Investigations into β-ketoacyl-ACP Synthase III and enoyl-ACP reductase of plant fatty acid synthase

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Investigations into β-Ketoacyl-ACP Synthase III and Enoyl-ACP Reductase of Plant Fatty Acid Synthase

by

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Abstract

An antisense transgenic approach was taken in order to assess the importance of β-ketoacyl-ACP-Synthase III (KAS III) in regulation and initiation of *de novo* fatty acid synthesis *in planta*. The transgenic plants were generated in order to show whether alternative pathways exist to initiate fatty acid synthesis if the most direct route is lost.

In order to develop such a study, a KAS III cDNA clone of 1622bp was isolated from a *Brassica napus* embryo library. This clone was used to generate an antisense construct containing a strong double 35S promoter and a CaMV poly A tail. The antisense vector was transformed into both *Brassica napus* and *Arabidopsis thaliana*. Sixty-three antisense KAS III lines were generated in *Brassica napus*, along with 10 transgenic KAS III *Arabidopsis thaliana* lines. The open reading frame of the KAS III cDNA clone was overexpressed in *E.coli* to yield a novel protein of 45kDa. It was subsequently purified and used to raise KAS III polyclonal antisera. This provided an immunological tool that could detect KAS III in leaf and seed extracts in wild type *Brassica napus*.

To analyse whether KAS III caused any pleiotropic effects on other FAS components such as down regulation of other FAS enzymes, a quantitative ELISA assay was developed to Enoyl-ACP-Reductase (ENR). This was the first such assay for the detection of ENR in plants and its development has required careful consideration of sample preparation. Levels of KAS III, ENR and β-Keto-ACP-Reductase (BKR) were assessed in developing leaf of wild type cultivars, along with total fatty acid levels in the growing leaf.

Analysis of transgenic KAS III lines was performed in conjunction with the analysis of transgenic sense and antisense ENR *Brassica napus* that had become available during the study. Transgenic KAS III lines were seen to be slower growing, but were still able to grow to maturity, flower and set seed. This suggests that the KAS III down regulation may lead to the use of alternative pathways in the synthesis of the initial condensation product acetoacetyl-ACP. Down regulation of ENR in some antisense KAS III lines suggest that a pleiotropic effect may have been exerted as a result of the lowering of KAS III levels. The analysis of the T1 generations for both KAS III and ENR during this study showed that certain lines possessed a phenotype which is characterised by a slower growing plant, where a change of seed morphology was observed along with a change in testa colour and a decrease in overall fatty acid levels in the seed.

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Finally, I would like to thank my Mum, Dad, Robert and Laura without whom this thesis would never have been! And to Ale, whose love and support has helped me to find the funnier side of it all!

Hayley, September 1999

Preface

The work described within this thesis was undertaken between 1995 and 1998 whilst the author was a research student under the supervision of Professor Antoni Slabas in the Department of Biological Sciences at the University of Durham.

Some of the work was carried out in collaboration with the following staff Dr. A.J.White and Dr P. O'Hara (Chapter 5, section 5.3). The major part of the work presented is, however, the author's own work. This work has not been submitted for any other degree at the University of Durham or at any other university.

Abbreviations

ACP	-	Acyl-Carrier-Protein
dATP	-	deoxy-Adenine-Triphosphate
BSA	-	Bovine serum albumin
CAPS	-	3-[cyclohexylamino]-1-propanesulphonic acid buffer
dCTP	-	deoxy Cytosine triphosphate
DAE	-	Days after emergence
DAF	-	Days after flowering
DEPC	-	Diethyl pyrocarbonate
DNA	-	Deoxyribose nucleic acid
cDNA	-	complementary DNA
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetracetic acid
ELISA	-	Enzyme linked immunosorbant assay
FPLC	-	Fast Protein Liquid Chromatography
GLC	-	Gas Liquid Chromatography
dGTP	-	deoxy Guanine Triphosphate
HPLC	-	High Performance Liquid Chromatography
IPTG	-	Isopropyl thio-β-D-galactoside
LB	-	Luria-Bertani Medium
MOPS	-	3-(*N*-morpholino) propanesulphonic acid
M-PBS	-	Milk-Phosphate buffer saline
MQ	-	Milli-Q water
mRNA	-	messenger RNA
MS	-	Murashige and Skoog salts
NADH	-	Nicotinamide adenine dinucleotide (reduced form)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced form)
OD	-	Optical density
pNPP	-	4-Nitrophenylphosphate.disodium salt
dNTPs	-	dioxynucleotide phosphate

Abbreviations cont.

PBS	-	Phosphate buffer saline
PBS-T	-	Phsosphate buffer saline – Tween 20
PCR	-	Polymerase Chain Reaction
PVP	-	Polyvinylpyrrolidine
RNA	-	Ribose nucleic acid
SDM	-	Site directed mutagenesis
SDS	-	Sodium Dodecoyl Sulphate
SDS/PAGE	-	SDS-polyacrylamide gel electrophoresis
SSC	-	Salt Sodium citrate buffer
TAE	-	Tris-acetic acid buffer
TE	-	Tris-EDTA buffer
TEMED	-	*N'N'N'N'*-tetramethylethylenediamine
dTTP	-	deoxy-thymine triphosphate
T-TBS	-	Tween 20 – Tris buffer saline
TCA	-	Trichloroacetic acid
μE	-	micro Einstein
UTR	-	Untranslated region
UV	-	Ultraviolet light
(v/v)	-	volume:volume ratio
(w/v)	-	weight:volume ratio

Enzyme Abbreviations

ACAT	Acetyl-CoA:ACP transacylase
ACBP	Acyl-CoA binding protein
ACCase	Acetyl-CoA carboxylase
ACL	ATP-citrate lyase
ACP	Acyl carrier protein
βKR	β-ketoacyl ACP reductase
DAGAT	Diacylglycerol acyltransferase
DHR	β-hydroxyacyl ACP dehydratase
ENR	Enoyl-ACP reductase
FAE	Fatty acid elongase
FAS	Fatty acid synthase
GPAT	Glycerol 3-phosphate acyltransferase
KAS I	β-ketoacyl ACP synthase I
KAS II	β-ketoacyl ACP synthase II
KAS III	β-ketoacyl ACP synthase III
LPAAT	Lysophosphatidyl acid acyl transferase
MCAT	Malonyl-CoA:ACP transacylase
pACS	plastidial Acetyl-CoA synthetase
PDC	pyruvate dehydrogenase
pPDH	plastidial Pyruvate dehydrogenase
SAP	Salmon alkaline phosphatase

Lipid Abbreviations

CDP-DAG	Cytidine 5' triphosphate
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
MGDG	Monogalactosyldiacylglycerol
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
SL	Sulpholipids
TAG	Triacylglycerol

Table of Contents

Chapter 1 General Introduction.....1

1.1 Fatty Acid Structure and Function.....1

1.2 Fatty Acid Synthesis – Introduction.....5

1.3 The Source of Acetyl-CoA.....7

1.4 The Individual Enzyme Reactions.....11

1.4.1 Acetyl-CoA Carboxylase.....12

1.4.2 Acyl-Carrier-Protein.....17

1.4.3 Malonyl-CoA:ACP Transacylase.....20

1.4.4 The Condensing Enzymes - β-Ketoacyl-ACP Synthases.....20

1.4.5 β-Ketoacyl-ACP Reductase.....26

1.4.6 β-Hydroxyacyl-ACP Dehydratase.....26

1.4.7 Enoyl-ACP Reductase.....27

1.4.8 Desaturation - Δ9- Stearoyl-ACP Desaturase.....29

1.4.8 Termination.....31

1.5 Further Metabolism of Fatty Acids.....34

1.5.1 Elongation of Fatty Acids.....34

1.5.2 Acyl-CoA Binding Proteins.....35

1.6 Glycerolipid Synthesis in Plants.....35

1.6.1 Membrane Lipid Synthesis.....35

1.6.2 Lipid Desaturation.....38

1.6.3 Storage Lipids.....39

1.6.4 Acyltransferases.....40

1.7 Oil storage.....41

1.8 Regulation of Fatty Acid Synthesis.....42

1.9 Outline of Thesis.....46

Chapter 2 Materials and General Methods.....51

2.1 Bacterial Strains.....51

2.2 Cloning Vectors.....51

2.3 Primer Sequences....52
2.4 Buffers and Solutions....53
2.5 Plant Material....56
2.5.1 *Brassica napus*....56
2.5.2 *Arabidopsis thaliana*....56
2.6 Bacterial Manipulations....57
2.6.1 Preparation of Competent *E.coli* subcloning Cells....57
2.6.2 Preparation of Electro-competent *Agrobacterium tumefaciens* Cells....57
2.6.3 Transformation of Competent *E.coli* cells with plasmids....58
2.6.4 Electroporation of *Agrobacterium* Cells....58
2.6.5 Isolation of Plasmid DNA....59
2.6.6 Overexpression of *Brassica napus* Ketoacyl-ACP Synthase III (KAS III) in *E.coli* BL21*(DE3)*....60
2.6.7 Overexpression of Malonyl-CoA:ACP Transacylase (MCAT) in *E.coli*....60
2.6.8 Purification of Over-Expressed Malonyl-CoA:ACP Transacylase (MCAT) from *E.coli*....61
2.7 General DNA and RNA Techniques....62
2.7.1 Small Scale DNA Preparation from *Brassica napus* Leaf....62
2.7.2 Preparation of RNA from *Brassica napus* Leaf....63
2.7.3 Restriction Endonuclease Digestions....64
2.7.4 General Method for the Polymerase Chain Reaction (PCR)....64
2.7.5 Southern Hybridisiation....65
2.7.6 Northern Hybridisation....66
2.7.7 Synthesis of Radioactive Probes....67
2.7.8 Washing Filters Hybridised with Radioactive Probes....68
2.7.9 Developing X-Ray Films....69
2.7.10 cDNA Library Screening....69
2.7.11 Plasmid Rescue from λZAP cDNA Library....71
2.7.12 Colony Hybridisation....71

2.7.13 DNA Sequencing....72
2.8 Protein Techniques....73
2.8.1 Crude Protein Extracts from *Brassica napus* Seed and Leaf....73
2.8.2 Protein Estimation by Bradford Micro-assay....73
2.8.3 Methanol/Chloroform Precipitation of Proteins....74
2.8.4 Quantitative Antigen-Inhibition ELISA Assay for Enoyl-ACP Reductase....74
2.8.5 SDS/Polyacrylamide Gel Electrophoresis (SDS/PAGE)....76
2.8.6 Coomassie Blue Staining of SDS/PAGE Gels....77
2.8.7 Silver Staining of SDS/PAGE Gels....77
2.8.8 Chromophore Green Staining of SDS/PAGE....78
2.8.10 Western Blotting....79
2.8.11 Western Dot Blotting....80
2.8.12 Amino Acid Sequencing using Problott Method....81
2.8.13 Determination of Absolute Concentration of Pure Protein Sample by Hydrolysis....82
2.8.14 Gel Filtration of *Brassica napus* Crude Protein Extracts....83
2.9 Biological Activity Assays....83
2.9.1 Enoyl-ACP Reductase Enzyme Assay....83
2.9.2 Malonyl-CoA:ACP Transacylase (MCAT) Enzyme Assay....84
2.9.3 Ketoacyl-ACP Synthase III (KAS III) Enzyme Assay....85
2.10 Fatty Acid Analysis....85
2.10.1 Extraction of Fatty Acid Methyl Esters from *Brassica napus* seeds....85
2.10.2 GLC Analysis of Fatty Acid Methyl Esters....86
2.11 Plant Transformation Methods....87
2.11.1 Callus-*Agrobacterium* Mediated Transformation of *Brassica napus*....87
2.11.2 Selection of *Brassica napus* Transformants....88

2.11.3 *In Planta Agrobacterium* mediated transformation by infiltration of adult *Arabidopsis thaliana* plants.....88

2.11.4 Selection of *Arabidopsis* Transformants.....89

Chapter 3 Isolating a Full-Length KAS III cDNA from *Brassica napus*.....91

3.1 Introduction.....91

3.2 Results.....95

3.2.1 Preparing of KAS III DNA Probe.....95

3.2.2 Genomic Southern of *Brassica napus*, *Brassica oleracae* and *Brassica campestris* to calculate isoform number in *Brassica napus*.....95

3.2.3 Screening the Rape Embryo cDNA Library for a full-length KAS III cDNA.....100

3.2.4 PCR Analysis of Putative Positive Plaques.....100

3.2.5 Complete Sequencing of KAS III Clone 13.....103

3.2.6 Identification of KAS III Isoforms by PCR Analysis.....111

3.2.7 Northern Analysis of RNA Samples to Determine Transcript Size.....120

3.3 Discussion.....123

3.4 Summary.....125

Chapter 4 Raising an Antibody to *Brassica napus* KAS III.....126

4.1 Introduction.....126

4.2 Results.....128

4.2.1 Generation of KAS III Insert Containing Cloning Restriction Sites by PCR.....128

4.2.2 Preparation of KAS III pET11d KAS III Construct.....129

4.2.3 Analysis of Plasmids for Presence of KAS III Insert.....131

4.2.4 Transformation of KAS III pET11d into BL21*(DE3)* and Attempted Overexpression.....134

4.2.5 Further Overexpression Experiments of KAS III pET11d in BL21*de*.....135
4.2.6 Scale up of Overexpression of KAS III pET11d.....138
4.2.7 Disruption of Cells Containing Overexpressed KAS III.....142
4.2.8 Extraction of KAS III Protein by Gel Electroelution.....144
4.2.9 Sequencing of Purified KAS III Protein.....147
4.2.10 Raising an Antibody to Purified KAS III Protein.....147
4.2.11 Analysis of Test Bleeds.....148
4.2.12 Antibody Recognition of KAS III in Crude Plant Extracts.....152
4.3 Discussion.....157
4.4 Summary.....161

Chapter 5 Development of a Quantitative ELISA and Developmental Analysis of Wild Type *Brassica napus* Leaf.....163
5.1 Introduction.....163
5.2 Results.....166
5.2.1 Development of an ELISA assay to Quantify ENR Levels in Crude Rape Extracts.....166
5.2.2 Optimising Antigen Coating Levels and Concentrations of Primary and Secondary Antibody.....167
5.2.3 Initial Antigen Inhibition ELISA Experiment.....170
5.2.3.1 Preparation of Plates.....170
5.2.3.2 Antigen-Antibody Incubation in Solution.....170
5.2.4 Spiking Crude Extract with Pure Enoyl-ACP Reductase.....175
5.2.5 Antibody Recognition Experiment – 1: Mechanical Manipulation.....175
5.2.6 Antigen Recognition Experiment – 2: The Effect of Denaturation.....179
5.2.7 Confirmation of True Positive ELISA Results from Guanidine-HCl Treated Extracts.....182

5.2.8 Improving the Speed of Removal of Guanidine-HCl from Denatured Extracts....182
5.2.9 Using ELISA to Examine ENR Levels in Leaf and Seed Extracts....184
5.2.10 Using the ELISA in analysis of Transgenic *Brassica napus* ENR Lines....188
5.2.11 ENR-ELISA of a Crude Rape Seed Extract after Fractionation by Gel Filtration....189
5.3 Developmental Standard Profile of Brassica napus Leaves....193
5.3.1 Growth of Plant Material and Preparation of Protein Extracts...193
5.3.2 ELISA Assays for ENR and BKR Quantification....201
5.3.3 Western Blot Analysis of KAS III....203
5.3.4 Fatty Acid Analysis of Leaf One of Brassica napus DH RV28...204
5.4 Discussion....210
5.5 Summary....215

Chapter 6 Antisense KAS III *Brassica napus* Plants....216
6.1 Introduction....216
6.2 Results....221
6.2.1 Construction of an antisense KAS III Vector....221
6.2.1.1 Preparation of antisense KAS III Fragment....222
6.2.1.2 Insertion of KAS III cassette from pJKD1.1 into pSCVnos....225
6.3 Creation of Antisense KAS III *Brassica napus* and *Arabidopsis thaliana*....227
6.3.1 Transformation into *Brassica napus*....227
6.3.2 PCR analysis to determine KAS III Construct Presence in *Brassica napus*....227
6.3.3 Transformation into *Arabidopsis thaliana*....230
6.3.4 Selection of Transformed *Arabidopsis* lines....231

6.3.5 PCR analysis to determine KAS III construct presence in *Arabidopsis thaliana*.....234
6.4 Transgenic *Brassica napus* ENR lines.....236
6.5 Analysis of Transgenic *Brassica napus* lines.....237
6.5.1 FAME analysis of T1 Seed.....237
6.5.2 Analysis of KAS III levels in transgenic lines.....242
6.5.3 Analysis of ENR levels.....242
6.5.4 Determination of copy number by southern hybridisation.....245
6.5.5 Phenotypic Comparison of Transgenic Lines to wild type.....251
6.5.5.1 Germination and Growth rates.....251
6.5.5.2 Plant Physiology.....251
6.5.5.3 Seed Morphology.....252
6.6 Discussion.....266
6.6.1 Creating Transgenic Lines.....266
6.6.2 Analysis of Transgenic *Brassica napus*.....267
6.6.2.1 FAME Analysis.....267
6.6.2.2 KAS III Protein Levels in Transgenic *Brassica napus*...269
6.6.2.3 Analysis of ENR Protein Levels in Transgenic *Brassica napus*.....269
6.6.2.4 Transgene Copy Number in Transgenic *Brassica napus*.....270
6.6.2.5 Changes in Plant Phenotype.....270
6.6.2.6 Germination Rates.....273
6.6.3 Future Experiments.....274
6.7 Summary.....275

Chapter 7 General Discussion.....276

7.1 A Full-Length KAS III cDNA Clone was Isolated.....276

7.2 Polyclonal KAS III Antisera Recognises 38kDa and 45kDa Proteins in *Brassica napus*.....277

7.3 A Quantitative Enoyl-ACP Reductase Antigen Inhibition ELISA was Developed.....278

7.4 Examination of a Standard Profile for *Brassica napus* cv. DH RV28....279

7.5 Some Transgenic FAS Plants can be seen to be Slower Growing.....280

7.6 Implications in Regulation.....282

7.7 Future Direction.....284

Appendix A - List of KAS III Transformants with Phenotype.....286

Appendix B – List of Suppliers.....288

Bibliography.....290

Chapter 1

General Introduction

1.1 Fatty Acid Structure and Function

A fatty acid is an organic compound consisting of a hydrocarbon chain and a terminal carboxyl group (figure 1.1). Fatty acid chain length is known to range from one to thirty carbon atoms but the most common fatty acids found in plants are between twelve and twenty two in length. Fatty acids are an essential component of all living organisms. They serve structural, metabolic and storage functions and are mainly found in the cell as constituents of lipids, prominently glycerolipids, sterols and waxes.

Commercially and scientifically there is considerable interest in plant fatty acids and lipids. The reasons for this include the high variation of plant lipids which differ considerably from those found in animal cells. Mammals cannot synthesise many polyunsaturated fatty acids (e.g. α-linoleic acid) and so certain plant lipids are essential dietary components for mammals.

Plant storage lipids provide a large concentrated reserve that can be readily utilised and provide a good source of oil for both the chemical and food industries. It has been estimated that the chemical industry alone utilises 9,500 kilotonnes of oils and fats per year (Somerville,

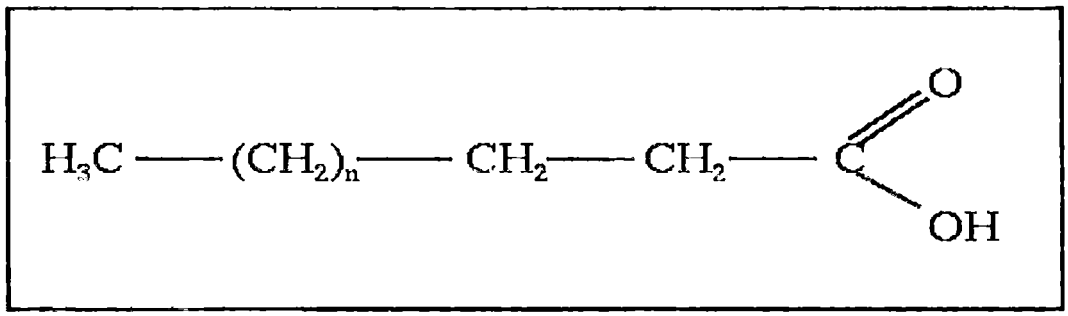


Figure 1.1 Structure of a Straight Chain Fatty Acid. This organic compound consists of a hydrocarbon chain and a terminal carboxyl group.

1993). This source could also in the long term provide a suitable, renewable alternative to fossil fuels.

Glycerolipid is the generic term for a variety of lipids that are esters containing a glycerol backbone to which three individual residues are esterified. One such important class of glycerolipid is the phospholipid. Phospholipids are the main structural components of cell membranes and fatty acids can be found be at the *sn*-1 and *sn*-2 positions with a phosphate ester attached at the *sn*-3 position (figure 1.2 (a). Within the structure of a biological membrane, it is thought that most of the lipid is present as a bimolecular sheet with the fatty acid chains in the inner part of the bilayer. The lipid bilayer serves as an environment for insertion of membrane proteins due to the ionic interactions between the phosphate headgroups of the phospholipid and the membrane proteins. These interactions, plus hydrophobic interactions between the fatty acid chains and hydrophobic regions of the proteins, create an environment which can act as a physical barrier and at the same time allow

the membrane to serve as a selective filter and a device for active transport (Albert *et al.*, 1989).

A second important glycerolipid group, which are of particular importance in plants, is the galactolipid (figure 1.2 (b). This class has a galactose residue at the *sn*-1 position and fatty acid moieties at the *sn*-2 and *sn*-3 positions. Galactolipids are a main constituent of chloroplastic membranes. The third main class of glycerolipid is the acylglycerol (figure 1.2 (c). Acylglycerols are a major storage lipid, the most common of which is the triacylglycerol. Three fatty acids are esterified to the glycerol backbone and this molecule provides a high-density energy store which can then be easily utilised via β-oxidation.

There are a variety of other diverse, less well defined functions involving fatty acids and lipids. In plants there is some evidence to show that lipid molecules play a key role in signalling and recognition in host range specificity of nodulating bacteria (Lerouge *et al.*, 1991). Slabas *et al.* (1992) have shown that there is significant homology of the amino acid sequence of β-ketoacyl-ACP reductase to the *NodG* gene product, which previously had no function assigned. Also *NodE* has been shown to be highly homologous to a condensing enzyme. These findings further indicate that it is likely that there is a need to synthesise fatty acids during nodulation (Hopwood and Sherman, 1990). Fatty acids can also act as pre-cursors in the synthesis of jasmonic acid (Vick and Zimmerman, 1984) and fatty acids are a main constituent of the epicuticular wax layer in leaves (Post-Beittenmiller, 1996), which can act as a barrier against fungal attack (Kolattukudy, 1987).

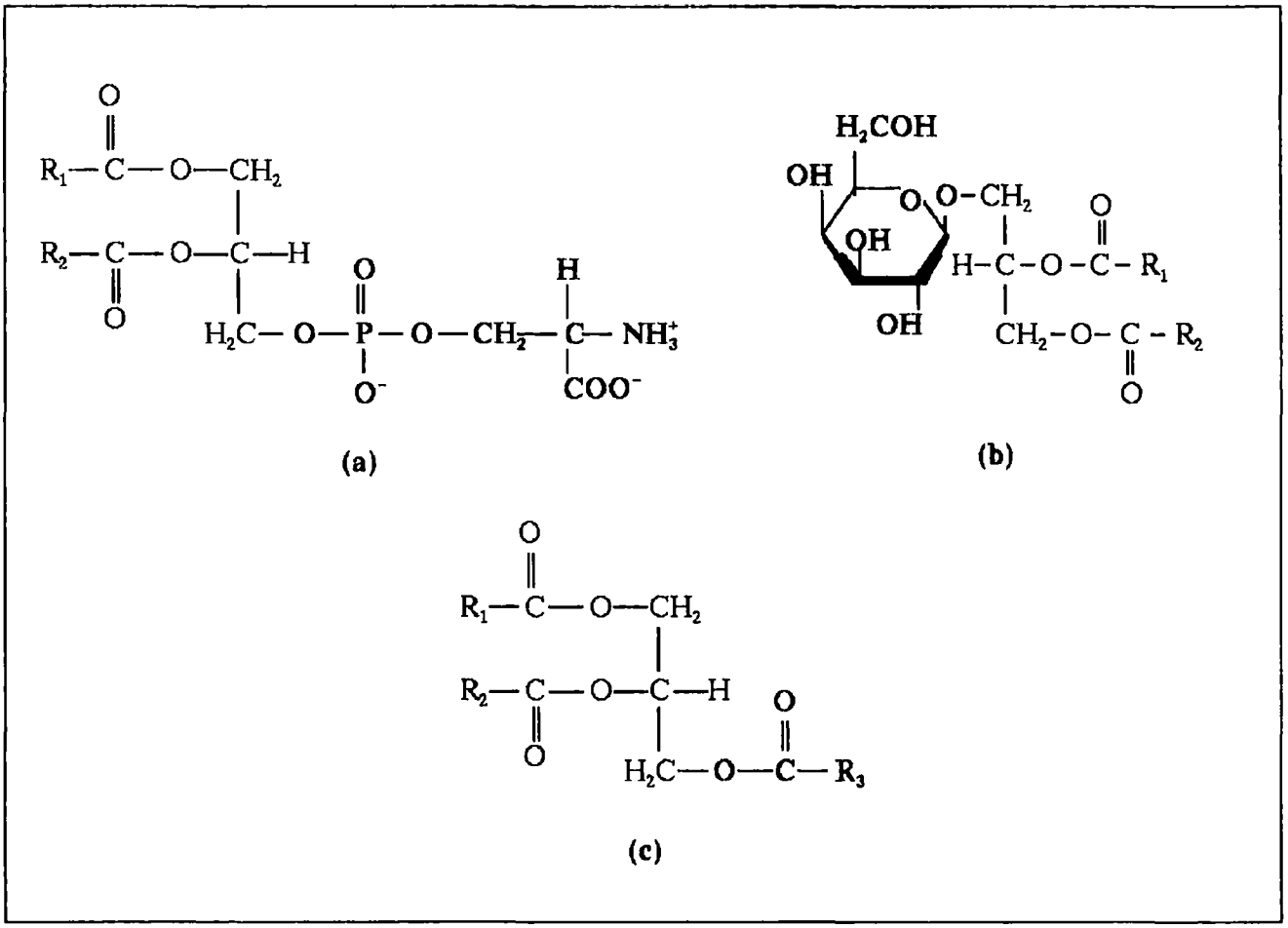


Figure 1.2 The Molecular Structures of the Main Glycerolipids.

(a) Phosphatidylserine, an example of a phospholipid – Positions *sn*-1 and *sn*-2 have acyl chains and *sn*-3 has a phosphate head group attached. (b) Monogalactosyldiacylglycerol an example of a galactolipid - Positions *sn*-2 and *sn*-3 have acyl chains attached, with a galactose residue at *sn*-1. (c) Triacylglycerol – Fatty acids are esterified at all three positions on the glycerol backbone. R denotes an acyl chain.

1.2 Fatty Acid Synthesis - Introduction

Fatty acids are essential to the growth and maintenance of every cell and this has led to the extensive investigation of the fatty acid synthesis pathway. The basic biochemistry is similar in all organisms and the enzymes which catalyse the reactions are collectively known as fatty acid synthases. The *de novo* synthesis of fatty acids requires the co-ordination of several enzyme activities. The synthesis is seen to proceed in a cyclic manner with the fatty acid chain elongated by the sequential addition of two-carbon units (figure 1.3).

Two types of fatty acid synthase (FAS) organisation are known. Type I FAS is found predominantly in animals (Amy *et al.*, 1989) and yeast (Schweizer *et al.*, 1984, Schweizer *et al.*, 1986, Chirala *et al.*, 1987). It is made up of one or two multifunctional polypeptide chains (Stoops *et al.*, 1975, Witkowski *et al.*, 1991). Separate domains on the peptide catalyse individual reactions of fatty acid synthesis. Type II FAS is found in plants and some bacteria, such as *Escherichia coli,* the enzymatic activities of this type of FAS exist as discrete monofunctional polypeptides. It is thought that the enzymes form a complex in order to synthesise fatty acids and each enzymic component can be readily dissociated and purified (Volpe and Vagelos, 1973).

FAS in animals has been shown to be a homodimer of 450-550kDa, with a subunit of 263kDa. A subunit is defined as the acyl carrier protein site, with a 4'phosphopantetheine group along with the seven catalytic activities required to synthesise fatty acids. A model for Type I FAS in yeast has been suggested from the study of yeast FAS mutants (Schweizer *et al.*, 1984). The

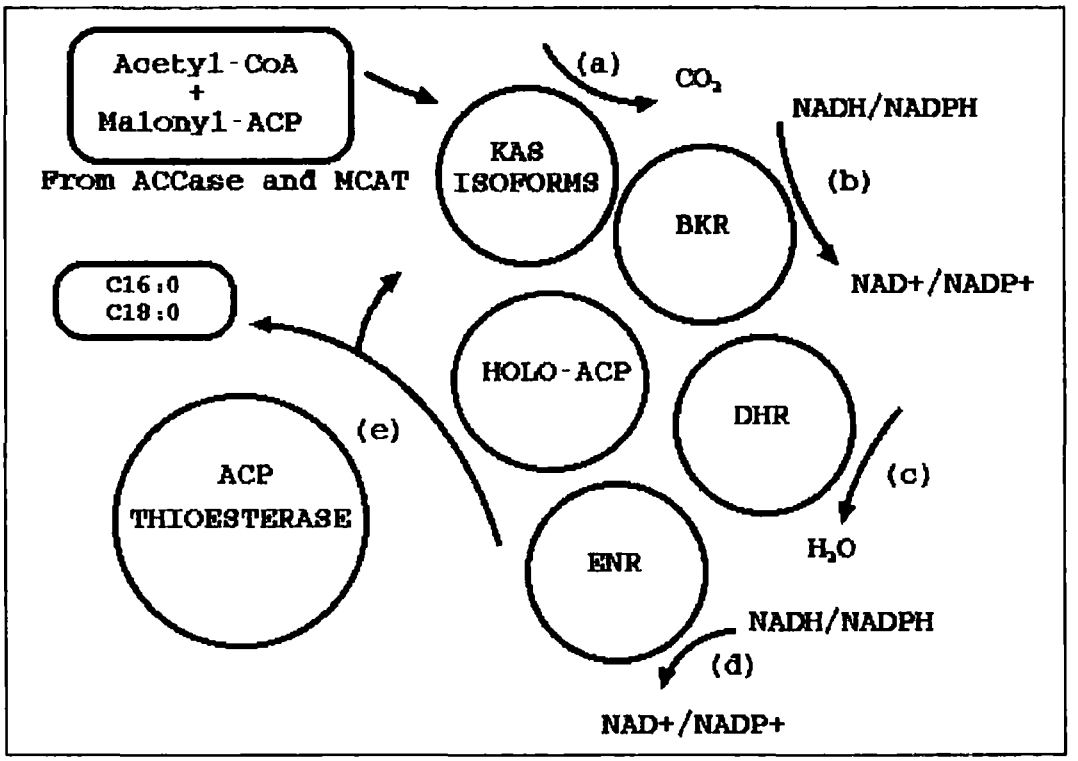


Figure 1.3 The synthesis of fatty acids by the sequential addition of two carbon units in type II fatty acid synthase.

Condensation of acetyl-CoA and malonyl-ACP and subsequent condensation reactions releases carbon dioxide (a). Reduction catalysed by BKR by the oxidation of a co-factor (b). This is followed by the removal of a water molecule by DHR (c) and a further reduction step (d). This cycle is repeated until chain length termination by ACP thioesterase (e). See section 1.4 for a detailed description of individual roles of each enzyme.

KEY: KAS - Ketoacyl-ACP synthase, BKR - β-Ketoacyl-ACP reductase, ENR – Enoyl-ACP reductase, DHR - β-Hydroxyacyl-ACP dehydratase, ACCase – Acetyl-CoA carboxylase, MCAT – Malonyl-CoA:ACP transacylase.

model proposes that the yeast FAS is made up of 2 chains of heterodimers ($\alpha_2\beta_2$), where half of the dimer is functional at any one time. The α chain contains two enzyme activities plus ACP and the β chain contains five different activities. This is reviewed in more detail in Wakil *et al.* (1983).

In prokaryotes fatty acid synthesis occurs in the cytosol. This is the same for the synthesis of palmitic acid in yeast and animals. However, in plants it has been shown that all the fatty acids that a plant cell requires are formed in the chloroplast or plastid. A two-compartmental model for *de novo* fatty acid synthesis in plants has been put forward to take into account this plastid localisation (Stumpf, 1980). Upon conversion of acetate or a derivative (see section 1.3) to acetyl-CoA within the plastid, the molecule is carboxylated to malonyl-CoA by ACCase (section 1.4.1). Both acetyl- and malonyl-CoA are then used to synthesise fatty acids. The growing acyl chain is attached to an acyl carrier protein (ACP) and once a fatty acid of up to C18:0 has been synthesised, a thioesterase terminates chain elongation. Desaturation occurs and the fatty acid is either incorporated into the lipids required by the plastid or exported to the cytoplasm after hydrolysis into free fatty acids. The fatty acids are metabolised further and used to synthesise such molecules as triacylglycerols.

1.3 The Source of Acetyl-CoA

Acetyl-CoA is the pre-cursor for a variety of biochemical plant components and all the carbon atoms used to synthesise fatty acids are derived from this pool of acetyl-CoA. In animals and yeast the accepted precursor for acetyl-CoA synthesis is generated by ATP:citrate lyase

(Wakil *et al.*, 1983). Although in plants acetyl-CoA is ultimately derived from products of photosynthesis, the way in which this pre-cursor is derived has been the subject of intensive investigation.

Acetyl-CoA cannot cross membranes and so it must ultimately be synthesised in the plastid for use in *de novo* fatty acid synthesis. Although acetate has been shown to play a role in the synthesis of the acetyl-CoA pool (Ohlrogge *et al.*, 1978) it may not be the direct precursor for plant lipid synthesis. Observations by Heintz *et al.*, (1990) also see acetate as the preferred pre-cursor for fatty acids in developing barley leaves. Roughan (1995) analysed acetate concentration in spinach leaves and found that levels are two to three times higher in the light than in the dark. It was suggested by this study that these levels were high enough to drive *in vivo* fatty acid synthesis at maximum rates.

Several other models for other possible pre-cursor substrates have been put forward, the first of which involves the use of cytoplasmic acetate (Liedvogel and Stumpf, 1982, Murphy and Stumpf, 1981). Acetate was incorporated into fatty acids at three times the rate of pyruvate and is preferentially used in the synthesis of fatty acids in spinach leaf. Mitochondria have an active pyruvate dehydrogenase (PDC) and it has been suggested that acetyl-CoA is synthesised here and hydrolysed to acetate in order to cross the mitochondrial membrane. It is then converted back to acetyl-CoA once it has been translocated into the plastid.

A second model involves the production of acetyl-CoA from pyruvate and CoA catalysed by the chloroplastic form of pyruvate dehydrogenase (PDC). Chloroplastic PDC activity has

been demonstrated in pea (Camp *et al.*, 1985.), maize and spinach (Treedle *et al.*, 1986). Further studies by Kang and Rawsthorne (1994) have showed that a range of substrates can be used for starch or fatty acid synthesis by plastids isolated from the embryos of oil seed rape. This work showed that pyruvate was the most effective for fatty acid synthesis. It is possible that different synthesis of acetyl-CoA occurs in different tissues in the plant.

Masterson *et al.* (1990) have also attempted to answer the precursor problem. The experiment tested four precursors that had the potential to create acetyl-CoA for fatty acid synthesis in pea leaf extracts. From the four precursors, the study suggested that L-acetylcarnitine was the most likely precursor. L-acetylcarnitine is converted to acetyl-CoA in a readily reversible reaction catalysed by a carnitine acetyl-transferase. Mclaren *et al.*, (1985) have reported the existence of a chloroplastic carnitine acetyltransferase in pea and Burgess and Thomas (1986) have demonstrated the existence of a mitochondrial form in pea. The model suggests that the acetyl-CoA in the mitochondrial matrix could be converted to L-acetylcarnitine and then exported out of the mitochondria via a carnitine:acetylcarnitine translocator. This molecule could then enter the chloroplast via a carnitine acetyltransferase. However, further experiments carried out by Roughan *et al.* (1993) have implied that acetyl-carnitine is not as an important precursor of acetyl-CoA as first thought. They found that 0.4mM L-acetylcarnitine could not compete with 0.2mM acetate as a substrate for fatty acid synthesis. In addition to this they were unable to detect carnitine acetyl-transferase activity in chloroplast preparations.

The use of malate has also been explored as a substrate for lipids (Smith *et al.*, 1992), where it has been proposed that once sucrose is imported into developing endosperm, it is converted to

malate. Smith *et al.* (1992) showed that in leucoplasts of developing castor endosperm malate, and to a lesser extent pyruvate, gave the greatest rates of fatty acids *in vitro*. Eastmond *et al.*, (1997) examined the processes by which malate and pyruvate are taken up across the leucoplast envelope and propose that leucoplasts from developing castor endosperm contain a malate/Pi translocator that imports malate for fatty acid synthesis. Malate enters the plastid and is subsequently converted to pyruvate and then to acetyl-CoA by PDH. Kang and Rawsthorne (1994) have also proposed glucose 6-phosphate (G-6-P) as a potential pre-cursor for plastidial acetyl-CoA. They showed that G-6-P was used at 70% of the rate of pyruvate in fatty acid synthesis.

Ohlrogge *et al.*, 1993 suggested that the involvement of ATP:citrate lyase in generating acetyl-CoA should be investigated further in plants. Wurtele *et al.* (1998) have recently identified two *Arabidopsis* cDNA clones for ATP:citrate lyase (ACL). Upon Northern analysis, these two cDNAs were seen to be upregulated during silique development. The mRNAs accumulated in flower buds and developing siliques as well as in epidermal cells of growing organs. It was observed that the pattern of accumulation was similar to that of cytosolic ACCase and distinct from plastidial ACCase (section 1.4.1). This study also included an investigation of two mechanisms which may be involved in plastidic acetyl-CoA synthesis; pyruvate dehydrogenase (pPDH) and acetyl-CoA-synthetase (pACS). Both cDNAs were cloned from *Arabidopsis* and pPDH mRNA was seen to accumulate during the heart and torpedo stages of embryogenesis, indicating that it may play an important role in the generation of acetyl-CoA within the plastid. Conversely pACS mRNA accumulated at low levels. Acetyl-CoA synthetase has been purified from spinach (Zeiher and Randall, 1991) and

seen to be localised in the chloroplast and as well as being inhibited by the accumulation of acetyl-CoA (Kuhn *et al.*, 1981). Hence this enzyme has been assumed to play a key role in chloroplastic acetyl-CoA synthesis. As pACS mRNA expression levels in *Arabidopsis* were reported to be low, it is possible that alternative pathways to plastidial acetyl-CoA synthesis, other than acetyl-CoA synthetase, exist. However, Northern analysis may indicate when transcripts are present but may not necessarily correlate to enzyme levels or longevity of the enzyme within the cell.

Therefore the debate into acetyl-CoA synthesis and its likely precursor in fatty acid synthesis still continues and currently there is no consensus opinion for this pathway in plants. Due to the high demand of acetyl-CoA in the synthesis of such phytochemicals as flavonoids and D-amino acids, as well as fatty acids, it is highly likely that plants have a number of mechanisms in order to synthesise this important molecule. Indeed Wurtele *et al.*, (1998) suggest that the multiplicity in acetyl-CoA generating systems could enable plants to differentially regulate the supply of this pre-cursor to each biosynthetic pathway which requires it. It is also possible that acetyl-CoA is synthesised in a various differing ways according to the plant species and tissue type.

1.4 The Individual Enzyme Reactions

This section will discuss the individual reactions required to make a fatty acid, particularly those of the type II FAS enzymes. The overall biochemistry is the same for both type I and type II FAS complexes. Due to both *E.coli* and plants containing the type II FAS, research into

plant lipids has been propelled by the vast research performed on *E.coli*. This is best demonstrated by the discovery of β-Ketoacyl-ACP synthase (KAS III) in *E.coli* (Jackowski and Rock, 1987), which led to the illustration of KAS III presence in plants (Jaworski *et al.*, 1989).

1.4.1 Acetyl-CoA Carboxylase

The first committed step in fatty acid biosynthesis is the carboxylation of acetyl-CoA to malonyl-CoA catalysed by acetyl-CoA carboxylase (ACCase), which is a member of a class of biotin containing enzymes. The generation of malonyl-CoA is a two step process (figure 1.4). First, the biotin carboxylase sub-unit transfers carbon to a nitrogen molecule present in the biotin ring; this step is ATP dependent. The intermediate formed is a carboxybiotin. The second step transfers the activated carbon to acetyl-CoA to form malonyl-CoA; this step is catalysed by the carboxyl-transferase.

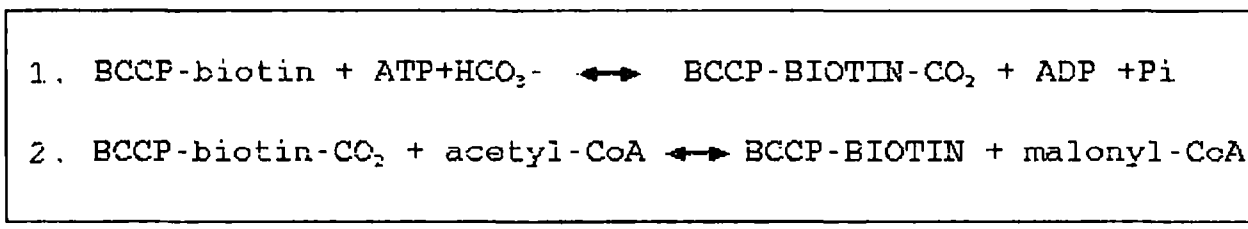


Figure 1.4 The two reactions of ACCase. (1) This ATP dependent step transfers carbon to the biotin ring catalysed by the biotin carboxylase sub-unit, to form a carboxybiotin intermediate. (2) The activated carbon is transferred to acetyl-CoA to form malonyl-CoA as catalysed by the carboxyl-transferase sub-unit.

In *E.coli* ACCase consists of four independent dissociable components; the biotin carboxyl carrier protein (BCCP), a biotin carboxylase sub-unit and the carboxyl-transferase component that is composed of an α and β sub-unit (Magnuson *et al.*, 1993). In *E.coli* cell extracts, the overall acetyl-CoA carboxylase reaction is lost as the enzyme easily dissociates and only the separate BCCP-biotin carboxylase and carboxyl-transferase components are detected. The overall reaction can be reconstituted *in vitro* by using high concentrations of the purified components (Gucchait *et al.*, 1974 and Polakis *et al.*, 1973). The genes encoding BCCP, biotin carboxylase and carboxyl-transferase subunits have been cloned (see table 1.1 for list of all fatty acid synthesis genes from *E.coli*). Genes designated *accB* (BCCP - Li and Cronan, 1992a) and *accC* (biotin carboxylase - Li and Cronan, 1992a) have been shown to be co-transcribed. Kondo *et al.* (1991), also independently observed that the *accC* ORF was located just downstream of the *accB* gene. The *accA* and *accD* genes which code for the α and β subunits of the carboxyl-transferase are not closely located to each other or to the *accB/C* operon (Li and Cronan, 1992b). The regulation of *E.coli acc* genes is unclear, *accB* and *accC* are co-transcribed and transcription of all four genes appears to be under growth rate control (Li and Cronan, 1993). This dissociable ACCase is commonly known as the prokaryotic form.

The multimeric form of ACCase is not only present in *E.coli*, it is also present in the plastids of many plants and the multimeric form of ACCase has been isolated from the chloroplasts of pea (Alban *et al.*, 1994). There has for sometime been much confusion as to the type of ACCase that was found in plants, although because of the need for malonyl-CoA in non-plastid reactions it was long thought that subcellular isoforms existed (Harwood, 1996). This

was confirmed by the isolation of a eukaryotic isoform from maize (Egli *et al.*, 1993, Herbert *et al.*, 1994), it differs from the prokaryotic form in that the components of the enzyme complex are present on a single, large multifunctional polypeptide similar to yeast and animal forms (Iverson *et al.*, 1990).

Which isozyme is present depends upon the type of plant. Many dicotyledonous plants possess both types; the prokaryotic form, which is situated in the plastid, and the eukaryotic form that is present in the cytosol (Konishi and Sasaki, 1994). This however is not the case for all plants and some plant families have been shown to contain the eukaryotic form in both compartments (Egli *et al.*, 1993, Konishi and Sasaki, 1994). This explains the observation of resistance of some herbicides by dicotyledonous plants and sensitivity in others such as the Graminaceae family. The herbicides, aryloxyphenoxypropionates and cyclohexanediones, have been shown to act on the eukaryotic form and ACCase is a much used target for herbicide attack (this is reviewed in more detail in Harwood, 1996).

The malonyl-CoA in the plastid is used in *de novo* synthesis of fatty acids in plants. It was considered that the prokaryotic form is of major importance in fatty acid synthesis, with the cytosolic form being utilised by other pathways, such as in the synthesis of flavonoids and anthocyanins (Hahlbrock, 1981) as well as production of cuticular lipids (Kolattukudy, 1987). However recent analysis of antisense ACCase I (eukaryotic form) in *Brassica napus* has shown that fatty acid levels are down regulated in embryos despite the antisense being directed to the eukaryotic form only (White *et al.*, 1998). This would suggest that ACCase I plays a greater role in *de novo* fatty acid synthesis than first thought. Schulte *et al.*, (1997) have also

examined the isoforms of ACCase, of which there are seen to be five, and observed that a possible transit peptide motif is present on a clone which encodes for an ACCase I isozyme. Therefore the role and position of the two ACCase isoforms is now further complicated and it is possible that both ACCase forms play a role in fatty acid synthesis depending upon the flux of malonyl-CoA required by the plant. ACCase I is known to supply malonyl-CoA to the secondary pathway of flavonoid synthesis. Flavonoid levels were seen to be affected by the down regulation of ACCase I as observed by the lack of accumulation of anthocyanin pigments in the testa of the seed (White *et al.*,1998). It is possible that this is an observation that malonyl-CoA in the cytosol is being directed to the more important primary fatty acid synthesis pathway in the transgenic lines in order to synthesise essential fatty acids or that the antisense of ACCase I is also affecting another isoform of ACCase I which may be targeted to the plastid.

Gene	Protein
aas	Acyl-ACP synthetase
accA	ACCase - Carboxyltransferase α-subunit
accB	ACCase - BBCP subunit
accC	ACCase - Biotin carboxylase subunit
accD	ACCase - Carboxyltransferase β subunit
acpP	ACP
acpS	ACP synthetase
fabA	β-Hydroxydecanoyl-ACP dehydratase
fabB	β-Ketoacyl-ACP synthase I
fabD	Malonyl-CoA:ACP transacylase
fabF	β-Ketoacyl-ACP synthase II
fabG	β-Ketoacyl-ACP reductase
fabH	β-Ketoacyl-ACP synthase III
fabZ	β-Hydroxyl myristoyl-ACP dehydratase
plsB	sn-glycerol 3 phosphate acyltransferase
plsC	1-acylglycerol 3 phosphate acyltransferase

Table 1.1 The genes mapped in *E.coli* that are involved in fatty acid synthesis.

There is currently considerable investigation into ACCase, as it has long been thought to be a regulatory enzyme in the synthesis of fatty acids in animals (Wakil *et al.*, 1983) and it is thought that a regulatory step in the *de novo* fatty acid synthesis in plants is highly likely to be exerted by ACCase (Post-Beittenmiller *et al.*, 1992). Studies by Page *et al.* (1994) used the herbicides fluazifop and sethoxydim to alter the activity of ACCase and measure the changes in flux through the lipid pathway. This demonstrated that ACCase exerts a strong flux control over lipid synthesis in plants. The vast interest in this enzyme has led to isolation of a variety of genomic and cDNA clones of ACCase isoforms from a number of plant species including *Arabidopsis* (Elborough *et al.*, 1994a) alfalfa (Shorrish *et al.*, 1994) maize (Somers *et al.*,

1993) and *Brassica napus* (Elborough *et al.*, 1994b). The enzyme has been isolated from many plant tissues including maize (Egli *et al.*, 1993) and wheat (Rendina and Felts, 1988). This is discussed in more detail in the reviews by Harwood (1996) and Töpfer and Martini (1994).

Studies into regulation of ACCase have shown that this enzyme is regulated by light in a possible redox modulation (Sauer and Heise, 1983). This was shown by incubating chloroplasts in both dark and light and observing the activity rate of the two samples. Continuation of this work to prove that the ACCase is regulated by a redox reaction had until quite recently failed to demonstrate this, however studies by Sasaki *et al.*, (1997) using dithiothreitol and thioredoxin as reductants in *in vitro* assays showed that plastidial ACCase was activated in the reductants presence. They suggest that the light directly regulates a site on the plastidial ACCase and the stromal pH and magnesium ion concentration, two other factors known to affect ACCase activity, are regulated by proton pumping in photosynthesis.

1.4.2 Acyl-Carrier-Protein

Acyl-Carrier-Protein (ACP) was the first protein involved in the synthesis of fatty acids to be purified from *E.coli* (Majerus *et al.*, 1964) and plants (Simoni *et al.*, 1967). It is a small molecule which varies from 8-10kDa depending up on the organism it has been isolated from. ACP is necessary to synthesise all *de novo* fatty acids and as its name suggests it is required to carry the growing acyl chain during the entirety of its synthesis. A thiol ester linkage attaches every fatty acid to an ACP molecule; this in turn activates the fatty acid and allows its involvement in metabolic reactions within the plastid (Majerus *et al.*, 1965).

In order for ACP to bind to an acyl chain it must be in its *holo* form. This requires that the ACP is attached by a serine residue to a 4'phosphopantetheine group. A thiol group connects the acyl chain. If the ACP is present in its *apo* form (i.e. minus the 4'phosphopantetheine group), then it cannot be utilised in fatty acid synthesis. *Holo*-ACP synthases have been found in both the cytosol and plastid of plants (Elhussein *et al.*, 1988, Fernandez and Lamppa, 1990). These enzymes are thought to play a role in the production of *holo*-ACP as well as have a protective role by re-forming *holo*-ACP that has lost its prosthetic group.

ACP has been purified from a variety of plants, including avocado mesocarp and spinach (Simoni *et al.*, 1967), barley (Hoj and Svedson, 1983) and oil seed rape (Slabas *et al.*, 1987). Slabas *et al.* (1987) demonstrated that ACP activity in oil seed rape was correlated with the deposition of storage lipid. The ACP activity rises rapidly and reaches a maximal rate but does not fall away again (at 46 DAF ACP activity is approximately 5.5dpm $\times$ 10^{-4}/seed). Lipid accumulation was observed later in the developing seed and at 46 DAF lipid deposition ceases to yield approximately 1.1mg of fatty acid per seed. As ACP activity was shown to increase with the onset of storage lipid biosynthesis this has suggested that the ACP genes could be regulated both in a tissue and temporal specific manner.

Protein purification and Western blots have unveiled the vast complexity of ACP isoforms in plants. Barley has been shown to contain at least three isoforms in the leaf alone (Hoj and Svedson, 1983). Comparison of the amino acid sequences of various ACPs reveals high sequence homology. Amino acid sequence information enabled the protein to be the first

central FAS component to be cloned from a number of plant species including spinach (Scherer and Knauf, 1987) and *B.napus* (Safford *et al.*, 1988). However, when *Brassica napus* seed and leaf ACPs were compared on a northern blot only the embryo RNA was highlighted by the embryo ACP cDNA probe (Safford *et al.*, 1988). This demonstrated that although there is strong homology between amino acid sequences from different species it is not reflected at the nucleotide level.

A genomic clone for ACP has been isolated from *Arabidopsis thaliana* (Post-Beittenmiller, 1986) and two from *B.napus* (de Silva *et al.*, 1990). The analysis of the clones revealed all of them to be nuclear encoded based on the observation that no sequences were seen on a tobacco plastid genome (Ohlrogge *et al.*, 1991).

Overexpression of ACP in transgenic tobacco has shown that high ACP levels do not appear to cause any alteration in lipid components (Post-Beittenmiller *et al.*, 1989). The study also demonstrated that the overexpressed *apo*-form was seen to be present in the chloroplast and therefore a phosphopantetheine group was not necessary for ACP uptake. However, experiments on the import of phosphopantethenylated precursor-ACP into spinach chloroplasts showed that if the chloroplasts were incubated simultaneously with pre-*apo*-ACP and pre-*holo*-ACP, that pre-*holo*-ACP was imported more efficiently (Savage and Post-Beittenmiller, 1994).

1.4.3 Malonyl-CoA:ACP Transacylase

In order for malonyl-CoA to be used in fatty acid synthesis the acyl group must first be transferred to ACP. This reaction is carried out by malonyl-CoA:ACP transacylase (MCAT). MCAT was first cloned from *E.coli* by complementation cloning (Verwoert *et al.*, 1992) utilising a heat sensitive strain known as the *E.coli fab*D mutant. MCAT has been purified from a variety of plants including spinach (Stapleton and Jaworski, 1984) and soybean (Guerra and Ohlrogge, 1986) and recently the isolation of MCAT cDNA clones from rape and maize has been reported (Simon and Slabas, 1998). The crystal structure for bacterial MCAT has been solved and shows that this protein belongs to a class of folded proteins of α/β type (Serre *et al*, 1995). Shimakata and Stumpf (1983a) showed that MCAT is highly active in spinach leaf and therefore is not thought to be a rate limiting step. This statement was supported when *E.coli* MCAT was overexpressed in oil seed rape (Verwoert *et al.*, 1994). Despite the level of MCAT activity increasing by as much as 55 times that of wild type, it did not did not effect the accumulation of storage lipid nor change the type of fatty acids produced.

1.4.4 The Condensing Enzymes - β-Ketoacyl-ACP Synthases

The first condensation reaction in plant and bacterial fatty acid synthesis was originally thought to be between acetyl-ACP and malonyl-ACP; the acetyl-CoA being converted to acetyl-ACP by acetyl-CoA:ACP transacylase (ACAT). The condensation reaction was established by isotopic experiments that showed that the acetyl groups from acetyl-ACP are exclusively located at the methyl end of a fatty acid (Gurr and Harwood, 1991). Initial studies

into the condensation reactions of FAS isolated two proteins; β-ketoacyl-ACP synthase I (KAS I) and β-ketoacyl-ACP synthase II (KAS II). Shimakata and Stumpf (1982a and 1983b) used spinach leaf extracts to show that KAS I readily utilises short fatty acid chains up to but not including palmitic acid (C16:0), to synthesise a β-ketoacyl-ACP. KAS II extends palmitic acid to stearate. Such substrate specificity was verified by the fact that KAS I is more sensitive to the antibiotic cerulenin than KAS II (Shimakata and Stumpf, 1983b). KAS I's increased sensitivity to cerulenin also aided in the purification of the two enzymes (Shimakata and Stumpf, 1983b). A pathway for the initial condensation reactions was put forward where KAS I condenses acetyl-ACP with malonyl-ACP to form acetoacetyl-ACP. The product is metabolised further by the other FAS components (figure 1.3). This is repeated up to 7 times where the appropriate condensing enzyme can add a 2-carbon unit to the growing acyl chain.

Until 1987 ACAT providing acetyl-ACP for utilisation by KAS I was the accepted model for the initial priming step in *de novo* fatty acid synthesis. However Jackowski and Rock, (1987) illustrated that a third condensing enzyme, insensitive to cerulenin, was present in *E.coli*. Acyl-ACP formation *in vivo* was not blocked by cerulenin and short chain (C4-C8) acyl-ACPs accumulated. This inferred the presence of a short-chain condensing enzyme. Its activity was demonstrated in plants (Jaworski *et al.*, 1989) and the protein has been purified from spinach (Clough *et al.*, 1992) and avocado (Gulliver and Slabas, 1994). The significance of this third condensing enzyme (KAS III) is that it was shown to use exclusively acetyl-CoA, not acetyl-ACP (Jackowski *et al.*, 1989). This obviates the need for ACAT, which until this discovery was thought to play a role in overall flux control of fatty acid synthesis. Jaworski *et al.*, (1993) analysed how acetyl-ACP and acetyl-CoA compared in the priming of fatty acid synthesis and

showed that acetyl-CoA was the most effective. KAS III is now accepted as catalysing the initial priming step in *de novo* fatty acid synthesis.

Experimentation has demonstrated that acetyl-ACP is not a major intermediate of fatty acid synthesis (Jaworski *et al.*, 1993) The exact role of ACAT is not known and there is evidence that KAS III has intrinsic ACAT activity (Tsay *et al.*, 1992, Clough *et al.*, 1992). Despite this it does appear that a specific ACAT enzyme exists in plants, which has been chromatographically separated from KAS III (Gulliver and Slabas, 1994, Harwood, 1996) and acetyl-ACP is still found in the chloroplast (Jaworski *et al.*, 1993). A possible explanation for this is that acetyl-ACP is a minor intermediate of fatty acid synthesis which is metabolised over a longer period. Winter *et al.* (1997) have put forward a model for regulation which proposes that the KAS enzymes have a decarboxylation activity which can convert malonyl-ACP to acetyl-ACP. The acetyl-ACP could be utilised in fatty acid synthesis or alternatively be converted to acetyl-CoA via the reverse reaction of ACAT and returned to the acetyl-CoA pool.

The roles of all three condensing enzymes have been the subject of thorough investigation. *E.coli* KAS I has been analysed and loss of a functional KAS I resulted in an unsaturated fatty acid auxotroph (D'Agnolo *et al.*, 1975). In *E.coli*, KAS I is thought to be the only condensing enzyme involved in the elongation of *cis*-3-decenoyl-ACP to palmitoleoyl-ACP, (KAS II can assist in the elongation of decanoyl-ACP to palmitoyl-ACP). The evidence for this is provided by the observation that when KAS I is overexpressed in *E.coli* there is an increase in *cis*-vaccenic acid content (deMendoza *et al.*, 1983).

In plants, KAS I has been purified and characterised in barley (Siggaard-Anderson *et al.*, 1991) and shown to be similar to the *E.coli* homologue and KAS I has also been purified from oil seed rape (Mackintosh *et al.*, 1989). KAS II has been partially purified from oil seed rape and is thought to have a sub-unit mass of 45kDa (Mackintosh *et al.*, 1989).

A variety of cDNA clones have been isolated for KAS III, the first of which was from *E.coli* (Tsay *et al.*, 1992). This was achieved using the antibiotic thiolactomycin to isolate a mutant shown to have decreased KAS III activity. This antibiotic is known to inhibit all KAS enzymes (Nishida *et al.*, 1986). The remaining KAS III appeared to be resistant to thiolactomycin treatment. The *E.coli* mutant did not possess a growth phenotype which also suggests that there are other mechanisms of initiating fatty acid synthesis. However this mutant was not completely lacking KAS III activity and it is possible that the low levels of KAS III were sufficient to drive the priming step of fatty acid synthesis. KAS III cDNA clones have been isolated from a number of plants including leek (Chen and Post-Beittenmiller, 1996), spinach (Tai and Jaworski, 1993), *Cuphea* (Slabaugh *et al.*, 1995) and also from an alga *Porphyra* (Reith, 1993). KAS III is the subject of much investigation as it is thought to have a role in the regulation of fatty acid synthesis as implied by analysis of reaction substrates and products (Post-Beittenmiller *et al.*, 1991) as well as a demonstration by Heath and Rock (1996c) that this enzyme is the subject of regulation by long chain acyl-ACPs (discussed further in section 1.8). Finally, there is now much discussion of the existence of a fourth condensing enzyme, particularly in plants which synthesise a considerable amount of medium

chain fatty acids. Dehesh *et al.* (1998) have recently reported the purification of such an enzyme from *Cuphea wrightii.*

A model for the mechanism of condensing enzymes' active sites has been suggested by Siggaard-Anderson (1993) which proposes that there are three partial reactions involved in a condensation, this involves five basic residues. The first step in condensation involves the transfer of the substrate to be elongated from the pantotheine moiety of an acyl carrier a substrate-binding residue. This residue has been identified as a cysteine. The first two bases are involved in substrate binding by enhancing the electrophilic nature of the substrate and heightening the nucleophilic nature of the cysteine residue. The following step forms a carbanion by decarboxylation of a second substrate, malonate. The second two bases enhance decarboxylase activity where the bases position the substrate and stabilise the intermediate in order for the final reaction to occur. Nucleophilic attack by the carbanion on the first bound substrate then produces a new carbon-carbon bond. The fifth central base (cysteine) improves the electrophilicity of the intermediate substrate and stabilises the product before its release. Site–directed mutagenesis (SDM) has been used to manipulate the active sites in β-ketoacyl ACP-synthases. When the conserved cysteine was changed to an alanine or serine residue in spinach KAS III, it was seen that the product of KAS III condensation reactions, acetoacetyl-ACP, did not form and the alanine mutant could not perform acetylations (Jaworski and Hinneburg-Wolf, 1998). Both mutants retained their ability to decarboxylate malonyl-ACP.

Further studies defining the function of highly conserved residues in β-Ketoacyl-ACP-Synthases (Siggaard-Anderson *et al.*, 1998) appears to have confirmed the hypothesis for the

first two partial reactions of a condensing enzyme. When *E.coli* KAS I was mutated by turning the cysteine residue at position 163 to an alanine it was seen that it could not transfer the acyl group as it lacked the substrate binding residue. However it was observed that the decarboxylation reaction of this mutant condensing enzyme was favoured due to the inability of the enzyme to carry out acylations. When the cysteine residue was mutated to a serine the ability to carry out acylations was heightened and there was a much reduced activity for decarboxylation compared to wild type *E.coli*.

The cysteine at position 111 of a recombinant KAS III from *Cuphea wrightii* has been put forward as the active site residue by Abbadi *et al.*, (1998). To confirm the cysteine's involvement in acetyl-CoA binding, the amino acid was converted to serine and alanine residues in SDM experiments. While both of these mutations led to a complete loss of condensing activity, the serine mutant was still able to covalently bind the substrate. During this study the histidine residue at position 261 was also mutated. It was seen that when the amino acid was mutated to alanine the KAS III activity was undetectable which was consistent with the suggested role of this residue to act as a general base which removes a proton from a sulphur of the cysteine at position 111. When histidine^{261} was replaced with an arginine (a hydrophilic amino acid with basic side chains, as is histidine) there was attenuation of the condensation activity suggesting that arginine can to a point mimic the role of histidine. These studies can assign precise roles to individual residues and so move towards an understanding of specificity in β-ketoacyl-ACP synthases and the mechanisms of catalysis.

1.4.5 β-Ketoacyl-ACP Reductase

After each condensation reaction the initial product is a β-ketoacyl-ACP. This product is reduced at the carbonyl group by a β-ketoacyl-ACP reductase (βKR) to produce a β-hydroxyacyl-ACP. The enzyme uses NADPH or NADH as the electron donor, but the plant form uses NADPH specifically. This is the first of two reductive steps in fatty acid synthesis. βKR has been purified from spinach (Shimakata and Stumpf, 1982b), avocado (Sheldon *et al.*, 1988) and oil seed rape (Sheldon *et al.*, 1992).

In rape seed βKR activity is induced at the same time as many of the other components of FAS, when storage lipid biosynthesis is at a maximum. The enzyme level decreases during the desiccation stage of seed development. The amino acid sequences of *Brassica napus* and *Arabidopsis thaliana* have been compared with the *NodG* gene product of *Rhizobium meliloti* where considerable homology can be seen (Slabas *et al.*, 1992). cDNA clones have been isolated from *Arabidopsis*, *Brassica napus* (Slabas *et al.*, 1992) and *Cuphea lanceolata* (Klein *et al.*, 1992).

1.4.6 β-Hydroxyacyl-ACP Dehydratase

β-Hydroxyacyl-ACP dehydratase carries out the removal of water from β-hydroxyacyl-ACP to form 2,3-*trans*-enoyl-ACP. In *E.coli*, β-hydroxyacyl-ACP dehydratase has been isolated in two different forms. These proteins, known as FabA (Birge and Vagelos, 1972) and FabZ (Heath and Rock, 1996a), have been shown to have different substrate specificities. FabA not

only demonstrates dehydratase activity but also isomerase activity. The isomerase activity converts *trans*-2-decanoyl-ACP into *cis*-3-decenoyl-ACP and this product is the direct precursor of synthesis of unsaturated fatty acids in *E.coli*. FabZ substrate specificity has been studied, and the enzyme is shown to prefer short chain *trans*-hydroxyacyl-ACPs. However the main difference between the two proteins is that FabA is inactive in the elongation of *cis*-unsaturated fatty acids (Heath and Rock, 1996a). β-hydroxyacyl-ACP dehydratase was amongst the first FAS enzymes to be purified from a plant (Shimakata and Stumpf, 1982b) but no more work was carried out on this enzyme until recently within the University of Durham laboratory. A cDNA clone has now been isolated from oil seed rape using a sequence derived from a castor bean EST database and was shown to be 75% identical and 83% similar at the amino acid level to the *E.coli* FabZ protein (Doig *et al.*, 1998).

1.4.7 Enoyl-ACP Reductase

Fatty acid reduction is completed by the final enzyme in the FAS complex, enoyl-ACP reductase (ENR). This enzyme reduces the *trans*-2 double bond to form a saturated fatty acid. ENR was first isolated from spinach and safflower (Shimakata and Stumpf 1982b) and it was demonstrated that safflower possessed two isoforms with either a preference for NADH or NADPH as a co-factor. Both isoforms of ENR are also present in *E.coli* (Weeks and Wakil, 1968). Studies by Slabas *et al.* (1986) purified the NADH-specific ENR to homogeneity and demonstrated that it was a tetramer consisting of an α_4 structure with a subunit mass of 35kDa. Kater *et al.* (1991) isolated a full length cDNA of ENR from *Brassica napus*. Southern analysis revealed there to be 4 genes, obtaining two from each ancestral parent. Fawcett *et al.*,

(1994) performed 2-D Western blots on *B.napus* seed and leaf and illustrated that all four isoforms were expressed in both tissue types.

Kater *et al.* (1994) used a hybrid genetic system to study the functional relationship between prokaryotic and plant FAS. The antibacterial agent diazaborine is a specific inhibitor of the bacterial ENR, whereas the plant enzyme is insensitive. The study involved genetically complementing an *E.coli* ENR mutant with the ENR from *Brassica napus*. They showed that *B.napus* ENR could functionally replace the lost ENR in *E.coli*. Upon analysis of the fatty acid composition of the phospholipids in the recombinant strain, it was observed that C18:1 content was significantly reduced and C16:1 accumulation had increased. In *E.coli*, therefore, it has been suggested that ENR is a rate limiting step in the conversion of C16:1 to C18:1.

