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**Examination of the *Brassica napus* β -Keto-Acyl
Carrier Protein Reductase Promoter for
Regulatory *Cis*-Acting Elements**

**Thesis Submitted for the Degree of Doctor of Philosophy at the
University of Durham**

By

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Department of Biological Sciences
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18 JUN 2003

September 2002

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Examination of the *Brassica napus* β -Keto-Acyl Carrier Protein Reductase Promoter for Regulatory *Cis*-acting Elements

Major interest has focused on the identification of regulatory factors involved in lipid biosynthesis. This study examined the *B.napus* β -Keto-ACP reductase 5' sequence for potential regulatory *cis*-acting elements. The 5' sequence of the most highly expressed *Brassica napus* β -Keto-ACP reductase isoform was fused to the reporter gene β -glucuronidase (GUS) and its expression pattern examined within transgenic *Arabidopsis*. The construct was shown to act as a functional promoter and direct transcription within embryos, cotyledons and roots. There was no apparent staining within the true leaves, but staining was visible within the cotyledons. Overlapping fragments of the promoter were analysed in gel mobility shift assays and all six showed the formation of protein-DNA complexes. Competition analysis suggested that the same trans-acting factor binds to a number of regions along the promoter. The protein-DNA complex appeared to be competed away by the *Arabidopsis* enoyl-ACP reductase (EnR) promoter sequence, but not the lipid transfer protein (LTP) promoter. A common 9bp *cis*-element (CGCANTAAA) was identified in four of the six promoter fragments. Deletion analysis of the β -Keto-ACP reductase promoter in transient expression experiments into *B.napus* tissue, suggested the promoter could still direct transcription upon deletion to 132bp within embryos. The GUS expression appeared to show more than one decrease in expression upon subsequent deletions of the promoter within embryos, suggesting that more than one *cis*-element may be involved in the control of transcription. At least one of these suspected decreases correlated with the deletion of one of the 9bp boxes identified. Differences were observed for expression of the constructs within leaves and embryos suggesting that different elements may be involved in transcriptional control within these tissues. The identification of a potential *cis*-acting element within this study could be used to isolate a potential regulatory trans-acting factor that binds to the *B.napus* β -Keto-ACP reductase promoter.

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Publications Arising from this Thesis

Identification of Protein-DNA Interaction Sites Common to the Promoters of the *Brassica napus* Lipid Biosynthesis Genes. Clare Young, Tina Barsby, Jeroen Wilmer and Kieran Elborough. *Abstract. 6th International Congress of Plant Molecular Biology, Quebec, Canada, 2000. S20.*

Abbreviations

ABA	abscisic acid
ACCase	acetyl Co-A carboxylase
ACP	acyl carrier protein
β-Kr	β-Keto-ACP reductase
bp	base pair
CaMV35S	cauliflower mosaic virus 35S RNA gene
CDNA	complementary DNA
dATP	2' -deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DAG	days after germination
ddH₂O	deionised water
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleotide 5'-triphosphates
EnR	enoyl-ACP reductase
GMSA	gel mobility shift assay
GUS	β-glucoronidase
LB	Luria-Bertani medium
LTP	lipid transfer protein
MCAT	malonyl-CoA ACP transacylase
mRNA	messenger RNA
MS10	murashige and skoog medium containing 10g/l sucrose
NaHPO₄	disodium hydrogen orthophosphate
NaH₂PO₄	sodium dihydrogen orthophosphate
NaOH	sodium hydroxide
ng	nanogram
NOS	nopaline synthase
OD	optical density
PCR	polymerase chain reaction

RNA	ribonucleic acid
T-DNA	transferred DNA
Ti Plasmid	tumour-inducing plasmid
Tris	tris(hydroxymethyl)aminomethane
µg	microgram
X-Gluc	5-brom-4-chloro-3-indoyl β-D-glucuronic acid

Introduction

Introduction

1.1 Plant Lipids

1.1.1 Importance of Plant Lipids

Plant oils have a huge economic importance, with a value of more than \$24 billion and over 82 million tonnes produced annually (Kaufman and Ruebusch, 1990). Animals and humans consume two thirds of these vegetable oils, whilst industries use the remaining third (Ohlrogge and Browse, 1998). Plants produce most of the world's lipids, the main component of these being the acyl lipids. Plant lipids are important within industry both as a raw material and within the food industry, forming the basis of a number of edible products, for example the 6-9-polyunsaturated fatty acids that form an important part of the human diet. Commercially some of the most significant plants, in terms of their oil, are soybean, sunflower, coconut, groundnut (peanut), olive, oil palm, and rapeseed, all of which are important edible oils, whereas those oils important for industrial purposes are linseed, castor and tung. The importance of plant oils means their commercial value is enormous and therefore the genetic engineering of the amount and type of oil produced is economically extremely important. Recently plants have been successfully engineered to produce completely new types of oil as discussed in section 1.1.4 (Murphy *et al.*, 1994, Knutzon *et al.*, 1992, Voelker *et al.*, 1992). The genetic modification of plant oils requires an understanding of the fatty acid biosynthesis pathway. Modification of the amount of oil will require an increased understanding of the regulation of the individual components of the pathway.

1.1.2 Composition of Plant Lipids

Lipids have various roles, including metabolic, structural and storage functions, and are essential constituents of all plant cells. Lipids are a diverse group characterised by their low solubility in water. One class of lipids are the glycerolipids, which are comprised of a glycerol molecule esterified with fatty acids. Epidermal cells produce cuticular lipids involved in the formation of membrane lipids to act as a barrier for protection and to prevent water loss. Diacylglycerols are the main component of the membrane lipids, having two fatty acids esterified to a glycerol molecule at the *sn-1* and *sn-2* positions. Lipids are also the major form of carbon storage in the seeds of many plant species, which are important energy stores for germination and growth. The main storage lipids are the triacylglycerols, composed of a glycerol molecule esterified with three fatty acids. Oil, carbohydrate and proteins are the major components of plant seeds. However the proportion of these varies considerably, depending on the species. The storage oil can be up to 60% of the dry weight of the seed within certain species. For example, castor seeds are more than 50% oil, and less than 25% protein or carbohydrate, whereas in wheat up to 80% of the seed by weight is starch, less than 10% protein and less than 5% oil (Ohlrogge and Browse, 1998). Therefore depending on the species, plant seeds are useful for different purposes and it is the oil producing seeds like castor, rape and sunflower that are important within the oil industry.

The composition of fatty acids within seeds varies enormously between species, unlike those forming the membrane lipids. Storage lipids contain fatty acids that differ both in their degree of saturation and also their chain length, in contrast to the membrane lipids where little variation is observed. A table of plants important commercially for their oil is shown in figure 1.1, highlighting both the oil content of the plant and the constituent fatty acids of these lipids. The predominant fatty acids within edible oils are oleic acid (18:1), linoleic acid (18:2) and palmitic (16:0). The composition of plant oils is important for their properties and therefore the genetic manipulation of plant fatty acids for industry is very important.

Oilseed	Prod (10 ⁶ m tonnes)	Oil content (% weight)	<16:0	16:0	18:0	18:1	18:2	18:3	Other
Soybean	15.3	19	-	11	3	22	55	8	1
Palm	8.4	48	-	47	4	38	10	-	-
Rapeseed	7.5	43	-	4	2	59	23	8	3
Sunflower	7.2	40	-	7	3	14	75	-	-
Cotton	3.3	19	1	25	3	51	31	-	2
Peanut	2.8	48	-	11	2	51	31	-	2
Coconut	2.7	66	80	8	4	5	3	-	-
Palm kernel	1.1	48	74	9	2	15	1	-	-
Castor bean	-	44	-	1	1	3	4	Tr	90

Figure 1.1 Table of Important Fatty Acids

A table of commercially important plant oils is shown, outlining their component fatty acids. The different oils show considerable variation in their fatty acid composition, the predominant fatty acids being the 16:0, 18:1 and 18:2. The fatty acid composition of the lipids account for the oil's properties, thereby determining the suitability of the oil for different purposes (Ohlrogge and Browse (1998)).

1.1.3 Fatty Acid Biosynthesis

Fatty acid biosynthesis is a primary metabolic pathway, being a fundamental process in all cells. Within animals, bacteria and fungi the fatty acid biosynthetic components are found in the cytosol, whereas in plants they are located within the plastidal stroma (chloroplasts within leaves and proplastids within the seed). However there is some evidence of fatty acid biosynthesis within the mitochondria of plants. Analysis of leaf mitochondria from pea has shown the presence of all the enzymes required for fatty acid biosynthesis (Wada *et al* 1997). The synthesis of *de novo* fatty acid biosynthesis was shown to occur within the mitochondria. However the products of plant mitochondrial fatty acid biosynthesis were lipoic acid precursors that differ from the 18C products synthesised within the chloroplasts. This may therefore be an alternative pathway for the production of different fatty acids. However the exact role of mitochondrial fatty acid biosynthesis still remains to be elucidated. The regulation of fatty acid synthesis is poorly understood (as discussed in section 1.1.5), but tight regulation of the pathway occurs both temporally and spatially. Different amounts of lipids are found within the leaf, roots and seeds, and changes in the amount of lipid production are also observed during the development of the seed. Compartmentalisation of the pathway also increases the need for tight regulation. The *de novo* synthesis of plant fatty acids occurs within the plastid, the acyl chains then pass out of the plastid and assemble on the endoplasmic reticulum (ER) as glycerolipids. Some acyl chains remain in the plastid (Roughan *et al.*, 1982), and some of the glycerolipids from the ER then re-enter the plastids. Because of this interorganelle exchange, tight regulation is crucial, requiring communication between the plastid and cytosol. The reactions of the fatty acid biosynthetic pathway occur in a cyclical fashion (reviewed by Ohlrogge and Browse, 1995, Harwood, 1988 and Slabas and Fawcett, 1992). It is thought that over 30 enzymatic reactions are involved in the formation of the end fatty acid of 16C (palmitate) or 18C (oleate, linoleate, and α -linoleate) (Ohlrogge and Browse *et al.*, 1998). The process and enzymatic reactions involved in fatty acid biosynthesis are shown in figure 1.2.

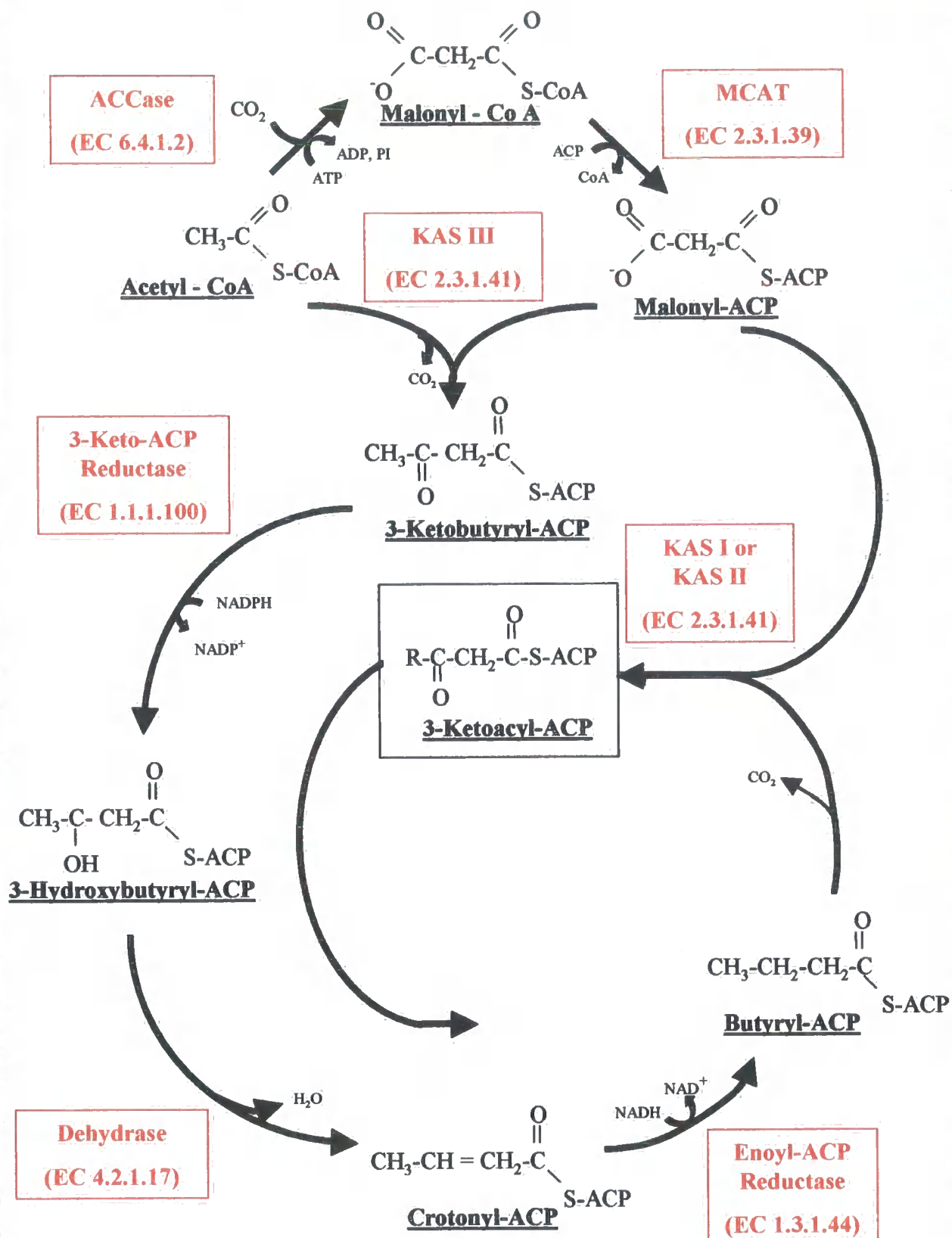
Figure 1.2 Fatty Acid Biosynthetic Pathway

The fatty acid biosynthetic pathway is outlined, showing the enzymes involved in the formation of the fatty acid chain (adapted from Ohlrogge & Browse, 1995). The process begins with the production of malonyl ACP; which is firstly fed into the pathway by a condensation reaction with acetyl-CoA and then used for the subsequent elongation of the acyl chains. The reactions involve a number of condensations, reductions and a dehydration to form the 3-ketoacyl-ACP. The chain is then elongated until the final product of 18:0 or 16:0 is obtained. The growing acyl chain is attached to the acyl carrier protein for the majority of the pathway.

ACCase = Acetyl-CoA Carboxylase

MCAT = Malonyl-CoA ACP Transacylase

KAS = 3-Ketoacyl-ACP Synthase



1.1.3.1 Acyl Carrier Protein (ACP)

The Acyl Carrier Protein (ACP) is a low molecular weight acidic protein (8.8Kda). As its name suggests, it is a carrier protein for the acyl chains and is involved in almost all stages of fatty acid biosynthesis. It is covalently, posttranslationally modified with a phosphopantetheine prosthetic group attached to a serine residue near to the middle of the protein (Ohlrogge, 1987). The end of the pantetheine group contains a sulfhydryl group, to which the fatty acid attaches as a thioester via its carboxyl carbon. A number of ACP clones from different species have been identified and different isoforms examined. ACP was the first of the plant fatty acid synthesis proteins to be purified, originally from avocado mesocarp and spinach leaf (Simoni *et al.*, 1967) and the first plant fatty acid synthesis gene to be cloned (Scherer and Knauf, 1987 and Rose *et al.*, 1987). Comparison of the *E.coli* ACP sequence allowed the identification of ACP clones from spinach leaf (Scherer and Knauf, 1987) and rape embryos (Safford *et al.*, 1988). Different isoforms of ACP within the same species have been identified and their expression pattern examined (Hlousekradojcic *et al.*, 1992 and Safford *et al.*, 1988). Two different ACP isoforms from spinach were identified and shown to be differentially expressed (Ohlrogge *et al.*, 1991). Both isoforms were present within the leaves however only one of them was located within the seed. Differential expression of the *Arabidopsis* ACP isoforms was attributed to a response to light (Bonaventure and Ohlrogge, 2002). Within *B.napus* multiple ACP genes exist. Two seed expressed ACPs from *B.napus* were isolated and sequenced, southern blot analysis suggested that this sub-family of seed expressed ACP's were encoded for by a multigene family consisting of 35 genes (de Silva *et al.*, 1990). The activity of different ACP isoforms was examined by the ectopic expression of an ACP seed specific isoform into leaf, which was shown to change the composition of fatty acids within the leaves (Branen *et al.*, 2001). The tissue specific regulation of the *Arabidopsis* ACP was examined by successive deletions of the ACP promoter, thereby identifying important sequences for transcriptional control (Baerson *et al.*, 1994). The expression pattern of ACP from soybean and rape was examined and

shown to increase prior to the onset of lipid synthesis within the seed (Ohlrogge and Kuo, 1984, Slabas *et al.*, 1987 & de Silva *et al.*, 1992).

1.1.3.2 Acetyl-CoA Carboxylase (ACCase)

The first committed step in fatty acid synthesis is carried out by the enzyme Acetyl-CoA Carboxylase (ACCase) (EC 6.4.1.2). Two isoforms of ACCase have been identified (Sasaki *et al.*, 1995). The first is a multifunctional eukaryotic ACCase, consisting of a polypeptide (200KDa) which contains functional domains for the Biotin Carboxyl Carrier Protein (BCCP), the Biotin Carboxylase (BC) and the CarboxylTransferase (CT) (Roessler and Ohlrogge, 1993). The second isoform is the prokaryotic ACCase, made up of multi subunits and found in the plastids of plants. The prokaryotic ACCase is comprised of at least four subunits forming a 600-700KDa complex (Sasaki *et al.*, 1993 & 1995 and Alban *et al.*, 1994). The β -carboxyltransferase subunit is plastid encoded, whereas the other three subunits are nuclear encoded (Sasaki *et al.*, 1995 and Shorrosh *et al.*, 1995). Plants were thought, as in animals and yeast to contain only the multifunctional form of ACCase, however the presence of a similar CT subunit within the pea chloroplasts genome to that of the multi subunit ACCase in *E.coli* was identified (Sasaki *et al.*, 1993). It is now known that the two ACCase isoforms are found in all dicots and most monocots (Sasaki *et al.*, 95). The multisubunit ACCase appears to be only present in the plastids and the multifunctional form in the cytoplasm. It is thought the cytosolic form may be involved in other pathways such as flavonoid biosynthesis and also involved in the production of cuticular lipid biosynthesis. The exception is found in the gramineae family where they appear to only have the multifunctional ACCase and therefore both the cytosolic and plastidal forms are the eukaryotic ACCase (Egli *et al.*, 1993, Sasaki *et al.*, 1995 & Konishi and Sasaki, 1994).

The ACCase from a number of species have been purified, for example rape and pea (Hellyer *et al.*, 1986), and the multisubunit ACCase from soybean (Reverdatto *et al.*, 1999). Work has also been carried out on the individual components of ACCase

including BC (Shorrosh *et al.*, 1995), BCCP (Choi *et al.*, 1995), and the alpha CT subunit from pea (Shorrosh *et al.*, 1996). The ratios of the individual subunits is not thought to be critical as the overexpression and antisense of the BC subunit did not alter the expression of BCCP (Shintani *et al.*, 1997). The ACCase subunits exhibit similar expression patterns and therefore appear to be co-coordinately regulated, for example the *B.napus* BCCP and CT subunits (Elborough *et al.*, 1996) and the four *Arabidopsis* ACCase subunits (Ke *et al.*, 2000). Similar expression patterns for the plastidal *Arabidopsis* ACCase BC subunit (*CAC2*) (Sun *et al.*, 1997) and BCCP subunit (*CAC1*) (Choi *et al.*, 1995) were also shown suggesting they might be coordinately regulated (Sun *et al.*, 1997). Two different isoforms of the BCCP from *Arabidopsis* were identified (Thelen *et al.*, 2000), however the other three subunits appeared to be encoded for by a single gene (Mekhedov *et al.*, 2000). The two BCCP isoforms were shown to be differentially expressed; one was only expressed in the developing seeds and the other one was shown to be constitutively expressed (Thelen *et al.*, 2000). ACCase is thought to be an important regulatory enzyme in the process of fatty acid biosynthesis as discussed in section 1.1.5.

ACCase catalyses the first reaction of the fatty acid biosynthetic pathway. This involves the two-step process of forming malonyl-ACP from acetyl-CoA, via the malonyl-CoA intermediate. Upon the formation of malonyl Co-A, ACP is required for all the following reactions to produce fatty acids. Malonyl-ACP is the principle substrate for fatty acid biosynthesis and forms the basis of the initial condensation reaction with acetyl-CoA and also serves to provide acetyl units to the growing acyl chain in further elongation reactions. The formation of malonyl-ACP from malonyl-CoA is catalysed by the enzyme Malonyl-CoA:ACP Transacylase (MCAT) (EC 2.3.1.39), here the malonyl group from the malonyl CoA is transferred onto the SH group of the phosphopantetheine of ACP. The CO₂ required is attached to the biotin of the BC domain and is then transferred to the acetyl-CoA by ACCase. MCAT was originally purified from avocado by Hilt in 1984 (cited by Stumpf *et al.*, 1987) and the MCAT analogue (FabD) identified within *E.coli* (Verwoert *et al.*, 1992). The first plant MCAT was cloned by Simon and Slabas (1998) from *Brassica napus*.

1.1.3.3 Fatty Acid Synthetase (FAS)

Two types of Fatty Acid Synthetase (FAS) have been identified, namely FAS I and FAS II. The type I FAS found in animals and yeast systems differs from the type II FAS found in plants and bacteria (Stumpf, 1981). The type I FAS consists of the different enzymes located on one or two multifunctional polypeptides. Whereas the type II consists of at least eight separate soluble enzymes that each carry out a separate reaction (Caughey and Kekwick, 1982; Hoj and Mikkelsen, 1982; Shimakata and Stumpf, 1982a). The individual FAS components were first examined in avocado (Caughey and Kekwick *et al.*, 1982), safflower (Shimakata and Stumpf, 1982a) and spinach (Shimakata and Stumpf, 1982b).

Fatty acid synthase adds malonyl ACP to the growing acyl chain, involving a number of condensation reactions by the enzymes 3-ketoacyl-ACP synthase I, II and III (KAS) (E.C. 2.3.1.41) (Jaworski *et al.*, 1989). The acyl chains produced are attached to the acyl carrier protein during these reactions. The initial condensation of the acetyl-CoA and malonyl-ACP is carried out by the KAS III enzyme, resulting in the four-carbon product 3-ketobutyryl-ACP (Jaworski *et al.*, 1989). The formation of 3-ketobutyryl-ACP by KAS III involves the release of the CO₂ that was added upon the formation of malonyl-CoA (thereby making the reaction irreversible due to the release of CO₂). KAS III was purified from avocado (Gulliver and Slabas, 1994) and spinach (Clough *et al.*, 1992). The *E.coli* KAS III homologue FabH was characterised (Tsay *et al.*, 1992) and the active site identified upon examination of its crystal structure (Davies *et al.*, 2000). The medium chain acyl ACP end products of fatty acid synthesis have been shown to inhibit the KAS III enzyme (Abbadì *et al.*, 2000). KAS I and II are involved later on in the fatty acid synthase cycle. Shimakata and Stumpf (1982c) studied spinach leaf extracts and found two KAS isoforms. KAS I was shown to be responsible for synthesising fatty acids up to C₁₆ and KAS II for C₁₆-C₁₈ products. The KAS I enzyme catalyses the condensation of a malonyl-ACP to the growing acyl chain and KAS II is involved in the formation of stearyl-ACP (18C) from palmitoyl-ACP (16 carbon) (Garwin *et al.*, 1980). The end product of these

condensation reactions is the 3-ketoacyl-ACP. The KAS I and KAS II genes have been identified within *Perilla* (Hwang *et al.*, 2000) and *B.napus* (Makintosh *et al.*, 1989).

1.1.3.4 β -Keto-ACP Reductase

The fatty acid biosynthesis pathway requires a series of reductions and a dehydration by the enzymes β -Keto-ACP reductase (EC 1.1.1.100), 3-hydroxyacyl-ACP dehydrase (EC 4.2.1.17), and enoyl-ACP reductase (EC 1.3.1.44). These three enzymes were first purified and characterized by Shimakata and Stumpf (1982d). The first of these reductions is carried out by the enzyme β -Keto-ACP reductase. This enzyme uses NADPH as a co-factor forming 3-hydroxy-acyl ACP from 3-keto-acyl ACP by reduction of the carbonyl group. It has been shown that the only β -Keto-ACP reductase enzyme involved in fatty acid biosynthesis is the NADPH dependent isoform (Sheldon *et al.*, 1992), however other experiments have identified an NADH specific isoform also present within the plastids (Caughey and Kekwick, 1982). The β -Keto-ACP reductase enzyme has been purified to homogeneity from spinach (Shimakata and Stumpf, 1982d), avocado (Sheldon *et al.*, 1990) and rape (Sheldon, 1988 PhD thesis University of Birmingham) and the cDNAs have been isolated from both *Arabidopsis* (Slabas *et al.*, 1992) and *Cuphea Lanceolata* (Klein *et al.*, 1991). Rafferty *et al.*, (1995) showed that upon examination of the rape β -Keto-ACP reductase there was 25% homology with the amino acid sequence of the *Arabidopsis* β -Keto-ACP reductase. The *B.napus* β -Keto-ACP reductase gene has also been shown to exhibit similar sequence homology to the *R.melilotia nodG* gene (Slabas *et al.*, 1992 & Sheldon *et al.*, 1990). Plants with antisense β -Keto-ACP reductase showed a distinct phenotype with reduced seed and leaf fatty acid accumulation, and stunted growth with wrinkled seeds (O'Hara *et al.*, 2000).

1.1.3.5 Dehydrase

Upon reduction by the β -Keto ACP-reductase the next reaction is the subsequent dehydration of the acyl chain. This is catalysed by 3-hydroxyacyl-ACP dehydrase by the removal of a water molecule to form trans 2-enoyl acyl ACP. Very little work has been published on this enzyme it was however purified from spinach leaf and safflower (Shimakata and Stumpf, 1982d) and from *B.napus* (Doig, Ph.D. Thesis 2001, University of Durham). Within bacterial fatty acid synthesis two different dehydrases have been identified that show similar functions, FabA and FabZ (Block 1970 and Mohan *et al* 1994). The enzymes were shown to have different substrate specificities, with FabA showing preference for long chain fatty acids and FabZ for short chain fatty acids.

1.1.3.6 Enoyl-ACP Reductase

The final enzyme in the cycle involves the subsequent reduction of the trans-2-enoyl acyl ACP via the enoyl-ACP reductase enzyme. This enzyme reduces the double bond to form the saturated acyl ACP derivative and involves either NADH or NADPH as a co-factor. Isolation of the enzyme has shown the presence of two distinct isoforms with different co-factors, the NADPH and NADH dependant isoforms (Shimakata and Stumpf, 1982d). Both the NADPH and NADH isoforms were identified within the seed whereas the leaf only contained the NADH form (Shimakata and Stumpf, 1982d). The NADH specific form was purified from avocado (Caughey and Kekwick, 1982) and spinach (Shimakata and Stumpf, 1982d). The enoyl-ACP reductase purified from rape (Slabas *et al.*, 1986 & 1991 and Kater *et al.*, 1991) was shown to contain several different isoforms (Kater *et al.*, 1991, Fawcett *et al.*, 1994). However in other species such as *Arabidopsis* there appears to be only one gene encoding the enzyme.

1.1.3.7 Stearoyl-ACP Desaturase

Butyryl-ACP is formed upon the completion of one cycle of the fatty acid biosynthetic pathway, subsequent cycles elongate the butyryl-ACP by the sequential addition of 2C malonyl-ACP. The chain undergoes keto reduction, dehydration and enoyl reduction for each complete cycle. The final products of fatty acid synthesis are acyl ACPs (C16 or C18), these are saturated fatty acids, however the majority of plant fatty acids are unsaturated therefore they are further modified by stearoyl-ACP desaturase (EC 1.14.99.6). The stearoyl ACP desaturase is involved in the addition of a double bond in the end product of fatty acid synthesis to form 18:1 fatty acids. Therefore the plastids themselves produce both palmitate and oleate. The Δ^9 stearoyl-ACP desaturase introduces a double bond between the 9 and 10 carbons, forming oleoyl-ACP (Jaworski and Stumpf, 1974), this reaction requires O_2 and an electron donor. Stearoyl-ACP desaturase was cloned and purified by Shanklin and Somerville (1991) and Thompson *et al.*, (1991). The desaturase is unique to plants and its 3D structure was determined by crystallography upon cloning (Shanklin *et al.*, 1991). The desaturase from *B.napus* has also been cloned (Slocombe *et al.*, 1992). Two isoforms of the stearoyl-ACP desaturase from oil palm were identified and shown to exhibit the same expression pattern, correlating with the increase lipid production (Shah *et al.*, 2000). The modification of plants to show seeds with 30-40% stearate were obtained in rape by antisense of the stearoyl-ACP desaturase enzyme (Knutzon *et al.*, 1992).

1.1.3.8 Termination of Fatty Acid Biosynthesis

The termination of the acyl chain elongation is carried out by the removal of the chain from ACP. This can occur in two ways, either by the transfer of the acyl chain to glycerol-3-phosphate via an acyl transferase or by the hydrolysis of the chain to free fatty acids by the acyl-ACP thioesterase, which then leave the plastid (Ohlrogge and Browse, 1995). The way in which the fatty acid is released from ACP determines its

fate as to whether or not it leaves the plastid. There are two pathways for the formation of glycerolipids, the eukaryotic and prokaryotic pathways (Roughan *et al.*, 1987). The eukaryotic pathway involves the free fatty acids produced leaving the plastid and forming glycerolipids on the ER (Roughan and Slack, 1982). The acyl groups are exported from the plastid and converted to Acyl CoA, the exact way in which acyl groups are exported from the cytoplasm is unclear. However, it is thought that the export may in some way be coupled to Acyl CoA Synthesis. Evidence for this comes from the work on spinach chloroplasts that identified an Acyl CoA Synthesis within the outer wall of the chloroplasts that converts free fatty acids to Acyl CoA in an ATP dependent manner (Block *et al* 1983). The prokaryotic pathway involves the transfer of the acyl chains from the ACP to glycerol and the glycerolipids are synthesised within the plastid (Roughan and Slack, 1982 & Somerville and Browse, 1991). The acyl thioesterase from rape has been purified and cloned (Hellyer and Slabas, 1990). Two types of thioesterases have been identified: one for saturated ACP and the other for 18:1 (Jones *et al.*, 1995). Two acyltransferases are also thought to exist: one that is specific for palmitoyl ACP and the other specific for oleoyl ACP.

1.1.3.9 Triacylglycerols (TAG) Biosynthesis

There are two pathways involved in the formation of glycerolipids: the eukaryotic pathway that is located on the ER and the prokaryotic pathway that occurs within the plastid (Roughan *et al.*, 1987). The termination of the acyl chain elongation is carried out by the removal of the chain from ACP. The way in which the fatty acid is released from ACP determines its fate as to whether or not it leaves the plastid and therefore determines whether it goes down the eukaryotic or prokaryotic pathway. The prokaryotic pathway involves the transfer of the acyl chain to glycerol-3-phosphate via an acyl transferase which therefore retains the fatty acid within the plastid. These glycerolipids lead to the prokaryotic type diacylglycerols for the synthesis of plastid membrane components. The eukaryotic pathway involves the free fatty acids produced leaving the plastid and forming glycerolipids on the ER. This occurs by the hydrolysis of the chain to free fatty acids by the acyl-ACP thioesterase which then

leave the plastid and go to the ER (Ohlrogge and Browse, 1995). The eukaryotic pathway on the ER is involved in the formation of phospholipids and triacylglycerols. Formation of triacylglycerol synthesis (TAG) within the ER utilises the 16:0 and 18:1 end products from the plastid. Two fatty acids are acylated onto glycerol-3-phosphate to form phosphatidic acid (PA), by the enzymes glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase. The PA is then converted to a diacylglycerol by dephosphorylation via the enzyme phosphatidic acid phosphatase. The diacylglycerols produced are then either used within the plant mainly as a protection function within cell membranes, or they are used to produce triacylglycerols using the enzyme diacylglycerol acyltransferase (DAGAT). DAGAT is the only enzyme that is unique to the TAG biosynthetic pathway. The final acylation occurs at the sn-3 position to be converted into a triacylglycerol. The membrane lipids and the triacylglycerides often consist of the same basic fatty acids and therefore the production of these membrane lipids are important in the final production of the triacylglycerides. It is thought that over 300 different fatty acids occur in triacylglycerols within the seed (Harwood, 1980).

1.1.4 Genetic Manipulation of Plant Fatty Acids

With the huge economical importance of lipids, the genetic manipulation of plant fatty acids is a growing area. Recent interest has focused on the production of plants with improved lipid characteristics that increase their commercial value. A review by Murphy, 1994 describes the genetic modification of the fatty acid composition in plants to produce "designer oils". Changes in the composition of storage triacylglycerols have been made, and this has been shown not to interfere with the plants themselves (Ohlrogge *et al.*, 1991). Modifications in the saturation of lipids and their chain length have been achieved in transgenic plants, by altering levels of thioesterases and desaturases (Kinney, 1994; Murphy, 1994; Ohlrogge, 1994; Topfer *et al.*, 1995). Altering the number of double bonds or the chain length of the fatty acids can greatly affect the properties of the oil. The presence of double bonds and the length of the fatty acid chain affects the melting point and the solubility of the oil;

therefore being able to alter these properties will affect their commercial value and have huge economical importance. A number of transgenic oil seed rape plants have been engineered for industrial use. Rape plants were engineered to produce more lauric acid by the introduction a single gene encoding a medium chain acyl-ACP thioesterase under the control of the napin promoter. This resulted in the production of transgenic plants with fatty acids of altered chain length (Voelker *et al.*, 1992 & 1996). The modification of the stearate levels within the oil has also been achieved within *B.napus* seeds (Knutzon *et al.*, 1992). Reduction of the stearoyl-ACP desaturase activity was achieved by antisense constructs, resulting in *B.napus* transgenic plants with an increase in stearate levels from 2% to 40%.

Other properties of the plants have been modified by the genetic modification of the fatty acid composition. Glycerol-3-phosphate acyltransferase is thought to increase the levels of unsaturated fatty acids, thereby increasing the chilling resistance of the plants. Murata *et al.*, (1992) engineered tobacco plants with increased glycerol-3-phosphate acyltransferase from *Arabidopsis* and Squash, thereby altering the chilling sensitivity of the plant by increasing the degree of unsaturated fatty acids in the membranes. Discovering the mechanism involved in the regulation of lipid biosynthesis will influence the value of the plant, for both industrial and dietary uses. The majority of study on the regulation of the fatty acid biosynthetic pathway has concentrated on the individual enzymes that regulate the pathway controlling the flux through the pathway. The overriding cis-acting elements involved in the regulation of the pathway have yet to be elucidated. A greater understanding of these cis-acting elements involved in fatty acid biosynthesis regulation will be required to engineer plants with increased lipid biosynthesis.

1.1.5 Regulation of Fatty Acid Biosynthesis

Fatty acid synthesis has been widely researched and work carried out on a number of the individual enzymes, however there is little understanding of how the pathway itself is regulated. An understanding of this regulation will be vital if the amount of lipid production within plants can be increased. A number of the enzymes within the pathway have been examined to see if any of them are rate limiting. A considerable amount of interest has focused on ACCase as the possible regulatory step in fatty acid biosynthesis. Evidence has shown that this is the major regulating enzyme in animals (Goodridge, 1985) and yeast (Wakil *et al.*, 1983). Experiments provide some evidence that this may also be the case in plants. The analysis of the substrate and product sizes implicates ACCase in the light/dark regulation of fatty acid synthesis within chloroplasts (Nakamura and Yamada, 1979 and Post-Beittenmiller *et al.*, 1991 & 1992). One suggestion is that the changes in pH, Mg, and ATP/ADP levels between light and dark chloroplasts may account for the change in the activity of ACCase. Examination of the flux co-efficient of ACCase from leaves of barley and maize by inhibition studies also led to the conclusion that ACCase exerts major control over fatty acid synthesis in maize leaves (Page *et al.* 1994).

If ACCase is the major control point of the fatty acid biosynthesis pathway then examination of how ACCase itself is regulated is important. Within animals this regulation has been shown to occur via phosphorylation and citrate, and within yeast it is thought that one possibility may involve acyl-CoAs. However, neither phosphorylation nor citrate have been shown to play a significant role in the regulation of the plant ACCase. It is thought that the plant ACCase may be affected by changes in the co-factors within the plastid upon illumination (Post-Beittenmiller *et al.*, 1991 & 1992). Feedback regulation has been associated with a number of metabolic pathways, this type of regulation often exerts its effects on the first enzyme of the pathway and therefore ACCase was examined as a candidate. One product of the fatty acid pathway considered as feedback inhibitors were the long chain acyl Co-As. They were shown to be capable of inhibiting the ACCase (Goodridge, 1972).

However, acyl Co-A binding proteins were shown to be present in plants reducing them to a concentration that may not be inhibitory (Engeseth *et al.*, 1996). Pea chloroplast ACCases were also shown to not be significantly inhibited by long chain acyl-ACPs. Feedback inhibition of ACCase was shown by the addition of lipids to tobacco cells (Shintani and Ohlrogge, 1995). When the acyl ACP profiles were observed during feedback inhibition an increase in acetyl-ACP, and a decrease in long chain acyl-ACPs was seen, whereas the medium chain acyl-ACP levels remained constant. These observations would be predicted if there was a decrease in fatty acid synthesis due to malonyl-CoA being a limiting factor, indicating that ACCase may be an important step in feedback inhibition.

Other steps in the pathway were examined to see if they have any control over the flux through the pathway. One idea is that other enzymes, such as the condensing enzymes may be important regulators when there is a high flux through ACCase. The medium chain acyl ACP end products of fatty acid synthesis have been shown to inhibit the KAS III enzyme (Abbadi *et al.*, 2000). However, it is thought that altering the rate of one of the condensing enzymes would have little effect on the overall rate of fatty acid synthesis, as one of the other condensing enzymes would be limiting.

The supply of substrate to a pathway has also been thought to be involved in a regulatory capacity. Acetyl Co-A is one of the main substrates for fatty acid biosynthesis and has been suggested to be involved in regulating fatty acid biosynthesis within animals. However, it is unclear whether this is the case in plants. Evidence has suggested that even with differing rates of fatty acid biosynthesis both in the light and dark and within different tissues the acetyl CoA remains relatively constant. Thereby suggesting that the levels of acetyl CoA do not differ with changing rates of fatty acid biosynthesis (Post-Beittenmiller *et al* 1993). Spinach chloroplasts were incubated in the light and levels of acetyl coA measured, the chloroplasts were then transferred to the dark and the level of acetyl Co A dropped and then returned to the same level as found within light incubated chloroplasts. Therefore it is thought that the level of acetyl Co A remains at a constant level to

supply a number of biosynthetic pathways including fatty acid biosynthesis, isoprenoid biosynthesis, and amino acid production. The role of carbon supply into the fatty acid biosynthetic pathway has been studied by redirecting it into other pathways to visual the affect on the synthesis of fatty acids. Within *Arabidopsis* the plastidal acetyl CoA was redirected into the polyhydroxylbutyrate pathway by targeting the enzymes of the PHB pathway into the chloroplasts thereby utilising the plastidal acetyl CoA (Nawrath *et al* 1994). However even with the acetyl CoA being redirected there was no obvious affect on fatty acid biosynthesis. Thereby suggesting that the plant has the potential to increase acetyl CoA production in response to an increase in demand for the substrate. However other experiments have supplied evidence that this is not the case for example within *Brassica napus* the carbon supply was redirected into the production of starch resulting in an increase in starch production but a reduction in the fatty acid biosynthesis (Boddupalli *et al* 1995).

Evidence has been put forward that suggests that the control of lipid biosynthesis may involve the supply of fatty acids to the pathway, suggesting the capacity of triacylglycerol synthesis is below maximum. An increase in the production of triacylglycerol has been shown by the addition of exogenous fatty acids. Greiner showed a 10% increase in triacylglycerol by the addition of exogenous phosphatidylcholine added as liposomes to cultured *Chlamydomonas reinhardtii*. The demand for fatty acids has also been forward as a possible regulatory role. An increase in fatty acid production was also observed with the release of feedback inhibition on ACCase with the overexpression of the plant ACP thioesterase in *E.coli*. This removal of the fatty acids produced released feedback inhibition and therefore increased the fatty acid production by 10 fold.

The plant fatty acid biosynthesis genes appear to be both temporally and spatially regulated. Every plant cell requires fatty acid biosynthesis, as this is a vital component of cell membranes. Therefore all genes involved in fatty acid biosynthesis must be constitutively expressed as a housekeeping role (Slabas and Fawcett, 1992).

However other plant cells, for example within the seed upregulate this basal level as an increase in fatty acid biosynthesis is needed for the deposition of lipids. Therefore different tissues at different stages of development require different amounts of lipids. The overall regulation of these genes is essential and tight co-ordination is required. It is thought that this regulation may occur at the level of transcription. Northern blots were carried out on enoyl-ACP reductase, β -Keto-ACP reductase, stearyl-ACP desaturase and the biotin carboxylase of the heteromeric ACCase in *B.napus* seeds. During seed development the mRNA levels were shown to increase until 29 DAF, with mRNA levels 15-30 times greater than in leaf. These mRNA transcripts were seen just before the increase of the protein themselves, and were shown to occur at their maximum level prior to maximum lipid synthesis. They also appeared to show a coordinate expression pattern (Fawcett *et al.*, 1994 and 2002). These experiments have therefore shown a correlation in the expression of a number of the lipid biosynthetic genes, suggesting that these genes may be under a common co-ordinate control.

