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The Regulation of Fatty Acid Synthetase Genes in *B. napus*

Helen Hooper

A thesis submitted to the Department of Biological Sciences, University of Durham in accordance with the requirements for the degree of Doctor of Philosophy

Department of Biological Sciences University of Durham

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DECLARATION

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Abbreviations used in text.

A_{280}	absorbance at 280nm
ACC	acetyl- CoA carboxylase
ACP	acyl carrier protein
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
cpm	counts per minute
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DMF	dimethyl formamide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
IPTG	isopropyl- β- D- thio- galactopyranoside
kDa	kilodaltons
µg	microgram
mg	milligram
ng	nanogram
OD_{600}	optical density (absorbance) at 600nm
oligo	oligonucleotide
pH	hydrogen ion potential
PCR	polymerase chain reaction
PMSF	phenyl methyl sulfonyl fluoride
RNA	ribonucleic acid
SDS- PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
TAG	triacyglyceride
TE	10mM Tris. HCl, pH 8.0, 1mM EDTA buffer
TEMED	N, N, N', N'- tetramethethylenediamine
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
v/ v	volume for volume ratio
w/ v	weight for volume ratio
X- GAL	5- bromo- 4- chloro- 3- indoyl- β- D- galactosidase

CONTENTS

The Regulation of Fatty Acid Synthetase Genes in *B. napus*

ABSTRACT

The main aim of this work was to analyse a cloned ACP gene promoter, which had been shown to contain regulatory information that directed both spatial and temporal expression. Gel retardation assays were used initially to define protein binding sites within the ACP05 promoter. Following such analysis, a DNA motif that interacted with a sequence specific binding factor was identified. This factor was detected in embryo extracts and was not present in leaf extracts. The stability and binding characteristics of the ACP promoter binding protein were studied after heparin agarose chromatography, using gel retardation assays to follow the protein. A binding site oligonucleotide was synthesised and used as a probe to screen an expression library, in a "South Western" cloning experiment. A single positive clone, λBS2xi, was isolated. The DNA binding specificity of the recombinant protein was determined by gel retardation. The clone was confirmed to encode a functional sequence specific DNA binding domain. Northern hybridisations demonstrated the mRNA was expressed and in a tissue specific manner: levels of message were high in seed, low in root and not detected in leaf tissue. Endonuclease restriction of the lambda clone with *Eco*RI excised a 3.0kbp fragment that corresponded to the entire cDNA. The fragment was subcloned in pSK^+ and several strategies were used to characterise and sequence it. A set of nested deletions was generated, but reliable sequence data was not obtained from the first half of the cDNA. A section of readable sequence data was obtained approximately 1.5Kbp from the 5' end of the deleted cDNA. Exhaustive databank searches using the sequence data demonstrated that it corresponded to β- galactosidase. Southern analysis further demonstrated that this sequence was present in λBS2xi. Further work required to characterise λBS2xi is discussed.

A second related subject of this thesis concerns a second member of the FAS complex, enoyl- ACP reductase (ER). Prior to this work it was shown that there

were four ER isoforms, expressed in both leaf and seed. The levels of expression of individual isoforms were different, there being two major forms and two minor forms. One leaf expressed clone, pERL8 had been isolated and characterised. A DNA probe that encompassed the 3' untranslated region (3'UTR) was generated from pERL8 and used to screen a cDNA library generated from embryo. Eleven positives were isolated and ten were successfully subcloned by plasmid rescue. The clones were sequenced with internal primers. Exhaustive searches of databanks using the sequence data demonstrated that five clones corresponded to seed storage proteins and five were confirmed as ER. The ER clones were divided into two groups on the basis of sequence differences. One clone was identical to pERL8 and represented an isoform expressed in both leaf and seed. The sequences of the clones within a group was identical, apart from an "insert" present in the 3' UTR of one clone from both groups. Whether these inserts were real or artifacts could not be demonstrated conclusively as further sequencing showed both clones with inserts in the 3' UTR were 5' truncated. In order to determine whether all four ER isoforms had been isolated further work would be required. This is also discussed.

Chapter 1

General Introduction

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1.1 Plant lipids

Acyl lipids are major constituents of plant tissue (Harwood, 1988; Harwood *et al.*, 1990; Gunstone and Heslof, 1992; Ohlrogge *et al.*, 1991; Murata and Nishida, 1987; Slabas and Fawcett, 1992; Slabas *et al.*, 1984; 1987; 1993; 1994; Somerville and Browse, 1991). They are a group of hydrophobic compounds which are derivatives of fatty acids coupled to alcohols (in particular glycerol), bases, phosphate esters sugars and sterols, or with combinations of these (Gunstone, 1992; Gunstone and Heslof, 1992). The main component of the lipid fraction in most plants are glycerides (derived from glycerol), however waxes, cutins, sterols and sphingolipids also occur commonly (Gunstone and Heslof, 1992; Murata and Nishida, 1987). The glycerides are subdivided into three groups, triglycerides (or triacylglycerides), phosphoglycerides and glycosylglycerides, which serve distinct roles within the plant (Slabas and Fawcett., 1992). The general structures of plant lipids are presented in figure 1.1.

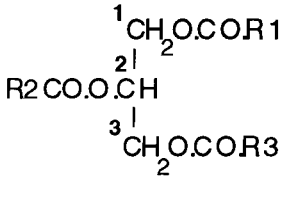
1.2 Structure and functions of plant lipids

The main constituent of lipids are fatty acids, the composition of which effectively defines the physical properties of the lipid. The nomenclature of fatty acids (Gunstone and Heslof, 1992) is normally based on the hydrocarbon chain having the largest number of carbon atoms. Double bonds are usually defined from the carboxyl end of the molecule and are indicated as Δ (or as ϖ, when numbered from the methyl end).

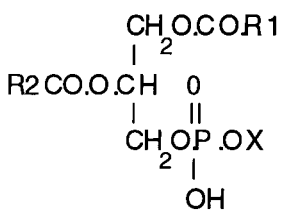
In total over five hundred fatty acids have been identified, most of which are rare, being found only in a single, or few species (Gunstone and Heslof, 1992; Hilldítch and Williams, 1964). Many of the common fatty acids have trivial names which are listed in table 1.1. Analysis of the composition of membrane fatty acids reveal little variation in acyl side chains, which are mainly long

Figure 1.1: The general structure of acylglycerides

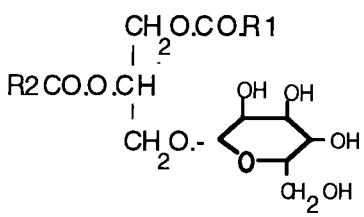
A: General structure of triglycerides. R1, R2 and R3 denote the fatty acid (acyl) chain esterified to the sn- **1**, sn- **2** and sn- **3** positions, respectively, of the glycerol backbone. **B:** phosphoglyceride. **C, D** and **E:** glycosylglycerides. MGDG, monogalactosyl diacylglyceride, DGDG, digalactosyl diacylglyceride and SQDG, sulphoquinovosyl diacylglyceride (Gunstone and Heslof, 1992).



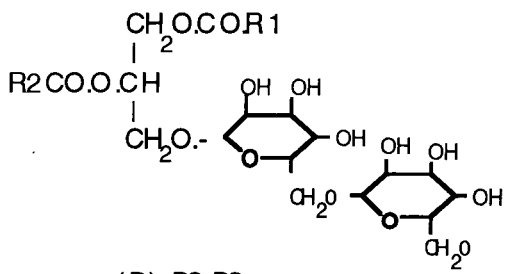
(A) Triglyceride



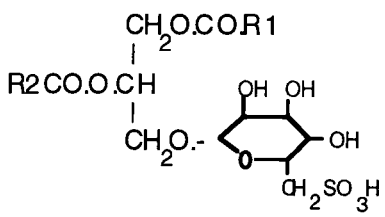
(B) Phosphoglyceride



(C) MGDG



(D) DGDG



(E) SQDG

chain (C16- 18). This invariance reflects the importance of fatty acids in maintaining fully functioning membranes (Somerville and Browse, 1991; Murata, 1983). In contrast, storage lipids have widely varying fatty acid side chains (Murata and Nishida, 1987; Somerville and Browse, 1991). Conservation of structure is not as important when the primary function of the molecule is to act as an energy reserve and is eventually degraded to acetyl CoA.

The functions of lipids are diverse (Gibson *et al.*, 1994). Among the most important is their structural role in membranes (Briskin, 1994). The analysis of the lipids isolated from different subcellular membranes reveals distinct profiles. For instance glycosylglycerides are a major component in the thylakoid membranes of the chloroplast (Douce and Joyard, 1980). Evidence indicates that lipids provide the necessary environment for biological activity of membrane proteins, such as ATPase (Mitchell, 1979) and glycerol- 3-phosphate- acyl transferase (Green and Bell, 1984; Larson *et al.*, 1980). Membrane lipids are also critical in determining the chilling sensitivity of certain plants (Murata, 1983; Murata et al., 1992) and cyanobacteria (Wada *et al.*, 1990; 1994). It has been demonstrated that in chilling tolerant species there is an increase in the degree of unsaturation of membrane lipids following a decrease in temperature (Gombos *et al.*, 1994; Murata, 1983; Murata *et al.*, 1992; Wada *et al.*, 1994). In this manner plants are able to adapt to environmental stress by altering membrane fluidity (and therefore maintaining biochemical function) (Moon *et al.*, 1995; Murata and Wada, 1995). An additional structural role is that of cutins and waxes on the outside of the plant that act as physical barriers against pathogen attack (Kolattakudy, 1987).

Lipids have many biosynthetic functions. As acyl Acyl Carrier Proteins (ACP) and acyl Coenzyme As (Co A) they act as intermediates in the synthesis of complex lipids and as substrates for acyl transfer and desaturation (Frentzen *et al.*, 1983; 1990). In addition there is evidence that suggests sulphated lipo-oligo saccharides elicit root nodulation in *Rhizobium meliloti*, (Lerouge *et al.*, 1991; Spaink, *et al.*, 1991).

Table 1.1 Commonly occurring plant fatty acids

Trivial name	Systematic name	Formula
Saturated		
capric acid	decanoic acid	C10:0
lauric acid	docanoic acid	C12:0
myristic acid	tetradecanoic acid	C14:0
palmitic acid	hexadecanoic acid	C16:0
steric acid	octadecanoic acid	C18:0
arachachic acid	eicosanoic acid	C20:0
Monounsaturated		
palmitoleic acid	cis- 9- hexadecenoic acid	C16:1 [Δ9c]
oleic acid	cis- 9- octadecenoic acid	C18:1 [Δ9c]
vaccenic acid	cis- 11- octadecenoic acid	C18:1 [Δ11c]
erucic acid	cis- 13- docosenoic acid	C22:1 [Δ13c]
Polyunsaturated		
linoleic acid	cis, cis- 9, 12, octadecadienoic acid	C18:2 [Δ9, 12c]
γ linolenic acid	all cis- 6, 9, 12, octadecatrienoic acid	C18:3 [Δ6, 9, 12c]
α linolenic acid	all cis- 9, 12, 15, octadecatrienoic acid	C18:3 [Δ9, 12, 15c]

Lipids also have an important role in storage. Many plants accumulate large amounts of storage lipids in fruits or seeds as triglycerides (Murphy, 1990; Pryde and Rothfus, 1989). Field crops such as rapeseed, flax, sunflower, sesame and peanut accumulate oil up to ~45% of the seed weight, concentrated in oil bodies. Such stores represent latent energy and fatty acid sources for seed development and are subject to considerable commercial exploitation (Pryde and Rothfus, 1989).

1.3 Biosynthesis of plant acyl lipids

Fatty acid synthesis is an ubiquitous feature of living organisms as it is essential for membrane biogenesis and growth. The same pathway is also used to synthesise fatty acids which are incorporated into triacylglycerides, which represent a concentrated energy store. The mechanism of fatty acid synthesis is essentially the same in all organisms, however the Fatty Acid Synthase (FAS) complex exists in a variety of structural forms. In plants (Ohlrogge, 1982; Shimakata and Stumpf, 1982a; Stumpf and Shimakata, 1983) and most bacteria (Vagelos, 1974) there is a type II, or dissociated FAS system, in which each of the individual partial reactions are carried out by separate proteins. This is in contrast to the type I FAS system found in vertebrates (Smith, 1994; Witkauski *et al.*, 1991), fungi (Schweizer *et al.*, 1987) and yeasts (Stoops and Wakil, 1980), in which components are combined within one or two high molecular weight multi- functional polypeptides.

The major site of fatty acid biosynthesis in higher plants is the chloroplast (Stumpf, 1981). The close similarity between cynaobacterial and plant FAS systems has led to the suggestion that type II FAS may represent a vestige of the procaryotic origins of the chloroplast (McCarthy *et al.*, 1983). There is evidence which suggests that type I FAS may have arisen by gene fusion events of component type II enzymes during evolution (Werkmeister *et al.*, 1983; Witkauski *et al.*, 1991).

1.3.1 Precursors of biosynthesis

Fatty acids are synthesised from acetyl- CoA and malonyl- CoA precursors, the C1 and C2 carbon atoms of the fatty acid are derived from acetyl- CoA and the 2-C units required for elongation are provided by malonyl- CoA (Stumpf, 1980; 1981). Most or all of *de novo* biosynthesis of C16- 18 fatty acids occurs in the plastids of both leaves and developing seeds (Ohlrogge *et al.*, 1979; Weaire and Kekwick, 1975). Subsequent elongation and modification occur in membrane associated systems (Harwood *et al.*, 1990). The source of acetyl- CoA has not yet been demonstrated unequivocally. It has been proposed that the generation of free acetate by the mitochondrial pyruvate dehydrogenase complex coupled to acetyl CoA hydrolase could produce free acetate, which could then enter plastids and be incorporated into lipids (Stumpf, 1980). In the chloroplast acetate would then be rapidly converted to acetyl- CoA via acetyl- CoA synthetase in the stroma (Harwood, 1988).

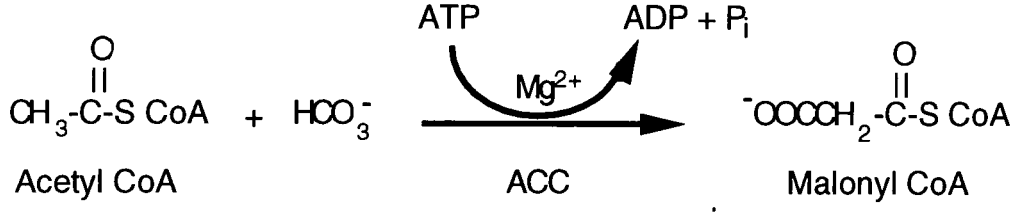
More recently it was suggested that L- acetylcarnitine was a likely precursor for plastid lipid synthesis (Masterson *et al.*, 1990). This was based on the five fold greater increase in fatty acid synthesis of isolated pea chloroplasts fed acetylcarnitine as compared to those fed with acetate. However, in complete contrast Roughan and colleagues (1993) found that the rate of long chain fatty acid synthesis in isolated plastids from several species (including pea) fed with acetylcarnitine was less that 2% of those fed with acetate. Carnitine acyltransferase activity was not detected in isolated plastids and they concluded that it could not be a possible precursor.

The first committed step of *de novo* fatty acid synthesis is the ATP dependent carboxylation of acetyl- CoA to form malonyl- CoA (see figure 1.2). Malonyl CoA is an important central metabolite and is utilised for the synthesis of flavenoids, very long chain fatty acids and stilbenoids (Stumpf, 1980). The reaction is catalysed by Acetyl- CoA Carboxlase (ACC), the structure and subunits of which vary between species. In mammals the enzyme consists of a single large multifunctional polypeptide of at least 200 kDa. The three functional domains are biotin carboxylase, biotin carboxylase carrier protein

and carboxyltransferase. In *E.coli* the enzyme consists of a complex of polypeptides, on which the composite reactions reside (Wood and Barden, 1977). All four polypeptide subunits have been purified to homogeneity and the acyl carrier protein has been found to be the only biotin containing protein in *E. coli* (Fall *et al.*, 1975; Fall, 1979).

Both the large multifunctional type I ACC and the multisubunit bacterial type II ACC are present in plants (Li *et al.*, 1992; Saskai *et al.*, 1993). Both types have different cellular and subcellular locations (Alban *et al.*, 1994; Ohlrogge et al., 1979). Type I ACC is found in epidermal cells and is cytosolic and type II is found in mesophyll cells and is chloroplastic. The type I ACC is induced by uv light and fungal elicitors and as a result is thought to be involved in flavanoid and wax biosynthesis (Shorrosh *et al.*, 1994). Type II ACC is thought to be associated with *de novo* lipid synthesis in the chloroplast (Ohlrogge *et al.*, 1979). All ACCs have a biotin moiety covalently bonded to a specific lysine residue, which is thought to act as a molecular arm passing the carboxyl group between subunit active sites (Samols *et al.*, 1988).

Figure 1.2 Carboxylation of acetyl- CoA by ACC



In mammalian systems ACC activity is the rate limiting enzyme for *de novo* fatty acid biosynthesis and is regulated by phosphorylation and polymerisation/ depolymerisation (Numa and Tanabe, 1984). In plants there is also evidence which suggests ACC is the rate limiting enzyme for FAS. A study of the acyl CoA and acyl ACP pools in spinach chloroplasts suggested a regulatory role (Post-Beittenmiller *et al.*, 1992). Also a correlation between ACC activity and the deposition of lipid in developing *Brassica napus* seeds was reported (Harwood,1988). However a more recent study of ACC activity in developing *B. napus* embryos found a negative correlation with lipid deposition (Kang *et al.*, 1993). The exact mechanisms which regulate ACC in plants are not clearly defined (Slabas and Fawcett, 1992).

1.3.2 The fatty acid synthetase system

Comparatively little was known about the enzymes involved in the *de novo* biosynthesis of fatty acids in plants until the 1960s. In the 1950s the incorporation of ^{14}C- acetate into palmitic and oleic acids in plant tissue extracts was demonstrated (Newcomb and Stumpf, 1953; Stumpf and Barber, 1957; Squires *et al.*, 1958). Subsequently, isolated chloroplasts were demonstrated as being capable of incorporating ^{14}C - acetate into ^{14}C - palmitic acid and ^{14}C - oleic acid (Mudd and MacManus, 1962; Weaire and Keckwick, 1975). In 1967 ACP was isolated from three different plant species (Simoni *et al.*, 1967). This was the first evidence that implied plant FAS was a type II or dissociated FAS system, like that from *E. coli.* However, it was not until 1982 that the partial or complete purification of individual component enzymes of FAS was published from barley leaves (Hoj and Mikkelsen, 1982), avocado mesocarp (Caughey and Kekwick, 1982), safflower seeds (Shimakata and Stumpf, 1982a), spinach leaves (Shimakata and Stumpf, 1982b; 1982c and 1982d) and parsley suspension culture (Schulz *et al*, 1982). Many of the enzymes that are involved in lipid biosynthesis are minor, membrane bound components and are difficult to purify using standard techniques (Topfer and Martini, 1994). Thus obtaining antibody or oligonucleotide probes for cDNA cloning (which require purified protein for their generation) is often technically difficult. Several alternative cDNA cloning strategies which circumvent the problem of protein purification exist and have

been used successfully to clone FAS components. These include T- DNA tagging (Browse *et al.*, 1993), chromosome walking (Lemieux *et al.*, 1990) and complementation cloning (Brown *et al.*, 1995).

De novo synthesis of C16 and C18 fatty acids is catalysed by the combined action of ACC and the cyclic FAS system (Slabas and Fawcett, 1992; Slabas *et al.*, 1994). In addition, the cofactor ACP is required to act as a shuttle for the growing acyl chains. The intermediates in the pathway are attached to ACP as thioesters via a phosphopantetheine prosthetic group (Ohlrogge, 1987). The component reactions of the FAS system are shown in table 1.2.

The exact mechanism of the initial reaction of fatty acid biosynthesis is unclear and there are three main possibilities. The first is the production of acetoacyl- ACP from the condensation of acetyl- CoA and malonyl- ACP, catalysed by 3- Ketoacyl- ACP Synthetase (KAS) III (Jaworski and Rock, 1987; Jaworski *et al.*, 1989; Walsh *et al.*, 1990). The second is the conversion of acetyl- CoA to acetyl- ACP by either Acetyl- CoA: ACP transacylase or KAS III. The acetyl- ACP produced would be condensed with malonyl- ACP by KAS I to form acetoacetyl- ACP. The last possibility is the decarboxylation of malonyl- ACP by KAS I to form acetyl- ACP, which is then condensed with malonyl- ACP (Magnuson *et al.*, 1993). Following the initial condensation, subsequent condensations between acyl- ACP intermediates and malonyl- ACP are catalysed by KAS I. The 3-ketoacyl- ACP intermediates are then reduced (by 3- ketoacyl- ACP- reductase), dehydrated (by 3- hydroxyacyl- ACP- dehydratase) and reduced again (by enoyl- ACP reductase) to yield the saturated acyl- ACP intermediate. The final condensation, from C16 to C18, which yields the end product of fatty acid synthesis requires yet another 3- ketoacyl- ACP- synthase (KAS II) (Shimikata and Stumpf, 1982c).

A carbon chain of 18 atoms is the longest acyl chain produced by the plastid located cyclic FAS system. However all plants produce long chain saturated fatty acids for the formation of cutins, suberin and waxes. Some plants such as *Brassica juncea* synthesise long chain monounsaturated fatty acids (C22:1, erucic acid) in their seed triglyceride oil fraction. Long chain fatty acids are elongated and modified by membrane associated enzyme systems

Table 1.2: Individual reactions of the fatty acid synthetase system

1) Acetyl- CoA: ACP transacylase (KAS III?)

Acetyl- CoA + ACP <------> Acetyl- ACP + CoA

2) Malonyl- CoA: ACP transacylase (MCAT)

Malonyl- CoA + ACP <------> Malonyl- ACP + CoA

3) 3- Ketoacyl- ACP synthase III (KAS III)

Acetyl- CoA + Malonyl- ACP <------> Acetoacyl- ACP + $C0_2$ + CoA

4) 3- Keto- ACP synthase I (KAS I)

nAcyl- ACP + Malonyl- ACP <------> (n + 2) Acyl- ACP + $C0_2$ + ACP

5) 3- Keto- ACP synthase II (KAS II)

Palmitoyl- ACP + Malonyl- ACP <------> 3- ketooctadecanoyl- ACP + $C0_2$ + ACP

6) 3- Ketoacyl- ACP reductase

3- ketoacyl- ACP + NADPH <------> 3- Hydroxyacyl- ACP + NAD(P)

7) 3- Hydroxyacyl- ACP dehydrase

3- Hydroxyacyl- ACP <------> Enoyl- ACP + H_20

8) Enoyl- ACP reductase

Enoyl- ACP + NAD(P)H <------> Acyl- ACP + NAD(P)

which are well characterised in animal systems. ACP does not appear to be involved in these elongation reactions but the 2C- donor is malonyl- CoA (Harwood *et al.*, 1990).

1.4 The acyl carrier protein

1.4.1. Historical perspectives

The original studies of Acyl Carrier Protein (ACP) were in *E. coli* (Prescott and Vagelos, 1972), where ACP was initially described as a heat stable cofactor to which the intermediates of fatty acid synthesis were attached (Prescott and Vagelos, 1972). Following this discovery the structures of the intermediates in fatty acid biosynthesis were elucidated over several years (Bloch,1970; Bloch and Vance, 1977; Prescott and Vagelos; 1972; Volpe and Vagelos, 1976; Wakil *et al.*, 1970).

The role of ACP in plants was demonstrated initially by ammonium sulphate fractionation studies of avocado mesocarp extracts. FAS activity could be separated into two components, which were inactive alone, but able to synthesise palmitic and stearic acids when recombined. One fraction was heat and acid stable, but protease sensitive and could partially substitute for the analogous *E. coli* fraction in the stimulation of *E. coli* FAS. This fraction was subsequently demonstrated to contain ACP and was taken as evidence for the similarity between plant and bacterial FAS systems. ACP is currently the most completely studied protein in both plant and bacterial lipid metabolism (Ohlrogge, 1987).

1.4.2 Functions of ACP

ACP is an absolute requirement for de novo fatty acid biosynthesis in bacteria (Cronan and Rock, 1987) and plants (Slabas and Fawcett, 1992). Probably its best known role is as the cofactor to which the intermediates of fatty acid biosynthesis are attached (Ohlrogge, 1987). ACP contains a 4 phosphopantetheine prosthetic group which is linked to the protein via a phosphodiester bond with a specific serine residue. Acyl groups (the

substrates, intermediates and products of fatty acid synthesis) are then attached via a thioester bond to the terminal sulphydryl of the prosthetic group. In the *de novo* biosynthesis of C16 and C18 fatty acids ACP acts as the carrier of the growing acyl chains between active sites of the various enzymes involved. The prosthetic group is donated to apo ACP from coenzyme A in a reaction catalysed by a chloroplast located holo ACP synthase (Jaworski and Rock, 1983; Post-Beittenmiller*et al.*, 1989b). The reverse reaction, the formation of apo- ACP is catalysed by holo- ACP hydrolase. Evidence for the importance of maintaining a fully phosphopantetheinylated ACP is provided by the *E. coli* mutant MP4. This mutant has reduced holo- ACP synthase activity and subsequently grows at only half the rate of the parent strain (Polacco and Cronan, 1981).

ACP also functions as an acyl donor in transfer, desaturation and hydrolysis reactions. A strong selectivity for acyl- ACPs rather than acyl CoAs is shown. by stearoyl- ACP desaturase (McKeon and Stumpf, 1982), glycerol- 3-phosphate- acyl transferase (Frentzen *et al.,* 1983) and an acyl- ACP hydrolase (McKeon and Stumpf, 1982) at physiological substrate concentrations. ACP has been shown to function in several other distinct areas. In *E. coli* it acts as a cofactor in the synthesis of membrane derived lipo- oligo saccharides (Therisod and Kennedy, 1987) and polyketides (Shen *et al.,* 1992) and has a putative role the activation of the membrane toxin, haemolysin (Issortal *et al.,* 1991). In *Rhizobia meliloti* ACP is essential in the induction of nitrogen fixing nodules, via the synthesis of acylated oligosaccharides (Geiger et al., 1994; Spaink *et al.,* 1991).

1.4.3 Structure of ACP

ACP has been purified and sequenced from many sources including *E. coli* (Vanaman *et al.*, 1968), barley leaf (Hoj and Svendson, 1983), spinach leaf (Kuo and Ohlrogge, 1984), castor seed, soybean (Ohlrogge and Kuo, 1985), *B. napus* seed (Slabas *et al.*, 1987), rabbit (McCarthy *et al.,* 1983) and goose (Poulose *et al.,* 1984). ACP has not yet been purified from leaf and seed in the same species. All ACPs analysed so far are characterised by their small size (81- 117 amino acid residues), and amino acid composition, which is high in

acidic residues and low in hydrophobic and aromatic residues. The *E. coli* ACP has been extensively studied, it is 68 amino acids long, with an isoelectric point of 4.1 and represents one of the most abundant proteins in *E. coli* (Fall, 1979). However, it is difficult to observe on SDS- gels due to its acidic nature and low binding capacity for coomassie blue dye (Cronan and Rock, 1987). The three dimensional structure of ACP from *E. coli* has been resolved using NMR techniques. It is strikingly asymmetric and contains four alpha helical regions connected by beta turns and random coils (Holak *et al.*, 1988; Kim and Prestegard, 1989; 1990).

A comparison of the amino acid composition and sequence data of ACP between different species illustrates extensive homology, especially in the area corresponding to the prosthetic group attachment site. Such sequence comparisons reveal that ACP is highly conserved between plant species, for example spinach and barley ACPs share 70% homology at the amino acid level. It is also apparent that plant ACPs are more closely related to bacterial ACPs than those isolated from animals. For example at the amino acid level spinach and *E. coli* ACPs share 40% homology as compared to 25% homology between spinach and rabbit ACP. Such a high degree of conservation is probably due to the fact that ACP is cofactor which interacts with at least 10 enzymes, thus any alteration in structure would not be easily accommodated (Ohlrogge, 1987).

1.4.4 Distribution

Plant ACP is predominantly located in the plastid (Ohlrogge *et al.*, 1979; Ohlrogge and Kuo, 1985) but following its discovery in the mitochondria of *Neurosporra crassa* (Brody *et al.*, 1988) was also detected in plant mitochondria (Chuman and Brody, 1989). In bovine heart muscle ACP is found as a component of NADH:ubiquitone oxidoreductase complex (I) (Runswick *et al.*, 1991). Fatty acid synthesis has never been demonstrated in the mitochondria, therefore its exact function is unknown. Mitochondrial acyltransferases suggests that this organelle be partially autonomous with respect to lipid synthesis (Frentzen *et al.*, 1990), however ACP can participate as an acyl carrier in reactions other than fatty acid synthesis, so the

mitochondrial form may be involved in secondary acylation reactions.

1.4.5 ACP isoforms

The initial sequencing of spinach ACP revealed heterogeneity in amino acid composition of some peptide fragments. This was taken as evidence to suggest the existence of two forms of ACP (Matsumura and Stumpf, 1968). Initial evidence for differential gene expression controlling fatty acid biosynthesis came from ACP in spinach (Kuo and Ohlrogge, 1984) and barley (Hoj and Svendson, 1984). In both species there are two isoforms of ACP in the leaf and a single form in the seed. Such sequence heterogeneity has been found in all plant ACPs subsequently examined. The analysis of the pattern of expression of ACP isoforms in many plant species using immunoblotting techniques revealed a common pattern of organ specific expression (Schmid and Ohlrogge, 1990). There is one form that is expressed in seeds and root and usually two or more ACPs expressed in leaf.

Redundant oligonucleotides were designed against the highly conserved region around the ACP prosthetic group attachment site. These were used as probes to clone ACP cDNAs from spinach leaf (Scherer and Knauf, 1987) and root (Schmid and Ohlrogge, 1990), barley leaf (Hansen, 1987; Hansen and Kauppinen, 1991), *Brassica campestris* seed (Rose *et al.,* 1987) and *Brassica napus* seeds (Safford *et al.,* 1988). Sequence heterogeneity was also found at the nucleotide level, confirming the existence of a multigene family.

1.4.6 Putative roles for ACP isoforms

The presence of only one ACP in unicellular algae and cyanobacteria infers a role in tissue specificity for ACP isoforms in higher plants (Battey and Ohlrogge, 1990). This would mean that some ACP isoforms are constitutively expressed to serve ɔhousekeeping functions and some are regulated in an organ/ tissue specific fashion (Schmid and Ohlrogge, 1990). In support of this hypothesis several *B. napus* isoforms that are preferentially expressed in developing seeds have been characterised (Safford *et al.*, 1988). However in

spinach, there are two ACP isoforms, ACP I is expressed only in leaves and ACP II in leaves, roots and seeds, which is contradictory. It would have been predicted that seeds would have two forms of ACP, a core FAS component and one involved in triacylglyceride biosynthesis and that leaf would only have one. Further experiments using ACP as substrates for acyltransferases and acylthioesters have drawn no conclusions. It was found that oleoyl- ACP I was the preferred substrate for oleoyl thioesterase, which results in oleate being fed into triacylglyceride synthesis. Oleoyl ACP- II was shown to be more reactive with glycerol-3-P- acyl transferase which directs oleate along lipid synthesis within the plastid. These results are inconsistent with the fact that ACP II is more abundant in the seeds, where triacylglyceride synthesis should be more active due to the lipid storage role of this organ (Guerra *et al.*, 1986). The true physiological role of the different isoforms therefore still remains to be elucidated.

1.4.7 ACP in *Brassica napus*

1.4.7.1 Pattern of induction and expression in the seed

Safford and colleagues (1988) determined the activity of ACP and correlated this with lipid deposition in developing embryos from *B. napus*. A correlation was found, with ACP activity preceeding lipid deposition (which is associated with fatty acid biosynthesis). This pattern of induction is consistent with ACP having a central role the regulation tissue specific and temporal control of lipid deposition.

1.4.7.2 cDNA cloning

There is only one molecular weight form of ACP in *B.napus* embryos, but cDNA cloning revealed a more complex situation. Mixed oligonucleotide probes were designed against the highly conserved sequence surrounding the prosthetic group attachment site. These were used to screen a mid-development embryo cDNA library (Safford *et al.*, 1988). From ten cDNAs, six unique clones were isolated, that confirmed ACP is encoded by a multigene family. Five mature protein sequences were encoded by the different cDNAs,

which confirmed heterogeneity observed in N- terminal sequencing of protein isolated from rape seed embryo (Slabas *et al.*, 1987).

The clones were divided into two subclasses, on the basis of DNA sequence differences. These differences, which also occur at the amino acid level, probably reflect the fact that *B. napus* is amphidiploid and originated from a cross of *Brassica campestris* and *Brassica oleracea*. Analaysis of sequence data demonstrated that ACP from *B. napus* is synthesised as a 134 amino acid cytoplasmic precursor and contains a 51 amino acid N- terminal extension. This transit sequence directs targeting to the plastid and is processed to give the mature protein of 83 amino acids (MW 9 200). The amino acid composition of rapeseed embryo ACP is typically high in acidic residues (24% ASP/ GLU) and extensive homology with ACPs from divergent species is apparent; the percentage of identical amino acid sequence between rapeseed embryo ACP and *E. coli*, barley leaf and spinach leaf ACPs is 31, 61 and 62, respectively (Safford *et al.*, 1988).

The expression of the ACP cDNAs isolated from embryos were analysed by Northern hybridisations. When the insert from a full length cDNA clone was used as a probe, it did not hybridise to leaf mRNA, even at low stringency (Safford *et al.*, 1988). This was unexpected, due to the high degree of sequence homology between leaf and seed ACPs. It was concluded that seed expressed ACP isoforms were encoded by a multigene family that was not expressed in leaf tissue. This result is coincident with the fact that during *B. napus* seed development, the level of ACP significantly rises prior to the start of storage lipid synthesis (Safford *et al.*, 1988). This could suggest that a specific subset of ACP genes are activated, to fulfil the role of lipid deposition.

1.4.7.3 Genomic cloning

The first genomic data for a plant FAS protein was that for an *Arabidopsis thaliana* ACP gene, AD4 (Post- Beittenmiller *et al.*, 1989a). Subsequently two seed- expressed *B. napus* genes (de Silva *et al.*, 1990), a seed specific *B. campestris* gene (Rose *et al.*, 1987; Scherer *et al.*, 1992) and another two linked genes from *A. thaliana* (Lamppa and Jacks, 1991) were isolated.

The *B. napus* genomic clones ACP05 and ACP09 were isolated using a probe generated from an ACP cDNA clone (Safford et al., 1988). Both ACP05 and ACP09 are seed expressed cDNAs and are very closely related. The clones share 94% homology within their coding sequences and 96% and 94% (respectively) homology to a *B. campestris* ACP cDNA sequence. This level of homology suggests that the *B. campestris* seed ACP gene is the progenitor of these two clones (de Silva *et al.*, 1990). The general organisation of the genes is the same, for example there are three introns; The first is in the transit peptide, the second at the transit peptide cleavage point and the third at the centre of the highly conserved prosthetic group attachment point. The *A. thaliana* and *B. campestris* clones also have introns in analogous locations (Lamppa and Jacks, 1991; Post-Beittenmiller *et al.*, 1989a; Scherer *et al.*, 1992).

ACP05 was subsequently studied in greater detail by de Silva and colleagues (1990) who used transgenic studies to investigate its tissue specific expression pattern. A construct was made in which a 1.4kb restriction, that contained the 5 upstream region of the gene plus 50bp of 5 non-coding was transcriptionally fused to the β– glucuronidase (GUS) reporter gene. The chimaeric gene was transformed into tobacco and expression monitored in developing seeds and in leaves. GUS activity was found to increase through seed development and reached a maximum that was coincident with the most active phase of storage lipid synthesis (on average 100 fold higher than in leaf). It was concluded that the 1.4kb 5 flanking region of ACP05 contained strong promoter elements that directed both spatial and temporal expression (de Silva *et al.*, 1992).

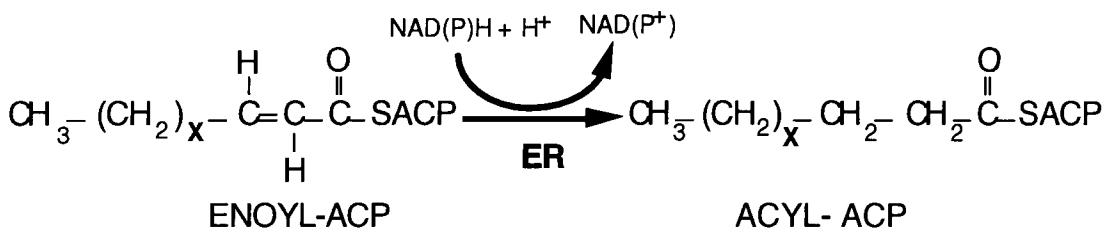
B. napus contains approximately 35 seed expressed ACP genes per haploid genome (deSilva *et al.*, 1990). This large number may reflect different isoforms being compartmentalized, having different physiological roles, or tissue- specific FAS functions. It was envisaged that a more detailed examination of the seed- expressed ACP05 gene promoter would provide an insight into this aspect of ACP regulation.

1.5 Enoyl ACP Reductase

1.5.1 Partial reaction catalysed

Enoyl ACP- Reductase (ER) is an integral component of the plant type II fatty acid synthetase complex, catalysing the second reductive step of each cycle. ER catalytically reduces the *trans*- 2, 3 double bond of a 3- ketoacyl- ACP intermediate, to form a saturated acyl- ACP (figure 1.3), which in turn can serve as the substrate for the next condensation reaction (Slabas and Fawcett, 1992).

Figure 1.3: Reduction of ketoacyl intermedates by ER



1.5.2 Isoforms and expression of ER

ER is a well characterised member of the FAS complex. In plants, two enzyme activities have been detected (Slabas *et al.*, 1984). Discrimination between the two forms may be achieved using several criteria. The forms have different substrate specificities (Shimakata and Stumpf, 1982a). One form is NADH-dependent and the other prefers NADPH (Weeks and Wakil, 1968; Harwood, 1988). The two forms also have distinct patterns of expression. Both are detected in safflower, castor bean and *B. napus* seeds (Slabas *et al.*, 1984), but only the NADH dependent form is detected in spinach leaf and avocado

mesocarp (Shimakata and Stumpf, 1982a; Caughey and Keckwick, 1982).

NADH dependent ER was purified to near homogeneity from *B. napus* embryos and the molecular mass shown to be approximately 35kDa (Slabas *et al.*, 1986). The enzyme is homo- tetrameric in structure (Slabas *et al.*, 1986; 1990) and was over expressed in *E.coli* (Kater *et al.*, 1991) and crystallised (Rafferty *et al.*, 1994). The crystals were analysed by X- ray diffraction and the tertiary structure was resolved to 1.9A (Rafferty *et al.*, 1995). Southern analysis of ER in *B. napus* demonstrated the presence of four genes, two inherited from each of it parents, *B. oleracae* and *B. campestris* (Kater *et al.*, 1994). The expression of ER mRNA and protein isoforms was analysed by hybridisation studies and two dimensional Western blots (Fawcett *et al.*, 1994). These experiments demonstrated that ER was regulated temporally during seed development. The increase in message preceded the increase in protein levels, which in turn preceded the deposition of lipid (which is associated with fatty acid deposition), as expected. Four isoforms were detected in both leaf and seed tissue, with expression in the seed at much higher levels than in the leaf. The pattern of expression was similar within the seed and leaf, there being the same two major isoforms and two minor isoforms (Fawcett *et al.*, 1994).

1.5.3 Cloning ER

NADH- dependent ER was cloned from *B. napus* cv Rafael embryos, by immunoscreening a λgt11 expression library using antisera raised against purified protein (Kater *et al.*, 1991). The identity of the cDNA pEAR7 was confirmed by *in vivo* expression of the clone in *E.coli*, the vast overproduction of enoyl- ACP reductase expression resulting in extremely high levels of enzyme activity (Kater *et al.*, 1994). The cDNA was subsequently used to isolate a full length genomic clone from *Arabidopsis* (Kater *et al.*, 1994).

A leaf cDNA library from *B. napus* cv Jet Neuf leaf mRNA was screened with the cDNA pEAR7 (A. Fowler, unpublished results). Nucleotide sequencing confirmed that all the positive clones were identical and represented a single

isoform, pERL8.The full length was 1.3kb and a 73 amino acid leader peptide was present. There was no evidence to prove whether all four ER genes present in *B. napus* were expressed and correspoded to the four detected isoforms. An objective of this work was therefore to re-screen a *B.napus* cv jet Neuf library for addtional clones with the aim of relating any information derived from their study, back to the differential expression of ER.

1.6 Regulation of gene expression

Initially most data on gene regulation was derived from studies on the organisation and expression of bacterial and mammalian systems. Techniques developed during the study of these genes were successfully applied to plants and allowed the analysis of many unique plant systems (Benfey and Chua, 1989; Coen and Meyerowitz, 1991; Consonni *et al.*, 1993; Dooner *et al.*, 1991; Flurh *et al.*, 1986; Gilmartin *et al.*, 1990; Hake, 1992; Huworth and Dugham, 1993; Katagiri *et al.*, 1992; Katagiri and Chua, 1992; Kuhlemeier, 1992; Purugganan *et al.*, 1995; Schindler and Cashmore, 1990; Tobin and Silverthorne, 1985). Plant genes are differentially regulated in response to a complex set of environmental and developmental stimuli. Specific gene products accumulate in specialised cell types, at certain stages of development or following a distinct environmental cue. Several different inducers can activate a single gene, for example uv light and fungal elicitors both induce phenylpropanoid genes (Kaulin *et al.*, 1986; Bell *et al.*, 1986) and the type I ACC gene (Shorrosh *et al.*, 1994). In contrast, a single stimulus may differentially regulate related genes. For example, abscisic acid induces the synthesis of the alpha subunit of β- conglycin specifically, the genes for the other subunits are not affected (Bray and Beachy, 1985). A major challenge is therefore to elucidate the molecular mechanisms underlying gene regulation, that is to determine exactly how gene expression is regulated in a temporal and spatial way during the life cycle of a plant.

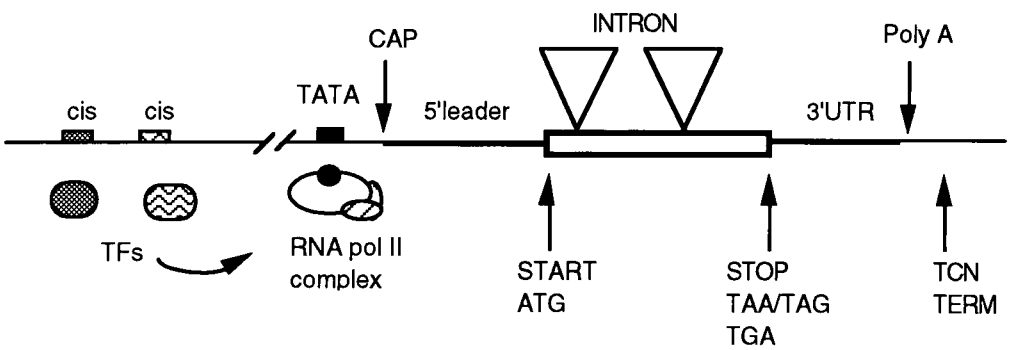
The regulation of fatty acid biosynthesis is of interest as it has dual functions, both a housekeeping role and a temporally regulated organ specific role (Slabas and Fawcett, 1992). The synthesis of membranes is essential in all cells, therefore the FAS genes must be expressed constitutively as a

housekeeping function. However during seed development, fatty acids in the form of triglycerides are deposited as a major carbon store. Deposition of storage lipid is also temporally regulated and requires expression of the FAS system at significantly higher levels than in leaf or root tissue. As FAS proteins must function in both housekeeping and oil production their study offers a system to examine how plants regulate genes that are needed for two divergent functions.

1.6.1 Control points at which regulatory mechanisms act

The general structure of eukaryotic genes is the same (see figure 1.4). For example, features such as introns are ubiquitous in protein coding genes (transcribed by RNA polymerase II). In principle, similar parallels can be drawn between the mechanisms that regulate gene expression.

Figure 1.4: Schematic view of an eukaryotic nuclear gene



The beginning and end of the DNA actually transcribed into RNA are indicated by CAP and TCN TERM. These encompass the 5 leader region, which is often important in product compartmentalisation and the 3 untranslated region (3 UTR), which may be involved in differential mRNA stability. Directly upstream is the TATA box, which is the binding site for RNA pol II transcription complex. The more distal upstream cis sequences can bind a variety of transcription factors (TFs) that may be regulatory. The coding region is defined by the translational initiation and stop codons and is usually split by introns, which are present in the primary RNA transcript but are subsequently spliced out, so are absent from mRNA.

Transcription is a major level at which expression is regulated and is discussed in the following section. Regulation is also possible and has been observed at the post-transcriptional level (Gallie, 1993). Control is exerted over the correct processing and transportation of primary nuclear RNA transcripts (Sanfacon, 1992). Differential stability and turnover of mRNA maintain steady state levels of message in the cytoplasm (Hagan et al., 1995). Following translation of mRNA a variety of post- translational modifications to the resultant polypeptides can be important. All of the stages outlined provide points at which control of gene expression could be exerted (Decker and Parker, 1994; Nanbu *et al.*, 1994; Raghow, 1987; Xu and Cohen, 1995). Thus a given protein is produced in a cell- type specific or inducible manner.

Transcriptional regulation is primarily under the control of the gene promoter, which contains regulatory *cis* acting sequences (detailed in section 1.6.2). Regulation of transcription is augmented by a number of other factors which include DNA methylation (Ingelbrecht *et al.*, 1994), the chromatin structure of the DNA (Prioleau *et al.*, 1994) and proteins that are able to relax supercoiled DNA (Merino *et al.*, 1993).

Core histones are basically octamers that contain 2 molecules each of histones H2A, H2B, H3 and H4. Two superhelical turns (165bp) of DNA are wrapped around this core. One molecule of H1 subsequently binds the exterior of each core and its carboxy terminus interacts with the DNA in the linker region (0- 80bp) (Chaboute *et al.*,1993). This polynucleosome filament is further compacted *in vivo*, by coiling to form a solenoidal structure with about six nucleosomes per turn. The resultant fibre is about 30nm in diameter. Higher levels of compaction are observed, but less well understood (Smith *et al.*, 1995).

The role of the nucleosome as a general repressor of transcription is well established (Felsenfeld, 1992). For example tissue specific genes are transcribed when transfected as purified DNA into non- expressing cell lines or into cell extracts (Weintraub, 1985). In yeast it has been demonstrated that the alteration of the stoichiometry of core histones alters transcription patterns. When histone H4 is depleted the activation (or derepression) of specific

genes in the absence of induction is observed (Clark- Adams *et al.,* 1988; Han *et al.,* 1988). Mutational analyses of histones points to the linker histone H1 being important in the differential repression of transcription. Histone H1 in turn is regulated by the core histone amino terminal (Zlatanova and van Holde, 1992; Juan *et al.,* 1994).

The way in which histones participate in the activation of genes is not clear. Activation must involve a sequential unfolding of chromatin structure if transcription is to occur (Laybourn and Kadonga, 1991; reviewed by Felsenfeld, 1992). The initial step involves the removal of molecules that maintain genes in a silent state, a process known as anti- or de- repression (Croston *et al.,* 1991). The second step represents true activation, in which levels of expression of particular genes are increased well above basal levels. Evidence points to competition during the initiation of transcription as being an important point at which histones act. Competition between chromatin and the assembly of transcription complexes has been shown to regulate gene expression in several systems (Prioleau *et al.,* 1994). However, the presence of bound core histone octamers however is not necessarily detrimental to transcription. It has been shown *in vitro* at least that RNA polymerase is able to transcribe through histones associated with the gene body (Lorch *et al.,* 1987).

Proteins that are able to relax supercoiled DNA have been implicated to have a role in the regulation of transcription (Merino *et al.,* 1993). Topoisomerases are often associated with transcriptionally active genes and have been shown to be involved in both repression and activation (Kretzschmar *et al.,* 1993; Merino *et al.,* 1993).

1.6.2 The structure and organisation of promoters and enhancers

The term promoter generally defines the group of control elements that are clustered around the initiation site for RNA polymerase II, whereas enhancers may be located at a more distal position, but both are often structurally and functionally analogous. An early insight into how promoters and enhancers were organised was provided by the study of the early transcription unit from

the DNA tumour virus SV40 (Weiher *et al.*, 1983; Gruss and Scoler, 1984). Enhancers were detected as cis-acting genetic elements that increased transcription from a distal position, in an orientation independent manner and with heterologous promoters. Subsequently it was demonstrated that enhancers are organised much like promoters, with multiple individual DNA motifs each of which bind one or more proteins (McKinght and Tjian, 1986). Such motifs can function at various distances from the TATA box and are have also been found in leader sequences (Elliot *et al.*, 1989; Hetherington and Quatrano, 1992, Marcotte *et al.*, 1989) downstream of the translational start site (Thompson *et al.*, 1988) and in introns (Luehrsen and Walbot, 1991). It is thought that motifs which occur in "unusual" positions may not function entirely at the transcriptional level. There is evidence that suggests roles in mRNA stability or the stability of the transcript during translation (Hagan *et al.*, 1995).

1.6.3 Cis- acting sequences

In terms of transcriptional regulation plant promoters are large, complex arrangements of short DNA sequence elements (cis sequences, boxes or motifs), as illustrated in figure 1.4. Mutation of promoters has defined two classes of DNA sequences, both of which represent binding sites for protein transcription factors: core promoter elements and regulatory motifs.

1.6.3.1 Core promoter elements

The TATA box, or functionally related sequence represents this class of cis acting sequence and is essential for the initiation of transcription by RNA polymerase II (RNA pol II). It is recognised by a sequence specific TATA binding protein (a component of the basal transcription factor TFIID complex). which determines where transcription will start from (in general approximately 30bp downstream from where it binds) (Buratowski *et al.*, 1994). In the case of TATA- less promoters sequences that flank the site of transcriptional initiation are important for the assembly of a preinitiation transcriptional complex (Aso *et al.*, 1994; Ellis *et al.*, 1993; Pugh and Tjian, 1991). When TATA boxes are transcriptionally fused to reporter genes, low transcript levels are usually

observed. However, there is evidence that the TATA box (or surrounding sequences) is involved in the light regulated expression of pea *rbcS* genes (Morelli *et al.*, 1985) which suggests a role for core promoter elements in the regulated expression of some genes.