Observations of sensitivity of bacterial ENR to diazaborine and the ability to crystallise this enzyme from *E.coli* (Baldock *et al.*,1998) and in plants (Rafferty *et al.*, 1995) has led to a flurry of investigation concerning the mode of action of this antibiotic using ENR as the model enzyme. Baldock *et al.* (1996) revealed that not only is ENR a target for diazaborines but also a target for the anti-tuberculosis drug; isoniazid. Structural analysis showed that when ENR is incubated with NADH and a diazaborine, a tight non-covalent bound bi-substrate analogue is formed. This study has vast implications in the structure base design of inhibitors of ENR and other reductases. This may eventually have applications in medicine, especially with the emergence of isoniazid resistant *Mycobacterium tuberculosis* strains (Baldock *et al.*, 1998) as well as the design of enzyme inhibitors for agricultural purposes (Stuitje *et al.*, 1998).

Stuitje *et al.* (1998) have examined the dynamic structure of ENR by the analysis of plant-ENR mutants in *E.coli*. Two groups of mutants were examined, one which affected active site residues and the other which affected arginine residues. The quaternary structure, affinity towards ACP and ability to interact with other mutant enzymes in order to restore *in vitro* activity was assessed for each mutant. The study revealed that all of the active site group residues were essential for *in vitro* enzyme catalysis. One arginine residue (Arg^{227}) was shown to be essential for proper folding and *in vivo* function. Substitution of two of the conserved residues (Arg^{193}/ Arg^{214}) did not affect the functional complementation *in vivo*. When these two mutants were overexpressed however ENR activity was not detected. Once the mutant proteins were mixed together in the presence of NADH and crotonyl-CoA, enzyme activity was restored. The finding that for active formation of an ENR heterotetramer a NADH co-factor is required, indicated the possibility that ENR activity *in vivo* may be controlled allosterically by the local NADH concentration.

1.4.8 Desaturation - Δ9- Stearoyl-ACP Desaturase

The main products of *de novo* fatty acid synthesis are saturated fatty acids but over 75% of fatty acids in plants are unsaturated. Further reactions must therefore occur that create the unsaturation. The main plant unsaturated fatty acids come from aerobic desaturation. Stearate is converted to oleate (C18:1) and this initial desaturation is carried out by the Δ9-stearoyl-ACP desaturase to place a double bond between the C9 and C10 carbon atoms. This can be followed by a second desaturation to form linoleic acid (C18:2), a double bond is placed between positions 12 and 13 (Δ12-desaturase). A final desaturation can occur to give α-

linolenic acid (C18:3); a double bond is positioned between C15 and C16 (Δ15-desaturase). These three C18 compounds constitute over 85% of total membrane acids and 80% of storage oils (Harwood, 1996). The second two desaturation steps occur once the acyl-ACP has been transferred onto a glycerol backbone and will be discussed later in section 1.6.

The Δ9-stearoyl-ACP desaturase is a soluble enzyme, found in the stroma of the plastid. Most other desaturases are membrane bound a known exception being Δ6-desaturase purified from *Thunbergia alata* (Cahoon *et al.*, 1994). On purification of Δ9-stearoyl-ACP desaturase from safflower (McKeon and Stumpf, 1982), it was found to be a homodimer of 68kDa. Shanklin and Somerville (1991) purified this enzyme to homogeneity from avocado, raised antibodies to this protein and immunoscreened castor and cucumber cDNA libraries and consequently isolated full-length clones. On comparison to animal and fungal homologues of Δ9-stearoyl-ACP desaturase (Thiede *et al.*, 1986, Stukey *et al.*, 1990), it was seen that these enzymes were structurally unrelated at the nucleotide level to the plant sequences. Confirmation of a Δ-9 stearoyl desaturase performing the same function was demonstrated by overexpression of the castor bean homologue in yeast. This conferred soluble desaturase activity upon the organism and so this work inferred that yeast and animal stearoyl-desaturases evolved independently of each other.

Studies by Thompson *et al.* (1991) demonstrated that if safflower Δ9-stearoyl-ACP desaturase is overexpressed in *E.coli*, there is no change in fatty acid composition. An *in vitro* assay showed that activity was only detected upon addition of ferredoxin as an electron donor. cDNAs have been isolated from other plant species including spinach (Nishida *et al.*, 1992),

Thunbergia alata (Cahoon *et al.*, 1994) and *Brassica napus* (Slocombe *et al.*, 1992). Analysis of temporal regulation of the *B.napus* embryo mRNA for Δ9-stearoyl-ACP desaturase showed that transcription was induced 25 DAF, peaked at 45 DAF and tailed off thereafter (Slocombe *et al.*, 1992). Analysis of the effect of temperature on the activity of the Δ9-stearoyl-ACP desaturase in soybean showed that as the temperature decreased, activity of the enzyme increased (Cheesbrough, 1993). Thus demonstrating that it is possible that plants can respond to environmental changes by increasing the levels of desaturation within their fatty acids. Analysis of an *Arabidopsis* mutant demonstrated to have increased levels of stearate showed this by severely affecting the morphology of the plant causing an extreme miniature growth (Lightner *et al.*, 1994a). It was shown that the whole plant contained higher stearate levels. Once moved to an higher temperature, the plant was restored to near normal growth. The authors suggest that the increase in C18:0 may have disrupted membrane function by decreasing membrane fluidity. Analysis of fatty acid composition of leaf lipids isolated from both the chloroplastic and extrachloroplastic membranes showed increased stearate content (Lightner *et al.*, 1994b). The level of unsaturated fatty acids in membranes is thus an important factor in survival at low temperatures.

1.4.9 Termination

Fatty acid synthesis is terminated by the removal of the fatty acid from the sulphydryl group of ACP. This can be carried out by an acyl-ACP hydrolase, a thioesterase. This free fatty acid is then subsequently exported to the cytosol and converted to acyl-CoA by acyl-CoA synthetase that is found on the chloroplast envelope (Joyard and Stumpf, 1982) and taken up by the

eukaryotic pathway to synthesise lipids. Alternatively it can be used as a substrate for complex lipid synthesis in the plastid using glycerol phosphate acyltransferases (section 1.6). It has been established that the chloroplast stroma contains a long chain acyl-ACP thioesterase. This enzyme has high specificity to oleoyl-ACP but very low activity towards palmitoyl- and stearoyl-ACP (Harwood, 1996).

Thioesterases have been studied in a variety of plants including oil seed rape (Helleyer *et al.*, 1990), safflower (Knutzon *et al.*, 1992), leek (Liu and Post-Beittenmiller, 1995) squash (Imai *et al.*, 1992) *Arabidopsis* (Dörmann *et al.*, 1995) and a host of species that predominantly synthesise medium chain fatty acids (Dörmann *et al.*, 1993).

ACP-thioesterase has been purified to homogeneity from oil seed rape (Helleyer *et al.*, 1992). It has a sub-unit of 38kDa and a native molecular weight of 70kDa. It has high specificity towards oleoyl-ACP, the product of the Δ9-stearoyl-ACP desaturase. Examination of the activity level of the thioesterase showed it to rise markedly prior to the onset of lipid accumulation. cDNA clones to the *B.napus* acyl-ACP thioesterase were subsequently isolated (Loader *et al.*, 1993). In members of the genus *Cuphea* the most abundant fatty acids in triacylglycerols ranges from C8-C14, depending on the species. Dörmann *et al.*, (1993) have partially purified two acyl-ACP thioesterases from *Cuphea lanceolata*. They were shown to be very different in their substrate specificity. One showing preference for medium chain acyl-ACPs, whilst the other had preference for oleoyl-ACP. These two isoforms were also found in *Cuphea wrightii*.

California bay laurel (*Umbellularia californica*) synthesises medium chain fatty acids (Pollard *et al.*, 1991) and when this gene was expressed in *Arabidopsis*, the resulting fatty acids were predominantly of medium chain length – C12:0 (Voelker *et al.*, 1992). The fatty acyl content of developing cotyledons of *Umbellularia californica* have been shown to change from a long chain composition to the major acyl content consisting of C10:0/C12:0 just five days after pollination, and thereafter be the dominant fatty acid (Maelor-Davies *et al.*, 1991). Jones *et al.* (1995) have determined two distinct categories of thioesterase (*Fat A* and *Fat B*), where they suggest *Fat A* is designated as the 18:1 thioesterase group. *Fat B* represents thioesterases which have a preference for saturated acyl groups. Upon isolation of a 16:0 ACP thioesterase cDNA from *Cuphea hookeriana*, which predominantly accumulates C8:0 and C10:0, the study proposed that this type of *FatB* gene is ancient and ubiquitous in plants. Comparison of all known thioesterase sequences placed the thioesterases in one of these two evolutionary linearages and the authors hypothesise that both of these are derived from the C16:0 thioesterase.

Medium chain fatty acids are of considerable interest biotechnologically due to their vast use in the food and chemical industries. It is thought that the cDNA clones isolated from such medium chain fatty acid synthesising plants can be expressed in commonly used crops such as oil seed rape without any adverse effect to the plant's physiology or future seed viability (Voelker *et al.*, 1992). High levels of C8:0 and C10:0 fatty acid in *Brassica napus* of any acyl-ACP thioesterase cDNA from *Cuphea hookeriana* has been reported (Dehesh *et al.*, 1996). The increase in these medium chain fatty acids resulted in a decrease in the typical C18:2 and C18:3 fatty acids normally detected in *Brassica napus* oil.

1.5 Further Metabolism of Fatty Acids

1.5.1 Elongation of Fatty Acids

Very long chain fatty acids (VLCFAs) are present in plants, particularly as constituents of wax, suberin and cutin (Kolattukudy, 1987). VLCFAs are important in the chemical industry (e.g. euricic acid – a lubricant within the plastics industry). Elongation beyond C18:0 occurs in the microsomes catalysed by fatty acid elongases. Each enzyme has chain length specificity (Harwood, 1996). Plant elongases have been studied extensively in microsome preparations from leek epidermal cells. These elongases preferentially utilise saturated fatty acids over unsaturated C18 substrates (Agrawal *et al.*, 1984).

Lessire *et al.*, (1989) examined the incorporation of malonyl-CoA into stearate and showed that in the presence of exogenous acyl-CoAs elongation to C24:0 occurred in a sequential manner in leek epidermal cells. This implied that elongation occurred in a cyclic manner similar to that of *de novo* fatty acid synthesis. Studies in developing *Lunaria annua* seed have demonstrated that intermediate products of such reactions can be detected by TLC analysis (Fehling and Mukherjee, 1991). A fatty acid elongase gene (FAE1) has been cloned by transposon tagging and found to share homology with KAS III and other condensing enzymes confirming that it is also a condensing enzyme (James *et al.*, 1995).

1.5.2 Acyl-CoA Binding Proteins

Free fatty acids are highly toxic to the cell and analysis of several types of mammalian cell has ascertained the existence of proteins with high affinity for free fatty acids and their CoA derivatives, an acyl-CoA- and fatty acid-binding protein. (Spener *et al.*, 1989, Knudsen, 1990). Acyl-CoA binding proteins are characterised by their small molecular weight (10kDa) and their higher affinity to bind to acyl-CoAs over non-esterified fatty acids. Functional homologues for acyl-CoA binding proteins (ACBP) in plants have been isolated in oat (Rickers *et al.*, 1984) and *B.napus* (Ostergaard *et al.*, 1993) and a cDNA cloned from *B.napus* (Hill *et al.*, 1994). Northern analysis showed that the genes encoding for ACBP were strongly expressed during embryo development. Johnson *et al.* (1998) suggest that acyl-CoA binding proteins may assist in regulation of *de novo* fatty acid synthesis by feedback regulation by demonstrating that ACBP stimulates fatty acid synthesis when glycerol 6-phosphate was used as an acetyl-CoA precursor. The study proposes that uptake of glycerol 6-phosphate as a fatty acid precursor is inhibited by acyl-CoA and stimulated by acyl-CoA-binding proteins.

1.6 Glycerolipid Synthesis in Plants

1.6.1 Membrane Lipid Synthesis

Five major fatty acids are utilised in lipid synthesis 18:1, 18:2, 18:3, 16:0 and 16:3 and constitute over 80% of the acyl chains in structural glycerolipids (Ohlrogge and Browse, 1995). The acyl chains form the hydrophobic portion of glycerolipid molecules. Higher plants

possess two distinct pathways for the synthesis of glycerolipids as demonstrated by Heinz and Roughan (1983). The first is known as the prokaryotic pathway and occurs within the inner envelope of the chloroplast. Lipids synthesised in the plastid characteristically have a C16:0 fatty acid placed at the *sn*-2 position of the glycerol backbone. This is due to the acyl-group specificity of acyltransferases found in various organelles. The second pathway for lipid synthesis, the eukaryotic pathway, begins with phosphatidic acid synthesis in the endoplasmic reticulum (ER). Lipids synthesised here have a C18 fatty acid at the *sn*-2 position and utilise acyl-CoAs rather than acyl-ACPs. Heinz and Roughan (1983) isolated chloroplasts from plants containing predominantly C18:3 or C16:3 and showed that 18:3 chloroplasts direct less fatty acids into phosphatidic acid and diacylglycerol synthesis than 16:3 chloroplasts. Analysis of monogalactodiacylglycerol (MGDG) formation showed very low levels accumulated in 18:3 chloroplasts and high rates in 16:3. This evidence proposed that there are differences between eukaryotic and prokaryotic pathways of MGDG biosynthesis, where 18:3 plants have lost the ability to synthesise prokaryotic MGDG and both types have the ability to synthesise eukaryotic MGDG.

Glycerolipid synthesis commences with two acylation steps. Fatty acids are transferred to glycerol 3-phosphate, which is carried out by the glycerol 3-phosphate acyltransferase (GPAT). This results in the formation of monoacylglycerol-3-phosphate. Lysophosphatidic acid acyltransferase (LPAAT) then transfers either 16:0 (prokaryotic) or 18:1 (eukaryotic) to the *sn*-2 position of the glycerol backbone to produce phosphatidic acid (PA). This molecule is the precursor for all lipids whether they are synthesised in the eukaryotic or prokaryotic pathway. PA provides the diacylglycerol portion of the lipid. Diacylglycerol (DAG) is

produced from PA by dephosphorylation carried out by a specific phosphatase. Alternatively it is produced by the reaction of phosphatidic acid with cytidine 5'-triphosphate to form CDP-DAG. It is DAG or CDP-DAG that is utilised in order to add the head group to position 3 of the glycerol moiety (Ohlrogge and Browse, 1995). The head group defines the glycerolipid and its role within the cell and the energy to drive attachment of the head group during *de novo* glycerolipid synthesis is supplied by nucleotide activation (CDP-DAG) or alternatively the headgroup is activated (DAG).

PA made in the prokaryotic pathway is used to synthesise phosphatidylglycerol (PG), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulpholipid (SL). Whereas, in the eukaryotic pathway PA is used to produce the phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). These eukaryotic pathway products are characteristic lipids found in extra-plastidial membranes. The relative flux through these two pathways varies from species to species and it is possible that a substantial proportion of the lipids that are synthesised in the ER are imported back into the chloroplast. Some plants have dispensed with the prokaryotic pathway altogether and other plants use the two pathways equally, and as a consequence may have greater metabolic flexibility. Heinz and Roughan (1983) suggest that from an evolutionary perspective 18:3 chloroplasts represent highly integrated organelles due the loss of prokaryotic features. Generally primitive angiosperm and lower plants have the highest amount of flux through the two pathways, but there is no taxonomic grouping of angiosperm families based on 18:3/16:3 plants. This implies that the loss of the prokaryotic pathway has occurred independently on a number of occasions (Somerville and Browse, 1991).

1.6.2 Lipid Desaturation

Desaturation reactions require molecular oxygen and so occur under aerobic conditions. This desaturation in glycerolipids is essential for the proper functioning of biological membranes (Los and Murata, 1998). Studying the desaturation of fatty acids has generally proved difficult, as apart from the Δ-9 stearoyl-desaturase, these enzymes are membrane bound and so troublesome to purify (Slabas and Fawcett, 1992). This led to a genetic approach in studying desaturation and lipid function (Gibson *et al.*, 1994). Mutant *Arabidopsis* lines were developed via ethyl methane sulphonate (EMS) and each viable plant analysed for fatty acid composition (Somerville and Browse, 1991). Seven mutants were isolated, which upon visual inspection in normal growth conditions did not vary from wild type. A number of mutants had reduced levels of a certain unsaturated fatty acid and consequently had an increase in a less unsaturated pre-cursor. Analysis of the acyl composition of various lipids within each mutant showed that some did not have the correct fatty acid moieties on the glycerol backbone. For example *fad A* (now known as *fad 4*) lacked 16:1 at *sn*-2 for phosphatidylglycerol (PG) and 16:0 levels were seen to increase. This inferred that the mutant lacked a desaturase, which has sole specificity for converting C16:0 at *sn*-2 in PG to C16:1 (Browse *et al.*, 1985). Of the seven desaturase mutants, five were seen to affect chloroplast lipid desaturation and two affected extra-plastidial lipids and genes for these have now been isolated either by chromosome walking or T-DNA tagging (see Ohlrogge and Browse, 1995).

There are three distinct desaturase groups; acyl-CoA desaturases, acyl-ACP desaturases and acyl-lipid desaturases. It has been found that most plant and cyanobacteria utilise acyl-lipid

desaturases, whereas in animal, fungal and yeast cells, acyl-CoA desaturases are abundant (Los and Murata, 1998). Unlike the Δ-9 stearoyl-ACP-desaturase, acyl-lipid desaturases have cytochrome b_5 as the electron donor (Kearns *et al.*, 1991).

1.6.3 Storage Lipids

Storage lipids are a storage form of carbon which offer no structural role to the cell. In oil seeds the vast majority of storage lipids are synthesised during a short period during seed maturation in order to provide a reserve of energy for the germination of a new seedling.

The main storage lipid is a triacylglycerol (TAG). TAG is synthesised in the cytoplasm of the cell in two main ways. The first involves the Kennedy pathway, where TAG is formed from PA that has been dephosphorylated to DAG. Upon the formation of DAG, a third fatty acid is esterified to the *sn*-3 position, such esterification is catalysed by the only enzyme found to be unique to TAG synthesis; diacylglycerol acyltransferase (DAGAT) (Frentzen, 1998).

The second, more complex synthesis of TAG utilises phosphatidylcholine (PC). After synthesis, fatty acids are not immediately available for TAG synthesis. The fatty acid is instead esterified to a glycerol molecule to form PC. Once modified by acyl-lipid desaturase it is thought that a free acyl-CoA trades places with the modified fatty acid attached to PC (Ohlrogge and Browse, 1995). Such a reaction is thought to be carried out by acyl-CoA-PC-transacylase (Stymne and Stobart, 1987). The modified acyl-CoA can now be used in TAG synthesis. Alternatively PC can donate its entire DAG portion to TAG synthesis, as it has been

observed that synthesis of PC from DAG and CDP-choline is reversible (Slack *et al.*, 1983). This may allow the DAG portion to be used in TAG synthesis.

1.6.4 Acyltransferases

Acyltransferases are an essential component of the synthesis of glycerolipids. Members of this class of enzyme aid in the formation of lipids by transferring a fatty acid to the glycerol backbone in a position specific manner and the roles of certain acyltransferases have been discussed in sections 1.6.2 and 1.6.3.

With the exception of GPAT, most acyltransferases are integral membrane proteins of the envelope membranes of plastids and ER. Acyltransferases have been shown to differ in their in fatty acid selectivity (Frentzen, 1998). DAGAT has broad acyl-CoA specificity and the composition of the fatty acids placed at positions *sn*-1 and *sn*-3 are dependent upon the type of acyl-CoA pool that can be utilised. The fatty acid placed at the *sn*-2 position is controlled by LPAAT, an enzyme that has pronounced acyl-CoA specificity. This specificity varies between species as determined by the analysis of the number of plants (Slabas and Brough, 1997).

In many *Brassiceae*, erucic acid (22:1) is rarely found at the *sn*-2 position, however the enzyme encoded by LPAAT isolated from *Limanthes douglasi* was seen to utilise erucoyl-CoA (Brown *et al.*, 1995). This cDNA was introduced into *B.napus*, where trierucin (erucic acid at all three positions) was found to be present (Brough *et al.*, 1996). Proposals of acyltransferases playing an important role in chilling sensitivity or resistance has led to

transgenic plants being created using GPAT overexpression constructs from a chilling resistant plant being transformed into tobacco. Upon chilling the tobacco plants were seen to become resistant (Murata *et al.*, 1992). Therefore the genetic manipulation of acyltransferases and utilisation of these genes from other species may aid in the improvement of crops and increase yield of desired products.

1.7 Oil storage

Seed storage oils are synthesised in the endoplasmic reticulum (ER). They are thought to accumulate in the centre of the ER lipid bilayer and then eventually stored in a densely packed oil body. As the activity of the enzymes involved in TAG synthesis increases there is an increase in the amount of TAG accumulating in the ER lipid bilayer. Eventually a point is reached where the quantity of TAG present in the bilayer causes the membrane to swell and create a droplet. The two leaflets of the lipid bilayer are forced apart to create this droplet and so it is bound by a monolayer of phospholipids. This structure is then thought to implant itself in a protein known as oleosin. Huang (1992) has put forward a structure for the oil body. The oleosin molecule consists of a hydrophobic stalk that embeds in the hydrophobic acyl moieties of the phospholipids and into the core of the TAG droplet. The remaining oleosin molecule protrudes from the oil body and may cover it. Once the oil body reaches a size of approximately 1μM it will bud off from the ER. The accumulation of oil during TAG biosynthesis is accompanied by the accumulation of these oil bodies (Ohlrogge and Browse, 1995).

Huang (1998) has described the synthesis of novel lipid bodies in *B.napus* tapetum cells; tapetosomes. This organelle is not enclosed by a membrane but has internal membranous vesicles. TAGS are distributed amongst these vesicles and store lipids until required to cover the pollen coat surface.

1.8 Regulation of Fatty Acid Synthesis

Although the synthesis of fatty acids has been well characterised, in comparison little is known about how plants control and regulate this supply. Due to the compartmentalisation of the FAS pathway in plants, every cell must produce fatty acids (Roughan and Slack, 1982). The ability for each cell to do this means that it can control its own membrane biogenesis and supply fatty acids needed for repair. Therefore regulation of the supply of fatty acids is imperative (Ohlrogge and Jaworski, 1997). The number of reactions necessary in order to synthesise a fatty acid plus the number of co-factors and pre-cursors required means that there is potential for fatty acid synthesis to be regulated at many points.

De novo fatty acid synthesis involves the enzyme systems ACCase and FAS. ACCase has long been thought to play an important regulatory role in fatty acid synthesis rates as it catalyses the production of the first and most highly demanded metabolite. Therefore it is thought that regulation of this enzyme provides the earliest point at which control can be exerted. In eukaryotic cells containing the type I FAS complex, ACCase has been shown to be controlled by citrate (the pre-cursor of cytoplasmic acetyl-CoA) and long chain acyl-CoAs (Wakil *et al.*, 1983) as well as by glucagon stimulated cAMP dependent phosphorylation (Goodridge, 1972).

In plants, there has been extensive investigations as to whether ACCase is a regulatory enzyme. ACCase has been shown to be light regulated (Sauer and Heise, 1983). This observation could be due to a lack of supply of co-factors during dark periods (Eastwell and Stumpf, 1984) as ACCase isolated from light incubated chloroplasts were up to 4 times more active than chloroplasts incubated in the dark (Sauer and Heise, 1983). Sasaki *et al.*, (1997) used dithiothreitol and thioredoxin as reductants in *in vitro* assays to show that plastidial ACCase was activated in the presence of these reductants thus demonstrating that ACCase is regulated by a redox reaction.

Analysis of the acyl-CoA and acyl-ACP pools has also inferred that ACCase exerts regulation over fatty acid synthesis (Post-Beittenmiller *et al.*, 1991). Levels of malonyl-ACP were seen to be lower than acetyl-ACP and it was assumed that this would be the same for the CoA derivatives, inferring that malonyl-CoA was limited by the activity of ACCase. Comparison of light and dark incubated acyl-ACP pools in spinach showed that acetyl-ACP levels increased in the dark, whereas malonyl-ACP levels did not. This was thought to reflect the regulatory effect of ACCase, demonstrating the diurnal effect of fatty acid synthesis rates in plants. Further analysis of acyl-ACP and acyl-CoA pools put forward more evidence that ACCase is a regulatory point. By analysing levels of the respective metabolites in spinach and pea chloroplasts, Post-Beittenmiller *et al.*, (1992) found that both malonyl- and acetyl-CoA were present in light incubated chloroplast but only acetyl-CoA was maintained in dark incubated chloroplasts. Page *et al.* (1994) examined ACCase by measuring changes in flux through the lipid pathway using herbicides to alter ACCase activity and observed strong alterations in flux

when ACCase was inhibited by using metabolic flux theories to calculate its importance. Further to this White *et al.*, (1998) have shown that when a cytosolic form of ACCase was down regulated, fatty acid synthesis and therefore lipid accumulation in *B.napus* seed is severely affected. Flavonoid synthesis was also affected showing strong metabolic flux control is exerted over the channelling of substrates for the appropriate pathway i.e. the important primary pathway of fatty acid synthesis takes precedence over the secondary flavonoid synthesis pathway for the malonyl-CoA supply or alternatively both are equally affected.

ACP is a necessary component along the entire synthesis of fatty acids and lipids. Overproduction of ACP in *E.coli* was shown to be lethal to the cell (Magnuson *et al.*, 1993) The prosthetic group of ACP undergoes metabolic turnover and the *apo*-protein is functionally inactive. To be reactivated the 4'pantetheine group is removed from CoA and transferred to apo-ACP by *holo*-ACP synthetase. The CoA pool is so large however that most of the ACP is in its *holo* form and so it appears that the supply of prosthetic groups does not limit fatty acid synthesis (Jackowski and Rock, 1981). It has also been suggested that the ACP pool in *E.coli* must be severely depleted before an effect on FAS is detected (Jackowski and Rock, 1983).

Other points of regulation are thought to be exerted throughout the synthesis of fatty acids. It had been thought for some time that ACAT was a regulatory enzyme in fatty acid synthesis (Shimakata and Stumpf, 1983a), but with the emergence of the third condensing enzyme, KAS III, as the initiator of the priming step in fatty acid synthesis ACAT is not thought to play an important role. KAS III has however been suggested as a potential rate limiting step due to its role in the initial condensation step (Jackowski *et al.*, 1996c, Post-Beittenmiller *et al.*, 1991).

By stimulating ACCase activity with the addition of Triton X100 levels of malonyl-ACP were seen to increase by five fold, but fatty acid synthesis rates did not increase in a similar manner. This suggested that FAS components were now becoming regulatory as supply of this metabolite was outstripping the ability and demand by FAS components to synthesise fatty acids. Examination of the effect of accumulation of long chain acyl-ACPs in a reconstituted fatty acid synthase system showed that *E.coli* KAS III is the subject of feedback regulation as determined by the accumulation of malonyl-ACP (Heath and Rock, 1996c). Overexpression of *E.coli* KAS III in *B.napus* however did not cause severe effects in fatty acid composition with only a slight alteration in 18:1 and 18:2 levels (Verwoert *et al.*, 1995) and the authors propose that fatty acid biosynthesis is not controlled by one rate limiting enzyme but shared by a number of FAS components. In addition to this when increases in KAS III activity was seen in spinach by 20-40 fold of wild type, KAS I became the limiting step as observed by the accumulation of 4:0ACP in transgenic leaves (Ohlrogge and Jaworski, 1997).

It is likely that supply and demand of metabolites affect the rates of synthesis and that feedback regulation plays a part in controlling fatty acid synthesis. Bruck *et al.* (1996) have proposed that KAS III is regulated by feedback inhibition in plants which synthesise medium chain fatty acids. They showed that KAS III activity was affected by the presence of NADH and NADPH, which allowed reduction of acetoacetyl-ACP to butyryl-ACP. Further investigations showed that this decrease was a result of the presence of product acyl-ACPs. Winter *et al.* (1997) propose a regulatory pathway for medium chain fatty acid synthesis, as the requirement for fatty acids is met, the unused malonyl-ACP induces decarboxylation activity in the condensing enzymes. This converts the malonyl-ACP to acetyl-ACP, which in

turn is restored to the acetyl-CoA pool by ACAT. This proposes that all the condensing enzymes play a role in regulation of malonyl-ACP levels and hence fatty acid synthesis rates, as well as putting forward a role for ACAT in returning unused metabolites to the acetyl-CoA pool for further metabolism.

1.9 Outline of Thesis

The surprising discovery of KAS III and its role in priming fatty acid synthesis (Jackowski and Rock, 1987) has led to the possibility of this enzyme possessing a role in the regulation of fatty acid synthesis. Previous work by Gulliver and Slabas (1994) identified two separate enzymes involved in the initial reaction of fatty acid synthesis in avocado; ACAT and KAS III. The question raised by the presence of a distinct ACAT activity, clearly separable from KAS III, is why does this ACAT activity exist if the initial reaction of plant FAS is determined by KAS III? Gulliver and Slabas have suggested that the plant has built in plasticity in its metabolism in order to allow it to respond to environmental stress.

Plant metabolism and growth are flexible due to several factors. These include the complexity of regulation and the possible built in redundancy due to alternative pathways (Sonnewald and Stitt, 1995). In the initial transition from dark to light there are two possible reactions to initiate fatty acid synthesis. First, ACAT and KAS I could catalyse the primary condensation reactions for FAS. Acetyl-ACP levels are shown to remain constant and even increase slightly (Post-Beittenmiller *et al.*, 1991) during the dark period, whereas malonyl-CoA and -ACP levels fall. The ACAT/KAS I initiation of fatty acid synthesis would allow all the available

acetyl-CoA to be directed towards synthesis of malonyl-CoA and therefore malonyl-ACP. KAS I would utilise the acetyl-ACP synthesised by ACAT. The second reaction would involve KAS III, if this were the case then the flux of acetyl-CoA to malonyl-ACP would be reduced (see figure 1.5 for the two schemes).

Analysis of KAS III substrates and products in spinach leaf has indicated that this condensation reaction is displaced from equilibrium and so is a potential point of regulatory control (Post-Beittenmiller *et al.*, 1991). Studies by Heath and Rock (1996c) showed that KAS III is inhibited by long chain acyl-ACPs. KAS III in *E.coli* is implicated as a regulatory site when the accumulation of malonyl-ACP was observed as a result of the inhibition of a reconstituted fatty acid synthase system by acyl-ACPs (Heath and Rock, 1996b). Further to this, Tsay *et al.* (1992a and b) observed that a mutant of KAS III, with severely decreased levels of KAS III activity, was still able to grow normally, exhibiting no growth phenotype. These observations suggests indirectly that KAS III may have a regulatory role in fatty acid synthesis and that there is also the potential for more than one pathway to initiate fatty acid synthesis.

Until the discovery of KAS III, ACAT was thought to play an important regulatory role in fatty acid synthesis. Its relegation to a minor role questions its existence and has put KAS III forward as a potential regulatory enzyme as it catalyses one of the initial reactions in *de novo* fatty acid synthesis. This thesis describes the development of a transgenic approach in *Brassica napus* to determine directly the significance of KAS III in the initiation and regulation of fatty acid synthesis. Such a study will assist in the understanding of the

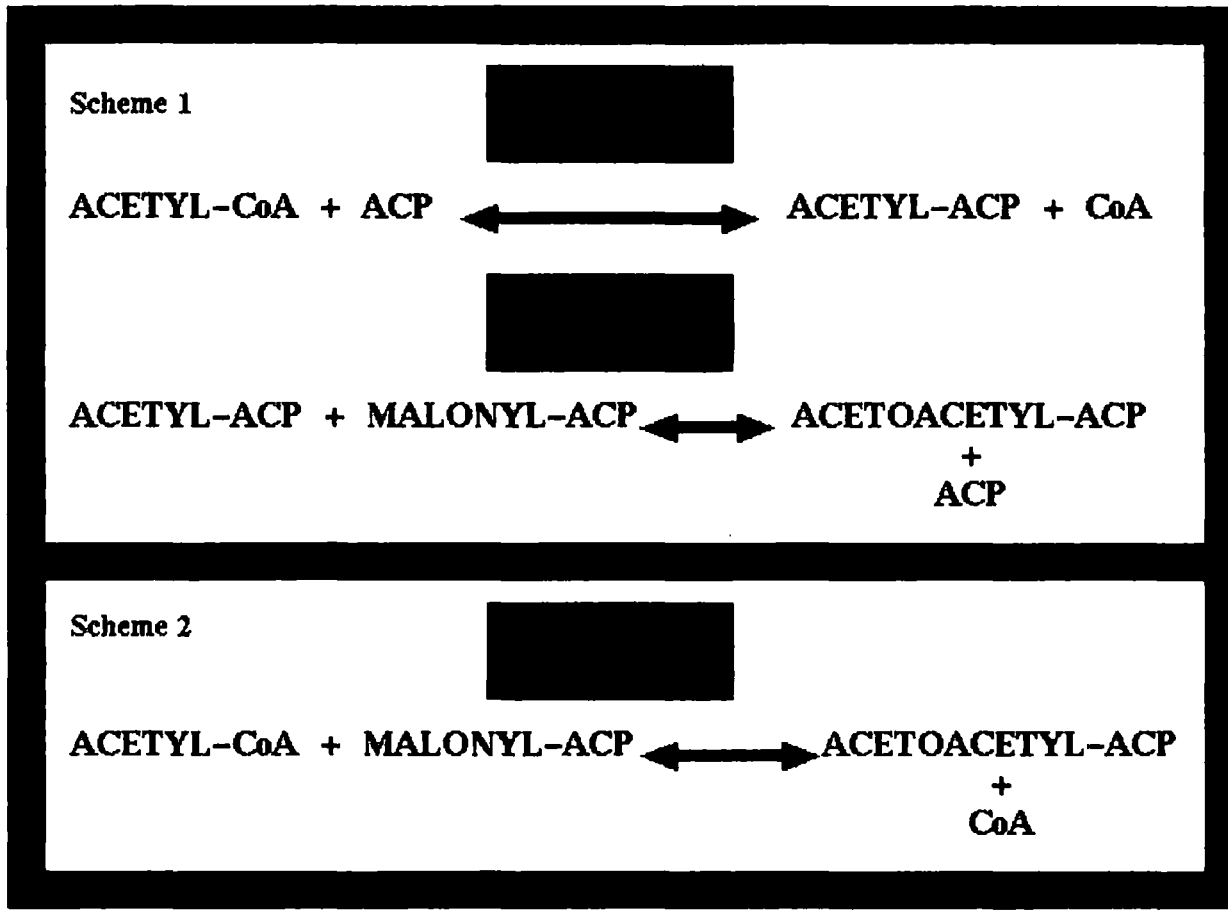


Figure 1.5 The two schemes for the intiation of fatty acid synthesis of plants.

Scheme 1 is a two step process which comprises of ACAT synthesising acetyl-ACP from acetyl-CoA and ACP and KAS I then catalysing the condensation of acetyl-ACP with malonyl-ACP to form acetoacetyl-ACP. The second scheme shows KAS III condensing acetyl-CoA with malonyl-ACP to form acetoacetyl-ACP directly, which obviates the need for ACAT.

importance of KAS III in terms of regulation and the effect down regulation has on pathway end products and other FAS components.

The generation of antisense KAS III plants could then be used to answer the question of the significance of ACAT within fatty acid synthesis, as down regulating KAS III may lead to little change, as the initial reaction may be taken over by KAS I and ACAT. If this is the case the rate of fatty acid synthesis may decrease as ACAT has been shown to have lower activity compared to other FAS components (Shimakata and Stumpf, 1983a, Jaworski *et al.*, 1993). Alternatively down regulation of KAS III may severely affect the plant and exert a pleiotropic effect over other FAS components. The development of antisense KAS III transgenic lines would therefore move towards observing what happens *in planta* when this gene is down regulated.

In chapter 3, the isolation and sequencing of a full length KAS III cDNA clone from *Brassica napus* is described, along with determination of gene copy number and transcript size. *Brassica napus* was selected as the organism in which to perform the transgenic study as it produces up to 40% dry weight lipid in its seed and is an important commercial crop. The *Brassica napus* KAS III DNA sequence and the derived amino acid sequence are compared to other known KAS IIIs from higher plants and *E.coli*. The isolation of such a clone is necessary in order to be able to generate an antisense KAS III construct for transformation into plants.

Chapter 4 describes the overexpression of the KAS III cDNA in *E.coli*, its subsequent purification and use in immunisation trials in rabbits. The production of polyclonal KAS III

antisera can be used to analyse both wild type and transgenic *Brassica napus* by determining KAS III levels *in planta.*

In order to assess any pleiotropic effects, the development of a quantitative enoyl-ACP reductase (ENR) ELISA assay is described in chapter 5. Such a high throughput, sensitive assay could determine ENR levels in both seed and leaf material, and the technique can be used to analyse transgenic KAS III plants. The chapter also determines a standard leaf developmental profile of wild type *Brassica napus* cultivars in order to pinpoint the optimum time at which to take samples from a plant. FAS enzymes are assessed in order to examine overall levels in the leaf as it matures using the ELISA assay described within the chapter and Western blot analysis. Leaves are also examined for overall fatty acid content using GLC analysis of derived fatty acid methyl esters (FAME). The determination of such a profile forms a baseline from which to perform experiments involving transgenic lines.

Chapter 6 takes the *Brassica napus* KAS III cDNA clone previously isolated and places the open reading frame in an antisense vector. Transformation of the resulting construct into *Brassica napus* and *Arabidopisis thaliana* is described along with selection of positive transformants. Analysis of T1 antisense KAS III plants using the ENR-ELISA assay, the KAS III antibody, Southern hybridisation and FAME analysis is compared against phenotypic traits observed within transgenic lines. This investigation is carried out in conjunction with analysis of transgenic ENR plants which became available during the study. Conclusions are summarised in chapter 7, where the potential implications of the findings are discussed as well as proposing future studies which can be carried out as a result of this work.

Chapter 2

Materials and General Methods

2.1 Bacterial Strains

All bacterial glycerol stocks originally supplied by Novagen unless otherwise stated.

***E.coli* Host Strain**	**Genotype**
DH5α	*sup*E44 Δ*lac*U196 *[φ80lacZ*ΔM15] *hsd* R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1
XL1 Blue	*rec*A1,*end*A1,*gyr*A96,*thi*-1,*hsd*R17,*sup*E44,*rel*A1,*lac*[F'proAB lacI^{q}ZΔM15 Tn10 (tet^r)]^c
JM101	*supE thi*-1 (Δ*9lac-proAB*) [F' *traD*36 *pro*AB *lacI^q*ZΔM15]
TOP10F' (supplied by Invitrogen)	F' {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (str^R) *end*A1 *nup*G
BL21*(DE3)*	*E.coli* B F$^-$ *dcm ompT hsd*S (r_{B-} M_{B-}) galλ(DE3)

2.2 Cloning Vectors

Vector	**Size**	**Selection**	**Reference**
pBluescript + (Stratagene)	2.96kb	Ampicillin	Short *et al.*, (1988)
TA Topo (Invitrogen)	3.9kb	Amp/Kan/X-gal	Invitrogen (1998)
PGEM-3Z (Promega)	2.74kb	Ampicillin	Promega (1996)

2.3 Primer Sequences

Primers were synthesised by MWG-Biotech or PE Applied Biosystems.

Name	Sequence (5'-3')
M13 Forward	GTA AAA CGA CGG CCA GT
M13 Reverse	GGA AAC AGC TAT GAC CAT G
T7	GTA ATA CGA CTC ACT ATA GGG C
T3	AAT TAA CCC TCA CTA AAG GG
HC1R	AAT GCG AAG CAG AAC CCA CCAT CC
HC1F	TCT ACT TCC CAG TTC AAC CGG
HC3R	AAG CCC TTG AAA TGG CGG
HC3F	GCA GAC TCA AGA GAT TGG GG
HC4R	GGT TGT TCA GGC TTG TGA CA
HC5F	GCC TGA TAC AAC GCG GT
HC5R	GCC GGT TTA ACT TGG GGT TC
HCNAP1	GTA CCC ATG GTC ATC TCC ACC TGA TAA TTG C
HCNAP2	TAT CCC GGG ATG GCG AAT GCA TCT GGC TTC
HC35S1	CCC AAG CTT TCA TCT CCA CCT GAT AAT TGC
HCOE1	TAC CAT GGC GAA TGC ATC TGG CTT C
HCOE2	CGG GAT CCT CAT CTC CAC CTG ATA ATT GC
ISOHC1	GTG AAA ATG AAA CGA AAT CAA AC
ISOHC2	TCT GCT TTA CAA AAA GCT GGT
ISOHC3	CTC TTT GGA GAT GCT GCT GGT
ISOHC4	GGG GAA CGC ATT TGA CAG C
KAN1	CGC AGG TTC TCC GGC CGC TTG GGT GG
KAN2	AGC AGC CAG TCC CTT CCC GCT TCA G
BN9F	TGG GAA CCG GAG CTC AAT
BN9R	GA TCC ATA GTT GCC TGA CTC CCC G
BLAF	TCC ATA GTT GCC TGA CTC CCC G
BLAR	TGG GAA CCG GAG CTC AAT GA

2.4 Buffers and Solutions

All chemicals were analar grade supplied by the following companies; Sigma, Merck (BDH) or Fisher Chemicals unless otherwise stated.

Buffer/Solution	Constituents
AB Media	0.2M K_2HPO_4, 0.1M NaH_2PO_4, 0.2M NH_4Cl, 0.02M KCl
Blocking buffer	2% Marvel Milk Powder, 1 × PBS
Blotting buffer	1.5M NaCl, 0.4M NaOH
Coating buffer	15mM Na_2CO_3 and 35mM $NaHCO_3$ (pH9.6)
Coomassie Blue Stain (I)	25% propan-2-ol, 10% acetic acid, 1% Coomassie blue R-250
Coomassie Blue Stain (II)	10% propan-2-ol, 10% acetic acid, 0.125% Coomassie blue R-250
Coomassie Blue Stain (III)	10% acetic acid, 0.125% Coomassie blue R-250
Coomassie Blue Destain	10% acetic acid, 1% glycerol
Denaturing solution	0.4M NaOH, 1M NaCl
50 × Denhardt's Reagent	1% ficoll, 1% PVP, 1% BSA
Developing Solution (silver stain)	250mM Na_2CO_3, 0.001% formaldehyde
DNA Extraction buffer	200mM Tris.HCl pH 7.5, 250mM NaCl, 25mM EDTA and 0.5% SDS
(6×) DNA Gel loading buffer	0.25% bromophenol blue, 0.25% xylenecyanol FF, 15% Ficoll (Type 400; Pharmacia) in MQ water.
Electrode running buffer	25mM Tris.HCl, 250mM glycine (electrophoresis grade), 0.1% SDS.
Fixing buffer (silver stain)	40% ethanol, 10% acetic acid
Homogenisation Buffer	0.1M KPO_4 pH 7.2, 5mM DTT, 1mM EDTA.

Incubation buffer (silver stain)	30% ethanol, 1.3% glutardialdehyde, 125mM sodium acetate, 3mM sodium thiosulphate
LB medium	10g bactotryptone, 5g bactoyeast extract, 10g NaCl, pH 7.0 with 5N NaOH
Ligation Buffer	0.5M Tris.HCl (pH7.6), 100mM $MgCl_2$, 100mM DTT, 500μg/ml BSA
MG/L Broth	0.001M sucrose, 1 ×10-5M $CaCl_2$, 0.04M $MgSO_4$, 0.5 x 10^{-6}M Fe $SO_4 7.H_20$
M-PBS	2% Marvel milk powder, 1 × PBS
5 × MOPS buffer	200mM MOPS, 50mM sodium acetate, 5mM EDTA
MS media	1.1g Murashige and Skoog salts pH 5.7 with KOH, 4g Fermtech agar (Fisher), 1× B5 vitamins in 1 litre
Northern Pre-hybridisation buffer	50% Formamide, 2 × Denhardt's reagent, 0.1% SDS, 100μg/ml Herring sperm DNA, 5 × SSPE
Neutralising solution	1.5M NaCl, 0.5M Tris.HCl pH 7.4
PBS	0.15M NaCl, 10mM $Na_2 HPO_4$, 25mM KCl, 2mM KH_2PO_4
PBS-T	1 × PBS, 0.05% Tween 20
Ponceau S Stain	0.1% Ponceau Stain, 1% glacial acetic acid
Preserving buffer (silver stain)	10% glycerol
Preparation buffer	0.5 × MOPS, 7% formaldehyde, 50% deionized formamide and 0.025μg ethidium bromide
Problott Transfer buffer	1 × CAPS, 10% HPLC grade methanol
(6×) RNA Loading buffer	50% glycerol, 10mm sodium phosphate buffer ph7.0, 0.25% bromophenol blue, 0.25% xylenecyanol ff

Rooting Media	MS Salts, 3% sucrose, 2μg/ml indolebutyric acid, 0.7% phytoagar and 50μg/ml carbenicillin
(1×) Sample buffer	50mM Tris.HCl (pH6.8), 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol
Shoot Elongation Media	MS Salts, 3% sucrose, 0.7% phytoagar pH5.8, 50μg/ml carbenicillin and 15μg/ml kanamycin
Silver solution (silver stain)	5mM Silver nitrate, 0.0002% Formaldehyde
SOB Medium	2% bactotryptone, 0.5% bactoyeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$
SOC Medium	SOB media + 20mM glucose
Southern pre-hybridisation solution	2 × Denhardt's reagent, 0.1% SDS, 100μg/ml Herring sperm DNA, 5 × SSPE
20 × SSC	3M NaCl, 0.3M sodium citrate, pH7.0
SM buffer	100mM NaCl, 1mM $MgSO_4(7H_20)$, 1M Tris-Cl (pH7.5) 50ml, 2% gelatin.
20 × SSPE	3M NaCl, 0.2M $NaHPO_4.H_2O$, 25mM EDTA, pH7.4
STET solution	0.1M NaCl, 10mM Tris-HCl, 1mM EDTA, 5% Triton-X100
Stop solution (silver stain)	50mM EDTA
TAE buffer (50×)	2M Tris base, 6% glacial acetic acid, 50mM EDTA (pH8.0)
TBS	130mM NaCl, 2mM KCl, 25mM Tris pH 7.4
TE	pH7.4; 10mM Tris.HCl (pH 7.4),1mM EDTA (8.0) pH7.6 ;10mM Tris.HCl (pH 7.6),1mM EDTA (8.0) pH8.0 ;10mM Tris.HCl (pH8.0),1mM EDTA (8.0)
T-TBS	130mM NaCl, 2mMKCl, 25mM Tris pH 7.4, 0.05% Tween-20
Transfer Buffer	25mM Tris, 200mM glycine, 0.1% SDS
2 × YT media	16g bacto-tryptone, 10g bacto-yeast extract, 5g NaCl, pH 7.0 in 1 litre

2.5 Plant Material

2.5.1 *Brassica napus*

Brassica napus plants were grown under a 16 hour light/8 hour dark regime at a temperature of 20°C during light and 15°C during the dark period at a constant humidity of 60% in a Sanyo Growth Room at a light intensity of 250μEm^{-2}sec^{-1} (μE = micro Einstein) measured using a Macam Quantam Radiometer/Photometer No. Q101. Plants were grown in Levingtons M3 compost soil mix, which had been autoclaved as a treatment for insect egg removal. The compost was treated with Phostrogen fertiliser according to the manufacturer instructions (Phostrogen) at fortnightly intervals during the first eight weeks of growth.

2.5.2 *Arabidopsis thaliana*

Arabidopsis thaliana plants of the ecotype Columbia-0 were grown in 3.5 inch pots, at the time of sowing a thin cloth netting was placed over the surface of the soil and the cloth secured with an elastic band. The plants were then grown for four weeks on a short day regime (10 hour light/14 hour dark) followed by four weeks on long day regime (16 hour light/8 hour dark). The light intensity was 250μEm^{-2}sec^{-1} (μE = micro Einstein) measured using a Macam Quantam Radiometer/Photometer No. Q101. After eight weeks the primary bolts that emerged were removed. This induced side bolting and the plants were ready for infiltration eight days after this treatment.

Any siliques or open flowers were removed from the plants prior to the vacuum infiltration process (section 2.11.3).

2.6 Bacterial Manipulations

2.6.1 Preparation of Competent *E.coli* subcloning Cells

A glycerol stock of cells (DH5α, JM101, XL1 blue) was taken and a sample streaked out onto a LB-Agar plate and allowed to grow overnight at 37°C. A single bacterial colony was used to inoculate a 5ml LB media and grown overnight at 37°C with shaking. A 1ml aliquot of this culture was taken and sub-cultured into 100ml of fresh LB media and grown at 37°C with shaking until an OD_{600nm} of 0.8 was reached. The cells were chilled on ice for 15 minutes and harvested by centrifugation at 3000 × g for 10 minutes at 4°C. The pellet was resuspended in 0.1 × vol. of 50mM $CaCl_2$, stored on ice for 30 minutes and subsequently centrifuged. The cells were resuspended in 1ml of ice-cold 50mM $CaCl_2$/glycerol (85:15), dispensed into 50µl aliquots and snap frozen in liquid nitrogen. The competent cells were stored at -80°C.

2.6.2 Preparation of Electro-competent *Agrobacterium tumefaciens* Cells

The *Agrobacterium tumefaciens* strain C58 C3 was used to make the electro-competent cells. A 5ml LB media culture containing 25µg/ml nalidixic acid and 100µg/ml streptomycin was inoculated with a C58 C3 colony and grown at 28°C overnight. A 100µl aliquot of this culture was then added to 50ml of 2 × YT media containing 25µg/ml nalidixic acid and 100µg/ml streptomycin at 28°C. The cells were

grown to an OD_{600} 0.4 and then chilled on ice for 30 minutes. The culture was centrifuged at 4000 × g for 10 minutes and the pellet resuspended in 50ml of 10% glycerol. Centrifugation and re-suspension was repeated another three times with cells being re-suspended in 25ml, 1ml and finally 500µl of 10% glycerol. The suspension was aliquoted into 40µl fractions, snap frozen in liquid nitrogen and stored at -80°C until required.

2.6.3 Transformation of Competent *E.coli* cells with plasmids

An aliquot of competent cells as prepared in section 2.6.1 were thawed on ice for 5 minutes. 450µl of ice-cold 50mM calcium chloride was added and the cells mixed gently. A 100µl aliquot was taken and 10ng of plasmid DNA added. The cells were then incubated on ice for 30 minutes and heat shocked at 42°C for 45 seconds before being returned to ice for 2 minutes. 0.9ml of LB media was added and the culture incubated at 37°C for one hour. 100µl and 200µl of the culture was then spread onto an LB-Agar plate containing 50µg/ml of ampicillin or an LB-Agar plate containing 50µg/ml ampicillin and 40µg/ml IPTG and incubated at 37°C overnight. Colonies were picked and grown for plasmid preps as described in section 2.6.5. When colour selection was used, recombinants were identified by formation of white colonies as opposed to the blue colonies formed by religated vector.

2.6.4 Electroporation of *Agrobacterium* Cells

10ng of plasmid DNA was mixed with a thawed aliquot of electro-competent *Agrobacterium* cells made as previously described in section 2.6.2. The cells were

then placed in an 1ml electroporator cuvette and put in a Bio-Rad Electroporator, which was set at 2.5kV with a capacitance of 25 Farads and a resistance of 400Ω. The cells were then subjected to one pulse of current with a time constant of 9.6. 1ml of 2 × YT media was immediately added to the cells and placed on ice. The culture was then grown for 3 hours at 28°C. 50µl and 100µl aliquots were spread on to LB-Agar plates containing 25µg/ml nalidixic acid and 70µg/ml gentamycin and incubated at 28°C for 2-3 days until colonies could be easily seen on the plate.

2.6.5 Isolation of Plasmid DNA

A single bacterial colony containing the desired plasmid was inoculated into 5ml of LB media containing 50µg/ml of ampicillin and grown at 37°C overnight with shaking. 1.5ml of this culture was then placed in an Eppendorf tube and the cells collected by centrifuging for 2 minutes at 10,000 × g. The supernatant was discarded and the cells resuspended in the media remaining in the Eppendorf tube. 350µl of STET solution was added and the tube was mixed by inversion. Lysozyme was added to a final concentration of 20µg/ml and the tube placed on ice for 10 minutes. The solution was then boiled for 1 minute followed by centrifugation for 20 minutes at 10,000 × g. The supernatant was taken and 200µl of 4M ammonium acetate added. 900µl of isopropanol was added, the tube mixed by inversion and incubated at -20°C for 30 minutes. The plasmid DNA was pelleted by centrifugation at 10,000 × g for 30 minutes and the supernatant was discarded. The pellet was washed with 500µl of 70% ethanol and then dried by centrifuging *in vacuo* for 10 minutes at 3000 × g. The plasmid was re-dissolved in 20µl of sterile distilled water. If the pellet did not re-

dissolve easily it was placed at 60°C for 10 minutes. A 1μl aliquot of the plasmid DNA was examined on an agarose gel to ensure only the plasmid DNA had been isolated and not contaminated with any genomic DNA or RNA.

Alternatively plasmid DNA was isolated using either the Promega Wizard Miniprep or the Hybaid Miniprep Kit, according to the manufacturer's recommended method.

2.6.6 Overexpression of *Brassica napus* Ketoacyl-ACP Synthase III (KAS III) in *E.coli* BL21*(DE3)*

Brassica napus KAS III plasmid pETKAS was introduced into *E.coli* BL21*(DE3)* as in section 2.6.3 and grown overnight at 30°C. A single colony was used to inoculate 5ml of LB media containing 50μg/ml ampicillin and grown at 30°C overnight with shaking. 1ml of this culture was used to inoculate a 500ml LB media culture containing 50μg/ml ampicillin in a 2L-baffled conical flask. The culture was incubated at 37°C until an OD_{600} of 0.5 was reached. 400μl of 50mg/ml ampicillin was then added and expression of the protein induced by the addition of IPTG to a final concentration of 4mM. The culture was grown for a further 3 hours to allow expression of the protein. Cells were harvested by centrifugation at 5,000 × g for 10 minutes at 4°C and the pellet stored at –80°C.

2.6.7 Overexpression of Malonyl-CoA:ACP Transacylase (MCAT) in *E.coli*

Overexpression of MCAT protein was essentially as for KAS III in section 2.6.6. Overexpression MCAT plasmids (pMIC6 and pETK50) were kindly provided by

Prof. A.R.Stuitje. Plasmid pMIC6 was a high copy number plasmid based on pUC19 and introduced into *E.coli* JM101 and transformants selected on 50μg/ml ampicillin LB-agar plates. A single colony was used to inoculate 5ml LB-media containing 50μg/ml ampicillin and incubated at 37°C. No induction was required and the culture was either incubated for 6 hours and the cells harvested or alternatively the culture was used to inoculate a 400ml LB–media culture containing 50μg/ml ampicillin which was grown to an OD_{600} of 1.0 and the cells harvested by centrifugation at 5,000 × g for 10 minutes and the pellet stored at -80°C.

Plasmid pETK50 was introduced into competent BL21*(DE3)* cells and transformants selected on 50μg/ml ampicillin LB-agar plates. A single colony was used to inoculate 5ml LB media containing 50μg/ml ampicillin and grown overnight at 30°C. A 0.5ml aliquot was then used to inoculate 50 ml LB-media, containing 50μg/ml ampicillin, which was grown at 37 °C to an OD_{600} of 0.5. At this point expression of MCAT was induced by the addition to IPTG at a final concentration of 4mM and the cells grown for a further three hours. The cells were harvested by centrifugation at 5,000 × g and stored at -80°C.

2.6.8 Purification of Over-Expressed Malonyl-CoA:ACP Transacylase (MCAT) from *E.coli*

Pelleted cells grown as in section 2.6.7 were thawed on ice and resuspended in a solution of 100mM Tris pH 8.1, 2mM DTT and 1mM EDTA. Lysozyme was added to the cells at a final concentration of 4mg/ml and the cells incubated on ice for 1 hour before addition of DNAse I at a final concentration of 20μg/ml. Lysate was vortexed

and centrifuged at 10,000 × g for 5 minutes. The supernatant was filtered through a 0.2μm acrodisc before loading onto a 1ml Mono-Q column attached to a Pharmacia FPLC. The column was equilibrated in 100mM imidazole-HCl pH 7.4, 1mM DTT and 1mM EDTA. The proteins were eluted using a 0-0.5M sodium chloride gradient and 0.5ml fractions collected. Each fraction was assayed for MCAT activity (section 2.9.2) and analysed by SDS/PAGE (section 2.8.5).

Fractions containing MCAT activity were pooled and protein concentration estimated by Bradford assay (section 2.8.2). These partially purified samples were used in KAS III assays as described in section 2.9.3.

2.7 General DNA and RNA Techniques

General DNA and RNA techniques were carried out according to Molecular Cloning – A Laboratory Manual (Sambrook *et al.*, 1989).

2.7.1 Small Scale DNA Preparation from *Brassica napus* Leaf

The second leaf was harvested from each plant 4 days after emergence, placed in an Eppendorf tube and ground using a motorised hand held sterile pestle. 400μl of DNA extraction buffer was added prior to incubation at room temperature for 1 hour. Samples were centrifuged at 10,000 × g for 5 minutes and the supernatant transferred to a tube containing 400μl of isopropanol. The tube was mixed by inversion and spun at 10,000 × g for 5 minutes. The supernatant was removed and the pellet was resuspended in 400μl of TE buffer pH 7.5.

If a DNA prep of higher purity was required for use such as in genomic Southern blots the Qiagen Plant DNeasy kit was used and the manufacturer's recommended method followed, with the modification of grinding the leaf as described above prior to adding to the DNeasy spin columns.

2.7.2 Preparation of RNA from *Brassica napus* Leaf

A day 4 leaf was taken and weighed. Trizol (Gibco BRL) reagent was added at the ratio of 1ml of Trizol to 100mg of tissue. The tissue was homogenised with a hand held homogeniser fixed with a sterile DEPC treated pestle. The homogenate was incubated for 5 minutes at room temperature. The Trizol reagent was used according to the manufacturer's instructions, where for every 1ml of reagent used in the preparation, 0.2ml of chloroform was added to the solution and shaken vigorously for 15 seconds. The sample was incubated at room temperature for 3 minutes and then centrifuged at 12,000 × g for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh RNAse free Eppendorf tube, which contained 0.5ml of isopropanol for every 1ml of Trizol used in the initial homogenisation. The sample was incubated at room temperature for 10 minutes and centrifuged at 12,000 × g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed in 1ml of 75% ethanol. The sample was vortexed and centrifuged at 7500 × g for 5 minutes. The pellet was vacuum dried for 10 minutes and the RNA re-suspended in 50µl of DEPC treated water by incubating at 55°C for 10 minutes. The samples were stored at -70°C until required.

2.7.3 Restriction Endonuclease Digestions

Restriction endonucleases were purchased from Boehrhinger-Mannheim. Generally DNA was cleaved with the appropriate amount of enzyme (1 unit of enzyme/1 μg of DNA) in the reaction buffer supplied, according to the manufacturer's recommendations. Reactions contained 0.1-1μg of plasmid DNA or up to 20μg of genomic DNA in a volume of 10μl (250μl for genomic digests) containing 1 × restriction buffer with 2-10 units of enzyme. Digests were incubated at 37°C for 2-3 hours or overnight for genomic digests. A sample was taken and run on the appropriate percentage agarose gel to determine the success of the digest.

2.7.4 General Method for the Polymerase Chain Reaction (PCR)

Primers for PCR were synthesised by MWG Biotech or PE-Applied Biosystems UK. The polymerase chain reaction conditions were optimised for each individual experiment. However a general method was followed for all PCR procedures. The standard PCR reaction mixture consisted of 0.2mM dNTP mix (equal mixture of dCTP, dTTP, dGTP and dATP), 2mM $MgCl_2$, 1 × Taq Ammonia buffer, Bioline) and primers at a final concentration of 10 pmoles. Approximately 10ng of DNA template was added and the reaction mixture was overlaid with 50μl of mineral oil. PCR was carried out using a Perkin Elmer 460 PCR machine. Initial denaturation of the reaction was performed at 94°C for 4 minutes and 1 unit of Taq DNA polymerase (Bioline) was added. Subsequent denaturation was carried out at 94°C for 30 seconds. Annealing and extension temperatures were optimised for each target DNA and the

temperature cycling programme for each PCR is cited in the relevant sections of each chapter. A sample from each reaction was run on the appropriate percentage agarose gel to determine the success of the PCR.

2.7.5 Southern Hybridisation

The DNA to be used in the Southern hybridisation was first precipitated by the addition of 0.1 × volume of 3M Sodium acetate pH 5.2 and 2.5 volumes of ice cold ethanol. This was followed by an incubation at –80°C for 15 minutes. The DNA was pelleted by centrifugation at 10,000 × g for 30 minutes at 4°C. The pellet was washed in 0.5ml of 75% ethanol, followed by centrifugation at 10,000 × g for 5 minutes. The supernatant was removed and the DNA pellet was dried by placing at 60°C or put under vacuum for 10 minutes. The DNA was resuspended in the required amount of sterile water and placed in a water bath at 50°C for 15 minutes, and then stored at 4°C. The DNA was then electrophoresed on a 1% agarose gel and run at 50V overnight. After electrophoresis, DNA bands were visualised using a short-wave Stratagene UV transilluminator and photographed. The DNA was depurinated by incubating the gel in 0.25M HCl for 15 minutes and soaked in blotting buffer.

For the transfer a piece of Whatman 3MM paper was placed on a support over a large pyrex dish containing blotting buffer to just below the top of the support to act as a wick. Any air bubbles in the 3MM paper were removed and it was ensured that the paper was thoroughly wet. The gel was transferred to the support in an inverted position and the orientation noted. Nylon membrane (Hybond N – Amersham) was cut to the same size as the gel and placed on top of it ensuring no air bubbles were

trapped. The gel/membrane was then surrounded on each side with strips of Saranwrap. Two pieces of Whatman 3MM, cut to the gel size and previously soaked in blotting buffer, were then placed on top of the gel/membrane assembly, followed by a further four pieces of Whatman 3MM paper and extra absorbent cotton wool. A weight was then placed on top of the assembly and left to allow DNA transfer to occur by capillary action overnight.

After transfer the membrane was marked with the positions of the wells of the gel, washed in 2 × SSC and DNA fixed by irradiation in a Stratalinker 1800 UV crosslinker (Stratagene) on an auto setting of 1200 Joules, or alternatively, baked for 90 minutes at 80°C. The membrane was placed in pre-hybridisation solution at 65°C for one hour prior to the addition of a radioactive probe (section 2.7.7) and incubated overnight at 65°C with rotation in a Hybridisation oven. The membrane was then washed to remove any unbound probe (section 2.7.8) and exposed to x-ray film and developed as in section 2.7.9.

2.7.6 Northern Hybridisation

All solutions used in this method were treated with diethyl pyrocarbonate (DEPC) or made up using DEPC-treated MQ water. All apparatus was baked at 140°C for 3 hours prior to use. Both of these treatments said in the removal of any contaminating RNases which may degrade the RNA.

A 1% agarose gel was made in 5 × MOPS buffer and 8% formaldehyde and allowed to set on a level surface in a fume hood. The RNA samples analysed (as prepared in

section 2.7.2) were pre-incubated in preparation buffer at 56°C for 15 minutes. RNA loading buffer was added to the sample and the sample briefly spun in a microfuge. The gel was placed in a RNase free gel electrophoresis tank and 1 × MOPS buffer added until level with the surface of the gel. The samples were then loaded and the gel run at 50V for 15 minutes. More 1 × MOPS buffer was added so as to immerse the gel and then electrophoresis carried out for 4 hours at 100V. The gel was then rinsed in DEPC treated distilled water and photographed by visualisation on a Stratagene UV Transilluminator. The RNA was transferred to zeta-probe membrane (Bio-Rad) by inverting the gel onto a piece of pre-soaked 3MM Whatman paper which had been placed on a support in a large dish containing 20 × SSC. The zeta-probe membrane was then placed on top and the gel/membrane assembled as described for Southern Hybridisation in section 2.7.5.

After transfer, the membrane was rinsed in DEPC treated 2 × SSC and irradiated in a Stratalinker 1800 UV crosslinker at an auto setting of 1200 Joules. The membrane was then placed in northern pre-hybridisation buffer and incubated for 4 hours at 42°C with rotation in a Hybridisation oven. A radioactive probe was added (section 2.7.7) and the membrane incubated at 42°C overnight. The membrane was then washed as in section 2.7.8 and then exposed to x-ray film (section 2.7.9).

2.7.7 Synthesis of Radioactive Probes

Radioactive probes were synthesised using the Amersham Rediprime Random Labelling Kit according to the manufacturer's instructions. [αP^{32}] dCTP was used as the source of radioactivity as it is readily incorporated into the DNA being synthesised

whereas nucleotide analogues with non-radioactive probes are incorporated less efficiently. [αP^{32}] dCTP emits β particles which are readily detected on x-ray film using an intensifying screen. [αP^{32}] dCTP also has the advantage of having a relatively short half-life. This means after approximately 3 months the radiation has decayed and is easy to dispose of. 50ng (conc. 5ng/μl) of template DNA was denatured by boiling for 2 minutes and snap cooled on ice before use in the reaction. Probe reaction mix was prepared by adding 50μCi of [αP^{32}] dCTP to a rediprime tube which already contained the appropriate enzyme buffers and dNTPs in a final volume of 45μl. Upon addition of 5μl of template DNA, the reaction was incubated for 1 hour at 37°C. 50μl of sterile water was added. The unlabelled DNA was separated from un-incorporated nucleotides by passage through a Bio-spin P6 column (Bio-Rad). The labelled DNA was then boiled for a further 5 minutes and briefly chilled on ice prior to use.

2.7.8 Washing Filters Hybridised with Radioactive Probes – General Protocol

For Southern hybridisations, filters were twice washed in 50ml of 2 × SSC containing 0.1% SDS for 10 minutes at room temperature. Filters were then washed in 1 × SSC containing 0.1% SDS for 15 minutes at 65°C followed by a final wash in 0.2 × SSC and 0.1 % SDS for 10 minutes at 65°C.

For Northern Hybridisation, filters were washed at 42°C with 1 × SSC, 0.1% SDS for 15 minutes.

The washed filters for both methods were exposed to Fuji x-ray film for as long as required and films developed as in section 2.7.9.

