Toward the characterisation of the protein-protein interactions of the type II fatty acid synthase of Brassica napus.

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Abstract.

The availability of cDNA and antibodies for the proteins of type II fatty acid synthase (FAS) of *Brassica napus* allowed an investigation into the protein-protein interactions of the putative type II FAS complex. Using the yeast two-hybrid system, four components were assayed for interactions: acyl carrier protein (ACP), enoyl-ACP reductase (ENR), oleoyl-ACP thioesterase (TE), and stearoyl-ACP desaturase (DES). Direct interactions were detected between DES and ENR. A possible, very weak, interaction may have been detected between TE and ENR. The relative affinity of DES for ENR was greater than that of TE for ENR. Both were much weaker than ENR homo-tetrameric interactions. No interaction was found between ACP and any other FAS enzyme tested by this technique.

Toward the characterisation of the protein-protein interactions of the type II fatty acid synthase of *Brassica napus*.

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Abbreviation List.

3-AT, 3-aminotriazole

ACCase, acetyl-CoA carboxylase

ACP, acyl carrier protein

AD, activation domain

AD/ACP, acyl-acyl carrier protein fused to the GAL4 AD

AD/ENR, enoyl-ACP reductase fused to the GAL4 AD

Amp, ampicillin

AT, acyltransferase

BC, biotin carboxylase

BCCP, biotin carboxyl carrier protein

β-HAD, β-hydroxyacyl-ACP dehydratase

β–HAS, β– holo-ACP synthase

β-KR, β-ketoacyl-ACP reductase

β-ME, β-mercaptoethanol

BSA, bovine serum albumin

CEB, chloroplast extraction buffer

CPRG, chlorophenol red-β-D-galactopyranoside

CT, carboxyltransferase

Ctp, chloroplast targetting peptide

Cyhx, cyclohexamide

DAG, diacylglycerol

DBD, DNA binding domain

DBD/ACP, acyl-acyl carrier protein fused to the GAL4 DBD

DBD/DES, stearoyl-ACP desaturase fused to the GAL4 DBD.

DBD/ENR, enoyl-ACP reductase protein fused to the GAL4 DBD

DBD/TE, oleoyl-ACP thioesterase fused to the GAL4 DBD

dBEST, expressed sequence tag database

DES, stearoyl-ACP desaturase

DGDG, digalactosyl diacylglycerol

DMF, dimethylformamide

DNP, dinitrophenyl

dNTPs, deoxynucleotides

DTT, dithiothreitol

EDTA, ethylenediamine tetraacetic acid

ENR, enoyl-ACP reductase

EST, expressed sequence tag

FabA, *E. coli* β-hydroxyacyl ACP dehydrase

FabB, *E. coli* ketoacyl-ACP synthetase I

FabG, *E .coli* β-ketoacyl-ACP reductase

FabH, *E. coli* KAS III

FabI, *E. coli* enoyl reductase

FabZ, *E. coli* β-hydroxyacyl ACP dehydrase

FAS, fatty acid synthase

FATA, fatty acid thioesterase type A (preference for oleoyl-ACP substrate)

FATB, fatty acid thioesterase type B (saturated substrates preferred)

FRET, fluorescent resonance energy transfer

G-3-P, sn-glycerol-3-phosphate

G-3-PAT, glycerol-3-P acyltransferase

Gluc-6-P, glucose-6-phosphate

H2O, purified sterile water

–His, minus histidine

HPLC, high performance liquid chromatography

ID, interdomain

IPTG, isopropyl-[β]-D-thiogalactopyranosideKAS I, β-ketoacyl-ACP synthetase I

KAS II, β-ketoacyl-ACP synthetase II

KAS III, β-ketoacyl-ACP synthetase III

LB, luria broth

Leu, leucine

–Leu, minus leucine

LiAc, lithium acetate

LPA, lysophosphatidic acid

MALDI-TOF, Matrix-Assisted, Laser Desorption/Ionization Time-Of-Flight

MCA, metabolic control analysis

MCAT, malonyl-CoA:ACP transacylase

MCS, multiple cloning site

MGDG, monogalactosyl diacylglycerol

NMR, nuclear magnetic resonance

ONPG, o-nitrophenyl β-D-galactopyranoside

PA, phospatidic acid

pACT2/ACP, acyl-acyl carrier protein cDNA cloned into Y2H vector pACT2

pACT2/ENR, enoyl-ACP reductase cDNA cloned into Y2H vector pACT2

PAGE, polyacrylamide gel electrophoresis

pAS2-1/ACP, acyl-acyl carrier protein cDNA cloned into Y2H vector pAS2-1

pAS2-1/DES, stearoyl-ACP desaturase cDNA cloned into Y2H vector pAS2-1

pAS2-1/ENR, enoyl-ACP reductase cDNA cloned into Y2H vector pAS2-1

pAS2-1/TE, oleoyl-ACP thioesterase cDNA cloned into Y2H vector pAS2-1

PC, phosphatidylcholine

PCI, phenol/chloroform/isoamylalcohol

PCR, polymerase chain reaction.

PEG, polyethylene glycol

PKS, polylketide synthases

SDO or SD, synthetic dropout selection media,

SDS, sodium dodecyl sulphate

SRK, S receptor kinase

TAG, triacylglycerol

TBS, tris-buffered saline

TCA, trichloroacetic acid

TE, oleoyl-ACP thioesterase

Tet, tetracycline

–Trp, minus trytptophan

Trp, tryptophan

URA, uracil

X-gal, 5-bromo-4-chloro-3-indolyl-[[β]]-galactoside

YNB, yeast nitrogen base

YPD, complete liquid media for yeast

Declaration.

Acknowledgements

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Chapter 1 - Introduction.

1.1 - *De novo* fatty acid synthesis of higher plants.

De novo fatty acid synthesis in plants and animals is performed by a set of sequential enzymatic reactions. The structure and location of the enzymes catalysing the reactions differ between different species. In fungi and yeast, all enzyme activities are located within the domains of two polypeptides. In vertebrates and certain bacteria, all enzyme activities are located within a single polypeptide. Both fungal and vertebrate structures are referred to as 'eukaryotic' type I FAS and are localized in the cytosol (Perham, 2000; Bloch and Vance, 1977). In plants and most prokaryotes, *de novo* biosynthesis of C16 to C18 fatty acids takes place in the plastid in both leaves and seeds (Ohlrogge *et al.*, 1991) and is carried out by a 'prokaryotic' type II FAS or 'dissociable' fatty acid synthase. This is a series or a complex of 'soluble' enzymes that may be purified independently from the other enzymes in the pathway (Shimikata and Stumpf, 1983 and 1982; Hoj and Mikkelsen, 1982). Elaborate biochemical and genetic studies in the 1960s and the early 1970s on the individual enzymes of *E. coli de novo* fatty acid biosynthesis facilitated the elucidation of all the intermediates and the general concept of fatty acid biosynthesis (Wakil, 1970; Prescott and Vagelos, 1972; Volpe and Vagelos, 1976). The subsequent prospect of producing tailor-made fatty acids by genetic manipulation of oilseed crops spurred molecular genetic research towards fatty acid and general lipid metabolism in plants (Slabas and Fawcett, 1992; Thelen and Ohlrogge, 2002).

1.1.1. Initiation of chain growth.

Initiation of fatty acid chain growth in plants begins with acetyl-CoA. The source of the acetyl-CoA substrate precursor varies depending on species and tissue and stage of development. For example, metabolic feeding experiments have shown that in *Brassica napus* seed, pyruvate and glucose-6-phosphate are the preferred substrate. For example, it can be generated from the pyruvate dehydrogenase complex during embryogenesis (Kang and Rawsthorne, 1994; Rawsthorne, 2002). In chloroplasts, acetate is the preferred substrate. This is due to the ability of isolated chloroplasts to incorporate [^{14}C]acetate into their fatty acids. The acetate is converted into acetyl-CoA by the ubiquitous enzyme acetyl-CoA synthase (Harwood, 1979). Evidence for the channeling of acetate led to the hypothesis it was the physiological precursor of fatty acid synthesis (Roughan and Ohlrogge, 1996). This was supported by results from other groups showing that acetate is a better substrate than acetyl-CoA for fatty acid synthesis in disrupted spinach chloroplasts (Roughan *et al.*, 1979) and in sonicated plastids from avacado (Weaire and Kekwick, 1975). The acetyl-CoA is then converted to malonyl–CoA by acetyl-CoA carboxylase (ACCase).

1.1.2. Acetyl-CoA carboxylase, the committed step.

The ATP-Mg-dependent carboxylation of acetyl-CoA to form malonyl-CoA by ACCase is the first committed step in the synthesis of fatty acids (Ohlrogge and Jaworski, 1997). In an analogous manner to the fatty acid synthase enzymes, the molecular organization of ACCase varies depending on the source of the enzyme. For

example, in *E. coli* the 'prokaryotic' ACCase is composed of four distinct subunit types that readily dissociate into three components: a homodimer of 49 kDa subunits (biotin carboxylase, BC), a 17 kDa homodimer (biotin carboxyl carrier protein, BCCP), and a carboxyltransferase (CT) consisting of $\alpha 2\beta 2$ subunits of 33 and 35 kDa, respectively (Li and Cronan, 1992). In animals, fungi and yeasts, these entities are located on a single multifunctional polypeptide. The structure(s) of plant ACCase was the subject of some confusion. The occurrence of cytosolic reactions and the fact that the plastid envelope is not permeable to malonyl-CoA led to the hypothesis that at least two isoenzymes of ACCase (cytosolic and plastidic) were present in plants. Early experiments indicated that ACCase in spinach and barley chloroplasts and avocado plastids had a multisubunit structure. However, the subsequent inclusion of proteinase inhibitors in purification media and the development of avidin-affinity matrices allowed the rapid isolation of homomeric ACCase, similar to that found in other eukaryotes (Gornicki *et al.*, 1994). Thus, it was generally accepted that the occurrence of low-molecular mass biotnylated polypeptides was largely due to severe degradation of the high molecular mass polypeptide. Consequently, the concept of a multisubunit (prokaryotic) form if ACCase fell into disfavour. It is now evident that the confusion surrounding the structure of ACCase arose because plants contain structurally different forms of the enzyme. One of which rapidly loses activity during purification because it readily dissociates into its' component subunits. Another reason for the confusion was that the enzymes from *Graminae* were mainly studied. Most plants other than *Graminicae* (which only has the multifunctional form in both plastid and cytosol) are now known to have a type II multisubunit (prokaryotic form) and type I multifunctional

(extraplastidial form). *Brassica napus* is an exception to this. It has both forms in the plastid (Elborough *et al.*, 1996). The prokaryotic form of ACCase in plants is organised into two functional domains that interact through ionic interactions and are readily dissociable and reassociable according to the laws of mass action (Alban *et al.*, 1994; Shorrosh *et al.*, 1995). It has been suggested that the prokaryotic form of chloroplastic ACCase may interact with type II FAS and form what has been termed a 'supramolecular structure' (Roughan and Ohlrogge, 1996; Roughan, 1997).

1.1.3. Acyl carrier protein.

Acyl carrier protein (ACP) was the first fatty acid biosynthesis protein of plants to be studied in detail. In plants, the nuclear encoded ACP is synthesized in the cytosol as a larger precursor protein and then is imported into the plastid where it is processed to a smaller mature form (Elhussein *et al.*, 1988). It is a small acidic molecule of 10 kDa. The protein is synthesised as an apo-ACP and is subsequently covalently modified to holo-ACP by holo-ACP synthase. This enzyme adds a 4-phosphopantetheine prosthetic group that is in turn, covalently attached to a specific serine residue. In *E. coli,* this prosthetic group is attached to the serine 36 residue of ACP. The important functional group is the terminal sulphydryl group of the phosphopantetheine. This modification has the same critical function throughout its multifarious roles: to hold the biosynthetic intermediate in a thioester linkage while it is modified and extended by other enzymes of the system with a one-to-one correspondence between the actions of successive enzymes and the structure of the product. The intermediates of the growing fatty acid

chains are attached to the terminal sulphydryl group (Shimikata and Stumpf, 1982; Slabas and Fawcett, 1992; Slabas *et al*, 2001), Figure 1.0.

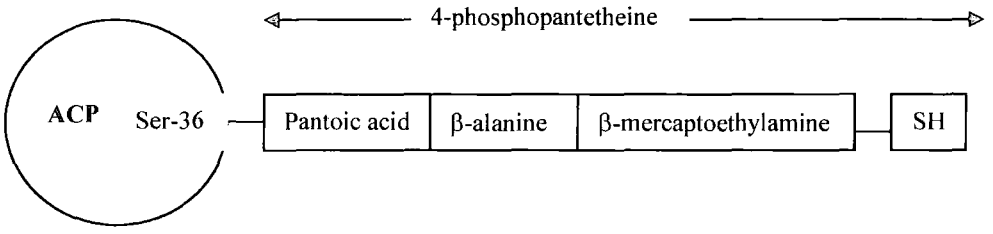


Figure 1.0. Simplified schematic diagram of the important features of holo-ACP. The first salient feature to note is the attachment of the prosthetic group to a specific serine residue, Ser-36 *in E. coli.* Secondly, the terminal sulfhydryl (-SH) group to which the growing fatty acid chain is attached.

In the type I FAS system, the prosthetic group, with attached fatty chain, swings between the active sites of the multifunctional complex (Perham, 2000). In the type II system, the ACP moiety sequentially delivers the lipid intermediates to the active site of each enzyme in the pathway (Roujeinikova *et al.*, 2002). The malonyl-CoA from ACCase and holo-acyl carrier protein is subsequently used by malonyl-CoA:ACP transacylase (MCAT) to make malonyl–ACP. The C2 units from malonyl-ACP are used in the subsequent condensation reactions.

1.1.4. The condensation reactions.

Acyl-chains are synthesized by the stepwise condensation of C2 units from the malonyl-ACP to the acyl chains. For each elongation cycle, four seperate reactions are

necessary. In the first step, β-ketoacyl ACP synthase III (KAS III) catalyses the condensation of acetyl-CoA and malonyl–ACP to form acetoacetyl–ACP. In the process, a molecule of CO_2 is lost from the α-carbon of the malonyl-ACP. The acetyl group is transferred to a cysteine thiol on KAS III and this is used as the acetyl donor for the subsequent condensation reaction (Gulliver and Slabas, 1994). The acetoacetyl-ACP is subsequently reduced to 3-hydroxyacyl-ACP using NADH or NADPH as the reductant, the reduced nucleotide preference varies from species to species (Sheldon *et al.*, 1992; Harwood, 1988). Subsequently, a molecule of water is removed by β-hydroxyacyl–ACP dehydratase (β-HAD) producing trans 2-enoyl acyl-ACP. This molecule is reduced to form the saturated acyl-ACP by enoyl-ACP reductase using NADH, or NADPH, as the reductant to form butyryl-ACP (Harwood, 1988; Slabas *et al.*, 1986). Elongation of the fatty acid chain through to C16:0-ACP (palmitoyl-ACP) is achieved by subsequent rounds of condensation reactions of 4:0-ACP with malonyl-ACP, catalysed by KAS I. Each addition of a C2 unit liberates a CO_2 unit and makes a β-ketoacyl-ACP substrate, which is subsequently converted to the saturated form, via the enzymes β-KR, β-HAD, and ENR. Subsequent formation of 18:0-ACP is achieved by the condensation of palmitoyl-ACP with malonyl-ACP, catalysed by KAS II (Mackintosh *et al.*, 1989).

1.1.5. Fatty acid desaturation and termination of synthesis.

The C18:0 may be rapidly desaturated at C9 within the chloroplast by a soluble and ferredoxin-dependent stearoyl-ACP desaturase (DES) to form oleoyl-ACP (C18:1

ACP)(Shanklin and Somerville, 1991). As this enzyme has a critical role in forming unsaturated lipids, this has led to it being the target of anti-sense experiments (Knutzon *et al.*, 1992). Acyl-ACPs synthesised in the plastid can be substrates for the synthesis of complex lipids by acyltransferases or can be hydrolysed by thioesterases. In plants, two different acyl-ACP thioesterases, FATA and FATB, may hydrolyze the C18:1 product. The FATA enzyme predominantly hydrolyzes 18:1-ACP with minor activities toward 18:0-ACP and 16:0-ACP. The FATB enzyme preferentially acts on 16:0-ACP but will also hydrolyze 18:1-ACP (Jones *et al.*, 1995; Voelker and Kinney, 2001). The fatty acid synthesis reactions are set out in Figure 1.1 and Table 1.0.

The 18:1-ACP thioesterase of *Brassica napus* has a low K_m for all three substrates, C16:0-ACP, C18:0-ACP and C18:1-ACP (Table 1.1, Figure 1.2). This was taken to imply that the binding affinity of thioesterase for the substrate is not a function of the chain length of the alkyl group but thioesterase is recognising the ACP moiety for binding. Once bound, the length and degree of saturation determine the reaction velocity (Hellyer *et al.*, 1992; Table 1.1; Figure 1.2). The twelve fold and six-fold higher V_{max} of TE for C18:1 over C16:0 or C18:0 respectively, may explain why few C16:0 fatty acids are exported from the plastid. In some plants, thioesterase enzymes cause premature termination of the chain-lengthening cycle and medium-chain products are produced. Normally, palmitoyl-ACP and stearoyl-ACP (in a ratio of about 1: 4 *in vivo*) are the products of plant fatty acid synthases (Voelker and Kinney, 2001).

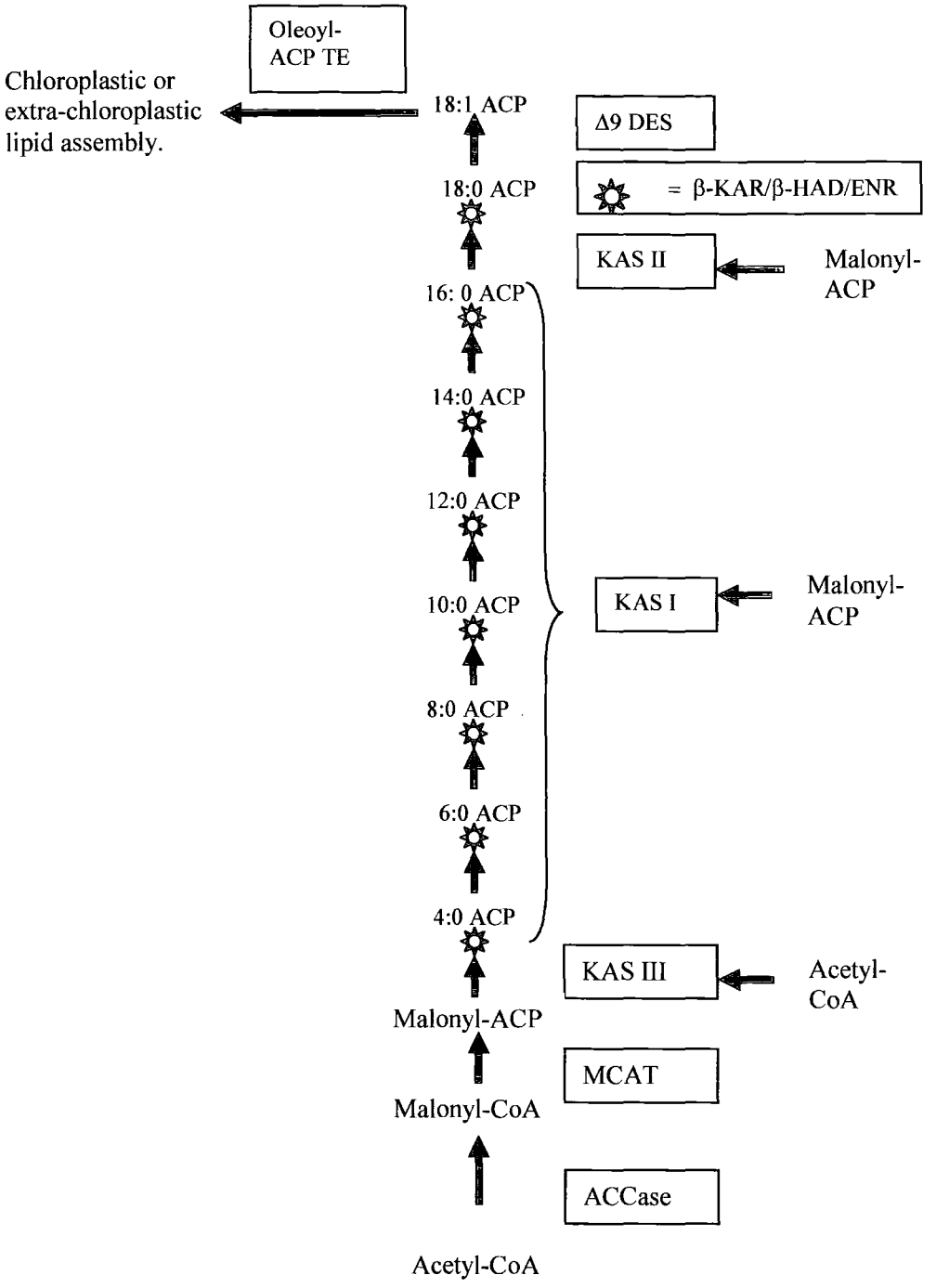


Figure 1.1. The *de novo* fatty acid biosynthesis pathway of *Brassica napus*.

The ✲ symbol represents the β-ketoacyl-ACP substrate formed by the condensing enzyme is subsequently converted to the saturated form, via the enzymes β-KR, β-HAD and ENR. Abbreviations: please refer to the abbreviation list.

Table 1.0. Overall reactions of *de novo* fatty acid biosynthesis in plants.

Enzyme	Abbreviation	Reaction catalysed
Acetyl-CoA carboxylase 1.Biotin carboxylase 2.Carboxyltransferase	ACCase BC CT	BCCP-biotin +HCO_3^- + ATP ⇔ BCCP-biotin-CO_2 + ADP + Pi BCCP-biotin-CO_2 + Acetyl-CoA ⇔ BCCP-biotin + malonyl-CoA
Malonyl-CoA:ACP transacylase	MCAT	Malonyl-CoA + ACP ⇔ Malonyl-ACP + CoASH
β-ketoacyl ACP synthase III	β-KAS III	Acetyl-CoA + Malonyl-ACP ⇔ Acetoacetyl-ACP + CO_2 + CoASH
β-ketoacyl ACP synthase I	β-KAS I	Acyl-ACP + Malonyl-ACP ⇔ β-ketoacyl-ACP + CO_2 + ACP
β-ketoacyl ACP synthase II	β-KAS II	Palmitoyl-ACP + Malonyl-ACP ⇔ β-ketooctadecanoyl-ACP +CO_2 +CoA
β-ketoacyl ACP reductase	β–KAR	β-ketoacyl-ACP + NAD(P)H ⇔ β-hydroxyacyl-ACP + NAD(P)
β-hyrdoxyacyl-ACP dehydrase	β-HAD	β-hydroxyacyl-ACP ⇔ Enoyl-ACP + H_20
Enoyl-ACP reductase	ENR	Enoyl-ACP + NAD(P)H ⇔ Acyl-ACP + NAD(P)
Acyl-ACP thioesterase	TE	Acyl-ACP + H_2O ⇔ Non-esterified fatty acid + ACP
Stearoyl-ACP desaturase	DES	Stearoyl-ACP + O_2 + Ferredoxin ⇔ Oleoyl-ACP + Ferredoxin

Table 1.1. The K_m and V_{max} estimations for partially purified *Brassica napus* oleoyl-ACP thioesterase. Abbreviations: not determined - n.d. (Data taken from Hellyer *et al.*, 1992).

Chain Length of Substrate	K_m (μM)		V_{max} (μmol/min X 10^6)	
	Primary plot	Lineweaver-Burke	Primary plot	Lineweaver-Burke
C8:0 ACP	n.d	7.14	n.d.	1.5
C10:0 ACP	4.50	n.d.	34.7	n.d.
C12:0 ACP	1.25	2.20	0.5	0.6
C14:0 ACP	2.50	5.00	1.5	1.9
C16:0 ACP	1.20	7.70	4.1	11.1
C18:0 ACP	1.75	3.12	9.6	18.2
C18:1 ACP	1.90	3.85	79.8	125.0
C18:1 CoA	1.30.	1.70	22.7	28.7

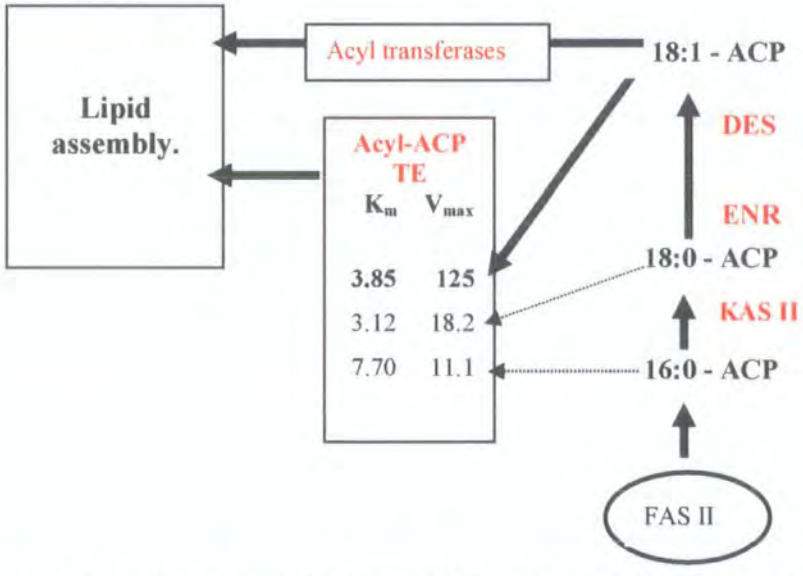


Figure 1.2. Schematic diagram to show that the main flux (thick arrows) of the *de novo* biosynthesis of developing seeds of *Brassica napus* is toward production of oleic acid (C18:1). ENR carries out the final reductive step before DES and TE can act on the acyl-ACP substrate, hence the interplay between ENR, TE and DES may have an important role in determining the principle type of fatty acid formed in *de novo* fatty acid biosynthesis. The acyl-ACP may also be directly incorporated into the chloroplast lipids by acyl transferases. Units of K_m:- μM; units of V_{max}:- μmol/min X 10^6. Thioesterase enzymatic data from Hellyer *et al.* 1992.

1.1.6. Lipid assembly pathways.

Type II FAS carries out the elongation of an acyl chain up to C16 or C18. In leaf, once elongation of the acyl chain is complete, the long-chain fatty acids can go on to become a component of the plastid membrane. This can occur via one of two pathways responsible for glycerolipid synthesis. In the eukaryotic (or 'extra-chloroplastic') pathway, the acyl-ACPs are hydrolysed to 'free' fatty acids by thioesterases, move through the plastid envelope, and are converted to coenzyme A thioesters in the outer envelope membrane by acyl-CoA synthase. The 'free' fatty acids are then incorporated into the backbone of complex lipids such as sphingolipids, phospatidylcholine, via acyltransferases. These enzymes catalyse the transfer of acyl groups onto acceptor molecules such as glycerol in the case of glycerolipids. This gives rise to the 'eukaryotic lipids' that have C18 fatty acids at the *sn*-2 position (Browse *et al.*, 1986). The second route the fatty acids can follow is the 'prokaryotic' or 'chloroplastic' pathway, where the fatty acids are retained in the plastid. In this pathway, the plastidial acyltransferase is a membrane-bound enzyme that leads to a 'prokaryotic' type diacylglycerol (DAG), which has an 18-carbon fatty acid at the *sn-1* position and a 16-carbon fatty acid at the *sn-2* position. In the chloroplast, the glycerol-3-phosphate acyltransferase competes with the acyl-ACP thioesterase for the acyl-ACP substrate. Therefore, the substrate specificity of the plastid enzyme is a major determinant of unsaturation of plastid membranes and chilling sensitivity (Murata *et al.*, 1992).

Plastids can also contain eukaryotic type lipids, where the DAG only contains the 18-carbon fatty acids. The proportion of prokaryotic and eukaryotic lipids in a plastid varies according to the plant species (Browse *et al.*, 1986). Diacylglycerols are converted to the major thylakoid membrane lipids, mono- and digalactosyl diacylglycerol (MGDG and DGDG) by synthase enzymes that have been cloned (Shimojima *et al.*, 1997). The spatially separate synthesis of eukaryotic and prokaryotic lipids has been confirmed by experiments using stable isotopes, Figure 1.3 (Pollard and Ohlrogge, 1999).

In seeds, depending on the species, fatty acids of C_6 and upwards can go on to form triacylglycerols (TAG, the main component of storage oils) via two main pathways:1) the phosphatidic acid phosphatase pathway, whereby diacylglycerol (DAG) is formed from phosphatidic acid by the reactions of the Kennedy pathway, then DAG transferase catalyses the conversion of DAG to TAG; 2) via choline phosphotransferase (Stobart *et al.* 1986). The second process begins with *sn*-glycerol-3-phosphate (G-3-P) backbone undergoing two acylations catalyzed by acyltransferases. The final acylation of *sn*-1, 2-DAG occurs after removal of the phosphate group from the *sn*-3 position of the glycerol backbone. The DAG backbone component for some membrane lipids (e.g. phosphatidylcholine [PC], phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine) are also synthesized via this pathway except for the last reaction, which is responsible for TAG formation. To generate PC, *sn*-1, 2-DAG receives a phosphocholine group at the *sn*-3 position. The acyl chains are further desaturated while esterified to PC, and PC may undergo acyl-exchange at the *sn*-2 position. The

phosphocholine group can in turn be removed to return desaturated/remodelled *sn*-1,2-DAG to TAG biosynthesis. It has been demonstrated that in castor and safflower (*Carthamus tinctorius* L.) seeds oilseed TAG can also be generated from two molecules of *sn*-1, 2-DAG and that the reverse reaction participates in the remodelling of TAG, Figure 1.3 (Mancha and Stymne, 1997). Recent studies indicated that TAG can be formed by transfer of an acyl group from PC to DAG, (Dahlqvist *et al.*, 2000).

1.2. Macromolecular organisation and compartmentalisation of metabolism.

De novo fatty acid synthesis and lipid assembly is compartmentalised into organelles. Evidence also exists of compartmentalisation of metabolism at the molecular level. For example, in isolated permeabilised spinach and pea chloroplasts, the enzymes of *de novo* fatty acid synthesis have been found to retain some substrates and intermediates (Roughan and Ohlrogge, 1996; Roughan, 1997). This indicated the enzymes of type II FAS of plants might have a higher order structure or what has been termed 'supramolecular' organisation in order to facilitate the retention of substrates and intermediates. *In vitro*, the proteins of type II FAS are easily dissociable from each other. Consequently, the higher orders of structures of type II FAS have escaped characterisation. Examining protein behaviour in the *in vivo* environment may help in obtaining this characterisation. Understanding the higher order structure may help to

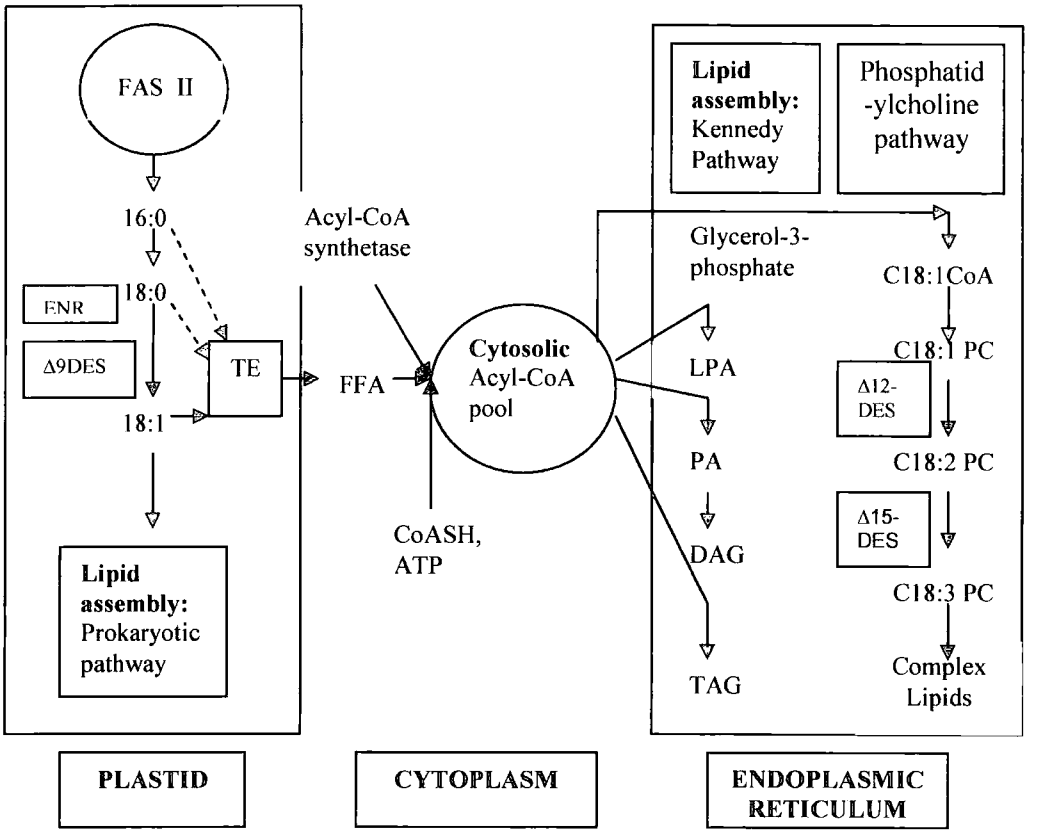


Figure 1.3. Simplified diagram indicating the compartmentalisation of lipid biosynthesis. After activation to CoA, fatty acids formed in the plastid can be sequentially esterified directly to glycerol 3-phosphate (G-3-P) to produce lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol, and triacylglycerol. Synthesis of storage lipid triacylglycerol (TAG) (as well as ' eukaryotic' membrane lipids) takes place predominantly via the glycerol 3-phosphate (or Kennedy) pathway in the endoplasmic reticulum. However, in most oilseeds the major flux of acyl chains involves movement through phosphatidylcholine (PC) pools where modifications such as further desaturation and hydroxylation occur. The spatially separate synthesis of prokaryotic and eukaryotic lipids has been confirmed by stable-isotope methodology. Other abbreviations: FAS, fatty acid synthase; ENR, enoyl reductase; DES, desaturase; TE, thioesterase; FFAs, free fatty acids (Pollard and Ohlrogge, 1999; Thelen and Ohlrogge, 2002; Slabas *et al,* 2002).

begin to identify the subtleties of regulation fatty acid metabolism (Martinez-Force and Garces, 2002; Slabas *et al.*, 2002; Thelen *et al.*, 2002).

1.2.1 – Macromolecular crowding (the excluded volume effect).

I*n vivo*, the environment is far from homogeneous and aqueous. Protein concentration is high in eukaryotes (up to 350 mg/ml) (Mathews, 1993) and even higher in prokaryotes and organelles such as mitochondria and chloroplasts (700 mg/ml) (Mendes *et al.,* 1995). Indeed, it has been suggested that, because protein crystals can form with less than 10 % protein, the cytoplasm is compact to the extent that a protein in the cytoplasm would only have slightly more freedom than would proteins in a crystal (Fulton, 1982). Yet the protein content of cells is generally higher than this, for example, red blood cells – 35 %, muscle cells- 23 %, actively growing cells 17-26 % (Loewy and Siekevitz, 1968). Therefore, as cells contain approximately 20-35 % protein by weight, this supports the hypothesis that the behaviour of the proteins in the cell may more closely resemble that of a crystal than the dilute solution of biochemists experiments, in which proteins are 0.1 % or less. This phenomenon has been termed as "macromolecular crowding" or, more precisely, 'the excluded volume effect'. The latter is more correct as it emphasises that it is a purely physical non-specific effect originating from steric repulsion. The effective concentration, or thermodynamic activity, of each macromolecular species inside cells is thus greater than its actual concentration and this difference has kinetic and thermodynamic consequences for the properties of that macromolecule (Ellis, 2001; Minton, 1997).

The two major qualitative manifestations of cellular crowding are enhancement of macromolecular associations and hindrance of marcomolecular diffusion (Zimmerman and Minton, 1993). For example, the equilibrium constant in dilute solution for a spherical homodimer and a monomer of molecular weight 40 kDa will shift towards dimerisation by a factor in the range 8 - 40 fold (depending on the partial specific volume of the protein) if the protein is expressed inside *E. coli*. In other words, the presence of 10-20 % of any other protein in a dilute (0.1 %) solution of myoglobin will drive the myoglobin into dimers (Wilf and Minton, 1981). Consequently, the actual enzyme activity *in vivo* is not only a function of which enzyme is there but also a function of all other proteins. For a tetramer, the shift in equilibrium towards tetramerisation would be in the range $10^3 - 10^5$. Thus, this aspect of the crowding effect is exerted by large molecules on large molecules and can be very large (Ellis, 2001).

The early studies detected the self-association of proteins such as myoglobin, aldolase and ovalbumin in concentrated solutions of these proteins. However, it is only recently that a method has been developed to measure the self-association of proteins. In this work, self-association was induced by the crowding effects of high concentrations of a second macromolecule to which the dilute proteins did not bind. This method, so-called 'tracer sedimentation equilibrium', was used to measure the self-association of two proteins fibrinogen and tubulin in solution. The concentration of fibrinogen was set at 0.25 - 1.0 g/ l, the protein was labelled with either ^{125}I or fluorescein isothiocyanate (FITC), and bovine serum albumin (BSA) was used as the crowding agent at 0-100 g/l. It was found that, at BSA concentrations exceeding 40 g/l, fibrinogen formed

homodimers. At 80 g/l BSA, the activity coefficient (defined as the ratio of its thermodynamic concentration to its actual concentration) of fibrinogen was calculated to be 10. As the latter concentration of BSA is close to the total protein concentration in blood plasma, this observation suggested that the activity of fibrinogen in its natural environment is an order of magnitude larger than that exhibited in the uncrowded solutions in which its properties are usually studied. When the concentration of tubulin was set at 0.2-0.6 g/l, labelled with rhodamine, and dextran was used as the crowding agent at 0-100 g/l. The average molar mass of tubulin was found to increase monotonically with increasing dextran concentration to produce soluble tubulin oligomers. Based on the latter observation, it was suggested that, inside the cell, such soluble oligomers might be intermediates in the assembly of insoluble microtubules (Rivas *et al.,* 1999). The common misunderstanding encountered is that crowding makes all macromolecules bind to one another. It needs underscoring that crowding enhances the inherent tendency of macromolecules to bind to one another, but it does not create this tendency *de novo*, if it did, the cytoplasm would be solid. The nature of the cytoplasm is still under debate, but crowding considerations suggest that it might be considered to be like a gel in the sense that most macromolecules may exist as components of large complexes, rather than as independent entities (Ellis, 2001).