1.6.3.2 Regulatory motifs

Regulatory DNA motifs represent binding sites for proteins that interact with the RNA pol complex. Interactions between individual elements are complex and motifs may function independently, cooperatively or competitively (Donald and Cashmore, 1990). The spacing between motifs is often not critical and promoter function may be maintained when individual elements are inverted or moved relative to one another (Benfey and Chua, 1990). These DNA elements are often regulatory, they enhance or repress transcription under specific cellular or environmental conditions. A classic example is the heat shock element, which only increases transcription of a reporter gene at high temperatures (soybean heat shock gene, Baumann *et al.,* 1987; maize heat shock gene, Rochester *et al.*, 1986). The analysis of more complex promoters from higher plants has discovered cis motifs that are involved in the regulation of expression by many factors. For example: light (pea *cab* gene, Simpson *et al.,* 1986; pea *rbc*S gene, Flurh and Chua, 1986; Gilmartin *et al.,* 1990), uv light (chalcone synthase gene from *A. majus*, Kaulin *et al.,* 1986; Type I ACC from *Alfalfa,* Sharrosh *et al.*, 1994), anaerobic stress (maize *Adh* 1 gene, Ellis, 1987), wounding (Ryan, 1988) and developmental queues such as hormones (Lincoln and Fischer, 1988), embryo specific development (soybean storage gene protein, Chen *et al.,* 1988; wheat glutenin gene, Thomas and Flavell, 1990; pea lectin gene, dePater *et al.,* 1993; reviewed in Bevan *et al.*, 1993) and nodulation (reviewed in Verma and Delauney, 1988).

1.6.3.3 "Regulated" and "Constitutive" promoters

The most comprehensive studies of promoters have included those regulated by light and those expressed in the seed (Flurh *et al*, 1986). These have revealed the complexity of cis-acting elements through which regulated

expression is mediated. Dissection of several light regulated promoters has identified conserved motifs such as the GT box and the GATA motif (Gilmartin *et al.*, 1990). The conservation of such motifs between light regulated promoters from different genes and even species, is taken as evidence for a partially common pathway of regulation. The analysis of promoters that are developmentally regulated has revealed that both negative and positive domains are required for the correct spatial and temporal pattern of expression (Bustos *et al.*, 1991).

Regulated promoters are often contrasted with so called constitutive promoters. An interesting example of an apparent strongly constitutive promoter is the Cauliflower Mosaic Virus 35S promoter (CaMV 35S). Initial analysis involved transcriptionally fusing 1Kbp of promoter to a reporter gene which resulted in high levels of constitutive expression in transformed plants (Odell *et al.*, 1985; Ow *et al.*, 1987). However, further analysis of defined promoter deletions revealed several things. Only 350bp upstream of the TATA box was required for constitutive expression at high levels, the remaining promoter sequence could be deleted, inverted or even replaced without a reduction in the level of expression (Benfey and Chua, 1990). However, if the promoter was deleted to within 90bp of the TATA box, expression became tissue specific (Benfey and Chua, 1990). This demonstrates the point that a "constitutive" promoter is as complex as a "regulated" promoter. All promoters consist of an array of cis- elements, the sum of which results in a specific pattern of expression.

1.6.4 Trans acting factors

Transcription of protein coding genes in eukaryotes is complex and is carried out by a multi enzyme complex that includes RNA pol II and a number of accessory transcription factors. These accessory factors fall into two classes. The first are the general or basal transcription factors, which act through core promoters elements (TATA box and related motifs). General transcription factors are required for basal levels of transcriptional initiation at all RNA pol II promoters. The second class of factors are sequence specific DNA binding proteins that recognise the regulatory motifs, integrate the encoded regulatory

information and subsequently interact with the RNA pol II complex. These factors determine transcription rates and result in gene specific expression.

1.6.4.1 RNA polymerase and associated basal transcription factors

RNA pol II transcribes nuclear protein encoding genes and has been isolated from a number of plant species (Guilfoyle *et al.,* 1990). A similar general structure is seen, with two large and eight small subunits. The largest subunit contains 35- 40 tandem repeats of the heptapeptide PTSPSYS at the carboxy terminus. Phosphorylation may regulate the activity of this subunit in terms of its ability to interact with accessory proteins and chromatin (Guilfoyle *et al.*, 1990). Mutation of these repeats suggest they directly contact transcription factors and thus may, in part determine the response to enhancer signals (Kim *et al.*, 1994; Scafe *et al.,* 1990; Seipel *et al.*, 1994).

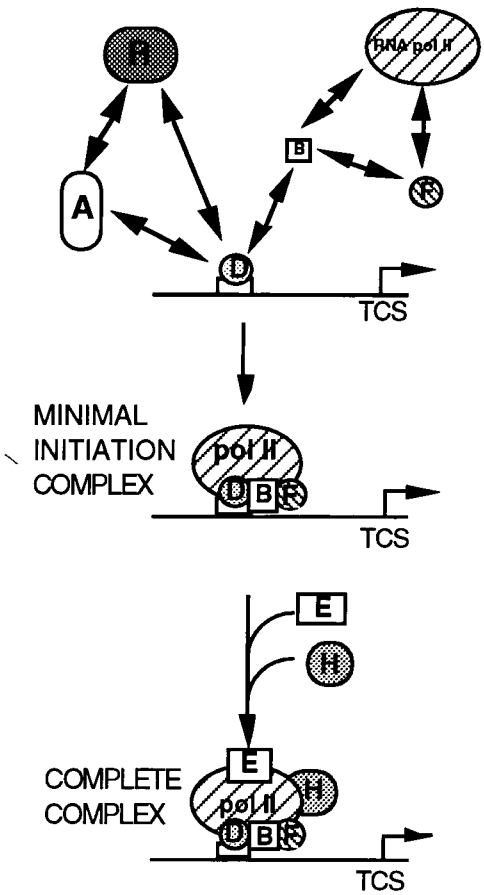
The general transcription factors isolated from HeLa cells that are necessary to form a stable preinitiation complex with RNA pol II are TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. Of these only TFIIA and TFIID have been characterised in plants. TFIIA is homologous to the mammalian equivalent (Burke *et al.,* 1990) and TFIID shows some homology to bacterial sigma factor (Gasch *et al.,* 1990). The TATA binding protein is highly conserved between species (Haas *et al.*, 1994; Heard *et al.*, 1993; Hernandez, 1993) and has a saddle like DNA binding domain, which sits in the minor groove of DNA at the TATA box. It has been demonstrated that the outer surface is involved in protein-protein interactions (Klug, 1993)

For RNA pol II to transcribe a gene, up to 20 accessory factors may be required (George *et al.*, 1995). A variety of reconstitution experiments (Buratowski *et al.*, 1989; 1994; Conaway and Conaway, 1993; Zawel and Reinberg, 1993; 1995) were used to reconstruct the order of assembly of a pre- initiation complex at the promoter. The resultant model is outlined in figure 1.5, (adapted from Buratowski, 1994).

The first step that nucleates assembly of the transcription complex consists of TFIID binding the TATA box. TFIIA can then bind to the TFIID- promoter

Figure 1.5: Schematic view of the initiation of transcription

Transcription factors are represented by their letter designations (for example TFIID=D). Double headed arrows represent protein- protein interactions and bent arrows indicate the site of initiation into complex assembly. Complex initiation is nucleated by TFIID binding to the TATA box (open square), which is usually located approximately 30bp 5' to the transcription initiation site (TCS). The TFIID- promoter complex may be stabilised by TFIIA (which may block repressor (R) activity). The assembly of a minimal initiation complex is augmented by the interaction of TFIIE and TFIIH, which complete the preinitiation complex.



complex (Yokomori *et al.*, 1994). This interaction is not necessary for transcription *in vitro* with purified factors, but is thought to function in maintaining the complex under physiological conditions. There is evidence to suggest this might be as a result of blocking the action of transcriptional repressors, that displace TFIID (Auble and Hahn, 1993). The next factor to join the complex is TFIIB, which binds the TFIID- promoter complex and recruits RNA pol II, acting as a "bridging protein". The order of these interactions is not known. TFIIF greatly stimulates RNA pol II entry into the complex, possible via an interaction with TFIIB (Tyree *et al.*, 1993, Ha *et al.*, 1993) and also affects transcriptional elongation (Price *et al.*, 1989). The minimal complex necessary to recruit and accurately position RNA pol II consists of TFIID, TFIIB and TFIIF. The complete complex has a further two factors. TFIIE is necessary to recruit TFIIH (Flores *et al.*, 1991; Maxon *et al.*, 1994), which is probably the factor that mediates activation of the complex and powers elongation by ATP hydrolysis. There is evidence to suggest that TFIIE requires zinc for full activity (Maxon and Tjian, 1994). However the initiation of transcription is possible without TFIIE and TFIIH *in vitro* so it may be that TFIIE and TFIIH function to convert initiation complexes in to elongation complexes (Flores *et al.*, 1991).

1.6.4.2 Regulatory transcription factors

The spectrum of transcription factors present in a cell type and their own levels of expression therein, determines which genes, or sets of genes are transcribed. The ratio between transcription factors is often crucial, especially if competing positive and negative factors are involved (Brindle *et al.*, 1990; Grierson *et al.*, 1994). Additional factors other than those that directly bind DNA may be involved such as protein cofactors, (Sakurai *et al.*, 1994; Martin, 1991) and accessory molecules such as metal ions (Berg, 1990).

The most highly studied transcription factors are multifunctional and highly modular proteins. It is commonly found that different functions, such as DNA binding, dimerisation and transcriptional activation are contained on discrete domains (Keegan *et al.*, 1986). The function of individual domains is often preserved when swapped among various factors or fused to heterologous protein sequences (Ptashne, 1992). Transcription factors with novel

sequence specificities have been designed by combining known DNA binding domains. For example, a fusion protein that contained a binding domain constructed from zinc fingers and a homeodomain was found to bind optimally to sequences that contained adjacent homeodomain (TAATTA) and Zinc finger (NGGGNG) binding sites. An activator domain was subsequently fused onto this binding domain and the resultant fusion protein regulated promoter activity in a sequence specific manner (Pomerantz *et al.*, 1995).

1.6.4.3 Characterised transcription factor protein motifs

The salient features of some of the best characterised motifs (Frampton *et al.*, 1989; Grussem, 1990) found in plant DNA binding proteins are discussed below.

The basic helix- loop- helix: bHLH. This motif was first identified in animal systems as a conserved region shared by the *myc* proto- oncogene and the immunoglobulin enhancer binding factor (Murre *et al.*, 1989). The maize regulatory protein *Lc* was the first plant protein shown to have this domain (Ludwig and Wessler, 1990). The bHLH domain is multifunctional, the HLH region consists of two amphipathic helixes connected by a loop. This serves as a dimerisation interface for other bHLH proteins. The basic region lies immediately upstream of the HLH domain and is the DNA binding domain (reviewed in Lusher and Eisenman, 1990)

The helix- turn- helix: HTH. The HTH motif is typified in the yeast MAT $\alpha 2$ protein (Sauer *et al.*, 1988) and bears resemblance to the homeobox domain (Gehring *et al.*, 1994), which is a highly conserved region in homeotic genes (so called as the identity of an organ is altered in homeotic mutants). This homology led to the suggestion that HTH proteins could be DNA binding factors (Laughon and Scott, 1984). Plant homeotic mutants like *deficiens* in *Antirrhinum* (Schwazsommer *et al.*, 1990) and *agamous* in *Arabidopsis,* (Yanofski *et al.*, 1990) were cloned and do have considerable sequence homology to the human and yeast DNA binding domains of transcription factors. In addition the *def* and *agamous* proteins seem to be very precise regulators of steps in the pathway of floral development, although their target

genes are unknown (Rounsley *et al.*, 1995).

The zinc finger: The zinc finger is probably the best known and most well characterised DNA binding motif (reviewed by Klug and Rhodes, 1993). Zinc (Zn) fingers were found originally as short, repeated motifs in the TFIIA factor from *Xenopus* oocytes (Brown *et al.*, 1985). Each repeat contained an invariant pair of cysteine and histidines, which coordinate with a zinc atom in the tertiary structure (Johnston, 1987). Zn fingers are ubiquitous (Takatsuji *et al.*, 1994) and are found in plant factors such as *En*-1 from maize and *Tgm*5 from soybean (Vodkin and Vodkin, 1989).

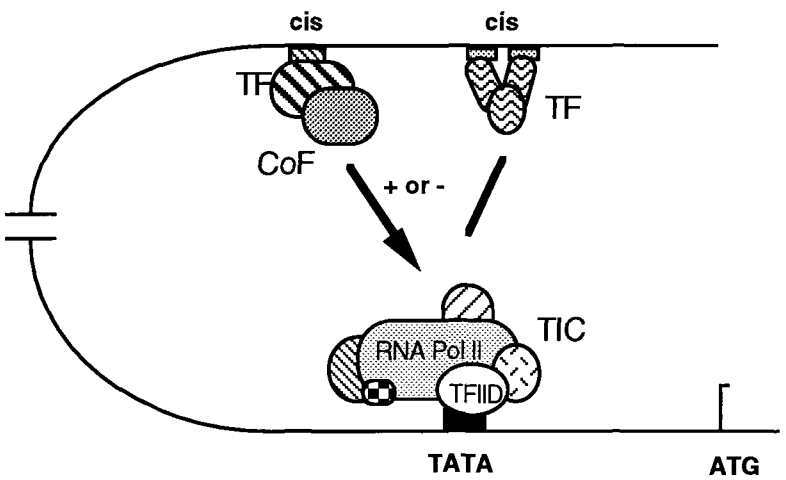
bZIP motif: This class of proteins is particularly well represented in putative plant transcription factors. The domain consists of a leucine zipper dimerisation motif and a basic DNA binding region (Hai *et al.*, 1989; Hurst, 1994). The leucine zipper has been well characterised in such proteins as *jun* and *fos* oncoproteins (Glover and Harrison, 1995) and the yeast GCN4 transcriptional activator (Struhl., 1989). Plant proteins that contain this domain include *opaque* 2 (Schmidt *et al.*, 1987), HBP- 1 (Tabata *et al.*, 1989), EmBP-1 (Guiltinan *et al.*, 1990), TGA1a/ TGA1b (Katigiri *et al.*, 1989) and OCSBF- 1 (Singh *et al.*, 1990). bZIP proteins are highly homologous over the basic DNA binding domain. Analysis of the cis-sequences used to isolate such proteins reveal a core palindromic consensus motif: CACGTG (Foster *et al.*, 1994). This means that bZIP factors are capable of binding to more than their cognate cis sequence (Armstrong *et al.*, 1992). This is exemplified by TAF-1 binding to its own cis motif in the ABA-regulated rice *rab*16 gene and also various G boxes from photoregulated genes (Oeda *et al.*, 1991)

1.6.5 DNA looping and potential targets for activators and repressors

The principal model for the way in which transcription factors bound to distal cis-acting DNA elements influence the rate of transcription involves the intervening DNA being looped out (Martin *et al.*, 1990). Transcription factors bound to distal elements are then brought into contact with the RNA pol II complex at the TATA box. The contact may then occur directly or via bridging molecules (Martin, 1991). The basics of this model are outlined in figure 1.6.

DNA looping has been described for several procaryotic systems including cooperative repressor binding over a distance, site specific recombination and DNA replication (Dunn, 1984; Hochschild and Ptashne, 1986; Griffith *et al.*, 1986). Several lines of evidence support the theory of DNA looping in eukaryotic systems. Roy and colleagues (1991) demonstrated the cooperative interaction between an initiation binding factor and the USF activator. The addition of an odd number of half helical turns between the SV40 early

Figure 1.6: DNA looping



Evidence suggests the way in which distal regulatory motifs exert their influence on the rate of transcription involves DNA looping. This would allow transcription factors (TF) bound to distal cis-sequences to be brought into close proximity with their targets- basal transcription factors in the transcriptional initiation complex (TIC), when the intervening DNA sequence is looped out, as shown above. Contact may be direct or via protein cofactors (CoF).

promoter upstream elements and the TATA box reduced the level of transcription (Takahashi, 1986). This implies cooperative binding between factors and thus looping out of the intervening DNA. Also the SV40 enhancer or cytomegalovirus (CMV) enhancer can stimulate transcription *in vitro* when linked to its promoter via a streptavidin- or avidin- biotin bridge (Muller *et al.*, 1989).

Several classes of effecter domains have been identified in transcription factors. Conserved activation domains include glutamine rich, proline rich and acidic motifs (Lieberman and Berk, 1994; Lin *et al.*, 1991; Ptashne, 1988; Tjian and Maniatis, 1994). The molecular mode of activation of one transcriptional activator, VP16 has been elucidated. It interacts directly with the basal transcription factor TFIIB. This contact disrupts intramolecular bonds between the C- and N- terminii of TFIIB which induces a conformational change and results in its recruitment into the preinitiation complex. The change in conformation also exposes binding sites for RNA pol II and TFIIF which are subsequently recruited into the preinitiation complex (Roberts and Green, 1994). Thus, most models suggest activators either stabilise and facilitate the formation of the preinitiation complex or modify the complex after assembly to increase its stability. Negative regulators of transcription have been shown to displace TFIID from the promoter or to block interactions between TFIID and other basal transcription factors, thus preventing active preinitiation complex formation (Auble and Hahn, 1993; Auble *et al.*, 1994). There is evidence which suggests that the general transcription factor TFIIA may "shield" TFIID from repressor action (Auble and Hahn, 1993).

1.7 Detection, purification and cloning of plant DNA binding proteins and (putative) transcription factors

1.7.1 *In vitro* detection methods

The initial characterisation of sequence specific DNA binding proteins used *in vitro* detection methods such as filter binding assays (Jones and Berg, 1966, Riggs *et al.*, 1968), electrophoretic mobility shift (EMSA) or gel retardation assays (Green *et al.*, 1988; Lane *et al.*, 1992) and footprinting

assays (Green *et al.*, 1988). Using these assays DNA binding is detected either through the separation of bound from unbound DNA or by the protection of bound, as opposed to unbound sequences.

1.7.1.1 Separation methods

Filter binding and gel retardation techniques are based on the separation of bound from unbound DNA. A labelled DNA fragment is mixed with protein under conditions that favour specific complex formation. The mixture is then either spotted onto a filter that will only retain complexed DNA following subsequent washing steps; or electrophoresised through a non- denaturing gel matrix. During a mobility shift assay binding of protein to a DNA fragment usually leads to a reduction in the electrophoretic mobility of the fragment and is viewed in comparison to free DNA. The specificity of an interaction is monitored by the addition of an excess of unlabelled DNA to the binding reaction. DNA with a sequence related to the cis- acting element will compete for binding (unrelated or mutated will not). The theory behind gel retardation/ EMSA assay is presented in figure 1.7.

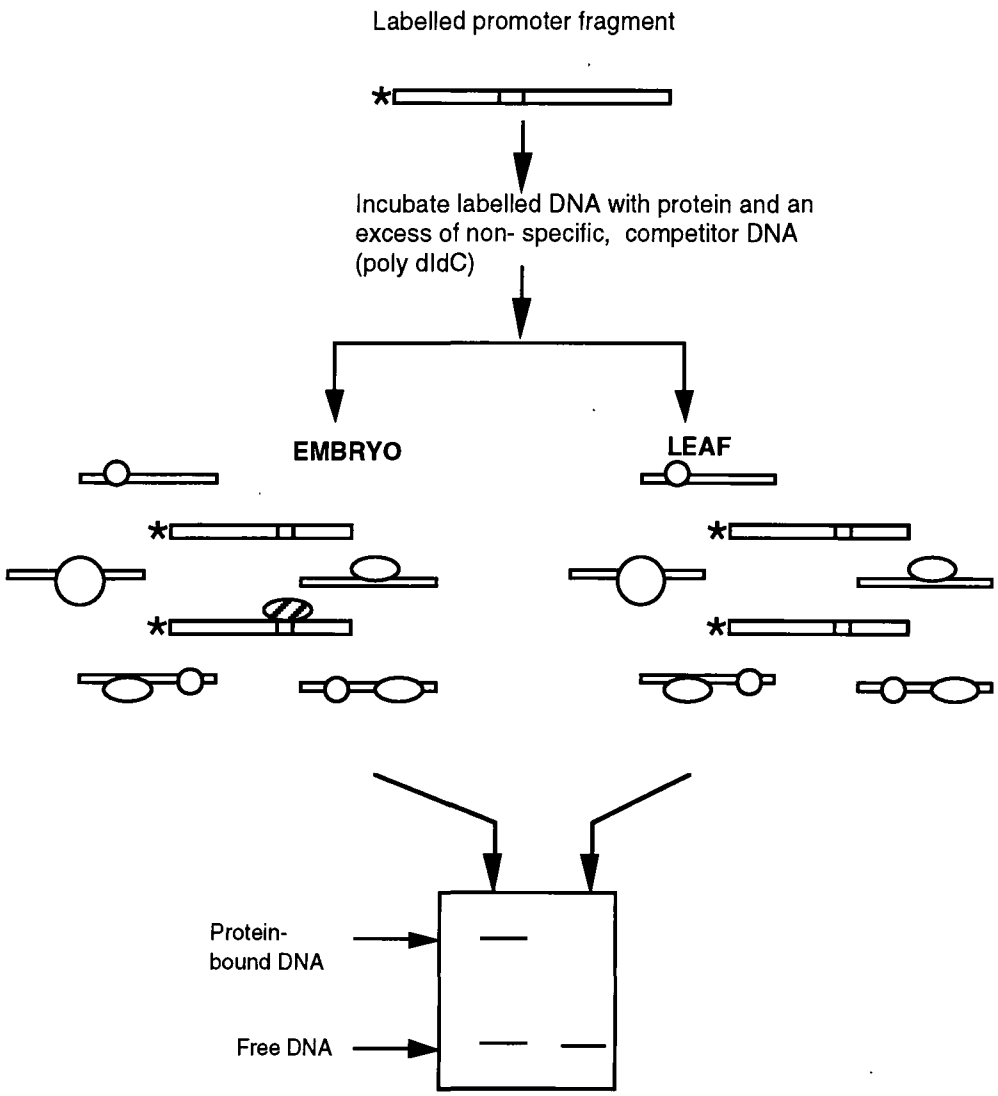
The use of gel retardation to analyse DNA- protein interactions has its roots in early work studying rRNA- protein interactions (Jones and Berg, 1966). Its widespread use dates from its development for studies on transcriptional regulation in bacteria (Fried and Crothers, 1981; Garner and Revzin, 1981). Separation methods for the detection of DNA:protein interactions are sensitive. Small amounts of material are required and low abundance interactions can be located. Additionally, the mobility shift assay can detect multiple protein binding sites along a DNA fragment. This assay has proved successful in elucidating the regulation of plant genes by many factors, such as light (Gilmartin *et al.*, 1990; Lam *et al.*, 1990; Lam and Chua, 1989), uv light (Kaulin *et al.*, 1986) and anaerobic stress (Ellis, 1987).

1.7.1.2 Protection methods

DNA foot printing techniques rely on the protection of DNA by bound protein. Reagents used to attack naked DNA include DNase I, Exonuclease III,

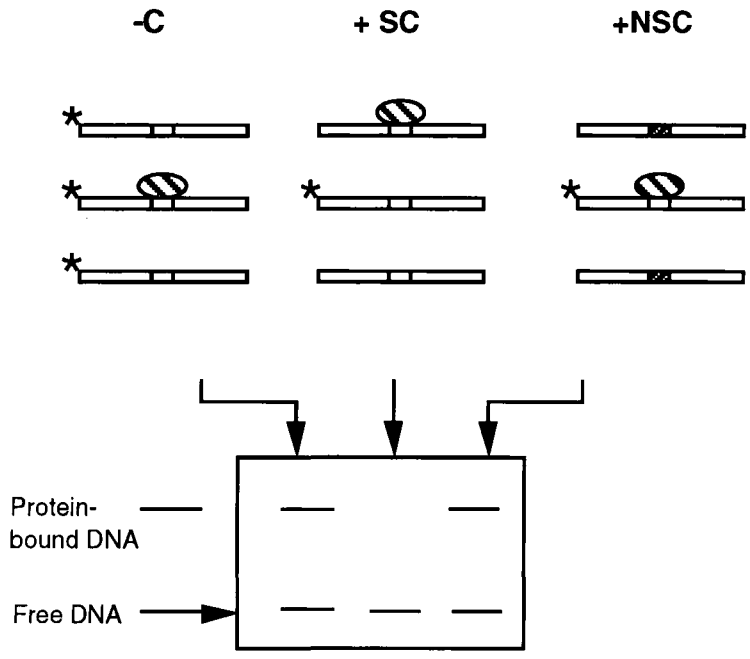
Figure 1.7: The principles of the gel retardation assay

Panel 1:



A labelled (indicated by star) DNA fragment is mixed with protein (represented by circles) under conditions that favour binding. Specific complex formation is ensured by including an excess of non- specific competitor such as poly (dIdC:dIdC). In the example above, an embryo specific factor (striped circle) binds to its cis motif (open box), forming a complex. During electrophresis, complex migration is retarded, relative to free probe. Subsequently a retarded band is seen on the autoradiograph. The second panel shows a competition assay, in which an excess of unlabelled Specific Competitor (SC) or NonSpecific Competitor (NSC) was added. Competitors are usually oligonucleotides, designed to contain putative binding motifs (open box) or altered versions of the binding sites (striped box) as control competitors. The excess of a specific competitor motif will compete for the factor of interest and the retarded band will disappear on the autoradiograph.

Panel 2: Competition



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dimethylsulphate (DMS) and methidium propyl-EDTA-Fe (MPE) (Galas and Schmitz, 1978; Green *et al.*, 1988; Neilson 1990). Footprinting can identify protein binding sites on DNA with high (single base pair) resolution

1.7.2 Cloning techniques

Both separation and protection methods have been used as assays to follow DNA binding proteins during biochemical purification (Briggs *et al.*, 1986; Chodosh *et al.*, 1986; Lee *et al.*, 1987). Pure protein is a pre- requisite for the

conventional methods of cDNA cloning, which utilises specific antiserum or oligonucleotide probes. In either case the minimal amount of purified protein required for probe preparation is 100pmol- 1μmol which represents a massive amount of starting material, due to the rarity of most transcription factors. An alternative cloning strategy that relies upon the use of radiolabelled binding sites as probes to screen expression libraries was developed (Singh *et al.*, 1988; 1990 and detailed in section 4.1). This method is commonly know as "SouthWestern" cloning and has proved particularly successful in isolating plant DNA binding proteins, many of which are structurally related in terms of their dimerization and DNA binding domains (described elsewhere). Several other techniques have also been used successfully to isolate DNA binding proteins and putative transcription factors (a number of which are listed in table 1.3). The flower specific homeotic gene *agamous* was isolated from *Arabidopsis* by T- DNA insertional mutation. The floral sex organs in the mutant are transformed to a "flower within a flower" morphology that is sterile. *Agamous* belongs to a large family of regulatory genes that possess a characteristic DNA binding domain known as the MADS- box (Pnueli *et al.*, 1991). Members of this family are homologous to transcription factors found in several animal and fungal species: DEF A (*Antirrhinum majus*), SRF (humans), MCM I and ARG80 (yeast). Phylogenetic analyses indicate that members of the plant MADS gene family are organised into several distinct groups: *Agamous*, APETAL3/ PISTILLATA and APETAL 1/ AGL9. These groups are thought to reflect the roles that these genes play in flower development (Purugganan *et al.*, 1995; Rounsley *et al.*, 1995). It is thought that the *agamous* gene encodes a transcription factor that regulates stamen and carpel development in the wild type flower (Yanofski *et al.*, 1990).

The homologous transcription factors *R* (Dellaporta *et al.*, 1988) and *delia* (Goodrich *et al.*, 1992) were both cloned by transposon tagging. They are involved in the regulation of expression of anthocyanin biosynthesis genes (pigmentation) in maize and *Antirrhinum*, respectively. *R* belongs to a small gene family, the other members, *Lc*, *Sn* and *B* also regulate pigment accumulation, but in different parts of the maize plant (Dooner *et al.*, 1991; Tonelli *et al.*, 1994). Their gene products are all highly homologous, which suggests that their own expression patterns specifies the distinct pigmentation

patterns they dictate (Consonni *et al.*, 1993, Radicelli *et al.*, 1992).

1.7.3 Plant DNA binding proteins and transcription factors

The number of cases in which evidence has shown that DNA binding proteins do actually regulate transcription are few in comparison to the number of DNA binding proteins that have been isolated. TGA- 1 has been demonstrated to stimulate transcription in both HeLA cell and in plant *in vitro* systems (Katagiri *et al.*, 1990; Yamazaki *et al.*, 1990).

An alternative approach at proving transcriptional regulation was taken with TAF- 1, which was transformed into plants as a cDNA. In plants that were cotransformed with a reporter gene that carried the cognate cis- sequence, expression was significantly increased (Oeda *et al.*, 1991)

1.8 **Aims and objectives**

Plant biotechnology has generated an enormous interest, based on the potential of genetically modified transgenic plants (Vanderleij and Witholt, 1995). An area of research that has important implications for both industry and agriculture is the genetic modification of oilseed crops (Topfer *et al.*, 1995).

Currently the oleochemical industry has a wide and increasing demand for oils, to act as substrates in the manufacture of greases, plasticisers, lubricants and detergents (Gunstone, 1992). Obvious targets for genetic manipulation would be increases in yield and quality of oil, the ability to produce oil of uniform character being of significant interest. The modification of oilseeds requires a fundamental understanding of the genetics of fatty acid biosynthesis and also a knowledge of the biochemical factors that govern chain length, degree of unsaturation and esterification of fatty acids (Knutzen *et al.*, 1992).

The genes that synthesis fatty acids have divergent functions. They are involved in synthesising membranes, a housekeeping function and also are

Table 1.3: Plant DNA binding proteins and transcription factors

Factor	Class	Target sequence	Reference
TFIIA	general	-	1
TFIID- 1	general	TATA box	2
TFIID- 2	general	TATA box	2
3AF- 1	zinc finger	AT- rich	3
ASF- 2		GATA	4
GA- 1		GATA	5, 6
GC- 1	Sp1- like	GC- rich	6
GT- 1		GTGG	6,7
GT- 2		GTGG	8
Knotted- 1	homeobox		9
Athb- 1	HD- ZIP		10
Athb- 2	HD- ZIP		10
HSF8	heat shock	GAAnnTTC	10
HSF24	heat shock	GAAnnTTC	11
HSF30	heat shock	GAAnnTTC	11
TGA1a+b	bZIP	TGACG	12, 13, 14
OCSTF	bZIP	GACGTA	15
TAF1	bZIP	ACGTG	16
Deficiens	MADS		17
Agamous	MADS		18
TM3- TM8	MADS		19
R	bHLH	CAGGTGC	20
B1	bHLH		21
Myb- like	bHLH		22
Lc	bHLH		23
C1	bHLH		24,25
delia	bHLH		26

References: [1]Burke *et al.*, 1987; [2]Gasch *et al.*, 1990; [3]Lam *et al.*, 1990; [4]Lam and Chua, 1989; [5]Donald and Cashmore, 1990; [6]Schindler and Cashmore, 1990; [7]Green *et al.*, 1988; [8]Dehesh *et al.*, 1990; [9]Tobin and Silverthorne, 1985; [10]Ruberti *et al.*, 1991; [11]Scharf 1990; [12]Katagiri *et al.*, 1989; [13]Katagiri *et al.*, 1990; [14]Weisshaar *et al.*, 1991; [15]Schmidt *et al.*, 1987; [16]Oeda *et al.*, 1991; [17]Singh *et al.*, 1990; [18]Coen and Meyerowitz, 1991; [19]Pnueli *et al.*, 1991; [20]Dellaporta *et al.*, 1988 ; [21]Goff *et al.*, 1990; [21]Jackson *et al.*, 1991; [22]Ludwig and Wessler, 1990; [23]Dooner *et al.*, 1991; [24]Paz- Ares *et al.*, 1990; [25]Cone *et al.*, 1986; [26]Goodrich *et al.*, 1992

Abbreviations used: HD-ZIP, homeodomain- leucine zipper; bZIP, basic domain- leucine zipper; MADS, MCM1- Agamous, Deficiens- SRF1 family; bHLH, basic helix- loop- helix.

required for the production of storage lipid, a developmentally regulated function. A major challenge of current research is therefore to determine the mechanism by which plants discriminate between the pathways of genes with divergent roles.

It is well established that regulation of gene expression at the level of transcriptional initiation is mediated by DNA motifs that represent binding sites for transcription factors. The main aim of this research was to define a characterised ACP gene promoter in terms of sequence specific DNA- protein interactions. Previously the ACP05 promoter was shown to contain regulatory information sufficient to direct its correct spatial and temporal expression (deSilva *et al.*, 1992). Gel retardation assays were used to define, *in vitro,* protein binding sites contained within this promoter. As it was known that this was a seed expressed promoter, assays were performed with both embryo and leaf extract, with the aim of identifying binding interactions with embryo specific proteins. It was envisaged that binding sites identified in this manner could be isolated on an oligonucleotide and used as a probe to screen an expression library for the corresponding DNA binding protein.

The second subject of this thesis concerns a second member of the FAS complex, enoyl- ACP reductase (ER). It has been demonstrated that there are four ER isoforms, expressed in both leaf and seed. A probe was generated from an existing leaf expressed clone, pERL8, isolated from *B. napus* cv Jet Neuf. This was used to screen a Jet Neuf embryo library for additional ER clones. It was envisaged that any additional clones would be characterised to generate new information on the differential gene expression of ER proteins.

Chapter 2

General Materials and Methods

Chapter 2

General materials and methods

2.1. Chemicals and reagents.

All general laboratory chemicals and biological reagents were from the Sigma chemical company Ltd or BDH Ltd and were AnalaR grade or the best grade obtainable. Other chemicals and reagents are as listed below.

Deoxyribonucleotide triphosphates, Taq DNA polymerase and poly (dIdC:dIdC); Bohringher Mannheim UK, Lewes, Sussex, UK.

Electrophoresis grade agarose; GIBCO- BRL Ltd, Paisley Scotland.

Fuji RX X- Ray film; Fuji Photo Film Co. Ltd, Japan.

Nitrocellulose filter discs BA85 (0.45mm); Schleicher and Schluell, Dassel, FRG.

Radiochemicals, hybridisation membranes ("Hybond C" and "Hybond N"); Amersham International Ltd, Bucks, UK.

Restriction endonucleases, DNA modifying enzymes, IPTG and X- GAL; Northumbria Biologicals Ltd, Cramlington, Co. Durham, UK, New England Biolabs Inc, Bishops Stratford, Harts, UK

Other commercially supplied consumables and equipment are acknowledged at the first reference to use.

The water used for this work was double deionised (Milli Q: 17- 18 MV/ cm resistivity; Millipore, Watford, UK). Water used for DNA manipulation was autoclaved before use.

2.2 Experimental plant material.

Brassica napus (cv. Jet Neuf) plants were greenhouse grown. Growth conditions were maintained at a 16h light period and a minimum temperature of 10°C. Leaf and root samples were harvested at the four- leaf stage, snap frozen in liquid nitrogen and stored at -80°C. Staged embryo samples were harvested from tagged plants generated in the following way: the terminal flowers of plants were dated with colour coded tags every 2 days. Embryos were then dissected from pods directly into and subsequently stored in liquid nitrogen.

2.3 *Escherichia coli* strains

The *E.coli* strains used during the course of this research were:

Strain	Genotype
JM101	*sup*E, *thi* , Δ(lac-*pro*AB) F' [*tra*D36, *pro* AB^+, *laq* I^q, lacZ Δ M15], Messing, 1979
"SURE"	*mcr* A, (*mcr* BC - *hsd* RMS - *mrr*) 171, *sup*E44 thi-1, λ-, *gyr*A96, *rel*A1, *lac*, *rec*B, *rec*J, *sbc*C,*umu*C :: Tn5 (kan^r) *uvr*C [F', *pro*AB,laq^qZΔM15, Tn10, (tet^R)] Stratagene
XL1- BLUE	*sup* E44, *hsd* R17, *rec* A1, *end* A1, *gyr* A46, *thi*, *rel* A1, *lac*$^-$ F' [*pro* AB^+, *lacI*^{q}, lacZΔM15, Tn10(tet^R) Bullock *et al.*, 1987
Y1089	*ara*D 139, Δ*lac* U169, *pro* A+ Δ*lon* , *rps*L*hfl*A150 [*chr* :: Tn10 (tet^R), pMC9, Huynh *et al.*, 1985
Y1090	*hsd*R, *sup* F, *ara*D 139, Δ *lon*, Δ *lac*, U169, *rps*L *trp*C22 :: Tn10 (tet^R), pMC9, ($r_k^+m_k^+$), Huynh *et al* .,1985

2.4 Plasmids

Plasmid	Comments	Reference
pUC19	amp^R	Vieira and Messing, 1982
pBluescript	amp^R, with T7 promoter	Stratagene
λgt11	amp^R	Young and Davis, 1983

Other plasmids used in the course of this research were:

ACP29C08	ACP cDNA clone in pBR322	Safford *et al.*, 1988
pTZ5PS	1.7kb ACP05 genomic fragment in pTZ18R	de Silva *et al.*, 1992
pTZ5PA	916bp ACP05 promoter fragment in pUC19	constructed during the course of this work
pOBS4	multimerised oligonucleotide binding site (8 copies) in pUC19	constructed during the course of this work
λBS2xi	sequence specific ACP05 binding protein cDNA, in λgt11	isolated from a λgtII library during course of this work
pBF2	3.0kb cDNA insert from λBS2xi in pSK^+	constructed during the course of this work
pERL8	*B.napus* enoyl- ACP reductase cDNA clone in pSK^-	A. Fowler, unpublished results
pERE plasmids	*B.napus* enoyl- ACP reductase cDNA clones in pSK^-	isolated from a λZAPII library during the course of this work

2.5 Bacterial media and growth conditions

Bacterial strains were routinely grown in luria broth (LB) (Sambrook *et al.*, 1989) or on LB- agar or LB- agarose at either 37°C (*E. coli*) or 42°C (phage hosts). If LB were required for phage host growth 2g of $MgSO_4.7H_2O$ per litre was added. The media was then autoclaved as detailed in section 2.6. For purification of bacteriophage DNA, λgt11 clones were grown on NZYCM media (Sambrook *et al.*, 1989). Plates were stored in the dark at 4°C for up to 1 week. If plates were required for blue/ white screening, X- GAL was made as a 2% stock in DMF and 200µl added for every 100ml autoclaved LB. IPTG was made as a 100mM stock in water and added to a final concentration of 1mM. When antibiotic selection was required, stock solutions were prepared, filter sterilised and the appropriate volume added to cooled, autoclaved media as detailed in the table below.

Antibiotic	Stock solution (mg/ml)	Solvent	Final Conc (µg/ml)
Ampicillin (Amp)	50	H_2O	50
Kanamycin (Km)	25	H_2O	25
Tetracyclin (Tc)	12.5	EtOH	10

2.6 Sterilisation procedure

All glasswear, plasticwear and other equipment required for aseptic manipulation was autoclaved or 20 minutes at 120°C, 15psi. Solutions were autoclaved or filter sterilised through 0.2µm filters before use.

2.7 Stock solutions and buffers

2.7.1 Denatured and sonicated DNA

Calf thymus DNA or herring sperm DNA was dissolved in water at a concentration of 10mg/ ml. The DNA was sheared to approximately 1kb

fragments by passing the solution 12 times through a 17- gauge hypodermic syringe. The DNA was then heat denatured by boiling for 10 minutes, then quenched on ice for 5 minutes before use or storage at -20°C. In prehybridisation solutions DNA was used at a concentration of 100µg/ ml.

2.7.2 DNase free RNase A

Pancreatic RNase A was dissolved at a concentration of 10mg/ ml in 15mM NaCl, 10mM Tris.HCl pH7.5. Any DNases present were inactivated by boiling the solution for 15 minutes and allowing it to cool slowly to room temperature, before aliquots were stored at -20°C.

2.7.3 PMSF

PMSF (polymethysulfonylfluoride) was made freshly, prior to use as a 100mM stock in acetone and kept at 4°C for no longer than 20minutes. It was added to a solution by injection underneath the surface of the liquid, while stirring rapidly.

2.7.4 Poly (dIdC:dIdC): non- specific competitor DNA

Lyophilised poly (dIdC:dIdC) was resuspended in TE at a concentration of 1.0- 5.0 µg/µl. To ensure the polyanion was double stranded the solution was heated to 55°C and allowed to cool to room temperature, then placed on ice for 15 minutes. Working aliquots were stored at -20°C.

2.7.5 Restriction endonuclease and DNA modifying enzyme buffers

Restriction enzyme and DNA modifying enzyme reaction buffers were supplied with the enzymes used. Those buffers described in individual protocols were as described by Sambrook (1989), or detailed at the first reference to use.

2.8 General molecular biology methods

The methods not described in detail in this section were performed as described by Sambrook, (1989).

2.8.1 **Isolation of plasmid DNA**

2.8.1.1 Mini- prep

A single colony of bacteria was grown overnight in 5ml of LB, with appropriate antibiotic selection. 1.5ml of this culture was pipetted into a sterile eppendorf tube and the cells harvested by centrifugation for 30 seconds at top speed in a microfuge (MSE MicroCentaur, approximately 12,000g). The supernatant was discarded and the tube was inverted. 100µl of ice-cold solution 1 (1% glucose, 10mM EDTA pH8.0, 25mM Tris.HCl pH8.0) was added and briefly vortexed. Following incubation at room temperature for 5 minutes, 200µl of solution 2 (0.2M NaOH, 1% SDS) was added and mixed by inversion. After 5 minutes on ice, 150µl of ice-cold solution 3 (11.5ml of glacial acetic acid and 28.5ml of distilled water added to 60ml of 5M potassium acetate. The solution has an overall pH of 4.8 and is 3M with respect to potassium and 5M with respect to acetate) was added to the mixture and the tube vortexed briefly before being placed on ice for a further 5 minutes. The tube was then microfuged for 5 minutes to remove bacterial debris. The supernatant was transferred to a fresh tube and extracted with an equal volume of TE (10mM Tris.HCl pH8.0, 1mM EDTA pH8.0) - saturated phenol:chloroform:isoamyl alcohol (25:24:1, v/v), (section 2.9.2). Following centrifugation for 2 minutes in the microfuge, the aqueous phase was transferred to a fresh tube and the DNA precipitated by the addition of 2.5 volumes of ethanol. The tube was left for 30 minutes on ice and the DNA collected by centrifugation for 5 minutes. The DNA pellet was washed in 70% ethanol and air dried at room temperature for 5 minutes. The final pellet was resuspended in 50µl of TE with RNAase A (2.7.2) added to a concentration of 20µg/ ml.

2.8.1.2 "Quick" mini- prep

When a large number of plasmid mini- preps had to be isolated from *E.coli* strains, this quicker method was used. Plasmid DNA of lower yield and purity was obtained (the nicked forms of plasmids were commonly seen and preps were usually contaminated with bacterial chromosomal DNA), relative to DNA prepared by alkaline lysis. However this method was most often used to

rapidly screen large numbers of clones for an increase or decrease in insert size (for example in selecting nested deletions). Hence once the correct plasmid containing strain had been identified by this method, alkaline lysis minipreps were then carried out to obtain large amounts of pure DNA.

Individual bacterial colonies were streaked onto LB- agar plates, with the appropriate antibiotic selection. 90% of the streak was scraped off the masterplate and transferred to an eppendorf containing 40µl solution A (100mM NaCl, 20mM Tris.Cl pH 7.5, 10mM EDTA). The cells were resuspended by brief vortexing and 40µl phenol: chloroform (1:1 v/v) added. The cells were lysed by vortexing for 30 seconds and the phases separated by centrifuging for 3 minutes at top speed in a microfuge. 20µl of the upper aqueous phase was transferred to a fresh tube and RNAse A added to 20µg/ml. Following a 20 minute incubation at room temperature the entire 20µl was analysed by gel electrophoresis, with appropriate plasmid controls as size markers.

2.8.1.3 Midi- prep

This larger scale preparation of plasmid DNA was essentially a scale- up of the mini- prep described in section 2.8.1.1, with an additional PEG precipitation step to selectively purify supercoiled plasmid.

A fresh 5ml bacterial culture, grown with the appropriate selection, to an OD_{600} of approximately 0.6 was subcultured into a fresh volume of prewarmed LB, in the ratio of 1:100 (culture: LB) and grown overnight. The cells were chilled on ice for 10 minutes and then harvested by centrifugation at 6 000rpm for 10 minutes at 4^0C in a Sorvall GS3 rotor. The supernatant was decanted and the tube inverted over tissue. The pellet was then vortexed and 3.0ml ice- cold solution I (section 2.8.1.1) added and vortexing continued to resuspend the cells. Following incubation at room temperature for 5 minutes, 6.0ml of solution 2 was added and the contents of the tube mixed by inversion. After 10 minutes on ice, 4.5ml of ice-cold solution 3 was added and the tube vortexed briefly before incubation on ice for a further 15 minutes. Bacterial debris was pelleted by centrifugation at 6000rpm in a sorvall GS3

rotor. The supernatant was transferred to a fresh tube and extracted with an equal volume of phenol:chloroform (2.9.2). After centrifuging for 5 minutes, the aqueous phase was transferred to a fresh tube and the DNA precipitated by the addition of an equal volume of isopropanol (pre- chilled to -20^{0}C). The tube was left for 2 hours in icy water or at 4^{0}C overnight and the DNA collected by centrifugation for 10 minutes at 10 000rpm in a Sorvall SS34. The supernatant was removed and the DNA pellet was washed in 70% ethanol, air dried and then resuspended in 500µl TE. RNase A was added to a final concentration of 20µg/ ml and incubated for 20 minutes at 37^{0}C. An equal volume of solution 4 (1.6M NaCl containing 13% (w/ v) PEG- 8, 000) was added and mixed by inversion. The DNA was precipitated by incubation on ice for 1 hour and the plasmid recovered by centrifugation at top speed for 10 minutes in a microfuge. The supernatant was removed and the pellet dissolved in 500µl TE. The solution was extracted once with an equal volume of phenol:chloroform (2.9.2) followed by an equal volume of chloroform. The supernatant was transferred to a fresh eppendorf and precipitated on the addition of 125µl 10M ammonium acetate and 2 volumes of ethanol. The tube was incubated on ice for 1hour and plasmid DNA recovered by centrifugation at 12 000g for 10 minutes. The final pellet was washed twice with 70% ethanol, air dried for 10 minutes and resuspended in 200µl of TE.

2.8.2 **Transformation of bacteria**

2.8.2.1 Calcium chloride method for preparing competent cells

This method is a modified version of Mandel and Higa (1970) and was used when large numbers of transformations were to be carried out or when competent cells were made for long term storage.

One milliltre of an overnight culture of *E. coli* was subcultured into 100ml prewarmed LB and grown until the OD_{600} was approximately 0.4 (2- 3 hours). The cells were chilled on ice for 10 minutes and then harvested by centrifugation at 4,000g for 5 minutes at 4^{o}C. The supernatant was discarded and the pellet gently resuspended in 10ml of ice- cold buffer R (100mM

$CaCl_2$, 10mM Tris.HCl pH8.0). The cell suspension was maintained on ice for 30 minutes then centrifuged as before. The supernatant was removed and the pellet resuspended in 1.0ml buffer R` (100mM $CaCl_2$: 100% glycerol; 85:15 v/v), then dispersed into 100µl aliquots and snap frozen in liquid nitrogen. Cells were stored at -80°C.

2.8.2.2 Transformation of *E.coli* competent cells

An aliquot of frozen cells was thawed on ice and diluted with 0.9ml ice- cold buffer R. The cells were mixed gently and dispersed into 100µl aliquots. The required DNA or ligation mix was added to a diluted aliquot and maintained on ice for 30- 90 minutes. Up to 40ng DNA was used per tube (following which transformation efficiency was reduced). The cells were then heat shocked at 43.5°C for 45 seconds then placed on ice for 2 minutes. 0.9ml LB was added and the cells incubated at 37°C for 30 minutes (Tc selection) or for 1 hour (Amp/Km selection) to allow antibiotic resistance to be expressed. Appropriate aliquots (generally 1/10th and 9/10ths of the tube) were spread on selective agar plates. Controls of undigested vector and cut vector were included. Dilutions were plated on LB to determine transformation efficiency and ligase efficiency was determined with cut and re- ligated vector controls.

2.9 DNA manipulations

2.9.1 RNase A treatment

Contaminating RNA was removed from a DNA solution by the addition of RNase A (section 2.7.2) to a final concentration of 20µg/ml and incubation at 25°C or 37°C for an appropriate length of time. Usually for RNase A treatment of plasmid DNA this incubation was carried out along with restriction endonucleases. For larger amounts of DNA, contaminating RNA was removed by digestion with 50µg/ ml RNAase A for 1 hour at 37°C. The enzyme was then removed by phenol-chloroform extraction (2.9.2) before precipitation of the DNA.

2.9.2 Phenol- chloroform extraction

A 25:24:1, v/v/v solution of phenol:chloroform:isoamyl alcohol was

equilibrated 3 times with TE buffer and stored under TE in a light-proof bottle at 4°C. To remove proteins from DNA solutions an equal volume of phenol:chloroform:isoamyl alcohol was added, the solutions mixed by vortexing for 30 seconds and the phases separated by centrifugation for 2 minutes in a microfuge. The aqueous phase was transferred to a fresh tube. This was repeated until no further protein was visible (as a white precipitate) at the boundary of the two phases. A final extraction with chloroform:isoamyl alcohol (24:1 v/v) was carried out to remove any traces of phenol from the DNA solution.