1.2 Regulation of Gene Expression

The differential expression of an organism's genes is responsible for the different cell types that arise, and therefore both the temporal and spatial regulation of their expression is crucial for the correct functioning of that organism. The regulation of these genes can be achieved at a number of stages along the pathway from DNA to protein, including transcriptional control (when a gene is transcribed), RNA processing control (for example RNA splicing), RNA transport control (which of the mRNA transcribed are transported into the cytoplasm to be translated), translational control (which of these transported mRNAs are translated), mRNA degradation control (which of the mRNAs is degraded rather than being translated) and finally protein activation control (whether the proteins are activated, deactivated or compartmentalised). All of these steps are important control mechanisms controlling when and how frequently genes are expressed. However, transcriptional control has

shown to be the major level at which gene expression is controlled in eukaryotes for nuclear encoded gene. This therefore ensures that no unwanted intermediates are synthesised. A cell can change the expression of its genes in response to external stimuli for example in humans hormones affect gene expression, in plants this is also the case with certain stimuli for example abscisic acid. The expression of plant genes are controlled both temporally and spatially as well as being affected by external stimuli and therefore the regulation of genes is a complicated process making the elucidation of the regulatory elements involved very difficult.

Transcription is the production of RNA from a DNA template, involving the enzyme RNA polymerase. Three different RNA polymerases are found within eukaryotes, RNA polymerase I, II and III. RNA polymerase II is the most common form and this transcribes genes encoding the majority of proteins. RNA polymerase I is involved in transcribing genes that encode ribosomal RNAs (Sommerville, 1984) and RNA polymerase III is involved in the transcription of transfer RNA (Cilberto *et al.*, 1983). The TATA box is a conserved sequence that is located approximately 30bp upstream of the transcription start site. It is here that the RNA polymerase binds along with other DNA binding proteins to form the transcription initiation complex and is important in positioning where transcription initiation occurs. The region surrounding the TATA box is termed the promoter and it is this region that contains the general cis-acting elements to which the general transcription factors bind to initiate and regulate transcription of the gene (Goodwin *et al.*, 1990). The RNA polymerase II basal transcriptional complex consists of a number of elements, firstly the RNA polymerase II that is itself a multisubunit complex. This then interacts with a number of other protein factors, known as general transcription factors (e.g. the TATA binding protein (TBP) and TFIID). The transcription initiation complex then interacts with the TATA box within the promoter region to initiate transcription.

Transcription is also influenced by other sequences that are found within this region, other than the transcription initiation complex. These sequences, known as regulatory

sequences are where gene regulatory proteins bind, and affect the basal level of transcription. They are located by the promoter, or some distance upstream or downstream. Specific DNA binding proteins can have positive or negative effects on the basal transcription activity, acting as individual proteins or as a complex. The regulatory proteins differ between genes, to allow cell specific transcription of certain genes, whereas the general transcription factors that associate with the promoters are very similar for all genes transcribed by RNA polymerase II. Figure 1.3 shows a diagrammatic representation of promoter region showing the TATA box and other regulatory sequences.

1.2.1 Transcription Factors

Transcription factors are important proteins required to initiate or regulate transcription in eukaryotes. Examination of the PEDANT database containing functionally assigned genes showed that on average 2-3% of the prokaryotic and 6-7% of the eukaryotic genome encodes proteins that bind to DNA (Luscombe *et al.*, 2000). More than 1500 transcription factors have been identified in *Arabidopsis* making up 5% of the genome, these have been identified with the completion of the *Arabidopsis* genome project (Riechmann *et al.*, 2000).

Two types of transcription factors exist as previously mentioned:

- **General transcription factors** - these are involved in the transcription of all genes, and bind to the core promoter elements to form the transcription initiation complex. They are required for a basal level of transcription.
- **Sequence specific DNA binding proteins** - these transcription factors bind to specific regulatory motifs to modify the basal transcriptional activity. They are involved in determining transcription activity, and also gene specific activity.

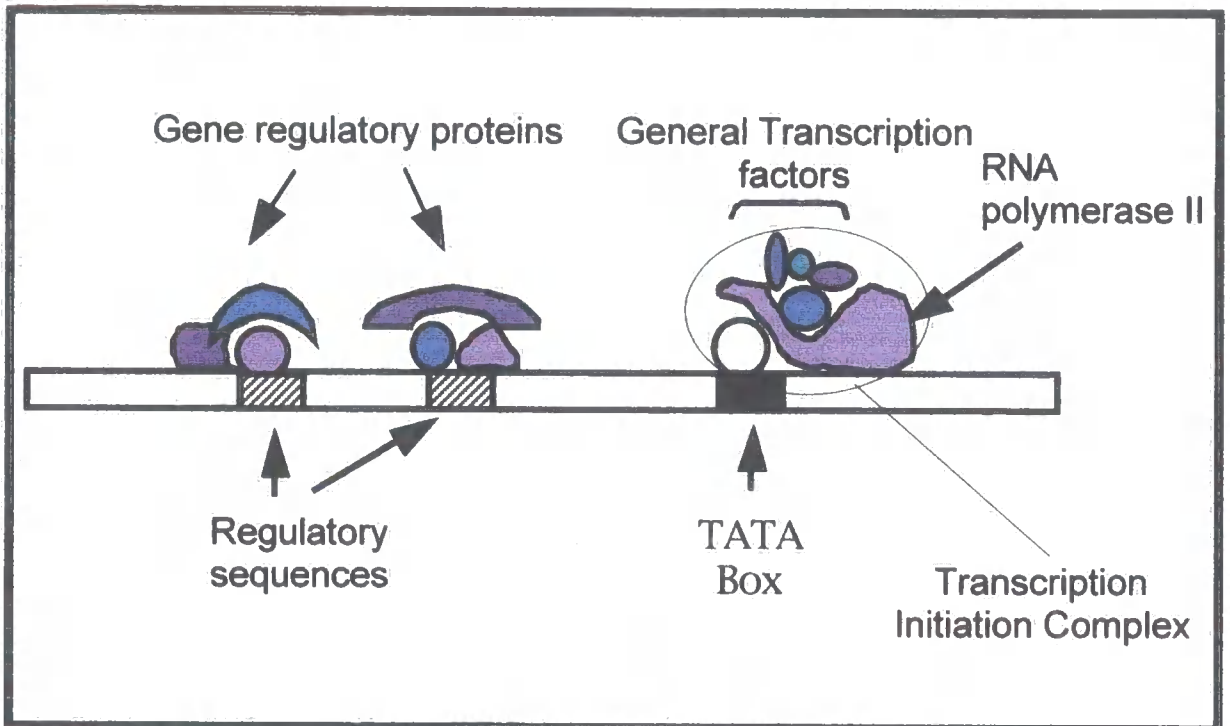


Figure 1.3 The Basal Transcriptional Machinery

Diagrammatic representation of the promoter and surrounding regions. The TATA box is where the RNA polymerase and general transcription factors bind. The gene regulatory proteins bind to regulatory sequences upstream or downstream from the TATA box and affect basal transcriptional activity.

1.2.1.1 Transcription Factor Domains

Most transcription factors are comprised of a DNA binding domain, an oligomerisation domain, a transcriptional regulation domain and a nuclear localisation signal (Latchman, 1990; Mitchell and Tjian, 1989; Ptashne *et al.*, 1988). These four domains confer certain features that the transcription factor requires to carry out its function. Firstly they must be able bind specifically to DNA in a sequence dependent manner (DNA binding domain). Upon binding they must be able to affect regulation either in a positive or negative way (transcriptional regulation domain). This occurs by the transcription factor interacting with the RNA polymerase II and or other transcription factors. Therefore transcription factors must be able to bind to other proteins and form hetero and homo dimers (oligomerisation domain). The transcription factor itself must also be regulated to some extent either before or after it has been synthesised. The variation in the activity of transcription factors and their specificity is thought to be due to the different combinations of transcription factors binding to the DNA (Izawa *et al.*, 1993). It is thought that a number of novel transcription factors within plants have been brought about by domain swapping, This is a common feature in the homeodomain proteins from *Arabidopsis* where a novel set of domains come together to form a distinct type of transcription factor only found in plants and not within animals (Reichmann *et al.*, 2000). The four different domains of a transcription factor are discussed individually below and an example of a typical transcription factor containing these domains shown diagrammatically in figure 1.4.



1.2.1.1a Transcriptional Regulation Domain (Activation Domain)

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amino acids within the domain does not affect the activity of the transcription factor. Liu *et al.* (1999) suggested that the activation of the transcription factor is brought about by conformational changes due to intramolecular interactions, however no clear indication of the way the activation domains function is at present known. A number of transcription factors have been shown to have more than one activation domain, for example the bZIP protein GCN4 (Drysdale *et al.*, 1995), however it is thought that the extra domain has no additional affect on the activity of the transcription factor. The acidic family of activation domains appears to be the largest so far identified within yeast and animals. Members of this family have a high number of acidic amino acids, producing a strong negative force. Although they have a large number of acidic amino acids, they do not appear to share high amino acid sequence homology. A number of plant transcription factors have been identified that fall into this class of acidic activation domains. Examples include the AtMYB2 (Urao *et al.*, 1996), ORCA3 (AP2/ERF) from *Catharanthus roseus* (van der Fits and Memelink, 2001) and the C1 MYB transcription factor from maize (Goff *et al.*, 1991 and Sainz *et al.*, 1997). The glutamine rich domains again, as with the acidic domains, do not appear to show a great deal of sequence homology, but contain a high proportion of glutamine residues. Examples include the Spl (McKnight and Tjian, 1986) and ARR1 and 2 (Sakai *et al.*, 2000). The final group of activation domains are the proline rich domains; examples include the *Arabidopsis* GBF1 (Schindler *et al.*, 1992) and the CPRF1 and 4 from parsley (Sprenger-Haussels and Weisshaar, 2000).

1.2.1.1b Oligomerisation Domain

The affinity and DNA binding specificity of a transcription factor is affected by the ability to form hetero and homo oligomers (Guiltinan and Miller, 1994). Therefore transcription factors must have the ability to bind to other proteins to affect the basal rate of transcription as well as binding to the specific DNA sequence. There appears to be high conservation between the sequences of the oligomerisation domains, however the length of the domain often varies and it is this that accounts for the

differences observed between the members of a family. The stabilisation between transcription factors is thought to be due to hydrophobic and hydrophilic interactions (Huang *et al.*, 1996). However a bZIP protein from tobacco (TGA1a) has been shown to contain a novel domain involved in stabilisation of the protein upon the formation of dimers (Katagiri and Chua, 1992).

1.2.1.1c DNA binding domain

The DNA binding domain determines the specificity of the transcription factor, as it is these domains that contact the specific *cis*-acting elements via their amino acid residues (Huang *et al.*, 1996). There are a number of contact points between the transcription factor and the DNA, forming a very specific and strong bond. The bonds are formed by hydrophobic interactions, ionic bonds and hydrogen bonds. Other phosphate or deoxyribose moieties also interact non-specifically with the DNA thereby helping to increase the binding of the transcription factor to the promoter (Huang *et al.*, 1996). The residues that recognise and bind to the DNA are very conserved (Huang *et al.* 1996), and it is the spatial arrangement of these residues that are responsible for the recognition of specific DNA binding sites and therefore account for the specificity of the transcription factor. Within the MADS transcription factor, family the conserved recognition residues are lysine and arginine (Huang *et al.*, 1996). The secondary structure of the DNA binding domain is thought to primarily affect their selectivity and affinity for DNA. Several copies of the DNA binding domain may be present, although there is only ever one type. A number of the MYB proteins contain two DNA binding domains, and the APETELA2 (AP2) transcription factors often show a conserved sequence in between two DNA binding domains (Jofuku *et al.*, 1994). The presence of more than one DNA binding domain means that the transcription factor can interact co-operatively with the same *cis*-element.

1.2.1.2 Transcription Factor Families

Based on their conserved DNA binding domains the plant transcription factors have been classified into families (Katagiri and Chua, 1992, Menkens *et al.*, 1995, Pabo and Sauer, 1992, Harrison, 1991, Luisi, 1995 and Luscombe *et al.*, 2000). Examples of the main plant transcription factor families are outlined below.

1.2.1.2a Basic Leucine Zipper (bZIP)

The basic leucine zipper (bZIP) proteins are one of the largest family of transcription factors identified within plants (Harrison, 1991). They are not plant specific unlike other transcription factor families but have been identified in all eukaryotes examined, examples within animals are CREB or Jun/Fos. However they do seem to be amplified within plants with four times as many bZIP transcription factors apparent within *Arabidopsis* than found within humans and yeast (Riechmann and Ratcliffe, 2000). By the completion of the *Arabidopsis* genome project, around 81 members of the *Arabidopsis* bZIP family have been identified (Riechmann and Ratcliffe, 2000), however others have since reported only 75 members (Jakoby *et al.*, 2002). The main structure of the bZIP domain is the alpha helix containing two main regions, the first of which is the 16 amino acid basic region within which a nuclear localisation signal is located. The region that contacts the DNA is also found within this basic region consisting of the following motif N-x₇-R/K (Ellenberger, 1994 and Hurst, 1995). The second region of the bZIP proteins is the leucine zipper region that forms an amphipathic helix, consisting of a heptad repeat of leucines located nine amino acids towards the C-terminal end. Two of these subunits interact via their hydrophobic helices to produce the coiled coil zipper structure. These proteins acting as dimers are held together by interactions between two alpha helices, one from each monomer. The bonds are formed by the interactions between the hydrophobic amino acid side chains.

The bZIP proteins can either form homodimers or heterodimers, allowing an increase in the number of DNA specificities of the protein. The conserved DNA binding sequence recognised by these proteins is the ACGT core sequence (Foster *et al.*, 1994). Work on the DNA binding specificity indicated that the nucleotides surrounding this core motif affect the specificity and affinity of binding by these proteins (Izawa *et al.*, 1993). Three boxes have been identified to which these plant bZIP proteins appear to bind, the G-box (CACGTG), the C-box (GACGTG) and the A-box (TACGTA) (Izawa *et al.*, 1993). The G-box is a *cis*-acting element that binds to G-box-binding factors (GBF) (Menkens *et al.*, 1995). GBFs are involved in the plants' response to hormones and light, and are associated with a variety of promoters and processes. Most GBFs have been shown to be members of the bZIP family.

The similarities between the basic regions, along with other conserved groups and the size of the zipper, allows these *Arabidopsis* bZIP proteins to be subdivided into ten different groups (Jakoby *et al.*, 2002). The classification of bZIP proteins into groups due to their similarities in their basic DNA binding region assumes that members of the same family will probably bind to similar promoter sequences. Within plants bZIP proteins have shown to be involved with a range of processes including flower and seed development, light and stress response and pathogen related defense (Jakoby *et al.*, 2002). The maize opaque 2 protein (O2) is a member of the bZIP family of transcription factors and is involved in the regulation of a certain classes of endosperm genes. The O2 was shown to bind to two sequences within the promoter of the alpha-coixin gene. The promoter was analysed and two binding sites identified within the promoter of this gene, containing the core motif TGAC. The O2 transcription factor was shown to bind as dimers to these two *cis*-acting elements, and act synergically in transient expression experiments (Yunes *et al.*, 1998). The rice bZIP protein RITA-1 protein is highly expressed during seed development, and appears to show specificity for A, C and G boxes but not T boxes (Izawa *et al.*, 1994). A number of the bZIP proteins have been shown to be regulated by certain stimuli, for example the following bZIP proteins are thought to be regulated by light: the parsley CPRF4a (Wellmer *et al.*, 2001) and the *Arabidopsis* HY5 (Chattopadhyay *et*

al., 1998), whereas the ABRE1/2 transcription factor was shown to be regulated by ABA.

1.2.1.2b MYB

MYB transcription factors are a large family (Riechmann *et al.*, 2000), widely classified within animals. The maize *cl* gene was the first plant transcription factor described that encoded a MYB protein, and is involved in the regulation of anthocyanin biosynthesis in seed development. (Paz-Ares *et al.*, 1987). The MYB transcription factors have been shown to act as both repressors and activators. The N terminal half of the MYB protein contains the DNA binding domain. The characteristics of the MYB domain are three imperfect repeats of about 53 amino acids (R1-3) (Jin and Martin, 1999) each one forming a helix-turn-helix structure, a common feature of these being the tryptophan cluster containing three tryptophan residues (Ogata *et al.*, 1992). It is the tryptophan cluster that stabilises the DNA binding domain by forming a hydrophobic core. Each repeat encodes three alpha helices with the 2nd and 3rd helices forming a helix-turn-helix structure. DNA binding requires only the presence of R2 and R3, although it is thought that R1 may be involved in the specificity, and stabilisation of this binding (Tanikawa *et al.*, 1993). The plant MYB transcription factors have been subdivided into three subfamilies classified according to the number of MYB repeats (Jin and Martin, 1999, Rosinski and Atchley, 1998). These are the MYB1R factors (one repeat), R2R3-type MYB factors (two repeats) (Lipsick *et al.*, 1996 and Martin and Paz-ares, 1997) and MYB3R factors (with three repeats) (Stracke *et al.*, 2001).

The largest of these families within plants are the MYB R2R3. So far between 125 (Stracke *et al.*, 2001) and 131 (Riechmann *et al.*, 2000) members of this subfamily have been identified within *Arabidopsis*. Whereas within animals the most common type is the MYB-R3, of which there are only 5 in *Arabidopsis*. Comparison of these repeats within the MYBR2R3 subfamily showed that R3 appears to be slightly more conserved than that of R2 (Stracke *et al.*, 2001). The *Arabidopsis* R2R3 group has

been characterised further into 22 subgroups (Kranz *et al.*, 1998). MYB proteins have been identified in a number of species including maize (Grotewold *et al.*, 1991), barley (Marocco *et al.*, 1989), Petunia (Avila *et al.*, 1993), *Arabidopsis* (Oppenheimer *et al.*, 1991; Shinozaki *et al.*, 1992) and *Antirrhinum* (Jackson *et al.*, 1991). The function of this subfamily appears to lie in the regulation of plant secondary metabolism and also involved in the identity and fate of plant cells. The R2R3 family plant MYB proteins have been associated with plant metabolism (Romero *et al.*, 1998), flavanoid biosynthesis (Mol *et al.*, 1998) control of phenylpropanoid metabolism within *Arabidopsis* (Borevitz *et al.*, 2000), differentiation of epidermal cells to trichomes (GL1) (Oppenheimer *et al.*, 1991), development of leaf form (Waites *et al.*, 1998), response to water stress (Urao *et al.*, 1993), and the strawberry FaMYB1 protein has been shown to regulate biosynthesis of anthocyanins and flavonols (Aharoni *et al.*, 2001).

The diverse range of processes in which MYB proteins are involved, was examined by Kranz *et al.* (1998), who examined the expression of the R2R3 genes under more than 20 different physiological conditions. The expression pattern of the genes showed that they were expressed under a number of conditions and within different tissues. The MYB genes have also been shown to interact with other transcription factors from different families. For example, the HvGAMYB barley transcription factor expressed during endosperm development was shown to interact with another endosperm specific transcription factor that was a member of the Dof family (BPBF) (Diaz *et al.*, 2002)

1.2.1.2c AP2/EREBP

The AP2/EREBP family of transcription factors show very similar DNA binding specificities, however they differ as to whether they activate or repress gene transcription. Therefore the concentration of the transcription factor is important, as depending on whether the repressor or activator is high depends on whether there is transcription of the gene (Fujimoto *et al.*, 2000). Over 100 members of the AP2/ERF

family of transcription factors have been identified (Riechmann and Ratcliffe, 2000). The AP2/ERF domain (Riechmann and Ratcliffe, 1998) appears to be unique to plants and has not been identified within other eukaryotes. It is the AP2 region that is highly conserved within this plant family made up of a stretch of 60 to 70 amino acids. Within this domain is an N-terminal region comprising of 20 basic and hydrophilic amino acid residues (YRG element), it is this element that is thought to be important for DNA binding. The RAYD element, consisting of an amphipathic alpha-helix within the C-terminus, is thought to be important for protein-protein interactions.

The AP2/EREBP family was divided into three classes depending on the number of AP2/ERF domains. The family was shown to bind specifically to the Drought Responsive Element (DRE) (involved in the adaption of plants to drought conditions) containing the core sequence TACCGACAT (Yamaguchi-Shinozaki and Shinozaki, 1994). The 4th and 7th C and the 5th G are thought to be the bases with which the DREB proteins specifically interact (Sakuma *et al.*, 2002), however they do appear to recognise slightly different analogues of the core motif. So far the DRE binding factors that have been identified appear to belong to the AP2/ERF family of transcription factors. Both ABA dependent and independent DRE transcription factors have been identified. The DREB proteins involved in regulating genes in response to stress appear to function independently of ABA (Kizis *et al.*, 2001). The members can be either repressors or activators but have similar DNA binding specificities to the GCC box. Examples of the AP2/EREBP family are the DREB1A and DREB2A which were shown to bind to the genes involved in cold and drought stress in *Arabidopsis* (Sakuma *et al.*, 2002) and ORCA3 which is thought to be activated by jasmonate and involved in primary and secondary metabolism in *Catharanthus roseus* (Van der Fits and Memelink, 2001).

1.2.1.2d DoF

The DNA binding with One Finger (Dof) proteins (Yanagisawa, 1996 & Yanagisawa and Schmidt, 1999) are a recently discovered class of transcription factor, which like many other transcription factors, share a unique DNA binding domain (Yanagisawa, 1995 & 1996). These transcription factors are thought to be specific to plants (Yanagisawa *et al.*, 1996), as they have not yet been identified in yeast and animals. They appear to have a diverse role within plants and have been shown to bind to a wide range of plant promoters (Yanagisawa, 1995 and 1996). The first Dof transcription factor to be identified was the maize Dof1 (MNB1a) (Yanagisawa and Izui, 1993 and Yanagisawa and Sheen, 1998).

The processes that are regulated by Dof proteins appear to be plant specific, for example pathogen response, photosynthetic genes and seed specific genes, this would complement the idea that they are unique to plants. The Dof DNA binding domain involved in recognition is apparently composed of a highly conserved region of 52 amino acids. Within this Dof domain, a zinc finger composed of the following motif $CX_2CX_{21}CX_2C$ was identified to be important for DNA recognition (Chen *et al.*, 1996, Yanagisawa, 1995). Yanagisawa and Schmidt (1999) analysed four different Dof proteins to look at their binding sites and they appeared to bind to the core sequence (A/T)AAAG. The flanking regions were also examined and were shown to have a limited effect on the specificity of the binding therefore it is thought that protein-protein interactions may be involved in the specificity of binding (Yanagisawa and Sheen, 1998). This supports the idea that these proteins interact with other families of transcription factors (Vincente-Carbajosa *et al.*, 1997 and Yanagisawa, 1997). Certain Dof transcription factors have been shown to interact with other transcription factors; which enhance the efficiency of the DNA binding of that protein. The OBP1, an *Arabidopsis* transcription Dof factor, binds to the OBF bZIP transcription factor involved in the plants response to stress and increases the binding of the protein to the specific *cis*-acting elements (Zhang *et al.*, 1995). The PBF endosperm specific protein that binds to the prolamin box, has been shown to

interact with the bZIP protein O2 in the zein gene (Vincente-Carbajosa *et al.*, 1997). Both activators and repressors of the Dof family have been identified, for example DoF1 and 2. Dof1 is a transcriptional activator and Dof2 blocks the activation of Dof1 (Yanagisawa and Sheen, 1998). The DNA binding domains of Dof1 and Dof2 proteins are very similar, however the differences occur within the transcriptional activation domain with the C-terminal sequence (Yanagisawa, 1996).

1.2.1.2e MADS

The MADS family of DNA binding proteins have been shown to be conserved within animals, plants and fungi (Schwartz-Sommer *et al.*, 1990). The name was originally derived from the original members of the family MCM1 (yeast protein), AGAMOUS and DEFICIENS (plant proteins) and SERUM RESPOSE FACTOR (from mammals). The plant MADS proteins have been shown, as in animals and yeast, to form dimers. Over 80 members of the *Arabidopsis* MADS genes are thought to exist (Riechmann and Ratcliffe, 2000). The MADS proteins consist of four domains (MIKC). Firstly the MADS domain (M) which is located at the N-terminal region. The MADS box domain is composed of a 180bp conserved sequence. This domain is involved with the DNA binding and also dimerisation of the protein. This is then followed by the intervening region (I) that is not highly conserved, the K box involved in protein-protein interactions and dimerisation (Theißen *et al.*, 1996), made up of a series of amphipathic alpha-helices and finally the C-terminal domain that confers specificity and is thought to be involved in the formation of MADS multimers.

The floral organ identity genes were the first plant MADS genes to be identified (ABC model) (Coen and Meyerowitz, 1991); examples from *Arabidopsis* are the APETALA1 (AP1) (Mandel *et al.*, 1992) and SEPALLATA1 (SEP1) (Flanagan and Ma, 1994). MADS box genes are not just involved in the regulation of floral identity but also regulate a variety of other processes, for example ovules, fruits, leaves and

roots (Riechmann and Meyerowitz, 1997). The MADS box proteins have also been associated with the formation of roots and pollen from *Arabidopsis* (Alvarez-Buylla *et al.*, 2000), vegetative shoot development, PKMADS1 in *Paulownia Kawakamii* (Prakash and Kumar, 2002) and the FLM (flowering locus M) involved in gene inhibition (Scortecci *et al.*, 2001).

1.2.1.2f WRKY

The WRKY family of transcription factors is a very large and important family. It has only been identified within plants and therefore appears to be plant specific (Robatzek and Somssich, 2001). The family contains 75 members from *Arabidopsis*, originally identified by Eulgem (2000). The region that separates these proteins into a family are a stretch of 60 conserved amino acids. The family name is obtained from a region of 7 amino acids within the N-terminus namely the WRKGQK peptide, and within the C-terminus is a C-C-H-H/C zinc finger motif. There are three subgroups of the WRKY family containing over 100 members of the WRKY super family. The WRKY proteins have shown to interact with the *cis*-acting element containing the core sequence TGAC (Eulgem *et al.*, 1999 & 2000), for example the *Arabidopsis* NPR1 (Yu *et al.*, 2001). The precise functioning of these genes is unknown. They are thought to be involved in the regulation of the immediate early response genes, however they have been identified in a wide variety of plant processes (Eulgem *et al.*, 2000). They have been associated with pathogen response and wounding of the plant within parsley and tobacco (Eulgem *et al.*, 2000 and Chen and Chen, 2000). WRKY 1-3 from parsley were shown to be important in the signal transduction pathway that leads to the activation of the pathogen related protein (Rushton *et al.*, 1996). The WRKY6 from *Arabidopsis* has been associated with plant defense response (Robatzek and Somssich, 2001), and the regulation of leaf senescence (Hinderhofer and Zentgraf, 2001). ZAP1 was the first described WRKY factor from *Arabidopsis*, showing high expression within flowers and roots (de Pater *et al.*, 1996).

Animals and yeast show specific transcription factors not found in plants, this is also the case for plants with some families of transcription factors being plant specific for example the WRKY (Eulgem *et al.*, 2000) and AP2/EREBP (Riechmann *et al.*, 1998). Other families for example the MYB and MADS families have been identified within plants and animals, however they appear to be amplified in plants.

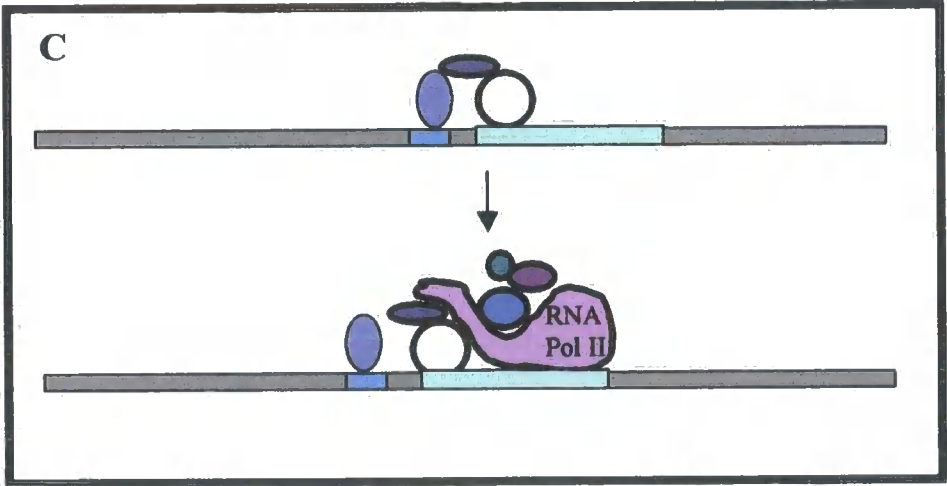
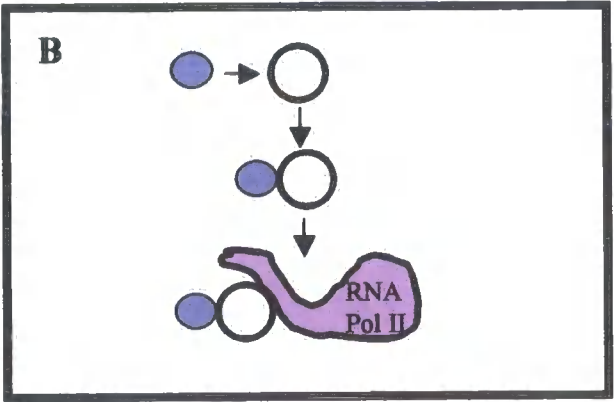
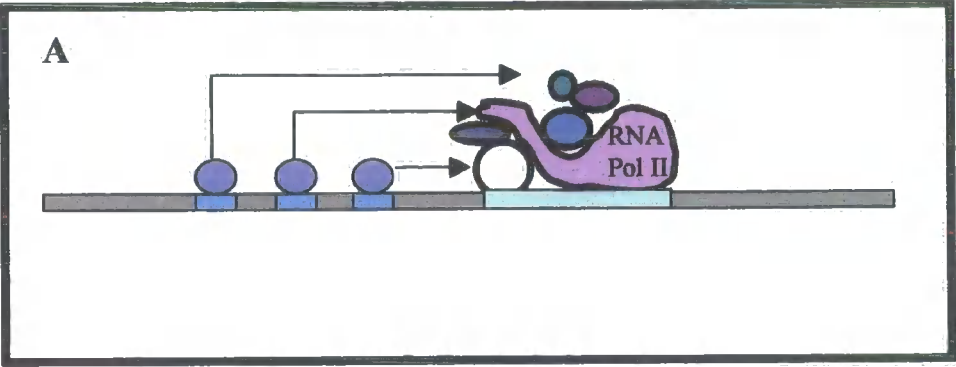
1.2.1.3 Transcription Factors as Activators and Repressors

The exact mechanism of how individual transcription factors either activate or repress transcription is unknown; more is known about the functioning of transcription factors in animals than plants and it is thought that similar mechanisms probably apply. Once the transcription initiation complex has been formed, then the activators can increase transcription by increasing either the activity or stability of the complex (Choy and Green, 1993). Transcription factors have been proposed to activate transcription in a number of ways (figure 1.5). The first is by the transcription factor binding to one of the basal transcription factors that forms the initiation complex, thereby enhancing the binding of this general protein to the transcription initiation complex, and so increasing transcription. The general transcription factor TFIIB binds after TFIID; this TFIIB binds RNA polymerase and other general transcription factors. Choy and Green (1993) showed that the binding of another activator to this TFIIB promotes the binding of TFIIB to the promoter, thereby increasing the association of the transcription initiation complex. It is also thought that the binding of this activation domain also increases the binding of other factors such as the RNA polymerase and general transcription factors to this TFIIB. Secondly the transcription factor can bind to the preformed transcription initiation complex, thereby increasing the stability or activity of the complex and affecting the transcription rates. Transcription factors can also work by helping the binding of other transcription factors by altering the structure of the chromatin, thereby facilitating the binding of other proteins to the DNA (Travers, 1994). A model of DNA looping has been proposed for the activation of transcription factors that bind at some distance from the

Figure 1.5 Activation of Transcription

Transcriptional activators are to work in one of the following ways:

- A) The transcription factors bind to the pre-assembled transcription initiation complex and in doing so stabilises the complex. Thereby increasing the rates of transcription.
- B) The transcription factor binds to one of the components of the transcription initiation complex. This binding helps the protein to form the transcription initiation complex, thus increasing the rate of transcription.
- C) The transcription factor binds to the promoter before the assembly of the complex and helps to initiate transcription by enhancing the binding of the general transcription factors.



promoter. If the regulatory *cis*-element is upstream or downstream of the TATA box, the transcription factor associates with the transcription initiation complex by looping out the intervening DNA, thereby contacting the transcription initiation complex directly (figure 1.6).

Transcription factors can also work as repressors (figure 1.7). As with activators, they are thought to work in a number of different ways. The factor can bind to the specific DNA sequence, thereby interfering and stopping the binding of the activation transcription factor by competing with the activator for the *cis*-element. The protein can also bind to the activator and form a complex that is unable to bind to the *cis*-acting element thereby hindering transcription rates. Thirdly the negative transcription factor can interfere with the rate of transcription by binding to the transcription initiation complex, thereby decreasing the transcriptional activity. Examples of transcriptional repressors are the two rice bZIP proteins, OsZIP 2a and 2b, which bind to and repress the action of the wheat bZIP EMBP1 (Nantel and Quatrano, 1996) and the *Arabidopsis* HY5 which regulates COP1. In the dark the COP1 protein interacts with the HY5 and prevents it from binding to its *cis*-element. In the light the COP1 releases the HY5 and therefore the HY5 is active and able to initiate transcription of its target genes (Ang and Deng, 1994 & Ang *et al.*, 1998).

1.2.1.4 Regulation of Transcription Factors

Transcription factors must themselves in some way be regulated, as they are important in regulating where and when a gene is switched on. Therefore they must be able to act constitutively and also specifically where they are switched on or off due to stimuli or developmental controls. There are two ways in which this regulation can be achieved: Either by regulating the production of the transcription factor (by controlling when and where it is synthesised) or by controlling the activity of the protein after it has been synthesised, (therefore it is only active at a certain time or within a certain tissue).

Figure 1.6 Looping of the DNA

Transcription factors often bind at some distance upstream or downstream of the transcription initiation complex and therefore it is thought that one way these transcription factors can influence the levels of transcription is by the looping of the DNA. Here the transcription factor binds at some distance from the TATA box, and for the trans-acting element to contact the basal transcriptional machinery the intervening DNA must be looped out. In this way the transcription factor can come into direct contact with the transcription initiation complex and affect the transcription rates.

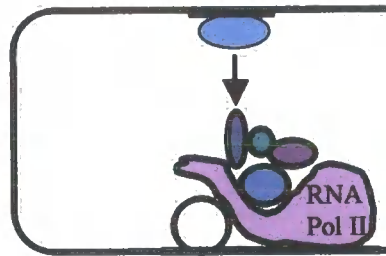
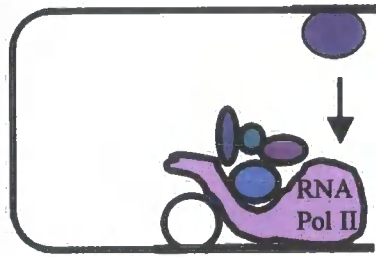


Figure 1.7 Repression of Transcription

The repression of transcription is also thought to occur via a number of possibilities

- A) Here the activator has bound to the DNA thereby initiating transcription
- B) The repressor binds to the activator itself thereby stopping the activator from binding to the DNA to initiate transcription
- C) The repressor binds to the DNA sequence, thereby competing with the activator for the *cis*-element and therefore no transcription occurs.
- D) Here there is constitutive transcription of the gene but the binding of the repressor to the DNA causes this transcription to stop and inhibits the transcription of the gene.
- E) The repressor binds to the activator already bound to the *cis*-element and stops the activation and therefore no transcription occurs.

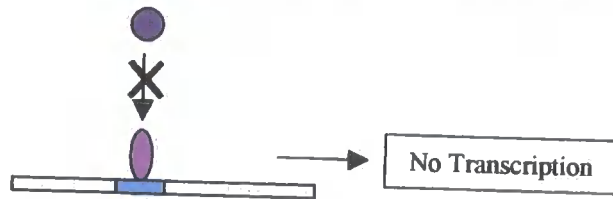
A



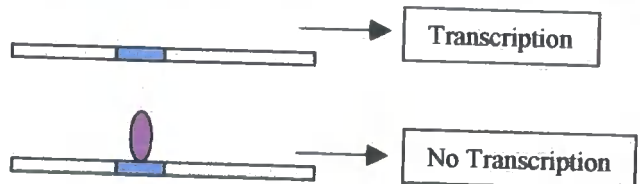
B



C






D



E



 = Repressor TF
 = Activator TF
 = Cis-acting element

Transcriptional control of transcription factors is not widely used for signal induced expression, where there would be a time delay between the signal and the synthesising of the transcription factor before transcription of the required gene was accomplished. Therefore these transcription factors are usually regulated by post-translational methods. The transcription factors therefore are already synthesised but remain in an inactive form until required. One way this is achieved is by a stimulus binding as a ligand to the transcription factor, thereby activating the protein. The dimerisation of transcription factors with other proteins is often important in the regulation of their activity. The C1 MYB transcription factor needs to bind to the helix-loop-helix transcription factor in order for it to activate transcription of its target genes (Martin *et al.*, 1996) and the interaction of the transcription factor COP1 with HY5 plays an important role in the regulation of HY5. In the dark the COP1 protein interacts with the HY5 and prevents it from binding to its *cis*-element. In the light the COP1 releases the HY5 and therefore the HY5 is active and able to initiate transcription of its target genes (Ang and Deng, 1994 & Ang *et al.*, 1998).

Transcription factors may also be activated by modification. One of the main proposed ways that transcription factors themselves are thought to be regulated is through phosphorylation. Phosphorylation is thought to affect the activity of the transcription factor, affinity for the DNA and also by interfering with its nuclear import. Phosphorylation of the bZIP transcription factor GBF1 from *Arabidopsis* was shown to increase the DNA binding activity of the protein (Klimczak *et al.*, 1992). Ciceri *et al.* (1997) also examined the affect of phosphorylation on the O2 transcription factor and showed that only the dephosphorylated form showed DNA binding activity and that the phosphorylated form was inhibited. Evidence has also been put forward for the trafficking of transcription factors between cells via their plasmodesmata during the development of plants, for example Knotted 1 (Jackson and Hake, 1997). This may have important consequences in their regulation, however the significance of this is not yet known.

1.2.1.5 The Study of Transcription Factors

Transcription factors have been studied *in vivo* and *in vitro*, their different domains have also been examined along with their interactions with other proteins and DNA. Reporter constructs are a common method for examining the regulation of a promoter. The promoter is attached to a reporter construct to examine its activity. A deletion series of the promoter can be examined, or mutagenesis analysis can be carried out to identify sequences important for the expression of that gene. Examples are the enoyl-ACP reductase (de Boer *et al.*, 1999) and the ACP promoter (Baerson *et al.*, 1994). Both of these involved sequential deletion analysis of the promoter to examine the sequences important for expression within different tissues. A number of *in vivo* experiments look at the functions of transcription factors using overexpression studies in transgenic plants. This allows the effect of excess transcription factor to be observed (Kasuga *et al.*, 1999). The modular nature of transcription factors has been examined using experiments such as domain swapping. To test a domain, if it is thought to be an activation domain, it can be fused to a known DNA binding domain. The *Arabidopsis* ARR1 and 2 transcription factor domains were studied in this way (Sakai *et al.*, 2000). The transactivation domains were identified within the C-terminal part of the protein by fusing them with the yeast GAL4 DNA binding domain. The interaction of transcription factors with other proteins is very important in their activation and DNA binding specificity. One of the main techniques employed to study these interactions is the yeast two-hybrid system. The interaction of the GAMYB protein from barley with the BPBF Dof transcription factor was identified using the yeast two hybrid system (Diaz *et al.*, 2002).

Transcription factors and their interaction with DNA can be studied in a number of ways. Their interaction with promoters can be studied using Gel Mobility Shift assays (GMSA) and DNA footprinting. The technique of GMSA shows the binding of a specific protein to a particular DNA sequence. This technique relies on the fact that binding of the protein to the DNA alters the DNA's mobility in non-denaturing acrylamide gel. Examples where the GMSA has been used to identify DNA binding

are the parsley PR1 to show the binding of nuclear proteins to its W boxes (Rushton *et al.*, 1996), the characterisation of the pea mitochondrial binding protein (Hatzack *et al.*, 1998) and the binding of the HY5 *Arabidopsis* bZIP protein to the G box (Chattopadhyay *et al.*, 1998). Another procedure for studying the interaction of transcription factors and DNA is that of DNA footprinting. This allows the identification of the sequence to which the protein is binding, unlike the GMSA. The DNA is radioactively labeled on one end and incubated with the protein. It is then cleaved randomly and run out on a gel. The region to which the protein binds will be protected from cleavage, resulting in a gap in the DNA ladder. This is used to identify sites in the DNA that bind specific proteins, in particular determination of promoter sequences, to which transcription factors bind. Quantitative DNase footprinting was used to show binding of the opaque 2 bZIP protein from maize in a cooperative manner (Yunes *et al.*, 1998) and to examine trans factors binding to the *Arabidopsis* PR1 gene (Lebel *et al.*, 1998).

The purification of transcription factors by affinity chromatography is a popular method for identifying DNA binding proteins (Hu *et al.*, 2000, Kadonaga, 1991, Jarrett, 1993, Gabrielsen *et al.*, 1993 and Hatzak *et al.*, 1998), and is often used in conjunction with the GMSA. The DNA sequence containing the *cis*-element of interest is attached to a column and used to isolate the DNA binding protein of interest. The protein can then be washed and eluted off. A recent review described the identification of DNA binding proteins by Mass spectrometry (Nordhoff *et al.*, 1999). Here the immobilised DNA containing the *cis*-acting element of interest is incubated with an extract containing the protein. The protein is then analysed for a molecular weight by MALDI.

South western blotting is another popular technique for the identification of potential transcription factors. The technique involves screening an expression library with the DNA sequence of interest to identify the cDNA clone that encodes the binding protein. This technique is therefore often used in conjunction with the gel mobility shift assay, which identifies the DNA sequences to which proteins bind. This

sequence can then be used to screen the expression library to isolate the protein of interest. An example of where this technique has been used to successfully isolate plant DNA binding proteins is in parsley (Rushton *et al* 1996). The parsley PR1 gene encodes a pathogenesis related protein. Gel mobility shift analysis identified 3 cis-acting elements (W1-3) that bound trans-acting factors. Upon identification of these boxes the DNA binding proteins that specifically bound to these W boxes were identified by south-western screening. DNA probes containing these W boxes were used to screen a parsley expression library and three cDNA clones encoding proteins that bind to these W boxes were identified. The proteins (WRKY 1-3) were shown to be members of the WRKY transcription factor family.

