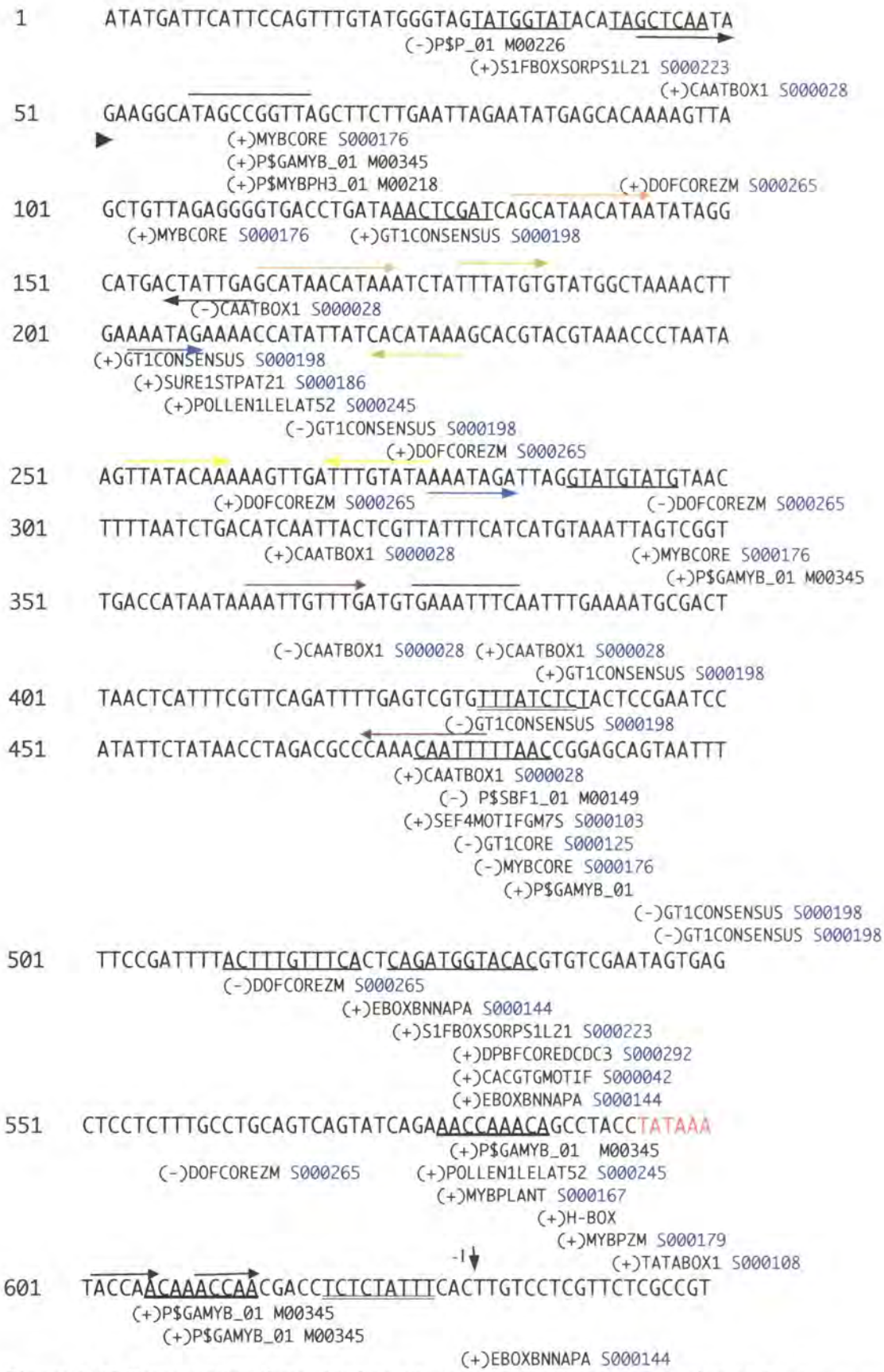

Figure 6.10 Transcription factor binding sites within the 5'-upstream region of the *F. ananassa* gene ACP-G24. Below the nucleotide sequence are potential transcription factor binding sites. Directional arrows above the sequence indicate inverted and direct repeats and are colour co-ordinated. An overhead line indicates an imperfect/perfect palindrome sequence. Sequences with dyad symmetry are underlined. The TATA-box is highlighted red and an arrow indicates the putative transcription start site. Accession numbers are highlighted blue.

Motif	Occurrence	Database description	Database(accession number/Ref.)
Ps ADH-1 DOF element	1	A 16 base homology sequence with alcohol dehydrogenase gene promoter from pea.	BLASTN EPD. (17009)
Hv α'amylase 2 TATA-box	1	A 14 base sequence homology with the promoter of α'amylase 2 from barley.	BLASTN EPD. (16018)
Ph chalcone synthase TATA-box/DOF element	2	A 16+13 base homology sequence to the chalcone synthase promoter of petunia.	BLASTN EPD. (35056)
MYBPH3	1	Binds product of petunia MYB.Ph3. Found in flavonoid Biosynthetic genes e.g. chalcone synthase.	Transfac (M00218)
TATA-BOX	1	An element which directs RNA Polymerase II to the correct transcription initiation site.	Plant Cis-acting Regulatory DNA Elements. (PLACE) (S000109)
MYBPLANT	1	Plant MYB transcription factor binding site which is related to the P box activation of phenylpropanoid biosynthesis genes e.g. chalcone synthase.	PLACE(S000167)
POLLEN1LELAT52	2	Element found in pollen specific promoter from tomato	PLACE(S000245)
EBOXBNNAPA	6	Element found in the promoter of the protein seed storage gene napA of *B.napus*.	PLACE(S000144)
CACGTMOTIF (G-BOX)	1	The G-BOX is bound by GBF (a bZip transcription factor) found in plant promoters including chalcone synthase.	PLACE (S000042) Transfac (M00182)
DPBFCOREDCD	1	A carrot embryo specific element.	PLACE(S000292)
S1FBOXSORPS1L21	2	A conserved element which is responsible for the regulation of spinach plastid ribosomal proteins S1 and L21	PLACE(S000223)
SEF4MOTIF GM7S	1	An element found in the promoter of b-conglycinin (a seed storage protein) from soybean.	PLACE(S000103)
CAAT BOX	6	An element responsible for controlling the frequency of transcription initiation.	PLACE(S000028)
GT1CONSENSUS	6	Consensus GT-1 binding site from many gene promoters e.g. RUBISCO and bean chalcone synthase.	PLACE(S000198)
SURE1STPAT21	1	Sucrose responsive element from potato.	PLACE(S000186)
DOF3, DOF2, DOF1 (DOFCOREZM)	6	DOF are single Zn finger DNA binding proteins identified in plants. Found in a diverse number of plant promoters.	PLACE (S000265). Transfac(M00352, M00353, M00354).
GAMYB	7	A MYB transcription factor binding element, which is expressed in cereal aleurone, cells in response to GA.	Transfac (M00345)
P-element (MYBPZM)	1	P-element is found in maize anthocyanin biosynthesis genes.	Transfac (M00226) PLACE (S000179)
SBF	1	A silencer element identified in the bean chalcone synthase promoter.	Transfac (M00149)
H-Box	1	An element found upstream of the TATA-box in the chalcone synthase gene	Arias *et al.* (1993)

Table 6.4. Potential transcription factor binding sites identified in the strawberry ACP-G24 gene promoter. The promoter sequence was screened against of three transcription factor binding site databases.

6.2.3.6 Common regulatory elements

The TATA-box was identified by the PLACE and BLASTN databases. BLASTN found homologies with the chalcone synthase and α-amylase genes, which are involved in flavonoid biosynthesis and the breakdown of starch, respectively. Another domain of the chalcone synthase promoter was found in the strawberry ACP 5' upstream region that also partially covered the pea ADH-1gene promoter sequence. Six CAAT box elements were found by the PLACE database search (Table 6.4, Fig. 6.10). CAAT boxes are believed to bind constitutively expressed transcription factors that allow gene transcription to occur (Latchman, 1995). Two of these CAAT elements were found in the genomic ACP-G24 clone sequence alignments with the strawberry apx26r and apple ACC oxidase gene promoters at nucleotides 319 and 389, respectively (Figs. 6.9 and 6.10). The CAAT-box sequences at nucleotides 46, 158, 364, 383 and 472 were found in or near inverted (5'- GCTCAATAG-'3) direct (5'-AGCATAACATAA-'3), inverted (5'-AAAATTGTTTG-'3), palindromic (5'-TGAAATTTCA-'3) or dyad (5'-CAATTTTTAAC-'3) repeats, respectively. Interestingly, CAAT boxes at 46/158 and 364/472 were also involved in inverted repeat base pairing (Fig. 6.10).

Tissue specific promoter sequences (Table 6.4) in the ACP-G24 genomic clone 5' upstream region could be separated into two groups containing the unrelated and related gene promoter elements.

Ps ADH-1 gene promoter

```
Query: 284 ttaggtatgtatgtaa  299
           ||||||||||||||||
Sbjct: 517 ttaggtatgtatgtaa  502
```

Ph chalcone synthase.

```
Query: 290 atgtatgtaacttttaa  306
           |||||||||||| ||||
Sbjct: 86  atgtatgtaactcttaa  102
```

Figure 6.11. BLASTN eukaryotic promoter database analyses of the 5' upstream region of strawberry ACP-G24.