2.9.3 Ethanol precipitation

DNA was precipitated from solution by the addition of 0.1 volumes of 3M sodium acetate (pH4.8) and 2 volumes of ethanol, unless specified otherwise. The sample was mixed by vortexing and plasmid DNA solutions were placed on ice or at -20°C for at least 30 minutes. The DNA was pelleted by centrifugation in a microfuge at 12 000g for 10 minutes. After which the supernatant was removed, the pellet washed twice in 70% ethanol, air dried and finally resuspended in TE buffer or sterile distilled water.

2.9.4 Quantification of DNA solutions

2.9.4.1 Spectrophotometric determination

The absorbance of an appropriate dilution (usually 1:50) of the DNA sample in sterile H_2O was read at 260nm and 280nm on a Beckman DU7500 spectrophotometer, using sterile distilled water as a blank. As an A_{260nm} of 1.0 is equivalent to a concentration of 50µg/ ml of double stranded DNA or ~33µg/ ml of single stranded oligonucleotides, sample DNA concentrations could be calculated.

2.9.4.2 Ethidium bromide fluorescence determination: "Dot" method

When the amount of DNA to be quantified was limited or at a low concentration then the following method was used (adapted from Sambrook *et al.*, 1989), which could detect as little as 1-5ng DNA. A standard solution of λ DNA was made in dH_2O and a dilution series of the standard DNA spotted

onto a circular 9cm agar plate. 1- 5µl of the DNA sample was then spotted on. An equal volume of TE containing 2µg/ ml ethidium bromide was added to every sample then viewed through a trans illuminator (UVP Inc). Concentration of the DNA is determined by comparison of the intensity of fluorescence in the sample with that of the standard solutions.

2.9.5 Restriction endonuclease digestions

Digestions were carried out according to the enzyme manufacturer's instructions. Generally plasmid DNA was digested at a concentration of 0.01-0.1µg/ µl) in a total volume of 10-50µl, with 5 units of restriction endonuclease, 0.1 volumes of the supplied 10x concentrated enzyme buffer and sterile H_2O to make up the volume. The reaction was incubated at the recommended temperature (usually 37°C) for 1-2 hours. If more than one restriction enzyme was to be used in the same reaction and the buffers supplied differed, the reaction was buffered using one-phor-all buffer PLUS (Pharmacia).

If the digestions were to be analysed by gel electrophoresis, 0.2 volumes of 6x gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% sucrose) was added prior to loading.

2.9.6 **Electrophoretic separation of DNA fragments**

2.9.6.1 Agarose gel electrophoresis

Gel electrophoresis was carried out with large gels 100x 80mm (volume 70ml) or minigels 105x 60mm and 50x 60mm (volume 40 and 20ml respectively). Electrophoresis was performed in horizontal gel tanks (Pharmacia). The concentration of agarose within a gel was varied depending on the size of DNA to be separated (Sambrook *et al.*, 1989). Usually 0.7-1.0% agarose gels were used, which efficiently separated linear DNA between 10- 0.8kb. The required amounts of agarose and 1x TAE (Sambrook *et al.*, 1989) buffer were mixed and the agarose dissolved by microwaving the mixture. The solution was cooled to about 60°C, ethidium bromide was added to a final concentration of 0.2mg/ ml, and the agarose poured into the gel mould with a well comb in place. Once set, the gel was put in a tank and

covered with 1x TAE buffer containing 0.2mg/ ml ethidium bromide. Samples plus appropriate size markers were loaded and electrophoresis carried out at 5-10 V/ cm for the required amount of time. DNA within the gel was visualised on a trans illuminator (UVP Inc.), and photographed with a video copy processor (Mitsubishi), using a red filter.

2.9.6.2 Polyacrylamide gel electrophoresis

Polyacrylamide gels were used for resolving proteins and low molecular weight DNA fragments. Electrophoresis was performed in either the Bio-Rad Protean I or Protean II tanks using 0.5x or 1x TBE buffer (Sambrook *et al.*, 1989) for DNA gels and SDS- running buffer (Laemmli., 1970) for protein gels. The acrylamide used in the gels was diluted from a 30% acrylamide stock which was made by the addition of 29g acrylamide and 1g N,N'-methylenebisacrylamide to 60ml distilled water. The solution was heated to 37°C to dissolve the chemicals and the volume then adjusted to 100ml with distilled water. The percentage of acrylamide used in resolving gels was varied depending on the size of the fragments to be fractionated (Sambrook *et al.*, 1989) and either 3% or 5% stacking gels were used. 0.07% Ammonium persulphate (APS: made fresh and kept for a week at 4°C) was added to the acrylamide and polymerisation catalysed by the addition of TEMED- 1 µl/ ml of gel. Samples and appropriate size markers were loaded and electrophoresis carried out at 5-10V/ cm for the required amount of time.

2.9.6.3 Non-denaturing (native) polyacrylamide gel electrophoresis

The separation of protein- DNA complexes for gel mobility shift or gel retardation analysis was achieved using native polyacrylamide gel electrophoresis. Thoroughly washed (to remove any residual SDS) Protean I or mini-Protean II (Bio-Rad) vertical gel equipment was assembled according to the manufacturers instructions. 3- 5% polyacrylamide gels, with a ratio of acrylamide : bisacrylamide of 30: 1 were used (suitable for proteins with a molecular mass of 15- 500kDa and DNA fragments 12- 600bp in length). The gel was made in 0.5x TBE, supplemented with 2.5% glycerol, 0.07% APS and the gels were polymerised using TEMED- 0.5 µl/ ml of gel. Gels were pre- run at 100V for 20minutes. If DNA probes were used the wells were washed out with incubation buffer prior to loading and the running buffer was doubly

recirclated from anode to cathode during electrophoresis. Following electrophoresis, gels were frozen at -80 °C or dried and the DNA located by autoradiography with an exposure from 1 hour to overnight (section 2.9.12.3).

2.9.7 **DNA fragment isolation**

If DNA was to be recovered from an agarose gel then the specific fragment was excised in the smallest possible volume and isolated be one of the following methods.

2.9.7.1 Electroelution using Biotrap B-1000

Electroelution of DNA from an agarose gel slice was performed using the Biotrap B1000 (Schleicher & Schuell) apparatus. The Biotrap apparatus was assembled according to the manufacturers instructions and placed into an horizontal electrophoresis tank (Pharmacia). 1x TAE was added to the apparatus chambers, then poured into the electrophoresis tank until a complete circuit was made. The gel slice was submerged in the sample chamber and electrophoresis was at 100V for at least 1 hour. The current was then reversed for 1 minute and the TAE from the collection chamber taken up and placed into an eppendorf. DNA was then ethanol precipitated (2.9.3). Recovery was dependent on the size and initial amount of DNA, but on average was approximately 70%.

2.9.7.2 "Freeze- squeeze"

The excised DNA fragment was placed in an 0.5ml eppendorf tube which had been pierced, then plugged with siliconised glass wool. The gel slice was frozen at -80°C for 30 minutes then the tube was placed in a 1.5ml eppendorf and centrifuged for 10 minutes at top speed in a microfuge. The DNA solution which collected in the 1.5ml eppendorf was extracted once with an equal volume of chloroform and then ethanol precipitated (2.9.3).

2.9.8 Filling in 3'- recessed termini

If it was necessary to convert a DNA restriction fragment with 3' recessed ends to one with blunt ends, the following method was used (Sambrook *et al.*, 1989). The DNA fragment (maximum of 500ng) was resuspended in 10-

15µl of sterile distilled water. 1µl of a stock dNTP solution (each at 1mM in H_2O) was added to the DNA with 2µl of Klenow buffer. The volume made up to 19µl with H_20 and the reaction was started with addition of 1µl (1 Weiss unit) of Klenow fragment. The reaction was incubated at ambient temperature for 30 minutes then at 70°C for 5 minutes, if the Klenow enzyme had to be inactivated.

2.9.9 Ligation of DNA

T4 DNA ligase was used to ligate DNA fragments with compatible cohesive or blunt termini. The fragments of insert and vector DNA were usually mixed at a ratio of 3:1 (insert:vector) with a maximum of 300ng DNA. 0.1 volume of 10x ligase buffer (0.66M Tris.HCl pH7.5, 50mM $MgCl_2$, 50mM DTT, 10mM ATP - (stock ATP solution - 100mM disodium salt, pH adjusted to 7.0 by adding 1/10th volume 1M Tris.Cl pH 9.5 and stored at -20°C)) was added and for cohesive termini 1 unit of DNA ligase added. This was then incubated at room temperature for 2 hours or overnight at 4°C. For blunt-ended termini, 3 units of ligase were added and the reaction incubated at 15°C overnight. The ligation mix was then used to transform competent *E. coli* cells.

2.9.10 **DNA sequencing**

DNA sequencing of double stranded plasmid templates was performed by the dideoxy- sequencing method of Sanger and colleagues (1977), using fluorescent dye- linked universal M13 primers. Sequences were analysed using an Applied Biosystems 373A DNA Sequencer. Plasmids were sequenced in both directions using forwards and reverse primers. Custom-synthesised primers (section 2.14) were used in conjunction with the Applied Biosystems Taq dyedeoxy terminator cycle sequencing kit. Reactions were prepared according to protocols described by the manufacturer. Standard control reactions supplied by the manufacturer were prepared in tandem with each batch of sequencing reactions.

2.9.10.1 Preparation of double stranded DNA template

For optimum sequence data the purity of the template was important and was

prepared using a modified Promega Magic Miniprep™ kit method. A 10ml culture with appropriate selection was grown overnight and the cells harvested by centrifugation for 5 minutes at 2 500rpm in a MSE minor. The supernatant was discarded and the cells were resuspended in 300µl buffer 1 (50mM Tris.Cl, pH 7.5, 10mM EDTA, 100µg/ ml RNAse A) in a sterile eppendorf. Lysis solution 2 (300µl of 0.2M NaOH, 1% SDS) was added and the tube inverted several times until the suspension cleared (lysis), then 300µl of neutralisation solution 3 (1.32M potassium acetate pH4.8) was added and mixed by gentle vortexing. The tube was then centrifuged for 3 minutes at top speed in a microfuge to remove bacterial debris. The supernatant was transferred to a fresh tube and the last step repeated. The clear supernatant was divided between two tubes and 500µl Magic miniprep DNA purification resin added to each. The resin /DNA slurry was incubated at room temperature for 5 minutes, with occasional mixing by inversion and then pipetted into a syringe attached to a magic miniprep mini column. The slurry was injected into the column, then washed with 3ml solution 4 (200mM NaCl, 20mM Tris.Cl pH 7.5, 5 mM EDTA, 55% ethanol) via the syringe, which was removed and the column transferred to a fresh eppendorf. The column was centrifuged for 1 minute at 12 000g, transferred to a fresh tube and air dried for 5 minutes. The DNA was eluted with 100µl sterile milli Q water, preheated to 70^{0}C. This was applied and eluted after one minute by spinning at top speed in a microfuge for 1 minute. The eluent was reapplied to the column and centrifuged as before. The purity and quantity of the DNA was assessed by comparison against known standards.

2.9.10.2 DNA sequence analysis

Primary DNA sequence analysis was carried out by entering the DNA sequence into the DNA-Strider program (Marck., 1988). Additionally, the DNA sequence was down-loaded to the Daresbury SERC SEQNET facility and an analysis using the UWGCG (Devereux *et al.*, 1984) package was performed. This included database searches and determination of DNA sequence features, such as terminators, start and stop codons and searches for upstream regulatory elements at the appropriate distance from a given start

site. When required deduced protein sequence analysis was compared to previously published sequences, searching the OWL non-redundant database on SEQNET with NEWSWEEP and a daily updated GenEMBL database with TFASTA and FASTA, alignment programs based on the algorithm of Lipman and Pearson, 1985.

2.9.11 **Preparation of radiolabelled probes**

2.9.11.1 Random primer reactions

DNA fragments were labelled with [α-^{32}P] dCTP by the random primer labelling method using an Amersham Megaprime kit. 50- 200ng of DNA in a total volume of 45µl was boiled for 5 minutes with 5µl of random hexanucleotide primers. 10µl of labelling buffer, 5µl [α^{32}P] dCTP (specific activities of both 500Ci/ mmol and 3000 Ci/ mmol were used) were added with H_2O to make the total volume 48µl. The labelling reaction was started by the addition of 2µl of Klenow enzyme and left to proceed for 1 hour at room temperature. The labelled DNA was boiled for 5 minutes, then quenched on ice immediately before use. If required, unincorporated label removed by chromatography using a Bio spin P-30 (Bio-Rad) column according to the manufacturors instructions. Following chromatography at 1, 100g for 4 minutes through the P-30 gel matrix most of the unincorporated nucleotide was retained and the purified probe was eluted.

2.9.11.2 Oligonucleotide end labelling

Synthetic binding sites for gel retardation assays were made as complementary oligonucleotides. These were labelled at their 5' ends using poly nucleotide kinase (PNK), according to a modified method of Chaconas and Van de Sande (1980). The following were incubated in an eppendorf at 37°C for 30 minutes:

oligonucleotide (3.5pmol/ µl)	1.0 µl
10X kinase buffer	1.0 µl
γ^{32}P- ATP(5 000 Ci/mmol)	1.0 µl
H_2O	to 10 µl
T4 PNK(8- 10U/l)	1.0 µl

Individual labelling reactions were combined then 50 µl light mineral oil was layered on. The mixture was briefly microfuged and then placed in a 92°C water bath which was switched off and subsequently allowed to cool to room temperature. The oligonucleotides were then placed on ice for 15 minutes and stored at -20°C. Purified oligonucleotides were counted by liquid scintillation and diluted such that 1.0µl (0.35pmol=0.5ng=10, 000 - 50, 000cpm) and 1.0µl was used in each gel retardation assay.

When oligonucleotides were used as competitors, the above method was followed, with the substitution of 1mM ATP instead of γ^{32}P- ATP in the kinase reaction.

2.9.11.3 Filling in 3'- recessed terminii with α^{32}P- dNTP's

The 3' recessed ends of gel purified DNA restriction fragments were filled in using Klenow DNA polymerase using the standard method (2.9.8). The stock dNTP solution was substituted with 2µl of each α^{32}P- NTP (specific activity 5 000 Ci/mmol) plus the appropriate nonradioactive dNTPs at 1mM.

2.9.12 **DNA hybridisation procedures**

2.9.12.1 Transfer of DNA to membranes

The following method is a modification of that of Southern (1975). DNA was transferred to Hybond- N (nylon) membranes or Hybond- C (nitrocellulose) membranes (Amersham), as described below. The agarose gel containing the DNA samples was photographed with a fluorescent calibration rule. If DNA fragments greater than 10kb in size were present, the gel was soaked in 0.25M HCl for 15 minutes, to partially depurinate the DNA, then rinsed twice with distilled water. For blotting, the gel was soaked in denaturation buffer (1.5M NaCl, 0.5M NaOH) for 45 minutes, then rinsed twice with distilled water and soaked in neutralisation buffer (1.5M NaCl, 0.5M Tris.HCl pH7.2, 0.001M EDTA) for 45 minutes. After rinsing the gel with distilled water, then 20X SSC (3M NaCl, 0.3M sodium citrate) the gel was blotted overnight. For one-way blots a platform was placed over a reservoir of 10X SSC. A wick made of

Whatman 3MM paper was put on this platform with its ends in the reservoir. The gel was placed, wells uppermost, on the 3MM paper followed by a piece of Hybond-N and 3 sheets of Whatman 3MM paper (all cut to the same size as the gel). Finally 2 layers of disposable nappies were placed on top, the stack was covered with a glass plate and a 0.5kg weight placed on top. Double sided (two-way) blots were created by sandwiching the agarose gel between two membranes. Both types of blot were left for at least 16 hours to allow DNA transfer, afterwhich the apparatus was dismantled and the positions of the wells marked on the filters and air dried. DNA was fixed to the membranes according to the manufacturers instructions.

2.9.12.2 Hybridisation of radio-labelled probes to Southern blots

Hybridisation was carried out in Techne Hybridisation tubes using a Techne Hybridiser HB-1 oven. Blots were incubated in 50ml of pre-hybridisation solution (5x SSC, 5x Denhardt's solution [50x Denhardt's solution is 1% ficoll, 1% polyvinylpyrrolidone, 1% BSA fraction V], 0.5% SDS and 100μg/ ml of denatured salmon sperm DNA) (section 2.7.1) at 65°C for 2- 4 hours. A fresh 10ml aliquot of prehybridisation buffer that contained denatured, labelled probe was then added and incubation continued at 65°C for at least 2 hours. Blots were subsequently washed twice in 2x SSC, 0.1% SDS for ten minutes at room temperature, followed by one wash in 0.- 1.0 x SSC, 0.1% SDS for 15 minutes at 65°C. Lower stringency washes were done with 2x SSC alone at 42°C. After each washing solution was removed, the filter was checked with a Geiger counter and the washing continued until sufficient (apparent) non-specific radio-labelled probe was removed. Finally the filters were wrapped in clingfilm and hybridising bands detected as described below.

2.9.12.3 Detection of hybridising probes

This was achieved in one of two ways.

a: The wrapped filter was taped onto a larger piece of Whatman 3MM paper and radioactive ink orientation marks spotted onto the edges. Radioactive bands were detected on exposure to pre- flashed Fuji RX-100 X-ray film, at -80°C, in cassettes fitted with tungstate intensifying screens, for varying amounts of time. Aligning the radioactive ink spots on the film to those on the 3MM paper allowed the position of the wells on the filter to be marked on the

film. Hence the size of any hybridising fragments could be calculated using the original gel photograph (with fluorescent calibration rule included).
b: The filter was taped onto a GS-250 high sensitivity phosphoimager plate and exposed for varying lengths of time at room temperature. The plate was then screened and the result visualised and displayed using the manufacturors software and instructions (GS- 250 Molecular Imager, BioRad). Bands were oriented using pre-existing grids on the phosphoimager plates.

2.9.12.4 Removal of radioactive probes from filters

To strip radioactive probes from Hybond- C filters for reprobing, the filter was washed with 0.4M NaOH at 45°C for 30 minutes. This was followed by a wash with 0.1x SSC, 0.1% SDS and 0.2M Tris.HCl pH7.5 for 30 minutes at 45°C. To strip radioactive probes from Hybond- N filters, boiling 0.1xSSC, 0.1% SDS was poured over the blot and left to cool to room temperature. Successful removal of probes from nylon filters only occurred if the filter had never been allowed to dry out completely after hybridisation/ washing. Filters were checked for probe removal by re-exposure to X-ray film, as described above.

2.9.13 Nested deletions of double stranded DNA

The nested deletion system for double stranded DNA (Pharmacia P-L Biochemicals) was designed to carry out unidirectional deletions with Exo III nuclease. Exo III will digest blunt and 5' protruding termini, but not 3' protruding termini. Plasmids were double digested with two restriction enzymes; one enzyme created a 5'-protruding or blunt end (nuclease-sensitive), adjacent to the target sequence and one that produced a 3'-protruding termini (nuclease resistant). The linearised DNA was then incubated with Exo III according to the manufacturer's recommendations, using reagents supplied with the kit. Samples were removed at intervals then treated with S1 nuclease to remove single- stranded region. Half of the deletion mix was then analysed by agarose gel electrophoresis and half was recircularised using T4 DNA ligase and transformed into calcium competent *E. coli* cells (2.8.2).

2.9.14 Polymerase Chain Reaction for *in vitro* amplification of DNA

The polymerase chain reaction (PCR) for *in vitro* amplification was carried out essentially as described by McPherson *et al.*, 1991 with modifications to avoid mispriming as described by Don *et al.*, 1991. Template DNA made according to the standard protocols (2.8.1 and 2.12.9) was used as target sequence for amplification.

For a single 100µl PCR reaction the following components were added to a 0.5ml sterile eppendorf tube on ice:

. 1x Taq polymerase buffer (10mM Tris pH8.3, 1.5mM $MgCl_2$, 50mM KCl, 0.1mg/ml gelatin)
. dNTP's - 0.2mM (dATP/ dTTP/ dCTP/ dGTP)
. 0.1- 10ng of template DNA
. Primers 5' and 3' to a final concentration of 0.1- 1µM
. H_2O to 100µl

The reaction was spun briefly to mix the components prior to the addition of 100µl of light mineral oil. The tubes were placed in a programmable heating block and the first round of denaturation and annealing was performed. 1.0U Taq DNA polymerase was then added and amplification allowed to proceed. Cycling parameters were varied according to the annealing temperature and length of target sequence, but the basic method was as follows:

		Temp (^{0}C)	Time (mins)
Twenty five cycles:	denaturation	92	0.5
	annealing	50- 55	0.5
	extension	72	0.5- 2
One cycle:	denaturation	92	0.5
	annealing	50- 55	0.5
	extension	72	1- 4

Control reactions were performed in tandem to monitor contamination by foreign DNAs. These consisted of reactions with no template DNA, no enzyme and no primers. A method of "touchdown" PCR (Don *et al.*, 1991) was adopted when shorter non- specific bands were seen in the PCR product (often products associated with mispriming). The correct primer annealing

temperature ("touchdown" temperature) was calculated, then the PCR was initiated at 5°C above this. Every second cycle the annealing temperature would be dropped 1°C until the "touchdown" temperature was reached. A further 15 cycles were then performed. This method effectively prevents mispriming (and subsequently only specific products are amplified).

Custom primers used during were synthesised (2.14) and are listed below.

Table 2.1: Sequence of oligonucleotides used during this work

A: Forward and reverse primers used with pUC based plasmids

Oligo	Sequence 5' to 3'	Position in pUC19
Forward	GTTTTCCCAGTCACGAC	359 to 375
Reverse	CAGGAAACAGCTATGAC	465 to 481

B: Primers used for gel retardation probe amplification

Oligo	Sequence 5' to 3'	Position in ACP05 promoter
For 1	TGGTAAGATATGGGTACTGT	-263 to -282
Rev 1	CTCGTCGTCATTTATAAGCTTG	-13 to -34
For 2	TGCGAATTGTGAGTGGTACTA	-643 to -663
Rev 2	ACAGTACCCATATCTTACCA	-263 to -282

C: Internal primers used to sequence pUC5PA

Oligo	Sequence 5' to 3'	Position in promoter
701	TGCGAATTGTGAGTGGTACTA	-663 to -641
730	TAGTACCACTCACAATTCGCA	-641 to -663
320	TATAATTAGTCTTTGTTTTATTT	-368 to -346
321	AAATAAAACAAAGACTAATTATA	-346 to -368

D: Internal primers used to sequence ER

Oligo	Sequence 5' to 3'	Position in pERL8
367N	GGCTTCATAGACACCAT	977 to 995
371	AATCTCCATCAATGAC	451 to 468
776	GAAGTCCAGAAGAAGCCTTG	239 to 259

2.9.15 **The gel retardation/ gel mobility shift assay: Binding reaction**

Various conditions of the basic assay were varied to optimise specific binding. The optimised assay conditions were then adopted as standard and used consistently to maintain an extent of uniformity between different assays.

2.9.15.1 Assay used with oligonucleotide probes

In the standard binding assay (final volume 10µl), 1.0µl (5- 10µg) protein extract (section 2.11.6) was incubated for 5 minutes on ice with 1- 5µg poly (dIdC:dIdC) (2.7.4) in 20mM Tris-HCl, pH 7.5, 1mM DTT, 1mM EDTA, 100mM

KCl and 3% glycerol. 10 000- 50 000CPM ^{32}P-labelled substrate DNA was then added and incubation continued for 10 minutes. Samples were loaded onto 3- 5% polyacrylamide native gels and subjected to electrophoresis as detailed in section 2.9.6.3. Sample dye was not added to the assays as the glycerol content in the assay buffer provided sufficient density for loading. Bromophenol blue and xylene cyanol (0.25%) in 3% glycerol was added to a spare track as marker during electrophoresis.

2.9.15.2 Assay used with DNA probes

High levels of background were observed when using promoter probes as substrate. This could have been due to non specific protein- DNA interactions or disintegration of complexes during electrophoresis. A number of modifications to the standard assay conditions were made to reduce the problem. The electrophoresis buffer used was 0.5x TBE which may result in a pH shift through electrolysis of the buffer which could interfere with the stability of the protein- DNA complex. Therefore the buffer was double recirculated from the anode to the cathode during electrophoresis. Also differences in the composition of the binding buffer and the gel running buffer potentially could alter protein binding during loading and entry of complex into the gel. To avoid these problems the wells of the gel were washed out with the binding buffer (made up without glycerol) before the assays were loaded. Immediately after which they were rapidly run in to the gel matrix at high voltage (500v, for approximately 30 seconds), before electrophoresis at 100V for an hour.

2.10 RNA procedures

2.10.1 General techniques

Due to the high stability of ribonucleases, care was taken to create a ribonuclease-free environment whenever possible. Aqueous solutions which were first treated with diethyl pyrocarbonate (DEPC) which is a non-specific inhibitor of RNases. DEPC was added to 0.1% v/v, incubated at ambient temperature for 16 hours then autoclaved. Solutions containing Tris could not be treated directly with DEPC, as the two compounds react to form a stable

complex. Instead Tris buffers were made with autoclaved DEPC-treated distilled water and then re-autoclaved.

2.10.2 Northern blotting

Total RNA was isolated from leaf and seed material by the method of Hall *et al* (1978). Poly A^+ enriched RNA was prepared by one round of chromatography on oligo- dT cellulose (Pharmacia), using the recommended manufacturors procedure and solutions. RNA was quantified by spectrophotometry. RNA was fractionated on 1% formaldehyde containing MOPS buffered gels as described in Fawcett *et al.*, 1994 and transferred to Hybond-N nylon membranes as described in section 2.9.12.1.

Pre-hybridisation and hybridisation were carried out as described in 2.9.12, except SSPE was used instead of SSC (at the same concentration) and formamide was added to a final concentration of 50%. The incubation temperature was always 42°C.

Washing was carried out with 0.1- 1xSSPE, 0.1% SDS at 42°C for 2- 5x 10 minute intervals. After each wash the blot was checked using the Geiger counter and this step repeated until excess (apparent) non- specific probe had been removed. Hybridising bands were visualised as described in 2.9.12.3.

2.10.3 Laser densitometry

An LKB Ultroscan XL densitometer was used for laser densitometry. Two exposures of an experiment were analysed to ensure that data in the linear range of film were obtained.

2.11 Protein procedures

2.11.1 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) for proteins was performed in either the Bio-Rad Mini Protean I or Protean II equipment using the method of Laemmli, 1970. The equipment was assembled according to

the manufacturers instructions and the gels were electrophoresised under the conditions outlined in section 2.9.6.2.

Resolving gels were made using 5- 10% acrylamide, 0.1% SDS, 0.07% ammonium persulphate (APS) and 0.375M Tris.HCl pH8.8. Stacking gels were made using 3- 5% acrylamide, 0.1% SDS, 0.07% APS and 0.125M Tris.HCl pH 6.8. Gels were polymerised with TEMED- 1 µl/ ml of gel. Before loading, the samples were mixed with an equal volume of 2x sample buffer (4% SDS, 20% glycerol, 120mM Tris.HCl pH 6.8 and 0.005% bromophenol blue, with 10% β-mercaptoethanol added immediately before use), then boiled for 5 minutes and spun down in the microfuge before loading with a Hamilton syringe.

2.11.2 Staining polyacrylamide gels with Coomassie Brilliant Blue

Stain was prepared by dissolving 0.25g of Coomassie Brilliant Blue in 90ml of methanol:distilled water (1:1 v/v) and 10ml of glacial acetic acid. The solution was filtered through Whatman no. 1 filter paper. This solution was poured over the gel in a plastic tray, a lid put on the tray, and the tray microwaved for 30 seconds at medium power. The stain was allowed to cool for 10 minutes then remicrowaved. The gel was allowed to cool to room temperature then destained overnight in 90ml of methanol:distilled water (1:1 v/v). Destaining was accelerated by placing sponge in the tray which absorbed stain it as it leached from the gel. The gel was photographed or dried between cellulose sheets.

2.11.3 Determination of protein concentrations:Bradford method

The protein concentration of cell extracts was determined by a Bradford microassy (total assay volume 300µl) using Bio-Rad reagent (Bradford., 1976). The dye reagent was diluted 1 in 5 with distilled water and mixed with 100µl of sample (diluted as necessary). The assays were carried out in a microtitre plate (Falcon) and the A_{595nm} measured using a Titertek Multiscan plate reader. The protein concentrations were calculated from the absorbance readings using a calibration curve generated from known concentrations of ovalbumin.

2.11.4 Precipitation of proteins

Protein samples were concentrated using chloroform- methanol precipitation (Sambrook *et al.*, 1989). 400µl methanol was added to 100µl protein sample and mixed briefly by vortexing. The solution was then centrifuged at top speed in a microfuge for 10minutes. 100µl chloroform was then added and the mixture was vortexed and recentrifuged. 300µl distilled water was then added, mixed and recentrifuged. The aqueous (upper) phase was discarded and another 300µl of methanol was added, vortexed and centrifuged as before. The supernatant was discarded and the protein pellet dried under nitrogen.

2.11.5 Electrophoretic blotting of proteins

Electrophoretic blotting of proteins (Western blotting) was performed in a Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturers specifications. All steps were performed at room temperature in a Techne hybridiser oven, unless otherwise stated. Following SDS- PAGE (section 2.11.1), gels were equilibrated in 100 ml transfer buffer (10mM CAPS, pH 11.0, 10% methanol) for 30 minutes. PVDF membrane was cut to size and briefly submerged in methanol. The activated membrane was then equilibrated in transfer buffer. The transfer apparatus was assembled, avoiding the enclosure of air-bubbles and electrophoresis was performed at 100 V/ 150mA for 1-2 hours. To assess protein transfer the membrane was stained with Ponceau S (0.2% Ponceau S in 3% TCA) for 5 minutes and then destained with H_20. The membrane was blocked in 10ml TBS- T (20mM Tris, pH 7.6, 137mM NaCl, 0.1% Tween- 20) supplemented with 5% carnation non-fat milk powder for 1hour. The membrane was then washed four times in 100ml TBS- T for 15 minutes. Primary antibody was diluted as appropriate, in 10ml TBS- T and incubated with the blot for 1hour. The blot was washed as described above, incubated with diluted secondary antibody and washed again. Bound antibodies were visualised using the Enhanced ChemiLuminescence (ECL) kit (Amersham). Detection reagents supplied with the kit were diluted prior to use and added to the membrane, followed by exposure to blue light sensitive film (varying from 10 seconds to 1 minute).

2.11.6 **Cell free extracts**

2.11.6.1 *Brassica napus* embryo (cv. Jet Neuf).

All solutions and equipment, unless otherwise stated, were pre- chilled to 4^0C before use. Embryos from staged plants (2.2) were excised from the testa then snap frozen in liquid nitrogen. For each protein extract, between 2 and 5 grams embryo was ground to a powder in liquid nitrogen using a pestle and mortar. Extraction buffer (10mM TrisHCl pH7.5, 1mM EDTA pH8.0, 250mM KCl, 0.5mM DTT, 10% glycerol, 1 mM PMSF) was added in the ratio of 1ml to 1g embryo. The embryo was homogenised with 5x20 second passes of a polytron blender in a glass beaker surrounded with a packing of icy water. The high lipid content of the extract prevented protein precipitation, so the extract was centrifuged at 15 000g for 30 minutes, to separate the phases. The supernatant was carefully removed through the lipid pad and transferred to a fresh tube and recentrifuged if necessary. The extract was incubated on ice for 30 minutes then 0.4g powdered ammonium sulphate was added per ml (65% saturation w/v) while stirring, and left for 1 hour on ice. Protein was pelleted by centrifugation at 15 000g for 30 minutes and resuspended in 1 ml R buffer (10mM TrisHCl pH7.5, 1 mM EDTA pH8.0, 0.5mM DTT, 10% glycerol, 1mM PMSF) per gram embryo.

To reduce salt for binding assays the extract was dialysed in spectraphor dialysis membrane (molecular weight cut- off limit of 6 000D, which was prepared by rinsing thoroughly in H_20), in 1000 fold excess of R buffer with constant agitation.The buffer was changed 4x1.5 hour intervals or 2 x 1.5 hour intervals followed by dialysis overnight.

As embryo material was limited it was investigated using gel retardation assays whether individual protein aliquots could be re- used (see section 3.2.5). It was found that multiple rounds of freeze- thawing resulted in rapid loss of DNA binding activity. A strategy was adopted where individual drops of protein were aliquoted into liquid nitrogen, using a finnpippette. The protein drops could be stored at -80^0C and individually thawed.

2.11.6.2 *B. napus* Leaf (cv. Jet Neuf)

Whole cell extracts were prepared as described above (section 2.11.6.2) using young leaf tissue harvested from green house grown 4 leaf stage plants (2.2).

2.11.7 **Chromatography**

All solutions and equipment were pre- chilled to 4°C and chromatography was at 4°C, unless otherwise stated.

2.11.7.1 Heparin agarose chromatography

1.0- 2.0ml heparin immobilised agarose affinity matrix (Sigma) was packed into an 0.5 cm diameter column (Bio-Rad). The column was equilibrated with 10X column bed volumes R buffer (20mM Tris.Cl, pH7.5, 1mM EDTA, 10% glycerol, 0.5mM DTT, 1mM PMSF) under gravity. A further 10X column bed volumes R buffer was pumped through, at 100µl/ minute. 20- 50mg *B. napus* embryo cell free extract (section 2.11.6) diluted to 1.0ml in R buffer, was then loaded. The column was subsequently washed with 10X column bed volumes R buffer at 100µl/ minute. Protein was eluted with stepped salt solutions, consisting of 1ml aliquots of KCl in R buffer (0- 1M KCL, in 100mM steps) at 100µl/ minute. Fractions of 100µl were collected, snap frozen in liquid nitrogen and stored at -80°C.

2.11.7.2 Gel filtration chromatography

A 2.4ml (3.2x3000mm) superose 12 (optimun separation of 1-300kDa) column (Pharmacia) was equilibrated and run in 150mM NaCl. Buffers were pumped through the column at a rate of 40µl/min. The column was calibrated with 5µg each molecular weight markers (β- galactosidase, BSA, ovalbumin and trypsin) and protein was detected at 280nm. The column was re-equilibrated and 100- 250µg of the protein sample was injected. 100µl fractions were collected, snap frozen in liquid nitrogen and stored at -80°C.

2.12 Cloning DNA binding proteins using recognition site probes: "Southwestern" cloning

Genes that encode DNA binding proteins, or DNA binding motifs can be isolated by screening λgt11 expression libraries with binding site DNAs. This strategy was developed by Singh and colleagues (1988) from the use of antibody probes to screen expression libraries. The protocol described below was adapted from Singh *et al.*, (1989), with a denaturation/ renaturation protocol from Vinson *et al.,* (1988). Lysogens were generated using the method of Cowell and Hurst (1993).

2.12.1 Plating cells: preparation of cells for bacteriophage infection

The *E.coli* host Y1090 cells are *sup* F, (section 2.3: *E. coli* strains), which suppresses the normally defective lysis of λgt11, leading to a high frequency of lysis, which facilitates screening. Y1090 is also *lon* protease deficient, which increases the stability of the β- galactosidase fusion protein and allows proteins to accumulate. Y1090 also carries the plasmid pMC9 (AmpR), which encodes the *lacI* gene. This allows regulated expression of β- galactosidase fusion proteins, which may be harmful to the growth of the bacterial host. Y1090 are used to plate out and screen λgt11 libraries. *E. coli* Y1089 was used primarily to generate preparative amounts of β- galactosidase fusion protein. This is possible due to the *hfl* A150 and Δlon mutations, which result in a high frequency of lysogeny and decreased proteases activity (respectively).

Both strains were prepared for infection in the same manner. A single bacterial colony was inoculated into 20ml LB that contained 10mM $MgSO_4$ and 0.2% maltose and was grown overnight with selection. Aliquots of the plating cells were used directly and kept on ice for further use for up to 24 hours.

2.12.2 Bacteriophage titration

100μl plating Y1090 were mixed with 3ml top agarose and poured onto a

prewarmed and dried 100mm LB ampicillin plate and left to solidify at room temperature. Serial dilutions of the phage were made in SM buffer and 10µl of each was spotted, in an ordered array onto the *E. coli* Y1090 lawn. The phage were allowed to adsorb for 10 minutes the the plate was inverted and incubated overnight at 37°C. The plaques were then counted to determine the original pfu/ml.

2.12.3 Plating phage for screening and preparation of protein replica filters

500µl aliquots of *E. coli* Y1090 plating cells were infected with 3- $5x10^4$ pfu of λgt11 library and incubated at 37°C for 20 minutes to allow phage adsorption. 10ml of melted top agarose that had been maintained at 50°C was added to each 500µl aliquot and mixed by inversion. The mixture was spread evenly onto prewarmed and dry 22cm² LB- amp plates. The plates were then incubated at 42°C until small plaques were visible (approximately 3- 4 hours). 20cm² Hybond-C filters (presoaked in 10mM IPTG for 30 minutes and air dried) were carefully layered onto the surface of the plate. The plates were then incubated at 30°C for a further 6 hours then chilled at 4°C for 10 minutes. The filters were numbered and their position on the plate marked by piercing orientation holes using a sterile hypodermic needle. The filters were lifted and air dried, protein side up for 15 minutes. For each filter 50ml binding buffer (25mM HEPES, pH 7.9, 25mM $MgCl_2$, 0.5mM DTT) supplemented with 6M guanidine hydrochloride (GuHCl) was added and incubated with slow shaking at 4°C for 10minutes. This was repeated once more then the filters were transferred to binding buffer supplemented with 3M GuHCl and incubated as before. This was repeated a further 4 times, with binding buffer supplemented with a 2- fold dilution of the GuHCl from the previous step. The filters were then incubated in binding buffer for 5 minutes at 4°C, this step was repeated an additional time. The filters were blocked by immersion in 5% carnation non- fat milk powder, 50mM Tris HCl, pH7.5, 1mM EDTA, 1mM DTT, for 30minutes at 4°C, with swirling, then in binding buffer supplemented with 0.25% carnation non- fat milk powder for 1 minute at 4°C.

2.12.4 Screening protein replica filters

Each blocked filter was incubated in 25ml binding buffer to which 125µg denatured sonicated calf thymus DNA (2.7.1) and 1x 10^7 cpm of ^{32}P- labelled DNA recognition site probe had been added, at room temperature for 60 minutes. Each filter was washed four times at room temperature for 10 minutes with 50ml of binding buffer. The filters were briefly dried with blotting paper and subsequently autoradiographed (2.9.12.3). Short versus long (18 and 36 hours) exposure times were generated to help distinguish between true (fuzzy, comet shaped) and false (intense centres) positives.

2.12.5 Identification and purification of positive plaques

Putative positives were identified by aligning the autoradiographs with the LB plates and removed with the wide end of a sterile Pasteur tip. Each plug was placed in 1ml SM buffer, vortexed and left for several hours to allow the phage to diffuse out. Due to the plaque density on the original plate it was not possible to remove a single plaque without taking non- positives as well. A second round of screening was therefore performed on dilutions of the phage plug containing the positive signal so that individual positive plaques could be isolated. 200µl *E. coli* Y1090 plating cells were mixed with dilutions of the primary phage suspension. Following adsorption 3ml top agarose was added and the mixed solution poured onto 9cm LB- amp plates. Plates with well isolated plaques were selected to prepare protein filter replicas using 80mm nitrocellulose discs as described overleaf. The filters were screened as described above, using 10ml aliquots of all the buffers mentioned. Positive plaques were stored in SM at 4°C, with a drop of chloroform to prevent bacterial growth.

Secondary filters representing true positives were purified to homogeneity by a further round of plaque purification and then rescreened with the wild- type recognition site DNA probe, plus the following controls: a DNA probe that lacked the binding site and α- ^{32}P dCTP.

2.12.6 Generation of phage stock

To provide a concentrated phage stock for the generation of lysogens, for the

purification of phage DNA and for long term storage a plate lysate was made.

The purified phage were inoculated at a high density (approximately 10^5pfu per 100µl plating cells) such that total lysis of the Y1090 lawn occurred overnight (9cm LB agarose plates with 3ml top agarose overlay). Confluent plates were cooled and 3ml prechilled SM buffer was added to each plate and swirled at 4°C for 4 hours. The supernatant was transferred to a sterile tube and a further 1ml SM was added to the plate, swirled briefly then tilted, drawn off and pooled with the rest of the lysate. The lysate was then centrifuged at 8, 000g for 10minutes at 4°C, to remove bacterial debris and transferred to a new tube to which a drop of chloroform was added. The plate lysate was titred before use (routinely 10^{10} pfu/ml) and stored at 4°C, for up to 6 months.

2.12.7 Isolation of recombinant phage lysogens

E.coli Y1089 plating cells (2.12.1) were diluted 100 fold in LB supplemented with 10mM $MgCl_2$. 200µl of the diluted culture was then mixed with 10^7 pfu of recombinant phage stock and incubated at 32°C for 20 minutes. The infected cells were then diluted to 10ml with LB. 15µl of this dilution further diluted to 10ml with LB. 200µl aliquots were spread onto LB- ampicillin plates and incubated at 32°C overnight. At 32°C the temperature sensitive λgt11 encoded repressor is functional and establishes the lysogenic state. Individual colonies were tested for lysogeny by replica plating. The master plate was incubated at 32°C and the replica at 42°C. Clones that grew at 32°C but not at 42°C represent lysogens. Stocks were made by adding 15% glycerol to log phase cultures which were aliquoted and stored at -80°C, however protein extracts were always generated from freshly plated colonies/ cultures.

2.12.8 Preparation of cell free extracts from phage lysogens

Overnight cultures of phage lysogens and wild type *E.coli* Y1089 (control extract) were grown in LB with selection. 20µl of each overnight culture was

added to 2ml LB and grown with selection at 32°C until an OD_{600}=0.5 was reached. The cells were then immersed in a 43°C water bath to raise the temperature rapidly, then incubated with vigorous shaking at 43°C for a further 20 minutes. IPTG was added to a final concentration of 10mM, from a stock of 100mM, in order to induce expression of the β- galactosidase fusion protein. The cells were shaken at 38°C for an hour, for fusion protein to accumulate. From each culture 1.5ml was transferred to two sterile eppendorfs and the cells harvested by centrifugation at top speed in a microfuge for 1 minute. The supernatant was discarded and the cell pellet resuspended in 100µl extraction buffer (50mM Tris.Cl, pH 7.5, 1mM EDTA, 5mM DTT, 1mM PMSF). The aliquots were recombined, snap frozen in liquid nitrogen and thawed on ice, this freeze-thaw lysis was repeated twice more. Lysozyme was added to 0.5mg/ml and incubated for 15minutes on ice, following which NaCl was added to 1M and the cells shaken gently for 15minutes at 4°C. The lysates were then centrifuged for 30minutes in a microfuge at 4°C and the supernatant transferred to a fresh eppendorf. The supernatant was a crude bacterial protein extract which contained the induced recombinant protein. The wild type Y1089 provided an appropriate control extract (lacked specific recombinant proteins) and was assayed in tandem. To reduce salt for retardation binding assay extract were dialysed in Spectrapor dialysis membrane (molecular weight cut- off of 6 000Da), against 1litre of extraction buffer supplemented with 10% glycerol for 3x60 minutes at 4°C in a roller bottle. The protein was aliquoted as described in section 2.11.6.1. and stored at -80°C.

2.12.9 **Preparation of phage DNA**

Both the methods used were adapted from Sambrook *et al.*, (1989).

2.12.9.1 Plate lysate method

Lysate from 5- 10 confluent plates (2.12.5) was pooled and made 10% with respect to PEG- 8000 and 4% with respect to NaCl, from 40% stock solutions. The tubes were incubated on ice for 1 hour to precipitate the phage particles and pelleted by centrifugation at 15 minutes at 10 000g at 4°C. The

supernatant was discarded and the pellet resuspended in 200µl SM buffer. To remove bacterial DNA and RNA, RNAase A and DNAase were added to 200µg/ ml and incubated at 37°C for 30minutes. The solution was phenol extracted (2.9.2) then extracted with an equal volume of chloroform. DNA was precipitated by the addition of 0.1 volumes 3M sodium acetate and 2 volumes of ethanol. The solution was incubated in icy water for 2 hours and the DNA pelleted by centrifugation at 10 000g for 10 minutes. The pellet as washed twice in 70% ethanol, air dried and resuspended in 100µl TE.

2.12.9.2 Liquid culture method

A single phage plaque was taken using a Pasteur pipet and expelled into 100µl SM buffer, which was left overnight at 4°C. A single *E. coli* Y1090 colony was inoculated into 5ml LB supplemented with 10mM $MgSO_4$ and 2% maltose and grown overnight at 37°C. 500µl of the overnight Y1090 culture was infected with 20µl of the top agar plug eluate and incubated at 37°C for 20 minutes. 500µl of the infected culture was transferred to 100ml prewarmed LB, 10mM $MgSO_4$ and grown at 37°C for 5- 7 hours until lysis occured at which point 500µl of chloroform was added and shaking resumed for a further 15 minutes. The lysate was centrifuged at 8 000g for 10minutes to remove bacterial debris and the supernatant drawn off and transferred to a fresh tube. RNAase A and DNAase A were added to 20µg/ ml and incubated at 37°C for 30minutes. An equal volume of precipitation buffer (20% PEG- 8000, 2M NaCl, in SM) was added, mixed thoroughly and incubated on icy water for an hour. The phage were pelleted by centrifugation at 10 000g for 10 minutes and resuspended in 1.5ml TE. 15µl 10% SDS was then added and the solution incubated for 5 minutes at 68°C after which 30µl 5M NaCl was added. The phage were purified by extraction once with an equal volume of phenol:chloroform (2.9.2) and once with chloroform. The supernatant was precipitated with an equal volume of isopropanol at -70°C for 15minutes and the DNA recovered by centrifugation at 12 000g for 15 minutes. The pellet was then washed twice with 70% ethanol, air dried and resuspended in 50µl TE.

2.13. Screening a λZapII cDNA library

The advantages of high efficiency of lambda library construction and convenience of a plasmid system are combined in λZapII. Either DNA or antibody probes may be used for screening and *in vivo* excision of the pBluescript phagemid allows positive clones to be analysed in a plasmid.

2.13.1 Preparation of cells for bacteriophage infection

The *E.coli* host cells XL1Blue are *rec* A^- and contains the DM15 mutation of the *lac* Z gene on the F' episome, required for complementation of the amino terminus of the *lac* Z gene in λZapII, which subsequently allows blue- white selection. XL1Blue was used as plating cells and were prepared for infection as described in 2.12.1. Bacteriophage were titrated as described in 2.12.2.

2.13.2 Screening the library

All buffers used are listed in section 2.9.12. Hybridisation and washes were performed in a Techne hybridisation oven at 65°C, unless otherwise stated. 500µl aliquots of *E.coli* XL1Blue plating cells were infected with $5x10^4$ pfu of the λZap II library and incubated at 37°C for 20 minutes to allow phage adsorption. Each aliquot was then spread onto $22cm^2$ plates and incubated as described in 2.12.3 until plaques were beginning to touch. The plates were then chilled at 4°C, then Hybond- N $20cm^2$ membranes were layered on for 1 minute. Orientation marks were made and a second lift of the primary screen was performed (to help distinguish false positives). The second filter was incubated for 2 minutes. Filters were transferred (face up) to sheets of 3MM paper which had been soaked in denaturing buffer, for 5 minutes, then transferred to 3MM paper soaked in neutralising buffer, for 5 minutes. Finally the filters were transferred to 3MM soaked in 2xSCC solution for 5 minutes. The filters were then air dried and the DNA from the phage plaques was bound to the nylon by exposure to U.V. irradiation for 3 minutes.

Filters were prehybridised for 5 hours in 50ml buffer/ filter. The buffer was then removed and replaced by 10ml hybridisation buffer, containing 10^6 CPM

denatured DNA probe for 5 hours. Filters were washed with 2xSCC for 5 minutes at room temperature followed by two one hour washes in 1xSCC, 0.1% SDS at 65°C. After the final wash filters were wrapped in cling film and hybridised probe detected as described in section 2.9.12.3. Putative positives were identified by comparing both lifts and purified to homogeneity with a second round of screening on 9cm plates. Hybridisation and washes were as described above. Tertiary screening was performed with purified phage. Positives were stored at 4°C in SM buffer, with a drop of chloroform for up to 6 months.

2.13.3 Plasmid rescue

An insert cloned in λZapII may be excised and recircularized *in vivo* to form a phagemid that contains the insert. *In vivo* excision is dependent upon f1-bacteriophage derived proteins, which are produced by a f1- "helper" phage. The f1- protein initiator and terminator recognition sites for phage DNA synthesis were subcloned either end of the λZapII polylinker region. λZapII phage are made accessible to the f1- proteins by simultaneously infecting *E. coli* with both λ vector and the f1- helper phage. The helper f1 proteins recognise the initiator site in the λ vector and nick the strand, to initiate DNA replication. DNA synthesis continues through the cloned insert until the terminator signal is reached. The single stranded DNA is recircularised and packed for secretion with other f1- gene products. Conversion to the phagemid represents subcloning as it contains all sequences of the phagemid, pSK^- and the insert, whereas all λ sequences are positioned outside of the initiator and terminator signals and are not contained within the circularised DNA.

200µl of an overnight culture of XL1Blue, grown with selection in LB supplemented with 10mM $MgSO_4$ and 0.2% maltose was used to seed 5ml LB which was grown with vigorous shaking for 3 hours. 200µl aliquots of the culture were infected with $5x10^7$ pfu positive phage and 10^6 pfu R408 helper phage (Stratagene). After incubating the cells at 37°C for 15minutes 3.0ml YT (Sambrook *et al.*, 1989) was added, incubation was then continued for a

further 3 hours, with shaking. The cells were then transferred to 70°C for 20 minutes. Bacterial debris was removed by centrifuging at 4,500g for 5 minutes. The supernatant contained the recircularized phagemids. A serial dilution of the supernatant was performed with 200µl XL1Blue (generated in the first step) and incubated at 37°C for 10 minutes. The cells were then spread onto LB Tc- Amp plates and incubated overnight at 37°C. Controls of XL1Blue cells with helper phage only (no lambda phagemids) and XL1Blue cells, with λ phagemids only (no helper phage) were performed in tandem. Plasmid DNA was isolated from recovered plasmids by standard methods (2.8.1)

2.14 Synthesis of oligonucleotides.

Oligonucleotides were synthesised by using an Applied Biosystems 381A DNA synthesiser operated with a standard synthesis programme as reccommended by the manufacturer. After cleavage and deprotection the oligonucleotides were dried under vacuum, twice resuspended in H_20 and vacuum dried. Oligonucleotides were stored dry at -20°C or as an aqueous solution at 4°C and were used without further purification.