2.7.9 Developing X-Ray Films

X-ray films were developed by placing the film in a solution of photographic developer D19 (Ilford) for up to 5 minutes. The film was rinsed in distilled water and fixed with Ilford Hypam Fixer for 1 minute. The film was then washed with distilled water and left to dry at 37°C. Alternatively the film was developed using a X-ograph Imaging Systems x-ray film processor on standard settings.

2.7.10 cDNA Library Screening

The λZAP oligo (dT) oil seed rape embryo cDNA library used in the screening was previously prepared by Mr. Johan Kroon using a Pharmacia Time Saver cDNA library kit. A titre of 1.5×10^8 plaque forming units (pfu/ml) was determined for the cDNA library.

XL1 blue *E.coli* cells were grown at 37°C in LB media containing 0.2% maltose and 10mM magnesium sulphate, until an OD_{600} 1.0 was reached. The cells were harvested by centrifugation at 3,500 × g for 10 minutes and resuspended in 0.1 × vol. 10mM magnesium sulphate and stored at 4°C until required. A 10 fold dilution of the cDNA library was made in SM buffer for use in the primary screen. 400µl of XL1 blue *E.coli* cells were mixed with 10µl of phage dilution and placed at 37°C for 20 minutes. The mixture was added to 40ml of top-agar (LB Media and 0.7% agarose) and poured

immediately onto dry LB-agar plates (19cm × 19cm). The plates were incubated at 37°C for 16 hours followed by incubation at 4°C for 30 minutes. Nylon membrane (Hybond N - Amersham) was cut to the same size as the plates and placed on the plate for 1 minute, the membrane orientation was noted. The nylon membrane was then placed on a piece of Whatman 3MM paper soaked in denaturing solution for 1 minute. Subsequently membranes were transferred to neutralising solution for 5 minutes and 2 × SSC for 5 minutes. The membrane was then allowed to dry before baking at 80°C for 90 minutes. A duplicate lift was performed for each plate screened.

The membranes were placed in 20 ml of pre-hybridisation solution at 65°C for one hour. 10 ml of prehybridisation buffer was removed and the ^{32}P labelled radioactive probe (section 2.7.7) was added and hybridised overnight at 65°C. After hybridisation the filters were washed to remove unbound probe (section 2.7.8) and then exposed to x-ray film overnight. The film was developed as in section 2.7.9 and used to identify potentially positive plaques.

Hybridising plaques were picked from the plates using the broad end of a sterile Pasteur pipette and placed in 550µl SM buffer/chloroform (10:1). These were stored at 4°C until needed for a further round of screening. In the secondary and tertiary screens disposable 90mm plastic petri dishes were used and the process repeated as previously described for the primary screen, using dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of the plaque solution. Screening continued until positive plaques were purified and then plasmid rescue performed (section 2.7.11) in order to obtain the clone of interest. The clone was then sequenced as described in section 2.7.13

2.7.11 Plasmid Rescue from λZAP cDNA Library

The plaque of interest was placed in 100μl of SM Buffer and 10μl of chloroform and stored at 4°C until required. A 100μl aliquot of the plaque mixture was added to a 15ml falcon tube containing 200μl of OD_{600} 1.0 competent XL1 blue *E.coli* cells (as described in section 2.6.1) and 1μl of R408 helper phage (~1×10^6pfu/μl). The mixture was incubated at 37°C for 15 minutes, 5ml of 2 × YT media added and then incubated a further 3 hours at 37°C. The tube was then heated for 20 minutes at 70°C before centrifugation at 4,000 × g for 5 minutes and the supernatant decanted into a sterile falcon tube. This contained the cDNA clone as a filamentous phage particle. At this stage the solution could be stored at 4°C until required.

In order to rescue the phagemid, 200μl of the stock was incubated with 200μl of *E.coli* XL1 blue cells for 15 minutes at 37°C. The solution was then plated out on to LB-Agar plates containing 50μg/ml of ampicillin and incubated at 37°C overnight. Colonies were then selected (section 2.7.12) and grown in 5ml LB media cultures containing 50μg/ml ampicillin. The plasmid was then isolated from these cells by the methods described in section 2.6.5.

2.7.12 Colony Hybridisation

In order to determine which colonies contained the desired plasmid, colonies which had been transformed as in section 2.6.3 were then selected at random and streaked onto a fresh LB-Agar plate containing 50μg/ml of ampicillin. A grid had been marked

on the plate and a colony was streaked into each square on the grid. The plate was incubated at 37°C overnight. A piece of nylon membrane was placed directly on the plate's surface for 1 minute and the orientation noted. The membrane was subsequently placed for 1 minute on a piece of Whatman 3MM paper that had been pre-soaked in denaturing solution. This was followed by incubation in neutralising solution for 5 minutes and a final incubation in 2 × SSC for 5 minutes. The membrane was then irradiated in a Stratalinker 1800 UV crosslinker at an auto setting of 1200 Joules. The membrane was then hybridised to a ^{32}P labelled a radioactive probe added (section 2.7.7) and then washed and developed as in section 2.7.8 and 2.7.9. Colonies, which had hybridised to a probe, were then selected and plasmid preparations made as in section 2.6.5.

2.7.13 DNA Sequencing

DNA sequencing was carried by the departmental sequencing suite using an Applied Biosystems 373 A DNA Automated sequencer using the fluorescent dideoxy-chain termination method (Sanger *et al.*, 1977.). Primers were supplied at a concentration of 3.2pmoles/μl and up to 700 bases was sequenced within one reaction. In order to sequence DNA which was longer than 700 bases, nested primers were designed to sequence determined from the first round of sequencing and the DNA sequenced again from the 5' terminus of the new primer.

2.8 Protein Techniques

2.8.1 Crude Protein Extracts from *Brassica napus* Seed and Leaf

All extracts were made in the following way unless otherwise stated. The material was weighed and homogenisation buffer added at a ratio of 5:1 (v/w). A hand held homogeniser or a Polytron (Ultra-Turrax, Fischer Scientific) was used to grind up the material until the extract was completely homogenised. The extract was centrifuged at 13000 × g at 4°C for 15 minutes. Any oil layer present was removed. The supernatant was removed and spun at 40,000 × g for 15 minutes at 4°C. The supernatant obtained from this spin was used in biological activity, Western blotting and ELISA. Protein concentration was estimated by a Bradford assay (section 2.8.2).

2.8.2 Protein Estimation by Bradford Micro-assay

Several dilutions of bovine serum albumin (BSA) protein standard were prepared from 1 to 25μg/ml in order to create the standard curve necessary each time the assay was performed. The Bradford assay is dependent on the observations that the absorbance readings at 595nm of an acidic solution of the Bradford dye reagent (Coomassie brilliant blue G250) will shift when the dye is bound to protein. It has been shown that by choosing the correct ratio of dye to sample enables quantitation of the protein accurately but not in an entirely linear response. Pure BSA is readily available and relatively inexpensive and was therefore chosen to perform the standard assay when relative protein values were needed as recommended by Bio-Rad Labs Ltd. 0.8ml of the standards and appropriately diluted samples were placed in dry clean

test tubes. 0.8ml of sample buffer was used as a 'blank'. 0.2ml of Bio-Rad Bradford Dye Reagent Concentrate was added to each tube and vortexed briefly. After 5 minutes samples were transferred to clean disposable plastic cuvettes, the optical density at 595nm was measured against the 'blank'. The OD_{595} versus concentration of standards was plotted and the unknown protein concentrations calculated from this graph.

2.8.3 Methanol/Chloroform Precipitation of Proteins

The volume of sample to be precipitated was measured and 4 × volume of methanol added to the sample. The sample was vortexed and briefly spun at 10,000 × g in a microfuge. 1 × volume of chloroform was added and the sample vortexed and spun briefly at full speed in a microfuge. Three volumes of distilled water were added and the sample mixed and spun at 10,000 × g for 5 minutes. The upper phase was discarded and 3 × volumes of methanol were added. The sample was briefly vortexed and centrifuged at 10,000 × g for 3 minutes. The supernatant was discarded and the pellet dried for 10 minutes in a Jouan rotary vacuum. The pellet was then resuspended in the appropriate buffer.

2.8.4 Quantitative Antigen-Inhibition ELISA Assay for Enoyl-ACP Reductase

A microtitre plate (Life Technologies) was coated with 1μg/ml of pure enoyl-ACP reductase (from lab stock purified by Ms S. Bithell) in coating buffer (15mM NaCl, 0.4M NaOH) and incubated overnight at 4°C. The plate was washed three times with PBS-T and excess buffer removed by shaking. The microtitre plate was blocked for

one hour in M-PBS and subsequently washed three times with PBS-T. Guanidine-HCl was added to crude *B.napus* extracts prepared as in section 2.8.1 at a final concentration of 2.6M and incubated for 30 minutes on ice. The sample was then desalted using a Bio-spin P6 column (Bio-Rad) and final protein concentration was estimated via a Bradford assay using BSA as the standard (section 2.8.2).

Samples were adjusted so that the final concentration of total protein was 100μg/ml. A 100μl aliquot of each treated sample was incubated with 100μl of 1:3000 dilution of sheep anti enoyl-ACP reductase antibody and placed at 4°C for 1 hour. In each experiment described two, four and eight fold dilutions of each sample were also analysed. 100μl of each sample was taken and added to a well of the microtitre plate and incubated for 1 hour at room temperature. The plate was washed three times with PBS-T. 100μl of 1:6000 dilution of donkey-anti sheep IgG antibody conjugated to alkaline phosphatase (Serotec) made up in M-PBS was added to each well. The plate was incubated for one hour at room temperature and then washed three times with PBS-T. pNPP substrate tablets (Sigma) were dissolved in 10% diethanolamine ACS reagent at pH 9.8 to a concentration of 1mg/ml. 100μl of this solution was added to each well and incubated at 37°C. The plate was read on a microplate reader at 405nm (Bio-Rad) every 30 minutes after the addition of this substrate for up to two hours. Absolute standards of enoyl-ACP reductase (section 2.8.12) ranging from 20μg/ml to 4ng/ml were treated in an identical manner to the samples being analysed and used to create a standard calibration curve. The amount of enoyl-ACP reductase present in each crude extract could then be estimated from this curve in a quantitative manner.

2.8.5 SDS/Polyacrylamide Gel Electrophoresis (SDS/PAGE)

Proteins were analysed by the Laemelli method of SDS/PAGE (Laemelli, 1970) using a Bio-Rad mini gel system. Gels were cast between glass plates (inner glass plate = 7.3 × 10.2cm and outer glass plate = 8.3 × 10.2cm) separated by 0.75mm spacers in the Bio-Rad clamp stand apparatus. Gels were composed of 2 layers; a lower resolving gel (usually 10%) and an upper stacking gel of 4%. For a 10% bis-acrylamide resolving gel 3.33ml of 30% *bis*-acrylamide stock solution (Bio-Rad) was mixed with 2.5ml 1.5M Tris.HCl pH 8.8, 10µl of 10% SDS, 100µl of 10% ammonium persulphate and 10µl of TEMED in a final volume of 10ml. The gel was overlaid with water saturated butan-1-ol to complete polymerisation by excluding oxygen and to ensure a level surface.

The gel was allowed to set for 30 minutes during which time the stacking gel was prepared by taking 1.3ml of Bis-acrylamide, 2.5ml of 0.5M Tris.HCl pH 6.8, 100µl of 10% SDS, 100µl of ammonium persulphate and 10µl of TEMED in a final volume of 10ml. The butan-1-ol overlay was removed from the resolving gel, the stacking gel was cast and a comb positioned to create the wells. The gel was allowed to set for 30 minutes before use. Samples of up to 25µl were boiled for 1 minute at 95°C, cooled briefly and then loaded onto the gel. Electrophoresis was performed in 1 × electrode running buffer (25mM Tris.HCl pH 8.3, 250mM glycine, 0.1% SDS) at 200V for 1 hour. After electrophoresis, gels were removed and either used in western blots (section 2.8.9) or the proteins fixed with Coomassie Blue (2.8.6) or silver stained (2.8.7).

2.8.6 Coomassie Blue Staining of SDS/PAGE Gels

Immediately after electrophoresis (as described in section 2.8.5) the gel was immersed in Coomassie blue I stain (25% Propan-2-ol, 10% acetic acid, 1% (w/v) Coomassie blue R-250) and heated on medium power in a microwave for 1 minute and then agitated for 15 minutes at room temperature. Heating the solution eliminates the need for a separate fixing step and speeds the diffusion of the stain into the gel. The Coomassie dye also acts as a co-fixative which assists in fixing of highly soluble proteins to the gel whereas in an alcohol/acid fixative the proteins may diffuse out. To gently destain the gel, it was first incubated in Coomassie blue II (10% Propan-2-ol, 10% acetic acid, 0.125% (w/v) Coomassie blue R-250), this aided in removing background staining and ensured complete fixing of the proteins. The gel was then placed in Coomassie blue III (10% acetic acid, 0.125% (w/v) Coomassie blue R-250) which contains no fixative and a low amount of Coomassie blue. This destained the gel further allowing visualisation of the bands present. It is thought that this method of staining and destaining aids in visualising proteins that do not readily interact with the Coomassie blue stain. The gel was then destained in 10% acetic acid, 1% glycerol overnight at room temperature in the absence of methanol which can strip off some of the protein bound stain. This would reduce overall sensitivity of the procedure. The gel was then dried in order to keep it as a permanent record.

2.8.7 Silver Staining of SDS/PAGE Gels

Immediately after electrophoresis (as described in section 2.8.5) the gel was placed in fixing buffer and incubated at room temperature for 30 minutes with agitation in a

clean glass dish. The gel was then placed in incubation buffer (30% ethanol, 1.3% glutardialdehyde, 125mM sodium acetate, 3mM sodium thiosulphate) for 30 minutes. The gel was washed three times with distilled water. The silver reaction was carried out by incubating the gel with silver solution (5mM silver nitrate, 0.0002% formaldehyde) for 40 minutes. The gel was then placed in developer solution (250mM Sodium Carbonate, 0.001% formaldehyde) and the gel developed until the proteins appeared to be sufficiently stained. This normally occurred within 5 minutes, however gels were left for a maximum of 15 minutes if the image was not developing quickly. The gel was then placed in stop solution (50mM EDTA) for 10 minutes and washed twice with distilled water. The gel was then placed in preserving buffer (10% glycerol) for 20 minutes prior to drying.

2.8.8 Chromophore Green Staining of SDS/PAGE

An SDS/PAGE was cast, placed in electrode buffer and samples loaded as described in section 2.8.5. In the central compartment of the electrophoresis equipment 200µl of chromophore green stain (Promega) was added and the gel electrophoresed at 100V until the bromophenol blue dye front had reached the end of the gel. The chromophore green stain ran through the gel with the dye front and lightly stained the proteins which was then be visualised under a light transilluminator.

2.8.9 Western Blotting

A 10% SDS/PAGE mini-gel was run as described in section 2.8.5. Western blots were carried using a Wet Transfer Kit supplied by Bio-Rad according to the manufacturers instructions. Six pieces of Whatman pre-cut filter paper were soaked in transfer buffer for 10 minutes, along with two pieces of transfer gauze and a piece of Hybond C extra membrane (Amersham) which had been cut to the same size as the gel. The apparatus was assembled with the gel sandwiched between the membrane, filter paper and gauze and the proteins electroblotted for 16 hours at a constant current of 40mA or alternatively for 2 hours at a constant current of 200mA in which case the transfer was carried at 4°C to avoid overheating of the equipment.

After transfer the apparatus was disassembled and the efficiency of transfer examined by soaking the membrane in Ponceau S stain (0.1% Ponceau S, 1% glacial acetic acid) for two minutes. Excess stain was rinsed away with distilled water and protein molecular weight markers visualised and marked on the membrane with a pencil. The membrane was then fully destained with 1% acetic acid or PBS-T. The gel used in the transfer was stained in Coomassie Blue (as described in section 2.8.6) to check that no protein remained in the gel.

All Western blots were treated in the following way unless stated otherwise. All incubations and washes were carried out with agitation. The membrane was incubated in 50ml of blocking buffer (M-PBS) for 2 hours to block unoccupied binding sites on the membrane before being transferred to a previously determined dilution of primary

antibody in 10ml of blocking buffer for 1 hour at room temperature. The membrane was then washed twice with PBS-T for 20 minutes.

A working dilution of the secondary antibody of between 1 in 1000 – 1 in 30,000 was prepared in blocking buffer and the membrane incubated for one hour at room temperature (generally a donkey anti-sheep IgG or goat anti-rabbit IgG both linked to alkaline phosphatase was used or for chemiluminesence development, goat-anti rabbit IgG or donkey anti-sheep IgG linked to horse radish peroxidase was used). The membrane was washed twice with PBS-T for 20 minutes.

Secondary antibodies had either Horseradish Peroxidase or Alkaline Phosphatase as the conjugate. In Westerns using Horseradish Peroxidase conjugates proteins were visualised using either an Amersham ECL Kit or a Pierce Supersignaling Kit. In both cases the westerns were developed according to the manufacturers recommended method. Blots which were probed using alkaline phosphatase conjugates were developed using BCIP/NBT tablets (Sigma) dissolved in MQ water at a concentration of 1mg/ml. The enzymatic reaction was terminated by transferring the membrane to excess distilled water. The membrane was then air-dried and could be stored as a permanent record.

2.8.10 Western Dot Blotting

A dot blot was carried out using the Bio-Dot Micro-filtration apparatus (Bio-Rad) and assembled according to the manufacturer's instructions. The nylon membrane (Hybond C – Amersham) was pre-soaked in TBS buffer for 10 minutes and then

placed in the dot blot apparatus and sealed under vacuum. Samples were prepared by boiling in the presence of 1% SDS for 2 minutes and 100µl added to each well of the dot blot and the sample allowed to filter through the membrane by gravity flow for 30 minutes. Any remaining solution was then pulled through by a partial vacuum. The membrane was then washed twice in T-TBS whilst still in the apparatus to remove any unbound antigen. The membrane was then removed and treated as in section 2.8.9 to visualise the protein.

2.8.11 Amino Acid Sequencing using Problott Method

A 10% SDS/PAGE gel was prepared as in section 2.8.5 and stored overnight at 4°C. The gel was then placed in 800ml of electrode running buffer in an electrophoresis tank and 150µl of 200µM thioglycolic acid added to the inner chamber of the apparatus and the gel pre-run at 50V for 30 minutes. Once the samples were treated and electrophoresed according to section 2.8.5 The proteins on the gel were then transferred to Problott membrane in the same manner as section 2.8.9 except that the transfer buffer used was 1 × CAPS, 10% HPLC grade methanol and the transfer carried out for 30 minutes at 50V. Once transfer was complete the Problott membrane was rinsed in distilled water and then saturated for a few seconds in 100% HPLC grade methanol The membrane was stained in 0.1% Coomassie blue R-250, 40% HPLC grade methanol and 1% acetic acid for 1 minute. The Problott membrane was then de-stained in 50% HPLC grade methanol for 1 hour. The membrane was rinsed in distilled water and allowed to completely dry. The stained protein band to be sequenced was then cut out of the membrane and sequenced using a ABI 477A pulsed

liquid protein microsequencer with 120A on-line analyser for up to 7 cycles, in order to confirm the identity of the protein. The sequencing was performed by Mr.J. Gilroy.

2.8.12 Determination of Absolute Concentration of Pure Protein Sample by Hydrolysis

This was performed in order to determine an absolute concentration of pure ENR and βKR samples for use in the quantitative ELISA assay described in section 2.8.4. A small glass screw top hydrolysis tube was acid washed, allowed to dry completely and 100μl of protein sample was added to the tube. The protein sample's concentration had been estimated by Bradford assay (section 2.8.2) so that the concentration was approximately 1mg/ml. 10nMoles of Norleucine were added to the sample to act as an internal standard. The sample was then freeze dried overnight in a dessicator. 100μl of 6M HCl was added to the sample and the tube flushed with nitrogen. The tube was then sealed with PTFE tape and a Teflon cap placed in the lid of the tube and placed at 110°C for 24 hours, this allowed complete hydrolysation of the protein in to its individual amino acids. After incubation the tubes were cooled to room temperature and freeze-dried overnight in the presence of sodium hydroxide pellets. The samples were then sent to Dr A.K.Allen at Charing Cross Hospital, London, where the samples were loaded onto an amino acid analyser in order to determine the absolute protein concentration.

2.8.13 Gel Filtration of *Brassica napus* Crude Protein Extracts

Gel filtration of crude extracts of oil seed rape was carried out using a Pharmacia superose 12 column on a Pharmacia SMART HPLC system. The column was stored in 20% ethanol and this was removed by washing the column in filtered distilled water at 40µl/min for 1 hour. The column was then equilibrated with 0.05M sodium phosphate buffer at pH 7.0 prior to use.

In order to determine retention times for different sized proteins, five protein standards were passed through the column: ferritin (440kDa), catalase (232kDa), bovine serum albumin (67kDa), ovalbumin (43kDa) and ribonuclease (13.7kDa) and a retention time was calculated for each. A graph was plotted of the log of the molecular weight versus the retention and used to calculate the molecular weight of each separate fraction collected from the run using a protein extract. The crude protein samples were passed through the column and 100µl fractions collected until 2ml of buffer had washed through. Each fraction was then analysed by an ELISA assay (section 2.8.4) or a Western dot blot (section 2.8.10).

2.9 Biological Activity Assays

2.9.1 Enoyl-ACP Reductase Enzyme Assay

80µl of 50mM sodium phosphate buffer pH6.2 was placed in a 100µl glass cuvette and the spectrophotometer set at 340nm and the spectrophotometer reference set. 10µl of NADH (1mg/ml) was added to the buffer and thoroughly mixed. The reading on

the spectrophotometer was allowed to stabilise before the addition of sample. 10µl of sample was then added and the assay initiated by the addition of 10µl of crotonyl-CoA (1.2mM stock). Crotonyl-CoA has been shown to a suitable replacement for crotonyl-ACP in *in vitro* assays for enoyl-ACP reductase (Slabas *et al.*, 1986). The oxidation of NADH to NAD^+ was visualised by using a graph tracer attached to the spectrophotometer. The activity of enoyl-ACP reductase was defined as the oxidation of 1µM of NADH per minute under the conditions of the assay.

2.9.2 Malonyl-CoA:ACP Transacylase (MCAT) Enzyme Assay

MCAT activity was assayed by measuring the transfer of the malonyl group from [^{14}C] malonyl-CoA to ACP as estimated by acid precipitable radiolabelled products.

Assays were started by the addition of 13.4µM of [^{14}C] malonyl-CoA to a reaction mixture containing 100mM Tris.HCl pH 8.0, 5mM DTT, 200µg ACP and 10µl of the extract to be assayed. The mixture was incubated for 2 minutes at 30°C and the reaction terminated by the addition of 100µl of ice cold 10% perchloric acid and 50µg of BSA. The reaction mixture was incubated on ice for 5 minutes and then centrifuged at 10,000 × g for 10 minutes. The supernatant was discarded and the pellet was washed twice with 150µl of ice cold 2% perchloric acid and resuspended in 100µl of 1N NaOH. The solution was subsequently neutralised with 100µl of 1M HCl and transferred to a scintillation vial. 4ml of ecoscint was added and the solution gently mixed. The sample was then counted on the [^{14}C] program of a scintillation counter.

2.9.3 Ketoacyl-ACP Synthase III (KAS III) Enzyme Assay

KAS III activity was assayed by measuring acid precipitated [^{3}H]acetoacetyl-ACP formed from the condensation of [^{3}H] acetyl-CoA to malonyl-ACP.

A reaction mixture (0.1M sodium phosphate pH 7.4, 1mM DTT, 20μM ACP, 50μg BSA, 1mM malonyl-CoA, 4μg MCAT), was incubated with the sample to be assayed and 100μM cerulenin at room temperature for 10 minutes. The assay was initiated by the addition of 1mM [^{3}H] acetyl-CoA and incubated at 30°C for 10 minutes. The reaction was terminated by the addition of 200μl of ice cold 10% tri-chloroacetic acid (TCA) and 50μg BSA and placed on ice for 10 minutes. The reaction mixture was centrifuged at 10,000 × g for 3 minutes, and the pellet washed twice in 150μl of ice cold 10% TCA. The pellet was resuspended in 100μl of 1N NaOH and neutralised with 1M HCl. The sample was then placed in a scintillation vial and 4ml of ecoscint was added. The sample was then counted on a [^{3}H] program in a scintillation counter.

2.10 Fatty Acid Analysis

2.10.1 Extraction of Fatty Acid Methyl Esters from *Brassica napus* seeds

Fatty acid methyl esters were prepared and extracted using a method described by Browse *et al.* (1986). Seeds were imbibed overnight by placing them on damp blotting paper. The testa was then removed from two seeds and the embryos combined and ground up in the presence of 1ml of methanolic HCl reagent using a polytron (Ultra-

Turrax, Fisher Scientific). 10µl of C17:0 fatty acid (10mg/ml) was added as an internal standard. The sample was placed in a screw-capped glass tube, sealed with a Teflon-lined cap and was heated at 80°C for 1 hour. The sample was then allowed to cool to room temperature and 0.3ml of hexane and 1ml of 0.9% NaCl added. The tube was then vigorously shaken and centrifuged at 1000 × g for 1 minute. The upper layer containing the FAMEs was removed to a clean glass vessel and the volume reduced to 200µl by purging with nitrogen. The sample was then stored at -20°C until required for analysis.

2.10.2 GLC Analysis of Fatty Acid Methyl Esters

Gas liquid chromatography was used to separate out the FAMEs isolated in section 2.10.1. A 2µl aliquot of FAME extract was loaded onto a Polar Wcot Fused Silica column (30m) with 0.25mm internal diameter and 0.25µM film, using a split injection system of a ratio of 27:1. The FAMEs were eluted in nitrogen at 40cm/min at a pressure of 100kPa in a total elution time of 20 minutes, with an acquisition delay time of 2.5 minutes. The column was heated in 4°C steps from 140°C to 212°C during the elution. The injector was set at a temperature of 250°C and the detector at 270°C. The samples were compared against an internal C17:0 standard (10µg/ml) within each sample and Nu-Check FAME Standard Marker combinations numbers 63 and 65 were run at the beginning and at the end of each batch run. The fatty acid composition of each sample was calculated by programming the computer to perform internal normalisation of peak areas and the amounts of fatty acid determined by extrapolating back using the internal standard set at 10µg/ml.

2.11 Plant Transformation Methods

2.11.1 Callus-*Agrobacterium* Mediated Transformation of *Brassica napus*

Transformation of *Brassica napus* was essentially as described by Moloney *et al.* (1989).

Seeds of *Brassica napus* cv RV28, a double haploid line, were surface sterilised in 1% sodium hypochlorite for 20 minutes and then rinsed thoroughly three times in distilled water. Seeds were placed on MS media containing 3% sucrose and 0.8% phytoagar and placed at 25°C in a 16hour light/8hour dark regime to germinate for five days (light intensity 40-60μEm^{-2}sec^{-1}). Bacterial cultures were produced from a single colony in 5ml culture grown overnight in AB medium at 28°C. 50μl of this culture was used to inoculate 5ml culture of MG/L broth that was grown overnight at 28°C in 25μg/ml nalidixic acid and 70μg/ml gentamycin. The cells were pelleted by centrifugation at 10,000 × g and resuspended in 10ml of MS media containing 3% glucose.

After 5 days the cotyledons were removed from the stem ensuring that approximately 2mm of petiole was attached. Each petiole was then immersed in a suspension of the *Agrobacterium* strain containing the construct for 5 seconds. Once the petiole had been immersed it was immediately embedded on a plate of MS medium, 3% sucrose and 0.7% phtyoagar which was enriched by the addition of 20μM benzyladenine. The plates were returned to the growth chamber and the cotyledons co-cultivated with the *Agrobacterium* for three days.

2.11.2 Selection of *Brassica napus* Transformants

Cotyledons which had been co-cultured with *Agrobacterium tumefaciens* were transferred to fresh MS medium containing 20μM benzyladenine, 3% sucrose, 0.7% phytoagar, pH5.8, 50μg/ml carbenicillin and 15μg/ml kanamycin. Only ten explants were placed on any one petri dish to avoid overcrowding which would lower the regeneration rate. The explants were transferred to fresh plates once a week for three weeks. In the fourth week green shoots which had emerged from the callus were excised and placed on to shoot elongation medium (MS salts, 3% sucrose, 0.7% phytoagar, pH5.8, 50μg/ml carbenicillin and 15μg/ml kanamycin). Dominant shoots from this selection were then transferred to rooting media (MS salts, 3% sucrose, 2μg/ml indolebutyric acid, 0.7% phytoagar and 50μg/ml carbenicillin).

Once a strong root system had formed plantlets were transferred to sterile peat pellet jiffy and placed in Magenta pots. The plants were grown for a further two weeks and then leaf samples taken and analysed for presence of the construct using PCR (section 2.7.4). Positive plants were then transferred to larger pots and allowed to grow to maturity and to set seed as described in section 2.5.1.

2.11.3 *In Planta Agrobacterium* mediated transformation by infiltration of adult *Arabidopsis thaliana* plants

This method is based on Bechtold *et al.* (1993). An *Agrobacterium* suspension containing the appropriate construct was prepared by plating the strain out on a LB-Agar plate containing 25μg/ml nalidixic acid and 70μg/ml gentamycin. The plate was

incubated at 28°C overnight and then a colony picked and used to inoculate a 50ml culture of LB media containing 25μg/ml nalidixic acid and 70μg/ml gentamycin. This culture was grown overnight at 28°C with shaking and used to seed a 400ml culture of LB media containing 25μg/ml nalidixic acid and 70μg/ml gentamycin. The culture was grown for 2 days at 28°C with shaking. The cells were harvested once an OD_{600} of 2.0 was reached, by centrifugation at 5000 × g for 10 minutes at room temperature. The cells were resuspended in approximately 1.2 litres of infiltration media to give an OD_{600} of 0.8 and kept at room temperature until required.

A*grobacterium* suspension was placed in a 1 litre beaker. The beaker was placed in large dessicator and a pot of the prepared *Arabidopsis* plants (section 2.5.2) were inverted into the suspension ensuring that all the plants were submerged. The dessicator was then closed and a vacuum applied using a diaphragm vacuum pump at a pressure of 27 inches of Hg for 5-7 minutes. The vacuum was then quickly released, the plant was removed from the beaker and placed in horizontal position on a tray that was then loosely covered with a plastic bag. The treated plants were placed overnight in a growth chamber with illumination and allowed to recover. The following day the plants were placed upright and grown under a long day regime until the plants had set seed. Once the seed was mature it was collected and allowed to dry in a dessicator for 2-3days and thereafter stored at 4°C.

2.11.4 Selection of *Arabidopsis* Transformants

To select for transformed lines, the seed was first sterilised by washing for 2 minutes in 70% ethanol. This was followed by soaking the seed for 3 minutes in 20%

Domestos. The wash was repeated a further two times with fresh solution in each case. The seed was then thoroughly rinsed in sterile distilled water and allowed to dry in a laminar flow hood. The seed was transferred to sterile Eppendorf tubes and plated out on selection media within two days of this treatment. Selection was carried out by sprinkling the seed onto plates of MS media (0.5 × Murashige and Skoog salts pH 5.7, 0.8% agar, 1 × Gamborg' B5 vitamins) containing 40μg/ml of kanamycin and placed at in 25°C plant tissue culture chamber with constant illumination. Within 10 days putative positives could be identified and transferred to sterile peat pellet jiffys contained within Magenta pots. The plants were then returned to the plant tissue culture chamber until the plantlet had grown to fill the pot. The plantlet was then transferred to soil in a plant growth room with a long day regime (16 hoursday/8hours dark, as described in section 2.5.2). For the first 24 hours the plantlet was covered in a transparent plastic pot to allow it to acclimatise to the new growth conditions. A leaf sample was taken from the plant in order to confirm the presence of the construct via PCR (section 2.7.4). Seed from these lines were collected and stored as previously described in section 2.11.3.

Chapter 3

Isolating a Full-Length KAS III cDNA from *Brassica napus*

3.1 Introduction

β-Ketoacyl-ACP synthase III (KAS III) catalyses the condensation of malonyl-ACP with acetyl-CoA to produce acetoacetyl-ACP (C4) and CO_2. This Claisen condensation reaction was first observed in *E.coli* (Jackowski and Rock, 1987), where despite the irreversible inhibition of KAS I and KAS II by cerulenin (D'Agnolo *et al.*, 1973), *in vivo* acyl-ACP formation continued with short chain (C4-8) acyl-ACPs increasing to 60% of the total ACP pool. These observations inferred the existence of a cerulenin insensitive condensing enzyme capable of catalysing the initial steps in chain elongation. Further work also demonstrated that KAS III possesses a preference for acetyl-CoA over acetyl-ACP (Jackowski *et al.*, 1989). This was surprising as until then it had been thought that C4 acyl-ACP was synthesised by the condensation of malonyl-ACP and acetyl-ACP with the latter molecule being produced by acetyl-CoA:ACP transacylase (ACAT). Thus the discovery of this enzyme has removed the necessity of ACAT to produce the primer molecule for fatty acid synthesis.

Thiolactomycin is an antibiotic, which specifically inhibits type II fatty acid synthase in a reversible manner (Nishida *et al.*, 1986). The study showed that KAS enzymes were especially affected by this inhibitor. On the discovery of KAS III activity, thiolactomycin was used to

isolate an *E.coli* mutant, CDM5, which possessed a severely depressed KAS III activity that was still able to synthesise acyl-ACPs (Jackowski *et al.*, 1989). The mutant strain, CDM5, was shown to lack KAS III activity yet at the same time synthesise acyl-ACPs. This observation was attributed to the malonyl-ACP decarboxylase activity of both KAS I and KAS II, which forms acetyl-ACP on decarboxylation, this could in turn be utilised by a functional KAS I present in the mutant. This inferred that KAS III activity was not essential, however the rate of synthesis of acetyl-ACP by decarboxylase activity of KAS I has been shown to occur at a much lower rate and to be generally induced when high levels of malonyl-ACP are present (Alberts *et al.*, 1972). When the mutant was inhibited with cerulenin no acyl-ACPs were detected in an *in vitro* assay further corroborating that this mutant lacked a KAS III protein and also that this enzyme is required to enable the incorporation of acetyl-CoA into fatty acids. However Tsay *et al.* (1992a) showed that the mutation in strain CDM5 results in the production of a KAS III condensing activity that is resistant to thiolactomycin but is much less stable to *in vitro* manipulations than the wild type enzyme and therefore there are no known null mutants of KAS III in *E.coli*.

As KAS III can directly utilise acetyl-CoA these observations removed the absolute necessity for an ACAT enzyme. Shimakata and Stumpf (1983a) had previously demonstrated that ACAT had the lowest enzyme activity in *de novo* fatty acid synthesis. On demonstration of KAS III activity in spinach (Jaworski *et al.*, 1989) ACAT activity was compared to FAS activity by two acid precipitable radioactive assays, which were used to calculate the initial velocities of the *in vitro* synthesis of acyl-ACPs. Jaworski *et al.* (1989) reasoned that if the ACAT step was bypassed by a more active KAS, the overall rate of fatty acid biosynthesis

would exceed the rate limiting ACAT step. KAS III was partially purified by ammonium sulphate precipitation and when assayed in the presence of fatty acid synthase co-factors and cerulenin, the principal fatty acids produced were butyric (C4) and hexanoic acids (C6). When ACAT activity was examined in this experiment it was seen to be five fold lower than the KAS III condensation reaction. This characteristic has been observed in other higher plants including oil seed rape (Walsh *et al.*, 1990). It was also shown that, as in *E.coli*, the spinach KAS III used acetyl-CoA in preference to acetyl-ACP implying that KAS III could by pass the ACAT step, rendering the latter enzyme superficial to fatty acid synthesis. It is now accepted that the initial priming reaction in fatty acid synthesis is carried out by KAS III, and ACAT, once thought to be a regulatory point in *de novo* fatty acid synthesis, now appears to have little importance in the synthesis of fatty acids.

The *E.coli* mutant, CDM5, was used to locate and characterise the KAS III gene; *fab*H (Tsay *et al.*, 1992b). A series of interrupted mating experiments between strain CDM5 and different Hfr donor strains carrying specific Tn10 insertions were performed. The conjugants were assayed for KAS III activity and on analysis of these recombinants it was seen that the *fab*H allele was located 24.5min region of the *E.coli* chromosome. The *fab*H gene was shown to encode a protein of 33.5kDa and when this protein was purified both KAS III activity and ACAT activity were demonstrated to be present on the same protein. When overexpressed in *E.coli* it was seen to have a significant impact on the membrane lipid composition where the amounts of *cis*-vaccenate were severely depressed and there was an increase in the amount of myristate. Following the isolation of the *fab*H gene, a cDNA clone was isolated from spinach (Tai and Jaworski, 1993) using information derived from the amino acid sequence of the

purified spinach KAS III (Clough *et al.*, 1992) to design degenerate primers using the *fab*H sequence from *E.coli* (Tsay *et al.*, 1992) as a guide. A variety of KAS III cDNAs isolated from a number of different plant species have been reported, these include *Arabidopsis thaliana* (Tai *et al.*, 1994) leek (Chen and Post-Beittenmiller, 1996) *Cuphea wrightii* (Slabaugh *et al.*, 1995) and as well as a putative KAS III from a strain of alga; *Porphyra umbilicalis* (Reith, 1993).

In higher plants KAS III and ACAT activity have been separated from each other providing evidence that these two discrete activities do exist (Gulliver and Slabas, 1994). This raises the possibility that fatty acid synthesis in plants could be plastic and down regulation of KAS III may be alleviated by a new initial condensation reaction requiring ACAT and KAS I activity. The question of KAS III playing a regulatory role could be answered by the examination of transgenic plants that have had KAS III levels down regulated. In order to do this an antisense construct is required, and it was decided that transformations should be performed on *Brassica napus*, a plant whose seed contains approximately 40% oil. Therefore there was a need for a full-length clone of KAS III from *Brassica napus* not only to make antisense constructs but also to be able to make overexpression constructs in order to raise KAS III antibodies (Chapter 4). A KAS III cDNA from this plant has not been reported in the literature and this chapter describes the isolation of a *Brassica napus* KAS III cDNA, along with an examination of the gene copy number. The *B.napus* clone is compared to other known KAS III proteins and the expression of KAS III transcripts and transcript size examined by Northern analysis.

3.2 Results

3.2.1 Preparing of KAS III DNA Probe

A partial KAS III clone, TF Clone 2, (Figure 3.1) was isolated from a rape embryo library (prepared by Mr J.Kroon) using a KAS III PCR product derived from a spinach cDNA library (by Dr.T.Fawcett). This clone of 500bp showed high homology to the 5' region of the open reading frame of the known *Arabidopsis thaliana* KAS III cDNA sequence. A plasmid prep of TF Clone 2 (Figure 3.1) was digested using *Hind* III and *Eco* RV following the standard protocol described in section 2.7.3 to yield a fragment of 387bp. This fragment was excised from a low melting point agarose gel and an equal volume of TE pH 8.0 was added. This solution was subsequently boiled and 10µl used in a rediprime labelling reaction (section 2.7.7) to radioactively label the DNA with [^{32}P] dCTP.

3.2.2 Genomic Southern of *Brassica napus*, *Brassica oleracea* and *Brassica campestris* to calculate KASIII gene copy number in *Brassica napus*

In order to determine the possible number of KAS III gene copies in *Brassica napus*, a Southern blot was performed against genomic DNA obtained from *Brassica napus* and its two parents; *Brassica oleracea* and *Brassica campestris*. Digesting the genomic DNA with a restriction enzyme creates different restriction profiles for each species. The restriction enzymes may cut within the gene of interest or alternatively there may not be the appropriate restriction site present. By digesting the genomic DNA separately with a number of different

```
1/1                                                 31/11
GAA TTC GCG GCC GCT GAT AAC AAT GCT TCT CCT TCT CCT TCT CAA TAC CGA CCA CCC AGG
E   F   A   A   A   D   N   N   A   S   P   S   P   S   Q   Y   R   P   P   R
61/21                                               91/31
CTA GTA CCG AGC GGC TGC AAA CTT ATT GGA TCT [redacted] CTT CTG ATT
L   V   P   S   G   C   K   L   I   G   S   G   S   A   V   P   S   L   L   I
121/41                                              151/51
TCT AAT GAT GAT CTC GCA AAG ATT GTC GAT ACT AAT GAT GAG TGG ATA GCT ACT CGC ACT
S   N   D   D   L   A   K   I   V   D   T   N   D   E   W   I   A   T   R   T
181/61                                              211/71
GGT ATC CGC AAC CGC AGA GTT GTA TCA GGC AAA GAT AGC TTG GTT GGC TTA GCA GTG GAA
G   I   R   N   R   R   V   V   S   G   K   D   S   L   V   G   L   A   V   E
241/81                                              271/91
GCA GCA ACC AAA GCC CTT GAA ATG GCA GAA GTT GCT CCT GAG GAT ATT GAC TTA GTC TTG
A   A   T   K   A   L   E   M   A   E   V   A   P   E   D   I   D   L   V   L
301/101                                             331/111
ATG TGT ACC TCC ACT CCT GAT GAT CTC TTC GGT GCT GCT CCA CAG ATT CAG AAG GCG CTT
M   C   T   S   T   P   D   D   L   F   G   A   A   P   Q   I   Q   K   A   L
361/121                                             391/131
GGT TGC ACA AAG AAC CCT TTG GCA TAT GAT ATC ACA GCT GCT TGT AGT GGA TTT GTT TTG
G   C   T   K   N   P   L   A   Y   D   I   T   A   A   C   S   G   F   V   L
421/141                                             451/151
GGT CTA GTC TCA GCT GCT TGT CAT ATA AGA GGA GGC GGT TTT AAG AAT GTT TTG GTG ATT
G   L   V   S   A   A   C   H   I   R   G   G   G   F   K   N   V   L   V   I
481/161                                             511/171
GGA GCT GAT TCT TTA TCT CGG TTT GTT GAT TGG ACT GAT AGA GGA ACT TGC ATC CTC TTT
G   A   D   S   L   S   R   F   V   D   W   T   D   R   G   T   C   I   L   F
541/181                                             571/191
GGA GAT GCT GCT GGT GCT GTG GTT GTT CAG GCT TGT GAC ATT GAG GAT GAT GGG TAT ATA
G   D   A   A   G   A   V   V   V   Q   A   C   D   I   E   D   D   G   Y   I
601/201                                             631/211
GTT TTG ATG TGC ACA GCG ATG G*G ACG GGT CGT AGA AAT TTG AAT *CT TCT GTT T
V   L   M   C   T   A   M   X   T   G   R   R   N   L   N   X   S   V
```

Figure 3.1 - The sequence of TF Clone 2; a partial clone of *Brassica napus* KAS III cDNA isolated from a rape embryo cDNA library.

The region in bold was excised using *Eco* RV and *Hind* III restriction enzymes (Boehrhinger-Mannheim). This fragment was used to probe the genomic Southern blot (Figure 3.2) and also to re-screen the *Brassica napus* embryo cDNA library. The boxed region is the annealing position of primer SDK2A used in PCR selection described in the text section 3.2.4.

restriction enzymes and then performing a Southern blot a comparison of the restriction profiles between the species is possible. This aids in determining the copy number of the gene of interest.

20μg of each type of DNA was digested in a final volume of 100μl with 30 units of one of the following enzymes, *Bam* HI, *Eco* RI, *Hind* III and *Xba* I in the appropriate restriction enzyme buffer (Boehrhinger-Mannheim). Each digest was incubated at 37°C overnight, ethanol precipitated and resuspended in 25μl of 1 × DNA gel loading buffer. The samples were loaded onto a 1% agarose gel and examined by electrophoresis at 50mA overnight. The gel was washed in 0.25M HCl for 15 minutes to depurinate the DNA and rinsed in blotting buffer for 10 minutes. The DNA was transferred to Hybond N membrane (Amersham) overnight via Southern blot (section 2.7.5) and the membrane was probed in Hybridisation buffer at 65°C with the KAS III fragment isolated in section 3.2.1. The membrane was washed to a final stringency of 0.1 × SSC, 0.1% SDS and exposed to x-ray film.

The autoradiograph (Figure 3.2) indicates that *B.napus* contains between two and four gene copies. Although no genomic DNA sequence data for KAS III *B.napus* is currently available, from the cDNA it is known that the open reading frame does not have any of the restriction sites present in its sequence which were used in the digests. When *B.napus* was restricted with *Bam* HI four bands can be seen, the higher two bands have a greater intensity than the other bands, this could suggest that the probe had greater homology to two of the possible four isoforms. When the genomic DNAs were digested with *Eco* RI there appears to be no identity between the three restriction profiles, *B.campestris* highlights 6 bands all with low intensity,

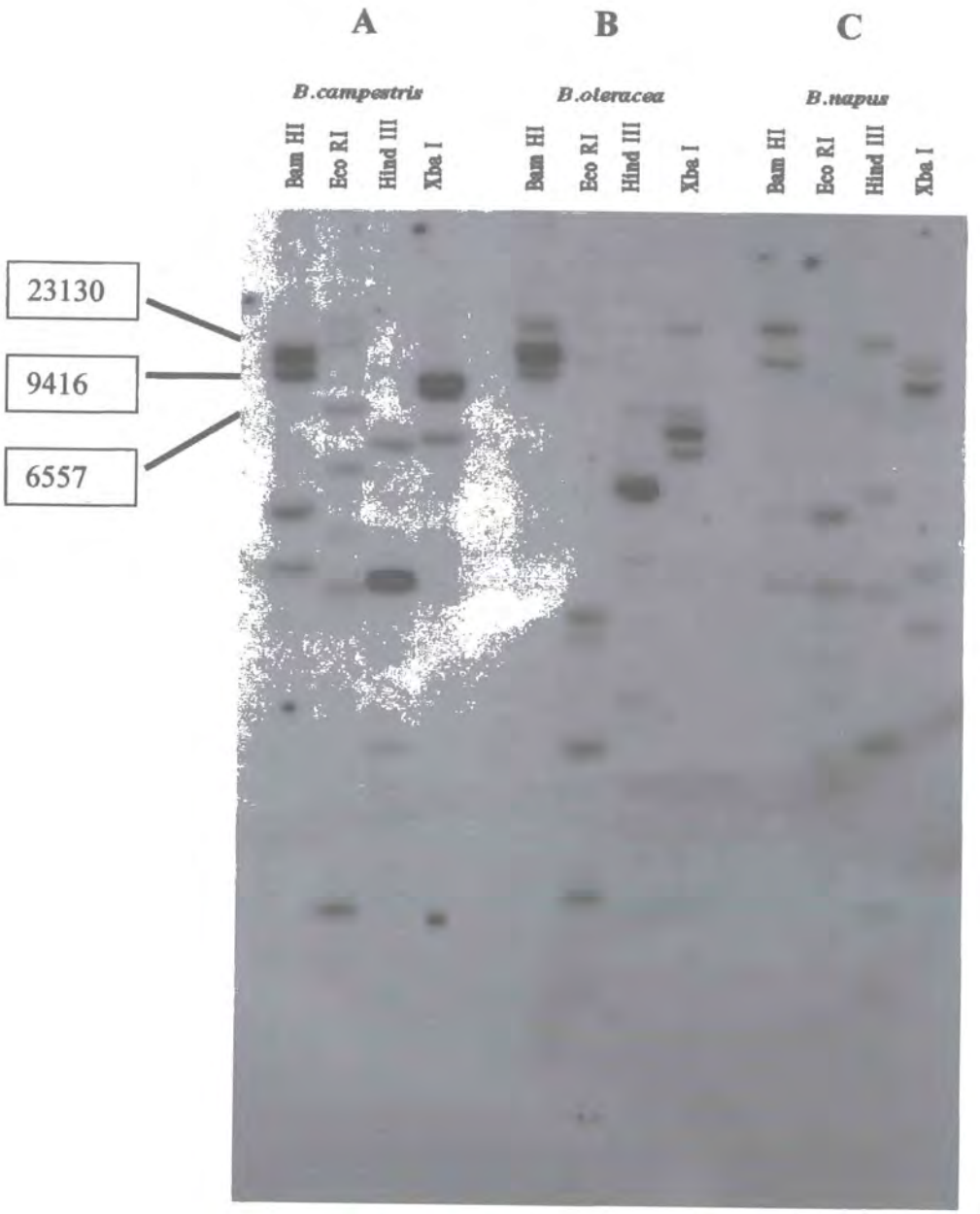
Figure 3.2 - Genomic Southern of *Brassica napus, Brassica campestris and Brassica oleracea* to determine gene copy number of KAS III.

The KAS III probe synthesised in section 3.2.1 was used in this analysis. The Southern indicates that at least two copies of the gene are present in *B.napus* but it is likely that there are at least four isoforms, as has been observed for enoyl-ACP reductase (Kater *et al.*, 1991 and Fawcett *et al.*, 1994).

Section A – The four individual enzyme digests of *Brassica campestris* genomic DNA.

Section B – The four individual enzyme digests of *Brassica oleracea* genomic DNA.

Section C – The four individual enzyme digests of *Brassica napus* genomic DNA.


A
B
C
B.campestris
B.oleracea
B.napus
Bam HI
Eco RI
Hind III
Xba I
Bam HI
Eco RI
Hind III
Xba I
Bam HI
Eco RI
Hind III
Xba I
23130
9416
6557

B.oleracea has 2 and *B.napus* has 4. This could simply mean that in a genomic clone of KAS III an *Eco* RI site is present. As a small cDNA fragment was used in this screen and as no genomic DNA information for KAS III *B.napus* is available it is not certain whether all the bands containing a KAS III region have been highlighted however this work demonstrates the existence of more than one copy of the KAS III gene in *B. napus*.

3.2.3 Screening the Rape Embryo cDNA Library for a full-length KAS III cDNA

150,000 pfu per plate were screened by inoculating *E.coli* XL1 blue cells with the rape embryo cDNA library in λ phage, and the mixture added to sterile LB top agarose and poured on to a LB-agar plate. This was duplicated and the plates incubated at 37°C overnight until plaques had formed. Plaque lifts were carried out in duplicate for each plate. Following overnight hybridisation of the membranes with the probe synthesised in section 3.2.1, the filters were washed to a final stringency of 1 × SSC at 65°C prior to autoradiography. Twenty-two putative KAS III clones were identified and agar plugs taken and placed in SM buffer/chloroform (10:1) and stored at 4°C.

3.2.4 PCR Analysis of Putative Positive Plaques

To allow preliminary analysis of putative positive plaques and to eliminate small non full-length clones, PCR was carried out on each primary plaque. The primer SDK2A 5' GGT TCA GCT GTC CCA AGT 3' was designed using the sequence from TF Clone 2 orientated in such a manner to enable extension to the 5' end of the clone. The PCR was set up in a total volume

of 50µl, which included 10µl of each primary plaque mixture isolated in section 3.2.3 and 20pmole of either primer T3 or T7 along with 20pmole of primer SDK2A. Following an initial denaturation for 4 minutes at 94°C the reaction was initiated by the addition of 1 unit of *Taq* DNA Polymerase (Bioline) and subsequently subjected to 25 cycles of the following conditions:

Denaturation	95°C	30 seconds
Annealing	50°C	30 seconds
Extension	72°C	2 minutes

A final extension cycle of 10 minutes at 72°C completed the PCR. These conditions were selected as the DNA denatures into single strands at 95°C. The primer annealing temperature is dependent on the oligonucleotide used and the extension temperature of 72°C is the optimum temperature for the Taq DNA polymerase to extend the DNA. A sample of each reaction was analysed on a 0.8% agarose gel (Figure 3.3) and the molecular weight of the PCR products calculated by plotting distance moved from the origin against the log of molecular weight of known standards that were also run on the gel. The KAS III open reading frame from *Arabidopsis thaliana* (Tai *et al.*, 1994) has been shown to be 1200bp therefore those plaques which yielded PCR products greater than 800bp (six in total) were chosen for further rounds of screening until plaque pure. The phagemids were isolated by the plasmid rescue technique described in section 2.7.11 and the purified plasmids sequenced using the M13 forward and reverse primers (section 2.3). From the initial sequencing of the clones, a clone that was derived from primary plaque 1.5, renamed as KAS III Clone 13 (pKAS13), was selected for further sequencing. The initial sequence of pKAS13 from the M13 forward and reverse sequence produced 267 bases and 333 bases of reliable sequence respectively. The sequence obtained from the M13 reverse primer contained the first 206 bases of the KAS III

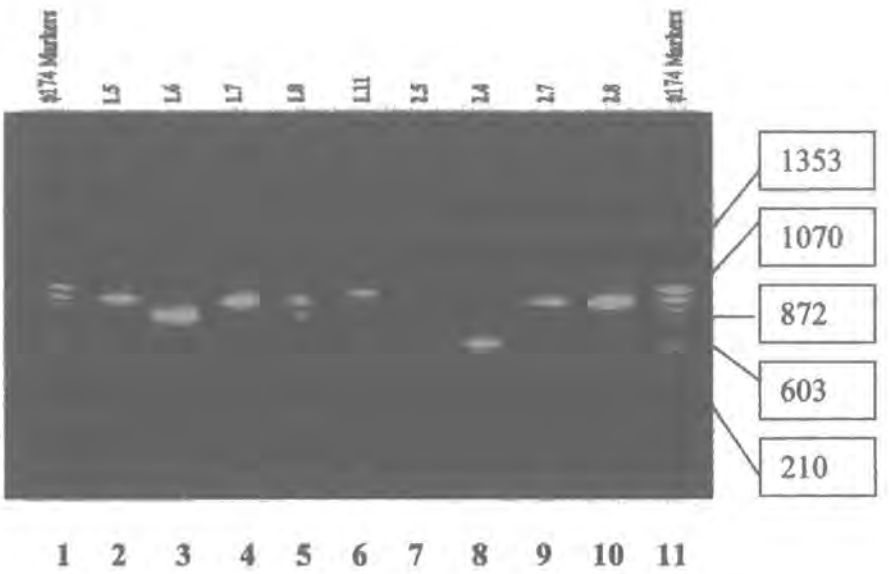


Figure 3.3 - PCR products of plaques from primary cDNA screen.

The DNA from the plaques were amplified using the primers SDK2A and T7. PCR analysis assisted in determining the size of the clone from each plaque. Plaques which produced PCR products of 800bp or greater were selected for further screening and sequencing.

Lane 1	ϕ174 Markers		
Lane 2	Plaque 1.5	-	1222bp PCR Product
Lane 3	Plaque 1.6	-	630bp PCR Product
Lane 4	Plaque 1.7	-	1122bp PCR Product
Lane 5	Plaque 1.8	-	1122bp and 776bp PCR Product
Lane 6	Plaque 1.11	-	1273bp PCR Product
Lane 7	Plaque 2.5	-	691bp PCR Product
Lane 8	Plaque 2.4	-	316bp PCR Product
Lane 9	Plaque 2.7	-	933bp PCR Product
Lane 10	Plaque 2.6	-	933bp PCR Product
Lane 11	ϕ174 Markers		

ORF along with 126 of the 5'UTR. It could be seen from the PCR that it was a cDNA of at least 1222bp and the initial sequence had shown strong homology to the *Arabidopsis thaliana* KAS III cDNA (data not shown).

3.2.5 Complete Sequencing of KAS III Clone 13

KAS III Clone 13 (pKAS13) was fully sequenced in both orientations using primers designed according to the sequence obtained from the previous round of sequencing (Table 3.1). The full sequencing strategy can be seen in figure 3.4. The cDNA was 1622bp and consisted of the entire 3' UTR region (281bp), open reading frame (1215bp) and 126bp of the 5'UTR region. (Figure 3.6). Comparison with the transcript size observed in the Northern analysis (Figure 3.9), estimated at 1600bp, suggested that the cDNA may be a full-length clone. Figure 3.6 shows the whole sequence and places a putative leader sequence of 222 bases at nucleotide positions 127 to 349. Comparison of the open reading frame sequence with the KAS III cDNA sequence of *Arabidopsis thaliana* revealed 86% homology at the nucleotide level and 75% identity at the amino acid level.

The predicted amino acid sequence for pKAS13 is 405 amino acids in length starting at the ATG initiation codon at position 43 and finishing at a TGA stop codon at position 447 giving a predicted theoretical molecular weight of 42.8kDa and a pI of 7.02. On comparison of the protein sequences of other known KAS III proteins (Figure 3.7) pKAS13 was shown to contain the highly conserved active site region, DITAACSGF demonstrated to be present in many condensing enzymes (Siggaard-Anderson, 1993).

A hydrophobicity plot (Figure 3.5) of the open reading frame region was calculated according to the method by Kyte and Doolittle (1982), the initial 50 amino acids show little hydrophobicity which is normally considered to be characteristic of a signal peptide. It is anticipated that a transit peptide exists on this protein as *de novo* fatty acid synthesis occurs in the chloroplast. The first 50 to 100 amino acids are rich in serine which is also known to be characteristic of signal peptides. In order to determine the signal peptide region of the clone, Von Heijne's (1986) signal peptide method was employed using the Daresbury sigpep package. Signal peptides on average vary between 13-26 amino acids. The programme highlighted a variety of possible cleavage sites (Figure 3.6) at amino acid positions 15-16, 16-17, 20-21, 28-29, 32-33 and 85-86. These regions all appear to follow the (-3-1) rule suggested by von Heijne, in that residues at these positions around the cleavage site are small at –1 and are not aromatic, charged or large and polar at position –3. It is interesting to note the number of suggested cleavage sites between 16 and 33 amino acids, although sigpep suggested that at position 85-86 was the most likely. Analysis of the derived amino acid sequence using the CBS chloroplast transit peptide computer package at the Center for Biological Sequence Analysis shows that the sequence has a high score for containing a chloroplast transit peptide (0.58) and proposes that the cleavage site is at position 75-76.

On examination of the *Poryphyra* and *E.coli* amino acid sequences they were seen to have approximately the first 70 amino acids missing (Figure 3.7). This could infer that this region is the signal peptide that transports KAS III into the chloroplast in plant KAS IIIs. Indeed Tai and Jaworski (1993) cleaved off this region in spinach KAS III and upon overexpression in *E.coli* obtained an active protein. Analysis of pKAS13 using the Expasy Chloroplast Transit

peptide prediction package suggested a cleavage site at 75-76. Cleavage of this position would indicate the signal peptide is approximately 7.1kDa and the mature protein is 34.7kDa.

Therefore the high number of possibilities predicted by computer packages make it difficult to ascertain the position of the cleavage site. Von Heijne (1990) states that although there appears to be a pattern of an N-terminal region which is positively charged, a central region which is hydrophobic and a carboxy terminal domain which is more polar, there are no precise sequence conservations to be found and so this means that signal peptides are highly variable and possibly rapidly evolving structures. Only purification and sequencing of the entire protein will determine the true length of the signal peptide.

PRIMER	**SEQUENCE 5'-3'**
M13 Reverse	GGA AAC AGC TAT GAC CAT G
M13 Forward	GGA AAC AGC TAT GAC CAT G
HC1R	AAT GCG AAG CAG AAC CCA CCT CC
HC1F	TCT ACT TCC CAG TTC AAC CGG
HC3R	AAG CCC TTG AAA TGG CGG
HC3F	GCA GAC TCA AGA GAT TGG GG
HC4R	GGT TGT TCA GGC TTG TGA CA
HC5R	GCC GGT TTA ACT TGG GGT TC
HC5F	GCC TGA TAC AAC TCT GCG GT

Table 3.1 - Primer sequences used to determine the sequence of pKAS13. The annealing positions can be seen in Figure 3.4.

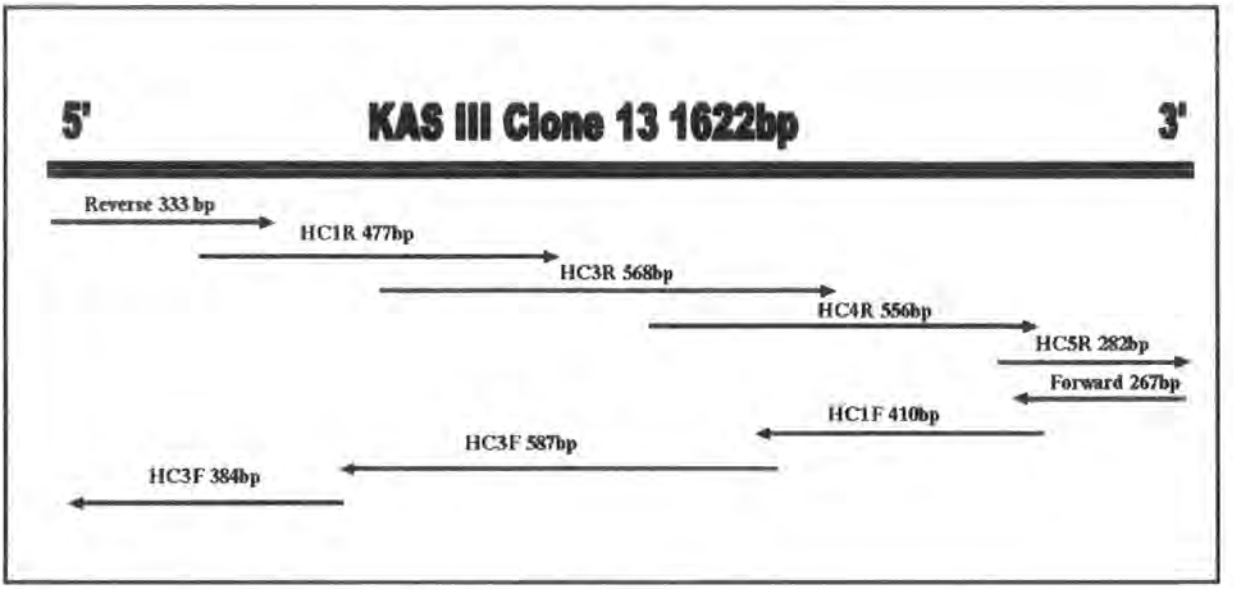


Figure 3.4 - A diagrammatic representation of the sequencing strategy of pKAS13.

The arrows indicate the direction of the sequencing primer and the length of each arrow is directly proportional to the amount of sequence obtained in each case. pKAS13 was initially sequenced using the M13 forward and reverse primers. Primers were then designed to reliable sequence towards the 3' end of the known initial sequence. Subsequent analysis using the new primer revealed more of the KAS III sequence and further primers were designed. This was repeated until the clone had been sequenced in its entirety in both directions. This ensured the accuracy of the sequence obtained, where each base was checked at least twice.

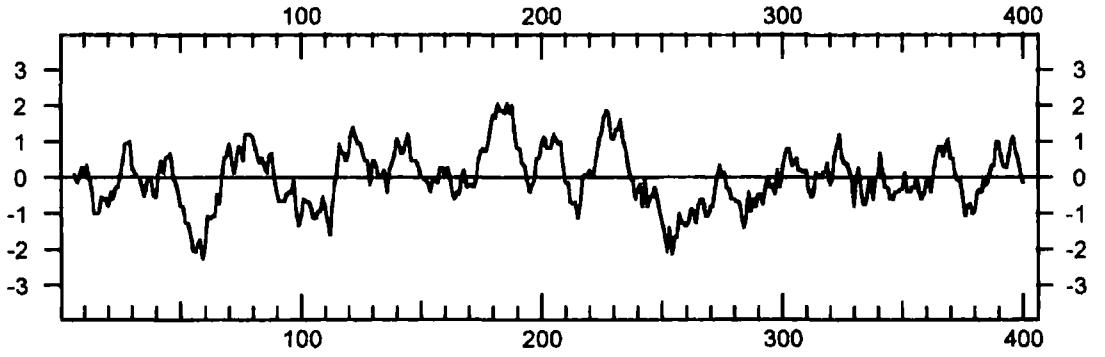


Figure 3.5 - A hydrophobicity plot of the open reading frame of pKAS13 as determined by the method of Kyte and Doolittle (1982).

Analysis of the nucleotide sequence reveals a TATA box positioned 47 bases upstream of the initiation ATG codon. No consensus polyadenylation site, AATAAA could be found in this clone (Joshi, 1987). It is also interesting to note that there are highly conserved residues between amino acid position 331 and 380 of all known KAS III proteins. What importance this region may have in maintaining the integrity of a fully functional KAS III is not known. When this region of the *Brassica napus* clone was used to search the NCBI genbank database (Alschul *et al.*, 1997) not only did it align to the other known KAS III proteins but it also had homology of between 34-39% to chalcone synthase proteins that have been isolated from a variety of plants. Although this homology is not as significant as the homology between all KAS III proteins, the fact that other condensing enzymes were found when the region 331-380 was used in a database search may imply that this region is conserved and may serve an important role in condensing enzymes structure or function.

Figure 3.6 - Nucleotide and deduced amino acid sequence of pKAS13 and flanking regions.

The clone is 1622bp with an open reading frame of 1215bp. The amino acid sequence is shown in the single letter format below the nucleotide sequence. The sequence in bold at the 5' end is put forward as a putative signal peptide of 225 nucleotides. All possible cleavage sites as determined by the computer analysis packages are indicated by a ∨ above and between the two appropriate codons. The sequence in italic is primer SDK2A used to select clones by PCR along with T7 or T3. The sequence blocked in yellow is the conserved active site region of known plant condensing enzymes (Siggaard-Anderson, 1993).