Both alignments in Figure 6.11 have an overlapping sequence of 5'-ATGTATGT-3' which contains the direct repeat ATGT. The chalcone synthase alignment also contains the DOF core element AAAG (located on the 3' to 5' DNA strand), in which it has been

associated with a diverse number of plant promoters (Yanagisawa and Schmidt, 1999). DOF are plant transcription factors that possess a single Zinc finger motif. The TF database analysis identified six DOF elements, 5'-AAAG-'3, that exist on both the 5'-3' (+) and 3'-5' (-) DNA strands (Table 6.4; Fig. 6.10). Five of these DOF elements lie in or at, dyad (512, 300, 94) and inverted repeats (261, 229). The DOF element, at nucleotide 94, also occurs between both strawberry gene promoter alignments of ACP-G24 and Apx 26r (Fig. 6.9).

6.2.3.7 Gene promoter elements associated with unrelated functions.

A number of apparently unrelated promoter elements were identified in ACP-G24, using the TF binding site databases, including elements from a potato sucrose responsive (SURE1STPAT21), ubiquitously expressed (GT1CONSENSUS), a spinach ribosomal (S1FBOXSORPS1L21), a carrot embryo (DPBFCOREDCD) and a tomato pollen (POLLEN1LELAT52) gene promoters. These promoter elements are located at nucleotides 204 (SURE1STPAT21), 499, 498, 431, 389, 217, 201 and 120 (GT1CONSENSUS), 527 (S1FBOXSORPS1L21), 532 (DPBFCOREDCD) and 576 (POLLEN1LELAT52) (Table 6.4; Fig. 6.10). The alignments from the strawberry ACP 5' upstream gene sequence and the tomato lipoxygenase promoter region (Fig. 6.9), at nucleotides 204 and 498, coincide with the motifs SURE1STPAT21 and GT1CONSENSUS, respectively. However, the GT1CONSENSUS motif was also found in the promoter of apple ACC oxidase at nucleotide 389. Seven elements were located near direct (nucleotides 204 and 201) or dyad repeats (at nucleotides 576, 532, 527, 431 and 120) (Fig. 6.10).

6.2.3.8 Gene promoter elements having related functions

Three seed specific promoter motifs, GAMYB (nucleotides 612, 608, 583, 483, 345 and 59), SEF4MOTIF GM7S (nucleotide 476) and E-BOXBNNAPA (nucleotides 533 and 533) were identified in the ACP-G24 gene promoter region (Table 6.4; Fig. 6.10), with the GAMYB, at nucleotide 345, not occurring within a repeat element (Fig. 6.10). The remainder of the TF sequence motifs identify with promoters of genes concerned with

flavonoid biosynthesis. In particular, the H-box (nucleotide 589), SBF (nucleotide 478), G-box (nucleotide 530) and MYBPLANT (nucleotide 579) elements (Table 6.4; Fig. 6.10) occur in the chalcone synthase gene promoters of maize (Grotewold *et al.*, 1994), bean (Arias *et al.*, 1993) and petunia (Solano *et al.*, 1995). The P-element (nucleotide 589) is found within the A1 gene (dihydroflavonol 4-reductase) promoter of maize and is involved in anthocyanin biosynthesis (Bernhardt *et al.*, 1998). The MYBPH3 binding site (nucleotide 56), identified from petunia, is also found within the promoter of flavonoid biosynthetic genes (Solano *et al.*, 1995). The SBF, G-BOX (CACGTGMOTIF), MYBPLANT and MYBPH3 elements were found within repeat sequences, at nucleotides 478, 530, 579 and 56, respectively. The MYBPLANT element is also found between an alignment of ACP-G24 and *B. napus* ACP05gene promoters (Fig. 6.9).

6.2.3.9 Intron sequence analysis

Because TF binding elements contained in enhancer and silencer regions can exist within introns (Latchman, 1995) the three introns of strawberry ACP-G24 were analyzed using the Transfac database (Table 6.5).

Table 6.5. Transcription factor binding sites located in the intronic regions of the strawberry gene ACP-G24.

Motif	Occurrence		
	Intron I	Intron II	Intron III
P$DOF_01	1	4	11
P$GAMYB_01	4	4	4
P$MYBPH3_01	0	0	1
P$P_01	1	2	2
P$SBF1_01	0	1	0

The number of potential TF binding sites, in descending order, within intron III, II and I, are 18, 11 and 6 (for a description see Table 6.4). The DOF TF binding site is the most recurring sequence element within intron III, followed by introns II and I. All the intron sequences have approximately an equal number of the elements P$GAMYB_01 and

P$P_01. Transcription factor binding motifs P$SBF1_01 and P$MYBPH3_01 are unique to intron sequences II and III, respectively. A very small number of repeats were observed within introns I and III (not shown). Intron II contained six repeats (data not shown), however, only three transcription factor binding elements coincide with a repeat sequence.

6.3 Discussion

The results presented here relate to the identification and analysis of *Fragaria ananassa* ACP genes and their 5' flanking regions. To further understand the genetic regulation of ACP in strawberry, the isolation and sequence analysis of genomic ACP isoforms are investigated.

A transcript encoding ACP has been found to be up-regulated in strawberry fruit but the gene from which it has been transcribed have yet to be isolated. Although ACP is required as a cofactor in fatty acid biosynthesis, ripe strawberry fruit contain only slightly more lipids that unripe fruit (Couture *et al.*, 1988). The reason for the sudden increase of ACP transcription at the stage of fruit ripening is unknown. Currently, the induction of ACP in plants is observed to precede the onset of fatty acid biosynthesis, as observed in developing seeds from *B. napus* (Slabas *et al.*, 1987), and one possibility is that ACP may be required for lipid turnover in cell membranes during fruit maturation. ACP is synthesised in a developmental and tissue specific manner in strawberry fruit and it is of interest to identify genetic isoforms that are expressed during strawberry fruit ripening. In order to isolate the gene corresponding to the ripening-enhanced ACP cDNA, a genomic library was prepared and screened with a probe derived from the cDNA. The isolated genomic ACP clones were partially sequenced, with one individual isoform fully sequenced, including its 5' upstream region.

The primary *F. ananassa* genomic library was shown to be 5.2×10^5 pfu, which was 1.4 times the minimum theoretical titre required to isolate a particular genomic clone with a 99% certainty. Of the nine putative ACP genomic clones isolated two isoforms were identified but these had no homology to the ripening-enhanced form. The genomic clone ACP-G11 had 100% homology to the CFLP ACP clone 19wf, over a short length of sequence, and could therefore be an expressed gene. ACP-G24 was not homologous to any of the ACP cDNAs isolated from strawberry and represents a potential seventh form. However, the ACP-G24 gene contained perfectly preserved consensus intron/exon borders, and specifies a cDNA and protein that is consistent with other strawberry ACPs isolated to date. The intron/exon structure of this gene is comparable to ACPs identified

in other plants and it is likely that this gene is expressed. It is uncertain why only two genomic isoforms of strawberry ACP were isolated when at least six isoforms are expressed in the fruit alone. One possibility is that the amplification of the primary genomic library introduced bias against a genomic subset of clones effectively decreasing the likelihood of isolating the gene of interest.

Introns often divide the protein coding regions of a gene into well defined structural domains, with each domain encoded by an individual exon (Watson *et al.*, 1992). This mechanism is particularly evident in alternative splicing, where different exon domains can be 'shuffled' to produce a variation in the function of the protein e.g. immunoglobulin genes (Latchman, 1995). Interestingly, intron I interrupts the transit sequence. This indicates the transit peptide consists of two domains. The first domain may act as a binding precursor protein to receptors (Mullet, 1997) located on the plastid membrane. The second domain may act as a target sequence for soluble proteases, which cleave the transit peptide releasing the mature protein into the stroma. Intron II occurs after the first codon of the mature peptide, separating the transit and mature sequence domains. Intron III interrupts the conserved phosphopantetheine sequence of the mature peptide. Introns may act to interrupt coding regions to reduce the likelihood of a crossover event occurring within an exon during replication (Watson *et al.*, 1992).

The analysis of the 5' upstream region of the strawberry indicates the transcription start site of the ACP-G24 clone to be 28 bases longer that the cDNA transcripts isolated thus far. This places the potential TATA-box 32 nucleotides further upstream of the transcription start site, which falls near the excepted distance of 20-30 bp (Lefebvre and Gellatly, 1997). Promoter regions are 100-200 nucleotides in length, proximal to the transcription start site, but enhancer regions, also 100-200 bp in length, can be located thousands of bases away from the transcription start site (Latchman, 1995). The high percentage of A+T residues of 5' upstream region ACP-G24 is observed in other promoter sequences and may act to lower the DNA melting temperature to allow the formation of secondary structures. Secondary structures and their sequences could be recognized by protein factors for the initiation of transcription. This idea is supported by the fact that four, 8 to 11bp, inverted repeat elements were identified in the 5' upstream

region of ACP-G24, which could form loops from 2 to 362 nucleotides in length. Other repeat elements, including dyad, palindrome and direct, were found throughout the 5' upstream sequence, indicating the close proximity of an enhancer region. These repeat sequences coincide with putative transcription factor binding sites that may be capable of inducing gene transcription. Many transcription factor binding sites exhibit dyad symmetry, such as the estrogen response element, which may bind a dimeric protein form (Latchman, 1995) e.g.

5'-AGGTCANNNTGACCT-'3
3'-TCCAGTNNNACTGGA-'5.