Chapter 3

Detection of DNA- Protein Interactions

Chapter 3

Detection of DNA- Protein Interactions.

3.1 **Introduction.**

3.1.1 The gel retardation assay

Identifying the DNA sequences that mediate regulated transcription is an essential step in understanding the fundamental processes that govern the molecular biology of gene expression. The gel retardation assay provides a means by which the difference in transcription factor content may be correlated with difference in gene behaviour (Hendrickson and Schleif, 1984; Gilmartin and Chua, 1990). The theory behind the assay is discussed fully in section 1.7, but essentially it entails the electrophoretic separation of protein-DNA complexes from unbound DNA. A DNA fragment is incubated with protein under conditions favourable for binding. The formation of specific complexes depends on the presence of motifs in the DNA and on detectable levels of the cognate binding factor in the extract.

3.1.2 Types of gel retardation assay

Three types of gel retardation assays were performed with each promoter fragment, to determine different parameters.

1: Poly (dIdC:dIdC) titration

When labelled DNA substrate is added alone to a cell extract, most of the radioactivity is found as a smear up the gel, bound in very large complexes with many different proteins. It is therefore necessary to add non- specific competing DNA such as poly (dIdC:dIdC). The optimal amount of competitor is determined experimentally using a poly (dIdC:dIdC) titration. Basically, an increasing amount of competitor is added to the assay, until the level of radioactivity in complexes at the top of the gel diminishes and the intensity of specific complex(es) and the free DNA band rise (for example, compare the retardation assays in lanes 2 and 3 in panel A of figure 3.8).

2: Competition assays

Synthetic oligonucleotides that contained a putative binding site were used as specific competitors and oligonucleotides that contained a mutated version of the same motif were used as control competitors. This allows the sequence specificity of DNA binding protein(s) in individual complexes to be examined. When competition assays were performed, assays were set up as standard, and the oligonucleotide added to the assay immediately after probe DNA.

3: Enzyme controls

Enzyme controls were performed in order to examine the nature of the factor that was involved in the formation of complexes. Proteinase K was added to analyse the role of protein factors in complex formation and stability. Enzyme assays were performed at room temperature. SDS was added to a final concentration of 0.5%, 50ng of Proteinase K and 20ng of RNAse A per assay were added, directly after probe DNA.

3.1.3 Factors that determine the detection of DNA- protein interactions

Detection of protein- DNA complexes within a gel depends on a number of factors. Eukaryotic transcription factors are rare proteins, varying from 10^3 molecules per cell (retinoic acid receptors) to 10^5 molecules per cell (GATA- 1 factor in erythroid cells) (Nicolas and Goodwin, 1993). The exact detection limit of gel retardation is not known but sensitivity can be maximised by labelling substrate DNA to a high specific activity and careful preparation of protein extract.

Protein extracts suitable for gel mobility shift assays may be prepared from isolated nuclei or whole cells. In general the use of nuclear extracts is preferable due to the relatively high concentration of transcription factors they contain. However, intact nuclei from *B. napus* embryos could not be prepared due to limited material (nuclear membranes are disrupted on freezing). As an alternative, the preparation of extracts from whole cells has several advantages. It enables the entire DNA- binding protein content of the cell to be examined rather than those transcription factors with access only to the

DNA. In addition, fewer manipulations involved in the preparation of whole cell extracts minimises loss and damage of transcription factors.

Once bound, the protein- DNA complexes must be resolved from unbound nucleic acid, which involves maximising their stability within the gel during electrophoresis. In addition, the significance of detectable complexes depends largely upon the specificity of the interactions involved, assay conditions must therefore favour the formation of specific complexes. The stability of these complexes is also an important factor to consider when optimising electrophoresis conditions.

3.1.4 The ACP05 promoter

Prior to this work, Safford and colleagues (1988) demonstrated that ACP isoforms in *B. napus* seed tissue were encoded by a seed specific multigene family. A genomic clone, ACP05 that corresponded to a seed- expressed cDNA was isolated (de Silva *et al.*, 1990). A 1.8kb *Pst* I- *Sst* I restriction fragment from the ACP05 gene promoter was subcloned into the plasmid vector pTZ18R to generate the subclone pTZ5PS. This contained 928bp ACP 5' flanking promoter DNA and ~0.8kb of the transcriptional unit of the ACP gene. pTZ5PS (de Silva *et al.*, 1992) was made available for this study and a restriction map is presented in figure 3.2. The expression pattern of the ACP05 gene was analysed in transgenic tobacco transformed with promoter-reporter gene fusions. 1.4Kb of 5' ACP05 sequence was sufficient to direct tissue specific and temporal expression (de Silva *et al.*, 1992). It was concluded that the isolated ACP05 promoter contained all the regulatory information required for the correct pattern of expression (de Silva *et al.*, 1992).

3.1.5 **Aim of experiment**

The aim of this work was to build up a profile of the protein- binding sites in ACP05 promoter using the gel retardation assay to detect sequence specific interactions. Of particular interest in this study were tissue specific interactions, as the ACP05 gene was known to correspond to a seed

expressed ACP isoform (deSilva *et al.*, 1990). Each assay was therefore performed with extract from embryo and leaf tissue, on the assumption that the profile of transcription factors directing spatial expression would be different in each extract. It was envisaged that this should enable the identification of complexes specific to seed extract.

3.2 **Results**

3.2.1 Subcloning and sequencing the ACP05 promoter

The ACP05 subclone pTZ5PS was not fully sequenced in the promoter region (deSilva *et al.*, 1992). The object of the study was to determine putative DNA binding motifs so a restriction fragment from the ACP05 promoter (see panel A of figure 3.2) was subcloned and fully sequenced in both directions.

pTZ5PS was digested with *Ava* I and *Pst* I to liberate a 916bp promoter fragment which was gel purified (section 2.9.7.2) and ligated into pUC19 to generate pUC5PA. The size of clones was estimated by PCR amplification (section 2.9.14) and analysed for internal restriction sites (figure 3.1). Uncut PCR product was run in lane 1 and a single band of approximately 1038bp was seen, which corresponds to amplified promoter (plus primer sequences). The *Eag* I digestion was run in lane 2. Two bands of the expected size (578 and 437) were observed. The *Pvu* II digestion was run in lane 3 and again produced the expected fragments (536 + 479bp). It was concluded that the promoter fragment had been subcloned successfully in pUC5PA and was subsequently sequenced (section 2.9.10). The sequencing strategy is indicated in panel B of figure 3.2.

3.2.2 Sequence analysis of the ACP05 promoter

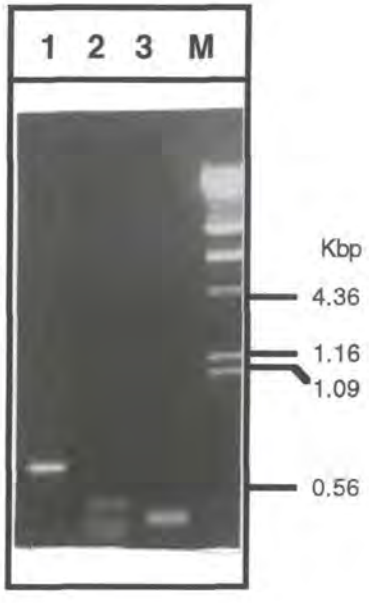
The nucleotide sequence data from each set of primers (approximately 300bp after editing) overlapped (panel B), was collated and is presented in panel C of figure 3.2. The nucleotide sequence of pUC5PA was compared to the published pTZ5PS data and no differences were found. pUC5PA was analysed for repeat sequences and palindromes (see section 2.9.10.2).

Figure 3.1

PCR analysis of pUC5PA

10µg pTZ5PS was digested with *Ava* I and *Pst* I to excise a 916bp promoter fragment. This was gel purified, recovered by freeze squeeze and ligated into *Ava*I- *Pst*I cut pUC19 to generate pUC5PA. The size of clones was determined by PCR amplification using the standard forward and reverse primers for pUC19 (listed in table 2.1A, section 2.9.14). 50ng of the PCR product was digested with *Eag* I and *Pvu* II (both known to have internal sites in the promoter) and analysed on a 1% agarose gel.

The lanes contained: **1- 3**, 50ng PCR product of pUC5PA; **1**, uncut (1.38kb); **2**, *Eag* I cut (578 + 437bp); **3**, *Pvu* II cut (536 + 479bp); **M**, 1.0µg *Hin* DIII cut λ DNA.



Several features of interest were identified and are indicated in panel C of figure 3.2. A motif, the core of which was AAGAC was noted because of the high frequency which it appeared and its palindromic arrangement. The presence of multiple binding sites is a common feature of eukaryotic enhancers (Schaffner *et al.*, 1988), for example the light responsive elements of pea ribulase bisphosphate carboxylase are repeated (Green *et al.*, 1987; Kuhlemier *et al.*, 1988) as are the heat shock elements in soybean (Bauman *et al.*, 1987). Within 500bp of the start of transcription, the sequence AAGAC appears five times (both orientations included). A specific 5bp sequence would only be expected to appear once every 1024bp by random chance, so the number of times that it did appear was significantly greater than expected by random chance. Each AAGAC motif (both orientations) with the immediate flanking sequence is given below in table 3.1. The motif is numbered from the first A in the motif (or first G in the complementary sequence, GTCTT) relative to the start of transcription.

Table 3.1: The AAGAC core motif in the ACP promoter

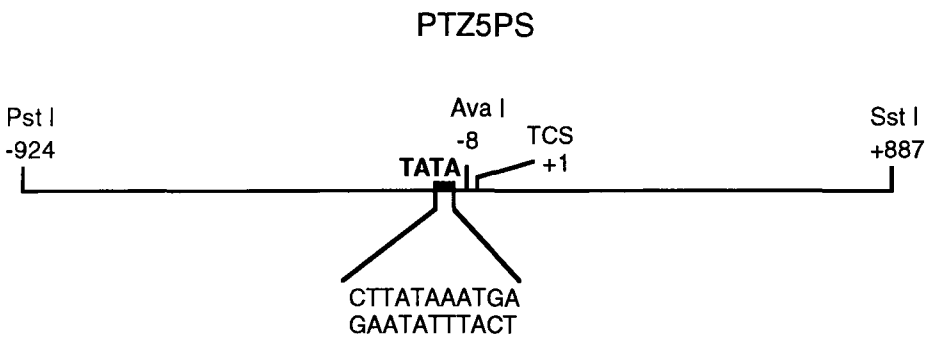
Motif number	DNA Sequence	Position in ACP promoter
1	AAAGG**GTCTT**TCTTTC	-470
2	TGGTT**AAGAC**TGGTT	-385
3	AATTA**GTCTT**TGTTT	-360
4	CGGTT**AAGAC**CGGTT	-190
5	AATTT**AAGAC**TTAAC	-93

Figure 3.2

Subcloning and sequencing the ACP promoter

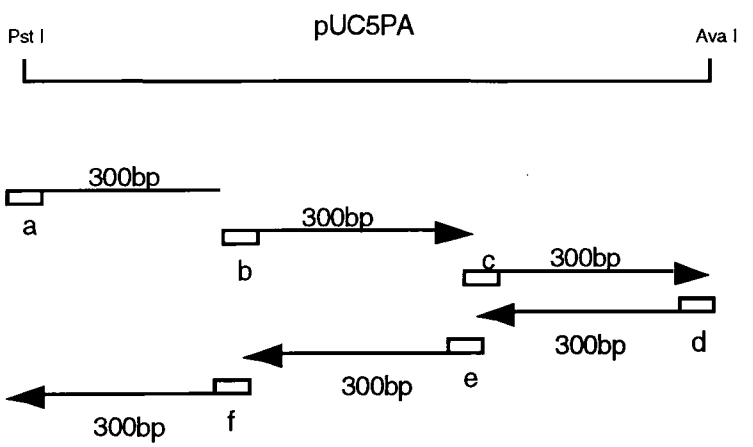
A: Subcloning

pTZ5PS contained a ~1.8kb *Pst* I- *Sst* I fragment from the genomic clone ACP05(de Silva *et al.*, 1992). A map is given below, with restriction sites numbered relative to the start of transcription (TCS= +1). A 916bp *Pst* I- *Ava* I restriction fragment that contained the TATA box (as indicated) was subcloned into pUC19 to form pUC5PA.



B: Sequencing strategy of pUC5PA

pUC5PA was sequenced using universal M13 primers and the specific internal primers that follow: Sequencing primers: a - M13 Reverse primer; b - oligo 701; c - oligo 320; d - M13 -20 primer; e - oligo 321; f - oligo 730. The sequence of primers is given in table 2.1C (section 2.9.14).



C. Nucleotide sequence of pUC5PA

The sequence is numbered relative to the start of transcription (+1). The TATA box is underlined and the motif AAGAC and its compliment GTCTT are in bold typeface.

```
|    10       |    20       |    30       |    40       |    50       |    60       |
CTGCAGCCAG AAGGATAAAG AAATTTTGGA CGCCTGAAGA AGAGGCAGTT CTGAGGGAAG -865
GAGTAAAAGA GTATGTCTCC TTAACTCTAC TATCAAGTTT CAAGAAGCTG AGCTTGGCTC -805
TACCTTGATA TGTTTATTGC TGTTGTGCAG GTATGGTAAA TCATGGAAAG AGATAAAGAA -745
TGCAAACCCT GAAGTATTCG CAGAGAGGAC TGAGGTGAGA GAGCATGTCA CTTTTGTGTT -685
ACTCATCTGA ATTATCTTAT ATGCGAATTG TGAGTGGTAC TAAAAAAGGT TGTAACTTTT -625
GGTAGGTTGA TTTGAAGGAT AAATGGAGGA ACTTGGTTCG GTAGCCGTAA CAAGTTTTTG -565
GGAATCTCTT GGGTTTTAAA TTGCTATGGA GTTTTTTTTT GCCTGCGTGA CAACATATCA -505
TCAGCTGTTG AGAAGGAAGA TGGTATTAGA AAGGGTCTTT CTTTCACATT TTGTGTTGTG -445
GACAAATATT AAAGTCAAAT GTGGCACATG GATTTTAATT CGGCCGGTAT GGTTTGGTTA -385
AGACTGGTTT AACATGTATA ATTAGTCTTT GTTTTATTTG GCTCAGCGGT TTGTTGGTGT -325
TGGTTAGGAA CTTAGGCTTG TCTCTTTCTG ATAAGATCTG ATTGGTAAGA TATGGGTACT -265
GTTTGGTTTA TATGTTTTGA CTATTCAGTC ACTATGGCCC CCATAAATTT TAATTCGGCT -205
GGTATGTCTC GGTTAAGACC GGTTTGACAT GGTTCATTTC AGTTCAATTA TGTGAATCTG -145
GCACGTGATA TGTTTACCTT CACACGAACA TTAGTAATGA TGGGCTAATT TAAGACTTAA -85
CAGCCTAGAA AGGCCCATCT TATTACGTAA CGACATCGTT TAGAGTGCAC CAAGCTTATA -25
TGACGACGAG CTACCTCGGGGCA                                          +1
```

Extended sequence homology between motif numbers 1 and 3 and 2 and 4 was noted and is shown below (the core motif in bold typeface).

1 TAGAAAGG**GTCTT**TCTTTCACATTTTG

3 TAT AAT TA **GTCTT**TGTTT -A- - TTT- G

2 GGTT**AAGAC**TGGTTT AACATG

4 GGTT**AAGAC**CGGTTTGACATG

The gel mobility shift analysis of the ACP05 promoter was designed to address whether this motif represented a protein binding site and if interactions were detected, to determine whether they were tissue specific.

3.2.3 Promoter probe preparation for gel retardation

The preparation and labelling of DNA probes for gel retardation analysis is complicated by several factors. These include size of the DNA fragment (the optimal size is below 200bp, to enable clear distinction of the free probe from complexes) and exposure to factors that might alter structure and (therefore) capability to bind transcription factors accurately. For example, it has been reported that small DNA fragments can denature following ethanol precipitation (Svaren *et al.,* 1987). The resultant single stranded species migrates aberrantly during PAGE and strongly binds proteins found in nuclear extracts (Svaren *et al.,* 1987). Such restrictions complicate probe preparation by standard techniques.

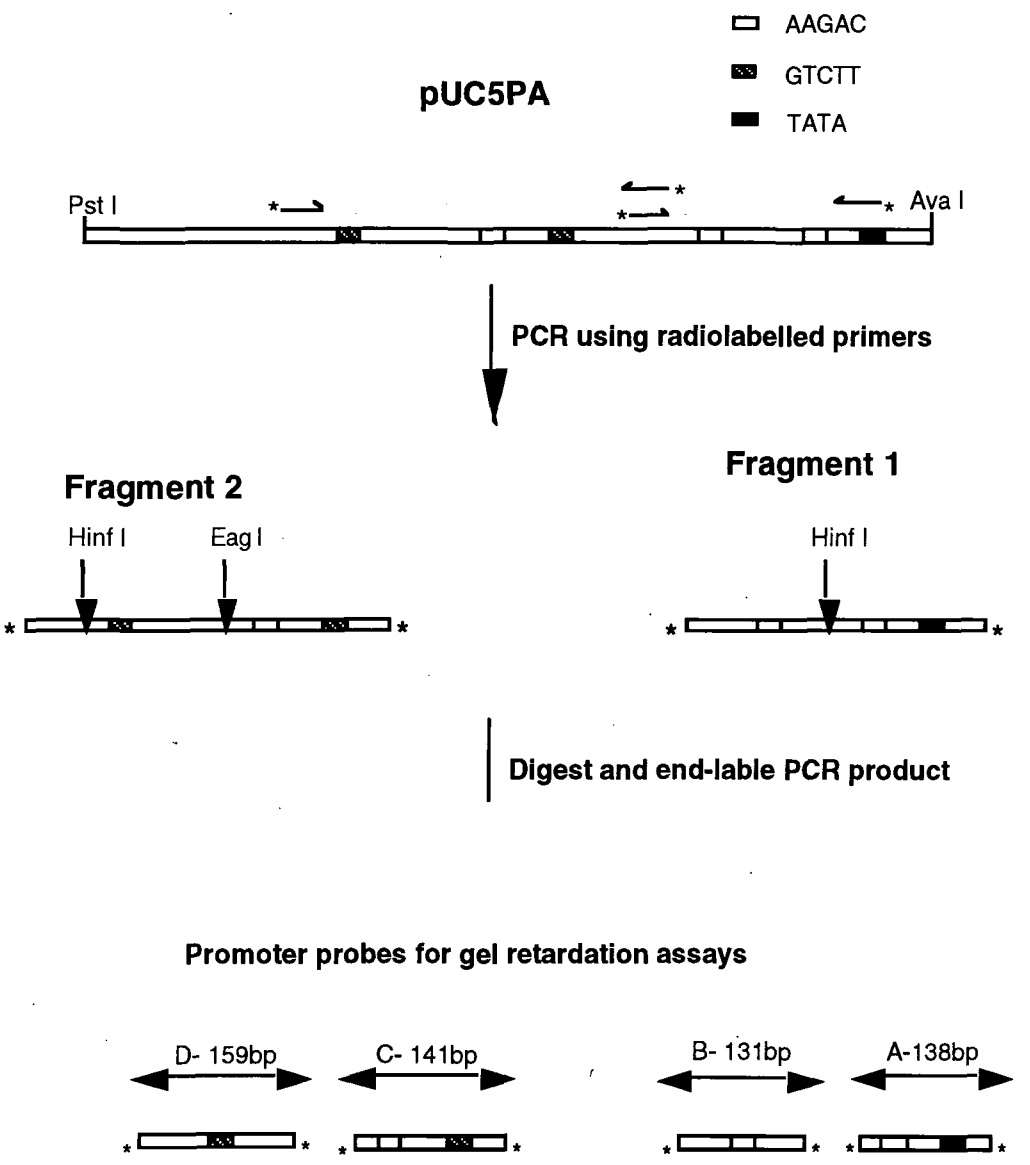
3.2.3.1 Strategy

A strategy based on the PCR amplification of promoter probes was developed. An overview of the protocol is shown in figure 3.3. Two promoter probes, fragments 1 and 2, were amplified using endlabelled

Figure 3.3

Overview of probe preparation for gel retardation.

Promoter probes for gel retardation analysis were generated by PCR amplification. The strategy is illustrated below. Radiolabelled (indicated by star) primers were used to amplify two promoter fragments, which were purified, and restricted to generate four probes for gel retardation assays.



oligonucleotides as primers. The PCR products were gel purified, then digested and end- filled with radiolabelled nucleotides, to generate the four probes A, B, C and D, each of which were assayed by gel retardation. Each probe contained at least one copy of the sequence AAGACT/C (or the complimentary sequence: G/AGTCTT).

3.2.3.2 PCR for probe amplification

Two pairs of oligonucleotides complimentary to internal sequences were used as PCR primers. The sequences of these and their position within the ACP promoter relative to the start of transcription are given in table 2.1B (section 2.9.14). Two PCRs were performed:

Reaction 1: For 1 and Rev 1 --------- fragment 1 (269bp)

Reaction 2: For 2 and Rev 2 --------- fragment 2 (400bp)

Two PCR reactions for each set of primers were performed. One reaction was primed with non- radiolabelled oligonucleotides and the second with radiolabelled oligonucleotides. Tandem reactions allowed analysis of reactions by standard techniques (using the non- radioactive reaction).

3.2.3.3 Primer preparation

Each primer was diluted to 5.0μM with H_2O. Ten pmol (2.0 μl of each diluted primer) was kinase labelled with 20μCi γ^{32}P ATP (>5, 000 Ci/mmol) (section 2.9.11.2) or 0.1mM ATP. The primers were ethanol precipitated, washed twice with 200μl 80% ethanol and resuspended in 6μl of H_2O. One μl of the radioactive reactions was counted by liquid scintillation. Oligonucleotides were routinely labelled to a specific activity of $1x10^7$ cpm/pmol. The remaining 5.0μl was used directly in the PCR as primer.

3.2.3.4 PCR conditions

The standard PCR method (section 2.9.14) was used to amplify pUC5PA. An internal size control PCR was performed in tandem using pUC forward and reverse primers (table 2.1A, section 2.9.14) to amplify a defined insert (280bp product).

3.2.3.5 Analysis and purification of PCR products

Following amplification each non- radioactive PCR product was sequenced (section 2.9.10) to confirm their integrity before subsequent use as probes in gel retardation assays. Sequence differences were not observed in the amplified fragments (data not shown).

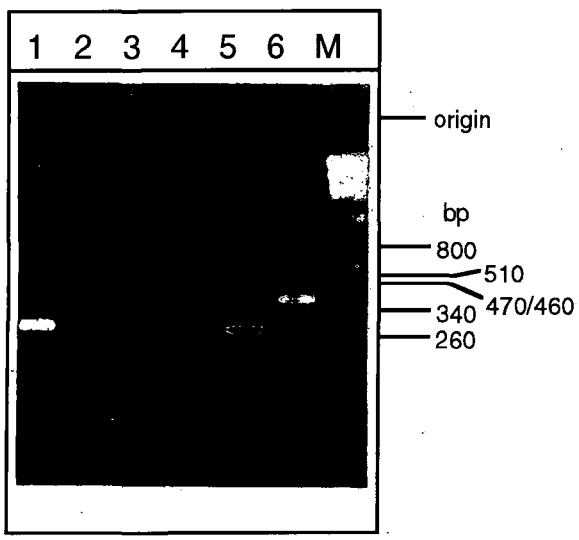
Each non- radioactive PCR was analysed by gel electrophoresis (panel A, figure 3.4). A product of the expected size (280bp) was observed in lane 1, which contained the positive control reaction. The PCR with primers for1 and rev1 amplified a fragment of 269bp (expected size for promoter fragment 1), as can be seen in lane 5. The PCR with primers for 2 and rev 2 amplified a fragment of 400bp (expected size for promoter fragment 2) as can be seen in lane 6. Negative controls failed to amplify any product (lanes 2- 4).

The remaining PCR products were gel purified (section 2.9.7.1), resuspended in 40µl TE and quantified by standard methods (2.9.4.2). The purified promoter fragments were analysed for internal restriction sites (see table 3.2 below). The digestions were fractionated by gel electrophoresis (panel B of figure 3.4).

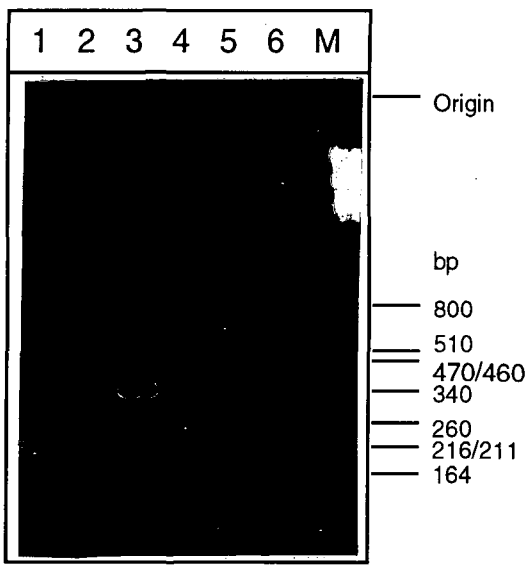
Figure 3.4 Probe preparation for gel retardation assays

Two fragments were amplified from pUC5PA using internal primers (table 2.1B, section 2.9.14). 20µl of each non- radioactive PCR was analysed by electrophoresis through a 1% agarose gel (panel A). The fragments were gel purified and analysed for internal restriction sites. Digestions were fractionated on a 1.5% agarose gel (panel B). Gels were calibrated with *Pst* I digested λ DNA (lane M).

A: PCR



B: Analysis of purified PCR products



The lanes contained:

Panel A: 1-6, 20µl PCR; **1**, size control template (280bp); **2**, no primer control; **3**, no template control; 4, no enzyme control; **5**, pUC5PA template, primers For 1 and Rev 1 (269bp---fragment I); **6**, pUC5PA template, primers For 2 and Rev 2 (400bp---fragment 2); **M**: 1.0µg *Pst* I cut λ– DNA.

Panel B: 1: 50ng fragment 1 (269bp); **2**: 50ng *Hinf* I cut fragment 1 (138 and 131 bp); **3**: 100ng fragment 2 (400bp); **4**: 100ng *Hinf* I cut fragment 2 (300 and 100bp); **5**: 100ng *Eag* I cut fragment 2 (259 and 141bp); **6**: 100ng *Hinf* I / *Eag* I cut fragment 2 (159, 141 and 100bp); **M**, 1.0µg *Pst* I cut λ– DNA.

Table 3.2: Restriction Sites in Fragments 1 and 2

PCR Product	Enzyme	Digestion Fragments (bp)	Lane
Fragment 1		269	1
Fragment 1	HinfI	138 (probe A) 131 (probe B)	2
Fragment 2		400	3
Fragment 2	HinfI	300 and 100	4
Fragment 2	EagI	259 and 141	5
Fragment 2	HinfI + EagI	141 (probe C) 159 (probe D) 100	6

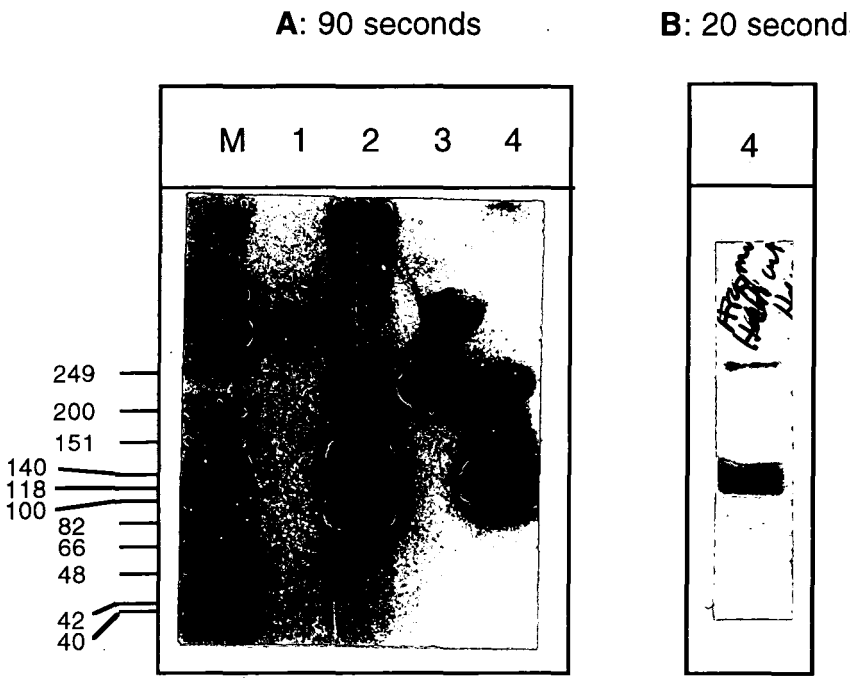
3.2.3.6 Purification of promoter probes

Radiolabelled fragments 1 and 2 were digested with *Hinf* I and *Eag* I and the overhangs generated were end filled *in situ* using α^{32}P dCTP and dATP (section 2.9.11.3). The digests were gel fractionated (2.9.6.2) and the probes were located by autoradiography (figure 3.5). A 20 second exposure of the gel is shown in panel B and probes A (138bp) and B (131bp) are indicated. A 90 second exposure is shown in panel A and probes C (141bp) and D (159bp) can be seen. Probes were excised from the gel and electroeluted (2.9.7.1). Purified probes were diluted to 20 000 CPM/µl and quantified using standard methods (2.9.4.2). Typically 1.0 µl DNA =10- 20,000CPM = 1ng was used per assay.

Figure 3.5

Gel purification of promoter probes A, B, C and D

Purified, radiolabelled fragments were digested as follows: 500ng of fragment 1 DNA was digested with 1U of *Hinf* I at 37^{0}C for 2 hours. 500ng of fragment 2 DNA was double digested with 1U of *Hinf* I and 1U of *Eag* I at 37^{0}C for 2 hours. The *Eag* I and *Hinf* I overhangs generated were end filled *in situ* using 20μCi each of α^{32}P dCTP (>5 000Ci/mM) and α^{32}P dATP (>5 000Ci/mM), 1.0μl 1mM dGTP, 1.0μl 1mM dTTP and 0.5 U Klenow fragment. The digests were fractionated on a 15% TBE buffered acrylamide gel, calibrated with radiolabelled *Hinf* I digested ϕX174 markers.



The lanes contained: **1**, 50ng unrestricted fragment 2; **2**, 500ng *Hinf* I/ *Eag* I restricted fragment 2; **3**, 50ng unrestricted fragment 1 DNA; **4**, 500ng *Hinf* I restricted fragment 1; **M**, *Hinf* I digested θX174 markers. Panel **A**: autoradiograph generated from 90 second exposure; panel **B**: autoradiograph generated from 20 second exposure.

3.2.4 Optimisation of gel retardation assay incubation conditions

The detection of protein- DNA interactions depends both on the formation and subsequent stability of the complex within the gel, which in turn depend upon the specific incubation (in large part the binding buffer) and electrophoresis conditions. The TATA box from the ACP promoter was incorporated in a pair of synthetic complimentary oligonucleotides TATA1 and TATA2 which were used as probes in gel retardation assays. It was envisaged that the annealed oligonucleotides would form a sequence specific complex with the TATA binding factor (which is a component of the basal transcription factor TFIID). This interaction could then be used as a index when optimising binding conditions.

3.2.4.1 Substrate DNA

The single stranded oligonucleotides TATA1 and TATA2 were complimentary and encompassed the TATA box from the ACP05 promoter. The sequence of oligonucleotides used as probes is presented is table 3.3. TATA oligonucleotide was labelled and annealed as described in section 4.2.3 and 10,000cpm double stranded DNA was used as substrate DNA per assay (section 2.9.15.1).

3.2.4.2 Parameters varied.

The basic assay was performed in 20mM Tris.HCl pH 7.5, 1mM DTT, 1mM EDTA, 3% glycerol for 15 minutes. The pH, incubation time, salt concentration (stringency) and additon of loading dye were varied and the effect on DNA binding analysed (figure 3.6). The assay run in lane one contained no protein and the mobility of the unbound probe is indicated. Non- specific competitor, poly (dIdC:dIdC), was excluded from lane 2 and the majority of the probe is shifted in non- specific complexes which are seen as a smear of radioactivity up the gel. Assays with the various buffers (listed in the figure) were run in lanes 3- 8, respectively. A single retarded complex (indicated with an arrow) was formed. The formation of this complex was consistent with it corresponding to probe bound- TATA binding factor. The complex is sensitive

to extremes of pH (compare intensity of retarded band in lanes 3 and 4 with that in lane 6) and the addition of loading dye (lane 5). The complex was stable in 100mM KCl (lane 6) and no additional complexes formed when incubation time was doubled from 10 to 20 minutes (lane 7 compared to 8). The assay was repeated using leaf protein extract, with identical results (not shown). Buffer 5 was used in subsequent assays. Further modifications to these assay conditions are listed at the first instance of use.

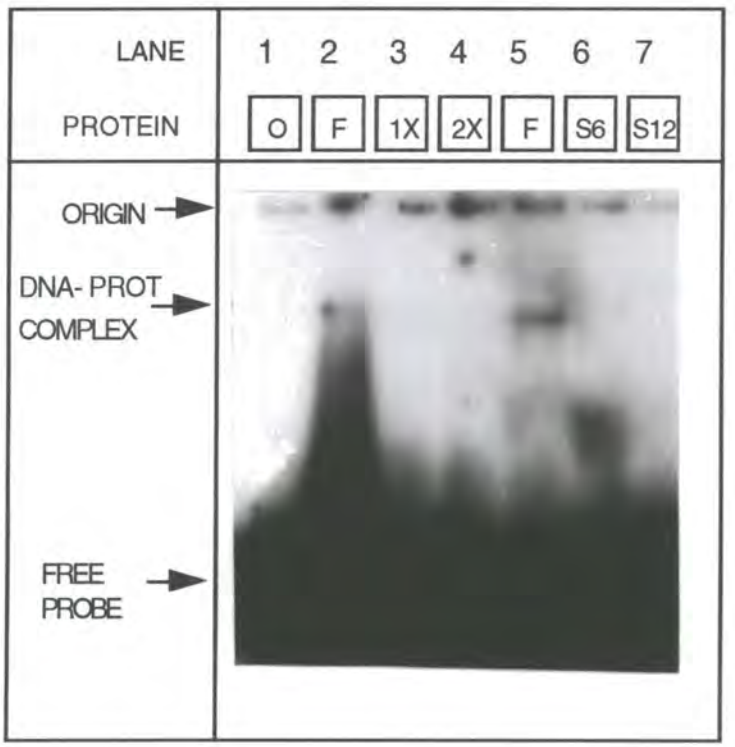
3.2.5 Analysis of protein extracts from *Brassica napus*

Deposition of lipid is at a maximum in mid maturation embryos (Norton and Harris, 1983; Safford *et al.*, 1988). In developing seeds the level of ACP has been show to rise prior to the onset of storage lipid synthesis (Safford *et al.*,1988), which would be consistent with the activation of specific members of the ACP gene family. It should be expected that maximal levels of transcription factors involved in the regulation of expression of ACP should also be present at this stage. In particular, those transcription factors involved in tissue specific regulation should be abundant. Tissue specific factors should be absent or present in very low abundance in leaf extract, which was used as a control to investigate this aspect of ACP expression.

Aliquots of protein extract (2.11.6) from both embryo and leaf were analysed by SDS- PAGE (2.11.1) as shown in panel A of figure 3.7. Staged embryo tissue was limited, therefore it was determined whether individual protein aliquots could be re- used. The effect of prolonged storage at -80°C on DNA binding activity was also investigated. The formation of the specific DNA-protein complex using TATA containing oligonucleotide (table 3.3) was again used as an index to assay DNA binding activity in the extracts.

Three aliquots of the same batch of embryo extract were assayed for the effects of freeze- thawing (to mimic re- use of a sample). One aliquot was kept at -80°C while the second aliquot was thawed on ice, refrozen in liquid nitrogen, then transferred back to -80°C. The third aliquot was thawed and refrozen twice. All three samples were then assayed for the effects of sample re- use on binding activity. The effects of prolonged storage on the activity of

B: Storage and re- use of protein extracts.



The lanes contained: **1,** 1.0µg poly (dIdC:dIdC), no protein (**O**); **2,** no poly (dIdC:dIdC), 1.0µl fresh embryo protein (**F**); **3- 7** 1.0µg poly (dIdC:dIdC); **3**, 1.0µl 1X thawed protein (**1 X**); **4,** 1.0µl 2X thawed protein (**2 X**); **5**,1.0µl fresh protein (**F**); **6,** 1.0µl protein that had been stored for 6 months (**S 6**); **7**, 1.0µl 12 month stored protein (**S 1 2**).

DNA binding proteins in embryo extract was analysed using different extracts, prepared in an identical manner, that had been stored at -80^{0}C for 6 and 12 months.

The results are presented in panel B of figure 3.7. Lane one contained no protein and the mobility of the free probe is indicated. Poly (dIdC:dIdC) was omitted from the assay in lane two and the non- specific shift of substrate DNA was observed. Fresh embryo extract was added to the assay in lane 5. A single retarded complex (indicated with an arrow) was observed as seen before. The formation of this complex was significantly reduced in extracts that had been thawed and re-used (lanes 3 and 4) and in extracts that had been stored for a prolonged period at -80^{0}C (lane 6, 6 months stored and 7, 12 months stored).

3.2.6 Gel retardation assays with ACP05 promoter probes

Three types of gel retardation assay: poly (dIdC:dIdC) titrations, competition assays and enzyme controls (discussed fully in section 3.1.2) were performed with each promoter fragment. Each type of assay was repeated at least 3 times with each probe to ensure that complexes were consistent.

3.2.6.1 Poly (dIdC:dIdC) titrations

To a series of standard assays an increasing amount of the non- specific competitor poly (dIdC:dIdC) was added in order to detect sequence specific complexes. Figure 3.8 is divided up into four panels, each presents a typical poly (dIdC:dIdC) titration for promoter fragments A- D, respectively.

A: Fragment A

A poly (dIdC:dIdC) titration (2.9.15.1) with fragment A using both embryo and leaf protein is shown in the first gel in panel A of figure 3.8. The assay in lane 1 contained no protein and the mobility of the free probe is indicated. In lanes 2 and 6 poly (dIdC:dIdC) was excluded from the assay and it can be seen that the probe is shifted in a smear of non- specific interactions. On the addition of poly (dIdC:dIdC) non- specific interactions are competed out as shown by the

titration in lanes 3- 5, with embryo protein and in lanes 7- 9 with leaf protein. Two specific complexes were formed with embryo extract (indicated with arrows). These complexes had similar mobilities and were difficult to resolve from each other. A single specific shift was observed with leaf extract. The mobility of this complex was approximately the same as the embryo complexes.

A much higher level of background was observed when promoter probes were used (as compared to oligonucleotide probes), which could have been as a result of an increased number of non- specific DNA binding interactions, or disintegration of complexes during electrophoresis. The electrophoresis buffer used was 0.5x TBE. A low ionic strength is required because at high salt concentration protein- DNA complexes carry a small fraction of the current and therefore do not migrate very far. Also a high salt concentration may lead to heating during electrophoresis. However a low salt buffer may result in a pH shift through buffer electrolysis which could reduce the stability of the protein- DNA complex.

anode: H_2O ---------> $2e^- + 2H^+ + 1/2\,O_2$ pH decreases

cathode: $2e^- + 2\,H_2O$ ---------> $2OH^- + H_2$ pH increases

In order to circumvent such problems, the buffer was double recirculated from the anode to the cathode and vice verse during electrophoresis. Protein-DNA complexes that dissociate quickly in binding buffer can be subjected to electrophoresis for several hours without substantial dissociation. This is possibly due to "caging" effects of the gel matrix (Hendrickson 1985; Revzin, 1989) or that the affinity of the protein for the DNA is increased in low ionic strength buffers used for electrophoresis. However differences in the composition of the binding and running buffers could alter protein binding during loading and entry of complex into the gel. To avoid these problems the wells of the gel were washed out with the binding buffer (made up without glycerol) before the assays were loaded. Immediately after which they were rapidly run in to the gel matrix at high voltage (500v, for approximately 30 seconds), before electrophoresis at 100V for an hour.

A second set of assays were performed using the modified conditions described above (section 2.9.15.2) and are presented in the second gel of panel A. Protein was excluded from the assay in lane 1 and the mobility of the free probe is indicated. Poly (dIdC:dIdC) was omitted in lanes 2 and 4 and it can be seen that substrate DNA was shifted in a smear of non- specific interactions. On the addition of the non- specific competitor poly (dIdC:dIdC) to a leaf assay one complex (as indicated) was observed (lane 3). This assay was repeated with embryo extract (lane 5) and two complexes were observed to form. Fragment A contains both an AAGAC sequence and the TATA box. It should therefore be expected to form a TFIID- bound complex with both leaf and embryo extract. However, if the AAGAC box was recognised by a specific factor then an additional band should be observed in tissues that contain this factor. From the poly (dIdC:dIdC) titrations (described above) one complex one complex formed with leaf extract and two formed with embryo extract. This would be consistent with one complex in both extracts corresponding to TFIID- bound DNA and the second, embryo specific complex corresponding to an interaction with a tissue specific factor. Competition experiments (presented in the following section) should demonstrate the binding specificity of each of the retarded bands.

The modified electrophoresis conditions were used in all subsequent assays when promoter fragments were used as substrate DNA.

B: Fragment B

Panel B of figure 3.8 shows a poly (dIdC:dIdC) titration with promoter fragment B. Protein was omitted from the assay in lane 1 and the mobility of unbound probe is indicated. Poly (dIdC:dIdC) was excluded from lanes 2 and 6, which were with embryo and leaf assays respectively. In both assays DNA was shifted non- specifically, bound in very large complexes. Lanes 3- 5 show a poly (dIdC:dIdC) titration with embryo extract. A single specific complex was resolved from non- specific interactions. The poly (dIdC:dIdC) titration was repeated with leaf extract, in lanes 6- 9. Probe was not retarded. The DNA-protein complex was therefore formed with an embryo specific factor. In subsequent assays 2.0μg poly (dIdC:dIdC) per assay was used.

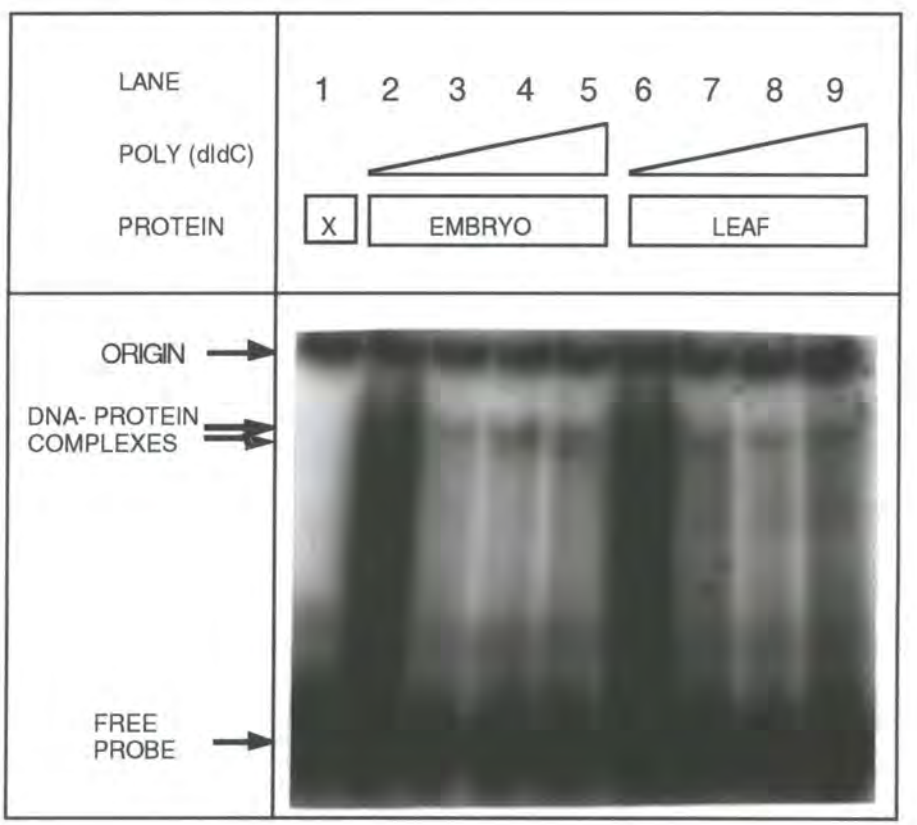
Figure 3.8: Poly (dIdC:dIdC) titrations

The figure is divided up into four panels, each presents a typical poly (dIdC:dIdC) titration for promoter fragments A- D, respectively. An increasing amount of poly (dIdC:dIdC) was added to a series of standard assays in order to detect sequence specific complexes. Assays were fractionated on 3% polyacrylamide native gels. In order to decrease the level of non- specific DNA- protein interactions several modifications were made to the basic electrophoresis conditions (details are given the text).

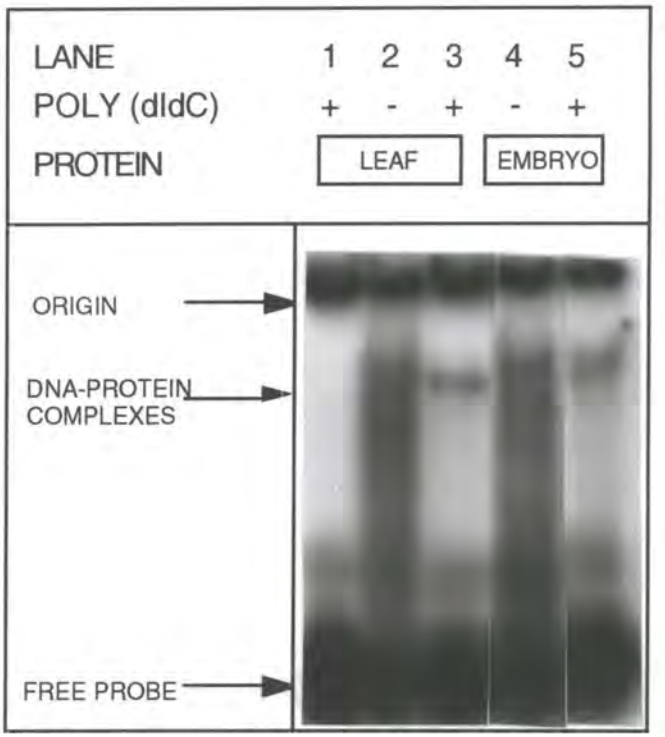
A: Promoter fragment A

The lanes contained: **1**, no protein, 1.0µg poly (dIdC); **2- 5,** 1.0µl embryo extract and 0, 1.0, 2.0, 3.0µg poly (dIdC), respectively; **6- 9**, 1.0µl leaf extract and 0, 1.0, 2.0, 3.0µg poly (dIdC), respectively.

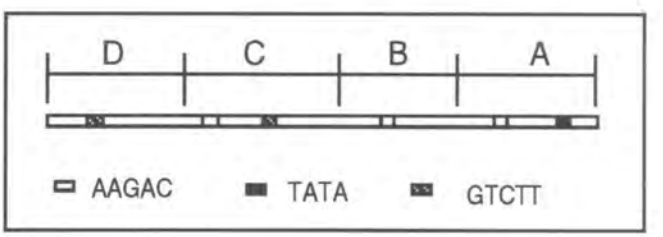
Gel 1: Unmodified Conditions



Gel 2: Modified conditions

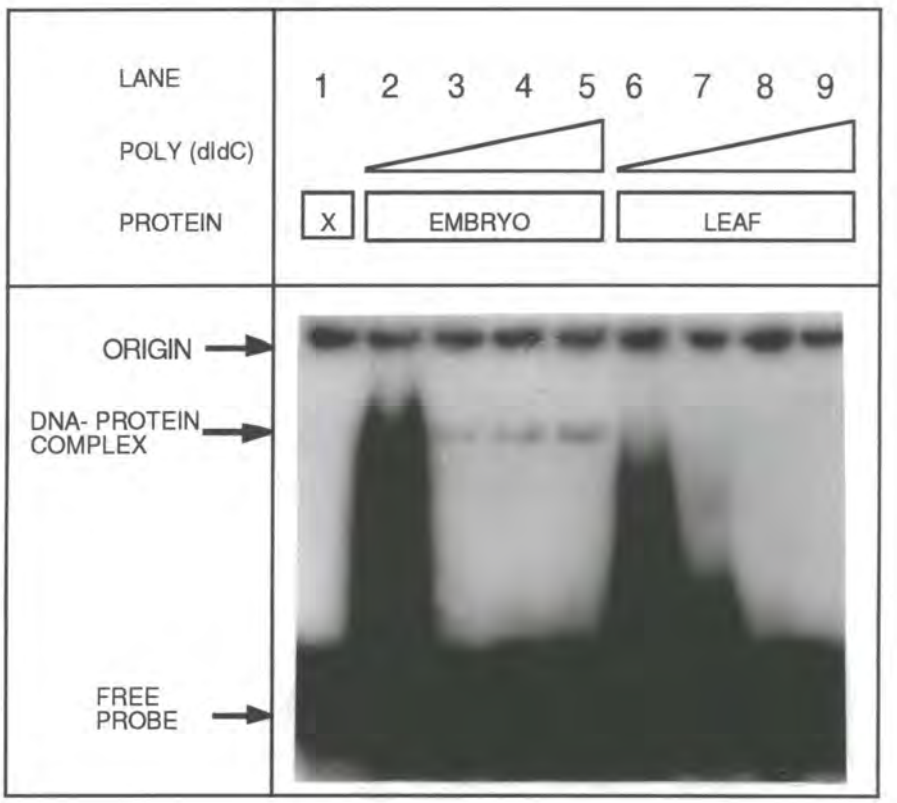


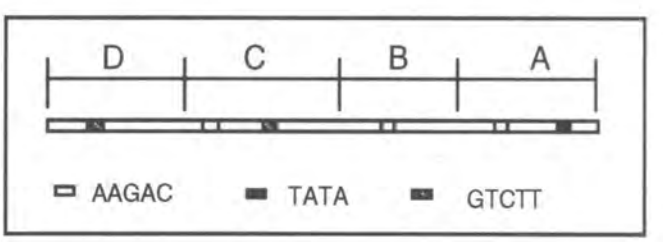
The lanes contained: **1**, no protein, 2.0µg poly (dIdC:dIdC); **2**, 1.0µl leaf protein, no poly (dIdC:dIdC), **3**, 1.0µl leaf protein, 2µg poly (dIdC:dIdC); **4**, 1.0µl embryo protein, no poly (dIdC:dIdC); **5**, 1.0µl embryo protein, 2µg poly (dIdC:dIdC).