The second major consequence affects the dynamics of the protein. The proteins within the cell are in constant motion. Due to crowding, diffusion coefficients (D) will be reduced by factors up to 10-fold. As the average time a molecule takes to move a certain distance by diffusion varies as D^{-2}, so if D is reduced 10-fold, it will take 100

times as long for a molecule to move a certain distance. This reduction applies to both small and large molecules, so the rate of any process that is diffusion-limited will be reduced, whether the process involves small molecules, large molecules or both. The diffusion of large molecules will, however, be impeded more than that of small molecules. For example, if a 160 kDa protein were uninhibited by surrounding molecules, it would travel at 500 cm s^{-1} at 300 K (calculated for an ideal gas). The molecule would move a distance comparable to its own size of 10 nm in 2 ns. However, *in vivo,* the protein would randomly change direction thus the protein would require approximately two milliseconds to cover 10 nm, a thousand times longer (Goodsell, 1991). There are differences between cell types, for example, in the cytoplasm of eukaryotic cells, diffusion of both large and small molecules is slowed three to fourfold. However, in the cytoplasm of *E. coli,* the diffusion of green fluorescent protein (GFP) is slowed eleven-fold. Beyond this, it is important to note that mobility inside cells is affected not just by crowding, but by other factors, such as binding to other molecules, including relatively immobile structures such as cytoskeletal components (Elowitz *et al.,* 1999).

Not only does the protein behaviour alter between *in vitro* and *in vivo* conditions, the rationalization of discrepancies between phenomena observed *in vitro* and *in vivo* can vary. For example, the binding of the *lac* repressor to the *lac* operator (Barkley, 1981) and RNA polymerase binding to the λPR operator (Roe *et al.,* 1984) are both very sensitive to salt inhibition *in vitro*. Yet, no significant salt effect was found *in vivo* (Richey *et al.,* 1987). Two groups, Zimmerman and Trach (1991) and Cayley *et al.*

(1991) agreed that crowding was one of the keys to resolving the apparent discrepancy. However, as set out below, their respective analyses differ in the type of data on which their conclusion were based and in the emphasis from various factors. The first group (Zimmerman and Trach, 1991) calculated activity co-efficients for test particles of arbitrary size under cytoplasmic crowding conditions over a range of values of the effective specific volume parameter. Application of these activity co-efficients to parameters characterising the dilute solution behaviour of the *lac* repressor-operator system resulted in calculated trends of *lac* operator function that agreed with Richey *et al.* (1987) *in vivo*: crowding greatly decreased the expected salt dependence of the *lac* operator function. According to this interpretation, crowding-effects *in vivo,* at any external osmolarity, caused very large increases in the affinity of *lac* repressor for both specific and non-specific binding sites in DNA. Hence, under crowded conditions, an increase in internal salt concentration caused by external shifts in salt levels will not cause significant dissociation of *lac* repressor from DNA binding sites – even though such salt concentrations readily cause dissociation in dilute solution conditions *in vitro*. If essentially all protein is bound, then the expression of *lac* operator is largely controlled by the salt-independent ratio of specific to non-specific DNA binding sites (Zimmerman and Trach, 1991). The interpretation of the second group (Cayley *et al.*, 1991) was based upon the decreased cytoplasmic volumes observed as extracellular salt concentrations were raised. The decrease led to increased concentrations of cytoplasmic macromolecules and hence increased crowding effects. These increased crowding effects were suggested to balance the protein-dissociating effects of increased salt, resulting in approximate salt independence *in vivo*. Estimated changes in the

activity co-efficient of RNA polymerase of at least two orders of magnitude in response to an increase in (RNA + protein) of 275 to 440 mg/ml, yielded final values consistent with the salt independence of RNA polymerase-λP_R promoter interactions observed by Richey *et al.* (1987) and Cayley *et al.* (1991).

1.2.2. Multienzyme complexes.

There are multiplicities of metabolic reactions within the crowded cell. A unique way of visualising the multitude of reactions in the cell was constructed by Alberts *et al.* (1983). Instead of giving the individual reactions, as is usually done in metabolic maps, the reactions were reduced to a line and dot diagram. Each intermediate is a black dot and each enzyme is a line (Figure 1.4). This was used to help reveal the fact that that many intermediates had multiple fates and interactions. Calculations were made in the following way. The chart contained about 520 intermediates. The intermediates were classified by the number of enzymes acting on them. A dot connected to only one line is either a nutrient or an end-product. A dot connected with two lines is an intermediate with just one fate in metabolism. A dot with three lines has two metabolic fates and so on. The analysis is summarised in Table 1.2. This indicated that about 80 % of the shown metabolic intermediates have just one use in the cell. It appeared that it might be a wasteful process if these intermediates each had to fill the water volume of the cell to attain its operating concentration. Consequently, it was suggested that, if the solvation capacity of the cell was a limiting factor in cell structure, a viable strategy during

evolution was the formation of sequential multienzyme complexes and their attendant benefits (Srere, 1987).

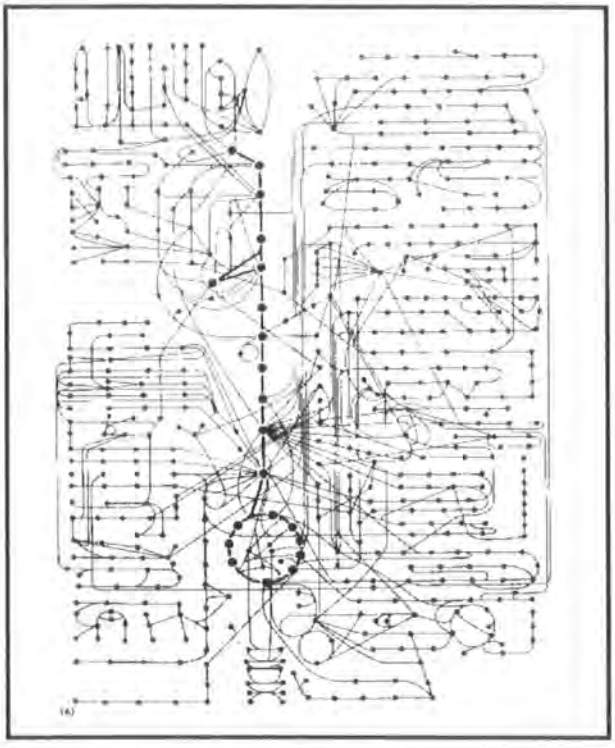


Figure 1.4. Schematic representation of about 500 related metabolic reactions. Black dots represent intermediates, lines represent enzymes (Alberts *et al.*, 1983).

Table 1.2. The number of fates of the intermediates of metabolism. Calculated by Srere (1987) from the metabolic map of Alberts *et al.* (1983) - Figure 1.4

Lines		**Dots**
1 or 2		410
3		71
4		20
5		11
6 or more		8

Assuming that most of the cytoplasm is crowded it may be logical to assume that a mature, functioning protein molecule within the cytoplasm may exist as part of a complex rather that as a free-floating moiety. Consequently, it would have a greatly reduced rate of long-range diffusion. However, diffusion of small molecules is affected only slightly by marcomolecular crowding (Zimmerman and Minton, 1993; Elowitz *et al.,* 1999). Therefore, metabolism may be expected to proceed predominantly by the diffusion of the small-molecule metabolites between the various active sites of the relatively immobile enzymes catalyzing the sequential reactions. In other words, the crowding of the cell does not necessarily mandate the formation of ordered complexes of enzymes catalyzing successive metabolic reactions. However, there is widespread use of multienzyme complexes in fundamental biological process such as DNA replication (Kornberg, 1988) and translation (Elenbaas *et al.,* 1996). Therefore, one may postulate that there was an evolution towards the co-localisation of sequential enzymes. The widespread use of multienzyme complexes may have evolved because of the potential benefits of multisubunits over a large single polypeptide (Klotz, 1975). Such advantages include that they are more economical to build (i.e. to use simple sub-units rather than code for multiple copies of coding information of an oligomer). Translation of a large protein could be more error prone and replacing a faulty sub-unit can more easily eliminate errors. The sub-units could also diffuse and assemble throughout the cell more easily, allowing faster diffusion and hence sub-compartmentalisation. They may also protect very unstable, very reactive or very unreactive intermediates e.g. tryptophan synthase. Multienzyme complexes can be: 1) *static*, the complex can exist in the absence of the intermediate metabolite or 2)

dynamic, the complex can only exist when the intermediate metabolite is bound or 3) catalytically induced enzyme associations (Mendes *et al.*, 1995; Spivey and Ovadi, 1999).

1.2.3. Metabolic channeling.

The consecutive enzymes of static, dynamic or catalytically induced complexes that drive metabolism may be able to 'channel' their intermediate from one enzyme to the next. The channeling of a substrate is defined as the transfer of the substrate between consecutive enzymes of a metabolic pathway without its release to the bulk solvent. In other words, it does not equilibrate with the surrounding medium. Channeling also implies restricted access to exogenous intermediates. Furthermore, as the intermediate metabolite is available to the next enzyme with a higher probability, overall catalytic efficiency increases without there being a change in the catalytic properties of the individual (though catalytic efficiency may be affected in complexed enzymes) (Srere, 1987). Channeling has the potential to confer several other benefits that include: 1) the protection of scarce or unstable metabolite; 2) isolating intermediates from competing reactions; maintenance of a substrate concentration gradient even in the absence of membrane bound organelles to compartmentalize substrates; 3) enzymes can operate at peak efficiency even if the intracellular concentration of the substrates is below that required for saturation of the enzymes; 4) conserving the scarce solvation capacity of the cell. Channeling may also provide new means of metabolic regulation by modulation of enzyme associations and increased sensitivities to regulatory signals (Mathews, 1993; Ovadi, 1991; Spivey and Ovadi, 1999).

Channelling is not restricted to a specific molecular mechanism. A well-documented mechanism is the 'direct transfer' of covalently bound intermediates by tryptophan synthase of *Neurospora crassa*. This enzyme has a two sub-unit structure that carries out the conversion of indole 3-glycerol phosphate via indole to tryptophan. The intermediate indole is formed more rapidly than it is converted to tryptophan, an effect ascribed to the presence of a tunnel between the active sites of the α and β sub-units of the structure, (Hyde, 1988). Another well documented mechanism are the 'swinging arms' of the multienzyme assemblies such as type I fatty acid synthases, α-keto-dehydrogenases, and polyketide synthases (PKS). The 'swinging arms' are lipoamide in the case of dehydrogenases, and phosphopantetheine in the cases of fatty acid synthases and PKSs. These swinging arms are flexible, long (10 –15 Å) tethers which 'channel' the covalently bound intermediates between successive active sites (Perham, 2000; Tsuji *et al.*, 2001).

The process of channeling can also occur when the active sites of two enzymes are transiently brought into contact with each other, forming an enclosure that permits direct transfer of the intermediate and sterically prevents its escape into the bulk phase. This mechanism, compartmentation without rigid clustering, was addressed in detail by Fredriech (1979, 1987) and remains topical. The notable feature of the hypothesis was that consecutive enzymes may accomplish direct metabolic transfer i.e. channeling, even if their complex is very loose (large K_d) and the rate constants for both association (K_{+1}) and dissociation (K_{-1}) were high. This implied that any individual complex is short lived (residence time small). In other words, the system may be characterised as

two enzymes engaged in random, almost elastic collisions; the slight 'stickiness' of the two enzymes gives rise to a finite residence time. Based on this doctrine, models of different complexity were built by Fredriech (1979) and Fredriech (1987). The first model was direct metabolite transfer in a 'complementary cage'. The following assumptions were made: (a) the contact areas of functionally adjacent enzymes, designated as foreign recognition sites, are located in the vicinity, or even around, the active site. Hence, in the complex the two active sites become juxtaposed, so forming the 'complementary cage'. In spite of the high K_d, the affinity towards adjacent enzyme(s) is significantly greater than toward functionally irrelevant enzymes in the same macrocompartment. (b) the frequency of productive i.e. complex-forming collisions between E_1 and E_2 is at least as high as the frequency of product release from the first enzyme, viz. the rate of catalytic reaction. The combination of the above two conditions may result in metabolite compartmentation as follows. If the formation of complementary cages were properly timed, the release of product from E_1 would occur into the cage where its diffusional freedom is severely restricted and the metabolite will bind to its active site of E_2 with a high probability. As this system is dynamic, there will always be free active sites on E_1 to pick up its substrate. As collision frequencies are statistical, not all products from E_1 would be expected to be channelled this way. However, if E_2 was in excess, there may be little chance for any E_1-product to go astray.

The second model proposed was 'alternating complementarily' in an enzyme sequence. In a metabolic sequence consisting of three steps, catalysed by $E_1E_2E_3$, both the

substrate and product of E_2 can be compartmented if E_2 possesses foreign recognition sites for E_1 as well as E_3. This may occur if the enzyme structure is rigid, but it seems more feasible if E_2 undergoes conformational change during catalysis. Three assumptions were made: 1) the intermediate enzyme, E_2, can exist in three different conformations E_0, E_S and E_P; 2) the foreign recognition sites on E_2 for E_1 and E_3 are created when the enzyme assumes conformations E_S an E_P respectively; 3) transition into one or the other conformation is promoted by both appropriate metabolite and enzyme partner. The model asserted that the appropriate ligand (metabolite or enzyme) promoted conformational adjustment in an induced-fit manner. An important consequence was predicted to follow from the adjustment of conformation by the partner enzyme. That is, if state E_2 plus substrate was induced by E_3, then E_3 may energetically contribute to the completion of the catalytic cycle, which in turn meant that complex formation between the two enzymes would trigger the release of the substrate. Such an arrangement was suggested to increase the efficiency of metabolite channeling.

Finally, it is possible that just the close proximity of active sites of two enzymes can provide a substantial channeling in addition to the random diffusion path. In principle proximity of active sites might be sufficient for channeling between the sites on soluble multifunctional enzymes, though it is likely that local interactions are also important in retaining at least a portion of the intermediates from escaping into the bulk phase. A specific example of such a process is electrostatic channeling, which uses the favorable electrostatic field between adjacent enzyme sites to constrain a significant fraction of

the intermediate within the channeling path. This occurs although the active sites are 40 to 60 Å apart. Brownian dynamic simulations demonstrate that this is much too far apart for channeling by random diffusion mechanisms alone. The correlation of experimental and theoretical studies has helped establish this mechanism (Shatalin *et al.*, 1999).

In summary, several different general mechanisms are involved in substrate channeling. First in the manner by which the enzymes interact with each other, and second, in the mechanisms providing the channeling once the enzymes associate. In addition, the fraction of the reaction flux going by the channeled path is virtually 100 % for some of the channeling processes, but significantly less for others. The latter are called "leaky channels" (Spivey and Ovadi, 1999).

1.3. Evidence that the components of type II FAS are associated within a multienzyme complex.

1.3.1. Metabolic channeling of type II FAS.

Roughan and Ohlrogge (1996) first presented evidence of metabolic/substrate channeling from permeabilised chloroplasts of spinach and pea. Based on this observation of channeling, they suggested that the componenets of type II FAS might be organised into a multienzyme complex. In that study, isolated spinach chloroplasts were permeabilised by transferring them from 330 mM sorbitol to a range of hypotonic

media. In the hypotonic medium, the organelles become swollen and the envelopes ruptured. These permeabilised chloroplasts expanded to approximately twice the volume of intact chloroplasts and lost about 50 % of their stromal proteins into the medium. Many functions of the chloroplast ceased, for example, CO_2 fixation was reduced by 80 % as was indicated by various parameters, such as the total loss of bicarbonate-dependent oxygen-evolving activity. However, fatty acid synthesis, as measured by the incorporation of [1^{-14}C] acetate into long chain fatty acids, still occurred. The measurements revealed that, in spinach, fatty acid synthesis increased as sorbitol concentration decreased, being greater at 66 mM (hypotonic medium) than 330 mM. In pea shoot, synthesis increased as the concentration of sorbitol decreased from 330 mM to 230 mM, and was about 70 % of maximum at 66 mM. In other words, isolated and permeabilised spinach and pea chloroplasts (from expanding spinach leaves and pea shoots) were capable of high rates of fatty acid synthesis from acetate.

In this system of permeabilised chloroplasts, three sets of results led to the suggestion that channeling was occurring. In the first set, results indicated that in 66 mM and 33 mM sorbitol there was no significant utilisation of acetyl-CoA and malonyl-CoA. In this method, known as isotope dilution (Spivey and Ovadi, 1999), it was found that in the permeabilized chloroplasts, neither [1^{-14}C] acetyl-CoA nor [1^{-14}C] malonyl-CoA (0.05 - 0.2 mM), could be significantly incorporated into chloroplast fatty acids nor did they inhibit acetate incorporation into fatty acids. Therefore, the mechanism of substrate channeling was invoked to explain the lack of interference from acetyl-CoA

and malonyl-CoA during fatty acid synthesis from acetate. The second set of results, to suggest channeling, was observed when the effect of adding cofactors was tested by adding ACP, ATP, CoA, NAD and NADPH to a basal reaction medium. The rate of synthesis was unaffected. Therefore, fatty acid synthesis in permeabilised chloroplasts was independent of exogenous cofactors. The results are presented in Table 1.3. The third result to suggest channeling stemmed from the fact that the putative complex of enzymes appeared to have compartmentalized endogenous intermediates. In other words, as swollen chloroplasts would render the membranes permeable to small molecules, such as ACP, they might be expected to leave the organelle, however, this had not occurred, because the rate of fatty acid synthesis was unaffected.

In addition to these findings, results indicated that the enzymes required for the synthesis of oleate from acetate might be an integral part of the type II FAS complex. The products of acetate incorporation from isolated chloroplasts incubated in 66 mM or 330 mM sorbitol were examined. Two facts were established. Firstly, glycerol 3-P, which had to be added, and Triton X-100 reduced acetate incorporation into unesterified fatty acids while increasing incorporation into glycerides in both intact and disrupted chloroplasts. Secondly, there was a marked increase in the ratio of unsaturated to saturated fatty acids in 66 mM sorbitol compared to 330 mM.

The light dependency of acetate incorporation (Roughan and Ohlrogge, 1996) of this putative complex indicated a link with the thylakoids which could, in theory, channel metabolites such as ATP and NADPH, the synthesis of which are also light-dependent,

to type II FAS. This hypothesis may be supported by the findings of Sasaki *et al.* (1997). In that study, it was found that partially purified pea plant ACCase could be

Table 1.3. Effect of fatty acid synthase cofactors on acetate incorporation into chloroplasts incubated in 66 mM sorbitol (Taken from Roughan and Ohlrogge, 1996).

Treatment	Acetate Incorporation nmol $h^{-1}mg^{-1}Chl$	
	Spinach	Pea
No cofactors, 330 mM sorbitol	1010	640
No cofactors, 66 mM sorbitol	1235	529
Cofactors, 66 mM sorbitol	1000	518
Cofactors, 33 mM sorbitol	495	144
Cofactors, 66 mM sorbitol, dark	21	17

The rate of fatty acid synthesis in spinach chloroplasts was unaffected by the addition of cofactors. Basal reaction medium: 330 mM sorbitol, 25 mM HEPES/NaOH pH 8, 10 mM $KHCO_3$, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4 and 0.2 mM [1-^{14}C] acetate (10 Ci/mol. Cofactors added were: MgATP (2 mM), CoA (0.25 mM), NAD(+) (0.01 mM), NADPH (0.2 mM) and ACP (10 μM). The 10 - minute reactions were stopped by adding methanolic KOH. The long chain fatty acids were recovered following saponification and were purified by TLC before scintillation counting.

activated by the reducing powers of thioredoxin, a transducer of the redox potential during photosynthesis. Therefore, these authors also suggested that there might be a direct link between the photosynthetic apparatus and fatty acid biosynthesis.

A subsequent study by Roughan (1997) provided 'classic' evidence for substrate channeling i.e. a clear discrepancy between substrate concentrations known to exist *in situ* and those required to drive individual enzymes at rates commensurate with the rate of that overall process (Ovadi, 1991). Specifically, it was found that the stromal concentrations of CoA and it esters were insufficient to account for measured rates of chloroplast fatty acid synthesis. This work is described below, However, it must be borne in mind that the rates used for calculations were taken from *in vitro* studies on isolated enzymes and these may have different characteristics *in vivo*. Using isolated, intact chloroplasts from expanding leaves of spinach and pea shoots, total CoAs were obtained by two different acid extraction methods and two different solvent extraction methods. This yielded a 90 %, 80 % and 80 %, recovery for acetyl-CoA, malonyl-CoA and CoASH (as determined by HPLC) respectively. The concentration of total CoA's in freshly isolated spinach and pea chloroplast was calculated to be 10-20 μM (assuming a stromal volume of 66 μl/μg of chlorophyll). The acetyl-CoA was found to account for at least 90 % of the total CoAs in freshly isolated chloroplasts. As the concentrations of fatty acid substrates in the intact chloroplasts were so far below the K_m for the various enzymes (Table 1.4) this indicated channeling had to be occurring to sustain rates of fatty acid biosynthesis. For example, the greatest concentration of malonyl-CoA measured in pea during fatty acid biosynthesis was 4.5 μM, yet the K_m for the MCAT enzyme purified from spinach was 400 μM. The V_{max} for the reaction becomes irrelevant with the realization that total stromal concentrations of malonyl-CoA during fatty acid synthesis may be several orders of magnitude less than the K_m for the MCAT reaction *in vitro*. Therefore, the calculated rate of malonyl-ACP

formation would be 'negligible' (see Table 1.4) compared with rates of acetate incorporation into fatty acids. In conjunction with the abnormally high K_m values reported for malonyl-CoA and ACP in the MCAT reaction *in vitro* (Hoj and Svendsen,

Table 1.4. Consequences of the concentrations of CoA and CoA derivatives in intact spinach or pea chloroplasts during fatty acid synthesis from acetate, and the calculated effect on the rates of fatty acids (Roughan, 1997).

CoA ester	Stromal concentration (μM)	Appropriate enzymatic reaction	Calculated rate of fatty acid synthesis at substrate concentration (% of acetate incorporation)
[CoASH]	<0.1-0.5	Acyl-CoA synthase	10-20%
[Acetyl-CoA]	10-15	ACCase	2-5%
[Malonyl-CoA]	0.1	MCAT	negligible
Abbreviations: ACCase: acetyl-CoA carboxylase; MCAT: malonyl-CoA:ACP transacylase.			

1983), this led to the suggestion that, *in situ,* the MCAT enzyme resides within a multienzyme complex. A complex where localized concentrations of substrates can be much higher than those revealed by Roughan (1997).

The results from the studies by both Roughan and Ohlrogge (1996) and Roughan (1997) led to the concept that type II FAS exists as part of a multienzyme assembly that may preferentially channel acetate for incorporation into fatty acids. The complex

might sequester pools of cofactors such as ATP, ACP, CoA and nicotanimide as indicated by the independence of fatty acid synthesis of permeabilised chloroplasts from such cofactors. As the end-products of fatty acid synthesis in permeabilised and intact chloroplasts were the same, the type II FAS multienzyme complex appeared to include enzymes required for the synthesis of oleic acid. Thus, the complex could, in theory, include enzymes such as acetyl-CoA carboxylase and the enzymes of type II FAS including stearoyl-desaturase and oleoyl-ACP thioesterase.

1.3.2. The co-purification of components of type II FAS of *B. napus.*

The type II FAS complex is defined by its easily separable components (Shimikata and Stumpf, 1983). However, there is evidence in the literature of co-purification of some of the enzymes of type II FAS of plants. These enzymes include β–hydroxyacyl–ACP dehydrase (β-HAD), enoyl reductase (ENR), stearoyl-ACP desaturase (DES), and oleoyl-ACP thioesterase (TE). This means that even *in vitro* there may be an association occurring between the essential terminal enzymes of *Brassica napus.* Evidence of this association of type II FAS enzymes came form the work to purify ENR from *Brassica napus* seed. A 100 K supernatant fraction was passed down a DEAE column and an ACP-affinity column. The DEAE binds thioesterase very tightly; consequently thioesterase would be missing from fractions with affinity towards ACP. The columns were treated with increasing salt concentrations. The retained fraction was eluted with 0.3 M potassium phosphate (pH 6.8) and found to have both ENR and stearoyl-ACP desaturase activity, indicating the enzymes had co-purified through two

chromatographic steps. On fractionating the pooled fractions by SDS-PAGE, a 34 kDa component, a minor component of 37 kDa and higher molecular weight bands at 64 and 90 kDa were obtained. The use of N-terminal sequencing revealed the 34 kDa band as the ENR tetramer subunit (Table 1.5, Kater *et al.*, 1991). Mckeon and Stumpf (1982) when attempting to purify stearoyl-ACP desaturase from developing safflower seeds used ACP-affinity chromatography but did not report co-purification of desaturase and ENR i.e. their SDS-PAGE analysis revealed no such bands. However, they did not assay for ENR activity, therefore its presence cannot be ruled out from those experiments.

Table 1.5. Purification procedure for stearoyl-ACP desaturase and enoyl-ACP reductase from *Brassica napus* seeds* (Kater *et al.*, 1991).

Fraction	Total Protein (mg)	Total Activity		Specific Activity		Purification (fold)		Recovery of activity (%)	
		A (mu)	B (u)	A (mu/mg)	B (u/mg)	A	B	A	B
Crude Extract	158	4.8	190	0.03	1.2	1	1	100	100
DEAE-Sepharose	60.2	13.4	154.2	0.22	2.6	7.3	2	279	81
ACP-Sepharose	0.18	0.9	45.1	5	250.6	167	209	19	24

* Starting from 30 g of immature seeds (5 weeks after flowering). A: stearoyl-ACP desaturase; B: enoyl-ACP reductase; mu: milliunits; u: units.

The strongest biochemical evidence for an association of type II FAS enzymes came from experiments to purify acyl-ACP thioesterase from *Brassica napus* to

homogeneity. During the purification of the acyl-ACP thioesterase from *Brassica napus*, a 38 kDa and a 33 kDa polypeptide were consistently identified on SDS-PAGE as associated with the thioesterase activity. Two other bands at 20 kDa and 34 kDa were also present. Furthermore, they found that under *native conditions*, the four molecular weight species would remain together through four additional steps, eight in total. When an antibody was prepared against the 38 kDa component, it also recognised the 33 kDa polypeptide in highly purified (2,000 fold) preparations. Subsequent immunoblotting of a crude extract identified one band at 38 kDa consistent with the 33 kDa component being a degradation product generated during purification. Other immunoblots and N-terminal sequencing revealed that the 34 kDa band was ENR. In other words, thioesterase and ENR co-purified. Additional N-terminal sequencing was unsuccessful in identifying the 20 kDa band. (Table 1.6; Hellyer *et al.*, 1992).

Table 1.6. Purification of acyl-ACP thioesterase from developing seeds of *Brassica napus*. * Protein determination by amino acid analysis (Hellyer *et al.*, 1992).

Fraction	Total Protein (mg)	Total Activity (milliunits)	Specific Activity (mU/mg)	Recovery of activity (%)	Purification (-fold)
Crude Extract	2586	226.8	0.09	100	1.0
$(NH_4)_2SO_4$ 30-50%	1408	160.7	0.11	71	1.3
Q-Sepharose	390	118.1	0.3	52	3.4
ACP-Sepharose	3.15	23.2	7.36	10	84.6
Mono-P	n.d.	11.9	n.d.	5.2	n.d.
Superose 12 gel filtration	0.024*	5.1	213.75	2.3	2457

The 20 kDa N-terminal sequence was recently identified as that of β-dehydrase, from comparison of the newly available partial amino acid sequence of β-HAD. In this work, the amino acid sequence derived from an *E. coli* β-hydroxyacyl ACP dehydrase (Fab Z) was used to search the expressed sequence tag (EST) database (dBEST). A homologous sequence from *Ricinus communis* was identified which showed 45 % homology to the *E. coli fabZ* sequence. Subsequently, the EST was amplified from a developing castor endosperm cDNA library and the amplified product used to screen a developing *Brassica napus* embryo library. A number of clones were identified on the primary screen and were purified. One clone contained a single open reading frame that showed 40.4 % homology to the EST sequence used as probe. Analysis of the amino acid deduced from SD4 revealed that the sequence contained a putative chloroplast target sequence at the N-terminus. Calculation of the cleavage site of the target sequence indicated that the mature protein of 19 kDa, which was similar in size to a β-hydroxyacyl-ACP-dehydrase previously purified form *Spinacia oleracea* (Doig, 2001).

Finally, it has been demonstrated separately that two enzymes involved in *Brassica napus* fatty acid biosynthesis, ENR and TE would elute from ACP-sepharose under the same conditions, 0.3 M potassium phosphate (pH 8.0). It was subsequently shown that the enzymes co-purify, indicating that either they had similar charge and hydrophobic properties or may have been interacting. In terms of the possible ACP interaction with ENR, it was reported that the nature of conditions for dissociation of enoyl reductase from ACP suggested that the two FAS components might be associated by an ionic interaction (Slabas *et al.*, 1990).

1.3.3. The interactions of *E. coli* ACP with other fatty acid synthase enzymes.

Despite evidence existing for the enzymes of type II FAS being associated, and/or organised into a multienzyme complex, it is only the interactions between *E. coli* ACP and its' fatty acid biosynthetic enzymes that have been characterised in detail. However, the molecular details of the interactions between *E. coli* ACP and its' enzymes of fatty acid biosynthesis are highly relevant to plant systems. This is because both *E. coli* and plants have a type II FAS system whereby the ACP moiety sequentially delivers the lipid intermediates to the active site of each enzyme in the pathway (Roujeinikova *et al.*, 2002). The components of *E. coli* and plant ACP share a high degree of structural homology. For example, Roujeinikova *et al.* (1999) demonstrated that when crystals of enoyl reductase from *Brasscia napus* and *E. coli* were overlapped they had 209 Cα in common, yet it was previously shown that they only have a 35 % sequence identity between them (Kater *et al.*, 1991). Another important aspect is that the ACP of *E. coli* can replace plant ACP in assays of fatty acid synthesis and for the synthesis of acyl–ACP substrates (Harwood *et al,* 1990).

The molecular details of the type II FAS enzyme and ACP-substrate associations have been elusive. This may be because the ACP association must be specific enough to deliver the acyl-chain cargo to the active site of the enzyme but be weak enough in order to allow rapid on and off rates for the substrates. The weak interaction may have made co-crystallization studies difficult. For example, the K_d between ACP-thioesters and FabH (*E. coli* KAS III) is ca. 5 μM (Heath and Rock, 1996). However, the

purification of type II FAS components has allowed crystallization of the components, such as enoyl reductase, from both *E. coli* and *Brassica napus*, allowing the details to begin to be unravelled (Roujeinikova *et al.*, 1999).

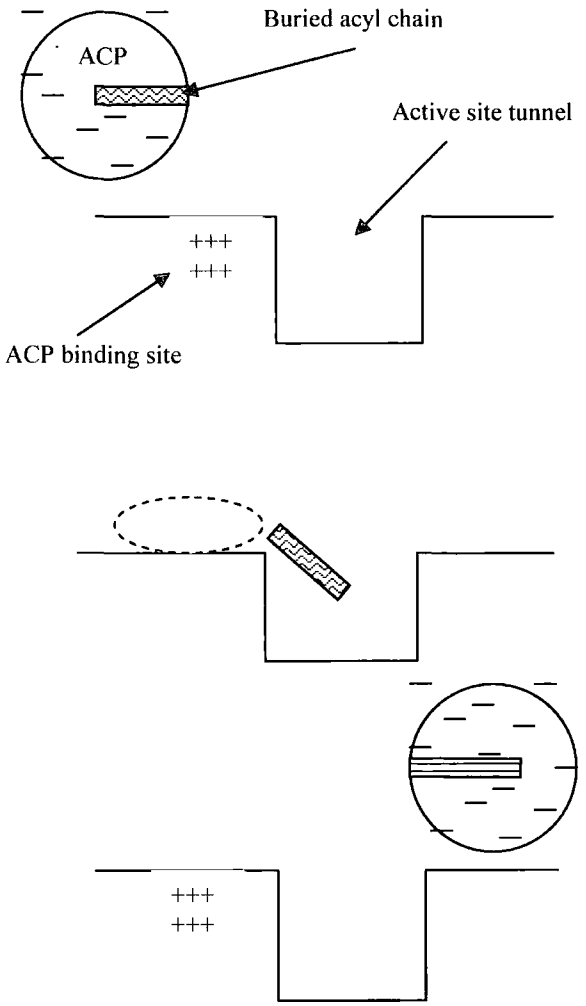
Of great import, is that the first crystal structures of an acylated form of ACP from *E. coli*, that of butyryl-ACP have become available. In that study, computer analysis of the molecular surface of ACP revealed a plastic hydrophobic cavity in the vicinity of the phosphopantetheinylated serine 36 residue that becomes expanded and occupied by the butyryl and mercaptoethylamine moieties of the acylated 4-phosphopantetheine group. When the acyl group was not present the cavity was contracted. Therefore, it was proposed that the protein adopted the conformation after delivery of substrate into the active site of a partner enzyme (Roujeinikova *et al.*, 2002). This hypothesis was supported by earlier computational and biochemical experiments. By comparing results from an algorithm, Surfdock, (which predicted acyl-ACP and FabH binding regions) with biochemical experiments (using spectrophotometric assays and surface plasmon resonance) of mutant and wild type FabH, the acyl-ACP was found to dock to a positively charged hydrophobic patch adjacent to the active site tunnel on FabH. An arginine – 249 residue was critical for the interaction. A conformational change of ACP was proposed to be the key to the facilitation of entry and exit of the acyl substrate into the enzyme pocket. An interesting speculation was that the proposed conformational changes were driven by the release of the acyl group into the FabH tunnel. Furthermore, its removal was a reverse of the conformational change stabilised by the new acyl intermediate. From this, it was concluded that the dynamic ACP binding

process involves three steps. The first step involves the weak but specific interaction between the target protein and the rigid three-helix bundle of ACP and effectively aligns Ser-36 and the prosthetic group with respect to the active site entrance. In the second step, a conformational change in ACP injects the substrate attached to the prosthetic group into the active site. These movements include the unfolding of an extended flexible loop and might be driven by the release of the acyl group from its interactions with ACP after the initial binding step. Finally, the modified substrate is removed from the active site by a reversal of conformational changes and the ACP structure is stabilized through interactions with the new lipid intermediate. This model allowed a way of easily introducing and extracting the prosthetic group from the active site openings without forming a tight complex between the two proteins (Zhang *et al.*, 2001).

Similarly, in the model proposed by Roujeinikova *et al.* (2002), the activated part of the substrate is buried within the carrier protein and thus is not available for interaction with the active sites of the FAS enzymes. This suggested that the initial step of any ACP-dependent reaction would involve specific recognition and binding of the negatively charged ACP by an enzyme rather than recognition of the particular acyl chain substrate. The enzyme-ACP interactions triggering a conformational change in ACP leading to delivery of the substrate into the enzyme's active site. The modified substrate could be subsequently released by the enzyme and reburied in the fold of the ACP. An interesting speculation was that a major function of the protein moiety of ACP might be to shield the acyl substrate attached to its phosphopantetheine arm from

unwanted recognition by the enzymes of other pathways that utilize fatty acyl chains, such as fatty acid degradation (oxidation) or regulatory signaling pathways. In a way, a form of channeling, protection of very reactive intermediates (Roujeinikova *et al.*, 2002).

Both Zhang *et al.* 2001 and Roujeinikova *et al.* (2002) arrived at a similar three-step process of ACP binding and both of their models involved conformational/structural changes of ACP (Figure 1.5). The key difference between them is that their experimental evidence is for different stages of this three-step process. Roujeinikova *et al.* (2002) found physical evidence that ACP would undergo a conformational change on binding of an acyl group. They then proposed that as the acyl chain is buried within the ACP it could not be available for the initial interaction with an enzyme, as such the enzyme must recognize the protein part of the acylated ACP (Roujeinikova *et al.*, 2002). This complemented the earlier results from Zhang *et al.* (2001) revealing a location of an ACP binding site on *E. coli* FabH. However, Zhang *et al.* (2001) could only speculate that a conformation change in ACP would occur, in order to allow the acyl chain cargo to reach the enzyme's active site, a speculation based on the distance the acylated ACP binding site was from the entrance of the FabH tunnel. A schematic diagram indicating the complementary nature of the evidence and models of Roujeinikova *et al.* (2002) and Zhang *et al.* (2001) is given below (Figure 1.5.).



Step 1. Recognition of negatively charged ACP by enzyme (acyl group not recognised).

Evidence from Zhang *et al.* (2001) later proposed as necessary by Roujeinikova *et al.* (2002).

Step 2. Conformational change in ACP to deliver acyl chain cargo.

Zhang *et al.* (2001) postulates that there is a conformational change in FabH. Roujeinikova *et al.* (2002) reveals evidence that ACP does undergo a conformational change when releasing an acyl group.

Step 3. Release of modified substrate.

Zhang *et al.* (2001) proposes removal of acyl group driven by reversal in conformational changes.
Roujeinikova *et al.*, (2002) reveals evidence that ACP undergoes a conformational change when binding to an acyl group.