B-DNA is regarded as the native form of DNA *in vivo* and 10 nucleotides represent a complete turn of the right-handed double helix (Voet and Voet, 1990). The direct and dyad repeats located within the ACP-G24 upstream region are placed in multiples of 10bp apart (Fig. 6.10) positioning them on the same face of the DNA helix. This could be important for the transcription factors to act in concert (Lefebvre and Gellatly, 1997). Further evidence of the presence of TF binding sites comes from the alignments of the 5' upstream regions of the ACP-G24 gene from strawberry with various genes from tomato, apple, strawberry and *B.napus*. All alignments coincide with a repeat element or/and a TF binding site. Together, this evidence strongly suggests the presence of regulatory sequences in ACP-G24. The ripening-enhanced cDNA sequence contains 26 repeat elements and 19 transcription factor binding sites (data not shown). However, only seven of these TF binding sites coincide with a repeat sequence, indicating that potential false positive TF binding site identification can occur. This is evidence that the identification of regulatory elements in any sequence should be treated cautiously.

The intron sequences within the ACP-G24 gene contain several potential TF binding sites. Only intron II contains both sequence repeats (11) and TF binding sites (11), with only three of these overlapping. It is unlikely that any of the introns contain enhancer elements assuming TF binding sites posses symmetry within their respective sequence. The enhancer regions of the apple ACC-oxidase (Atkinson *et al.*, 1998), *B. napus ACP05* (de Silva *et al.*, 1992), *Arabidopsis* ACP *Acl1.2* (Baerson *et al.*, 1994) and tomato

lipoxygenase (Beaudoin and Rothstein, 1997) genes are all located within 2.4kb, 1.4kb, 1.0kb and 2.4kb, of their proximal 5' upstream sequences, respectfully.

CAAT-box elements that are commonly found upstream of the TATA-box in a wide variety of genes, an exception being the *B.napus* ACP gene ACP05 (de Silva *et al.*, 1992). DOF and GT-1 TF binding elements, which are found in diverse gene promoters and plants (Yanagisawa and sheen, 1998; Zhou, 1999), are located within the 5' flanking region of ACP-G24,

Other putative TF promoter elements in ACP-G24 include SURE1STPAT21 that is found in the sucrose responsive promoter of the potato patatin gene that encodes a major tuber protein (Grierson *et al.*, 1994). Sucrose levels in strawberry fruit are low in the first ten days after anthesis then rapidly increases toward the turning stage, as does ACP. Increasing levels of soluble sugars stimulate the formation of secondary metabolites, including anthocyanins (Zabetakis and Holden, 1997) and generally modulate gene expression (Kock, 1996) through signalling mechanisms (Lalonde *et al.*, 1999). Therefore, sucrose may act to stimulate ACP transcription. The S1FBOXSORPS1L21 element is conserved in the promoter region of many plastid-related genes, including rbcS and ribosomal protein L21 acting both constitutively and tissue specifically, negatively regulating gene expression (Zhou *et al.*, 1992). As ACP is a plastid-related gene, S1FBOXSORPS1L21 may control ACP expression according to plastid development. The elements DPBFCOREDCD and POLLEN1LELAT52 control embryo and pollen specific expression in carrot (Kim *et al.*, 1997) and tomato (Bate and Twell, 1998), respectively.

The promoter elements GAMYB (Gubler *et al.*, 1999), SEF4MOTIF GM7S (Lessard *et al.*, 1991) and E-BOXBNNAPA (Stalberg *et al.*, 1996), identified in genes expressed in seeds, lie within the 5' upstream region of the ACP-G24 gene, indicating that this gene may be expressed within the strawberry achene. The SEF4MOTIF GM7S has also been reported in the promoter region of *B. napus ACP05* (de Silva *et al.*, 1992). Northern analysis using a closely related ACP cDNA probe (see chapter 5) did not show detectable levels of ACP expression in seeds or flower tissues of strawberry. ACP expression is

expected to occur in all tissues, but basal levels may be too low for its detection. GAMYB is a MYB transcription factor that is expressed in cereal aleurone cells, in response to gibberellin (GA) (Gubler *et al.*, 1999). However, exogenous GA applied to strawberry fruit delayed ripening events, such as colour development (Martinez *et al.*, 1996), but a combination of GA and 1-NAA promoted growth and ripening (Manning, 1993). It is uncertain what effect GA would have on ACP levels in strawberry although treatment that accelerate or inhibit ripening correspondingly induce or suppress the expression of ripening enhanced genes including ACP (Manning, 1998).

Other TF elements, putatively identified within the strawberry ACP-G24 gene promoter, include: H-box, SBF, G-box, MYBPLANT, MYBPZM and MYB.PH3, all of which are found in gene promoters concerned with flavonoid biosynthesis. The H-box and G-box elements are found in the chalcone synthase gene of bean (Arias *et al.*, 1993). Chalcone synthase is a key enzyme in the formation of flavonoids including anthocyanins (Ellis, 1997). A putative chalcone synthase has been isolated from strawberry (*F. ananassa*) and is upregulated during fruit ripening (Manning, 1998; Wilkinson *et al.*, 1995). MYBPLANT, MYBPZM and MYB.PH3 transcription factors are implicated in the regulation of flavonoid biosynthetic genes within floral tissues of snapdragon (Sablowski *et al.*, 1994), maize (Grotewold *et al.*, 1994) and *petunia* (Solano *et al.*, 1995), respectively. The SBF element is found within the promoter of the bean defence gene CHS15. SBF acts as a transcriptional silencer in electroporated soybean cells protoplasts (Lawton *et al.*, 1991). Together this information suggests that the flavonoid promoter elements found in the 5' upstream region of the ACP-G24 gene could regulate ACP expression in relation to the expression of other genes involved in phenylpropanoid metabolism and in particular anthocyanin biosynthesis. A fruit-specific putative dihydrophenol 4-reductase gene is up-regulated during commercial strawberry ripening and is thought to be involved in anthocyanin biosynthesis (Moyano *et al*, 1998). The co-ordination of ACP expression with anthocyanin appears to be an integral part of strawberry fruit ripening.

The number of ACP isoforms in plants may reflect the need to control ACP levels within specific tissues or cells in response to varying demands for lipid biosynthesis, such as

may occur in organogenesis, in which the expression of the *Arabidopsis* Acl1.2 ACP gene is resulted (Baerson *et al.*, 1994). However, except for the ripening enhanced form the expression patterns of ACP isoforms in strawberry tissues are unknown.

The information presented here indicates the difficulty in isolating the exact genomic homologues from strawberry, especially when dealing with closely related members of a likely multigene family. Library amplification may also distort the proportions of each recombinant clone (Frischauf, 1991). Therefore, primary libraries are the preferred method for screening purposes. Promoter analysis indicates the presence of regulatory sequences in the 5' upstream region ACP-G24 gene. However, analysis of cDNA sequences has shown that TF-like binding sites are not exclusively found within promoter regions. The proportion of A+T residues and the distribution of repeat elements and transcription factor binding sites together may define important motifs within a promoter. Further analysis of other ripening related genes from strawberry should reveal important domains necessary for gene expression, but ultimately, deletion analysis will be required to precisely define the regulatory elements.

Chapter 7

Final Conclusion and Future Studies

7.1 Final Conclusion

This study describes the isolation and partial characterization of ACP cDNA and genomic clones from strawberry. It was prompted by the first report linking enhanced ACP expression with fruit ripening (Manning, 1998). In the past there have been few studies on lipid metabolism in fleshy fruits, including strawberry, as there was little reason to investigate an area of biochemistry seemingly unrelated to fruit ripening.

It has long been established that ACP is important in the synthesis of fatty acids within the cells of both animals and plants. Fatty acids are important storage compounds, they provide energy for growth, and are integral membrane components (Ohlrogge and Browse, 1995). Although a few fruits, such as avocado, are rich in lipids it was generally assumed that lipid metabolism in these organs was largely concerned with 'house-keeping' functions. More recently, attention has focused on the role of lipids in maintaining membrane integrity, particularly in the later stages of fruit ripening characterised by cellular disorganisation.

Lipids are very diverse and include phospholipids and glycolipids, triacylglycerols, sterols, pigments, and waxes. The most studied lipids are probably the glycolipids and the phospholipids as these are essential components in the structure of all biological membranes; the glycolipids are particularly important in the thylakoid membranes of the chloroplast (Murphy, 1993). During the development of some fruits, such as tomato, chloroplasts retain their integrity but become chromoplasts in which lipophilic pigments accumulate. In strawberry fruits the chloroplasts lose their integrity and become disorganised during ripening. It is not known if lipid-derived pigments in fruits are associated with ACP as a cofactor. Sterols are, however, known to be esterified to fatty acids (Post-Beittenmiller, 1996). Although sterols are found in fruits their presence in strawberries has not been reported. Changes that occur in the fatty acids and sterol

moieties of tomato during normal ripening are absent in mutants with impaired ripening (Post-Beittenmiller, 1996). It has been suggested that acyl sterols may provide cells with sterols or fatty acids for maturation, ripening and senescence, or that they may affect membrane fluidity (Izzo *et al.*, 1995 and Post-Beittenmiller, 1996). Sterols may have a role in membrane stability during ripening and senescence (Izzo *et al.*, 1995). One hypothesis suggests that changes in the cell membrane during ripening increases their fluidity resulting in decompartmentalisation of cellular components. This would lead to an increase in catabolic processes and accelerate fruit senescence (Izzo *et al.*, 1995). The phase properties and composition of membrane may critically determine enzyme activity, transport capacity and their permeability to metabolites.