1.2.1.6 Previous Studies on Transcription Factors

A number of transcription factors have been shown to exert controlling affects on genes. The overexpression of a single transcription factor has been shown to upregulate genes and in some cases to upregulate a whole pathway. Therefore by identifying the controlling factor for a pathway it can have a huge affect on the upregulation of the genes and important consequences for the plants properties. Recent work on transcription factors has shown to improve stress tolerance by the overexpression of a single stress-inducible transcription factor (Kasuga *et al.*, 1999). Previous work showed that the dehydration response element, a *cis*-acting promoter element, was an important regulatory feature in the role of plant tolerance to drought, salt and freezing stress. The expression of these tolerance genes was found to be under the control of DREB1A, a transcription factor that binds to the DRE. The overexpression of the DREB1A cDNA in transgenic plants was shown to increase stress tolerance in the plant. The overexpression of this one transcription factor upregulated a number of genes involved in stress tolerance and therefore if a potential regulator of the fatty acid genes could be identified then the overexpression of this factor may upregulate the genes involved in the fatty acid biosynthetic pathway.

Other examples of studies on transcription factors include the overexpression of the cDNA for SCOF1, a cold inducible zinc finger protein from soybean which was shown to increase cold tolerance in *Arabidopsis* (Kim *et al.*, 2001). The genetic modification of flavonoid biosynthesis in maize, by the ectopic expression of a MYB transcription factor (Grotewold *et al.*, 1998). The ectopic expression of one of the *Arabidopsis* MYB transcription factors (AtMYB23) was shown to give rise to ectopic trichomes (Kirik *et al.*, 2001). Finally the ectopic expression of an *Arabidopsis* seed specific transcription factor AB12 (involved in freezing tolerance) within vegetative tissues showed the accumulation of seed specific transcripts and also an enhanced freezing tolerance.

1.2.2 Transcriptional Regulation of the Fatty Acid Synthesis Genes

The control of gene expression by examination of the *cis*-elements and trans-acting factors is an area of fatty acid synthesis currently being examined to try to increase our understanding of the regulation of the pathway. The enzymes involved in fatty acid synthesis are encoded for by a variable number of genes, some by a single gene (*Arabidopsis* enoyl-ACP reductase) and others by a number of genes (*B.napus* enoyl-ACP reductase). It is thought that the regulation of the lipid biosynthesis may be at the level of transcription, and a number of the lipid biosynthetic genes have shown to appear to be co-coordinately regulated both temporally and spatially (Elborough *et al.*, 1994 and Fawcett *et al.*, 1994 & 2002).

Little is currently known about the transcription factors involved in regulating the plant lipid biosynthetic genes, however work has been carried out on other systems. For example in mammals and *Drosophila* the sterol regulating element-binding protein (SREBP) has been identified. These transcription factors have been shown to activate the lipid biosynthetic genes (Goldstein *et al.*, 2002) and be involved in the regulation of membrane lipids. The SREBP from *Drosophila* has been examined and shown to be under the control of feedback from the phospholipid, phosphatidylethanolamine (Dobrosotskaya *et al.*, 2002). However it is thought that

the mammalian SREBP is regulated by feedback from sterol. SREBP is thought to be activated by the cleavage of the SREBP by proteases within the Golgi, thereby producing basic helix-loop-helix-leucine-zipper domains (Goldstein *et al.*, 2002). This process requires a SCAP (SREBP cleavage-activating protein), which transports the protein to the Golgi. The SREBP proteins are thought to be involved in regulating membrane lipid synthesis, by monitoring the cell wall and adjusting the rate of lipid synthesis accordingly. A second transcription factor FadR from *E.coli* was shown to be involved in the regulation of the fatty acid biosynthetic genes (DiRusso *et al.*, 1998). FadR was shown to bind to a number of genes involved in FAS, repressing those involved in degradation, and activating those involved with fatty acid biosynthesis. The binding of this transcription factor was inhibited by long chain acyl-CoA thioesters, but not by free fatty acids. The results suggested that the FADR ligand binding was affected by both the length of the acyl chain and also the presence of double bonds

To investigate *cis*-acting elements involved in the regulation of genes, the promoter of the gene is examined. This often involves designing deletions of the promoter and fusing them to a GUS construct. Transgenic plants can then be examined for the effects of these deletions on expression, thereby identifying which regions of the promoter are important for expression. A number of studies have been carried out on the promoter regions of the genes involved in fatty acid synthesis. The 5' region of the *B.napus* ACP gene (ACP05) was fused to GUS and its expression examined (de Silva *et al.*, 1992). GUS activity was found to be seed specific and showed an expression pattern that correlated with lipid biosynthesis by increasing during the development of the seed. Maximum activity was apparent at the highest lipid synthesis stage and compared to leaves was on average 100 times higher. Deletion analysis of the *Arabidopsis* Ac11.2 promoter was studied to examine important *cis*-acting elements controlling the expression of the gene within different organs (Baerson *et al.*, 1994). Deletions of the promoter were fused to GUS and examined in transgenic tobacco. They found that a different region of the promoter appeared to be responsible for seed specific expression than that for roots and leaves. A region between -235 and -55

when deleted was shown to produce the greatest reduction in gene expression within seeds. Therefore a 180bp domain was shown to contain all the *cis*-acting information necessary for ACII.2 promoter activity in seeds.

Work by de Boer recently carried out on the *Arabidopsis* enoyl-ACP reductase promoter (de Boer *et al.*, 1999), provided information on important promoter elements involved in the regulation of transcription within different tissues. de Boer made GUS constructs of the enoyl-ACP reductase promoter using a region of 1470bp upstream of the translational start codon. Deletions were performed and GUS expression was examined in transgenic tobacco. The aim was to examine the promoter for sequences required for transcription within different tissues. The experiment revealed three important regions within the enoyl-ACP reductase promoter. Firstly the sequence between -329 and -201 upstream of the translational start codon was shown to be required for expression in young leaves. Root expression was still observed when deletions were made up to -19bp. The root expression was however, shown to be increased by the removal of the intron within the untranslated leader sequence. Lastly he concluded that deletions up to -47 of the transcription start site did not significantly affect activity in seeds. Therefore the elements essential for high seed expression of the enoyl reductase enzyme were thought to be located close to the transcription start site.

1.3 Objectives of the Study

Due to the economic importance of plant oils there is considerable amount of interest in the genetic modification of oil crops for use within industry. To date most of this genetic engineering has concentrated on the modification of the fatty acid content, thus modifying the properties of the oil (Murphy *et al.*, 1994, Knutzon *et al.*, 1992, Voelker *et al.*, 1992). However the oil content of the plant is of huge economical importance and therefore understanding the regulation of the fatty acid synthesis pathway is of critical importance. The pathway itself is well documented, however there is currently little knowledge of its regulation. There are two approaches to

examining the regulation of the fatty acid synthesis pathway, firstly much work has been carried out on the metabolic flux of the pathway and trying to identify the enzymes that may be rate limiting and in some way regulate flux through the pathway, for example by feedback inhibition (Page *et al.*, 1994, & Shintani and Ohlrogge, 1995). From these studies certain genes have been implicated to be involved in a regulatory capacity for example ACCase (Post-Beittenmiller 1991 & 1992). The second approach involves the examination of regulators of gene expression and identification of factors involved in the transcriptional control of the pathway. As previously mentioned, coordinate regulation of the genes is thought to occur, and therefore an overall regulator of the enzymes of fatty acid synthesis may exist. If this control can be identified then this would open the way for the production of transgenic plants with manipulated lipid content. Upregulation of a pathway of genes has been shown by the overexpression of one transcription factor (Kasuga *et al.*, 1999). Therefore there is great interest to find transcription factors that may be involved in the regulation of the genes in the fatty acid biosynthesis pathway.

If an overall control factor is shown to be present then it would be expected to bind to all of the genes that it is regulating and therefore a common *cis*-acting element should exist. This is however complicated by the fact that transcription factors may recognize and bind to slightly different sequences (Sakuma *et al.*, 2002) and therefore direct sequence comparisons of the promoter may not reveal the answer. Therefore work has been carried out on dissecting *cis*-elements important in transcriptional activation within the promoters of the fatty acid biosynthetic gene promoters, and certain regions important for differential expression of the gene have been identified (de Boer *et al.*, 1999 and Baerson *et al.*, 1994). Currently no transcription factors involved in the regulation of the plant fatty acid synthesis pathway have been identified, however more is known for other systems such as *E.coli* and *Drosophila* (DiRusso *et al.*, 1998 and Doborosotskaya *et al.*, 2002).

The aim of this study is to examine the *B.napus* β -Keto-ACP reductase 5' sequence for potential *cis*-acting elements involved in the regulation of this enzyme. It is hoped

that the identification of any potential regulatory elements within this promoter could then be used to isolate a potential regulatory trans-acting factor that binds to this sequence. To carry out this analysis, firstly it needs to be found whether the 5' sequence will act as the functional promoter, using stable transformations of the 5' sequence fused to the GUS reporter gene in *Arabidopsis*. This will also identify the expression pattern of the gene, as the aim is to identify elements involved in regulation of a seed expressed isoform. Upon identification of the 5' sequence functioning as the promoter the gel mobility shift assay will then be used to identify any potential *cis*-elements to which proteins bind. The use of a lipid and non-lipid biosynthetic gene promoter in competition gel mobility shift analysis will be employed to analyse the specificity of any complexes identified. The minimal promoter sequence to direct transcription will then be identified by construction of a deletion series of the promoter for transient expression experiments into *B.napus* tissues. Any potential *cis*-acting elements identified in this study could then be used to analyse and clone a potential trans acting factor that binds to the β -Keto-ACP reductase promoter. Therefore this analysis is aimed at increasing our knowledge of the factors that are involved in regulating the *B.napus* β -Keto-ACP reductase gene.

Chapter Two

Materials and Methods

The materials and methods used for this study are outlined below, however any that are specific for individual chapters are outlined within that chapter.

2.1 Materials

2.1.1 Chemicals and Reagents

All the chemicals used in this study were obtained from one of the following companies unless otherwise stated: Sigma Aldrich Ltd. (Poole, UK), ICN Pharmaceuticals Ltd. (Basingstoke, UK), Fisher Scientific (Loughborough, UK), Bio-Rad laboratories (Hemel Hempsted, UK), Fisons Scientific Equipment (Loughborough, UK) or Merck Ltd. (Poole, UK).

Intercept from Levington (Ipswich, UK), agarose from Boehringer Mannheim Biochemica UK Ltd. (Sussex, UK), X-Gluc and IPTG from Melford Laboratories (Suffolk, UK), dNTPs and X-Gal from Bioline (London, UK), Silwett (Lehle Seeds, Texas, USA) and pUC19 from GIBCO.

2.1.2 Molecular Biology Kits

The following molecular biology kits were used for this study: Qiagen mini and midi plasmid isolation kits, Hybaid and Sigma plasmid isolation kits, the TOPO-TA cloning kit from Invitrogen (Groningen, the Netherlands), DNA extraction kit from Qiagen, and the GFXTM PCR DNA and Gel band purification Kit from Amersham Pharmacia biotech (Buckinghamshire, UK), Colony/Plaque Hybridisation Transfer membrane from NEN Research Products, Du Pont (UK) Ltd, (Hertfordshire).

2.1.3 Oligonucleotides

Oligonucleotide primers used for PCR reactions were ordered from MWG-Biotech (Ebersberg, Germany). The primers were resuspended to a concentration of 100pmol/ μ l with ddH₂O and used at 10pmol/ μ l.

2.1.4 Enzymes

Restriction enzymes were obtained from Boerringer Mannheim Biochemica UK Ltd. (Sussex, UK), T4 DNA ligase and klenow from Promega (Southampton, UK), Biotline Taq DNA polymerase from Biotline (London, UK), Amplitaq gold from Roche and Shrimp Alkaline Phosphatase from Sigma-Aldrich (Poole, UK).

2.1.5 Radioactivity

Radioactivity was ordered from ICN Pharmaceuticals Ltd (Basingstoke, UK). The [³²P] α -dATP and [³²P] α -dCTP were provided at a concentration of 10mCi/ml and a specific activity of 3000Ci/mmol.

2.1.6 DNA Sequencing

All DNA sequencing was performed at the University of Durham's DNA sequencing unit, using an ABI 373 DNA sequencer and dye terminator labelling reactions (Perkin Elmer Applied Biosystems). The primers were supplied at a concentration of 3.2pmol/ μ l and the DNA supplied as a plasmid preparation using either the Sigma or Hybaid plasmid prep kits (as in the manufacturers instructions) at a concentration of 0.2 μ g/ μ l.

2.1.7 Bacterial Strains

The *E.coli* strains DH5- α and XL1-Blue were used as bacterial strains for cloning experiments, and were made chemically competent as in section 2.7.2. They were routinely grown at 37°C overnight. The *Agrobacterium* C58C3 used for the plant transformation experiments (Dale, *et al.*, 1989) was selected for by nalidixic acid and streptomycin and grown at 30°C for 48 hours. This strain has been modified to still contain the virulence factors for insertion of its T-DNA into the plants genome, but not to cause crown gall disease. Long-term storage of the all the bacterial strains was obtained by producing glycerol stocks, containing 0.5ml of the bacterial strain and 0.5ml of sterile 50% glycerol, and stored at -80°C until required.

2.1.8 Plasmids

The plasmids used for the cloning experiments and their antibiotic selection are shown below:

Plasmid	Characteristics	Resistance
pGEM-T Easy	3.01Kb <i>E.coli</i> cloning vector	Amp ^R
pCR 2.1 TOPO	3.9Kb <i>E.coli</i> cloning vector	Amp ^R
pUC19	2.7Kb <i>E.coli</i> cloning vector	Amp ^R
pBI101.1	GUS vector	Kan ^R

The vectors were obtained from the following companies: pGEM-Teasy from Promega, pCR 2.1 TOPO from Invitrogen, pUC19 from GIBCO and pBI101.1 was kindly donated by Dr J. Kroon from the University of Durham.

2.1.9 Antibiotics

The addition of antibiotics to liquid media or agar, for selective growth, was carried out after autoclaving. A stock solution of the antibiotic was prepared, filter sterilised and stored at -20°C until required. The table below outlines the antibiotics used and the final concentration required:

Antibiotic	Stock Concentration	Final Concentration
Ampicillin	50mg/ml	50 $\mu\text{g/ml}$
Streptomycin	100mg/ml	100 $\mu\text{g/ml}$
Kanamycin	50mg/ml	50 $\mu\text{g/ml}$
Nalidixic Acid	25mg/ml	25 $\mu\text{g/ml}$

Blue/white colony selection was used to identify positive clones containing the required insert for certain vectors. Thus allowing for selection due to the inactivation of the β -galactosidase gene, by insertion of DNA fragments into the multiple cloning sites of the plasmid. When Blue/White colony selection was required 40 μl of X-gal (20mg/ml) and 12 μl of IPTG (100mM) were included into each agar plate.

2.2 Bacterial Growth Media, Conditions and Procedures

2.2.1 Sterilisation Procedures

Sterile conditions were maintained throughout all experimental work, particularly that involving bacteria. Solutions unable to be autoclaved were filter sterilised into a sterile container, through a 0.22 μm nitro-cellulose filter. All other solutions and any glassware, or other equipment to be used in bacterial procedures, were autoclaved at 121°C , 15p.s.i for 20 minutes.

2.2.2 Growth Media

Bacterial growth media were sterilised by autoclaving at 121°C for 20 minutes before the addition of the appropriate antibiotic.

- **LB media:** 10g/l Bactotryptone, 5g/l Bacto-yeast extract, 10g/l NaCl and pH adjusted to pH7.5 with NaOH
- **LB agar:** for solid agar plates, 6g of agar was added to 400ml of LB media
- **2xYT Media:** 16g/l Bacto-Tryptone, 10g/l NaCl, 10g/l yeast extract and adjusted to pH7.5 with NaOH

2.2.3 Bacterial Growth Conditions

Sterile cocktail sticks were used to inoculate liquid cultures with single colonies. *E.coli* bacterial cultures were grown by incubating at 37°C overnight, with continuous shaking. Bacterial cultures were spread onto agar plates using a glass spreader, sterilised by immersing in 70% ethanol and flaming. Agar plates containing bacterial cultures were kept at 4°C for short term usage and glycerol stocks were maintained at -80°C as long term stocks.

2.3 Plant Material and Growth Conditions

2.3.1 Plant Material

The *Arabidopsis thaliana* Columbia variety were use for the stable transformations. The *B.napus* variety Jet neuf plants were used for genomic DNA isolation and supplied by Prof. Slabas at the University of Durham. The *B. napus* tissues used in the transient expression experiments were kindly supplied by Dr Wallington from Biogemma Ltd. (Cambridge Science Park).

2.3.2 Plant Culture Media

- **MS10:** 1 x Murashige and Skoog medium (Sigma #M55124), 3% sucrose, 0.05% MES, adjusted to pH5.7, 8% agar added and sterilised by autoclaving.

Kanamycin was added (50µg/ml) after sterilisation and for the T-1 plants augmentin was also added (200mg/l) to kill any *Agrobacterium*.

2.3.3 Plant Growth Conditions

Arabidopsis plants were grown for the transformation experiments in 4:1 soil to sand (to allow for drainage) in the greenhouse with 16 hour day lengths at 22°C. The soil was treated with intercept, an insecticide upon planting. The transgenic seeds were plated onto selective plates and vernalised for 4 days at 4°C in the dark. They were then transferred into the plant growth room and left to grow with 16 hours light a day at 25°C, and a photon flux of 50-150µmol/m²/s.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Genomic DNA Isolation

B.napus variety Jet neuf plants were grown from seed and young leaves removed for genomic DNA isolation. The genomic DNA was isolated using the DNA isolation kit from Qiagen as in the manufacturers instructions. This DNA was used in subsequent PCR reactions with addition of 100ng of DNA per reaction.

2.4.2 PCR Components

The table outlines the various components of the PCR reaction, the final concentration of some components were varied for optimal conditions depending on the PCR. Two DNA polymerases were used, the Bioline Taq was used for confirmation PCR and the Amplitaq gold for PCR of the β -Keto-ACP reductase fragments involved in the main experiments. The Bioline Taq DNA polymerase was supplied with a 10x reaction buffer (Mg^{++} free) and 50mM $MgCl_2$ solution. The Amplitaq gold was supplied at 5u/ μ l (diluted 1:5 and 1 μ l used per reaction), with a 10x reaction buffer. The $MgCl_2$ was supplied at 25mM and the concentration altered depending on the primers (3-6 μ l of the solution was generally used). The primers were made up to 10pmol/ μ l and 1 μ l used per 50 μ l reaction. The following table shows the typical concentrations of the components of the PCR reaction for both the Amplitaq gold DNA polymerase and the Bioline Taq DNA polymerase:

Component	Bioline Taq	Amplitaq Gold
10x reaction Buffer	5.0 μ l	5.0 μ l
$MgCl_2$ (50mM/25mM)	1.5 μ l	3-6 μ l
dNTPs (10mM)	1.0 μ l	1.0 μ l
Primer 1 (10pmol/ μ l)	1.0 μ l	1.0 μ l
Primer 2 (10pmol/ μ l)	1.0 μ l	1.0 μ l
Template DNA (100ng/ μ l)	1.0 μ l	1.0 μ l
DNA Polymerase	1.0 μ l (1:2)	1.0 μ l (1:5)
ddH ₂ O	made up to 50 μ l	made up to 50 μ l

Mineral oil was placed on the surface of the reaction mixture to reduce evaporation. The hot start method of PCR was used to commence the reaction. The DNA polymerase was added when the tubes were in the 94°C heating block. This method reduces the amount of non-specific binding of the primers. 5µl of the PCR reaction was then run out on an agarose gel alongside the appropriate markers to confirm the size of the product.

The cycles of a typical PCR reaction are shown below:

	1 cycle	30 cycles	1 cycle
Denature	95°C 5mins	95°C 30secs	72°C 5mins
Anneal		*65°C 30secs	
Extend		72°C **2mins	

*The Annealing temperature was varied according to the T_m of the primers used.

**The extension time was adjusted according to the length of the fragment in the PCR reaction, typically 1min/1Kb.

2.4.3 PCR Primers

Primers were designed from genomic DNA sequences usually between 20 and 30 nucleotides in length. Care was taken to design primers with equal numbers of A and T's, to G and C's, also the formation of primer dimers was taken into consideration and similar regions with which the primers may anneal together were avoided. The primers were usually designed with restriction sites for directional cloning into plasmids. The sequences of the primers are given within the specific chapters.

2.5 Agarose Gel Electrophoresis

2.5.1 Solutions:

1xTAE buffer: 40mM Tris-acetate pH8.0, 1mM EDTA

10x loading buffer: 0.25% (w/v) acridine orange, and 0.25% (w/v) Bromophenol blue.

2.5.2 Preparation of Agarose Gels

Gel electrophoresis was carried out in Pharmacia tanks. The agarose concentration of the gel varied according to the size of the fragment being separated, as shown below:

% (w/v) Agarose	DNA molecules (Kb)
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Gels were made by dissolving the appropriate amount of agarose in 1xTAE buffer and heating in a microwave. The solution was then cooled to approximately 60°C, and ethidium bromide added to a final concentration of 0.5µg/ml. The agarose was poured into a gel mould containing a comb and left to set. The comb was removed and the gel placed in a tank containing 1x TAE. DNA samples were loaded into the wells alongside appropriate markers. The gels were run at 100V until the bromophenol blue was two-thirds of the way down the gel. Visualisation of the DNA was achieved using a UV transilluminator.

2.5.3 DNA Molecular Weight Markers

The two main markers used in gel electrophoresis were lambda and Phi X174, prepared by digesting with *Hind* III and *Hae* III respectively. Typically 50µg of DNA was incubated with 50 units of the respective restriction enzymes and the appropriate buffer in 250µl total. The digest was left overnight at 37°C and then 50µl of 6x nucleic acid dye was added with 200µl of ddH₂O. The markers gave bands of the following sizes:

- **Lambda /*Hind*III:** 23130, 9416, 6557, 4361, 2322, 2027, 564bp.
- **Phi X174 / *Hae* III:** 1353, 1078, 872, 603, 310, 281,241bp.

2.5.4 Purification of DNA Fragments from Agarose Gels

Isolation of the DNA fragment was achieved by excising the band from the agarose gel using a sterile razor blade, and purifying the DNA fragment using the GFXTM PCR DNA and Gel band purification Kit, according to the manufacturer's instructions. The DNA was re-suspended in sterile ddH₂O. The PCR products were also be purified directly from solution using the GFXTM PCR DNA purification kit, again as in the manufacturers instructions.

2.6 Cloning of PCR Products

2.6.1 Digestions of DNA

The DNA to be cloned and the plasmid were digested with the appropriate restriction enzyme(s). Digestions were typically set up with 1µg DNA, 2µl of the appropriate restriction buffer, 1µl of enzyme 1 (10u/µl) and 1µl of enzyme 2 (10u/µl) if required, the reaction was then made up to 20µl and left for 1-4 hours at

37°C. The digests were then purified using the GFX™ PCR DNA purification kit as in the manufacturers instructions.

2.6.2 Dephosphorylation of Vector DNA

Upon digestion of plasmid with one restriction enzyme the religation of the vector was prevented by dephosphorylation of the 5' ends prior to the ligation reaction. This was carried out by using shrimp alkaline phosphatase. One unit of shrimp alkaline phosphatase was added to the digestion reaction mixture. After incubation at 37°C for 1 hour the phosphatase was inactivated by incubating at 70°C for 10 minutes. The digested vector was then purified from the agarose gel as detailed in section 2.5.4.

2.6.3 DNA Ligations

DNA fragments were ligated using T4 DNA ligase. The DNA ligase catalyses the formation of a covalent phosphodiester bond between a 5'-phosphoryl group and an adjacent 3'-hydroxyl group. Digested DNA was mixed in a molar ratio of 3:1 insert to vector (this varied depending on the ratio of the size of the vector to insert DNA), along with 0.1 volume of 10x ligase buffer (supplied with the ligase), 1 unit of DNA ligase and made up to 10µl with ddH₂O. The reaction mixture was left overnight at 14°C to incubate, and then transformed into competent *E.coli* cells.

2.6.4 Ligation of PCR Fragments into the TOPO 2.1 Vector

One of the vectors used for cloning of PCR products was the TOPO2.1 vector. The vector is pre-digested and contains 3' thymidine overhangs for efficient ligation. The enzyme ligation is the topoisomerase enzyme that is important in fast efficient ligation. Restriction sites are not required for this ligation and therefore the DNA does not need to be digested prior to the ligation. 1-2 μ l of the PCR product was added to 1 μ l of the pCR TOPO 2.1 vector and made up to 5 μ l in volume. The reaction was mixed, left for five minutes and then transformed into *E. coli* cells.

2.7 Transformation of *E. coli* Competent Cells

2.7.1 Solutions:

- **TfbI:** 30mM KOAc; 100mM RbCl, 10mM CaCl₂; 50mM MnCl₂, 15% (v/v) glycerol, pH to 5.8 with 0.2M acetic acid, filter sterilised and store in the dark
- **TfbII:** 10mM MOPS, 75mM RbCl; 15% (v/v) glycerol, pH to 6.5 with 1M KOH; filter sterilised and stored in dark

2.7.2 Production of Competent Cells

A 5ml culture of LB media (with appropriate antibiotic selection) was inoculated with a pure culture of the appropriate bacterial strain. The culture was grown overnight, with aeration, at 37°C. 1ml of the culture was transferred to 100ml of fresh LB media and grown at 37°C until an OD₅₅₀ of 0.5 was obtained. The cells were chilled for 5 minutes and then spun at 400g, for 5 minutes at 4°C. The cells were resuspended in 40ml ice-cold TfbI solution and placed on ice for 5 minutes. The cells were then harvested by centrifugation, resuspended in 4.0ml of TfbII

solution and left on ice for 15 minutes. Finally 100 μ l aliquots of the cell suspension were made, frozen in liquid nitrogen and stored at -80°C .

2.7.3 Transformation of *E.coli*

An aliquot of *E.coli* competent cells were thawed by hand, 1.7 μ l of β -mercaptoethanol (0.5M) added and the cells left on ice for 10 minutes. The ligation reaction was transferred into the tube of competent cells, and left for a further 30 minutes on ice. The cells were heat shocked for 30 seconds at 42°C , and placed on ice for 5 minutes, followed by the addition of 900 μ l of LB media. This was then left, with continuous shaking, at 37°C for 1 hour. The cells were then spread onto selective agar plates and left overnight at 37°C .

2.7.4 Isolation of Plasmid DNA

5ml of LB media, containing the appropriate antibiotic selection was inoculated with a single bacterial colony and grown overnight at 37°C . The cells were harvested by centrifugation in a micro centrifuge, and the plasmid DNA isolated using a Hybaid recovery plasmid Cosmo miniprep kit or Sigma plasmid miniprep kit, according to the manufacturer's instructions. When isolating plasmids from the *Agrobacterium* strain C58C3, either 50ml or 250ml flasks were inoculated and grown at 30°C for 48 hours and then either a Qiagen maxi or midi prep kit was carried out according to the manufacturers instructions.

2.8 Colony Hybridisation

2.8.1 Solutions:

- Denaturing solution: 0.5M NaOH, 1.5M NaCl.
- Neutralisation solution: 0.5M Tris-HCl pH8.0, 1.5M NaCl.

Upon cloning of the insert into the appropriate vector the positive colonies were identified by colony hybridisation. The colonies to be identified were streaked onto both an agar plate and also a sheet of 70mm Colony/Plaque Screen Hybridisation Transfer Membrane (NEN). One hundred colonies for each of the constructs were streaked out using a sterile toothpick and where labelled accordingly with pencil. The hybridisation membrane was placed colony side up the following solutions containing soaked Whatmann 3MM filter paper: denaturing solution for 3 minutes, neutralising solution for 3 minutes and finally washed in 2xSSC. The DNA was covalently linked to the hybridisation membrane by vacuum drying at 80°C for 2 hours.

2.8.2 Hybridisation

The hybridisation to the radioactively labelled probe was carried out according to the manufacturers instructions that accompanied the Colony/Plaque Screen Hybridisation Transfer Membrane (NEN). The pre-hybridisation stage blocks any non-specific binding sites to which the probe may bind. The hybridisation step involves incubation of the radioactively labelled probe with the membrane. Finally the membrane was washed in 2xSSC, 1xSSC and 0.1xSSC solutions. The membrane was removed, wrapped in Saran wrap and exposed.

2.9 *Arabidopsis* Plant Transformations

The dipping method as originally used by Clough and Bent (1998)

2.9.1 Solutions and Media

- 5% sucrose (w/v)/0.05% Silwett L-77 (v/v)
- MS10 agar plates: supplemented with 50µg/ml kanamycin sulphate

2.9.2 Preparation of *Arabidopsis*

Arabidopsis Columbia plants were grown in pots covered by plastic netting, containing on average 10 plants per pot. The plants were grown and upon the production of flowers the primary bolts were cut back to produce more secondary shoots. The plants were then grown until a number of immature unopened buds were formed. Prior to the dipping of the *Arabidopsis* any open buds were removed to leave only unopened transformable buds.

2.9.3 Preparation of Competent *Agrobacterium*

The *Agrobacterium* strain C58C3 was used as the bacterial strain for the introduction of the plasmid into the plant cells. This strain of *Agrobacterium* confers both nalidixic acid and streptomycin resistance. Therefore the cells were grown at 30°C for 48 hours in LB supplemented by 100mg/l streptomycin, and 25mg/l nalidixic acid. The *Agrobacterium* strain C58C3 were made chemically competent by the following procedure. The culture was grown until an OD at 600nm was in the region of 0.45. The cells were then chilled on ice for 30 minutes and centrifuged at 4000g for 10 minutes at 4°C and resuspended in 1 volume 10% sterile chilled glycerol. The cells were then centrifuged (4000g, 10 minutes at 4°C) and resuspended in 0.5 volumes of chilled sterile 10% glycerol. They were centrifuged and spun twice more the first time they were resuspended in 0.02

volumes of 10% glycerol and the finally in 0.01 volumes of 10% glycerol. Finally they were aliquoted out, snap frozen and stored at -80°C .

2.9.4 Transformation of the *Agrobacterium* by Electroporation

The *Agrobacterium* were transformed by electroporation. The cells were thawed and 0.5 μl of the DNA was added to 40 μl of competent *Agrobacterium*. This was then mixed and added to an electrocuvette. The cells were then electroporated for one pulse using:

- 2.5 Volts
- 25 Capacitance
- 400 Ω resistance

250 μl of the 2xYt media was then added to the cuvette and withdrawn into an eppendorf and made upto 1ml with 2xYt media. The cells were then left at 30°C with continuous shaking for 3 hours and plated onto selective plates and left to grow at 30°C for 2 days.

2.9.5 Transformation of the *Arabidopsis*

The *Agrobacterium* containing the plasmid of interest was grown in LB supplemented with 50mg/l kanamycin, 25mg/l nalidixic acid, and 100mg/l streptomycin at 30°C for 48 hours and centrifuged to pellet the *Agrobacterium*. The cells were resuspended in 1 litre of sucrose 5% and a final concentration of silwett L-77 of 0.05%. The plants were dipped into the sugar solution and agitated to coat all the inflorescences. The plants were then covered lightly in Saran wrap to produce a humid environment and left in the greenhouse under the bench to produce a shaded environment overnight. The *Arabidopsis* were re-dipped one week later as above except that the buds were not removed prior to dipping. The

plants were left to set seed, which were collected and left to dry out for 2 weeks at 25°C.

2.9.6 Seed Sterilisation and Selection of Positives

The *Arabidopsis* seed was sterilised by firstly washing in 70% ethanol for 1 minute. This was followed by two to three washes in detergent (20% with 0.05% Tween20) for 2 minutes. The *Arabidopsis* seeds were then rinsed to remove any traces of the detergent by washing with sterile water. This was usually carried out at least six times to remove any remaining detergent. The seed was then transferred onto sterile selective media containing kanamycin to select for positive transformants. They were left to vernalise in the dark at 4°C for 3-4 days and transferred to germinate in the growth room at 16 hour day length at 25°C. The positive plants that grew were transferred to soil and left to grow in the green house.

2.9.7 Alkaline Lysis PCR of Transformants

A leaf from the transformant was removed and placed into an eppendorf, and the lid pierced. The leaf was bruised slightly with the end of a tip upon the addition of 40µl of NaOH. The sample was then boiled for 30 seconds and kept on ice. Then 40µl of HCl and 20µl of detergent (0.5M Tris-HCl pH 8.0, and 0.25% Ipegal) was added and the sample re-boiled for two minutes and returned to ice. A small sample of the leaf (1-2mm) was placed into the PCR solution. The following PCR reaction was set up for each leaf using the Bioline Taq DNA polymerase:

Buffer	5µl
MgCl ₂ (50mM)	1.5µl
dNTPs (10mM)	1µl
Primer a (10pmol)	1µl
Primer b (10pmol)	1µl
Taq DNA polymerase	0.2µl
ddH ₂ O	Upto 50µl

The following conditions were used within the PCR reaction

	1 cycle	30 cycles	1 cycle
Denature	94°C 5mins	94°C 30secs	72°C 10mins
Anneal		58°C 45secs	
Extend		72°C 60secs	

2.10 Analysis of GUS Expression

(Stomp *et al.*, 1990)

2.10.1 Solutions:

- X-Gluc Buffer: 0.5mM potassium ferricyanide (K₃(Fe(CN)₆)), 0.5mM potassium ferrocyanide (K₄(Fe(CN)₆)), pH7.0, 100mM NaH₂PO₄, 10mM EDTA, 0.1% (v/v) Triton X-100.
- X-Gluc stock: 20mM X-Gluc (5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide) in N,N-dimethyl formamide, and stored at -20C until required.
- X-Gluc solution: a final concentration of 1mM X-Gluc was obtained by mixing 19 volumes of the X-Gluc buffer with 1 volume of the X-Gluc stock. This was used as the solution to analyse for GUS staining.

- Chloral hydrate solution: This was used to mount the embryos for visualisation and was prepared using 8g chloral hydrate, 3ml of water and 1ml of glycerol.

2.10.2 GUS Analysis of the *Arabidopsis* Seedlings

The *Arabidopsis* seedlings (8-14 days old) were incubated in the 1mM X-Gluc staining solution and left at 37°C overnight (however the GUS intensity was examined after 1 hour). The plants were then placed into 70% ethanol, with two washes to remove any chlorophyll for examination of the GUS staining. The seedlings were then placed onto slides and visualised using the Olympus SC35 type 12 camera and photographs taken using the Ektachrome 160 tungsten-balanced film from Kodak.

2.10.3 GUS Analysis of the *Arabidopsis* Embryos

Siliques from a range of developmental stages were obtained by taking siliques from the length of one stalk, therefore obtaining a range of ages the youngest being at the top. The siliques were secured to a dissection microscope by cellotape and were pierced down the length of the silique to reveal the embryos inside. The siliques were then placed into the X-Gluc staining solution and left to incubate under a vacuum for 5 minutes this was repeated and then the siliques were left in the GUS solution overnight. The embryos were then removed and cleared in 95% ethanol, and mounted onto slides in chloral hydrate solution for visualisation.

2.11 Gel Mobility Shift Analysis

2.11.1. Solutions:

- **R Buffer** - 150mM KCl, 50mM Tris pH 7.5, 0.5mM EDTA, 10% glycerol, 0.5mM DTT
- **R Buffer (-KCl)** - 50mM Tris pH 7.5, 0.5mM EDTA, 10% glycerol, 0.5mM DTT
- **10x GMSA Buffer:** 20mM Tris-HCl, pH 7.5-7.9, 50% glycerol, 10mM DTT, NaCl, 20mM EDTA

2.11.2 Preparation of Protein Extract

Embryos from stages two and three were harvested from *B. napus* and frozen in liquid nitrogen. The embryos were placed into a cooled mortar filled with liquid nitrogen and ground into a fine powder, whilst constantly kept frozen in liquid nitrogen. The ground tissue was then transferred into a 50ml falcon tube on ice. Two volumes of ice-cold R buffer were then added to the ground tissue, and homogenised for three 10 second periods, whilst on ice. The extract was passed through cheese cloth and centrifuged for 2 minutes at 5,000xg. The supernatant was disregarded and the pellet re-suspended in R Buffer (-KCl).

Q-sepharose beads were placed into a falcon tube and left to settle, the buffer was removed and replaced with 4mls of Buffer R (-KCl). The matrix was shaken and left to settle. The procedure of washing the matrix with R Buffer (-KCl) was repeated. The matrix was left overnight with the embryo extract on a shaker. A column was then assembled, and protein fractions were collected by passing increasing amounts of KCl buffer (50mM KCl, 100mM KCl, 150mM KCl, and 200mM KCl) through the column. The fraction containing the highest amounts of protein was identified using the Bradford assay and used in subsequent gel mobility shift assays.

2.11.3 Bradford Protein Assay

The Bio-Rad Protein Assay ascertained the fraction containing the highest concentration of proteins. The assay was carried out according to the manufacturer's instructions. The fraction containing the highest protein concentration was aliquoted out and stored at -80°C.

2.11.4 Preparation of Acrylamide Gels

Low ionic strength acrylamide gels were used in gel mobility shift assays to minimize the dissociation of protein-DNA complexes. The glass plates were thoroughly washed with distilled water and ethanol, and placed in a clamp separated by spacers. Gels were made to a concentration of 4% acrylamide in 1x TBE buffer. The gels were pre-electrophoresed at 75V for 30 minutes, prior to the addition of the sample.

4% acrylamide gel (100ml)

10ml	40% acrylamide
10ml	10x TBE buffer
6.7ml	2% Bis-acrylamide
100µl	TEMED
1.5ml	5%APS

The gels were run in mini-protean II gel tanks, manufactured by Bio-Rad.

2.11.5 3' Labelling of Probes

Probes were labelled to a high specific activity using the Klenow fragment of DNA polymerase I. The following reaction mixture was incubated at 37 °C for 30 minutes:

6µl of *Eco*R1 digested DNA fragment

1µl Klenow Buffer

1µl Klenow

1µl dTTP

1µl ³²P dATP

The reaction mixture was then made up to 40µl with ddH₂O, and purified using a Micro Biospin 6 chromatography column, according to the manufacturer's instructions. The probe was diluted to between 1:20 and 1:100, depending on the specific activity.

2.11.6 Ethanol Precipitation of DNA Probes

The DNA probes were excised from the gel and two volumes of TE buffer added to the sample. 100µl of 7.5M ammonium acetate was added and vortexed, followed by the addition of 600µl of 100% ethanol. The solution was placed at -20°C for a minimum of 1 hour, and the precipitated DNA pelleted using a micro centrifuge on high speed for 20 minutes. The supernatant was removed and the pelleted DNA left to dry, and resuspended in ddH₂O.

2.11.7 Gel Mobility Shift Reaction

The gel mobility shift assay consisted of buffer, competitor DNA, labelled DNA fragment and protein extract. The volume of the assay was kept to 10 μ l and consisted of 0.1-0.5ng of labelled DNA (approximately 5-10,000 cpm) and 5-10 μ g of protein. A titration of competitor DNA was carried out but usually in the region of 0.1 μ g to 2 μ g was added. The reaction mixture consisted of the following components.

1 μ l 10x binding buffer

0.1-2.0 μ l poly d(I-C)

0.5-1 μ l additional competitor DNA

1 μ protein extract

1 μ l probe

Made up to 10 μ l with ddH₂O

The gel mobility shift assays were carried out at 4°C, as certain DNA-Protein complexes are heat labile and breakdown under high temperatures. It also keeps the gel at a constant temperature to allow reproducibility. Firstly the 10x buffer, competitor DNA and ddH₂O were incubated for 10 minutes, after which the protein sample was added and left for 10 minutes. The diluted radioactive probe was then incubated with the reaction mixture on ice for 30 minutes. The reaction was loaded into the gel with 1 μ l of 6x loading buffer, and electrophoresed at 100V for 45–60 minutes for the small gels, and 3 hours at 120V for the large gels. The gel was then wrapped in cling film and placed in a lead cassette at –80°C with an autorad film, for between 1 day and one week depending on the specific activity of the radioactivity.

2.12 Transient Expression Experiments

2.12.1 Preparation of the Tissue

Ten day old *B.napus* plants were grown and for each construct 1 leaf and 1 section of root were placed onto an agar plate, alongside 50 embryos (30DAF). The constructs were cloned into the pUC19/GUS vector and midi plasmid preparations were carried out and the DNA made up to a final concentration of 1µg/µl.

2.12.2 Bombardment of the Plant Material

The particle bombardment experiments were carried out at Biogemma Ltd, Cambridge according to the specifications quoted in *Finer et al* 1992. The gold particles coated with the plasmid DNA were prepared in the following way: The gold particles were firstly washed, 30mg were taken and mixed in a sterile eppendorf with 500µl of 95% ethanol and left for 20 minutes. This was centrifuged at 14000rpm for 1 minute, washed four times in sterile water and finally re-suspended in 500µl of sterile 50% glycerol. 25µl of the re-suspended gold particles were added to 5µl of DNA (1µg/µl) and mixed, to which 25µl of 2.5M CaCl₂ was added and vortexed, followed immediately by 10µl of 100mM spermidine and vortexed well. The mixture was allowed to settle and 25µl of the supernatant was discarded. The mixture was then placed on ice for 5 minutes and then used immediately. For the bombardments, the particles were vortexed and 2µl added into the gun cartridge. The petri dish containing the *B.napus* tissue was located 20cm from the gun cartridge containing the DNA, and a baffle placed 12cm from the petri dish. The pressure was set to 80 psi. The door of the chamber was closed and the vacuum turned on until it reached 100kPa at which point the particles were released. Each of the tissues (roots, embryos and leaves) were bombarded individually. The tissues were then left at 25°C for 2 days and then analysed for GUS staining. The tissues were stored in 70% ethanol for visualisation.

Chapter Three

Transformations of the *B. napus* β -Keto-ACP Reductase 5' Sequence into *Arabidopsis*.