Figure 1.5. Schematic diagram of a possible three-step process of ACP interacting with a fatty acid biosynthetic enzyme. The complementary nature of the evidence and models from both Zhang *et al.*, (2001) and Roujeinikova *et al.*, (2002) allowed this three-step model to be constructed. **Model of Zhang *et al.* (2001).** Step 1. ACP binding to FabH (*E. coli* KAS III) at the arginine 249 location (evidence from correlation of biochemical and computer based data). Step 2. Conformational change in ACP and FabH to allow ACP to enter active site in FabH tunnel (a theory based on an assumed necessity for ACP to change shape in order to allow the acyl chain to reach the FabH active site). Step 3. Removal of acyl chain by reversal of conformational change. (theoretical). **Model of Roujeinikova *et al.* (2002).** Step 1. Conformational change in ACP when acyl group present and/or absent (evidence from computer analysis of crystal structures of acylated ACP). Step 2. Recognition of the protein moiety of the negatively charged ACP by enzyme (theory based on the fact that the acyl chain would be buried within ACP therefore, it is not available for interaction with enzymes). Step 3. Binding causes conformational change in ACP leading to delivery of acyl chain to enzyme active site (theory based on a necessity to release the acyl cargo buried within ACP).

Taken together, the results of both groups suggest the interactions between ACP and the fatty acid biosynthetic enzymes of type II FAS may be weak, transient, stabilised by the acyl-chain, and as such, are dynamic protein-protein interactions.

ACPs from highly diverse organisms function interchangeably in the *E. coli* FAS II system. For example domains of ACPs from distantly related organisms (*E. coli* and Rhizobium) can be interchanged without affecting the protein's conformation and function (Ritsema *et al.,* 1998). Recently, primary sequence analysis by Zhang *et al.* (2003a) identified a motif that is conserved in ACP family members and found that several amino acids in this region of the protein, aspartate 35, serine 36, leucine 37, glutamine 41, and glutamine 47 were completely conserved in fifty ACP sequences analyzed. Such observations, led Zhang *et al.* (2003a) to predict that enzyme-ACP interactions were achieved through a conserved set of electrostatic and/or hydrophobic contacts. Acyl carrier proteins are asymmetric monomers consisting of four -helices packed into a bundle held together by interhelical hydrophobic interactions (Holak et al, 1988). The conserved acidic residues are arrayed along helix II with serine 36, the site of prosthetic group attachment in *E. coli* (Figure 1.0), located on a loop at the N-terminus of helix II. This conserved region of the protein was postulated to represent a 'recognition helix' that accounts for the ability of the ACPs from widely diverse organisms to interact with target enzymes and to function interchangeably in FAS II systems. Direct support for the 'recognition helix' theory came from the results of the co-crystallation of ACP from *Bacillus subtilis* in complex with AcpS. The contacts between ACP and AcpS were predominantly ionic occurring between the positively

charged residues of AcpS helix 1 and the negatively charged residues of ACP helix 2. The hydrophobic residues on ACP helix 2 (methionine-44 and alanine-45) were complemented by hydrophobic counterparts on AcpS. Thus, the AcpS-ACP structure provided direct support for the hypothesis that the conserved helix 2 ("recognition helix") of ACP functions as a universal protein interaction domain (Parris *et al.*, 2000)

Unlike ACP itself, ACP-interacting proteins do not share a primary signature sequence that defines an ACP binding motif. Therefore, to explore the idea, that the enzymes of the type II system share 3-D surface features that account for their specific recognition of ACP and its thioesters, Zhang *et al.* (2003b) explored the recognition helix hypothesis by examining the protein-protein interactions of *ACP* and FabG (an *E .coli* ketoacyl-ACP reductase). In this instance, FabG was selected because of the availability of the crystal structures of *E. coli* FabG (Price *et al.*, 2001) and the *Brassica napas* FabG•NADP+ binary complex (Fisher *et al.*, 2000). Zhang *et al.* (2003) subsequently used site-directed mutagenesis to determine the residues on FabG required for high affinity ACP binding and protein NMR (nuclear magnetic resonance) spectroscopy to identify the ACP residues that contribute to the formation of the FabG•ACP complex. To specifically test the recognition helix hypothesis, two surface residues, arginine-129 and arginine-172, located in a hydrophobic patch adjacent to the active site entrance on FabG were mutated. Subsequent enzymatic analysis showed that the mutant enzymes were compromised in their ability to utilize ACP thioester substrates but were fully active in assays with a substrate analog. Fluorescent resonance energy transfer (FRET) assays and surface plasmon resonance using BIAcore

equipment) and competitive inhibition experiments showed that the FabG mutant proteins had reduced affinities for ACP. Finally, chemical shift perturbation protein NMR experiments showed that FabG-ACP interactions occurred along the length of ACP helix 2 and extended into the adjacent loop-2 region to involve isoleucine-54. These data confirmed a role for the highly conserved electronegative/hydrophobic residues along ACP helix 2 in recognizing arginine residues embedded in a hydrophobic patch on the surface of its partner enzymes. Thus, the results supported the 'recognition helix' hypothesis. i.e. highly conserved helix 2 of ACP recognizes an electropositive/hydrophobic surface feature adjacent to the active site entrances on the enzymes of type II fatty acid synthesis. Based on these results, Zhang *et al.* (2003b) postulated that the recognition helix hypothesis could explain the interchangeability of the ACPs and type II enzymes from difference species.

Perhaps more interesting to the thesis, was the subsequent question raised: do the individual acyl moieties attached to ACP may alter the protein structure to subtly change the affinity of a particular intermediate for its complementary enzyme? This question was derived from the fact that such alterations in ACP are the basis conformationally sensitive gel electrophoresis (Heath and Rock, 1995). If such a mechanism occurs in type II FAS systems, this may have direct ramifications for the design and interpretation of protein-protein interaction experiments for type II FAS components.

1.4. The *in vitro* analysis of protein-protein interactions.

Cellular crowding can enhance of molecular associations and in doing so stabilise weak, transient protein-protein interactions maintaining the structure of a dynamic multienzyme complex (Chapter 1.2.1; Zimmerman and Minton, 1993; Ellis, 2001). As a consequence, the factor most responsible for dissociation of interactions of weakly associated multienzyme complexes is the dilution of cell contents that usually accompanies cell disruption on purification (Srere and Mathews, 1990). This dilution factor varies from 5 to 10. For example, a hypothetical protein complex has a K_d of 10^{-6} M and components have a total protein concentration of 5 x 10^{-6} M. In the cell excluding other effects, 60 % of the proteins of this hypothetical system are complexed. After a 5-fold dilution, only about 10 % of these proteins are present as a complex (Figure 1.6). Disruption of the cells without dilution is possible, but the gel-like material obtained is difficult to use in ordinary physical seperations such as centrifugations and filtrations. Another consequence of dilution is the reduction of total protein concentration from normal cellular levels (20-30 % in the cytosol and 40-50 % in the mitochondria and chloroplasts) to levels that are one-fifth to one-tenth of normal levels. It has been shown that in concentrated protein solutions (20% and greater), a volume-excluding effect caused a change in the activity co-efficients of interacting proteins such that complex formation is favoured (Wilf and Minton, 1981). Dilution negates this force. As small molecules (metabolites) have a role in the stability of multienzyme complexes, dilution may also cause the loss of stabilising substrates in a dynamic multienzyme complex and thus affect the ability to isolate the holocomplex.

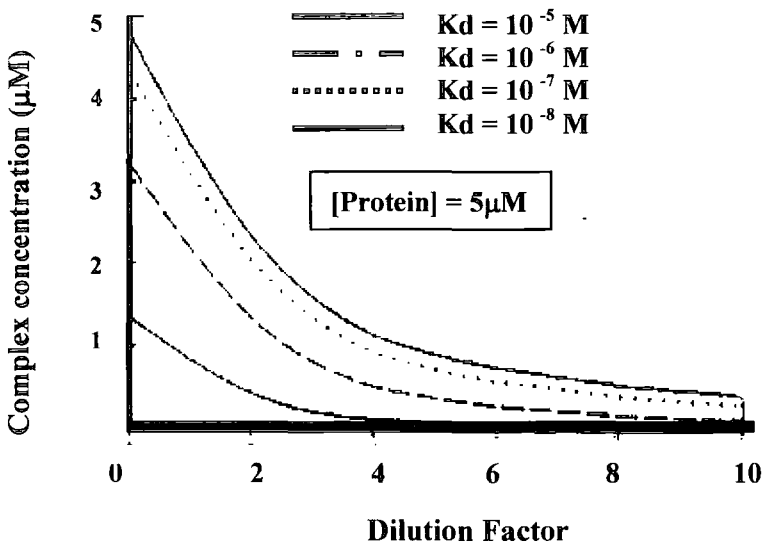


Figure 1.6. Computer-generated curves for the dissociation of a bienzyme (E_1 and E_2) complex. Whereby, $E_1E_2 \Leftrightarrow E_1 + E_2$ (Srere and Mathews, 1990).

Some metabolic pathways use stable multienzyme complexes that are easily identifiable. This includes complexes such as pyruvate dehydrogenase from *E. coli* and the type I FAS from animals, both of which channel intermediates and substrates (Perham, 2000). For example, in animals, including humans, the seven partial activities of the type I FAS and ACP are linked together covalently in a single polypeptide chain encoded by a single gene. The native enzyme has a molecular weight of 544,000 kDa and is composed of two dimers, each containing all of the component activities plus ACP. The dimer formation is essential for FAS function. The organization of these

activities along the multifunctional polypeptide from the N-terminus to the C-terminus is as follows: β-ketoacyl synthase, acetylymalonyl transacylase, β-hydroxyacyl dehydrase, enoyl reductase, β-ketoacyl reductase, ACP, and thioesterase. In addition, these activities are grouped in three separate domains, domain I (DI), domain II (DII), and domain III (DIII). The phosphopantetheine arm of the ACP domain swings between these domains (a form of channeling) (Stoops and Wakil, 1981; Perham, 2000). The preferred mode of interaction is between domains from different subunits of the homodimer, although recent studies with the vertebrate FAS have demonstrated that the ketosynthase and ACP of the same subunit can also interact, albeit with lower kinetic efficiency. The post-condensational reductions and dehydrations are carried out on a single polypeptide. The active sites of the ketosynthase and ACP from opposing monomers can be cross-linked, suggesting that they are within 20 Å of each other (the phosphopantetheine moiety is approximately 20 Å in length). In contrast, fluorescent energy transfer experiments have estimated the ACP-thioesterase and ketoreductase-enoylreductase distance between active sites to be 37 - 48 and > 49 Å, respectively, suggesting that a conformational change is likely to be required for catalysis (Tsuji *et al.*, 2001).

Such results were corroborated by the recent work, using the yeast two-hybrid system, where it was discovered that the catalytically inactive interdomain (ID) regions of type I FAS interact and so may facilitate movement within the complex. In this work, it was shown that the fatty acid synthase activity could not be reconstituted when the ID sequences present in the two recombinant halves were deleted, suggesting that these ID

sequences were essential for fatty acid synthase dimer formation. By using the yeast two-hybrid system, the ID sequences were found to be the only regions of fatty acid synthase monomers that showed significant dimer formation. Therefore, it was proposed that the ID regions, which have no known catalytic activity, associate readily and hold together the two dynamic active centers of the fatty acid synthase dimer, playing an important role in the architecture of catalytically active fatty acid synthase (Subrahmanyam *et al.,* 2001). Thus, in conjunction with the "swinging arm", protein-protein interactions are likely to play an important role in intramodular chemistry within a type I FAS homodimer. The relative contributions of the swinging arm, interdomain interactions, linkers, and active site-substrate interactions to the type I FAS catalytic cycle, as well as their precise mechanisms, remain to be elucidated in type I FAS. However, it is of some note to this work that protein-protein interactions may have a role in the channeling of intermediates in the 'stable' type I FAS.

As evidence is present for more than one mechanism of channeling in 'stable' complexes, it might be logical to assume some facilitating mechanism such as protein-protein interactions would be desirable for dynamic or easily dissociable complexes such as that of *Brassica napus.* However, the evidence in current literature for such direct protein-protein interactions is not strong. A possible reason may be that the forces that arise from cellular crowding and which supported the weak interactions *in vivo* were lost on the massive dilution of plant preparations for *in vitro* studies. As the putative interactions of type II FAS may be dynamic (Chapter1.3.3), another reason might be instability of interactions during purification due to loss of transiently

associated specific substrates (acyl-ACP), again, a loss that occurs on the massive dilution of plant preparations for *in vitro* studies.

1.5. Aims of the thesis.

Type II FAS is composed of separable soluble enzymes but *in vitro,* under native conditions, the *Brassica napus* enzymes ENR, TE and β–HAD have been found to co-purify (Hellyer *et al.,* 1992). In seperate work, ENR and DES have been found to co-purify (Kater *et al.,* 1991). Hence, individual component enzymes may form transient interactions perhaps through shared intermediates. This may be important in functions (such as channeling) and regulation of the pathway. Using the yeast two-hybrid system (an *in vivo* protein-protein interaction assay), this thesis aimed to present evidence to support the idea of association of the components ACP, ENR, DES, and TE of the type II FAS of *Brassica napus.* The *B. napus* ACP was intended as a positive control. A direct interaction was detected between ENR and DES. A possible interaction may have occurred between TE and ENR. The *B. napus* ACP failed to show any interaction with any other enzyme tested. This may have been because the yeast two-hybrid system could not detect the kind of dynamic interactions that ACP may have with other enzymes of fatty acid synthesis (i.e. a false negative result).

Chapter 2. Material and methods.

2.1. Yeast two-hybrid system.

All reagents were supplied by Sigma unless otherwise stated. Where necessary, materials were sterilised by autoclaving for 15 minutes at 121 °C or filter sterilised through a 0.22 micron filter. All water was prepared by autoclaving distilled and purified (Millipore system) water (H_2O). Phenol (AquaPhenol, Appligene Oncor) was equilibrated with Tris-HCl buffer pH 8, 10 mM; EDTA 1 mM. Yeast Nitrogen Base (Difco) was supplied as a powder and was dissolved in H_2O. The chemical 3-aminotriazole (3-AT) was dissolved in H_2O and stored at –20 °C, in containers wrapped in foil to exclude light. Lithium acetate was prepared as a 1 M x 10 stock, pH 7.5, for the yeast transformation. Polyethylene glycol (PEG 4000- actual MW 3350) was dissolved in H_2O to a final concentration of 50 %. Acid-washed glass beads (425 – 600 μm) were prepared by washing the beads with nitric acid (10 M). The washed beads were stored at 4 °C. The GAL4-AD (activation domain) and GAL4-DBD (DNA binding domain) monoclonal antibodies were supplied at a concentration of 2.1. μg/μl (20 μg total) and 2.74 μg/μl (25 μg total), respectively in storage buffer (Clontech, UK). These were shipped in dry ice and immediately stored in 2 μl aliquots at –20 °C.

2.1.1. Antibiotics, X-gal stocks and media.

Ampicillin (Amp) solution (50 mg/ml in H_2O) and cycloheximide (Cyhx) solution (1 mg/ml in H_2O) were filter sterilised through a 0.22 micron filter. Tetracycline (Tet)

was dissolved in 50:50 ethanol:H_2O to give a stock solution with a concentration of 12.5 mg/ml. Isopropyl-[β]-D-thiogalactopyranoside (IPTG) (100 mM stock – 238 mg/10 ml H_2O) was filter sterilised through a 0.22 micron filter.

Luria Broth (LB) broth (10 g/L Bacto-tryptone (Difco), 5 g/L Bacto-yeast extract, 5 g/L NaCl in H_2O, pH 7.0) was made weekly. The broth was autoclaved and stored at room temperature. Where necessary, ampicillin was added to a final concentration of 50 μg/ml. For LB/amp plates, agar at 18 g/L was added. Plates were covered and stored at 4 °C.

Complete liquid media for yeast (YPD) was prepared as follows: 20 g/L peptone, 10 g/L yeast extract, H_2O, pH 5.7. The media was autoclaved and stored at 4 °C. For YPD plates, agar at 18 g/L was added. Plates were stored at 4 °C. For YPD containing cycloheximide, the YPD was prepared as described and allowed to cool to 55 °C and cycloheximide stock solution added. The cooling was necessary, as cycloheximide is heat-labile. The amount added depended on the yeast strain used, whereby CG1945 and Y190 will grow in the presence of cycloheximide at concentrations of 1 and 10 μg/μl, respectively. Synthetic dropout (SDO) selection media for yeast, these lacked the appropriate nutrient(s) to ensure plasmid selection and, in the case of '–His medium', selection for a two-hybrid interaction. SDO medium contained 2 % glucose as the carbon source for optimal growth, and SDO supplements. The following abbreviations refer to specific formulations of the various SD media used in the work: SD/–Trp (minus tryptophan), SD/–Leu (minus leucine), SD/–His (minus histidine). A combination of

these was used where appropriate. For 3-aminotriazole (3-AT) containing medium: the concentration of 3-AT used in the medium depended on the yeast strain. Whereby, CG1945 and Y190 require concentrations of 5 mM and 25 mM 3-AT, respectively, to prevent leaky expression of histidine and, as a consequence, prevent residual growth of the yeast. The 3-AT is heat-labile and was added after the SDO medium was autoclaved and cooled to ~ 55 °C.

2.1.2. Plasmids.

The Clontech Matchmaker II vectors pAS2-1 and pACT2 provided are shuttle vectors that can replicate in yeast and *E. coli* (Figures 2.0 and 2.1). They each contain a unique multiple cloning sites (MCS) which allows for cloning of the cDNA of the protein under investigation. These proteins were expressed fused with either the GAL4 transcription factor DNA binding domain (DBD) or activation domain (AD) when expressed from pAS2-1 or pACT2, respectively. An important development of the vectors pAS2-1 and pACT2 (derived from pACT1 and pAS2 respectively) was a more adaptable multiple cloning site allowing cDNA inserts to be cloned from one vector to another and remain in frame. The full length ADH1 promoter (pAS2-1) or a truncated version of it (pACT2) adjacent to a section of pBR322 (which acts as a transcriptional enhancer in yeast) was used to drive expression of the fusion proteins (Tornow and Santaneglo, 1990). The use of a full-length ADH1 promoter or the truncated-ADH1/pBR322-enhancer combination provided high and medium expression of the fusion proteins respectively (Ammerer, 1983; Clontech data).

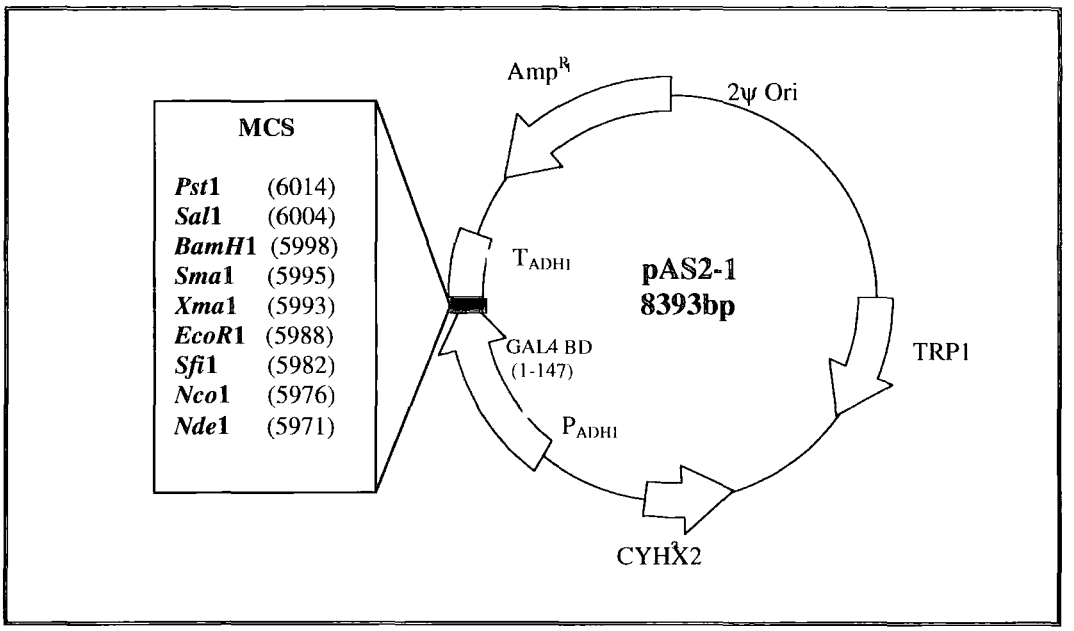


Figure 2.0. Vector map of the DNA binding domain (DBD) plasmid pAS2-1. (Genbank Accession number #U30497). Unique sites are shown in bold. The vector was supplied by Clontech, UK.

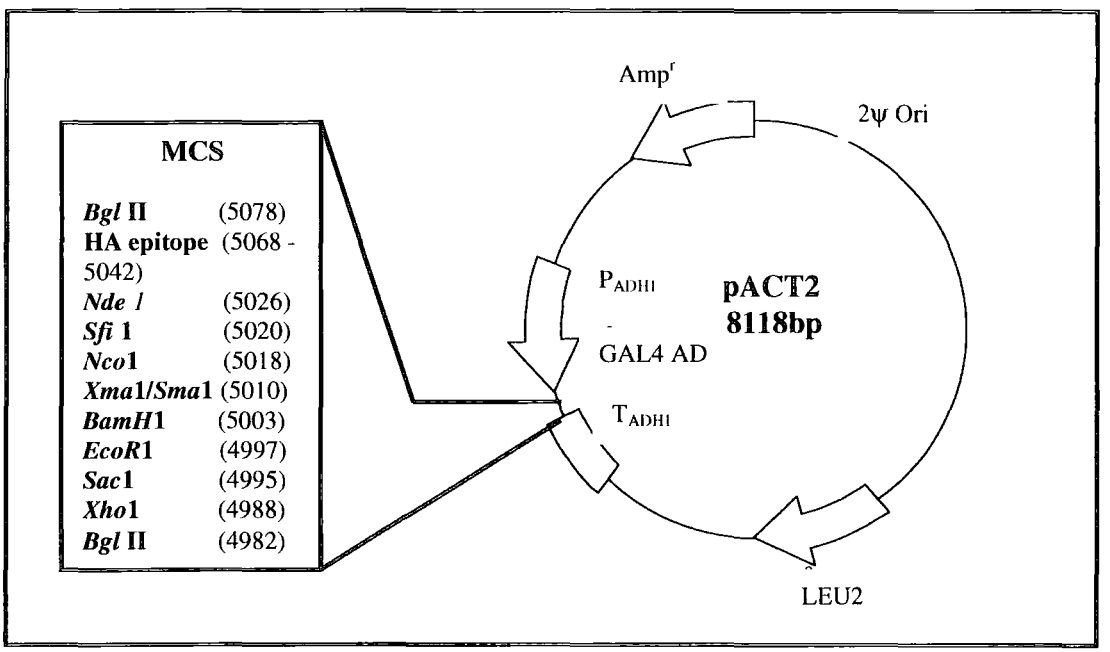


Figure 2.1. Vector map of the activation domain plasmid (AD) pACT2. Unique sites are shown in bold. (Genbank Accession number #U29899). The vector was supplied by Clontech, UK.

2.1.3. Bacterial Strains.

Cloning was carried out in *E. coli* DH5α . The genotype for DH5α is as follows: ϕ80d*lacZ*ΔM15, *rec* A1, *end* A1, *gyr* A96, *thi*-1, *hsd* R17, (r_k-,m_k^+), *sup* E44, *rel* A1, *deo* R, Δ(*lacZ*YA - *arg*F)U169. Strain DH5α containing plasmids were maintained by streaking onto a LB plate containing ampicillin or other appropriate antibiotic. These plates were incubated overnight. For liquid cultures, 5 ml or 50 ml of LB broth and the appropriate antibiotic were inoculated with an isolated colony from an LB plate and incubated at 37 °C for 8 hours to overnight with rotation at 250 rpm on an orbital shaker. For glycerol stocks, glycerol was added to a final concentration of 20 % and 1 ml aliquots were frozen at –80 °C until use.

2.1.4. Yeast strains.

Yeast strains Y187, CG1495 and Y190 (Clontech, UK) were stored at –80 °C as glycerols. Details of these strains are provided in Table 2.0. Yeast strains were streaked on YPD plates with appropriate antibiotic and incubated at 30 °C. For liquid cultures, several yeast colonies, 2–3 mm in diameter were resuspended in 1 ml of YPD or SDO. This was used to inoculate 5 ml or 50 ml of YPD broth or SDO media with the appropriate antibiotic. Yeasts were incubated at 30 °C at 200 rpm on an orbital shaker until the appropriate OD_{600} was obtained. The phenotype of the yeast strains provided was checked before using them in interaction assays. From frozen glycerol stocks, a fresh working stock plate of each strain was made. To recover a strain, a small amount of cells was scraped from the frozen stock with a sterile loop or wooden stick and

streaked onto YPD plates. The yeast strains were propagated on the YPD plates by incubating cells at 30 °C for 3–5 days until colonies appeared. Additional cultures were propagated only from isolated colonies on this plate. Yeast strains were then tested for appropriate nutritional requirements as described in Chapter 3. Interaction assays only proceeded if the strain had the expected phenotype. Colonies from the verified working stock plate were used to inoculate liquid cultures for preparing competent cells for subsequent transformation. The verified working stock plate(s) was sealed with Parafilm and stored at 4 °C.

2.1.5. Cloning of the cDNA of ACP, ENR, TE, and DES into vectors pAS2-1 and pACT2.

Acyl carrier protein (ACP), Genbank Accs. nos. X13128 (Safford et al., 1988), was cloned from the vector pBR322/SK$^+$ (Boehringer Manheim). Enoyl-ACP reductase (ENR) Genbank Accs. nos. X95462 (direct submission, Fowler, A., Fawcett, T., and Slabas,T. 1996), was cloned from the T7 expression vector pET11d (Promega). Stearoyl-ACP desaturase (DES), Genbank Accs. nos. X63364 (Slocombe et al, 1992), was cloned from the T7 expression vector pET13b (Promega). Oleoyl-ACP thioesterase (TE), Genbank Accs. nos. X73849, was cloned from the vector termed 'pNL2' (Loader *et al.*, 1993). Further details of the plasmids used in the yeast two-hybrid system are described in Chapter 3.

Following isolation of these plasmids from their host, the cDNA of ACP, ENR, TE, and DES, were directionally cloned into the vectors pAS2-1 or pACT2. Clones for the yeast two-hybrid system were created first, by attaching restriction sites to the cDNA via PCR.

The plasmid and insert cDNA were then digested with the appropriate restriction enzyme and ligated together. These plasmid constructs were used to transform *E. coli* DH5α

Table 2.0. The reporter genes and transformation markers of the three yeast strains provided in the Clontech Matchmaker II yeast two-hybrid kit. CG1945 is a derivative of HF7c, the *LYS2* gene is non-functional. **References**: 1) Harper *et al.* 1993; 2) Feilotter *et al.* 1994; 3) Harper *et al.* 1993.

Strain	Genotype	Reporter Gene(s)	Nutritional Marker	Mating Type	Uses
[1] Y187	MATα, *ura*3-52, *his*3-200, *ade* 2-101, *trp*1-901, *leu*2-3, 112, Gal4Δ, *met*, *gal80*Δ URA3::$GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ*	*lacZ*	*trp*1, *leu*2	MATα	Two hybrid test of known proteins. Quantitative β-gal assay. Mating partner with CG1945 or Y190.
[2]CG1945	MATa, *ura*3-52, *his*3-200, *lys*2-801, *ade*2-101, *trp*1-901, *leu*2-3, 112, Gal4-542, *gal*80-538, *cyh*^{r}2, LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3 URA3::$GAL4_{17mers(x3)}$-$CyC1_{TATA}$-*lacZ*	HIS3, *lacZ*	*trp*1, *leu*2, CYHX2	MATa	Two hybrid library screening. Highly sensitive HIS3 reporter. Cycloheximide counter selection.
[3] Y190	MATa, *ura*3-52, *his*3-200, *lys*2-801, *ade*2-101, *trp*1-901, *leu*2-3, 112, Gal4Δ, *gal80*Δ, *cyh*^{r}2, LYS2::$GAL1_{UAS}$-$HIS3_{TATA}$-HIS3 URA3::$GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ*	HIS3, *lacZ*	*trp*1, *leu*2, CYHX2	MATa	Two hybrid library screening. Quantitative β-gal assay. Cycloheximide counter selection.

2.1.5a. DNA isolation.

Small-scale extractions of plasmid DNA (mini-preps) were performed using the Hybaid DNA isolation kit, as per the manufacturer's instructions. Larger scale extractions (midi-preps) were performed using the Qiagen Midi-kit, as per the manufacturer's instructions. Alternatively, DNA was isolated using phenol/chloroform/isoamylalcohol 25:24:1 (PCI) followed by ethanol precipitation. The PCI was added to the biological sample contained in a 1.5 ml micro-centrifuge tube and the tube vortexed for 15-30 seconds. The sample was centrifuged for 5 minutes at room temperature and the upper, aqueous layer was removed to a clean tube. The DNA was precipitated out of solution by adding two volumes of cold 95 % ethanol and one-tenth of a volume of 3 M NaOAc (pH 5.2). The mixture was placed at –70 °C for at least 30 minutes or at –20 °C overnight. The sample was then spun at 12 000 rpm for 15 minutes and the supernatant decanted. The DNA pellet was washed with 70 % ethanol to remove residual salt and moisture, then air dried for about 5-10 minutes. DNA was dissolved in 10 mM TE buffer (pH 7.6-8) or H_2O. In larger scale isolations, such as midipreps, the DNA was aliquoted out to avoid repeated freezing and thawing.

2.1.5b. Polymerase chain reaction (PCR).

PCR was used for attaching directional cloning sites to the cDNA of ACP, ENR, TE, and DES and to identify the presence of these cDNA from transformed yeast. Deoxynucleotides (dNTPs, 100 mM) were supplied by MWG–Biotech and were stored

at –20 °C. A working stock of nucleotides (10 mM) was made by dissolving each dNTP in the appropriate volume of H_2O. *Taq* polymerase (MBI Fermentas) and *Vent* polymerase (New England Biolabs) enzymes were stored at –20 °C in the appropriate x10 storage buffer. The manufacturer of the enzymes supplied a x 10 PCR reaction buffer. The basic PCR conditions are given in Table 2.1. Any variation from this basic procedure is described in the appropriate section. The reaction tubes were overlaid with mineral oil. Reactions were carried out using a Stratgene Robocycler or Perkin Elmer cycler.

Table 2.1. Basic PCR conditions used for amplification of DNA fragments.

Component	Final concentration
Sterile deionised water	—
10 x PCR buffer	1x
2 mM dNTP mix	0.2 mM of each dNTP
Primer 1	25 pmol
Primer 2	25 pmol
Taq DNA polymerase	2 U/100 µl
25 mM $MgCl_2$	1-4 mM
Template DNA	2 ng

Step	Time	Temperature	Number of cycles
Hot Start	3-5 minutes	94 °C	1
Denaturation	1 minute	94 °C	25
Annealing	1 minute	55 °C	
Extension	1 minute	72 °C	
Run-off	10 minutes	72 °C	1

2.1.5c. Restriction digests, ligations, and transformation of *E. coli.*

Restriction enzyme digestions were performed by incubating DNA with an appropriate amount of restriction enzyme in its respective buffer as recommended by the supplier. Typical digestions included one unit of enzyme per microgram of starting DNA. These reactions were incubated at 37 °C for 1-3 hours. The enzyme(s) were inactivated by heating at 70 °C for 10 minutes. The DNA was purified from the mixture using PCI and ethanol precipitation. The subsequent ligation procedure was as follows. To a sterile microfuge tube, the following was added: 1 µl (100 ng) of enzyme cDNA fragment, X ng of plasmid (this varied with the ligation ratio), 1 µl of reaction buffer, 1 µl (0.5 units) of T4 DNA ligase, and 6 µl of H_2O. The mixture was subsequently incubated for 6 hours, or overnight, at 16 °C. All enzymes and buffers were supplied by Boehringer Manheim.

If the ligation of the ACP, ENR, TE or DES cDNA into pAS2-1 and pACT2 was unsuccessful, additional adenine overhangs were added onto the cDNA fragments for subsequent cloning into T-vectors (pGEMT, Promega). To do this the insert cDNA was incubated with 1 mM ATP and *Taq* polymerase (5 units) at 72 °C for 10 minutes. The adenylated insert cDNA was then mixed with the pGEM-T plasmid with 1 unit of ligase and 1 x ligation buffer. This was brought to 20 µl with H_2O and the mixture incubated at 15 °C overnight. The DNA was purified from the mixture using PCI and ethanol precipitation. The T-vector/cDNA constructs were digested, using restriction enzymes

appropriate to the restriction sites on each end of the cDNA. The cDNA was subsequently cloned into vectors pAS2-1 and pACT2.

The pAS2-1 and pACT2 clones containing ACP, ENR, TE, or DES were used to transform *E. coli*. A 1 µl aliquot of DNA was added to 30 µl of competent DH5α cells (previously prepared using rubidium chloride and stored at –80 °C). The mixture was incubated for one hour on ice, heat-shocked in a water bath at 42 °C for 30 seconds and immediately removed onto ice. The transformed cells were removed from the ice, and 0.8 ml LB media (preheated to 37 °C) was added. This was followed by an incubation step of 1 hour at 37 °C. A 100 µl aliquot of the transformed cells was spread on LB (Amp 50) plates (or other appropriate selection media). The plates were left at room temperature until the liquid was absorbed (~ 30 - 60 minutes). The plates were inverted and incubated at 37 °C overnight. The remaining transformant mix was spun for 5 minutes at 7500 rpm in a microcentrifuge and the supernatant decanted. Remaining cells were gently resuspended in 100 µl LB and spread on the appropriate media and incubated at 37 °C overnight.

2.1.5d. DNA sequencing.

A 5 µl aliquot of construct DNA (3.2 pmol/µl) in H_2O was sequenced by method of Sanger using the primers described. To sequence the cDNA inserts of pAS2-1 constructs, the GAL4-DBD sequencing primer (0.1 µg/µl): 5'–TCATCGGAAGAGAGTAG–3' (forward) was used for sequencing toward the junction

of the GAL4-DBD and cloned insert. The primer binding site is 59 bp from the first restriction site in the MCS (multiple cloning site). The GAL4-DBD sequencing primer (0.1 μg/μl): 5' - CGTTTTAAAACCTAAGAGTCA C - 3' (reverse) was used for sequencing in the reverse direction. To sequence the cDNA inserts of pACT2 constructs, the GAL4-AD sequencing primer (0.1 μg/μl): 5'–TACCACTACAATGGATG–3' (17-mer) was used for sequencing toward the junction of the GAL4-AD and the cloned insert; the primer -binding site was 107 bp from the first restriction site in the MCS. The GAL4-AD sequencing primer (0.1 μg/μl): 5' - GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG AT CG -3' was used for sequencing in the reverse direction. All sequencing primers were designed by Clontech, UK. All primers were manufactured by MBG-Biotech and were stored at –20 °C.

2.1.6. Yeast transformations.

Transformations were performed using the technique of heat-shock in the presence of polythene-glycol/lithium acetate (PEG/LiAc). This process weakens and permeabilises the yeast cell wall (Ito *et al.*, 1983). Sonicated herring sperm DNA was used as the carrier of the plasmid DNA. This non-specific, sonicated or fragmented DNA makes the transformation more efficient by protecting the plasmid DNA from cellular nucleases that would otherwise destroy the plasmid. For optimal performance, immediately prior to use, the carrier DNA was boiled and cooled on ice. The following protocol was adapted from the Clontech Matchmaker II manual. Sterile 1 X TE (0.01 M Tris-HCl, 1 mM EDTA, pH 7.5.); PEG/LiAc (5% PEG, 0.1 M LiAC, pH 7.5); solution and 1 X

TE/LiAc (1 x TE, 0.1M LiAC, pH 7.5) buffer were prepared immediately prior to use from X 10 stocks.

Yeast cultures were grown by inoculating a flask containing YPD or SDO (50 ml) with several yeast colonies, 2–3 mm in diameter. The flask was incubated at 30 °C with shaking (250 rpm) till the yeast reached log growth phase (OD_{600} = 0.6). Cells were aseptically transferred to sterile 50 ml Falcon universal tubes and centrifuged at 1000 g for 5 minutes at 21 °C. The supernatant was discarded and the pellet resuspended by vortexing in sterile TE buffer or H_2O (50 ml). The centrifuge step was repeated and the cell pellet was resuspended in freshly prepared, sterile 1 X TE/LiAc (1.5 ml) to give competent yeast cells.

To 0.1 ml of competent cells the following was added: DBD vector construct (0.1-5 μg), AD vector construct (0.1-5 μg), herring sperm DNA (0.1-1 mg) and 0.6 ml of PEG/LiAc. Each tube was gently flicked to mix the contents. The cells were then incubated at 30 °C for 30 minutes with shaking (200 rpm), heat shocked (42 °C) for 15 minutes in a water bath and immediately removed onto ice for 1–2 minutes. The cells were centrifuged for 5 seconds at 7000 rpm and the supernatant was discarded. The cells were resuspended in 100 μl of 1 x TE and a 50 μl aliquot was plated onto the appropriate media. Plates were incubated at 30 °C until yeast colonies were evident (3-10 days).

2.1.7. Interaction assay.

Yeast strains Y187 and CG1945 and vectors pAS2-1GAL4-DBD (DNA binding domain), TRP1, amp^r pACT2 GAL4-AD (activation domain), LEU2, amp^r were from the Clontech Matchmaker II kit (Clontech, UK). Using PCR, restriction sites were attached to the cDNA of the following FAS II components: acyl carrier protein (ACP), enoyl-ACP reductase (ENR), acyl-ACP thioesterase (TE), and stearoyl-ACP desaturase (DES). All the cDNA was then directionally cloned into the vectors pAS2-1. The cDNAs for ACP and ENR were also directionally cloned into the vector pACT2. All constructs were sequenced across their full length in both 5' and 3' directions. Yeast strains Y187 (-leu, -trp, *lacZ*) and CG1945 (-leu, -trp, -His, *lacZ*) were grown to an OD_{600} of 0.6 and pairwise transformations were carried out. Transformed Y187 were plated out on –Leu, -Trp media. Transformed CG1945 were plated on –Leu, -Trp, -His + 5 mM 3-amino-1,2,4 triazole (3-AT). The chemical 3-AT is a competitive inhibitor of histidine synthesis and was used in order to suppress any residual expression of histidine by CG1945. Protein-protein interactions were detected by β-galactosidase activity in Y187 and by both β-galactosidase activity and histidine expression (allowing growth) in CG1945.