KAS III Clone 13 Full Sequence

```
1/1
*TAACTTCCGAAGATTCGATATTTTTACTAATAAACTATTTCGAAATTCTTCCTCTCCTCTCAATCTCTGTAATCACAATATAG
ACAGCAAAGCTTCTTCCTTTCTTCGATTCCTTTGTT
                                                    151/51                  v   v
CAA ACA ATG GCG AAT GCA TCT GGC TTC TTC ACT CAT CCT TCG ATT TCC TCA ATG CGA AGC
      M   A   N   A   S   G   F   F   T   H   P   S   I   S   S   M   R   S
181/61 v                                        v  211/71       v
AGA ACC CAC CTC CCG ATT CAA GTT TCT GGA TCT GGG TTT TGC GTC TCG AAA CGA TTC TCC
R   T   H   L   P   I   Q   V   S   G   S   G   F   C   V   S   K   R   F   S
241/81                                          271/91
AAA AGG GTC CTC TGC TCT AGC CTC AGC TCC CTC GAT GAC AAT GCT TCT CGT TCT CCC TCT
K   R   V   L   C   S   S   L   S   S   L   D   D   N   A   S   R   S   P   S
301/101                                         331/111                 v
CAA TAC CGA CCA CCC AGG CTA GTT CCG AGT GGC TGC AAA CTG ATT GGA TCT GGT TCA GCT
Q   Y   R   P   P   R   L   V   P   S   G   C   K   L   I   G   S   G   S   A
361/121             v                           391/131
GTC CCA AGT CTT CTC ATT TCT AAT GAT GAT CTC GCG AAG ATC GTC GAT ACT AAT GAT GAA
V   P   S   L   L   I   S   N   D   D   L   A   K   I   V   D   T   N   D   E
421/141                                         451/151
TGG ATA GCT ACT CGC ACT GGT ATC CGC AAC CGC AGA GTT GTA TCA GGC AAA GAT AGC TTG
W   I   A   T   R   T   G   I   R   N   R   R   V   V   S   G   K   D   S   L
481/161                                         511/171
GTT GGC TTA GCA GTG GAA GCA GCG ACC AAA GCC CTT GAA ATG GCG GAG GTT GCT CCT GAG
V   G   L   A   V   E   A   A   T   K   A   L   E   M   A   E   V   A   P   E
541/181                                         571/191
GAT ATT GAC TTA GTC CTG ATG TGT ACT TCC ACT CCT GAT GAT CTC TTT GGT GCT GCT CCA
D   I   D   L   V   L   M   C   T   S   T   P   D   D   L   F   G   A   A   P
601/201                                         631/211
CAG ATT CAG AAG GCG CTT GGT TGC ACA AAG AAC CCT TTG GCG TAT GAT ATC ACA GCT GCT
Q   I   Q   K   A   L   G   C   T   K   N   P   L   A   Y   D   I   T   A   A
661/221                                         691/231
TGT AGT GGA TTT GTT TTG GGT CTA GTC TCA GCT GCT TGT CAT ATA AGA GGA GGT GGT TTT
C   S   G   F   V   L   G   L   V   S   A   A   C   H   I   R   G   G   G   F
721/241                                         751/251
AAG AAT GTT TTA GTG ATT GGA GCT GAT TCT TTA TCT CGC TTT GTT GAT TGG ACT GAT AGA
K   N   V   L   V   I   G   A   D   S   L   S   R   F   V   D   W   T   D   R
781/261                                         811/271
GGA ACT TGC ATC CTC TTT GGA GAT GCT GCT GGT GCT GTG GTT GTT CAG GCT TGT GAC ATT
G   T   C   I   L   F   G   D   A   A   G   A   V   V   V   Q   A   C   D   I
841/281                                         871/291
GAG GAT GAT GGG TTA TAC AGT TTT GAT GTG CAC AGC GAT GGA GAC GGT CGT AGA CAT TTG
E   D   D   G   L   Y   S   F   D   V   H   S   D   G   D   G   R   R   H   L
901/301                                         931/311
AAT GCT TCT GTT AAA GAA TCC CAA ACC GAT GGT GCC TTG AGC TCC AAT GGG TCA GCG TTG
N   A   S   V   K   E   S   Q   T   D   G   A   L   S   S   N   G   S   A   L
961/321                                         991/331
GGT GAC TTT CCA CCG AAG CAA TCT TCA TAC TCT TGC ATT CAG ATG AAT GGA CAA GAA GTG
G   D   F   P   P   K   Q   S   S   Y   S   C   I   Q   M   N   G   Q   E   V
1021/341                                        1051/351
TTT CGC TTT GCT GTC AAA TGC GTT CCC CAA TCT CTT GAG TCT GCT TTA CAA AAA GCT GGT
F   R   F   A   V   K   C   V   P   Q   S   L   E   S   A   L   Q   K   A   G
1081/361                                        1111/371
CTT CCT GCT TCC TCC ATT GAT TGG CTC CTC CTT CAT CAG GCA AAC CAG AGA ATA TTA GAC
L   P   A   S   S   I   D   W   L   L   L   H   Q   A   N   Q   R   I   L   D
1141/381                                        1171/391
TCT GTG GCT ACA AGG CTT CAG TTT CCA CCG GAA CGA GTG ATA TCA AAC CTG GCT AAT TAC
S   V   A   T   R   L   Q   F   P   P   E   R   V   I   S   N   L   A   N   Y
1201/401                                        1231/411
GGT AAC ACA AGC GCT GCT TCT ATC CCT CTA GCT CTT GAT GAG GCG GTG AGA AGC GGG AAA
G   N   T   S   A   A   S   I   P   L   A   L   D   E   A   V   R   S   G   K
```

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1261/421                                    1291/431
GTT AAA CCG GGA CAT ACC ATA GCC ACT TCC GGT TTT GGA GCC GGT TTA ACT TGG GGT TCA
V   K   P   G   H   T   I   A   T   S   G   F   G   A   G   L   T   W   G   S
1321/441                                    1351/451
GCA ATT ATC AGG TGG AGA TGA ATGGTGAAAGTCCAACATGTAAGTTTAACTTCAGAGCCAAAGTTTACTTTT
A   I   I   R   W   R   *

CTCTTCCACAGGGTAGAGGAACCACTGGAAATTGGTTTAAACCGGTCTGGTTTAATCCGGTTGAACTGGAAGTAGAGCGCATTT
TGTTTGATTTCGTTTCATTTTCACTTTCGAGGTTTCATTGTTTACTTGTTAGTTATTGTTTCCTTGGATCTTTTTCTGTTCTTG
AAAACCTTTTTTTTTGTTGGTGATAGATGAATAGTTCTTCAAAAAAAAAAAAAAAAAAAAAA
```

3.2.6 Identification of KAS III Isoforms by PCR Analysis

A similar PCR was carried out on all the primary plaques isolated in section 3.2.4 to ascertain which plaques were KAS III positive but may also be a different isoform from pKAS13. Two sets of PCR reactions were performed one set contained 20pmole of primer ISOHC1 - 5' GTG AAA ATG AAA CGA AAT CAA AC 3' and 20pmole primer ISOHC2 - 5' TCT GCT TTA CAA AAA GCT GGT 3' in a final volume of 50µl. This PCR was performed on each plaque to determine whether the 3'UTR of each plaque was identical to pKAS13 since ISOHC1 was designed to be positioned in the 3'UTR of pKAS13.

The second PCR reaction used primer ISOHC3 5' CTC TTT GGA GAT GCT GCT GGT 3' and primer ISOHC4 5' GGG GAA CGC ATT TGA CAG C 3' in order to confirm that the clone did contain at the very least 200bp of the open reading frame. The PCR products were analysed separately on a 0.8% agarose gels. The two experiments were compared to (a) confirm its KAS III status and (b) if they contained the 3'UTR region found in pKAS13. A positive results could however also be seen if primer ISOHC1 shared homology to another isoform and hence would anneal and amplify the DNA, so it is important to acknowledge that there may not be significant differences at the 3'UTR.

Results showed that four of the plaques shown to contain KAS III did not produce a PCR product for the 3'UTR. The possible interpretations of this PCR analysis are (i) the 3'UTR of the clone was not the same as that of pKAS13, (ii) the PCR had failed or (iii) that the 3' UTR had been truncated during the creation of the library, although this possibility is limited by the

use of a library formed by the use of oligo dT primers. The four primary plaques 1.9, 2.1, 2.6 and 2.8 were examined further by PCR using primer HCISO3 in conjunction with either Primer T7 or T3. Plaque 1.9 would not yield any PCR products with either T3 or T7 primers, but PCR products were obtained from the other primary plaques. Plaques 2.1 and 2.8 yielded PCR products of approximately 600bp, whereas plaque 2.6 gave a much larger PCR product of approximately 1kb.

The PCR products from 2.1, 2.8 and 2.6 were each ligated into a *Topo* TA cloning vector (Invitrogen) by transformation into competent *E.coli* TOP10F' cells following the manufacturer's recommended method. Transformants were selected on LB-agar plates containing 50μg/ml ampicillin and 40μg/ml IPTG and incubated overnight at 37°C. White colonies indicated that the insert had ligated into the vector and eight white colonies from each clone were examined by the PCR protocol described above to verify insert presence. Two positive colonies from each clone were cultured and the plasmid DNA isolated and then sequenced in both the forward and reverse direction using ISOHC3 and either the T3 or T7 primer as appropriate. These plasmids were then designated p2.1KAS, p2.6KAS and p2.8KAS.

Analysis of the sequences indicated p2.1KAS and p2.8KAS to be initially identical to each other and to pKAS13. The sequence of p2.1KAS on the otherhand, although highly homologous to pKAS13, differed at the 3'UTR. Unfortunately p2.1KAS was not a full-length clone but the sequence suggested this was the partial clone of a different KAS III isoform

Figure 3.7 The pKAS13 predicted amino acid sequence compared against all other known KAS III amino acid sequences from plants, *E.coli* and the alga *Porphyra* accessed at the NCBI genbank database (Alschul *et al.*, 1997).

The highly conserved active site region starts at position 181 with the cysteine at position 186, which is present in all 11 sequences. There are other highly conserved regions notably from 331 to 380 although no function has as yet been determined for this region. It is also a highly conserved region in other condensing enzymes in particular chalcone synthases. A * denotes identical amino acids amongst all the KAS III proteins and • denotes where the proteins may have different amino acids but the property of the amino acids is similar.

Key	Origin Of KAS III	Accession Number From NCBI Database/ Reference
Cuphea (a)	*Cuphea wrightti*	U15934 (Slabaugh *et al.*, 1995)
Cuphea (b)	*Cuphea wrightii*	U15935 (Slabaugh *et al.*, 1995)
Perilla(b)	*Perilla frutescens*	AF026150
Perilla(a)	*Perilla Frutescens*	AF026151
Arab	*Arabidopsis thaliana*	L31891 (Tai *et al.*, 1994)
Brassica	*Brassica napus*	pKAS13
Spinach	*Spinacea oleracea*	Z22771 (Tai, 1993)
E.Coli	*E.coli*	P24249 (Tsay *et al.*, 1992)
Porpurpure	*Porphyra purpurea*	Z14034 (Reith, 1993)
Porumbil	*Porphyra umbilicalis*	P31176 (Reith ,1993)
Leek	*Allium porrum*	U30600 (Chen *et al.*, 1996)

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                        10         20         30         40         50         60
Cuphea (a)      -MANASGFLG SSVP-ALRRA TQPQHSISSS RGSSSDFVFK R--VFCCSAV QGSDRQSLGD
Cuphea (b)      -MANAYGFVG HSVP-TMKRA AQFQQMGSG- -FCSADSISK R--VFCCSVV QGADKPASGD
Perilla(b)      -MANVSGLVV PTVP-SLRRR FSPSSDIYRS GFWFYDGVSR R--TVCSSAA QGAEKLSPAE
Perilla(a)      -MANASGLFT PAAP-SVRRR CSPCIGIYRS GFWFSEGVSR R--IVCSSTT QGAEKLSPSE
Arab            -MANASGFFT HPSIPNLRSR IHVPVRVSGS GFCVSNRFSK R--VLCSSVS SVDKDASSSP
E.Coli          ---------- ---------- ---------- ---------- ---------- ----------
Brassica        -MANASGFFT HPSISSMRSR THLPIQVSGS GFCVSKRFSK R--VLCSSLS SLDDNASRSP
Spinach         -MATSYGFFS PSVPSSLNNK ISPSLGINGS GFCSHLGISK R--VFCSSIE ASEKHAAAGV
Porpurpure      ---------- ---------- ---------- ---------- ---------- ----------
Porumbil        ---------- ---------- ---------- ---------- ---------- ----------
Leek            MAAASIGFTT PSANPRIRAR NFGNFGALGF LCFKSERSFK RNWVGCCSVS ESSSSLSYST

                       70         80         90        100        110        120
Cuphea (a)      S----RSPRL VSRGCKLIGS GSAIPSLQIS NDDLAKIVDT NDEWISVRTG IRNRRVLTGK
Cuphea (b)      SRTEYRTPRL VSRGCKLVGS GSAMPALQVS NDDLSKIVDT NDEWISVRTG IRNRRVLTGK
Perilla(b)      S----KVPRL ASKGCKLVGC GSAVPSIQIS NDDLSKIIDT SDEWISVRTG IRNRRILSRK
Perilla(a)      S----RASRL VSRGCKLVGC GSAVPSLQIS NDDLSKIVDT NDEWISVRTG IRNRRILSGK
Arab            S--QYQRPRL VPSGCKLIGC GSAVPSLLIS NDDLAKIVDT NDEWIATRTG IRTRRVVSAK
E.Coli          ---------- --MYTKIIGT GSYLPEQVRT NADLEKMVDT SDEWIVTRTG IRERHIAAPN
Brassica        S--QYRPPRL VPSGCKLIGS GSAVPSLLIS NDDLAKIVDT NDEWIATRTG IRNRRVVSGK
Spinach         SSSESRVSRL VNRGCKLVGC GSAVPKLQIS NDDLSKFVET SDEWIATRTG IRQRHVLSGK
Porpurpure      ---------- --MGVHILST GSSVPNFSVE NQQFEDIIET SDHWISTRTG IKKRHLAPSS
Porumbil        ---------- --MGVHILST GSSVPNFSVE NQQFEDIIET SDHWISTRTG IKKSILPLLL
Leek            N----RTKRL VGMGSKLIGS GSAVPKLQIS NDDMAKIVET SDEWISVRTG IRNRRVLTGN
                           ... . ** .*      *      ..*  *  *. *** *.   .

                       130        140        150        160        170        180
Cuphea (a)      DSLTNLASEA ARKALEMAQ IDADDVDMVL MCTSTPEDLF G-SAPQISKA LGCKKNPLSYD
Cuphea (b)      ESLTNLATVA ARKALEMAQ VDANDVDMVL MCTSTPEDLF G-SAPQIQKA LGCKKNPLAYD
Perilla(b)      DSLTALATEA AQKALQMAE VDPDDVDLVL LCTSTPEDLF G-SAPQIPKA LGCKNNPLAYD
Perilla(a)      DSLTDLAVEA ARKALEMAE VDPDEVDLVL MCTSTPEDLF G-SAPQIQKA LGCKNNPLAYD
Arab            DSLVGLAVEA ATKALEMAE VVPEDIDLVL MCTSTPDDLF G-AAPQIQKA LGCTKNPLAYD
E.Coli          ETVSTMGFEA ATRAIEMAG IEKDQIGLIV VATTSATHAF PSAACQIQSM LGIKG-CPAFD
Brassica        DSLVGLAVEA ATKALEMAE VAPEDIDLVL MCTSTPDDLF G-AAPQIQKA LGCTKNPLAYD
Spinach         DSLVDLAAEA ARNALQMAN VNPDDIDLIL MCTSTPEDLF G-SAPQVQRA LGCSRTPLSYD
Porpurpure      TSLTKLAAEA ANDALSKAS INAEDIDLII LATSTPDDLF G-SASQLQAE IGATS-STAFD
Porumbil        PSLTKLAAEA ANDALSKAS INAEDIDLII LATSTPDDLF G-SASQLQAE IGATS-STAFD
Leek            ENLNGLAVEA AKGALRMAE VEAENVDLVI FWSSTPDDLF G-GASRIQGD LGCKS-ALAFD
                  .  .   * *  *.  *  .    . ...   ...    *    * ..    .*      ..*

                       190        200        210        220        230        240
Cuphea (a)      ITAACSGFVL GLVSAACHIR GGGFNNVLVI GADSLSRYVD WTDRGTCILF GDAAGAVVVQ
Cuphea (b)      ITAACSGFVL GLVSAACHIR GGGFNNILVI GADSLSRYVD WTDRGTCILF GDAAGAVLVQ
Perilla(b)      ITAACSGFLL GLVSAACYIR GGGFNNVLVI GADALSRYVD WNDRGSCILF GDAAGAVLVQ
Perilla(a)      ITAACSGFVL GLVSAACYIK GGGFKNVLVI GADALSRYVD WTDRGSCILF GDAAGAVLVQ
Arab            ITAACSGFVL GLVSADCHIR GGGFKNVLVI GADSLSRFVD WTDRGTCILF GDAAGAVVVQ
E.Coli          VAAACAGFTY ALSVADQYVK SGAVKYALVV GSDVLARTCD PTDRGTIIIF GDGAGAAVLA
Brassica        ITAACSGFVL GLVSAACHIR GGGFKNVLVI GADSLSRFVD WTDRGTCILF GDAAGAVVVQ
Spinach         ITAACSGFML GLVSAACHVR GGGFKNVLVI GADALSRFVD WTDRGTCILF GDAAGAVVVQ
Porpurpure      ITAACSGFII ALVTASQFIQ AGSYNKVLVV GADTMSRWID WSDRSSCILF GDGAGAVLIG
Porumbil        ITAACSGFII ALVTASQFIQ AGSYNKVLVV GADTMSRWID WSDRSSCILF GDGAGAVLIG
Leek            ITAACSGFVV GLITATRFIK GGGYKNVLVI GADALSRFVD WTDRGTCILF GDAAGAVLVQ
                ..***.**    *  *   ..  *     **. *.* ..*  *   ** . *.* ** *** ..
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                    250         260         270         280         290         300
Cuphea (a)      SCDAEEDGLF AFDLHSDGDG QRHLKAAIKE D-EVDKALGS NG-SIRDFPP RRSSYSCIQM
Cuphea (b)      SCDAEEDGLF AFDLHSDGDG QRHLKAAITE N-GIDHAVGS NG-SVSDFPP RSSSYSCIQM
Perilla(b)      SCDSEEDGLF SFDMHSDGGG QRHLNASMKE N-KIDHELGS NG-SFLGLLP KGSSYSCIQM
Perilla(a)      ACESDEDGLF AFDMHSDGEG QRHLNATMKE KEKENHEVGT NG-SLLGLLP KGSSYSCIQM
Arab            ACDIEDDGLF SFDVHSDGDG RRHLNASVKE S-RNEGESSS NGSVFGDFPP KQSSYSCIQM
E.Coli          ASE-EPGIIS THLHADGSYG ELTLLPNADR ---------- ---VNPENSI H------LTM
Brassica        ACDIEDDGLY SFDVHSDGDG RRHLNASVKE S-QTDGALSS NGSALGDFPP KQSSYSCIQM
Spinach         ACDSEEDGMF AFDLHSDGGG GRHLNASLLN D-ETDAAIGN NG-AVTGFPP KRPSYSCINM
Porpurpure      ESS--INSIL GFKLCTDGRL NSHLQLMNSP S-----DSQQ FG--LTTVPK GR--YDSIRM
Porpumbil       ESS--INSIL GFKLCTDGRL NSHLQLMNSP S-----DSQQ FG--LTTVPK GR--YDSIRM
Leek            ACSEDEDGLL GFDLNSDGSG QRHLNAFVSD A-EHEAISNT NG--APLFPP KRSTYSCIKM
                 .      .     . .**      *                                    ..*

                    310         320         330         340         350         360
Cuphea (a)      NGKEVFRFAC RCVPQSIESA LGKAGLNGSN IDWLLLHQAN QRIIDAVATR LEVPQERIIS
Cuphea (b)      NGKEVFRFAC RCVPQSIESA LGKAGLNGSN IDWLLLHQAN QRIIDAVATR LEVPQERVIS
Perilla(b)      NGKEVFRFAV RVVPHSIELA LEKAGLAASN IDWLLLHQAN QRIIDAVATR LEVPPERVIS
Perilla(a)      NGKEVFRFAV RVVPQSIELA LAKAGLTGSS IDWLLLHQAN QRIIDAVATR LELPPERVIS
Arab            NGKEVFRFAV KCVPQSIESA LQKAGLPASA IDWLLLHQAN QRIIDSVATS LHFPPERVIS
E.Coli          AGNEVFKVAV TELAHIVDET LAANNLDRSQ LDWLVPHQAN LRIISATAKK LGMSMDNVVV
Brassica        NGQEVFRFAV KCVPQSLESA LQKAGLPASS IDWLLLHQAN QRILDSVATR LQFPPERVIS
Spinach         NGKEVFRFAV RCVPQSIEAA LQKAGLTSSN IDWLLLHQAN QRIIDAVATR LEVPSERVLS
Porpurpure      NGKEVYKFAV FQVPIVIKNC LNDVNISIDE VDWFILHQAN IRILEAIATR LSIPLSKMIT
Porpumbil       NGKEVYKFAV FQVPIVIKNC LNDVNISIDE VDWFILHQAN IRILEAIATR LSIPLSKMIT
Leek            NGNEVFRFGW RCVPQTIQAS LDDAGLSSSN IDWLLLHQAN QRIIDAVSTR LEIPSEKVIS
                 * **..      .   . . *  ..     .** . **** **. . .   *      ..

                    370         380         390         400         410
Cuphea (a)      NLANYGNTSA ASIPLALDEA VRSGNVKPGH VIATAGFGAG LTWGSAIIRWG
Cuphea (b)      NLANYGNTSA ASIPLALDEA VRGGKVKAGH LIATAGFGAG LTWGSAIVRWG
Perilla(b)      NLANYGNTSA ASIPLALDEA VRSGKVQAGH TIATAGFGAG LTWGSAIVRWG
Perilla(a)      NLANYGNTSA ASIPLALDEA VRSGKVQAGH TIAAAGFGAG LTWGSAIVRWG
Arab            NLANYGNTSA ASIPLALDEA VRSGKVKPGH TIATSGFGAG LTWGSAIVRWR
E.Coli          TLDRHGNTSA ASVPCALDEA VRDGRIKPGQ LVLLEAFGGG FTWGSALVRF-
Brassica        NLANYGNTSA ASIPLALDEA VRSGKVKPGH TIATSGFGAG LTWGSAIIRWR
Spinach         NLANYGNTSA ASIPLALDEA VRSGKVKPGN IIATSGFGAG LTWGSSIIRWG
Porpurpure      NLENYGNTSA ASIPLALDEA IKEKKIQPGQ VVVLAGFGAG LTWGAIVLKWQ
Porpumbil       NLENYGNTSA ASIPLALDEA IKEKKIQPGQ VVVLAGFGAG LTWGAIVLKWQ
Leek            NLANYGNTSA ASIPLALDEA VRNGKVKAGD TIATAGFGAG LTWGSAIVKWG
                .*   ***** **.* ***** ..   .. *   .    ** *  ***. ...
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Figure 3.8 Comparison of pKAS13 and p.2.1KAS isolated by PCR against the partial clone; TF Clone 2.

All the clones were isolated from the same rape embryo cDNA Library. In the section of open reading frame compared between pKAS13 and p2.1KAS there was a total of 41 nucleotide differences, the majority of changes did not constitute an amino acid change, but 15 codons were seen to be different. The 3'UTRs do show regions of homology but p2.1KAS appears to have a truncated version of pKAS13's 3'UTR. TF Clone 2 does appear to be a different isoform from both other clones and although there is only a minor overlap (121bp) in sequence data between TF Clone 2 and p2.1KAS there are 9 nucleotide difference which would imply that these two are different isoforms.

Key

p2.1KAS	-	(Section 3.2.6)
pKAS13	-	(Section 3.2.5)
TF Clone 2	-	(Section 3.2.1)

```
                                                                                                    60
Consensus     ---------- ---------- ---------- ---------- --XAATTC-- ----------
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        TAACTTCCGA AGATTCGATA TTTTTACTAA TAAACTATTT CGAAATTCTT CCTCTCCTCT
TF Clone 2    ---------- ---------- ---------- ---------- --GAATTC-- ----------
                                                                                                   120
Consensus     ---------- ---------- ---------- ---------- ---------- ----------
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        CAATCTCTGT AATCACAATA TAGACAGCAA AGCTTCTTCC TTTCTTCGAT TCCTTTGTTT
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                   180
Consensus     --------GC GX-------- ---------- ---------- ---------- ----------
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        AAACAATGGC GAATGCATCT GGCTTCTTCA CTCATCCTTC GATTTCCTCA ATGCGAAGCA
TF Clone 2    --------GC GG-------- ---------- ---------- ---------- ----------
                                                                                                   240
Consensus     ---------- ---------- ---------- ---------- ---------- ----------
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        GAACCCACCT CCCGATTCAA GTTTCTGGAT CTGGGTTTTG CGTCTCGAAA CGATTCTCCA
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                   300
Consensus     ---------- -CXGCT---- ---------- --GATXACAA TGCTTCTCXT TCTCCXTCTC
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        AAAGGGTCCT CTGCTCTAGC CTCAGCTCCC TCGATGACAA TGCTTCTCGT TCTCCCTCTC
TF Clone 2    ---------- -CCGCT---- ---------- --GATAACAA TGCTTCTCCT TCTCCTTCTC
                                                                                                   360
Consensus     AATACCGACC ACCCAGGCTA GTXCCGAGXG GCTGCAAACT XATTGGATCT GGTTCAGCTG
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        AATACCGACC ACCCAGGCTA GTTCCGAGTG GCTGCAAACT GATTGGATCT GGTTCAGCTG
TF Clone 2    AATACCGACC ACCCAGGCTA GTACCGAGCG GCTGCAAACT TATTGGATCT GGTTCAGCTG
                                                                                                   420
Consensus     TCCCAAGTCT TCTXATTTCT AATGATGATC TCGCXAAGAT XGTCGATACT AATGATGAXT
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        TCCCAAGTCT TCTCATTTCT AATGATGATC TCGCGAAGAT CGTCGATACT AATGATGAAT
TF Clone 2    TCCCAAGTCT TCTGATTTCT AATGATGATC TCGCAAAGAT TGTCGATACT AATGATGAGT
                                                                                                   480
Consensus     GGATAGCTAC TCGCACTGGT ATCCGCAACC GCAGAGTTGT ATCAGGCAAA GATAGCTTGG
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        GGATAGCTAC TCGCACTGGT ATCCGCAACC GCAGAGTTGT ATCAGGCAAA GATAGCTTGG
TF Clone 2    GGATAGCTAC TCGCACTGGT ATCCGCAACC GCAGAGTTGT ATCAGGCAAA GATAGCTTGG
                                                                                                   540
Consensus     TTGGCTTAGC AGTGGAAGCA GCXACCAAAG CCCTTGAAAT GGCXGAXGTT GCTCCTGAGG
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        TTGGCTTAGC AGTGGAAGCA GCGACCAAAG CCCTTGAAAT GGCGGAGGTT GCTCCTGAGG
TF Clone 2    TTGGCTTAGC AGTGGAAGCA GCAACCAAAG CCCTTGAAAT GGCAGAAGTT GCTCCTGAGG


                                                                                                   600
Consensus     ATATTGACTT AGTCXTGATG TGTACXTCCA CTCCTGATGA TCTCTTXGGT GCTGCTCCAC
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        ATATTGACTT AGTCCTGATG TGTACTTCCA CTCCTGATGA TCTCTTTGGT GCTGCTCCAC
TF Clone 2    ATATTGACTT AGTCTTGATG TGTACCTCCA CTCCTGATGA TCTCTTCGGT GCTGCTCCAC
                                                                                                   660
Consensus     AGATTCAGAA GGCGCTTGGT TGCACAAAGA ACCCTTTGGC XTATGATATC ACAGCTGCTT
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
PKAS13        AGATTCAGAA GGCGCTTGGT TGCACAAAGA ACCCTTTGGC GTATGATATC ACAGCTGCTT
TF Clone 2    AGATTCAGAA GGCGCTTGGT TGCACAAAGA ACCCTTTGGC ATATGATATC ACAGCTGCTT
                                                                                                   720
Consensus     GTAGTGGATT TGTTTTGGGT CTAGTCTCAG CTGCTTGTCA TATAAGAGGA GGXGGTTTTA
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        GTAGTGGATT TGTTTTGGGT CTAGTCTCAG CTGCTTGTCA TATAAGAGGA GGTGGTTTTA
TF Clone 2    GTAGTGGATT TGTTTTGGGT CTAGTCTCAG CTGCTTGTCA TATAAGAGGA GGCGGTTTTA
```

```
                                                                                                  780
Consensus     AGAATGTTTT XGTGATTGGA GCTGATTCTT TATCTCGXTT TGTTGATTGG ACTGATAGAG
p2.1KAS       ---------- ---------- ---------- ---------- ---------- ----------
pKAS13        AGAATGTTTT AGTGATTGGA GCTGATTCTT TATCTCGCTT TGTTGATTGG ACTGATAGAG
TF Clone 13   AGAATGTTTT GGTGATTGGA GCTGATTCTT TATCTCGGTT TGTTGATTGG ACTGATAGAG
                                                                                                  840
Consensus     GAACTTGCAT CCTCTTTGGA GATGCTGCTG GTGCTGTGGT TGTTCAGGCT TGTGACATTG
p2.1KAS       ---------- -CTCTTTGGA GATGCTGCTG GTGCTTTGGT TGTTCAGGCT TGTGACATTG
pKAS13        GAACTTGCAT CCTCTTTGGA GATGCTGCTG GTGCTGTGGT TGTTCAGGCT TGTGACATTG
TF Clone 2    GAACTTGCAT CCTCTTTGGA GATGCTGCTG GTGCTGTGGT TGTTCAGGCT TGTGACATTG
                                                                                                  900
Consensus     AGGATGATGG GTTATATAGT TTTGATGTGC ACAGCGATGG AGACGG-TCG TAGACATTTG
p2.1KAS       AGGATGATGG GTTATTTAGT TTCGATGTAC ACAGCGATGG AGACGG-TCG TAGACATTTG
pKAS13        AGGATGATGG GTTATACAGT TTTGATGTGC ACAGCGATGG AGACGG-TCG TAGACATTTG
TF Clone 2    AGGATGATGG GT-ATATAGT TTTGATGTGC ACAGCGATGG AGACGGGTCG TAGAAATTTG
                                                                                                  960
Consensus     AATGCTTCTG TTAAAGAXTC CCAAACCXAT GGTGXXTTGA GCTCCXAXGG XTCTGXGTTG
p2.1Kas       AATGCTTCTA TTAAAGACTC CCAAACCAAT GGTGAGTTGA GCTCCCACGG CTCTGTGTTG
pKAS13        AATGCTTCTG TTAAAGAATC CCAAACCGAT GGTGCCTTGA GCTCCAATGG TTCTGCGTTG
TF Clone 2    AATGCTTCTG TTT------- ---------- ---------- ---------- ----------
                                                                                                 1020
Consensus     GGXGACTTTC CACCGAXXCA AXCTTCATAX TCTTGCATTC AGATGAATGG AXAAGAAGTX
p2.1KAS       GGAGACTTTC CACCGAGACA AGCTTCATAT TCTTGCATTC AGATGAATGG AAAAGAAGTA
pKAS13        GGTGACTTTC CACCGAAGCA ATCTTCATAC TCTTGCATTC AGATGAATGG ACAAGAAGTG
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                 1080
Consensus     TTTCGCTTTG CTGTCAAAXG CGTTCCCCAA TCTXTTGAGT CTGCTTTACA AAAAGCTGGT
p2.1KAS       TTTCGCTTTG CTGTCAAAAG CGTTCCCCAA TCTATTGAGT CTGCTTTACA AAAAGCTGGT
pKAS13        TTTCGCTTTG CTGTCAAATG CGTTCCCCAA TCTCTTGAGT CTGCTTTACA AAAAGCTGGT
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                 1140
Consensus     CTTCCTGCTT CXTCCATTGA TTGGCTCCTC CTTCAXCAGG CAAACCAGAG AATAXTAGAC
p2.1KAS       CTTCCTGCTT CTTCCATTGA TTGGCTCCTC CTTCACCAGG CAAACCAGAG AATAATAGAC
pKAS13        CTTCCTGCTT CCTCCATTGA TTGGCTCCTC CTTCATCAGG CAAACCAGAG AATATTAGAC
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                 1200
Consensus     TCTGTGGCTA CAAGGCTTCA GTTTCCXCCX GAACGAGTXA TATCAAACXT GGCTAATTAC
p2.1KAS       TCTGTGGCTA CAAGGCTTCA GTTTCCGCCX GAACGAGTCA TATCAAACTT GGCTAATTAC
pKAS13        TCTGTGGCTA CAAGGCTTCA GTTTCCACCX GAACGAGTAA TATCAAACCT GGCTAATTAC
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                 1260
Consensus     GGXAACACAA GCGCTGCTTC XATXCCTCTA GCTCTTGATG AGGCGGTGAG AAGCGGXAAA
p2.1KAS       GGGAACACAA GCGCTGCTTC GATTCCTCTA GCTCTTGATG AGGCGGTGAG AAGCGGAAAA
pKAS13        GGTAACACAA GCGCTGCTTC TATCCCTCTA GCTCTTGATG AGGCGGTGAG AAGCGGGAAA
TF Clone 13   ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                 1320
Consensus     GTTAAACCGG GACATACCAT AGCXXCXTCC GGTTTTGGAG CCGGTTXAAC TTGGGGTTCA
p2.1KAS       GTTAAACCGG GACATACCAT AGCTGCGTCC GGTTTTGGAG CCGGTTCAAC TTGGGGTTCA
pKAS13        GTTAAACCGG GACATACCAT AGCCCCTTCC GGTTTTGGAG CCGGTTTAAC TTGGGGTTCA
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                 1380
Consensus     GCAATTATXA GGTGGAGATG AATGGXXAAX XTCCAXCATG TAAGTTTACT TCAGAGCCAA
p2.1KAS       GCAATTATTA GGTGGAGATG AATGGCTAAG TTCCA-CATG TAAGTTTACT TCAGAGCCAA
pKAS13        GCAATTATCA GGTGGAGATG AATGGYGAAA GTCCAACATG TAAGTTTACT TCAGAGCCAA
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                 1440
Consensus     AGTTTXXTTX XXXXXXXXTT CTCTXTXCAC XXXGTAGAGG AAXCACTGGA AATTGGTTTA
p2.1KAS       AGTTTTTTTG GTTTTATGTT CTCTCTTCAC TAAGTAGAGG AAGCACTGGA AATTGGTTTA
pKAS13        AGTTTACTT- --------TT CTCT-TCCAC AGGGTAGAGG AACCACTGGA AATTGGTTTA
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
```