Waxes have not been described in strawberry but they are known to be produced from fatty acids, the role of ACP being to provide C16 and C18 fatty acids (Post-Beittenmiller, 1996). Elongases are responsible for the further elongation of acyl-CoAs, but ACP isoforms may have a role in directing particular fatty acids to a particular metabolic pathway (Ohlrogge *et al.*, 1986 and 1991).

Fatty acids, such as linoleic acid, have been reported to activate the 20s proteosome from spinach leaves (Watanabe and Yamada, 1996), which may allow the translated proteins of particular mRNA species to be regulated. Fatty acids may act as physiological regulators in the cell controlling the levels of translated products of mRNA involved in ripening related biochemical pathways. Interestingly, a ripening-enhanced strawberry cDNA reported by Manning (1998) encodes Elongation factor 2, an essential component of eukaryotic translation systems.

Mostly neutral lipids are present in strawberry achene with a relatively high proportion of C18: 3 (35.0%) and C18: 2 (46.5%), and lower levels of C18: 1 (14.5%), fatty acid moieties (Kundu *et al.*, 1973). In comparison, the ripe fleshy receptacle contains more C18: 1 (22%) and less C18: 3 (29.6%) and C18: 2 (38.9%) fatty acids (Couture *et al.*, 1988) than the achenes. The distribution of these fatty acids within the tissues of the receptacle i.e. cortex and epidermis has not been determined. Thus, achene lipids have a higher degree of desaturation than receptacle lipids. In strawberry, lipids may be

exported from the receptacle or synthesised in the achenes themselves. Seed protoplasts in other species possess the enzymes necessary for synthesising their own lipid (Schmid *et al.*, 1997). A thioesterase, isolated from *B. napus*, has a high preference for hydrolysing C18: 1-ACP, consistent with the fact that C18: 1 (oleate) is the major fatty acid in rape oil (Loader *et al.*, 1993). Therefore it seems likely that the strawberry achene contains all the necessary enzymes for fatty acid synthesis. Achenes are known to actively synthesise other compounds, such as the hormone indole-3-acetic acid which regulates receptacle development. In ripening strawberry methyl jasmonate stimulates respiration and ethylene production *in vitro* and accelerates anthocyanin biosynthesis (Perez *et al.*, 1997). Unsaturated fatty acids may be required for the achenes to become fertile upon maturation. For example, *Arabidopsis* mutants lacking trienoic acids produced non-viable pollen and were male sterile (McConn and Browse, 1996). Linolenic acid is a substrate for the octadecanoid pathway involved in the synthesis of signal molecules, such as jasmonic acid. Treatment of the unopened flower buds with jasmonic acid produced mature siliques (a dry dehiscent fruit) with over 30 seeds. Jasmonic acid may act as a mediator of seed fertility (McConn and Browse, 1996) but also stimulates ethylene in ripening apple and tomato fruits (Sanieswky *et al.*, 1987).

Because strawberry ACP expression coincides with ripening events, it has been hypothesised that lipid biosynthesis may have a role in aroma formation. This is because ADH, SAAT, LOX and HPL (see section 1.2.2.3) are also up-regulated during maturation of strawberry fruit, which strongly suggests that these enzymes are acting in concert to generate aromas in the fruit.

The role of different ACP isoforms has not been established in strawberry, but it is conspicuous that ACP has not been identified as a ripening enhanced gene in other fruits. This may be related to the relatively high levels and complexity of aroma compounds formed in this fruit. The number of ACP isoforms may reflect the need for rapid fatty acid biosynthesis during ripening. Although several different ACP genes are expressed in the developing fruit only one ACP cDNA isoform predominates in the ripe fruit.

Six isoforms of ACP are expressed in strawberry fruit but only one of these (RE-ACP) has been determined to be up-regulated during ripening. This RE-ACP cDNA has been repeatedly isolated from ripe fruit using different techniques indicating it is the dominant form expressed during ripening. As multiple ACP isoforms have been isolated from other species, it is likely that other ACPs remain to be discovered in strawberry. The RE-ACP appears to be localised in the plastid and has structural features consistent with other ACPs examined. Two genomic ACP clones have been isolated; one of which represents a potential seventh form of ACP. This genomic clone contains four exons and three introns, resembling ACP genes in other species. However, it is not known if this gene is expressed. The genomic clone ACP-G24 has promoter elements common to those found in promoters that regulate genes in a temporal and spatial manner, such as those related to anthocyanin biosynthesis, that suggests that this gene may be ripening related. A recombinant protein encoded by RE-ACP was expressed in *E. coli* and partially purified following extraction by the freeze-thaw technique. The M_r of the mature protein estimated by SDS-PAGE was more than twice the predicted value, which is consistent with that observed for ACPs from other species.

In the context of other biochemical and molecular changes that occur during the ripening of strawberry fruit, it is likely that RE-ACP has a role in the biogenesis of volatile aroma compounds that originate via the lipid biosynthesis pathway.

7.2. Future studies

Although six ACP isoforms were isolated in the strawberry fruit it is not known if they are all fruit specific. Two genomic ACP isoforms were identified that were not related to the six cDNA clones isolated. Therefore, identification of all ACP isoforms will be of interest in order to determine the extent of this gene family in strawberry and the relationship of the forms expressed in fruit to those in other parts of the plant. A primary library is preferred for screening candidate clones to minimise bias. The ripening-enhanced ACP gene promoter is yet to be isolated and characterised. Promoter-reporter gene constructs will be important in characterising the RE-ACP gene in transgenic plants. Promoter deletion analysis will identify the regions of the promoter elements that control

temporal and spatial expression. An obvious experiment is to down-regulate the ripening-enhanced ACP to investigate its effects on lipid composition and aroma production in the fruit. It may not be possible to constitutively down-regulate ACP because the high sequence similarities of this gene family may prove to be lethal. Inhibitors of fatty acid biosynthesis are known to be lethal to the cell (Ohlrogge and Browse, 1995). To avoid detrimental effects of an ACP transgene in non-fruit tissues a fruit-specific promoter is required. A number of candidate genes for isolating fruit-specific promoters can be identified (Manning, 1998) from published data.

To examine the hypothesis that the function of ACP is providing fatty acid precursors for aroma biosynthesis, via the lipoxygenase pathway, labelling experiments will be a valuable analytical tool. Procedures to determine the relative concentrations of the major acyl-ACP intermediates *in vivo* have been developed (Post-Beittenmiller *et al*, 1991). Application of these techniques could be used to determine the role the strawberry ripening-enhanced ACP isoform has in aroma development. The formation of aldehydes and alcohols from labelled fatty acids has been described in tomato (Stone *et al*, 1975). Radiolabeled precursors, ^{14}C-linolenic and ^{14}C-linoleic acids, were injected into the stem of fully ripened fruit and incorporation of label analysed after a 24hr period. Tomatoes injected with ^{14}C-linolenic acid principally formed labelled cis-3-hexenal, whereas the label from ^{14}C-linoleic acid appeared in n-hexanal. Similarly, the production of furaneol was studied in *in vitro* developed strawberry fruit (Perez *et al*, 1999). This work showed that the addition of D-fructose in the incubation medium gave rise to an increase in furaneol, and its glucoside derivatives, with respect to the control fruits. This experiment may be duplicated with the addition of labelled fatty acid precursors, such as ^{14}C-malonate, with carboxylic acids, such as pyruvate, to examine the effects on aroma development. A biosynthetic pathway for the generation of esters in strawberry was also identified using plant tissue cultures (Zabetakis and Holden, 1997). Pulse-chase labelling experiments may enable the binding and release of label to and from the ACP to be determined. This is an important step in correlating fatty acid biosynthesis with the generation of aroma in strawberry. Different stages of fruit development could be examined in order to relate label distribution to volatile production.

In spinach leaf and seed tissue up to 60% of the ACP is non-esterified indicating a predominance of the free form (Post-Beittenmiller *et al*, 1991). Unbound ACP was also observed in *E. coli* (Rock and Jackowski, 1982). This suggests that ACP levels in plants are in excess to that required for fatty acid biosynthesis and a partial reduction of the total ACP concentration, through antisense technology, will not necessarily lead to complete inhibition of fatty acid biosynthesis. However, Rock and Jackowski (1982) observed that an inhibition of phospholipid synthesis at the acyltransferase step increased the content of its acyl-ACP substrate in the ACP pool. Therefore, it would be of interest to inhibit the lipoxygenase pathway in the strawberry fruit at the genetic level to test the effects this pathway has on its acyl-ACP substrate levels in the ACP pool. Modulating the lipoxygenase pathway by down regulating LOX, or HPL, in the fruit may provide additional information on how levels of fatty acids are regulated during ripening. It is anticipated that down regulation of LOX, or HPL, would cause the build up of excess fatty acids in the fruit, or divert them into other pathways. Alternatively, a feedback mechanism may exist that may inhibit fatty acid biosynthesis as the demand for fatty acids is reduced, and co-ordinately alter the levels of ACP transcription/translation as a result. These results may help to establish which acyl-ACP species are the acyl donors for aroma synthesis *in vivo* and correlate this to the development of the volatiles formed.

Expression of ACP determined from northern studies has not been conclusive. The high similarity that exists between these clones requires more specific methods to distinguish their expression profiles. Alignment of the cDNA sequences suggest that it may be possible to design specific primers for each isoform.