3.1 Introduction

3.1.1 *B. napus* β -Keto-ACP Reductase (β -Kr) Gene

The genes involved in fatty acid biosynthesis show a distinct expression pattern, both temporally and spatially, to accommodate for the different tissue requirements for lipid biosynthesis. This expression pattern could be due to a housekeeping gene that is differentially expressed, containing different *cis*-acting elements responsible for the temporal and spatial expression pattern. It could also be the result of a multi-gene family for an individual component of fatty acid biosynthesis, each of the genes being responsible for expression in different tissues. For example seed specific ACP isoforms from *B.napus* were identified that were part of a multigene family (de Silva *et al.*, 90). Within *B.napus*, four isoforms of both the β -Keto-ACP reductase (β -Kr) gene and the enoyl-ACP reductase (EnR) gene have been identified. They were found by screening a *B.napus* embryo cDNA library (Fraser *et al.*, in preparation). Upon identification of the four different isoforms, their sequence homology was examined. The EnR isoforms showed very strong sequence homology; their amino acid sequences were 95% homologous and their mature protein sequences were 97% homologous. The cDNAs of the four β -Kr isoforms share greater than 90% homology within the coding regions (Fawcett *et al.*, 2002). These homologies are shown in figure 3.1 (cDNA sequence alignments) and figure 3.2 (protein sequence alignments). The four isoforms of the β -Kr were divided into two groups according to their sequences; β -Kr1 and β -Kr4 (group 1) and β -Kr2 and β -Kr3 (group 2). The cDNAs of the four isoforms of β -Kr have been cloned from *B.napus* and their abundance estimated in plant tissues (Fraser *et al.*, in preparation). Quantitative RT-PCR analysis was carried out on *B.napus* embryo and leaf total RNA. The presence of unique restriction sites allowed the different β -Kr genes to be distinguished.

Figure 3.1 cDNA Alignments of the Four *B.napus* β -Kr Isoforms

The nucleotide sequences of the four β -Kr isoforms were aligned to examine the similarity between their cDNAs. The sequence comparisons show the homology between the four cDNA's, the main differences are observed within the most 5' region.

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Figure 3.2. Protein Sequence Alignments of the Four *B.napus* β -Kr Isoforms

Alignment of the four β -Kr protein sequences highlights the major similarities. The main differences between the protein sequences appearing within the 5' sequence, with the 3' sequence showing the major regions of homology.

1	MATT	-V	AATK	LTSL	KA	VK	-KL	G	F	R	E	I	R	Q	V	R	O	W	T	P	L	O	S	S	M	P	H	F	G	-	-	-	S	-	-	R	B.napus BKR1.pro														
1	MATT	-A	AATK	LTSL	KA	TAG	KL	G	Y	R	E	I	C	Q	V	R	O	W	S	P	L	O	S	A	M	P	H	F	G	M	L	R	C	-	-	R	B.napus BKR2.pro														
1	MATT	S	I	A	G	P	R	L	T	S	L	K	T	A	G	K	L	G	Y	R	E	I	C	H	V	R	O	W	A	P	V	H	S	F	M	P	H	F	G	M	L	R	C	G	S	R	B.napus BKR3.pro				
1	MATT	-V	AATK	LTSL	KA	TAG	KL	G	Y	R	E	I	C	Q	V	R	O	W	A	P	L	K	S	A	M	P	H	F	G	M	L	R	C	-	-	-	B.napus BKR4.pro														
44	Q	S	F	A	T	S	T	V	V	K	A	Q	A	T	A	V	E	Q	S	T	G	-	-	-	-	-	E	A	V	P	K	V	E	S	P	V	V	V	T	G	A	S	R	G	I	B.napus BKR1.pro					
48	Q	P	F	A	T	S	T	V	V	N	A	Q	A	Q	A	Q	A	T	A	T	G	V	E	Q	T	T	T	E	E	E	E	A	V	P	K	V	E	S	P	V	V	V	T	G	A	S	R	G	I	B.napus BKR2.pro	
51	R	P	F	A	T	S	S	S	T	V	V	Q	A	Q	A	Q	A	T	A	A	-	-	E	Q	S	T	G	E	E	E	E	A	V	P	K	V	E	S	P	V	V	V	T	G	A	S	R	G	I	B.napus BKR3.pro	
47	-	-	-	A	T	S	T	V	V	K	A	Q	A	Q	A	Q	A	T	T	-	-	E	Q	T	T	-	-	E	E	E	A	V	P	K	V	E	S	P	V	V	V	T	G	A	S	R	G	I	B.napus BKR4.pro		
85	G	K	A	I	A	L	S	L	G	K	A	G	C	K	V	L	V	N	Y	A	R	S	A	K	E	A	E	E	V	S	K	Q	I	E	A	Y	G	G	Q	A	I	T	F	G	G	D	V	S	K	E	B.napus BKR1.pro
98	G	K	A	I	A	L	S	L	G	K	A	G	C	K	V	L	V	N	Y	A	R	S	A	K	E	A	E	E	V	S	K	Q	I	E	E	Y	G	G	Q	A	I	T	F	G	G	D	V	S	K	E	B.napus BKR2.pro
98	G	K	A	I	A	L	S	L	G	K	A	G	C	K	V	L	V	N	Y	A	R	S	A	K	E	A	E	Q	V	S	K	Q	I	E	A	Y	G	G	Q	A	I	T	F	G	G	D	V	S	K	E	B.napus BKR3.pro
88	G	K	A	I	A	L	S	L	G	K	A	G	C	K	V	L	V	N	Y	A	R	S	A	K	E	A	E	E	V	S	K	Q	I	E	E	Y	G	G	E	A	I	T	F	G	G	D	V	S	K	E	B.napus BKR4.pro
135	A	D	V	E	A	M	M	K	T	A	I	D	A	W	G	T	I	D	V	V	N	N	A	G	I	T	R	D	T	L	L	I	R	M	K	K	S	Q	W	D	E	V	I	D	L	N	L	T	G	B.napus BKR1.pro	
148	A	D	V	D	A	M	M	K	T	A	V	D	K	W	G	T	I	D	V	V	N	N	A	G	-	-	-	D	T	L	L	L	I	R	M	K	K	S	Q	W	D	E	V	M	D	L	N	L	T	G	B.napus BKR2.pro
148	A	D	V	D	A	M	M	K	T	A	V	D	A	W	G	T	I	D	V	V	N	N	A	G	I	T	R	D	T	L	L	L	I	R	M	K	K	S	Q	W	D	E	V	I	D	L	N	L	T	G	B.napus BKR3.pro
138	A	D	V	D	S	M	M	K	T	A	V	D	K	W	G	T	I	D	V	V	N	N	A	G	I	T	R	D	T	L	L	L	I	R	M	K	K	S	Q	W	D	E	V	I	D	L	N	L	T	G	B.napus BKR4.pro
185	V	F	L	C	T	Q	A	A	T	K	I	M	M	K	K	R	K	G	R	I	I	N	I	A	S	V	V	G	L	I	G	N	I	G	Q	A	N	Y	A	A	A	K	A	G	V	I	G	F	S	K	B.napus BKR1.pro
195	V	F	L	C	S	Q	A	A	T	K	I	M	M	K	K	R	K	G	R	I	I	N	I	A	S	V	V	G	L	I	G	N	I	G	Q	A	N	Y	A	A	A	K	A	G	V	I	G	F	S	K	B.napus BKR2.pro
198	V	F	L	C	T	Q	A	A	T	K	I	M	M	K	K	R	K	G	R	I	I	N	I	A	S	V	V	G	L	I	G	N	I	G	Q	A	N	Y	A	A	A	K	A	G	V	I	G	F	S	K	B.napus BKR3.pro
188	V	F	L	C	T	Q	A	A	T	K	I	M	M	K	K	R	K	G	R	I	I	N	I	A	S	V	V	G	L	I	G	N	I	G	Q	A	N	Y	A	A	A	K	A	G	V	I	G	F	S	K	B.napus BKR4.pro
235	T	A	A	R	E	G	A	S	R	N	I	N	V	N	V	V	C	P	G	F	I	A	S	D	M	T	A	K	L	G	E	D	M	E	K	K	I	L	G	T	I	P	L	G	R	Y	G	Q	P	E	B.napus BKR1.pro
245	T	A	A	R	E	G	A	S	R	N	I	N	V	N	V	V	C	P	G	F	I	A	S	D	M	T	A	K	L	G	E	D	M	E	K	K	I	L	G	T	I	P	L	G	R	Y	G	Q	P	E	B.napus BKR2.pro
248	T	A	A	R	E	G	A	S	R	N	I	N	V	N	V	V	C	P	G	F	I	A	S	D	M	T	A	K	L	G	E	D	M	E	K	K	I	L	G	T	I	P	L	G	R	Y	G	Q	P	E	B.napus BKR3.pro
238	T	A	A	R	E	G	A	S	R	N	I	N	V	N	V	V	C	P	G	F	I	A	S	D	M	T	A	K	L	G	E	D	M	E	K	K	I	L	G	T	I	P	L	G	R	Y	G	Q	P	E	B.napus BKR4.pro
285	D	V	A	G	L	V	E	F	L	A	L	S	P	A	A	S	Y	I	T	G	Q	A	F	T	I	D	G	G	I	A	I																B.napus BKR1.pro				
295	D	V	A	G	L	V	E	F	L	A	L	S	P	A	A	S	Y	I	T	G	Q	A	F	T	I	D	G	G	I	A	I																B.napus BKR2.pro				
298	D	V	A	G	L	V	E	F	L	A	L	S	P	A	A	S	Y	I	T	G	Q	A	F	T	I	D	G	G	I	A	I																B.napus BKR3.pro				
288	D	V	A	G	L	V	E	F	L	A	L	S	P	A	A	S	Y	I	T	G	Q	A	F	T	I	D	G	G	I	A	I																B.napus BKR4.pro				

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

The products of the digests were analysed by densitometry, to quantify the amounts present. β -Kr1 was shown to be the most highly expressed isoform. Upon the identification of β -Kr1 as the most highly expressed of the four isoforms, the genomic clone was isolated and a full length sequence was obtained. This cDNA clone was termed β -Kr 10.1 and the genomic clone, PG1A (Fraser *et al.*, in preparation). The putative transcription start site was determined using the database entry (Accession number AJ243091) of the genomic sequence of the *Brassica napus* β -Kr. Figure 3.3 shows the position of this putative transcriptional start site within the β -Kr gene. The location of the transcription start and start codon define the 5' UTR of the mRNA. It is unclear what the functions of the four isoforms are. One idea is that they may be responsible for differential expression, both temporal and tissue specific. In contrast, *Arabidopsis* contains only one enoyl-ACP reductase gene, therefore the gene is expressed in all tissues requiring fatty acid biosynthesis and must be differentially expressed within different tissues. The *Arabidopsis* EnR promoter was shown to contain a number of *cis*-acting elements to differentially express the gene in different parts of the plant (de Boer *et al.*, 1999). Deletion analysis of the promoter showed that certain *cis*-acting elements were responsible for expression of the gene in leaves, embryos and roots and therefore the one gene was thought to be responsible for the temporal and spatial control of the *Arabidopsis* EnR gene. This may be in contrast to the β -Kr and EnR genes of *B.napus* as the individual isoforms may be responsible for differential expression. However the exact role of the different isoforms is still unclear.

3.1.2 *Agrobacterium* Mediated Plant Transformation

The introduction of new genes into plants is often carried out via the natural process adopted by *Agrobacterium tumefaciens*. This technique is widely used to engineer transgenic plants, by exploiting the natural process that occurs between plants and *Agrobacterium*, producing crown gall tumours. *Arabidopsis* is a species widely used in plant transformations and a number of different techniques have been developed for this species. The first method devised using this technique involved the

transformation of imbibed seeds (Feldmann and Marks, 1987). Other methods include transforming flowering *Arabidopsis* by vacuum infiltration (Bechtold *et al.*, 1993) and transformation following removal of the plants inflorescence to induce wounding (Chang *et al.*, 1994). The gametes or zygotes are thought to be the targets leading to the best transformation (Feldmann, 1991, Bechtold *et al.*, 1993)

3.1.3 Aims

In this chapter, the 2Kb sequence was isolated upstream of the putative translational start site from the *B.napus* β -Kr1 genomic clone (PG1A). The consensus sequence was determined and this was used to devise a deletion series. The constructs were then fused to the β -Glucoronidase (GUS) gene, and transformed into *Arabidopsis* plants in order to determine if the 2Kb sequence would act as the fully functional promoter. The effect of the deletion series could then be analysed to see which part of the promoter was important for expression, that is, which part contained the necessary sequence for transcription to occur. Examination of the transgenic *Arabidopsis* plants transformed with the GUS gene under the control of this promoter would also allow the expression pattern of the β -Kr1 gene to be analysed.

3.2 Methods

All the standard molecular biology techniques used in this study are described in Chapter 2 (Materials and Methods). Any conditions and primers specific for this chapter are outlined below:

Table 3.1 – β -Kr Fragment Primers

Primer	Primer Sequence
Pr1	5' TGCTCTAGAGTCATACAAATTACCATCAAATC 3'
Pr2	5' TGCTCTAGAGATGACGTGGCAACTTCGGAC 3'
Pr3	5'TGCTCTAGACACATCCCTCTTCTGTATCGC 3'
Pr4	5' GGAGAAGGGGATCCGAGCTAGTG 3'
Pr5	5' AATGAACAGCATAAAGACAACC3'

Table 3.2 – GUS Primers

Primer	Primer Sequence
Pr6 (nested 1)	5' CGTAAGTCAGACCTAGCG 3'
Pr7	5' ATCTCGAGCTTCATGACGACCAAA 3'
Pr8	5' ATGTCGACCAGGAAGTGATGGAGCA 3'

Table 3.3 – Sequencing Primers

Primer	Primer Sequence
Pr9 (forward)	5' TTGTAAAACGACGGCCAGTG 3'
Pr10 (reverse)	5' CACACAGGAAACAGCTATGACC 3'

Table 3.4 - PCR Conditions

	1 cycle	30 cycles	1 cycle
Denature	95°C 5mins	95°C - 30secs	72°C 5mins
Anneal	-	*54°C - 30secs	-
Extend	-	72°C - 90secs	-

*Annealing temperature – PCR of β -Kr fragments, 54°C (Pr1-4), PCR using GUS primers, 58°C (Pr7 & 8), PCR using Nested 1 GUS primer (Pr6) in conjunction with β -Kr primers, 56°C (Pr1-3).

3.3 Results

This section describes: the amplification of the 2Kb 5' sequence from the *B.napus* β -Kr1 gene to obtain a consensus sequence; the design of a deletion series; and the cloning of these fragments into the GUS construct to allow the analysis of GUS expression in the transgenic *Arabidopsis*.

3.3.1 Consensus Sequence Data

A consensus sequence for the β -Kr1 5' sequence was determined to confirm that it was consistent with the previously sequenced genomic clone PG1A (Fraser *et al.*, in preparation). This also eliminated errors incurred by PCR based amplification techniques. A DNA extraction from the leaves of *B.napus* Jet neuf plants was carried out using the sigma genomic isolation kit (as in the manufacturers instructions). The 2Kb 5' sequence upstream of the putative translational start site was amplified from this genomic DNA and from the PG1A genomic clone (Pr1 and Pr4 – Table 3.1). The first PCR was carried out on genomic *B.napus* DNA, and the DNA of the 10 PCR's were pooled and sequenced. The second set of PCRs was performed on the original genomic clone of β -Kr1 (PG1A). The amplified product was cloned into the TOPO 2.1 vector (Invitrogen) and sequenced. The sequence data was compiled and a consensus sequence obtained which was consistent with the original sequence identified for the genomic clone PG1A. This sequence shown in figure 3.3 was taken as the consensus and used in this study to construct a deletion series of the β -Kr1 5' sequence.

Figure 3.3 Sequence Data for the 5' Region of the β -Kr1 Gene

The consensus sequence for the 2Kb region upstream of the translational start codon, ATG (boxed) is shown. The 4 primers (Pr1-4) used to PCR the three promoter fragments (KrI-KrIII), are shown in colour. The primers contained either Xba I (Pr1-Pr3) or BamH I (Pr4) restriction sites for directional cloning. These fragments were used to make the β -Kr/GUS constructs to transform into *Arabidopsis*.

3.3.2 Design of the Deletion Series

A deletion series of the β -Kr1 5' sequence was devised to identify whether the 500bp 5' sequence could function as the promoter. The putative translational start codon was identified from the genomic clone of the most abundant β -Kr isoform β -Kr1 (PG1A). The 5' region upstream of the presumptive translational start site was taken as the putative promoter and analysed for introns by comparison of the *Brassica napus* β -Kr genomic and full length cDNA sequences. No intron appeared to be present in the 5' region, unlike the *Arabidopsis* enoyl-ACP reductase gene, where a 468bp intron was identified. Primers were designed to amplify a deletion series originating 2Kb upstream of the coding sequence (Pr1-4), producing three differently sized fragments - 2Kb (KrI), 1Kb (KrII), and 0.5Kb (KrIII), actual sizes - 1917bp, 998bp, 536bp respectively. The primers contained either Xba 1 (5') or BamH 1 (3') restriction sites for directional cloning into the GUS vector. A schematic representation of the primer sites is shown below in figure 3.4, and the 5' sequence of the β -Kr1 gene and the primers devised to amplify the deletion series can be seen in figure 3.3.

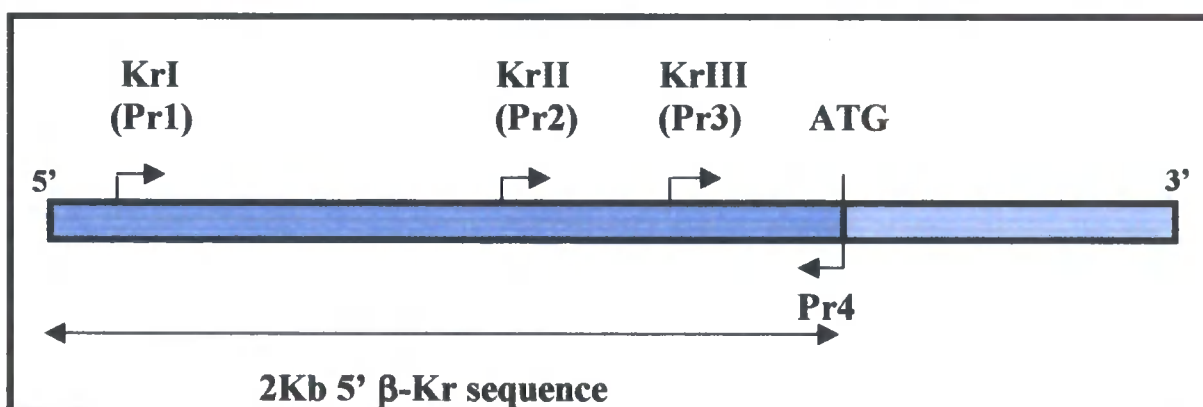


Figure 3.4 5' Region of the β -Kr Gene

Schematic representation of the 5' sequence of the β -Kr1 gene. The position of the primers (Pr1-4) used to amplify the fragments (β -KrI, β -KrII and β -KrIII) in relation to the putative translational start codon are shown.

3.3.3 Sub-Cloning of the β -Kr Fragments into TOPO 2.1

The fragments Kr I, II and III were sub-cloned into the TOPO 2.1 vector (Invitrogen) to allow confirmation of the correct sequence. The three β -Kr fragments obtained by PCR (figure 3.5(A)) were gel purified, cloned into the TOPO 2.1 vector and grown on selective ampicillin, X-gal and IPTG plates. Plasmids were obtained using the sigma plasmid DNA isolation kit (as in the manufacturers instructions) and positive colonies were identified by restriction digestions with Xba 1 and BamH 1 (figure 3.5 (B)). The cloned fragments were confirmed by sequencing in the TOPO 2.1 vector (Primers - Pr9 and Pr10).

3.3.4 Cloning of the β -Kr Fragments into the GUS Cassette.

Following the sub-cloning of the fragments into TOPO 2.1 and confirmation of the correct sequences, the fragments were cloned into the GUS vector ready for the transformations into *Arabidopsis*. The GUS cassette used for this analysis was pBI101.1; the map of this vector is shown in figure 3.6. pBI101.1 is a promoterless GUS cassette that was originally derived from the binary plasmid vector pBIN19.

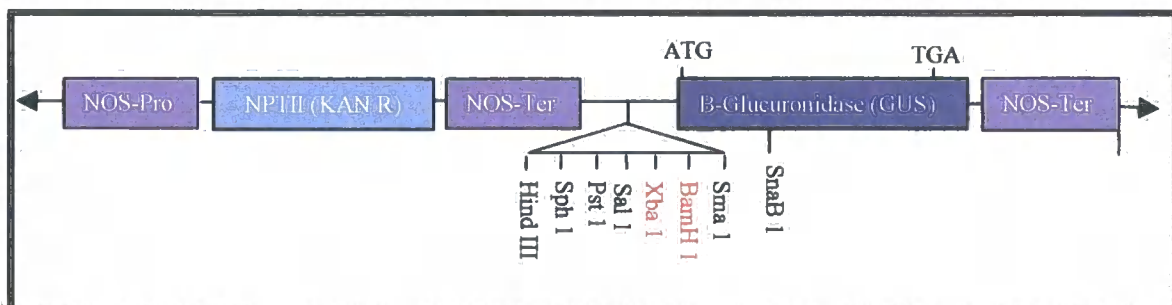
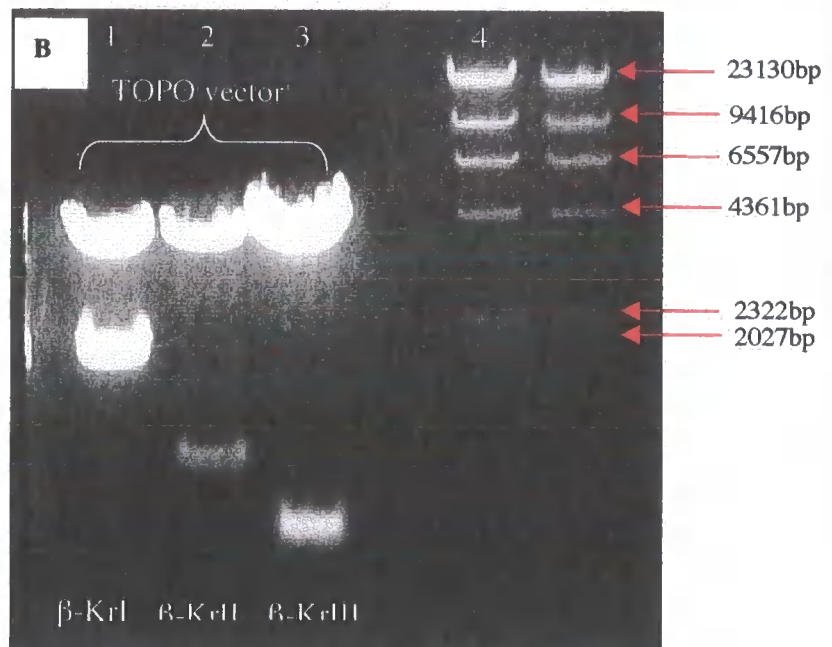
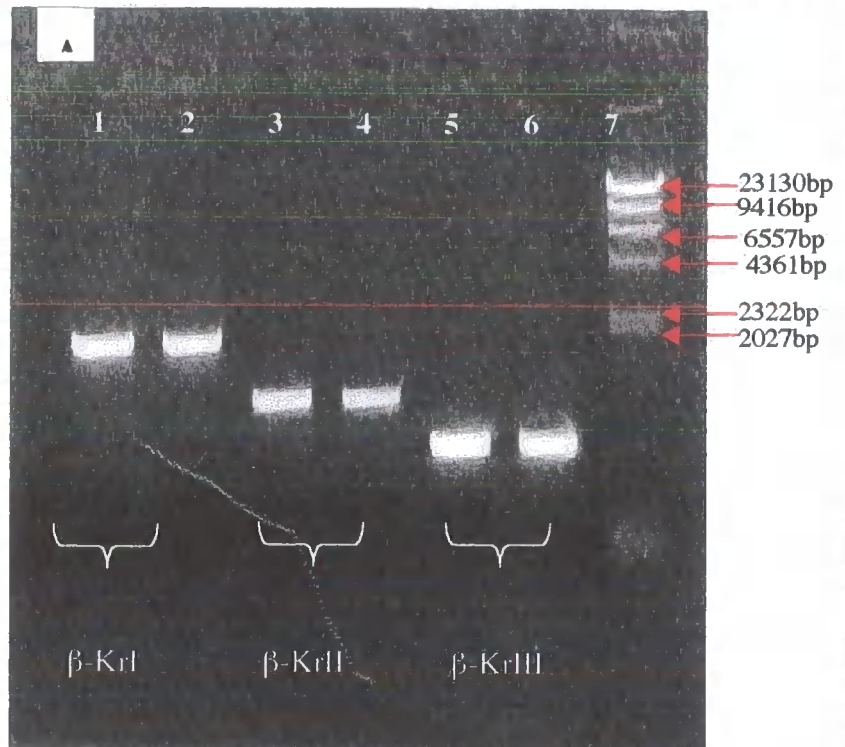


Figure 3.6 Map of the pBI101.1 GUS Cassette

Restriction sites Xba 1 and BamH 1 (highlighted in red) are positioned within the multiple cloning region. The GUS cassette is downstream from these restriction sites. The plasmid confers kanamycin resistance in both plants and bacteria

Figure 3.5 Gel Analysis of the β -Kr Fragments (KrI-KrII)

- A) Gel electrophoresis was carried out on the amplified β -Kr PCR products to confirm the correct sizes (2Kb, 1Kb, and 0.5Kb). The lanes contained: **1&2)** KrI (2Kb), **3&4)** KrII (1Kb), **5&6)** KrIII (0.5Kb), **7)** Molecular size marker – λ x *Hin* D III.
- B) The three β -Kr fragments were cloned into the TOPO 2.1 vector. Positive colonies were identified by restriction digests, using Xba I and BamH I to confirm that the correct insert was present. The lanes contained: **1)** KrI (2Kb), **2)** KrII (1Kb), **3)** KrIII (0.5Kb), **4)** Molecular size marker – λ x *Hin* D III.



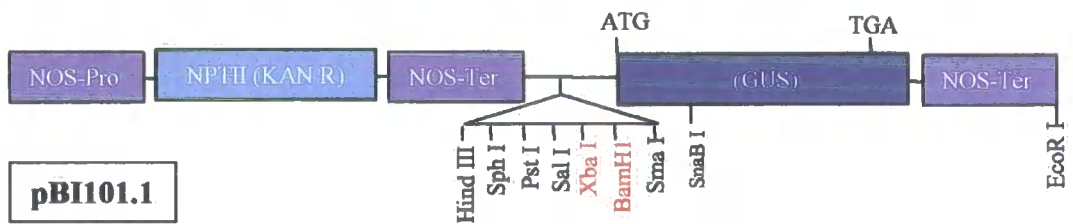
The fragments were obtained for cloning into the GUS vector, by digestion out of the TOPO 2.1 vector with Xba 1 and BamH 1 restriction enzymes. They were then isolated from a gel and purified. pBI101.1 was also digested with the restriction enzymes Xba 1 and BamH 1 and gel purified. Figure 3.7 outlines the cloning of the promoter fragments into the pBI101.1 vector. The β -Kr fragments were ligated into the pBI101.1 vector, using Xba 1 and BamH 1 restriction sites and transformed into XL-1 blue competent cells. They were grown on selective kanamycin plates, using blue/white selection, and one hundred colonies obtained for each fragment were probed to identify colonies containing the correct insert.

3.3.5 Identification of Positive Colonies Containing β -Kr/GUS Constructs

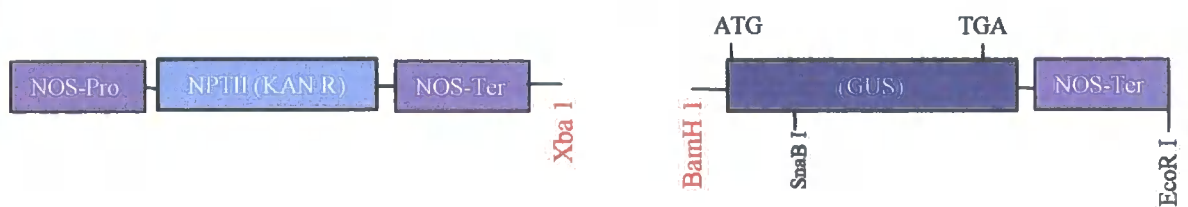
Positive colonies containing the transformed β -Kr/GUS constructs were identified using KrIII as a radioactive probe (section 2.8). Figure 3.8 shows the autoradiographs and indicates the positive colonies for the three different fragments. The difference in the number of positive colonies between the constructs can be seen, with KrI having the most efficient ligation and the KrIII having the least. Restriction analysis was carried out on the plasmid preparations obtained from the positive colonies identified, using Xba 1 and BamH 1 restriction enzymes (figure 3.9 (A)). All of the positive colonies identified (except one of the KrIII colonies) appeared to contain the insert of the correct size. The positive colonies were then sequenced using the Nested 1 primer (Pr6) and β -Kr primer Pr5. Figure 3.9 (B) shows the position of the Nested 1 primer within the GUS sequence (99bp downstream). This primer was used to check the 3' β -Kr/GUS overlap and primer Pr5 within the β -Kr 5' region was used to sequence the 5' β -Kr/GUS overlap, to confirm that the inserts had been cloned correctly. The sequence data confirmed that, from the colonies analysed, the β -Kr fragments had been correctly inserted in the right orientation into pBI101.1. Therefore the constructs were ready to be transformed into *Agrobacterium* for transformation into *Arabidopsis*.

Figure 3.7 Cloning of the β -Kr Fragments into the GUS Vector.

Diagrammatic representation outlining the cloning of the promoter fragments into pBI101.1. The pBI101.1 and the β -Kr fragments were digested with Xba 1 and BamH 1 (shown in red in the multiple cloning site of the plasmid) and gel purified. The plasmid and insert were then ligated together and transformed into X11-blue competent cells.



pBI101.1 and promoter fragment
cut with Xba I and BamH I



+

**Promoter
Fragment**



pBI101.1 and promoter fragment
ligated together

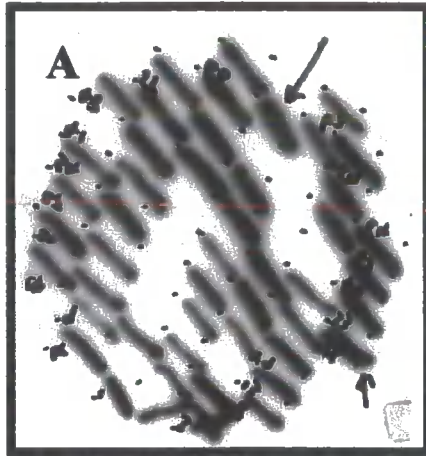


Transformed into *Arabidopsis*

Figure 3.8 Autographs to Show the Identification of Positive Colonies Containing the β -Kr/GUS Constructs

The KrlII fragment was radioactively labelled and used as a probe to identify positive colonies from the transformed competent cells. The difference in the number of positive transformants between the three constructs is apparent. The greatest number of transformants was obtained for the Krl insert, whilst the KrlII insert shows far fewer positive transformants.

- A) Krl -- 48 positive colonies
- B) KrlII -- 37 positive colonies
- C) KrlII -- 9 positive colonies

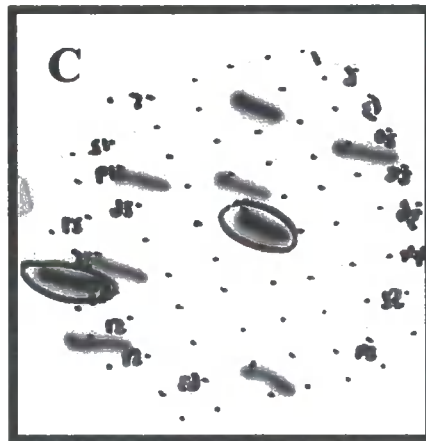


KrI

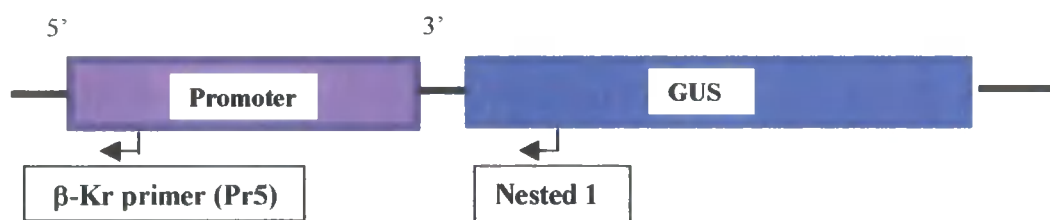
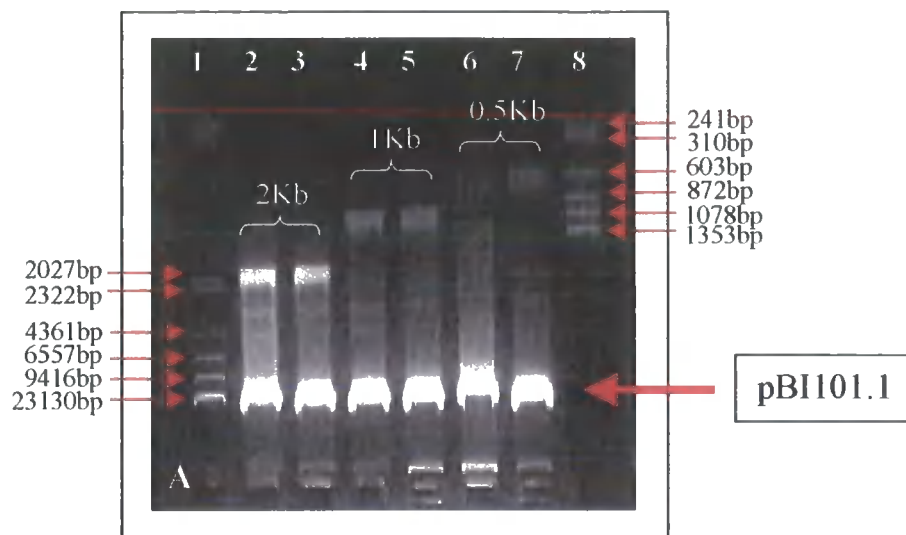


KrII

Positive Colony



KrIII



Nested 1 sequence: 3' CGTAAGTCAGACCTAGCG 5'

Figure 3.9 Confirmation of the β -Kr Inserts within pBI101.1

A) Restriction Analysis

The positive colonies identified were digested with Xba 1 and BamH 1 restriction enzymes to confirm that the correct β -Kr insert was present in the pBI101.1. The lanes contained the following: **2&3**) Krl (2Kb), **4&5**) KrII (1Kb), **6&7**) KrIII (0.5Kb), **1&8**) Molecular size markers – λ x *Hin* D III and ϕ X x *Hae* III respectively.

B) Position of the Nested 1 Primer within the GUS Gene.

The Nested 1 primer (Pr6) was used to sequence over the 3' β -Kr/GUS overlap to confirm that the fragments had been cloned correctly. The β -Kr Pr5 was used to sequence the 5' overlap. Therefore the positive colonies were confirmed to contain the β -Kr sequence correctly inserted into pBI101.1.

3.3.6 Transformation of β -Kr/GUS Constructs into *Agrobacterium*

Upon cloning the β -Kr fragments into pBI101.1, the constructs were transformed into *Agrobacterium*. The *Agrobacterium* strain C58C3, which confers streptomycin and nalidixic acid resistance, was used for the transformation with the pBI101.1 plasmid. The competent *Agrobacterium* (section 2.9.3) were transformed with the three β -Kr/GUS constructs (KrI, KrII and KrIII) and the empty pBI101.1 construct (used as a negative control) by electroporation (section 2.9.4). The transformed C58C3 cells were plated onto LB selective plates containing kanamycin, nalidixic acid and streptomycin. Positive colonies were confirmed by PCR analysis using a combination of primers. Two GUS internal primers (Pr7 and Pr8) were used to confirm that the negative control pBI101.1 construct was present. The GUS primer Pr8 was also used in combination with the β -Kr primers (Pr1-3), to confirm that the β -Kr/GUS construct was present in the transformed *Agrobacterium* (figure 3.10 and figure 3.11)

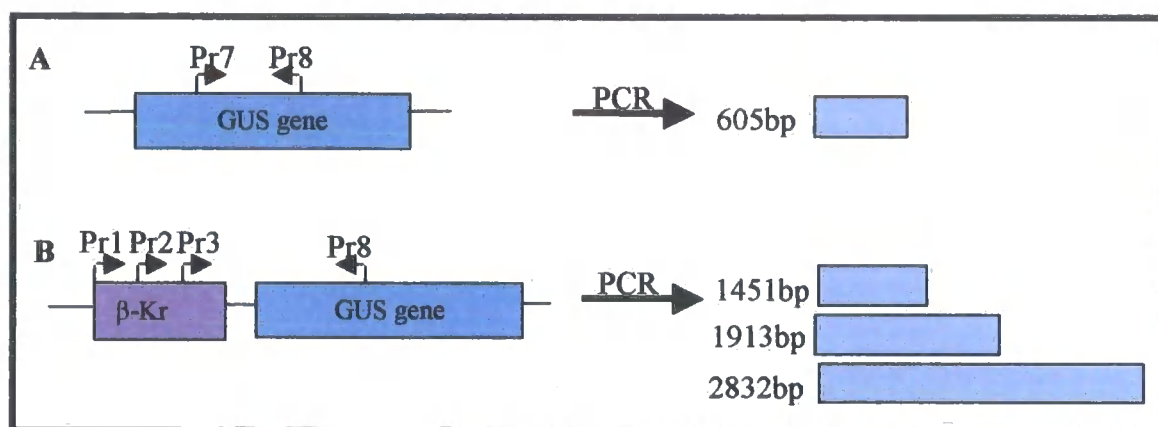


Figure 3.10 PCR Analysis on the Transformed *Agrobacterium*

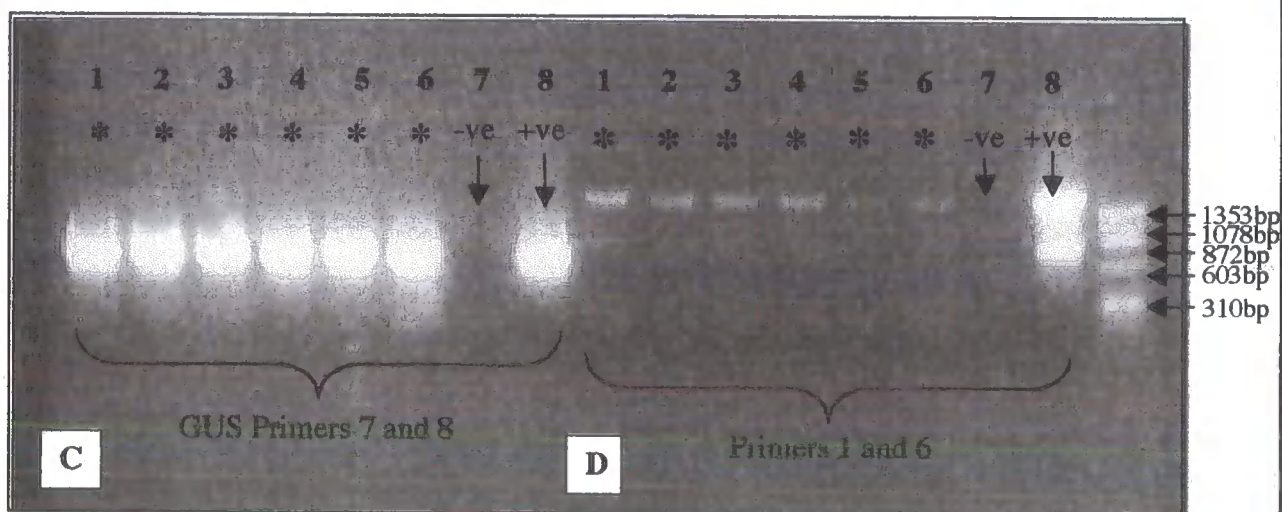
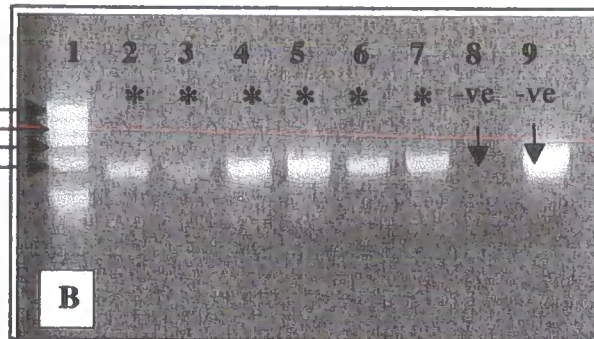
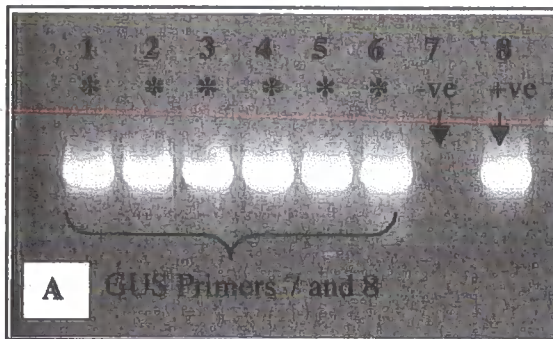
PCR analysis was carried out on the transformed *Agrobacterium* to identify positive colonies containing the β -Kr/GUS construct. (A) The GUS primers Pr7 and Pr8 were used to confirm the GUS construct was present. (B) The β -Kr primers (Pr 1-3) were used in combination with the GUS primer Pr8 to confirm that the correct β -Kr/GUS construct was present.



Figure 3.11 PCR Analysis on the Transformed *Agrobacterium* (C58C3) Containing the β -Kr/GUS Constructs

Positive colonies were identified by PCR analysis (lanes contain different *Agrobacterium* colonies isolated for PCR analysis). The colonies containing the correct construct are represented by *. A positive control (+ve) was performed alongside the PCR analysis using the original constructs in *E.coli* as the template, and a negative control containing no template (-ve).