```
                                                                    1500
Consensus     AXCCGGTCTG GTTTAXTCCG XXXXAXXXGX AXGTAXAXCG CATTTTGTXT GATTTCGTTT
p2.1KAS       A-CCGGTCTG GTTTAGTCCG ACAAACTAGA AGGTAAACCG CATTTTGTCT GATTTCGTTT
pKAS13        AACCGGTCTG GTTTAATCCG GTTGAACTGG AAGTAGAGCG CATTTTGTTT GATTTCGTTT
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                    1560
Consensus     XXCAXTTTXX XXTTXGAGGT TTCATTGTTT XXTTGTTAXT XXXTGXTXXX XXXXATCXTT
p2.1KAS       TTCACTTTTG TCTT-GAGGT TTCATTGTTT CTTTGTTACT C--TGAT--- ---AATCXTT
pKAS13        --CATTTTCA CTTTCGAGGT TTCATTGTTT ACTTGTTAGT TATTGTTXXX TTGGATCXTT
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                                                                    1620
Consensus     TXXXXXXXXX GAAAAXXXXX XTTTTXXTXX XXXAXAXAXX AAXAXXXXXX XAAAAAAAAA
p2.1KAS       TAAAA----- GAAAATAGCG CTTTTCATCA AAAAAAAAAA AAAAAAAAAA -AAAAAAAAA
pKAS13        TTCTGTTCTT GAAAACCTTT TTTTTTGTTG GTGATAGATG AATAGTTCTT CAAAAAAAAA
TF Clone 2    ---------- ---------- ---------- ---------- ---------- ----------
                         1634
Consensus     AAAAAAAAAA AAAA
p2.1KAS       AAAAAAAAAA AAAA
pKAS13        AAAAAAAAAA AAAA
TF Clone 2    ---------- ----
```

(ISO2) differing by having a truncated 3'UTR. p2.1KAS has high identity to the open reading frame of pKAS13, however it can be seen that this isoform has several base changes to that of pKAS13 that in some cases constitutes a change in codon, (there are 15 codon changes in total). On examination of the sequence from the previously isolated partial clone TF Clone 2, it was seen to be a different isoform to that of pKAS13. It is difficult to compare TF Clone 2 to p2.1KAS as the two clones only overlap by 121 bases. There are 9 nucleotide base changes in this region, which suggests that these two partial clones are different from each other as well as pKAS13. Only isolation of the entire cDNA for these two isoforms will ascertain the significance of these differences.

3.2.7 Northern Analysis of RNA Samples to Determine Transcript Size

Poly A+ RNA from *B.napus* root, leaf and embryo were taken from previously prepared stocks (by Mr.R.Swinhoe). All solutions used in this method were first treated with DEPC or made up with DEPC-treated MQ water and all glassware was baked at 140°C for 3 hours prior to use.

3µg of each of the polyA+ RNA samples were incubated in RNA gel loading buffer, ethidium bromide was added (1mg/ml) and the samples loaded onto a 1 % denaturing agarose gel. Electrophoresis was carried out at 100V overnight and the gel then rinsed in DEPC-treated distilled water and transferred to zeta-probe membrane (Bio-Rad) by the standard RNA Blotting procedure (section 2.7.6). The membrane was incubated in 20ml of Northern pre-hybridisation buffer for 4 hours at 42°C. The ORF of pKAS13 was excised from and used to

synthesise a radioactive probe using high activity [^{32}P]dCTP. 10ml of the Northern prehybridisation buffer was removed and 50µl of the KAS III probe was added. Subsequent hybridisation was carried out overnight at 42°C and the membrane was washed to a final stringency of 1 × SSC and 0.1% SDS at 42°C. The blot was exposed to x-ray film for seven days.

The autoradiograph showed that KAS III was expressed in all three tissue types and the transcript size was estimated to be 1600bp, although the transcript root size appeared to be slightly smaller. As the results indicated that the KAS III transcripts were present in all tissue types examined using the ORF of pKAS13, it would imply that a transgene construct using pKAS13 has the potential to affect KAS III isoforms present within the plant tissues examined.

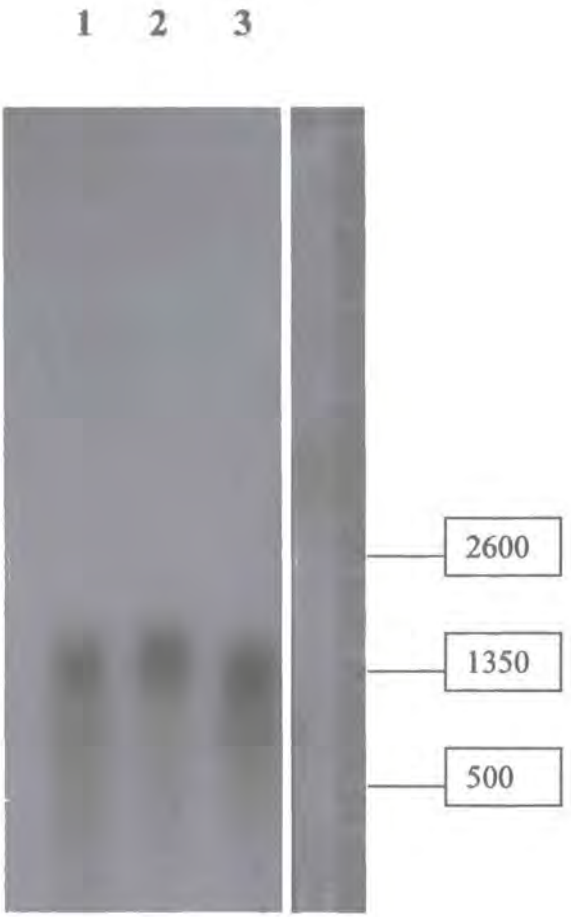


Figure 3.9 Northern analysis to determine the transcript size of KAS III using poly A^+ RNA isolated from root, leaf and embryo of *Brassica napus*.

The size of the transcript was calculated by plotting the distance moved by RNA standards against their molecular weight. The probe highlighted a transcript that has moved 5.7 cm which corresponds to a molecular weight of approximately 1.6kb.

Lane 1 - Embryo
Lane 2 - Young Leaf
Lane 3 - Root

3.3 Discussion

A 1622bp KAS III cDNA has been isolated from a *Brassica napus* embryo library for use in studies to further the understanding of regulation of fatty acid synthesis in plants. Antisense constructs can be created and transformed into *Brassica napus* as well as enable the overexpression of the gene product in order that antibodies to the protein can be raised and used in the analysis of transgenic plants.

The cDNA isolated showed high homology to a cDNA from its close relative *Arabidopsis thaliana*. This similarity, plus the close identity of the predicted amino acid sequence with known KAS IIIs infers that this cDNA encodes a KAS III protein. Northern analysis has demonstrated that it is highly likely that pKAS13 is, or is very close to being a full-length clone. A genomic Southern blot demonstrated that more then one gene copy of KAS III exists as seen by differences in restriction digest profiles of *B.napus* and its two parents *B.campestris* and *B.oleracae*. It is possible that four KAS III genes exist in *B.napus*, where it has inherited two genes from each parent. This is suggested as Kater *et al.*, (1991) showed that *Brassica napus* contained four copies of the enoyl-ACP reductase (ENR) gene, two obtained from each parent. This was further substantiated by the observations of Fawcett *et al.*, (1994) who demonstrated that four isoforms of (ENR) were present in the tissue examined as determined by 2D western blots. When the ENR isoform expression was compared in leaf and seed material no tissue specific expression was seen, although it was suggested that the same two isoforms were most abundant in both tissue types examined. The existence of other KAS III genes offers the opportunity to examine whether KAS III genes are tissue or temporally

regulated. TF Clone 2 is a different isoform compared to pKAS13 but there is only 121bp of overlapping sequence between TF Clone 2 and Isoform 2, as TF Clone 2 is severely truncated at the 3' end. In order to isolate further gene copies different regions of the KAS III cDNA could be used to reprobe this library plus libraries from different tissue sources.

ENR isoforms do not show tissue specificity and it is not known whether KAS III also possesses this trait, but probing a poly A+ RNA Northern blot containing root, leaf and embryo samples with the ORF of pKAS13 showed that the probe hybridised to all three tissue types. This is unlike the observations of Safford *et al.*, (1988) where ACP was seen to have leaf and seed specific isoforms that differed significantly at the nucleotide level. Their study showed that when a northern blot containing both tissue types was probed with an embryo ACP cDNA, it only hybridised to the embryo ACP.

From the full-length sequence of pKAS13 the highly conserved DITAACSGF region can be seen, which is known to be the active site of this condensing enzyme (Siggaard-Anderson, 1993). This region has been the subject of extensive investigation (Jaworski and Hinneberg-Wolf, 1998, Siggaard-Anderson *et al.* 1998, Abbadi *et al.*, 1998) and the cysteine residue is known to be the important in performing the Claisen condensation reaction as confirmed by site directed mutagenesis. Although site directed mutagenesis experiments on *B.napus* KAS III are beyond the scope of this overall study, the clone isolated during this study offers the opportunity to perform such experiments. As KAS IIIs are known to possess an ACAT activity, site directed mutagenesis could prove useful in manipulating this activity by examining the effects of loss or enhancement of function when KAS/ACAT mutants are

expressed in *E.coli*. This technique could also aid in understanding the importance of the conserved regions at position 360-380 of KAS III proteins and determine whether this region is important structurally or has an enzymatic function.

3.4 Summary

- A genomic Southern blot of *Brassica napus* and its ancestral parents *Brassica oleracea* and *Brassica campestris* determined that there is more than one copy of KAS III gene in *Brassica napus*
- A KAS III cDNA clone of 1622bp was isolated from a rape embryo library (pKAS13). This clone was seen to be 85% homologous to the *Arabidopsis thaliana* KAS III sequence. The derived amino acid sequence showed the conserved DITAACSGF region of plant condensing enzymes.
- A second partial clone was isolated and determined to be a different isoform to pKAS13 and TF Clone 2 by sequence comparison.
- Analysis of KAS III transcript size by Northern hybridisation showed the mRNA to be approximately 1600bp suggesting that pKAS13 is a full or near to full-length clone.

Chapter 4

Raising an Antibody to *Brassica napus* KAS III

4.1 Introduction

The advancement in the understanding of gene expression in *E.coli* and bacteriophage, and the importance of their various promoters and polymerases (Chamberlin *et al.*, 1970, Youderian *et al.*, 1982 and Deuschle *et al.*, 1986) has led to the development of a variety of expression vectors and host strains for the expression of foreign genes in *E.coli*. A common feature of all expression vectors is a strong regulatable promoter to which the gene to be expressed is fused. These promoters are normally obtained from bacteriophage and one of the most commonly used promoters is one from the bacteriophage T7 (Studier and Moffatt, 1986). These vectors utilise a T7 phage promoter, which is recognised by the T7 RNA polymerase but not the host *E.coli* RNA polymerase. Various systems have been developed which utilise the T7 promoter in the vector and induction of strains containing an integrated copy of the T7 RNA polymerase.

A number of commercially available vectors possess various different attributes to aid in the overexpression of a foreign gene in *E.coli* strains. These systems include pGEX (Smith *et al.*, 1986, Smith, 1993), pMAL (Maina *et al.*1988), pSKF (Rosenberg *et al.*, 1983) and pET (Studier *et al.*, 1990). pGEX and pMAL are vectors in which the foreign gene is fused to the vector DNA in such a manner as to create a fusion protein, with the glutathione-*S*-transferase and maltose binding protein respectively. This type of vector allows the expression and

recovery of a foreign protein in a soluble form, and the properties of the fusion protein can assist in the purification process by using maltose binding columns or glutathione-agarose beads to easily and specifically purify the foreign protein.

The vector pSKF utilises the λ bacteriophage promoter P_L, which is often used in order to eliminate 'leaky' basal expression of foreign genes which are found to be toxic to the bacterial cell. The λ promoter is highly efficient with the ability to be tightly controlled and so will not express until induced to do so. Induction can be performed once there are sufficient cells containing the construct present to efficiently produce the foreign protein before the culture is affected by its toxicity. These three systems tend to be employed if difficulties in expression or purification have arisen when a system, such as pET, has been used.

The pET system is a two component process that has been developed to utilise T7 promoter in the vector (Studier *et al.*, 1990). The T7 promoter is positioned at the 5' end of the foreign DNA and the construct is introduced into a strain of *E.coli*, BL21*(DE3)* which contains the gene for T7 RNA polymerase on its chromosome under the control of a *lac* promoter. Expression of the T7 RNA polymerase is induced by the addition of IPTG, which then allows the T7 promoter to express the foreign protein.

This chapter describes the overexpression of a KAS III isoform from *Brassica napus* using the expression vector pET11d, the subsequent purification of the protein and its use to raise a polyclonal antibody. An antibody to *B.napus* KAS III would be an excellent tool in the

analysis of this protein *in vivo* and a sensitive antibody would open up the possibility to use a wide range of immunological techniques in the examination of transgenic KAS III plants.

4.2 Results

4.2.1 Generation of KAS III Insert Containing Cloning Restriction Sites by PCR

As it was not possible to determine the correct transit peptide cleavage site of the KAS III ORF of pKAS13, it was decided to clone the whole KAS III ORF into an overexpression construct. The vector pET11d was chosen. The vector is 5651bp and contains an ampicillin resistance gene, a T7 promoter, a multiple cloning site for insertion of the foreign DNA into the vector, followed by a T7 terminator to stop translation of the gene. This system was selected as it allows cloning into the unique *Nco*I and *Bam*HI sites of the vector which are not present in the KAS III ORF. The KAS III insert was amplified by PCR using the primers HCOE1 5' TAC CAT GGC GAA TGC ATC TGG CTT C 3' and HCOE2 5' CGG GAT CCT CAT CTC CAC CTG ATA ATT GC 3' which incorporated an *Nco*I and *Bam*HI site in their design respectively. Vent DNA polymerase (New England Biolabs) was used instead of *Taq* DNA polymerase, as this enzyme has a high fidelity derived in part from the 3'-5' proof reading exonuclease activity, thereby minimising errors during DNA synthesis. The following PCR conditions were used:

1 cycle	94°C	4 minutes
30 cycles	94°C	30 seconds
	56°C	1 minute
	72°C	2 minutes
1 cycle	72°C	10 minutes

A sample of this PCR product was run on a 0.8% agarose gel to confirm size and purity of the product. The remainder was purified using Promega's Wizard PCR Direct Purification method following the manufacturer's instructions (Figure 4.1).

4.2.2 Preparation of pETKAS KAS III Construct

To enable access to the restriction sites generated in the PCR, and therefore allow ligation into the pET11d vector, the PCR product was digested with *Nco*I for 2 hours at 37°C. This was followed by digestion with *Bam*HI and subsequently the insert was purified using the Qiaex DNA purification kit (Qiagen).

Approximately 500ng of pET11d was digested with 10 units of *Nco*I and the appropriate restriction buffer in a final volume of 20μl and incubated at 37°C for two hours. The digest was run on a 1% low melting point agarose gel and the restricted vector purified using the Qiaex gel extraction kit. The purified vector was added to a digestion reaction containing 10 units of *Bam*HI along with the appropriate restriction enzyme buffer and incubated for a further 2 hours. This was followed by the addition of 5 units of salmon alkaline phosphatase (SAP), further incubation at 37°C for 15 minutes and subsequent heating at 70°C for 20 minutes to deactivate the SAP enzyme. This treatment dephosphorylates the free ends of the vector thereby reducing self re-ligation events. The vector was run on a 1% low melting point agarose gel, the DNA band excised and purified with the Qiaex gel purification kit.

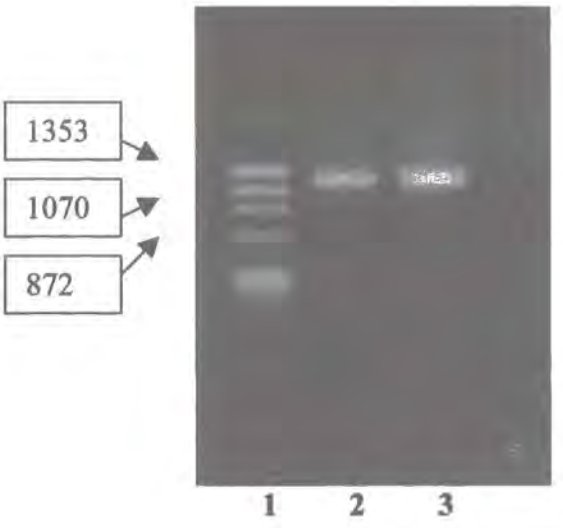


Figure 4.1 Agarose gel electrophoresis of PCR Products

PCR of KAS III ORF engineered to have a *Nco*I and *Bam*HI at the 5' and 3' end respectively. Amplification of the fragment was carried out using Vent DNA polymerase, an enzyme that has proof reading ability that ensures correct sequence amplification. The figure shows duplicates of the PCR reaction.

Lane 1	-	ϕ174 Molecular weight markers
Lane 2	-	PCR Product of KAS III ORF using Vent polymerase
Lane 3	-	PCR Product of KAS III ORF using Vent polymerase

Approximately 50ng of the *Nco*I/*Bam*HI digested PCR insert was incubated overnight at 16°C with 25ng of SAP-treated pET11d, 1 × ligation buffer (Promega) and 10 units of DNA ligase (Promega) in a final volume of 10μl. This ligation would form the construct shown in figure 4.2. Half of the ligation reaction was then used to transform 100μl of competent *E.coli* DH5α cells. Transformants were selected by plating on LB-agar plates containing 50μg/ml ampicillin and incubated at 37°C overnight. Colonies from the transformation were selected at random and streaked onto a fresh LB-Amp 50 plate, incubated at 37°C overnight and a colony blot performed (Figure 4.3). The membrane was probed with the KAS III ORF which had been radioactively labelled using [^{32}P]dCTP and the filters were washed to a final stringency of 0.1 × SSC, 0.1% SDS. Autoradiographs highlighted the colonies containing the insert, these were picked and grown overnight in 5ml LB containing 50μg/ml ampicillin. The plasmids were isolated by miniprep (section 2.6.5) and analysed for insert presence.

4.2.3 Analysis of Plasmids for Presence of KAS III Insert

Plasmids isolated after selection from the colony blot screen were analysed by restriction enzyme digests to confirm the presence of the KAS III ORF. Three separate digestions were performed. In the first, 100ng of plasmid DNA was restricted with 10 units of *Nco*I. In the second digest, 100ng of plasmid DNA was incubated with 10 units of *Bam*HI and the third restriction digest was performed using *Nco*I and *Bam*HI together in order to excise the KAS III insert. All three digests for each plasmid were examined on a 0.8% agarose gel. Plasmids shown to yield two bands when digested with *Nco*I and *Bam*HI, one of approximately 1,200bp the size of the KAS III insert and a second band of 5.6kb which was

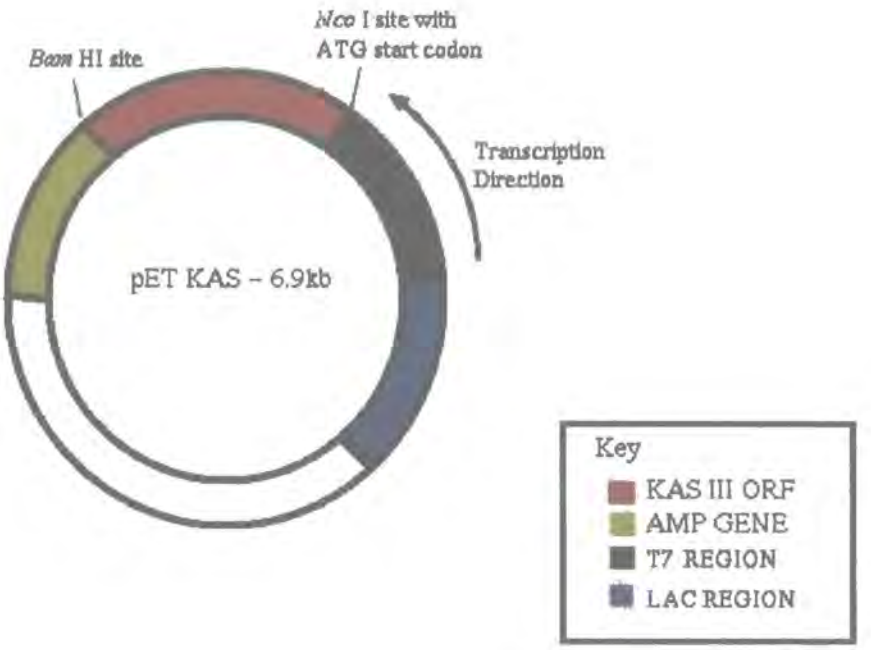


Figure 4.2 A diagrammatic representation of pETKAS overexpression construct.

The 1215bp KAS III ORF insert cloned into the pET11d vector created a plasmid of 6867bp. The construct has a T7/*lac* promoter/repressor system. The KAS III ORF is inserted at the multiple cloning site of pET11d using *Nco*I and *Bam*HI which ensures correct orientation of the cDNA.

Figure 4.3 Colony blot of *E.coli* DH5α cells transformed with the KAS III/pET11d ligation in order to determine which colonies contained the cloned KAS III ORF. The blot was screened with a ^{32}P[dCTP] labelled KAS III ORF and highlighted colonies were selected for further analysis.

the linearised vector were selected for sequencing. The plasmid was sequenced using primers designed to the T7 promoter and terminator, which confirmed that the plasmid contained a correctly ligated KAS III insert, that was in frame with the initial ATG codon of the vector, and contained a TGA terminator.

4.2.4 Transformation of pETKAS into BL21*(DE3)* and Attempted Overexpression

Upon confirmation that pETKAS had the KAS III ORF correctly ligated into the pET11d vector, the construct was introduced into a strain of *E.coli* BL21*(DE3)* which contains the T7 RNA Polymerase in its chromosome. 20ng of pETKAS was transformed into 100µl BL21*(DE3)* cells transformants selected on LB-Agar plates containing 50µg/ml ampicillin and incubated overnight at 37°C. A colony was picked and used to inoculate a 5ml culture of LB media containing 50µg/ml ampicillin and incubated overnight at 37°C. Subsequently this culture was diluted 1 in 50 in fresh LB media containing 50µg/ml ampicillin and the culture grown at 37°C until an OD_{600} 0.5 was obtained. At this point a 1ml sample was removed and IPTG added to a final concentration of 4mM and the culture incubated at 37°C for a further 3 hours. A second sample was taken and the cells incubated on ice for 15 minutes prior to collection by centrifugation at 5,000 × g for 10 minutes. The pellets were snap frozen and stored at -70°C. Samples were run on a 10% SDS/PAGE and stained with Coomassie Blue (section 2.8.6). This initial experiment did not indicate any overexpression of KAS III, so further experiments were carried out in order to optimise conditions to achieve expression.

4.2.5 Further Overexpression Experiments of pETKAS in BL21*de*

The experiment described in section 4.2.4 was repeated using only 5ml cultures, in which the cells were grown at three different temperatures, 30°C, 37°C and 42°C, and 200µl of samples taken every 60 minutes (Figure 4.4). Unfortunately no overexpression was observed. It is possible that this was due to the pETKAS expression vector being lost during the experiment. Therefore a fresh transformation was carried out and the plates incubated at 30°C overnight. This ensured that the colonies obtained were small and the formation of satellite colonies was prevented.

Ten to fifteen colonies were taken and used to inoculate a 5ml culture of LB media containing 50µg/ml of ampicillin and grown at 37°C until an OD_{600} 0.5 was reached. Expression of the construct was induced by the addition of IPTG to a final concentration of 4mM and a 200µl sample was taken. The culture was grown for a further three hours, a 200µl sample taken, and the remaining cells were harvested by centrifugation at 3,500 × g for 5 minutes and subsequently snap cooled and stored at -70°C. The samples were spun briefly in a microfuge and the cells resuspended in 1 × sample loading buffer electrophoriesed on a 10% SDS/PAGE gel and stained with Coomassie Blue. This experiment produced a novel band at approximately 45kDa in cultures containing the pETKAS construct (Figure 4.5). In order to confirm that the correct protein was being overexpressed, a second SDS/PAGE was performed, and the proteins transferred to Problott PVDF membrane to allow N-terminal sequencing of the protein. The expected amino acid sequence was (M)ANASGF, which is the first 6/7 amino acids expected if the KAS III ORF is expressed. This sequence was observed

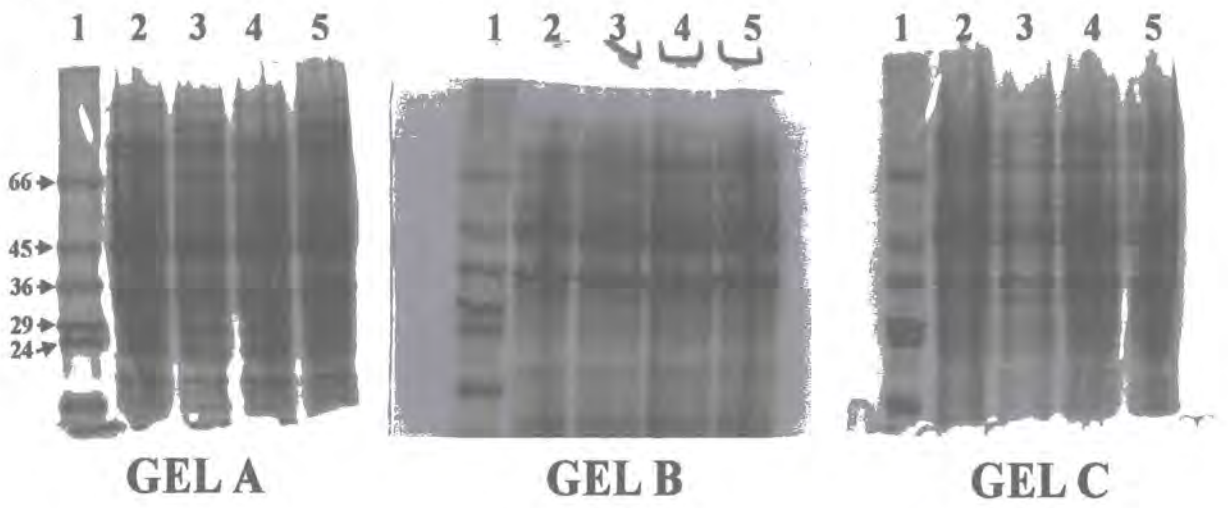


Figure 4.4 Overexpression attempts of pETKAS in *E.coli* strain BL21(*DE3*) at various growth temperatures.

No overexpression product was observed suggesting that during this experiment the pETKAS construct had been lost.

Gel A - 30 °C	**Gel B - 37 °C**	**Gel C - 42 °C**
Lane 1 - SDS7 Markers	Lane 1 - SDS7 Markers	Lane 1 - SDS7 Markers
Lane 2 - 2h - IPTG	Lane 2 - 2h - IPTG	Lane 2 - 2h - IPTG
Lane 3 - 2h + IPTG	Lane 3 - 2h + IPTG	Lane 3 - 2h + IPTG
Lane 4 - 3h -IPTG	Lane 4 - 3h -IPTG	Lane 4 - 3h -IPTG
Lane 5 - 3h + IPTG	Lane 5 - 3h + IPTG	Lane 5 - 3h + IPTG

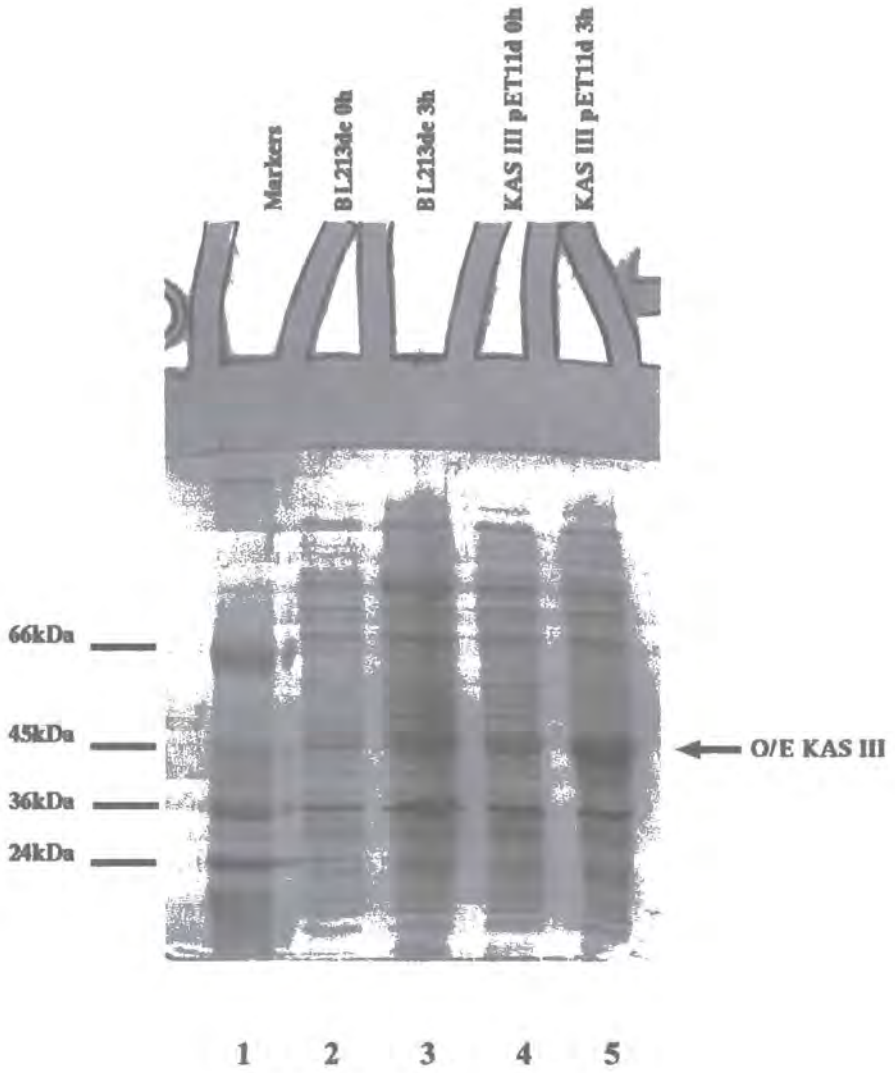


Figure 4.5 The overexpression of pETKAS in the *E.coli* strain BL21*(DE3)*. After an incubation of 3 hours following induction with IPTG a novel band at 45kDa can be seen in Lane 5. Overexpressed KAS III is indicated with the arrow labelled O/E KAS III.

Lane 1	SDS7 Markers
Lane 2	BL21*(DE3)* only before IPTG addition (0h)
Lane 3	BL21*(DE3)* only after IPTG addition (3h)
Lane 4	pETKAS before IPTG addition (0h)
Lane 5	pETKAS after IPTG addition (3h)

using Edman degradation confirming the identity of the overexpressed protein. The first methionine residue can be difficult to detect and generally when the overexpressed protein was sequenced the residues ANASGF were seen (Figure 4.6).

4.2.6 Scale up of Overexpression of pETKAS

A fresh transformation was carried out as in section 4.2.4 but grown overnight at 30°C. A colony was taken and used to inoculate a 5ml culture of LB media containing 50μg/ml ampicillin and grown at 30°C overnight. 1ml of this culture was used to inoculate a 500ml LB media culture that had been made up in a 2L-baffled conical flask containing 50μg/ml ampicillin. This culture was incubated at 37°C until an OD_{600} of 0.5 was obtained. At this point a 1ml sample was taken, 400μl of 50mg/ml ampicillin added and the expression of the construct induced by the addition of IPTG to a final concentration of 4mM. The culture was grown for a further 3 hours and a second 1ml sample taken. The remaining sample was snap frozen and stored at -80°C until required. The samples taken during the experiment were harvested by centrifugation at 5,000 × g for 10 minutes and the cells were spun down and resuspended in 200μl of 1 × sample loading buffer, 20μl of each sample was examined on a 10% SDS/PAGE gel and stained with Coomassie Blue. To examine the protein profile of BL21(*DE3)* without pETKAS, an experiment was performed concurrently with that described above using BL21(*DE3)* minus any overexpression vectors. A culture of pETKAS in BL21(*DE3)* minus IPTG induction was also grown. SDS/PAGE analysis of samples revealed that KAS III could be successfully overexpressed on a large scale (Figure 4.7), this overexpressed protein was again checked by amino acid sequencing of the first six residues to

Figure 4.6 Amino acid sequencing of overexpressed KAS III Isoform from *Brassica napus.*

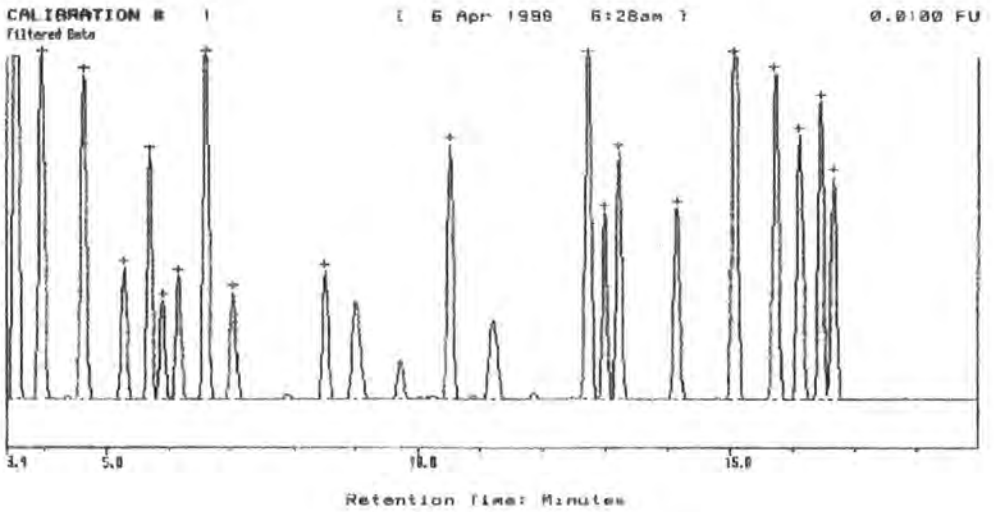
The overexpressed KAS III isoform sequence was determined using Edman degradation on an ABI 477 automated amino acid sequencer. This figure shows the calibration curve of a sequence run and the first 2 amino acids obtained from the KAS III protein; the full sequence obtained was ANASGF. Determination of the sequence of the first 6 amino acid from the overexpressed protein demonstrated that the correct protein was being overexpressed.

A - Standards

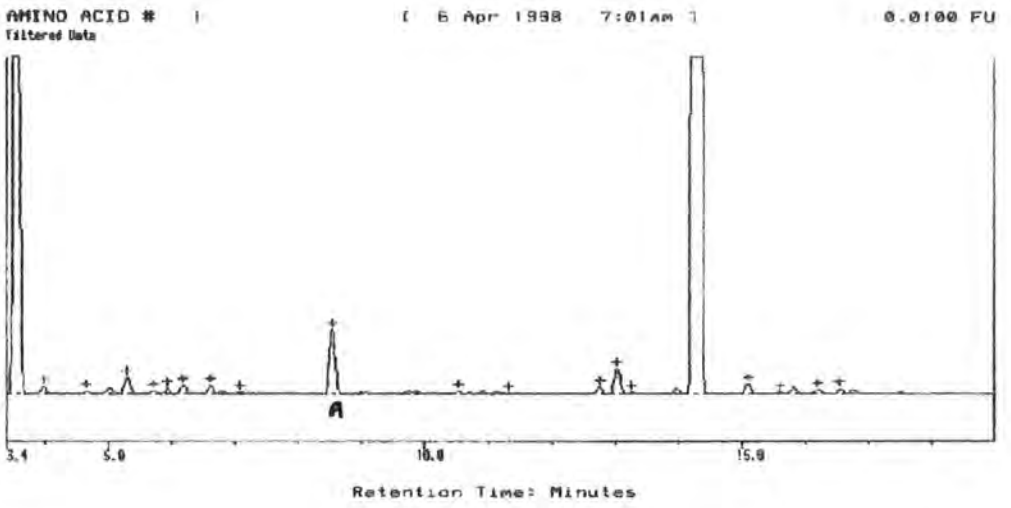
B - Cycle 1

C - Cycle 2

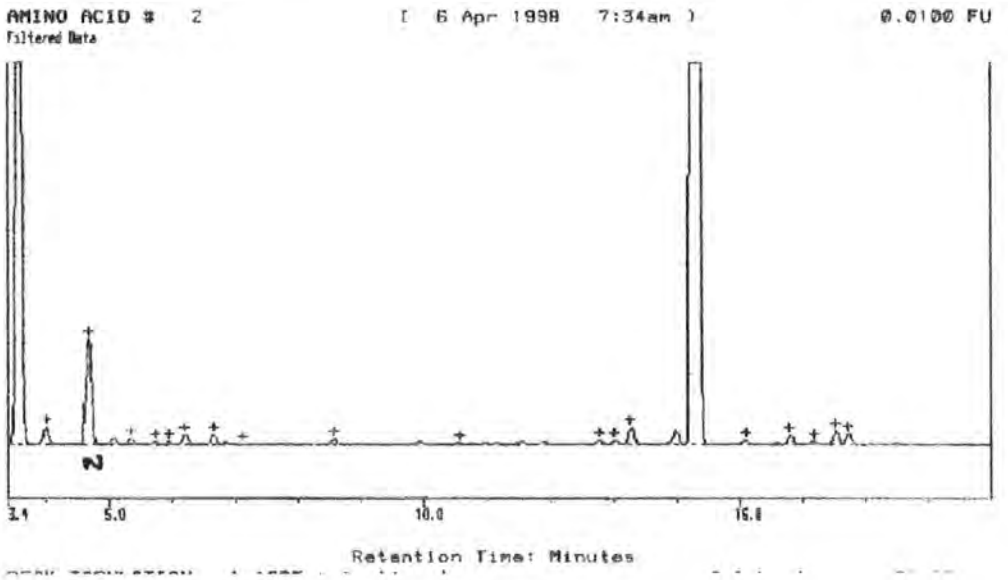
A


CALIBRATION #
Filtered Data
[6 Apr 1998 6:28am]
0.0100 FU
3.4
5.0
10.0
15.0
Retention Time: Minutes

B


AMINO ACID #
Filtered Data
[6 Apr 1998 7:01am]
0.0100 FU
A
3.4
5.0
10.0
15.0
Retention Time: Minutes

C


AMINO ACID #
Filtered Data
[6 Apr 1998 7:34am]
0.0100 FU
N
3.4
5.0
10.0
15.0
Retention Time: Minutes

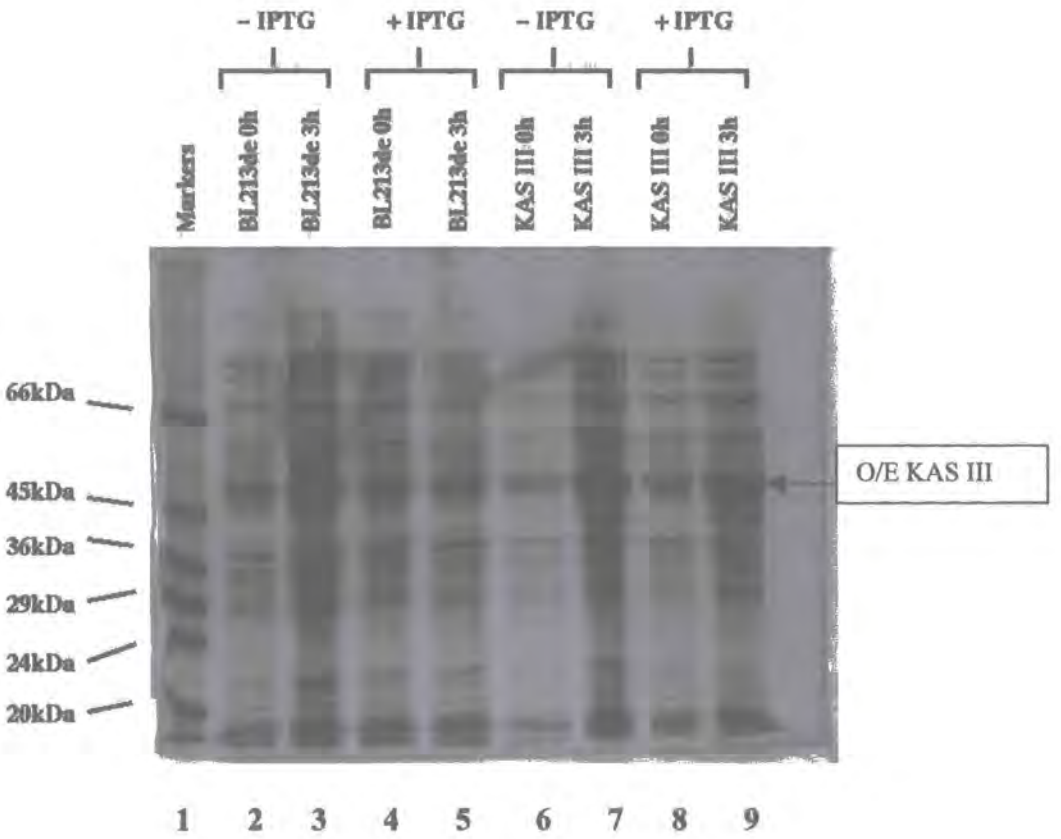


Figure 4.7 SDS-PAGE showing overexpression of pETKAS and BL21*(DE3)* induced (+IPTG) and non-induced (-IPTG).

A 10% SDS/PAGE stained with Coomassie Blue showing overexpressed KAS III against BL21*(DE3)* only cultures with and without the presence of IPTG. KAS III, which is indicated by the arrow annotated O/E KAS III, appeared to be expressed with and without IPTG. 1ml of cell culture was taken at the indicated time, pelleted, resupsended in 200µl of 1 × sample buffer and 20µl of this loaded into a well of the gel.

Lane 1	SDS 7 Markers
Lane 2	BL21*(DE3)* minus IPTG (0h)
Lane 3	BL21*(DE3)* minus IPTG (3h)
Lane 4	BL21*(DE3)* plus IPTG (0h)
Lane 5	BL21*(DE3)* plus IPTG (3h)
Lane 6	pETKAS minus IPTG (0h)
Lane 7	pETKAS minus IPTG (3h)
Lane 8	pETKAS plus IPTG (0h)
Lane 9	pETKAS plus IPTG (3h)

confirm its identity as a KAS III protein. Expression of this protein was specific to those samples containing the pETKAS construct. Expression could also be seen in the culture of pETKAS without IPTG induction and this can only be explained by basal expression of the T7 RNA Polymerase, which can occur despite the presence of a strong T7/*lac* promoter/repressor system in pET11d.

4.2.7 Disruption of Cells Containing Overexpressed KAS III

Each of the pelleted cells from section 4.2.6 were resuspended in 50ml of 0.1M $NaPO_4$ buffer pH 7.4 at 4°C. The cell suspension was then placed in a cell disrupter where the cells were subjected to a pressure of 25 Pascals. The effluent was collected and kept on ice until all the cells had been disrupted. The effluent was subjected to a further round of cell disruption to ensure all the cells had been broken and then centrifuged at 5,000 × g for 10 minutes and samples of the supernatant and pellet were analysed by a 10% SDS/PAGE gel and stained with Coomassie Blue. This showed that the protein was distributed between the pellet and the supernatant but indicated that the majority (60-70%) was insoluble (Figure 4.8). Two other methods of cell lysis were examined; freeze-thaw (Johnson and Hecht, 1994) and lysozyme treatment. The freeze-thaw method, as expected, did not appear to release as much total protein compared to the other two cell lysis methods, but nor did it release more recombinant protein than the other two methods. Lysozyme treatment appeared to release similar quantities of protein however the cell disruptor was selected for ease of use.

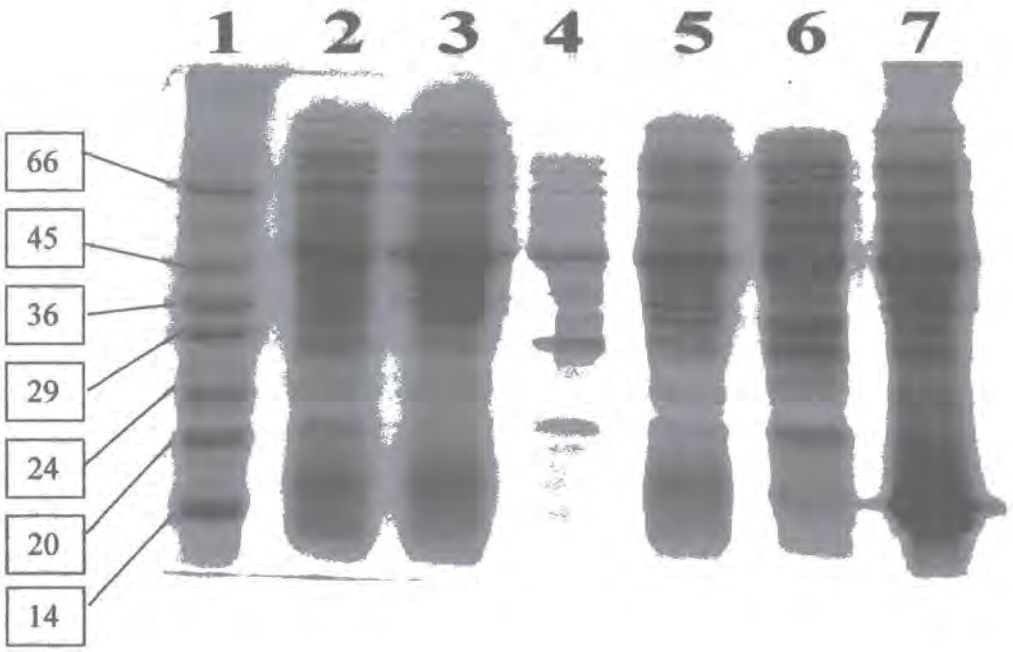


Figure 4.8 Analysis of the crude extract from various cell lysis techniques to examine whether the overexpressed protein was soluble.

A 10% SDS/PAGE stained with Coomassie Blue. Bracketed figures indicate the amount of overexpressed KAS III protein found in each sample as determined by densiometry of the overexpressed bands by comparing their densiometric values to the overall protein densiometric reading for each lane. The Freeze-thaw method (Lanes 4 and 5) did not appear to release as much recombinant protein as the other two techniques.

Lane 1	SDS 7 Markers
Lane 2	Supernatant of cell disruption method **(40%)**
Lane 3	Pellet of cell disruption method **(60%)**
Lane 4	Supernatant of Freeze-Thaw Method **(30 %)**
Lane 5	Pellet of Freeze-Thaw Method **(70%)**
Lane 6	Supernatant of Lysozyme Treated Extract **(40%)**
Lane 7	Pellet of Lysozyme Treated Extract **(60%)**

4.2.8 Extraction of KAS III Protein by Gel Electroelution

As the majority of the overexpressed KAS III protein was found to be insoluble, it was decided to purify the protein using a gel electroelution technique. Approximately 50µl of overexpressed pETKAS culture, lysed in section 4.2.7, was loaded into each lane of a 1.5mm thick 10% SDS/PAGE and electrophoriesed in electroelution running buffer (25mM Tris, 250mM glycine, 0.035% SDS, this buffer contains a lower concentration of SDS compared to standard electrophoresis buffer). The gel was stained during electrophoresis by addition of 200µl of Chromophore Green Stain (Promega) to the central compartment of the gel apparatus prior to electrophoresis, this stain ran with the bromophenol blue dye front (section 2.8.8). The overexpressed protein could be easily identified immediately after gel electrophoresis (Figure 4.9) and was excised from the gel. To release the protein from the gel, a gel electroelutor vessel was used which was assembled according to the manufacturer's instructions (Schliecher and Schuell). The electroelutor was placed in a gel electrophoresis tank containing electroelution buffer. The gel pieces were placed in the eluting section of the vessel and elution was carried out at 4°C at a constant current of 100mA for 16 hours. Polarity of the tank was reversed for 30 seconds and the eluted protein removed. A sample was electrophoresed on a 10% SDS/PAGE against known standards in order to calculate the concentration of the protein (Figure 4.10). The sample was on average 70% pure as calculated by densiometric analysis but it is possible that some of the sample could have degraded during the electroelution process. It was not possible to obtain 100% purity using this method as *E.coli* proteins of the same molecular weight would have been purified along with the protein of interest. Nevertheless it was decided to continue to raise antibodies using this prep, despite

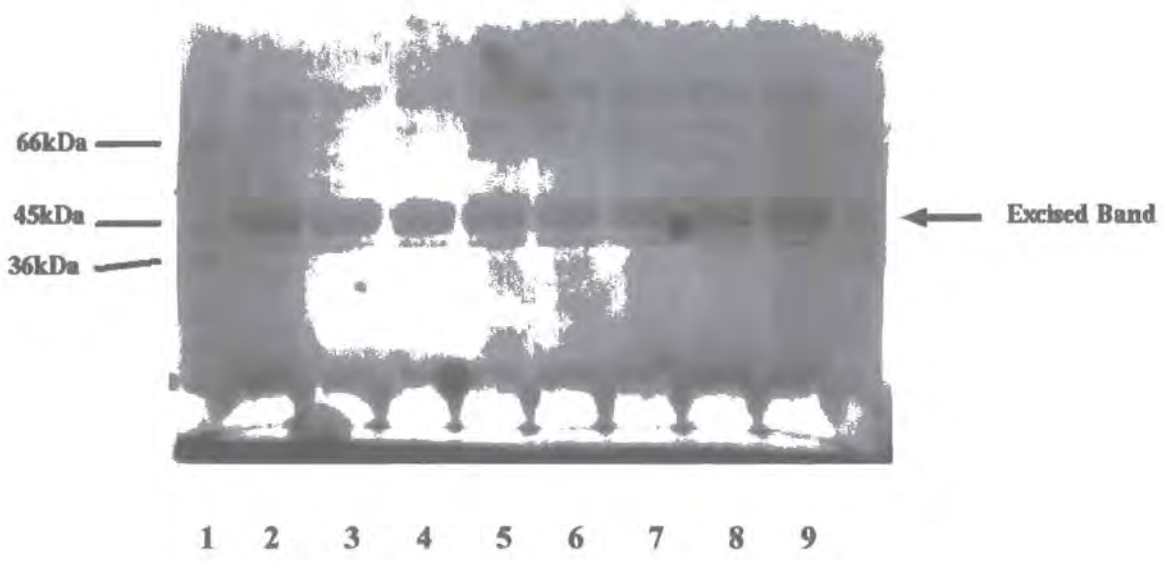


Figure 4.9 Chromophore Green Staining (Promega) of a 10% SDS/PAGE gel in order to excise the KAS III protein band for gel electroelution. This excised protein was confirmed to be KAS III by amino acid sequencing.

Lane 1 SDS 7 Markers

Lanes 2-9 pETKAS BL21*de3* overexpressed lysed cells.

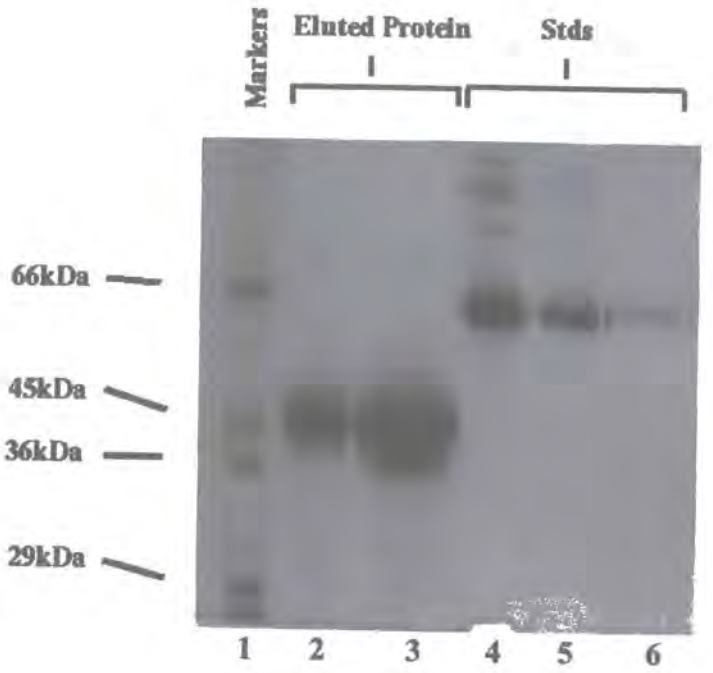


Figure 4.10 A 10% SDS/PAGE stained with Coomassie Blue to quantify eluted KAS III protein

The eluted protein was run on a gel to check the quality of the sample and the concentration was determined by densiometric analysis.

Lane 1	SDS 7 Markers
Lane 2	10µl of eluted KAS III protein
Lane 3	20µl of eluted KAS III protein
Lane 4	2µg BSA Standard
Lane 5	1µg BSA Standard
Lane 6	0.5µg BSA Standard

the presence of other proteins, any antibodies not specific to KAS III can be removed later if found to interfere with any experiment that requires the use of this antibody.

4.2.9 Sequencing of Purified KAS III Protein

To confirm that the protein being excised from the gel for electroelution was KAS III, the purified samples were transferred to Problott PVDF membrane and sequenced. However, no data was obtained from this sequencing, which inferred that the protein had become blocked during the electroelution process. This is where Edman degradation cannot occur as the N-terminus amino acid has been modified thus blocking the amino acid making it unavailable to Edman degradation. Therefore in order to confirm the identity of the protein, the excised protein band slices were placed in the wells of a second 10% SDS/PAGE gel and electrophoresed at 100V to return the isolated protein in the gel slices back into the SDS/PAGE gel. The samples were then transferred to Problott PVDF membrane. The sequence obtained was that of (M)ANASGF, the expected sequence for KAS III. This was repeated on a number of occasions in order to ensure that when KAS III was overexpressed and purified using the electroelution method, that the correct band was always being isolated.

4.2.10 Raising an Antibody to Purified KAS III Protein

The eluted protein was dialysed into PBS and 100μg of the purified protein was mixed with Freund's Complete Adjuvant (FCA). The immunogen emulsifies with the adjuvant to produce an antigen/water droplet within an oil phase. The FCA enhances and prolongs the antibody

response and forms a site of slow release of the immunogen when injected into an animal (Harlow, 1989).

The antibody was raised at Harlan Sera-Lab Ltd., Loughborough. The emulsion was injected into two rabbits subcutaneously at four sites. A rabbit was used rather than a mouse or rat as it has the potential to provide a significant amount of serum for the analysis of plants. It was necessary to immunise two rabbits in case they responded differently to the immunogen. Therefore each test sera obtained during the schedule were examined individually in order to determine the individual response of each rabbit. The immunisation schedule is shown in Table 4.1 and all bleeds obtained were assessed by Western blotting.

4.2.11 Analysis of Test Bleeds

Each bleed obtained from both rabbits was examined in order to follow the progress of the antibody. Pure KAS III samples at varying concentrations ranging from 100ng to 20ng along with 30μg of crude rape embryo extract were transferred to Hybond C-extra (Amersham). In order to ensure that the gel was correctly loaded with the varying levels of pure overexpressed KAS III, a 10% SDS/PAGE gel with the samples loaded was developed by silver staining (Figure 4.11). The following dilutions of the test bleeds were made up in 10ml of blocking buffer: 1 in 500, 1 in 1,000, 1 in 2,000, 1 in 4,000 and 1 in 8,000 and used to probe the membranes.

DAY	BLEED TAKEN	IMMUNISATION / BOOSTS
0	Pre-Immune Bleed	100μg of protein with Freund's Complete Adjuvant
28	Test Bleed 1	100μg of protein with Freund's Incomplete Adjuvant
35	Test Bleed 2 not taken	50μg of protein in incomplete adjuvant
49	Test Bleed 3	50μg of protein in incomplete adjuvant
63	Test Bleed 4	No Boost
81	Test Bleed 5	50μg of protein
89	Test Bleed 6	No Boost
96	Terminal Bleed	-

Table 4.1 Immunisation schedule to raise KAS III polyclonal antibodies in a rabbit

The Western blots were developed using BCIP/NPP (Sigma) tablets at a concentration of 1mg/ml and the colour development terminated by extensive washing in distilled water (or alternatively when using horseradish peroxidase linked secondary antibody; the Pierce Supersignalling Kit was used to develop the western). Monitoring of the immunisation trials in this manner showed that the pre-immune serum did not cross react with crude rape embryo extracts (Figure 4.12). As the trial continued there was increased sensitivity to the purified protein, where a 1 in 8,000 dilution could easily recognise 20ng of pure overexpressed protein. The antisera obtained from both rabbits could readily recognise the pure protein and so either could be used successfully. However in initial experiments the recognition of KAS III in plant extracts was not possible with either antibody.

Figure 4.11 Silver stained 10% SDS/PAGE gel to verify that the correct levels of purified KAS III protein were loaded, identical gels were then used in Western blots to analyse test bleeds during the immunisation trial.

Lane 1	SDS7 Markers
Lane 2	20 ng of KAS III purified by gel electroelution
Lane 3	50ng of KAS III purified by gel electroelution
Lane 4	100ng of KAS III purified by gel electroelution
Lane 5	30μg of Crude Rape Embryo Extract

Figure 4.12 Assessment of sixth test bleed on purified protein and crude extracts from Rabbit 092 using chemiluminescence Western blots.

20ng of pure KAS III protein could easily be detected by 1 in 8,000 dilution of antisera but KAS III could not be identified in crude plant extracts. Antisera from rabbit 093 showed a similar response to rabbit 092. Each blot has 20ng, 50ng, 100ng of purified KAS III immunogen and 30μg of crude rape embryo extract loaded from left to right as in lanes 2-5 in Figure 4.11.

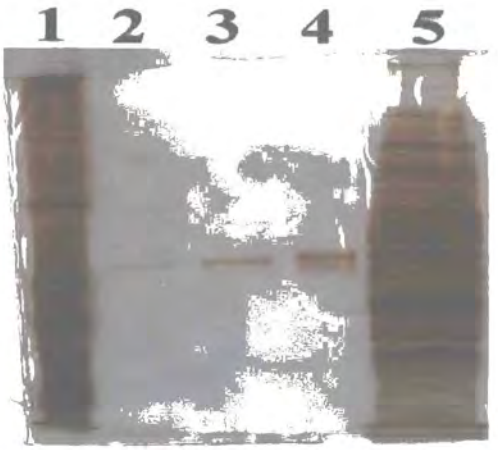


Figure 4.11

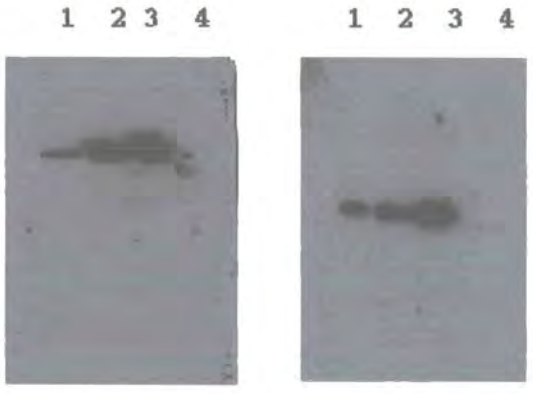


Figure 4.12

4.2.12 Antibody Recognition of KAS III in Crude Plant Extracts

The antisera obtained from both rabbits did not appear to recognise any proteins in crude rape embryo extracts. Therefore to examine the integrity of the crude extracts they were examined on a western blot probed with a sheep anti-ENR antibody which has very high affinity for ENR and it could be seen to recognise a 35kDa protein that corresponds to the molecular weight for the monomeric form of ENR (Figure 4.13). This indicated that the extracts were not degraded. Therefore this would suggest that either KAS III was not present, that the affinity of the KAS III antibody was not good enough to recognise KAS III in crude plant extracts or that the antibody was prevented from binding due to the KAS III protein being blocked by another protein. It was also possible that the extraction procedure used did not effectively isolate KAS III from plant material.

Therefore an embryo extract was used over the previously used mature seed extract to see if more KAS III is present in such samples. Also upon examination of the literature it was seen that Verwoert *et al.*, (1995) used a different extraction buffer to perform crude plant extracts in order to analyse KAS III activity. Therefore 1ml samples were prepared using this procedure. 1 ml of extraction buffer (50mM $NaPO_4$ buffer pH 7.2 and 2mM DTT stored at 4°C) was used to isolate the proteins from 10 oil seed rape embryos taken at 21 days after flowering. Samples were prepared using a hand held homogeniser, followed by centrifugation at 40,000 × g for 30 minutes. The supernatant was used in the following two ways:

1. 200µl of the extract was placed on a 0.5ml monoQ column which was equilibrated with 50mM $NaPO_4$, 2mM DTT. The column was washed with 1ml of 50mM $NaPO_4$, 2mM

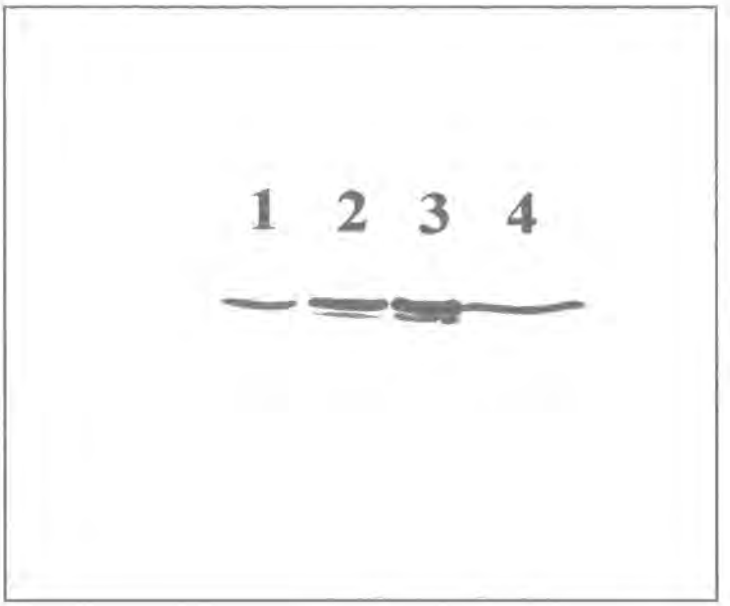


Figure 4.13 Western Blot analysis to determine integrity of the initial crude rape seed extract used to test the sensitivity of KAS III antisera test bleeds

This blot demonstrates that ENR is successfully recognised in the extracts by the Sheep-anti-ENR antibody, thereby confirming the integrity of these extracts.

Lane 1 20ng of pure ENR

Lane 2 50ng of pure ENR

Lane 3 100ng of pure ENR

Lane4 Crude rape seed extract.

DTT and any unbound protein was collected in 500µl aliquots. The column was then washed with 5ml of 50mM $NaPO_4$, 2mM DTT and 200mM NaCl. The sample was collected in 500µl aliquots and a Bradford assay performed on 5µl of each aliquot. The aliquot containing the highest level of protein was used in the western experiment described below.

2. 100µl of the extract was desalted using a Bio-Rad Bio-6 spin column. This desalted extract was also used in the Western experiment described below

The two extracts prepared as described above were examined on a 10% SDS/PAGE along with untreated extract and 100ng of pure KAS III protein (Figure 4.14) and a Western blot performed. A 1 in 1,000 dilution of antisera from rabbit 093 was made in blocking buffer and incubated with the Western blot for two hours. The membrane was washed twice in PBS-T and incubated with the secondary antibody (goat-anti rabbit IgG-Horse Radish Peroxidase) at a 1 in 20,000 dilution made in blocking buffer. The western was developed using a Pierce Supersignalling kit according to the manufacturer's instructions.

From figure 4.14 it can now be seen that the antibody recognises protein bands of approximately 45kDa and 38kDa. The cDNA expressed by the pETKAS construct encodes for a protein of approximately 42.8kDa and upon examination of the overexpressed protein on an SDS/PAGE a 45kDa protein was observed. If the proposed signal peptide of 7kDa were removed from this it would produce a 38kDa protein which can be seen in the Western blot. Since the KAS III protein can be seen in all three extracts it would appear that it is not

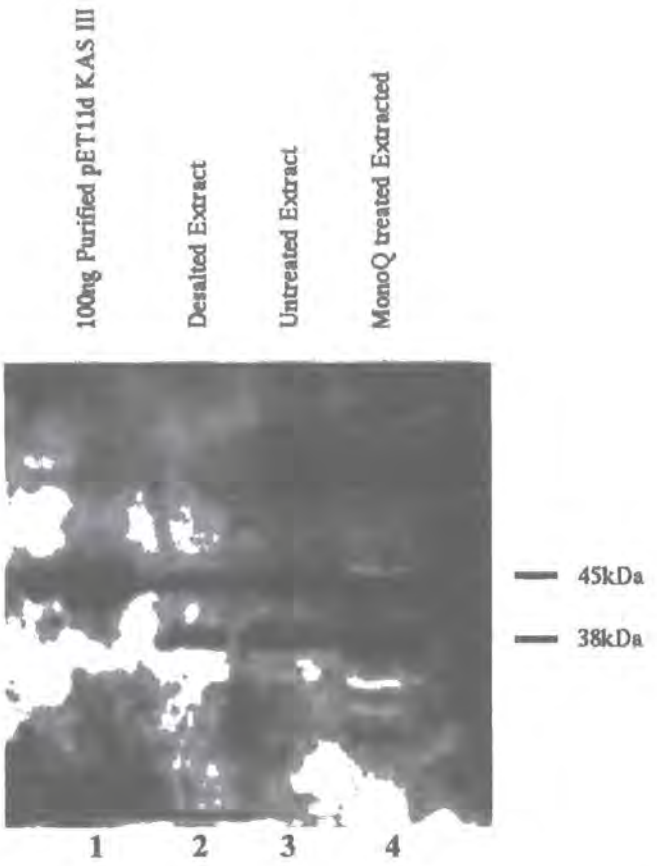


Figure 4.14 Use of KAS III antibody from rabbit 093 for recognition of KAS III in crude rape embryo extracts. Two bands are highlighted in all extracts suggesting that the antibody can recognise the pre-protein and mature protein of KAS III, if the transit peptide is the first 7kDa of the open reading frame.

Lane 1	100ng of pure KAS III protein used to raise the antibody
Lane 2	Desalted Crude Rape Embryo Extract
Lane 3	Untreated Crude Rape Embryo Extract (as prepared by Verwoert *et al.*, 1994)
Lane 4	Mono Q Treated Crude Rape Embryo Extract

necessary to desalt or partially purify the extract in order for the antibody to bind, but that a different extraction seems to have allowed the antibody to recognise KAS III. It is difficult to see how this could have occurred as there is little difference between the two extraction buffers. Both contained DTT which keeps the sample in the reduced form. This aids in the prevention of covalent modifications of amino acid residues particularly those at the active site of an enzyme which are likely to be in an ionised form and so prone to oxidation. It is therefore likely that using freshly prepared extracts aided in antibody recognition, as the protein may be very labile despite the use of DTT to protect the sample. The use of a fresh extract also decreases the length of exposure of the extract to proteases before use.

Extracts of rape embryo 21 DAF and the *Brassica napus* cv. DH RV28 leaf 2 at 4 DAE were made and examined by Western Blotting. Embryo extracts ranging from 25-150μg of total protein were loaded and probed with a 1 in 1,000 dilution of KAS III antibody from rabbit 093. A 1 in 30,000 dilution of goat anti-rabbit IgG linked to horseradish peroxidase was used as the secondary antibody and the protein detected using the Pierce Supersignalling Kit. The concentration of the secondary antibody was lowered to 1 in 30,000 in order to lower the background that rapidly builds up when using the Pierce Supersignalling Kit. This Western blot shows that the antibody binds in a quantitative manner (Figure 4.15). Densiometric analysis carried out on the blot. The blot contained known standard amounts of KAS III against protein extracts of known concentration. Densiometric readings of the highlighted bands in the Western blot calculated that in 3 Week old embryo, 0.14% of the total protein is KAS III.

Quantitative Western blots can be used in the analysis of transgenic antisense KAS III plants, where levels of KAS III could be lower and figure 4.15 demonstrates the antibody's ability to bind at differing amounts and therefore demonstrates the sensitivity of this antibody. Figure 4.15 also shows that the antibody recognises KAS III in *Brassica napus* leaf although it only binds to a single band of approximately 38kDa. Unfortunately due to the high background level of hybridisation it was not possible to accurately determine the quantity of KAS III in these extracts at this time but determination of these levels will be discussed in more detail in Chapter 5.

4.3 Discussion

This chapter reports the construction of a KAS III expression vector, the subsequent overexpression of the protein in *E.coli* BL21(*DE3)* and its use to successfully raise an antibody in rabbits. The antisera obtained recognises a protein of the expected size for KAS III and was successfully used in Western blots. The production of this antibody will also enable the development of other immunoassays such as ELISA and immunolocalisation of this protein within the plant cell.

Initial problems in overexpression were overcome by the use of fresh transformants in all overexpression experiments. Overexpression was scaled up by first growing a 5ml culture at 30°C and using this to inoculate a 500ml culture that was incubated at 37°C. 50μg/ml of ampicillin was added at the same time as IPTG induction and these measures ensured that the pETKAS construct was maintained in the bacterial cells. The overexpressed protein was found

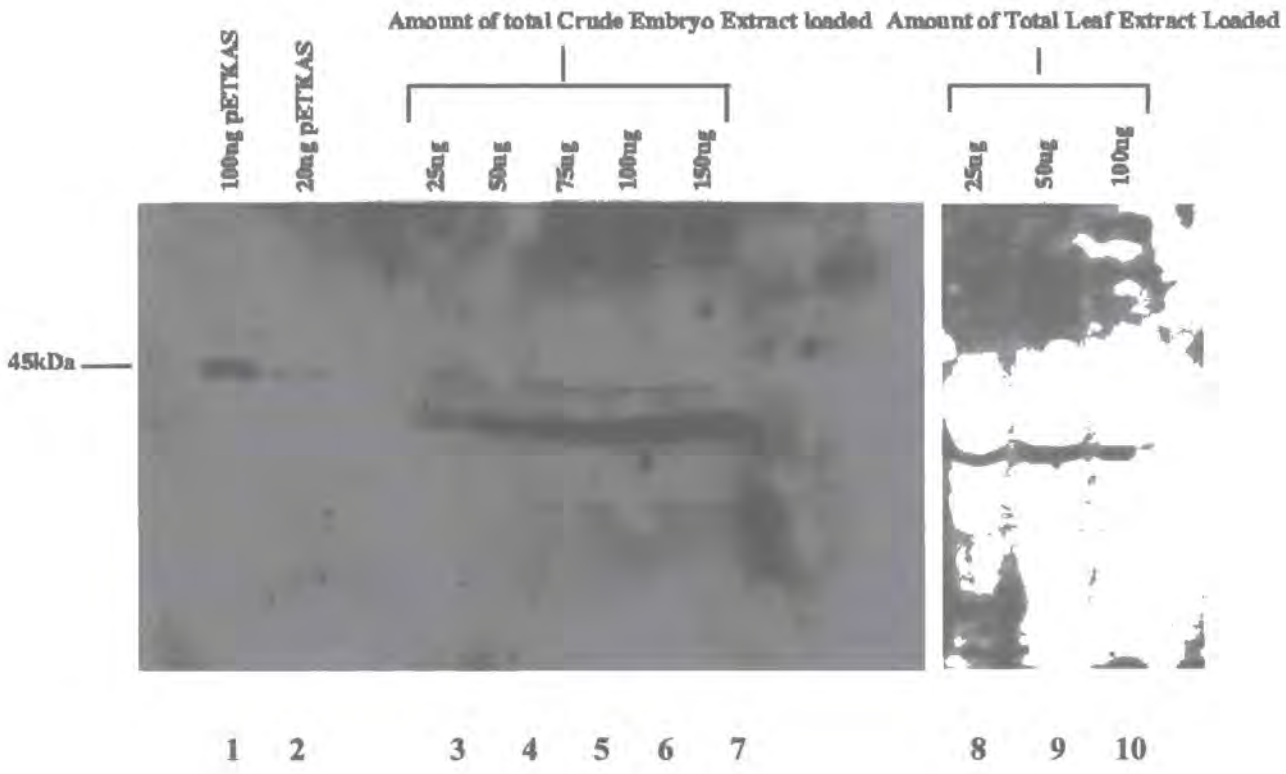


Figure 4.15 Quantitative Western Blot of KAS III from Crude *Brassica napus* Embryo and Leaf Extracts

These blots were developed using rabbit 092 serum. 150μg of total protein (Lane 7) appears to be overloaded and the detection of KAS III in this sample is beyond the limits of the Pierce Supersignalling Kit in that it has reached its maximum by 100μg of total crude embryo extract.

Lane 1	100ng of Pure KAS III
Lane 2	20ng of Pure KAS III
Lane 3	25μg of crude embryo extract (21 DAF) - 57ng
Lane 4	50μg of crude embryo extract (21 DAF) - 87ng
Lane 5	75μg of crude embryo extract (21 DAF) - 99ng
Lane 6	100μg of crude embryo extract (21 DAF) -108ng
Lane 7	150μg of crude embryo extract (21 DAF) -110ng
Lane 8	25μg of leaf extract (Leaf 2 Day 4 DH RV28)
Lane 9	50μg of leaf extract (Leaf 2 Day 4 DH RV28)
Lane 10	100μg of leaf extract (Leaf 2 Day 4 DH RV28)

to 60-70% insoluble and electroelution proved to be an effective way to purify enough protein for immunisation of rabbits to raise an antibody.

A typical immune response could be seen in both rabbits on examination of the test bleeds. The antibody response in an animal is the culmination of a series of interactions between macrophages, T-lymphocytes and B-lymphocytes that are reacting to the presence of a foreign protein. The initial immunisation induces a weak primary response of IgM antibodies. A final booster of 50μg of protein was given to the animal after a one month rest. This induces a more powerful secondary response that culminates in the production of a high number of IgG antibodies and test bleed 6, taken eight days after the boost, showed that the antibody had increased sensitivity to the purified protein (Figure 4.12). Seven days later terminal bleeds were taken. It was important to take them at this point before the secondary response died away. If this were allowed to occur, further immunisations would not induce such a high level of antibody specific to the KAS III protein as the animal would now be immunised or resistant to the foreign antigen.

The difficulty in initial recognition of KAS III using the antisera appears to have been solved by using a different extraction buffer and the use of freshly prepared crude extracts. However, it is probable that using freshly prepared extracts was more significant in obtaining antibody recognition than the use of a different extraction buffer as the extract is not subjected to prolonged exposure of proteases in the sample, which may degrade KAS III, whereas there is actually little difference in the properties of the two extraction buffers.

Western blots with embryos and leaf extracts did sometimes prove difficult to repeat. This problem may be overcome if the antibody was purified further, which could increase its sensitivity. Purification of the overexpressed protein using the electroelution procedures described in section 4.2.8 meant that any *E.coli* protein of a similar molecular weight could be isolated along with the recombinant KAS III, and sequencing of the eluted protein did show contamination with other proteins. Therefore it is very probable that antibodies to *E.coli* proteins are present. The antisera could be purified further by adsorbing out the *E.coli* proteins using a freeze dried preparation of total *E.coli* proteins. All *E.coli* antibodies would bind to the powder leaving the antibodies specific to plant KAS III in the solution.