B: Promoter fragment B

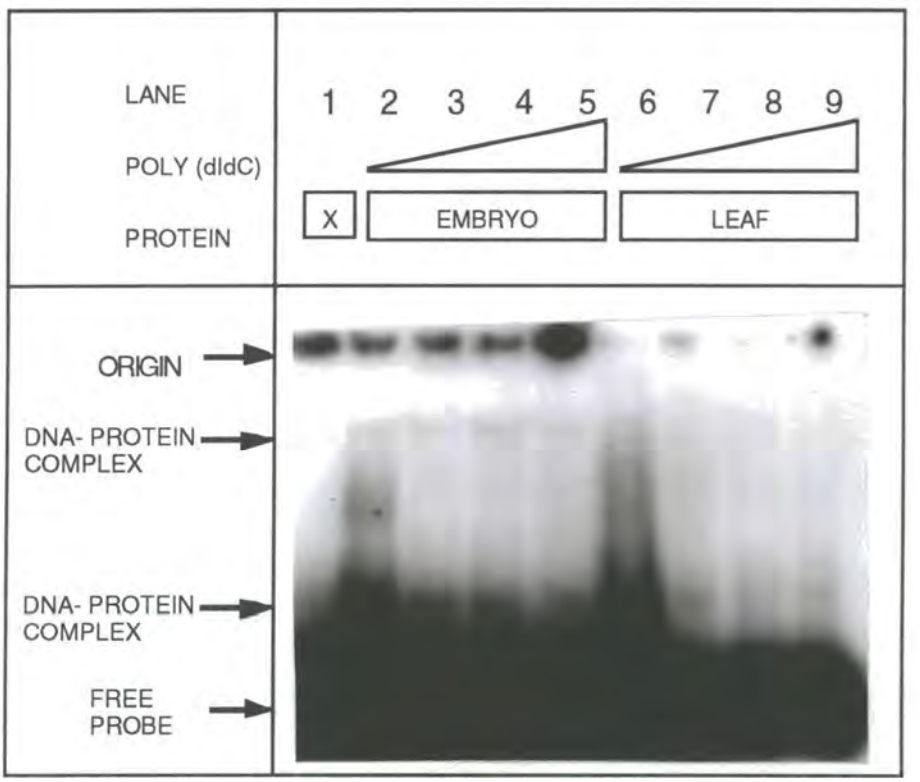
The lanes contained: **1**, no protein and 1.0µg poly (dIdC:dIdC); lanes **2-5** contained 1.0µl embryo extract and 0.0, 1.0, 2.0, 3.0µg poly (dIdC:dIdC) respectively; lanes **6-9** contained 1.0µl leaf extract and 0.0, 1.0, 2.0, 3.0µg poly (dIdC:dIdC), respectively.

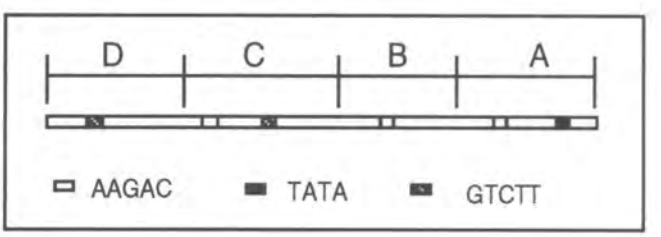




C: Promoter fragment C

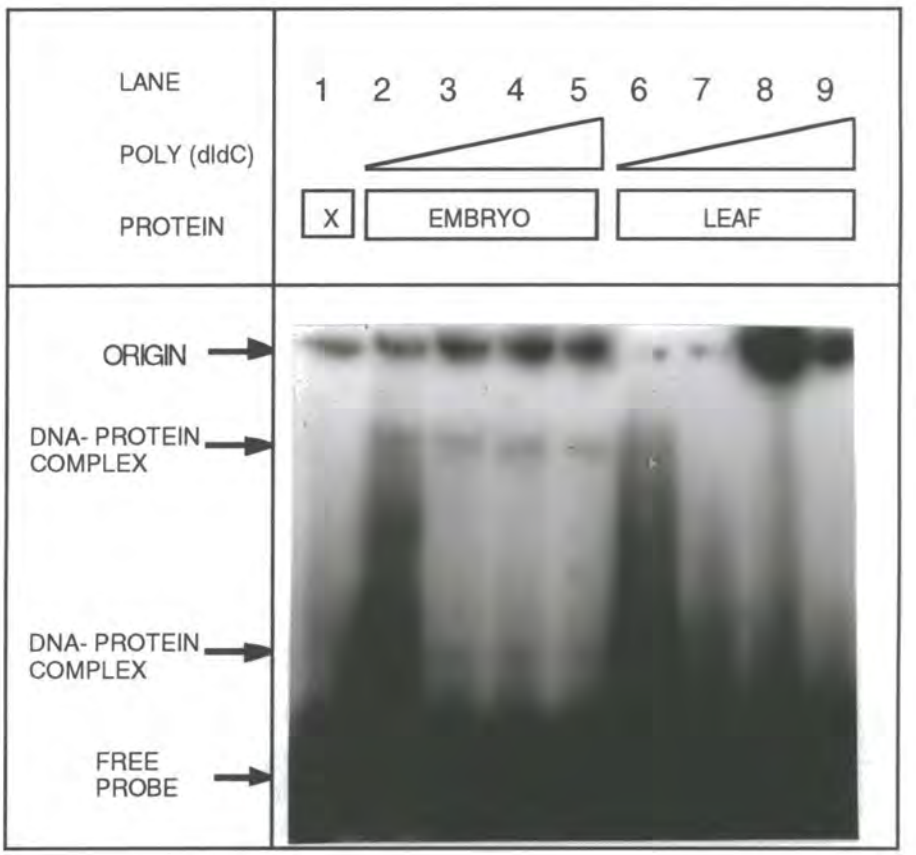
The lanes contained: **1**, no protein, 1.0µg poly (dIdC:dIdC); **2- 5**, 1.0µl embryo protein and 0, 1, 2, 3µg poly (dIdC:dIdC), respectively; **6- 9**, 1.0µl leaf extract and 0, 1, 2, 3µg poly (dIdC:dIdC), respectively.

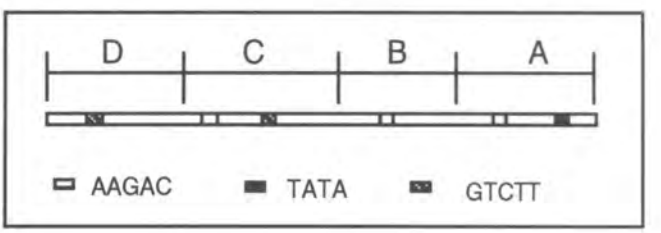




D: Promoter fragment D

The lanes contained: **1**, no protein, 1.0µg poly (dIdC:dIdC); **2- 5**, 1.0µl embryo extract, 0.0, 1.0, 2.0 and 3.0µg poly (dIdC:dIdC) respectively; **6- 9**, 1.0µl leaf extract with 0.0, 1.0, 2.0, 3.0µg poly (dIdC:dIdC) respectively.





C: Fragment C

A typical poly (dIdC:dIdC) titration with fragment C is presented in panel C of figure 3.8. Lane 1 contained no protein and subsequently free probe was observed. Poly (dIdC:dIdC) was not added to the assays in lanes 2 and 6 and the DNA substrate is shifted non- specifically. Increasing amounts (0.0- 3.0 μg) of non- specific competitor was added to lanes 3- 5 in a titration with embryo protein. Two complexes were observed and are indicated. The poly (dIdC:dIdC) titration was repeated with leaf extract (lanes 7- 9), and one complex was observed (with approximately the same mobility as the lower complex observed in embryo extract).

D: Fragment D

Panel D of figure 3.8 shows a poly (dIdC:dIdC) titration with fragment D, protein was excluded from the assay in lane 1 and unbound fragment D is indicated. Poly (didC:dIdC) was omitted from lane 2, and the typical non-specific shift of probe was observed. A poly (dIdC:dIdC) titration with embryo extract was performed in lanes 2- 5. Two sequence specific complexes formed and are indicated. The poly (dIdC:dIdC) titration was repeated with leaf extract in lanes 6-9. No specific complexes were observed with leaf proteins. In subsequent assays 2.0μg poly (dIdC:dIdC) per assay was used.

3.2.6.2 Competition assays

3.2.6.2.1 Design of oligonucleotides as probes and competitors

It was an objective of this analysis to determine whether the AAGAC motif participated in the formation of any of the embryo specific complexes observed following the poly (dIdC:dIdC) titrations with promoter probes (discussed in the previous section). Oligonucleotides that contained the AAGAC motif and a mutated version of the motif (listed in table 3.3) were synthesised and used as competitors to determine the role of this motif in complex formation.

Table 3.3: Complimentary oligonucleotides used as probes and competitors in gel retardation assays

Oligo	Sequence	Comments
TATA1:	5' CAAG**CTTATAAATGA**CGACGAG 3'	contained TATA box
TATA2:	3' GTTC**GAATATTTACT**GCTGCTC 5'	
BS1:	5' CGCATGCTT**AAGAC**TGGTAC 3'	contained AAGAC motif
BS2:	3' GCGTACGAA**TTCTG**ACCATG 5'	
MBS1:	5' GCGATGCTT**GATAC**TGGTAC 3'	identical to BS1:BS2, apart from altered bases in AAGAC
MBS2:	3' CGCTACGAA**CTATG**ACCATG 5'	

Oligonucleotides (oligos) TATA1 and TATA 2 were complimentary and encompassed the TATA box (in bold text) from the ACP05 promoter. Nine out of the eleven bases from the TATA box of the ACP promoter fit the consensus plant TATA box sequence (CCTATAAATTA). Oligos BS1, BS2 contained an AAGAC motif (in bold). Oligos MBS1, MBS2 were identical to BS1 and BS2 apart from the altered bases in the AAGAC motif (underlined). Complimentary oligos were annealed and referred to as BS (Binding Site), MBS (Mutated Binding Site) and TATA.

3.2.6.2.2 The preparation of oligonucleotides as probes and competitors

Oligonucleotide probes were kinase labelled and annealed using the standard method (2.9.11.2). Unincorporated label was removed by chromatography through a Bio-Rad P- 30 gel filtration column. Purified probe was counted by liquid scintillation and diluted to 10- 20,000 CPM/µl. Typically 1.0 µl (~0.3ng) double stranded DNA was used in each gel retardation assay. Oligonucleotides that were required as specific competitors were

phosphorylated with 1mM ATP and annealed by the standard methods (section 2.9.11.2).

3.2.6.2.3 Competition assays

The four panels in figure 4.9 present competition assays for each of the complexes detected.

A: Fragment A

Fragment A formed three specific complexes, two with embryo factor(s) and one with leaf factor(s) (see panel A of figure 3.8). Promoter fragment A contained the TATA box, and therefore it should be expected that fragment A form a TFIID bound complex with both embryo and leaf protein extracts. (The TATA binding protein is a component of the general transcription factor TFIID).

A competition assay is shown in panel A of figure 3.9. Competition of embryo complexes is shown in the first gel. Lane 1 contained no protein and the mobility of the free probe is indicated. Poly (dIdC:dIdC) was not added to the assay in lane 2 and the probe was shifted in non- specific interactions. Both non- specific competitor and leaf protein were added to the assay in lane 3 and the formation of the specific leaf complex was observed. The effect of the competitor oligo BS on this complex was observed in lane 4, the addition of a 10x molar excess of BS had no effect. This assay was repeated in lane 5, with embryo extract. It was observed that one complex was titrated out and the remaining band had the same mobility as the leaf complex. This would be consistent with the competed complex corresponding to an interaction between fragment A and an AAGAC- binding factor and the remaining complex corresponding to probe- bound TFIID. The specificity of this complex was analysed in lane 6, with the further addition of a 10X molar excess of competitor MBS. No effect on its formation was observed, however a repeat of this assay with a 10x molar excess of oligonucleotide TATA resulted in its competition. These results are consistent with this complex corresponding to an interaction between the TATA box and TFIID.

Competition of the specific leaf complex is presented in the second gel of

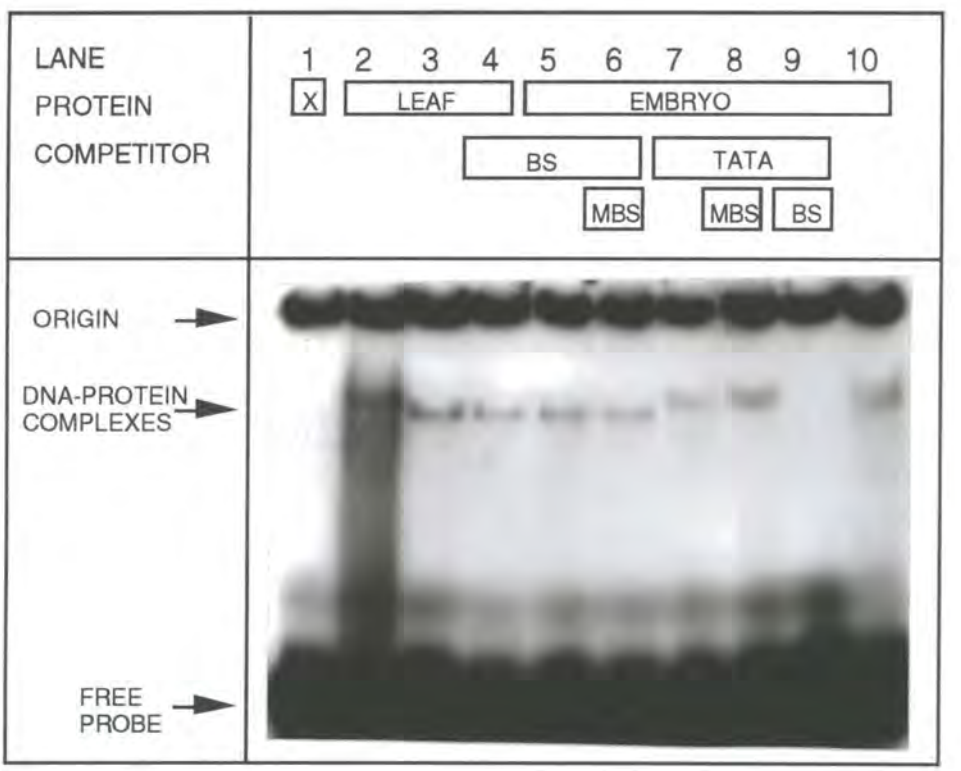
Figure 3.9 Competition assays

The figure is divided into four panels, each presents a competition assay for a complex formed with the promoter fragments. Sequence specificity was analysed with oligonucleotide (oligo) competitors. The sequence of these are given in table 3.3 (see text). A set of standard assays were performed and fractionated on 3% polyacrylamide native gels.

A: Promoter fragment A

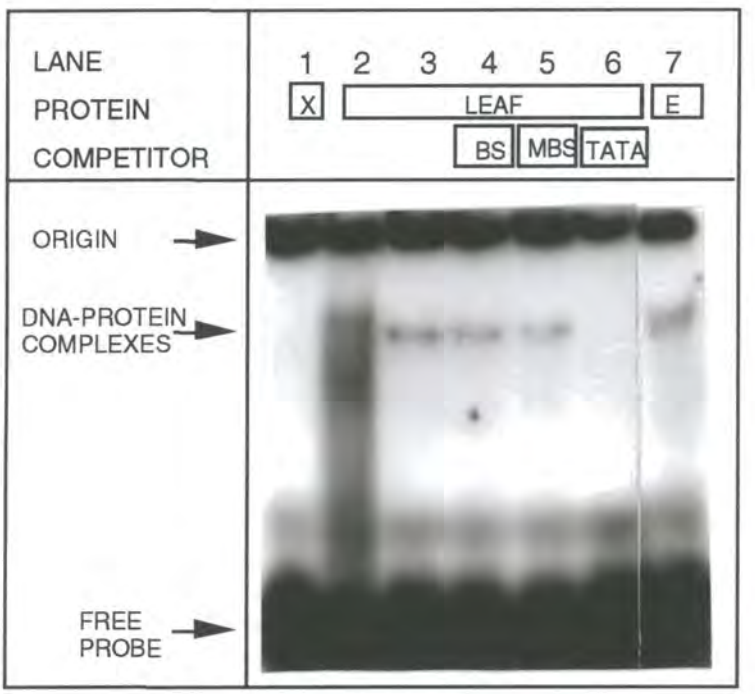
GEL 1- Competition of embryo specific complexes

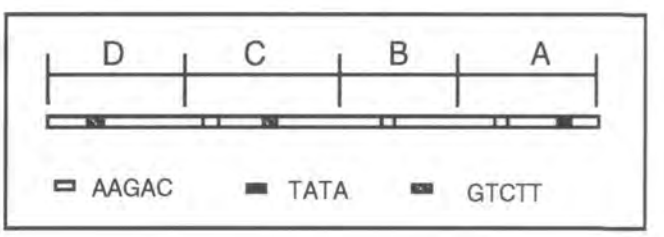
The lanes contained: **1**, no protein, 3.0μg poly (dIdC); **2**, 1.0μl leaf protein, no poly (dIdC); **3**, 1.0μl leaf protein, 3.0μg poly (dIdC); **4**, 1.0μl leaf protein, 3.0μg poly (dIdC), 1.5ng oligo competitor BS; **5- 10**, 1.0μl embryo protein, 3.0μg poly (dIdC); **5**, 1.5ng oligo BS; **6**, 1.5ng each BS oligo and MBS oligo; **7**, 1.5ng TATA oligo; **8**, 1.5ng each TATA and MBS oligos; **9**, 1.5ng each TATA and BS oligos; **1 0**, unmodified assay.



GEL 2- Competition of specific leaf complex

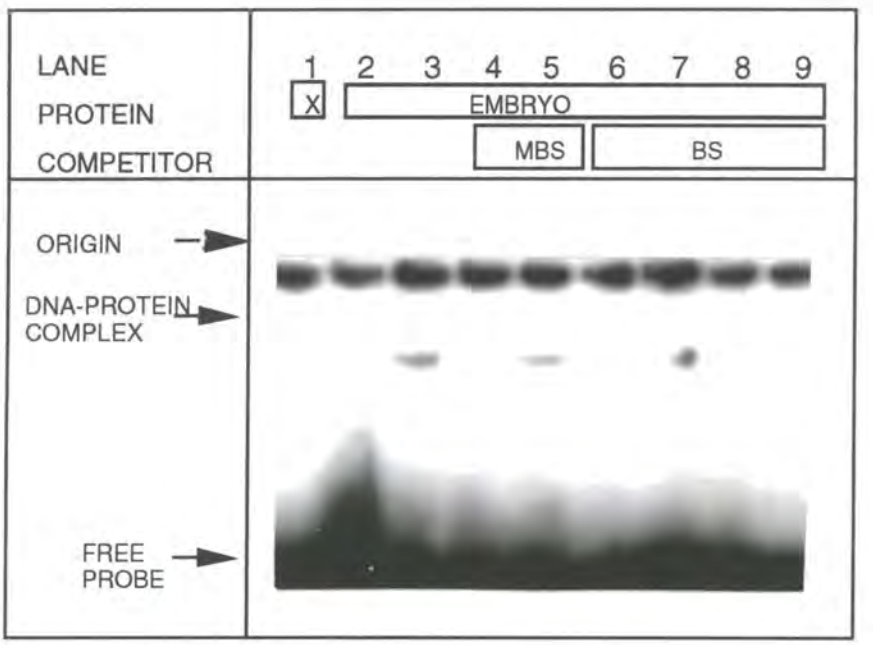
The lanes contained: **1**, no protein, 3.0μg poly (dIdC:dIdC); **2**, 1.0μl leaf protein and 0.0μg poly (dIdC:dIdC); **3-6**, 1.0μl leaf protein and 3.0μg poly (dIdC:dIdC); **4**, 1.0μl 1.5ng BS oligo competitor; **5**, 1.5ng MBS oligo; **6**, 1.5ng TATA oligo; **7**, 1.0μl embryo protein and 3.0μg poly.

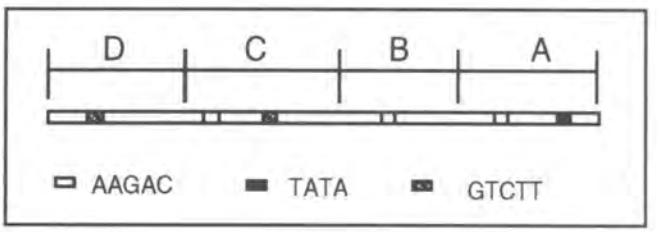




B: Promoter fragment B

The lanes contained: **1**, no protein and 2.0µg poly (dIdC:dIdC); **2**, 1.0µl protein and no poly (dIdC:dIdC); **3- 9** 1.0 µl protein and 2.0µg poly (dIdC:dIdC); **4- 5**, 0.3ng and 0.8ng of oligo MBS, respectively; **6- 9**, 0.15, 0.3, 0.8, 1.5ng unlabelled oligo BS, respectively.

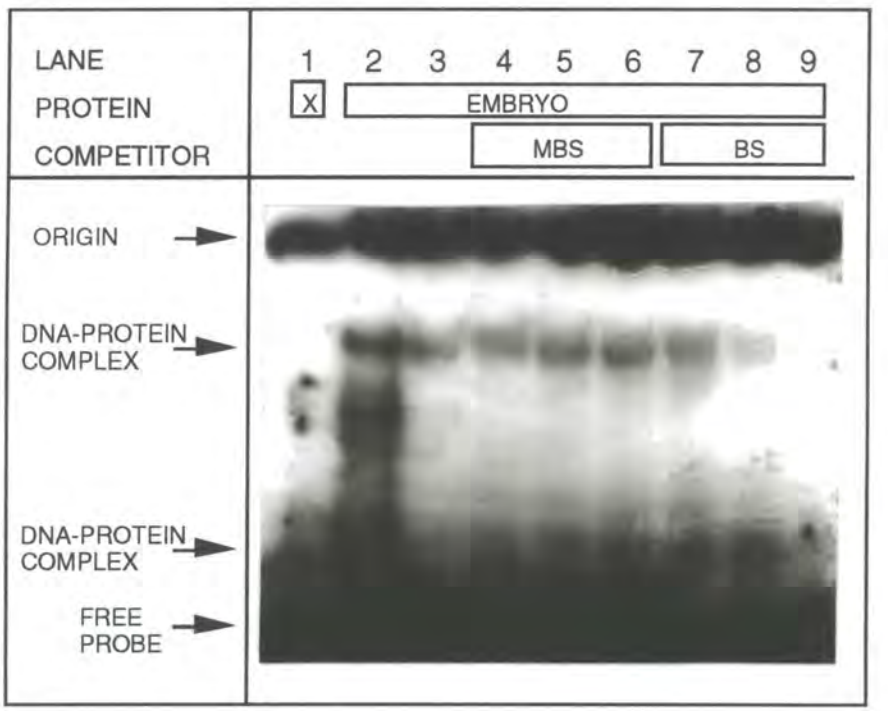


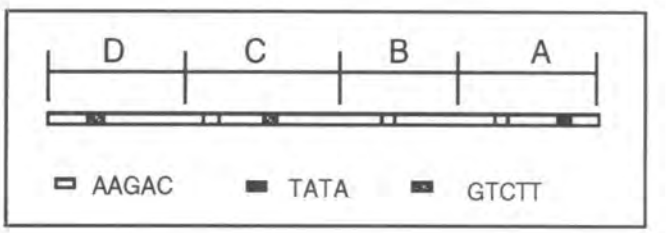


C: Promoter fragment C

Gel 1: Embryo DNA- protein complexes

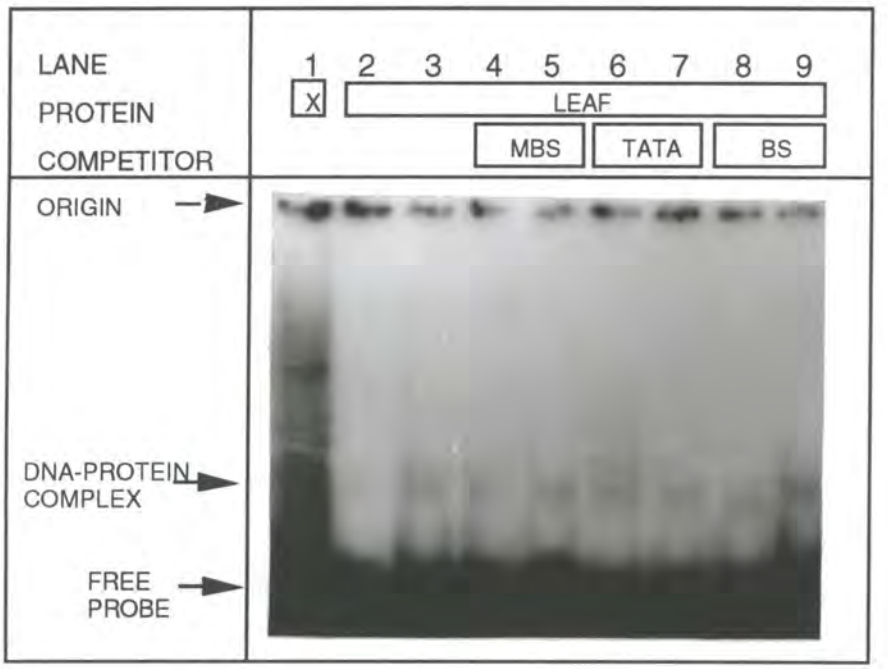
The lanes contained: **1**, no protein and 2.0µg poly (dIdC:dIdC); **2**, 1.0µl embryo protein and no poly (dIdC:dIdC); **3- 9**, 1.0 µl embryo protein and 2.0µg poly (dIdC:dIdC); **4- 5**, 0.15, 0.3ng and 0.8ng MBS oligo competitor, respectively; **6- 9**, 0.15, 0.3, 0.8ng BS oligo, respectively.





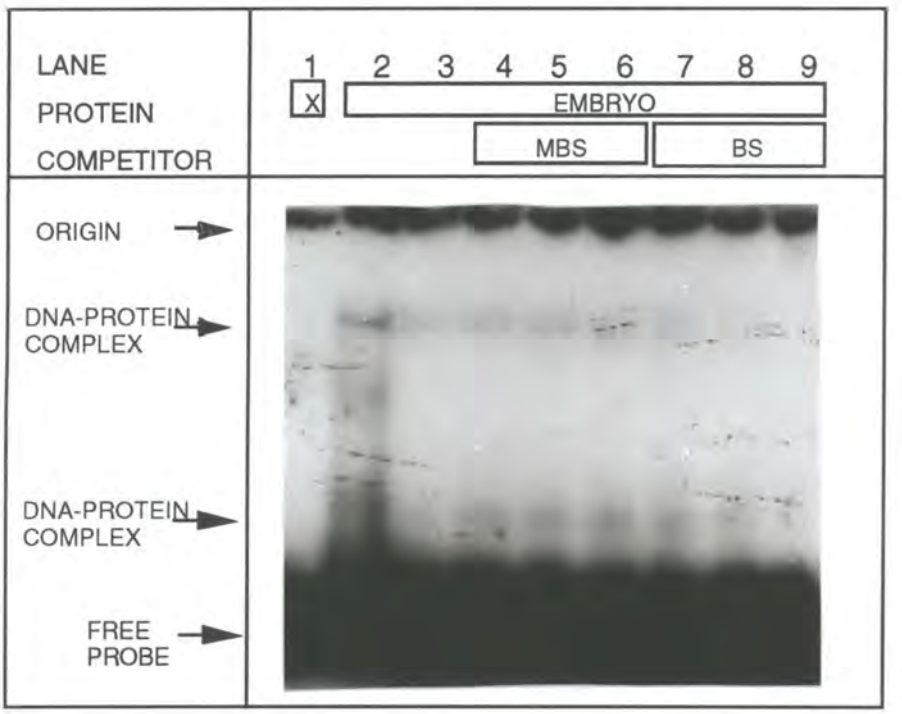
Gel 2: Leaf DNA-protein complex

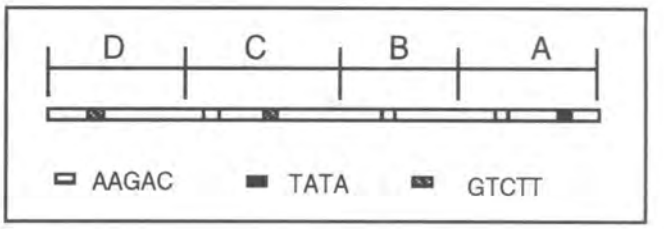
The lanes contained: **1**, 1.0µl leaf protein and no poly (dIdC:dIdC); **2**, no protein and 2.0µg poly (dIdC:dIdC); **3- 9**, 1.0 µl leaf protein and 2.0µg poly (dIdC:dIdC); **4- 5**, 0.3ng and 0.8ng BS oligo competitor, respectively; **6- 7**, 0.3, 0.8ng TATA oligo, respectively; **8-9**, 0.3ng and 0.8ng MBS oligo respectively.



D: Promoter fragment D

The lanes contained: **1**, no protein and 2.0µg poly (dIdC:dIdC); **2**, 1.0 µg protein and no poly (dIdC:dIdC); **3- 9**; 1.0 µl protein and 2.0µg poly (dIdC:dIdC); **4-6**, 0.15, 0.3 and 0.75ng MBS oligo competitor, respectively; **7-9**; 0.15, 0.3 and 0.75ng BS oligo, respectively





panel A. Free probe is indicated in lane 1 (contained no protein). The assay run in lane 2 contained no poly (dIdC:dIdC) and a non- specific shift of probe DNA was observed. Both leaf protein and non- specific competitor were included in lane 3 and the complex is indicated. In lanes 4, 5 and 6 a 10X molar excess of the oligonucleotide competitors BS, MBS and TATA were added, respectively. The complex was titrated out only with the TATA containing competitor andthus suggests that it arose as a result of an interaction between the TATA binding protein (which is a component of TFIID) and the TATA box in the probe.

B: Fragment B

Fragment B formed one sequence specific complex with an embryo factor. Panel B of figure 3.9 shows a competition assay using fragment B substrate DNA. The mobility of the free probe is indicated in lane 1, which contained no protein. Lane 2 was a control assay with no poly (dIdC:dIdC) and showed non- specific shift of the probe. Lane 3 contained both embryo protein and non- specific competitor and the specific shift of probe was observed. Oligonucleotide competitor MBS was added to lanes 4 and 5 (2X and 5X molar excess, respectively) No effect on the complex was observed. The competition was repeated in lanes 6-9 (1X, 2X, 5X, 10X molar equivalents respectively) with competitor oligonucleotide BS. The complex was competed and it was concluded that this complex is AAGAC bound fragment B.

C: Fragment C

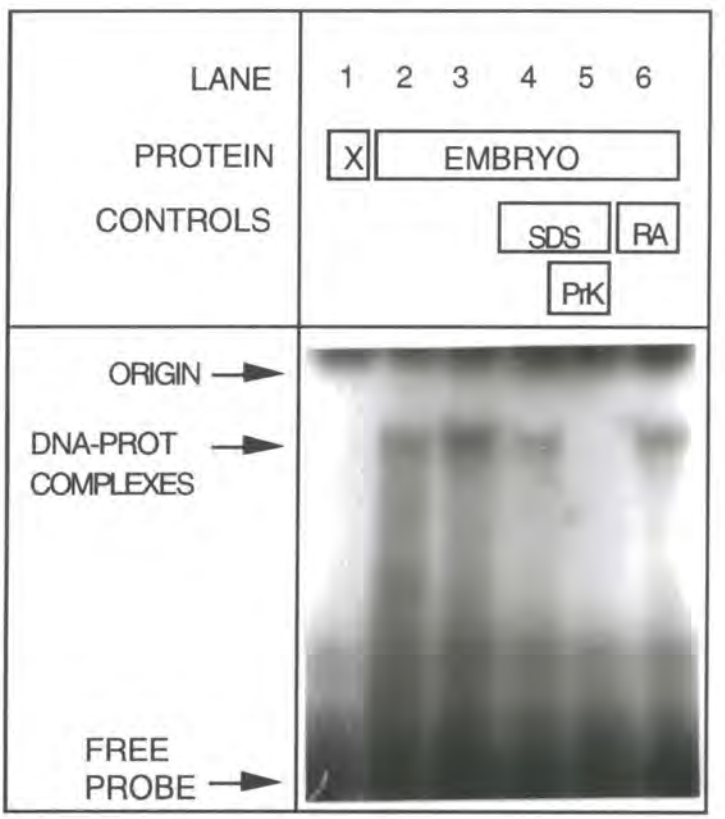
Fragment C formed three complexes, two with embryo DNA binding protein(s) and one with a leaf factor. A competition assay of the embryo complexes is presented in the first gel of panel C. Protein was omitted from the assay in lane 1 and free probe is indicated. Lane 2 contained no poly (dIdC:dIdC) and the probe is shifted in a non- specific interactions. Lane 3 was the positive control, containing both protein and poly(dIdC:dIdC) and the formation of the upper complex was clearly observed. In lanes 4-6 a competition titration with oligonucleotide MBS (1x, 5x and 10x molar excess added, respectively) was performed. No effect on complex formation was observed. An identical titration with oligonucleotide BS was performed in lanes 7-9 and the upper band was titrated out. No effect on the high mobility complex was observed.

Figure 3.10: Analysis of the factors involved in complex formation

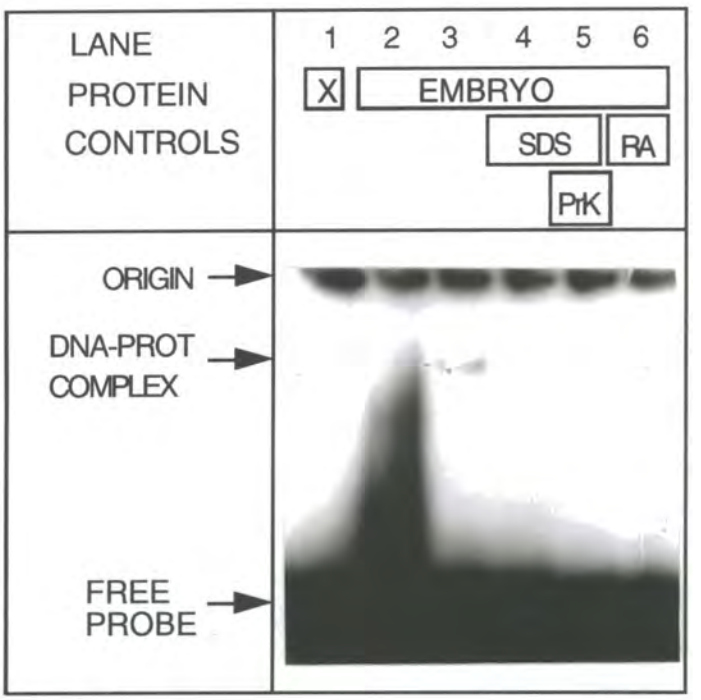
The figure is divided up into four panels, each presents a typical enzyme retardation assay for promoter fragments A- D, respectively. Lane 1 assays contained no protein and the mobility of unbound probe is indicated. Poly (dIdC:dIdC) was omitted from assays in lane 2. Lane 3 contained standard assays with both non- specific competitor and protein extract. The formation of specific complexes is indicated in each case. The standard assay was added to lane 4 and Proteinase K and SDS were both added to the assays in lane 5. RNase A was added to the standard assay and run in lane 6.

The lanes contained: **1**, no protein and 2.0µg poly (dIdC:dIdC); **2**, 1.0µl protein and no poly (dIdC:dIdC); lanes **3- 6**, 1.0µl protein and 2.0µg poly (dIdC:dIdC), **4**; SDS to 0.5%; **5**, 0.5% SDS and Proteinase K (PRK); **6**, RNase A (RA)

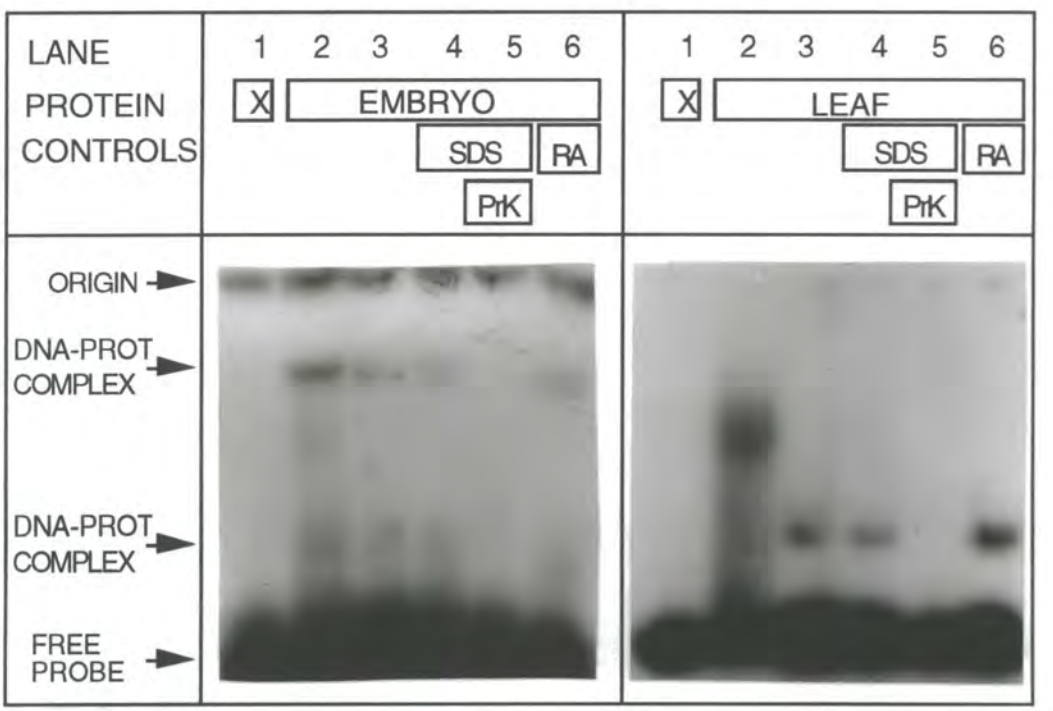
A: Fragment A



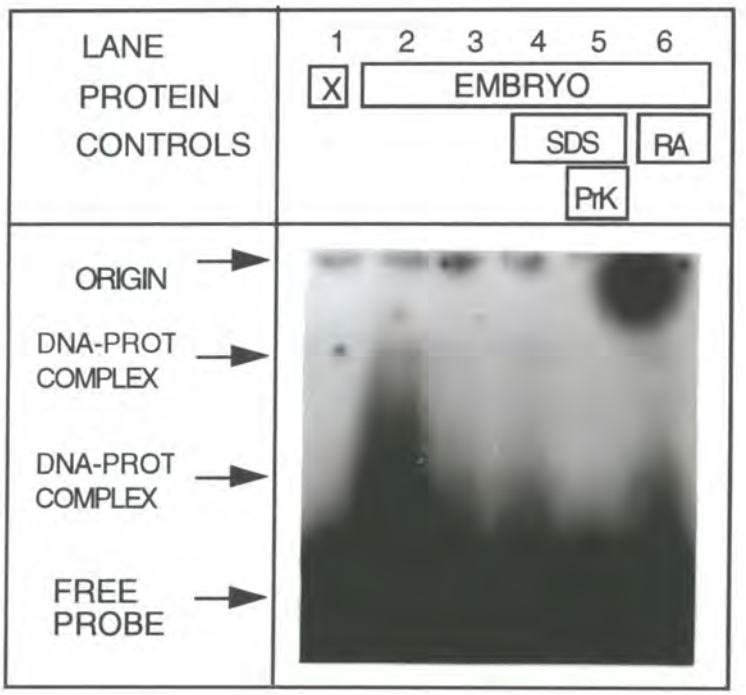
B: Fragment B



C: Fragment C



D: Fragment D


LANE
PROTEIN
CONTROLS
1 2 3 4 5 6
X
EMBRYO
SDS
RA
PrK
ORIGIN
DNA-PROT COMPLEX
DNA-PROT COMPLEX
FREE PROBE

A competition assay of the high mobility complex in leaf protein is presented in the second gel of panel C. The non- specific shift of substrate DNA was observed in lane 1, which contained no poly (dIdC:dIdC). Free probe is indicated in lane 2 (assay which contained no protein). Lane 3 contained both protein and poly (dIdC:dIdC) and the specific band shift is indicated. In lanes 4 and 5 the oligonucleotide competitor BS was added (5x and 10x molar excess respectively). This competition was repeated in lanes 6 and 7 with oligonucleotide TATA and in lanes 8 and 9 with MBS. No significant effect on the formation of this complex was observed. A factor, of unknown binding specificity resulted in the formation of this high mobility complex.

D: Fragment D

Fragment D formed two embryo specific complexes and a standard binding assay with oligonucleotide competitors is presented in panel D of figure 3.9. Lane 1 contained no protein and shows the mobility of unbound fragment D. Lane 2 contained embryo protein, but no poly (dIdC:dIdC). The typical non-specific probe shift was observed. Lane 3 contained embryo extract and poly (dIdC:dIdC) and the two specific complexes are indicated. To the assays in lanes 3- 5 oligonucleotide MBS competitor was added (1X, 2X and 5X molar equivalents of AAGAC respectively). It can be seen that there is no competition of either band. These assays were repeated in lanes 6- 9, with BS. Both complexes were competed out and it was concluded that the sequence AAGAC is involved in the formation of both specific band shifts.

3.2.6.3 Enzyme controls

To determine the nature of the factors that were involved in complex formation, the standard assay was modified to include enzymes. Proteinase K was used to confirm that protein factor(s) were integral to probe retardation and RNAse A was included to determine if RNA was involved.

Figure 3.10 is divided up into four panels A- D, each panel presents a typical enzyme retardation assay for promoter fragments A- D, respectively. The mobility of unbound probe is seen in the first lane of every gel. Poly (dIdC:dIdC) was omitted from assays in lane 2 and standard assays that

contained both non- specific competitor and embryo extract were loaded into lane 3. The formation of specific complexes is indicated in each case. The standard assay was modified in lane 4 with the addition of SDS to 0.5%, which had a similar effect on complex formation for each fragment. There was a reduction in complex formation but in all cases retarded probe was still observed. The effect of Proteinase K was analysed in the assays in lane 5 and was identical with all four probes. The retardation of probe was completely inhibited. This suggests that protein factors are responsible for complex formation resulting in probe retardation. RNAse A was added to the standard assay and run in lane 6. RNAse A had no discernible effect on the formation of any complex. This indicated that DNA-protein interactions were being observed.

3.3 **Summary**

An overview of the DNA- protein interactions detected with ACP05 promoter probes A- D is presented in table 3.4, which collates the number and type of complexes formed with each fragment. Prior to this work the ACP05 promoter was demonstrated to contain regulatory information that directed its spatial and temporal expression (deSilva *et al.*, 1992). An *Ava* I- *Pst* I restriction fragment from the ACP05 promoter was subcloned and sequenced. The sequence was analysed for direct repeats and palindromes, both of which are characteristic of cis-acting regulatory motifs. Nuclear factors, such as GT1, which is involved in light regulated expression, cannot bind significantly unless its cognate binding sequence is repeated at least three times (Green et al., 1989). Multiple palindromic copies of the core motif AAGAC were identified. Gel retardation assays using promoter fragments that contained this motif (or its compliment) demonstrated it was necessary and sufficient for stable complex formation with a sequence specific embryo DNA binding protein.

Similar regulatory elements have been identified in seed protein genes. For example multiple copies of the motif A A/G CCCA occur within the promoter of the α- subunit of the soybean β conglycinin gene (Chen *et al.*, 1986; 1988). This motif was shown to be the core of a protein binding site that interacts with

an embryo nuclear protein, SEF (Allen *et al.*, 1989).

Table 3.4: Summary of DNA- protein interactions with the ACP05 promoter

Probe	Motifs	DNA- Protein Complexes		Conclusion
		Embryo	Leaf	
A	AAGAC TATA box	TWO	ONE	Band shifts with an AAGAC protein BF and TATA BF
B	AAGAC	ONE	NONE	Band shift with an AAGAC protein BF
C	AAGAC GTCTT	TWO	ONE	Band shifts with an AAGAC protein BF and a high mobility factor
D	GTCTT	TWO	NONE	Band shift with an AAGAC protein BF

BF: DNA binding factor

The AAGAC motif was incorporated on synthetic oligonucleotides and multimerised to be used as a binding site probe to screen an expression library for the cognate binding protein. These experiments are described in chapter 4.

Chapter 4

Cloning DNA Binding Proteins with Recognition Site Probes: "Southwestern" Cloning

Chapter 4

Cloning DNA Binding Proteins with Recognition Site Probes: "Southwestern" Cloning

4.1 Introduction

4.1.1 Problems associated with cloning DNA binding proteins

The majority of transcription factors are present in very low quantities in the cell. A major difficulty associated with the purification of such rare proteins is the requirement for large amounts of starting material. This makes purification on a preparative scale very difficult for most DNA binding proteins. The development of DNA-affinity chromatography matrices by Kadonaga and Tjian (Kadonaga and Tjian., 1986) facilitated the biochemical purification of a number of DNA binding proteins using conventional techniques (Rosenfield *et al.*, 1986; Kadonaga and Tjian, 1986; 1987; Landschulz *et al.*, 1988). The method uses a column matrix to which a large number of DNA binding sites are covalently linked. Gel retardation assays, DNase I foot printing and filter binding assays are used to follow specific DNA binding proteins during their purification.

4.1.2 Alternative cloning strategy

An alternative cloning method which circumvented the problems associated with low abundance was developed by Singh and colleagues (Singh *et al.*, 1988; 1989). The first cDNA isolated by this method coined "Southwestern" screening corresponded to the human enhancer binding protein H2TF1/ NFκB (Singh *et al.*, 1988). The basic concept was derived from screening λgt11 expression libraries with antibody probes (Young and Davies, 1983; 1985) and many parallels exist between the techniques. Both methods rely upon the expression of functional recombinant protein in *E.coli*. During antibody screening positive clones are detected through a specific interaction between an epitope of the recombinant protein and the antibody probe.

During Southwestern screening positives are detected through the specific interaction of a DNA binding domain with its cognate binding site which is used as a probe (Singh *et al.*, 1988; 1989; Vinson *et al.*, 1988).

4.1.3 Parameters that limit Southwestern screening

Several factors limit the success of Southwestern screening experiments. The type of library used is of great importance. In order to maximise the chance of detecting rare clones the library should be generated from mRNA isolated from a tissue source with the highest levels of a given binding factor. In addition, a primary, unamplified library should be used for initial screening as each round of amplification can lead to a selective loss of low abundance bacteriophage. This appears to be the case especially for phage that encode DNA binding proteins (Singh *et al.*, 1989). It has been shown that following successive rounds of library amplification and probing for known DNA binding proteins, their numbers rapidly become unrepresentative (Singh *et al.*, 1989). The library should also be randomly primed rather than oligo-dT primed (Berger and Kimmel, 1987) as binding domains may lie at the C or N terminus. Thus random primed libraries give improved representation of mRNA species. Library complexity is also of importance when cloning low abundance transcripts. The frequency at which a cDNA clone of a given mRNA appears in a library is generally proportional to the abundance of that species in the mRNA population. It has been estimated that a typical eukaryotic cell contains 10^6 mRNA molecules, transcribed from 15 000 different genes (Berger and Kimmel, 1987). Low abundance mRNAs may only have 20 copies of message present. A library must therefore contain sufficient numbers of individual recombinants to ensure the DNA sequence of interest is represented.

A further consideration is the type of probe used. Single binding site probes have been used successfully to clone DNA binding proteins. However the use of multiple binding sites has been shown to increase the signal intensity of a positive clone (Staudt *et al.*, 1988; Vinson *et al.*, 1988). This is possibly due to several protein molecules binding the probe simultaneously and thus stabilising the interaction. Multiple binding site probes have been particularly

successful in cloning members of the leucine zipper family of factors (Katigiri *et al.*, 1989; Maekawa *et al.*, 1989; Poli *et al.*, 1990; Singh *et al.*, 1988) and other classes of factors including the helix- turn- helix and zinc finger (Kageyana and Pastan, 1989; Klemsz *et al.*, 1990; Lum *et al.*, 1990; Williams *et al.*, 1991).

A major limitation of Southwestern screening is that proteins which require additional factors for DNA binding will not be detected. For example, components of multi protein complexes and heterodimers cannot be detected using this strategy. Furthermore, full length cDNAs are often not isolated by this method. Binding site probes are used to detect the presence of binding domains and successful detection of a positive depends only upon the presence of a functional DNA binding domain.

4.2 **Results**

The ACP05 promoter was screened for protein binding sites using gel retardation assays, as described in chapter 3. The interaction between a promoter motif and an embryo DNA binding protein was characterised. Complimentary oligonucleotides that contained a copy of this sequence were synthesised for use in a Southwestern cloning experiment. BS1 and BS2 were complimentary and contained a single copy of the motif AAGAC. MBS1 and MBS2 were similar to BS1 and BS2, apart from two altered nucleotides in the AAGAC motif (see table 3.3; pp.92).