- A) pBI101.1 construct - the GUS primers (Pr7 and Pr8) were used to confirm the presence of the pBI101.1
- B) KrIII construct – the primers (Pr3 and Pr4) used to PCR the original KrIII fragment were used to confirm that the KrIII fragment was present.
- C) KrIII construct – the pBI101.1 construct was shown to be present using the GUS primers (Pr7 and Pr8).
- D) KrIII construct – the GUS primer – Pr8 was used in conjunction with Pr3 to confirm that the KrIII /GUS construct was present.



* = Positive Colonies
-ve = Negative Control
+ve = Positive Control

3.3.7 Transformation of *Arabidopsis*: The Dipping Method

The positive *Agrobacterium* identified by colony PCR were used to transform *Arabidopsis* plants. The floral dip method of transformation was used in this study (Clough and Bennet, 1998) (section 2.9). To optimise the number of transformants obtained, the plants were trimmed prior to the transformation, and any open flowers were removed to leave only buds, as opened flowers would not be transformed.

The transformed *Agrobacterium* containing the four GUS constructs (three β -Kr/GUS constructs and one negative control – pBI101.1 with no insert) were grown up in selective media containing kanamycin, nalidixic acid and streptomycin. The cells were then re-suspended in a sucrose media into which the *Arabidopsis* were dipped. The plants were re-dipped without being trimmed one week later and left to grow. The plants were left to set seed, which was collected at regular intervals. The first collection of seed was the most important, as this batch originated from the buds that were dipped and so these would be expected to contain the highest number of transformants. Later seed would come from buds produced after the dipping experiments. A further transformation was carried out two weeks later in a repeat experiment, therefore 8 pots of *Arabidopsis* were transformed for each of the four constructs.

3.3.8 Selection of Positive Transformants

The seeds from the transformed *Arabidopsis* plants were grown up and positive transformants selected. The seeds were sterilised (section 2.9.6) and plated onto kanamycin plates containing augmentin (to kill any *Agrobacterium*), vernalised and left to grow (as in section 2.3.3). The untransformed plants gave a bleached appearance and were unable to survive, as they were kanamycin sensitive, whereas the kanamycin resistant transformants grew as normal green seedlings. The positive plants were removed from the kanamycin plates onto non-selective MS10 plates, thus allowing them to recover before being planted into soil and grown up to produce the

T-1 plants. PCR analysis, using the alkaline lysis method on leaves was used to confirm that these plants were true positives and did contain the construct (section 2.9.7). Primers were used to check that the leaves contained both the GUS construct and the correct β -Kr insert (figure 3.12). The positive plants were then left to grow and produce siliques (some of which were used for embryo histochemical analysis). The seeds of the T-1 plants were grown under selective conditions to identify the positive T-2 plants, on which the histochemical analysis was carried out.

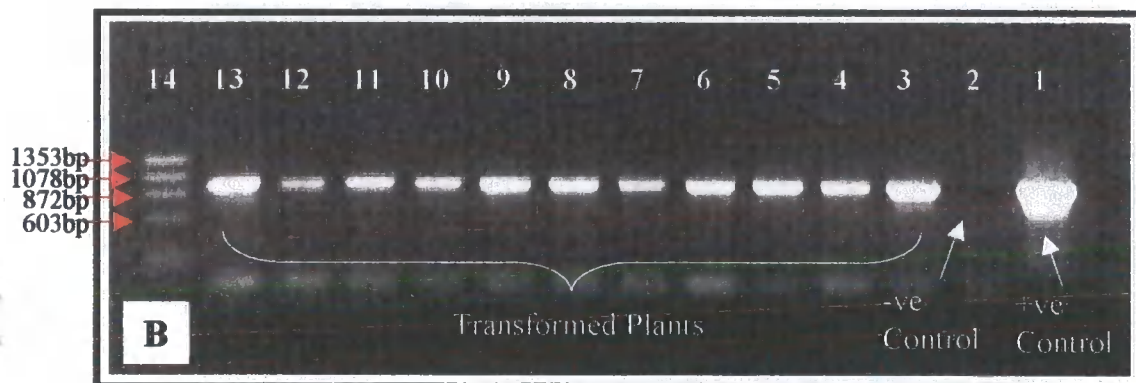
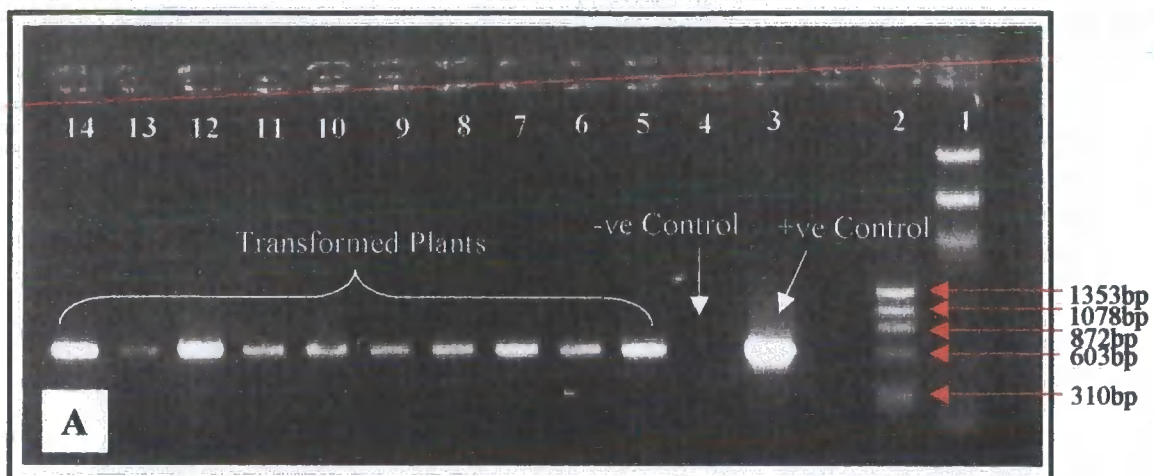
3.3.9 Transformation Efficiency

The average number of positive transformants expected for the floral dipping method is usually 1 in 100 (Clough *et al.*, 1998). However upon examination of the plates it was observed that a higher percentage of the plants appeared to be kanamycin resistant. The results of the transformations are shown in table 3.5, which gives the number of positive transformants obtained. The reason for the high number of transformants may be due to a combination of factors within the methodology to try and achieve the highest number of positive transformants. These include trimming any open buds off the plants before dipping, re-dipping the *Arabidopsis* one week later, and collection of the first seed to grow up to the T-1 plants. An example of one of the plates containing positive transformants can be seen in figure 3.13, which shows the high number of positive plants. Plate Ai is the selective plate, showing the kanamycin sensitive plants (bleached), unable to survive on the selective plates and the kanamycin resistant plants that are able to survive (green). Plate Aii shows the positive transformants plated out onto non-selective plates to allow them to recover before being transferred into soil.

Figure 3.12 PCR Analysis on the Kanamycin Resistant Plants

The kanamycin resistance plants were analysed by PCR for the β -Kr/GUS construct. A leaf was removed from the plant and alkaline lysis PCR analysis carried out. The GUS primers (Pr7 and Pr8) were used in conjunction with the β -Kr primers (Pr1-3) to allow confirmation.

- A) β -KrII construct – the GUS primers (Pr7 and Pr8) were used to confirm the presence of the GUS cassette. Lanes contain; **1&2)** molecular size markers, **3)** positive control using the original β -Kr/GUS construct in *E.coli*, **4)** negative control – with no template DNA, **5-14)** kanamycin resistant plants to confirm the presence of the β -Kr/GUS construct, using the GUS primers (Pr7 and Pr8)
- B) β -KrII construct – the β -Kr primers (Pr2 and Pr4) were used to perform PCR analysis to confirm that the β -KrII construct was present. Lanes contain; **1)** positive control – β -Kr/GUS construct in *E.coli*, **2)** negative control – no template DNA, **3-13)** kanamycin resistant plants – to confirm the presence of the correct β -Kr construct, using Pr2 and Pr4 **14)** molecular size marker.



Construct	No Transformants	Percentage (Average)
pBI101.1	11	4.4
	11	4.4
	20	8
	12	4.8
	13	5.2
KrI	16	6.4
	14	5.6
	11	4.4
	20	8
	6	2.4
	3	1.2
KrII	9	3.6
	11	4.4
	10	4
	4	1.6
	8	3.2
	9	3.6

Table 3.5 Percentage of Transformants Obtained for Constructs KrI, KrII and the Negative Control

The high number of positive transformants obtained for the different constructs is shown and a percentage has been worked out based on an average plate containing 250 seeds.

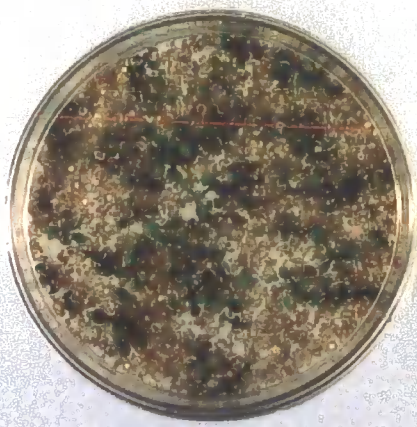
Figure 3.13 Selective Plates showing Positive Transformants

Ai) Kanamycin plate containing transformed and untransformed plants. The transformed plants were kanamycin resistance and therefore able to survive on the selective plates (green plants). Whereas those plants that did not contain the GUS construct were kanamycin sensitive and therefore unable to grow (bleached). The plants were 9 days old, and grown on 50 μ l/mg kanamycin.

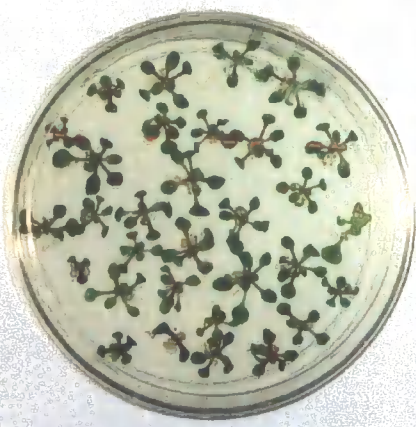
Aii) Non-selective plate – transformants from plate A were transferred onto normal MS10 plates with no kanamycin, to recover before being planted into soil.

B) Closer examination of plate Ai showing the positive green transformants that were kanamycin resistant and therefore able to survive and the untransformed, kanamycin sensitive plants that were unable to survive on the selective plates.

A



(i)



(ii)



B

3.3.10 Histochemical GUS Examination

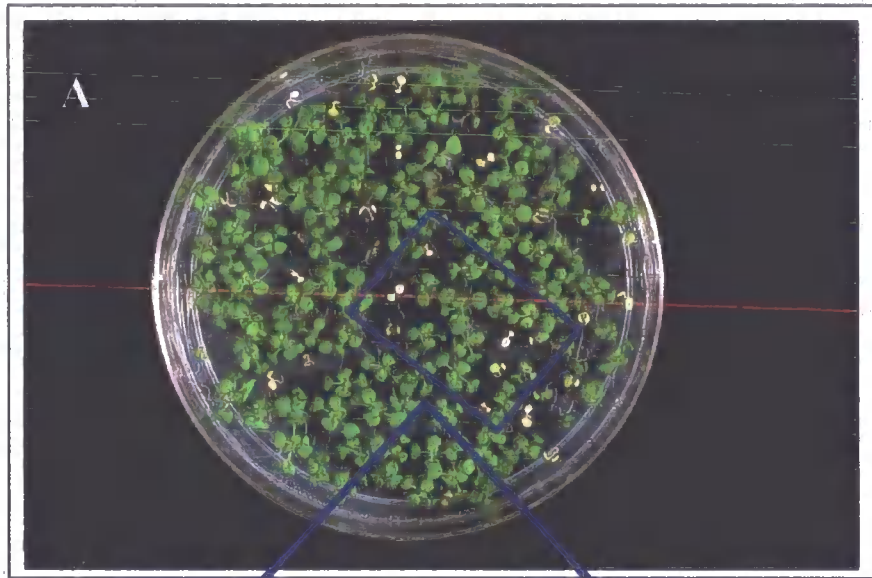
The T-2 seedlings were GUS stained for histochemical examination. Upon the production of siliques, the primary T1 transformants were left to dry out. The seed was collected and grown on selective plates containing kanamycin (section 2.9.6) to allow for the identification of positive plants (figure 3.14). These T-2 transformants were transferred to soil, apart from 20 of the plants for each construct, which were selected as small seedlings for histochemical GUS examination (8-10 days old). The seedlings were incubated in GUS buffer overnight, and were observed after one hour for the development of GUS staining. They were then left to clear in ethanol and visualised (section 2.10).

3.3.11 Differences in GUS Expression between Constructs

The plants transformed with only the pBI101.1 construct used as a negative control showed no GUS expression in any of the staining techniques carried out. This was expected as the GUS construct contained only the empty vector with no β -Kr insert. Positive transformants were obtained for both KrI and KrII. However no positive transformants were obtained for the β -KrIII construct, all these plants appeared to have no kanamycin resistance as all were bleached in appearance. One explanation for this may be due to a problem within the kanamycin resistant gene. The pBI101.1 plasmid contains a eukaryotic and prokaryotic kanamycin resistance gene; one for resistance in plants and the other one for bacteria. There appears to be no problem with the bacterial resistance gene, as the *Agrobacterium* grew with kanamycin selection, but there appears to be no kanamycin resistance within the plant. The other explanation could be due to a problem within the T-DNA borders required for insertion into the plant's genome. Therefore results were only obtained for the KrI and KrII constructs. Upon examination of GUS staining within the plants, there appeared to be no apparent difference in the intensity of staining between the two constructs.

**Figure 3.14 Selective Plates Containing T-2 Transformants
(Construct KrII)**

- A) Selective kanamycin plate showing the identification of positive T-2 plants. The kanamycin sensitive segregants (bleached) can clearly be seen within the kanamycin positive segregants. These T-2 plants identified were used for subsequent histochemical analysis. The plants were 10 days old and grown on 50µg/ml kanamycin selective plates.
- B) Closer examination of the selective plate, clearly showing the positive kanamycin resistant segregants, compared to kanamycin sensitive segregants that were therefore unable to survive.



Kanamycin Sensitive
Segregant

Kanamycin Resistant
Segregant

3.3.12 GUS Staining within the T-2 *Arabidopsis* Seedlings

The T-2 plants were stained for GUS expression and visualised. The seedlings were left to stain overnight, however some GUS staining was apparent after one hour. A distinct pattern of staining within the seedlings was observed, as shown in figure 3.15. GUS expression was observed within the roots and cotyledons but not the true leaves.

3.3.13 GUS Expression within Roots

Upon closer examination of the roots (figure 3.16) a distinctive pattern of GUS expression was observed. GUS expression appears to be absent in the root cap and meristem, but staining appears to begin in the region of elongation and intensifies above this region. The youngest part of the root shows strong homogeneous staining throughout the different tissues. However in the older regions of the root, the expression appears to diminish and be restricted to the vascular tissue and the endodermis, with little or no staining in the epidermis and cortex (figure 3.17). Figure 3.18 shows a schematic representation of the arrangement of tissues within the older regions of the root showing the pattern of GUS staining. The root hairs within the youngest parts of the root appear to show staining by the region of elongation (figure 3.17), however this appears to diminish higher up in the older part of the root. The staining pattern therefore seems to suggest that the main area of staining is within the youngest parts of the root, starting in the area of elongation, and that the GUS expression becomes more restricted within the different tissues higher up the root with age.

Figure 3.15 GUS Expression within the *Arabidopsis* Seedlings

- A) Negative control – as expected the seedling shows no positive GUS expression in any of the tissues. This control contains only the empty GUS vector pBI101.1 with no β -Kr insert.
- B) KrrII construct (overnight incubation) – the pattern of GUS staining within the seedling can be seen. The main areas of staining are within the roots and cotyledons, with no staining apparent in the true leaves.

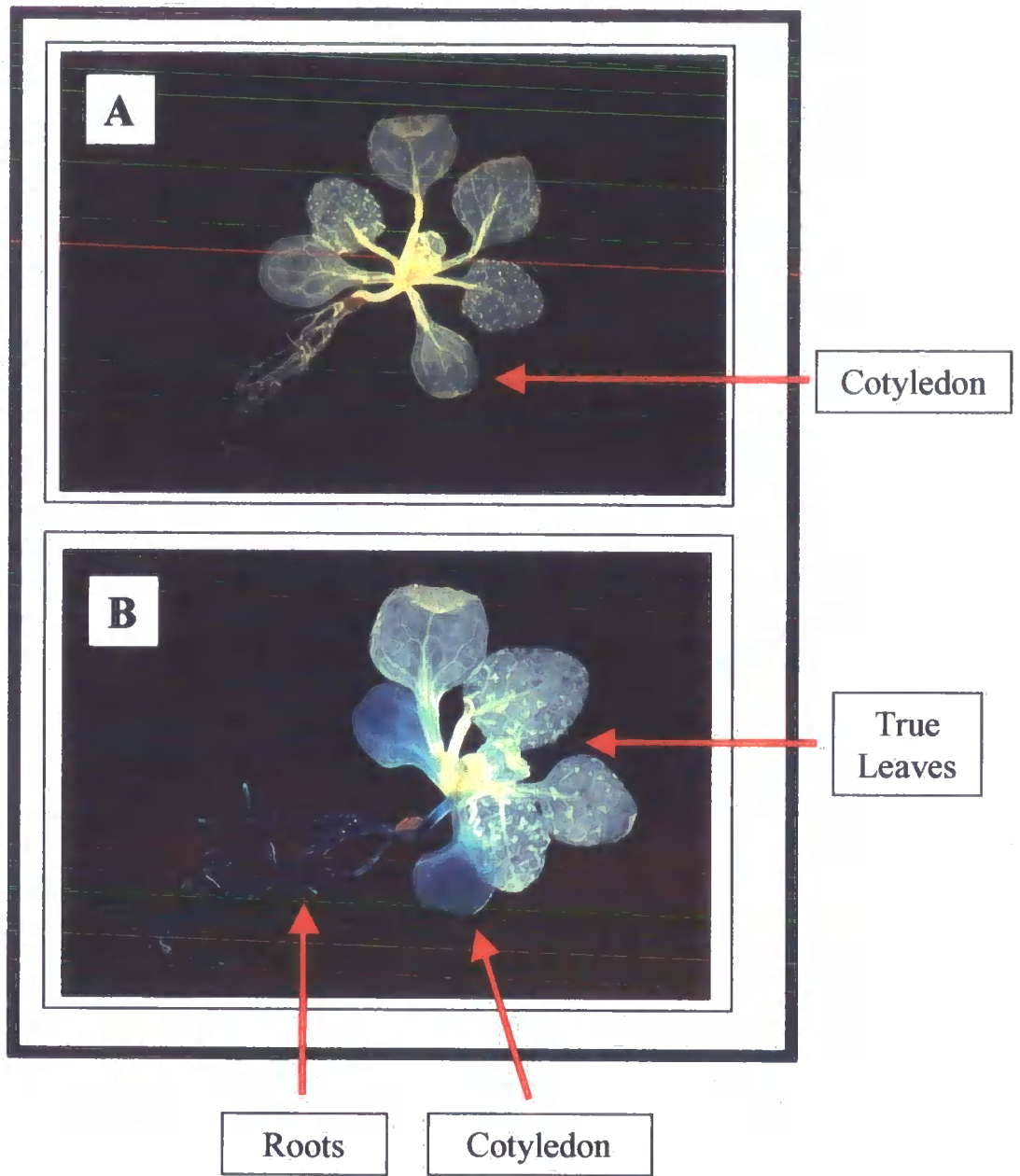
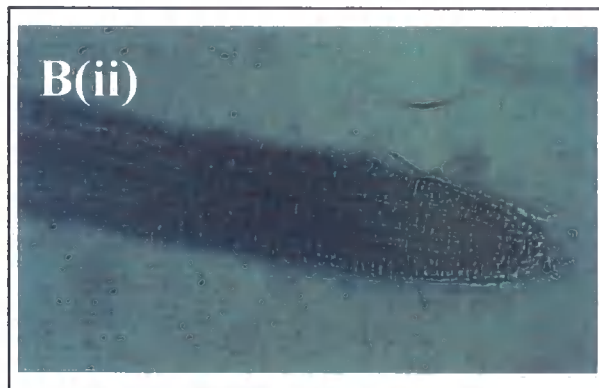
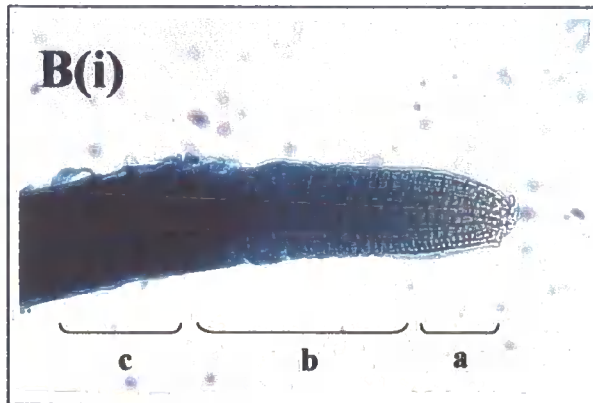
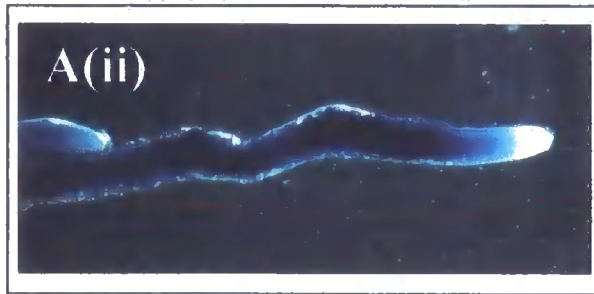
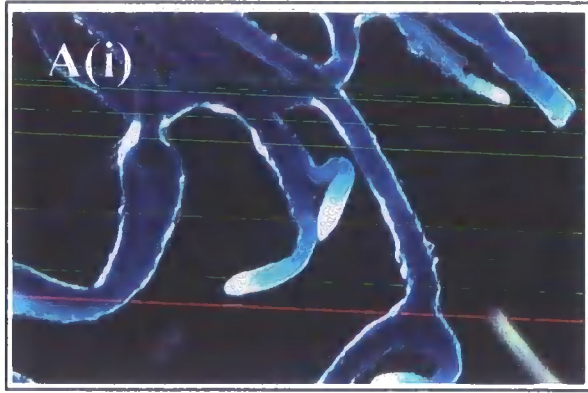


Figure 3.16 GUS Expression within the Root (Construct KrII)

- A) Examination of the roots showed staining in the majority of the root. However there was a distinct region within the root tip itself that appeared to show no GUS expression. Both Ai & Aii clearly show the lack of staining within the root tip.
- B) Higher magnification of the root cap shows the region up to the meristematic area appears to exhibit no GUS expression. However above the meristematic region in the area of elongation, the staining becomes apparent (B(i) & B(ii)).
- Bi) a) Root cap and meristematic region
 b) Region of elongation
 c) Differentiated root



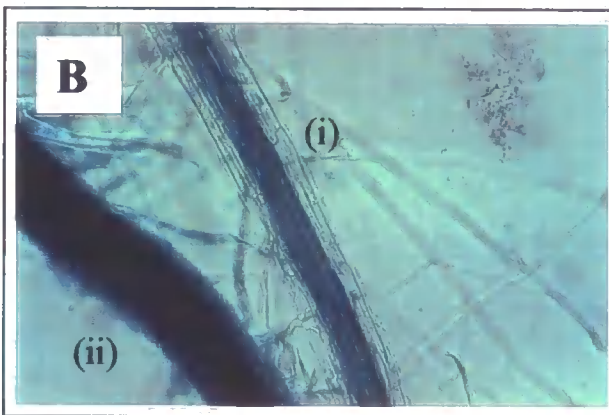
**Figure 3.17 Examination of GUS Expression within the Roots
(Construct KrII)**

- A) The older region of the root appears to show a more restricted expression pattern. The GUS staining appears to be more restricted to the inner tissues of the root, with the strongest staining in the endodermis and vascular tissue, and less staining in the epidermis and cortex. The root hairs appear to be devoid of any staining higher up the root.
- B) The older root (i); showing a restricted staining pattern, can be visualised alongside the younger part of the root (ii); showing homogenous staining throughout the tissues.
- C) The root hairs within the younger part of the root appear to show GUS staining but this staining seems to diminish in the older parts of the roots.



Root Hair

Vascular Cortex
and Endodermis



Root Hairs

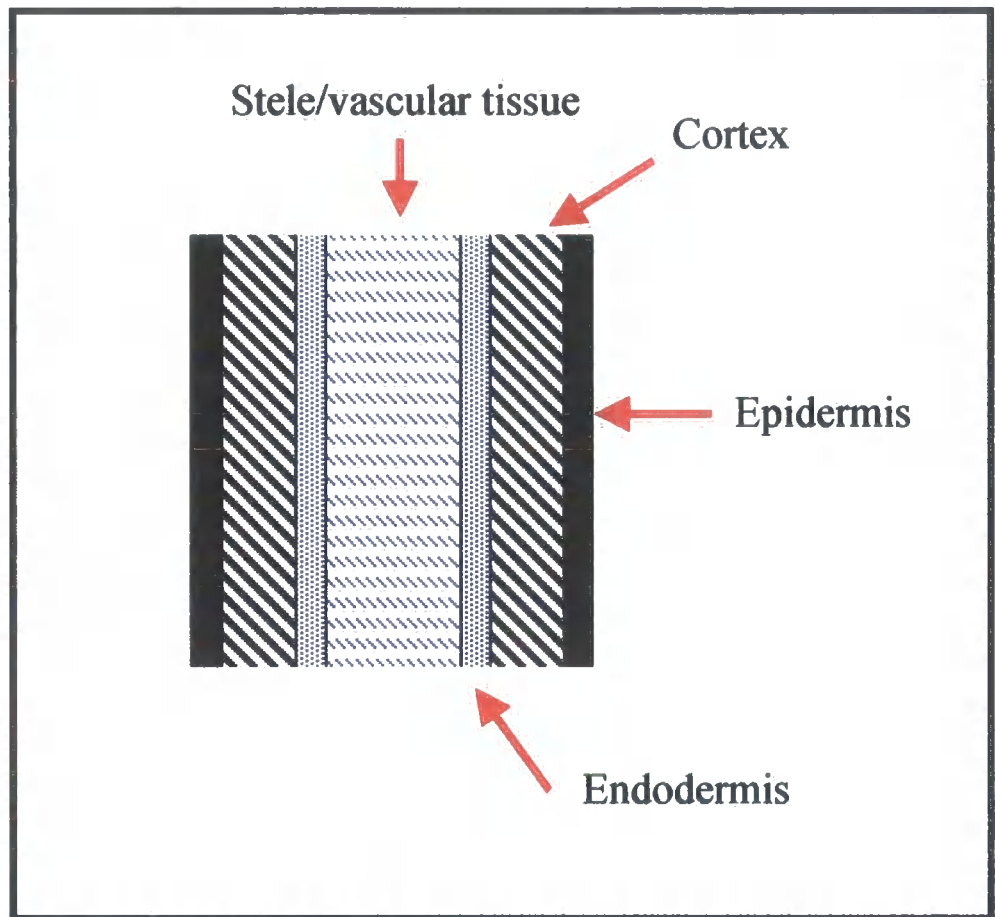


Figure 3.18 Tissues of the *Arabidopsis* Root (Older Region)

Schematic representation of the older regions of the *Arabidopsis* root, showing GUS expression within the tissues. The main regions of GUS staining observed are shown in blue (vascular and endodermis), whereas the regions devoid of any staining are represented in black (epidermis and cortex). Within the younger part of the root there is homogenous staining throughout the root (not shown).

3.3.14 GUS Expression within Leaves

There appears to be definite pattern of staining in the leaves of the *Arabidopsis* seedlings, with no obvious staining observed within the true leaves, but noticeable GUS expression within the cotyledons of the seedlings (figure 3.19). It is the cotyledons that are contained within the seed and will be present when the embryo is laying down lipid.

3.3.15 Histochemical Analysis of Embryos

Siliques were removed from the length of a single branch of the T-1 plants for each construct. A range of developmental stages were required for this study, to examine if there was any pattern in the expression of this gene through embryo development. The siliques at the top of the branch would be the youngest, gradually increasing in age to the bottom. The siliques were split down both sides to uncover the embryos inside and were placed into the GUS buffer under a vacuum. They were then left to stain overnight, cleared in ethanol and mounted in chloral hydrate for visualisation (section 2.10.3).

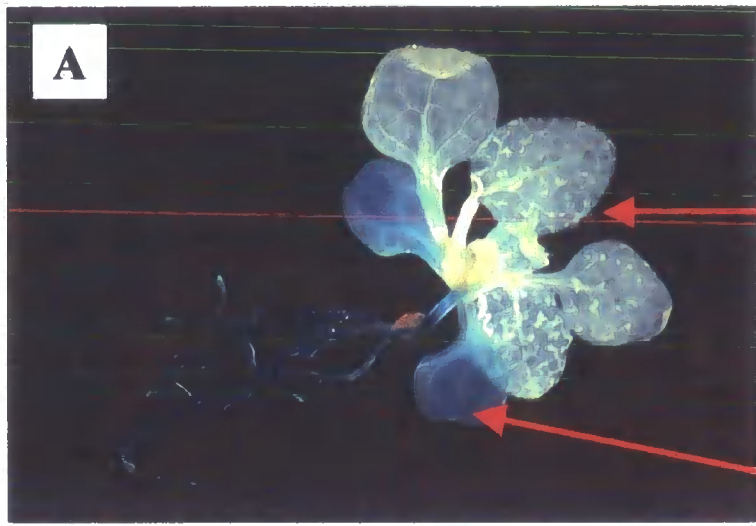
3.3.15.1 GUS Staining within Embryos

Figure 3.20(A) shows the negative control embryos within the siliques, showing no GUS expression as would be expected. Figure 3.20(B) shows a silique from construct KrII clearly showing the GUS positive segregants and GUS negative segregants within the silique. The segregation ratio of roughly 3:1 (positive to negative) can be seen, as would be expected. Different stages of embryo development were also examined for GUS expression (figure 3.21). All stages appeared to exhibit GUS expression, and the staining suggested that there may be some increase in staining throughout development (figure 3.21).

Figure 3.19 GUS Expression within Leaves (Construct KrII)

- A) Staining of the whole *Arabidopsis* seedling – there is a distinctive pattern of staining observed, with GUS expression apparent within the cotyledons but not within the true leaves.
- B) Closer examination of a cotyledon from construct KrII.

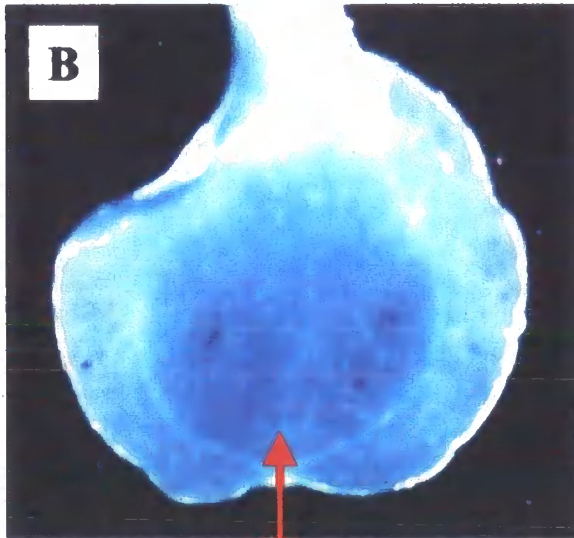
A



True
leaves

Cotyledon

B

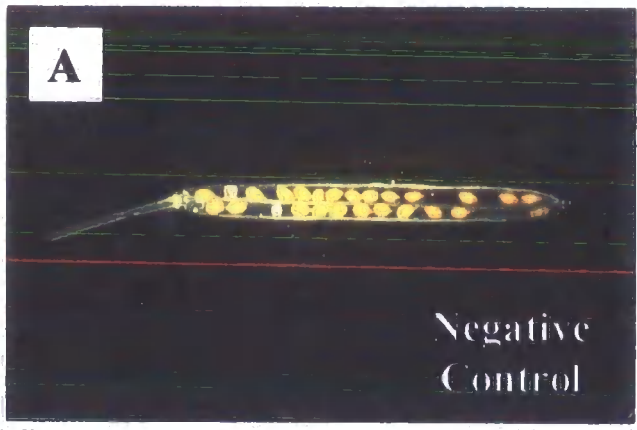


Cotyledon

Figure 3.20 GUS Expression within Embryos (Construct KrII)

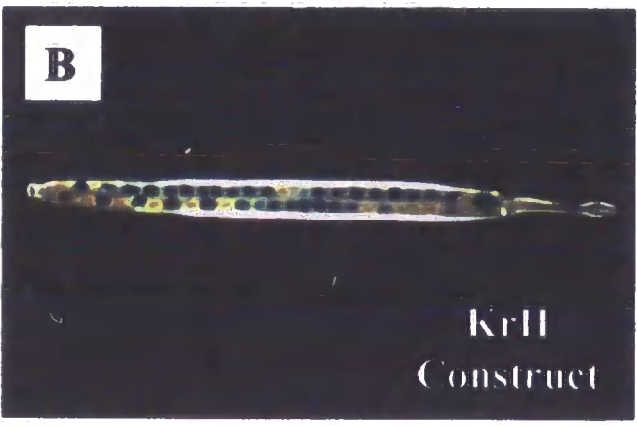
- A) Negative control – as expected the negative control embryos within the silique showed no GUS staining.
- B) KrII construct - examination of GUS expression of the embryos within the silique. The segregation ratio of 3:1 GUS positive segregants to GUS negative segregants would be expected, which is roughly what was observed with 35 positive segregants and 13 negative segregants.
- C) Higher magnification of the embryos within the siliques clearly showing the GUS negative segregants and the GUS positive segregants (blue).

A



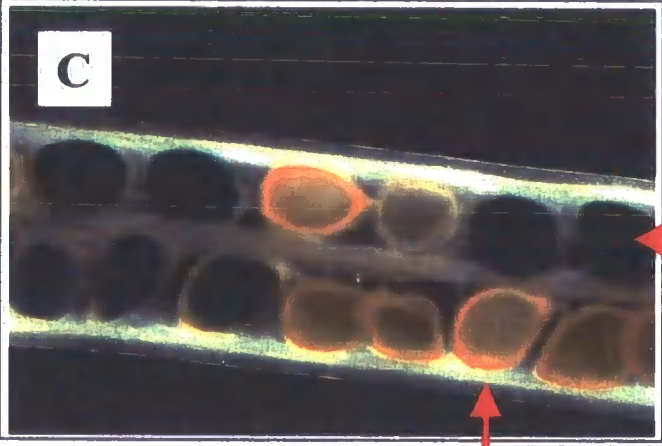
Negative
Control

B



KrII
Construct

C



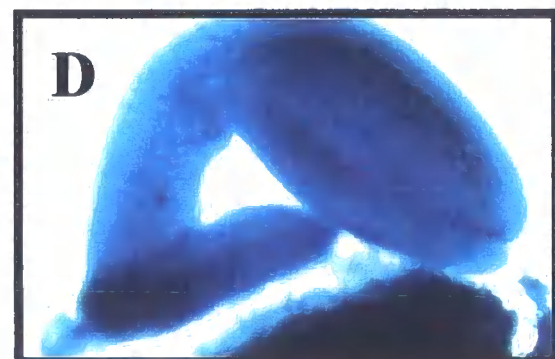
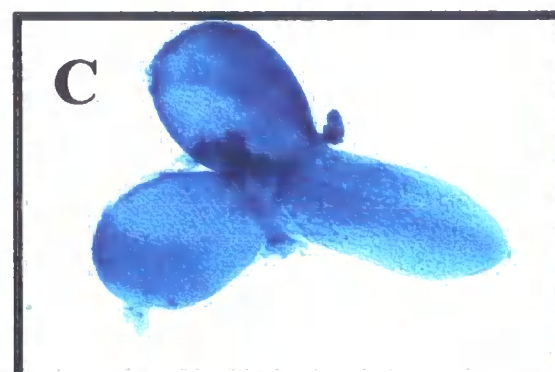
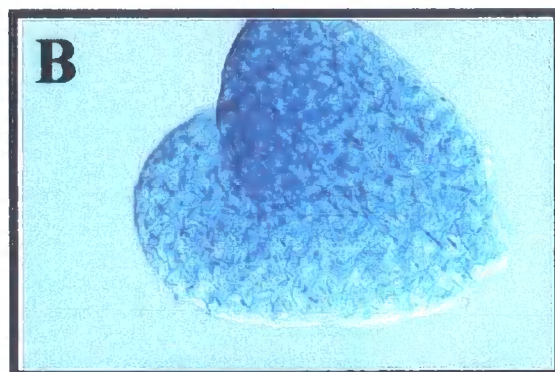
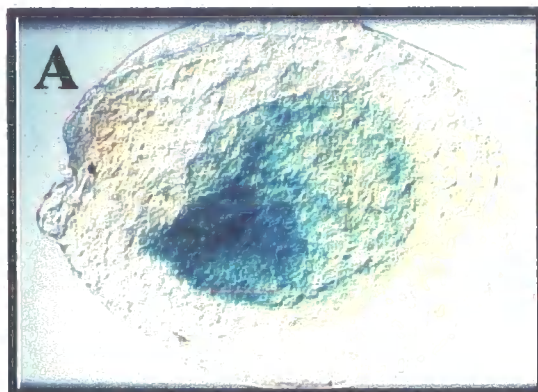
GUS
Positive
Segregant

GUS
Negative
Segregant

Figure 3.21 GUS Expression in the Developmental Stages of the Embryos (Construct KrII)

Examination of the different stages of embryo development, showing staining throughout development. It appears that there may be an increase of intensity of staining with age, however this is difficult to conclude and fluorometric analysis would be needed for quantitative analysis.

- A) Early heart stage
- B) Heart stage
- C) Torpedo
- D) Mature cotyledon stage



3.4 Discussion

The aim of this study was to examine whether the 2Kb 5' region of the *B.napus* β -KrI genomic clone would act as a functional promoter. *Arabidopsis* plants were transformed with four different *B.napus* β -Kr/GUS constructs (KrI (2Kb), KrII (1Kb), KrIII (500bp) and the negative control) and GUS expression examined. The KrI and KrII constructs both directed GUS expression, indicating that these sequences could initiate transcription and drive gene expression and therefore act as a promoter. Unfortunately no kanamycin resistant plants were obtained for construct KrIII and therefore no GUS examination could be obtained for this construct. The second aim was to examine if the deletion of this sequence had any affect on the intensity of expression. It appeared that the KrI and KrII showed no difference in the intensity of GUS expression. However unfortunately the effect of deleting the promoter down to 500bp could not be examined due to no positive transformants being obtained for construct KrIII. Therefore it appeared that the necessary machinery to carry out transcription was contained within the 1Kb sequence.

To examine the regulation of the genes involved in seed storage lipid biosynthesis, the isoform would need to be expressed within embryos. The expression pattern of the β -Kr gene was analysed in the transgenic *Arabidopsis*. The staining showed a distinctive pattern of expression, with expression observed in the roots, cotyledons and embryos. The staining within roots appeared to be highest within the youngest part of the root but absent from the root tip. It appeared that staining was mainly in the area of elongation and above. This would be expected, as if the cells are elongating they would require lipid production for cell membrane synthesis. The GUS staining appeared to become more restricted with age, with the staining more apparent in the vascular tissue than the epidermis and cortex in the older root. However this may be due to the intensity of staining within the younger root, and therefore the GUS staining may be leaking out into the surrounding tissues, giving the appearance of staining within the cortex and epidermis. However the staining does not appear to leak into the root tip, therefore it is unlikely that this accounts for the

homogenous staining within the younger parts of the root. The expression of the gene only within the cotyledons but not within the true leaves is interesting as this would agree with it being a seed expressed isoform, as the cotyledons are present within the seed where there is high deposition of lipid. The embryos appear to show an increase in GUS expression with age, however fluorometric analysis would be required for quantitative analysis.

In conclusion, this 2Kb 5' sequence of the *B.napus* β -Keto-ACP reductase gene has been shown to direct transcription and therefore function as a promoter. Deletion of the sequence to 1Kb appeared to have no effect on expression and therefore this sequence was shown to be sufficient to initiate transcription. This isoform does not appear to be expressed within the true leaves and therefore one of the other isoforms may be responsible for leaf expression. However expression was observed within embryos and also the cotyledons that are present within the seed. This fits in with the promoter being a fatty acid biosynthetic gene as it is expressed in areas that have high demand for lipid synthesis (embryos, cotyledons and expanding root). Further analysis of the 1Kb sequence could identify specific *cis*-acting elements within this region required for the regulation of this gene.

Chapter Four

Identification of Protein-DNA Interactions within the *B. napus* β -Keto-ACP Reductase Promoter

4.1 Introduction

Previous work has shown that a number of the lipid biosynthetic genes exhibit both temporal and tissue specific regulation (Elborough *et al.*, 1996). This has led to the idea that there may be an overriding control factor involved in the expression of the lipid biosynthetic genes. Transcription factors are often found to exert a controlling effect, therefore determining the specificity of the promoter. If there is an overall control factor that upregulates the genes involved in fatty acid biosynthesis then it would be expected to bind to all of the promoters that it upregulates. Therefore the promoters should contain the specific *cis*-acting element to which the protein binds. The gel mobility shift assay is a way of detecting protein-DNA complexes that are formed using a radioactively labelled *cis*-acting element to detect the binding of proteins to the DNA.

4.1.1 Aim

The aim of this study was to examine the protein-DNA interactions of the *B. napus* β -Keto-ACP reductase 1 promoter (β -Kr1), in order to identify specific *cis*-acting elements to which specific regulatory proteins may bind. In the previous chapter the 1Kb 5' sequence of the β -Kr1 gene was shown to be sufficient to initiate transcription. Therefore this sequence was thought to contain all the necessary *cis*-acting elements involved in the transcription of this gene. Gel mobility shift analysis was used to analyse this promoter sequence; using embryo extracts from *B.napus* to identify potential *cis*-acting elements within this β -Kr promoter. Competition analysis was also used to examine binding to other promoter fragments.