The function of a gene cannot be determined by the DNA sequence itself, even if similar genes are identified in the databases. If expressed recombinant ACP (discussed in chapter 4) can be crystallised its tertiary structure can be determined and this may provide important clues to its function. Antibodies to the recombinant strawberry RE-ACP have been raised (Manning, personal communication) and these will be useful in a number of ways. Firstly, western analysis using these antibodies will enable the abundance of ACP in fruit and other tissues to be correlated with RE-ACP gene expression. It is anticipated that the antibodies will not distinguish specific isoforms of ACP and similarly northern

analysis will only give an estimate of total levels. Similarly, the antibodies may enable the localisation of ACP within fruit tissues, e.g. cortex and epidermis to be determined by tissue blots for example. Antibodies to ACP will also be useful for monitoring ACP level in transgenic plants.

It would be of interest to determine if the recombinant ACP has any influence on the length of fatty acids formed in cell free fatty acid synthase reactions. Such studies may provide important evidence for the function of this protein during ripening.

Finally, it would be of interest to determine if ACP homologues are up-regulated in other ripening fruits, such as raspberry, tomato and banana particularly in relation to their volatile aroma.

References

Aharoni, A., Keizer, L.C., Bouwmeester, H.J., Sun, Z., Alvarez-Huerta, M., Verhoeven, H.A., Blaas, J., van Houwelingen, A.M., De Vos, R.C., van Der Voet, H., Jansen, R.C., Guis, M., Mol, J., Davis, R.W., Schena, M., van Tunen, A.J. and O'Connell, A.P. (2000). Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. *Plant Cell* **12**(5): 647-62.

Arias, J.A., Dixon, R.A. and Lamb, C.J. (1993). Dissection of the functional architecture of a plant defense gene promoter using a homologous in vitro transcription initiation system. Plant Cell, **5**(4): 485-96

Arumuganathan, K. and Earle, E.D. (1991). Nuclear content of some important plant species. *Plant Molecular Biology Reporter*, **9**(3): 208-219

Atkinson, R.G., Bolitho, K.M., Wright, M.A., Iturriagagoitia-Bueno, T., Reid, S.J., and Ross, G.S. (1998). Apple ACC-oxidase and polygalacturonase: ripening-specific gene expression and promoter analysis in transgenic tomato. *Plant Molecular Biology*, **38**(3): 449-460.

Atta-Aly, M.A., Brecht, J.K. and Huber, D.J. (2000). Ethylene feedback mechanisms in tomato and strawberry fruit tissues in realtion to fruit ripening and climacteric patterns. *Postharvest Biology and Technology*, **20**: 151-162.

Baerson SR, Vander Heiden MG, Lamppa GK. (1998). Overlapping yet distinct promoter activities of two Arabidopsis genes coding for nearly identical isoforms of the acyl carrier protein. *International Journal of Plant Sciences*, **159** (4): 533-538.

Baerson, S.R., Lamppa,G.K. (1993). Developmental Regulation of an Acyl Carrier Protein Gene Promoter in Vegetative and Reproductive Tissues. *Plant Molecular Biology*, **22**: 255-267.

Baerson, S.R., Vander Heiden, M.G., Lamppa, G.K. (1994). Identification of domains in an *Arabidopsis* acyl carrier protein gene promotor required for maximal organ-specific expression. *Plant Molecular Biology*, **26**: 1947-1959.

Bandyopadhyay, C. and Gholap, A.S. (1973). "Relationship of aroma and flavour characteristics of mango *(Mangifera indica* L.) to fatty acid composition." *Journal of Science, Food and Agriculture*, **24**(12): 1497-503.

Bao, X., Focke, M., Pollard, M. and Ohlrogge, J. (2000). Understanding in vivo carbon precursor supply for fatty acid synthesis in leaf tissue. *Plant journal*, **22** (1): 39-50.

Bate, N. and Twell, D. (1998). Functional architecture of a late pollen promoter: pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. *Plant Molecular Biology*, **37** (5): 859-869.

Beaudoin, N.,and Rothstein, S.J. (1997). Developmental regulation of two tomato lipoxygenase promoters in transgenic tobacco and tomato. *Plant Molecular Biology* **33** (5):835-846.

Belding, R.D., Blankenship, S.M. and Young, E. (1998). Composition and variability of epicuticular waxes in apple cultivars. *Journal of the American Society of Horticultural Science*, **123** (3):348-356.

Bernhardt, J., Stich, K., Schwarz-Sommer, Z., Saedler, H. and Wienand, U. (1998). Molecular analysis of a second functional A1 gene (dihydroflavonol 4-reductase) in *Zea mays*. *Plant Journal*, **14** (4):483-488.

Bienkiewicz, E.A. and Woody, R.W. (1997). Reexamination of the structural characteristics of the acyl carrier protein. *Biospectroscopy*, **3** (3):171-181.

Bojorquez, G., Gomezlim, M.A. (1995). Peroxisomal thiolase messenger-RNA is induced during mango fruit ripening. *Plant Molecular Biology* , **28** (5): 811-820.

Brady, C.J. (1987). Fruit Ripening. *Annual Review of Plant Physiology*, **38**:155-178.

Butt, A.D. and Ohlrogge, J.B. (1991). Acyl carrier protein is conjugated to glutathione in spinach seed. *Plant Physiology*, **96** (3): 937-942.

Cannon, M., Platz, J., O'Leary, M., Sookdeo, C., Cannon, F. (1990). Organ-specific Modulation of Gene Expression in transgenic Plants Using RNA. *Plant Molecular Biology*, **15**: 39-47.

Couture, R., Wiemot, C., Avezard, F., Gosselin, A. (1988). Improved Extraction of Lipids from Strawberry. *Phytochemistry*, **27**: 2033-2036.

Croft, K.P.C, Jüttner, F. and Slusarenko, A.J. (1993). Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringe pv phaseolicola.* Plant Physiology, **101**: 13-24.

Das, N. and Ghosh, S. (1995). Molecular-cloning and sequence-analysis of a seed-expressed acyl carrier protein (ACP) gene from *Brassica-campestris* (Agrani). *Biochimica et Biophysica Acta*, **1258**(1): 75-79.

Davies, C., Robinson, S.P. (1996). Sugar accumulation in grape berries. *Plant Physiology*, **111**: 275-283.

de Silva, J., Loader, N.M., Jarman, C., Windhurst, J.H.C., Hughes, S.G., Safford,R. (1990). The Isolation and Sequence Analysis of two seed expressed Acyl carrier proteins from *Brasica napus. Plant Molecular Biology*, **14**:537-548.

de Silva, J., Robinson, S.J., Safford, R. (1992). The isolation and functional characterisation of a *B.napus* acyl carrier protein 5' flanking region involved in the regulation of seed storage lipid synthesis. *Plant Molecular Biology*, **18**:1163-1172.

Dirinck, P.J., DePooter, H.L., Willaert, G.A., Schamp, N.M. (1981). Flavour quality of cultivated strawberries: the role of sulphur compounds. *Journal of Agriculture and Food Chemistry*, **29**:316-321.

Dobbeling, U., Boni, R., Haffner, A., Dummer, R. and Burg, G. (1997). Method for simultaneous RNA and DNA isolation from biopsy material, culture cells, plants and bacteria. *Biotechniques*, **1**(22): 88-90.

Douillard, C., Guichard, E. (1990). The Aroma of Strawberry (*Fragaria ananassa*): Characterisation of some cultivars and influence of freezing. *Journal of Science and Food Agriculture,* **50**: 517-531.

Dyas, L. and Goad, L.J. (1993). Steroyl Fatty Acyl Esters. *Plant Phytochemistry*, **34**:17-29.

Ealing, P.M. (1994). "lipoxygenase activity in ripening tomato fruit pericarp tissue." *Phytochemistry* **36** (3):547-552.

Ellis, B.E. (1997). Metabolism of defence and communication. In: Plant Metabolism (Eds: Dennis, D.T., Turpin, D.H., Lefebvre, D.D., Layzell, D.B.). Second Edition. Longman, pp 81-148.

Emes, M.J and Dennis, D.T. (1997). Regulation by compartmentation. In: Plant Metabolism (Eds: Dennis, D.T., Turpin, D.H., Lefebvre, D.D., Layzell, D.B.). Second Edition. Longman, pp69-80.

Evans, D. (1996). Produce-on-demand: what's good for US markets is good for world markets too. *Nat Biotechnol.* **14** (7): 802.

Forney, C.F., Breen, P.J. (1986). Sugar Content and Uptake in the Strawberry Fruit. *Journal of the American society for Horticultural Science*, **111**: 241-247.

Frischauf, A. (1991). Construction of genomic libraries in lambda and cosmid vectors. In: *Essential Molecular Biology* (Eds. Brown, T.A.) Oxford university press, **2**: pp 15-38.

Giovannoni, J.J. (1993). Molecular and Genetic Analysis of Tomato Fruit Development and Ripening. In: *Methods in Plant Biochemistry*. Academic Press, **10**:251-286.

Given, N.K., Venis, M.A. and Grierson, D. (1988). Phenylalanine ammonia-lyase activity and anthocyanin synthesis in ripening strawberry fruit. *Journal of Plant Physiology*, **133**: 25-30.

Grierson, C., Du, J.S., Zabala, M.D., Beggs, K., Smith, C., Holdsworth, M. and Bevan, M. (1994). Separate cis sequences and trans factors direct metabolic and developmental regulation of a potato-tuber storage protein gene. *Plant Journal.* **5**(6):815-826.