2.1.8. Colony filter assay.

The β–galactosidase activity was assayed by placing a filter paper over all colonies, lifting off and exposing the filter to liquid nitrogen. The filter was removed from the liquid nitrogen and placed on another filter in a petri-dish saturated with a buffer

containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-[[β]]-galactoside *(X-gal)*. A working stock of X-gal solution (20 mg/ml) was prepared in dimethylformamide (DMF). To develop colony colour, 1.67 ml of X-gal stock solution was added to 100 ml of Z–buffer ($Na_2HPO_4.2H_2O$, $NaH_2PO_4.H_2O$, KCl, $MgSO_4.7H_2O$) containing 0.27 ml of β-mercaptoethanol (β-ME) per 100 ml. The filters were left at 30 °C and monitored regularly for colour development. In the case of CG1945, where HIS3 reporter gene activity was first indicated by the growth of colonies, a filter lift was taken of these colonies to determine β-galactosidase expression.

2.1.9. Isolation of plasmid DNA from yeast (back extraction).

Yeast were grown in the appropriate SDO media for ~1.5 - 2 days ($0D_{600}$ ~1.6-1.9). Cells were pelleted by centrifugation at 13 000 rpm for five minutes and the supernatant decanted. A 100 µl aliquot of yeast DNA breaking buffer (1 % SDS, 2 % Triton X-100 (Biorad), 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) was added and the tube vortexed to resuspend the pellet. An equivalent volume of acid-washed glass beads was added and the tubes vortexed vigorously with intermittent breaks on ice, this was repeated three times. A 200 µl aliquot of phenol/chloroform/isoamylalcohol was added and the mixture vortexed vigorously for three minutes. The mixture was centrifuged at 16 000 rpm for 5 seconds and the supernatant containing the plasmid DNA was removed and stored at –80 °C.

2.1.10. Protein extraction from yeast.

Yeast was grown to saturation in SDO media (5 ml) thus under selection for the plasmid of interest. Cells were pelleted by centrifugation for 10 seconds at 15 000 rpm and stored at –80 °C. Cells were subsequently thawed on ice. An equivalent volume of acid washed glass beads, yeast protein breaking buffer (20 mM Tris pH 8.0, 1 mM EDTA, $MgCl_2$ 10 mM, glycerol 5 %, DTT 1 mM, 0.3 M ammonium sulphate and β-ME), plus protease inhibitor cocktail mix (Table 2.2), was added to the cells. The mixture was vortexed for 15 seconds at 15 000 rpm followed by an interval of 15 seconds on ice. This was repeated three times. The supernatant (containing the proteins) was removed and these proteins were fractionated by SDS-PAGE and subject to immunoblotting.

Table 2.2. Concentration of protease inhibitors used in yeast protein breaking buffer and the protease types they inhibit.

Protease Inhibitor	Concentration	Type of protease's inhibited
Pepstatin A	0.1 mg/ml	Carboxyl proteases
Leupeptin	0.03 mM	Some thiol and serine proteases
Benzamidine	145 mM	Trypsin, plasmin and thrombin
Aprotinin	0.37 mg/ml	Some serine poroteases
Pefabloc	0.1 mg/ml	Serine proteases

2.1.11. Determination of protein concentration

All protein determinations were performed by the method of Bradford (1976) with bovine serum albumin as the standard (Ausubel *et al.*, 1989). This method involves the binding of Coomassie Brilliant Blue G250 (BioRad) to protein, The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 nm to 595 nm (Neumann *et al.*, 1994). The increase in absorption at 595 nm was monitored.

2.1.12. Trichloroacetic acid (TCA) precipitation of yeast proteins.

Yeast proteins were extracted as described in section 2.1.10. A 100 µl aliquot of the protein sample was resuspended in 25 µl of ice cold TCA. This was mixed and incubated on ice for 1 hour. The protein precipitate was centrifuged at high speed (15 000 rpm) for 15 minutes at 4 °C and the supernatant discarded. The resultant pellet was washed with 1 ml of ice cold acetone and spun at 15 000 rpm in a microfuge for 5 minutes. The supernatant was discarded and the resultant pellet was air-dried. All precipitation procedures were carried out in a cold room (4 °C). The pellet was resuspended in 20 µl of SDS-PAGE loading buffer (25 mM Tris, 192mM glycine, 0.05 % SDS, 0.1% bromophenol blue) and the proteins seperated by electrophoresis.

2.1.13. SDS-polyacrylamide gel electrophoresis (PAGE) of proteins.

The following were kept as stock solutions for the preparation of SDS-PAGE mini-gels: 30 % acrylamide (BioRad) (made to a 37.5: 1 acrylamide: bis acrylamide ratio), 0.8 %

SDS, 1.5 M Tris pH 8.8, 1 M Tris pH 6.8, 0.28 % ammomium persulphate (BioRad), TEMED (BioRad). All gels were made using H_2O. Stacking gels were made to a final concentration of 3 % or 5 % acrylamide, pH 6.8. Resolving gels were made to a final concentration of 12 %, pH 8.8. The following SDS7 proteins were used as size markers: albumin; bovine (66 kDa), albumin; egg (45 kDa); glyceraldehyde 3-P dehydrogenase (36kDa); carbonic anhydrase; bovine (29 kDa), trypsinogen; bovine pancreas (24 kDa), trypsin inhibitor; soybean (20 kDa), α-lactalbumin; bovine milk (14.2 kDa), (BioRad, Laemmli, 1970). The gels were cast and run using a vertical mini-gel electrophoresis system (BioRad). Protein samples were resuspended in SDS–PAGE loading buffer and electrophoresed in SDS- running buffer (25 mM Tris, 192 mM glycine, 0.0 5% SDS). The voltage applied was initially set at 80 V until the marker entered the resolving gel, followed by 180 V for 1.5 hours at room temperature. Alternatively, proteins were electrophoresed at 30 V overnight at 4 °C, with continual magnetic stirring of the buffer in the gel tank.

2.1.14. Immunoblotting of SDS-PAGE seperated proteins.

SDS –PAGE was carried out as described in section 2.1.13. Gels were wet-blotted onto Hybond-ECL (nitrocellulose), either overnight at 30 V or for 2 hours at 200 mA in a cold room. The filter was stained with Poncaeu S (which stains proteins red; BioRad), and washed with tris-buffered saline (TBS, pH 7.4). The filter was blocked for 1 hour with 5 % milk powder in TBS/0.01 % Tween-20 (BioRad), pH 7.4. The filter was probed with primary antibody in freshly made blocking solution for 2 hours, then

washed for 3 x 10 minutes in 0.5 % milk powder, TBS. The filter was subsequently incubated with a 1: 20 000 dilution of a secondary antibody (in blocking solution). Finally, the filter was washed for 10 minutes in TBS (X 3). Proteins were detected by chemiluminescence using the Pierce Supersignal system and Hyperfilm- ECL (Kodak). After the initial exposure (0-10 seconds), the timing of further exposures was gauged empirically. Whereby, if the signal were weak the film would be left to develop for a longer length of time e.g. one minute.

2.2. Bandshift analysis using isolated *Brassica napus* chloroplasts.

2.2.1. Plant Growth.

In this project, *Brassica napus* seeds cv. Escort were stored at 4 °C in sealed plastic containers with silica beads to ensure a low humidity. Three trays with approximately 200 seeds per tray (50 cm x 35 cm) were sown in compost. These were kept under growth lamps (500-600 $\mu mol/m^2/sec$) giving 14 hours light and 10 hours dark at a constant temperature of 21 °C. An ongoing cycle of planting harvesting leaves weekly was set up to allow constant access to rapidly growing leaves.

2.2.2. Isolation of chloroplasts.

The isolation of chloroplasts required rapid and delicate extraction into a high osmiticum, typically containing 330 mM sorbitol at low temperatures (4 °C) and low ionic strength (Robinson *et al.*, 1979; Walker *et al.*, 1971). The high concentration of

sorbitol prevented the chloroplasts from swelling via osmosis and rupturing, termed 'osmotic shock'.

For isolation of *Brassica napus* chloroplasts, all solutions and equipment for both the isolation and oxygen electrode measurements were set out before isolation. All material and equipment was pre-chilled to 4 °C. All solutions were set to the correct pH with a dedicated pH meter. The Polytron homogenizer was rendered detergent free by repeated washes in 80 % acetone and water. To remove starch grains, the young plants were kept in the dark for 12-14 hours then exposed to light for 1 hour. The pre-illumination period increases the yield of intact chloroplasts and shortens the induction period (Walker *et al.*, 1971). Leaves were harvested 30 minutes to 2 hours after the beginning of the light cycle. The top halve of true leaves were removed from plants, thus avoiding material with a high cellulose content located near the stem. The leaves were immediately placed in ice cold H_2O. Approximately 50 g of leaves were picked per 500 ml of chloroplast extraction buffer (CEB: 330 mM sorbitol, 20 mM Hepes-NaOH pH 8.0, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$). The leaves were dried on kitchen towel, finely chopped and immediately placed into the semi-frozen buffer. This way the leaf homogenate may remain at approximately 4 °C. The leaves were homogenised for 2 x 3 seconds using a Polytron homogeniser (with PTA 20S attachment) in a thick walled glass container 20 cm x 5 cm x 5 cm. The resulting homogenate was filtered through four layers of acetone washed muslin. The filtrate was gently poured into pre-chilled Corex centrifuge tubes. The tubes were immediately transferred to a pre-chilled J2-HC or JA-20 rotor and then to a pre-chilled, Beckmann centrifuge. The filtrate was spun at 2,500 g for 2 minutes. The supernatant was removed and the remaining pellet washed in 5 ml of ice cold CEB.

This 'washing' removed many of the damaged chloroplasts round the edges of the pellet and on the side of the Corex centrifuge tubes. The remaining pellet was very gently resuspended in the smallest volume possible (0.5-1 ml) of CEB. This kept the chloroplast concentration high and may have helped confer a protective effect on the chloroplasts. A wide bore pipette was used to resuspend the pellet to decrease mechanical shearing. The resuspension of one pellet was then used to resuspend the next pellet. The final resuspension of isolated chloroplasts was immediately taken to the laboratory to measure intactness. The remaining chloroplasts were immediately frozen in liquid nitrogen at –80 °C (Roughan *et al.*, 1987; Kang and Rawsthorne, 1994; Eastmond *et al.*,. 1996).

2.2.3. Oxygen electrode measurement of isolated *Brassica napus* chloroplasts.

Oxygen evolution measurements were made in the DW1 oxygen electrode unit part of the Hansatech Oxygraph system (Figure 2.2.). It consisted of a volume adjustable, borosilicate glass reaction vessel (200 µl to 2.5 ml) with an integral electrode forming the chamber floor. A clear water jacket surrounded the reaction vessel and permitted good sample visibility and temperature control when connected to a suitable temperature controlled water circulator (flow-rate of 6-8 litres/minute). A 50 % saturated potassium chloride solution was used as an electrolyte (a 1+1 dilution of a saturated solution of potassium chloride with distilled water).

Calibration of the oxygen electrode was achieved by adding sodium hydrosulphite to oxygen saturated H_2O in the chamber. The concentration of oxygen in water at 25 °C is

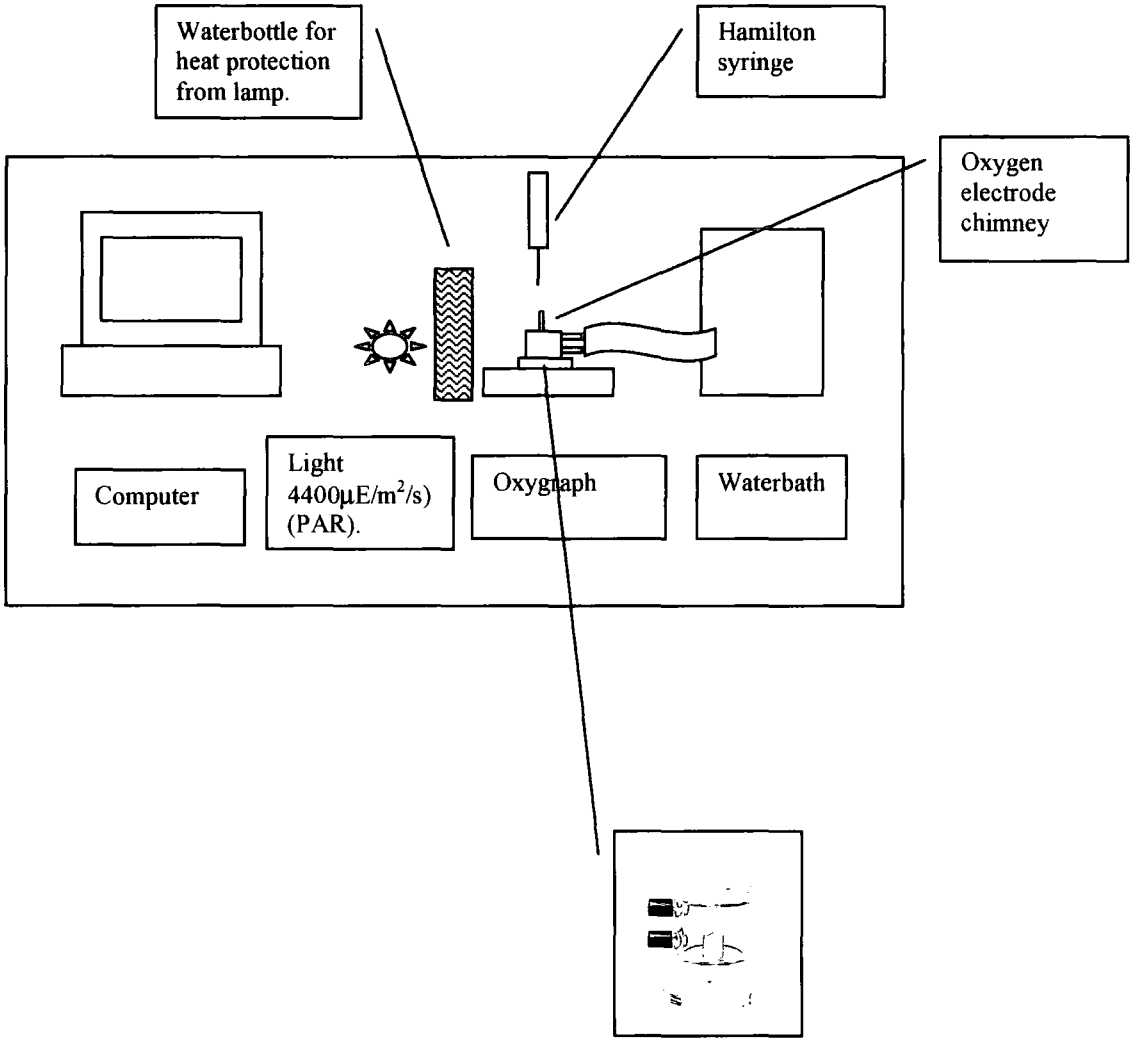


Figure 2.2. Schematic diagram of the equipment used for chloroplast intactness assays (adapted from Hansatech, UK).

0.28 μmoles/ml (Hansatech data). This may be effectively removed by the addition of the reducing agent (Delieu and Walker, 1972). It was imperative after calibration to remove all traces of sodium hydrosulphite that would interfere with subsequent measurements. This was done by ten washes with 1 ml of H_2O. A central control unit

operated the oxygen electrode, stirrer unit, and analogue to digital PC-interface. The instrument was controlled via a serial link to a Windows 98 PC. The data-acquisition/instrument control program (Oxyg32.exe, Version 2.17) was used to control signal-conditioning parameters (gain and back-off) and instrument calibration. Data was plotted as real-time chart-recorder emulation with facilities for event marking.

The following chemicals were used for subsequent oxygen electrode assays: D, L glutaraldelhyde (0.3 M, 25 °C); ammonium chloride (0.5 M, 25 °C). Potassium hexanocyanoferrate (0.5 M) was kept in a foil-covered eppendorf at room temperature. All solutions were made on the day of use.

2.2.4. Determination of chlorophyll content.

Chlorophyll content was spectroscopically determined in 80 % acetone as a solvent (Porra *et al.*, 1974). The chlorophyll concentration estimate allowed oxygen evolution to be calculated in absolute terms. In this project, 50 µl of isolated chloroplast was added to 10 ml of 80 % acetone and filtered by one pass through Whatman filter paper. This removed any insoluble material. The absorbance of the filtrate was measured at A_{652} against an 80 % acetone blank. Chlorophyll concentration was calculated by converting the absorbance by a factor of 100/9 (Table 2.3). This factor is needed to take into account the dilution factor and the extinction coefficient.

Table 2.3. Example of chlorophyll concentration estimation for isolated *Brassica napus* chloroplasts.

1. Treatment of extract	50 µl of chloroplasts into 10 ml 80 % of acetone, filtered into 10 ml 80 % acetone
2. Absorbance reading (652 nm)	0.095/µl
3. Chlorophyll estimation A X 100/9 (µg chl per µl suspension)	1.05

2.2.5. Immunoblotting to detect bandshifted proteins.

Native gels were prepared in the same way as SDS-PAGE gels with H_2O replacing the use of SDS in the stock solutions. The gels were cast and run using a vertical mini-gel electrophoresis system (BioRad). Purified *B. napus* proteins (previously purified in this laboratory) were co-incubated on ice in chloroplast extraction buffer for one hour and loaded onto the same native gel as the isolated chloroplasts. Protein samples were resuspended in x 1 native PAGE loading buffer (25 mM Tris, 192 mM glycine, 0.1% bromophenol blue) and electrophoresed in native running buffer. The voltage applied was 80 V for ~ 3 hours at 4 °C with continual magnetic stirring of the buffer in the gel tank. Alternatively, proteins were electrophoresed at 30 V overnight at 4 °C, with continual magnetic stirring of the buffer in the gel tank. Following fractionation of proteins by native PAGE, the gels were subsequently used for immunoblotting. Gels were wet-blotted onto Hybond-ECL (nitrocellulose), for 3 hours at 4 °C at 60-80 V. The filter was then probed as described in section 2.1.14. Filters were incubated in stripping buffer (2 % wv SDS, 62.5 mM Tris pH 6.8, 100 mM β-ME) for subsequent re-probing.

2.3. Computer-based methods.

DNA and protein sequence searches and comparisons were carried out using BLASTN and BLASTP search algorithms provided by the National Centre for Biotechnology Information (NCBI). Translations were carried out using DNA Star and/or DNA Strider packages. The transit peptide size and potential cleavage sites of oleoyl-ACP thioesterase were calculated using Daresbury SigSeq, part of the University of Wisconsin Genetics Computer Group sequence analysis suite, version 8. The parameters of SigSeq are based upon the principles and properties of transit peptides as described von Heijne. (1990). The neural network ChloroP (Emanuelsson *et al.*, 1999), was used to update the prediction of transit peptide cleavage site. The parameters of ChloroP are based upon the principles and properties of transit peptides as described by von Heijne. (1989. The ChloroP program was trained, by a separate group, with a test set of 75 known transit peptides containing chloroplast proteins excluding lumenal proteins and with a negative test set consisting of 75 proteins from other non-chloroplast localizations (Emanuelsson *et al.*, 1999).

Chapter 3. Results.

3.1. Analysis of protein-protein interactions of type II FAS of *Brassica napus* using the yeast two-hybrid system.

The yeast two-hybrid system uses the activation of transcription to indicate the interaction between two proteins (Fields and Song, 1989). Central to this technique is the fact that many eukaryotic transcriptional activators consist of two physically discrete modular domains. The DNA-binding domain (DBD) that binds to a specific promoter sequence and an activation domain (AD) that directs the RNA-polymerase II complex to transcribe the gene downstream of the DNA binding site. Both domains are required for normal activation. The two domains are normally part of the same protein (as in the case of the native yeast GAL4 protein). However, they can also function as two separate proteins - as long as the activation domain and DNA-binding domain are brought into close proximity (Fields and Song, 1989). In principle, any AD may be paired with a DBD to activate transcription. Experimentally, this involves the construction of two-hybrids: the DNA binding domain fused to some protein 'X' and a transcription activation domain fused to some protein 'Y'. The fusion proteins are expressed in the yeast cell, containing the reporter gene, if proteins X and Y interact the two domains of the transcription factor come together and there is consequent expression of the reporter gene (Fields and Song 1989), Figure 3.0.

The fundamental advantage the yeast two-hybrid system has over other techniques for studying protein-protein interactions is that the assay is performed in the highly concentrated environment of the yeast nucleus. Thus, it may be a more appropriate method to allow formation of weak and transient interactions. These interactions may be critical for the proper functioning of complex biological systems (Estojak *et al.*, 1995). For example, work on the binding of p16 proteins (which are present in tumour cells) to examine

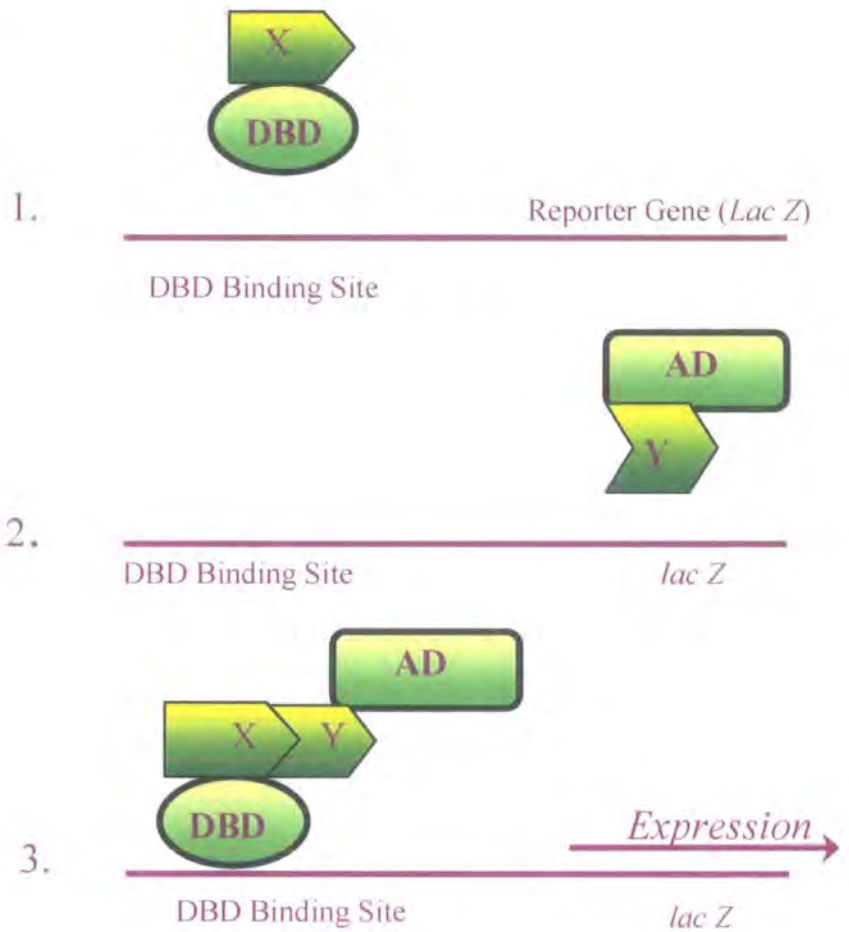


Figure 3.0. Principle of the yeast two-hybrid system. One protein 'X' is fused to the DNA binding domain (DBD), another protein 'Y' is fused to the transcriptional activation domain (AD) of a reporter gene e.g. *E. coli lac* Z. The fusions are expressed in the yeast cell, if the proteins interact, the two domains of the transcription factor come together, and there is expression of the reporter gene (Fields and Song, 1989).

the proteins involved in regulation of the cell cycle i.e. the cyclin dependent kinases:- CDK4 and CDK6, led to the suggestion that protein-protein interactions with dissociation constants (K_d) above ~ 70 μM may be detected using a GAL4-based two-hybrid assay (Yang *et al.*, 1995). This was achieved by comparing the binding affinities of mutant p16 proteins to CDK4 and CDK6 in the yeast two-hybrid system and from a number of tumour-specific p16 mutants that were impaired for CDK interaction. *In vitro* reconstitution studies with some of the mutants confirmed a defect in their ability to interact (Koh *et al.*, 1995). Similarly, based on different proteins binding to the retinoblastoma protein, it was estimated that the minimal binding constant required to detect an interaction was approximately 1 μM (Durfee *et al.*, 1993). The large differences in Kd's may be interaction-specific.

There are disadvantages with the yeast two-hybrid system. It is limited to proteins that can be localised to the nucleus, which may prevent its' use with extracellular proteins. Proteins must be able to fold and exist in a stable fashion in yeast cells as well as retain activity as fusion proteins. The use of protein fusions may also mean that the site of interaction may be occluded by one of the transcription factor domains. Interactions that depend on post-translational modifications that do not commonly occur in yeast (*Saccharomyces cerevisae*) cells will not be detected. For example, mono-specific tyrosine phosphorylation, which is a major modification in signal transduction in higher eukaryotes, has not been found to occur in yeast (Hunter, 1995). Many proteins, including those not normally involved in transcription will activate transcription when fused to a DNA binding domain (a false positive). However, it may be possible to get

round this by deleting a small region of the protein that activates transcription. Finally, the interaction between the two proteins may require the presence of a stabilising intermediate that is not found in the yeast nucleus.

Fields and Song (1989) exploited the GAL4 promoter. Later systems were based on both LexA promoters and GAL4 promoters. In the GAL4-based two-hybrid systems, sequences encoding the two functional domains of the GAL4 transcriptional activator have been cloned into the DBD and AD expression vectors. In the LexA two-hybrid system, the DBD is provided by the entire prokaryotic LexA protein, and the AD is a heterologous 88-residue acidic peptide that activates transcription in yeast. The latter system has the added advantages of regulating the level of expression of the proteins under study. Both systems allow mutagenesis and, in the case of a library screen, detection of novel interacting proteins from the same and other species. Clontech commercialized the GAL4 based system.

The important differences of the two systems are summarised in Table 3.0. The work in this study used the Clontech Matchmaker II as the system of choice. This was because the expression level of the constructs is relatively high therefore, there may be more chance that any weak interaction may be retained. Furthermore, the GAL4 system allows for the filter lift method of β-galactosidase detection, which is one of the more sensitive detection methods of β-galactosidase activity, Figure 3.1 (Breeden and Nasmyth, 1985; Clontech data, UK).

Table 3.0 Comparison of the Clontech Matchmaker GAL4 and LexA yeast two-hybrid systems.

	GAL4 system (Clontech)	LexA system (Clontech)
Bait vector marker	TRP1 (CYH^S2)	HIS3
Prey vector marker	LEU2	TRP1
Promoter of prey vector	ADH2 promoter (full length or truncated)	GAL1 promoter (inducible by raffinose/ galactose)
Reporter gene	*lacZ*	*lacZ*
Amino acid marker	HIS3	LEU2
Reporter stably integrated?	Yes -> leaky HIS3 expression	No -> more copies of reporter, so may be more sensitive, but need of a stable clone
ß-galactosidase assay	Filter lift method	On plate blue-white selection
Vector replication	2-µm based	2-µm based
Lambda vector	Yes	No
Cycloheximide counter-selection	Yes	No
Mating partner	Yes	Yes
Fusion protein expression level	High	Inducible AD[b]
Additional features	Constant level expression of fusion proteins	May be more suitable for working with toxic AD fusion proteins.

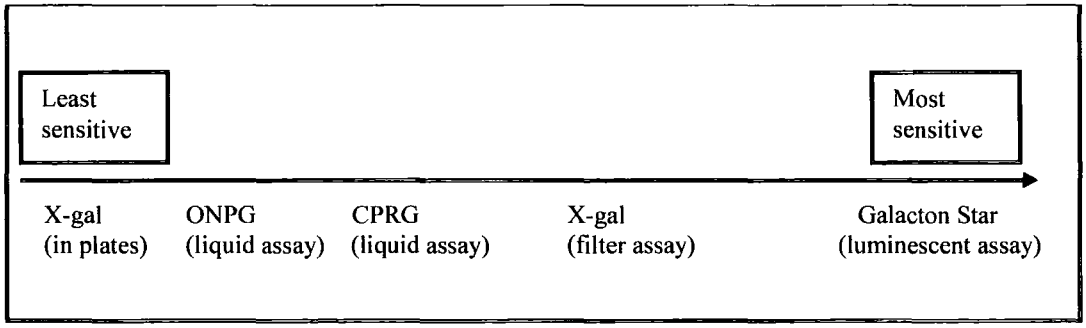


Figure 3.1. Relative sensitivity of the five types of β-galactosidase assays. The X-gal filter assays have been reported to be approximately 10^6 fold more sensitive than ONPG liquid assays and were subsequently used for this work. CPRG (chlorophenol red-β-D-galactopyranoside; ONPG, *o*-nitrophenyl β-$_D$-galactopyranoside (Breeden and Nasmyth, 1985; Clontech, UK).

This project used the yeast two-hybrid system to provide an environment of high protein concentration (the yeast nucleus), thus circumventing the problem of loss of associative forces of crowding, a problem that occurs during dilution. The second facet of the strategy for the yeast two-hybrid system stemmed from the availability of cDNA for most of the components of type II FAS of *Brassica napus*. This included some of the enzymes that were found to co-purify. These enzymes are involved in the terminal reactions of fatty acid synthesis: enoyl-ACP reductase (ENR), stearoyl-ACP desaturase (DES) and oleoyl-ACP thioesterase (TE).

3.1.1 Cloning.

The procedure followed for the first step in the yeast two-hybrid system, cloning the cDNA of ACP, ENR, TE and DES into the yeast two-hybrid vectors, is set out in Figure 3.2.

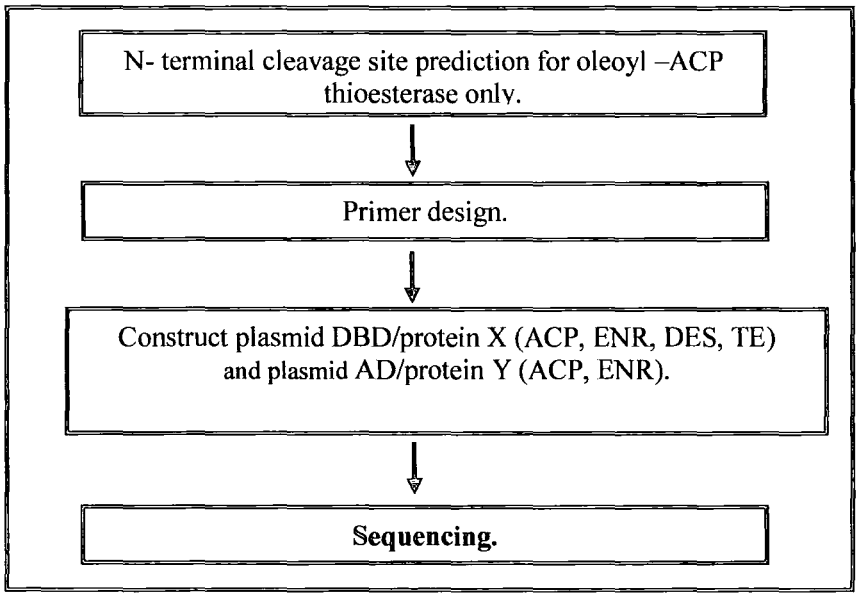


Figure 3.2. Flow diagram to indicate the procedure used for cloning the cDNA of ACP, ENR, TE and DES into vectors pAS2-1 and pACT2 and subsequent sequencing.

3.1.1a. The prediction of the chloroplast-targetting peptide cleavage site of oleoyl-ACP thioesterase of *Brassica napus.*

This project used the full-length cDNAs for *Brassica napus* ACP, ENR, TE, and DES. Plant type II FAS proteins are nuclear-encoded and transported to the chloroplast, this requires the use of a chloroplast-targetting peptide. This information comes from cellular fractionation and immunolocalisation studies. It is supported by analysis of cDNA clones of ACP, β-KASI, and ENR from *B. napus*, *Arabidopsis* and barley which have encoded the appropriate targetting peptide. The cleavage sites of the chloroplast targetting peptide of ACP, ENR and DES were already published (Slabas *et al.*, 1988; Safford *et al.*, 1988; Kater *et al.*, 1991; Slocombe *et al.*, 1992). Therefore, the first stage of cloning was the prediction of the cleavage site of the transit peptide for thioesterase (TE) before cloning into the vectors pAS2-1 and pACT2 could proceed. This was achieved using the algorithm 'SigSeq' (part of the Wisconsin suite). This programme is based upon the principles and properties of transit peptides as described von Heijne (1989). Whereby, chloroplast targetting sequences may be summarised into three main features - 1) an uncharged N-group; 2) a few acidic residues centrally positioned; 3) an amphiphillic β-strand found located in the last ten residues. The SigSeq programme required the submission of the complete protein sequence. This sequence was subsequently divided into sections and analysed for potential transit peptides and the potential cleavage site of these transit peptides. Each of these is in turn given a probability score from 0-1 of being the transit peptide. The full-length *Brassica napus* thioesterase cDNA when submitted presented a strong candidate, probability value of

1.0 (the highest possible score), for the cleavage site between amino acids 33/34 (Figure 3.3, Figure 3.4).

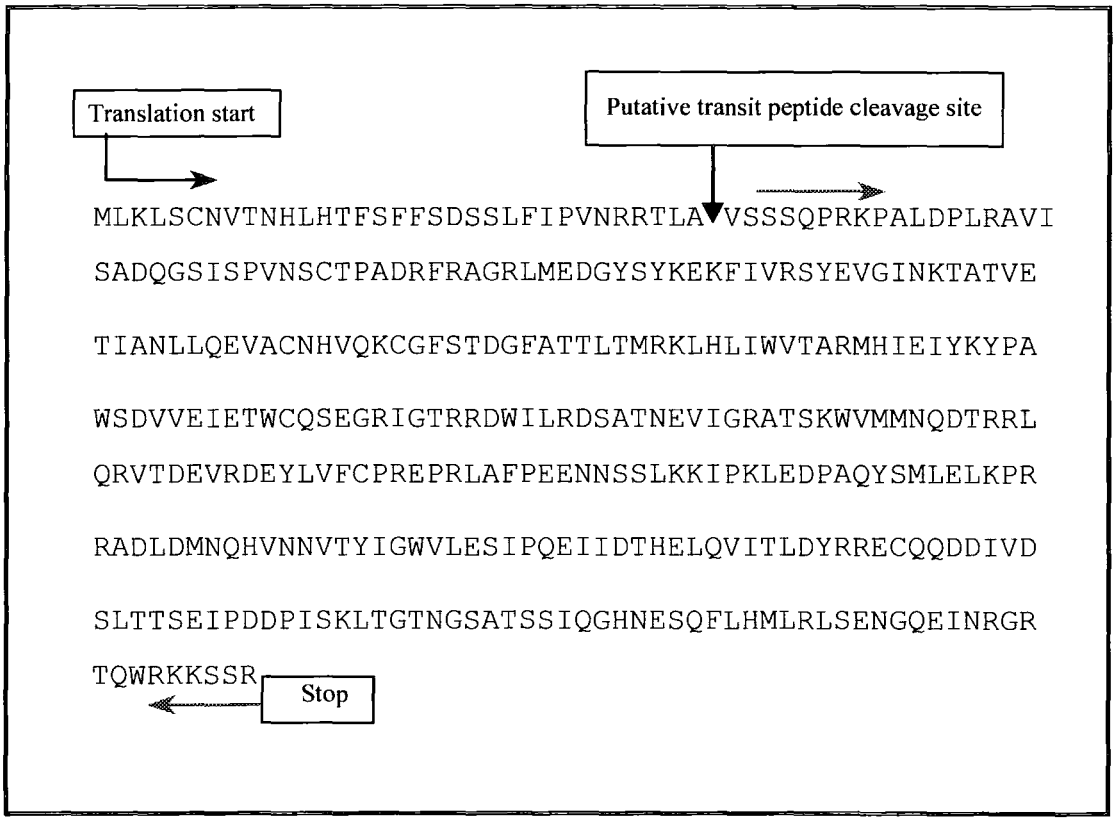


Figure 3.3. Full-length protein sequence of *Brassica napus* oleoyl-ACP thioesterase indicating transit peptide cleavage site. The arrows indicate hybridisation sites for primers following the prediction of the cleavage site of the transit peptide. The length of primer ensured high specificity of amplification and allowed restriction enzymes to bind to the DNA more easily.

POS	AA	Normalized Probability	
13	H		
14	T		0.000000
15	F		0.001344
16	S	xxxxxx	0.000000
17	F		0.110071
18	F		0.000000
19	S		0.000000
20	D		0.000000
21	S		0.000000
22	S	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx	0.786321
23	L		0.000000
24	F		0.001111
25	I		0.000000
26	P		0.000000
27	V		0.000000
28	N		0.000000
29	R		0.000000
30	R		0.000000
31	T		0.000000
32	L		0.000000
33	A		0.000000
34	V	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx	1.000000
35	S		0.000000
			0.001003

Figure 3.4. Prediction of the cleavage site of the chloroplast targeting peptide (cTP) of *Brassica napus* oleoyl-ACP thioesterase using the algorithm SigSeq, part of the Wisconsin Package. Programme manual for the Wisconsin Package, Version 8, August 1994, (Genetics Computer Group). POS- position; AA - amino acid.

The use of a more modern algorithm ChloroP (Emanuelsson *et al.*, 1999) agreed on the targetting peptide but gave a length of 35 αα not 33 αα as predicted by the SigSeq algorithm. Both of these programmes are based upon the principles and properties of transit peptides as described von Heijne (1990). However, ChloroP 1.1 may be more reliable as it uses a neural network. A neural network allows the programme to be trained to 'remember' characteristics of transit peptides. A neural network gives computational processes the ability to "learn"; in an attempt to approximate human learning, whereas most computer programs execute their instructions blindly in a sequential manner. The ChloroP program was trained, by a Emanuelsson *et al.* (1999) with a test set of 75 known transit peptides containing chloroplast proteins excluding lumenal proteins and with a negative test set consisting of 75 non-chloroplast proteins. This group found that 11 % of the test set were wrongly targetted and calculated this would transcribe into ~ 2500 false positives for the entire genome (Emanuelsson *et al.*, 1999). In this project, although there was a two amino acid difference, which may significantly affect protein-protein interactions, the primers had already been used in the cloning process and the yeast two-hybrid experiments carried out. The ChloroP neural network was extensively used by Peltier *et al.* (2000) to help in ascribing potential functions to proteins identified by MALDI-TOF from 2D gel analysis of thylakoid integral and lumenal proteins from pea. This group found ChloroP correctly ascribed all nuclear encoded photosynthetic proteins. However, as acknowledged by Peltier *et al.* (1999), this result gives somewhat misplaced confidence in ChloroP as those chloroplast proteins identified were part of the training set used by Emanuelsson *et al.* (1999).