4.1.2 Detection of Protein-DNA Complexes

The gel mobility shift assay (GMSA) is a method for observing protein-DNA interactions, which is both simple and sensitive. It shows the effect of protein binding to labelled nucleic acids, and relies upon the observation that protein-DNA complexes migrate more slowly through non-denaturing polyacrylamide gel, than unbound DNA. The protein-DNA complex can therefore be distinguished from unbound DNA electrophoretically. Using this technique it is possible to detect transcription factors that bind to regulatory sequences.

4.1.3 Factors Determining the Detection of Protein-DNA Interactions

Eukaryotic transcription factors are found in very small concentrations within cells, varying from 10^3 to 10^5 molecules per cell, therefore for their detection the assay needs to be very sensitive. The following factors need to be taken into consideration to maximise the sensitivity of the assay: preparation of a good protein extract, labelling the DNA to a high specific activity and titrating the binding conditions within the reaction to optimise binding. The DNA must be radioactively labelled to a high specific activity, as only a very low concentration of DNA is used due to the low concentration of proteins. The stability of the complex is crucial in the detection of protein-DNA complexes, which in turn depends on the conditions of the reaction, in particular the binding buffer and electrophoretic conditions. Stabilisation of the protein-DNA complex is achieved using low ionic acrylamide gels; the mobility may also be altered by the acrylamide percentage. Protein-DNA complexes also favour low salt concentrations as this decreases the dissociation rate of the complex. Therefore the components of the assay must be carefully considered to optimise the conditions for the formation of protein-DNA complexes. This often involves a titration of the components within the buffer solution to achieve the most favourable conditions for protein-DNA complex formation. Protein extractions can either be prepared from whole cell extracts or isolated nuclei. The preferable extract is from nuclei, due to the high concentration of transcription factors within the nucleus.

However there is no reliable method of isolating nuclei from *B.napus* and only limited material was available for this study, therefore whole embryo protein extractions were carried out.

4.1.4 Competitor Analysis

Non-specific competitor DNA is an essential component of the gel mobility shift assay when trying to select a specific binding protein from a crude extract that contains a huge number of potential binding proteins. Upon the addition of protein extract to labelled DNA, non-specific binding is observed, which appears as a smear along the length of the gel. This is due to ionic and hydrophobic interactions between the proteins and the DNA. Competitor DNA is required to compete this away, by providing a huge array of non-specific binding sites. Thereby removing the non-specifically bound proteins, allowing detection of the specific protein-DNA interactions. Figure 4.1 shows the effect of competitor DNA, by the disappearance of the non-specific binding, without competing away the specific protein-DNA complexes. A competitor titration can be carried out to observe the optimal concentration; this is achieved when the level of radioactivity along the length of the gel diminishes, without the disappearance of the specific protein-DNA complex. These specific protein-DNA interactions are unaffected by the addition of competitor DNA, as the competitor does not contain the specific binding site for the protein (figure 4.2). A huge competitor to probe ratio is used ensuring that any complexes formed are likely to be specific interactions. Specific competitor analysis can also be carried out to see if a fragment contains the same binding site as the probe. If this is the case and the competitor DNA binds the protein of interest, the protein-DNA complex will diminish (figure 4.3). Here the fragment contains the same *cis*-acting element as the probe, to which the protein binds, and therefore competes with the probe for the protein and because it is present in excess the protein-DNA complex diminishes. This can therefore be used to identify if a DNA sequence of interest contains the same protein-binding site as the probe.

Figure 4.1 Effect of Poly (d(I-C)) Competitor on Non-Specific Binding

Gel mobility shift assays were carried out to examine the effect of competitor DNA on non-specific binding. Upon the addition of protein extract to the probe non-specific binding appears as a smear along the length of the gel. This disappears in the presence of poly (d(I-C)) competitor, allowing the detection of the specific protein-DNA interactions. The lanes contain **1)** free probe **2)** probe and protein **3&4)** probe, protein and the addition of poly (d(I-C)) competitor DNA.

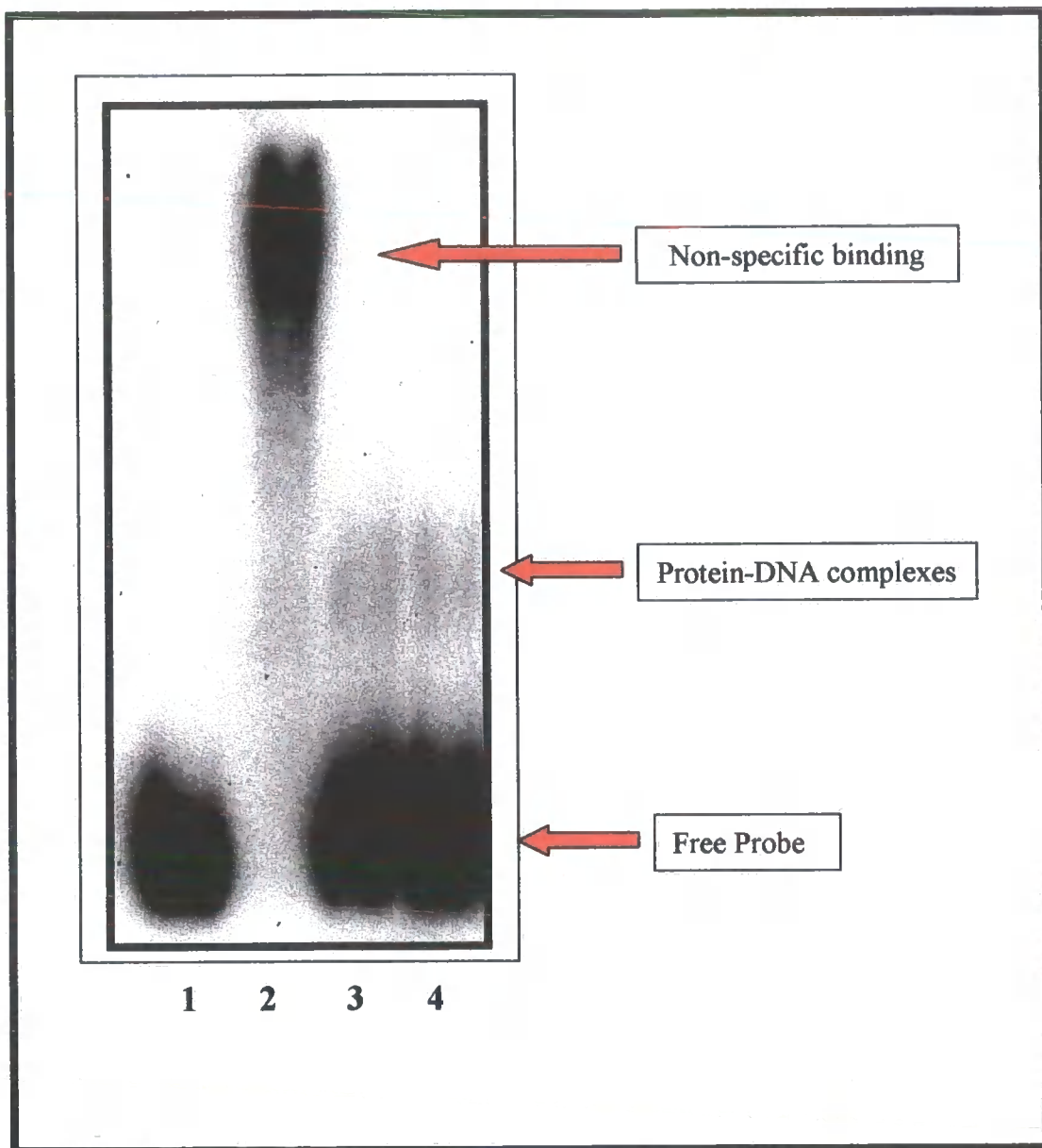


Figure 4.2 Gel Mobility Shift Assay (GMSA)

This figure outlines the basic principle of the GMSA. The labelled probe is mixed with unlabelled poly (d(I-C)) (non specific competitor) and then incubated with protein extract. The poly (d(I-C)) binds any non-specific proteins thereby leaving only the specific proteins binding to the probe. Therefore any protein-DNA complexes visualised will be specific. Without the protein only one band containing the free probe is seen, upon the addition of protein and competitor, specific protein-DNA complexes can be visualised.

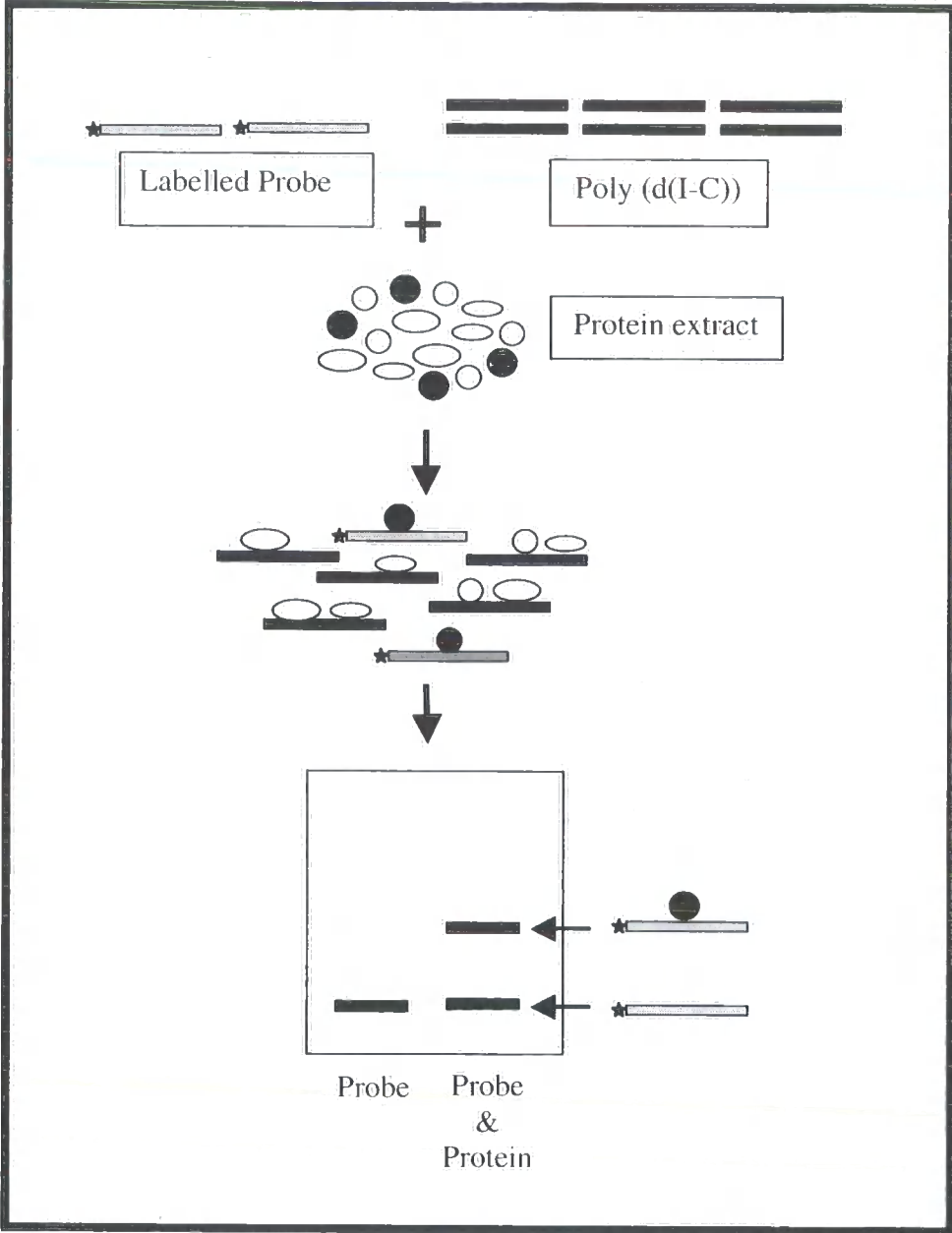
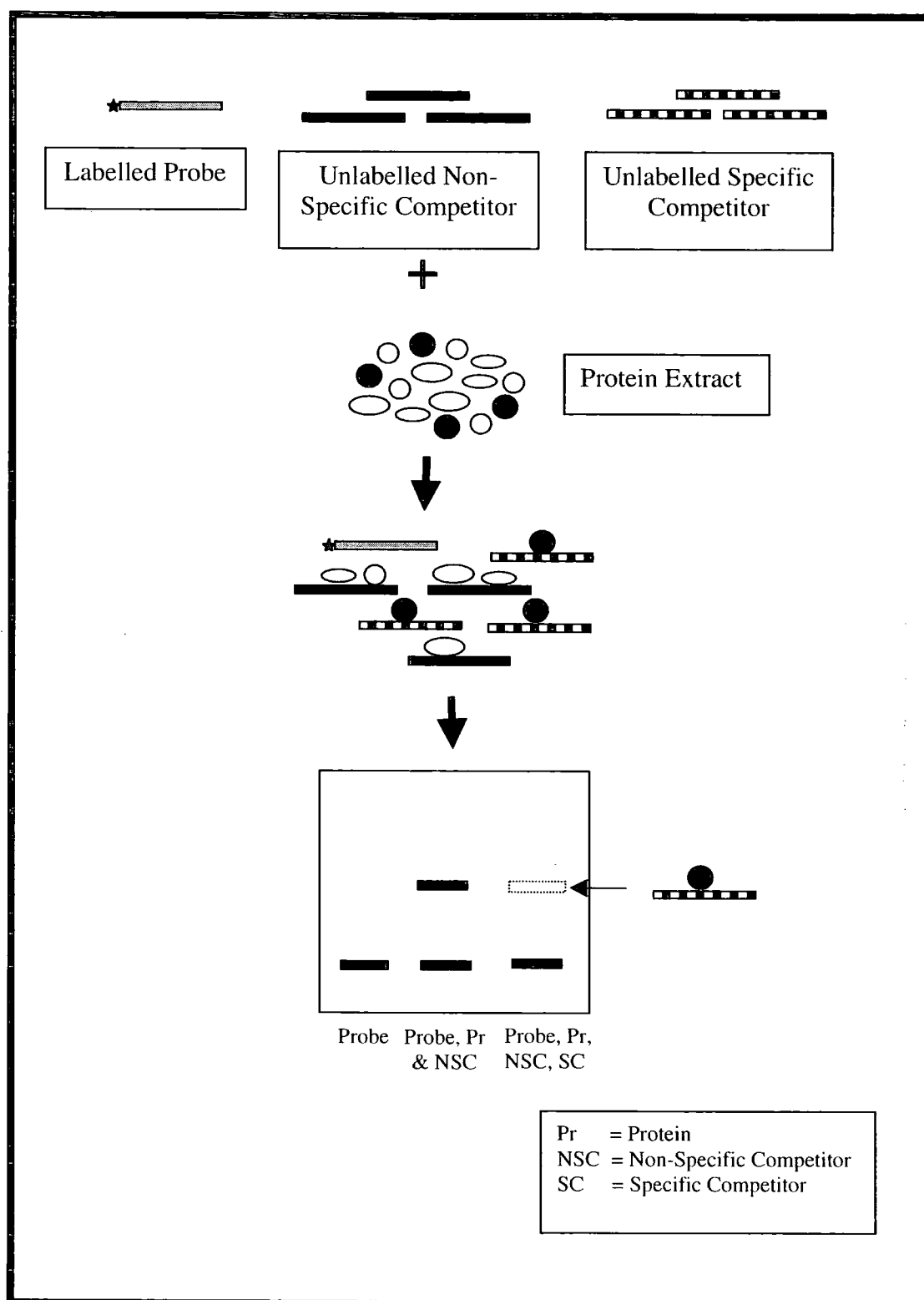


Figure 4.3 Competitor Analysis in GMSA

This figure shows the use of unlabelled competitor DNA within the GMSA. The labelled probe is mixed with unlabelled specific and non-specific competitors. The non-specific competitor will bind any non-specific proteins away from the probe leaving only specific proteins bound. However the specific competitor, if it contains the same binding site as the probe, to which the specific proteins bind will compete away the protein. Therefore the protein-DNA complex will diminish as the protein is competed away by the specific competitor that is present in excess.



4.2 Methods

The standard molecular biology techniques used in this study are described in Chapter 2. Any conditions and primers specific for this chapter are outlined below.

Table 4.1 – Primers used for Amplification of Fragments

Primer	Sequence
Pr11 (KrA)	GGAATTCCTAGCCTCCTTCCAACCTCTCTCG
Pr12 (KrA)	GGAATTCTTCGAAAAGCGATACGGACGAGG
Pr13 (KrB)	GGAATTC CGAAGCTCCAAC TACTGACTACC
Pr14 (KrB)	GGAATTCACACAGTGTAGAGAGAGAGAGAG
Pr15 (KrC)	GGAATTCTTAAGTTTACCCGCGCATGG
Pr16 (KrC)	GGAATTC CGAGAGAGTTGGAAGGAGGC
Pr17 (KrD)	GGAATTC CGACATTCAAGATTATACATGC
Pr18 (KrD)	GGAATTCTTAAATTTACTGCGCCATGCGC
Pr19 (KrE)	GGAATTCACACATCCCTCTTCTGTATCG
Pr20 (KrE)	GGAATTC CATGTATAAATCTTGAATGTCCG
Pr21 (KrF)	GGAATTCGTACATAGAATCAAGTTGTATCC
Pr22 (KrF)	GGAATCTTTAATGCGATACAGAAGAGGG
Pr23 (EnR)	GGAATTC AACCAGGATTCCGTTTTTTATAACAGAACCGATCGGTTTTGTGAATTCC
Pr24 (EnR)	GGAATTC ACAAACCGATCGGTTCTGTTATAAAAACCGAATCCTGGTTGAATTCC
Pr25 (LTP)	GGAATTC CACACCGTCGGATTGGGATTTC
Pr26 (LTP)	GGAATCTGGATGATGAAGAGTGTTAGGGG

Table 4.2 - PCR Conditions

	1 cycle	30 cycles	1 cycle
Denature	95°C 5mins	95°C - 30secs	72°C 5mins
Anneal	-	64°C - 30secs	-
Extend	-	72°C - 60secs	-

4.2.1 Preparation of Protein Extract

Protein extracts were prepared from *B.napus* embryos as outlined in section 2.11.1. Fractions were collected following elution by an increasing salt concentration through a Q-Sepharose column. Protein extracts from both embryo and seed were prepared, however the best binding results were achieved using the embryo extracts, therefore this was used in all the subsequent assays.

4.2.2 Gel Mobility Shift Assay

The gel mobility shift assay was carried out as described in section 2.11, care was taken to keep the reactions cold, on ice at all times and any movements that might disturb the gel were minimised. The buffer, competitor, protein and probe were incubated together to allow association of the proteins with the DNA. The protein-DNA complexes were then separated from free probe by gel electrophoresis, and autoradiography was used to detect any specific protein-DNA complexes. All of the reactions were carried out in a final volume of 10 μ l, thereby keeping the volume constant to eliminate any dilution effects.

4.3 Results

4.3.1 Design of the β -Kr Promoter Fragments

Chapter three identified the 1Kb 5' sequence of the *B.napus* β -Kr1 gene as the sequence containing the transcriptional machinery required to direct gene expression. Therefore the sequence upstream of the presumptive translational start site was used in this gel mobility shift analysis to identify potential *cis*-acting elements to which protein binds. The β -Kr promoter was analysed in the PLACE database (Plant Associated Cis-Acting Elements) to identify any known general transcription factor binding sites. This identified three putative TATA boxes within the promoter region

of the β -Kr1 gene. Primers were devised (Pr11–Pr22) to produce six overlapping fragments (~120bp in length) of the promoter (KrA–KrF). The fragments were designed up to 677bp upstream of the ATG and inclusive of the TATA boxes, as indicated in figures 4.4–4.5. *Eco*R1 sites were incorporated into the fragments during amplification by PCR, to allow radioactive 3' labelling of the fragments (section 2.11.2.2). The promoter fragments were cloned into the pGEMT-Easy vector and sequenced (Pr9 and Pr10) to confirm their sequences were correct.

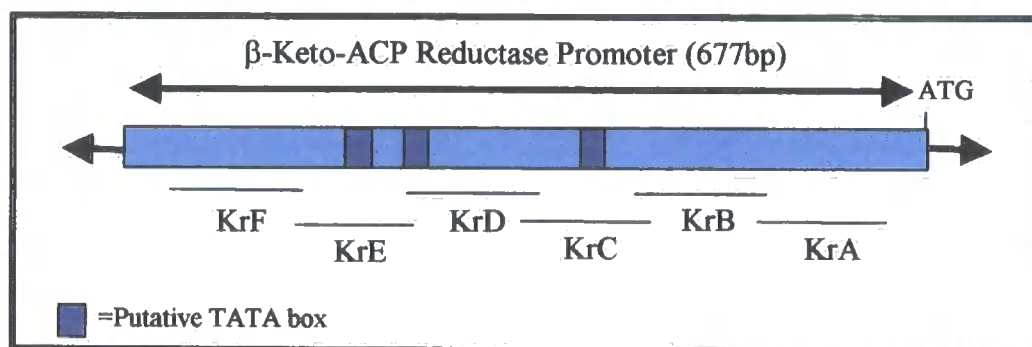


Figure 4.4 Schematic Representation of the β -Kr Fragments

The position of the six overlapping β -Kr promoter fragments are outlined. The translational start codon (ATG) and the putative TATA boxes are highlighted. The fragments covered a region 616bp, which was 677bp upstream of the putative translational start site and each fragment was ~120bp in length.

4.3.2 Preparation of the β -Kr Probes

The six β -Kr fragments (Kr1–6) were digested out of the pGEMT-Easy vector using the *Eco*R 1 restriction enzyme. They were then radioactively labelled to a high specific activity with ^{32}P dATP as detailed in section 2.11.2.2, using the klenow fragment of the DNA polymerase I. Klenow labels from the 3 prime end of each of the fragments produced by restriction endonuclease cleavage, using the 5' overhang of the corresponding chain as the template.

Figure 4.5 Sequences of the Six β -Kr Promoter Fragments

The sequences of the promoter fragments are shown. Six overlapping fragments of the *B.napus* β -Kr promoter (KrA-KrF) were amplified surrounding the three putative TATA boxes (as shown schematically in figure 4.4). These fragments were radioactively labelled and used for subsequent gel mobility shift analysis.

GTATTATTTTAAAAAAATTACAGGTATTGTGAACGATAGTTAGACT
ATATTATAGAGATTTTACTGTATATTAATTAAGAGCATTACAACCTT
TGAGTAAATCTGATTGGGTTTTTCAGAATCTTTAGTTAATAAAATATG
AAAGTTCTTTAAATATGACTAGTTAGTAATAGTATATGTTAAGAGAA
ATATATAAAGTATAGTTTAAAAAAATGTTGTACATAGAATCAAGT
TGTATCCAACCTTGTGATTCAAAATAGATTATAGATGATGTAAATA
AGATAAACCAAAACCATTTTGAACAGATAAATGGTAAATATTTTG
AAAGATAAGTGAACACACATCCCTCTTCTGTATCGCATTAAAGAA
ATATCACATTGTTATTAATCTGAGTGTTTTTATGTAATTTAAAAAAA
TGAACAGCATAAAGACAACCATAATATATACATAATCCGACATTCA
AGATTTATACATGCTTCAACCAAGAATTTTTTTTAATGTTTATGC
TTAATTTGAAAAGAAAACCTATTGTAGTTTTTTAAATTTAAGTTTCA
CCCGCGCATGGCGCAGTAAATTTAAGTTAGTTTTGATGTTATATAC
GGGTTGGATACTACTACAACTGGGAAAACCTTTAAAATCAGTTCGG
TCCGGTCCGGTACGGTTCAATCTAGCCTCCTTCCAACTCTCTCGTC
TGGCCGTCTAAATTCAACCGAAGCTCCAACCTACTGACTACCAACT
AAACACTGTACAACCTCGTCCGTATCGCTTTTCGAAGAATTAAAT
ATTCACACCTTTTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTACTGT
GTCGATCTTTACGAGGTTCACTAGCTCGGACGCCCTTCTCCACCTCCT
CCTCCGTTTCGATG

4.3.3 Competitor DNA

Two types of competitor were used in the gel mobility shift analysis, both non-specific and specific competitor DNA. The first, Poly (d(I-C)), a synthetic co-polymer is commonly used as a non-specific competitor in gel mobility shift assays. These co-polymers provide an extensive range of low affinity binding sites that bind any non-specific proteins to allow specific interactions to be observed. The promoter regions of two other plant genes were used as specific competitors to try and compete away the specific interactions, thus indicating whether those promoters also contain the specific binding site for the protein. If large amounts of unlabelled specific competitor DNA, that contain the binding site identical to the probe, are added to the assays, the sequence will bind to the protein and therefore the protein-DNA complex will diminish. However, upon the addition of non-specific competitor, that does not contain the specific binding site, the complex formed should not be affected and still be detected.

The three main specific competitors used for this study were:

- β -Keto-ACP reductase fragments (unlabelled) - *B. napus*
- Enoyl-ACP Reductase (EnR) promoter sequence – *Arabidopsis*
- Lipid Transfer Protein promoter (LTP) - *Arabidopsis*

The β -K_r promoter fragments themselves were used as unlabelled competitors to see if they would compete away from the other radioactively labelled β -K_r fragments, indicating whether the same protein binds at a number of regions within the promoter. The non-lipid biosynthetic, lipid transfer protein (LTP) promoter from *Arabidopsis* was also used as a competitor. The 300bp region surrounding the TATA box was examined for introns and was amplified and used as an example of a non-lipid biosynthetic promoter, to examine the effect of this competitor on any specific interactions. Competition analysis also involved the sequence surrounding the putative TATA box from the *Arabidopsis* enoyl-ACP reductase promoter. The 47bp sequence surrounding the TATA was used as a competitor as it was the region

surrounding the putative TATA boxes that was amplified from the β -Kr promoter for the GMSA. Deletions of the *Arabidopsis* EnR promoter by de Boer et al., 1999 also showed that deletion to -47bp of the promoter still directed transcription within seeds and was therefore shown to be sufficient for expression within the seed. Therefore anything upstream of this sequence appeared not to direct seed specific expression. The -47bp region upstream of the transcription initiation site was used as a specific lipid biosynthetic competitor. These competitors would therefore identify if any protein interacting with the β -Kr promoter would also bind to another fatty acid synthesis gene promoter or whether it was more of a general transcription factor that binds to other promoters for example the lipid transfer protein. Any protein-DNA complexes that are competed away by the LTP promoter are likely to be more general transcription factors, whereas interactions that are not susceptible to LTP, but competed away by the *Arabidopsis* EnR promoter, are indicative of a more specific transcription factor for the fatty acid biosynthetic genes.

4.3.3.2 Preparation of the Competitor DNA

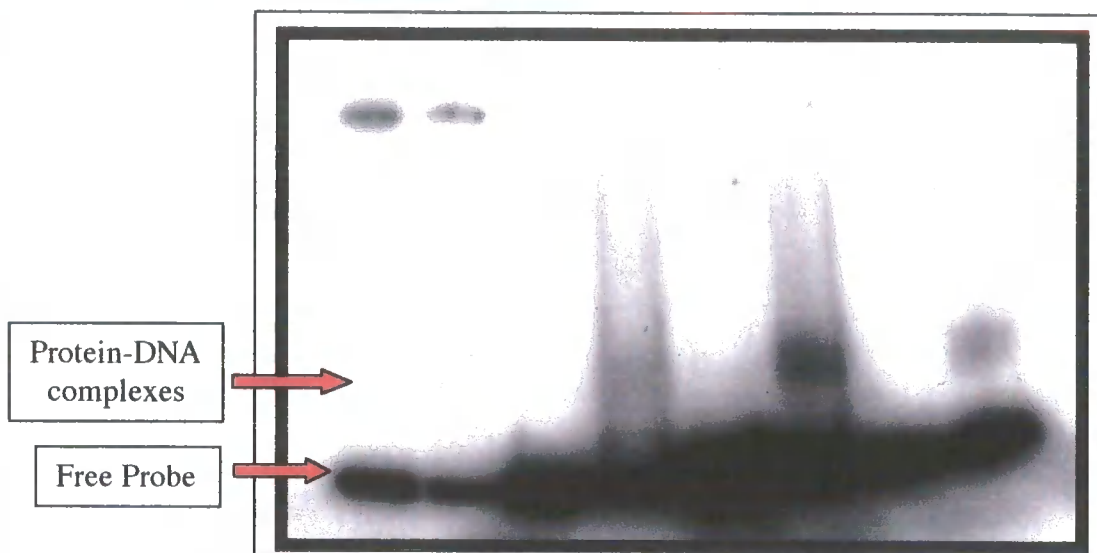
Poly (d(I-C)) was made up to a stock concentration of 1 μ g/ μ l, and a range of concentrations used (0.1-2 μ g). Oligonucleotides were ordered for both the *Arabidopsis* EnR promoter and LTP promoter sequences (Pr23-Pr26). The specific competitor DNA was made up to a final concentration of ~500ng/ μ l. and 0.5-1 μ l. All assays were carried out in a final volume of 10 μ l, and therefore the amounts given for the competitors within this study in terms of μ g are in a final volume of 10 μ l.

4.3.4 Protein-DNA Interactions

Gel mobility shift analysis was carried out on all six of the β -Kr fragments (KrA-KrF). All the fragments appeared to form specific protein-DNA complexes upon the addition of embryo protein extract, as indicated by figures 4.6 and 4.7. Upon addition of protein to the radioactively labelled probe the appearance of protein-DNA

Figure 4.6 Gel Mobility Shift Analysis

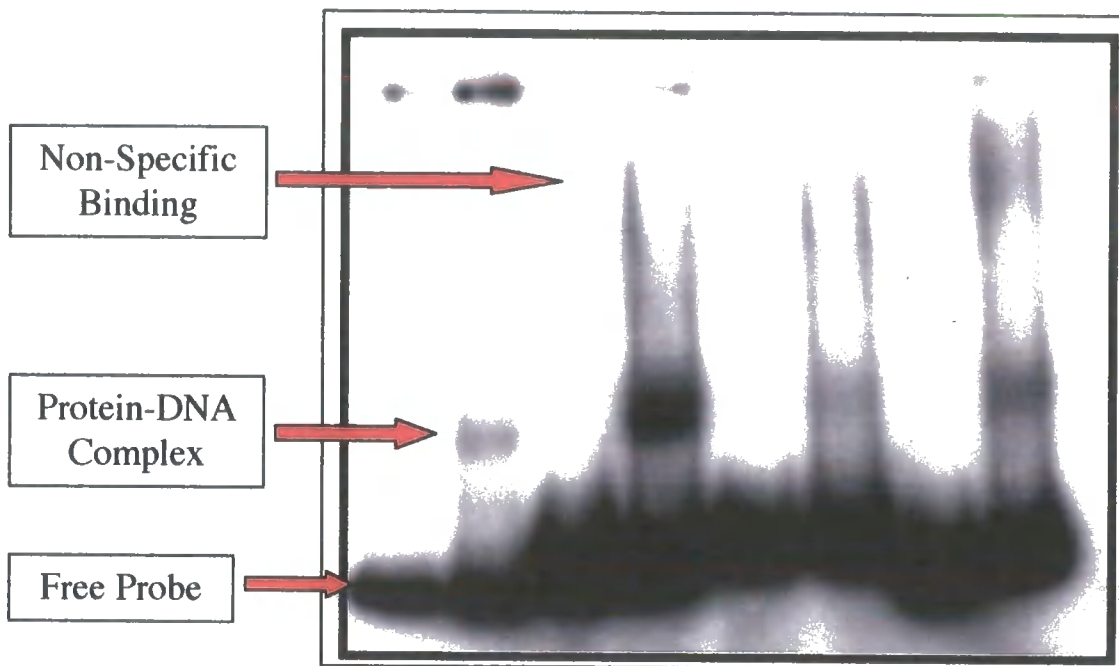
Gel mobility shift assays were carried out on all six β -Kr promoter fragments (KrA-F). All lanes contained 1 μ g of poly (d(I-C)) per 10 μ l reaction. The lanes contained the following probes **1&2)** KrA, **3&4)** KrB, **5&6)** KrC, **7&8)** KrD. Lanes 1, 3, 5, and 7 contained the free probe, with no protein extract. Lanes 2, 4, 6 and 8 show the effect of the addition of 1 μ l of protein extract to the probe. Upon the addition of protein, specific protein-DNA complexes were observed for all the fragments. Indicating that all the fragments appear to contain a specific *cis*-acting sequence to which protein is binding.



Lane	1	2	3	4	5	6	7	8
Probe	KrA	KrA	KrB	KrB	KrC	KrC	KrD	KrD
Protein	-	+	-	+	-	+	-	+
Poly (d(I-C)) (μ g)	1	1	1	1	1	1	1	1

Figure 4.7 Gel Mobility Shift Analysis

Gel mobility shift assays were carried out on all six β -Kr promoter fragments (KrA-KrF). All lanes contained 1 μ g of poly (d(I-C)) per 10 μ l reaction. The lanes contained the following probes **1&2)** KrA, **3&4)** KrE, **5&6)** KrF, **7&8)** KrB. Lanes 1, 3, 5, and 7 contained the free probe, with no protein extract. Lanes 2, 4, 6 and 8 show the effect of the addition of 1 μ l of protein extract to the probe. Upon the addition of protein, specific protein-DNA complexes were observed for all the fragments. Indicating that all the fragments appear to contain a specific *cis*-acting sequence to which protein is binding.



Lane	1	2	3	4	5	6	7	8
Probe	KrA	KrA	KrE	KrE	KrF	KrF	KrB	KrB
Protein	-	+	-	+	-	+	-	+
Poly (d(I-C))(μ g)	1	1	1	1	1	1	1	1

complexes were observed, even with a high ratio of competitor poly (d(I-C)) DNA to probe, suggesting that the binding is specific to that sequence. These results suggest that all six fragments contain specific *cis*-acting regions to which proteins bind. KrC and KrE both appeared to show the most noticeable binding, therefore the majority of the study concentrated on these fragments.

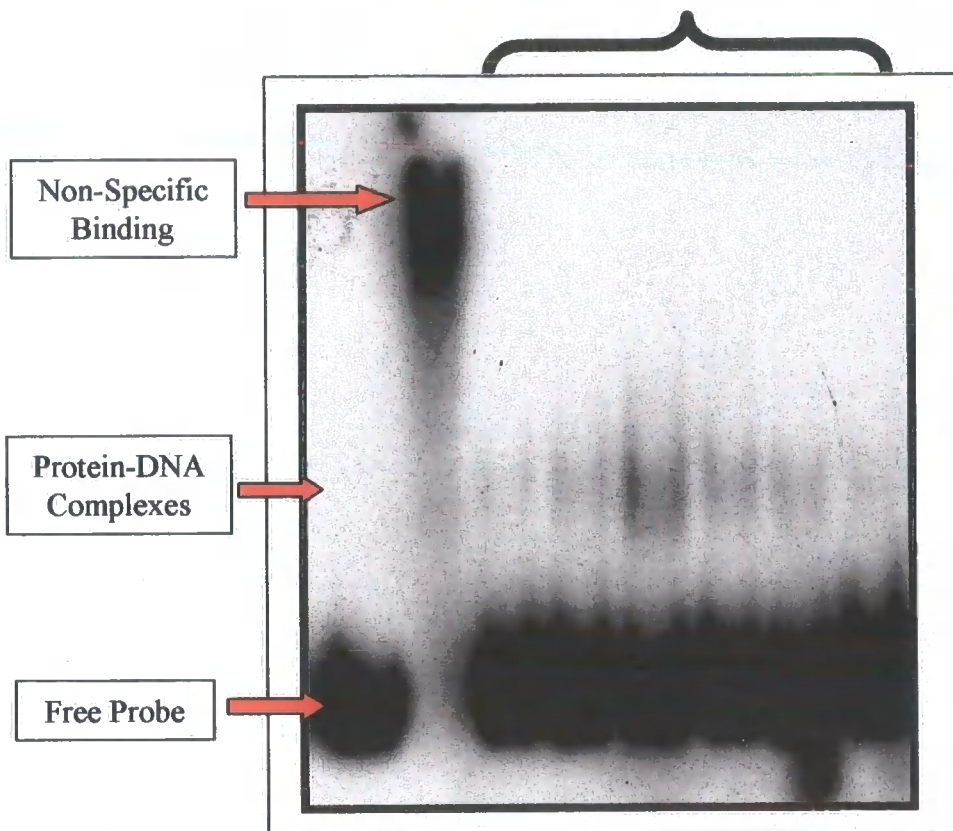
4.3.5 Poly (d(I-C)) Titrations

Without poly (d(I-C)) the detection of specific protein-DNA interactions is masked by non-specific binding. Therefore a titration of poly (d(I-C)) competitor DNA allows the detection of specific interactions. After examining the protein-DNA interactions of the fragments, and identifying binding to all six fragments, poly (d(I-C)) titrations were carried out to ascertain if they were specific protein-DNA interactions. The concentration of poly (d(I-C)) used ranged from 0.1 μ g-2 μ g within a 10 μ l reaction (therefore a final volume of 0.01 μ g/ μ l – 0.2 μ g/ μ l was achieved). Even at the lowest concentration of 0.1 μ g, the ratio of competitor to probe is enormous. Typically 0.3ng of probe is used in an assay, therefore with 2 μ g of competitor a ratio of 1:6667 is obtained. These titrations suggested that the complexes were specific, as the concentration and size of the competitor DNA present, would provide a huge array of low affinity binding sites that would compete away any non-specific interactions. The β -Kr fragments showed the disappearance of non-specific binding along the length of the gel, and the visualisation of specific protein-DNA complexes upon the increase in poly (d(I-C)). Figure 4.8 shows a typical poly (d(I-C)) gradient obtained using fragment KrC, even upon the addition of 2 μ g of poly (d(I-C)) competitor DNA a protein-DNA complex can still be observed. The gel shows how, with the addition of protein to the probe, non-specific binding is seen as a smear at the top of the gel, but upon the addition of the poly (d(I-C)) DNA this diminishes and detection of the specific protein-DNA band is achieved.


Figure 4.8 Poly (d(I-C)) Gradient on Fragment KrC

Gel mobility shift analysis was carried out on fragment KrC, using a poly (d(I-C)) gradient to examine specific protein-DNA interactions. The lanes contain **1)** free probe **2)** probe and protein extract with no poly (d(I-C)) competitor **3)** probe, 1 μ l of protein and 1 μ g of poly (d(I-C)) competitor **4)** probe, 2 μ l of protein and 2 μ g poly (d(I-C)) competitor **5-8)** represents a gradient of poly (d(I-C)) competitor ranging from 0.1 μ g-1 μ g. The results show the disappearance of any non-specific binding upon the addition of increasing levels of poly (d(I-C)). The detection of specific protein-DNA complexes can be observed even with a large concentration of 2 μ g of competitor DNA. The competitor to probe ratio is enormous as only 0.3ng of probe is being used in a typical binding assay with up to 2 μ g of competitor.

Disappearance of Non-Specific Binding



Lane	1	2	3	4	5	6	7	8
Protein (μ l)	-	1.0	1.0	2.0	1.0	1.0	1.0	1.0
Poly(d(I-C)) (μ g)	1.0	-	1.0	2.0	0.1	0.2	0.5	1.0


 Poly (d(I-C)) Concentration

4.3.6 Competition Analysis

Upon identification of specific protein-DNA complexes with all the fragments, competition assays were carried out to examine the binding of this protein to other promoter regions using the LTP and EnR. The EnR and LTP promoters were used as specific competitor DNA to examine if the binding protein was specific to other lipid biosynthetic genes. The fragments were also competed against each other, to observe if the same protein was binding at a number of different regions along the promoter.

4.3.6.1 Competition Analysis on Fragment KrC

Fragment KrE was used as a non-radioactively labelled competitor, against the labeled KrC probe. Figure 4.9 shows how upon the addition of unlabelled KrE to the radioactively labelled KrC probe, the specific protein-DNA interaction seems to disappear, suggesting the same protein that binds to KrC is also specific for KrE. Therefore suggesting that the binding protein may bind at more than one region along the β -Kr promoter. This multiple binding of transcription factors within a promoter is a common feature of eukaryotic promoters.

4.3.6.2 Competition Analysis on Fragment KrE

Competition assays were carried out on fragment KrE as the probe using KrC, EnR and LTP promoters as unlabelled competitors. Figure 4.10 shows the results of this analysis. The Poly (d(I-C)) gradient identifies the formation of a specific protein-DNA complex. This complex appeared to be competed away upon the addition of fragment KrC. Indicating that the same sequence to which the protein binds appears to be common both in KrC and KrE. The gel also shows how the protein-DNA complex seems to be competed away by the addition of the EnR promoter, again indicating that the protein also appears to bind to a sequence within this promoter. If there is an overriding control factor of the lipid biosynthetic genes, then it would be

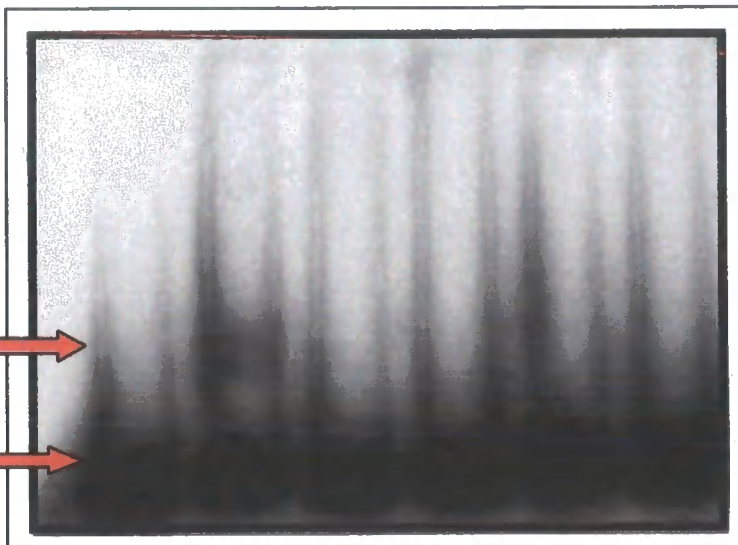
Figure 4.9 Analysis of Fragment KrC using KrE as Unlabelled Competitor

All lanes contain KrC as the radioactively labelled probe and 1 μ g of poly (d(I-C)) to compete away any non-specific binding. In addition the lanes contain **1)** free probe **2)** protein extract **3-6)** protein extract and a gradient of increasing concentrations of unlabelled KrE. Lane 2 shows the formation of specific protein-DNA complexes. Lane 3 shows how upon the addition of KrE the specific protein-DNA complexes are no longer visible, indicating that this band has been competed away by KrE, thereby suggesting they both contain a binding site for the protein.