It is interesting to note that two protein bands are detected in the Western blots performed on 3 week old embryo (Figure 4.15) and it is assumed that the larger band is the pre-protein prior to cleavage of the transit peptide. In leaf the 45kDa band is not always seen, suggesting that more KAS III is produced in young embryo than in young leaf, possibly due to the increased requirement for FAS proteins during the onset of lipid deposition or that the transit peptide is more readily removed. Alternatively this antibody has affinity to another protein present in embryo extracts. However the sizes of the two bands would lead to the former proposal. Confirmation of this could be provided by isolating chloroplasts and comparing a chloroplast extract to a total cell extract minus chloroplasts to verify the size of the proteins that would be detected. In chloroplast only extracts it would be anticipated that only the 38kDa band is observed due to the loss of the transit peptide. This would also aid in determining the size of the transit peptide which from this work thus far would suggest it to be 7kDa or the first 225 nucleotide bases of the KAS III ORF (Figure3.7).

There have been few reports of KAS III antibodies and this is the first antibody raised to an isoform of *Brassica napus* KAS III. An *E.coli* KAS III antibody was raised by Verwoert *et al.* (1995) to analyse *Brassica napus* modified to express the *fabH* gene from *E.coli*. However, the antibody did not appear to recognise the *E.coli* KAS III being expressed *in planta*, although KAS III activity was seen to increase when this gene was introduced to the plant.

It is interesting to note that when the *fab*H gene was overexpressed in *E.coli* it had severe effects on the growth behaviour of the organism, with the growth of the cells being halted upon induction of the *fab*H gene. This phenomenon was also seen in overexpression of *fab*H in *E.coli* by Tsay *et al.* (1992b). These problems were not encountered in the overexpression of *B.napus* KAS III possibly as the whole ORF was expressed including the transit peptide which may have rendered the protein inactive and therefore it would not affect any of the metabolic processes occurring in the overexpression strain. Thus the decision to overproduce the KAS III ORF and not attempt to estimate the position of the transit peptide cleavage site proved a successful way to aid in the protein's overexpression for use in the raising of an antibody, the further use of which is described in Chapters 5 and 6.

4.4 Summary

- An overexpression vector containing the KAS III ORF was constructed (pETKAS).
- pETKAS was successfully overexpressed in *E.coli* BL21*(DE3)* to produce a novel protein band at 45kDa as determined by SDS/PAGE.

- The protein was purified by gel electroelution and used to raise polyclonal antibodies.
- Polyclonal antibodies recognise KAS III in *Brassica napus* embryo and leaf protein extracts.

Chapter 5

Development of a Quantitative ELISA and Developmental Analysis of Wild Type *Brassica napus* Leaf

5.1 Introduction

The eventual aim of this project is to analyse transgenic plants containing an antisense KAS III in order to determine if this enzyme has a potential role in regulation, and to examine its overall importance in *de novo* synthesis of fatty acids. The effects of down regulating genes in plants have been shown by various techniques. Molecular techniques can ascertain the number of copies of the antisense gene present and determine expression levels. Ultimate analysis however, should also investigate the effect that down regulation has on the gene product and the pathway end products, as the antisense down regulation would be anticipated to affect both of these.

As this project is also part of a larger investigation into the down regulation of various enzymes involved in fatty acid synthesis, this chapter aims to describe two studies. First the development of an assay in order to quantify the amount of the FAS protein, enoyl-ACP reductase (ENR), by an enzyme linked immunosorbent assay (ELISA). An ENR quantitative ELISA was initially developed as this protein was the first FAS enzyme to be purified from *Brassica napus* (Slabas *et al.*, 1986) and as a result, sensitive polyclonal antibodies to this protein were readily available. The second part of this study establishes a developmental profile of certain FAS enzymes (KAS III, βKR and ENR), along with the total fatty acid

profiles in a developing leaf from two *B.napus* cultivars. It was intended that the information gathered from this study would then determine the optimal period to take leaf samples for the analyses in transgenic studies, which would minimise the analyses which had to be performed.

An ELISA was first described by Engvall and Perlmann (1971) and they are now widely used in a variety of biological disciplines. The assay provides a safe, simple, non-radioactive method for detecting and quantifying antigen specific antibodies (IgGs). The method relies on the ability of most proteins to bind to plastic surfaces, which is likely to be due to hydrophobic interactions between the non-polar amino acids in proteins and the non-polar plastic matrix. There are a variety of ELISA techniques described in the literature (see Catty, 1989) all of which involve (a) bound and free fractions of the antibody and (b) the detection of the antigen which is either directly or indirectly attached to the solid phase (Pathak *et al.*, 1997). Compared to quantitative Western blots, ELISAs have the potential to determine protein levels in a high throughput manner. This is because more samples can be analysed on one microtitre plate and the assay takes a shorter time to perform compared to Western blots. Therefore this assay can be useful in the analysis of transgenic plants in a high throughput manner.

The ENR ELISA was designed using a previously isolated polyclonal antibody raised to rape ENR which was overexpressed and purified from *E.coli*. The purified ENR was used to raise polyclonal antisera in a sheep. It was anticipated that once developed this assay could be used to determine any pleiotropic effects that a KAS III antisense gene might exert on another FAS component, as well as utilising this technique in the analysis of other antisense FAS lines.

The experimental development of the ENR ELISA was used as the basis for development of a β-ketoacyl-ACP reductase (βKR) ELISA and both assays were then used in the developmental study of *B.napus* leaf. Additionally novel KAS III Western blots were performed and total fatty acid content analysed by FAME analysis to determine a developmental profile of the *Brassica napus* leaf aged Day 2 - Day 14.

Two cultivars were examined in this study, Westar and a double haploid line RV28 (DH RV28), as the transgenic lines available within the Durham laboratory (transgenic βKR, ENR and KAS III) had been transformed into either Westar or DH RV28. The developmental profile obtained was used to determine the optimal time to take samples for the various assays described. This was so identical assays could be performed during transgenic studies. The results obtained within this developmental study in wild type plants could also be compared against findings in transgenic *B.napus* and hence determine any possible phenotypic similarities and differences. The profiles from the two cultivars were compared to assess any differences, this enabled cross-referencing of results from the different transgenic lines in subsequent analysis.

5.2 Results

5.2.1 Development of an ELISA assay to Quantify ENR Levels in Crude Rape Extracts

The ELISA assay design employed was a competitive antigen inhibition ELISA technique. This has the advantage of being quantitative, sensitive to 1ng of protein on optimisation and utilised the polyclonal sheep anti-ENR antibody available within the laboratory (courtesy of Prof. A.R.Slabas). However, this ELISA procedure does require a supply of pure antigen which in this case was a lab stock (purified by Miss S. Bithell) of recombinant rape ENR overexpressed in *E.coli* (Kater *et al.*1991).

After optimisation of the antigen coating level and dilution of primary antibody, an antigen inhibition ELISA can be employed, the principles of such an assay can be seen in figure 5.1. In this type of ELISA, the analyte is incubated with the antibody in an initial step before being incubated directly on the microtitre plate. This is essentially an affinity isolation step of any free ENR present in the solution as the ENR will bind to the antibody. This in turn prevents the antibody from binding to the ENR coated to the ELISA plate. On addition to the assay plate the remaining free antibody binds to the antigen coated to the wells in a manner which can then be quantified, this is essentially an affinity purification of the remaining free ENR antibody in the solution. The more antigen present in the analyte the less free antibody there is to bind in this second step. After incubation of the analyte/antibody mixture on the plate, a solution of conjugate antibody (donkey anti-sheep IgG linked to alkaline phosphatase) is

incubated in the wells. The conjugate binds in a quantitative manner to the primary antibody. The colormetric conversion of the *p*-phosphophenyl-nitrate (pNPP) substrate from colourless to yellow is then catalysed by the alkaline phosphatase and the absorbance of each sample measured at 405nm every 30 minutes over a two hour period.

5.2.2 Optimising Antigen Coating Levels and Concentrations of Primary and Secondary Antibody

To perform a quantitative antigen inhibition ELISA, the amount of antigen required to coat the microtitre plate and the dilution of primary antibody to use must be optimised[1]. If optimal conditions are not defined, less than adequate coating of the solid phase will reduce overall sensitivity and give high background, which will obscure the properties of the test and reference samples. If the plate is 'overcoated' it will lead to non-specific trapping of the antibody. These first experiments describe the optimisations.

A direct ELISA assay (Figure 5.2) was performed, 1μg/ml, 2μg/ml and 4μg/ml of pure ENR, which had been purified from overexpressed rape ENR in *E.coli*, were made up in coating buffer. Each concentration was bound to the wells of two lanes of a microtitre plate. A seventh lane was coated with 100μg/ml bovine serum albumin (BSA) and the plate incubated overnight at 4°C. The plate was blocked with 2% M-PBS for 2 hours. Serial dilutions of the

[1] This initial experiment was performed by Dr. J.Markham, who has allowed the author to utilise the data. All subsequent experiments and analysis in this section was carried out solely by the author.

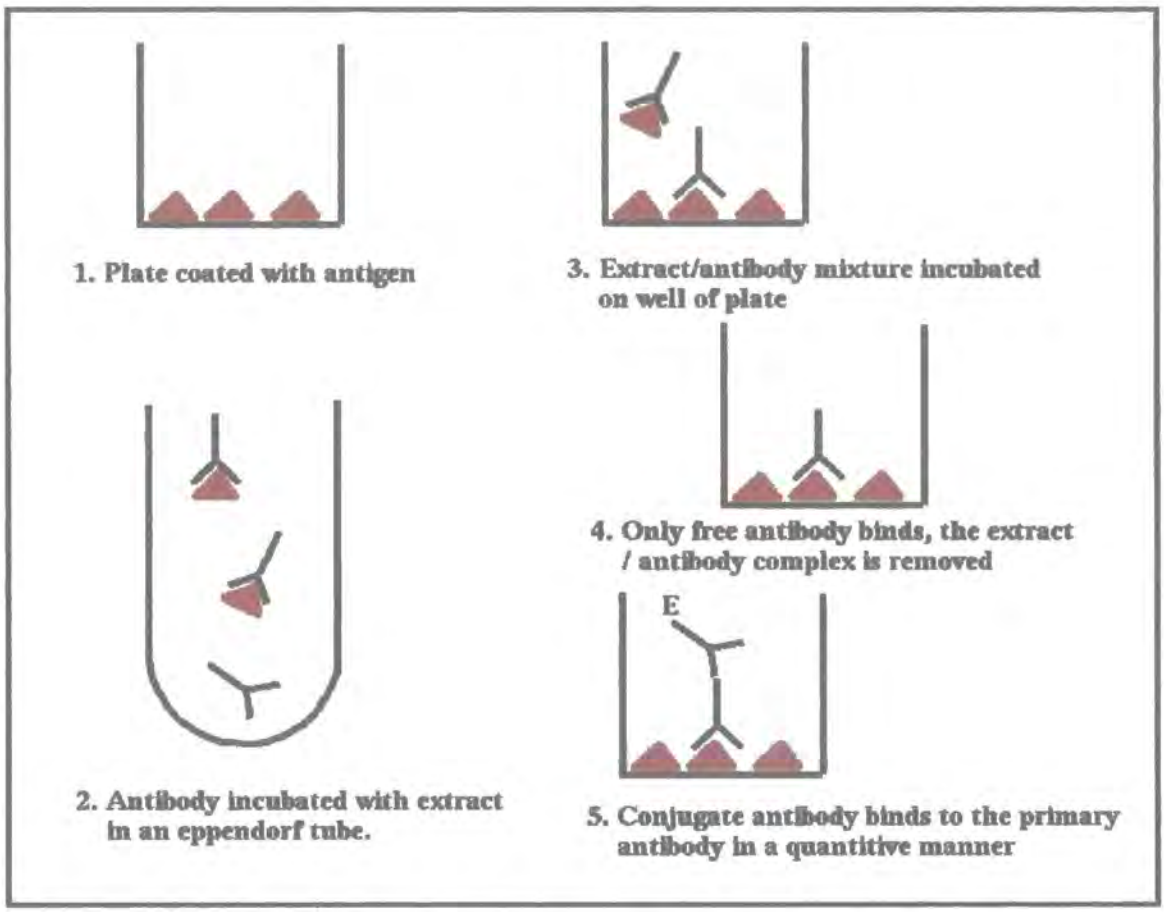


Figure 5.1 A diagrammatic representation of the steps involved in an antigen inhibition ELISA assay.

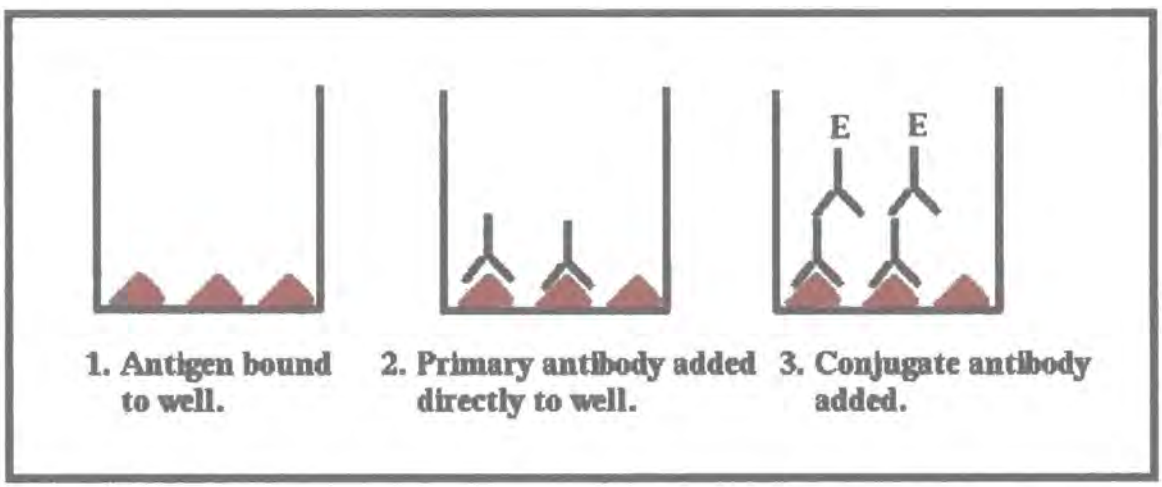


Figure 5.2 A diagrammatic representation of the steps involved in a direct ELISA assay.

primary antibody, sheep anti-ENR, were prepared ranging from 1 in 100 to 1 in 204,800 and 100µl of each dilution added to a well. After an incubation for 1 hour, the primary antibody was removed by washing the plate 3 × with PBS-T, and 100µl of 1 in 6,000 dilution of donkey anti-sheep IgG - alkaline phosphatase was added to each well. The plate was incubated for a further hour at room temperature. The solution was removed from the plate by washing 3 × with PBS-T, 100µl of 1mg/ml pNPP solution in 10% diethanolamine pH9.8 was added to each well, and incubated at 37°C for 30 minutes The plate was read at 405nm in a Bio-Rad microplate reader (Figures 5.3 and 5.4). The lowest antigen coating concentration that gives a combination of optimal dilution with low background readings should be selected (see Catty, 1989). Using these criteria, a 1 in 6,000 dilution of primary antibody was chosen against an antigen coating level of 1µg/ml.

Further studies by the author (data not shown) used ENR coating levels of 1µg/ml, 0.5µg/ml and 0.1µg/ml from a supply of ENR where absolute standard concentration had been determined by amino acid analysis as in section 2.8.12. The primary antibody levels used were 1 in 6,000, 1 in 12,000 and 1 in 24,000 and these further tests concluded that 1µg/ml and a 1 in 6,000 primary antibody dilution were the optimal conditions to use in the ELISA assay.

5.2.3 Initial Antigen Inhibition ELISA Experiment

5.2.3.1 Preparation of Plates

Six lanes of a microtitre plate were coated with 1μg/ml ENR, which had been prepared in coating buffer. One lane was coated with 100μg/ml BSA and the final lane used for duplicate controls in order to ensure that the ELISA was working correctly. Controls included two wells of each of the following; (i) no ENR coated to the well, (ii) no primary antibody incubated with the sample, (iii) no conjugate antibody incubated in the well, (iv) no ENR present in the sample and (v) no substrate added to the well. The plate was incubated overnight at 4°C and rinsed three times with PBS-T. Each well of the plate was then blocked with 200μl of 2% M-PBS and incubated for 1 hour at room temperature.

5.2.3.2 Antigen-Antibody Incubation in Solution

100μl of a 1:3,000 stock solution of the sheep anti-ENR primary antibody was added to known amounts of pure ENR (ranging from 20μg/ml to 4ng/ml), which had previously been placed in eppendorf tubes in 100μl aliquots These reference samples would create a standard calibration curve. A serial dilution of crude rape seed extract (section 2.8.1) was prepared and 100μl of each dilution incubated in an eppendorf tube with 100μl of stock primary antibody solution. The standards and test solutions were incubated at 4°C for 1 hour. 100μl of each solution was then added to a well of the ELISA plate, a set of the pure ENR standards were added to the 100μg/ml BSA lane in order to calculate background antibody binding levels.

The plate was incubated for 1 hour at room temperature, the solutions removed and the plate washed with 3 × PBS-T. A 1 in 6,000 dilution of conjugate antibody (donkey anti-sheep IgG linked to alkaline phosphatase) was added and incubated for 1 hour 30 minutes. Once the conjugate was removed, 100µl of 1mg/ml pNNP solution in 10% diethanolamine (pH9.8) was added to each well and the plate placed at 37°C for two hours. The plate was read on a Bio-Rad microplate reader every 30 minutes at 405nm to obtain optimal absorbance readings. This was where the highest readings obtained in the no inhibiting sample control wells were at least 10 times greater than the highest background reading (BSA coated lanes). This initial ELISA produced a standard inhibition calibration curve for the reference samples used but the antibody failed to recognise any ENR present in the crude rape seed extract (Figure 5.5). This failure was surprising and implied that the antibody could not bind to the appropriate epitopes of the ENR in a crude extract.

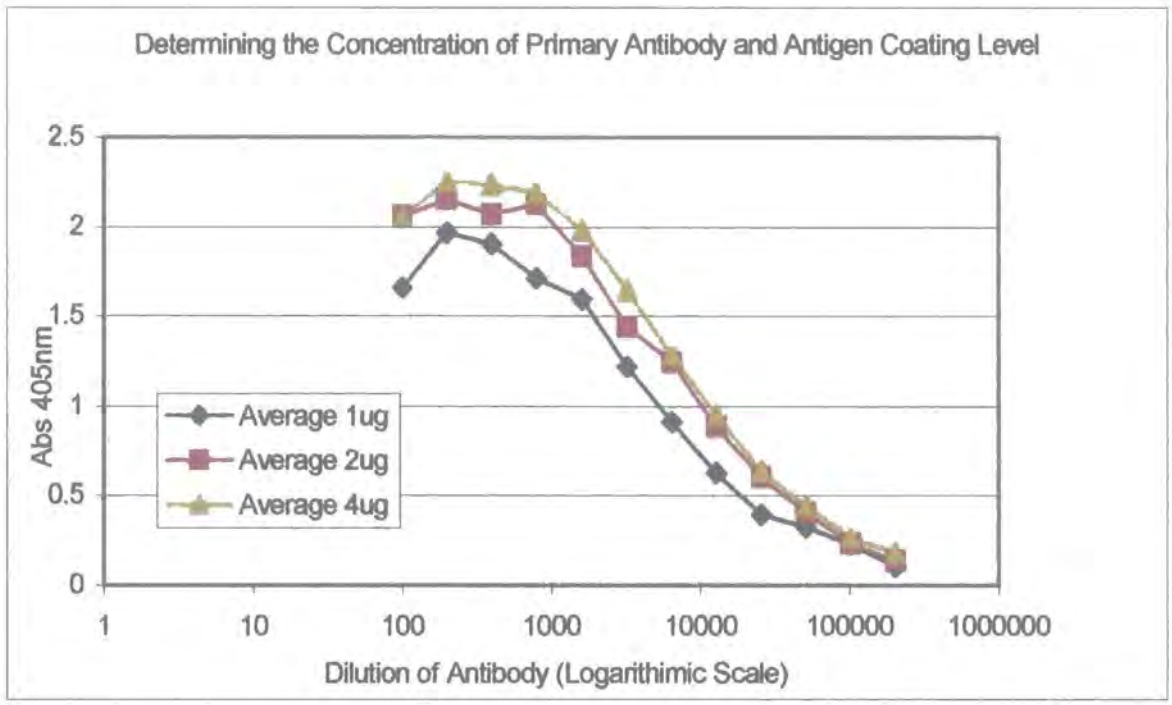
Despite numerous repetitions of the experiment described within this section with freshly prepared extracts, it was apparent that the antibody could not bind with the ENR in a crude *B.napus* extract. Previous work (Slabas *et al.*, 1990) had demonstrated via Western blots that ENR is present in such extracts. Therefore to confirm that ENR was present in this crude rape seed extract, and also in leaf extracts of *B.napus* cv. Westar, Western blots were performed as well as a ENR biological activity assay as described in section 2.9.1. This experiment confirmed that the extract contained ENR and so the extract had not deteriorated nor had it been prepared from a sample that had little ENR in it (see Figure 5.8 for quantitative western).

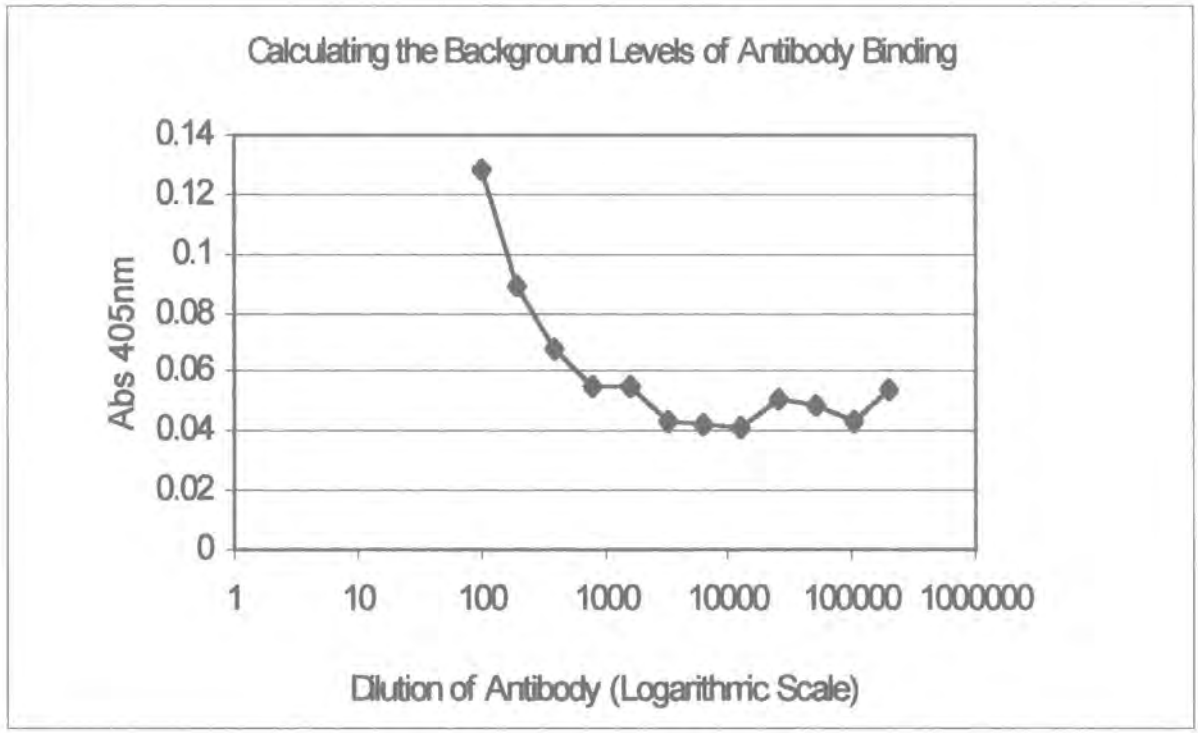
Figure 5.3 A direct Enoyl-ACP reductase-ELISA.

This graph shows the effect of three different antigen coating levels of ENR incubated with various dilutions of the polyclonal sheep anti-ENR antibody. The optimum antibody dilution should fall at an O.D of 1.0 (Catty, 1989). A 1μg/ml coating level was chosen for the antigen inhibition ELISA with a 1 in 6,000 dilution of the primary antibody.

Figure 5.4 The background levels of the direct ELISA assay shown in Figure 5.3.

The background was calculated from the absorbance readings of the control BSA coated lane, the lowest background falls at the 1 in 3,200 and 1 in 6,400 dilutions.


Determining the Concentration of Primary Antibody and Antigen Coating Level
Abs 405nm
2.5
2
1.5
1
0.5
0
Average 1ug
Average 2ug
Average 4ug
1
10
100
1000
10000
100000
1000000
Dilution of Antibody (Logarithimic Scale)


Calculating the Background Levels of Antibody Binding
Abs 405nm
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
10
100
1000
10000
100000
1000000
Dilution of Antibody (Logarithmic Scale)

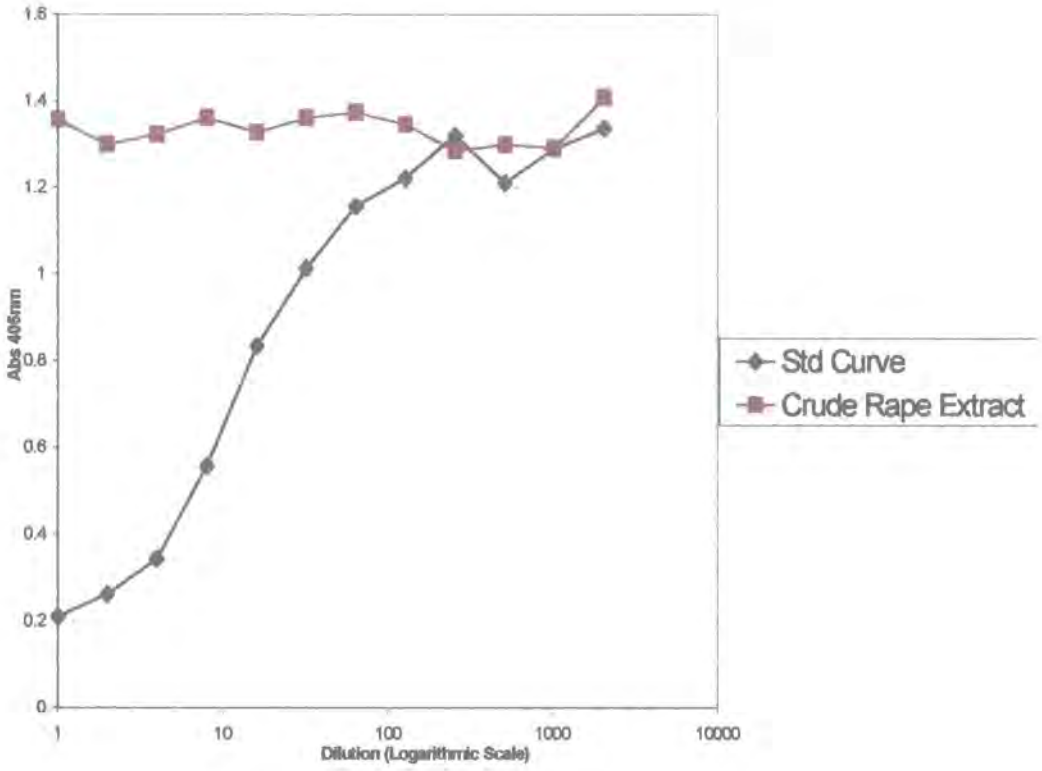


Figure 5.5 An Enoyl-ACP reductase - Antigen Inhibition ELISA.

Incubation of polyclonal Sheep anti-ENR serum performed with dilutions of pure ENR (♦) or crude rape extract (■) as described in the text section 5.2.3. The graph shows that the ENR in the crude rape seed extract has failed to interact with the sheep anti-ENR antibody.

5.2.4 Spiking Crude Extract with Pure Enoyl-ACP Reductase

The ELISA had been shown to work on pure ENR. It was decided that to ensure that a component in the crude rape seed extract was not affecting the antibody from binding, that such an extract should be spiked with pure ENR. Pure ENR was added to a crude rape seed extract at a concentration of 0.125μg/ml and an ELISA assay performed as in section 5.2.3 (Figure 5.6). The results showed that the antibody would recognise the pure ENR in a crude extract and the absorbance readings obtained indicated that the sample contained 0.125μg/ml of ENR as determined from the calibration curve. If the antibody had bound to the ENR already present in the crude extract, an absorbance reading giving an overall concentration greater then 0.125μg/ml would have been expected. As this was not the case, it was assumed that the ENR present in the crude extract is being prevented from interacting with the ENR antibody. It was also assumed that the pure ENR added was not interacting with anything in the crude rape seed extract and hence was recognised by the antibody.

5.2.5 Antibody Recognition Experiment – 1: Mechanical Manipulation

The antibody would not recognise ENR in a crude native extract, and it was assumed a component in the extract was preventing the antibody from binding to the ENR. It was imperative that the protein was in solution for the ELISA to work and the following describes methods that were employed to overcome this antigen recognition problem. Two methods of mechanical manipulation, centrifugation and microcon separation were employed in order to

see if the ENR could be recognised by the antibody after one of these treatments. It was thought that this may physically remove any component that was preventing recognition of ENR in the extract.

Mechanical Manipulation (i) The crude extract was centrifuged at 200,000 × g for 30 minutes. This would pool any membranes into the pellet and the ENR should be retained within the supernatant. Therefore if there was any component that was membrane bound and preventing the ENR epitopes from being recognised by the antibody, it would be removed to the pellet. Both the supernatant and pellet were used in an antigen inhibition ELISA as described in section 5.2.3.

Mechanical Manipulation (ii) The protein was centrifuged through a microcon with a molecular weight cut off of 30kDa. This would retain the ENR in the concentrate as the native tetrameric weight of ENR is 120kDa and if any small component was preventing antibody recognition of ENR in a crude extract it was thought that this treatment may remove it. Both the filtrate and concentrate were used in an antigen inhibition ELISA (section 5.2.3).

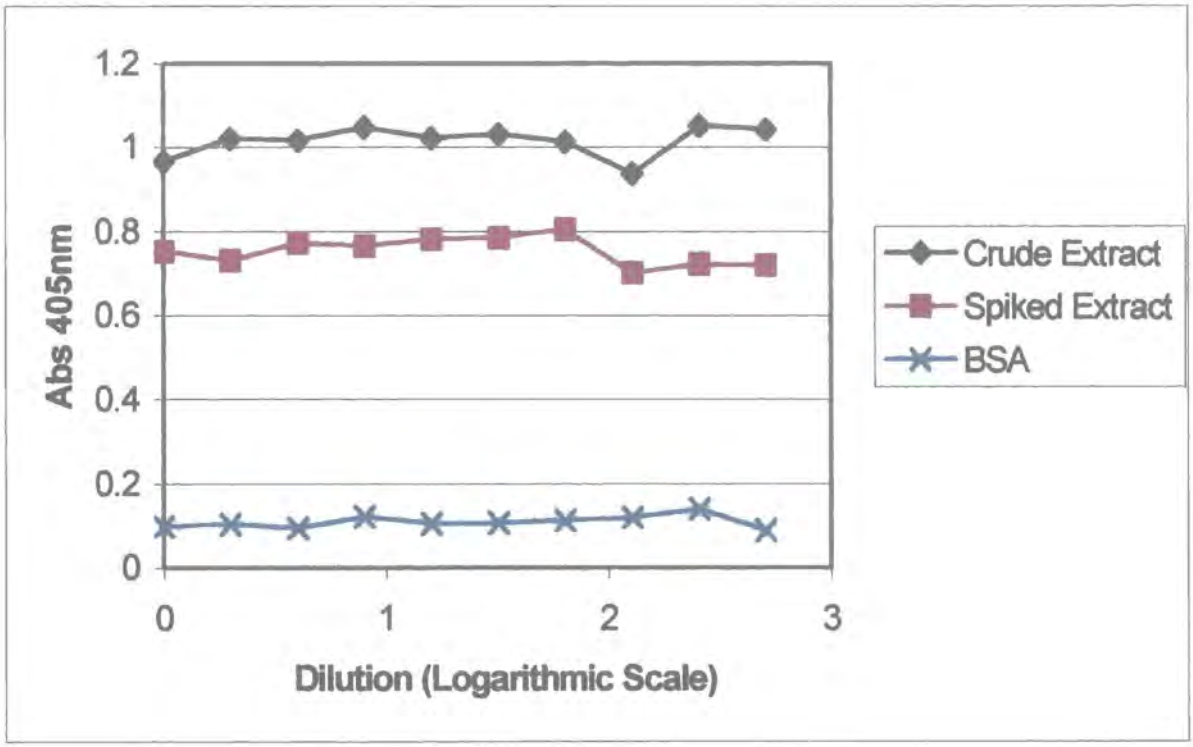
The ELISA performed on the samples provided by the two above methods both gave negative results (Figure 5.7), although a standard inhibition calibration curve was obtained. From these initial experiment the following conclusions can be made; (i) that the component preventing antibody inhibition is tightly bound (ii) centrifugation will not remove it (iii) the component is possibly larger than 30kDa as a microcon with 30kDa cut off will not remove it. Mechanical manipulation therefore, did not aid in antibody recognition of ENR in crude extracts.

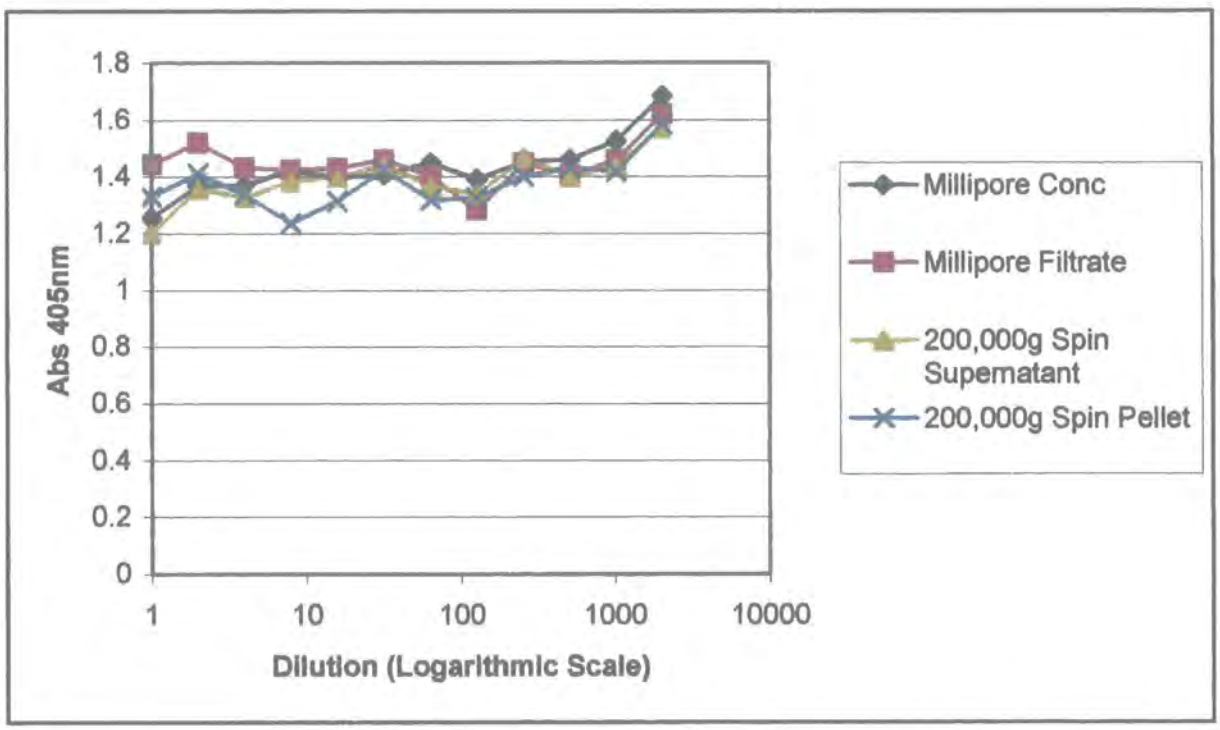
Figure 5.6 Enoyl-ACP reductase antigen inhibition ELISA of a crude *Brassica napus* seed extract spiked with pure ENR.

Inhibition observed in the spiked extract is equivalent inhibition to that of the amount expected for 125ng/ml of pure ENR alone (■). This shows that the antibody did not recognise any of the ENR in this extract, other than the amount used to spike the sample. This implies that there is no interaction between the pure ENR and the crude extract. When an untreated crude rape seed extract is analysed no inhibition is observed (◆).

Figure 5.7 Enoyl-ACP reductase antigen inhibition ELISA of a crude *Brassica napus* seed extract that has been mechanically manipulated.

When a crude rape seed extract is treated by centrifugation at 200,000 × g or if low molecular weight proteins are removed by centrifuging through a microcon as described in section 5.2.5, the antibody is still unable to bind to the ENR. This is confirmed by the lack of inhibition when an ELISA is performed with the samples treated in this way.


1.2
1
0.8
0.6
0.4
0.2
0
Abs 405nm
0
1
2
3
Dilution (Logarithmic Scale)
Crude Extract
Spiked Extract
BSA


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Abs 405nm
1
10
100
1000
10000
Dilution (Logarithmic Scale)
Millipore Conc
Millipore Filtrate
200,000g Spin Supernatant
200,000g Spin Pellet

5.2.6 Antigen Recognition Experiment – 2: The Effect of Denaturation

Mechanical manipulation of the crude extract did not confer antibody binding and yet at the same time the crude extract used in a western blot derived from a denaturing SDS/PAGE would recognise the ENR successfully (figure 5.8). This inferred the possibility that the antibody may recognise ENR in a crude extract if the protein was in a denatured form. It was important that denaturation was performed in such a manner that retained all the protein in solution. Strong denaturants are available which will still enable the proteins to be retained in the solution, this includes guanidine-HCl (Gu-HCl). This chemical is freely soluble in water and its low molecular weight enables its easy removal from a sample by dialysis.

A crude embryo extract was denatured in Gu-HCl at a final concentration of 2.6M and incubated on ice for 30 minutes. The denaturant was removed by dialysis overnight into PBS using a microdialysis system, and an ELISA assay was performed against the treated sample. This extract was compared against untreated extract and extract which had solely been dialysed (Figure 5.9). This showed that the antibody can recognise a crude extract once it has been denatured. Dialysis alone does not remove the inhibitor preventing antibody recognition. As the antibody cannot bind in a solution of native crude extract it is assumed that the antigen binding sites for the antibody are being 'hidden' and that denaturation of the extract reveals the epitopes. At this point it can only be speculated as to what is preventing antigen recognition in native crude extracts. It is possible that this particular antibody prefers to bind to a denatured form of the protein, but as it readily recognises pure native ENR this is not likely (data not shown). A second possibility, that a component is bound to the ENR and

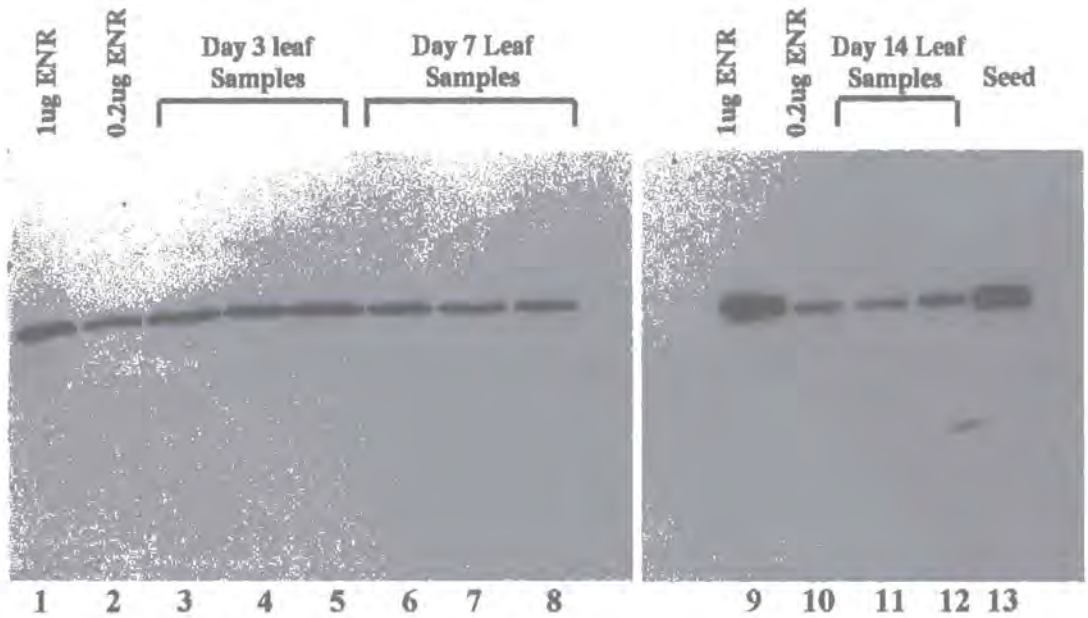


Figure 5.8 Enoyl-ACP Reductase Western blots of crude *Brassica napus* seed and leaf extracts.

The proteins were run on a 10% SDS/PAGE and the blots were developed using the sheep anti-ENR antibody at a 1 in 1,000 dilution, followed by an incubation with a 1 in 1,000 dilution of donkey anti-sheep conjugated to horse radish peroxidase antibody. This experiment demonstrated that the sheep anti-ENR antibody readily binds to ENR in crude plant extract on Western blots.

Lane 1 - 1μg of pure ENR
Lane 2 - 200ng of pure ENR
Lane 3 - Leaf Day 3
Lane 4 - Leaf Day 3
Lane 5 - Leaf Day 3
Lane 6 - Leaf Day 7
Lane 7 - Leaf Day 7
Lane 8 - Leaf Day 7
Lane 9 - 1μg of pure ENR
Lane 10 - 200ng of pure ENR
Lane 11 - Leaf Day 14
Lane 12 - Leaf Day 14
Lane 13 - Seed

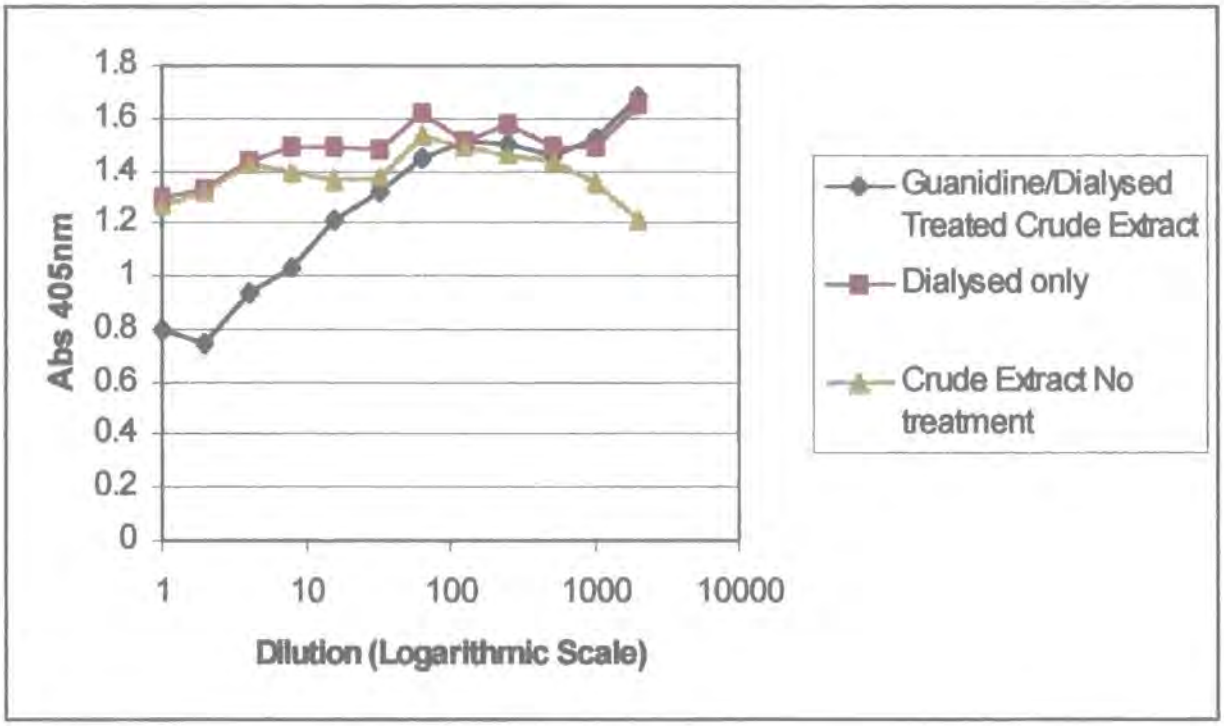


Figure 5.9 Enoyl-ACP reductase antigen inhibition ELISA of crude *Brassica napus* seed extract pre-treated with guanidine-HCl.

The experiment as described in the text (section 5.2.6) shows that significant inhibition is seen in the ELISA assay upon treatment of the crude extract with Gu-HCl (♦). Analysis of a sample which has been dialysed only (■) and an untreated extract (▲) show that no inhibition occurs in these two samples.

preventing recognition of the epitopes by the antibody, could be indicated by these results. The latter is explored further in section 5.2.11.

5.2.7 Confirmation of True Positive ELISA Results from Guanidine-HCl Treated Extracts

On repetition of the experiments described in section 5.2.6, the same inhibitory effect was seen when crude extracts were treated with Gu-HCl. To confirm that this was a true result i.e. that the antibody was indeed binding to ENR and the subsequent inhibition seen was not due to lack of removal of Gu-HCl (which would then denature the antibody), a BSA solution was incubated with Gu-HCl followed by overnight dialysis in PBS. An ELISA was performed on this sample and also on an untreated BSA sample which had solely been dialysed. Upon examination of the levels of inhibition it was shown that both treatments showed no inhibition (Figure 5.10). This indicated that the Gu-HCl is successfully removed by dialysis and that the inhibition observed in crude rape seed extracts is due to the ENR-antibody interaction.

5.2.8 Improving the Speed of Removal of Guanidine-HCl from Denatured Extracts

Microdialysis of the extract can prove problematic, as it is difficult to ensure that all the crude extract is retrieved and also dialysis must be carried out for a prolonged period to ensure removal of the Gu-HCl. An alternative method was investigated using a desalting column to remove the Gu-HCl. A crude extract was denatured with Gu-HCl at a final concentration of

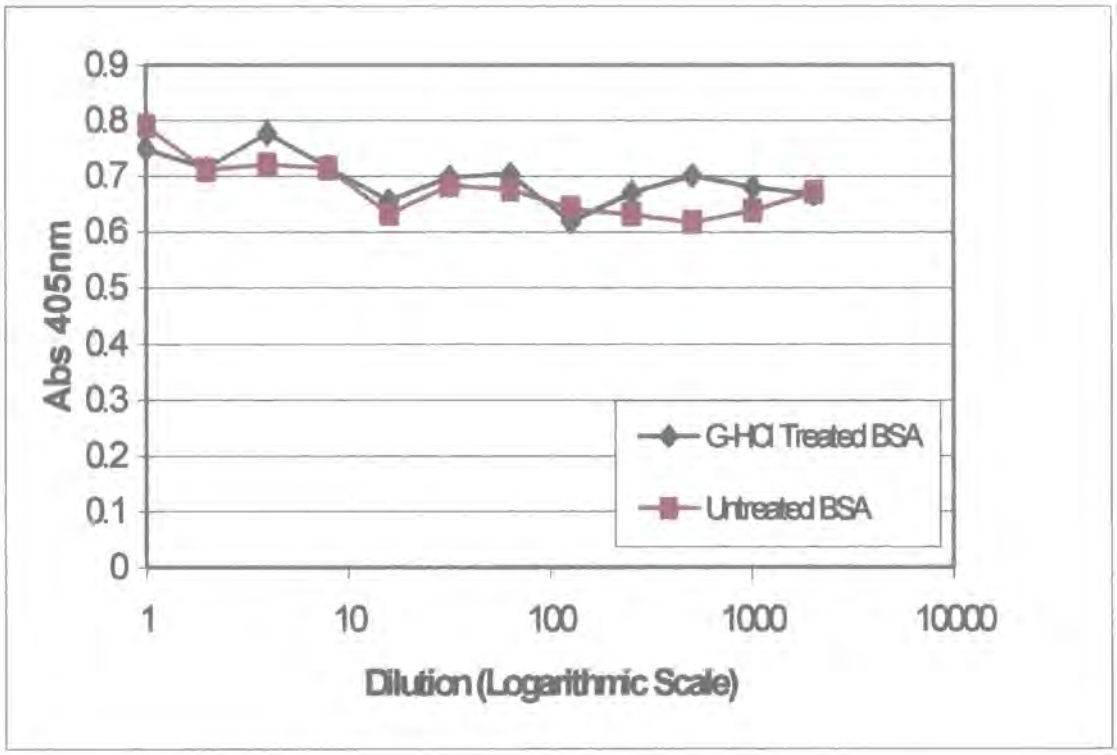


Figure 5.10 An Enoyl-ACP reductase antigen inhibition ELISA to verify that Gu-HCl is removed from extracts during the dialysis process.

The absorbance of a BSA sample treated with Gu-HCl (♦), followed by dialysis is compared against a BSA sample which has solely been dialysed (■). As the absorbance readings are similar for both samples it suggests that the Gu-HCl is being successfully removed during dialysis. Therefore it is not affecting the primary antibody and so not giving false inhibition.

2.6M and incubated on ice for 30 minutes, the solution was desalted in a Bio 6 spin column (Bio-Rad) equilibrated in PBS and the filtrate used in an ELISA (section 5.2.3). This was compared to a crude extract that had solely been desalted. This experiment showed that desalting also had the desired effect in removing Gu-HCl and that there is negligible differences between the dialysis and desalting. Identical calibration curves were also seen when treating pure ENR in this way. It also showed that desalting a crude rape seed extract through a spun column alone does not aid in antibody recognition. Hence the denaturing step in the protocol was no longer limiting the time taken to perform an ELISA as the desalting method was then incorporated for G-HCl removal.

5.2.9 Using ELISA to Examine ENR Levels in Leaf and Seed Extracts

After optimisation of the assay (see section 2.8.4 for final protocol) and confirmation of a highly reproducible standard inhibition calibration curve, which is sensitive to 30ng/ml (Figure 5.11) the technique was used to quantify levels of ENR in young leaf and seed. The leaf extract was made from 10 leaves harvested 3 days after emergence (DAE). A Bradford assay was performed and a total of 100μg of this extract used in an ELISA and compared against 100μg of seed extract. The samples were serially diluted from 1:1 to 1:32 (Figure 5.12) and duplicate assays performed. From this it was concluded that seed contains 0.771μg/ml of ENR in 100μg of total protein and day 3 leaf (DAE) contains 0.46μg/ml of ENR in 100μg/ml of total protein. The amount of ENR in a single leaf can be calculated to be 2.9μg, if a 3.2mg/ml leaf extract is prepared from a 3DAE leaf weighing 0.1g. This is equivalent to 0.77% of the total protein in seed and 0.46% in leaf, and indicates that seed

contains 1.6 times the percentage of ENR than 3 day leaf (DAE). The amount of ENR in young leaf is surprisingly high. However young leaf is being compared to mature seed here, and if young leaf is compared to developing embryo (data not shown) the amount present in developing embryo is considerably higher, with embryo 39 DAF containing 2.4% ENR, which is 5.3 times the percentage present in day 3 leaf.

If this data is compared to the study of Slabas *et al.*, (1990), where quantitative radioimmunoassays and quantitative Western blots were performed in order to determine absolute levels of ENR in 100g fresh weight of rape seed cv. Jet Neuf, figures of 1.84mg/100g seed and 1.92mg/100g seed of ENR were obtained respectively from the appropriate assay. These figures agree well with each other. The units of measurement in the Slabas *et al.* (1990) study are different to that of the ELISA performed in this section. The amount of ENR as calculated by an ELISA assay is calculated as the percentage of total protein or as amount of micrograms per 100 micrograms of total protein. If the calculations obtained from the ELISA are converted to the same units as those used by Slabas *et al.*, (1990). A figure of 1.2mg/100g seed is obtained, which is lower than that of Slabas *et al.* (1990). The study by Slabas *et al.* also suggests that the percentage of ENR present in seed tissue is 0.79% of the total, which is extremely close to the 0.77% measured in this study. Therefore it can be concluded that less ENR is present in this Westar seed compared to cv. Jet Neuf used in the Slabas *et al.* study and that contrasts may also be observed due to slight variance in levels of ENR in differing developmental stages of the two cultivars. Although the ELISA assay has given lower figures than Slabas *et al.*(1990), repetitions of this assay on young rape leaf extracts gave results

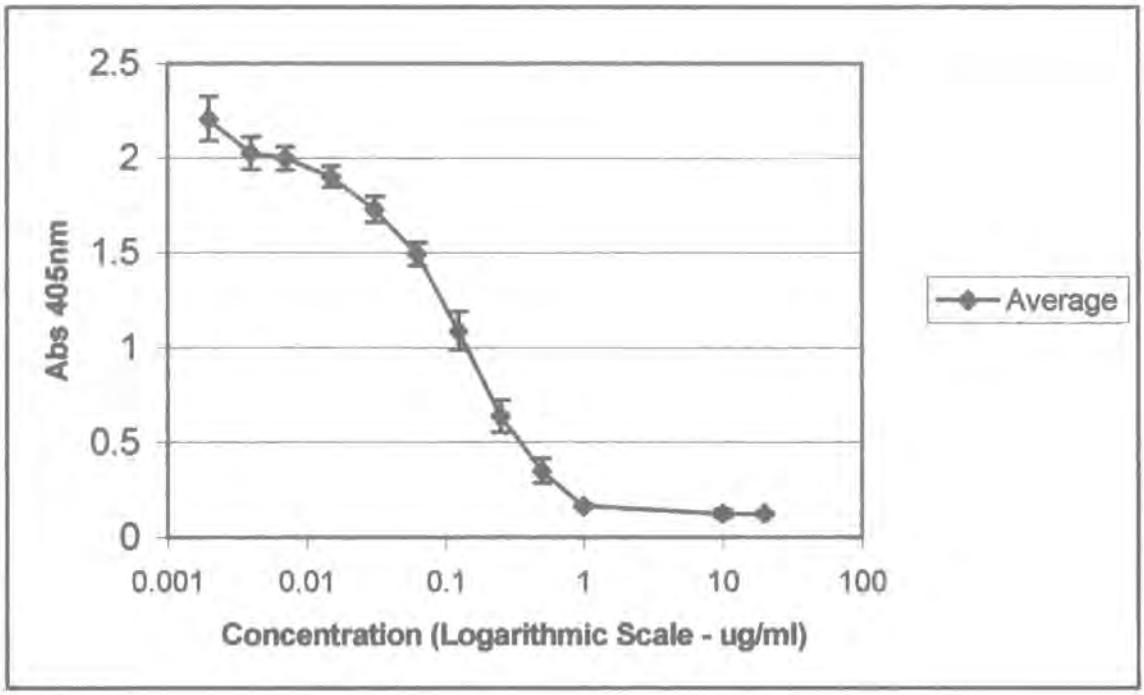


Figure 5.11 A standard calibration curve of the Enoyl-ACP reductase-antigen inhibition ELISA showing +/- standard error bars for each point.

The assay used pure ENR standards ranging from 20μg/ml to 4ng/ml. The calibration curve produced shown in this figure is an average of 30 individual ELISA standard curves after exposure to the substrate for 90 minutes at 37°C. The concentration is shown on a log scale along the abscissa, in order to spread the curve out and make accurate readings possible. The curve shows that each concentration gives consistent absorbance readings, which indicates that this is a reliable and highly reproducible part of the ELISA method. Absorbance readings vary a little from plate to plate due to the slightly differing absorption properties of the plastic surface of each plate.

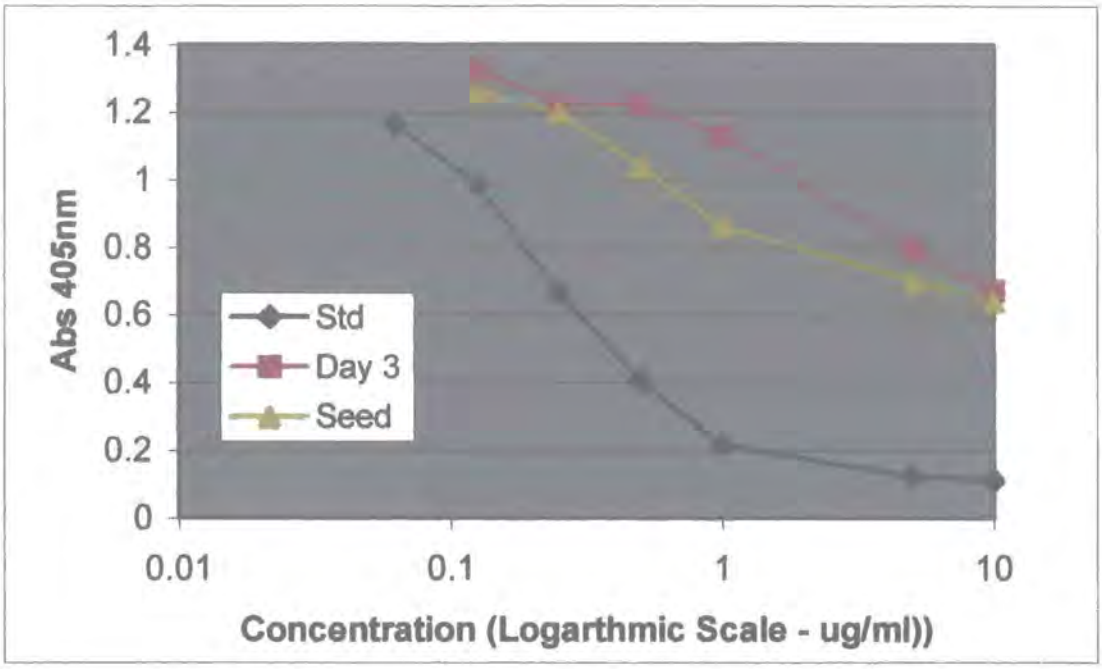


Figure 5.12 Quantification of *Brassica napus* cv. Westar seed (▲) and leaf 3 DAE (■) using the Enoyl-ACP reductase Antigen Inhibition-ELISA.

The graph shows inhibition curves for 6 dilutions of a *Brassica napus* cv. Westar seed sample (×) compared to a 3 DAE leaf (■). A standard curve (♦) was used to determine the levels of ENR in leaf and seed. From this experiment it was concluded that seed contains 0.77μg/ml of ENR in 100μg of total protein and day 3 leaf (DAE) contains 0.46μg/ml of ENR in 100μg/ml of total protein. This is 0.77% and 0.46% of the total protein respectively and indicates that seed contains 1.6 times the percentage of ENR than 3 DAE leaf (see section 5.2.9).

varying from 0.35μg/ml to 6.0μg/ml. These differences are in part due to the variation in the size of the leaves of the same age. The assay it is still a valuable tool as it is sensitive enough to analyse leaf extracts, as well as seed/embryo extracts and can be used in a high throughput manner. Hence it can be used in the analysis of a developmental profile of wild type *B.napus* leaf as well as in transgenic studies. The readings are being directly compared to a standard total amount of protein which will enable direct comparison of samples and would not therefore be reliant on consistent protein extractions from different samples.

5.2.10 Using the ELISA in analysis of Transgenic *Brassica napus* ENR Lines

In order to determine whether the ELISA assay could work successfully in a transgenic study where ENR levels were anticipated to be lower, eight lines of transgenic ENR, which became available during the study, were selected at random and ten seeds from each line sown and grown under the regime described in section 2.5.1. These ENR plants were available as sense and antisense lines (figure 6.5) and both types analysed in this initial study. A full description of these lines can be found in section 6.4. Leaf 1 day 5 was taken from all germinated plants confirmed to contain an ENR construct and a protein extract made as described in section 2.8.1. Each extract was denatured in Gu-HCl at a final concentration of 2.6M and was then estimated for protein concentration using a Bradford assay. The concentration was adjusted to 100μg/ml of total protein and used in an ELISA assay. The ELISA was performed on each individual leaf sample (5 DAE) in a series dilution ranging from 1:1 to 1:32. Figure 5.13 shows the various levels of ENR present for each line and demonstrates that it was possible to obtain meaningful data concerning the ENR levels in transgenic *Brassica napus*.

The analysis showed that line 107.17 has severely reduced ENR levels, however only 2 plants from this line could be examined due to the extremely low germination rate of this line (2/10). Lines 106 R4 and 106 R14 were also significantly down regulated and the remaining lines show slightly depressed levels of ENR. When the data is statistically analysed using the Student's T-test, which compares two separate data sets, it was seen that there was a 95% significant difference between the control Westar and the lines 107.17, 106 R4 and 106 R14. The remaining lines had a 90% significant difference to that of the Westar control. This demonstrated that the ENR constructs were affecting the ENR levels in the transgenic lines to varying degrees and also in such a manner which showed that the levels of ENR were significantly different to that of wild type Westar.

As this technique was able to quantify the various levels of ENR in rape plants it could now be employed in the determination of a developmental profile of ENR in *B.napus* and also be used in the analysis of transgenic KAS III plants as well as other transgenic FAS plants.

5.2.11 ENR-ELISA of a Crude Rape Seed Extract after Fractionation by Gel Filtration

To determine if a protein component was preventing binding of the antibody to ENR in a native crude extract a gel filtration experiment was performed as described in section 2.8.13. 50µl of untreated crude rape seed extract (cv. Westar) prepared as in section 2.8.1, which was used in the development of the ELISA assay, was loaded onto a Superose 12 column on a Pharmacia SMART HPLC system. The proteins were eluted off in 3ml of 50mM $NaPO_4$ pH 7.6 and collected in 100µl aliquots. An ELISA was performed on each aliquot in an identical

manner to that described in section 2.8.4 except that the denaturing step was not employed. This analysis was performed in order to see if partial purification of ENR enabled antibody recognition. This experiment was repeated three times using 50mM $NaPO_4$ buffer pH7.6 containing 0mM, 50mM, 100mM and 200mM NaCl respectively to see if stronger eluting conditions (i.e. increased salt strength) had any effect on the elution of the proteins. Stronger salt conditions did not appear to affect where the ENR was eluted and no prevention of ENR-antibody binding could be seen when an ELISA was performed. Figure 5.14 shows that the main inhibition is seen in fraction 7 (100-200kDa) for all 4 different salt concentrations. This fraction correlates with the molecular weight for ENR and hence ENR is binding with the antibody.

It is interesting to note that purification of the crude sample by gel filtration enabled the ENR antibody to bind to the ENR in solution. Gel filtration allows dissociation of the proteins and as the whole process is slower than for rapid desalting columns, it allows time for any interacting components to dissociate fully. Also this method physically separates proteins by size and it can be assumed that this treatment aids in ENR/antibody recognition by removing the component preventing the antibody/antigen complex forming. However dialysis does not remove the inhibiting component, so the recognition may also be due to the removal of a component concealing the ENR epitopes and this component is at least 10kDa as it is not removed during dialysis. Also the inhibitor does not appear to be removed by spun column, high speed centrifugation (200,000 × g) or the use of a microcon. It can only be

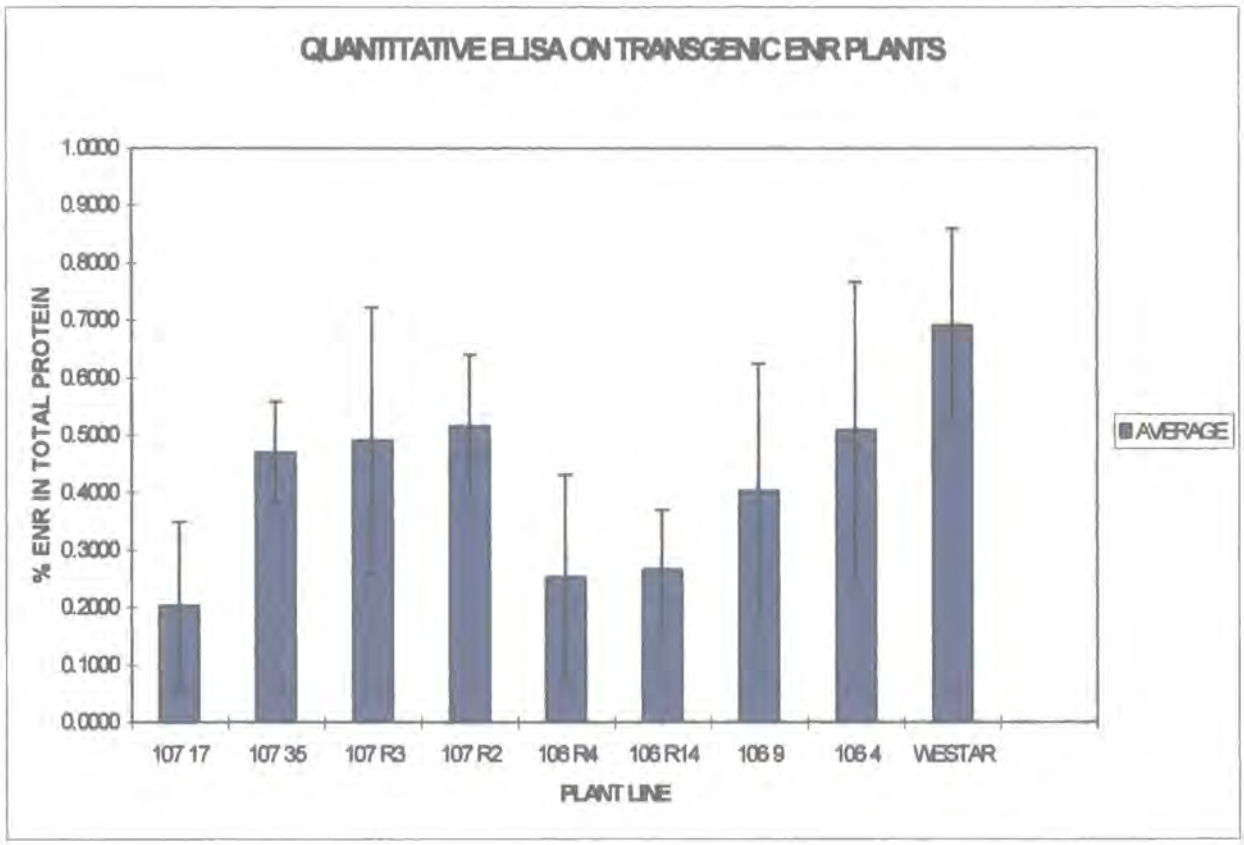


Figure 5.13 Levels of Enoyl-ACP reductase in transgenic ENR lines using the quantitative antigen inhibition ELISA assay showing +/- standard deviation error bars.

Six plants were analysed for each line with the exception of line 107-17 where only two plants germinated. Lines 107-17, 106-R4 and 106-R14 have ENR levels down regulated to approximately a third of the wild type. Each line shows high variability amongst its individuals and as T1 plants were examined it is likely that segregation of the transgene is still occurring.

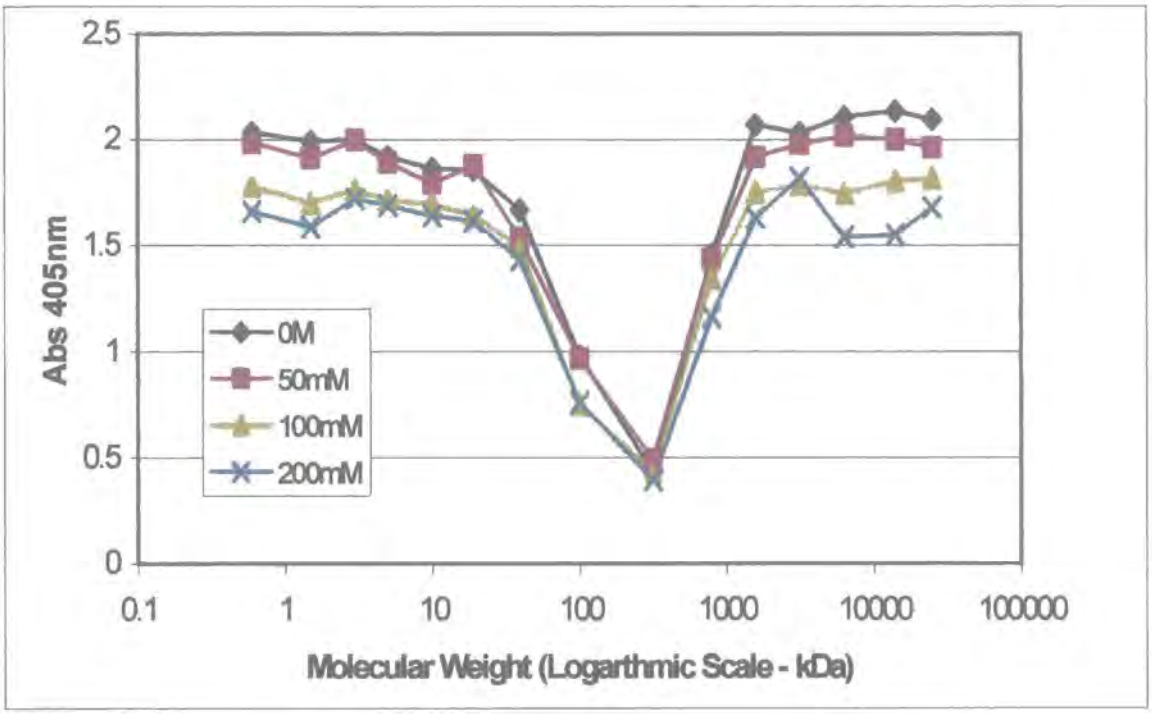


Figure 5.14 Enoyl-ACP reductase Antigen Inhibition ELISA on fractions of crude rape seed extract following gel filtration.

Gel filtration was performed using a Superose 12 Column on a Bio-Rad HPLC SMART system. Considerable inhibition can be seen in fraction 7 (approx. weight between 100-200kDa) for all four NaCl concentrations used in the filtration process. This inhibition is observed in the molecular weight fraction for ENR, suggesting that the inhibitory component has been removed by the gel filtration process.

deduced that the inhibitor is tightly bound and/or likely to be at least 30kDa in size as it was not removed by microcon treatment. Only further work in this area will reveal what is preventing the antibody from binding in native crude extracts.

5.3 Standard Profile of the development of *Brassica napus* Leaves

This study aimed to define a standard developmental profile for *Brassica napus* leaves in the wild type cultivars Westar and DH RV28 and also to provide data which would assist in the attainment of the optimal time to take samples from the growing plant in order to determine levels of FAS enzymes and fatty acid and lipid profiles when analysing transgenic lines.

In order to be able to analyse transgenic lines it is ideal to determine a developmental profile of the wild type plant so as to reduce the number of samples requiring analysis. Two of the enzymes assessed in this profile had transgene constructs transformed in to the *B.napus* cultivar Westar. These were βKR and ENR. KAS III was transformed into a double haploid line RV28. Double haploid lines are thought to have less variation between plants and so if plants are transformed with an antisense construct any effects caused by the introduction of a transgene can be more easily distinguished.

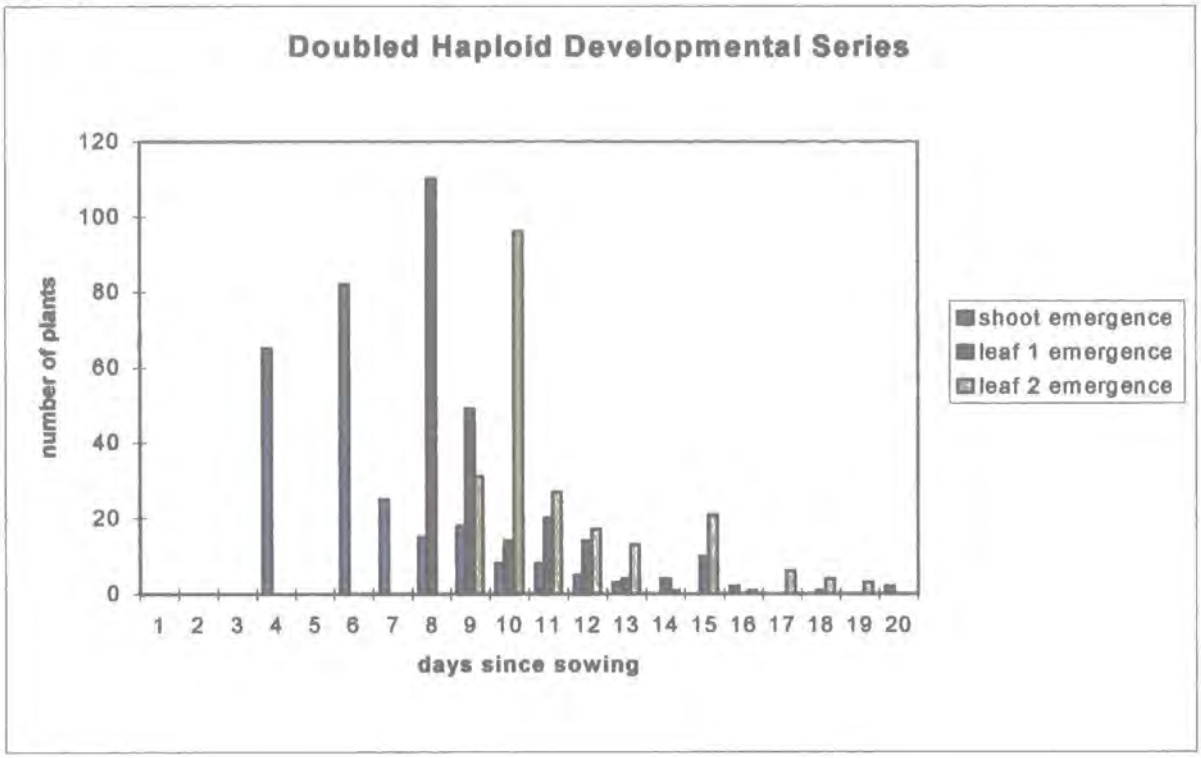
5.3.1 Growth of Plant Material and Preparation of Protein Extracts

In order to determine a preliminary developmental profile ELISAs were performed on ENR and βKR and Western blots performed on KAS III (due to the lack of a supply of soluble KAS III to coat to the wells of an ELISA plate). All protein assays were performed on the second

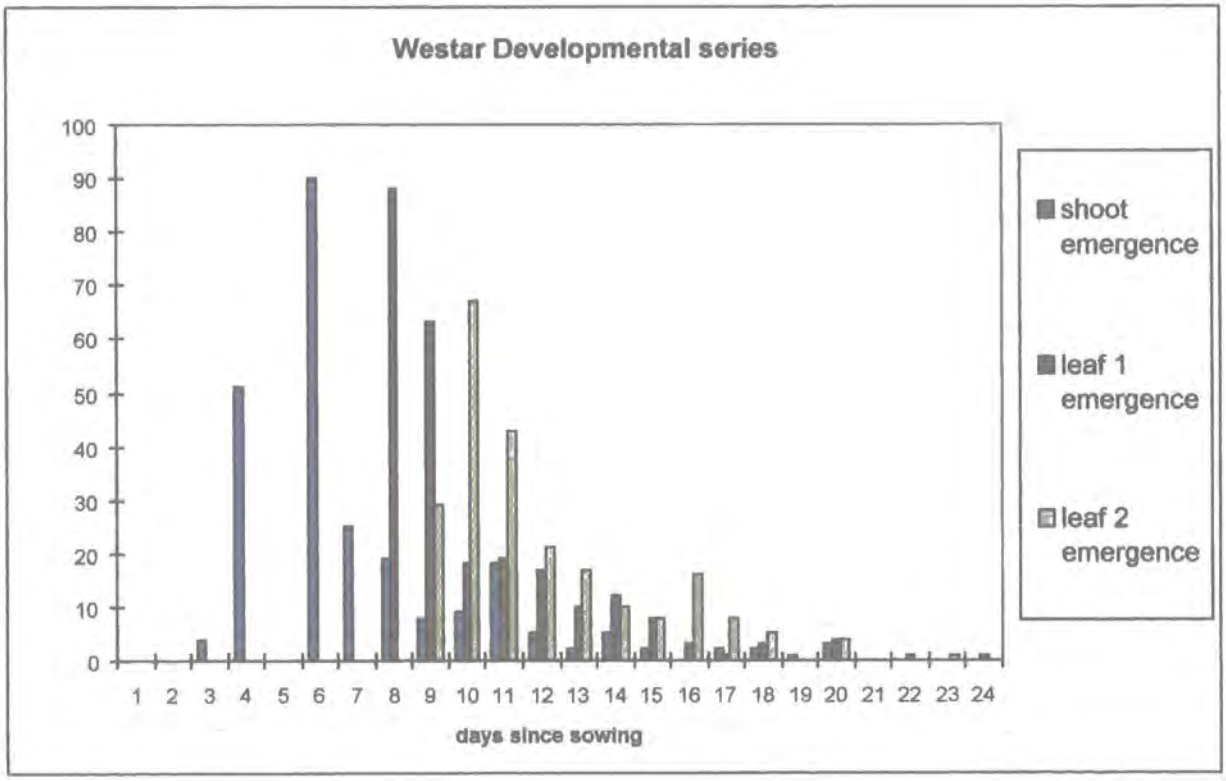
leaf which emerged from the seedling at ages ranging from 2 DAE to 14 DAE. The first leaf to develop was used in FAME and complex lipid analysis again at ages ranging from 2 DAE and 14 DAE. A total of 600 seeds (300 from each cultivar) were sown under the regime described in section 2.5.1 The plants were examined at the same time every day and any harvesting of the leaves was also performed at this time. The time of germination and the emergence of leaf 1 and leaf 2, which were used in the following analyses, were noted and subsequently the size of the leaf 1 and 2 taken once harvested by measuring the length of the midrib of the leaf. A high number of seed was sown for each cultivar so to obtain enough material from young leaves, and overall to provide enough material to perform all the separate assays. More than one leaf would be required to make any extract for two and three day old leaves and so three leaves were harvested for these two time points compared to only 1 leaf needed for the remaining ages. These samples were then used in an overall profile experiment described within this section.

Figure 5.15 shows the developmental series of the two cultivars, by noting when each plant had germinated and when leaf 1 and 2 emerged. The majority of both cultivars had germinated by day 8, although some seed did not germinate until later, the last seed noted (Westar) took 19 days to germinate. Leaf 1 was shown to emerge on average two days after emergence of the cotyledons, followed by leaf 2, two to three days later. Calculation of the mean length of leaf 1 and 2, at each specific age in both cultivars, showed that there is a steady increase in leaf length from day 2-14 (Figure 5.16). When the morphology of the two cultivars is compared there appear to be slight differences with cv. DH RV28 producing a more oblong

A


Doubled Haploid Developmental Series
number of plants
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
days since sowing
shoot emergence
leaf 1 emergence
leaf 2 emergence

B


Westar Developmental series
100
90
80
70
60
50
40
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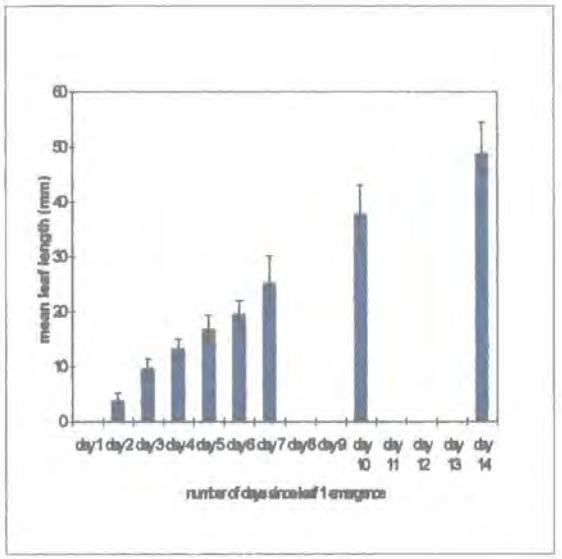
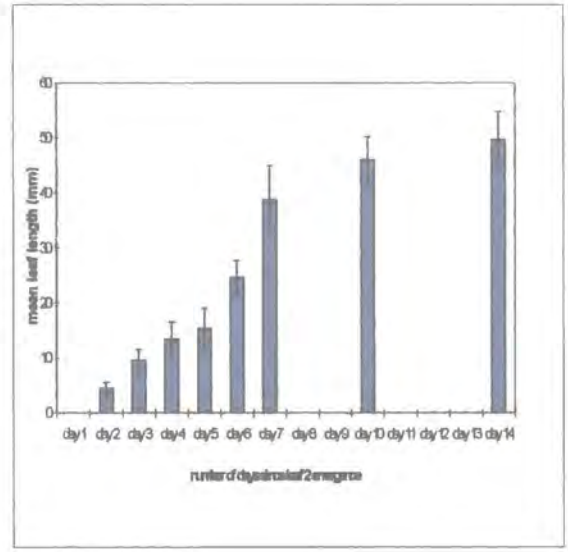
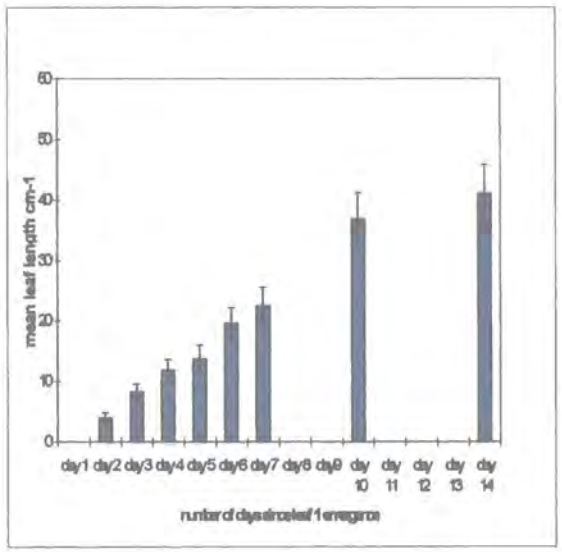
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
days since sowing
shoot emergence
leaf 1 emergence
leaf 2 emergence



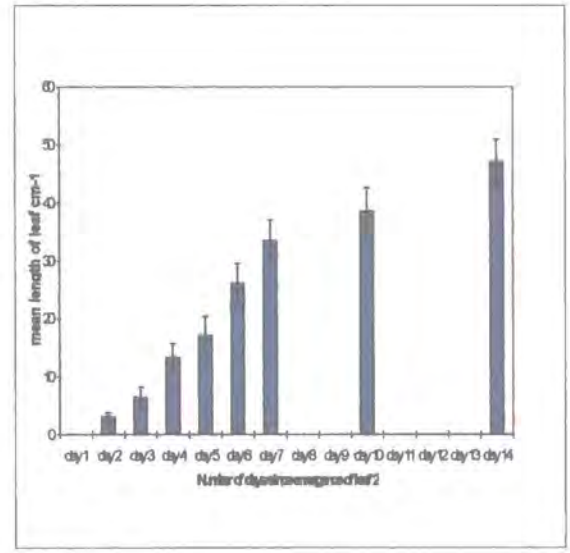
A - DH RV28 Leaf 1



B - DH RV28 Leaf 2



C - Westar Leaf 1



D - Westar Leaf 2

Figure 5.17 Morphology of wild type *Brassica napus* cv.DH RV28 leaves from Day 2 to Day 7 after emergence.

A to scale (approx. 0.5 × actual size) photograph of wild type *Brassica napus* cv. DH RV28 leaves, which shows that this cultivar has a slightly larger more rectangular morphology than compared to cv. Westar (Figure 5.18).

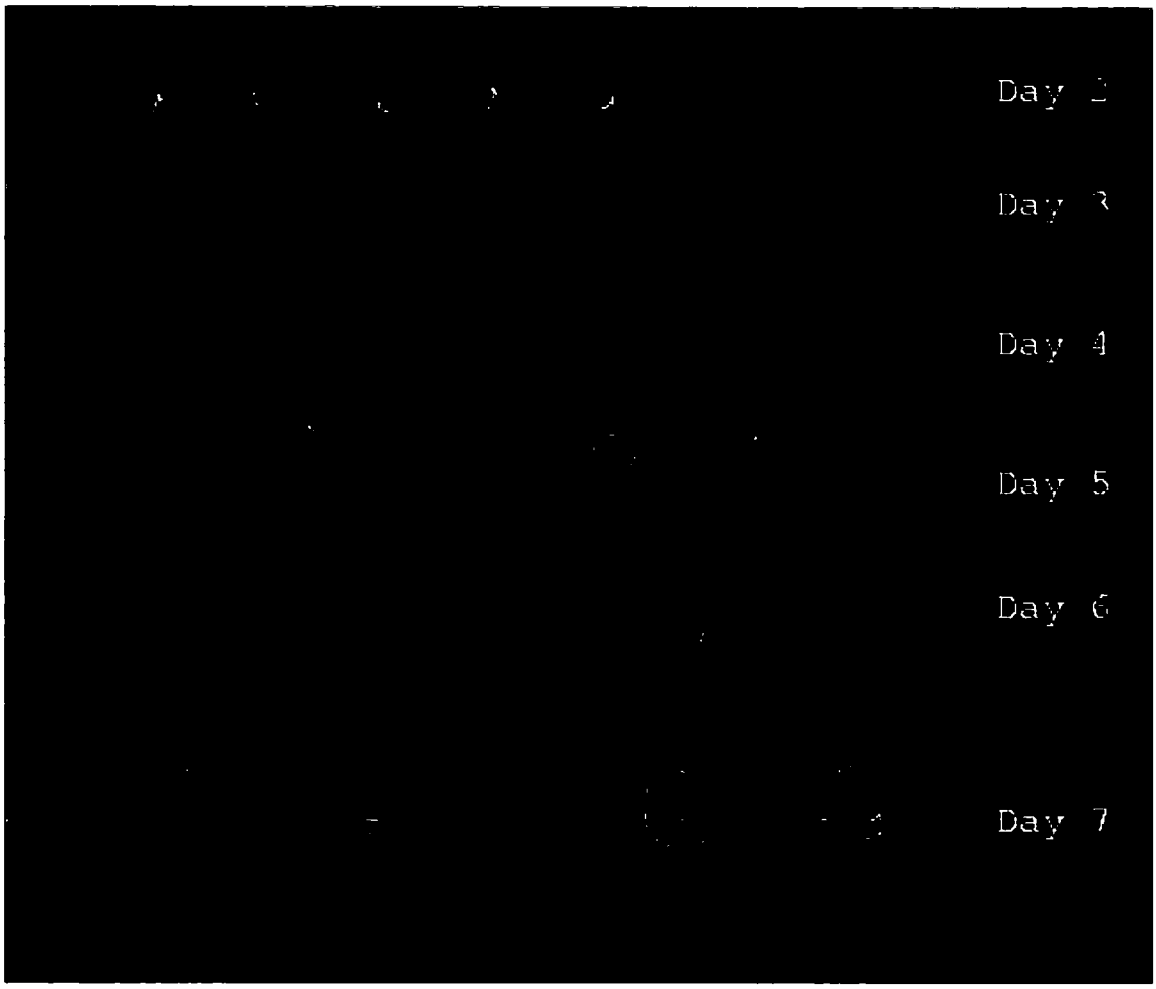


Figure 5.18 Morphology of *Brassica napus* cv. Westar Leaves from Day 2 to Day 7 after emergence.

A to scale (approx. 0.5 × actual size) photograph of *Brassica napus* cv. Westar leaves, which shows that this cultivar is slightly smaller rounder than compared to cv. DH RV28 (Figure 5.17).

leaf (Figure 5.17) compared to the rounder shape of Westar (Figure 5.18). This is in line with the mean length of cv. DH RV28 which is slightly longer than cv. Westar.

5.3.2 ELISA Assays for ENR and BKR Quantification

Protein samples were prepared as in section 2.8.1 for each individual leaf; the extracts were then pooled in order to have enough material to perform all the assays. The concentration of protein was determined by Bradford assay (section 2.8.2) using BSA as the standard and the samples aliquoted and stored at -80°C until required. The ELISA for ENR quantification was carried as previously described in section 2.8.4 and βKR quantification using an antigen inhibition ELISA assay was adapted from the development of the ENR ELISA. βKR protein was purified from *E.coli* overexpressing the rape βKR, its concentration calculated by amino acid analysis as described in section 2.8.12 and coated to the microtitre plate at a concentration of 1μg/ml. The primary antibody was a rabbit anti βKR polyclonal which was used at a 1 in 6,000 dilution as determined by direct ELISA assays, and the conjugate antibody was anti rabbit IgG linked to alkaline phosphatase (Sigma) at a dilution of 1 in 9,500. The ELISA was performed in an identical manner to that of ENR except that there was no need to denature the crude extract before performing the ELISA assay.

Analysis of the cv. DH RV28 line shows that the amount of ENR and βKR present in the leaf falls as the leaf matures (Figure 5.19). This cannot be seen for cv. Westar where approximately equal amounts are seen through out the entire leaf age range, with the exception of day 2 βKR, where levels can be seen to be three times higher than the other time points. As

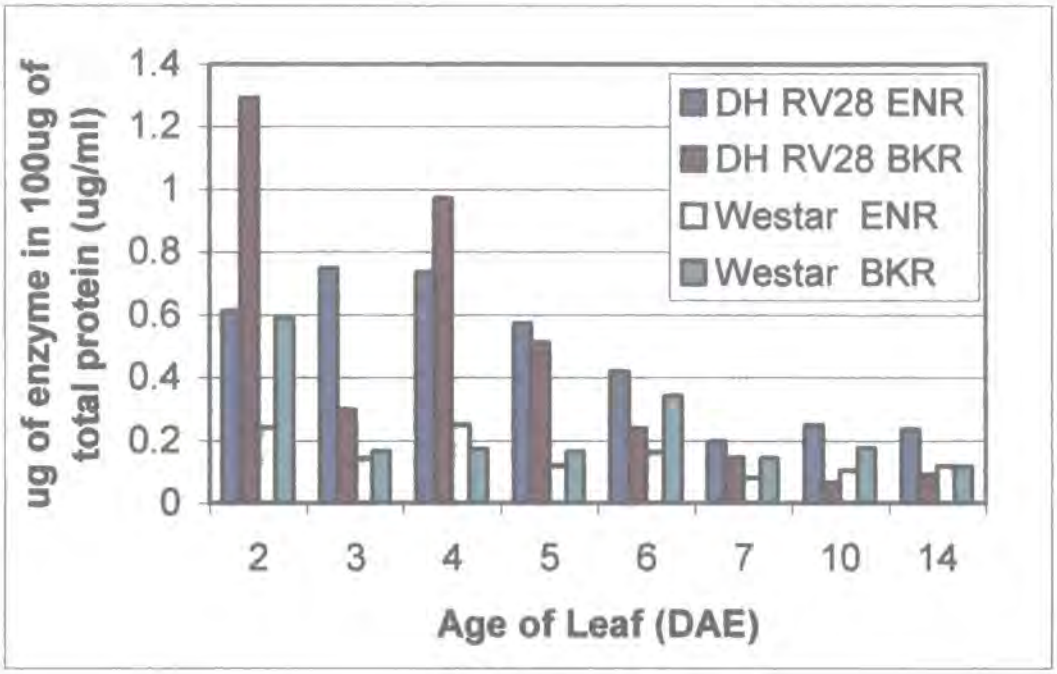


Figure 5.19 A comparison of enoyl-ACP reductase and β-ketoacyl-ACP reductase levels in the *Brassica napus* cultivars Westar and DH RV28.

The graph illustrates the levels of ENR and βKR of ten pooled leaves from each leaf age. The levels were compared using ELISA assays as described in the text (section 5.3.2). ENR in DH RV28 slowly decreases as the leaf ages as does the βKR for this line (day 3 is assumed to be an anomalous result). Analysis of the Westar cultivar proved disappointing as it appears that the protein samples used have degraded during the experiment, as only basal levels of ENR and βKR were generally seen, with the exception of a two days (βKR Day 2 and day 6) sampled where levels are higher. Although the graph shows a general trend where ENR and βKR levels gradually fall no rigid conclusions can be drawn.

the profiles for cv. Westar ENR and βKR both show a steady state low level of the two proteins. This could imply that degradation of the two proteins has occurred, as it was anticipated that a similar profile to cv. DH RV28 should have been seen.

5.3.3 Western Blot Analysis of KAS III

SDS/PAGE and Western blots were performed as described in sections 2.8.5 and 2.8.9. 30μg of total protein was loaded in duplicate for each leaf age except for Day 2 leaf (Westar only) where only 20μg could be loaded. After transfer the membrane was incubated in 5% M-PBS overnight to ensure sufficient blocking. A 1 in 1,000 dilution of the primary antibody was made up in 5% M-PBS and incubated with the membrane for 2 hours at room temperature. This was followed by excess washing in PBS-T before an incubation with a 1 in 40,000 dilution of goat anti–rabbit IgG in 5% M-PBS for two hours. The membrane was extensively washed with 3 changes of PBS-T for 1 hour to ensure all unbound antibody was removed. The Western blots were developed using the Pierce Supersignalling Kit and the bands that developed were quantified densiometrically.

The data showed that the KAS III antibody highlighted a 38kDa protein only in cv. Westar. Densiometrically the highest levels of KAS III are seen in Day 2 leaf and the levels fall by day 7 and at Day 10 the levels of KAS III are too low to be detected by the antibody (Figure 5.20a, Table 5.1). Several attempts were carried out on the double haploid developmental series of leaf extracts using a 1 in 1,000 dilution of the antibody, but it was not until a 1 in 500 dilution was used that the antibody successfully highlighted KAS III in double haploid lines (Figure

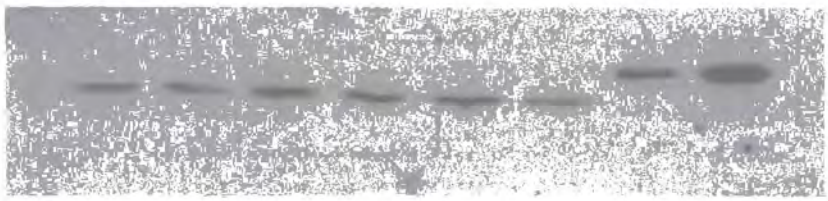
5.20b, Table 5.2). However, the higher concentration of antibody used also meant the detection of a 45kDa band (which is thought to be the KAS III pre-protein, prior to the removal of the transit peptide). A similar developmental pattern was observed with the double haploid line (only the 38kDa band was assessed densiometrically), where KAS III levels were highest in the youngest leaf ages and levels fell as the leaf aged. KAS III was undetectable at day 7 in this cultivar's case.

If KAS III levels in DH RV28 are compared to the respective ENR and βKR levels (Figure 5.21), the percentage of KAS III appears to be much lower. However as a different method for quantitation was used this may explain the observations. Further repetitions of these experiments, plus the development of KAS III ELISA in order to make the comparisons between all three enzymes fairer, are required. The development of a KAS III ELISA would also assist in seeing whether the results obtained using this technique agree with those of the Western blots.

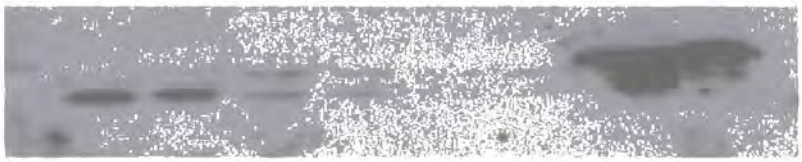
5.3.4 Fatty Acid Analysis of Leaf One of *Brassica napus* DH RV28