3.1.1b. Primer design for cloning the cDNA of ACP, ENR, TE, and DES into the shuttle vectors.

The cDNA of ACP, ENR, TE, and DES was directionally cloned into either the DBD vector (pAS2-1) or AD (pACT2) vectors of the Clontech Matchmaker II yeast two-hybrid system. A directional cloning strategy (Figure 3.5) was employed to increase the probability of obtaining the correct orientation. The primer design process is shown in Figure 3.5 (stage 1), using the cloning of the cDNA of ACP into the DBD vector pAS2-1, as an example. The aim was the addition of restriction sites on 5' and 3' ends of the cDNA of ACP, ENR, TE, and DES, for subsequent cloning into vectors pAS2-1 and pACT2. The forward and reverse primers for the shuttle vector constructs are show in Table 3.1. If necessary, additional bases were included to keep the GC content around 50 %, thus keeping the melting temperature (Tm) of the two primers approximately the same but also avoiding GC hairpins. The plasmids in DH5α containing the cDNA for ACP. ENR, TE, and DES were stored as glycerols at –80 °C. These were streaked on plates containing the appropriate antibiotic and incubated overnight at 37 °C. An individual colony from the inoculated plate was picked and grown in liquid media. Plasmid DNA was isolated using the Hybaid DNA extraction kit (Chapter 2.1.5a). Inserts were amplified by PCR using *Vent* DNA polymerase (Boehringer Mannheim). This was used because it has proofreading ability (Chapter 2.1.5b). An 8 μl aliquot of the product was directly loaded onto a 0.8 % gel to confirm size and purity of the fragments (Figure 3.6). To remove enzymes, salts and other impurities the cloning inserts were purified using the Qiaex gel extraction kit or by phenol/chloroform and ethanol precipitation.

Stage 1:

a) Acyl Carrier Protein coding sequence for *B. napus* (Genbank Accs. nos. X13128)

MSTTFCSSVSMQATSLAATTRISFQKPALVSRTNLSFNLSRSIPTRLSVSCAAKPETVEKVSKI

VKKQLSLKDDQNVVAETKFADLGADSLDTVEIVMGLEEEFHIEMAEEKAQKITTVEEAAELIDE

LVQAKK

b) Transit peptide:

MSTTFCSSVSMQATSLAATTRISFQKPALVSRTNLSFNLSRSIPTRLSVSC

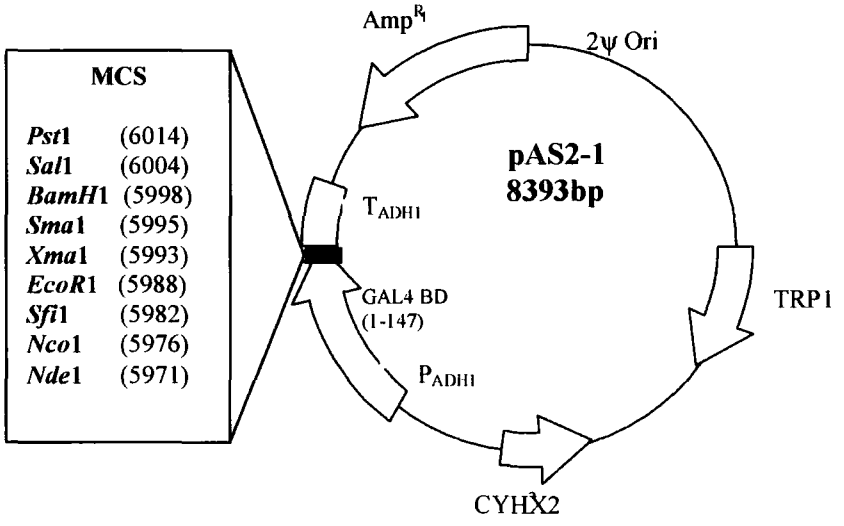
c) Mature sequence, the arrows indicate primer hybridisation sites:

5'AAKPETVEKVSKIVKKQLSLKDDQNVVAETKFADLGADSLDTVEIVMGL

EEEFHIEMAEEKAQKITTVEEAAELIDELVQAKK 3'

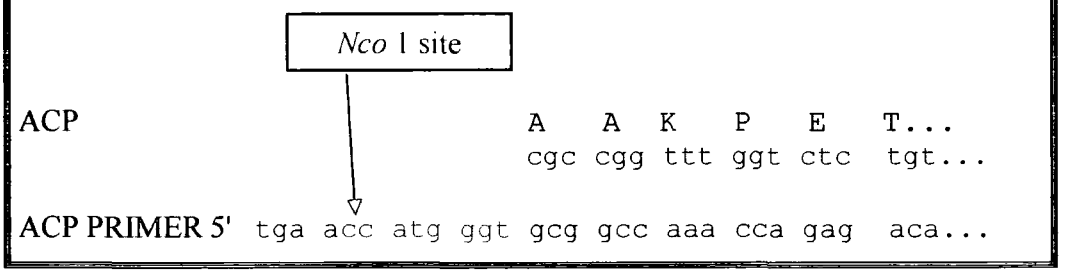
Stage 2.

Choose unique restriction site in vector MCS e.g. *Nco*1 for subsequent restriction digest.



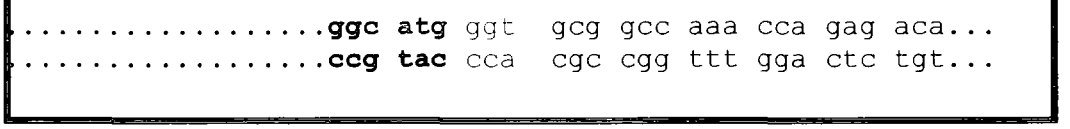
Stage 3.

Nco 1 restriction site introduced by PCR on to the 5' end of the cDNA of ACP.



Stage 4.

Ligation of PCR modified ACP cDNA to digested pAS2-1.



Stage 5.

Expression of DBD/ACP fusion protein.

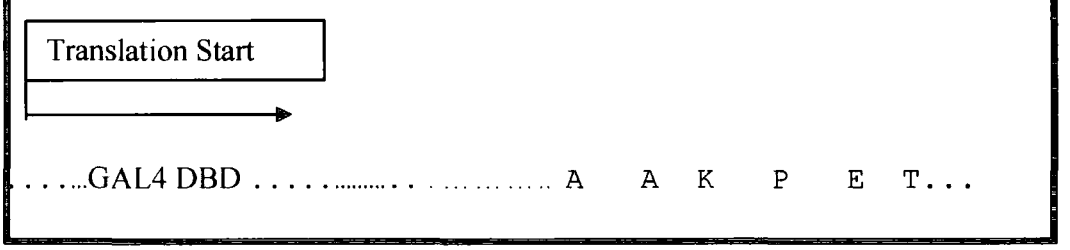


Figure 3.5. Example of the stages of directional cloning of seed specific cDNA of *B. napus* ACP into the shuttle vector pAS2-1 from the Clontech Matchmaker II yeast two-hybrid system. Stage 1: transit peptide prediction (if necessary); stage 2: primer design to amplify mature protein cDNA; stages 3 and 4: restriction digest and ligation into the shuttle vector; stage 5: transformation of construct into yeast gives subsequent expression of fusion proteins. Primers were designed to have approximately equal denaturing temperature. Expression starts from the pADH1 promoter. DBD, DNA binding domain; MCS, multiple cloning site.

Table 3.1. Primers designed for directional cloning of the cDNA for *Brassica napus* ACP, ENR, TE, and DES into the shuttle vectors pAS2-1 and pACT2 of the Clontech Matchmaker II yeast two-hybrid system. The red type indicates the restriction site.

FAS II Protein	Accs. nos.	Plasmid carrier	Vector Destination	Primers		Restriction site
ACP	X13128	pBR322	pAS2-1 and pACT2	tga acc atg ggt gcg gcc aaa cca gag aca	F	*Nco*1
				ctg gat ccg tca ctt ctt ggc ttg cac ga	R	*Bam*H1
ENR	X95462	pET11d	pAS2-1 and pACT2	tga acc atg ggt tct gaa tcc agc gaa agc aag	F	*Nco*1
				cgg gat ccg tta ttt gtt gag gtc ttt gaa	R	*Bam*H1
TE	X73849	pNL2 (Unilever)	pAS2-1 and pACT2	tag aat tcg tct cgt ctt ctc agc caa	F	*EcoR*1
				tag gat ccg tca tcg tga gca ttt ctt	R	*Bam*H1
DES	X63364	pET13b	pAS2-1	cga att cca tat ggc ttc ttc ttc tcc tgc t	F	*Nde*1
				cga att ctc act gca ctt ctc tgt cat	R	*EcoR*1

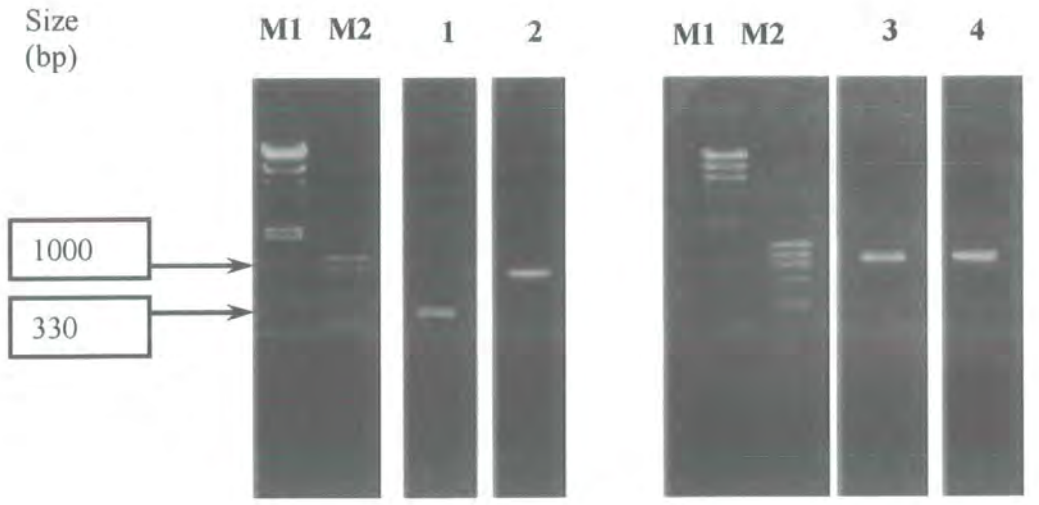


Figure 3.6. Amplification of the cDNA of ACP, ENR, TE, and DES with restriction sites for subsequent cloning into the Clontech Matchmaker II yeast two-hybrid shuttle vectors pAS2-1 and pACT2. The cDNAs were amplified using *Vent* DNA polymerase (Boehringer Mannheim). Restriction sites were introduced by adding 25 pmol of primer to the PCR reaction mixture. Reactions contained 2 µl of plasmid DNA (1 ng/µl), 0.1 mM dNTPs, 1 X *Vent* polymerase buffer, one unit of *Vent* polymerase and the total reaction mixture was brought to 50 µl with H_2O. General cycle conditions were used (Chapter 2.1.5b). An 8 µl aliquot of the product was directly loaded onto a 0.8 % gel, to confirm size and purity of the fragments. M1: λHind III marker; M2: ϕX174 marker; lane 1: 330 bp fragment of ACP cDNA for cloning into pAS2-1 and pACT2; lane 2: 1000 bp ENR fragment for cloning into pAS2-1 and pACT2; lane 3: 1019 bp TE fragment for cloning into pAS2-1; lane4: 1112 bp DES fragment for cloning into pAS2-1.

3.1.1c. Preparation of DBD/ACP, DBD/ENR, DBD/TE, DBD/DES and AD/ACP, AD/ENR plasmid constructs.

The shuttle vectors, pAS2-1 and pACT2, were subject to the appropriate restriction digest (Chapter 2.1.5c). The double digested vectors were run on 0.8 % agarose gel, the DNA band excised and purified using phenol/chloroform followed by ethanol precipitation. Following removal of impurities, ACP and ENR cloning inserts were

digested with the appropriate enzymes and ligated into the double digested vectors, pAS2-1 and pACT2, and TE and DES were ligated into pAS2-1. The initial direct cloning of the inserts into the shuttle vectors was unsuccessful. This could have been due to the incomplete digestion of the PCR fragments. Although extra bases were included in the primer design to allow suffice area for the enzyme to obtain its DNA substrate, complete digestion of both ends cannot be determined by gel analysis. This led to use of an intermediate T-vector 'pGEM-T' (Promega). This vector is prepared by cutting Promega's pGEM-5Zf(+) vector with *Eco*RV and adding a 3' terminal thymidine (T) to both ends. The single 3'-T overhangs at the insertion site greatly improve the efficiency of PCR product ligation into the plasmid (Robles and Doers, 1994). Ligation takes advantage of the template-independent addition of a single adenosine (A) to the 3' end of PCR products by thermostable polymerases e.g. *Taq* DNA polymerase (Clark, 1988). Single adenosines were thus added to the PCR products by incubating the cDNA with 1 mM ATP, with *Taq* polymerase (5 units) at 72 ^{o}C for 10 minutes and successfully ligated to the T-vector. Once the PCR fragments were in the T-vector this allowed the restriction enzymes better access to the actual site. More importantly, completely digested inserts should be easily distinguishable on a gel i.e. if only one site is digested the plasmid will linearise and it will be larger than the double digested fragment. The procedure is described in Chapter 2.1.5c. The inserts were cut out from the T-vectors and purified using the Qiaex gel extraction kit or by phenol/chloroform extraction and ethanol precipitation. Subsequently, ligations of the ACP, ENR, TE, DES cloning inserts and the vectors pAS2-1 and pACT2 were carried out using 3:1, 1:1, and 1:3 insert:vector ratios (Chapter 2.15c). The ligation mixture was used to transform *E. coli* DH5α (Chapter 2.1.5c) and 100 µl aliquots of the transformed cells were spread onto

LB-Amp^{50} plates. The plates were incubated at 37 °C overnight and individual colonies selected and used to innoculate 5 ml of LB-media cultures containing 50 μg/ml ampicillin. The plasmid constructs were isolated as described in Chapter 2.1.5a and digested with restriction enzymes to check for the presence of the inserts. Figure 3.7 shows that inserts of the correct size were present. The constructs prepared for the yeast two-hybrid system are shown in Table 3.2.

Table 3.2. Plasmid constructs and their encoding fusion protein tested in the yeast two-hybrid system. The DBD and AD proteins are 27 kDa and 19 kDa respectively. All fusions are N-terminal to GAL4.

Plasmid	Encoding	Project Acronym	Fusion Protein Size (kDa)
pAS2-1/ACP	Acyl-acyl carrier protein fused to the GAL4 DBD (DNA binding domain).	DBD/ACP	37
pAS2-1/ENR	Enoyl-ACP reductase protein fused to the GAL4 DBD	DBD/ENR	61
pAS2-1/TE	Oleoyl-ACP thioesterase fused to the GAL4 DBD	DBD/TE	65
pAS2-1/DES	Stearoyl-ACP desaturase fused to the GAL4 DBD.	DBD/DES	61
pACT2/ACP	Acyl-acyl carrier protein fused to the GAL4 AD (activation domain)	AD/ACP	29
pACT2/ENR	Enoyl-ACP reductase fused to the GAL4 AD (activation domain)	AD/ENR	53

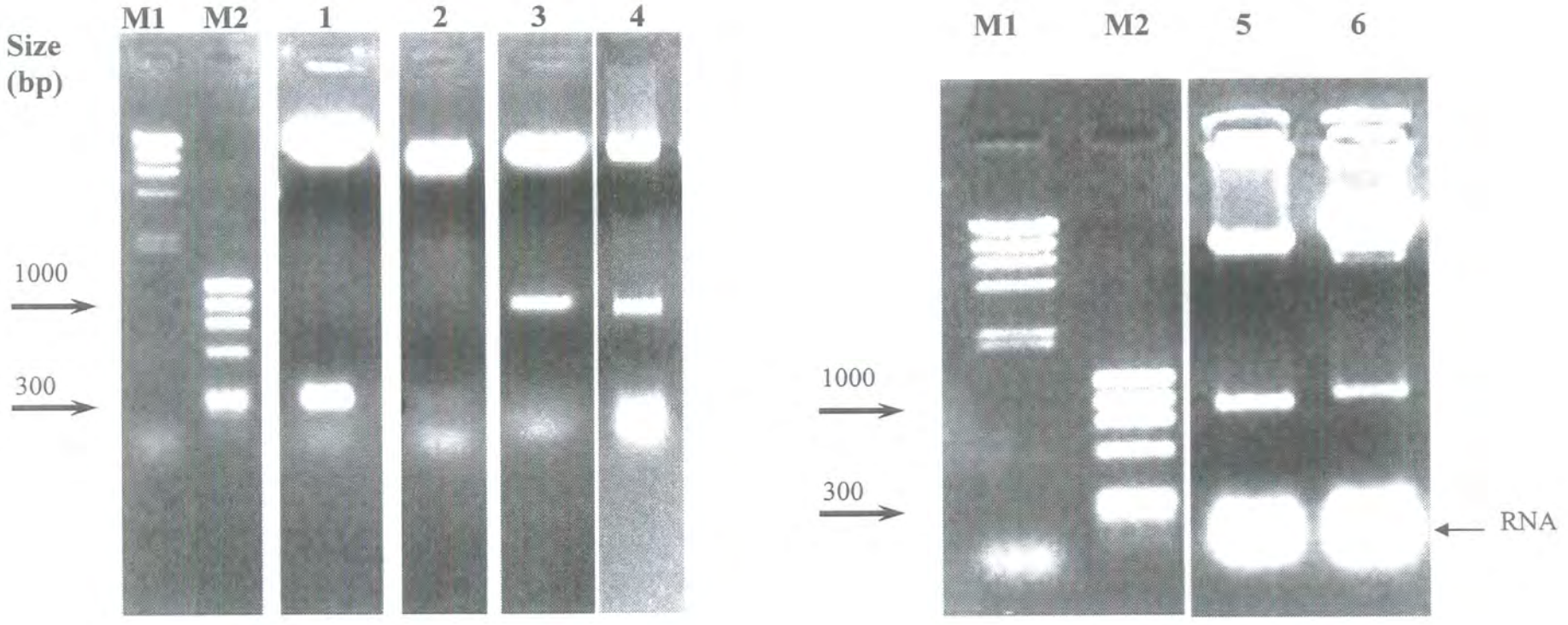


Figure 3.7. Restriction digest of pAS2-1 and pACT2 constructs. DNA constructs were subjected to restriction digest to confirm the presence of the correct insert. The presence of the correct insert was confirmed by the bands on the gel at the correct size. RNA present was kept in the DNA preparation to act as a carrier for the DNA in subsequent yeast transformations. 5 µl from 300 µl of midiprep DNA was digested and the product loaded on to a 0.8 % agarose gel. M1: λHind III marker; M2: ΦX174 Hae III marker; lane 1: pAS2-1/ACP digested with *BamH*1/*Nco*1; lane 2: pACT2/ACP digested with *Bam*H1/ *Nco*1; lane 3: pAS2-1/ENR digested *BamH*1/ *Nco*1; lane 4: pACT2/ENR digested with *Bam*H1/ *Nco*1; lane 5: pAS2-1/TE digested with *Eco*R1/*Bam*H1; lane 6: pAS2-1/DES digested with *EcoR*1/*Nde*1.

3.1.1d. Cloning: Sequencing of constructs - identification of an anomaly in the Genbank database for the DNA sequence deposited for ACP of *Brassica napus*.

Following the cloning of the cDNA of ACP, ENR, TE and DES into the vectors pAS2-1 and pACT2, all constructs were subsequently sequenced in both directions, across the whole encoding region, to ensure no errors in the sequence were present. A 5 µl aliquot of plasmid construct DNA (3.2 pM) was sequenced by primer extension using forward and reverse primers specific for pAS2-1 or pACT2 (Table 3.3).

Table 3.3. Sequencing primers used for sequencing the cloned inserts of pAS2-1 constructs (DBD sequencing primers) and pACT2 constructs (AD sequencing primers).

GAL4-DBD Sequencing Primers	**Primer sequence**	**Tm (°C)**
Forward	5'–TCATCGGAAGAGAGTAG–3'	44.2
Reverse	5' - CGTTTTAAAACCTAAGAGTCA C - 3'	47.8
GAL4-AD Sequencing Primers		
Forward	5'–TACCACTACAATGGATG–3'	41.8
Reverse	5' - GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG AT CG -3'	62

An Entrez and Swiss-Prot database search using Genbank BLAST2 algorithm identified the pAS2-1/ACP and pACT/ACP constructs as being mismatched to the Genbank deposited sequence (Accs. nos. X13128, Safford *et al.*, 1988). The mismatch occurred at the highly conserved site 'LEEEFH'. The Genbank sequence yielded 'LDEEFD' for the sequence (Figure 3.8). This region is near the critical phosphopantetheine-binding site.

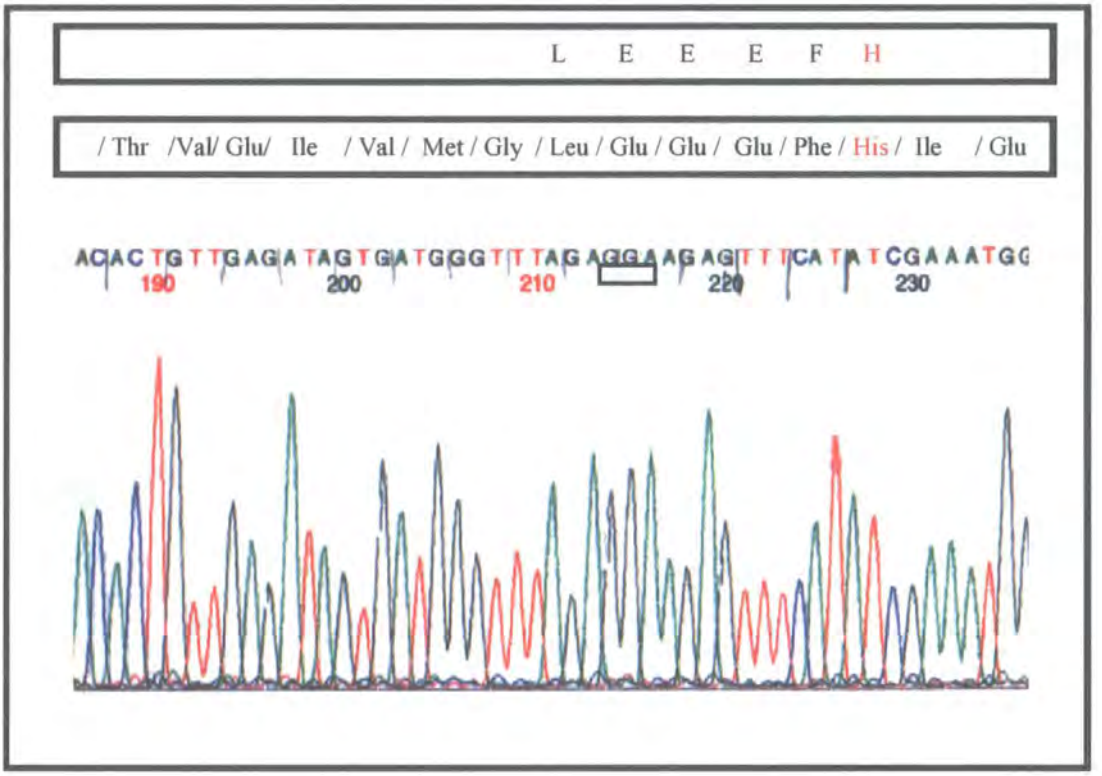


Figure 3.8. Example of sequence analysis output of clone pAS2-1/ACP using the forward and reverse primers for sequencing across the pAS2-1 multiple cloning site/insert junctions (Table 3.3). The DNA triplets coding for LEEEFH are shown and the translated protein sequence is shown in single letter code.

For this reason, it was imperative to ascertain if the base change could have been a mutation that occurred whilst cloning ACP into the pAS2 and pACT2-1 vectors. Subsequently, the sequence was checked to Swissprot, to the authors' own paper (Safford *et al.*, 1988) and to an independent reference (Suh *et al.*, 1999). This confirmed that 'LEEEFH' was the correct sequence. The incorrect entry in the Genbank database and the high degree of sequence conservation are shown in Table 3.4. This degree of sequence conservation relates to the idea that the ACP proteins diverged from a common ancestor (McCarthy *et al.*, 1984).

Mutations may directly cause the loss of any potential interaction of ACP with the FAS enzymes. Furthermore, holo-ACP is the pre-requisite for all uses of ACP in the cell (Lambalot and Walsh, 1991). This includes fatty acid synthesis and degradation, the synthesis of membrane-derived oligosaccharides (Therisod *et al.*, 1986), polyketides (Shen *et al.*, 1992) and in the activation of haemolysin an *E. coli* membrane-targeted toxin (Issartel *et al.*, 1991). Therefore, interactions may also be lost indirectly by alteration of the structure in such a way that the ACP cannot be phosphopantetheinlyated. The phosphopantetheinylation of apo-ACP is dependent on the overall structure of ACP, rather than being sequence specific (Perham, 2000).

Table 3.4. Sequence comparison of ACP in, and, between species.

ORGANISM	ACP nomenclature	Sequence surrounding highly conserved LEEF site	GENBANK Acc. Nos.	References*
Brassica napus	pAS2-1/ACP (29C08) this project	GAD **S** LDTVEIVMG LEEEFH IEMAEE	CAA31519	1
	Genbank Database entry 29C08 (Dec. 2000)	GAD **S** LDTVEIVMG LDEEFD IEMAEE	-	1
	29C08 (Ref. 1)	GAD **S** LDTVEIVMG LEEEFH IEMAEE	-	1
	22C01 (Ref 1.)	GAD **S** LDTVEIVMG LEEEFD IEMAEE	CAA31518	1
	28f10 (Ref. 1.)	GAD **S** LDTVEIVMG LEEEFG IQMAEE	CAA30782	1
Spinach	-	GAD **S** LDTVEIVMN LEEEFG INVDED	AAA34023	2
Barley	-	GAD **S** LDTVEIVMG LEEEFN ITVDET	AAA32923	3
E. coli	-	GAD **S** LDTVELVMA LEEEFD TEIPDE	AAB27925	4

Analysis at the region surrounding the phosphopantetheine binding site (serine residue-S) revealed the Genbank entry for ACP (Genbank accession nos. X13128) was incorrect at the highly conserved residues. The entry should read as LEEEFH and not LDEEFD as stated. This was confirmed by the study of the paper from which the database entry is derived (Safford *et al*, 1998) and confirmed in a seperate reference (Suh *et al*, 1999). *References: 1- Safford *et al.* 1988; 2- Scherer and Knauf; 1987; 3 - Hansen L., 1987; 4 - Jones *et al.* 1993.

3.1.2 Interaction assay.

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When the sequences were checked and found to have no mutation, interaction tests proceeded. This involved three steps: phenotype verification of the yeast strains, transformation of the yeast strains with the control plasmids, interaction tests of the construct proteins (Figure 3.9 and 3.10).

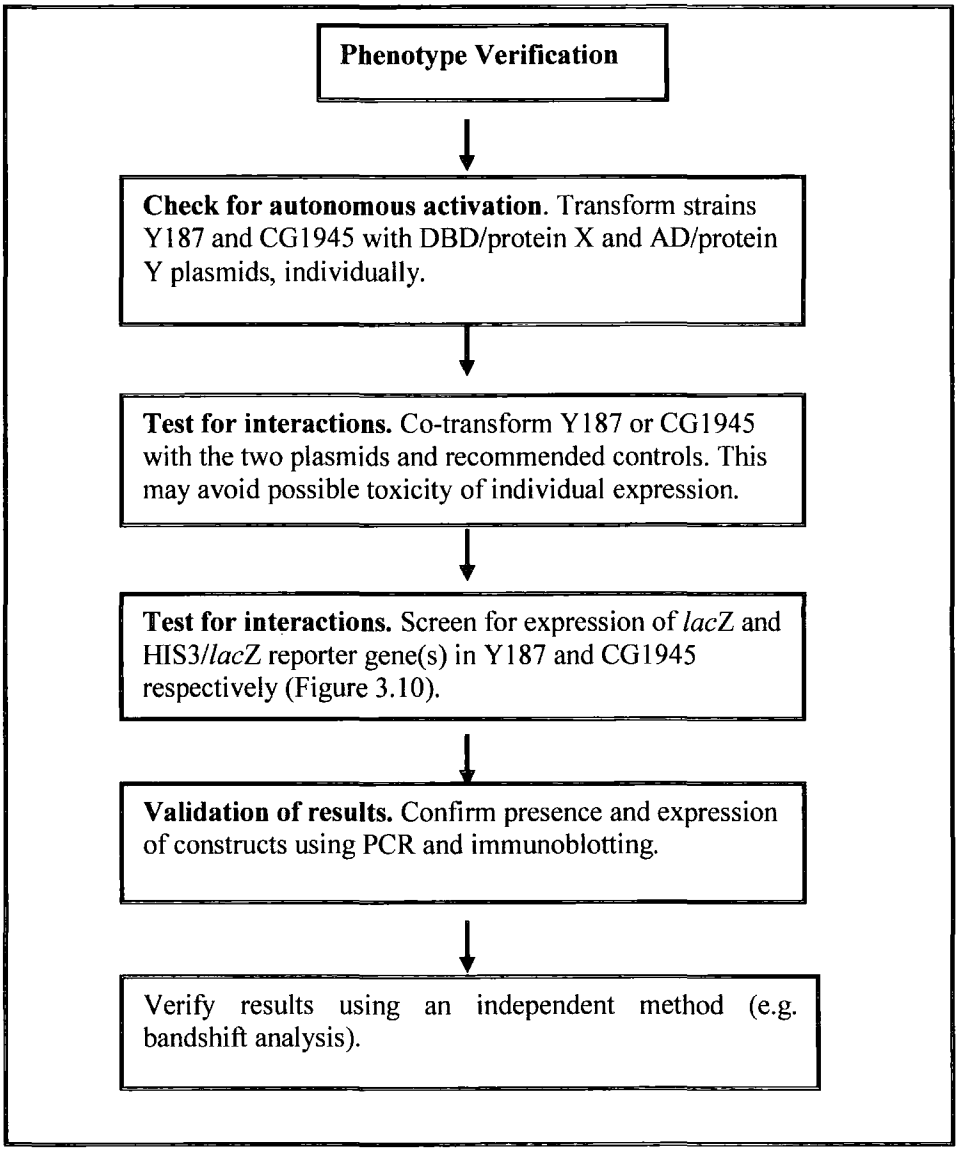


Figure 3.9. Flow chart for performing a two-hybrid protein-protein interaction assay.

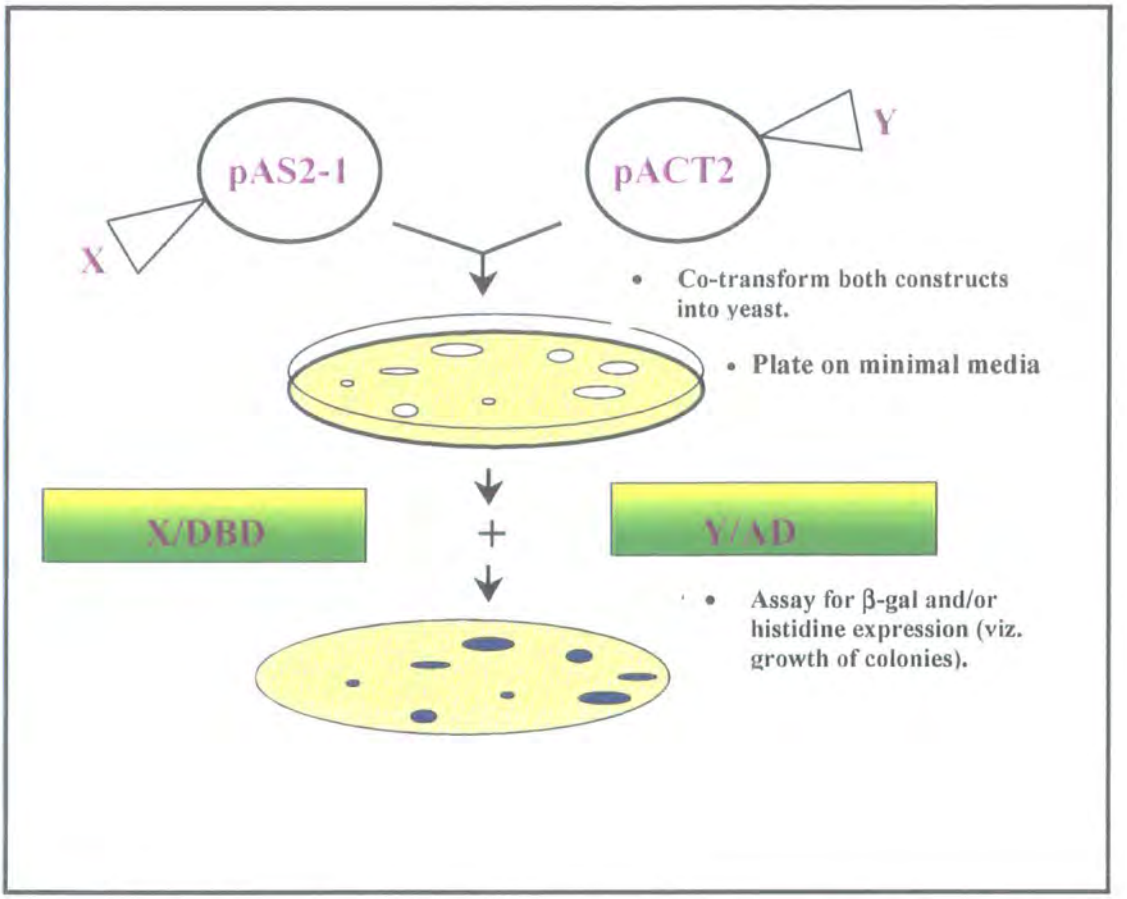
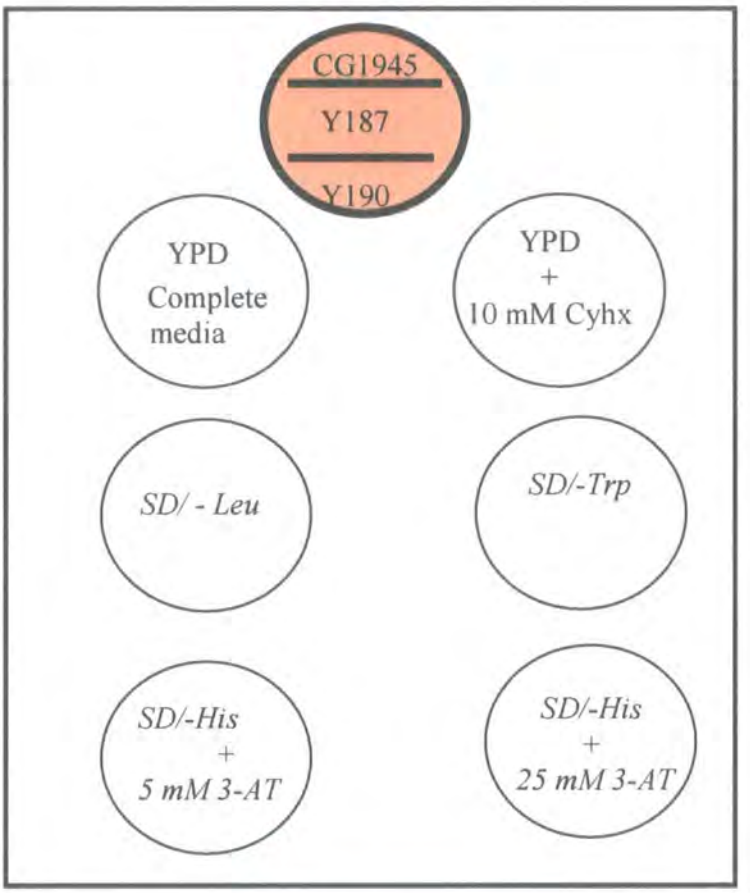


Figure 3.10. Schematic diagram of the yeast two hybrid assay. X and Y, cDNA of proteins to be cloned in to Y2H vectors. DBD, DNA binding domain; AD, activation domain.

3.1.2a. Phenotype verification of yeast strains Y187, CG1945 and Y190.