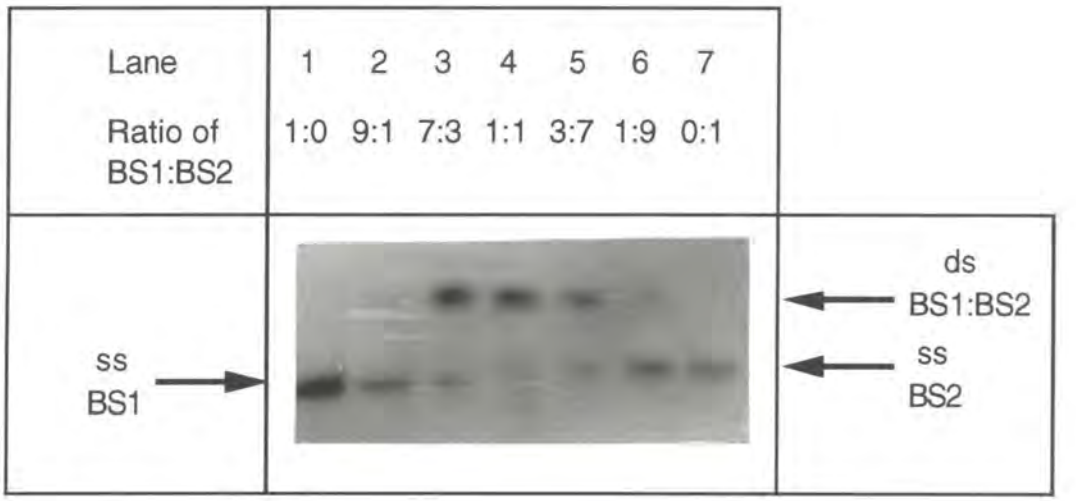
4.2.1 Analysis of probes

Single stranded DNA forms have been shown to bind strongly to nuclear proteins, forming anomalous complexes (Svaren *et al.*, 1987). Binding site oligonucleotides were synthesised as single strands which were annealed (section 2.9.11.2) and analysed by gel electrophoresis (figure 4.1). Both forms were clearly resolved from each other and are indicated. The formation of double stranded probe was maximal when equimolar amounts of complimentary oligonucleotides were annealed using this method.

Figure 4.1

Analysis of oligonucleotide annealing conditions

Oligonucleotide probes were analysed as follows. Different ratios of 5' labelled, single stranded complimentary oligos annealed over a temperature gradient. The products were subsequently fractionated through a 15% polyacrylamide gel. Single stranded (ss) and double stranded (ds) forms were clearly resolved from each other.



4.2.2 Protein interactions with binding site oligonucleotide

Gel retardation assays were performed to determine the binding characteristics and specificity of the isolated AAGAC core motif in BS. Typical poly (dIdC:dIdC) titrations with embryo and leaf extracts are presented in panels A and B, respectively of figure 4.2. Protein was excluded from the assays in lane 1 and the mobility of the free probe is indicated. Poly (dIdC:dIdC) was excluded from the assays in lane 2 and non-specific shift of substrate DNA was observed. In these assays the majority of probe was bound in very large complexes at the top of the gel. Increasing amounts of the non- specific competitor, poly (dIdC:dIdC) were added to the remaining assays (lanes 3- 9) to titrate out non- specific interactions. One specific complex (indicated) was observed to form with an embryo factor. No specific complexes were observed with to form when incubated with leaf extract.

The specificity of the embryo binding factor was analysed by competition with the unlabelled oligonucleotides BS and MBS (table 3.3) and is shown in panel C of figure 4.2. Increasing amounts of the specific competitor BS (AAGAC motif) was added to the assays in lanes 4-8 and the DNA- protein complex was competed out. Competitor oligonucleotide MBS (altered AAGAC) was added to the assays in lanes 11 and 12, with no effect complex formation.

Poly (dIdC:dIdC) assays showed that a binding site motif that was incorporated on an oligonucleotide specifically bound an embryo DNA binding protein. Furthermore, competition assays demonstrated that the formation of the DNA- embryo protein complex was dependent upon the AAGAC motif. It was concluded that the binding site oligonucleotide BS behaved in an analogous manner to a promoter fragment that contained an AAGAC motif.

4.2.3 Polymerisation of binding site oligonucleotide

The presence of multiple binding sites in a probe has been shown to increase the signal intensity of a positive clone (Staudt *et al.*, 1988; Vinson *et al.*,

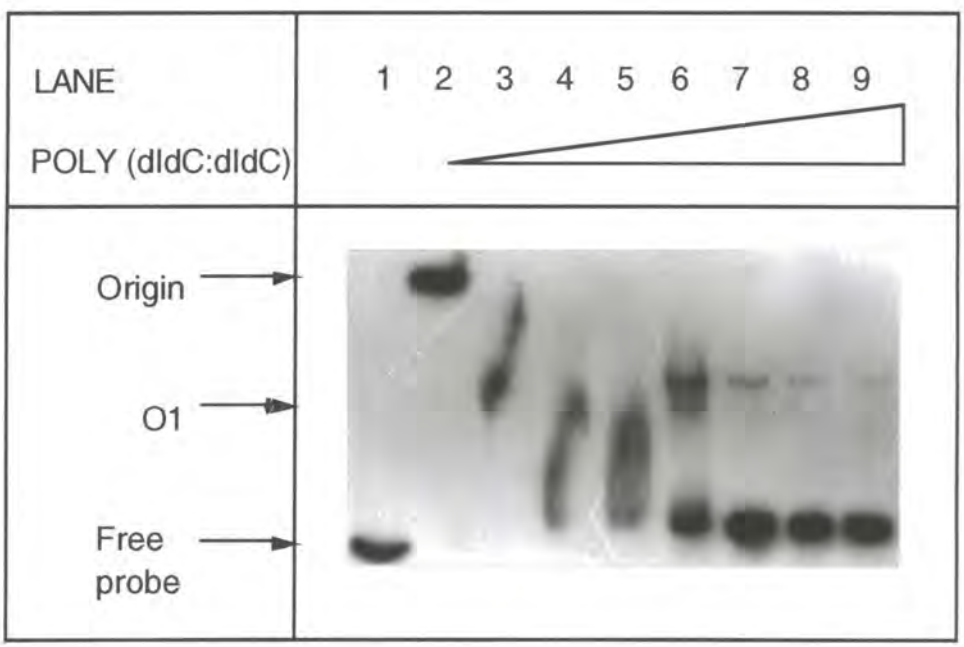
Figure 4.2

A binding motif identified in the ACP05 promoter was incorporated in a pair of complimentary oligonucleotides. These were labelled and annealed and used in gel retardation assays. Poly (dIdC:dIdC) titrations were performed with both embryo and leaf extracts. The specificity of complexes was analysed by competition with unlabelled oligonucleotides. Assays were fractionated on 4% polyacrylamide native gels.

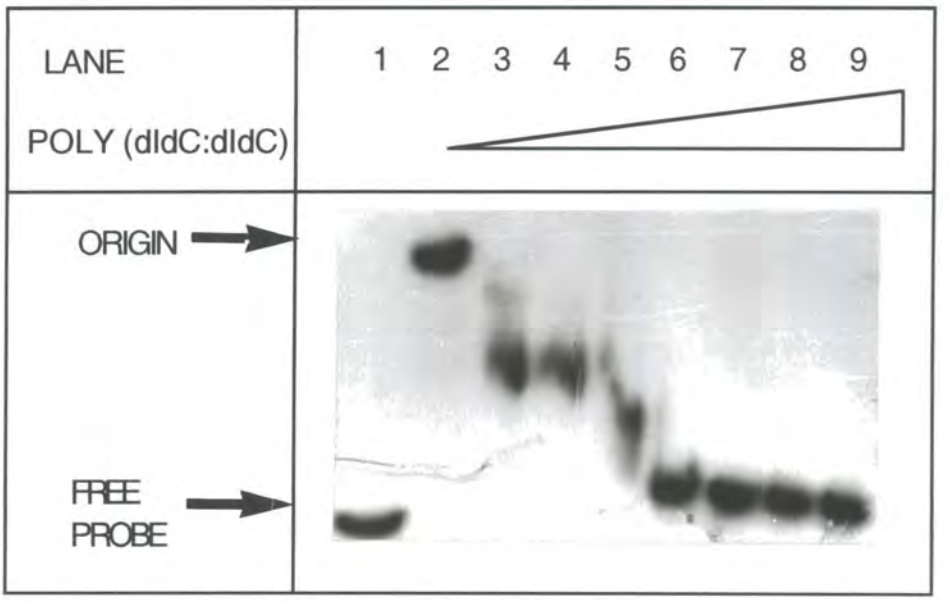
Gel retardation assays with binding site oligonucleotide

The Lanes contained: **1**, 1.0µg poly (dIdC:dIdC); **2- 10**, 1,0µl protein extract; **3-10**, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10µg poly (dIdC:dIdC) respectively.

Panel A: Poly(dIdC:dIdC) titration with embryo extract.

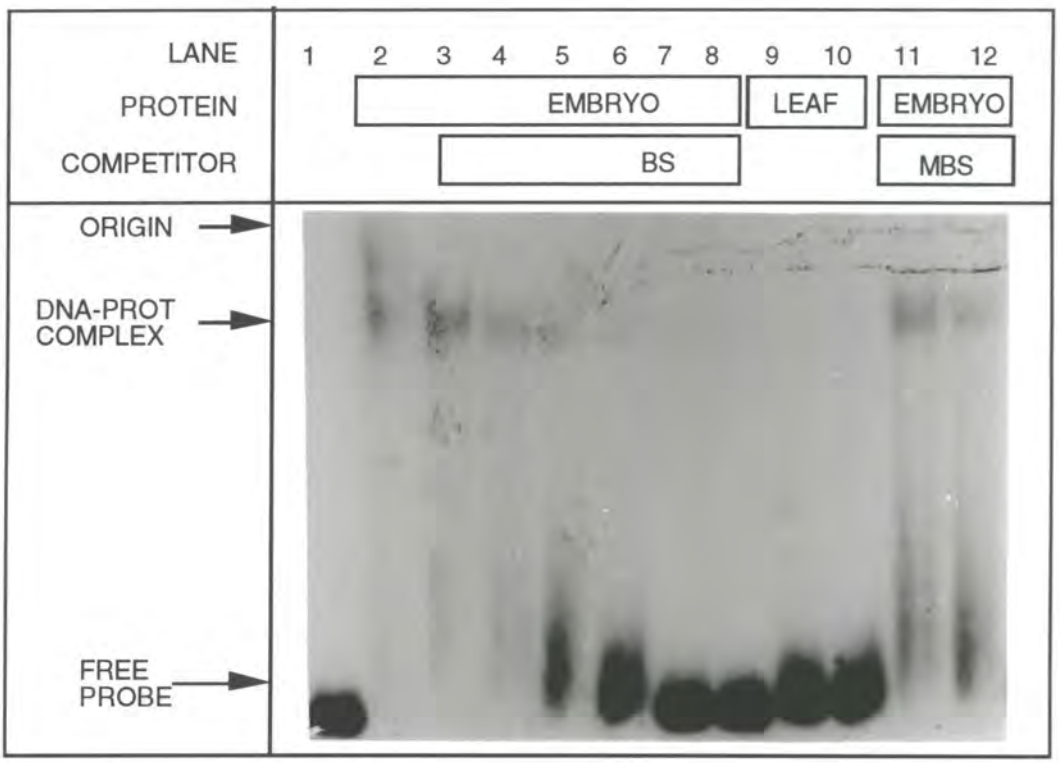


Panel B: Poly(dIdC:dIdC) titration with leaf extract.



Panel C: Competition of DNA- protein complex

The lanes contained: **1**, 1.0μg poly (dIdC:dIdC); **2**, 1,0μl embryo extract; **3- 8**, 1.0μg poly (dIdC:dIdC) and 1,0μl embryo extract ; **4- 8**; 0.25, 0.5, 1.0, 2.5, 5.0ng oligo BS, respectively (0.5X, 1X, 2X, 5X and 10X molar excess); **9 and 10**, 1,0μl leaf protein with 0.5 and 1.0μg poly (dIdC:dIdC) respectively; **11 and 12**, 1,0μl embryo, 1.0μg poly (dIdC:dIdC) and 2.5 and 5.0ng oligo MBS, respectively (5X and 10X molar excess).



1988). However probes longer than 250bp give higher background signals (Singh *et al.*, 1989). It was therefore decided to generate a defined 5- 10 mer of BS for use as a probe in a SouthWestern cloning experiment.

BS1 and BS2 were ligated (section 2.9.9) and gel fractionated (figure 4.3) and a gel slice that contained 5-10mers was taken and cloned into pUC19 to generate the pOBS series of plasmids. Insert sizes were determineded by PCR amplification, using the standard method (2.9.14) and primers listed in table 2.1 (panel A of figure 4.4). Insert sizes (and the corresponding number of copies of cloned oligos) are presented in table 4.1.

Table 4.1: PCR amplification of pOBS clones

Lane	pOBS clone	Insert size(bp)	Corresponding copies of cloned oligo BS
1	1	340	11
2	2	280	8
3	8	340	11
4	4	280	8
5	6	240	6
6	5	200	4
7	3	200	4
8	12	160	7
9	9	220	5

4.2.4 Sequencing multiple binding site clone pOBS4

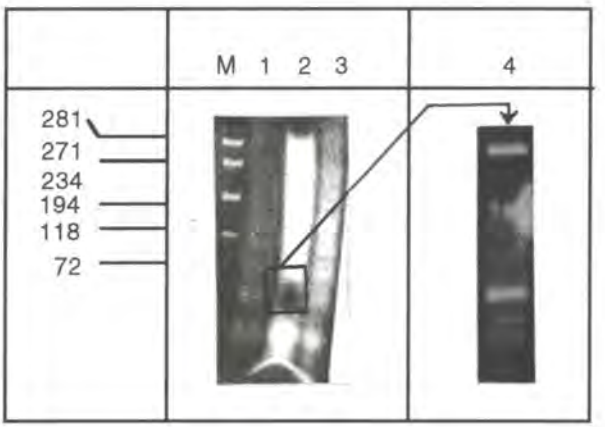
From PCR analysis it was estimated that there were 8 copies of BS cloned into pOBS 4, which was sequenced using universal M13 primers (section 2.9.10). Approximately 300bp of overlapping nucleotide sequence was obtained and is presented in panel B of figure 4.4. pOBS4 contained 8

Figure 4.3

Generating a multimerised binding site probe

Two micrograms each of phosphorylated BS1 and BS2 were annealed and ligated. Products were fractionated on a 2.0% LMP agarose gel, with molecular weight markers in order that a gel slice that corresponded to 5-10mers could be isolated. DNA was recovered by freeze squeeze (lane 4).

The lanes contained: **1**, **2**, **3**: 0.3, 1.0 and 0.7μg (respectively) annealed and ligated BS oligo; **4**, 20μl gel slice eluent. **M**: 1.0μg *Hae* III cut ϕX174 DNA.



complete copies of oligonucleotide BS, with the end copy motif cloned in a reversed orientation relative to the other seven (as indicated by the direction of the arrows above the motifs in figure 4.4).

4.2.5 Preparation of radiolabelled binding site probe

Theoretically, a probe with a specific activity of 10^7cpm/ pmol could detect 10^{-2} fmol of active protein in a plaque (1pg if MWt was 170kDa). This assumes a 1:1 stoichiometry for the protein:DNA interaction. The level of expression of an overexpressed lacZ fusion protein in a plaque should result in the accumulation of 100pg of fusion protein (Cowell and Hurst, 1993). If there are 10^5 infected cells/plaque and the β- galactosidase fusion represents 1% of the total protein mass. Therefore the sensitivity of detection is well within limits and similar to that of ^{125}I- labelled primary antibody or a secondary antibody system based on a secondary antibody conjugated with alkaline phosphatase (Broome and Gilbert, 1978; Leary *et al.*, 1983; Singh *et al.*, 1989).

pOBS 4 was digested with *Eco*RI and *Hin* DIII to excise the multiple binding site (211bp). The fragment was gel purified (panel A of figure 4.5) and end labelled (section 2.9.11.3) using 50μCi of α^{32}P dATP and α^{32}P dCTP (3 000Ci/ mmol). Unincorporated label was removed by a Bio spin P- 30 column (2.9.11) and the specific activity calculated from liquid scintillation (routinely 10^7 cpm/ pmol).

4.2.6 Preparation of a control DNA probe

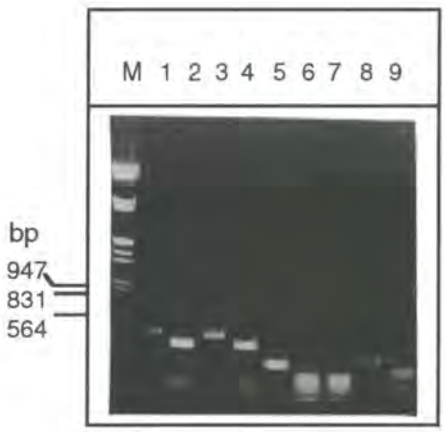
A control DNA was required to screen putative positive phage for sequence specificity. A general DNA binding protein would hybridise to any DNA probe used, whereas a specific protein would only hybridise to its cognate binding site. A 141bp *Hin*DIII- *Pvu*II pUC19 restriction fragment was chosen. This fragment did not contain AAGAC motifs and it included the polylinker region that was also present in the binding site probe. Therefore it could be used to discriminate between a specific and general DNA binding clone. pUC19 was digested with *Hin* DIII and *Pvu* II (restriction fragments of 2058, 305, 181 and

Figure 4.4: Analysis of cloned concatimers

Oligonucleotide binding sites were polymerised and fractionated on a 2.0% agarose gel. The DNA was recovered by freeze squeeze and ligated into *Sma* I cut pUC19 to generate pOBS clones. The exact number of cloned copies was determined by PCR amplification with 1ng template and 05μM each primer per reaction. PCR reactions were analysed n a 1% agarose gel and and pOBS4 (lane 4- 8mer) was subsequently sequenced (panel B).

A: PCR

The lanes contained: Lane **1- 9**, 20μl each PCR **1**, pOBS1; **2**, pOBS2; **3**, pOBS 8; **4**, pOBS 4; **5**, pOBS 6; **6**, pOBS 5; **7**, pOBS 3; **8**, pOBS 12; **9**, pOBS 9; **M**, 1.0μg *Eco*R I cut λ DNA.



B: Nucleotide sequence of pOBS4

The orientation of the binding motif AAGAC is indicated with an arrow.

1 CGCATGCTTAAGACTGGTAC CGCATGCTTAAGACTGGTAC 40

41 CGCATGCTTAAGACTGGTAC CGCATGCTTAAGACTGGTAC 80

81 CGCATGCTTAAGACTGGTAC CGCATGCTTAAGACTGGTAC 120

121 CGCATGCTTAAGACTGGTAC GTACCAGTCTTAAGCATGCG 160

141bp) and gel fractionated (see panel a of figure 4.5). The 141bp restriction fragment was gel purified and 100ng end labelled (section 2.9.11.3) using 50μCi of α^{32}P dATP (5 000Ci/ mmol) and α^{32}P dCTP (3 000Ci/ mmol). Unincorporated label was removed by centrifugation through a Bio spin p- 30 column (2.9.11) and the specific activity calculated from liquid scintillation (routinely 10^7 cpm/ pmol).

4.2.7 Description of library used

A λgt11 *Brassica campestris* (cv. RV500) embryo cDNA library was provided by Prof. A. R. Slabas. The library was generated from mRNA extracted from mid- maturation embryos (coincident with both maximum lipid synthesis and ACP expression). The library was randomly primed and unamplified. cDNA was cloned into the unique λgt11 *Eco* RI site (53 amino acids from the amino terminus in the lac Z gene) with *Eco* RI adapters. Fusion proteins expressed in λgt11 usually contain all but 53 N terminal amino acids of β- galactosidase, which is thought to increase the stability of recombinant fusion protein. The library was titred before use, according to the standard protocol in section 2.12.2 and was 4x 10^7 pfu/ ml.

4.2.8 Screening the library

Four aliquots of competent cells were infected with 5x10^4 pfu and screened with 1x 10^7cpm ^{32}P- labelled DNA binding site probe as described in section 2.12. A primary filter is shown in panel A of figure 4.6. Eight putative positives with the characteristic "comet" shaped plaque were chosen for further analysis. The ringed positive corresponded to BS2xi, which was the only phage that remained positive after a second round of screening (panel B). λBS2xi was purified to homogeneity by a tertiary round of screening. Clone λBS2xi was re- screened with two controls: A 141bp pUC19 *Hin*DIII- *Pvu* II restriction fragment (4.2.6) was used to determine whether the putative positive was a specific or general DNA binding protein. In order to demonstrate that λBS2xi did not have a general nucleotide binding site it was

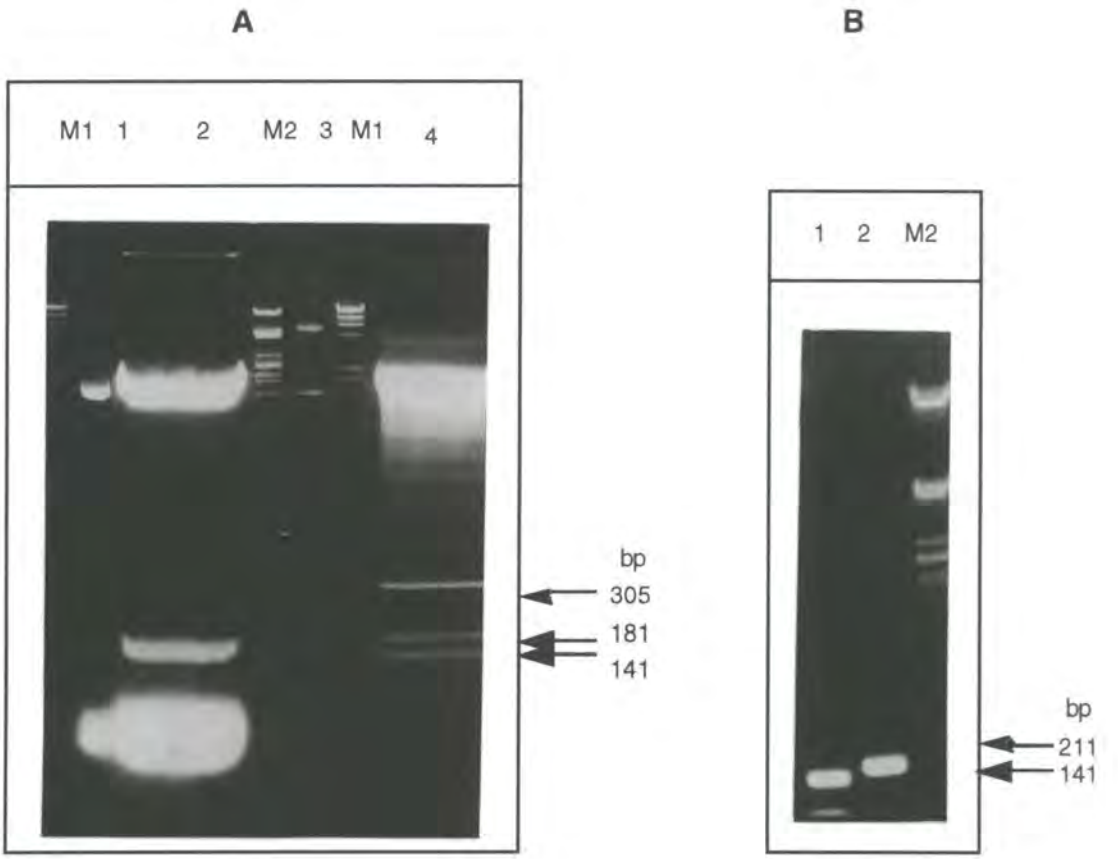
Figure 4.5

Preparation of probes for screening expression library.

10µg of pOBS DNA was double digested with *Eco*RI and *Hin* DIII to excise the 211bp insert. The digest was gel fractionated on a 1.5% LMP agarose gel and the DNA was recovered by electroelution. 100ng of the purified DNA was subsequently end- filled with radionucleotides. A second DNA was needed as a control to screen putative positive phage for their DNA binding specificity. A pUC19 restriction fragment that contained the polylinker sequence included in the binding site probe was chosen. 10µg of pUC19 DNA was double digested with *Pvu* II and *Hin* DIII and the 141bp fragment was purified as described above.

A: The lanes contained: **1**, 1.0µg pOBS 4; **2**, 20µg *Eco*RI- *Hin* DIII restricted pOBS4 (211bp insert); **3**, 0.5µg pUC19; **4**, 20µg *Hin* DIII- *Pvu* II restricted pUC19 (2058, 305, 181, 141bp); **M1**, 0.5µg *Hin* DIII restricted λ DNA; **M2**, 1.0µg *Pst* I restricted λ DNA.

B: Gel purified probes
The lanes contained: **M2**, 1.0µg *Pst* I restricted λ DNA; **1**, *Hin* DIII- *Pvu* II fragment D (141bp); **2**, *Eco*RI- *Hin* DIII fragment B (211bp).



also screened with 0.5µl α^{32}P- dCTP. The control filters are shown in panel C of figure 4.6. Clone λBS2xi only hybridised with the multimerised binding site probe. These results suggested that λBS2xi contained a sequence specific DNA binding domain.

4.2.9 Generation of bacteriophage lysogens

A high titer phage stock of λBS2xi was made (2.12.6) and used to generate lysogens (described in section 2.12.7) which were noted as L1- L7. Protein extracts from L1- L7 and Y1089 were made (2.12.8) and the concentration was determined by the Bio-Rad assay (2.11.3). On average the concentration of lysogen extracts (typically 1µg/µl) was lower than that of Y1089 (typically 5-10µg/µl) as induced recombinant cultures always exhibited partial cell lysis.

4.2.10 Analysis of lysogen protein extracts

4.2.10.1 SDS- PAGE

Protein extract was analysed by SDS- PAGE (panel A of figure 4.7). Control extract (Y1089) can be observed in lane 1 and lysogen extracts L1- L7 in lanes 2- 8, respectively. Differences in the polypeptide composition of lysogen extracts and Y1089 were observed. Several additional bands in the range 66-116kDa and a prominent band at approximately 40kDa were observed in the induced lysogens.

4.2.10.2 Gel retardation assays

Lysogens and Y1089 were assayed for DNA binding activity using the binding site oligonucleotide BS as a probe (sequence given in table 3.3). Assays were performed using the standard method (2.9.15.1) in a modified incubation buffer that was made with a lower salt concerntration than the standard buffer. DNA binding activity was not observed when the standard incubation buffer was used. A short exposure (4hours) of s set of typical

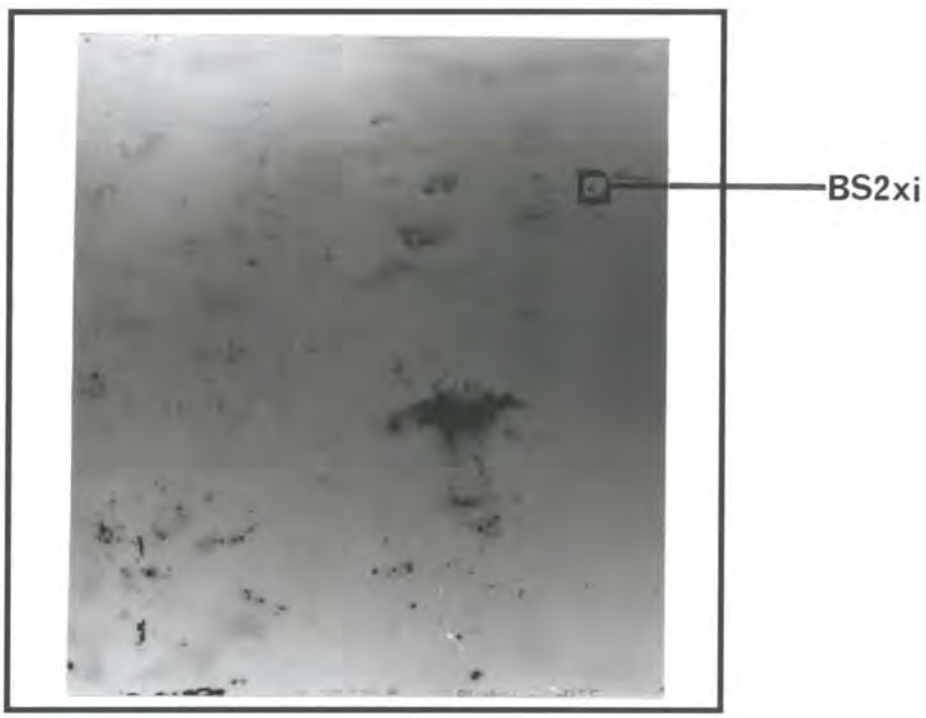
Figure 4.6

South western screen of a λgt11 library

500µl aliquots of competent *E. coli* Y1090 cells were each infected with $5x10^4$ pfu of the λgt11 library. Each was plated out and screened with $1x\ 10^7$cpm ^{32}P-labelled DNA binding site probe. A primary screen filter which hybridised to λBS2xi (ringed) is presented in panel A. Agar plugs containing eight putative positives were isolated, however each plug also contained non- positives, due to the high plating density. Following the second round of screening only BS2xi remained positive (see panel B). This phage was purified to homogeniety by a third round of screening and was re- screened with control DNAs (panel C).

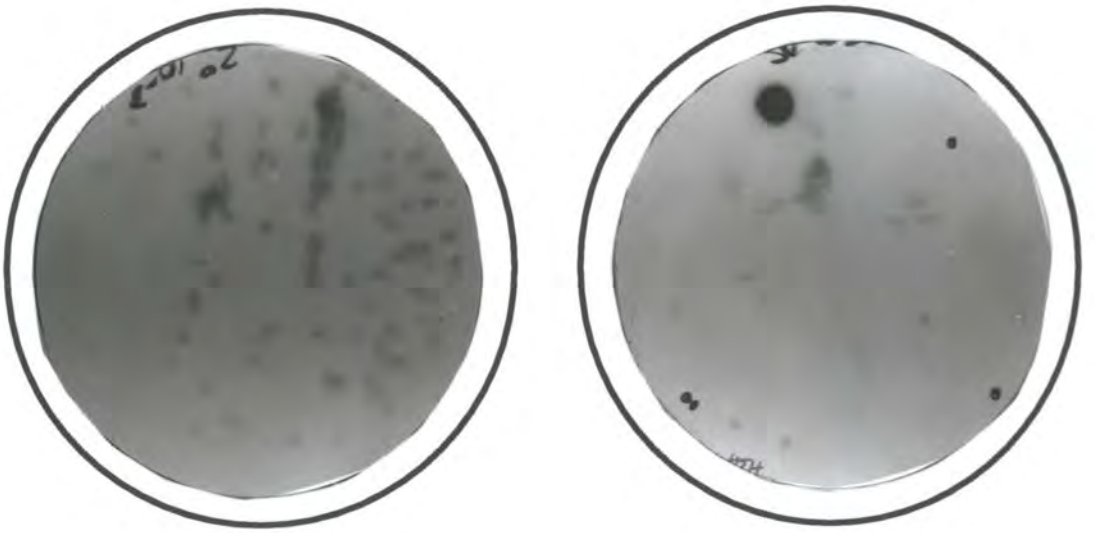
A: Primary screen filter

The positive 'phage BS2xi is ringed



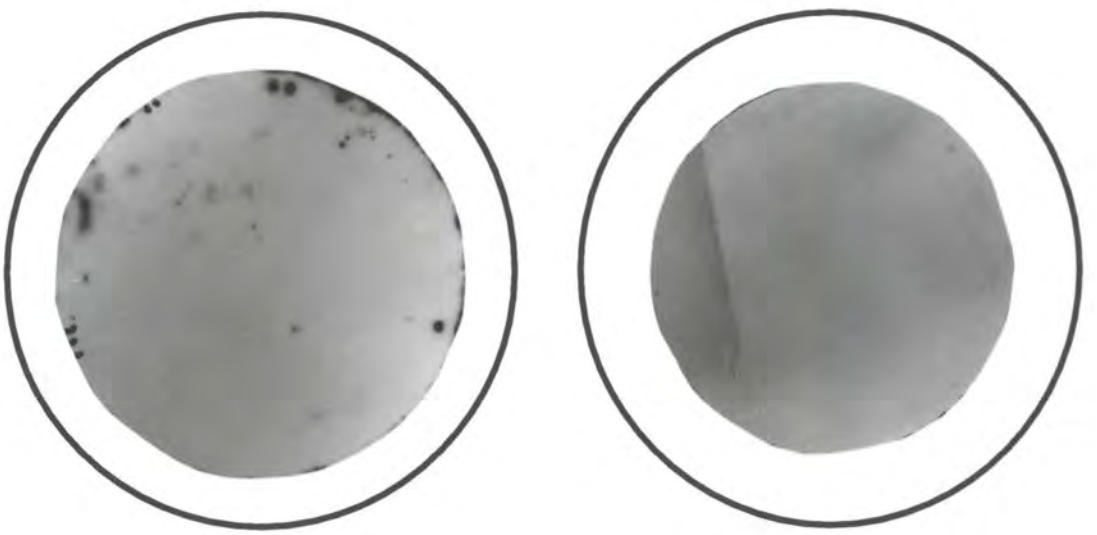
B: Secondary screen. Two dilutions of phage BS2xi were screened with binding site probe

10^{-3} 10^{-5}



C: Tertiary control screen. Purified phage BS2xi was screened with two control probes (described in the text).

Control DNA **Free nucleotide**



assays is presented in panel B of figure 4.7. Y1089 extract was used as a control to determine whether there was any endogenous sequence specific DNA- binding activity. Y1089 was assayed in lanes 1- 3. Poly (dIdC:dIdC) was omitted from the assay in lane 1 and several non- specific interactions that were competed out (lane 3) were observed. Lane 2 contained no protein and free probe is indicated. A standard assay that included poly (dIdC:dIdC) and lysogen extract was repeated in lanes 4- 10. The highest binding activity was noted in L6, with the formation of two DNA- protein complexes (indicated with arrows). The same pattern of retarded bands were detected in the other lysogen extracts (L1- 6) with longer exposures (data not shown).

The binding specificity of the two complexes formed with factor(s) in L6 was investigated with competition assays (the sequence of oligonucleotide competitors is given in table 3.3). The results of competition are shown in panel C of 4.7. Free probe is indicated in the assay in lane 1, from which protein had been excluded. Poly (dIdC:dIdC) was excluded from lane 2 and the probe DNA was shifted non- specifically. A standard assay with lysogen L6 protein and poly (dIdC:dIdC) was run in lane 3 and the two specific complexes are indicated. Non- specific competitor oligonucleotide MBS was added in increasing amounts to the assays in lanes 4-6. Complex formation was not inhibited. Competition was repeated in lanes 7- 10 with the addition of an excess of BS. Both complexes were titrated out. These results are consistent with a specific interaction between the AAGAC motif and the induced recombinant protein in lysogen L6.

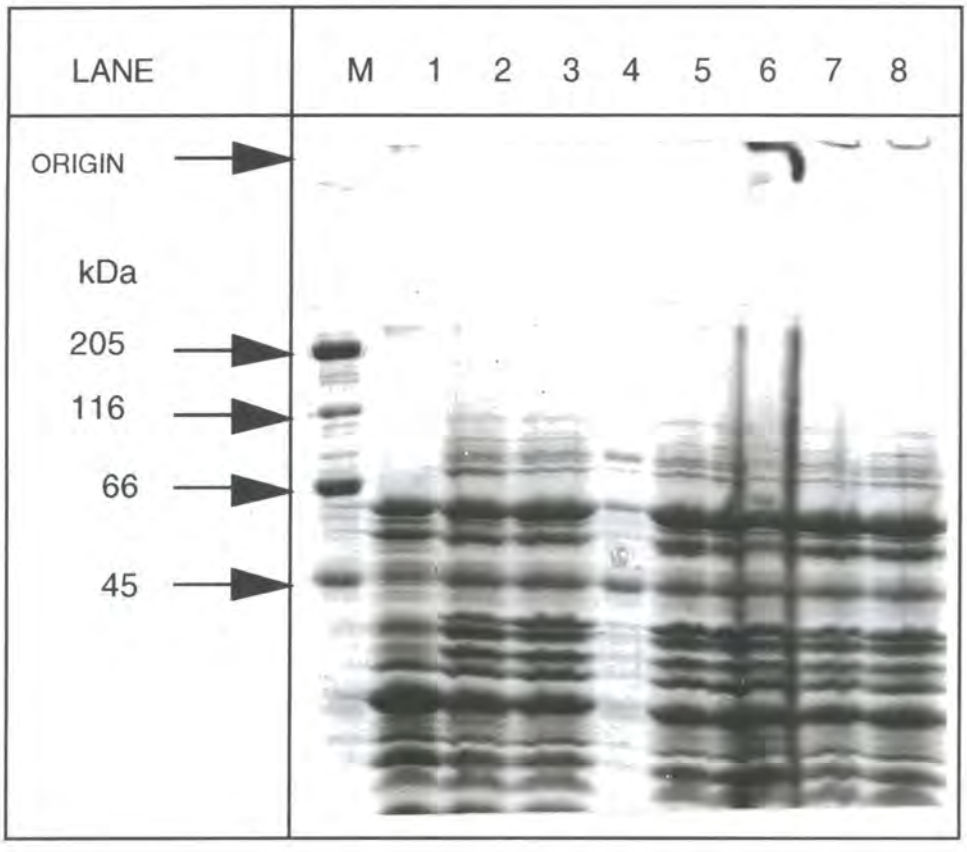
4.2.10.3 Western blotting

In λgtII the unique *Eco* RI cDNA cloning site in *lac* Z is located 53 amino acids from the carboxy terminus. Assuming translation of the recombinant fusion protein occurred from the normal initiation codon, an induced recombinant protein would be fused to all (but 53 amino acids) of β- galactosidase (molecular weight of 116kDa). Western blots, using an anti β- galactosidase antibody (Sigma) were performed in order to determine whether the DNA binding activity in lysogen L6 was a recombinant fusion protein.

Figure 4.7: Analysis of Y1089 lysogen extracts

Lysogens were generated from the DNA binding clone λBS2xi and analysed by SDS- PAGE and gel retardation assays. Y1089 extract was used as a control to determine whether there was any endogenous bacterial DNA binding activity. A modified low salt incubation buffer was used in these assays: (20 mM Tris.HCl, pH7.5, 1mM DTT, 1mM EDTA, 20mM KCl).

A: SDS- PAGE
Protein extracts were concentrated by chloroform- methanol precipitation and electrophoresised through 5% acrylamide gels. The lanes contained: **1**, 2.0μl Y1089 cell free extract; **2- 8**, 5.0μl Y1089 lysogen extract L1- L7 respectively. **M**, 1.0μg each of myosin (205 kDa) and BSA (66kDa). 0.5μg each of β-galactosidase (116kDa) and BSA (66kDa).



Primary antibody (Sigma) was titred to determine its detection limit and specificity. β- galactosidase (Sigma) was run on SDS- PAGE gels and blotted onto PVDF filters as described in 2.11.5. Blots were developed using ECL (Enhanced Chemi Luminescence), Amersham as described in 2.11.5 and are presented in panel A of figure 4.8. A prominent band at 116kDa was observed in the lanes that contained β- galactosidase (10ng was detected at 20 second exposure of blots) and there was no cross reaction with the molecular weight standards in lane M.

Chloroform- methanol precipitated (2.11.4) protein extracts from Y1089 and lysogen L6 were analysed as described above. The results are presented in panel B of figure 4.8. A 116kDa band was observed in lanes that contained β-galactosidase (lane M and 1). A single band at approximately 100kDa was observed in lane 4 (lysogen L6). This band corresponds to truncated β-galactosidase, the translation of which must have been terminated by an in-frame stop codon in the cloned cDNA. From this result it was concluded that the detected DNA binding activity must reside on a polypeptide translated from an internal ATG codon. This has been found most commonly in cloned cDNAs complete at their 5' end, as 5' leader regions often contain in frame stop codons, thus preventing read- through from β- galactosidase.

4.2.11 Characterisation of λBS2xi

The cDNA clone λBS2xi was analysed by several methods. λDNA was prepared as described in section 2.12.9 and digested with *Eco* RI (lane 2, figure 4.10). A 3.0kb fragment that corresponded to the entire cDNA insert was excised. Preparative *Eco* RI digests (2.9.5) were performed and the 3.0kbp fragment was recovered by electroelution (lane 3, figure 4.10).

4.2.11.1 PCR

PCRs were performed with phage BS2xi template and a characterised λgtII clone, λgt6 (provided by C. Lilley). λgt6 was used as a positive control (cDNA

insert of 0.9kb). PCRs were analysed by gel electrophoresis (figure 4.9). Negative controls failed to amplify a product (lanes 1 and 2) and a 0.9kb band was amplified from λgt6, as expected (lane 4), however no product was amplified from λBS2xi (lane 3). As amplification of the template cDNA did not occur using standard conditions, several modifications to the basic method were made. A magnesium titration is presented in figure 4.9. Several reaction buffer formulations have been published, but the standard $MgCl_2$ final concentration is 1.5mM (McPherson *et al.*, 1991). In many circumstances different concentrations of $MgCl_2$ are necessary for successful amplification. On this basis it was decided to titrate the magnesium concentration. 10x reaction buffers were made up with a range of magnesium concentrations. In each case the positive control PCR amplified a band at 0.9kb (as indicated), however no products were produced in any PCR when λBS2xi was used as template (lanes 5, 6 and 8).

4.2.11.2 Sub- cloning the *Eco* RI cDNA restriction fragment

An overview of the subcloning strategy and production of nested deletions from λBS2xi is given in panel A of figure 4.13. The 3.0kbp *Eco*RI cDNA insert was ligated (2.9.9) into pSK^+ (2.9.5), to generate pBF2. Transformants were digested with *Eco* RI and a fragment of the correct size (3.0kb) was excised (compare lanes 3 and 4 with lane 5 in figure figure 4.10). However PCR analysis of pBF2 also failed to amplify the insert.

4.2.11.3 Nested deletions

To facilitate sequencing pBF2 a series of nested deletions were generated (section 2.9.13). Unidirectional nested deletions rely upon the presence of a nuclease sensitive site (from which deletions proceed) and a nuclease resistant site. A *Kpn* I digestion of pBF2 produced two fragments (see panel B of figure 4.10, lane 7). Fragment A was excised from the gel and and an in gel self ligation performed (section 2.9.9) to generate pBFK. pBFK was digested with a variety of restriction endonucleases to map nuclease sensitive and

Figure 4.9

PCR amplification of phage BS112xi

PCR was used to amplify the DNA binding clone BS2xi. Ten nanograms of phage template DNA and 0.5μM each λgtII forward and reverse primers (Promega) were added to each PCR. The magnesium concentration of the buffer was varied and a characterised λgtII clone, λgt6 was used as a positive control (cDNA insert of 0.9kb). Once the cycling programme had been completed, 20μl aliquots of each PCR were analysed on a 1% agarose gel.

The lanes contained: 20μl PCR; **1**, no template control; **2**, no primers control; **3**, PCR with λBS2xi template,1.5mM $MgCl_2$; **4**, PCR with λgt6, 1.5mM $MgCl_2$; **5**, λBS2xi, 0.5mM $MgCl_2$; **6**, λBS2xi, 3.0mM $MgCl_2$; **7**, λgt6, 0.5mM $MgCl_2$; **8**, λBS2xi, 7mM $MgCl_2$; **9**, λgt6, 3.0mM $MgCl_2$; **10**, λgt6, 7.0mM $MgCl_2$. The gel was calibrated with 1.0μg *Hin* DIII cut λ- DNA molecular weight markers in lane M.

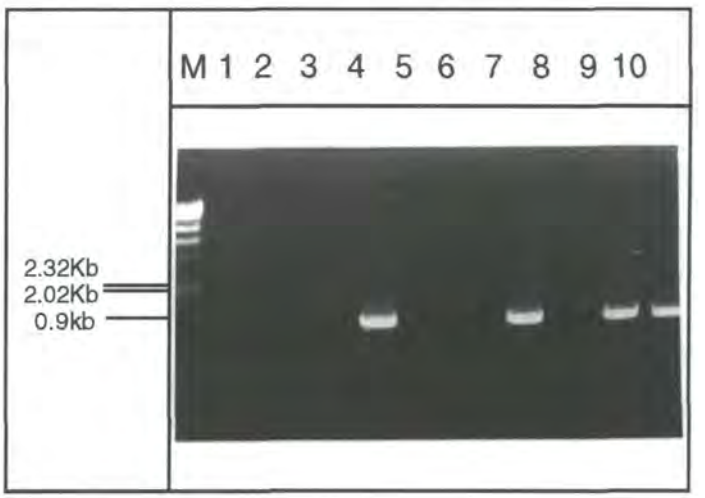
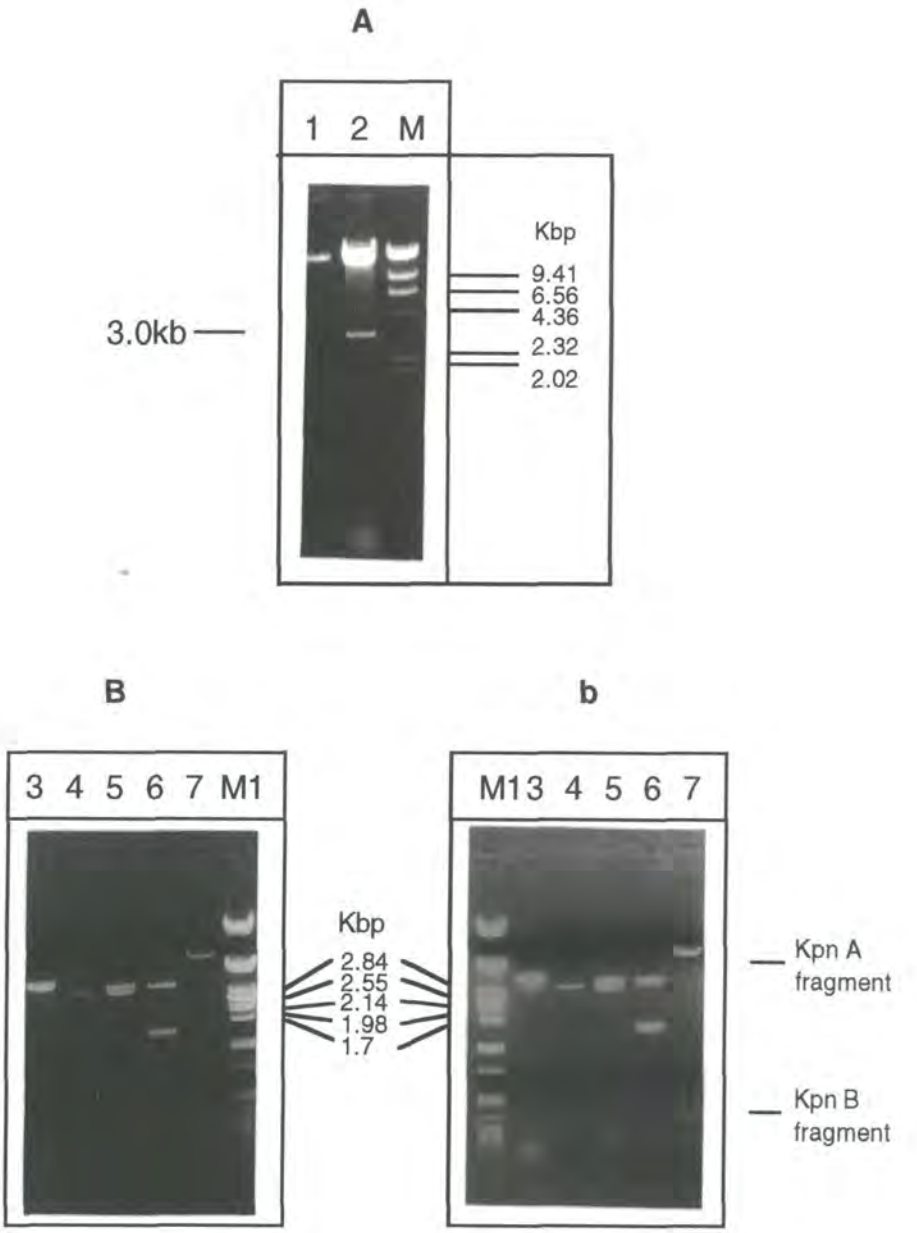


Figure 4.10

Subcloning the EcoRI cDNA insert from λBS2xi

The entire cDNA insert from DNA binding protein clone λBS2xi was excised by *Eco* RI digestion (A). The insert was gel purified and subcloned into *Eco* RI-cut pSK$^+$ to generate pBF2 (B). The lanes contained: **M** 1.0μg *Hin* DIII- cut λDNA. **M1** 1.0μg *Pst* I cut λDNA. **1**, 100ng uncut λBS112xi; **2**, 1.0μg *Eco* RI-cut λBS112xi. Panels **b** and **B** are different exposures of the same gel.The lanes contained: **3**, 100ng gel purified *Eco* RI fragment from λBS2xi; **4**, 10ng purified *Eco* RI- cut pSK$^+$; **5**, 100ng *Eco* RI cut pBF2; **6**, 100ng *Pst* I- cut pBF2; **7**, 100ng *Kpn* I- cut pBF2



resistant sites (figure 4.11). pBFK had a single *Eco* RI site (5' overhang therefore nuclease sensitive), see lane 2 and a single *Sac* I site (3' overhang, therefore nuclease resistant), see lane 8. These two sites were in the correct orientation for use in the generation of nested deletions from the *Eco* RI site.

pBFK was double digested with *Eco* RI and *Sac* I (figure 4.12, panel A). *Exo* III was added and a set of nested deletions, pBFND, were generated (section 2.9.13). pBFND clones were annonoted with the time point from which they were taken, for example as pBFND28 was generated following 28 minutes treatment with *Exo*III. Timed samples from the *Exo* III digestion mix were analysed by gel electrophoresis (figure 4.15, panel B) and appropriately deleted reactions were transformed into competent *E. coli* SURE cells (2.8.2.2).