Each age of leaf 1 (Day 2 – Day 14) was prepared for fatty acid analysis by homogenising a whole leaf in the presence of 2ml of isopropanol. 1ml of the sample was taken in order to perform the fatty acid extraction as described in section 2.10.1. The fatty acid methyl esters were extracted by hexane and used directly in GLC analysis (section 2.10.2).

The analysis showed that total FAME levels rose as the leaf aged and then levels dropped significantly by day 14 when a μg/mg fresh tissue weight is compared (Figure 5.21). This is most likely due to the increase in leaf size, where in young leaf the abundance of fatty acid is higher as the cells do not contain large vacuoles that exist in more mature leaf cells. However there appears to be little difference in the FAME content of the two cultivars (Dr. P O'Hara personal communication).



1 2 3 4 5 6 7 8



(a) 9 10 11 12 13 14 15 16

17 18 19 20 21 22 23 24

(b) 25 26 27 28 29 30 31 32

cv. Westar Leaf Age	Amount of KAS III per 30μg of total protein (ng/30μg)	Average amount of KAS III per 30μg of total protein (ng/30μg)	Amount of KAS III in 100μg of total protein (ng/100μg)	Percentage of KAS III Protein in total protein (%)
Day 2	32/29	31	103	0.1
Day 3	28/24	26	87	0.08
Day 4	25/17	21	70	0.07
Day 5	18/17	18	60	0.06
Day 6	6 /2	4	13	0.01
Day 7	0.5/1	0.7	2	0

Table 5.1 Levels of KAS III in crude *Brassica napus* leaf cv. Westar extracts. This is calculated as ng/30μg loaded on to the gel. This is then converted into a percentage of total protein. As the leaf ages the percentage of KAS III in the total protein falls.

cv. DH RV28 Leaf Age	Amount of KAS III per 30μg of total protein (ng/30μg)	Average amount of KAS III per 30μg of total protein (ng/30μg)	Amount of KAS III in 100μg of total protein (ng/100μg)	Percentage of KAS III in total protein (%)
Day 2	22/25	23	76	0.07
Day 3	17/18	17	56	0.05
Day 4	12/16	14	46	0.04
Day 5	26 /10	18	60	0.06
Day 6	5/12	8	26	0.02
Day 7	Not detectable	-	-	-

Table 5.2 Levels of KAS III in a crude *Brassica napus* leaf cv. DH RV28 extracts. This is calculated as ng/30μg loaded on to the gel, measuring the 38kDa band only. This is then converted into a percentage of total protein. As the leaf ages the percentage of KAS III in the total protein falls with the exception of one sample at day 5 which raises the average level of KAS III in day 5 leaf to 0.062% of total protein, which is slightly higher than for day 4 leaf.

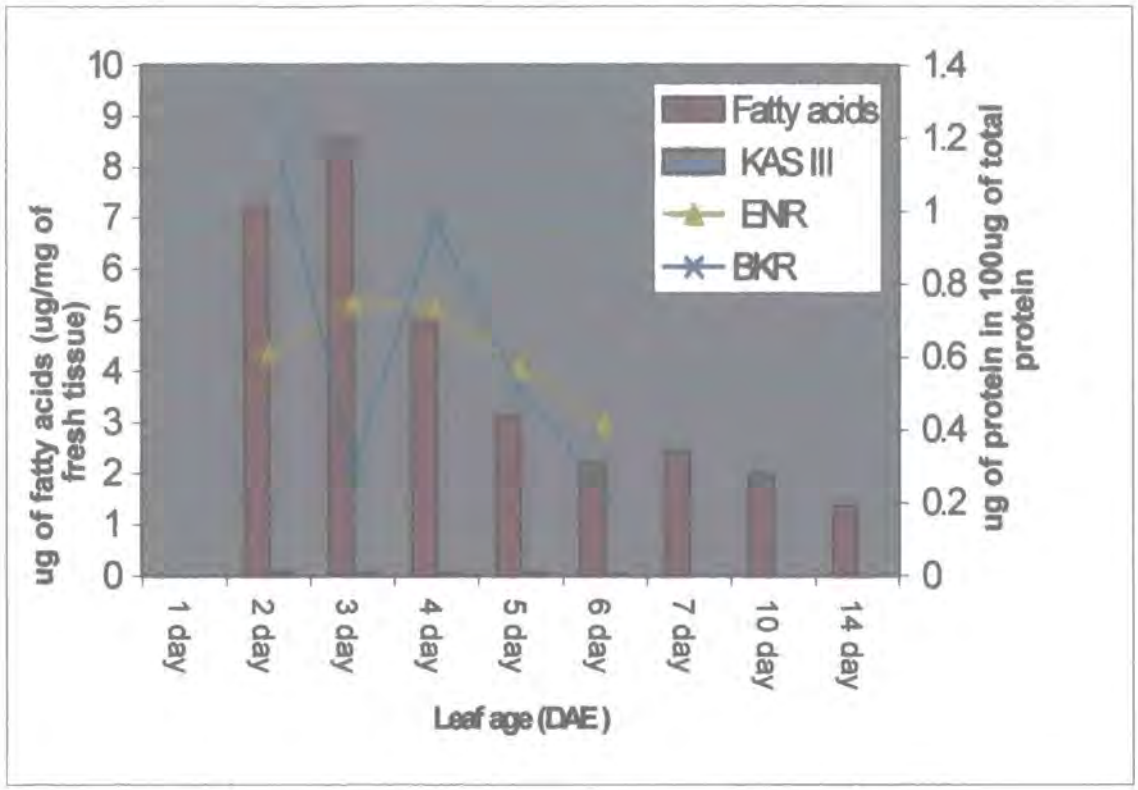


Figure 5.21 Comparison of levels of KAS III, ENR and βKR in *Brassica napus* cv DH RV28 against total fatty acids content for each leaf age. The protein levels are the determination of one reading from ten leaf extracts pooled together for each age.

The data shows that KAS III is present at a considerably lower level compared to the other two enzymes, being only 10% of ENR levels. can be seen to be slowly decreasing as the leaf ages. The results for ENR and βKR protein levels was obtained by quantitative ELISA, whereas KAS III was obtained from a quantitative Western blot. However direct comparison of KAS III levels to ENR and βKR protein levels is not possible due to the different quantitation methods used. Total fatty acid levels in the leaf drop as the leaf ages when a comparison of μg/mg fresh tissue weight is used.

5.4 Discussion

This chapter reports the development of a quantitative antigen inhibition ELISA assay for ENR. This technique is generally used as a tool for the detection of viruses and other pathogens and has been successfully adapted for use in transgenic *Brassica napus* lines. The sensitivity of the ELISA is crucial in observing any down regulation of the protein which may be observed in transgenic lines and the ELISA can be seen to be sensitive to 30ng/ml which is equivalent to 3ng of protein. Upon analysis of transgenic lines, it is sensitive enough to detect differences in ENR levels in transformants.

The initial difficulty in antibody/antigen recognition was overcome by denaturing the extract prior to antibody addition. Although an attempt has been made to explain this phenonomen by the use of a simple gel filtration experiment, results are inconclusive and can merely speculate as to the requirements of the denaturing step. The possibilities being the preference of the antibody for a denatured form of the protein, which is less likely as the antibody will recognise pure native ENR and also highlights ENR on a native Western blot (performed by author, data not shown). The second plausible explanation is that epitope sites on the plant protein are concealed by another molecular component which is removed on denaturation. Experiments during the development of the ELISA assay unveiled that this component could not be removed by dialysis or desalting via a spun column alone. It is removed however, by gel filtration experiments where the sample is loaded on and off the column slowly allowing a longer period for conformational change in the sample to occur. Also proteins are physically separated by size using this method and the component preventing recognition is removed.

Mechanical manipulation of the crude extract by high centrifugation and by the use of a microcon (30kDa cut off) to separate high and low molecular weight proteins revealed a number of characteristics concerning the features of this component. First it is tightly bound as it is not removed by dialysis or spun column alone. Second, it is likely that the protein is at least 10kDa and probable that it is at least 30kDa, as it was not removed by dialysis or Microcon treatment. Third it is probably not membrane bound, as high centrifugation to remove the membranes did not result in antibody/ENR recognition. It is possible that this is an observation of ENR interacting in a complex with other proteins, potentially other FAS components. Brief studies using crude gel filtrated *B.napus* protein samples where each fraction was incubated with ACP appeared to reveal little interaction when analysed by ELISA (data not shown). Therefore there is further scope to study this phenomenon and explore whether ACP is the molecular component concealing ENR epitopes in native crude extracts.

In comparison to the ENR ELISA no denaturing step was required in the competitive antigen inhibition βKR ELISA. However as actual epitope sites of a polyclonal antibody cannot be determined it may be that the βKR antibody recognises a site on βKR that is not involved in any interactions between different molecular components.

The ENR ELISA established levels for ENR in wild type *B.napus* cv. Westar leaf of 0.46μg/ml. It also determined a figure of 0.77μg/ml of ENR in cv. Westar seed, which upon conversion to the same units as used in the study by Slabas *et al.*, (1990) was equivalent to 1.2mg/100g of seed. This is lower than that of the Slabas study but their figures are determined on the *B.napus* cultivar Jet Neuf. The ELISA was also shown to successfully

enable direct comparison of wild type *B.napus* to transgenic ENR lines, where the Student's T-test showed the differences between the wild type and transformants to be statistically significantly different. In conclusion a reliable quantitative ELISA assay has been established that required careful consideration of sample preparation.

On surveyance of current literature, ELISAs are not reported as a commonly used tool to examine the effects of a transgene on a plant. This may be due to the necessary requirement of a good supply of pure antigen and/or the need for a sensitive antibody. Gendolff *et al.*, (1990) developed a quantitative ELISA to determine the levels of expression of chloramphenicol acetyl transferase (CAT) in transgenic tobacco. This reporter gene from *E.coli* is widely used for eukaryotic transformation systems, but in this method a monoclonal antibody was used and the levels of a protein not normally found in plants was being assessed. Other competitive ELISAs reports also use monoclonal antibodies (Albrecht *et al.*1993) which are much more complex and time consuming in their production. A report by Zhang *et al.*, (1997) has used a polyclonal antibody in a competitive ELISA to determine the concentration of acetaldehyde-protein adducts that accumulate in dry seeds during ageing. So this, along with the report in this chapter, demonstrates that a polyclonal antibody can be successfully used in facilitating accurate quantification of plant proteins in both seed and leaf material. Such reports may widen the scope and possibility of regularly incorporating this technique into analysis of transgenic plants.

This study has also moved towards the determination of a developmental profile of FAS enzymes in *B.napus* leaf cv. Westar and cv. DH RV28 along with fatty acid analysis. Leaves

from cv. DH RV28 were seen to have a slightly different morphology to that of cv. Westar, in that they were more rectangular. This explained the observation of a slightly longer leaf when measured. Both cultivars showed that the leaf increases in size in a steady proportional manner as it ages.

On comparison of the enzymes involved in the synthesis of fatty acids variation between the two cultivars was noted. No information regarding the fatty acid and complex lipid profile or the characteristics of FAS enzymes had been determined for cv. DH RV28 in leaf, and so examination of this cultivar was necessary as the antisense KAS III gene had been transformed into this line. Preliminary analysis was however hampered by the apparent degradation of the Westar samples where comparison of Westar to DH RV28 was not possible. The four separate ELISAs were performed on the same day and therefore precautions were taken to prevent such degradation occurring. Degradation was known to have occurred as previous results from ELISA assays with Westar had suggested that higher levels of ENR were present. Only repetition of this experiment with undegraded samples will reveal the differences of ENR and βKR levels in the two cultivars. ACCase activity levels were seen to be higher in the Westar samples than DH RV28 (Dr. A.J.White – personal communication) and the amounts of KAS III in Westar appear to be slightly higher than in DH RV28. As with ENR and βKR, the levels of KAS III fall as the leaf matures. A surprising result was seen with the Western blots of KAS III, where KAS III levels were suggested to be only 10% of ENR levels in DH RV28. However quantification comparisons between KAS III with ENR and βKR are difficult due to the different methods used so it is not possible to make direct comparisons of levels of these enzymes.

This study reveals that protein analysis should be performed on young leaves for maximum expression of the appropriate enzyme to be observed. This would then accentuate any differences in transgenic plants if expression was down regulated. Therefore it was decided that day 4 leaf should be used, as it was still relatively young yet large enough to obtain a good protein extract from.

The preliminary analysis of the enzymes KAS III, βKR and ENR showed there to be some differences in the profiles between the two cultivars, this however did not appear to affect the fatty acid content of either cultivar which were shown to be essentially identical. If the cultivars had severely differing levels of FAS components a change in the profile of the fatty acid content would be anticipated however this was not the case. Additionally there does not appear to be significant differences in their complex lipid profiles (Dr P' O'Hara – personal communication).

Studies by Rennie and Tanner (1991) of the fatty acid composition of oil from soybean leaves grown at extreme temperatures showed that unlike in *B.napus* seed (Deng and Scarth, 1998) and soybean seed, unsaturated fatty acids appear to be more concentrated at higher temperatures and saturated fatty acids present in greater concentrations at lower temperatures. The various cultivars of soybean tested did not fluctuate significantly in their fatty acid profiles intra-temperature. This implies that little difference in fatty acid and complex lipids would be seen in this study between the two cultivars of *B.napus* which were grown under identical conditions. This is confirmed by the observation of two identical fatty acid profiles

seen in the two cultivars used in this study. Therefore, it is likely that FAS components would have to be severely affected in the leaf before any effect of pathway end products would be seen (Sonnewald and Stitt, 1995).

5.4 Summary

- A sensitive quantitative ENR-ELISA was developed and used to determine ENR levels in wild type *Brassica napus* leaf and seed as well as used in to determine ENR levels in transgenic ENR lines.
- The ELISA required that crude extracts be denatured before use in the ELISA, as the antibody did not recognise ENR in native extracts.
- Attempts to characterise the component concealing the epitope binding sites for ENR/antibody recognition showed that it was tightly bound, at least 10kDa in size and not membrane associated.
- Gel filtration removed the component concealing the epitopes on the ENR to allow native extract/antibody interactions to occur.
- A standard profile of FAS components of *Brassica napus* leaf from cultivars Westar and DH RV28 was determined for use in deciding optimal sampling times for transgenic studies.

Chapter 6

Antisense KAS III *Brassica napus* Plants

6.1 Introduction

Naturally occurring antisense molecules have been reported in prokaryotes (Bovre and Szybalski, 1969, Mizuno *et al.*, 1984, Okamoto and Freundlich, 1986) and eukaryotes (Van Duin *et al.*, 1989, Dolfini *et al.*, 1993). The first observation of the transcription of two opposing DNA strands in the same region was reported by Bovre and Szybalski (1969) and they speculated that it was a means to regulate gene expression. In plants such an antisense molecule exits for α-amylase in barley (Rogers, 1988), although an *in vivo* role has yet to be proven, but both the sense and antisense transcripts have been shown to be developmentally regulated. Recently Cock *et al.*, (1997) demonstrated natural antisense transcripts of the S locus receptor kinase gene in *Brassica oleracea.*

The discovery of these naturally occurring molecules led to the development of antisense technology, where artificial antisense genes are used to manipulate cells or organisms in a target specific manner and so create mutant strains. It has proved an invaluable tool in unravelling metabolic pathways, where it has been used to determine the importance of individual enzymatic components in plants (Van der Krol, 1988) and commercially there has been vast interest in using this technique for crop improvement. (Grierson, 1994, Bourque, 1995).

The first engineered experiments in antisense were performed in mouse cells expressing complementary RNAs against thydimine kinase (Izant and Weintraub, 1984). However plants were the first multicellular eukaryotic organism transformed with a foreign antisense gene (Ecker and Davis, 1986). Using carrot cell cultures a chloramphenicol acetyltransferase (CAT) marker gene was electroporated into protoplasts and subsequent temporal synthesis of the CAT enzyme was seen. This was followed by the introduction of an antisense *cat* gene, which reduced CAT activity by up to 95% and consequently demonstrated the effectiveness of down regulating an enzyme by this method. Experiments by Rothstein *et al.* (1987) used the cauliflower mosaic virus 35S promoter in tobacco plants transformed with sense and antisense nopaline synthase (*nos*) genes.

The first wild type plant gene artificially regulated by an antisense RNA was the chalcone synthase (CHS) encoding gene from petunia and tobacco (Van der Krol, 1988). An antisense *chs*A gene was made using a 35S CaMV promoter and a nos 3' terminator fragment which provided a poly A adenylation signal to the antisense transcript. The antisense gene was introduced into both petunia and tobacco and the regenerated plants showed a high frequency of altered flower pigmentation. The petunia construct was also shown to alter flower pigmentation in tobacco, thus demonstrating that 100% homology with the sense gene was not required in order to cause an antisense effect.

How an antisense molecule causes a down regulatory effect is still open to debate (see Bourque, 1995), possible explanations include the formation of a duplex with the DNA

template that in turn blocks transcription. A second theory involves the formation of a duplex of the antisense RNA with the mRNA which either promotes rapid RNA degradation or prevents transportation of the mRNA. Antisense transcripts could also affect transcription by interfering with splicing of introns during mRNA maturation as observed with pectin methylesterase, introns were antisensed and subsequently were not excised (Tieman *et al.*, 1992). When the introns were placed in the sense orientation, they were excised. This points to post transcriptional regulation as a possible mechanism for antisense down regulation..

There are a variety of methods that have been developed to genetically transform plants with foreign genes by direct gene transfer into cells and protoplasts. These include electric discharge particle acceleration (Klien *et al.*, 1987 and Christou, 1990), which involves coating gold particles with the foreign gene and firing them with a finely tuned electric discharge apparatus into the chosen plant. DNA can be directly transferred into the plant protoplasts using microinjection techniques (Neuhaus and Spangenberg, 1990). Other successful methods include electroporation-induced DNA uptake into intact cells, silicon fibre-induced DNA uptake and LASER-mediated uptake (see Barcelo and Lazzeri, 1998).

The most commonly used method for modifying dicotlyedonous plants with an antisense construct is that of the tumour inducing bacteria *Agrobacterium tumefaciens.* This bacteria was first noted in 1907 (Smith and Townsend) for causing crown gall disease in plants. Further study of this pathogen showed that the crown gall tumour cells had the ability to synthesise nopalines in the absence of the bacterium. Nopalines are a group of amino acid derivatives that are not normally found in plant tissue and are utilised by the *Agrobacteria* in

order to grow (Braun and Wood, 1976, Schell and Van Montagu, 1977, Wullems *et al.*, 1981). Further research examining the genetic element or tumour inducing principle showed it to be transferred into the plant's genome. The tumour inducing principle is a plasmid borne trait (Ti plasmid) and is essential for virulence in crown gall disease (Van Larbreke *et al.*, 1974). A section of the Ti plasmid, the T-DNA, was shown to be physically inserted into the plant genome (Chilton *et al.* 1977). Genetic maps have been obtained which show that the T-DNA contains several oncogenic genes, as well as genes to synthesise the opines which are essential for the *Agrobacterium*'s growth (De Greve *et al.*, 1981). Disarmed Ti plasmids, which have lost their ability to cause cell proliferation, have been developed. These plasmids are capable of integrating foreign DNA into the plants genome in place of the nopaline or octopine synthase genes (Zambryski *et al.*, 1983, Bevan, 1984).

An antisense approach is described within this chapter aimed at understanding the importance of KAS III in the synthesis of fatty acids. Antisense plants which have KAS III levels down regulated may assist in determining whether KAS III has a regulatory role in *de novo* fatty acid synthesis. An *E.coli* mutant with severely down regulated levels of KAS III still grew (Jackowski *et al.*, 1989, Tsay *et al.*, 1992a), which suggests that the organism may have used an alternate step to prime fatty acid synthesis.

KAS III, however, has been shown to be the important priming step in fatty acid synthesis in plants and has significantly higher activity than acetyl-CoA:ACP transacylase (ACAT), the enzyme once proposed to prime fatty acid synthesis (Clough *et al.*, 1992). Regulation of metabolic pathways is often exerted at the beginning of the pathway (Stitt and Sonnewald,

1995) and Post-Beittenmiller *et al.*, (1991) suggest that such regulation may be imposed upon KAS III. The development of antisense KAS III transgenic lines can enable the examination of the effect KAS III down-regulation on fatty acid synthesis. Not only could these plants aid in understanding FAS regulation, they may also assist in revealing the significance of the role of ACAT in fatty acid synthesis. Prior to the discovery of KAS III, ACAT was considered to be a regulatory enzyme in FAS due to its lower activity than compared to other FAS enzymes (Shimakata and Stumpf, 1983a). After the discovery of KAS III, the absolute need for ACAT was obviated. Therefore antisense plants could also reveal whether there are alternative ways to synthesise fatty acids when the main priming enzyme is down regulated. Such alternate pathways include the synthesis of acetyl-ACP by ACAT for subsequent condensation with malonyl-ACP by KAS I.

An *Agrobacterium* based transformation is described where a KAS III antisense construct has been assembled under the control of the strong double 35S promoter and the CaMV poly A tail. The construct was transformed into the *Agrobacterium* strain C58 C3 (Dale *et al.*, 1989), by electroporation. This in turn was used to transform *B.napus*, by callus transformation (Moloney *et al.*, 1989). The callus was co-cultured with the *Agrobacterium* and transformants were then selected by transferring to media containing kanamycin. Confirmation of insert presence was determined by PCR analysis.

Arabidopsis thaliana was also transformed with the construct using vacuum infiltration (Bechtold *et al.*, 1993). The vacuum infiltration technique had previously not been used in the Durham laboratory and conditions were optimised during the creation of these transgenic

lines. Putative positives were selected by kanamycin and insert presence confirmed by PCR analysis.

Seed from 63 positive primary *B.napus* antisense KAS III transformants were collected along with seed from 10 transformed *Arabidopsis* lines. Seeds from 16 *B.napus* lines were examined for total fatty acid content by FAME analysis. From the data obtained five of these lines were then grown and analysed for copy number, a quantitative ENR ELISA assay was performed on day 4 leaf and KAS III levels examined by Western blots. The above is described along with analysis of transgenic ENR lines which had become available within the Durham Laboratory during the course of this study. The examination of two FAS components, one which primes the initial fatty acid synthesis and a second which is involved at the end of the cycle of synthesis, means that comparison of phenotypes and analysis of any pleiotropic effects can be used to determine FAS phenotypes in transgenic lines.

6.2 Results

6.2.1 Construction of Antisense KAS III Vector

The cloning strategy for the construction of the antisense KAS III with a double 35S promoter and a poly A tail with the selectable NPT II marker gene can be seen in Figure 6.1 a and b. The whole open reading frame (1215bp) was placed in the construct in the antisense orientation.

6.2.1.1 Preparation of Antisense KAS III Fragment: Subcloning into Plasmid pJKD1.1

A *Hind* III site was engineered to be positioned at the 3' end of the KAS III ORF and a *Sma* I site at the 5' end using primers designed with these sites incorporated in the following PCR. The KAS III ORF could then be ligated correctly in the antisense orientation into plasmid JKD1.1 at its multiple cloning site. Plasmid JKD1.1 contains a double 35S promoter and a 35S poly A terminator (Figure 6.1a). The fragment was produced by PCR, using 10 pmoles of primer HCNAP2 (*Sma* I site) 5 ' TCC CCC GGG ATG GCG AAT GCA TCT TTC 3' and Primer HC35S1 (*Hind* III site) 5' CCC AAG CTT TCA TCT CCA CCT GAT AAT TGC 3' and was subjected to the following PCR cycling conditions using 20ng of pKAS13 as the template DNA:

1 cycle	94°C	4 minutes
25 cycles	94°C	30 seconds
	59°C	30 seconds
	74°C	2 minutes
1 cycle	74°C	10 minutes

The PCR product was purified directly from the reaction using Promega's Wizard Purification Kit. The purified sample was digested with 10 units of *Sma* I and *Hind* III in the presence of restriction buffer A (Boehrhinger Mannheim) in a final volume of 30µl. The digested sample was examined on a 1% agarose gel and the fragment purified by Qiaex gel purification (Qiagen).

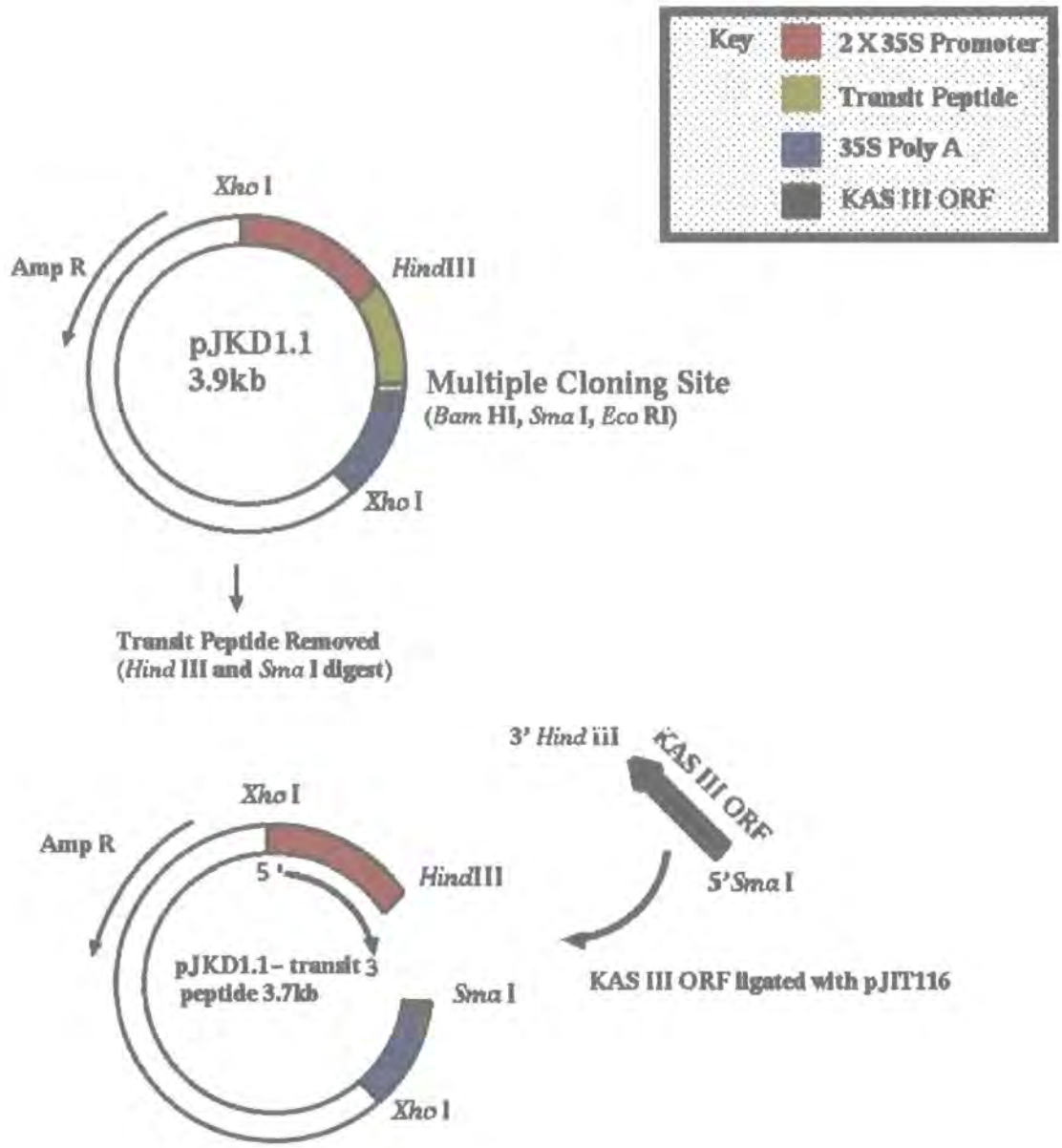


Figure 6.1a Cloning strategy for the KAS III ORF in the antisense orientation into pJKD1.1, a derivative of pJIT116. The antisense KAS III ORF is constitutively expressed by the strong double 35S promoter (Kay *et al.*, 1987) and terminated by the CaMV Poly A tail. This cassette can be excised from the plasmid using *Xho* I.

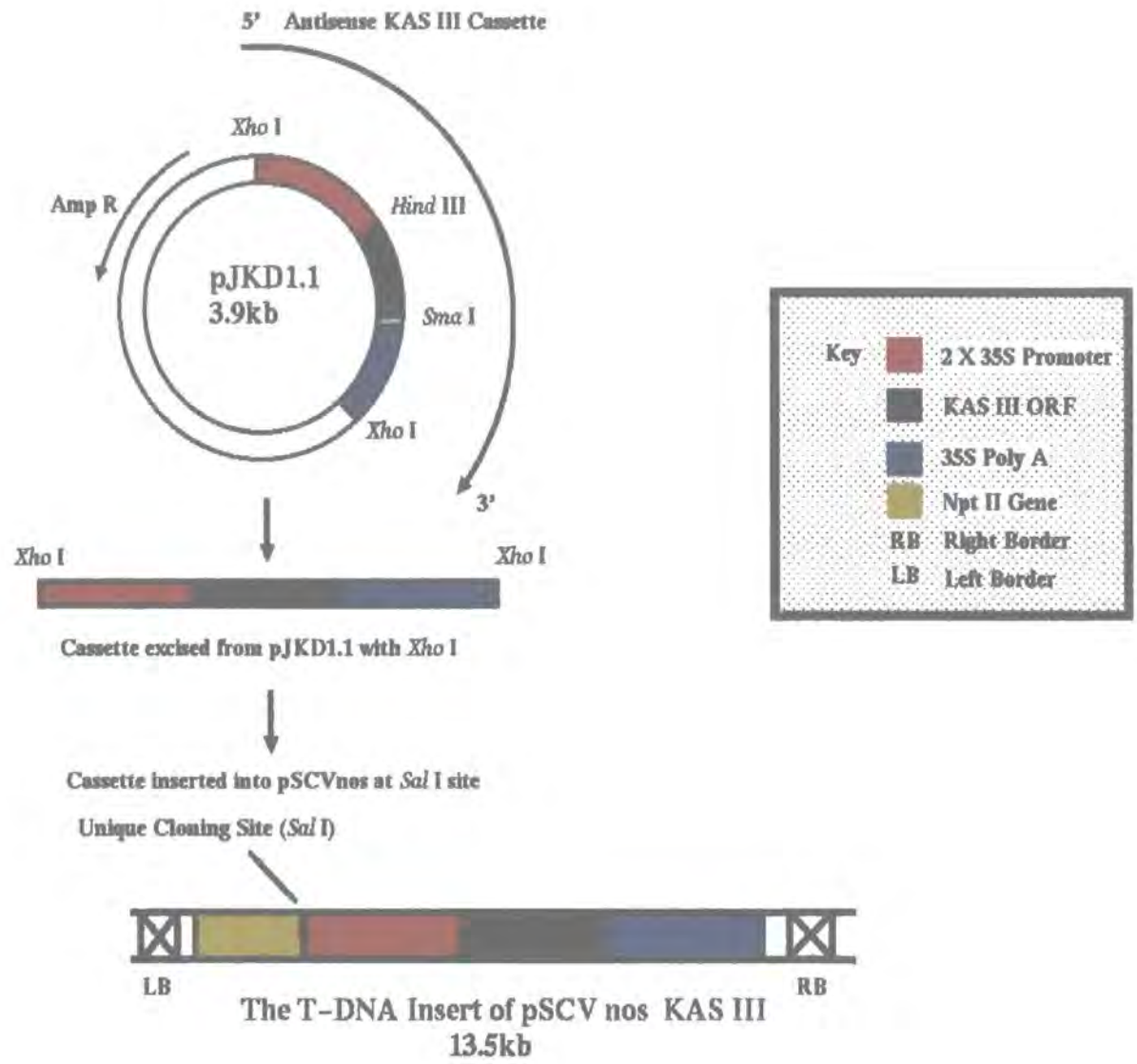


Figure 6.1b The cassette from pJKD1.1 containing the antisense KAS III cDNA is excised from the plasmid and ligated into the *Sal* I site of pSCVnos (courtesy of Biogemma UK), as described in the text section 6.2.1.2.

pJKD1.1 was digested with *Hind* III and *Sma* I to remove a 200bp transit peptide, which had been previously engineered into this plasmid. The restricted plasmid was purified from a 1% low melting point agarose gel using the Qiaex II gel extraction kit (Qiagen). 50ng of KAS III fragment was incubated with 25ng of pJKD1.1, 10 units of DNA ligase (Promega) and 1 × ligation Buffer in a final volume of 10μl at 15°C overnight. 5μl of the ligation was transformed into *E.coli* DH5α cells. Transformants were isolated on LB-Amp 50 plates; individual colonies were selected at random and used to inoculate 5ml LB-Media cultures containing 50μg/ml ampicillin to grow up sufficient plasmid for use in cloning into pSCVnos. The plasmid was isolated and digested with *Hind* III and *Sma* I to check for the presence of the KAS III fragment.

6.2.1.2 Insertion of KAS III Cassette from pJKD1.1 into pSCVnos

A successfully ligated KAS III pJKD1.1 plasmid created a cassette containing the antisense KAS III open reading frame cloned between a strong double 35S promoter and a 35S poly A tail, which would terminate transcription. Confirmation of plasmids containing this were obtained by restriction digests with *Hind* III/*Sma* I to release the 1215bp KAS III fragment and 3.7kb vector. A second restriction digests was performed using *Xho* I to yield bands 2.8kb (the antisense cassette) and the remaining vector band at 2.1kb. This whole cassette was then isolated by digesting 200ng of the plasmid with *Xho* I. The cassette was subsequently purified using the Qiaex gel extraction kit after electrophoresis on a 1% agarose gel.

500ng of pSCVnos (provided by Biogemma UK and utilised with their permission) was digested with 10 units of *Sal* I, and purified with the Qiaex DNA purification kit. 50ng of KAS III cassette was placed in a ligation reaction with 25ng of restricted pSCVnos, 10 units of DNA ligase and 1 × DNA ligase Buffer (Promega) in a final volume of 10µl at 15°C overnight (Figure 6.1b). 5µl of the ligation was transformed into competent *E.coli* DH5α and transformed cells selected by plating 100µl aliquots on LB-Amp 50 plates overnight at 37°C. Individual colonies were selected at random and used to inoculate 5ml LB media cultures containing 50µg/ml ampicillin. After isolation, the plasmids were digested with *Sma* I to determine insert presence and orientation by correctly yielding a band of 2.8kB (the cassette) and a vector band of 10.7kb. Also pSCVnos KAS III was digested with *Sma* I and *Hind* III to give 3 bands of the following expected sizes 11.5kb, 1.2kb and 0.8kb. The plasmids shown to contain the KAS III cassette in the correct orientation were used to transform the *Agrobacterium* strain C58 C3 (Dale *et al.*,1989). After transformation the cells were grown at 28°C for 3 days in LB media containing 25µg/ml nalidixic acid and 30µg/ml streptomycin. Colonies were then selected at random and checked for insert presence by PCR analysis with primers HC35S1 and HCNAP2 as described in section 2.7.4 The PCR product produced was subsequently sequenced to confirm its status as a KAS III cDNA. These two methods demonstrated that the antisense construct had been successfully transformed into *Agrobacterium* and could be transformed into plants.

6.3 Creation of Antisense KAS III *Brassica napus* and *Arabidopsis thaliana*

6.3.1 Transformation into *Brassica napus*

All transformations and plant tissue culture techniques on *B.napus* were performed by the author using the facilities at Biogemma UK, Cambridge.

Transformation was essentially the as described by Moloney *et al.* (1989) and the method is described in section 2.11.1. 1000 separate transformation events were performed. The plants derived from the calli were transferred to sterile peat jiffys and allowed to grow for two weeks and then analysed for construct presence by PCR (section 6.3.2). Positive plants were then transferred to larger pots and allowed to grow to maturity and set seed.

6.3.2 PCR Analysis to determine KAS III Construct Presence in *Brassica napus*

To determine T-DNA presence a PCR was performed using primers designed to the kanamycin (*kan*) and β-lactomase (*bla*) genes of pSCVnos KAS III. This would yield PCR products of 240bp and 352bp respectively (Figure 6.2). If the two products were produced, the reaction was repeated two weeks later to allow any *Agrobacterium* that may have been present to die, ensuring that if the PCR products had been formed by using the *Agrobacterium* DNA as a template, that upon repetition, any PCR products would likely be due to insertion of the cassette into the plant's genome. If two bands were seen on repetition of the PCR it was

assumed that the integration of the T-DNA had continued beyond the left border (Martineau *et al.*, 1995). Integration beyond the left border would allow the amplification of the *bla* gene which is situated upstream of the left border. The 240bp product alone indicated that integration had occurred successfully and no products indicated an untransformed plant. Crude DNA preps were prepared as described in section 2.7.1. 2.5µl of each DNA extract was added to a PCR reaction containing 1 × Perkin Elmer Buffer II, 2mM $MgCl_2$, 0.2mM dNTPs and 1 unit of LD Taq polymerase (selected for its high purity). Use of Taq DNA polymerase can give false positives due to small contaminants present in the solution. 20 pmoles of each of the following four primers were added to the reaction:

Kanamycin I	5'	CGC AGG TTC TCC GGC CGC TTG GGT GG	3'
Kanamycin II	5'	AGC AGC CAG TCC CTT CCC GCT TCA G	3'
Bla I	5'	TGG GAA CCG GAG CTC AAT GA	3'
Bla II	5'	TCC ATA GTT GCC TGA CTC CCC G	3'

The reaction mixture was made to a final volume of 50µl and subjected to the following PCR cycling conditions:

1 cycle	-	94°C	3 minutes
30 cycles	-	95°C	30 seconds
		55°C	30 seconds
		73°C	1 minute
1 cycle	-	73°C	5 minutes

10µl of the PCR reaction was examined on a 2% agarose gel. From these transformations, a total of 63 regenerated plants contained the construct, with 19 shown to have been transformed with integration beyond the left border of the T-DNA.

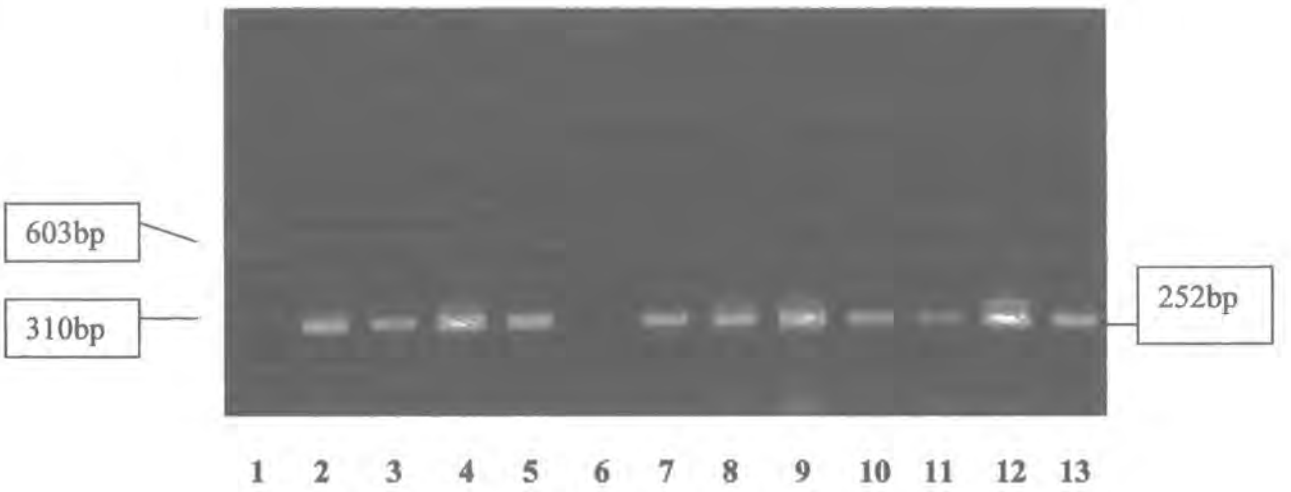


Figure 6.2 A 1% Agarose DNA Gel of PCR products from *Brassica napus* KAS III transformants. Primers designed to the NPT II gene present in the T-DNA produce a 240bp PCR product. A second set of primers was used to determine the presence of the β-lactomase gene which is also present in the construct and will yield a PCR product of 352bp.

Seven lines have T-DNA only inserts, 4 lines have integration beyond the left border and one is untransformed.

Lane 1	ϕ174 Markers	Lane 2	Line 104.35
Lane 3	Line 103.25	Lane 4	Line 103.29
Lane 5	Line 103.68	Lane 6	Line 104.21
Lane 7	Line 104.34	Lane 8	Line 104.39
Lane 9	Line 104.63	Lane 10	Line 104.31
Lane 11	Line 104.74	Lane 12	Line 104.17
Lane 13	Line 103.77		

6.3.3 Transformation into *Arabidopsis thaliana*

Transformation of the *B.napus* antisense KAS III pSCVnos construct was also carried out in *Arabidopsis thaliana* plants. Transformation of *Arabidopsis* with antisense KAS III would provide lines which could then be analysed alongside *B.napus*. Time constraints did not allow further analysis of these plants once made. However optimisation of the conditions required for this technique such as vacuum infiltration time, vacuum pressure, subsequent selection of transformants and growth conditions was necessary for other studies within the laboratory and this section describes the steps taken to achieve this.

This method is based on that of Bechtold *et al.*, (1993). An *Agrobacterium* suspension containing the antisense KAS III pSCVnos construct was prepared by plating the strain out on a LB-Agar plate containing 25μg/ml nalidixic acid and 70μg/ml gentamycin. The plate was incubated at 28°C overnight and a colony from this plate used to inoculate a 50ml culture of LB media containing 25μg/ml nalidixic acid and 70μg/ml gentamycin. This culture was grown overnight at 28°C and used to seed a 400ml culture of LB media containing 25μg/ml nalidixic acid and 70μg/ml gentamycin. The culture was grown for 2 days at 28°C and the cells were harvested by centrifugation at 5000 × g for 10 minutes at room temperature, once an OD_{600} of 2.0 was obtained. The cells were resuspended in approximately 1.2 litres of infiltration media to give an OD_{600} of 0.8. The suspension was kept at room temperature until required.

Approximately 250ml of the *Agrobacterium* suspension was prepared as described in section 2.11.3 in a 1 litre beaker. The beaker was placed in large dessicator and a pot of prepared

Arabidopsis plants (see section 2.5.2) were inverted into the suspension ensuring that all the plants were submerged. The dessicator was then closed and a vacuum applied using a diaphragm vacuum pump at various set pressures for a variety of time courses (Table 6.1). The vacuum was quickly released to allow the culture to infiltrate the plant. After this treatment, the plant was removed from the beaker and placed in horizontal position on a tray, which was then loosely covered with a translucent plastic bag. The treated plants were placed in a growth chamber with illumination and allowed to recover. After 24 hours the plants were placed upright and grown under a long day regime until they had set seed. Once the seed was mature it was collected and allowed to dry in a dessicator for 2-3 days and thereafter stored at 4°C.

6.3.4 Selection of Transformed *Arabidopsis* Lines

Putative transformants were selected using MS media/agar plates containing 40μg/ml of kanamycin as described in section 2.11.3. Within 10 days of sowing putative positives could be identified (Figure 6.3). Putative positives had a strong root system and more leaves. These plants were transferred to sterile peat pellet jiffys contained within Magenta pots and allowed to grow at 25°C in constant illumination until the plantlet had grown to fill the pot. The plantlet was then transferred to soil in a plant growth room with a long day regime. For the first 24 hours the plantlet was covered in a transparent plastic pot to allow it to acclimatise to the new growth conditions. A leaf sample was taken from the plant in order to confirm the presence of the construct via PCR (section 6.3.5). Seed from these lines were collected and stored as previously described in this section.

LENGTH OF TIME IN WHICH VACUUM WAS HELD (MINS)	PRESSURE EXERTED DURING INFILTRATION (IN. HG)	NUMBER OF TRANSFORMANTS OBTAINED
1 × 30	22	0
3 × 10	20	0
3 ×5	20	0
3 × 10	25	1
4 × 5	29	3
2 × 10	29	1
2 × 7	29	9
1 × 10	29	0

Table 6.1 Time course treatment of *Arabidopsis thaliana* and number of transformants obtained. A total of 15 transformants (10 KAS III pSCVnos and 5 pSCVnos only) lines were obtained.

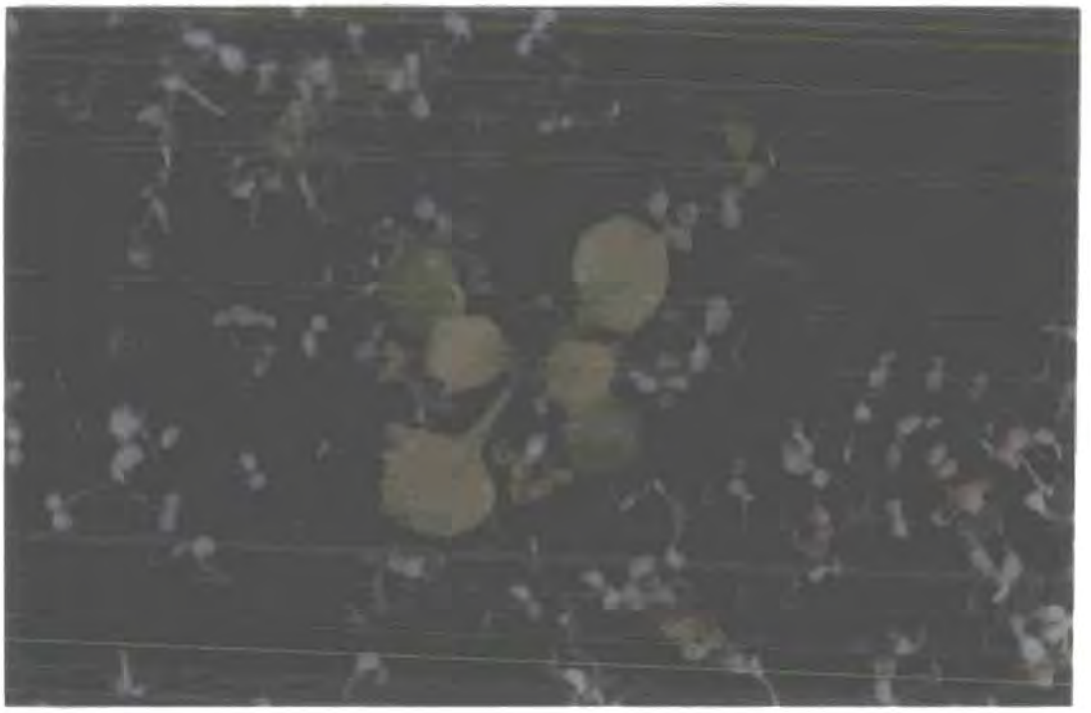


Figure 6.3 Putative antisense KAS III transformant. The *Arabidopsis* seed derived from vacuum infiltration experiments is sterilised and plated on MS media containing 40μg/ml kanamycin. The antibiotic will allow any plants containing the construct to grow, untransformed seeds germinate but the cotyledons become bleached and do not grow.

6.3.5 PCR Analysis to determine KAS III Construct Presence in *Arabidopsis thaliana*

A PCR technique was adapted from Klimyuk *et al.* (1993) and used to determine construct presence in treated *Arabidopsis* plants. Two sets of primers were used in the PCR, one set designed to the kanamycin gene and a second set designed to the Arabidopsis Δ-12 desaturase gene (BN9) for use as a control to confirm that the reaction has worked. The kanamycin PCR product is 252bp and the BN9 PCR product is 800bp (Figure 6.4). The BN9 PCR product should appear in all PCR reactions containing an *Arabidopsis* sample as this is a control PCR to check that the PCR reaction is occurring successfully. The kanamycin PCR product will only appear in samples where the line has been successfully transformed.

A rosette leaf was taken from each putative positive and 40µl of 0.25M NaOH added to the sample. The sample was incubated in a boiling water bath for 30 seconds and subsequently neutralised with 40µl of 0.25M HCl. 20µl of 0.5M Tris-HCl pH 8.0, 0.25% Nonidet P-40 (Sigma) was added and the sample was boiled for a further two minutes. A small sample of the tissue was then added to a PCR reaction containing 2mM $MgCl_2$, 0.2mM dNTPs, 20pmole of the following four primers in a final volume of 50µl:

Kanamycin I 5' CGC AGG TTC TCC GGC CGC TTG GGT GG 3'
Kanamycin 2 5' AGC AGC CAG TCC CTT CCC GCT TCA G 3'
BN9 Forward 5' TGA AGA GAA TCG CCA CGG TGA 3'
BN9 Reverse 5' AGC CTC TGA GGC ACG GAG GA 3'

Upon the addition of 1 unit of Taq DNA polymerase to each sample the reaction was subjected to the following PCR conditions:

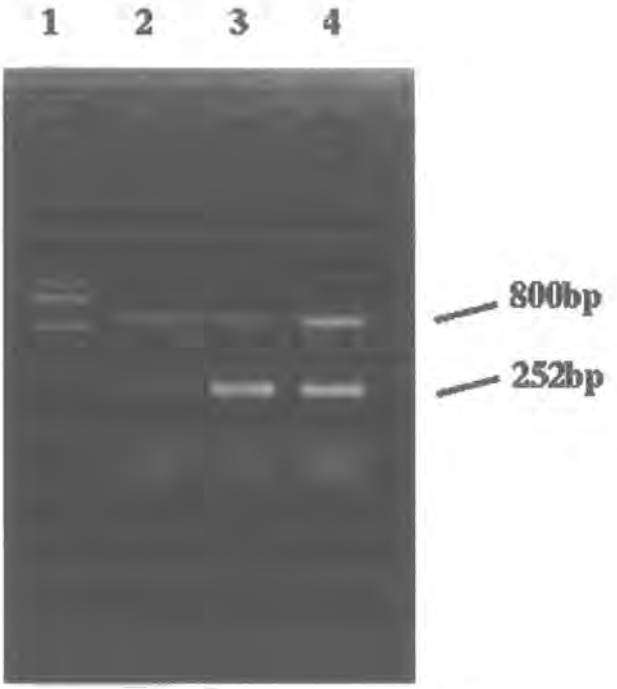


Figure 6.4 Alkali treated PCR of *Arabidopsis* putative transformants (Klimyuk *et al.*, 1993).

Two PCR products were expected. The first product is of 800bp produced by amplification of a section of the *Arabidopsis* Δ12 desaturase gene. This should always be produced as it is a control PCR to check that the reaction has worked. The second product of 252bp will only be produced if the T-DNA has been inserted into the genome of the plant. All three putative transformants here were shown to contain the T-DNA insert.

Lane 1 - ϕ 174 Markers

Lane 2-4 *Arabidopisis* transformants

1 cycle	94°C	4 minutes
30 cycles	95°C	30 seconds
	55°C	30 seconds
	72°C	1 minute
1 cycle	72°C	10 minutes

This analysis demonstrated that 10 lines contained an antisense KAS III pSCVnos construct and 5 lines were obtained which contained a pSCVnos construct only. It was found that more successful transformations occurred when plants received short vacuum bursts of 5-7 minute intervals for periods of no longer than 20 minutes, rather than longer uninterrupted periods of 30minutes (see table 6.1). During the release of the vacuum, the culture can infiltrate the plant's leaves due to the extra space formed by the removal of the air from the intracellular spaces of the leaf and if this action is repeated a number of times during the suggested time course more *Agrobacterium* has the opportunity to infiltrate the plant. Longer vacuum periods with no breaks appeared to exert more stress on the whole plant and have a more destructive effect upon the leaves which tended to die back quickly after treatment.

6.4 Transgenic *Brassica napus* ENR Lines

During the course of this study transgenic ENR *B.napus* plants became available. Four ENR constructs (Figure 6.5) made in the laboratory by Dr. C. Brough were transformed into *B.napus* cv. Westar (by Biogemma UK, Cambridge). Two constructs contained an ENR cDNA in the antisense orientation. These constructs differ in that one was ligated in the identical orientation to the NPT II marker gene (106) and the other opposed this (106 R). A further two constructs contained ENR cDNA in the sense orientation and again one construct

was in the identical orientation to the NPT II marker gene (107) and the other opposed this (107 R).

Eight of these lines were taken at random and FAME analysis performed upon T1 seed and ELISA assays performed on T1 plants. Three of the sense lines were then analysed in a duplicate study alongside KAS III antisense lines.

6.5 Analysis of Transgenic *Brassica napus* Lines

6.5.1 FAME Analysis of T1 Seed from Antisensed KAS III *Brassica napus* Lines

Seed was collected from all 63 positive primary transformants. To rapidly select lines for further analysis, 16 lines from KAS III primary transformants were selected at random and FAME analysis carried out as described in section 2.10.1 using seeds which had been allowed to imbibe water overnight. This treatment eased the removal of the testa (previous work within the laboratory has proved that this treatment does not affect the fatty acid content of the seed), fatty acids from three sets of two seeds from each line were extracted and the average total fatty acid content determined for each line. Eight lines of ENR were also assessed in this manner. Figure 6.6 compares the average contents of all lines analysed against wild type DH RV28 and figure 6.7 compares the average contents of the ENR constructs against cv. Westar. Five KAS III antisense lines were chosen from those that had been subjected to FAME analysis for further examination. Two lines which had severely down regulated levels of fatty acids were selected (104.34 and 104.31) along with two lines with medium levels of fatty acids (104.57 and 103.85) and one line which did not appear to have had its fatty acid levels

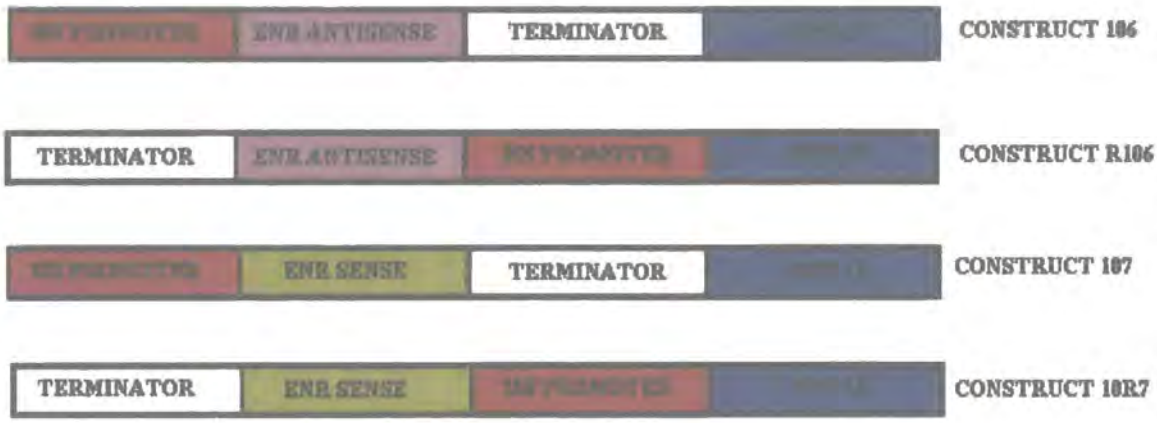


Figure 6.5 Enoyl-ACP Reductase Transgene constructs.

Four ENR transgene constructs, all contained within pSCVnos, were made by Dr. C. Brough and transformed into *Brassica napus* cv. Westar (at Biogemma UK). Two constructs contained ENR in the antisense orientation, whilst two contained ENR in the sense orientation. The two types of constructs only differed in their orientation to the NPT II marker gene.

affected by the antisense construct's presence (104.35). Three sense lines were taken from ENR transgenic lines examined (107.35, 107.R3 and 107.17) in order to compare the two different types transgenic plants and also examine the effect a sense FAS construct may have on *B.napus*. This was performed in order to determine what differences and similarities appeared between the two transgenic FAS plants and determining overall FAS phenotypes for down regulation of the two enzymes. On comparison of the two wild types cv. DH RV28 and cv. Westar it was seen that Westar on average contains 1600μg of total fatty acid per seed compared to only on average 1150μg per seed for DH RV28. This could link with the observation of lower ACCase activity and lower overall levels of KAS III observed previously in DH RV28 leaf (section 5.4).

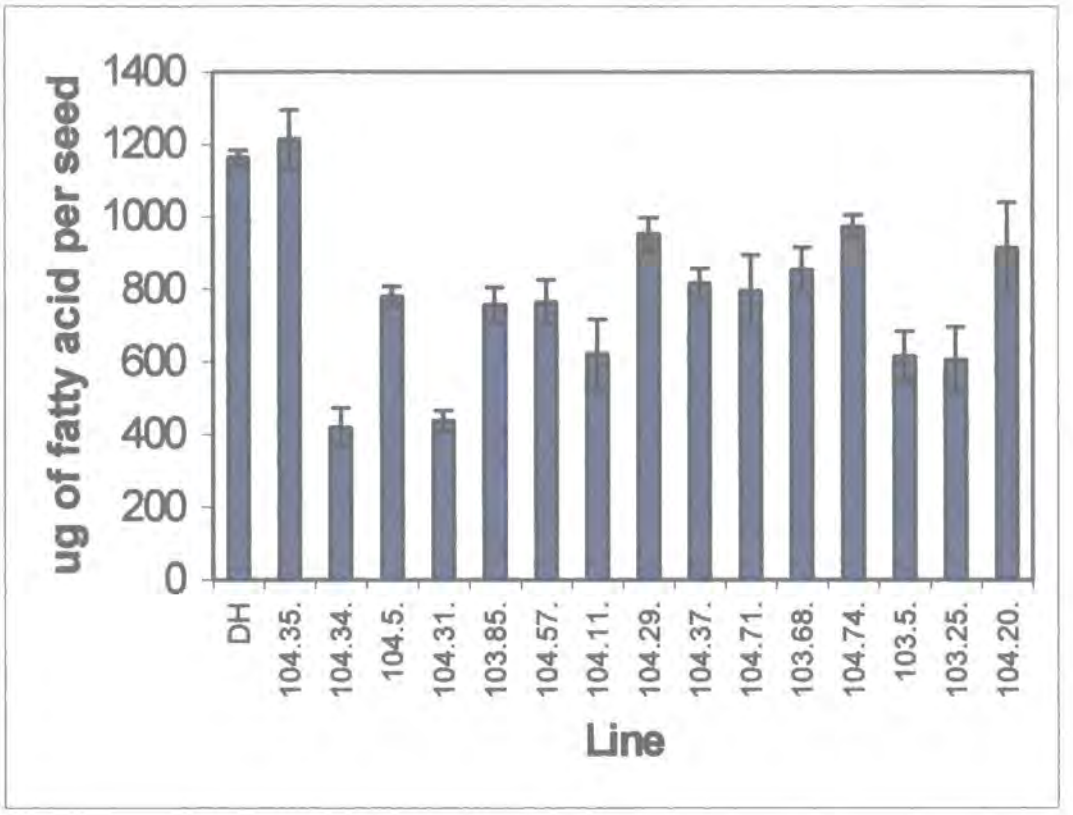


Figure 6.6 Total amounts of fatty acids per seed in transgenic KAS III lines showing +/- mean standard error bars for each line (n = 3).

Each line represented in the graph is the FAME analysis for three replicates each individually analysed on a GLC. Each sample consisted of FAMEs extracted from two seeds in each case. This figure shows how KAS III antisense lines vary in their total fatty acid content. This may be due to different expression levels of the KAS III transgene within the plants and also the number of copies within the plant may exert a greater or lesser effect (Hobbs *et al.*, 1993). This will depend on the position of integration and whether introduction of the transgene has induced the plant's gene silencing mechanisms (Stam *et al.*, 1997).

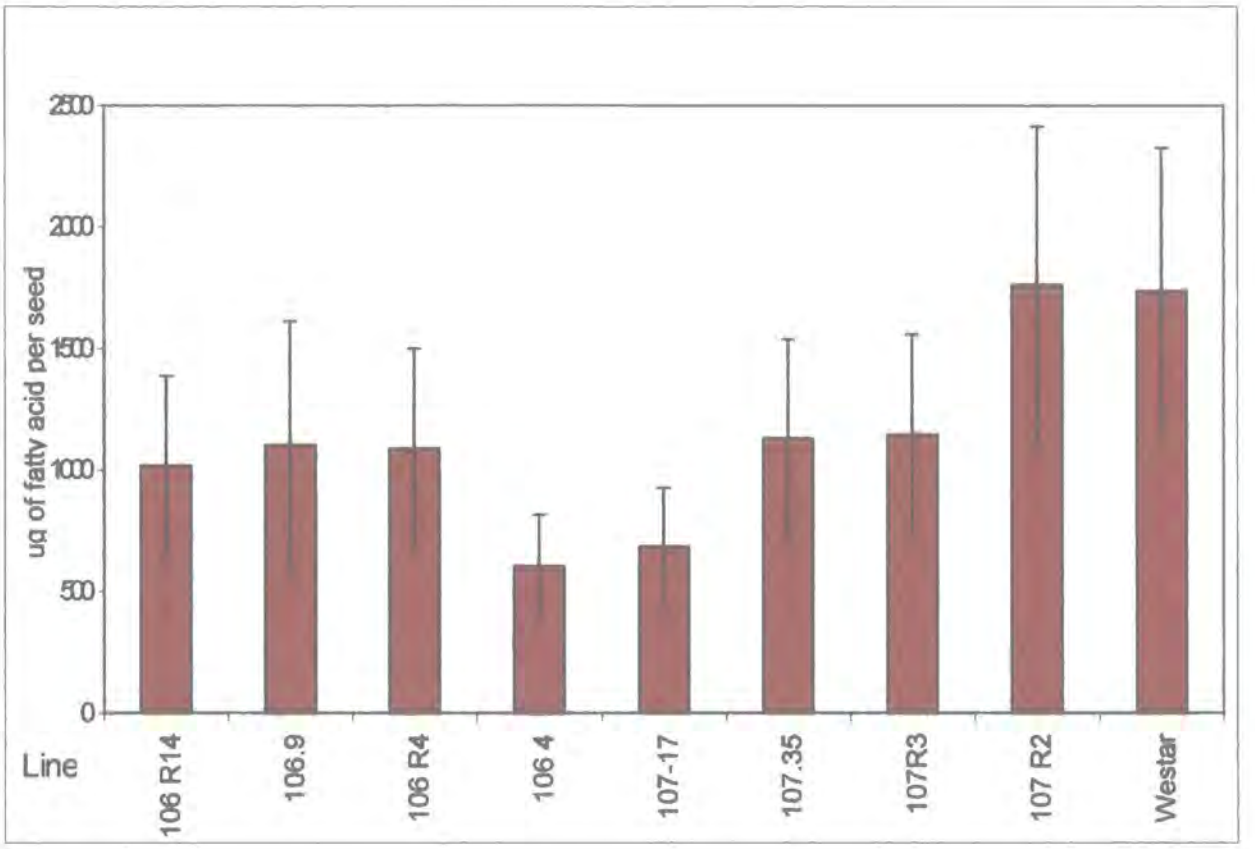


Figure 6.7 Total amounts of fatty acids per seed in transgenic ENR Lines showing +/- mean standard error bars for each line (n = 3).

Each line represented in the graph is the FAME analysis for three replicates each individually analysed on a GLC. Each sample consisted of FAMEs extracted from two seeds in each case. Eight transgenic ENR lines were compared against the wild type cultivar Westar to determine the effect the transgene may have on overall fatty acid content of T1 seed. Varying degrees of fatty acid content can be seen with both sense and antisense constructs.

6.5.2 Analysis of KAS III Levels in Transgenic Lines Using Quantitative Westerns

Western blots were carried out in identical fashion to section 5.3.3. Figure 6.8 shows that KAS III in the ENR line 107.17 are detectable with this technique. However the antibody was unable to detect KAS III in KAS III antisense lines at this point despite replication of the experiment three more times. The fact that no signal was seen may be due to the real low levels of KAS III in these lines, which were beyond the detection limits of the antibody. A second possibility is that the Western transfer was not 100% successful, this would again render the antibody unable to create a high enough signal for visual quantitative analysis. However the standard KAS III protein which was also loaded was detected by the antibody Improvement of detection of KAS III in crude plant extracts by Western blotting may be possible by enhancement of the signal (avidin-biotin treatment) or an ELISA assay could be developed which would mean more total protein could be analysed.

6.5.3 Analysis of ENR Levels by Quantitative ELISA Assay

To determine ENR levels in both transgenic KAS III and ENR lines nine leaf 2 4DAE samples were taken for each line examined and pooled into three groups of three. The proteins were extracted as described in section 2.8.1. Each sample was analysed by an ENR ELISA assay, which was carried out as described in section 2.8.4. Figure 6.9 demonstrates that in KAS III antisense lines there is an effect on ENR levels to various degrees in particular lines 104.57, 104.34 and 104.35. This would suggest that down regulation of KAS III has in certain cases

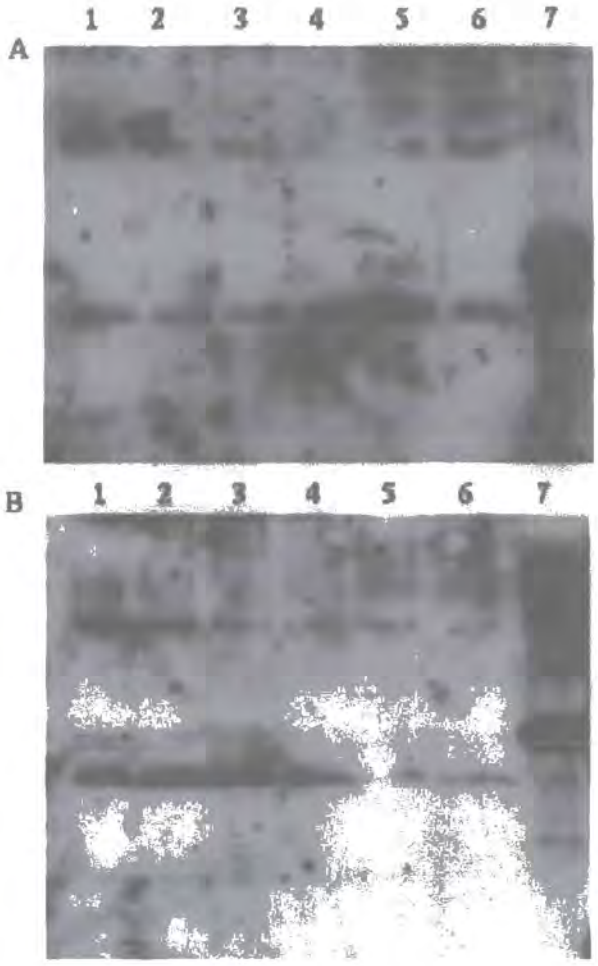


Figure 6.8 Western blot analysis of protein extracts from Leaf 2, 4DAE from three individual plants of the ENR Sense Line 107.17 (A) and Westar control (B), probed with the anti-KAS III polyclonal antibody. A total of 30μg of total protein was loaded onto each lane.

Blot A – Line 107.17, each lane has 30μg of total protein loaded from leaf 2 4DAE (lanes 1-6), each sample is loaded in duplicate. A 20ng KAS III standard is loaded in lane 7.

Blot B – cv. Westar control, each lane has 30μg of total protein loaded from leaf 2 4DAE (lanes 1-6), each sample is loaded in duplicate. A 20ng KAS III standard is loaded in lane 7.

had a pleiotropic effect on other FAS components as seen by down regulation of ENR in certain lines namely lines KAS III 104.34 and 104.57).

To determine if there was any correlation between ENR or KAS III protein levels against FAME levels determined in section 6.5.1, Spearman rank correlation statistical analysis was employed. This is a non parametric statistical test which examines whether there is a tendency for the two components to increase and decrease together (or for one to decrease as the other increases, Watt, 1993). If this test is employed to compare the levels of ENR to FAME levels in antisense KAS III T1 seed, there is no significant correlation to suggest that FAME levels and ENR levels are linked. If line 104.35 is removed from the data, (as it could possibly be an anomalous result due to it having the highest FAME levels, having no phenotypic differences to wild type, yet the lowest ENR levels), and a repeat of the Spearman Rank statistical analysis performed a 90% significant correlation between the FAME levels in the KAS III lines and the ENR levels in these plants is seen. This would imply that the KAS III transgene could be affecting the ENR protein levels.

Statistical analysis from transgenic ENR lines shows that if FAME and ENR levels for all eight lines are compared, there is 97% correlation between levels of fatty acids and ENR. If the three lines analysed again for ENR content (Figure 6.10) are assessed there is no significant correlation. This is most likely due to the low number of data sets available in the second set of analyses.

Within the scope of this study, the analysis shows however, that all three types of transgene construct affect ENR levels, where certain antisense KAS III lines appear to exhibit pleiotropic effects on ENR levels.

6.5.4 Determination of Copy Number by Southern Hybridisation