Gross, J. (1982). Changes of chlorophylls and carotenoids in developing strawberry fruits (*Fragaria ananassa*) cv. Tenira. *Gartenbauwissenschaft,* **47**: 142-144.

Grotewold, E., Drummond, B.J., Bowen, B., and Peterson, T. (1994). The myb-homologous p-gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. *Cell,* **76** (3):543-553..

Gubler, F., Raventos, N., Keys, M., Watts, R., Mundy, J. and Jacobsen, J.V. (1999). Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. *Plant Journal*, **17**: (1) 1-9.

Guerra, D.J., Ohlrogge, J.B. and Frentzen, M. (1986). Activity of acyl carrier protein isoforms in reactions of plant fatty-acid metabolism. *Plant Physiology*, **82** (2):448-453.

Hamilton-Kemp, T.R., Archbold, D.D., Loughrin, J.H., Collins, R.W. and Byers, M.E. (1996). Metabolism of natural volatile compounds by strawberry fruit. *Journal of Agricultural and Food Chemistry*, **44**: 2802-2805.

Hannapel, D.J. and Ohlrogge, J.B. (1988). Regulation of acyl carrier protein messenger-RNA levels during seed and leaf development. *Plant Physiology.* **86** (4):1174-1178.

Harwood, J.L. (1988). Fatty Acid Metabolism. *Annual Review of Plant Physiology*, **39**:101-138.

Harwood, J.L. (1996). Recent advances in the biosynthesis of plant fatty acids. *Biochimica et Biophysica Acta*, **1301**:7-56.

Heineke, D., Wildenburger, K., Sonnewald, U., Willmitzer, L., Heldt, H.W. (1994). Accumulation of hexoses in leaf vacuoles: studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. *Planta*; **194**: 29-33.

Hlousek-Radojcic, A., Postbeittenmiller, D. and Ohlrogge, J.B. (1992). Expression of constitutive and tissue-specific acyl carrier protein isoforms in *Arabidopsis*. *Plant Physiology*, **98**: (1) 206-214.

Hyodo, H. (1971). Phenylalanine Ammonia-Lyase in Strawberry Fruits. *Plant Cell Physiology*, **12**: 989-991.

Izzo, R., Scartazza, A., Masia,A., Galleschi,L.,Quartacci,M.F., Navari-Izzo,F. (1995). Lipid evolution during developement and ripening of peach fruits. *Phytochemistry*. **39**:1329-1334.

Johnson, B. H. and M. H. Hecht (1994). Recombinant proteins can be isolated from *E. coli* cells by repeated cycles of freezing and thawing. Biotechnology, **12**(13): 1357-60.

Kano, Y. and Asahira, T. (1978). Effects of some plant growth regulators on the development of strawberry fruits *in vitro* culture. *Journal of the Japanese Society of Horticultural Science*, **47**:195-202.

Keating, D.H. and Cronan, J.E. (1996). An isoleucine to valine substitution in *Escherichia coli* acyl carrier protein results in a functional protein of decreased molecular radius at elevated pH. *Journal of Biological Chemistry*, **271**(27): 15905-10.

Kim, S.Y., Chung, H.J. and Thomas, T.L. (1997). Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification

elements in the Dc3 promoter using a modified yeast one-hybrid system. *Plant Journal*, **11** (6):1237-1251.

Kim, Y. and Prestegard, J. H. (1990). Refinement of the NMR structures for acyl carrier protein with scalar coupling data. *Proteins-Structure Function And Genetics*, **8**(4): 377-85.

Knee, M., Sargent, J.A., Osborne, D.J. (1977). Cell Wall Metabolism in Developing Strawberry Fruits. *Journal of Experimental Botony.* **28**:377-396.

Kock, K.E. (1996). Annual Review of Plant Physiology, 47:509-540.

Kocher, T.D. and Wilson, A.C. (1991). DNA amplification by the polymerase chain reaction. In: Essential Molecular Biology (Ed. Brown, T. A.). Oxford university press, 2, pp185-207.

Konishi, T., Shinohara, K., Yamada, K. and Sasaki, Y. (1996). Acetyl-CoA carboxylase in higher plants: most plants other than gramineae have both the prokaryotic and the eukaryotic forms of this enzyme. *Plant Cell Physiology*, **37**(2): 117-22.

Kundu, M. K., Brychova, H. and Pokorny, J. (1973). Plant lipids. 4. Lipids in strawberry seeds. *Nahrung* **17**(6): 680-1.

Lalonde, S., Boles, E., Hellmann, H., Barker, L., Patrick, J.W., Frommer, W.B. and Ward, J.M. (1999). The dual function of sugar carriers: Transport and sugar sensing. *Plant Cell*, **11** (4): 707-726.

Lamppa, G. and Jack, S.C. (1991). Analysis of 2 linked genes-coding for the acyl carrier protein (ACP) from *Arabidopsis-thaliana* (Columbia). *Plant Molecular Biology*, **16** (3): 469-474.

Latchman, L. (1995). Transcriptional control-DNA sequence elements. In: Gene Regulation. Chapman & Hall, pp 142-177.

Lawton, M.A., Dean, S.M., Dron, M., Kooter, J.M., Kragh, K.M., Harrison, M.J., Yu, L., Tanguay, L., Dixon, R.A., and Lamb, C.J. (1991). Silencer region of a chalcone synthase promoter contains multiple binding-sites for a factor, SBF-1, closely related to GT-1. *Plant Molecular Biology*, **16** (2):235-249.

Lea, P.J., Leegood, R.C. (Ed). (1993). Plant lipids: their metabolism, function and utilisation in: Plant Biochemistry and Molecular Biology. Wiley. pp 113-128 and pp197-220.

Lefebvre, D.D and Gellatly. (1997). The control of metabolism. In: Plant Metabolism (Eds: Dennis, D.T., Turpin, D.H., , D.D., Layzell, D.B.). Second Edition. Longman, pp 81-148.

Lemieux, B. (1996). Molecular genetics of epicuticular wax biosynthesis. *Trends in Plant Science*, **1**(9): 312-316.

Lessard, P.A., Allen, R.d., Bernier, F., Crispino, J.D., Fujiwara, T. and Beachy, R.N. (1991). Multiple nuclear factors interact with upstream sequences of differentially regulated beta-conglycinin genes. *Plant Molecular Biology*, **16**(3): 397-413.

Loader, N.M., Woolner, E.M., Hellyer, A., Slabas, A.R. and Safford, R. (1993). Isolation and characterization of 2 *Brassica-napus* embryo acyl-ACP thioesterase cDNA clones. *Plant Molecular Biology*, **23**(4): 769-778.

Lois, L., Rodriguez-Concepcion, M.M., Gallego, F., Campos, N. and Boronat, A. (2000). Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. *Plant Journal*, **6**(22): 503-13.

Manning, K. (1998). Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits. *Planta*, **205**(4): 622-31.

Manning.K. (1994). Changes in gene expression during strawberry fruit ripening and their regulation by auxin. *Planta*, **194**:62-68.

Manning.K. (1993). Soft Fruits: In The Biochemistry of Fruit Ripening. Chapman and Hall. Ch 12. pp 347-377.

Martinez, G.A., Chaves, A.R. and Anon, M.C. (1996). Effect of exogenous application of gibberellic acid on colour and phenylalanine ammonia-lyase, chlorophyllase, and peroxidase activities during ripening of strawberry fruit (Fragaria x ananassa Duch.). *Journal of Plant Growth Regulation,* **15**: 139-146.

Matsui, K., Miyahara, C. Wilkinson, J., Hiatt, B., Knauf, V., Kajiwara, T. (2000). Fatty acid hydroperoxide lyase in tomato fruits: cloning and properties of a recombinant enzyme expressed in *Escherichia coli* [In Process Citation]. *Bioscience Biotechnology and Biochemistry,* **64**(6): 1189-96.

McConn, M., Browse, J. (1996). The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. *Plant Cell*, **8**(3): 403-416.

Medina-Escobar, N., Cardenas, J., Valpuesta, V., Munoz-Blanco, J. and Caballero, J. L. (1997). Cloning and characterization of cDNAs from genes differentially expressed during the strawberry fruit ripening process by a MAST-PCR- SBDS method. *Analytical Biochemistry*, **248**(2): 288-96.

MedinaSuarez R., Manning, K., Fletcher, J., Bird, C., Aked, J., and Seymour, G.B. (1997). Gene expression in banana peel and pulp during ripening. Plant *Physiology*, **111** (2): 517-517.

Mekhedov, S., de Ilarduya, O.M., and Ohlrogge, J. (2000). Toward a functional catalog of the plant genome. A survey of genes for lipid biosynthesis. *Plant Physiology,* **122**: (2) 389-401.

Mitchell, W.C. and Jelenkovic, G. (1995). "Characterizing NAD-dependent and NADP-dependent alcohol-dehydrogenase enzymes of strawberries." *Journal of the American society for Horticultural Science*, **120**(5): 798-801.

Montero, M., Mollà, E.M., Esteban, R.M., López-Andréu, F.J. (1996). Quality Attributes of Strawberry During Ripening. *Scientia Horticulturae*, **65**:239-250.

Moyano, E., Portero-Robles, I., Medina-Escobar, N., Valpuesta, V., Munoz-Blanco, J. and Caballero, J.L. (1998). "A fruit-specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process." *Plant Physiology*, **117**(2): 711-6.