The important difference between the yeast strains used in this project, apart from mating type and cycloheximide and 3-AT (3-aminotriazole) sensitivity, are the promoter differences. In GAL4 based Matchmaker two-hybrid systems, either an intact GAL4 UAS (which contains four GAL4-binding sites) or an artificially constructed UAS consisting of three copies of the 17-mer consensus binding sequence, is used to confer regulated expression of the reporter genes. The intact GAL1 promoter tightly regulates the *lacZ* reporter gene of Y187 and the HIS3 reporter gene of CG1945. The *lacZ*

reporter gene of CG1945 is under control of $UAS_{G\ 17\text{-}mer(x3)}$ and the extremely weak promoter of yeast cytochrome C1 (CYC1) gene. The *lacZ* reporter under the control of the intact GAL1 promoter can be expressed at approximately ten times the level of *lacZ* under the control of $UAS_{G\ 17\text{-}mer(x3)}$/ CYC1 minimal promoter construct (Chapter 2.1.4.; Table 2.0., Clontech data). Therefore, some weak or transient interactions not detectable in CG1945 may be detectable in Y187. Furthermore, if the interacting proteins can activate transcription from these two different promoters (strains) then this is a further validation of a true positive result. This is because Y187 and CG1945 have only the GAL4 responsive elements in common. The rest of the promoter sequences differ significantly. Therefore, any positive two-hybrid signals observed in both strains are likely to require binding of the GAL4-DBD specifically to the GAL4-responsive element (Bartel *et al.*, 1993). Before proceeding with pairwise interaction test, the phenotype of these yeast strains provided in the Clontech Matchmaker II kit was verified by growing the strains on selective media. Strains were taken from glycerol stocks stored at –80 °C and grown on complete yeast media (YPD) and subsequently plated onto synthetic dropout (SD) media. Strains CG1945 and Y190 will grow in the presence of cycloheximide at concentrations of 1 and 10 μg/μl, respectively. As CG1945 and Y190 use *lacZ* and HIS3 reporter genes, they were also plated on 3-AT at concentrations of 5 mM and 25 mM respectively. The 3-AT prevents leaky expression of histidine. Figure 3.11a, b confirmed that the phenotype of all yeast strains was correct. Although not apparent in Figure 3.11b, some residual growth was seen by Y190 on 25 mM 3-AT underscoring the need for phenotype verification to prevent misinterpretation of the result.



	Media				
	SD/-Trp	SD/-Leu	SD/-His	YPD	YPD + Cyhx
Strains					
Y187	-	-	-	+	+
[a]CG-1945	-	-	-[b]	+	+[c]
[a]Y190	-	-	-[d]	+	+[e]
a: These strains are cycloheximide sensitive; however, cycloheximide resistant colonies may appear as a result of spontaneous mutations.					
b: In the presence of 5-15 mM 3-AT					
c: In the presence of 1.0 µg/ml cycloheximide					
d: In the presence of 25-60 mM 3-AT					
e: In the presence of 10.0 µg/ml cycloheximide					

Figure 3.11a. Key and table for phenotype verification of yeast strains used in the Clontech Matchmaker II yeast two-hybrid system. The table indicates the expected results. Abbreviations: YPD, complete media; SD, synthetic dropout media; 3- AT, 3 – aminotriazole; Cyhx, cycloheximide; - Trp, minus tryptophan; - Leu, minus leucine; - His, minus histidine.

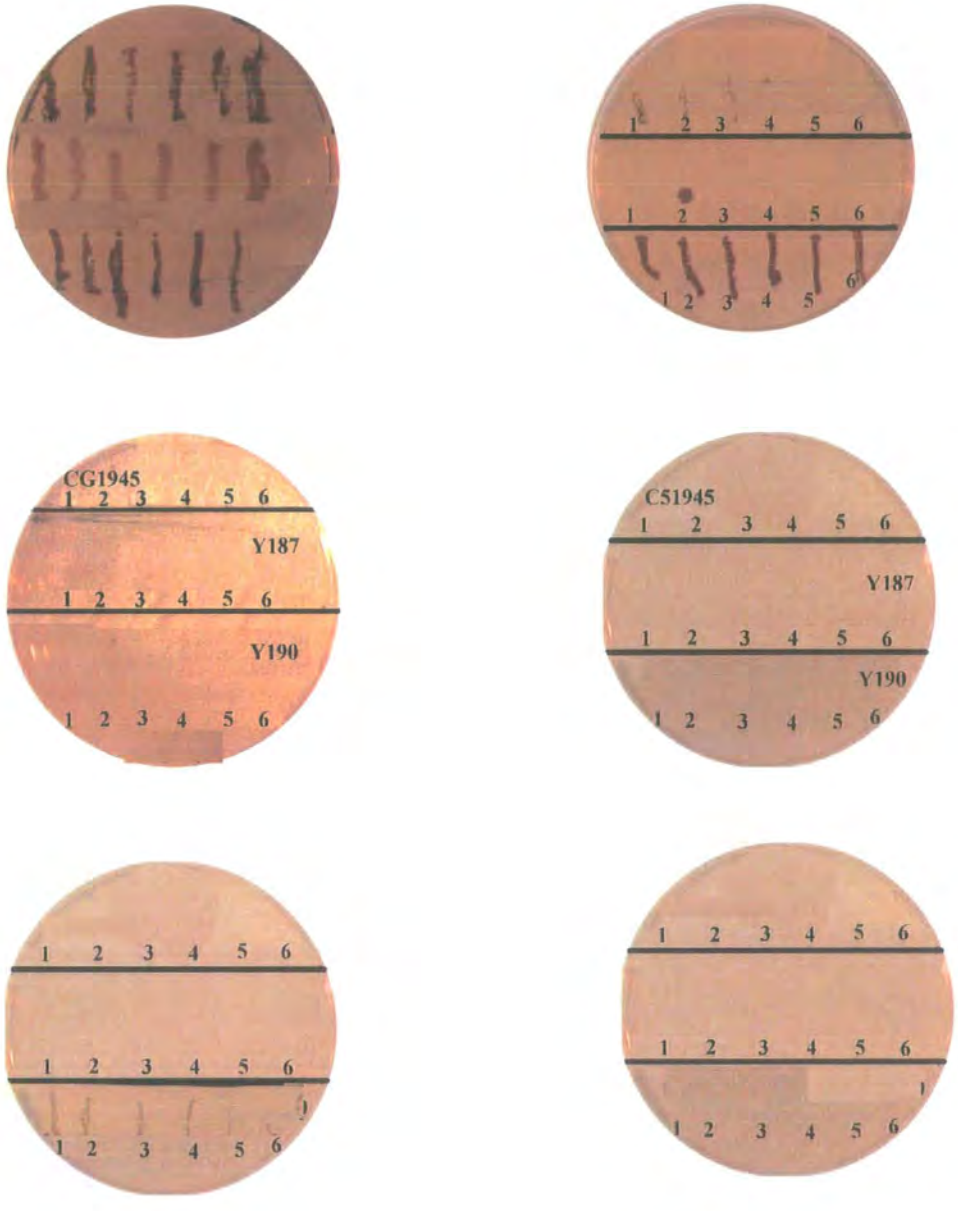


Figure 3.11b. Phenotype verification of yeast strains used in the Clontech Matchmaker II yeast two-hybrid kit. Yeast strains were recovered from glycerols by streaking on complete media (YPD) followed by streaking onto YPD containing cycloheximide (10 µg/ml) or synthetic drop-out media (SDO) minus the amino acid(s) indicated in Figure 3.11a. The expected results are shown in the table opposite and confirm that the yeast strains were suitable for use in interaction tests, Nos. 1-6 = repeats.

3.1.2b. Transformations with control plasmids.

Unlike transformation of bacteria, the transformation of yeast requires saturating amounts of DNA and a method to permeabilise the tough cell wall of yeast. For this reason, midi-preps of DNA were made of the shuttle vectors (pAS2-1 and pACT2) and the control plasmids supplied by Clontech. Plasmid DNA was isolated using the Qiagen Midi-prep extraction kit according to the manufacturers instructions. Spectrophotometer readings indicated that the DNA was highly concentrated, approximately 1 μg/μl. For the transformations, sonicated herring sperm DNA was added as a carrier, RNA was not removed from the midi-preps of the constructs, as this is also a carrier of DNA (Chapter 2.1.5c).

To confirm that the yeast strains would perform as expected, yeast strains Y187 and CG1945 were transformed with negative and positive control constructs provided by Clontech (UK). Two positive controls were provided. The first was the full length GAL4 protein carried in plasmid pCL1-1. The second was the interaction between the SV40 T-antigen and the tumour suppressor p53 (Li and Fields, 1993). The cDNA of these proteins was carried in the pTD1-1 and pVA3-1 plasmids, respectively (Table 3.5). Both positive controls caused the activation of the reporter *lacZ* in Y187 and *HIS3/lacZ* in CG1945 (Figure 3.12.). The results confirmed that the Y187 *lacZ* reporter was very much stronger than CG1945. The expression of the reporter genes in these control experiments and consequent colouration were used in a semi-quantitative fashion to

gauge the strength of any interactions that may be revealed by the yeast-two-hybrid system.

Table 3.5. Control plasmids used in the Matchmaker II yeast two-hybrid system (Clontech, UK).

Plasmid	Encoding	Purpose
pACT2	GAL4 AD (activation domain), LEU 2, amp^r	8.4 kb cloning vector; used to generate fusions of the bait protein with the GAL4 DBD.
pAS2-1	GAL4 DBD (DNA binding domain), TRP1, amp^r	8.1 kb cloning vector; used to generate fusions of a known protein (or a collection of random, unknown proteins) with the GAL4 AD.
pCL1-1	full-length GAL 4, LEU2, amp^r	15.3 kb positive control plasmid; encodes the full-length, wild-type GAL4 protein.
pLAM5'-1	GAL4 DBD/human laminC hybrid (in pAS2-1), TRP1, amp^r	9.1 kb false-positive detection plasmid; encodes a DBD/human lamin C fusion protein in pAS2-1.
pTD1-1	GAL4 AD-SV40 large T-antigen hybrid (in pACT2), LEU 2, amp^r	9.9 kb positive control plasmid used with pVA3-1; encodes an AD/SV40 large T-antigen fusion protein in pACT2.
pVA3-1	GAL4 DBD, murine p53 hybrid, TRP1, amp^r	9.4 kb positive control plasmid used with pTD1-1.

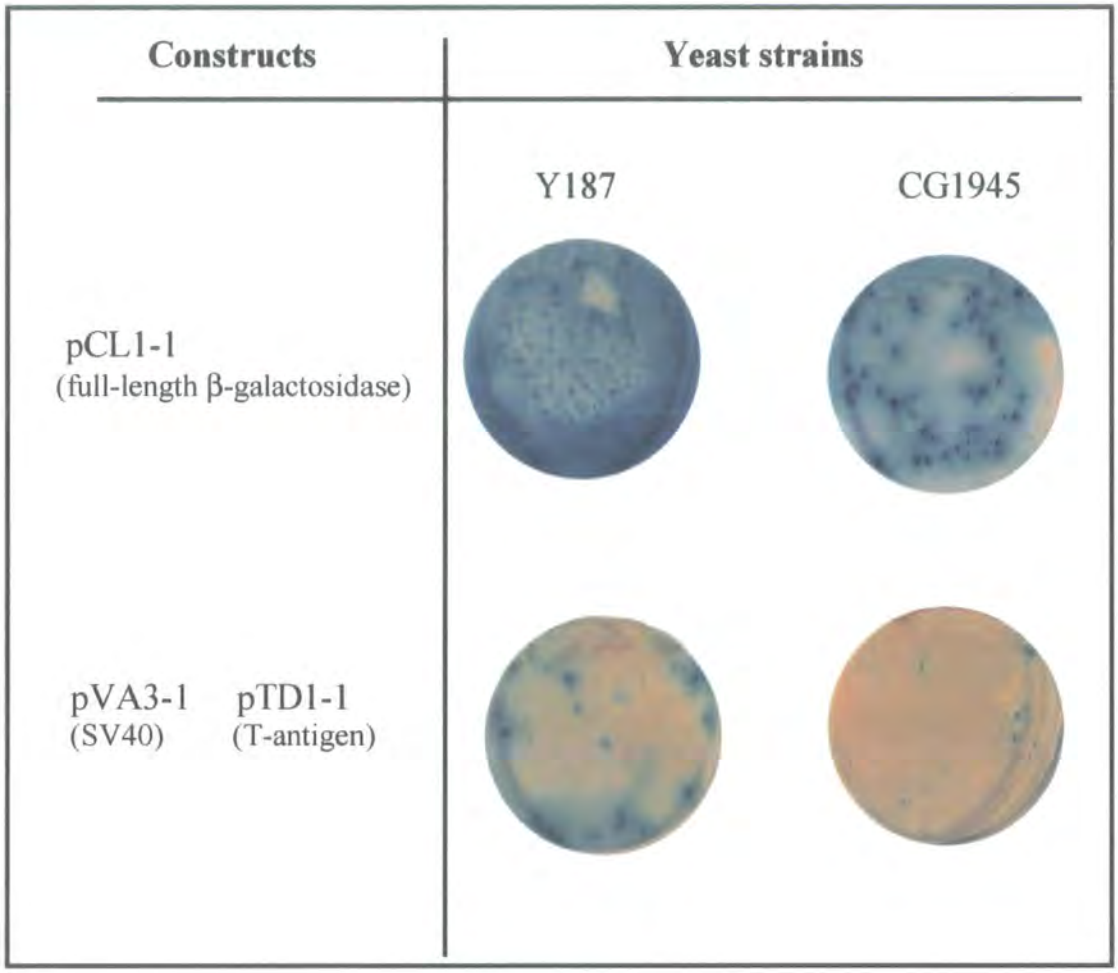


Figure 3.12. Yeast two-hybrid protein-protein interaction assays using the control plasmids for the Clontech Matchmaker II system. Yeast strains Y187 or CG1945 were simultaneously transformed with the plasmids indicated, using the lithium acetate/PEG protocol. An aliquot containing 1 μg of DNA of each of the positive and negative control plasmids was used. The results show representative colony filter lifts of the positive controls: pCL1-1 (the full-length β-galactosidase protein), pVA3-1 (DBD/murine p53 protein in pAS2-1) and pTD1-1 (GAL4 AD-SV40 large T-antigen hybrid in pACT2). The latter two plasmids expressed the SV40 protein and T-antigen, respectively. The results confirmed that: a) CL1-1 gave the strongest expression; b) the Y187 *lacZ* reporter was very much stronger than CG1945.

3.1.2c Pairwise interaction tests of the type II FAS enzymes of *Brassica napus.*

The constructs (Table 3.2) that had been checked by restriction digest and fully sequenced in both 5' and 3' directions were used to transform the yeast strains Y187 and CG1945. The most suitable strain and reporter system was unknown. Therefore, pairwise interaction tests were carried out using two different yeast strains, Y187 and CG1945.

The constructs were tested individually and pairwise along with the controls. Transformation of the individual plasmids was necessary because if there was autonomous activation i.e. activation of reporter genes from one fusion protein, meaningful results would be difficult to obtain. In the pairwise tests, simultaneous transformations of the constructs was done in order to avoid any possible toxicity effects that expression of these foreign proteins may have on yeast. The constructs were transformed at the same time as the control plasmids provided by Clontech. This allowed the detection and the estimate of strength of the interaction to be more accurately assessed. Transformations were carried out as described in Chapter 2.1.5c. Following transformations the plates were incubated until colonies appeared. Approximately 500 - 1000 transformants were obtained per microgram of DNA for the controls and the individual constructs. This was the standard for yeast but was relatively inefficient compared to *E. coli.* This is due to the cell wall of yeast (Ito *et al.*, 1983). Colonies of Y187 and CG1945 were assayed for β-gal activation as described in Chapter 2.1.8.

Earlier experiments from Li and Fields (1993) working with SV40 T-antigen and murine p53 showed that two-hybrid signals could be correlated with the affinities between interacting proteins. Clontech (UK) subsequently incorporated this interaction as a positive control for the yeast two-hybrid system. In both Y197 and CG1945, there is no direct correlation between K_d and expression of the reporter genes *lacZ* or HIS3 (Estojak *et al.*, 1995). However, the nature of the yeast two-hybrid system allows the *relative strength* of protein-protein interactions to be gauged between a related set of transformants within the same strain. In Y187, this can be gauged by the colour of the colony. This may range from an intense blue (strong interaction), blue, light blue, very light blue, to white (no interaction). In CG1945, the relative interaction strength between a set of related protein may be gauged from the number of colonies that grow. This is because the fusion protein interactions cause the expression of the HIS3 reporter gene allowing the yeast to grow. If these colonies subsequently tested positive for *lacZ*, this is a further indication of a relatively high strength of interaction. In this project, this allowed a semi-quantitative estimation of interaction strength based on how blue the colonies were (Y187) and the number of colonies that grew (CG1945). The related sets of protein-protein interactions in this project were the interactions of type II FAS proteins. Kit control plasmids provided by Clontech provided the simplest way for intra- and inter- experimental variability to be taken into account (Table 3.6, Table 3.7).

Due to the nature of the yeast two-hybrid system, using it in a semi-quantitative fashion may lead to misinterpretation of results. A major pitfall in the interpretation stems from the fact yeast two-hybrid system can generate both false positive and false negative results. False positives, for example, are commonly generated when an individual fusion

protein (DBD/X or AD/Y) will activate transcription from the reporter gene i.e. autonomous activation. However, it may be possible to get round this by deleting a small region of the protein that activates transcription. In this project, in order to look for any signs of autonomous activation from the fusion proteins, constructs were transfected, individually, into both Y187 and CG1945. Subsequent colony filter assays revealed no signs of autonomous activation (Table 3.6, Table 3.7). A false negative is when some protein pair that interacts in their native environment will not interact when fused to the DBD or AD. False negatives are generally harder to detect with novel protein pairs, for example when carrying out a Y2H library screen, as there will be no activation of reporter gene. However, it can be revealed if the two proteins have previously been shown to interact in another assay, but do not do so in the Y2H system. Due to false positives and false negatives, all results from the Y2H system, must be cross-checked with other experimental and/or published data.

In this work, the DBD/ENR and AD/ENR interaction produced a strong blue colour in Y187, and by the formation of many colonies in CG1945 (Figure 3.13; Table 3.8; Table 3.9). The *Brassica napus* ENR has been previously characterised as a stable 130 kDa homotetramer (Rafferty *et al.*, 1994). Therefore, the ENR/ENR interaction provided a positive control for these experiments whereby, the strong, consistent, interactions would allow the relative affinities of other hetero-enzymatic interactions to be compared to a type II FAS enzyme. These results also indicated that the native state of the protein may be retained in the yeast two-hybrid assay and the N-terminal fusion does not interfere with the interaction. A similar result was found by Heath and Rock (1996) on examining the type II FAS enzymes of *E. coli* i.e. ketoacyl-ACP synthetase I (FabB) and

β-HAD (FabA) for evidence of direct interactions. These authors reported positive results i.e. homodimerization occurred of FabB. As in this thesis, Heath and Rock (1996) took this to mean that the N-terminal fusion did not interfere with dimerization. Unlike the present project, these authors could not detect heteroenzymatic interactions.

In this thesis, the use of the yeast two-hybrid system allowed detection of direct hetero-enzymatic interactions between the enzymes of type II FAS from *Brassica napus* (Figure 3.13). In Y187, the hetero-enzymatic interaction of DES and ENR was indicated by a much weaker *lacZ* expression than the ENR homotetrameric association, producing a light blue colouration (Figure 3.13, Table 3.8). This was the first indication that this heteroenzymatic interaction was weak and perhaps transient. Interactions are a necessary requirement for the growth of CG1945 yeast colonies and when the DBD/DES and AD/ENR proteins were co-expressed in CG1945 there was growth (Figure 3.13; Table 3.9) but there was no detectable activity of *lacZ*. This further strengthened the proposition that the DES/ENR interaction was weak and/or transient. The fact that the DES/ENR interaction did not activate *lacZ* in CG1945 may have been because the *lacZ* expression in CG1945 is approximately ten times less strong than Y187, due to the difference in promoters between these strains. However, as there was no *lacZ* expression from the ENR/ENR subunit interaction, phenotype verification was repeated. No fault was found with CG1945, the nutritional selection, and control plasmids behaved as predicted presenting the same results as shown in Figure 3.11. The TE/ENR co-transformation only activated transcription in Y187 (Figure 3.13, Table 3.8). Furthermore, as the blue colouration was barely detectable it was possible that it may

have been a false positive. On the other hand, it is supported by previous biochemical evidence (Hellyer *et al.*, 1992). No interactions were detected between ACP and any other enzyme tested. The failure of ACP to show any interaction, as set out above, may have been a false negative result. Potential reasons for this include steric hindrance, and/or immeasurably weak and transient interactions and/or a requirement for a stabilsing cofactor/substrate (i.e. dynamic interactions). Such dynamic interactions of

Table 3.6. Yeast two-hybrid system transformation table for Y187.

Group 1 - Control plasmids

pAS2-1 (DBD)	pACT2 (AD)	Selection	No. of colonies	ß-gal assay development time
CL1-1		-Leu	1000	10minutes
AS2-1	ACT2	-Leu-Trp	1000	-
VA3-1	ACT2	-Leu-Trp	200	-
AS2-1	TD1-1	-Leu-Trp	300	-
VA3-1	TD1-1	-Leu-Trp	500	30 minutes
LAM5-1	TD1-1	-Leu-Trp	1000	-

Group 2 - Individual plasmids

pAS2-1 (DBD)	pACT2 (AD)	Selection	No. of colonies	ß-gal assay development time
ACP		--Trp	1000	-
ENR		-Trp	1000	-
TE		-Trp	1000	-
DES		-Trp	1000	-
-	ACP	-Leu	250	-
	ENR	-Leu	500	-

Group 3 - Co-transformants

pAS2-1 (DBD)	pACT2 (AD)	Selection	No. of colonies	ß-gal assay development time
ACP	ACP	-Leu-Trp	3	-
ACP	ENR	-Leu-Trp	~ 200	-
ENR	ACP	-Leu-Trp	6	-
ENR	ENR	-Leu-Trp	~ 300	12 hours
TE	ACP	-Leu-Trp	45	-
TE	ENR	-Leu-Trp	~ 200	24 - 48 hours
DES	ACP	-Leu-Trp	48	-

The table shows the constructs and controls that were employed for protein-protein interaction assays. All three groups of transformations were carried out together. Yeast was then incubated at 30 ^{O}C until colonies appeared. Subsequently, colony filter assays were taken. The '-'indicates no development of colour after 72 hours. No autonomous activation could be detected from yeast transformed with individual constructs.

Table 3.7. Yeast two-hybrid system transformation table for CG1945.

Group 1 - Control plasmids

pAS2-1 (DBD)	pACT2 (AD)	Selection	No. of colonies	Growth time (days)	ß-gal assay
CL1-1		-Leu	500	2	30 minutes
AS2-1	ACT2	(-LTH + 5mM 3-AT)			-
VA3-1	ACT2				-
AS2-1	TD1-1				-
VA3-1	TD1-1		155	2	2 hours
LAM5-1	TD1-1				-

Group 2 - Individual plasmids CG1945

pAS2-1 (DBD)	pACT2 (AD)	Selection	No. of colonies	Growth time (days)	ß-gal assay
ACP	-	-Trp	100	3	-
ENR	-	-Trp	34	3	-
TE	-	-Trp	41	3	-
DES	-	-Trp	44	3	-
-	ACP	-Leu	8	3	-
-	ENR	-Leu	37	3	-

Group 3 - Co-transformants CG1945

pAS2-1 (DBD)	pACT2 (AD)	Selection	No. of colonies	Growth time (days)	ß-gal assay
ACP	ACP	-LTH + 5mM 3-AT*	-	-	-
ACP	ENR	-LTH + 5mM 3-AT	-	-	-
ENR	ACP	-LTH + 5mM 3-AT	-	-	-
ENR	ENR	-LTH + 5mM 3-AT	105	6	-
TE	ACP	-LTH + 5mM 3-AT	-	-	-
TE	ENR	-LTH + 5mM 3-AT	-	-	-
DES	ACP	-LTH + 5mM 3-AT	-	-	-
DES	ENR	-LTH + 5mM 3-AT	50	6	-

The time of growth of colonies varied from 3 - 10 days. Growth occurred on SDO minus - LTH + 5 mM 3-AT and indicated that the fusion proteins were interacting and causing activation of the reporter gene. Colony filter assays were subsequently taken. *Abbreviations: - LTH + 5 mM 3-AT, SDO media minus leucine, tryptophan and histidine + 5mM 3-amino triazole (3-AT). The 3-AT was included to suppress any residual HIS3 expression of CG1945.

Figure 3.13(overleaf). Yeast two-hybrid analysis of FAS II components in two different yeast strains. Yeast strains Y187 or CG1945 were co-transformed with the DNA binding domain plasmid (pAS2-1) constructs and the activation domain plasmid (pACT2) constructs indicated. Co-transformation was carried out simultaneously with the transformation of individual constructs and the Matchmaker II kit controls. Transformed yeasts were then plated onto the appropriate synthetic drop out media. In Y187, interactions were indicated by *lacZ* expression. In CG1945 interactions were indicated by HIS3 and *lacZ* expression. Heteroenzymatic interactions were detected between ENR and DES. The level of *lacZ* expression from the TE/ENR co-transformation was extremely weak and was viewed as a possible interaction, rather than positive interaction. Abbreviations: ENR, enoyl reductase; TE, thioesterase; DES, stearoyl ACP-desaturase; ng, no growth.

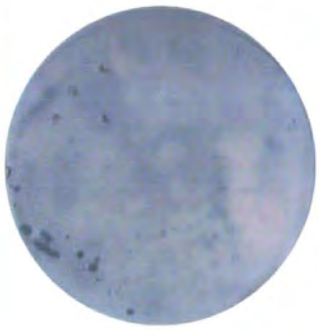
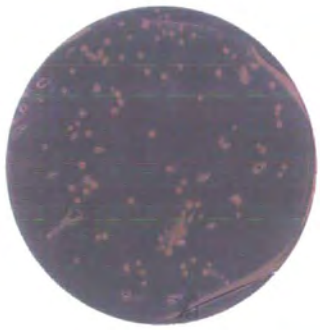
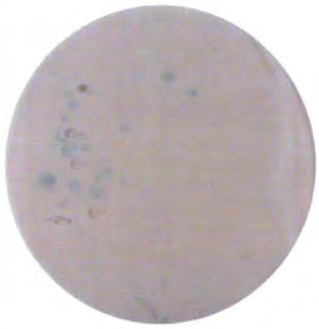
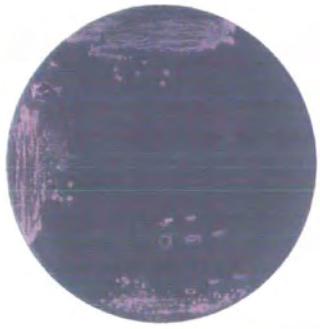
Construct		Yeast strains	
pAS2-1	pACT2	Y187	CG1945
ENR	ENR		
DES	ENR		
TE	ENR		 ng

Table 3.8 Y187 yeast two-hybrid interaction matrix of FAS II components of *Brassica napus*.

DNA binding domain	Activation Domain	Y187-*lacZ* development time	*LacZ* Phenotype. (colony color)	Nos. of times interactions detected
pCL1 - full length GAL4 protein		10-30 minutes		4/4
pVA3-1 (murine p53)	pTD1-1 (SV40 T-antigen)	30 -120 minutes		3/4
ENR	ENR	12 hours		3/4
DES	ENR	24-48 hours		2/3
TE	ENR	24-48 hours		2/3
ACP	ACP ENR TE DES	No *lacZ* expression		0/9*

Interactions assays were carried out in yeast strain Y187. The result represented the spectrum of strength of reporter gene expression (indicated by the intensity of colour). Whereby, ENR homotetrameric interactions were as strong as the positive controls (T–antigen and p53), the DES/ENR strength of expression was several fold lower than that of ENR, and TE/ENR interactions lower again.

Table 3.9. CG1945 yeast two-hybrid interaction matrix of FAS II components of *Brassica napus*.

DNA binding domain	Activation Domain	CG1945 colony formation time (days)	Nos. of colonies	Nos. of times interactions detected
pCL1 - full length GAL4 protein		2	500	5/5
pVA3-1 (murine p53)	pTD1-1 (SV40 T-antigen)	2	155	5/5
ENR	ENR	6	105	5/5
DES	ENR	6	50	2/3
TE	ENR	0	ng	0/3
ACP	ACP ENR TE DES	0	ng	0/9

Interactions assays were carried out in yeast strain CG1945. In CG1945, interactions were indicated by expression of the HIS3 marker. The stronger the interaction, the more colonies that grew. Therefore, ENR/ENR homotetrameric interactions appeared to be the strongest, followed by the DES/ENR interactions. Abbreviations: ng, no growth

ACP, or indeed any other enzyme of type II FAS, may be more readily detectable *in situ*, where the proteins would be in their native state and any stabilsiing cofactors or substrates could be present. Subsequently, bandshift analysis experiments using isolated chloroplasts were instigated. This is discussed in Chapter 3.2. In conclusion, the results from the yeast two-hybrid system have revealed a direct interaction between *B. napus* DES and *B. napus* ENR. The results indicated that the DES/ENR interaction of type II FAS of *Brassica napus* may be weak and/or transient.

3.1.2d. The toxicity of the AD/ACP fusion protein.

The fusion protein AD/ACP had a toxic effect on the yeast cells in which it was expressed, whereby growth was reduced to as little as five colonies per plate (Figure 3.14 and Figure 3.15). There are reports of the toxicity stemming from over-expression of many FAS enzymes. For example, in *E. coli* the overproduction of the condensing enzyme FabF (KAS II) has been shown to block fatty acid biosynthesis (Subrahmanyam and Cronan, 1998). Similarly, unmodified ACP (apo-ACP) was found to be a potent inhibitor of cell growth (Keating *et al.*, 1995). Another important fact is that the folding of apo-ACP has been shown to be less stable than that of holo-ACP (Jackowski and Rock, 1983). However, as the DBD/ACP fusion protein did not exhibit such toxicity, nor the activation domain alone (i.e. pACT2 control). This indicates the AD/ACP fusion protein alone, must have been responsible for the toxicity. It has been suggested that as

yeast uses a type I FAS system, the use of the yeast two-hybrid system will be a satisfactory tool to investigate type II FAS interactions (Subrahmanyam and Cronan, 1998), the results here indicate that in certain instances, the yeast two-hybrid system may not be appropriate.

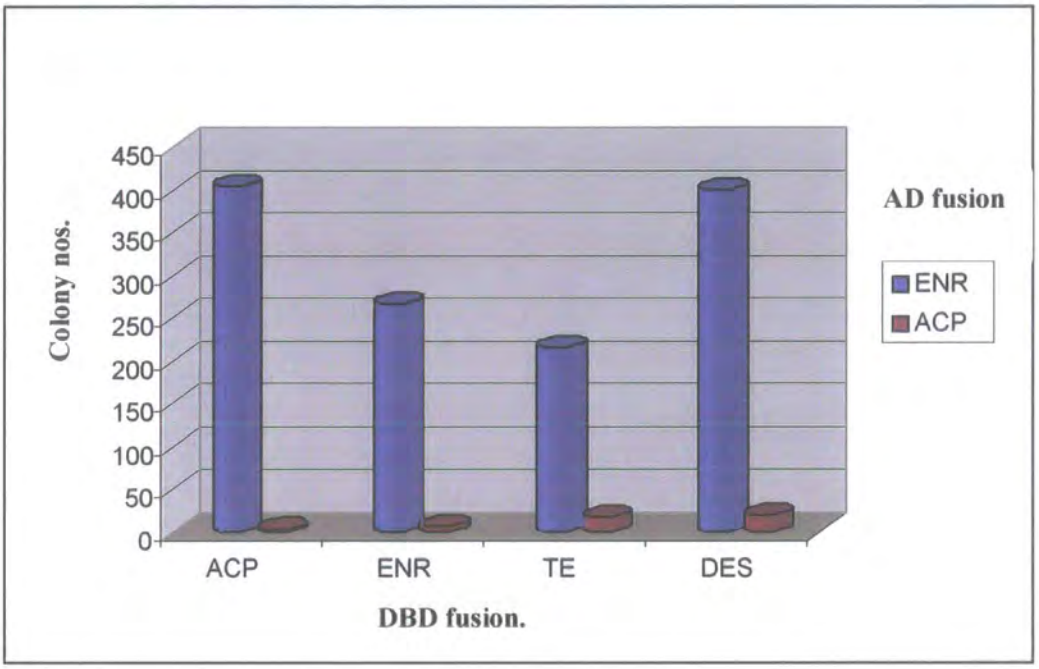


Figure 3.14. Bar graph demonstrating the toxicity conferred on yeast strain Y187 by the activation domain/acyl carrier protein fusion protein (AD/ACP).

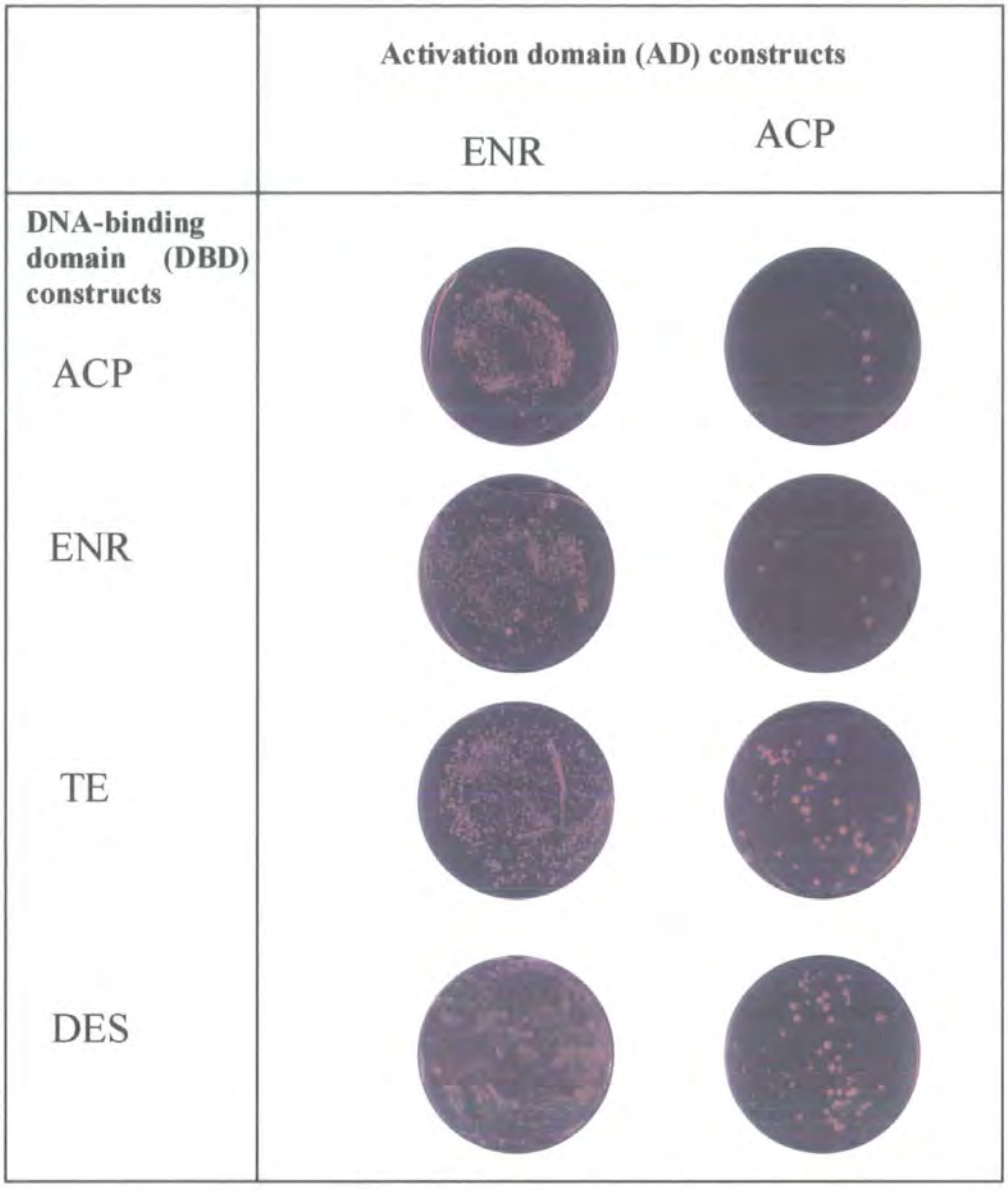


Figure 3.15. The toxicity of fusion protein AD/ACP in yeast. Y187 was co-transformed with the constructs indicated. A 50 µl aliquot of the transformed yeast was plated onto the appropriate selective media. Plates were incubated until yeast colonies were evident (3-7 days). This inhibitory effect on yeast growth was observed in all co-transformations where AD/ACP fusion protein was present. The underlying mechanism was not known. Yeast containing the AD/ENR fusion protein are shown for comparison.

3.1.2e. Confirmation of the presence of the two-hybrid plasmid constructs in yeast by PCR.

It was necessary to confirm that the positive and negative results were or were not due to the presence or absence of the construct plasmid. The extraction of plasmid DNA from yeast is not trivial because of the tough cell wall, the large plasmid size (> 6 kb) and the low copy number (~ 50/cell, Clontech). This may result in a very low plasmid yield. There are several methods to extract yeast DNA, all of which break the yeast chromosomes and release it from cellular material. Once isolated, there are several ways to identify the construct. A digest of backextracted DNA, if it works, may show an insert of desired size. However, the other plasmid and/or inserts may be digested producing overlapping bands and contaminating yeast genomic DNA will also be present.

The choice of method in this project was to use acid-washed glass beads to disrupt the cell wall. Briefly, transformants were grown to saturation in 5 ml of SDO media. The yeast was pelleted by centrifugation for 10 seconds at 15 000 rpm and stored at –80 °C until all samples were ready. Samples were thawed on ice and an equivalent volume of acid washed glass beads and yeast 'DNA breaking buffer' (containing SDS) was added (Chapter 2.1.9). The mixture was vortexed for 15 seconds followed by an interval of 15 seconds on ice. The vortexing and resting was done three times. A 200 µl aliquot of phenol/chloroform was added and the mixture vortexed vigorously for three minutes. The mixture was centrifuged at 16 000 rpm for 5 seconds and the supernatant containing the lysed yeast was removed.