Protein-DNA
Complexes



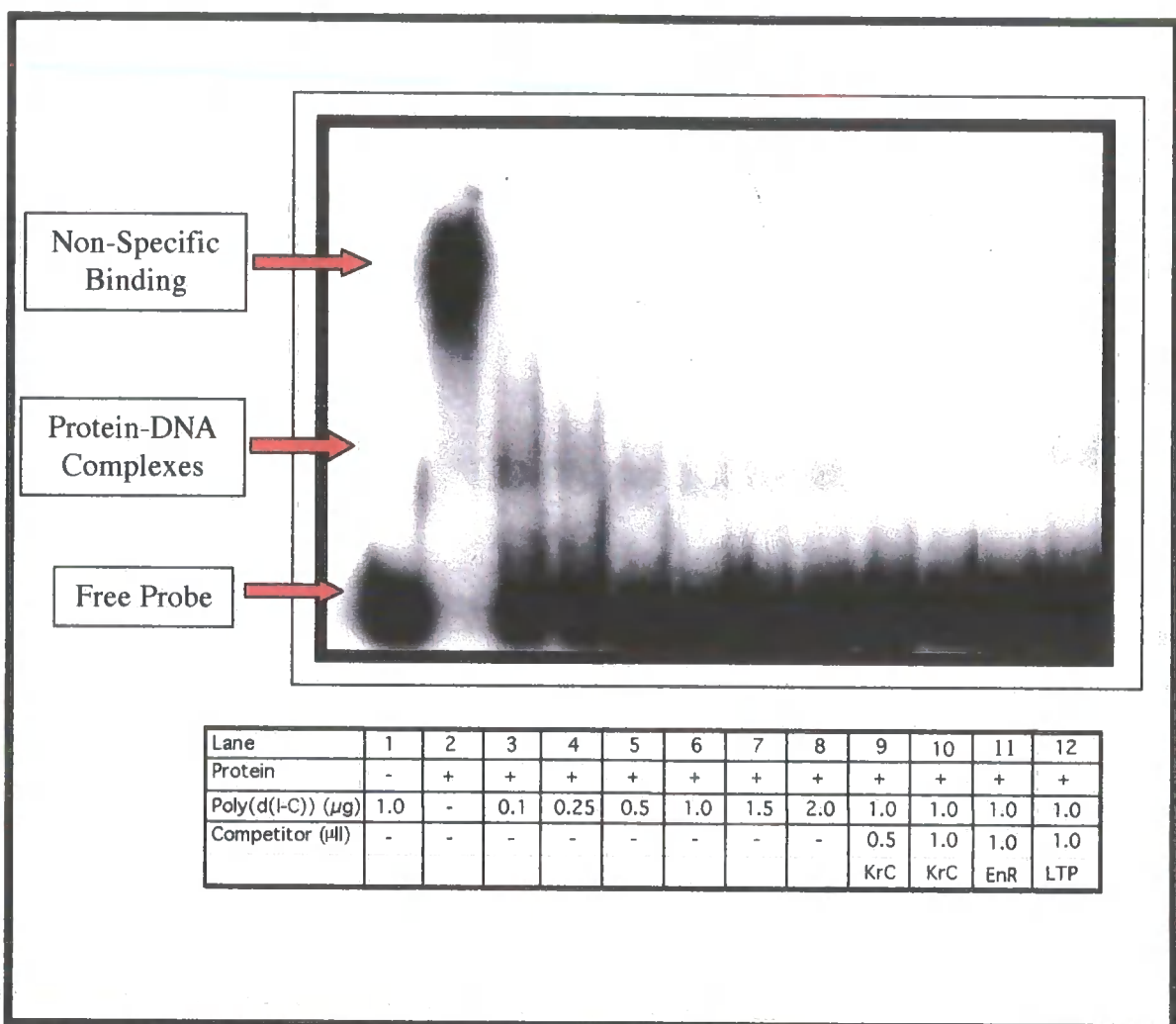
Free Probe



Protein	-	+	+	+	+	+
Poly(d(I-C)) (μg)	1.0	1.0	1.0	1.0	1.0	1.0
KrE (μl)	-	-	1.0	2.0	3.0	4.0

Figure 4.10 Competition Analysis on Fragment KrE

Gel mobility shift analysis on KrE, using KrC, EnR and LTP as competitors. Lanes contain **1)** free probe **2)** probe and protein extract **3-8)** poly (d(I-C)) gradient (0.1µg-2µg) **9-10)** poly (d(I-C)) and KrC as competitors **11)** poly (d(I-C)) and EnR as competitors **12)** poly (d(I-C)) and LTP as competitors. The results indicate the protein-DNA complex appears to be specific, as with 2µg of poly (d(I-C)) a protein-DNA complex is still visible. The KrC and EnR fragments appear to compete this band away, suggesting that they contain the same sequence present in KrE to which the protein is binding. However the LTP did not appear to have any affect on the protein-DNA complex.



expected to bind to both the EnR and β -Kr promoters. The LTP promoter was also used as an unlabelled competitor, upon the addition of LTP to the KrE fragment, there appeared to be no affect on the specific protein-DNA complex, indicating that the LTP promoter sequence does not appear to contain the same specific binding site for the protein that binds to the KrE fragment.

4.3.6.3 Competition Analysis on Fragments KrC, KrD, and KrF

Gel mobility shift competition analysis was also carried out on the labelled fragments KrC, KrD, and KrF as probes. Similar results were achieved as for fragment KrE. Figure 4.11 shows the results using the KrC fragment as the probe, specific protein-DNA interactions were observed upon the addition of protein. The disappearance of these specific protein-DNA interactions was shown upon the addition of the EnR promoter, but not the LTP promoter. These experiments were also carried out using fragments KrD and KrF as the probes and similar results were obtained (figure 4.12). Therefore all four of the fragments examined in competition analysis (KrC-KrF) appeared to show specific protein-DNA interactions that appeared to be competed away by the addition of the KrE/KrC unlabelled β -Kr fragments and also the EnR promoter, but were not susceptible to the LTP promoter.

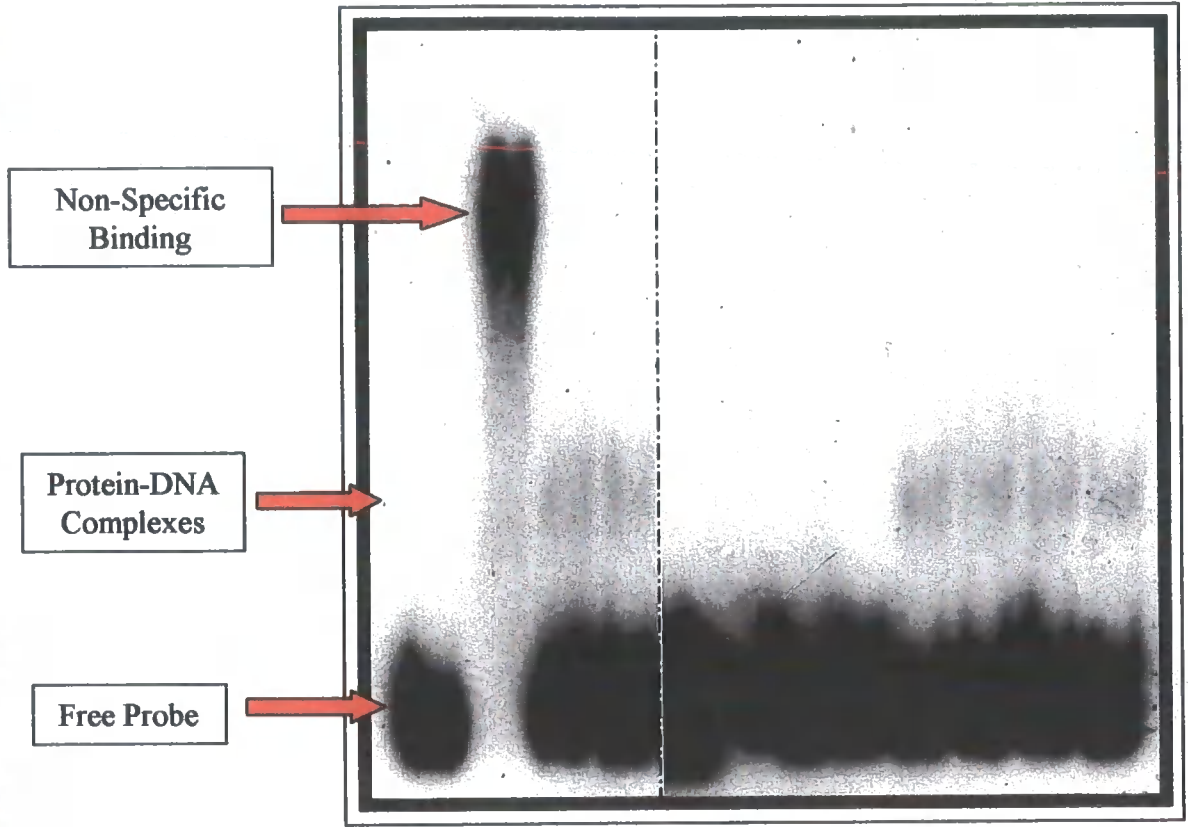
4.3.7 Sequence Comparisons of the β -Kr promoter Fragments

The results of the competition assays for the gel mobility shift analysis suggested that the same protein appears to binds at a number of points along the promoter region. This was shown by the β -Kr fragments (KrC-KrF) competing away specific protein-DNA complexes from one another. Computer analysis was therefore carried out on the fragments to see if a region of homology could be identified to which this protein was binding. A consensus 9bp sequence (CGCANTAAA) was identified in four of the six fragments (KrC-KrF). The chance of a 9bp sequence occurring randomly is

Figure 4.11 Competition Analysis on Fragment KrC

Gel mobility shift analysis on fragment KrC, using LTP and EnR promoters as competitors. The lanes contain 1) free probe 2) probe and protein extract 3-4) addition of poly (d(I-C)) to the probe and protein to detect specific interactions 5-8) poly (d(I-C)) and EnR as competitor DNA, showing the disappearance of the specific protein-DNA interactions previously observed 9-12) addition of LTP to probe, protein and poly (d(I-C)), here specific interactions can still be observed, indicating this protein appears not bind to the LTP.

(--- represents where lanes that have not been used due to the formation of poor wells have been removed, all the lanes originate from the same gel)

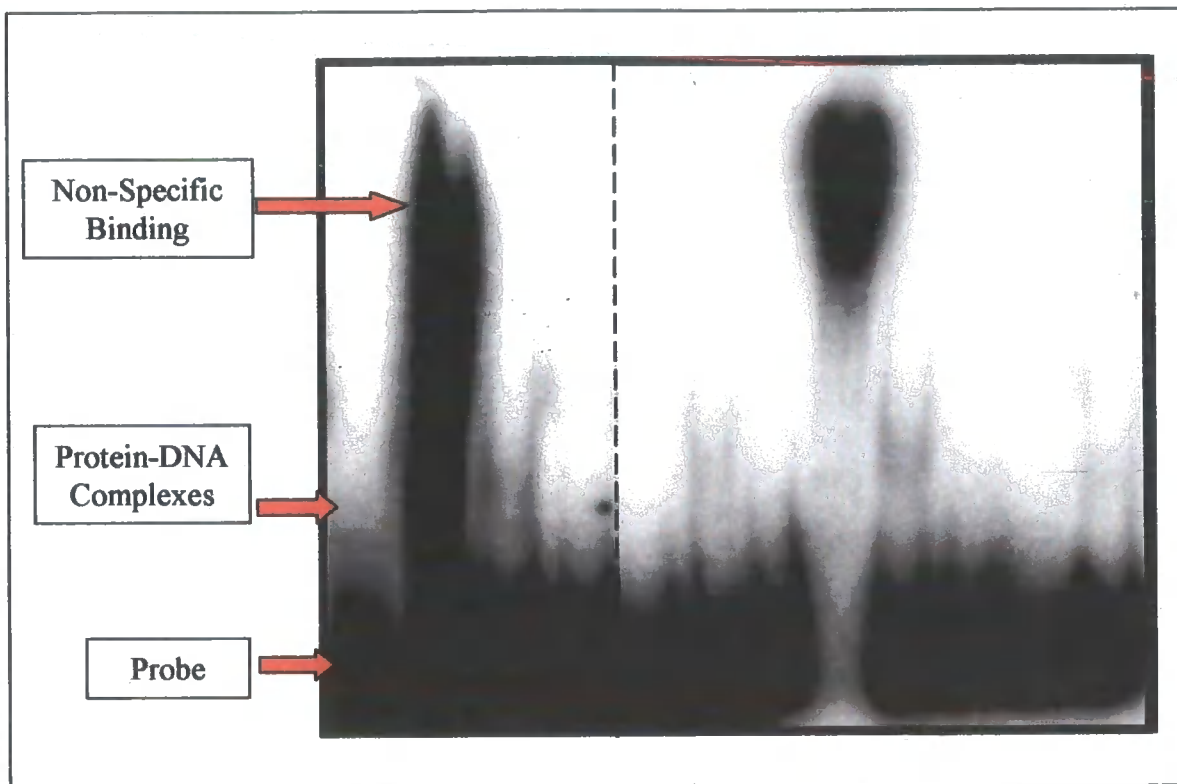


Lane	1	2	3	4	5	6	7	8	9	10	11	12
Protein	-	+	+	+	+	+	+	+	+	+	+	+
Poly d(I-C)	1	-	0.1	0.25	0.1	0.1	0.25	0.25	0.1	0.1	0.25	0.25
Competitor					0.5	1	0.5	1	0.5	1	0.5	1
					EnR	EnR	EnR	EnR	LTP	LTP	LTP	LTP

Figure 4.12 Competition Analysis on Fragments KrD and KrF

Competition analysis was carried out on the KrD and KrF fragments using the competitors poly (d(I-C)), KrE, EnR and LTP. Lanes 1-5 contain fragment KrD as the probe, and lanes 6-10 contain the probe KrF. The lanes contained **1&6)** free probe **2&7)** probe and protein **3&8)** probe, protein and poly (dI-C)) **4&9)** probe, protein, poly (d(I-C)) and EnR **5&10)** probe, protein, poly (d(I-C)) and LTP. Both fragments seem to be susceptible to the EnR promoter and the KrE fragment but not to the LTP promoter.

(--- represents where lanes that have not been used due to the formation of poor wells have been removed, all the lanes originate from the same gel)



Lane	1	2	3	4	5	6	7	8	9	10	11	12
Probe (Kr)	D	D	D	D	D	D	F	F	F	F	F	F
Protein	-	+	+	+	+	+	-	+	+	+	+	+
Poly d(I-C)(μ g)	1.0	-	1.0	1.0	1.0	1.0	1.0	-	1.0	1.0	1.0	1.0
Competitor				1.0 KrE	1.0 EnR	1.0 LTP				1.0 KrE	1.0 EnR	1.0 LTP

Very low and would be expected every 16577216bp. This sequence however appears twice within a few hundred base pairs. Figure 4.13 outlines the position of this sequence within the four fragments (KrC-KrF). This sequence only appears twice within the promoter sequence, however, by coincidence both times it appeared within the overlapping regions, therefore it was present in four of the fragments (KrC-KrF). It was these four fragments that were shown to compete away from one another. The exact 9bp consensus sequence was not identified within the enoyl-ACP reductase promoter sequence and so the sequence was examined in terms of Purines (R) and Pyrimidines (Y), and a similar sequence observed, as shown below. However the chance of this occurring is one in 512bp.

The 9bp consensus sequence was:

CGCANTAAA

This sequence was found to be present in KrC, KrD, KrE and KrF as shown below:

KrC CGCAGTAAA

| | | | | | | |

KrD CGCAGTAAA

| | | | | | | |

KrE CGCATTA AAA

| | | | | | | |

KrF CGCATTA AAA

EnR sequence TTATAACAGAA

| | | | | | | |

Consensus (Y and R) YRYR - YRRR

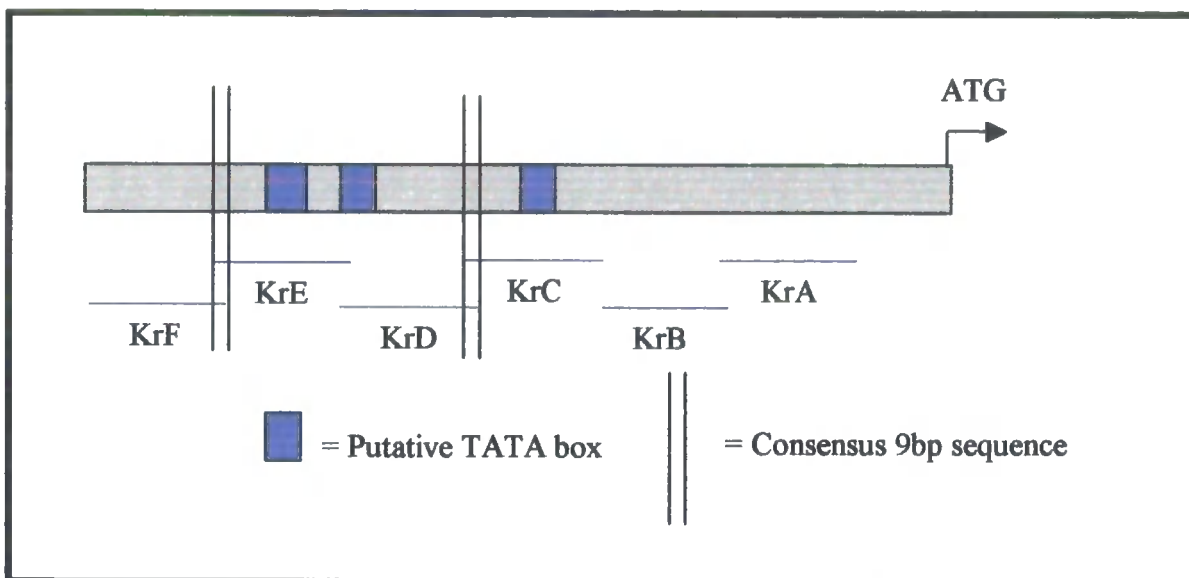


Figure 4.13 Schematic Representation showing the Position of the 9bp Consensus Sequence

The position of the six fragments within the β -Kr promoter is shown. Comparison analysis was carried out on these fragments and a 9bp consensus sequence identified within fragments KrC, KrD, KrE, and KrF. The position of this sequence is highlighted in the diagram.

The exact 9bp consensus sequence was not found within fragments KrA and KrB, however similar regions of homology within these fragments were found. Other lipid biosynthetic gene promoters were also analysed for the presence of this sequence from both *B.napus* and *Arabidopsis*. If this sequence is involved in the regulation of the lipid biosynthetic genes then it would be expected to be present within the promoters of these genes. The exact sequence was not shown to be present in any of the promoters examined, however similar sequences were identified. The identification of *cis*-acting elements is complicated by the fact that the protein may

sequence comparisons when searching the lipid biosynthetic gene promoters for this sequence more difficult, as the *cis*-element may differ by one or two base pairs but still bind the same protein.

4.4 Discussion

Six overlapping fragments were amplified from the *B.napus* β -Kr1 promoter upstream of the putative translational start site. All six of the fragments appeared to exhibit specific interactions with a *B.napus* embryo protein extract, indicating that they all contain potential *cis*-acting elements within their sequences, to which proteins bind. Competition analysis using unlabelled β -Kr promoter fragments demonstrated that fragment KrE appeared to compete away from KrC, KrD and KrF any specific protein-DNA interactions formed. Therefore suggesting that the fragments may share a common binding site to which the protein binds. The same protein therefore appears to bind at more than one point along the β -Kr promoter. This multiple binding observed is a common feature of eukaryotic transcription factors. An example of multiple copies of a motif being present within a promoter region, is the α -subunit of the soybean β -conglycinin gene. Within the promoter multiple copies of the motif A A/G C C C A are found (Chen. *et al.*, .1986; 1988). This motif was observed to be the core of the protein binding site that interacts with an embryo nuclear protein, SEF (Allen *et al.*, .1989). Another example is from the WRKY family of transcription factors. WRKY1, 2 and 3 have been shown to bind to W boxes showing slightly different sequences containing a core motif of TGAC surrounded by slightly different bases (Rushton *et al.*, 1996). Two WRKY transcription factors ABF1 and 2 bind to a Box 3 sequence, this element was shown to contain 3 repeats of the core W-box motif located within a region of only 22bp (Rushton *et al.*, 1992).

Competition analysis was carried out using fragments KrC-KrF as radioactively labelled probes, however due to problems with the protein-DNA binding conditions

within the assay and time constraints no competition analysis was carried out using fragments KrA and KrB. Therefore it was only observed that fragments KrA and KrB showed protein-DNA binding, it is not known whether these fragments would compete away the from the other fragments (KrC-KrF). It is therefore unknown whether these fragments are binding the same protein as fragments KrC-KrF appear to bind. However similar sequences were identified in both KrA and KrB, therefore it could be that it is the same protein that is binding to all the fragments, but that the protein does not need the whole sequence for recognition. Transcription factors are shown to have certain base pairs within a sequence that are more important for binding than the others. Therefore only four or five base pairs can be needed for the specific binding of the protein, and within the sequence one or two base pairs can differ and still the protein will bind. For example the DREB proteins are thought to specifically interact with certain bases, 4th and 7th C and the 5th G (Sakuma *et al.*, 2002).

The LTP was used as an example of a non-lipid biosynthetic promoter, therefore anything that binds to this fragment is likely to be more of a general transcription factor, and not specific to the lipid biosynthetic genes. Results indicated that the protein-DNA interactions formed were not susceptible to the addition of LTP and therefore the protein binding to the β -Kr fragments did not appear to bind to the LTP promoter competitor. The enoyl-ACP reductase promoter sequence was used as a competitor to examine if the protein binding was specific to other lipid biosynthetic promoters. Therefore if all of the lipid biosynthetic genes share the same controlling factor then binding to both the EnR and the β -Kr promoters may be indicative of a lipid biosynthetic specific transcription factor. The competition assays involving the EnR promoter showed that this fragment appears to compete away any specific binding from the β -Kr fragments (KrC-KrF), suggesting that the same protein that binds to the β -Kr may also bind to the EnR promoter.

Comparison of the promoter fragments showed four of the fragments possess a 9bp consensus sequence. However, along the length of this promoter region the sequence

only appears twice. This is because this 9bp consensus sequence is present within the overlapping regions of the fragments. Upon examination of the EnR promoter this common sequence was also found using Y (pyrimidines) and R (purines). Other fatty acid biosynthetic gene promoters have also been analysed for this element, however within *cis*-elements often only a few of the nucleotides are thought to be important that for recognition by the protein. Therefore the sequence can differ by one or two base pairs and still bind the same protein. This therefore complicates the identification of potential sequences within other promoters, when carrying out sequence comparison analysis.

The future analysis of the *cis*-acting elements within the β -Kr promoter would firstly involve the examination of KrA and KrB in competition analysis to ascertain if these sequences are binding the same protein as the other fragments. Also this 9bp consensus sequence could be multimerised and used as a competitor within the assays to examine if this sequence is important for protein binding. Future work could be carried out using the gel mobility shift analysis to examine protein binding to other lipid biosynthetic gene promoters. One disadvantage of the gel mobility shift analysis technique, is that it does not reveal the DNA binding sites that the protein actually binds to, therefore DNase I footprinting could also be used in conjunction with the GMSA assay.

Chapter Five

Transient Expression Experiments using a Deletion Series of the *B.napus* β -Keto-ACP Reductase Promoter

5.1 Introduction

5.1.1 Transient Expression Experiments

The transformation of plants cells using the natural process adopted by *Agrobacterium* was previously discussed in chapter three. This process of plant transformation is the most commonly used and requires a biological vector to deliver the DNA of interest into the plant cell. A second type of transformation, Direct Gene Transfer (DGT) uses either, chemical, physical or electrical techniques to introduce the DNA into the plant cell. This type of transfer is often used when the plant species is incompatible with the *Agrobacterium* method of transformation. However in this case the DGT method of transformation was carried out due to time constraints, as upon design of the constructs the results can be observed within days. Unlike stable plant transformations where there is a delay incurred due to the life cycle time of the *Arabidopsis* plant. Examples of DGT methods include: microinjection of DNA, electroporation induced uptake, particle bombardment and silicon carbide fibre-induced DNA uptake.

The DGT technique used in this study of the *B.napus* β -Krl gene involved the particle bombardment of whole plant tissues (leafs, roots and embryos) (Sanford *et al.*, 1987). This is the most popular of the DGT techniques used. The DNA of interest is targeted into the plant cell using dense microscopic particles coated with the plasmid of interest. The particles are delivered into the target cells using gas, air, gunpowder, or electrical discharge. Gold beads were used as the particles within this study and helium used as the method of delivery into the plant cells.

Transient Expression experiments have been used in studies to examine a number of plant promoters. The promoters are fused to a GUS construct and bombarded into plant material. The affect of a deletion series can then be examined. Examples include the deletion series of the *Arabidopsis* AVP1 gene (Mitsuda *et al.*, 2001). Deletion constructs were prepared and fused to GUS. The affect of the deletion series showed that the promoter could be deleted down to 418bp and still function as a promoter. The putative TATA box was also confirmed to be the functional one. The barley gene (*blt* 4.9) was also used in transient expression experiments to examine the affect of a deletion series on GUS expression (Dunn *et al.*, 1998). Both positive and negative *cis*-acting elements were identified and a 42bp sequence within these barley transient assays was found to be involved in enhancing the low temperature response of the plant.

Other studies have been carried out using stable transformations of plant promoters to examine the affect of a deletion series of the promoter on GUS expression within transgenic plants. Thereby identifying putative *cis*-acting elements important in the expression of the gene. An example of a fatty acid promoter studied is the EnR promoter (de Boer *et al.*, 1999). A deletion series of the EnR promoter from *Arabidopsis* was designed using the 1470bp upstream of the translational start codon. Deletion constructs were made and fused to a GUS cassette. The GUS expression was examined in transgenic tobacco within different tissues. The results analysed suggested the presence of different *cis*-acting elements involved in differential expression of the gene within tissues.

The ACP promoter from *Arabidopsis* was also examined for important regulatory elements by using deletion constructs fused to the GUS reporter gene and transforming them into tobacco. These promoter deletions showed that a 180bp domain was sufficient to drive expression within seeds and that other elements were required for expression within leaves and roots (Baerson *et al.*, 1994)

The napin promoter is an example of a non fatty acid promoter examined using transgenic tobacco for examination of regulatory *cis*-acting elements (Ellerstrom *et al.*, 1996). The deletion of the region from -309 to +44 of the *B.napus napA* promoter was analysed. The results suggested the presence of specific *cis*-acting elements for expression of the gene in both embryos and endosperm. Five different *cis*-acting elements were identified within the sequence from -309 to -152, one endosperm and four embryo specific elements. Therefore different elements were shown to be responsible for expression of this gene within different tissues.

5.1.2 Aim

Upon identification of the 1Kb 5' sequence functioning as the *B.napus* β -Keto-ACP reductase 1 (β -Kr1) promoter (Chapter 3) and identification of potential *cis*-acting elements (Chapter 4), this study was carried out to examine the minimal region of the β -Kr 5' sequence that would act as a promoter and direct transcription. Thereby identifying the minimum sequence containing all the necessary basal transcriptional machinery. Unlike the stable transformations in *Arabidopsis* (Chapter 3), these transient expression experiments were carried out in *B.napus* plant tissues. This chapter involved: the design of a deletion series from 998bp to 132bp of the 5' sequence, the amplification of the seven promoter deletion fragments and the cloning of these into a specifically designed pUC19/GUS vector. These GUS constructs were then used for subsequent bombardment experiments into *B.napus* tissues, and the GUS expression analysed in root, leaf and embryo material.

5.2 Method

All standard molecular biology techniques used in this study are described in chapter two. Any primers and conditions specific to this chapter are outlined below.

Table 5.1 – Primer Sequences

Primer	Sequence
Pr27 (Kr1)	5' TGCTCTAGACCAACTAAACACTGTACAACC 3'
Pr28 (Kr2)	5' TGCTCTAGAGGATACTACTACAAACTGGG 3'
Pr29 (Kr3)	5' TGCTCTAGAAGTTTTGATGTTATATACGGGT 3'
Pr30 (Kr4)	5' TGCTCTAGAAAGTTTCACCCGCGCATGG 3'
Pr31 (Kr5)	5' TGCTCTAGATGAACAGCATAAAGACAACC 3'
Pr32 (Kr6)	5' TGCTCTAGAACCATTTTGAACAGATAAATGG 3'
Pr34 (Kr7)	5' TGCTCTAGAGAGATGACGTGGCAACTTCGGAC 3'
Pr35 (SnaB 1)	5' AACGGTGATACGTACACTTTTCCCGGC 3'
Pr35 (For)	5' TTGTAAAACGACGACGGCCAGTG 3'
Pr36 (Rev)	5' CACACAGGAAACAGCTATGACC3'

Table 5.2 - PCR Conditions

	1 cycle	30 cycles	1 cycle
Denature	95°C 5mins	95°C - 30secs	72°C 5mins
Anneal	-	54°C - 30secs	-
Extend	-	72°C - 90secs	-

5.3 Results

5.3.1 Design of the Deletion Series

A deletion series was designed to produce fragments from 998bp to 132bp in length from the 5' region upstream of the putative translational start site from the *B.napus* β -Kr1 promoter. This was devised using the putative 9bp *cis*-acting element previously identified in the gel mobility shift assays (GMSA box - Chapter 4), and also three putative TATA boxes identified by PLACE. The fragments were designed so that upon subsequent deletions different *cis*-acting elements (either a GMSA box or a putative TATA box) were deleted. Thereby eliminating down to find the minimal sequence that would act as the β -Kr1 promoter, containing the functional TATA box. Therefore the importance of the putative *cis*-acting elements (GMSA and TATA boxes) could be examined. Figure 5.1 shows the position of the primers, the size of the fragments and the position of the GMSA and putative TATA boxes. Figure 5.2 shows the β -Kr1 5' sequence highlighting the position and sequence of the primers.

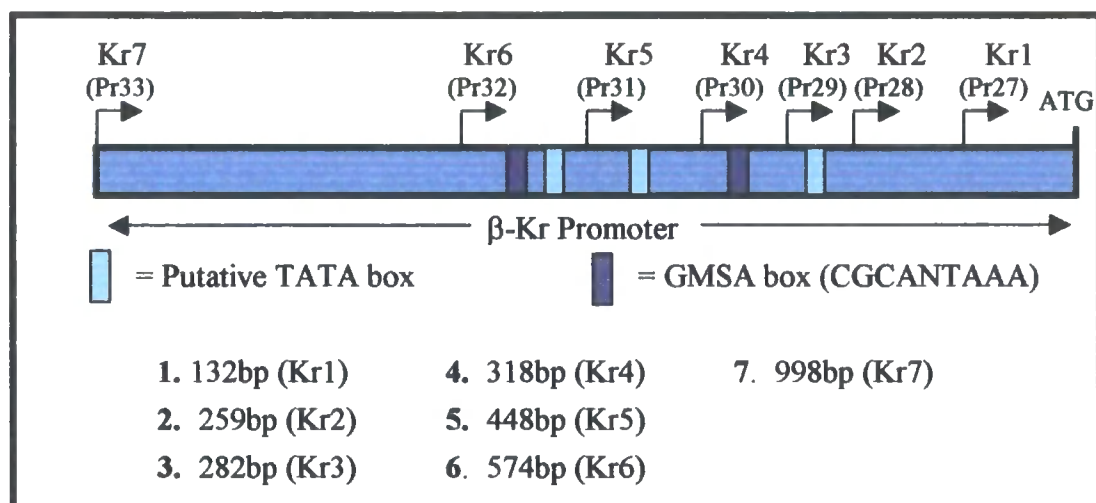












Figure 5.1 Diagrammatic Representation of the β -Kr1 Promoter

The position of the putative TATA boxes and GMSA boxes located within the 5' sequence of the β -Kr promoter are shown. The size and position of the seven promoter fragments are also given.

Figure 5.2 5' Sequence of the β -Kr Promoter Fragments

Sequence of the promoter fragments including the beginning of the GUS sequence is shown. The Xba primers (Pr27-Pr33) and also the SnaB 1 primer (Pr34) located within the GUS sequence (as discussed in section 5.3.4) are highlighted. The GMSA elements are boxed; the GUS sequence shown in bold and the primers are identified as in the key.

-1034 TTTTACGAAAAAATAAATGAGATGACGTGGCAACTTCGGACTCTCTGAT
 TGGACGATTTTTTGTGCCTACGTGGACACTCTAAAATGAGCTTATATCCT
 -934 CCTTTTAGTATAGTATAGATTTCAAAGTCCCAAAATTATTAATTTATAGA
 GATTTTACTGTATATTAATTAAAGAGCATTACAACCTTTGAGTAAATCTG
 -834 ATTGGGTTTTTTCAGAATCTTTAGTTAATAAAATATGAAAGTTCGTATTAT
 TTTAAAAAAATACAGGTATTTGTAACGATAGTTAGACTATATTTAAAT
 -734 ATGACTAGTTAGTAATAGTATATGTTAAGAGAAATATATAAAGTATAGTT
 TAAAAAAATGTTGTACATAGAATCAAATTGTATCCAACCTTTGTGATTCA
 -634 AATAGATTATAGATGATGTAAATAAGATAAACCAAAACCATTTTGAACAG
 ATAAATGGTAAATATTTTGAAAGATAAGTGAACACACATCCCTCTTCTGT
 -534 ATCGCATTAAGAAATATCACATTGTTATTAATCTGAGTGTTTTTATGTA
 ATTTAAAAAAATGAACAGCATAAAGACAACCATAATATATACATAATCC
 -434 GACATTCAAGATTTATACATGCTTCAACCAAGAATTTTTTTTTTAATGTTT
 ATGCTTAATTTGAAAAGAAAACCTATTGTAGTTTTTTTAAATTTAAGTTTCA
 -334 CCCGCGCATGGCGCAGTAAATTTAAGTTAGTTTTGATGTTATATACGGGT
 TGGATACTACTACAACTGGGAAAACCTTTAAAATCAGTTCGGTCCGGTC
 -234 CGGTACGGTTCAATCTAGCCTCCTTCCAACCTCTCTCGTCTGGCCGTCTAA
 ATTCAACCGAAGCTCCAACCTACTGACTACCAACTAAACACTGTACAACCT
 -134 CGTCCGTATCGCTTTTTCGAATTAAATATTCACACCTTTTTCTCTCTCTC
 TCTCTCTCTCTCTCTCTACACTGTGTCGATCTTTACGAGGTTCACCTAG
 -34 CTCGGATCCCGGGTGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCA
 ACCCGTGAAATCAAAAACCTCGACGGCCTGTGGGCATTGAGTCTGGATCG
 CGAAAACCTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAA
 GCCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTTCGCCGATGCA
 GATATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCGAAGTCTTTAT
 ACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCCGGTC
 ACTCATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTGATGGAGCATCA
 GGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCC
 GGGAAAAGTGACGTATCACCGTTTGTGTGAACAACGAAGTGAAGTGAAC
 TGGCAGACTATCCCGCCGGGAATGGTGATTACC

 = Pr33	 = Pr32	 = Pr31
 = Pr30	 = Pr29	 = Pr28
 = Pr27	 = Pr26	 = Pr34
 = GMSA box		

5.3.2 Construction of the GUS Vector (pUC19/GUS)

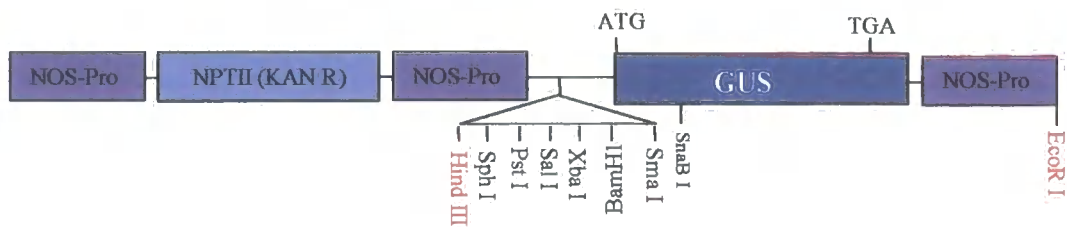
A GUS vector (pUC19/GUS) was constructed into which the seven promoter fragments were cloned. The cloning vector pUC19 was used as the basic template for these transient expression studies. The GUS cassette was digested out of the pBI101.1 vector (used in the previous stable transformations, Chapter 3) using Hind III and Ecor 1 restriction sites and ligated to the pUC19 vector (figure 5.3). This pUC19/GUS vector was sequenced to confirm that the GUS cassette had been correctly ligated into the pUC19 vector (Pr35 and Pr36). The pUC19/GUS vector was used as the negative control and also as a basic template vector into which the promoter fragments were inserted in front of GUS.

5.3.3 Cloning of the Promoter Fragment Kr7 into pUC19/GUS

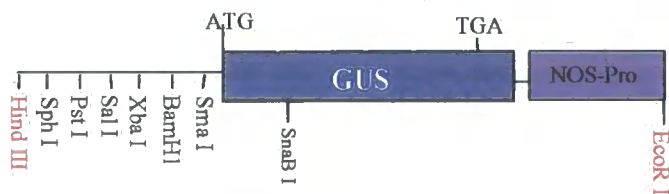
The designed primers (Pr27-Pr33) were used to amplify the deletion promoter fragments with restriction sites to enable cloning into the pUC19/GUS vector. Figure 5.2 shows the sequence of the β -Kr promoter fragments and the beginning of the GUS sequence, highlighting the position of the primers. However the largest fragment Kr7 was cloned into the pUC19/GUS vector using a different 3' restriction site to the smaller fragments (Kr1-Kr6). Construct Kr7 had previously been amplified, cloned (into pBI101.1) and sequenced in chapter 3. This was labelled KrII for chapter 3, but was the same sequence as Kr7 used for this study. Therefore this promoter sequence had previously been proven to act as a promoter within the *Arabidopsis* stable transformations, and was used to prepare the Kr7 construct within these transient expression experiments. The KrII fragment was digested out of the original KrII/GUS (pBI101.1) construct (Chapter 3) using Xba 1 and BamH 1 restriction sites (these restriction sites are found within the multiple cloning sites upstream of GUS) and cloned into the pUC19/GUS vector, as shown in figure 5.4 (and labelled Kr7). This construct was then transformed into *E.coli* and sequenced to confirm that the promoter insert was correct.

Figure 5.3 Preparation of the pUC19/GUS Vector

The GUS cassette was digested out of the pBI101.1 (using EcoR 1 and Hind III) and ligated into the pUC19 vector. This pUC19/GUS vector was used as the negative control and also the basic template into which the promoter fragments (Kr1-Kr7) were cloned.

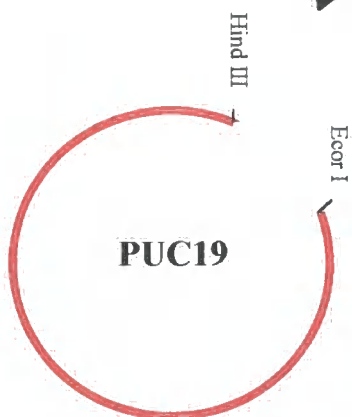


pBI101.1 digested with
Hind III and EcoR I



GUS Cassette

pUC19 digested with
Hind III and EcoR I



GUS cassette
ligated into pUC19

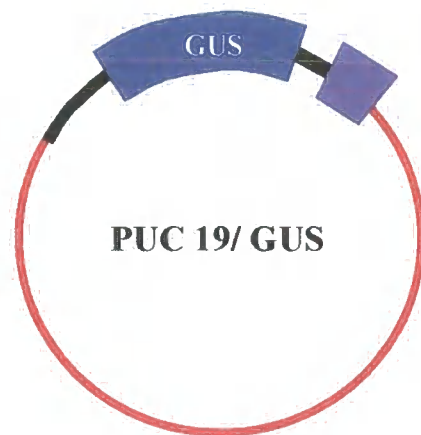
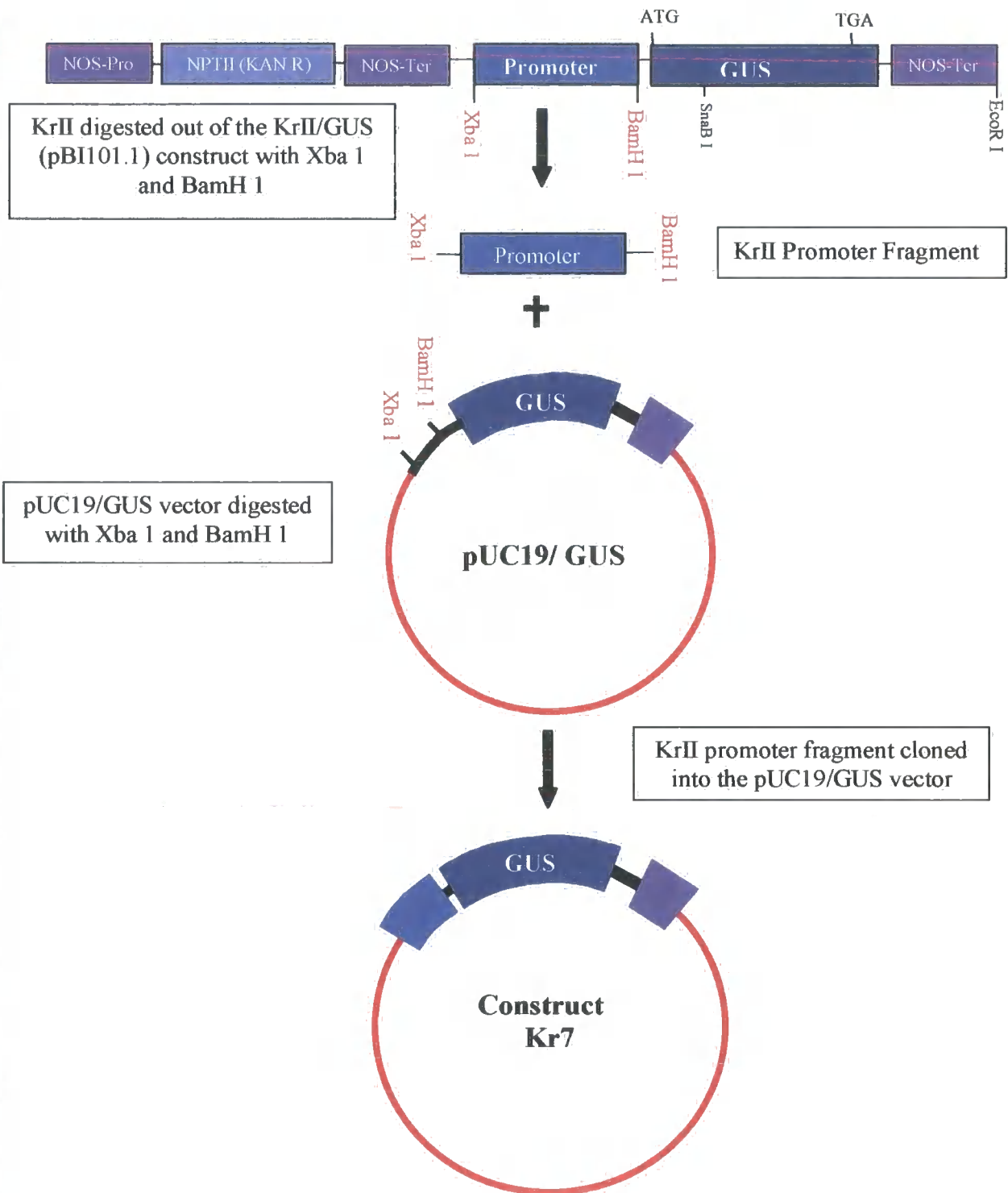


Figure 5.4 Preparation of Construct Kr7

The KrII promoter fragment was digested out of the KrII/GUS construct (in pBI101.1 - used in Chapter 3) with the restriction sites Xba 1 and BamH 1. The KrII promoter fragment was then ligated into the pUC19/GUS using the restrictions sites Xba 1 and BamH 1 and sequenced to confirm that it was correct (this construct was called Kr7 for these transient expression experiments).