Mullet, J.E. (1997). Fatty acid and lipid biosynthesis and degradation. In: Plant Metabolism. (Eds. Dennis, D.T., Turpin, D.H., Lefebvre, D.D. .and Layzell, D.B.) Longman, pp 260-273.

Murphy, D.J. (1993). Plant Lipids: Their Metabolism, Function and Utilisation. In: Plant Biochemistry and Molecular Biology (Eds. Lea, P.J. and Leagood, R.C.). John Wiley and Sons Ltd. pp114-128.

Mussinan, C.J., Walradt, J.P. (1975). Organic acids from fresh California strawberries. *Journal of Agriculture and Food Chemistry*, **23**(3): 482-484.

Nakai, K., Horton, P. (1999). PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends in Biochemical Science*, **24** (1):34-35.

Nam, Y.W., Tichit, L., Leperlier, M., Cuerq, B. and Marty, I. (1999). Isolation and characterization of mRNAs differentially expressed during ripening of wild strawberry (*Fragaria vesca* L.) fruits. *Plant Molecular Biology*, **39**(3): 629-36.

Nehra. N.S., Kartha, K.K., and Stushnoff, C. (1991). Nuclear-DNA content and isozyme variation in relation to morphogenic potential of strawberry (*Fragaria X ananassa*) callus-cultures. *Canadian Journal of Botony*, **69** (2): 239-244.

Ohlrogge, J.B, Post-Beitenmiller, D., Hlousek-Radojcic. (1991). Expression of constituative and tissue specific acyl carrier protein isoforms in *Arabidopsis*. Plant *Physiology*, **98**: 206-214.

Ohlrogge, J.B., Browse, J.(1995). Lipid Biosynthesis. *The Plant Cell*, 7:957-970.

Perez, A.G., Olias, R., Olias, J.M. and Sanz, C. (1999). Biosynthesis of 4-hydroxy-2,5-dimethyl-3(2H)-furanone and derivatives in *in vitro* grown strawberries. *Journal Of Agricultural and Food Chemistry,* **47**(2): 655-658.

Perez, A.G., Sanz, C., Olias, R. and Olias, J.M. (1997). Effect of methyl jasmonate on *in vitro* strawberry ripening. *Journal of Agricultural Food Chemistry*, **45**: (10) 3733-3737.

Pickenhagen, W., Velluz, A., Passerat, J., Ohloff, G. (1981). Estimation of 2,5-Dimethyl-4-hydroxy-3(2H)-furanone(FURANEOL) in Cultivated and Wild Strawberries, Pineapples and Mangoes. *Journal of Science and Food Agriculture*, **32**,1132-1134.

Poovaiah, B.W. and Veluthambi, K. (1985). Auxin Regulated Invertase Activity in Strawberry Fruits. *Journal of American society for Horticultural Science*, **110**:258-261.

Post-Beittenmiller, D. (1996). Biochemistry and molecular biology of wax production in plants. *Annual Review of Plant physiology. Plant Molecular Biology* **47**: 405-430.

Rock, C. O.; Jackowski, S. (1982). Regulation of phospholipid synthesis in Escherichia coli. Composition of the acyl-acyl carrier protein pool in vivo. *Journal Biolological Chemistry.* **257** (18) :10759-65.

Righelato, R.C. and Traill, B. (1996). Technology foresight in horticulture strawberries in the U.K. Prepared for the Ministry of Agriculture, Fisheries and Food.

Riley, J.C.M, Willemot, C. and Thompson, J.E. (1996). Lipoxygenase and hydroperoxide lyase activities in ripening tomato fruit. Postharvest Biology *Technology*, 7 (1-2): 97-107.

Sablowski, R.W.M., Moyano, E., Culianezmacia, F.A., Schuch, W., Martin, C. and Bevan, M. (1994). A flower-specific myb protein activates transcription of phenylpropanoid biosynthetic genes. *Embo Journal*, **13**: (1) 128-137.

Safford, R., Windust, J.H.C., Lucus, C., de Silva, J., James, C.M., Hellyer, A., Smith, C.G., Slabas, A.R., Hughes, S.G. (1988). Plastid localised seed acyl carrier protein of *Brassica napus* is encoded by distinct, nuclear multigene familly. *European Journal of Biochemistry*, **174**: 287-295.

Sambrook, J., Fritsh, E.F and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press.

Savage, L.J. and Post-Beittenmiller, D. (1994). Phosphopantethenylated precurser acyl carrier protein imported into spinach (*Spinacia oleracea*) chloroplasts. *Plant Physiology*, **104**: 989-995.

Schmid, K., J. Andrews, and Ohlrogge, J. (1997). Fatty acid and lipid biosynthesis and degradation. In: Plant Metabolism. (Dennis, D.T., Turpin, D.H., Lefebvre, D.D. and Layzell D.B.). Longman pp 414-429.

Schreir, P. (1980). Quantitative composition of volatile constiuants in cultivated strawberries, *Fragaria x ananassa* cv. *Senga sengana, Senga litessa* and *Senga gourmella. Journal of Scientific Food Agriculture*, **31**: 487-494.

Schutt, B. S., M. Brummel, Schuch, R. and Spener, F. (1998). The role of acyl carrier protein isoforms from *Cuphea lanceolata* seeds in the de-novo biosynthesis of medium-chain fatty acids. *Planta*, **205**(2): 263-8.

Shintani, D.K. and Ohlrogge, J.B. (1994). The characterisation of a mitochondrial acyl carrier protein isoform isolated from *Arabidopsis thaliana. Plant Physiology*, **104**: 1221-1229

Singer-Sam, J. (1994). Quantitation of specific transcripts by RT-PCR SNuPE assay. PCR Methods Appl, **3**(4): S48-50

Slabas, A.R., Harding. J., Hellyer, A., Roberts, P., Bambridge, H.E. (1987). Induction, purification and characterisation of acyl carrier protein from developing seeds of oil seed rape (*B.napus*). *Biochemica et Biophysica Acta.* **921**:50-59.

Solano, R., Nieto, C., Avila, J., Canas, L., Diaz, I. and Pazares, J. (1995). Dual DNA-binding specificity of a petal epidermis-specific MYB transcription factor (MYB.PH3) from Petunia-hybrida. *Embo Journal*, **14**: (8) 1773-1784.

Sreenivas, A. and Sastry, P.S. (1995). Purification and partial characterization of acyl carrier proteins from developing oil seeds of pisa (Actinodaphne hookeri) and ground nut (Arachis-hypogaea). *Indian Journal of Biochemistry and Biophysics,* **32**: (3) 137-146.

Stalberg, K., Ellerstom, M., Ezcurra, I., Ablov, S. and Rask, L. (1996). Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. *Planta,* **199**(4): 515-519.

Stone, E.J., Hall, R.M. and Kazeniac S.J. (1975). Formation of aldehydes and alcohols in tomato fruit from U-^{14} C-labeled linolenic and linoleic acids. *Journal of Food Science.* 40: 1138-1141.

Stumpf, P.K. (1969). Metabolism of fatty acids. *Annual Review of Biochemistry,* **38**:159-207.

Subagio, A., Morita, N. and Sawada, S. (1996). Carotenoids and their fatty-acid esters in banana peel. *Journal of Nutritional Science and Vitaminology* (Tokyo). **42**(6): 553-66.

Suh, M.C., Schultz, D.J. and Ohlrogge, J.B. (1999). Isoforms of acyl carrier protein involved in seed-specific fatty acid synthesis. *Plant Journal,* **17**: (6) 679-688.

Tian, M.S., Prakash, S., Elgar, H. J., Young, H., Burmeister, D.M. and Ross, G.S. (2000). Responses of strawberry fruit to 1-Methylcyclopropene (1-MCP) and ethylene. *Plant Growth Regulation*, **32** (1): 83-90.

Topfer, R. and Martini, N. (1994). Molecular-cloning of cDNAs or genes encoding proteins involved in de-novo fatty-acid biosynthesis in plants. *Journal of Plant Physiology*, **143**(4-5): 416-425.

Tucker, G.A (1993). Introduction. In: The Biochemistry of Fruit Ripening (Eds: Seymour, G.B., Taylor, J.E. and Tucker, G.A.). Chapman and Hall, pp 1-52.

Voet, D and Voet, J.G. (1990). Biochemistry (Ed: Stiefel, J.). John Wiley & Sons Ltd.

Voetz, M., Klein, B., Schell, J. and Topfer, R. (1994). Three different cDNAs encoding acyl carrier proteins from *Cuphea-lanceolata. Plant Physiolog,*. **106**(2): 785-786.

Wade, N.L. and Bishop, D.G. (1978). Changes in the lipid composition of ripening banana fruits and evidence for an associated increase in cell membrane permeability. *Biochim et Biophys Acta,* **529**(3): 454-60.

Watanabe, N., Yamada, S. (1996). Activation of 20s proteosomes from spinach leaves by fatty acids. *Plant Cell Pysiology*, **37**(2): 147-151.

Watson, J.D., Gilman,M., Witkopwski,J., Zoller,M. (1992). Recombinant DNA. (2nd ed). Scientific American Books, pp99-127 and 273-292.

Weier, E.T., Stocking, R., Barbour, M.G., Rost, T.L. (1982). Botany: An Introduction to Plant Biology. (Sixth Ed). Wiley, pp303-325.

Whitaker, B.D. (1988). Changes in the steryl lipid-content and composition of tomato fruit during ripening. *Phytochemistry*, **27**: (11) 3411-3416.