Plasmid DNA extracted from yeast will be contaminated with yeast genomic DNA. For this reason, many protocols (including Clontech) advocate the use of transforming *E. coli* before using the construct plasmid DNA in downstream work. The transformation of *E. coli* using the backextracted constructs was found to be inefficient. Subsequently, the crude plasmid DNA prep was used as the template for PCR. This was found to be much quicker and more efficient and construct analysis proceeded using this method. Initial trials of PCR using pAS2-1 and pACT2 forward and reverse sequencing primers were unsuccessful. The primers were subsequently changed to insert (ACP, ENR, DES and TE cDNA) specific primers (Table 3.1). Trials using all cloning primers simultaneously, were run under standard conditions: 94 °C for 5 minutes followed by 25 cycles of denaturation at 95 °C for 1 minute; annealing at 55 °C; extension at 72 °C for 1 minute. This was sufficient for CG1945 (Figure 3.16) but it did not produce a clear result for Y187. Cycle conditions were then changed. The extension time was increased to two minutes and the number of cycles increased to 32. This should, in theory, allow more efficient amplification of longer inserts e.g. 1000 bp. It should also increase the number of copies of the insert. The conditions were changed to: 94 °C for 5 minutes followed by denaturation at 95 °C for 1 minute; extension at 72 °C for 2 minutes; annealing at 55 °C for 1 minute, for 32 cycles. An 8 µl aliquot of the PCR products were directly loaded onto a 0.8 % agarose gel. The results confirmed size was as predicted. However, due to the similar sizes of the cloning inserts of ENR, TE and DES, the result was not clear. Further confirmation was given by using each cloning primer individually in the reaction. This again produced the expected band size with greater clarity. Non-specific amplification did not occur (Figure 3.17). These results confirmed the presence

of all the constructs. They also confirmed that the lack of interaction between ACP and any other enzyme tested was not due to the absence of either plasmid construct.

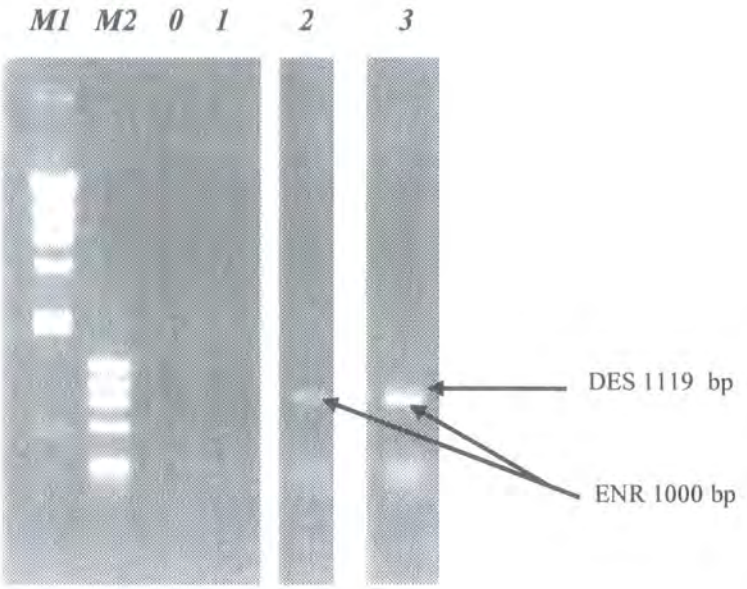


Figure 3.16. Amplification of the ENR and DES cloning inserts from yeast (CG1945) transformants. Plasmid DNA was isolated as described in the text. A 1 µl aliquot of the purified plasmid was used as the template. Inserts were amplified using *Vent* DNA polymerase (Boehringer Manheim). The reaction contained 1 µl of backextracted DNA, 0.1 mM dNTPs, 1X *Vent* polymerase buffer, 1 unit of *Vent* polymerase and the total reaction mixture was brought to 50 µl with water, primer concentration was 25 pmol. Cycle conditions are discussed in the text. An 8µl aliquot of the product was directly loaded onto a 0.8 % gel, to confirm size and purity of the fragments. The fragment sizes were correct for the size predicted. The DES insert, 1119 bp was seen to be present as a band just above that of the enoyl reductase 1000 bp (lanes 3a, 3b, 3c). Amplification of constructs was attempted in triplicate on three seperate colonies. M1: λHind III marker, M2 ϕX174 marker; lane 0: no DNA; lane 1: non-transformed CG1945; lane 2: co-transformation of pAS2-1/ENR and pACT2/ENR (ENR primers); lane 3: co-transformation of pAS2-1/DES and pACT2/ENR (DES and ENR primers).

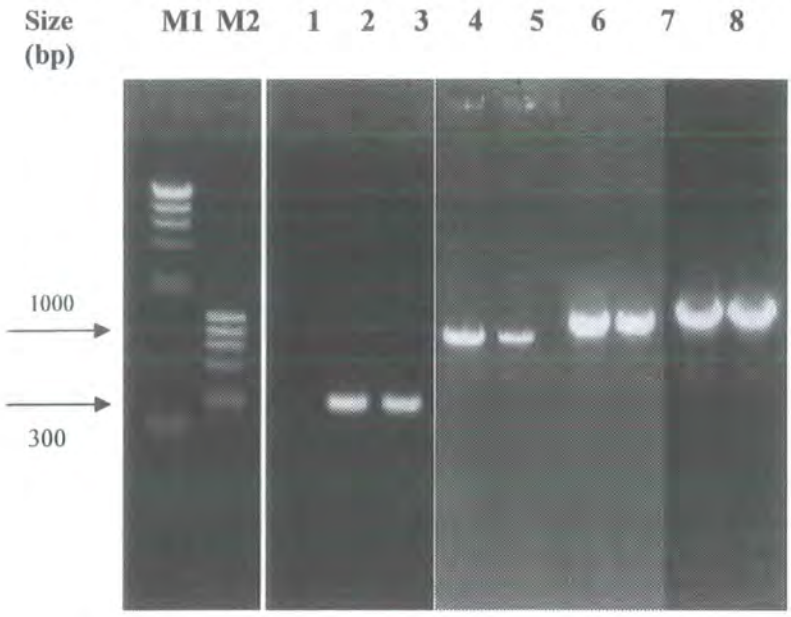


Figure 3.17. Amplification of ACP, ENR, TE, and DES cloning inserts from yeast (Y187) transformants. Plasmid DNA was isolated as described in the text. A 1 μl aliquot of the purified plasmid was used as the template. Inserts were amplified using *Vent* DNA polymerase (Boehringer Manheim). The reaction contained 1 μl of backextracted DNA, 0.1 mM dNTPs, 1 X *Vent* polymerase buffer, 1 unit of *Vent* polymerase and the total reaction mixture was brought to 50 μl with water, primer concentration was 25 pmol. Cycle conditions are discussed in the text. An 8 μl aliquot of the product was directly loaded onto a 0.8 % gel, to confirm size and purity of the fragments. M1: λHind III marker, M2 ϕX174 marker; lane 1: zero DNA (ACP primers); lane 2: co-transformation of pAS2-1/ACP and pACT2/ACP (ACP primers); lane 3: co-transformation of pAS2-1/ACP and pACT2/ENR (ACP primers); lane 4: co-transformation of pAS2-1/ENR and pACT2/ACP (ENR primers); lane 5: co-transformation of pAS2-1/ENR and pACT2/ENR (ENR primers); lane 5: pAS2-1/TE and pACT2/ACP (TE primers); lane 6: co-transformation of pAS2-1/TE and pACT2/ENR (TE primers); lane 7: co-transformation of pAS2-1/DES and pACT2/ACP (DES primers); lane 8: co-transformation of pAS2-1/DES and pACT2/ACP (DES primers). Primers: refers to those primers used to clone that enzyme (e.g. ENR) cDNA initially.

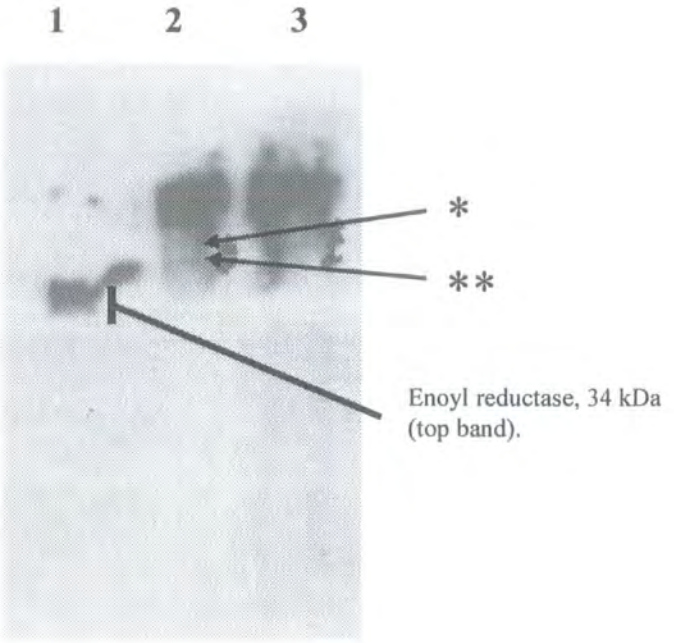
3.1.2.f Detection of fusion proteins by immunoblotting (*Brassica napus* ENR polyclonal antibodies).

Immunoblotting was used to ensure the fusion proteins were expressed. Polyclonal ENR antibodies were used in the first instance as they had been well characterised and were raised to the native form of the protein (Slabas *et al.*, 1990). Transformants were grown to saturation, pelleted and the proteins extracted with 'protein breaking buffer' that included a cocktail of protease inhibitors (Chapter 2.1.10). Proteins were fractionated by SDS-PAGE and subject to immunoblotting (Chapter 2.1.13, 2.1.14). On exposure to the polyclonal *Brassica napus* ENR antibody, two faint bands (indicated by arrows) cross-reacting at approximately 60 kDa, were present (Figure 3.18a,b). Enoyl reductase is 34 kDa (Figure 3.18a,b, lane 1, top band) and the DBD and AD proteins are 27 kDa and 19 kDa respectively. Therefore, these two faint bands closely correlated to the size predicted for DBD/ENR and AD/ENR fusion proteins. However, the control of yeast (Y187) with no vector also contained these bands (Figure 3.18a., lane 3) although the control yeast CG1945 with no vector (Figure 3.18b, lane 3) did not. Furthermore, the antibodies were hybridising (producing a smear) to a much greater molecular weight species. There may have been the possibility that the antibodies were crossreacting to the ENR of the yeast type I FAS complex. Yeast FAS is a 2500 kDA, $\alpha 6\beta 6$ multifunctional protein. On SDS-PAGE, it separates into 212 kDa and 203 kDa sub-units (Stoops and Wakil, 1978). However, unlike ACP and KAS I (Revill and Leadlay, 1991; Tai and Jaworski, 1993) there is no sequence homology between ENR in type I

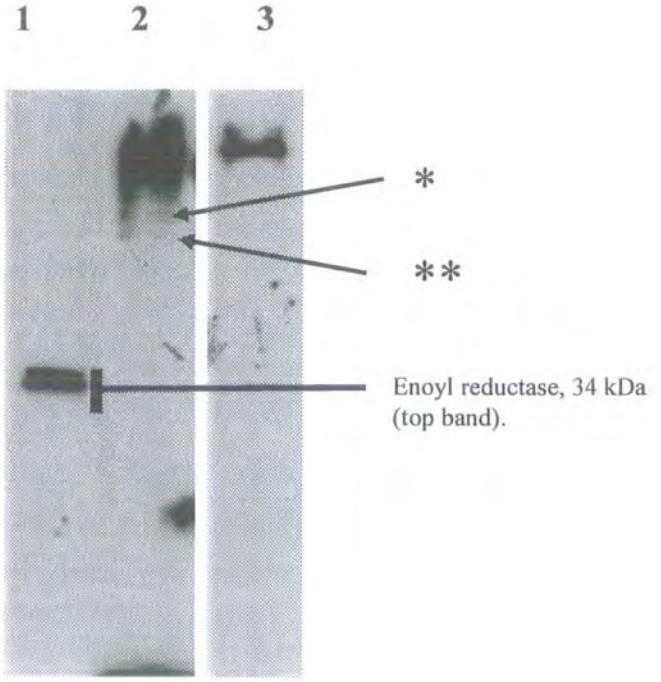
and type II FAS complexes (this project), therefore, it may have been due to a mass-action effect.

Figures 3.18a,b. (Overleaf). Immunoblots of SDS-PAGE separated proteins from yeast strain Y187 and CG1945 using polyclonal antibodies to *Brassica napus* enoyl reductase. Proteins were fractionated on a 12 % SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The membrane was probed using the rabbit anti-enoyl reductase as the primary antibody at a 1 in 10 000 dilution followed by an incubation with a 1 in 20 000 dilution of goat anti-rabbit IgG conjugated to the horse radish peroxidase (HRP) antibody. The blots were developed using the Pierce supersignal ECL Kit after a 30-second exposure. **A.** Lane 1: purified *Brassica napus* ENR (0.05 μg); lane 2: Y187 co-transformed with pAS2-1/ENR and pACT2/ENR (10 μg), possibly expressing DBD/ENR (*) and AD/ENR (**), respectively; lane 3: Y187 control (no plasmid) (10 μg); **B.** Lane 1: purified *Brassica napus* ENR protein (0.05 μg); lane 2: CG1945 co-transformed with pAS2-1/ENR and pACT2/ENR (8 μg), possibly expressing DBD/ENR (*) and AD/ENR (**), respectively; lane 3: CG1945 control (no plasmid) (13 μg). The results show that the polyclonal antibodies did not clearly detect proteins of the correct MW.

A: Y187



B: CG1945



3.1.2g. Detection of fusion proteins by immunoblotting (GAL4-AD monoclonal antibodies).

As the results from polyclonal antibody detection of fusion proteins were not clear, GAL4p DBD and AD monoclonal antibodies were used (Clontech). These were specific to the DNA binding domain and activation domain of the fusion proteins, respectively. Attention was initially focused on the ENR fusion protein, the presence of which could not be ascertained from the use of polyclonal antibodies. Attention was also focused on the AD/ACP as no interactions were detected between ACP and any other FAS enzyme tested. In addition to the protein extraction, a trichloroacetic acid precipitation procedure was employed to concentrate the proteins (Chapter 2.1.12). The final pellet was resuspended in 20 µl of SDS-PAGE loading buffer and the proteins fractionated by SDS-PAGE. The gel was subjected to immunoblotting. The results indicated expression of AD/ACP and AD/ENR fusion proteins (Figure 3.19). The AD/ENR fusion protein appeared (Figure 3.19, lane 3, top band) at the expected size of 53 kDa (Table 3.2) and a lower band of approximately 45-50 kDa was detected (Figure 3.19, lane 3). The AD/ACP fusion appeared at a slightly larger size than the estimated 29 kDa (Table 3.2) indicating that the acidity of ACP may have effected the migration of the fusion protein. Although ACP is 10 kDa in size, due to its high acidic content it will run at 18 kDa on SDS-PAGE (Elhussein *et al.*, 1988). This may indicate that the fusion proteins used in this project retained their function or at the least their native properties.

The doublet apparent in the AD/ENR lane may have stemmed from the action of proteases. This is supported by work on the purification of ENR. Although it is now accepted that the purified ENR protein has an α4 structure as definitively shown by X-ray crystallography (Rafferty *et al.*, 1994), an apparent α2β2 doublet was observed when ENR was first purified from the developing seeds of *Brassica napus* (Slabas *et al.*, 1986). This latter result was based on the presence of two separate bands on SDS-PAGE. Although the authors included a protease inhibitor cocktail mix, the doublet was still produced (Slabas *et al.*, 1986). However, subsequent analysis by immunoblotting using freshly homogenised rape seed indicated that ENR actually has an α4 structure. The lower β-band was shown to be an isolational artefact caused by an endogenous protease that cleaved between a serine and lysine residue (Slabas *et al.*, 1990). Similarly, in this project, although a protease inhibitor mix was included a doublet was still produced. Most likely, a single band may be achieved if proteins were immediately fractionated after extraction.

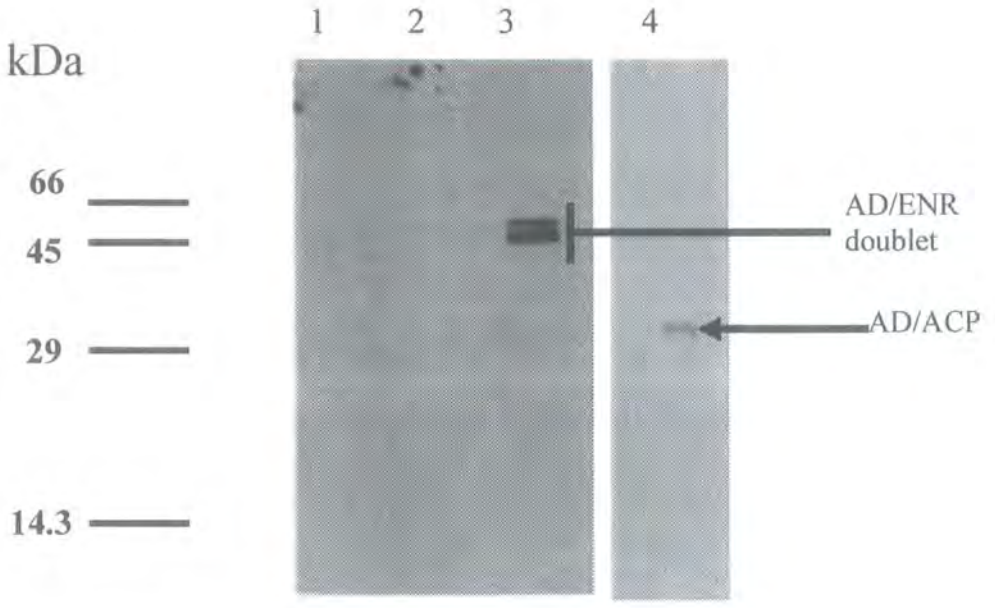


Figure 3.19. Immunoblot of proteins from yeast expressing the recombinant proteins AD/ACP and AD/ENR using the monoclonal antibodies to the GAL4p activation domain. Proteins were concentrated from yeast lysates using TCA precipitation and fractionated on a 12 % SDS-PAGE gel. Fractionated proteins were electroblotted onto a nitrocellulose membrane and probed with monoclonal antibodies to the GAL4p transcription activation domain (Clontech). Primary antibodies were detected with anti-mouse immunoglobulin (IgG) antibody (Pierce). This was followed by ECL chemiluminescent detection (Pierce Supersignal system). The results confirmed the presence of the fusion proteins and demonstrated that the insert cDNA was in frame producing fusion proteins of the predicted size. The sizes predicted were: AD + ENR (19 kDa + 34 kDa) = 53 kDa (top band) and AD + ACP (19 + 10 kDa) = 29 kDa. The blot also confirmed that a lack of interaction between AD/ACP and the other FAS II proteins was not due to a lack of expression of the ACP fusion protein. Lane 1: untransformed yeast CG1945 exposure time 12 hours; lane 2: transformed with pCLI-1 exposure time 12 hours; lane 3: expressing AD/ENR (top band, exposure time 12 hours); lane 4: expressing AD/ACP (exposure time 2 minutes).

3.2. Analysis of protein-protein interactions *in organello* using the bandshift technique.

In type II FAS, ACP is the substrate carrier and, as such, may be expected to interact with all type II FAS enzymes. In this work, using the yeast two-hybrid system, no interaction was detected between ACP and any other FAS enzyme tested (Chapter 3.1). This may have been a false negative. For example, the interaction may have may have required the presence of stabilising co-factors, or other intermediates, which were not present in the yeast nucleus. Bandshift analysis using isolated *Brassica napus* chloroplasts allowed a way round the potential problem of examining protein-protein interactions dependent on/or stabilised by the presence of cofactors and/or post-translational modifications and/or a membrane requirement. Furthermore, it presented a way to search for proteins interacting with a type II FAS multienzyme complexes or part complexes.

3.2.1. Bandshift analysis.

Bandshift (or mobility shift) analysis exploits the fact that a protein complex migrates on a native polyacrylamide gel with a different mobility than its individual components. Proteins are kept in their native state by not including reducing agents (dithiothreitol and β-mercaptoethanol found in most loading buffers), SDS and not boiling the sample before loading. Individual proteins are pre-incubated in binding buffer to allow complex formation. After dilution with sample buffer, free and complexed proteins, and proteins

from the cell lysate, are separated by electrophoresis. Protein bands may be detected using either Coomassie blue staining, autoradiography or antibodies.

Bandshift analysis was used in this project as total cell lysates can be tested without the need for further purification. Therefore, it is advantageous in terms of studying membrane or membrane associated proteins. This makes it especially advantageous in terms of overcoming perhaps the most significant hurdle(s) of studying dynamic interactions in the yeast two-hybrid system because post-translational modification such as phosphopantetheinylation of ACP would have occurred. Furthermore, any necessary cofactors and/or coenzymes were more likely to be present. Such factors may be required to maintain the structure of a dynamic complex and/or interactions of the proteins within that structure. In bandshift experiments, all proteins in the cell lysate compete for binding at their natural relative level of abundance, thus complementing the techniques using overexpressed binding partners, for example, the yeast two-hybrid system. Gel-based methods such as bandshift are valuable tools to achieve complete characterization of protein-protein interactions and/or multienzyme complexes. The caging effect of the gel is thought to keep local protein concentrations high (Coghlan, 1998). However, there are disadvantages to the technique. For example, there is no way to discern if the shift in mobility is due to direct or indirect interactions. It is not sensitive in its ability to detect rare interacting proteins, although cell lysates can be fractionated to increase the sensitivity of procedure. Only reduced proteins can be sized as each protein folds in different ways changing its mobility on PAGE gels. Finally, as separation on native gels is by a combination of charge and size, the position of the protein band can only give an indication of the size of the complex of protein(s).

However, it remains a powerful method to study of supramolecular organisation. The advantages and disadvantages of the yeast two-hybrid system and bandshifting analysis are compared in Table 3.10.

Table 3.10. Comparison of the advantages and disadvantages of the yeast two-hybrid system and bandshift techniques. Due to the ability of the bandshift technique to use crude cell lysate it was used to complement the work of the yeast two-hybrid system.

Technique	Advantages	Disadvantages
Yeast two-hybrid system	1) Can be used with a wide variety of proteins. 2) Deletions can be made in the DNA encoding one of the proteins to identify a minimal domain for interaction. 3) Point mutations can be assayed to identify specific residues critical for the interaction. 4) Interactions take place *in vivo.* 5) Highly sensitive. 6) Allows direct selection and screening of a large number of variants to detect those that act more or less strongly. 7) With a reporter gene such as the yeast HIS3 gene, the competitive inhibitor 3-aminotriazole can be used directly to select for constructs that yield increased affinity.	1) The interaction is limited to fusion proteins that can be localized to the nucleus. 2) Many proteins will activate transcription when fused to a DNA binding domain and this activation prevents a library screen from being performed.
Bandshift analysis	1) Total cell lysates can be tested without need for further purification. 2) The local protein concentration is kept high. 2) Cell lysates can be fractionated to increase sensitivity of procedure. 3) The precise binding polypeptide of a multimeric complex can be detected.	1) The biological activity of the protein on the membrane may be lost on a denaturing gel. 2) There is no way to discern if the shift in mobility is due to direct or indirect interactions. 3) It is not sensitive in its ability to detect rare interacting proteins. 4) Only reduced proteins can be sized as each protein folds in different ways changing its mobility on PAGE gels. 5) The position of the protein band can only give an indication of the size of a complex of proteins.

In these experiments (Figure 3.20), purified *Brassica napus* proteins, ACP and ENR, were available in the laboratory from previous projects. Due to the size of ACP (a 10 kDa polypeptide which migrates at 18 kDa on SDS-PAGE), its interaction with members of the putative FAS II complex may cause a considerable alteration in its mobility. Furthermore, interactions between ENR, ACP, and any other type II FAS enzymes of the same complex may be detected as a mobility shift and crossreaction to the same region on a gel. As antibodies were available for the detection of ACP and ENR, they were used to increase the sensitivity of the bandshift analysis whilst, and perhaps more importantly, retaining specificity of detection.

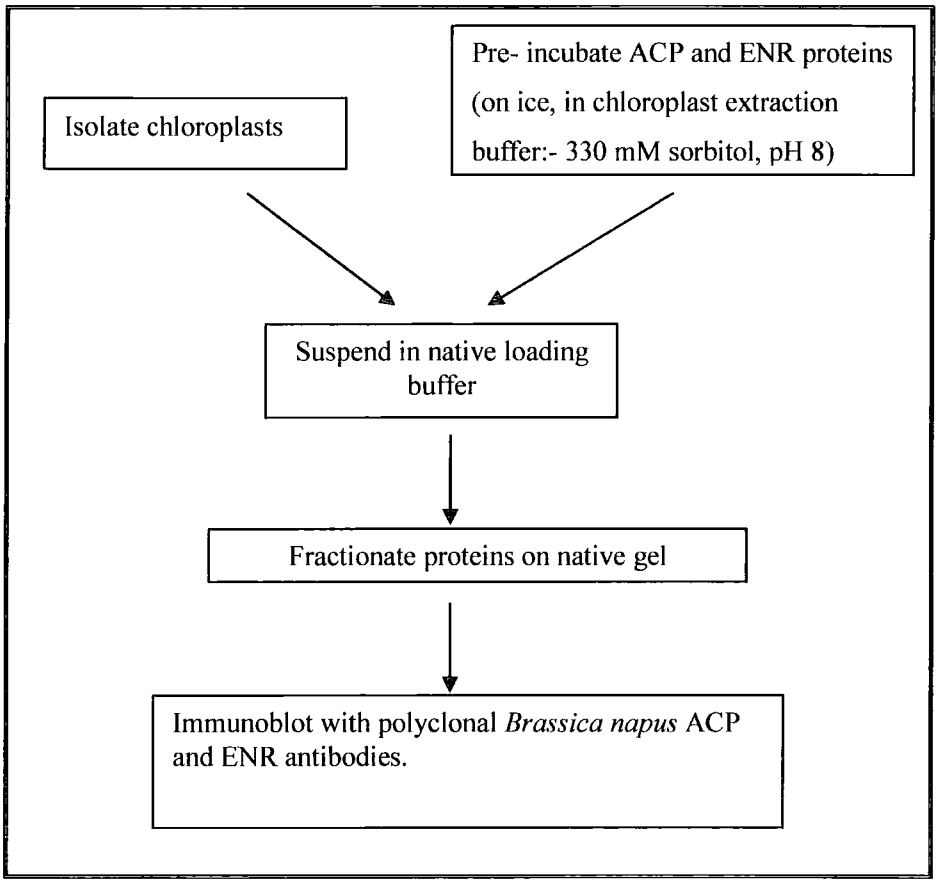


Figure 3.20. Flow diagram for bandshift analysis of type II FAS proteins of *Brassica napus*.

3.2.2a) Isolation of *Brassica napus* chloroplasts.

Most plant chloroplast work tends to use the more 'robust' chloroplasts of spinach and pea (Roughan, 1987). The chloroplasts from *Spinacia oleracea* are the preferred source due to the softness of the leaves and the normal absence of large quantities of phenolics, calcium oxalate and starch (Loomis, 1974). Acceleration during centrifugation may push the starch grains through the chloroplast envelope and thus rupture it. This is usually indicated by a large white ring around the chloroplast pellet (Robinson *et al.*, 1979). The problem of spinach is its' strict growth requirement for high rates of photosynthesis. Leaves are used when plants are 6-8 weeks old thus, a large stock of spinach plants is needed to provide a continuous supply. Pea (*Pisum Sativum*) is often used because it can be harvested when shoots are 10-12 days old.

The most successful chloroplast isolation depends on detailed attention from the very point of planting the seed. For example, Roughan (1987) (here referring to spinach and the activity of type II FAS) states: 'The most active chloroplasts seem to be isolated from plants grown in a completely controlled environment.' In addition, they found chloroplasts that were isolated from rapidly growing leaves of young (6-8 weeks), well grown plants are most likely to exhibit high rates of fatty acid synthesis from [1^{-14}C] acetate. Therefore, these authors although referring to spinach and pea recommended that plants were grown in tightly controlled conditions such as aerated solution culture. Observations such as this were supported by work from other authors. For example, Tambussi *et al.* (2000) found that even transient water stress could have a profound effect on the biosynthetic activities of chloroplasts from plants. Specifically, they found

that oxidative damage occurs to thylakoid proteins in water-stressed leaves of wheat (*Triticum aestivum*).

In this project, the focus was to develop a supply of intact chloroplasts. These would, in the future, be used as a starting point for a series of experiments that would involved gradual release of proteins to investigate the presence of type II FAS complexes/part complexes. Subsequently, a thorough protocol for chloroplast isolation and assaying their integrity was developed. The procedure is outlined in Figure 3.21.

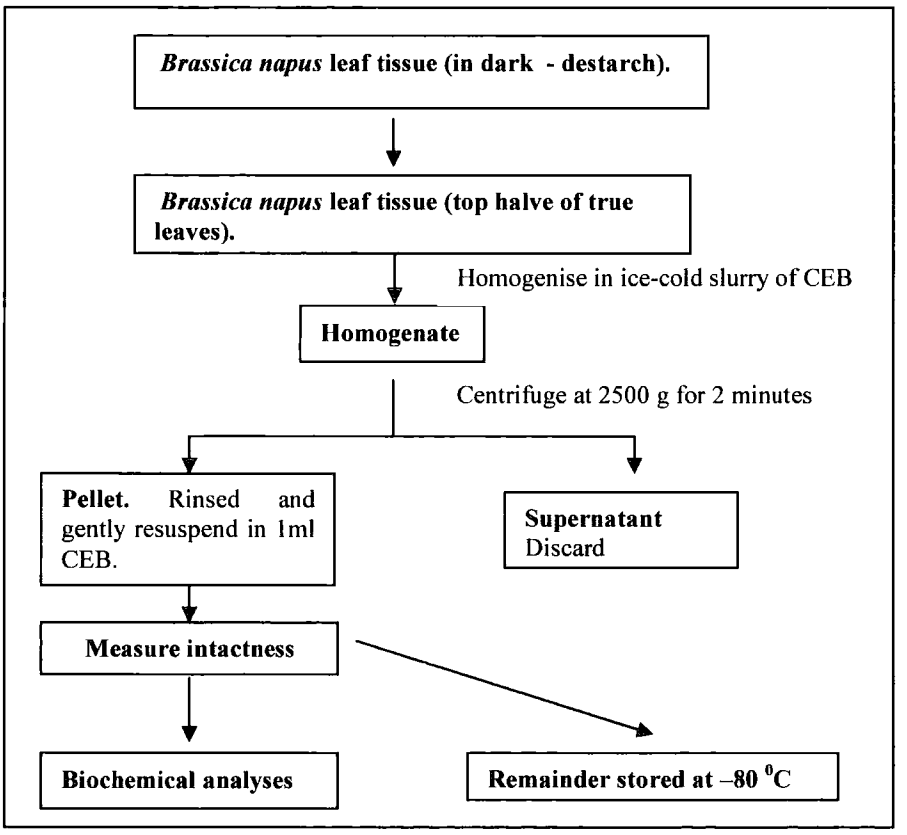


Figure 3.21. Schematic diagram outlining steps for isolation of *Brassica napus* chloroplasts. The chloroplasts were used for subsequent bandshift analysis of protein-protein interactions. CEB: chloroplast extraction buffer.

Although the isolation of *Brassica napus* chloroplasts was not a routine procedure, using only *Brassica napus* chloroplasts may minimize any potential errors from trying to extrapolate from, for example, pea. For example, Peltier *et al.* (2000) on the first significant foray and publication specifically on chloroplast proteomics used pea for ease whereas, most information in the databases is derived from *Arabidopsis*. This turned all their results into an extrapolation from another species. Although, this problem was addressed by the publication of a proteome of *Arabidopsis* seed (Gallardo *et al.,* 2001).

3.2.2b) Measuring chloroplast intactness: the oxygen electrode assay.

The chloroplast role is to supply energy to the plant in the form of ATP. The chloroplast has a system of converting light energy to the chemical energy of ATP. This system may be split into two cycles, a light cycle, producing ATP and NADPH. The second cycle is termed the 'dark cycle' during which the synthesis of carbohydrate takes place. The light cycle traps the photons using the membrane bound light harvesting complexes PS1 and PS II and transfers a released electron down a cytochrome chain with oxygen being the final acceptor (Mitchell, 1974). PSII splits water into O_2 and H^+. The electrons acquired from PSII water splitting are transported through a series of carriers such as plastoquinone (PQ/PQH_2) and cytochrome b/f complex to PSI, where the electrons are energized once again. This is called the Z-scheme. The electrons from PSI are then used by the soluble protein, ferredoxin, for production of NADPH that is essential for reduction of CO_2. This is the process of natural photosynthesis. Isolated chloroplasts evolve oxygen when they are illuminated in the presence of a suitable artificial electron acceptor e.g. potassium ferricyanide ($[Fe(CN)_6]^{3-}$). This is known as the Hill reaction.

The transfer of electrons takes place in the membrane and is coupled to a proton pump extruding protons out of the thylakoid. This proton efflux causes a translocation of electric charges. That way both a chemical proton gradient and an electric potential are generated. The tendency of the protons (H^+) to return to the inside of the compartment is therefore quite large and it is called the proton motive force (Mitchell, 1975). The energy set free when the protons flow back is used for the synthesis of ATP, through the action of ATP synthethase. Depending on whether the reaction takes place in chloroplasts or mitochondria it is called photophosphorylation or oxidative phosphorylation, respectively. A number of toxic substances like ammonium ions or dinitrophenyl (DNP) destroy the proton gradient so that no more energy is available for active transport. Such substances are called uncoupling agents. Lipid bilayers are relatively impermeable to ions. Thus, the addition of salts such as ammonium chloride to intact chloroplasts will not induce a significant rise in oxygen evolution in the presence of an artificial Hill oxidant such as potassium ferricyanide. However, rupturing the chloroplast membrane by exposure to hypotonic solutions will allow entry of the ammonium, causing a sharp rise in oxygen evolution.

In this project, oxygen evolution measurements were made in the DW1 oxygen electrode unit part of the Hansatech Oxygraph system (Chapter 2.2.3). The percentage estimation of intact chloroplasts was achieved by measuring oxygen evolution of completely ruptured versus newly isolated chloroplasts. The rate of oxygen diffusion to the cathode (and hence the current) depends on the oxygen concentration in the main incubation chamber. It also depends on several other factors: temperature, membrane thickness and

permeability, sample viscosity and stirring speed. The electrode was calibrated each time it was used.

The order of addition of components for lysed and intact measurements is shown in Table 3.11. The effect of adding the chloroplasts to water was to cause immediate swelling and rupturing of the chloroplast membranes. Oxygen evolution from ruptured chloroplasts was always measured first.

Table 3.11. The order of components used to measure oxygen evolution from ruptured and intact chloroplasts from *Brassica napus*.

Ruptured chloroplasts	Intact chloroplasts
0.9 ml of H_2O	1 ml of 2 x CEB
100 µl chloroplasts	0.9 ml of H_2O
1 ml 2 X CEB	100 µl chloroplasts
10 µl DL-glyceraldelhyde	10 µl DL-glyceraldelhyde
20 µl potassium hexanocyanoferrate	20 µl potassium hexanocyanoferrate
10 µl ammonium chloride	10 µl ammonium chloride

Chemicals were maintained at 25 °C by incubation in the water circulation unit that supplied the oxygen electrode unit waterjacket. Hamilton syringes added the following chemicals via the chimney of the oxygen electrode: DL-glyceraldehyde (0.3 M, 25 °C), this serves to inhibit CO_2 fixation. Potassium hexanocyanoferrate (0.5 M, room temperature) was used as a Hill oxidant. Chloroplasts were exposed to light at an intensity of 4400 µE m^{-2} s^{-1} $^{(PAR)}$. Ammonium chloride (0.5 M, 25 °C) was the uncoupler. Abbreviations: CEB- chloroplast extraction buffer.

Oxygen evolution occurred in both untreated and completely lysed chloroplasts (Figure 3.22). This finding was expected. Only chloroplasts that were 100 % intact would yield a very low evolution value.

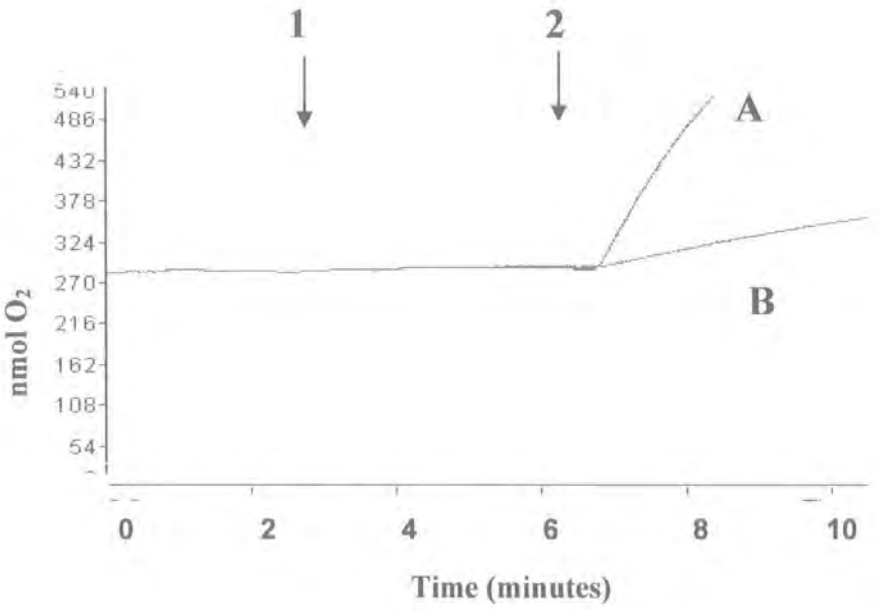


Figure 3.22. Trace of oxygen electrode measurements of isolated *Brassica napus* chloroplasts. The evolution of oxygen was measured using an Oxygraph computer controlled oxygen electrode (Hansatech). Measurements were taken in both coupled and uncoupled states from 100 µl of: A) osmotically shocked chloroplasts (diluted in H_2O). B): freshly isolated chloroplasts. Hamilton syringes added the following chemicals via the chimney of the oxygen electrode: DL-glyceraldehyde (0.3 M, 25 °C); ferricyanide (potassium hexanocyanoferrate 0.5 M, RT). (1) Exposure to light at an intensity of 4400 µE m^{-2} s^{-1}; (2) ammonium chloride (0.5 M, 25 °C) was the uncoupler. The percentage intactness was calculated as Ri-R_L/R_L x 100/1. The data presented here yielded 72 % intactness. These chloroplasts were used in subsequent bandshift analysis.