To confirm insert presence and determine if copy number of transgene is related to the severity of the phenotype seen, leaf five was taken at approximately four days old from each plant. DNA preps were made using the Qiagen Plant DNAeasy Kit (section 2.7.1). Three DNA samples (20μg of each) from each line were individually restricted with 20 units of *Xba* I in a final volume of 200μl and a genomic southern performed (section 2.7.5). The filter was probed with a ^{32}P kanamycin DNA fragment derived from a PCR using primers Kanamycin I and II using pSCVnos DNA as a template. The probe was made using high affinity $^{32}dCTP$ and the filter subjected to the wash treatment described in section 2.7.8. The autoradiograph was developed after a ten day exposure. Figure 6.11 shows the southern blot for KAS III lines 104.31, 104.57. 104.34 and 104.35 and shows that line 104.57 has a single insert, lines 104.31 and 104.34 contain 3 inserts and 104.35 has 2-5 copies of the transgene. 104.35 does not show any down regulation of fatty acids in the T1 seed, nor does there appear to be any phenotypic traits on the plant or in T2 seed. Therefore the high number of inserts present in this line may have induced silencing of the construct (Stam *et al.*, 1997) or alternatively the constructs have been incorporated into the plant's genome in such a manner as to prevent the expression of the transgene (Herman *et al.*, 1990, Jorgensen *et al.*, 1987).

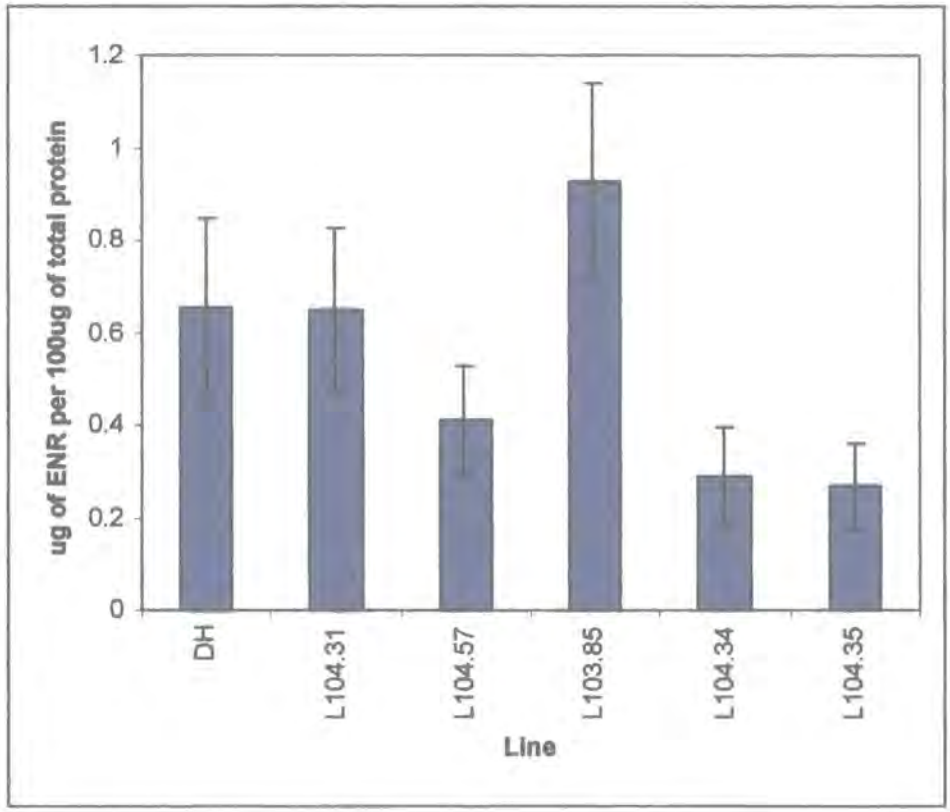


Figure 6.9 Quantitative analysis of enoyl-ACP reductase levels by ELISA in KAS III transgenic lines compared to the wild type cultivar DH RV28 showing +/- mean standard error bars for each line (n=3). Three leaves were pooled from each line and the assay performed on three different samples from the same line. Certain lines show down regulation of ENR despite being antisensed to KAS III. This may imply that a pleiotropic effect is being observed, where the down regulation of one enzyme is, as a consequence, affecting other enzymes in the same metabolic pathway.

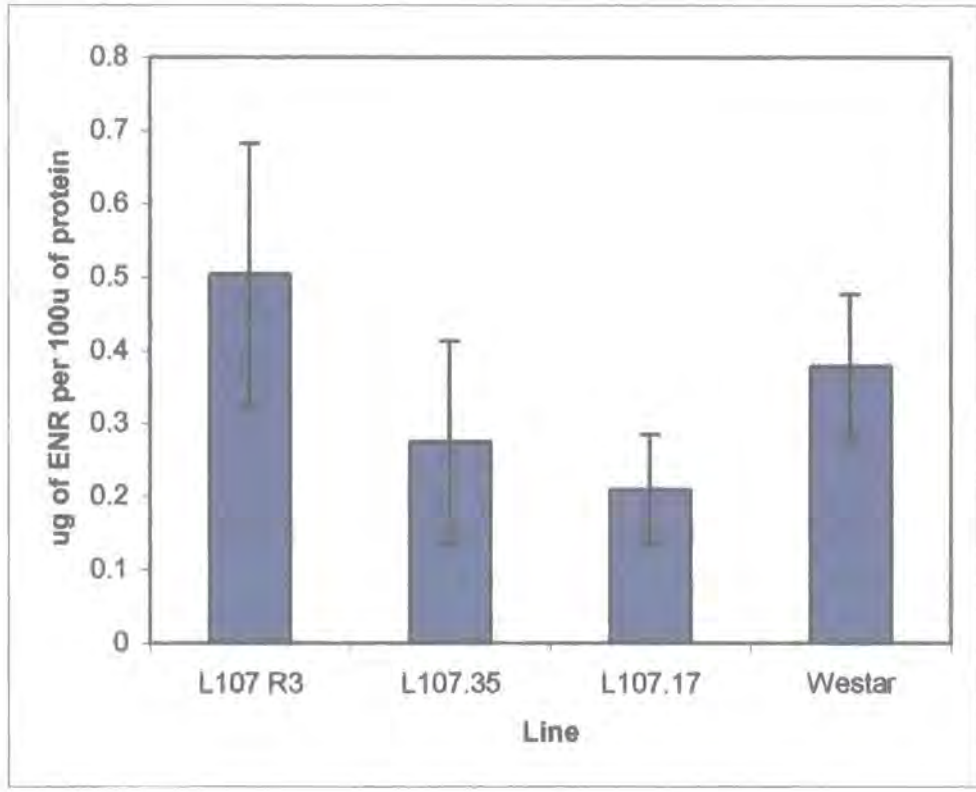


Figure 6.10 Quantitative analysis of enoyl-ACP reductase levels by ELISA in ENR sense plants showing +/- mean standard error bars for each line (n=3). Three leaves were pooled from each line and the assay performed on three different samples from the same line. One of the sense ENR lines has overexpressed levels of ENR compared to the wild type, however line 107.17 demonstrates that insertion of a sense transgene into a plant can cause cosuppression. This is where the plant not only silences the transgene but also down regulates the expression of the naturally occurring gene and causes similar effects to that of antisense methods.

All of the sense ENR lines examined by Southern analysis for copy number were each shown to contain one copy of the transgene. Lines 107 R3 and 107.35 did not appear to be affected by the transgene phenotypically as the plants grew normally. However the seed of 107 R3 (Figure 6.15) has changed colour from T1 to T2 generations. This seed had reddish-brown pigmentation in comparison to the typical black colour seen in wild type. Line 107.17 was severely affected by its single copy of the transgene. The line showed considerable stunting, extra large leaves compared to wild type and an inability to flower and set seed. These characteristics must all point to this line suppressing the plant's own copies of ENR as well as the sense ENR transgene in an attempt to silence the transgene and therefore this line exhibits cosuppression. This line was confirmed to have low ENR levels, yet surprisingly was the only line in which KAS III could be detected.

Figure 6.11 Lane Contents

Lane 1	-	Line 104.31a – 3 copies
Lane 2	-	Line 104.31b -3 copies
Lane 3	-	Line 104.31c -3 copies
Lane 4	-	Line 104.57a – 1 copy
Lane 5	-	Line 104.57b – 1 copy
Lane 6	-	Line 104.57c – 1 copy
Lane 7	-	Line 104.34a – 2 copies
Lane 8	-	Line 104.34b – 2 copies
Lane 9	-	Line 104.34c – 3 copies
Lane 10	-	Line 104.35a - 2 copies
Lane 11	-	Line 104.35b – no copies detected
Lane 12	-	Line 104.35c – 5 copies

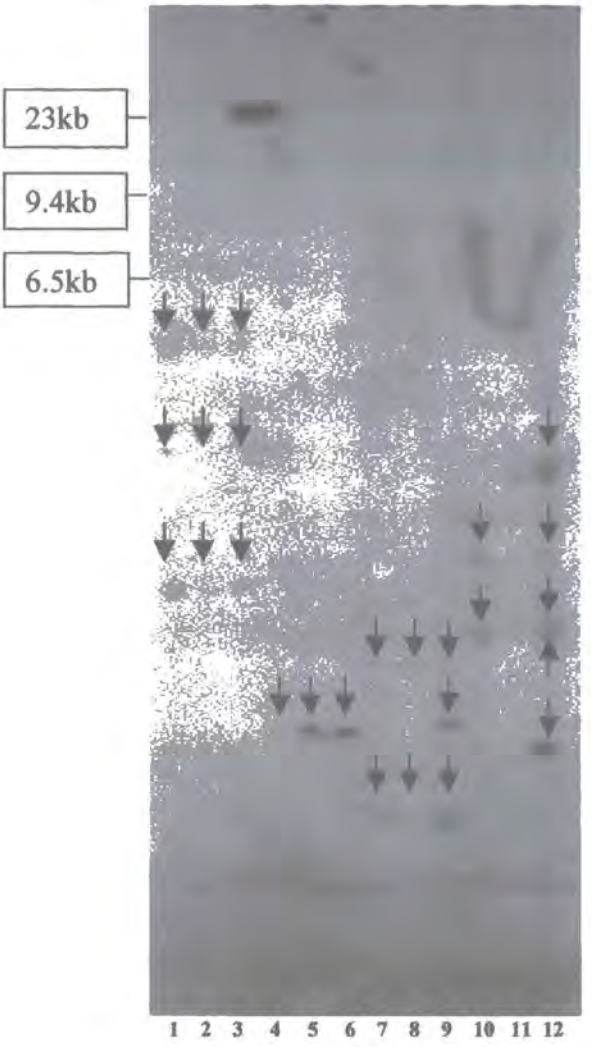


Figure 6.11 An examination of transgene copy number in *B.napus* transgenic lines by Southern blot analysis. The membrane was probed with a fragment of the kanamycin gene labelled with ^{32}P[dCTP], which is the selectable marker known to be present in all transformants. This Southern shows the copy number for 4 antisense KAS III lines where 20μg of genomic DNA was digested for 16hrs with *Xba* I. Arrows indicate where bands can be seen for each sample indicating where a copy of the transgene is present.These are lines that are still segregating and hence the copy number may be different in samples from the same line but different plants.

6.5.5 Phenotypic Comparison of Transgenic Lines to Wild type

6.5.5.1 Germination and Growth Rates

Analysis of germination rates showed that the two lines most affected by their transgene were ENR 107.17 and KAS III 104.34, showing 30% and 46% germination rates respectively, KAS III 104.31 also showed lower germination rates at 66%, however the remaining transgenic lines did not differ from the wild type lines (Table 6.2). Observations of the seedlings' growth showed that certain lines were growing more slowly than others. Leaf 6 from each plant was subsequently measured daily until it had reached a midrib length of 30cm. Figure 6.12 shows that some lines particularly 107.17 and 104.34 had delayed leaf growth compared to wild type lines. It also should be noted that line 107.17 only had a total of 6 germinated plants and all of these produced pods void of any seeds or any sign of embryo formation. Table 6.3 compares the time taken for a leaf of the lines examined to grow 30cm against the total average fatty acid content again showing 107.17 has severely down regulated levels of total fatty acids compared to wild type which seems to correlate with the time it takes for one leaf to grow 30cm (17 days).

6.5.5.2 Plant Physiology

Figure 6.16 compares seedlings from cv. DH RV28 to transgenic lines 104.31 and 103.85, where the wild type can be seen to be significantly larger despite the seeds being sown on the

same day. Comparison of fully grown plants which are setting seed also show the transgenic KAS III lines to be slightly stunted compared to the wild type (Figure 6.17). Although direct proof of down regulation of KAS III has not been obtained, presence of the construct in these lines has been confirmed, along with down regulation of the ENR FAS enzyme and also an indication of down regulation of KAS III was observed by the inability to detect KAS III antibody binding in crude transgenic extracts on Western blots. This may be indirect proof that down regulation of KAS III may be causing a decrease in the initial priming reaction in *de novo* fatty acid synthesis and hence affecting the plant's physiology by slowing down its development.

6.5.5.3 Seed Morphology

If the morphology of the seed is examined (Figure 6.14) it is seen that KAS III lines 104.31 and 104.34 vary in size, colour and shape to wild type (Figure 6.13). Lines 103.85 and 104.57 appear to be more regular in shape, but their pigmentation appears to be more reddish brown compared to wild type's black. Line 104.35 does not exhibit any morphological differences. Similar patterns of morphology are seen in T2 seed, 104.34 produced significantly less seed and of all the KAS III lines examined it appears to be the most affected line.

Seeds of ENR transgenic lines also show various morphologies in a similar manner to KAS III, with Line107.17 showing severely wrinkled T1 seed, and this line did not produce any T2 seed (Figure 6.15). The lack of seed production can be explained by the physiology of this plant (Figure 6.17B), where no flowering occurs and extra large leaves have formed. This

transgenic line may have produced these type of leaves in order to create the energy that was originally lacking in its seed. The loss of oil from the seed may have been caused by the down regulation of ENR in the primary transformant which in turn affected the T1 seed it produced. The poor quality of T! seed may have caused its initial slow growth and germination. However it appears that by the time it is large enough to flower and set seed, the plant either does not have enough energy, has highly down regulated *de novo* fatty acid synthesis or is stressed in such a manner that it cannot perform the important functions of flowering and setting seed.

LINE	% GERMINATION RATE
DH RV28	80
KAS III 104.35	90
KAS III 104.34	46
KAS III 104.57	86
KAS III 104.31	66
KAS III 103.85	80
ENR 107.17	30
ENR 107.R3	93
ENR 107.35	86
WESTAR	96

Table 6.2 Germination rates of each line grown. Germination rates were calculated from a total of 30 seeds sown from each line.

LINE	AVERAGE AMOUNT OF FATTY ACID PER SEED (MICROGRAMS)	DAYS TAKEN TO REACH 30CM IN LENGTH
DH RV28	1162	10
104.35	1213	12
104.34	419	14
104.31	437	11
104.57	764	9
103.85	756	10
107.17	682	17
107.35	1128	10
107.R3	1143	10
Westar	1672	13

Table 6.3 A comparison of average fatty acid content in the wild type and transgenic lines of T1 seed compared to the time taken for the T2 plants to produce a leaf of 30cm in length.

Lines 107.17 has significantly reduced levels of fatty acids compared to its wild type Westar and takes the longest to produce a leaf of 30cm. Of the antisense KAS III lines there is not much variation from the wild type double haploid line with the possible exception of line 104.34 which takes 14 days to grow to 30cm compared to 10 days for the wild type and has severely reduced fatty acid levels compared to DH RV28.

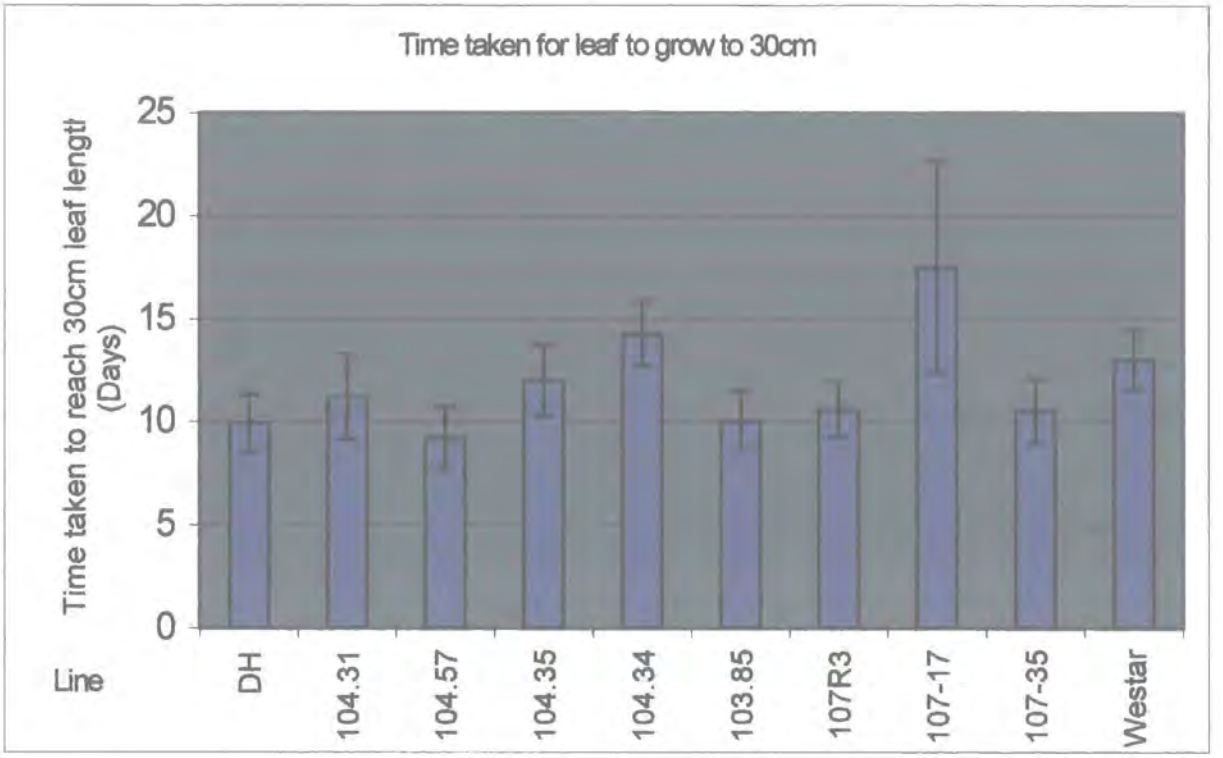
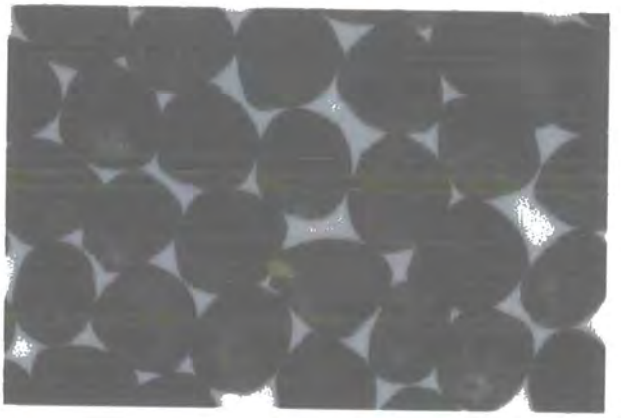
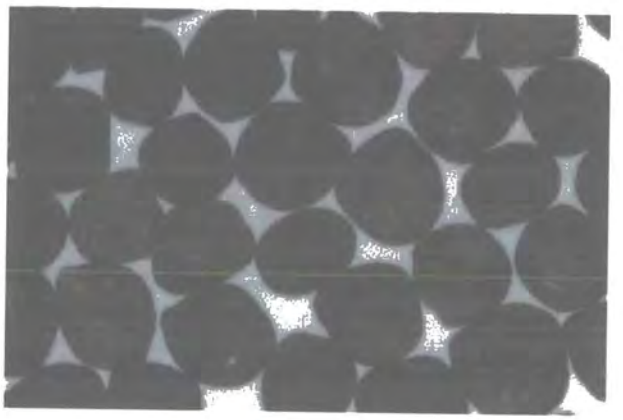


Figure 6.12 Time taken for the midrib leaf 6 from each plant of each line to reach 30cm in length showing +/- standard error bars. The number of individual analysed was dependent on the number of plants which germinated but was at least 10 replicates with the exception of line 107.17 where only 6 germinated. Line 107.17 (sense ENR) has the longest growth period, compared to wild type (cv. Westar) and line 104.34 (antisense KAS III) requires the longest growth period in order to reach 30cm compared to wild type (cv. DH RV28) in KAS III transgenic lines. This may be due to the lack of oil in the T1 seed, which then has an indirect effect on the growth and development of the resulting seedlings.

Figure 6.13 The phenotype of wild type rape seed from cv. Westar and cv DH RV28. Both cultivars show similar features, with a dark testa coat and well accumulated storage oils and thus no wrinkled phenotype observed. Both photographs are to the same scale (× 5).

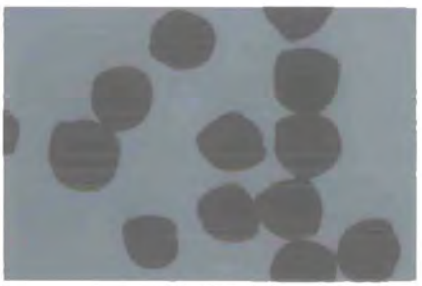


CV. DOUBLE HAPLOID RV28

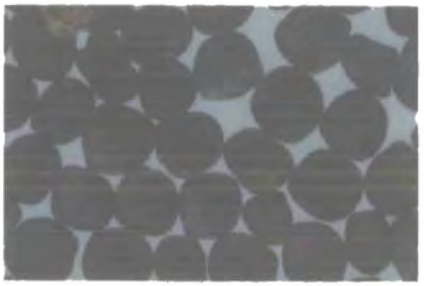


CV. WESTAR

Figure 6.14 Phenotype of T1 and T2 seed from KAS III antisense lines. Line 103.34 shows severe phenotype in that few seed are produced, with a considerable number discoloured and wrinkled. Line 104.35 does not show any difference in phenotype from wild type. Less severe phenotypes are observed in the other three lines examined. All photographs are to the same scale (× 3)



104.57 T1

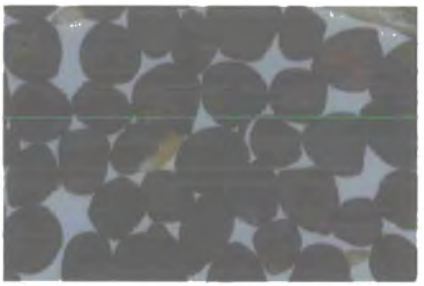


104.57 T2

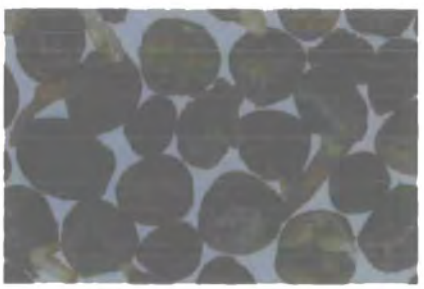
103.85 T1

103.85 T2

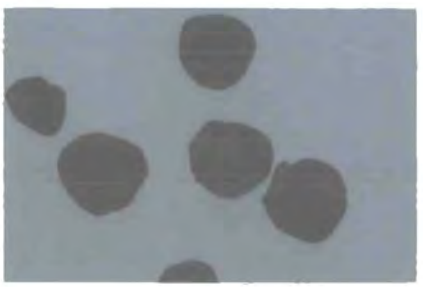
104.31 T1



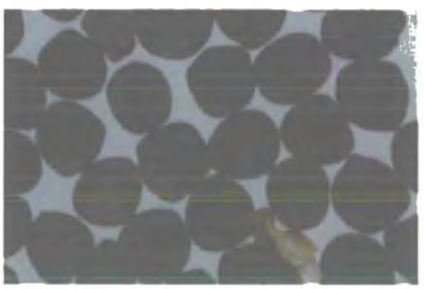
104.31 T2



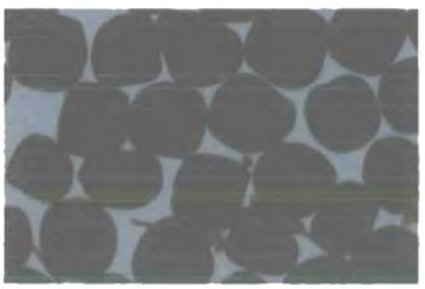
104.34 T1



104.34 T2

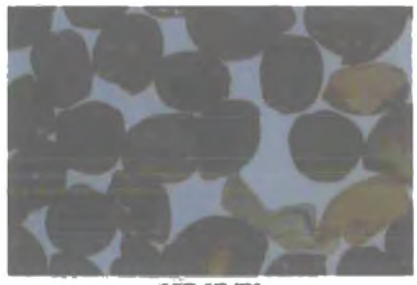


104.35 T1

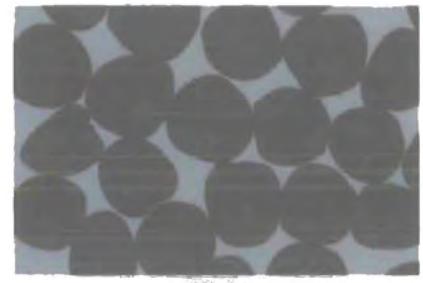


104.35 T2

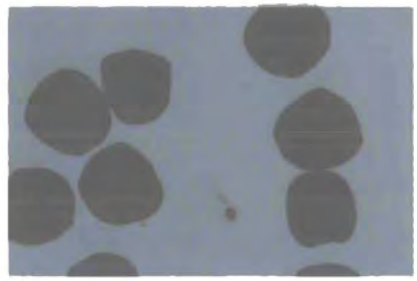
Figure 6.15 Transgenic T1 and T2 seed of the ENR lines examined within this study. Some lines were only examined at T1 only. Of the three lines examined at T2, 107.17 failed to produce any seed, 107R3 produces a reddish brown seed in the T2 generation and 107.35 does not appear to be affected. All photographs are to the same scale (× 3).



107.17 T1

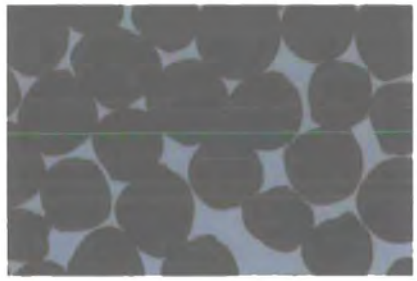


107.R2

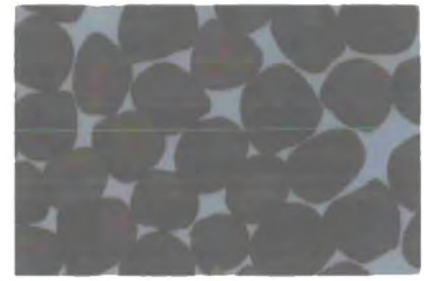


107.35 T1

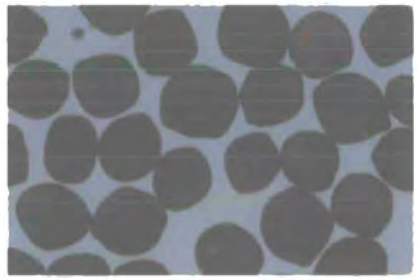
107.35 T2



107.R3 T1

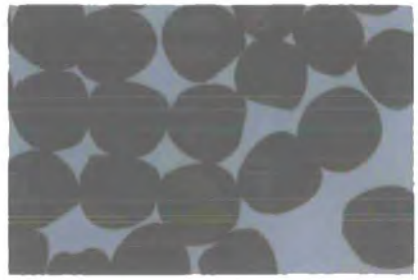


107. R3 T2

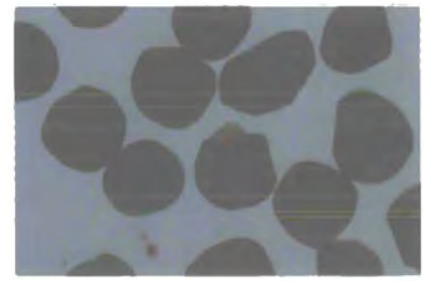


106.4 T1

106.9 T1



106.R14 T1



106.R4 T1

Figure 6.16 Physiology of Line 104.85 and Line 104.31 compared to wild type. The transgenic lines generally grew a lot slower than compared to wild type, but overall physiology did not alter.

A **Double Haploid Line 103.85**

B **Double Haploid Line 104.31**

Figure 6.17 A plant from line KAS III 104.34 compared to wild type cv. DH RV28 (A), which shows some stunting, less seed pods and fewer flowers. This resulted in a considerably lower yield of seed, which showed some wrinkling a loss of pigmentation. Figure 6.20 B shows line ENR 107.17 compared to wild type cv.Westar. The transgenic line shows a severe phenotype of severe stunting but the production of large leaves. Little flowering is seen and as a consequence no seed deposition occurs.

A **DH RV28** **104.34**

B **Westar** **107.17**

6.6 Discussion

6.6.1 Creating Transgenic Lines

An antisense KAS III construct was generated using a strong double 35S promoter. Transcription of the antisense cDNA was terminated by a CaMV poly A tail positioned at the 3' end of the antisense gene. This cassette was ligated into the *Agrobacterium* plasmid pSCVnos (Biogemma UK) and the construct electroporated into the *Agrobacterium tumefaciens* strain C58 C3. The resulting strain was used to transform *B.napus* cv. DH RV28 using conventional callus transformation (Moloney *et al.*, 1989). Plants were co-cultured with *Agrobacterium* and transformants were initially selected by their resistance to kanamycin, as the NPT II gene had also been engineered into the cassette to assist in selection. Putative positives were examined by PCR which confirmed that 63 lines had been successfully created to contain an antisense KAS III cassette from the 1000 separate transformations performed (Appendix A).

Creation of transgenic *Arabidopsis thaliana* using the vacuum infiltration technique proved successful in producing 10 lines containing the KAS III antisense cassette and a further 5 lines containing a pSCVnos NPT II gene only cassette. Optimisation of the technique showed that more transformants (14 in total) were produced from short periods (i.e. up to 14 mins) of infiltration with 1-2 breaks in the vacuum procedure, rather than 30 minute periods (1 produced) with no breaks in the exposure to the vacuum. Short infiltration incubations did not

appear to exert severe stress on the plants, whereas 30 minute treatments caused the plant to take longer to recover and also cause severe necrosis of the rosette leaves of the plant.

Once optimised, this procedure proved to be a labour saving method of producing transgenic lines and the technique was successfully used to introduce other constructs into *Arabidopsis thaliana* within the laboratory (Dr. F.MacDonald and Mr. J.Hamilton, personal communications). However, due to time constraints no further analysis of these lines could be performed. As the ORF of the KAS III cDNA used in this transformation shows 85% homology to the known ORF of *Arabidopsis*, the transgene should affect the *Arabidopsis* KAS III. This theory can be substantiated by the observations of Van der Krol *et al.* (1988), where a petunia chalcone synthase construct was transformed into tobacco and observed to affect flower pigmentation. So these transgenic *Arabidopsis* lines have the potential to aid in the understanding of the importance of KAS III in the regulation of *de novo* fatty acid synthesis in plants.

6.6.2 Analysis of Transgenic *Brassica napus*

6.6.2.1 FAME Analysis

FAME analysis of seed from transformants of both *B.napus* KAS III and ENR lines showed that each line varied in total fatty acid content and produced a wrinkled phenotype in some lines, but despite decreased levels the overall composition of fatty acids did not change. This is in line with observations from other transgenic plants down regulated with the FAS

component βKR (White *et al.*, 1996) and ACCase (White *et al.*, 1998), where a wrinkled seed phenotype has been demonstrated. ACCase provides the supply of malonyl-CoA for *de novo* fatty acid synthesis and is thought to be an important regulatory point in fatty acid synthesis (Post-Beittenmiller *et al.*, 1991, Post-Beittenmiller *et al.*, 1992, Page *et al.*, 1994). As the introduction of antisense FAS constructs into *B.napus* also appears to affect total fatty acid content in the seed, it would imply on current evidence (e.g. down regulating βKR, ENR and KAS III protein levels in *B.napus*) that down regulation of any FAS component has the potential to affect *de novo* fatty acid synthesis to a certain degree and hence affect total oil composition in the seed.

A study by Verwoert *et al.*, (1995) examined overexpression of *E.coli* KAS III in *Brassica napus*, where it was seen that the construct exerted a slight change in metabolic flux with an increase in 18:2 and a decrease in 18:1 fatty acids. However no overall change in lipid biosynthetic flux was observed and the authors suggest that overexpression of KAS III has caused more subtle changes in regulation and control of fatty acid synthesis. Other studies by Verwoert *et al.*, (1994) show that when MCAT is overexpressed in *B.napus* by up to 55 times no effect is seen on the fatty acid profiles at all, which seems to eliminate this enzyme from having a regulatory step in fatty acid synthesis. Verwoert *at al.* (1995) also propose that regulatory control is shared by a number of FAS components or products of fatty acid biosynthesis in a similar manner to that suggested in tryptophan biosynthesis in yeast (Niederberger *et al.*,1992).

6.6.2.2 KAS III Protein Levels in Transgenic *Brassica napus*

It was possible to detect KAS III expression in line 107.17 by Western blot analysis. However no comparisons could be made to other transgenic lines due to the inability of the antibody to bind to any of the other transgenic crude extracts. It is probable therefore that the phenotype of line 107.17 is produced solely due to the down regulation of ENR. Also as the seed of this line is severely lacking in storage products, which are needed in the seedlings' initial germination and development, it is likely that this factor has also affected the plant's metabolism and has aided in the overall phenotype of the progeny.

6.6.2.3 Analysis of ENR Protein Levels in Transgenic *Brassica napus*

Analysis of ENR levels of the transgenic lines using quantitative ELISA showed that KAS III lines affected the ENR levels in the plant to varying degrees. This indicates that it is possible that the introduction of KAS III antisense construct can have a pleiotropic effect on other FAS components or alternatively the transgene construct has been inserted into a region of DNA which has affected the plant's metabolism in some other aspect e.g. a regulatory gene or another fatty acid synthesis gene. It is unfortunate therefore that a direct comparison of KAS III levels and ENR levels could not be made, which highlights the need for an amplification of the antibody signal in KAS III Western blots, by possibly using a streptavidin/biotin method. Transgenic ENR lines also had affected ENR levels, as determined by ELISA.

6.6.2.4 Transgene Copy Number in Transgenic *Brassica napus*

Analysis of copy number showed that any phenotype assigned to down regulation of a FAS component is not linked to the number of copies of transgene present, but due to the position of transgene integration into the genome of the plant and whether the transgene has been silenced by the host. This is seen most strongly in line KAS III 104.35, where despite the presence of 5 transgene copies, no phenotypic effects were seen. Conversely, if lines have the same number of copies of transgene, they do not always exhibit the same phenotype. This is demonstrated most poignantly by the sense ENR lines where line 107.17 shows severely affected growth and physiology and lines 107.35 and 107 R3 are not affected.

6.6.2.5 Changes in Plant Phenotype

On examining the transgenic plants, phenotypic traits of each line were noted. This was in an attempt to build up an impression of a typical phenotype of plants which have had a FAS component downregulated. Of the lines examined, it appears that the whole plant growth pattern of both KAS III and ENR transgenic lines ranges from normal growth to slower growth, and the plants are generally still able to flower and set seed (with the main exception of ENR line 107.17). The amount of seed deposited varied amongst the lines as did the quality of the seed produced. Lines KAS III 104.34, 104.31 and 104.57 all produced discoloured seed with some wrinkled seed observed. From this study it is seen that some lines are slower growing plants, which produce a lower quality seed that has lost some colouration in its testa pigmentation.

The ENR sense line 107.17 has severely depressed fatty acid content levels in seed, T1 seed has 700μg of fatty acids per seed compared to 1600μg in wild type. This line also has low ENR levels in leaf as determined by ELISA and demonstrates that severe cosuppression of this enzyme has resulted in the inability of the plant to deposit high quantities of fatty acids and therefore lipids within the seed. This results in a drastic change in the resulting progeny's physiology, where the plant is extremely slow to grow, produces large leaves and few flowers and as a consequence does not produce T2 seed.

Certain transgenic lines within this study, despite confirmation of construct presence, did not affect the physiology of the seed or FAME content at all, line KAS III 104.35 was such a candidate. Various levels of total fatty acids were observed for the both ENR and KAS III transgenic lines. These variety of observations can be linked to a number of phenomena. Independent integration events will have occurred at random points within the plants' genome during transformation, which could determine whether expression of the construct occurs. If they are inserted in a transcriptonally active region, their expression can be influenced by other regulatory sequences (Herman *et al.*, 1990). The manner in which the T-DNA and number of copies of the T-DNA inserted at that integration point has also been demonstrated to affect expression levels (Jones *et al.*, 1987 and Jorgensen *et al.*, 1987). There is also much discussion as to the cumulative effect of a transgene *in planta* and it has been proposed that copy number can be either positively or negatively associated with transgene expression within a group of transformants (Hobb's *et al.*, 1993). Finally expression of the foreign gene can often be suppressed by the host's gene silencing mechanisms (Stam *et al.*, 1997) and all

of these will determine the overall effect the foreign gene will have on the plant and therefore in this study it will determine the overall effect the transgenes have on *de novo* fatty acid synthesis.

The majority of plant mutational studies in lipid synthesis have been characterised in *Arabidopsis thaliana* (Browse and Somerville 1991). Ten thousand mutants created by EMS were analysed for fatty acid composition which resulted in the finding of mutations that affected at least 12 of the steps in the glycerolipid pathway. None of the mutants were readily phenotypically identifiable from wild type under normal conditions. The majority of these mutants were found to be lacking certain desaturase activities (Browse and Somerville, 1991) which in turn was reflected in the chilling sensitive phenotype observed when these mutants were placed in cold growth conditions. The creation of mutant desaturase lines has resulted in an understanding of the role of desaturases in chilling tolerance/sensitivity in *Arabidopsis* as well as determining the flux of lipid synthesis through the plastid and endoplasmic reticulum. It is interesting to note that upon visual inspection a severe phenotype is not at first seen in mutant fatty acid or lipid synthesis plants. Once plants are analysed for composition, the seed inspected and a environmental stress exerted (e.g. a change in temperature), various differing phenotypes are observed (Lightner *et al.*, 1994). An interesting study would be to analyse the phenotypic effect on the KAS III and ENR transgenics when environmentally stressed.

Studies by Shintani *et al.* (1997) demonstrated that if the biotin carboxylase subunit of the plastidial form of ACCase is antisensed and the subsequent transgenics grown in optimal light conditions, the plants grew normally. If the plants were grown under low light conditions,

severely retarded growth of transgenic lines are seen. In the study described within this chapter, slow growing FAS transgenic plants are observed under normal conditions. ACCase is known to be light regulated which would explain the observations of Shintani *et al.* (1997). This is most likely due to the fact that levels of ACCase were insufficient in poor light, whereas normal growth conditions circumvented the need for high ACCase activity levels.

6.6.2.6 Germination Rates

Germination rates of three lines within the KAS III/ENR study (Sense ENR line 107.17 and antisense KAS III lines 104.31 and 104.34) were low and these plants grew slower than wild type. Knutzon *et al.*, (1992) created antisense Δ9 stearoyl-ACP desaturase constructs and transformed them into *B.napus* and *B.rapa*. Their findings found that *B.napus* had a normal phenotype with a change in oil composition and the seeds had normal germination rates. *B.rapa* had severely reduced oil levels and as a consequence germinated poorly. Stearoyl-ACP desaturase activity was detected in *B.napus* but not *B.rapa* and the authors suggest that the poor germination of *B.rapa* seed was due to some unknown aspect of lipid synthesis rather than an increased amount of saturated fatty acids.

The suggestion that poor germination rates are due to some unknown aspect of lipid synthesis is substantiated by the fact that higher plants can tolerate wide variation in lipid fatty acid composition without there being a phenotypic effect. Studies on transgenic *B.napus* containing the bay laurel thioesterase showed the seed to accumulate 60% medium chain fatty acids but not affect seed viability (Eccleston and Ohlrogge, 1998). This demonstrated that the various

components of the β-oxidation pathway are able to utilise a wide variety of substrates for fatty acid oxidation. Thus illustrating the adaptability capable by plants, when their natural storage products are manipulated.

6.6.3 Future Experiments

In future experiments, analysis of further generations of transgenic plants should isolate genetically stable lines. These can then be assessed for acyl-ACP levels in order to detect any changes in FAS metabolites as well as determining KAS III and ENR levels. This would help determine the importance of KAS III in regulation by examining which acyl-ACPs were dominant and whether this was different from wild type.

An activity assay for KAS III has been described in the literature (Jaworski *et al.*, 1989, Clough *et al.*, 1992, Gulliver and Slabas, 1994) and such an assay for use in transgenic studies has been investigated by the author (data not shown). KAS III activity was detected by acid precipitable radioactive acetoacetyl-ACP (relevant methods described in Chapter 2). A linear response was obtained for KAS III from *Brassica napus* embryos (21 DAF) in relation to the amount of extract added. However it was thought that further enhancement of the assay was required to ensure it was sensitive and suitably quantitative for use in *B.napus* leaf and transgenic material. Once further optimised this assay could also be used in transgenic studies.

6.7 Summary

- A KAS III antisense construct was created and transformed into *Brassica napus* cv DH RV28 by conventional callus transformation to yield 63 positive lines.
- The KAS III antisense construct was used to optimise the conditions of the vacuum infiltration technique for further use with the laboratory. The experiment yielded 10 KAS III transgenic lines for further analysis.
- FAME analysis of *Brassica napus* containing KAS III or ENR transgenes illustrated that lines had total fatty acid levels had been down regulated by varying degrees in each line.
- Indications of pleiotropy were observed when KAS III antisense *Brassica napus* ENR levels were analysed.
- Spearman rank correlation statistical analysis suggests that there is a correlation between the overall fatty acid levels of a line and the ENR level in the leaf in the sense that if the ENR level is low so is the total fatty acid level.
- Certain transgenic KAS III and ENR lines have a slower growing plant with some stunting and lower seed production which may indicate that manipulation of these FAS components can cause these effects.

Chapter 7

General Discussion

7.1 A Full-Length KAS III cDNA Clone was Isolated

Using a *Brassica napus* embryo cDNA library, a clone of 1622bp containing an open reading frame of 1215bp was isolated (pKAS13). It had 127bp 5' untranslated region along with 280bp 3' untranslated region with a poly A tail. Upon comparison to known plant KAS III sequences it was seen to have 85% homology and 75% identity with the KAS III sequence identified from *Arabidopsis thaliana* (Tai *et al.*, 1994). Examination of the derived amino acid sequence showed that the clone had the conserved DITAACSGF region, which is known to be present in all plant condensing enzymes (Siggaard-Anderson, 1993). Northern analysis showed that the messenger transcript was 1600bp in size, suggesting that pKAS13 is or is near to full-length.

Southern hybridisation showed that more then one gene copy of KAS III exists in *B.napus*, this is in line with observations for other FAS components including ENR (Fawcett *et al.*, 1994) and ACP (Safford *et al.*, 1988). Further analysis of clones isolated from the *B.napus* embryo cDNA library identified a partial clone. This was shown to have a truncated 3' UTR and 49 nucleotide differences in the ORF compared to pKAS13, which constituted 15 codon changes. Comparison of these two genes to the partial clone TF Clone 2 (which was used to isolate pKAS13), showed them both to have differences.

This is the first report of the isolation of a full length KAS III cDNA along with two partial clones from *Brassica napus*, all of which are different gene copies of KAS III.

7.2 Polyclonal KAS III Antisera Recognises 38kDa and 45kDa Proteins in *Brassica napus*

Overexpression of the ORF of pKAS13 resulted in the production of a novel 45kDa protein present in *E.coli* BL21*(DE3)* as determined by SDS/PAGE. The protein was confirmed to be KAS III by amino acid sequencing, it was subsequently purified by electroelution. Immunisation of two rabbits using the purified protein resulted in antisera which could detect 20ng of pure protein at 1 in 8,000 dilution. To the author's knowledge this is the first report of an antibody raised to a KAS III Brassica isoform.

Upon change of extraction method and use of freshly prepared samples the antisera could recognise 38kDa and 45kDa proteins in *Brassica napus* 3 week old embryo. These were assumed to be the pre- and mature proteins of KAS III as the overexpressed protein included the transit peptide. If the transit peptide is not cleaved it is proposed that a protein of an approximate molecular weight of 45kDa would be seen. Both bands were not always seen in *B.napus* leaf material (i.e. only 38kDa detected). The antisera could easily detect KAS III in *B.napus* embryo and leaf when 25μg of total protein extract was used. The antisera was subsequently used in the determination of the level of KAS III in developing leaf of *Brassica napus* cultivars, thus demonstrating that as the leaf matures KAS III protein levels fall.

7.3 A Quantitative Enoyl-ACP Reductase Antigen Inhibition ELISA was Developed

A quantitative ENR-ELISA was developed which was sensitive to 3ng of protein. There are few reports in the literature of using quantitative ELISA to assess levels of proteins in transgenic plants (e.g. Gendolff *et al.*, 1990 and Zhang *et al.*, 1997, see section 5.4). The technique required denaturation of the crude rape seed and leaf samples prior to incubation with a sheep anti-ENR antibody as in native extracts antigen/antibody recognition did not occur. The reasons for this may include that the antibody was prevented from binding to ENR epitope sites as they were concealed by another molecular component. Attempts to characterise this component suggest that it is not membrane bound, is at least 10kDa (and likely to be larger) and tightly bound to ENR. The component could not be removed by dialysis, high speed centrifugation, microcon separation or desalting, but is removed when the crude extract is subjected to gel filtration. The gel filtration allows a slower conformational change of proteins and physical separation which may account for the occurrence of ENR/antibody interactions after this treatment.

The ENR-ELISA was used to determine ENR protein levels in leaf 3 DAE, 7 DAE and 14 DAE as well as embryo and seed material. The ELISA was also successfully used to quantify ENR protein levels in transgenic studies. This is the first development of a

quantitative ELISA for ENR protein levels in plants and its development has required careful consideration of sample preparation.

7.4 Examination of Levels of FAS Components in *Brassica napus* cv DH RV28

Levels of certain FAS components were examined for the *Brassica napus* leaf of cultivar DH RV28. This showed that ENR and βKR protein levels fell as the leaf aged, as did KAS III protein levels. Comparison to the cultivar Westar was hampered by the apparent degradation of samples in the ENR and βKR analysis by ELISA. However KAS III protein levels were higher in this cultivar as are ACCase activity levels (Dr.A.J.White – personal communication). It was decided that in the analysis of transgenic lines that careful monitoring of the plant was necessary to ensure that it was taken at the correct developmental stage as determined by this study. Leaf 2 4DAE was selected as the sample to be taken for protein analysis, but transgenic material had to be of a similar size as wild type leaf 2 4DAE in order to take the sample and this was seen to take a further 2-3 days to be able to do this in some lines. FAME and complex lipid samples were not seen to differ between the two cultivars examined in the standard profile (Dr. P. O'Hara, personal communication). This work highlighted the need for careful monitoring of samples and plant development in order to ensure that samples were taken at the correct stage of development during experiments.

7.5 Some Transgenic FAS Plants can be seen to be Slower Growing

The study reports the successful development of sixty-three antisense KAS III transgenic lines by the introduction of an antisense KAS III construct under the control of the double 35S promoter by *Agrobacterium*/callus transformation into *Brassica napus*. Analysis to determine overall seed FAME levels by GLC proved a rapid way to select interesting lines for further examination. Of the lines analysed it was seen that KAS III 104.34, 104.57 and 104.31 along with ENR 107.17 were much slower growing than compared to wild type. They also had lower germination rates and produced less T2 seed. Detection of KAS III protein levels by Western blotting of transgenic KAS III lines resulted in no signal being detected despite several repetitions, suggesting that KAS III protein levels had fallen below that detectable by the antibody. Further optimisation of the KAS III antibody is required in order to detect the KAS III in transgenic lines, possibly using streptavidin/biotin methods to amplify the signal. Lowering the levels of KAS III protein could imply that this does not severely affect fatty acid synthesis and that it is possible that the plant has alternative pathways to initiate fatty acid synthesis, such as the slower ACAT/KAS I pathway, which could explain the observations of slower growing transgenic lines with lower overall fatty acid levels.

Up until the generation of these antisense plants, only reports of KAS III overexpression in plants have been described in the literature (Verwoert *et al.*, 1995, Jaworski and Ohlrogge, 1997). The advantage of an antisense approach described within this thesis is that evaluation of the effect of loss of the protein activity can be assessed more easily. As

has been observed, overexpression may just show that another enzyme becomes limiting as the overall pathway cannot utilise the initial products fast enough (Jaworski and Ohlrogge, 1997).

ENR protein levels were determined by ELISA. In some KAS III lines, ENR protein levels were lower than compared to wild type suggesting that KAS III down regulation may cause a pleiotropic effect and as a consequence affect ENR protein levels. Alternatively this effect may have been caused by the insertion of the transgene into an important site for regulation or cause disruption of another fatty acid synthesis gene. ENR protein levels in ENR transgenic lines were seen to be affected in certain lines. Correlation of fatty acid levels (as determined by FAME analysis) to ENR protein levels were compared for both KAS III and ENR lines by the use of statistical analysis. It was seen that there was high correlation between these levels i.e. low levels of ENR protein could be correlated to low fatty acid levels in the seed. This suggests that the low ENR protein levels affect seed fatty acid levels.

This study has shown that when KAS III and ENR protein levels are down regulated in *Brassica napus*, a slower growing plant results in some transgenic lines. These lines appear to have a lower germination rate, poorer quality seed and have a lower fatty acid content than compared to wild type.

7.6 Implications in Understanding the Regulation of Fatty Acid Synthesis in Plants

Other than the now accepted regulatory role of ACCase in fatty acid synthesis, little is known about the importance of other FAS components in regulation. ACCase is regulated by a redox reaction under the influence of light (Sasaki *et al.*, 1997) and is also affected by the stromal pH, magnesium ions and is ATP dependent. All of these factors indirectly affect FAS enzymes from synthesising fatty acids due to the supply of malonyl-CoA which is limited by the activity rate of ACCase. The levels of ACCase activity will in turn affect the rate in which lipids can be synthesised in the plant. Down regulation of ACCase I was seen to have an effect on lipid deposition in plants as well as affecting flavonoid synthesis in the leaf (White *et al.*, 1998). This demonstrated that the supply of malonyl-CoA is important in fatty acid and lipid synthesis. βKR and ENR require the co-factors NADH and NADPH and it is likely that the availability of these will also regulate fatty acid synthesis rates to some extent. The potential regulatory points have been discussed previously in section 1.8.

Investigations into the transgenic FAS plants created within this study observed that if KAS III is down regulated, slower growing plants resulted in some cases. Down regulation has thought to have occurred due to the inability to detect KAS III in transgenic lines with a KAS III antibody using Western blots analysis and in directly by possible pleiotropic effects observed when protein levels of ENR are examined by an ELISA assay. However, the plants were still able to reach maturity, flower and set seed.

If KAS III is down regulated there is the potential that the ACAT/KAS I pathway has a greater role in the initiation of fatty acid synthesis. ACAT is known to have lower activity than compared to KAS III (Clough *et al.*, 1992) and acetyl-ACP is not a major intermediate of fatty acid synthesis (Jaworski *et al.*, 1993). If KAS III is down regulated and the ACAT/KAS I pathway used there is the potential to observe a slower growing phenotype due to the decreased availability of acetoacetyl-ACP. The growth rate of the transgenic plants would be determined by the amount in which the antisense molecule has down regulated the endogenous KAS III activity. Some photosynthetic enzymes have had to be reduced by as much as 80% for there to be a major phenotypic difference of change in flux (see Stitt and Sonnewald, 1995).

An alternate pathway could generate acetyl-ACP by decarboxylation of the malonyl-ACP. The malonyl-ACP substrate pool may have increased as a result of lower turnover between acetyl-CoA and malonyl-ACP by KAS III. Condensing enzymes can be induced to decarboxylate when malonyl-ACP levels are high (Winter *et al.*, 1997). Therefore, theoretically there is the potential for more than one way to initiate fatty acid synthesis if the most direct route is somehow limited.

Down regulation of KAS III also may have affected ENR protein levels in certain transgenic lines. These observations can be compared to White *et al.*, (1998), where βKR protein levels were seen to fall when an antisense ACCase gene was introduced into *Brassica napus*. The significance of such possible pleiotropy is not yet known and could point to co-ordinate expression of the genes encoding FAS enzymes. However, it is clear

that the creation of the transgenic KAS III lines will be able to assist in determining the importance of this enzyme's function in control and regulation of fatty acid synthesis, as well as examining whether down regulation affects the other FAS enzymes.

7.7 Future Direction

This work has laid the foundations for continuing investigations into examining the importance of KAS III in the initiation and regulation of fatty acid synthesis in plants by generating antisense KAS III transgenic lines. It has also put in place analytical techniques in the form of a reliable highly reproducible ELISA assay and polyclonal antisera towards KAS III for use in Western blot analysis. Further development of the use of the KAS III antibody in the development of an ELISA as well as amplifying the signal foe use in analysing transgenic KAS III plants will mean that these techniques can be used in a high throughput manner to analyse transgenic lines. This may provide information on actual enzyme levels *in planta*. Understanding the profile of the wild type *Brassica napus* cultivars, along with the identification of possible pitfalls in sampling will assist in ensuring that future experimentation with these plants will take into account the phenomenon observed i.e. the variation in development of leaves of transgenic and wild type.

It is interesting to note that most FAS and lipid phenotypes in transgenic plants are not observed until the plants are exposed to an environmental stress e.g. cold (Somerville and Browse, 1991), light reduction (Shintani and Ohlrogge, 1997) and increased UV. levels

(White *et al.*, 1998). This may be due to the fact that the effect of an imbalance in the plant's metabolism (e.g. the down regulation of an enzyme caused by antisense) is not observed until the plant is required to respond to an environmental stress. An environmental stress may require the plant to up regulate this enzyme as a protective measure and if it can not do so an adverse phenotype may be observed. Therefore to examine the phenotypes of transgenic KAS III lines further, the plants could be exposed to a variety of abiotic stresses. Such experiments may accentuate phenotypes and determine whether these plants can find alternate ways to initiate fatty acid synthesis.

The production of KAS III polyclonal antibody could allow immunolocalisation studies to be performed. This could determine the exact position of KAS III in the plastid. Studies by Slabas *et al.*, (1990) have shown that the ENR is localised to the plastid in *Brassica napus*. A quantitative KAS III ELISA assay can also be set up if a supply of KAS III pure antigen can be made available and then subsequently be used in transgenic studies. The cDNA clone can be used to isolate further KAS III isoforms and also in site directed mutagenesis to determine the importance of the conserved region between positions 330-360, which is a highly conserved region in all plant condensing enzymes, possibly having a structural role.

Appendix A – List of KAS III Transformants T1 Seed with Phenotype

A total of 63 transformed B.napus lines were obtained. +/- indicate plants which are negative for the bla gene and positive for the kanamycin gene. Therefore no integration beyond the left border has occurred. +/+ indicates plants which are positive for both the bla gene and the kanamycin gene. Therefore integration beyond the left border has occurred.

Line	Transformant Type	Phenotype Of Seed
103.1	+/-	Normal
103.2	+/-	Slight colour change
103.3	+/+	Normal
103.5	+/-	Few seed, small and some wrinkling
103.7	+/-	Few Seed, various sizes, some wrinkling
103.8	+/-	Very few seed, wrinkled and discoloured
103.9	+/-	Very few seed, wrinkled and discoloured
103.15	+/-	Normal
103.20	+/-	Normal
103.21	+/-	Normal
103.23	+/-	Very few seed, slight discolouration, some wrinkled
103.25	+/-	Normal
103.28	+/-	Normal
103.29	+/+	Normal
103.34	+/-	Few seed, wrinkled
103.32	+/+	Normal
103.36	+/-	Normal
103.40	+/-	Very few seed/wrinkled
103.41	+/-	Normal
103.42	+/-	Normal
103.43	+/-	Normal
103.44	+/+	Normal
103.47	+/-	Few Seed/ Wrinkled
103.61	+/-	Few Seed/slight discolouration
103.62	+/+	Very few seed, discoloured, wrinkled
103.68	+/-	Normal
103.67	+/-	Some wrinkling
103.70	+/-	Small seed
103.76	+/-	Very few seed, small

103.77	+/-	Few seed, normal
103.81	+/-	Normal
103.85	+/-	Small, discolouration in some seed
104.6	+/+	Normal
104.11	+/-	Normal
104.16	+/-	Normal
104.17	+/+	Normal
104.20	+/+	Few seed, generally normal
104.23	+/+	Small seed
104.24	+/+	Normal
104.27	+/+	Normal
104.30	+/-	Normal
104.31	+/-	Small seed/wrinkled
104.32	+/+	Normal
104.33	+/+	Normal
104.34	+/-	Wrinkled
104.35	+/+	Normal
104.36	+/-	Normal
104.37	+/-	Few seed, some wrinkled
104.39	+/+	Normal
104.41	+/-	Normal
104.42	+/+	Few seed, small wrinkled
104.51	+/-	Few seed/normal
104.53	+/-	Normal
104.58	+/-	Normal
104.59	+/-	Normal
104.60	+/-	Few seed/wrinkled
104.63	+/+	Normal
104.65	+/-	Normal
104.66	+/-	Few seed/appearance normal
104.71	+/-	Few seed/appearance normal
104.74	+/-	Few seed/appearance normal
104.76	+/+	Very few seed, various sizes,some wrinkled
103.53	+/+	Normal

Appendix B - List of Suppliers

Amersham.Pharmacia Biotech Ltd..
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA

Bioline
16 The Edge Business Centre
Humber Road
London
NW2 6EW

Bio-Rad Labs Ltd.
Bio-Rad House
Maryland Avenue
Hemel Hempstead
Hertfordshire
HP2 7TD

Boehringher-Mannheim
Sandhofer Strasse 16
D-68305 Mannheim
Germany

Fisher Scientific UK
Bishop Meadow Road
Loughborough
Leicestershire
LE11 5RG

Harlan Sera-Lab Ltd.
Dodgeford Lane
Belton
Loughborough
LE12 9TE

Hybaid Ltd.
Acton Court
Ashford Road
Ashford
Middlesex
TW15 1XB

Invitrogen
PO Box 2312, 9
9704 CH Groningen
The Netherlands

Life Sciences International UK Ltd.
Unit 3 The Ringway Centre
Edison Road
Basingstoke
Hampshire
BG21 6YH

Life Technologies Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley
PA4 9RF

Merck (BDH) Ltd.
Hunter Boulevard
Magna Park
Lutterworth
Leicestershire
LE17 4XN

Millipore UK Ltd.
The Boulevard
Blackmoor Lane
Watford
Hertfordshire
WD1 8YW

MWG Biotech UK
Waterside House
Peartree Bridge
Milton Keynes
MK6 3BY

New England Biolabs UK Ltd.
Knowl Place
Wilbury Way
Hitchin
Hertfordshire
SG4 OTY

Novagen
601 Science Drive
Madison
W1 53711
USA

Nu-Check Prep Inc.
P.O.Box 295
Elysian Minnesota
56028-0295
USA

Perkin Elmer Biosystems
Kelvin Close
Birchwood Science Park North
Warrington
Cheshire
WA3 7PB

Pharmacia Biotech Ltd.
See Amersham Pharmacia Biotech Ltd.

Pierce and Warriner UK Ltd
44 Upper Northgate Street
Chester
CH1 4EF

Promega
2800 Woods Hollow Road
Madison
W1 53711-5399
USA

Qiagen Ltd.
Boundary Court
Gatwick Road
Crawley
West Sussex
RH10 2AX

Schliecher and Schuell UK Ltd.
Unit 11
Brunswick Park Industrial Estate
London
N11 1JL

Sigma Aldrich Co. Ltd.
Fancy Road
Poole
Dorset
BHH 4QH

Stratagene Europe
Gebouw California
Hogelhilweg 15
1101 CB Amsterdam Zurdoost
The Netherlands

X-ograph Imaging Systems Ltd.
Hampton Street
Tetbury
Gloucestershire
GL8 8LD

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