4.2.11.4 Sequencing pBFK and nested deletions

The universal M13 reverse and M13 (-20) forward primers consistently failed to generate appreciable sequence data from pBF2 and pBFK (despite standard control sequencing reactions carried out in tandem working consistently). When sequencing from the M13-20 primer site, the enzyme sequenced through the polylinker region into cloned insert then was unable to extend past a site located approximately 40bp into the cDNA (panel B, figure 4.13). Attempts at re- sequencing using different preparations of DNA were made, but sequence data was never obtained from this primer site. Sequence data generated in the reverse direction was of poor quality, with high background and was not of sufficient quality to be used for data bank searches or for the synthesis of internal primers.

4.2.11. 5 Sequencing clone BS2xi directly

λgtII forward and reverse primers (Promega) were used to sequence λBS2xi DNA directly. Various lambda DNA preparations were used, but reliable sequence data was not obtained from either primer site.

4.2.11.6 Sequencing nested deletions

Nested deletions sequenced from the M13 forward primer site produced the same result as seen from pBFK (panel B of figure 4.13). The sequencing enzyme was not able to extend through the cloned insert. Nested deletions also consistently failed to sequence in the reverse direction until approximately half way (1.5kbp) through the cDNA insert (panel C of figure 4.13). Low background nucleotide sequence data was obtained from clone pBFND28, which was edited and used in an exhaustive homology search through the GenEmbl data banks (2.9.10.2). An exact match with β-galactosidase sequence was given. The position and extent of this sequence within pSK^+ is indicated in panel C of figure 4.13.

4.2.12.7 Origin of β- galactosidase sequence

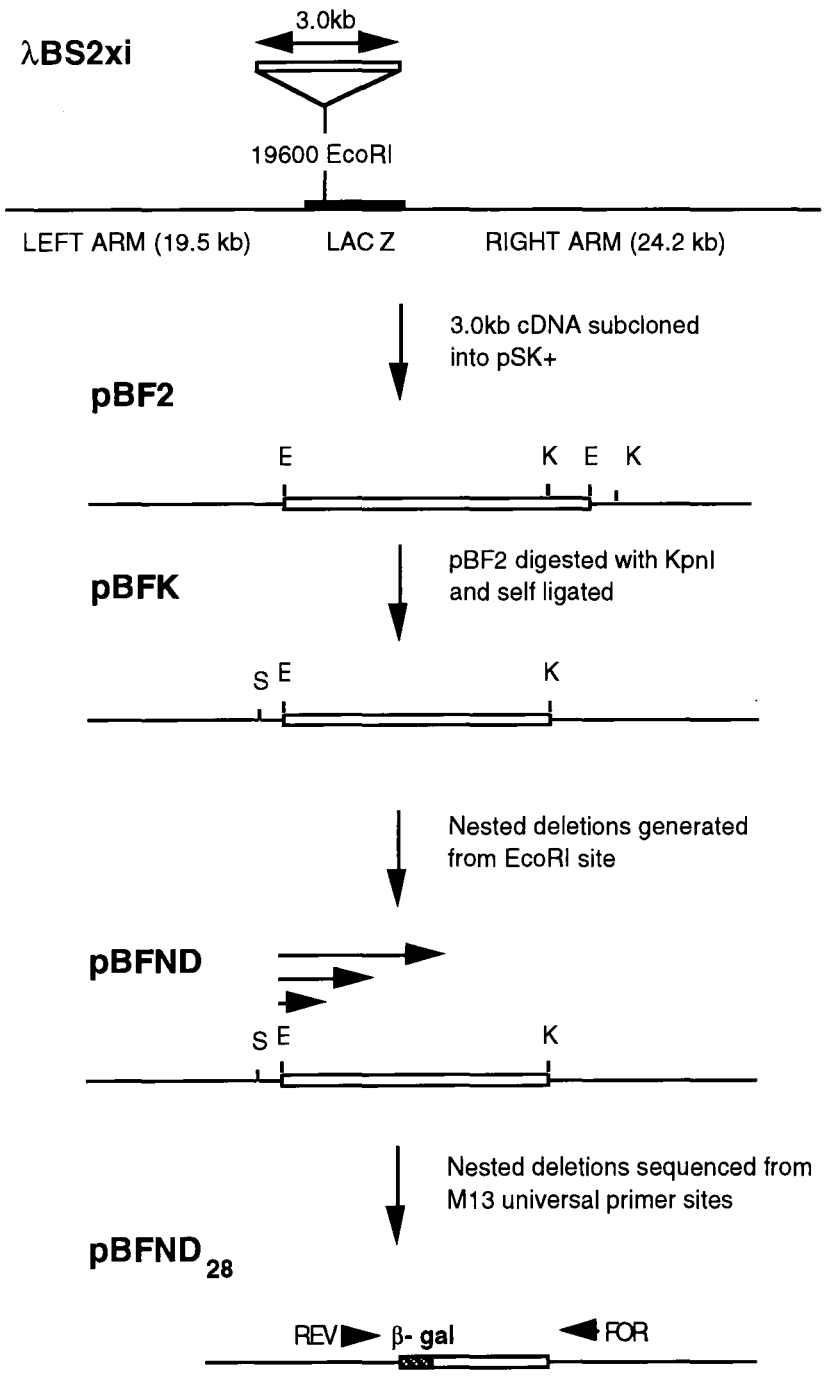
Southern analysis of λBS2xi was performed to determine whether β-galactosidase was present. pSK^+ was digested with *Fok* I (panel A of figure 4.14) and the 181bp fragment (which corresponded to the majority of pBFND28 sequence as indicated in panel D of figure 4.13) was used as a probe. The fragment was excised from the gel and randomly labelled (2.9.11.1). The specificity of the probe was determined by hybridising it to a southern blot that contained a dilution series of *Eco*RI cut pSK^+ (panel B of figure 4.14). The blot was blocked and then incubated overnight with the 181bp pSK^+ *Fok* I probe (described above). A single hybridising band of 2.9Kbp (linearised pSK^+) was observed, which did not cross react with the λDNA in the marker lane.

An agarose gel loaded with various restriction digests of phage λBS2xi was blotted and probed with the *Fok*I 181bp probe as described above (panel C of figure 4.14). The probe hybridised to the 3.0Kbp *Eco*RI fragment (lane 2) which confirmed that β- galactosidase sequence was present in λBS2xi.

Figure 4.13 : Analysis of nested deletions

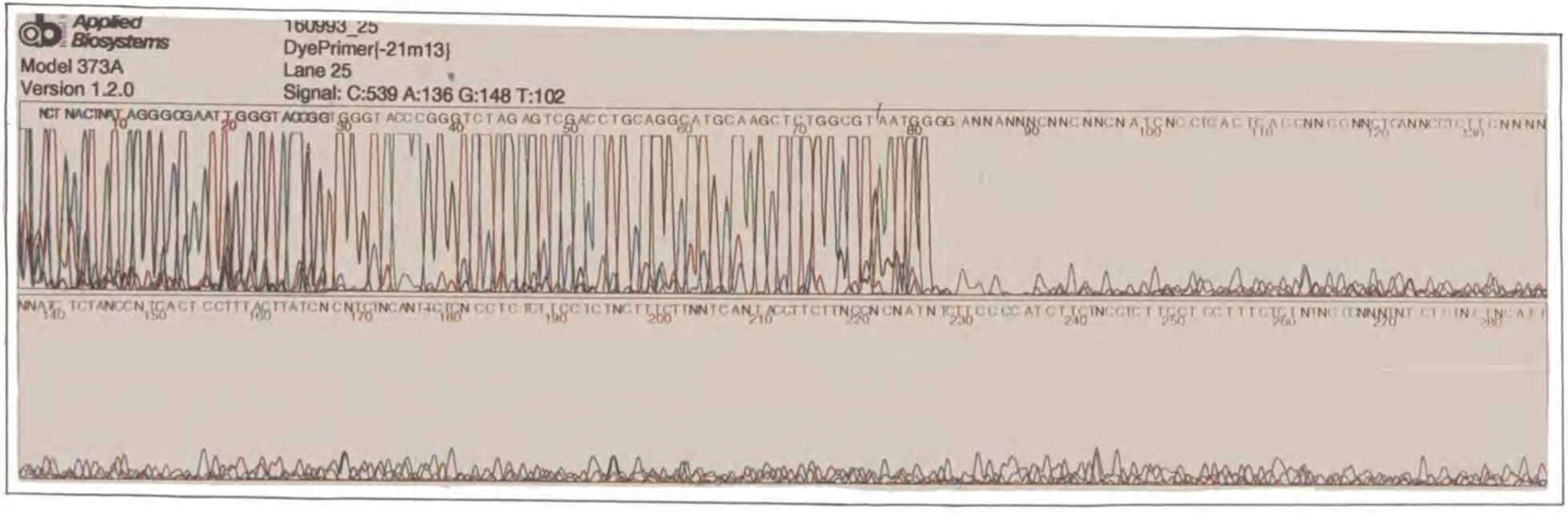
Panel A: Generation of nested deletions from λBS2xi

An overview of the generation of nested deletions from λBS2xi is given below. The 3.0kb cDNA is represented by an open box and vector sequences by a black line. **E** represents *Eco*RI; **K**, *Kpn* I and **S**, *Sac* I. The sites for the M13 universal primers are designated by FOR and REV. The location of β- galactosidase sequence detected in $pBFND_{28}$ is designated by a dashed box.

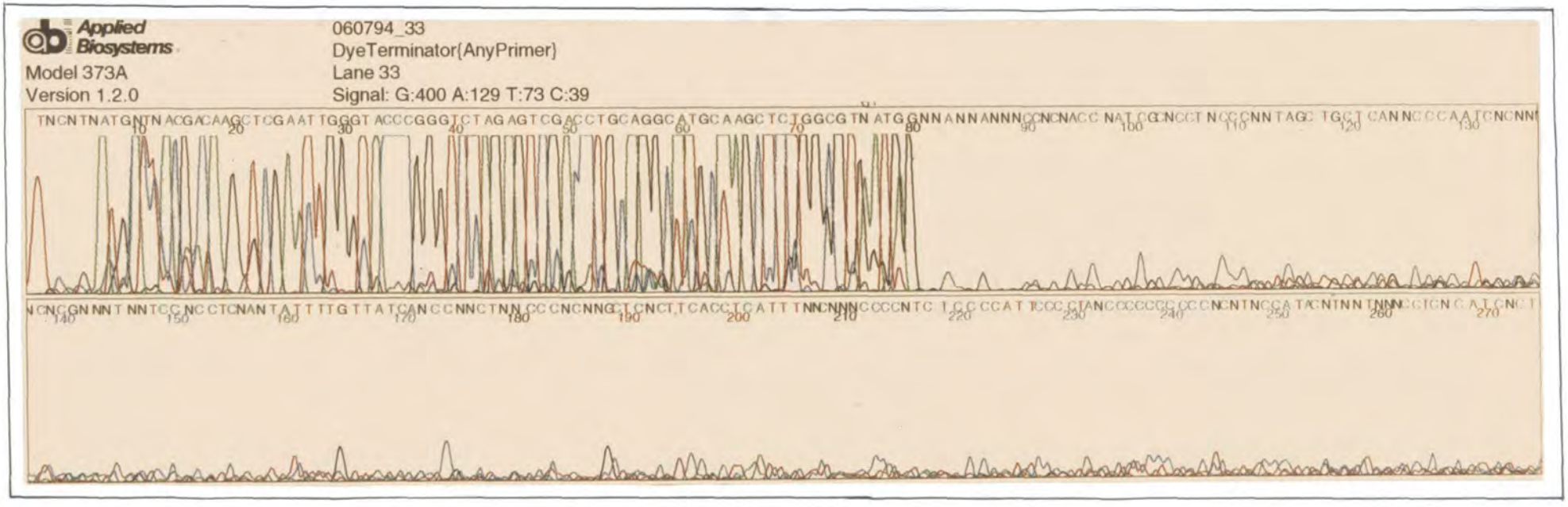


B(i): Nucleotide sequence of pBFK

Sequence data using the M13 universal forward primer with clones pBFK and pBFND28 (panel C) is presented below. Sequencing from this primer site consistently produced the same pattern of result, with the sequencing enzyme encountering a structure that it could not read through just outside the polylinker region. Nucleotide sequence data from the M13 universal reverse primer site was of consistently poor quality, until approximately half of the cDNA had been deleted. A stretch of good quality sequence was obtained from pBFK28. This sequence was demonstrated to correspond to β- galactosidase. The position and extent of this sequence in β-galactosidase in pSK+ is shown in panel D.

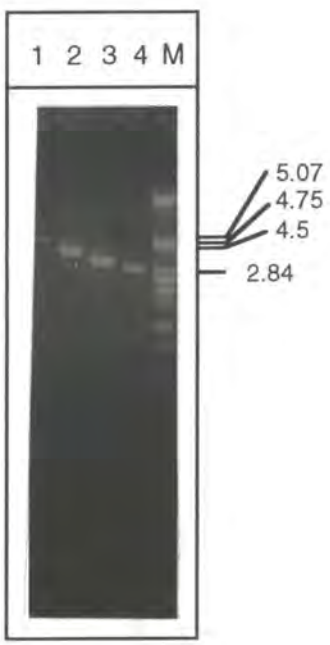


Panel B(ii): Nucleotide sequence of pBFND28



Panel C: pBFND28

The lanes contained: **1- 3**, 100ng *Bam*HI- cut pBFND clones. **1**, pBF16; **2**, pBFK26; **3**, pBFND28; **4**, 100ng *Bam*HI cut pSK$^+$. The gel was calibrated with 1.0μg *Pst* I- cut λ DNA in lanes **M**.



D: Extent of pBFND28 nucleotide sequence within pSK+

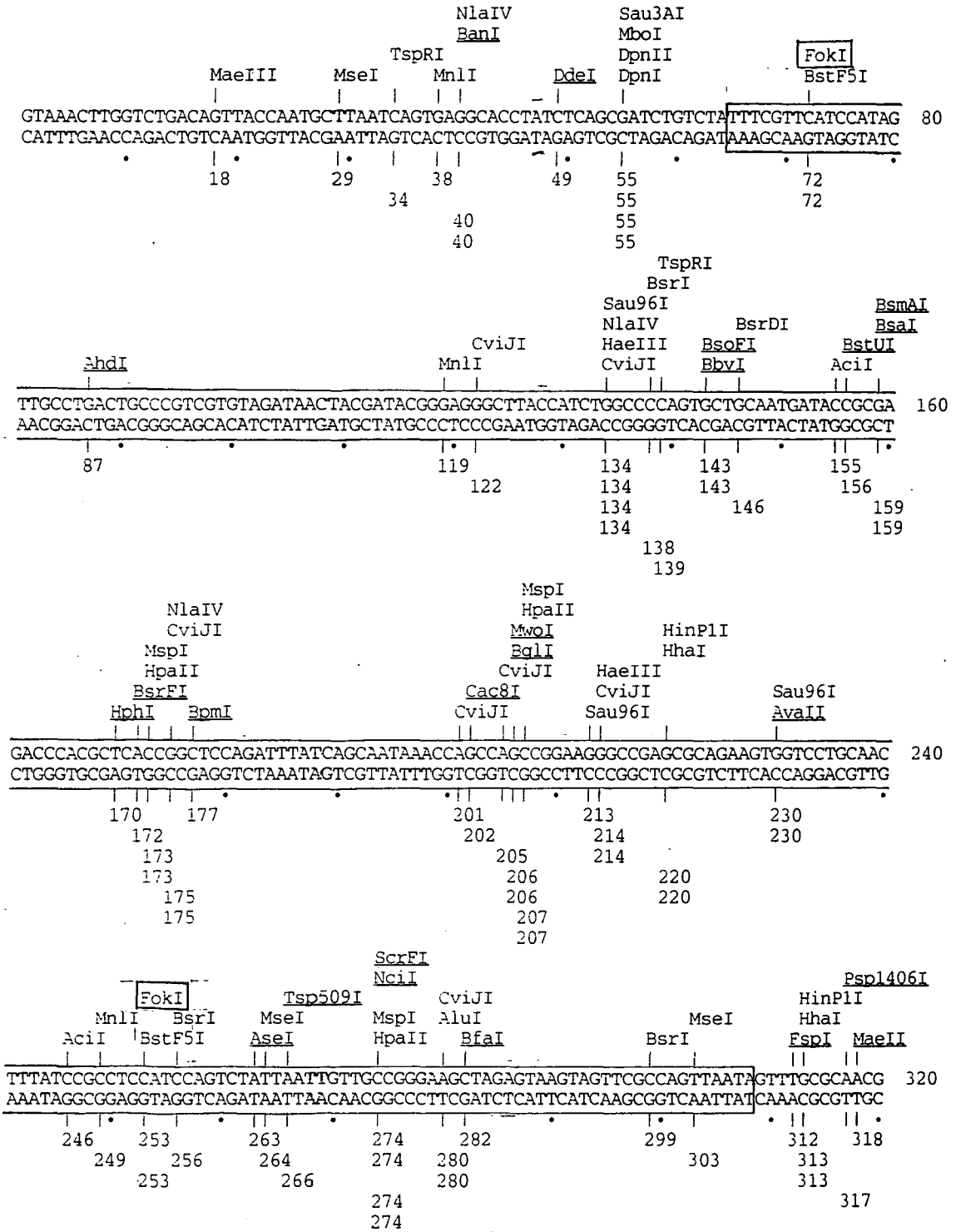


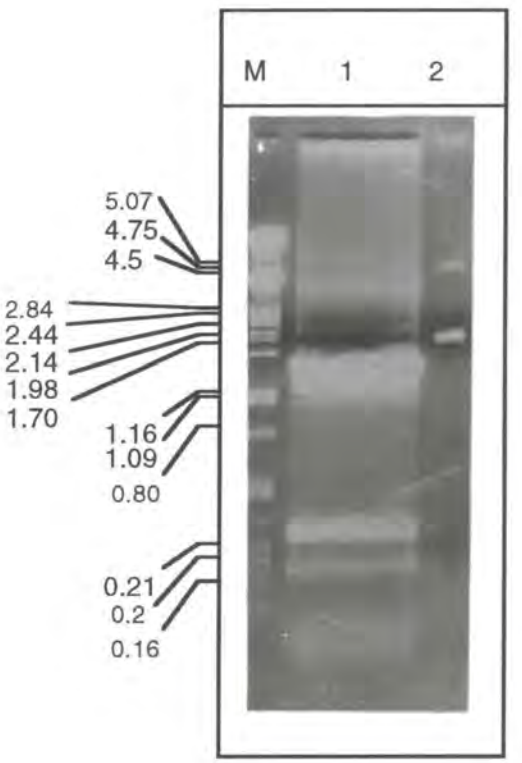
Figure 4.14

Southern analysis of DNA binding protein phage BS2xi

λBS2xi was analysed by Southern hybridisation with a pSK+ restriction fragment that corresponded to plasmid sequence detected in $pBFND_{28}$.

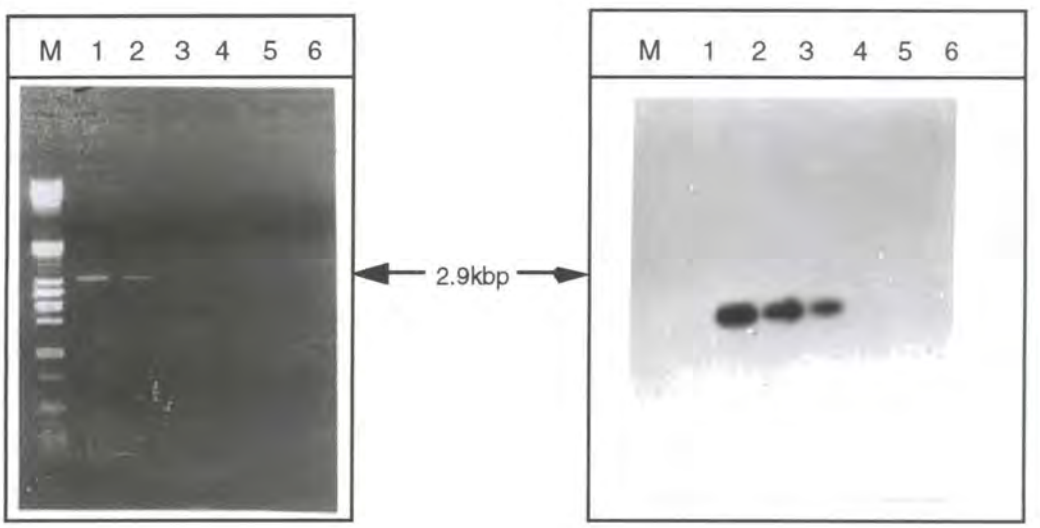
Panel A: Generation of probe

The *Fok* I 181bp fragment of pSK+ corresponded to the majority of pBF_{28} sequence data and was used as a probe. The lanes contained: **1**, preparative *Fok*I digest of pSK+ (1481, 1010, 287, 181bp fragments). **2**,1.0µg uncut pSK+. The gel was calibrated with 1.0µg *Pst* I- cut λDNA in lanes **M**



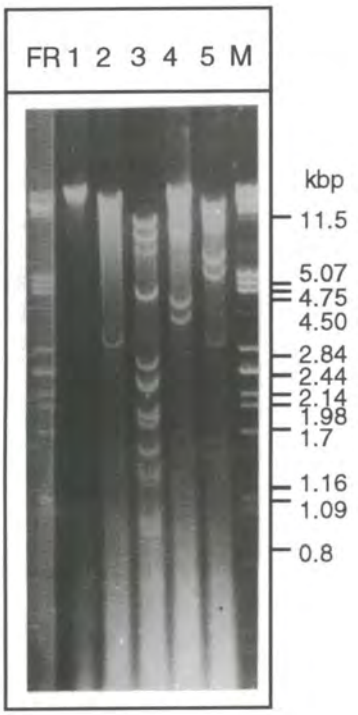
Panel B: Southern blot of *Eco*RI- cut pSK$^+$

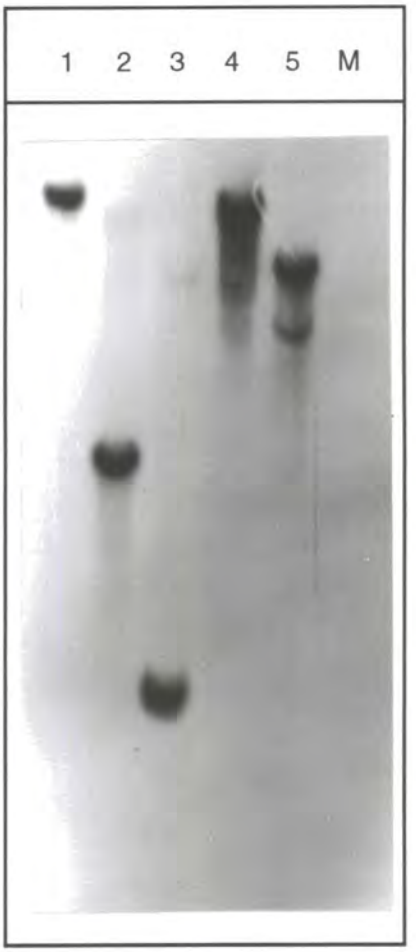
The specificity of the probe was determined by hybridising it to a southern blot that contained a dilution series of *Eco* RI cut pSK$^+$. A 1% agarose gel was blotted as described in materials and methods and then incubated with 10^6 cpm of the pSK$^+$ *Fok* I (181bp) probe. The lanes contained: **1- 6**, 10, 5, 1, 0.1, 0.01 and 0.001ng *Eco* RI cut pSK$^+$ respectively. The gel was calibrated with 1.0µg *Pst* I- cut λDNA in lanes **M**.



Panel C: Southern blot of λ BS2xi

The lanes contained: **1- 5**, 2.0μg λBS2xi DNA; **1**, uncut; **2**, *Eco*RI- cut; **3**, *Pst* I cut; **4**, *Hin* DIII- cut; **5**, *Bam*HI- cut. The gel was calibrated with a fluorescent rule in lane **FR** and 1.0μg *Pst* I cut λ DNA in lane **M**.





4.3 **Summary and further experiments**

Chapter three described the analysis of the ACP05 gene promoter in terms of its protein binding sites. A motif was identified that bound a protein detectable only in embryo. Complimentary oligonucleotides that contained this binding site were synthesised. These were multimerised and use as a probe to screen a library in a Southwestern experiment. A λgtII library generated from mid-maturation *B. campestris* embryos was screened. The decision to use a *B. campestris* library was made due to the fact that the *B.napus* ACP05 gene probably originated from *B. campestris*. ACP05 shares 96% homology, within its coding sequence to a *B. campestris* cDNA clone, which is strong evidence that the *B. campestris* seed ACP gene is the progenitor of the *B. napus* genomic ACP05 (*B. napus* originated from a cross between *B. campestris* and *B. oleracea*). One positive clone was isolated, phage BS2xi, which did not bind to control DNAs. This was taken as evidence that λBS2xi contained a sequence specific DNA binding clone. Lysogens were generated from λBS2xi and the encoded recombinant protein was analysed by gel retardation. Poly (dIdC:dIdC) assays demonstrated that a sequence specific DNA binding domain was present. Furthermore competition assays demonstrated that the formation of DNA- protein complexes was dependent on the presence of AAGAC. Western blots using anti β- galactosidase antibodies demonstrated that this DNA binding motif was translated from an internal initiation codon.

Several strategies were attempted to in order to characterise and sequence the cDNA. Endonuclease restriction of λBS2xi with *Eco* RI excised the entire 3.0kbp cDNA insert. This was subcloned into pSK^+, to generate pBF2 and maintained in SURE cells. However it was observed that when the subcloned cDNA insert was maintained in JM101 cells, the correct plasmid could not be obtained. In addition PCR amplification of phage BS2xi and subclones consistently failed.

A series of nested deletions, pBFND, were generated and sequenced. Data from the forward primer site was blocked, possibly by secondary structure and

reliable data from the reverse direction was not obtained until approximately 1.5 Kbp from the 5' end of the cDNA. This result is consistent with PCR not amplifying a product as the Taq polymerase would have encountered the same structure that prevented the sequencing enzyme extending through it. The sequence data from clone pBFND28 was used in an homology search through the GenEmbl databanks which demonstrated that it corresponded to β- galactosidase sequence. Southern analysis with a restriction fragment that encompassed the identified β-galactosidase sequence showed that it was also present in the original λBS2xi clone. The origin of this sequence was unknown.

The presence of contaminating plasmid DNA in λBS2xi obviated one strategy which could have been used to isolate a full length clone. This was to re-screen a λZap II library with the cDNA insert from λBS2xi. cDNA sequence is automatically subcloned following plasmid rescue in λZAPII. However the λZAPII vector contains β- galactosidase, so this approach was not possible. A possible strategy that could be tried in order to obtain clones that do not contain plasmid sequence is one of "shotgun cloning". This would involve digesting λBS2xi with a frequent cutter, such as *Sau* 3A and to clone the restriction fragments into a non pUC based vector such as pSU19 (from the pSU series of plasmids). Southern analysis of the recombinants with a probe that contained β- galactosidase sequence could discriminate between clones that contained plasmid sequence and those that did not. Clones that did not contain plasmid sequence could then be sequenced, or used directly to re-screen a λZAPII library. If this approach failed then a new library could be screened with the multimerised binding site probe to re- isolate the binding protein.

Chapter 5

Characterisation of the ACP Promoter Binding Protein

Chapter 5

Characterisation of the ACP Promoter Binding Protein.

5.1 Introduction

The analysis of the ACP05 promoter in terms of its protein binding sites was discussed in chapter 3. A motif that bound an embryo protein was characterised. This motif was incorporated into a pair of oligonucleotides which were polymerised and used to screen an expression library for the corresponding DNA binding protein. A single clone, λBS2xi was isolated from this Southwestern experiment and is described fully in chapter 4. Gel retardation assays were used to analyse the recombinant protein encoded by λBS2xi. Competition assays demonstrated that the cDNA encoded a sequence specific DNA binding domain. This chapter deals with the characterisation of λBS2xi and the recombinant protein that it encodes.

5.1.1 Expression of λBS2xi

The main objective of experiments described in this chapter was to determine whether clone λBS2xi corresponded to an expressed protein. The entire 3.0 kbp cDNA insert from λBS2xi was excised upon *Eco*RI digestion and used to probe northern blots in order to ascertain whether it hybridised to an expressed message. Gel retardation assays showed that detectable levels of the ACP05 promoter binding protein were only present in embryo extract (and not in leaf extract). Northern blots that contained RNA extracted from different tissues and from developing rapeseed were also probed to analyse the expression pattern of λBS2xi. A second probe was generated from a seed expressed ACP cDNA and was used to determine whether there was a correlation between the expression of λBS2xi and the protein it was proposed to be involved in regulating.

5.1.2 Chromatography

The stability and binding characteristics of the ACP05 promoter binding protein from embryo extract were examined following liquid chromatography. It was envisaged that if the stability and concentration of the binding protein remained high enough following a round of chromatography, parameters such a native molecular weight could be assigned. Embryo protein was limited, therefore the first round of chromatography had to represent a large purification step. It has been well documented that the polyanion heparin interacts with nucleic acid enzymes and DNA binding proteins (Losito *et al.*, 1981; Farooqui and Horrocks., 1983). Immobilised heparin has been used as a successful affinity matrix in the purification of several DNA binding proteins, such as reverse transcriptase (Bhikhabhai *et al.*, 1992) and RNA polymerases (Hammon and Holland., 1983). Gel retardation assays were used to analyse column fractions and thus follow the DNA binding protein through chromatography.

5.2 **Results**

5.2.1 Northern blots

In order to determine whether λBS2xi cDNA hybridised to an expressed RNA, northern blots were probed. Blots were prepared with poly $(A)^+$ enriched RNA as described in section 2.10.1. The blots were blocked and then hybridised with the probes described overleaf as detailed in section 2.10.2. Filters were washed and hybridising bands detected as outlined in section 2.9.12.3. The density of specific bands on autoradiographs was determined by laser densitometry (2.10.3).

5.2.2 Probe preparation

Probe 1: ACP promoter DNA binding protein cDNA clone λBS2xi

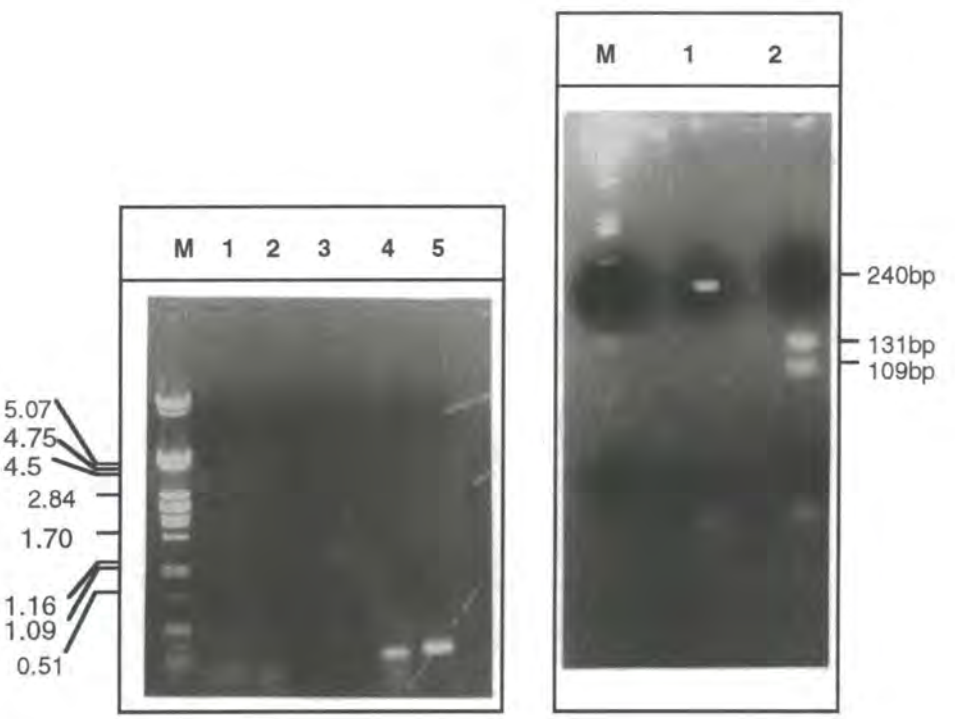
The entire 3.0kb cDNA insert of phage BS2xi was excised on digestion with

Figure 5.1

Analysis of probe 2
2ng of the seed expressed cDNA ACP29C08 was used as target template in each PCR. 0.5μM internal primers (see below) were used to amplify a 240bp 3' coding fragment (panel A). The 240bp PCR product was gel purified and the DNA recovered by freeze squeeze. 50ng of the gel purified product was further analysed by restriction digestion (panel B).

PCR primers used for probe preparation:

for: 5' GGCCTAGGTCACCTCTTGGCTTGCACGAGC 3'
rev: 5' GGCCATGGCCAAACCAGAGACAGTTGAG 3'



A: PCR
The lanes contained:**1- 5**, 20μl each PCR; **1**, no template DNA control reaction (CR); **2**, no enzyme CR; **3**, no primers CR; **4**, for and rev primers, ACP29C08 template DNA (240bp product); **5**, for and rev primers, pUC template (280bp product)

B: Analysis of Probes
1 and **2**, 50ng purified product from ACP29C08 reaction; **1**, undigested; **2**, *Hinf*I digested. The gel was calibrated in lane **M** with 1.0μg *Pst*I digested λ DNA

Eco RI (panel A of figure 4.10). Purified DNA was labelled by random primer reaction (2.9.11.1) and purified by centrifugation through a Biospin P-30 column (2.9.11.1) and the specific activity calculated from liquid scintillation (routinely 10^6 cpm/ng).

Probe 2: ACP cDNA 29C08

The seed specific ACP cDNA clone ACP29C08 (Safford *et al.*, 1988) was provided by Prof. A.R.Slabas. PCR was used for probe preparation, to amplify a 240bp 3' coding sequence fragment using internal primers. A size control PCR using a pUC plasmid with a characterised insert (280bp) was set up in tandem using pUC forward and reverse primers (2.9.14). Following amplification, PCRs were analysed by gel electrophoresis (panel A of figure 5.1). Negative control reactions performed to monitor contamination failed to amplify any product (lanes 1- 3) and the positive control reaction contained a product of the expected size (280bp, lane 5). The PCR of ACP29C08 amplified a fragment of the expected size, 240bp (lane 4). The remaining 240bp PCR product was gel purified (2.9.6.1) and analysed by restriction digestion. The correct product contains one internal *Hinf* I site, which would produce fragments of 131bp and 109bp on digestion. It can be seen in panel B of figure 5.1 that the PCR product produced two fragments of the expected size (lane 7) which was consistent with it being the correct product. The purified PCR product was labelled by random primer reaction (2.9.11.1) and purified from unincorporated radionucleotide by centrifugation through a P-30 spin column and the specific activity calculated from liquid scintillation (routinely 10^6 cpm/ ng DNA).

5.2.3 **Northern hybridisations**

These were performed using the two probes described above in section 5.2.2:

Probe 1: cDNA BS2xi

A 10 hour phosphoimager exposure of a northern blot hybridised with probe 1 is presented in panel A of figure 5.2. A single hybridising band of

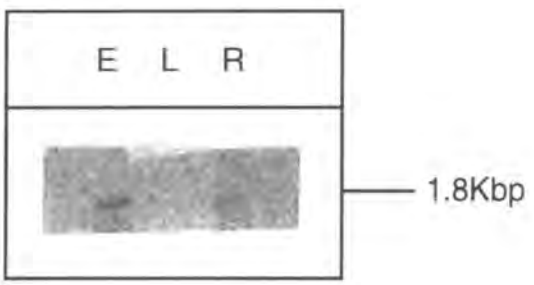
Figure 5.2

Expression pattern of the DNA binding clone λBS2xi

Poly A^+ enriched RNA was electrophoresised through 1.4 % formamide gels and blotted onto Hybond N membranes. Blots contained RNA extracted from leaf, root, seed and series of developing embryo. Blots were blocked and then hybridised with probe 1 (described in text), then stripped and reprobed with probe 2. A 10 hour phosphoimager exposure and a 36 hour autoradiograph of a blot probed in this way are presented in panel A and C respectively. The relative expression of the binding clone was determined by laser densitometry (panel B). A series of developing embryos were also hybridised with both probes (panel D).

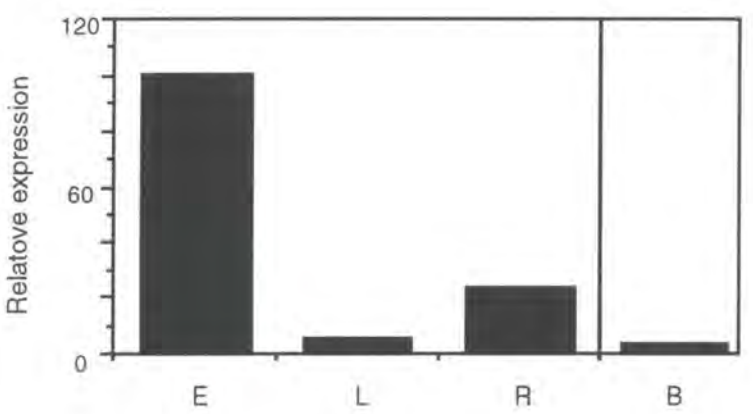
A: Northern blot hybridised with probe (1): cDNA λBS2xi

The lanes contained: 1.0μg mRNA from **E**, embryo; **L**, leaf and **R**, root.



B: Relative expression of DNA binding clone

B = Background



approximately 1.8kb was observed in both the embryo and root lanes. An appreciable level of expression was not observed in the leaf lane. This result demonstrated that λBS2xi cDNA hybridised to an expressed message, which was detected in embryo but not in leaf mRNA, at this exposure. The relative level of expression was measured by densitometry (2.10.3) and determined to be approximately five fold higher in embryo than in root tissue (as shown in panel B). This correlated with data obtained from gel retardation experiments (chapter 3), in which the activity of the ACP promoter binding protein was also only detected in embryo tissue.

Probe 2: ACP29C08 cDNA

Northern filters were stripped (2.10.2), reblocked then reprobed probe 2, the PCR fragment amplified from ACP29C08. The results are presented in panel C of figure 5.2, which shows a 36hour autoradiograph. A single hybridising band of approximately 0.75kb was observed in the embryo and root lanes, but not in the leaf lane. This band corresponds to full length ACP cDNA 29C08 as demonstrated previously (Safford *et al.*, 1988).

Northern blot using mRNA from developing rapeseed

Both probes were hybridised simultaneously to northern blots that contained mRNA from a series of developing rapeseed. Blots were blocked, hybridised and washed as described previously. Panel D of figure 5.2 shows a 20hour Phosphoimager exposure of a northern blot probed as described above. Two hybridising bands bands were observed; the 0.75kb represents full length rapeseed ACP29C08 mRNA and the second band at 1.8kb represented the ACP05 promoter binding protein transcript. It can be seen that the expression of ACP increases to a maximum around 50DAF, then decreases (as previously described by Safford and colleagues (Safford *et al.*, 1988). The level of expression of the DNA binding protein transcript is far lower than the ACP transcript (estimated to be approximately 20- 30 fold less as the level of background in double hybridised blots was too high to quantify the relative expression accurately by densitometry). The level of the DNA binding protein transcript also increased to a maximum at around 50DAF then decreased, but

was still detected at 63 DAF, at which point the ACP transcript was not detected.

5.2.4 **Preliminary characterisation of the ACP promoter binding protein from *B.napus***

5.2.4.1 Effect of salt on embryo protein-DNA binding interaction

A gel retardation assay performed over a range of salt concentrations with embryo extract (2.11.6.1) is presented in figure 5.3. Sequence specific DNA-binding is mainly mediated through electrostatic interactions. On the addition of salt, these interactions should be disrupted and subsequently at high salt concentration binding completely inhibited. Thus with an increase in the salt concentration the formation of retarded complex should disappear. The concentration of salt at which binding is inhibited was used as an approximate guide when assaying column fractions for eluted activity.

The AAGAC containing oligonucleotide BS (table 3.3) was used as probe in a set of standard assays (section 2.9.15.1). The only variant was the KCl concentration of the binding buffer (figure 5.3). Lane 1 is the no protein control and shows the mobility of the free probe (indicated as FP). The assays in lanes 2- 8 were incubated in binding buffer made with 0, 100, 200, 250, 300, 500, and 1000mM KCl, respectively. The formation of the DNA- protein complex was stable in KCl up to 100mM. Above this salt concentration the stability of the retarded complex was reduced, probably due to disrupted electrostatic interactions and subsequently was not observed. The titration was repeated using NaCl, with identical results (not shown).

5.2.4.2 Effect of different polyanion competitors on complex formation

It has been reported that by changing the non- specific polyanion competitor in a gel retardation assay, different DNA- protein complexes can form (Lee and Schwartz, 1992). In retardation assays using the same DNA probe and protein extract, but different polyanion competitors, different DNA complexes were observed with each competitor. For instance one complex was detected

with poly (dIdC:dIdC), but with poly (dGdC:dGdC) an additional, previously undetected complex was observed. It was concluded that multiple complexes formed as a result of differential affinities of the binding proteins in the extract used for each polyanion competitors (Lee and Schwartz, 1992).

The ACP promoter binding protein was assayed for its affinity towards different polyanion competitors. Leaf extract was also analysed in case a previously unobserved binding protein was present and detectable using an alternative competitor. Various poylanions (poly (dIdC:dIdC), heparin, poly (dGdC:dGdC) and *E.coli* DNA) were used as non- specific competitors in a set of standard assays (2.9.15.1) with oligonucleotide BS as probe (figure 5. 4). A single DNA- protein complex formed consistently with all the polyanions tested and no activity was detected in leaf extract with any of the alternative competitors. It was concluded that the AAGAC binding protein was embryo specific and did not possess differential binding affinities for any of the competitors analysed.

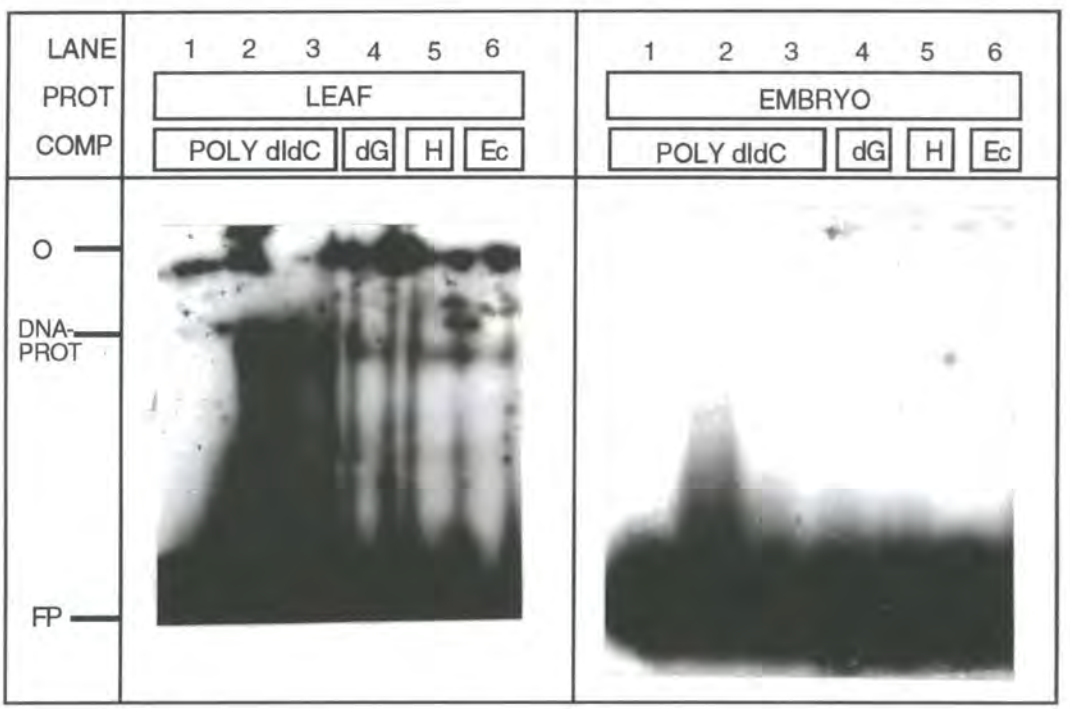
5.2.4.3 Heparin agarose affinity column chromatography

Heparin agarose liquid chromatography was performed as described in section 2.11.7.1. with embryo extract. The fractions were analysed using gel retardation assays (figure 5.5 panel B) in a modified binding buffer made without salt, in order to compensate for the increased salt in column eluent ("no salt": 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA and 3% glycerol). The protein concentration of each fraction was assayed by the Bradford method (2.11.3) and is presented in panel A of figure 5.5. A gel retardation assay with column fractions 25- 38. is presented in panel B. Lane 1 contained no protein and the mobility of the free probe is indicated (FP). Lanes 2-15 contain 1.0µl of the fractions 25- 38, respectively. DNA binding activity started to elute at approximately 250mM KCl (fraction 27) and the highest activity was contained in fractions 27- 30, which was in the main peak of eluted protein (panel A). Binding was not detected in fractions above 35.

Figure 5.4

Analysis of the binding characteristics of BS probe with different polyanion competitors

DNA binding proteins can possess differential affinities for polyanion competitors which can result in the detection of additional DNA- protein complexes. The following set of poly anion competitors (COMP) were used to assay the ACP promoter binding protein for differential binding activity: poly(dIdC:dIdC)- **polydIdC**; poly(dGdC:dGdC)- **pG**; heparin- **H**; *E. coli* DNA- **Ec**). Both leaf and embryo extract was analysed. The Origin (**O**) and mobility of the Free Probe (**FP**) and DNA- Protein complex (**DNA-PROT**) are indicated.



The lanes contained: **1**, no protein, 1.0µg poly (dIdC:dIdC), **2**, 1.0µl protein, no poly (dIdC:dIdC); **3**, 1.0µl protein, 1.0µg poly (dIdC:dIdC); **4**, 1.0µl protein, 1.0µg poly(dGdC:dGdC); **5**, 1.0µl protein, 1.0µg heparin; **6**, 1.0µl protein, 1.0µg *E. coli* DNA.

5.2.4.4 Analysis of the DNA binding protein detected in heparin column fractions

Heparin column fractions 27- 30 exhibited the strongest interaction with the AAGAC binding site probe. These were pooled and the specificity of the DNA binding interaction was determined by a set of competition assays (3.2.1), using the modified "no salt" binding buffer. The results of a typical competition assay are presented in panel C of figure 5.5. Lane 1 contained no poly (dIdC:dIdC) and lane 2 contained no protein. The mobility of the free probe is indicated. Lane 3 contained protein and poly (dIdC:dIdC) and the specific DNA - protein complex is indicated. A excess of the non- specific competitor oligo MBS was added to the assays run in lanes 4 and 5. MBS contained a modified AAGAC motif (table 3.3) and the addition of a large excess of this competitor has no effect on the formation of the specific complex. An excess of the specific oligonucleotide competitor BS were added to assays 6-8. BS bears an AAGAC motif (table 3.6) and the retarded DNA- protein complex was effectively competed with unlabelled BS. It was concluded that the DNA binding protein detected in fractions 27- 29 from the heparin agarose column was the embryo AAGAC binding protein.

5.2.4.5 Further chromatography

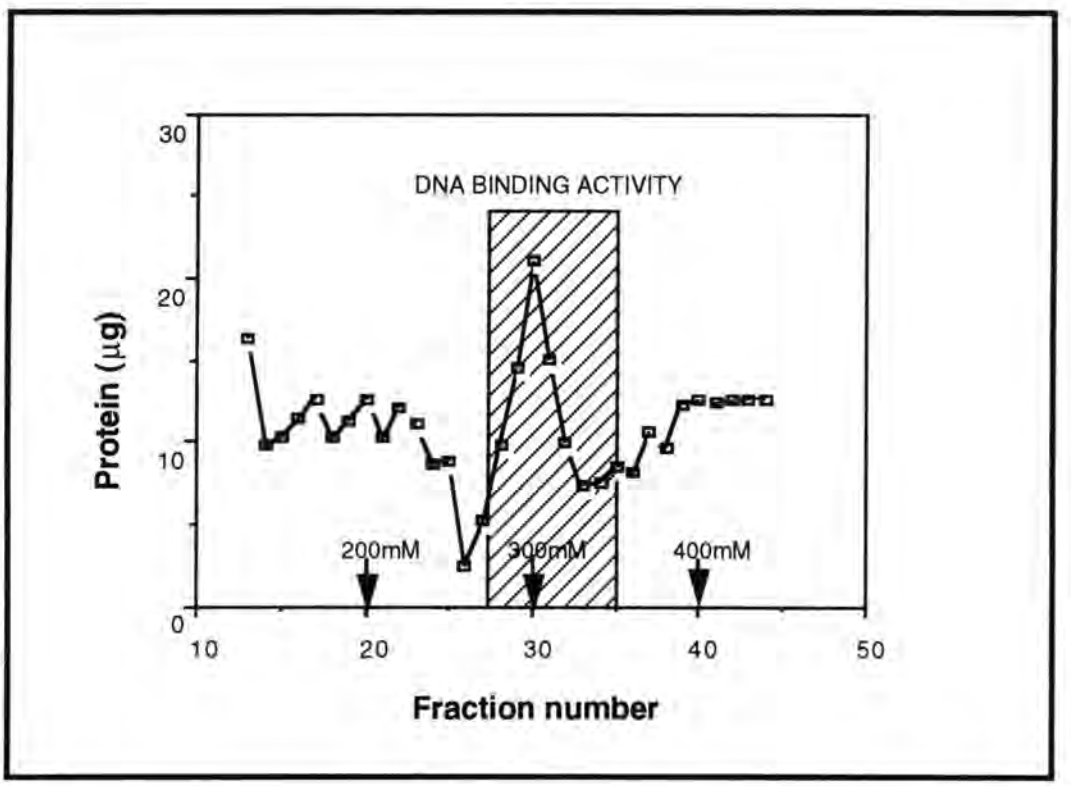
The fractions that contained the highest specific binding activity (27-29) from heparin column chromatography were pooled and used in an attempt to determine the native molecular weight of the binding protein. Fifty microlitres (approximately 250μg) of the pooled fractions was thawed on ice and immediately loaded onto a calibrated gel filtration column (2.11.7.2). The column was eluted as described in 2.11.7.2 and fractions were collected, snap frozen in liquid nitrogen and subsequently assayed by gel retardation with oligonucleotide BS, as described above. Gel filtration columns were repeated, but reproducible binding activity was not seen. Following heparin agarose chromatography the active column fractions were stored as frozen drops at -80°C (2.11.6). Periodic checks of activity showed that the stability of the DNA binding protein was significantly reduced, with most binding activity lost within a week.