The rate of oxygen evolution was measured for coupled and uncoupled states for intact and lysed chloroplasts and the percentage intactness was calculated. For example, R_{Lysed}- R_{intact}/ R_{Lysed} = (211- 77) /211 * 100/1 = 63.4 % (values were in nmolO_2/mg chlorophyll/ per hour) (Table 3.12). The isolation of intact chloroplasts ranged in value from 50-72 % (Table 3.13).

Table 3.12. Example calculations of oxygen evolution from isolated *Brassica napus* chloroplasts and subsequent calculation of percent intact chloroplasts.

% intactness calculations	
Rates: Lysed and Uncoupled	
nmol O_2 /39 μg chlorophyll/ min	55
nmol O_2 /39 μg chlorophyll/ per hour	3300
nmol O_2 /mg chlorophyll/ per hour	84615
μmol O_2 /mg chlorophyll/ per hour	84.6
Rates: Not lysed and uncoupled	
nmol O_2 /39 μg chlorophyll O_2 /min	15.4
nmol O_2 /39 μg chlorophyll / per hour	924
nmol O_2 /mg chlorophyll/ per hour	23692
μmol O_2 /mg chlorophyll/ per hour	23.7

The percentage intactness was calculated as Ri-R_L/R_L x 100/1 and therefore equal to 72 %.

The chlorophyll concentration was found to be relatively low compared to other published work (Roughan, 1987). This was ascribed to the large ratio of buffer to leaves picked i.e. 500 ml per 50 g of leaves picked and it is thought to vary according to the efficiency of homogenization (Roughan, 1987).

Table 3.13. Summary of intactness values of isolated *Brassica napus* chloroplasts.

			Rate of O_2 evolution			
			Lysed, uncoupled		Intact, uncoupled	
Organism	Percent Intactness	Chlorophyll concentration µg/µl	nmol /O_2/min	µmol O_2 /mg chlorophyll / per hour	nmol O_2/min	µmol O_2 /mg chlorophyll/ per hour
Pea	16.2	1.77	27.6	18.7	26.4	17.9
Brassica napus cv Escort	51.6	1.7	27.4	19.3	13.2	9.3
	29.9	1.61	29.9	22.3	20.98	15.6
	63.4	1.05	33.7	19.2	12.3	7.0
	27.1	0.3	9.0	36	6.57	26.3
	30.4	0.56	9.2	19.7	6.42	13.7
	72.0*	0.78	55.0	84.6	15.4	23.7
	55.0	1.2	32.0	32	21	21

Brassica napus chloroplasts were routinely isolated into a high osmiticum until a high degree of intactness was achieved for bandshift analysis (*72 %: These chloroplasts were used in subsequent bandshift analysis, Figure 3.23).

3.2.3. Immunoblotting to detect bandshifted proteins from isolated chloroplasts.

Brassica napus chloroplasts were isolated as described above, purified *B. napus* proteins (ACP and ENR) were available in the laboratory from previous projects. Native gels were prepared at pH 8 to simulate the pH in light conditions of the chloroplast. The purified proteins, ACP and ENR, were incubated together on ice. The chloroplasts were isolated. Subsequently, native PAGE was used to fractionate the individual proteins, pre-incubated proteins and the isolated chloroplasts (Chapter 2.2.4). The titre of the antibodies was held at a level known to detect ACP and ENR from plant homogenates i.e. 1: 10 000, (determined from SDS-PAGE) for the primary antibody.

Although native PAGE cannot accurately size the proteins, at pH 8 most proteins will acquire a net negative charge and in general follow their size. This may be seen from the difference in migration distances of the purified proteins of monomeric ACP (10 kDa) and the tetrameric ENR (140 kDa) (Figure 3.23). In both the freshly isolated and freeze thawed samples of chloroplasts, no bandshift could be discerned from the blots (Figure 3.23, lanes 4 and 5). This was due to the poor resolution of the gel. One potential reason for this was inhibition of protein migration by chloroplast lipids or other plant material. Another reason may have been the use of a fixed titre of antibody and the use of a maximal amount of sample may have led to overloading and smearing of the sample leading to poor resolution of the proteins.

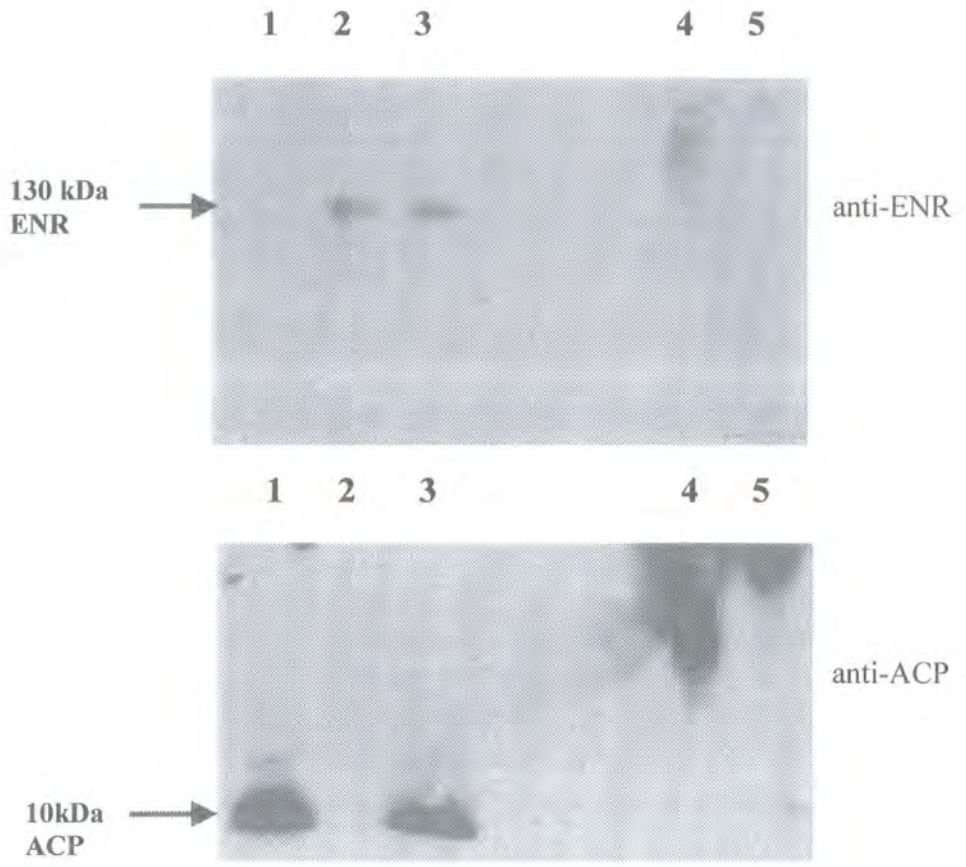


Figure 3.23. Bandshift analysis of ACP and ENR proteins from isolated *Brassica napus* chloroplasts. Proteins were fractionated on a non-denaturing 7.5 % PAGE (80 V, 4 °C, 3 hours). The proteins were electroblotted to nitrocellulose overnight at 4 °C and the blot exposed to rabbit polyclonal anti-ENR antibodies (1:10 000) then HRP-conjugated goat anti-rabbit antibodies (1: 20 000). The blot was developed using Pierce ECL Supersignal Kit. The blot was subsequently probed with anti-ACP. The poor resolution of the gel meant no conclusion involving interactions could be drawn. Lanes 1: 0.1 μg of purified ACP; 2: 0.1 μg of purified ENR; 3: 0.1 μg of ACP and ENR proteins incubated in chloroplast extraction buffer; 4: freeze/thawed chloroplasts (20 μg total protein); 5: freshly isolated chloroplasts (72 % intactness, 10 μg total protein).

Therefore, an attempt to resolve the bands by decreasing the polyacrylamide concentration to 4 % was attempted. However, the fragility of the gel was such that it made it unsuitable for immunoblotting. Similar observations have been described on testing the titre of the polyclonal *Brassica napus* ENR antibody on purified and crude plant extract. It was found that a high (1: 50) titre tested on 20 ng of the ENR protein would produce a smearing effect. At a 1: 10 000 titre, the slot-blot band was barely visible. Secondly, Slabas *et al.* (1990) reported similar immunoblotting problems whilst trying to characterise the ENR antibody, a fault they put down to overloading of the gel. This was ascribed to poor resolution or sample loading, which results in reduction of signal and can go as far as to result in complete loss of signal. This is due to the antigen being physically competed out from the area of detection. It was stated that doing multiple loading of samples onto the gel may circumvent this (Slabas *et al.*, 1990).

A freeze/thawed sample of chloroplasts was also run on the gel (Figure 3.23; Lane 4). Freeze/thawing will lyse the chloroplast membrane and is a more gentle way to disrupt protein-protein interactions (Srere and Mathews, 1990). However, due to the poor resolution of the gel any effect on any association of proteins of type II FAS enzymes by freeze/thawing could not be ascertained.

In conclusion, in this project, bandshift analysis did not show that *Brassica napus* ACP or ENR were interacting, or were associated with a putative type II FAS complex in the chloroplast. By extension, this bandshift analysis as it stands, cannot confirm that the lack of interactions with ACP with ENR in the yeast two-hybrid analysis was a false negative result. Nor does the result indicate whether the technique would be of any use

to examine protein-protein interactions *in organello.* As such the experiments would have to be repeated employing suitable controls to ensure clarity of result. For example, one would need to include pure proteins, proteins mixed with chloroplast lipids to ascertain their effect on protein mobility, crosslinked proteins to ascertain expected migration distance of interacting proteins. If *B. napus* antibodies were being used, then to check for nonspecific binding, chloroplasts from another source e.g. *Arabidopsis* could be included on the gel.

Alternatively, one could incubate isolated *Brassica napus* chloroplasts, or fractions thereof, with membrane permeable crosslinkers. Isolating the putative interacting proteins, before analysis, may avoid any bandshift artefacts that lipids, cellulose or other plant material may cause, the potential interference from such artefacts may be seen in Figure 3.23, lanes 4 and 5. Furthermore, such crosslinking, experiments may, in theory, stabilise any potential weak interaction occurring *in vivo*. Subsequently, any interacting proteins could be detected using SDS-PAGE and immunoblotting. SDS-PAGE would be more accurate and easy to use when trying to detect due to interacting proteins.

Chapter 4 – Discussion.

4.1. Protein-protein interactions of type II FAS.

In vitro, the complex of type II FAS enzymes are easily dissociable indicating interactions are weak. However, the nature of any potential association could not be defined from the co-purification of some of the enzymes of type II FAS of *Brassica napus*. For example, was this association a purification artefact, were they truly interacting, were they held together by a third protein, and/or were they stabilised by intermediates? Using the yeast two-hybrid system, this thesis has revealed a direct interaction between ENR and DES. There was also a possible, very weak, interaction detected between TE and ENR (Chapter 3.3.3). The former finding is supported by previous evidence for the co-purification of the *Brassica napus* enzymes: ENR and DES by Kater *et al.* (1991). The latter finding is supported by the co-purification of *Brassica napus* ENR, TE and β-HAD (Hellyer *et al.*, 1992; Doig, 2001). The substrate carrier, ACP, did not interact with any other protein tested.

In this project, there was a large variation in interaction strength. For this reason, repetition of the two-hybrid assay was used to confirm the relative strength of the weak hetero-enzymatic interactions (Chapter 3.1.2c). Mazzurco *et al.* (2001) also reported a large variation in interaction strengths whilst investigating protein-protein interactions involved in the signalling pathway controlling self-incompatibility of plant pollination. Having previously identified the *Brassica* S receptor kinase (SRK) interacting proteins

in a yeast two-hybrid library screen, it was hypothesised these proteins could represent potential downstream proteins involved in a signalling pathway and were further investigated. The two-hybrid assay was used to investigate the interactions between the *Brassica* S receptor kinase and three interacting proteins (ARC1, THL1 and THL2). In this case, OPNG was used as the chromogenic X-*gal* substrate and colour development was monitored from 0 hours to overnight. A very large variation in absorbence readings of *lacZ* expression, which ranged from 0.1 – 1000 'arbitrary units', indicated a very large variation in interaction strength. As the relatively low values of 0.1 may not be physiologically relevant, repetition was used to help confirm positive interactions. It was subsequently found that substituting a cysteine residue in SRK with a serine residue (UGU to UCU) resulted in the loss of the interaction of the THL1 and THL2 proteins (thioredoxins) with the receptor. This pattern was found for the same proteins in *Arabidopsis*. The cysteine residue was identified as being critical for the interaction of the THL1 and THL2 proteins with the receptor.

In this project, although there were no interactions between ACP with itself or any other FAS enzyme tested, back extractions revealed there was no problem in terms of presence of the plasmid construct (Figure 3.17.). Furthermore, immunoblotting confirmed the fusion proteins were expressed and were the size predicted (Figure 3.19).

The cDNA used in this work were known to be free of mutations and this was a critical factor in validating/having confidence in the results of the pairwise interaction tests. This was especially true in the case of ACP where an error in the database was detected (Chapter 3.2). The recent study of the interactions between the *Brassica* S receptor

kinase (SRK) and three interacting proteins (ARC1, THL1 and THL2) in the yeast-two hybrid system (previously described) provide a highly relevant example of how an interaction may be lost by an individual base change (Mazzurco *et al.,* 2001).

Holo-ACP is a pre-requisite for any cellular function involving ACP (Lambalot and Walsh, 1997). This includes fatty acid synthesis and degradation, the synthesis of membrane derived oligosaccharides (Therisod *et al.,* 1986), polyketides (Shen *et al.,* 1992) and in the activation of haemolysin an *E. coli* membrane-targeted toxin (Issartel *et al.,* 1991). ACP has also been found in the mitochondria of *Neurospora* (Brody *and* Mikolajczyk, 1988), plants (Chuman and Brody, 1989; Shintani and Ohlrogge, 1994) and bovine heart muscle (Runswick *et al.,* 1991). In yeast, there is no evidence that ACP from another species can be phosphopantetheinlyated i.e. the holo-ACP may be species specific. In other words, in this project, the lack of ACP interactions with any other FAS enzyme on test, may have resulted from the lack of post-translational modification of the ACP fusion proteins. Similarly, Mazzurco *et al.* (2001, previously described) hypothesised that the lack of ARC1 interactions with the SRK could be ascribed to a need for phosphorylation.

Another scenario for the lack of interaction between ACP and any other protein tested may stem from the fact that yeast two-hybrid proteins are N-terminal fusion proteins. As such, this may have caused steric hindrance. This is because ACP at 10 kDa, is smaller than the AD/ACP fusion protein and the DBD/ACP fusion protein, which are 29 kDa and 37 kDa respectively. Secondly, the necessary residues for ACP docking were found at Glu-41 (conserved in all 49 ACPs) was predicted to interact with the crucial Arg–249

of FabH (Ala–45 was also reported to be an important residue) (Zhang *et al.,* 2001). It may be have been possible that these residues were hidden and consequently the interaction was lost. On the other hand, Heath and Rock (1996) found that dimerization /homointeractions occurred with the *E. coli* dehydrase - a 20 kDa homodimer. In other words, each dimer is 10 kDa the same size as ACP. However, they did not detect a direct interaction between *E.coli* β-HAD and *E.coli* KAS I (Heath and Rock, 1996). It is notable that there have been no reports of the co-purification of *E. coli* type II FAS enzymes. This may indicate that the organization of *E. coli* type II FAS enzymes is quite distinct from a plant type II FAS.

It has been suggested that yeast two-hybrid system will be a suitable method to analyse type II FAS because yeast uses a type I fatty acid synthesis pathway, that is, the type II FAS fusion proteins would not interfere with yeast (Subrahmanyam and Cronan, 1998). The results from this project suggested a rather different scenario. The fusion protein AD/ACP was toxic to the yeast cells. The fact that yeast uses type I FAS machinery may be irrelevant and two-hybrid interaction analysis may only be appropriate in some instances. In this project, it was possible to envisage several mechanisms of toxicity. For example, it may be possible that the AD/ACP fusion protein had a novel structure or novel properties that adversely effected transcription machinery of yeast. This hypothesis was supported by the finding that the acidic activation domain of VP16 (the acidic activation domain of the herpes simplex virus) when fused to the GAL4-DBD conferred toxicity in yeast. In that work, using the toxicity of VP16 acidic activation domain to isolate the transcriptional adaptors (the bridge between activators and general

transcription factors) found the non-DNA binding forms of GAL4-VP16 not toxic. The toxicity required the integrity of both domains and high levels of GAL4-VP16. It was subsequently proposed that the inhibition/toxic process was one where the high level of AD fusion protein caused DNA binding at a secondary site in the yeast genome. The complex trapped at these sites depleting the availability of general transcription factors. Another model proposed included a direct interaction between GAL4-VP16 and one or more general transcription factors. The final model proposed was the inappropriate expression of cellular genes (Berger *et al.*, 1992).

The yeast two-hybrid system is a sensitive technique however, if interactions are weak and dependent on co-factors for stabilising interactions as proposed by Roujeinikova *et al.* (2002) and Zhang *et al.* (2001) (Chapter 1.3.3), then this approach may fail. Other approaches have been successful. For example, Fawcett *et al.* (2000) investigated interactions between ACP and a type II FAS enzyme. Specifically, they investigated the kinetics of ENR activity using ultrafiltration substrate binding assays. An interaction was detected between ENR and crotonyl-ACP (a four-carbon enoyl-ACP) as long as NADH was present. Using the same procedure, it was found there was no interaction between apo-ACP and ENR in the absence of NADH. No data was presented for these two statements (Fawcett *et al.*, 2000). However, ACP interactions represent a special case of protein-protein interactions as acyl-ACP is a substrate that has a protein moiety. Therefore, in light of the model proposed by Roujeinikova *et al.* (2002) and Zhang *et al.* (2001) (Chapter 1.3.3) ACP-FAS enzyme interactions may have occurred in the yeast two-hybrid system but were not stable enough to be detected.

If the interactions of type II FAS proteins are weak and/or substrate stabilised more suitable techniques may be ACP affinity chromatography (more sensitive) or cross-linking combined with co-immunoprecipitation (interactions captured and then precipitated). Both of these options are discussed more fully in Future Work (4.4).

4.2. Type II FAS as a dynamic multienzyme complex.

It is known that under native conditions extensive co-purification of type II FAS enzymes can be obtained (Hellyer *et al.*, 1992) and that channeling occurred in permeabilised chloroplasts (in an assay where intermediates were always present) (Roughan and Ohlrogge, 1996). This suggests that, where *in vivo* conditions are used or mimicked as far as possible and intermediates can be present, evidence of a multienzyme complex may be found to exist. Consequently, type II FAS may be characterised as having weak individual enzyme-enzyme interactions but, the multienzyme complex of proteins needs and/or is stabilised by, as yet, unidentified factors *in vivo*. In other words, type II FAS may be a dynamic multienzyme complex. The detection of direct weak interactions between ENR and DES in this thesis goes someway to support this hypothesis.

It has been suggested that an organism may evolve a stable ordered complex to guarantee advantages such as channeling at all times (Srere, 1987). This raises the question of what would be the advantage of a complex held together with weak and/or substrate stabilised interactions? In general, the advantage of multiple weak interactions

is that it allows for plasticity at the adhesive junctions (sites of interaction) and the formation of dynamic contacts. In other words, low affinity interactions, that are believed to exhibit short-lifetimes, requires multiple such interactions (Pierres *et al.*, 1996; Van der Merwe *et al.*, 1993). Consider the scenario whereby the type II FAS enzymes are in equilibrium, having similar amounts of interacting (bound) and non-interacting (free) states. In the free state they remain in close proximity due to the competing 'bound' state, a state which has been referred to as 'stickiness' (Fredriech, 1987). Therefore, when permeabilisation of the chloroplast occurred e.g. Roughan and Ohlrogge (1996) fatty acid synthesis may not have halted, as the total quanta of weak interactions were not simultaneously interrupted. In other words, the 'stickiness' of interactions was one factor that allowed retention and cohesion of structure and hence functions. However, at very low concentrations, a component(s) of type II FAS may disappear and synthesis may cease.

Another advantage of an easily dissociable complex may be found at the level of each individual enzyme. For example, in *Brassica napus*, enoyl reductase isoforms can exist in both the embryo and leaves, therefore, they must be able to exist and interact within the complex(es) (Fawcett *et al.*, 1994; de Boer, 1998). These isoforms could functionally replace their counterparts in a type II FAS complex, therefore, producing rarer fatty acids without the need for forming separate complexes. Due to the weak interactions of type II FAS enzymes, such isoform interchanges may easily occur and, in theory, be much more energetically favourable to the cell. In other words, there may be a modular approach to the type II FAS complex. Whereby a different module (isoform) produces a different fatty acid and it may be much more efficient than re-building a new complex.

Such a concept was previously raised in a discussion of the 'duplicate yet functionally different' components of *E. coli* FAS (Hoj and Svendsen, 1983). On the other hand, Suh *et al.* (1999) has argued that the isoforms of ACP could dictate the synthesis of unusual fatty acids via separate complexes. However, this would appear to be energetically expensive to the cell and thereby unnecessary.

The hetero-enzymatic interactions detected in this thesis indicate that the enzyme stearoyl-ACP desaturase is not, as commonly depicted, a discrete entity out-with the type II FAS complex. The enzyme could be an integral part of a dynamic type II FAS complex that directly interacts with enoyl reductase. It may be that if the co-purification of ENR and DES as carried out by Hellyer *et al.* (1992) can be faithfully reproduced, there may exist slightly more stable sub-complexes. For example, ACCase may be seen in this way. By extension, it may be possible that the termination/modification enzymes β-HAD/ENR/DES/TE are held together in a subcomplex by interactions which are stabilised by the acyl-ACP intermediate and other factors such as the membrane. Again, such a modular approach may be more energetically favourable to the cell. This implies that free subcomplexes may exist in a cell and can be recruited into an active complex when the limiting subunit becomes available.

Fatty acid synthesis has been shown to occur after approximately 50 % of stromal proteins were lost on permeabilisation (Roughan and Ohlrogge, 1996). This may indicate that membrane associations may play a role in enzyme-enzyme interactions and perhaps act as a scaffold between the sub-complexes. This hypothesis is supported by

the fact that ACP was localized to the plastids in avacado mesocarp (Slabas *et al.*, 1988), the maturing seeds and the chloroplast of *Brassica napus* by immunogold localization using rabbit anti-spinach ACP antibodies (Slabas *et al.*, 1988). It was subsequently found that around 80% of the ACP located at thykaloid membrane (Safford *et al.*, 1988). When this finding was taken in conjunction with the fact that acetyl-CoA carboxylase was also bound to the thykaloid membrane (due to a requirement for detergent to solubilise the protein) (Kannangaraand and Jensen, 1975), this indicated that two soluble components of *de novo* fatty acid biosynthesis were associated with a membrane. It has also been suggested that TE was membrane bound due to a requirement for detergent for its purification (Hellyer *et al.*, 1992). The channeling of the metabolites of type II FAS led to the suggestion that the putative fatty acid synthesising 'supercomplex' may provide a physical bridge that spans the stroma and joins the thylakoid to the plastid envelope (Roughan and Ohlrogge, 1996). This complex hypothesised by Roughan and Ohlrogge (1996) may be assumed to be very large (greater than 500 kDa). Whether this 'supercomplex' would associate with other complexes such as the photosynthetic apparatus remains an open question.

4.3. Protein-protein interactions and metabolic channeling of type II FAS.

Substrate channeling can be achieved by several different molecular factors or mechanisms (Chapter 1.2.3). In fact, it is currently thought that many of the examples of substrate channeling probably involve a combination of mechanisms. Therefore, in type

II FAS of *Brassica napus*, the extremely high V_{max} of TE for its oleoyl-ACP substrate (Hellyer *et al.,* 1992) may be one mechanism and, in *E. coli*, the shielding of the acyl chain by ACP may be another (Roujeinikova *et al.,* 2002). The recent discovery that protein-protein interactions may play a role in channeling even in 'stable' complexes such as type I FAS (Subrahmanyam *et al.,* 2000) suggests at the very least a contributory role for this mechanism in the 'dynamic' type II FAS. For example, specific and direct protein-protein interactions may allow the sequestering and formation of distinct metabolic pools of substrates. This may be achieved by the 'complementary cage' or 'alternating complementarily' as proposed by Fredriech (1987), Chapter 1.2.3.

The extent, if any, to which enzyme-enzyme interactions contribute to channeling in type II FAS remain to be discovered. An indication of the extent has been obtained from the application of a computer simulation of metabolic control analysis (MCA), using the GEPASI programme to investigate sunflower fatty acid synthesis (Martinez-Force *et al.,* 2002). In this work, data about the fatty acid content of normal and high-saturated seed formation and from the enzymatic characterization of the stearoyl-ACP desaturase, acyl-ACP thioesterase (TE) and fatty acid synthase II complex (FAS II) was obtained from earlier work by Martinez-Force *et al.* (1999). In this earlier work by Martinez-Force *et al.* (1999), it had appeared that reducing the activity of C16-thioesterase, or increasing the activity of KAS II, in the common pathway of *de novo* fatty acid synthase, could increase the level of oleic acid (C18:1). The economic significance of this lies in the fact that olive oil, with 70 % oleic acid and only 10 % palmitic, sells for at least six times the price of palm oil, with 40 % oleic and 45 % palmitic. It was subsequently discovered that the mutant sunflower plants (not transgenic) with low KAS II and high C16

thioesterase activities had abnormally high palmitic acid levels. However, the seeds also contained 3 unusual fatty acids: palmitoleic (C16:1 Δ9), palmitolenic (C16:2 Δ9 Δ12) and asclepic acid (C18:1 Δ11; i.e. with the double bond in a different place in the chain from oleic acid, which is C18:1 Δ9). It appeared that C16-ACP accumulated to high levels, despite the higher C16 thioesterase activity, and the enzyme (Δ9 desaturase) which normally desaturates C18-ACP to C18:1, also acted on C16-ACP, to give C16:1. Although KAS II activity was reduced, this enzyme acted on C16:1-ACP, adding 2 carbons to extend the chain to C18:1, but because the chain elongation has occurred after the desaturation instead of before, the double bond appeared in the 'wrong' place (Δ 11 instead of Δ 9). The third unusual fatty acid, C16:2, was formed when oleate desaturase, which normally desaturates C18:1 to C18:2, also acted on C16:1. Therefore, a single, apparently simple, change in a synthetic pathway had unexpected side effects. The authors also proposed that channeling for type II FAS was disrupted. To what extent these factors contributed to the desaturation of palmitoyl-ACP was not speculated upon at that time. (Martinez-Force *et al.,* 1999). In subsequent work using GESPASI (Martinez-Force *et al.*, 2002), the authors initially applied a free diffusion model to normal sunflower, high-palmitic and high stearic acid mutants. Subsequent predictions of high-stearic sunflower mutants could fit the data. i.e. the products found in the mutants via biochemical detection correlated well with the GESPASI model. However, when this 'free diffusion' model was applied to high-palmitic sunflower mutants the model had to be adjusted. The biochemical data (from the 1999 paper) was thus used to modify the theoretical model. The only model that could work, predicted the existence of dynamic channeling between the FAS II complex and DES. Whereby, the channeling

was responsible for an alternative pathway starting with the desaturation of palmitoyl-ACP by the DES. From this, it was hypothesised that there was a region on DES involved in the temporal interaction with the FAS II complex. The results of this project corroborate this proposal.

Heath and Rock (1996) examined the possibility of a link between protein-protein interactions and channeling in *E. coli* type II FAS. In that work, investigations were carried out into the substrate preferences of the two dehydrases, FabA and FabZ. Fatty acid synthesis was reconstituted using a cell-free extract with [2-^{14}C]- malonyl CoA plus or minus FabA or FabZ. This revealed that although FabA and FabZ have overlapping substrate specificities, FabZ was the most active on short chain β-hydroxyacyl ACPs. The FabA enzyme was most active on intermediate length chains. In *E. coli,* the products from both FabA and FabZ are converted to deconyl-ACP by *E. coli* enoyl reductase (FabI) and ultimately towards the manufacture of saturated fatty acids. FabA can also convert a small amount of the *trans*-3-deconyl-ACP product to *cis*-3-deconyl-ACP which is the substrate for the *E. coli* ketoacyl-ACP synthetase I (FabB). However, the authors noted that significant quantities of the *cis*-3-deconyl-ACP.product were only found in the presence of active FabB. Therefore, FabB appeared to be diverting the flow of intermediates towards production of unsaturated fatty acids i.e. channeling the *cis*-3-deconyl-ACP intermediate. A yeast two-hybrid analysis indicated there was no direct interaction between FabB and FabA. Channeling via protein-protein interactions was thus dismissed and the 'channeling' effect attributed to the affinity of FabB for *cis*-3-deconyl-ACP (Heath and Rock, 1996). This interpretation may need re-examining in

light of the growing understanding of channeling. This is because 'significant interactions' and channeling are not interdependent (Fredriech, 1987). It may have been the case that the interactions were too weak to detect and/or needed the *in vivo* environment and/or a stabilising intermediate and/or the enzymes changed structure on binding their substrate thus facilitating interactions. In other words, *in vitro*, where there are few competing substrates, utilising affinity as a channeling mechanism would likely work. In the crowded cell, affinity would work, but it seems logical that there may be a need for some sort of enzyme-enzyme interaction in order to maintain close proximity of enzymes and so facilitate channeling by 'affinity only' from one active site to another. For example, perhaps a mechanism such as 'alternating complementarily' (as described by Fredriech, 1987, Chapter 1.2.3) is in use *in vivo*.

Shared roles in channeling between enzyme-enzyme interactions, substrate affinity, and ACP affinity have been similarly documented for polyketide synthases (PKS). These complexes are similar to vertebrate FAS, and are organized in multidomain polypeptides containing a ketosynthase, acyltransferase, and ACP domain within each module (Zheng *et al.,* 2001). Such reports may become more widespread as the appreciation of the need for metabolic compartmentalisation via supramolecular structures becomes more common. In the case of rational metabolic engineering of type II FAS to obtain novel lipid products, this will need to take supramolecular organization and metabolic control into account if manipulations of products are to be successful.

4.4. Future Work.

This project has shown that the two-hybrid system can detect interactions of the putative type II FAS complex. Immediate work specific for the yeast two-hybrid system may include the cloning of DES and TE into the pACT2-1 vector and possibly β-HAD into both vectors pAS2-1 and pACT2 of the Clontech Matchmaker System. This would allow the most logical extension of the pairwise linkage map for type II FAS enzymes. In other words, do TE and DES interact? It may also be worthwhile to include a more sensitive indicator of *lac* Z expression such as the luminescent substrate, Galacton Star (Clontech), although this may give rise to protein-protein interaction artefacts. In this thesis, the programs Sigseq and ChloroP gave a tranist peptide for TE, but there was a two amino acid difference between these programs, when they predicted the cleavage site of the transit peptide of the TE chloroplast transit protein. This project used the primers that took into account the results of the Sigseq predicition. If however, this was wrong, this could have significantly affected protein-protein interactions, therefore a priority may be to redesign primers for cloning with respect to the result from the ChloroP program. In this thesis, the fusion protein AD/ACP was found to confer a level of toxicity on yeast cells, one possible solution to tackle this problem could be to use a two-hybrid system based on the inducible promoter LexA, to help regulate the level of expression. A three-hybrid system does exist and may be a possibility to co-express, for example, the ACP modifying enzyme β–holo-ACP synthase (β-HAS) in order to ascertain the necessity for this modification and its interaction of ACP with any other FAS component. One may express three FAS enzymes such as TE/ENR/DES and see

the difference to activation of the reporter gene but it would be difficult to ascertain if the interaction was direct.

Using the plasmid constructs prepared for the yeast two-hybrid system could allow the effect of mutagenesis to be investigated. This would involve the delineation of important binding regions or the pinpointing of critical residues. A high priority would be to study the effect of the ENR-MOD1 mutant on interactions. This *Arabidopsis* ENR mutant caused dramatic changes in plant phenotype due to a change of threonine to isoleucine (via C to T missense mutation) (Mou *et al.*, 2000). Furthermore, the results from the MOD 1 mutant demonstrated that a mutation need not be at the active site to have a dramatic effect on the cell. Therefore, one could ask, would a change from threonine to isoleucine (via a C to T missense mutation) in ENR effect the protein-protein interactions detected in this thesis? This may further the understanding of the links between protein-protein interactions, supramolecular structure and metabolism.

A recent example of the contribution to plant physiology by the yeast two-hybrid system and mutagenesis was given in the recent article by Mazzurco *et al.* 2001 (Chapter 4.1). Mutagenesis may also be used to identify structural motifs that might mediate enzyme-enzyme interactions of type II FAS. In this project, having identified direct interactions between the sequential enzymes of type II FAS, mutagenesis represents a way forward at identifying residues or segments involved in mediating them. For example, do TE and DES have similar or different ENR binding sites? In other words, is the binding of TE and DES to ENR mutually exclusive?

One of the most fundamental and common starting points of proteomics is the ability to identify novel interacting partners of a candidate protein by virtue of a yeast two-hybrid library screen. It also remains an important feature in beginning novel projects. The basis of this was achieved during the course of this project. The construction of four 'bait ' proteins i.e. the DBD/FAS fusion proteins and the extraction of RNA from seed at different stages of development which may provide the basis for enhancing the number of low abundance of messenger RNA for FAS molecules. This strategy will enhance the chance of identifying type II FAS cDNA from a library screen with one of the four baits constructed in this project (i.e. ACP, ENR, DES and TE). Time was taken to establish unequivocal positive interactions between ENR with DES. Thus, this has helped set the basis for future screening with the baits (DBD/ENR; DBD/DES and DBD/TE) that have been sequenced, expressed, characterised and their use validated

There are various ways to study levels of organization of enzyme-enzyme interaction from, for example: cofactor/coenzyme to sub-unit association to protein-protein interaction. The first level was extensively covered by Fawcett *et al.* (2000). This project allowed one to look at the next two levels simultaneously. However, available data indicate that the hetero-enzymatic interactions might not be 'stand alone' occurrences. The relatively weak interactions of found in this project, compared to the extensive co-purification of ENR and TE (Hellyer *et al.*, 1992) suggested a need for co-factors and perhaps other unidentified factors. That is, a necessity to mimic *in vivo* conditions as far as possible. Using isolated *Brassica napus* chloroplasts represented the logical way of avoiding such modification/microenvironment dependent interactions. The use of information from protein-protein interaction studies from the proteins in their natural

environment as far as it is possible, will be invaluable and an absolute requirement for definitive interaction map of type II FAS and for higher levels of supramolecular organization.

This project attempted to use bandshift analysis with isolated chloroplast in order to circumvent problems associated with the yeast two-hybrid system. This was unsuccessful (Chapter 3.2) but this approach may be used in the future with appropriate fractionation of chloroplasts and appropriate controls in place. Alternatively, one may use affinity chromatography. A technique which has the following advantages: 1) it tests against all extracts equally; 2) proteins that are detected have successfully competed against the rest of the population of proteins; 3) it is possible to test for the responsible interacting domains and residues by preparing mutant derivatives; 4) interactions that depend on a multisubunit tethered protein can be detected. A major problem comes from the technique being oversensitive i.e. there is detection of very weak interactions with a dissociation (binding) constant of 10^{-5} M which may not be physiologically relevant (Formossa *et al.*, 1991). Perhaps it may be employed to test the hypothesis that the type II FAS complex has the potential to link the thylakoid and plastid membrane by spanning the stroma (as suggested by Roughan and Ohlrogge, 1996). For example, a purified enzyme, such as ENR, may be attached to a column and plastid/thylakoid membrane fractions passed down the column. Strongly retained fractions may then be eluted by high salt conditions, cofactors, chaotropic solvents or SDS. Interacting membrane proteins could be identified immunologically or by mass-spectrometry.

Co-immunoprecipitation using total chloroplast extract, or fractions thereof, may represent a logical next step. If used with the ENR antibody as the precipitating agent, in theory, this may precipitate out interacting proteins with ENR with relatively minimal disruption to the *in vivo* conditions of the chloroplast. It may also allow the membrane to remain relatively intact (i.e. a low degree of permeabilisation) and thus sustain interactions of type II FAS that may require a lipid or other specific microenvironment. The great advantage of this approach over affinity chromatography is that dilution/permeabilsation experiments could be done using the co-immunoprecipitation buffer. This may facilitate a dilution series as was carried out by Roughan and Ohlrogge (1996) and subsequently one may observe if interactions were disrupted or not as the sorbitol concentration decreased. At this point, cross-linkers could be employed to stabilise the dynamic complex. The subsequent analysis to ascertain the nature of these proteins may be carried out regardless of the any such effect on protein concentration caused by the analytical system because, in theory, interacting proteins would have already been 'captured' by the co-immunoprecipitation. Furthermore, as Hellyer *et al.* (1992) and Kater *et al.* (1991) found co-purification of FAS enzymes, it may be the case that β–HAD and/or TE and/or DES would also co-precipitate. If there was no fractionation and consequently no loss of co-enzymes or co-factors, perhaps a larger complex may be precipitated. This would give much greater experimental confidence in the co-purification work. This was because a crucial and fundamental tenet of biology was still missing from the 'study' of type II FAS interactions: the extensive co-purification of the FAS II enzymes by Hellyer *et al.* (1992) has never been repeated.

The consensus is that the establishment of the presence or absence of protein-protein interactions for any given cellular process requires a multifaceted approach incorporating a spectrum of experimental techniques. Mass spectrometry based techniques have been used to identify many novel proteins in the chloroplast (Peltier *et al.*, 2000; Gallardo *et al*, 2001) but such work reveals little about their function. Thus, the mapping of protein-protein interactions may remain for some time, the crux of understanding cellular biochemistry. The study will require the best efforts to reproduce *in vivo* conditions before characterising behaviour. This project addressed that issue by focusing only on *Brassica napus* enzymes and *Brassica napus* chloroplasts. The former has provided solid evidence in favour of interactions of *Brassica napus* type II FAS enzyme interactions and the latter now provides the framework for future *in organello* work to proceed.

Finally, there appears to be a need of an appreciation of the significance of weak and transient interactions and their physiological role. These fragile protein-protein interactions may occur at degrees of subtlety that defies apparently thorough experimental detection. However, they must not be disregarded, they may be protein-protein interactions that are weak by design.

Chapter 5. References.

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