Wilkinson, J.Q., Lanahan, M.B., Conner, T.W., Klee, H.J. (1995). Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. *Plant Molecular Biology*, **27**:1097-1108.

Woodhead, M., Taylor, M.A, Brennan, R., McNicol, R.J. and Davies, H.V. (1998). Cloning and characteristion of the cDNA clones of five genes that are differentially expressed during ripening in the fruit of blackcurrent (*Ribes nigrum* L.). *Journal of Plant physiology*, **153**: 381-393.

Yanagisawa, S. and Schmidt, R.J. (1999). Diversity and similarity among recognition sequences of Dof transcription factors. *Plant Journal*, **17**(2): 209-214.

Yanagisawa, S. and Sheen, J. (1998). Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. *Plant Cell*, **10**: (1) 75-89.

Zabetakis, I. and Holden, M.A. (1997). Strawberry flavour: Analysis and biosynthesis. *Journal of Sci ence, Food and Agriculture*, **74**(4): 421-434.

Zhou, D.X. (1999). Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. *Trends in Plant Science*, **4**(6): 210-214.

Zhou, D.X., Li, Y.F., Rocipon, M. and Mache, R. (1992). Sequence-specific interaction between S1F, A spinach nuclear factor, and a negative cis-element conserved in plastid-related genes. *Journal of Biological Chemistry*, **267**(33): 23515-23519.

Appendix A

Composition of Solutions and Reagents

A.1.. 50 X TAE Stock Solution

For each litre of solution:

242 g	Tris Base
57.1 mL	Glacial Acetic Acid
100 mL	0.5 M EDTA

Mix Tris with stir bar to dissolve in about 600 mL of ddH2O. Add the EDTA and Acetic Acid. Bring final volume to 1 L with ddH2O. Store at room temperature.

Note: Final (1x) working concentration :

0.04 M Tris - Acetate

2.7 M EDTA

A.2. TE Buffer

For each litre of solution:

Add 10 ml of 1M Tris-HCl, pH 7.5 (or pH 8.0 if desired) and 2 ml of 0.5M EDTA, pH 8.0, with distilled water to one litre final volume.

Store at room temperature.

A.3. S.O.C.

For each 100 ml of solution:

2.0 g	2 % Bacto-Tryptone
0.5 g	0.5 % Yeast Extract
2.92 g	10 mM NaCl
20.20 g	20 mM MgCl2
3.73 g	2.5 mM KCl
24.60 g	20 mM MgSO4
9.00 g	20 mM Glucose

Mix above, bring to 100 ml, and filter through a sterile 0.45μm filter. Store the stock solution at -20 C or -70 C.

A.4. X-gal

Stock solution: 50mg/ml X-gal in dimethylformamide (DMF). Add 10 μl for each ml top agarose.

A.5. IPTG (isopropyl-ß-D-thio-galactopyranoside)

Stock solution: 0.5M IPTG (w/v) in water (0.25μ sterile filtered). Store at -20 °C. Add 1.5μl for each ml of top agar.

A.6. LB (Luria-Bertani) Broth

5 g	Bacto-Yeast Extract
10 g	NaCl
10 g	Bacto-Tryptone

Make up to 1L with ddH_2O. Adjust the pH7.5 with NaOH.

To prepare LB plates, add 7.5 g Bacto-agar / 500 mL media just prior to autoclaving. Let media cool to approximately 50° C before pouring plates. Each plate should be about 30 - 40 mL volume.

A.6. Antibiotic Stock Solutions

A.6.1. Carbenicillin (50 mg/ml)

2 g	Carbenicillin
40 ml	ddH_2O.

Mix and filter sterilise (0.22µm). Add 1µl per ml LB (50°C) for a working concentration of 50µg/ml. Store at -20°C.

A.6.2. Chloramphenicol (25 mg/ml)

1g	Chloramphenicol
40 ml	ethanol.

Add 1.36µl per ml LB (50°C) for a working concentration of 34µg/ml. Store at -20°C

A.6.3. Kanamycin (50 mg/ml)

2 g	Kanamycin
40 ml	dH_2O

Mix and filter sterilise (0.22µm). Add 1µl per ml LB (50°C) for a working concentration of 50µg/ml. Store at -20 oC.

A.6.4. Tetracycline (12.5 mg/ml)

125mg	tetracycline
10 ml	50% ethanol

Sterile filter (0.25µm) and cover tube with foil and store at -20 °C covered in light proof container. Working solution is 50 µg/ml.

A.7. 1 M Tris Solution

For a 500 ml solution:

Add 60.55 g Tris in approximately 300 mL ddH2O. Adjust pH to the desired point using concentrated HCl. Bring volume to 500 mL

Sterilize by autoclaving.

A.8. 20 X SSC

For a 1 L solution:

175.3 g	NaCl
88.2 g	Sodium Citrate

Dissolve in about 800 mL ddH2O. Adjust pH to 7.0 and bring volume to 1 L. Sterilize by autoclaving.

A.9. 30% (v/v)Tween 20 (Polyoxyethylene sorbitan mono-oleate)

Add 30 ml of Tween to 60 ml of water. Mix and dilute to a final volume of 100 ml with water.

A.10. SM (Phage suspension media)

For a 1 L solution:

5.8 g	NaCl
2 g	MgSO4-7H2O
1 g	Gelatin
50 ml	1M Tris 7.5

Add ddH_2O to a final volume of 1 L and autoclave.

A.11. 1M Magnesium chloride ($MgCl_2$)

For a 100ml solution:

Dissolve 20.33 g of $MgCl_2$ x 6H 2O in 60 ml of dH_2O. Adjust the volume to 100 ml. Pass through a 0.2µ filter.

Mg^{2+} is required by enzymes e.g. DNA polymerases, and is important for phage integrity and adsorption.

A.12. 0.5M EDTA Stock Solution

For a 500 ml solution:

add 93.05 g in 350 mL of ddH_2O. Add ~ 20 - 30 NaOH pellets to raise pH to 8. When dissolved, bring the final volume to 500 mL with ddH2O. Sterilize by autoclaving and store at room temperature.

A.13. 5M Ammonium acetate

For a 1 L solution:

Dissolve 385.4 g. of ammonium acetate in 500 ml distilled water by slowing adding the ammonium acetate solid into water stirring in a 2 L graduated cylinder. Adjust volume to 1 L and filter through 0.22 μm membrane. Store at room temperature.

A.14. 3M Sodium acetate (pH 5.2)

Dissolve 408.18 g. of sodium acetate (x 3H2O) in distilled water added to 900 ml. Adjust the pH to 5.2 with glacial acetic acid and make up the final volume to 1 L with distilled water and filter sterilize (0.22μm). Store at room temperature.

A.15. 1M Sodium acetate (pH 4.5 or pH 6.0)

Place 500 ml of dH_2O into a 2 L graduated cylinder with stirring. Pour 272.13g of sodium acetate (NaOAc x 3 H2O) into the water and stir until dissolved. Adjust pH to 4.8 (or pH 6.0 if desired) with glacial acetic acid. Adjust final volume to 2 L and filter sterilize (0.22μm). Store at room temperature.

A.16. 40% Acrylamide/Bisacrylamide

For a 1 L solution:

Combine 380 g of ultrapure acrylamide, 20 g of N,N-methylene-bisacrylamide and distilled water to 800 ml in a 2 L glass beaker. Cover and stir until dissolved. Deionize by stirring with Amberlite MB-1 resin at 5 g per 100 ml of solution for 1 hour at room temperature. Pass over a 0.45 μm filter to remove resin. Adjust volume to one liter. Store at 4 °C for up to two weeks.

A.17. 10X TBE Stock Solution

For each litre of solution:

108 g	Tris Base
55 g	Boric Acid
40 mL	0.5 M EDTA

Mix Tris Base and boric acid in about 600 ml of ddH2O to dissolve. Add EDTA and bring the volume to 1 L. Store at room temperature.

A.18. 100X KP buffer

For each 200ml of solution:

KH2PO4	18.1 g
K2HPO4	5.0 g

Adjust pH to 6.2 with 10 N KOH! Add ddH20 to 200ml and autoclave.

A.19. MOPS 10X buffer

For each litre of solution:

0.4M	3-[N-morpholino]-2-hydroxypropanesulfonic acid (MOPS), pH 7.0.
0.1M	sodium acetate
0.01M	EDTA, pH 8.0

Add 83.72g MOPS and 8.23g sodium acetate to 600mL ddH_20. Once dissolved, add 0.5M EDTA and adjust the pH to 7.0 with 10N NaOH. Bring the final volume to 1L. Autoclave. The solution will turn yellow.

A.20. 0.5M Sodium phosphate buffer ($NaPO_4$)

For each litre of solution:

Stock solution A:

Dissolve 276g of monobasic sodium phosphate to a 1L final volume in distilled water to give a 2M solution.

Stock solution B:

Dissolve 284 g of dibasic sodium phosphate to a 1L final volume in distilled water to give a 2 M solution.

For a 1.0M $NaPO_4$ buffer of pH 7.2, mix 28.0 ml of Solution A and 72.0 ml Solution B with 100 ml of distilled water.

A.21. 13% PEG/1.6M NaCl

For each litre of solution:

Place 500 ml of ddH2O into a 1 L graduated cylinder and stir. Add 130 g of polyethylene glycol (PEG-8000) and 93.6g of sodium chloride (NaCl) to the water. Stir until completely dissolved and adjust the volume to 1 L with ddH2 O. Filter sterilize over a 0.45 μm filter unit. Store at room temperature.