Figure 5.5

Gel retardation analysis of heparin column fractions for embryo DNA binding protein

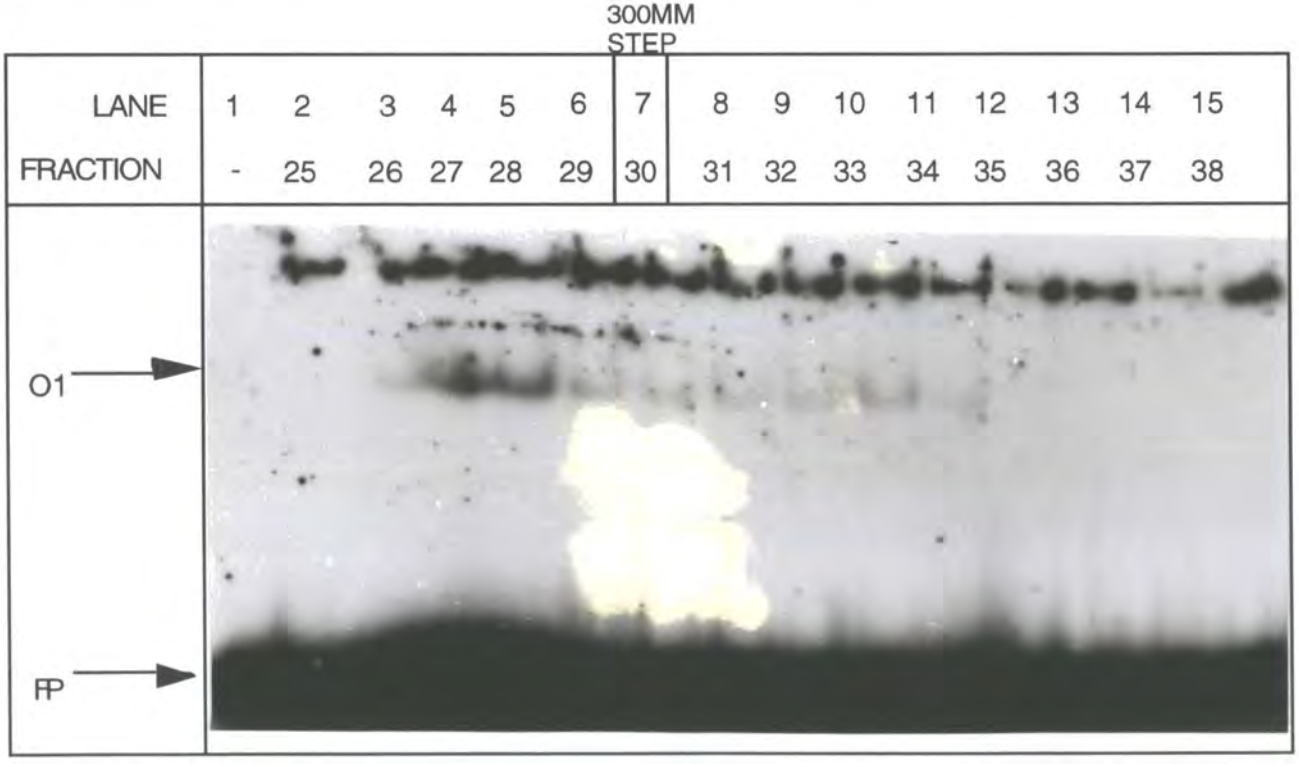
Heparin agarose affinity liquid chromatography was performed with 50mg embryo protein as described in materials and methods. 100µl fractions were collected and snap frozen in liquid nitrogen. The protein concentration of each column fraction was determined by the Bradford method, using ovalbumin to generate calibration curves (panel A). Fractions were also analysed using gel retardation assays, for the presence of ACP promoter binding protein (panels B and C).

Panel A: Protein concentration of fractions



Panel B: Gel retardation analysis of column fractions

Heparin agarose column fractions were analysed by gel retardation assays for DNA binding activity. The The lanes contained: **1-15**, 1.0µg poly (dIdC:dIdC), 10 000CPM labelled oligonucleotide BS; **1**, no protein; **2-15**, 1.0 µl fractions 15- 28, respectively. Incubation was in modified binding buffer: 20mM Tris.HCl pH 7.5, 1mM DTT, 1mM EDTA and 3% glycerol.



5.3 **Summary and further experiments**

Northern blots were probed with λBS2xi cDNA. A single hybridising band of approximately 1.8kb was detected. This showed that λBS2xi corresponded to an expressed message. It also demonstrated that the clone contained approximately 1.2kb unrelated DNA sequence. This result was consistent with Southern experiments that detected plasmid DNA sequence in λBS2xi (chapter 4). The expression of the DNA binding protein was not detected in leaf tissue, was expressed at a high level in embryo tissue and at a lower level in root tissue. This pattern of expression correlated with that of the seed specific ACP29C08 cDNA clone (Safford *et al.*, 1988). Such a correlation should be expected for a putative transcription factor and its cognate gene product. The expression of the DNA binding clone was also examined in developing rapeseed and was found to increase to a maximum level at approximately 50 DAF (Days After Flowering), then decrease. The timing of maximum expression again correlated with ACP29C08.

Embryo extract was fractionated by heparin agarose liquid chromatography. Fractions were assayed for the presence of the ACP promoter binding protein by the use of gel retardation assays. The protein was followed through one round of heparin agarose chromatography, however following chromatography there was a marked reduction in stability and characterisation of the protein was taken no further. This reduction in stability could have been as a result of dilution, the removal of stabilising protein factors during chromatography or mechanical damage from ice crystal formation. The main difficulty in the characterisation of the ACP05 promoter binding protein was the limited amount of starting material. The preparation of staged embryos was time consuming and bulk preparations could not be made. A future strategy which could possibly circumvent this limitation would be to optimise a protein extraction protocol using whole seed. If binding factors were still present at a detectable level in this extract, then alternative purification strategies could be attempted with the greater amounts of material available.

Chapter 6

cDNA Cloning of Enoyl- ACP Reductase

Chapter 6

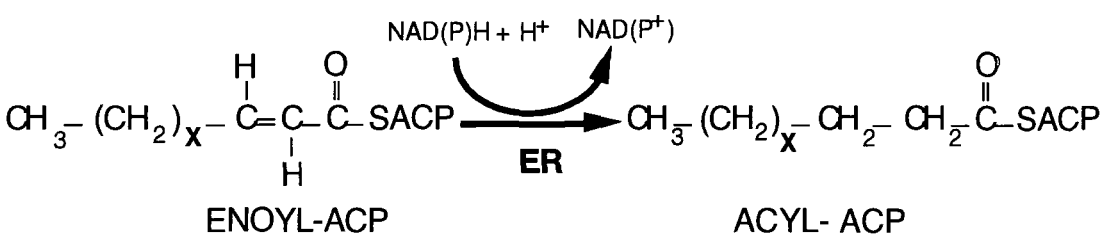
cDNA Cloning of Enoyl ACP- Reductase

6.1 **Introduction.**

6.1.1 Enoyl ACP- reductase

Enoyl ACP- reductase (ER) is a well characterised component of the plant fatty acid synthetase complex (Slabas *et al.*, 1986; 1990; Kater *et al.*, 1991; 1994; Slabas and Fawcett., 1992; Fawcett *et al.*, 1994). It catalytically reduces the *trans*- 2, 3 double bond of a 3- ketoacyl- ACP intermediate, to form a saturated acyl- ACP, which in turn can serve as the substrate for the next condensation reaction (the individual reactions of the FAS system are listed in table 1.2).

The Reduction of ketoacyl intermedates by ER



There are two detectable enzyme activities in plants which have different

patterns of expression and different substrate specificities, one form is NADH-dependent and the other prefers NADPH. (Shimakata and Stumpf, 1982a; 1982d; Slabas *et al.*, 1986; 1990). The expression of ER mRNA and protein isoforms was previously analysed by hybridisation studies and two dimensional Western blots (Fawcett *et al.*, 1994). These experiments demonstrated that ER was regulated temporally during seed development, with the increase in message preceding the increase in ER protein. This in turn preceded the deposition of lipid. Four isoforms (ERI- IV) were detected in both leaf and seed tissue, but not at equimolar amounts. There were two major and two minor isoforms, with the expression of all four proteins significantly higher in the seed than in the leaf (Fawcett *et al.*, 1994).

Southern analysis of ER in *B. napus* showed there were four genes present, two of which were inherited from each of its parents, *B. campestris* and *B. oleracae* (Kater *et al.*, 1991). Prior to this work, only two full length cDNA clones had been characterised from *B. napus*: pEAR7, isolated from a Rafael cv embryo library (Kater., *et al* 1991) and pERL8, isolated from a Jet Neuf cv leaf library (A. Fowler., unpublished results).

The main objective of experiments described in this chapter was to derive additional information about the differential expression patterns of ER isoforms. A prerequisite was to screen a *B. napus* library in order to isolate additional ER clones and analyse specific regions of interest. It is well established that the leader peptide sequence serves to direct the compartmentilisation of nascent polypeptides (Zimmermann and Meyer., 1986). Several lines of evidence currently show that there are one or possibly two membrane associated forms of ER (*pers. comm.* A. Fowler), so this area was of interest. In addition, the 3' untranslated area of transcripts has been shown to be important in the stability and turnover of message (Decker and Parker., 1994; Hagan *et al.*, 1994; Nanbu *et al.*, 1994). This area might therefore be implicated in the differential expression of the ER isoforms, so was also targeted.

6.2 **Results**

6.2.1 Probe preparation

The nucleotide sequence of pERL8 is presented in panel A, figure 6.1 (A. Fowler., unpublished results). It is approximately 1.3kb, with a 73 amino acid leader peptide. A 3' *Eco*RI restriction fragment that contained 16 codons and the entire 3' untranslated region (boxed in panel A) was used as probe. pERL8 was digested with *Eco*RI (section 2.9.5) to generate fragments of 2958, 1163 and 187 bp (panel B, figure 6.1). The digestion was gel fractionated (2.9.6.1) and the 187bp fragment was excised and labelled directly in the gel slice by a random primer reaction (section 2.9.11.1). The labelled DNA was purified by centrifugation through a Biospin p-30 gel filtration column and the specific activity was calculated from liquid scintillation. 4x 10^6 CPM was used to screen 200 000 pfu.

6.2.2 Description of library used

The library used was constructed from total *B. napus* cv. Jet Neuf RNA isolated from mid- maturation embryo. First round cDNA synthesis was poly A primed and adapters were used to directionally clone the cDNA into the lambda vector λZapII, such that the 5' end of the cDNA was cloned into the *Eco*RI site and the 3' end into the *Xho*I site.

6.2.3 Screening the library

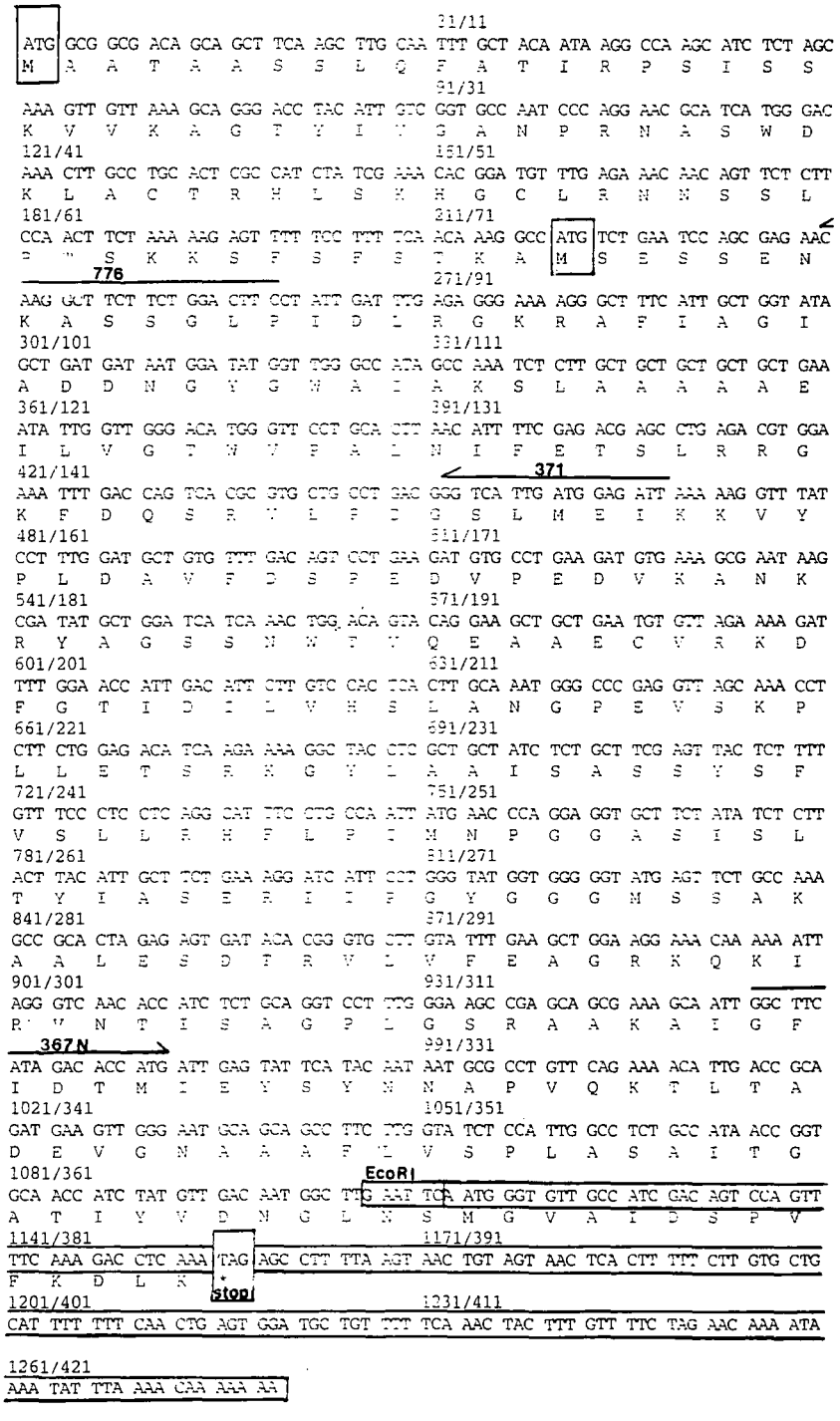
A total of 200, 000 pfu (50, 000pfu/ 22cm^2 plate) were screened as described in section 2.13. Ten putative positives that had the characteristic comet shaped plaque were isolated and taken through a further two rounds of screening. Plasmids that contained the positive cDNAs were generated by plasmid rescue (*in vivo* excision of bluescript phagemid from the λZAPII vector) as described in section 2.13.3, to generate the pERE series of plasmids.

Figure 6.1

ER cDNA clone pERL8

A: Nucleotide sequence

The complete nucleotide sequence of pERL8 is given below (A. Fowler, unpublished results). The sequence begins with the start codon for the leader peptide and the start and stop codons for the mature peptide are boxed. The position of internal sequencing primers is indicated above the complimentary sequence. The 3' *Eco*RI fragment used as a probe is outlined.



6.2.4 **Characterisation of the positive clones**

6.2.4.1 Determination of cDNA insert size

The size of the cDNA insert was determined by PCR amplification, using the standard method described in section 2.9.14 and forward and reverse primers for pSK (listed in table 2.1). Following amplification PCRs were analysed by gel electrophoresis (figure 6.2). A positive control using pSK^+ as template was performed in tandem (lanes 11 and 12: no insert=245bp) and the no-template control reaction (lane 13) failed to amplify any product. The size of the cDNA inserts were calculated from comparison with the molecular weight standards and are listed in table 6.1 below (the contribution from primer sequence has been subtracted).

Table 6.1: Size of cDNA insert for positive Enoyl- ACP reductase clones

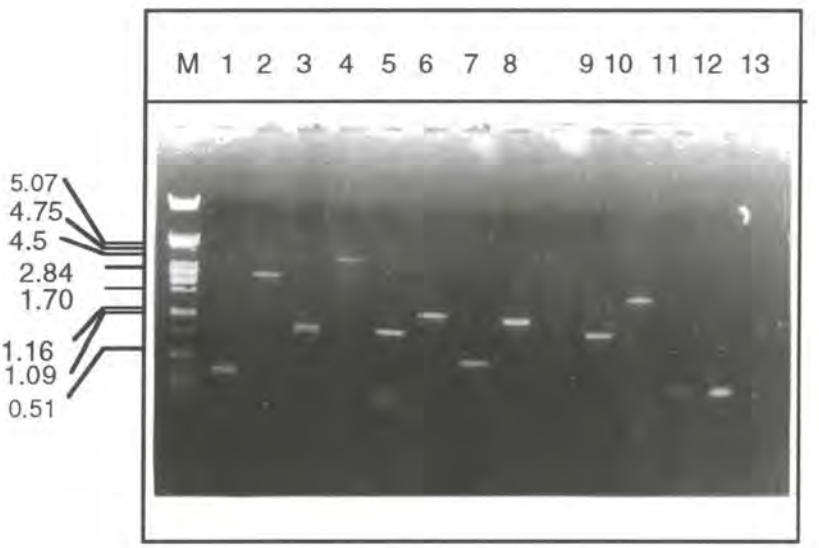
p ERE Clone	Size (Kbp)
1.9	0.35
1.9h	2.5
1.30	0.8
1.30h	2.9
1.31	0.76
4.0h	1.3
3.33	0.4
4.3	1.2
4.21	0.7
4.21h	1.6

From the determination of insert size it was noted that only pERE 4.0h (1.3kb), 4.21h (1.6kb), 1.30h (2.9kb) and 1.9h (2.5kb) could possibly contain full

Figure 6.2

PCR analysis of positive clones

The size of cDNAs was determined by PCR amplification. 2ng template DNA and 0.5μg pSK primers were used per reaction. Following amplification 20μl each reaction was analysed on a 1% agarose gel. The lanes contained: **1-12**, 20μl PCR reaction performed by the standard method with the following pERE clones as target templates; **1**, 1.9; **2**, 1.9h; **3**, 1.30; **4**, 1.30h; **5**, 1.31; **6**, 4.0h; **7**, 3.33; **8**, 4.3; **9**, 4.21; **10**, 4.21h; **11** and **12**, 20 and 30 μl respectively of positive size control PCR (245bp); **13**, no template control PCR. The gel was calibrated with 1.0 μg *Pst*I- cut λDNA in lanes marked **M**.



from the large size of these cDNAs) multiple ligation events had resulted in additional clones present at the 5' and/ or 3' end of the ER cDNA. FASTA searches (2.10) were conducted for the unknown cDNA sequences and the top matches are presented in table 6.3.

Table 6.2: Sequence analysis of artifacts: Top matches from FASTA search of GenEMBL database

pERE clone	Top Match from FASTA search
1.9	79.2% identity to *Brassica campestris* clone BE5 (storage protein napin clone)
1.30	91% identity with *B. campestris* clone pBC2SC (2S storage protein clone)
1.31	96.7% identity with *B. campestris* clone BE5 (storage protein napin clone)
3.33	98.1% identity with *Brassica napus cru*4 mRNA (cruciferin *cru*4 subunit clone)
4.21	99.1% identity with *BngNAP* 1 gene (*B. napus* napin gene)

6.2.4.5 Sequence comparisons of authentic clones

The clones were divided into two groups based on nucleotide sequence differences:

Group A: pERL8, pERE1.30h and 4.3

Group B: pERE1.9h, 4.0h and 4.21h

The nucleotide and corresponding translated amino acid sequences of the authentic ER clones are presented in figure 6.3 and the comparison of different regions is discussed further below.

Table 6.3: Sequence analysis of 5' and 3' cDNAs in multiple ligated clones: Top matches from FASTA search of GenEMBL database

pERE clone	Top match from FASTA search
1.9h 5' cDNA	expressed cDNA clone SBC4T7P (unknown function) 82.3% identity over 250bp
1.9h 3' cDNA	expressed cDNA clone SBB2T7P (unknown function) 78.6% match over 200bp.
1.30h 5' cDNA	GT- rich mouse period clock protein 40.2% over 150bp
4.21h 5'cDNA	*Arabidopsis* clone FAFJ60 (unknown function) 87.9% over 200bp

A: 3' Coding region

A comparison of the nucleotide sequences of the 3' coding region (160bp immediately preceding the stop codon) is presented in panel Ai of figure 6.3. The derived amino acid sequence is compared in panel Aii. Based on differences in this region (boxed for clarity in the figure) the clones were classified into two groups A and B (see above). pERL8 and pERE1.30h were identical and thus represent an isoform expressed both in embryo and leaf tissue. pERE 4.0 and 1.9h and 4.21h were identical to each other in this region and represented a new ER isoform. Sequence was highly conserved overall between the two groups, with only 3 amino acid changes out of 160 in this region.

Figure 6.3

Sequence comparisons of Enoyl- ACP reductase clones

Five clones were confirmed to contain authentic enoyl- ACP reductase: pERE 1.9h, 1.30h, 4.21h, 4.0h and 4.3. The nucleotide sequence was determined using both universal and internal primers (see text). After editing, approximately 2- 300bp of good quality nucleotide sequence data was initially analysed using the DNA strider programme (Marck, 1988). Alignments were carried out with the GAP and PILEUP programmes in the GCG package at SERC SEQNET. Sequence differences are boxed for clarity and provided the basis on which the clones were classified into two groups, A and B. Comparisons are presented below.

Panel A: Comparison of the 3' coding region

i: Nucleotide sequence comparison

```
                1                                                   50
      pERE130h  GTTCAGAAAA CATTGACCGC AGATGAAGTT GGGAATGCAG CAGCCTTCTT
A       pERE43  GTTCAGAAAA CATTGACCGC AGATGAAGTT GGGAATGCAG CAGCCTTCTT
        pERL8   GTTCAGAAAA CATTGACCGC AGATGAAGTT GGGAATGCAG CAGCCTTCTT
      pERE19h   ATTCAGAAAA CACTGACCGC AGATGAAGTT GGGAATGCAG CGGCCTTCTT
B   pERE421h    ATTCAGAAAA CACTGACCGC AGATGAAGTT GGGAATGCAG CGGCCTTCTT
      pERE40h   ATTCAGAAAA CACTGACCGC AGATGAAGTT GGGAATGCAG CGGCCTTCTT

                51                                                  100
      pERE130h  GGTATCTCCA TTGGCCTCTG CCATAACCGG TGCAACCATC TATGTTGACA
        pERE43  GGTATCTCCA TTGGCCTCTG CCATAACCGG TGCAACCATC TATGTTGACA
        pERL8   GGTATCTCCA TTGGCCTCTG CCATAACCGG TGCAACCATC TATGTTGACA
      pERE19h   GGTATCTCCA TTGGCCTCTG CCATAACTGG TGCAACCATC TATGTGGACA
    pERE421h    GGTATCTCCA TTGGCCTCTG CCATAACTGG TGCAACCATC TATGTGGACA
      pERE40h   GGTATCTCCA TTGGCCTCTG CCATAACTGG TGCAACCATC TATGTGGACA

                101                                                 150
      pERE130h  ATGGCTTGAA TTCAATGGGT GTTGCCATCG ACAGTCCAGT TTTCAAAGAC
        pERE43  ATGGCTTGAA TTCAATGGGT GTTGCCATCG ACAGTCCAGT TTTCAAAGAC
        pERL8   ATGGCTTGAA TTCAATGGGT GTTGCCATCG ACAGTCCAGT TTTCAAAGAC
      pERE19h   ATGGCTTGAA TTCAATGGGT GTTGCTCTGG ACAGCCCTGT TTTCAAAGAC
    pERE421h    ATGGCTTGAA TTCAATGGGT GTTGCTCTGG ACAGCCCTGT TTTCAAAGAC
      pERE40h   ATGGCTTGAA TTCAATGGGT GTTGCTCTGG ACAGCCCTGT TTTCAAAGAC

                151     162
      pERE130h  CTCAAATAG. ..
        pERE43  CTCAAATAG. ..
        pERL8   CTCAAATAG. ..
      pERE19h   CTCAACAAGT AG
    pERE421h    CTCAACAAGT AG
      pERE40h   CTCAACAAGT AG
```

ii: Derived amino acid comparison

```
                1                                                   50
     pERE19h   IQKTLTADEV GNAAAFLVSP LASAITGATI YVDNGLNSMG VALDSPVFKD
     pERE40h   IQKTLTADEV GNAAAFLVSP LASAITGATI YVDNGLNSMG VALDSPVFKD
A    pERE421h  IQKTLTADEV GNAAAFLVSP LASAITGATI YVDNGLNSMG VALDSPVFKD
     pERE130h  VQKTLTADEV GNAAAFLVSP LASAITGATI YVDNGLNSMG VAIDSPVFKD
B    pERE43    VQKTLTADEV GNAAAFLVSP LASAITGATI YVDNGLNSMG VAIDSPVFKD
     pERL8     VQKTLTADEV GNAAAFLVSP LASAITGATI YVDNGLNSMG VAIDSPVFKD

               51
     pERE19h   LNK*
     pERE40h   LNK*
     pERE421h  LNK*
     pERE130h  LK*.
     pERE43    LK*.
     pERL8     LK*.
```

Panel B: Comparison of the 3' untranslated region

i: Groups A and B

```
               1                                                   50
     pERE130h  .......... .......... ..TAGAGCCT TTTAAGTAAC TGTAGTAACT
A    pERL3     .......... .......... ..TAGAGCCT TTTAAGTAAC TGTAGTAACT
     pERE43    .......... .......... ..TAGAGCCT TTTAAGTAAC TGTAGTAACT
     pERE19h   TAGGGTCTTC TTGATCGACG AATAGAGCAT ATGATCTCCC CATCGGCTTT
B    pERE421h  TAGGGTCTTC TTGATCGACG AATAGAGCAT ATGATCTCCC CATCGGCTTT
     pERE40h   TAGGGTCT.. TTGATCGACG AATAGAGCAT ATGA..TCCC CATCGGCTTT

               51                                                  100
     pERE130h  CACTTTTTCT TGTGCTGCAT TTTTTTCAAC TGAGTGGATG CTGTTTTTCA
     pERL3     CACTTTTTCT TGTGCTGCAT TTTTTTCAAC TGAGTGGATG CTGTTTTTCA
     pERE43    CACTTTTTCT TGTGCTGCAT TTTTTTCAAC TGAGTGGATG CTGTTTTTCA
     pERE19h   GTTTTTCTTT TAAGTTTCA. ..AGAGAACA TGTTATGTTT CTAGTTTGTC
     pERE421h  GTTTTTCTTT TAAGTTTCA. ..AGAGAACA TGTTATGTTT CTAGTTTGTC
     pERE40h   GTATTTTCTT TCCCTTTCA. ..AGAGAACA TGTTATGTTT CTAGTTTGTC

               101                                                 150
     pERE130h  AACTACTTTG TTTTCTAGAA CAAAATAAAA TATTTAAAAC AAAAAAAAAA
     pERL3     AACTACTTTG TTTTCTAGAA CAAAATAAAA TATTTAAAAC AAAAAAAAA.
     pERE43    AACTACTTTG TTTTCTAGAA CAAAATAAAA TATTTAAAAC AATCAGAATA
     pERE19h   ACTTTAGCTG ATATGTAATA ACTGCTGCAC TATTTCTCTA ATGATTGAAG
     pERE421h  ACTTTAGCTG ATATGTAATA ACTGCTGCAC TATTTCTCTA ATGATTGAAG
     pERE40h   ACTTTAGCGA ATGTGTAATA ACTGCTGCAT TATTTCTCTG ATGATTGAAG

               151                                                 200
     pERE130h  AAAAAAAA.. .......... .......... .......... ..........
     pERL3     .......... .......... .......... .......... ..........
     pERE43    AATTCCAGTC AAATAAAAAA AAAAAAAAAA AAAA...... ..........
     pERE19h   TTTGNAAGTT TCTAAAAAAA AAAAAAAAAA AAAAA..... ..........
     pERE421h  TTTGCAAGTT TCTACTTTGT AATTGAACCA CTTTACATGT TTATGGTTTC
     pERE40h   TTTGCACGTT TCTAAAAAAA AAAAAAAAAA AAAA...... ..........

               201                                             242
     pERE130h  .......... .......... .......... .......... ..
     pERL3     .......... .......... .......... .......... ..
     pERE43    .......... .......... .......... .......... ..
     pERE19h   .......... .......... .......... .......... ..
     pERE421h  ATAGACGGCT TTGTTCAAGT TGTTAAAAAA AAAAAAAAAA AA
     pERE40h   .......... .......... .......... .......... ..
```

A clone within each group could be subdivided depending on the presence of an extra sequence in the 3' untranslated area. This "insert sequence" is boxed.

ii: Group A

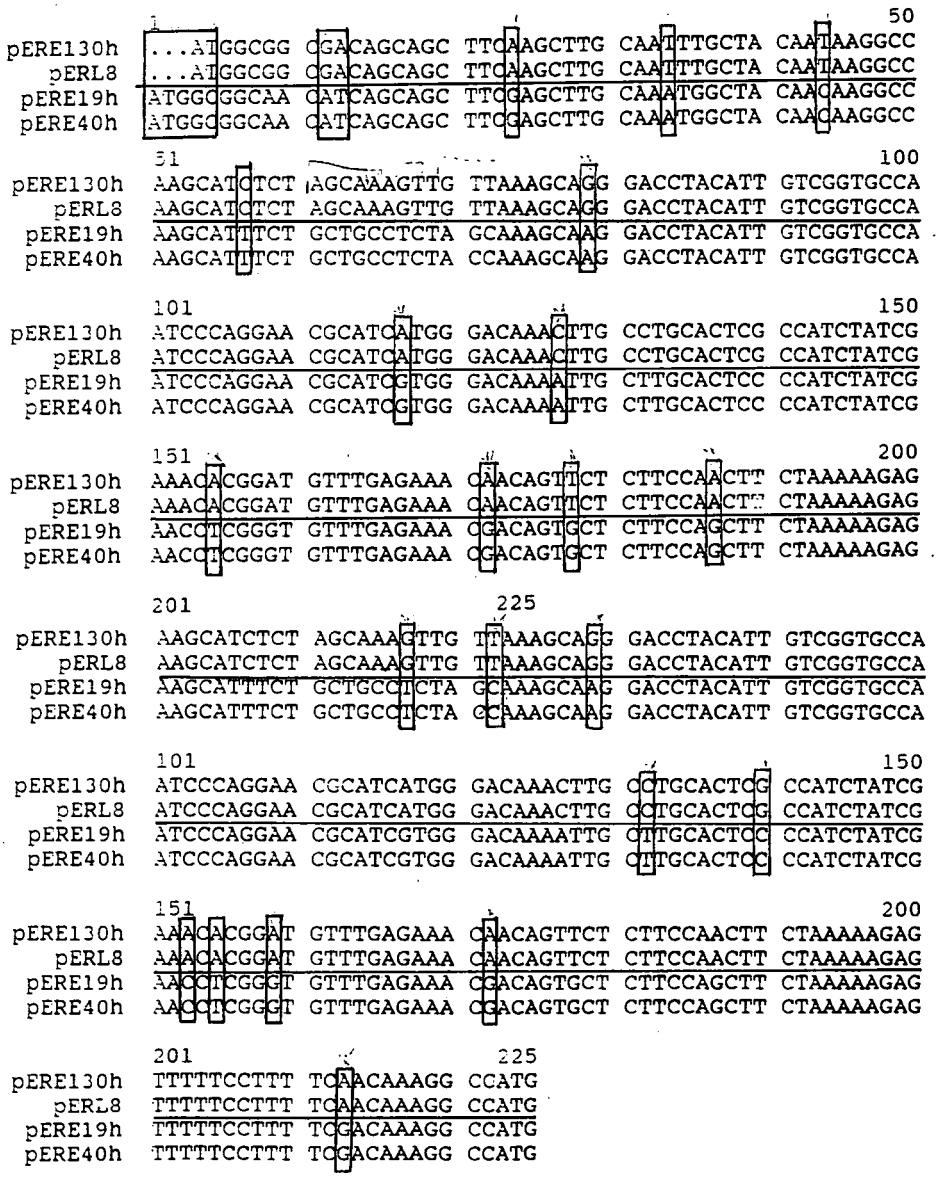
```
            1                                                    50
pERE130h    TAGAGCCTTT TAAGTAACTG TAGTAACTCA CTTTTTCTTG TGCTGCATTT
  pERL8     TAGAGCCTTT TAAGTAACTG TAGTAACTCA CTTTTTCTTG TGCTGCATTT
pERE43      TAGAGCCTTT TAAGTAACTG TAGTAACTCA CTTTTTCTTG TGCTGCATTT

            51                                                   100
pERE130h    TTTTCAACTG AGTGGATGCT GTTTTTCAAA CTACTTTGTT TTCTAGAACA
  pERL8     TTTTCAACTG AGTGGATGCT GTTTTTCAAA CTACTTTGTT TTCTAGAACA
pERE43      TTTTCAACTG AGTGGATGCT GTTTTTCAAA CTACTTTGTT TTCTAGAACA

            101                                                  150
pERE130h    AAATAAAATA TTTAAAACAA AAAAAAAAAA AAAAAA.... ..........
  pERL8     AAATAAAATA TTTAAAACAA AAAAAAA... .......... ..........
pERE43      AAATAAAATA TTTAAAACAA TCAGAATAAA TTCCAGTCAA ATAAAAAAAA

            151       162
pERE130h    .......... ..
  pERL8     .......... ..
pERE43      AAAAAAAAAA AA
```

iii: Group B

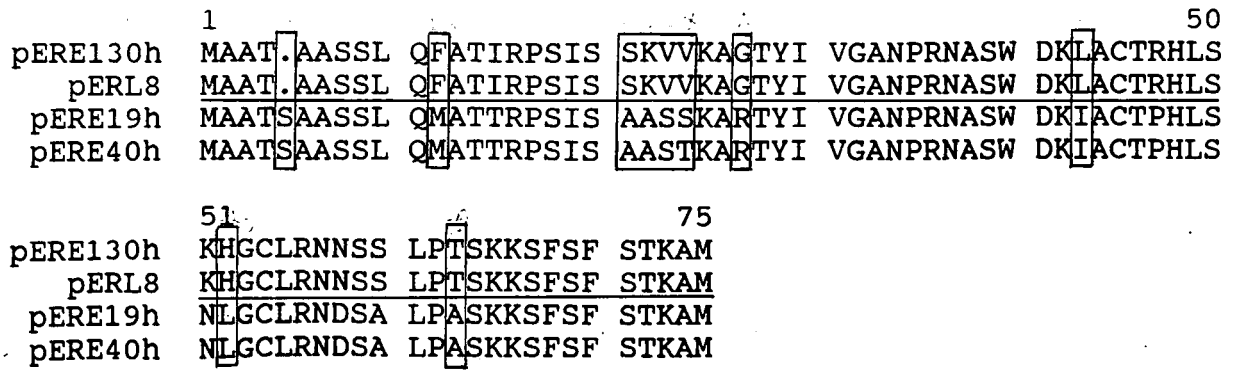
```
            1                                                    50
 pERE19h    TAGGGTCTTC TTGATCGACG AATAGAGCAT ATGATCTCCC CATCGGCTTT
pERE421h    TAGGGTCTTC TTGATCGACG AATAGAGCAT ATGATCTCCC CATCGGCTTT
 pERE40h    TAGGGTCT.. TTGATCGACG AATAGAGCAT ATGA..TCCC CATCGGCTTT

            51                                                   100
 pERE19h    GTTTTTCTTT TAAGTTTCAA GAGAACATGT TATGTTTCTA GTTTGTCACT
pERE421h    GTTTTTCTTT TAAGTTTCAA GAGAACATGT TATGTTTCTA GTTTGTCACT
 pERE40h    GTATTTTCTT TCCCTTTCAA GAGAACATGT TATGTTTCTA GTTTGTCACT

            101                                                  150
 pERE19h    TTAGCTGATA TGTAATAACT GCTGCACTAT TTCTCTAATG ATTGAAGTTT
pERE421h    TTAGCTGATA TGTAATAACT GCTGCACTAT TTCTCTAATG ATTGAAGTTT
 pERE40h    TTAGCGAATG TGTAATAACT GCTGCATTAT TTCTCTGATG ATTGAAGTTT

            151                                                  200
 pERE19h    GNAAGTTTCT AAAAAAAAAA AAAAAAAAAA AA..... .....
pERE421h    GCAAGTTTCT ACTTTGTAAT TGAACCACTT TACATGTTTA TGGTTTCATA
 pERE40h    GCACGTTTCT AAAAAAAAAA AAAAAAAAAA A..... .....

            201                                          239
 pERE19h    ..... ..... ..... .....
pERE421h    GACGGCTTTG TTCAAGTTGT TAAAAAAAAA AAAAAAAAA
 pERE40h    ..... ..... ..... .....
```

Panel C: Comparison of the leader peptide sequences of full length enoyl-ACP reductase clones

i: Nucleotide sequence



ii: Derived amino acid sequence



B: 3' Untranslated region

A comparison of the 3' untranslated regions of pERE clones is presented in panel Bi of figure 6.3. Poly A tails were identified in all the Jet Neuf clones and TAG was used as the stop codon. Differences in sequence in this area classify the same clones into the same groups as determined by differences in the coding region. Each group is compared separately in Bii and iii. One clone from each group could be subdivided due to the presence of an "insert sequence" in this region. In group A, pERE4.3 was identical to pERE 1.30h (pERL 8), apart from an extra 22bp, which occurred just before the poly A tail (boxed). Group B clones were subdivided in an analogous way. pERE 4.0 and 1.9h were identical (panel C ii) to each other and to pERE4.21h, apart from an extra 61bp, in 4.21h (boxed), in 4.21h which again occurred just before the poly A tail.

C: 5' Leader peptide region:

The 5' leader peptide region was sequenced with the internal primer 776 (see figure 6.1A for position of primers in ER) in order to help resolve whether the clones with "inserts" in the 3' untranslated regions were distinct. However no conclusions could be drawn for group A as pERE 4.3 was 5' truncated. Group B clones were also sequenced with 776 and pERE 4.21h and 4.3 were also 5' truncated. Comparisons of the 5' leader peptide region from the full length clones pERE clones are presented in panel C. It can be seen that the clones fall into the same two groups based on differences (boxed for clarity) in this region as well. The overall level of sequence conservation is again very high (with twelve amino acid changes between the two groups).

6.3 Summary and further experiments

A restriction fragment from the 3' region of the characterised ER cDNA clone pERL8 was used to screen a *B. napus* embryo library. Ten positives were isolated and subcloned by plasmid rescue of bluescript phagemids from λZAPII to generate pERE plasmids. The clones were sequenced with universal primers and five did not correspond to ER. Homology searches of sequence databanks were performed to determine the putative identity of these artifacts. The highest homology was with storage proteins. The level of

storage protein message in a library generated from mid- development embryos would be high, which could have facilitated isolation of false positives. Enoyl reductase sequence was identified in the remaining five clones pERE 1.30h, 1.9h and 4.0h, 4.21h and 4.3. However ER sequence was internal to unrelated cDNAs ligated at the 5' and 3' ends in pERE 4.21h, 1.30h and 1.9h. Furthermore, sequencing with a 5' internal primer demonstrated that only three clones were full length 1.30h, 1.9h and 4.0h. pERE 4.21h and 4.3 were 5' truncated.

Nucleotide sequence comparisons demonstrated that pERL8 was identical to pERE130h and provided a basis on which to divide the clones into two groups. Within a group one clone could be further subdivided, due to the presence of an "insert" in the 3' untranslated region (pERE 4.21h and 4.3). Both clones with "3' inserts" were 5' truncated so analysis of the leader peptide region could not be performed. Therefore a definite conclusion as to whether these represented an additional two new isoforms could not be made.

If the "inserts" in the 3' untranslated region were real features (and not artifacts that arose during library construction) this would mean that a total of four ER isoforms had been cloned from *B.napus* (pERL8 and three from this experiment). This would correlate with observed data for ER in *B. napus*, there being four genes and four isoforms detected in both leaf and seed tissue (Fawcett *et al.*, 1994). It was also shown that there were two "major" isoforms (ER II and IV), which were expressed at a greater level than the two "minor" isoforms (ER I and III). It is tempting to speculate at this stage that this could correlate to the two putative groups, A and B, of clones characterised in this expriment.

To ascertain whether the "inserts" in the 3' untranslated regions were real their presence in another population of mRNAs would have to be demonstrated. There are several possible strategies including the analysis of Northern blots with specific probes and 3' RACE (Rapid Amplification of cDNA Ends). This technique utilises a set of 3' nested primers, used in conjunction with a poly T primer to amplify the 3' end of target sequences. The presence

of "inserts" in the 3' regions would then be demonstrated by electrophoretically resolving different PCR products or by nucleotide sequencing.

General Discussion and Overall Conclusions

General Discussion and Overall Conclusions

The initial aim of this work was to analyse a temporally and spatially regulated gene promoter for protein binding motifs. Analysis of promoters in general has revealed that both negative and positive domains are required for the correct pattern of expression (Benfey and Chua, 1990). These regulatory domains are comprised of discrete functional units, or cis motifs, each consisting of small (4- 20bp) DNA sequences which represent recognition sites for transcriptional regulatory proteins.

Previous studies of the ACP05 promoter, included the analysis of transcriptional promoter- reporter gene fusions in transgenic plants. Experiments had shown that 1.4kb of 5' promoter sequence encoded all the regulatory information that directed correct expression within the seed. DNA sequence analysis of the ACP05 promoter for direct repeats and palindromes (both typical features of cis sequences) highlighted the presence of a motif, the core of which was AAGAC. The presence of multiple binding sites is a common feature of eukaryotic enhancers. For example the light response element of pea ribulase bisphosphate carboxylase is a repeat motif (Kuhleimer et al., 1988). Also it has been shown that the binding sites of certain classes of common DNA binding protein, for example factors with bZIP motifs, are arranged palindromically (Foster *et al.*, 1994).

Four probes that contained at least one copy of this motif (or its compliment) were generated from the ACP05 promoter. These were used in gel retardation assays and all formed specific complexes with embryo protein. Competition experiments using unlabelled oligonucleotides showed that the embryo DNA binding protein formed a sequence specific complex with the AAGAC motif. Binding activity with this motif was not detected with leaf protein extract. Similar motifs (repeated hexameric sequences in the α subunit of the soybean conglycin gene) that bind embryo specific nuclear proteins have been characterised previously (Chen *et al.*, 1986; Allen *et al.*, 1989). The AAGAC sequence was therefore of interest as it potentially represented a regulatory motif.

The motif was incorporated onto a binding site oligonucleotide, which was multimerised and used to screen an expression library in a Southwestern experiment. A positive clone was isolated, λBS2xi. Lysogens were generated from λBS2xi and the DNA binding specificity of the recombinant protein that it encoded was analysed using gel retardation assays. The clone was found to encode a DNA binding domain that formed a specific complex with the binding site oligonucleotide. Northern hybridisations demonstrated the clone was expressed in seed and root, and that the RNA transcript was approximately 1.8kbp. The highest level of hybridisation was detected in developing embryo at 50DAF. Hybridisation was not detected in leaf. A correlation between the expression of the DNA binding transcript and a seed specific ACP cDNA was noted (see chapter 5 for details); Northern hybridisations demonstrated that both transcripts were expressed in a similar pattern: hybridisation was detected in root, was highest in the seed and not detected in leaf. Such a correlation should be expected if the DNA binding clone λBS2xi was involved in the spatial regulation of ACP.

Several strategies were used to characterise λBS2xi. Both PCR amplification and direct nucleotide sequencing were unsuccessful. *Eco*RI digestion excised the entire 3.0kb cDNA fragment, which was subcloned into pSK+, to generate pBS2. The size of the cDNA indicated that it contained at least 1.2kb additional unrelated sequence as a 1.8kb RNA transcript was detected when Northern blots were probed. Nested deletions were generated from the subclone and sequenced. Sequencing past the polylinker region from the M13 forward primer site was not achieved and sequence data from the reverse primer site was of poor quality until approximately 1.5kb from the 5' end. The analysis of sequence data from clone $pBSND_{28}$ showed that it corresponded to β- galactosidase sequence. Southern analysis further demonstrated that this sequence was present in λBS2xi. Further work is required to characterise this clone and possible strategies include: re-screening a *B. napus* library with the multimerised binding site probe; shotgun cloning and sequencing of λBS2xi and protein sequencing induced peptides from lysogen extract. These strategies are discussed fully in chapter 4.

Other ACP promoters have been analysed using similar techniques. A study on a *B. campestris* ACP promoter, Bcg 4-4, used promoter- reporter gene fusions to analyse its pattern of expression in transgenic plants (Sherer *et al.*, 1991). Expression was high in the developing seed and very low in the leaf. The promoter region was characterised by the presence of three different types of direct repeats in the 200bp immediately 5' to the putative TATA box (these repeat motifs were not present in the promoter ACP05). Three AAGAC sequences (but not GTCTT) were also detected in this region. These direct repeats were deleted and analysed in transgenic plants to determine the effect on expression in the seed. It was found that expression was unaffected in both seed and leaf and was concluded that they did not play a part in the regulation of tissue specific expression (Sherer *et al.*, 1991).

Studies that combined gel retardation assays and promoter- reporter gene fusions was performed on the AcII.2 promoter from *Arabidopsis* (Baerson *et al.*, 1994; Baerson and Lamppa, 1993; Lamppa and Jacks, 1991) to define regulatory domains. There are five ACP isoforms in *Arabidopsis,* three of which have been characterised and are all expressed in seed, leaf and root. The fourth isoform is restricted to seed and the fifth is restricted to leaf. Baerson and colleagues (1993; 1994) conducted a deletion analysis of the AcII.2 gene promoter, which is a major isoform in the seed, but is also expressed at lower levels in the leaf and root. Transcriptional promoter-reporter gene fusions were made and transformed into tobacco. Expression of the undeleted promoter was highest in the seed and lowest in the leaf. Deletion analysis further defined three main domains responsible for different aspects of expression. A large domain from -466 to -55bp directed expression in the roots; An 85bp domain from -320 to -236bp directed expression within the leaf and also had an effect on the maximal expression within the root; and a 180bp domain from -235 to -55bp that directed expression within the seed (Baerson *et al.*, 1994). A 91bp probe that encompassed the domain that directed maximal promoter activity in the leaf and root was generated and analysed by gel retardation. A specific complex was detected in leaf and root (data not shown for seed) that bound to the core motif ACGT (which is part of a larger domain that contained AAGAC) (Baerson *et al.*, 1994).

This data contradicts the results from gel retardation analysis of the ACP05 promoter, in which the AAGAC motif interacted with an embryo specific factor. There are several possible explanations for this difference. Firstly the expression patterns of the *Arabidopsis* gene is distinct from that of the *B. napus* and *campestris* genes and as a result the regulatory motifs in such complex promoters are likely to be different. However the promoter sequences of ACP05 and Bcg 4-4, which are both seed expressed isoforms, are also distinct: Bcg 4-4 is characterised by direct repeats (Scherer *et al*, 1991) and ACP05 is characterised by palindromic repeats (chapter 3 for details). It has been shown that putative transcription factors bind to more than only their cognate binding site (Foster *et al.*, 1994). For example the G- box binding factor TAF1 binds to its cognate cis element in the *aba* regulated rice *rab* 16 gene but also to G boxes motifs in various light regulated genes (Foster *et al.*, 1994 ;Oeda *et al.*, 1991). This reflects the fact that binding affinities *in vitro* do not reflect those *in vivo*.

A major limitation of gel retardation assays is the inability to measure transcription rates. Usually a simplified situation is presented, factors such as chromatin structure have an important role to play in transcription. Genes may be masked by histones and other DNA binding proteins, thus rendering it inaccessible to transcription factors (Felsenfeld, 1992; Smith, 1995; Zlatanova and VanHolde, 1990). The results from mobility shift assays (and other *in vitro* techniques) therefore must be considered as indicators of what may be happening *in vivo* i.e. a binding site *in vitro* does not necessarily represent a binding site *in vivo*.

To unequivocally define the domains that regulate the expression of ACP05 additional transcriptional promoter reporter gene fusions would have to be constructed and analysed in transgenic plants. Constructs in which the AAGAC motif were deleted and mutated would demonstrate its importance in regulation of expression within the seed in *B.napus*.

A second related subject of this thesis concerned another member of the FAS complex in *B. napus*, enoyl- ACP reductase (ER). There are four ER isoforms

and four ER genes. The isoforms are all expressed in the leaf and seed (but not at equimolar levels). Expression is appreciably higher in the seed and there appear to be two major isoforms and two minor isoforms. Prior to this study two cDNAs had been characterised, but were isolated from different varieties. It was therefore unclear whether they actually represented different cDNAs.

A 3' probe was generated from pERL8, the existing Jet Neuf leaf expressed ER clone (A. Fowler., unpublished results). This was used to screen a cDNA library generated from Jet Neuf embryo. Ten positives were isolated and subcloned by plasmid rescue. Nucleotide sequencing showed that five clones corresponded to ER, and were divided into two groups on the basis of sequence differences, both in the coding and 3' untranslated regions. One clone, pERE130h, was identical to pERL8 and represented an isoform expressed in both leaf and seed. The remaining clone in group A, pERE 43 was identical to pERE130h, apart from an "insert" present in its 3' untranslated region. This pattern was repeated in group B where clones pERE 40 and 19h were identical to each other and to pERE 421h, apart from an insert in the 3' untranslated area of pERE421h. The clones with inserts were both 5' truncated, so differences could not be confirmed at the 5' end of the cDNA. Further experiments are needed to clarify whether the inserts were real features and strategies include the use of Northern blots or 3' RACE experiments. These are discussed fully in chapter 6. However, at this point it is tempting to speculate about a possible correlation between the two groups of clones isolated and the two families (major and minor expressed ER isoforms) of ER detected in *B. napus* by two dimensional western blotting (Fawcett *et al.*, 1994).

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