5.3.4 Cloning of Fragments Kr1-Kr6 into the pUC19/GUS Vector

The six smallest promoter fragments (Kr1-Kr6) were cloned into the pUC19/GUS vector using a different 3' restriction site to that used for the Kr7 construct. The KrII/GUS construct (within pBI101.1) used in Chapter 3 for the stable transformations was used as the PCR template for the amplification of Kr1-Kr6. A SnaB 1 restriction site was identified within the GUS sequence. The 5' primers were positioned to obtain the deletion series and were then used in combination with the 3' primer containing the SnaB 1 site to amplify the fragments (figure 5.5). The reason for using this SnaB 1 site was to increase the size of the fragments obtained (as they would contain part of the GUS sequence), thereby assisting with the cloning.

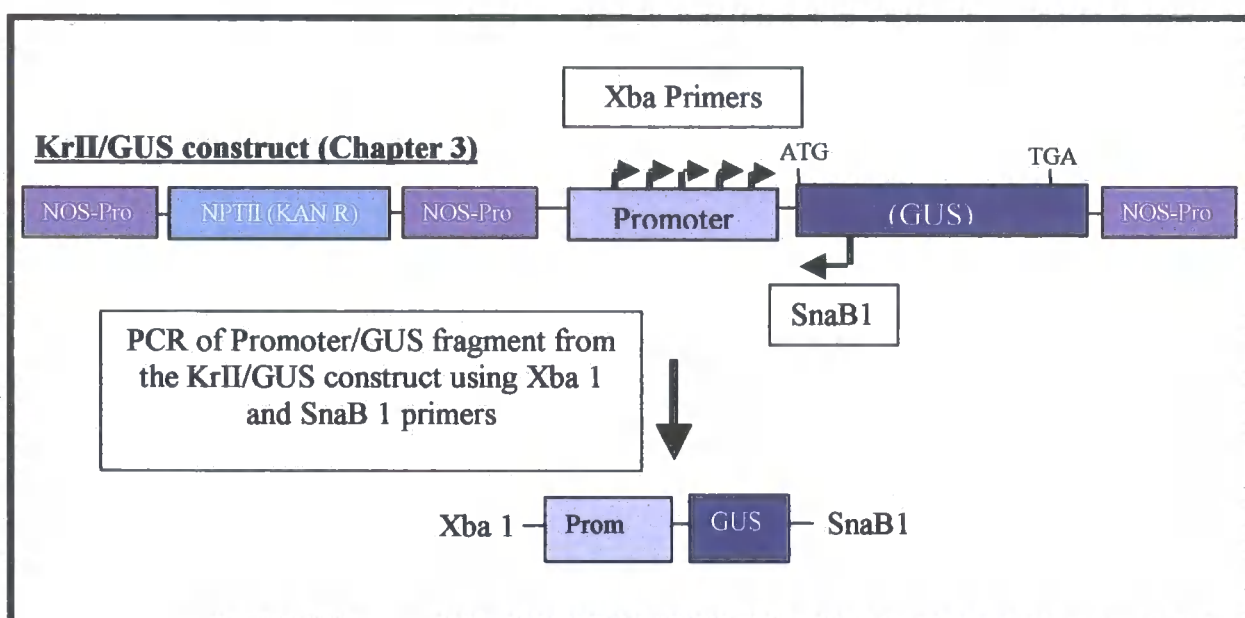


Figure 5.5 Amplification of the Promoter/GUS fragments (Kr1-Kr6)

Schematic representation showing the position of the Xba 1 and SnaB 1 primers (Pr27-Pr34) within the KrII/GUS construct (used for the stable transformations - Chapter 3). The primers were used to amplify by PCR the promoter/GUS fragments to be cloned into the pUC19/GUS vector (figure 5.7).

The promoter fragments (Kr1-Kr6) were amplified by PCR (figure 5.6A), gel purified (figure 5.6B), cloned into the pUC19/GUS vector using the Xba 1 and SnaB 1 restriction sites (figure 5.7) and transformed into Xl1 blue competent cells. Digestion analysis was performed (figure 5.6C) using Xba 1 and SnaB 1 restriction enzymes, to confirm that pUC19/GUS constructs contained an insert. The positive colonies identified were then sequenced (Pr35 and Pr36) to confirm that the correct promoter fragment was present. Midi plasmid preparations were carried out for all the constructs (Kr1-Kr7) to obtain a sufficient quantity of plasmid and diluted to 1µg/µl.

5.3.5 Transient Expression Experiments using the β-Kr Constructs

Two positive control promoters were used in the transient expression experiments, these were the pSCV 1.2 GI - CaMV 35S promoter and the pLH3 SCV - napin promoter (strong seed specific promoter) (both kindly donated by Dr Wallington, Biogemma Ltd., Cambridge). The largest promoter fragment Kr7 was also used as a positive control, as it had previously been shown to direct transcription within the stable transformations (Chapter 3). Therefore ten different constructs were used for these transient expression experiments, one negative and two positive controls and seven deletion constructs (Kr1-Kr7). *B.napus* plants were tagged and siliques obtained 30 days after flowering. The embryos were excised onto agar plates, along with leaves and roots from ten-day-old *B.napus* plants for the subsequent firings. Therefore each plate contained 50 embryos, 1 leaf and 1 section of root. The *B.napus* tissues were bombarded as in section 2.12, using gold particles coated with the plasmid preparations. The individual tissues were bombarded and left for 3 days, stained for GUS and analysed. One plate was used in these transient assays for each of the 10 constructs (with embryo, root and leaf tissue on each) and the experiment repeated the following day. The bombardments were also repeated one month later as no results were obtained for fragment Kr5; this was due to an aberration within the GUS sequence. Therefore the construct was remade and the experiment carried out in duplicate. The bombardments were therefore carried out four times for each construct, with new plasmid preparation dilutions each time.

Figure 5.6 Gel Analysis of the Promoter Fragments (Kr1-Kr7)

- A) Analysis of the amplified promoter fragments by PCR. The size of the five promoter fragments (Kr1-Kr5) were checked by gel electrophoresis and purified. The fragments contain part of the GUS sequence (414bp) and therefore are larger than just the fragment size. Lanes contained **1&2)** molecular size marker **3&4)** Kr5, **5&6)** Kr4, **7&8)** Kr3, **9&10)** Kr2, **11&12)** Kr1
- B) Upon purification quantitative analysis was carried out for both the promoter fragments and the pUC19/GUS vector for the subsequent ligations. Lanes contained **1)** Kr1, **2)** Kr2, **3)** Kr3, **4)** Kr4, **5)** Kr5, **6&7)** pUC19/GUS vector, **8-10)** molecular size marker.
- C) Digestion analysis was carried out on the positive colonies identified with the restriction enzymes Xba 1 and SnaB 1. Lanes contained: **1&11)** Molecular size markers **10)** Kr8 (this construct was prepared for the experiment but not used due to limited *B.napus* material, and it had already been shown to direct expression similar to Kr7 within Chapter 3 (Kr1)), **9)** Kr7, **8)** Kr6, **7)** Kr5, **6)** Kr4, **5)** Kr3, **4)** Kr2, **3)** Kr1, **2)** negative control

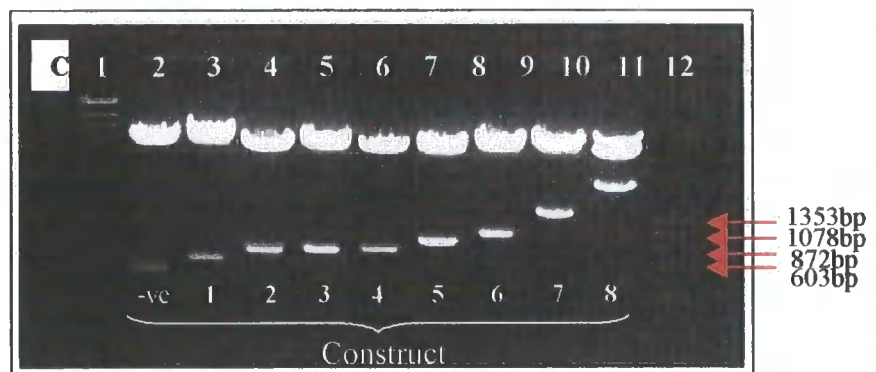
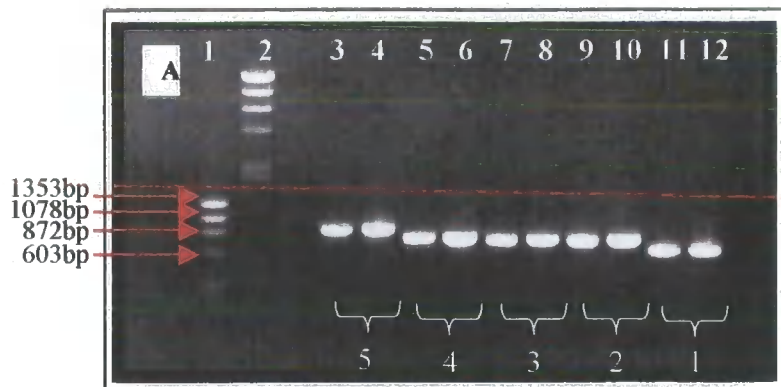
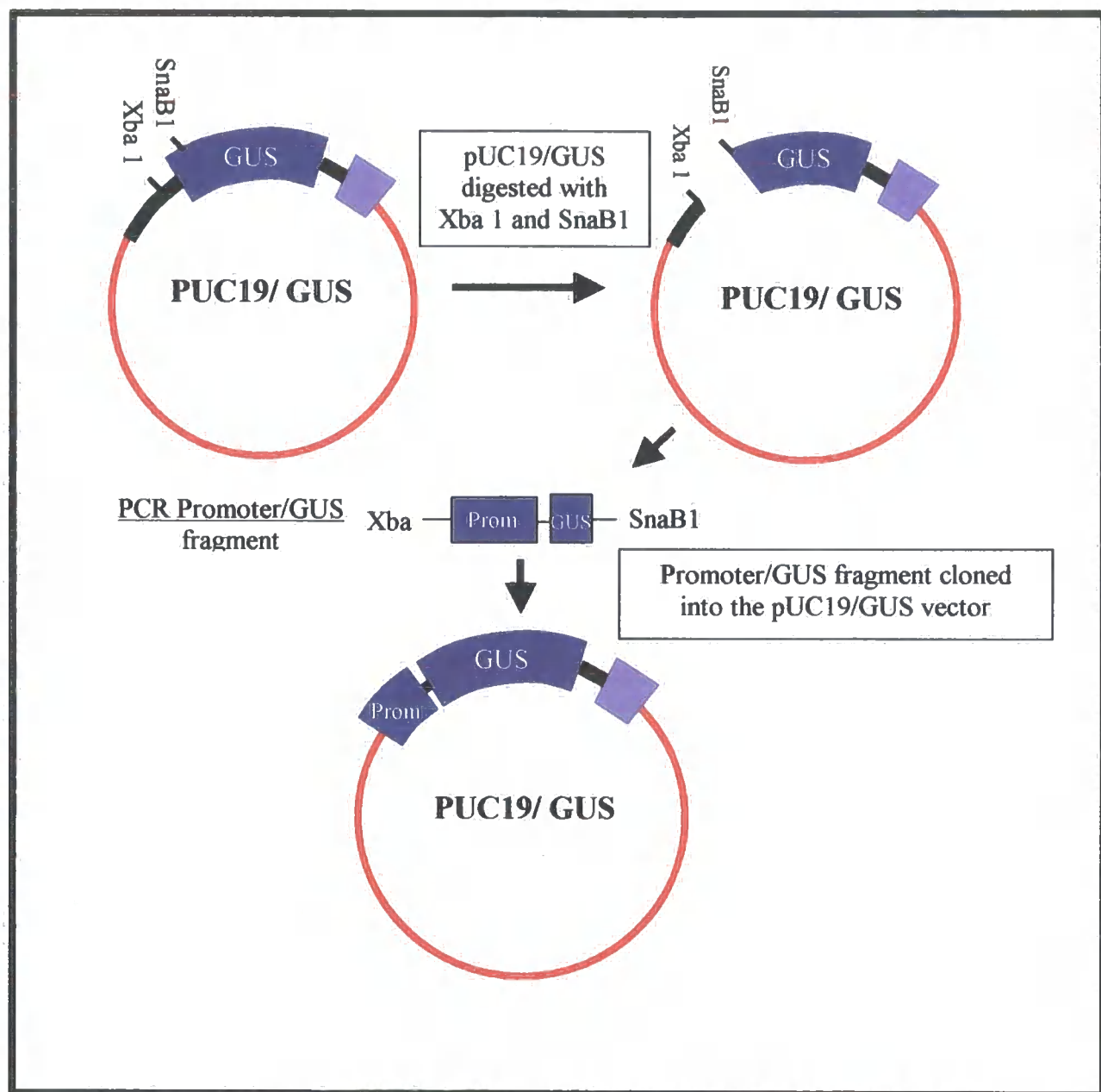


Figure 5.7 Cloning of the Promoter Fragments into pUC19/GUS (Kr1-Kr6)

The promoter/GUS fragments were amplified as outlined in figure 5.5 containing Xba I and SnaB I restriction sites. The promoter fragments were cloned into the pUC19/GUS vector using these restriction sites.



5.3.6 GUS Expression within the Control Constructs

The negative control construct, pUC19/GUS (containing no promoter insert) exhibited no GUS expression in both embryos and leaves as expected. An example of an embryo and leaf from the negative control is presented in figure 5.8. The brown spots confirm that the tissues have been bombarded, as these are where the gold particles have entered the cells, but no blue spots representing GUS staining can be observed. The napin promoter (PLH3 SCV) and the CaMV35S promoter were analysed as the positive controls (figure 5.9). The results for the napin promoter showed GUS expression within the embryos but expression was not observed within the leaves. This was expected as this promoter is highly seed specific in expression. CaMV35S, the second positive control exhibited expression in both leaves and embryos, this promoter is highly expressed within most tissues and so would be expected to show expression in this way. The dark brown spots are where the gold beads have entered the cells and the blue spots represent the areas of GUS expression.

5.3.7 GUS Expression within the Roots

The use of root material had never been performed before in transient expression experiments by this research group, therefore no positive root specific promoter was available. However the CaMV35S positive control promoter would be expected to show GUS expression within roots as this promoter is highly expressed within most tissues. The results of the stable transformations in Chapter 3 suggests that GUS expression would also be expected within the roots, as high expression was shown within the *Arabidopsis* transformants. However the results showed no GUS expression within roots for any of the β -Kr constructs or the CaMV35S positive control, which showed expression in both leaves and embryos but no expression within the roots. Therefore no results for the expression of the β -Kr gene in *B.napus* roots were obtained, and the results concentrated on the GUS expression within leaf and embryo material for the different promoter constructs.

Figure 5.8 Negative Control Construct within Embryos and Leaves

The pUC19/GUS vector was used as a negative control in these transient expression experiments. As expected the results showed no GUS expression in both embryos (A) and leaves (B). No areas of GUS expression (blue spots) were observed, however the brown spots where the gold beads have entered the tissues can be seen.

A



B

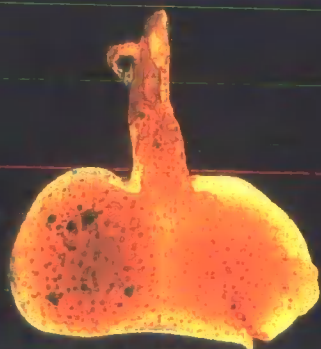


Figure 5.9 Positive Control Constructs within Embryos and Leaves

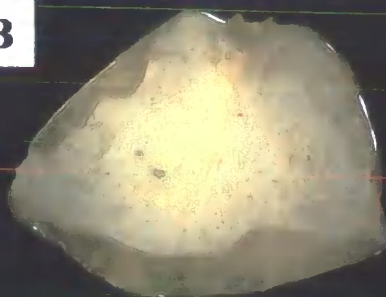
The CaMV35 and Napin promoter were used as positive controls in these transient expression experiments. A&B) = Napin, C&D) = CaMV35S

- A) Napin (embryo) – This promoter is highly seed specific and therefore GUS expression was observed within embryos.
- B) Napin (leaf) – No GUS expression was observed within leaves, this would be expected as the promoter is highly seed specific.
- C) CaMV35S (embryo) – GUS expression was observed within the embryo represented by blue staining.
- D) CaMV35S (leaf) – GUS expression was observed within the leaf represented by blue staining.

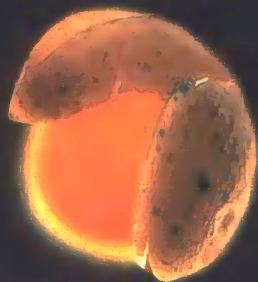
A



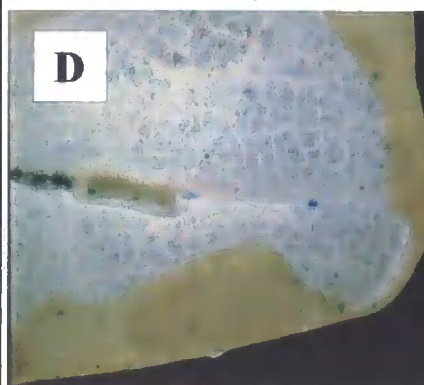
B



C



D



5.3.8 GUS Expression for Constructs Kr1-Kr7

The results obtained for the transient expression experiments are not quantitative as for the stable transformations in *Arabidopsis*. However it does give an answer as to whether or not the individual fragments would act as a functional promoter and direct transcription. For quantitative analysis a biolistic control would be required to be co-transformed alongside the β -Kr promoter constructs. This would allow the variation in particle penetration of the plant material to be taken into consideration. An example of a common control is the luciferase construct, thereby allowing the results to be normalised.

All the promoter deletion constructs showed GUS staining in both embryos and leaves except for Kr1 (132bp), the smallest promoter fragment. This construct showed GUS expression within embryos but no apparent expression within leaves. These results therefore show that the promoter can be deleted down to 132bp (Kr1) and still direct transcription with respect to embryos and down to 259bp to direct expression within leaves. This study was undertaken to examine the minimal sequence that could direct expression, however upon examination of the leaves and embryos noticeable differences between the promoter constructs were observed with respect to GUS staining. Therefore this 132bp sequence (Kr1) may direct transcription, but may only direct minimal expression. Therefore not containing the specific regulatory *cis*-acting elements that upregulate the basal expression of the β -Kr1 gene. However although differences were observed between the different constructs there are limits to the conclusions that can be made, as it is by no means quantitative. Therefore the following results only describe the observations of GUS staining and stable transformations would be required to obtain quantitative results. However the differences between the constructs appears to be more than a coincidence as the same concentration of plasmid was used for all of the fragments, a new dilution made up for each separate firing, and four firings carried out for each construct on different days. A similar pattern of GUS staining was observed each time; it could therefore be suggestive of a difference in the intensity of expression

between the fragments. The differences in staining were observed both in the size and number of spots and the number of embryos showing expression. The positive controls were taken as strong GUS staining to which the constructs (Kr1-Kr7) were compared.

5.3.9 GUS Staining within Embryos

The promoter constructs, Kr1-Kr7 all showed GUS staining within embryos. Therefore the promoter was shown to still direct transcription upon deletion to 132bp (Kr1), thereby containing the general transcription machinery necessary for basal transcription. However in comparison to the positive controls there appeared to be a reduction in GUS staining for Kr1, and therefore although this construct is able to direct transcription it may not contain the *cis*-acting elements responsible for upregulating the basal levels of transcription. Examination of the other constructs for GUS staining suggested a decrease in GUS staining upon certain deletions of the promoter within embryos. The two largest promoter fragments (Kr6 and Kr7) showed the highest GUS staining of all the constructs (figure 5.10). These constructs showed similar levels of staining to the napin positive control. Whereas construct Kr4 appeared to show a reduction in GUS staining compared to the positive controls. However it is difficult to conclude whether this apparent decrease in GUS staining occurred upon deletion to Kr5 (figure 5.11) or Kr4 (figure 5.12). Upon the deletion from construct Kr4 to Kr3 a further decrease in GUS staining was observed. Subsequent deletions from Kr3 seemed to have little affect on GUS staining and therefore the smallest GUS staining was observed for constructs Kr3, Kr2 and Kr1 (figures 5.13 & 5.14). These constructs showed very small areas of GUS staining on only 4 or 5 of the 50 embryos (figure 5.14). In comparison to the positive controls there was a noticeable reduction in GUS staining. Figure 5.15 shows a comparison of the embryos for the different constructs (Kr1-6).

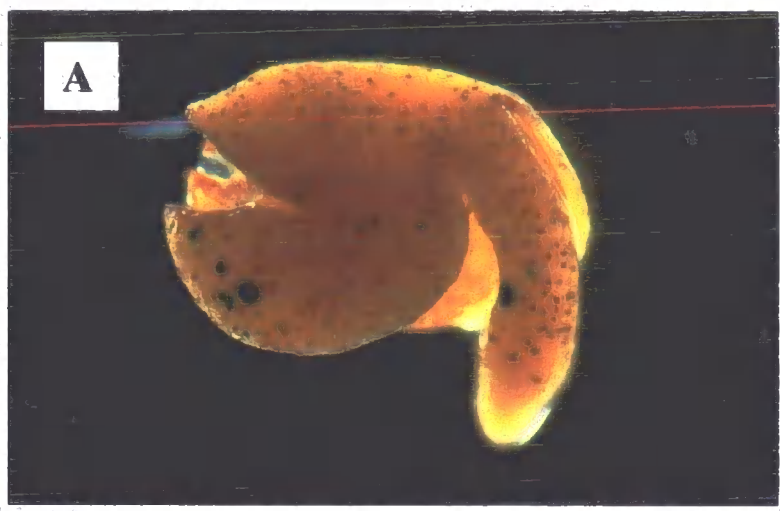
Figure 5.10 GUS Staining within Embryos for Constructs Kr6 & Kr7

Constructs Kr6 and Kr7 showed the largest areas of GUS staining for all the deletion constructs within embryos. Similar amounts of staining were observed as for the positive seed specific control (napin promoter). This suggests that these constructs may therefore contain all the general transcriptional machinery for basal transcription. However these sequences may also include specific *cis*-acting elements that upregulate the basal levels of transcription, as they appeared to show similar GUS staining to the napin promoter, an example of a strong seed specific promoter.

A) Construct Kr7

B) Construct Kr6

A



B

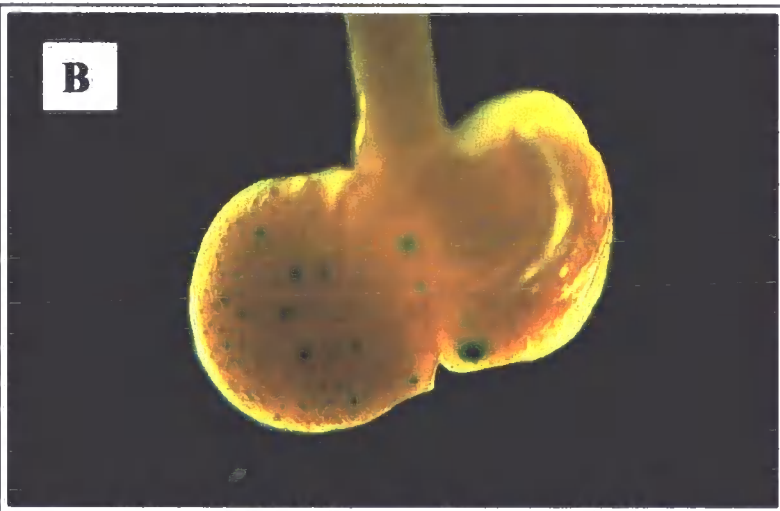
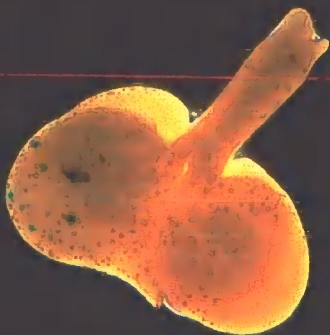


Figure 5.11 GUS Staining within Embryos for Construct Kr5

A-D are examples of GUS staining within the embryos for construct Kr5.

A



B



C



D



Figure 5.12 GUS Staining within Embryos for Construct Kr4

Examples of GUS staining within embryos for Kr4 can be seen in A-D. There appears to be a reduction in the GUS staining in comparison to the positive napin control promoter.

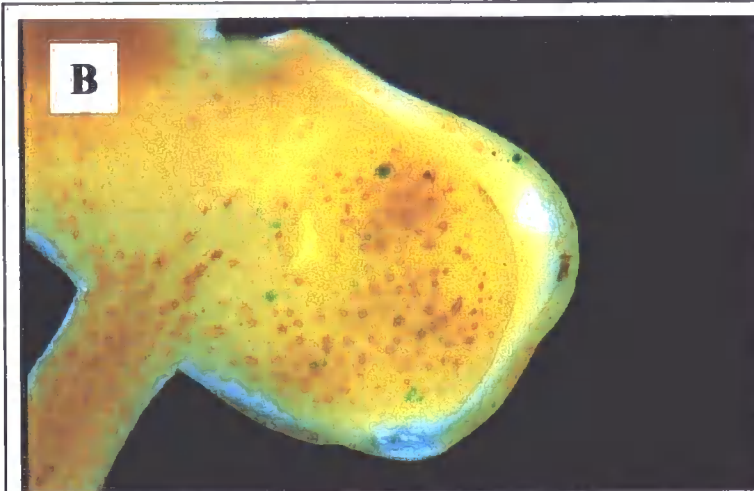
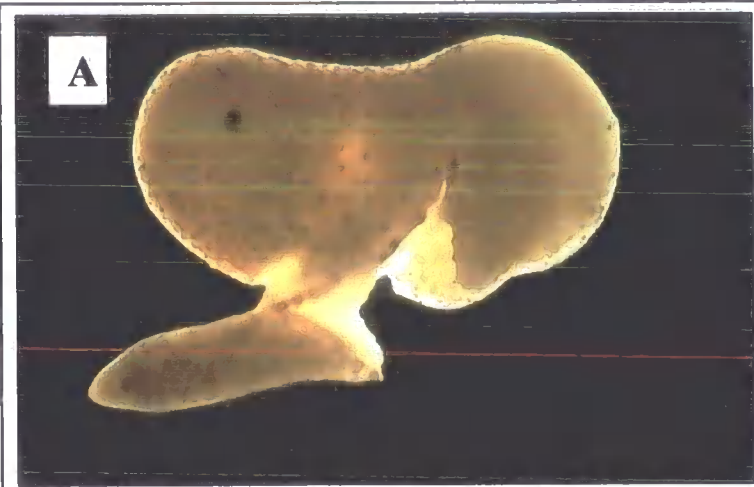


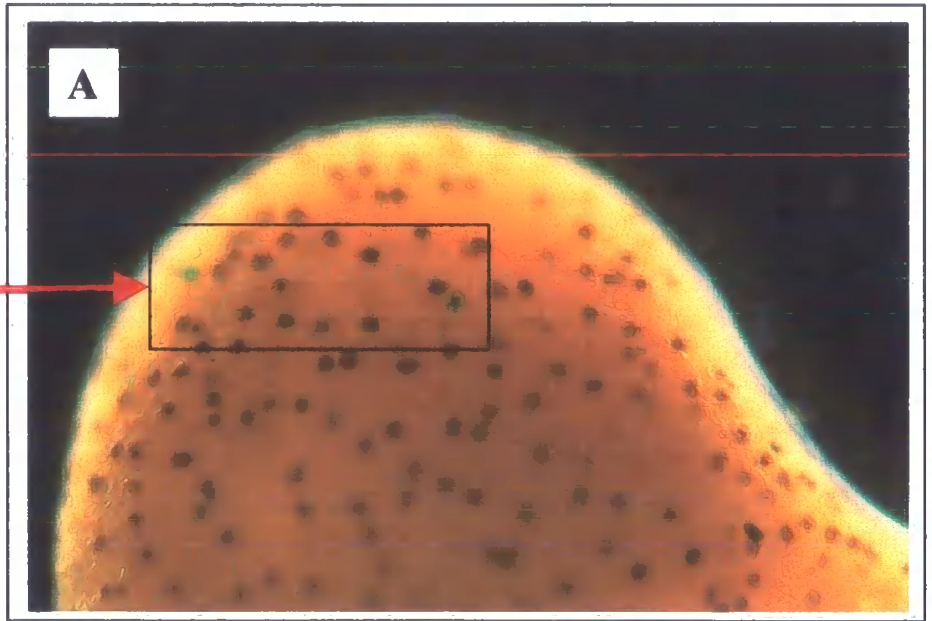
Figure 5.13 GUS Staining within Embryos for Constructs Kr2 & Kr3

There appeared to be a decrease in GUS staining upon deletion from Kr4 to Kr3. Constructs Kr2 and Kr3 showed similar amounts of GUS staining; this was very small and only observed within a few of the embryos. There was a noticeable difference in GUS staining between these constructs and the positive napin control promoter.

A) Construct Kr3 embryo

B) Construct Kr2 embryo

GUS
Expression



GUS
Expression

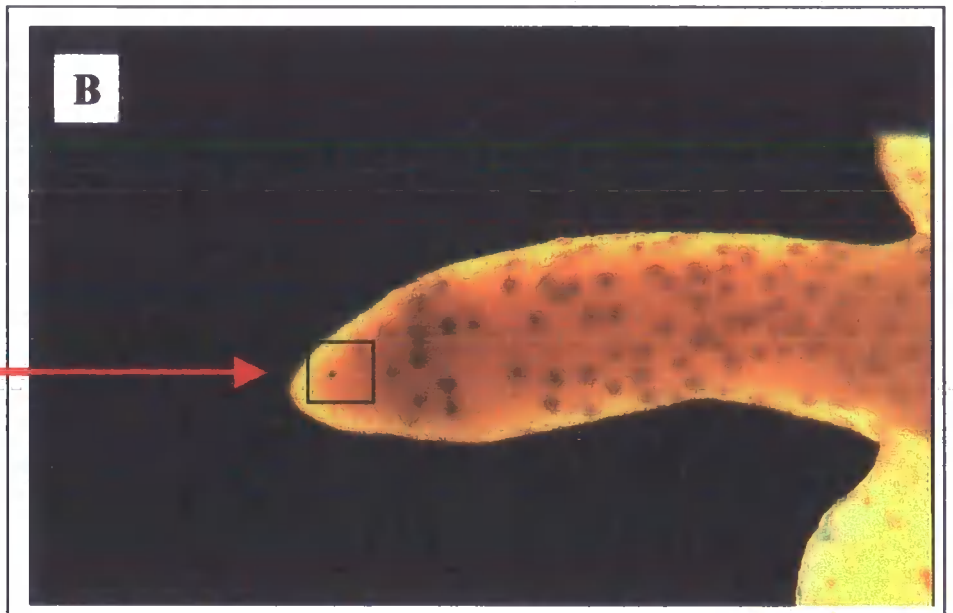


Figure 5.14 GUS Staining within Embryos for Construct Kr1

GUS expression was examined within the embryos for construct Kr1:

- A) Examination of Kr1 embryos showed very little GUS staining. Very small areas of GUS staining were observed for only 4 or 5 of the 50 embryos.
- B) Upon closer examination these areas of GUS staining can be observed. In comparison to the positive controls the GUS staining appears to be very small.

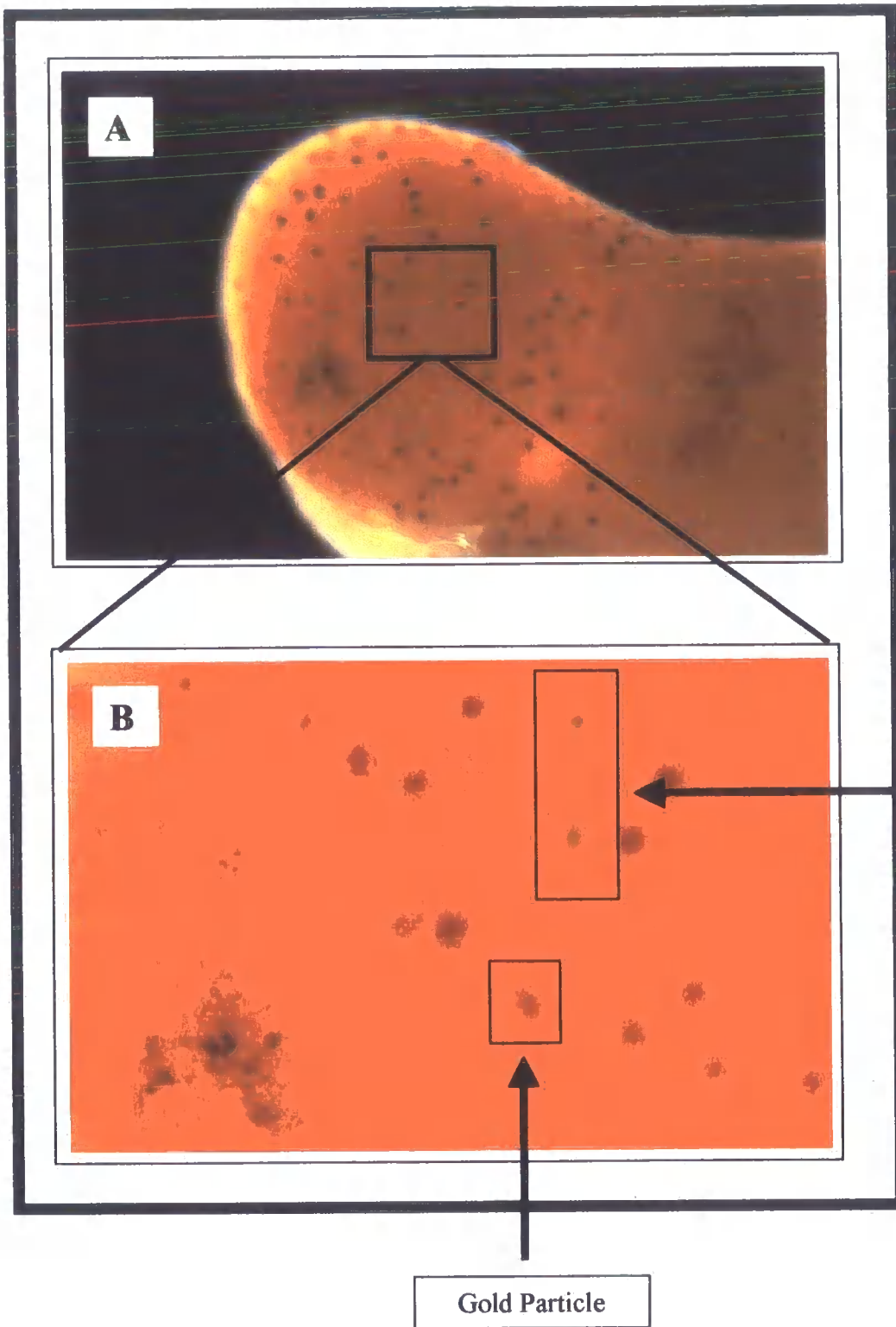
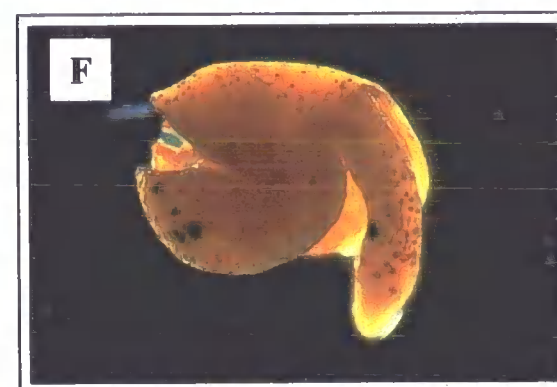
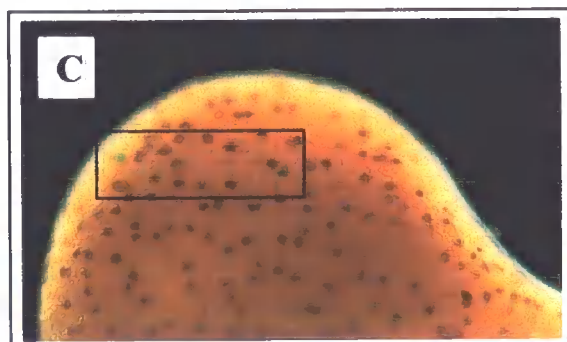
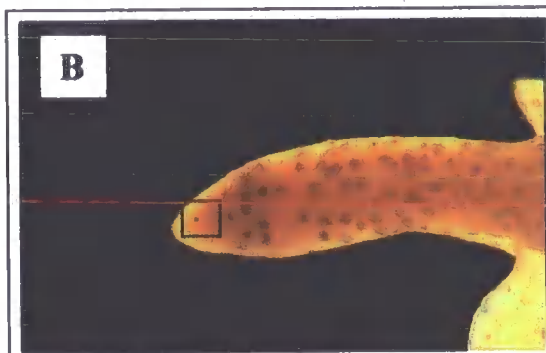
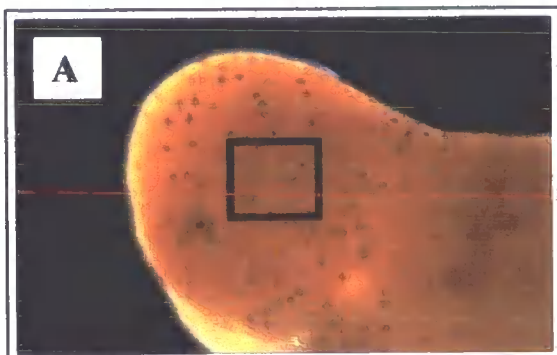


Figure 5.15 Comparison of GUS Staining within Embryos for Constructs Kr1-Kr6

The embryos for constructs Kr1– Kr6 were examined to compare the differences in GUS staining. Kr1-3 seemed to show the least GUS staining, with an apparent increase between constructs Kr3 and Kr4. The largest staining observed appeared to be for constructs Kr6 and Kr7, exhibiting similar GUS staining to the positive napin promoter.

- A) Kr1
- B) Kr2
- C) Kr3
- D) Kr4
- E) Kr5
- F) Kr6



5.3.10 GUS Staining within Leaves

The comparison of GUS staining within leaves was easier than for the embryos, as only four leaves were used in comparison to 200 embryos for each construct, however this does not give the same reproducibility. The three largest promoter constructs Kr5, Kr6 and Kr7 showed the largest GUS staining out of all the promoter fragments (figure 5.16 and figure 5.17). The staining was similar to that observed for the positive control (CaM35S). However upon deletion from construct Kr5 to Kr4 there was a noticeable decrease in GUS staining (figure 5.17). Kr5 exhibited GUS staining similar to that observed for the CaMV35S positive control, however Kr4 seems to show a large reduction in this staining with only one visible area of GUS staining. Subsequent deletions to constructs Kr3 and Kr2 showed no apparent further decrease in staining (figure 5.18 and figure 5.19). No areas of GUS staining were observed for Kr1. Therefore there appeared to be only one decrease in GUS staining observed, between constructs Kr5 and Kr4. Closer examination of a leaf for construct Kr2 can be seen in figure 5.19, showing where the gold beads have entered the cells, and the areas of GUS staining surrounding these particles can be observed. A comparison of GUS staining within the leaves for the different constructs can be seen in figure 5.20. Construct Kr2 was the minimal sequence shown to direct expression within leaves as construct Kr1 showed no visible GUS staining. It could be that construct Kr1 does not direct transcription unlike embryos or due to the staining not being obvious as it was so minimal. Within embryos, construct Kr1 showed very small GUS staining and only upon close examination was the staining visualised. Therefore it may be the case within leaves that the staining could not be seen. The leaf also appears semi-transparent upon incubation in ethanol and therefore it is more difficult to observe the areas of GUS staining.

Figure 5.16 GUS Staining within Leaves for Constructs Kr6 & Kr7

- A) Construct Kr7 – this construct showed similar GUS staining to the positive construct CaMV35S, suggesting this is strong GUS staining.
- B) Construct Kr6 – there appears to be no difference between constructs Kr6 and Kr7 in respect to GUS staining. Again this construct showed similar staining to the positive control CaMV35S. Therefore Kr6 and Kr7 both appeared to show high levels of GUS staining.

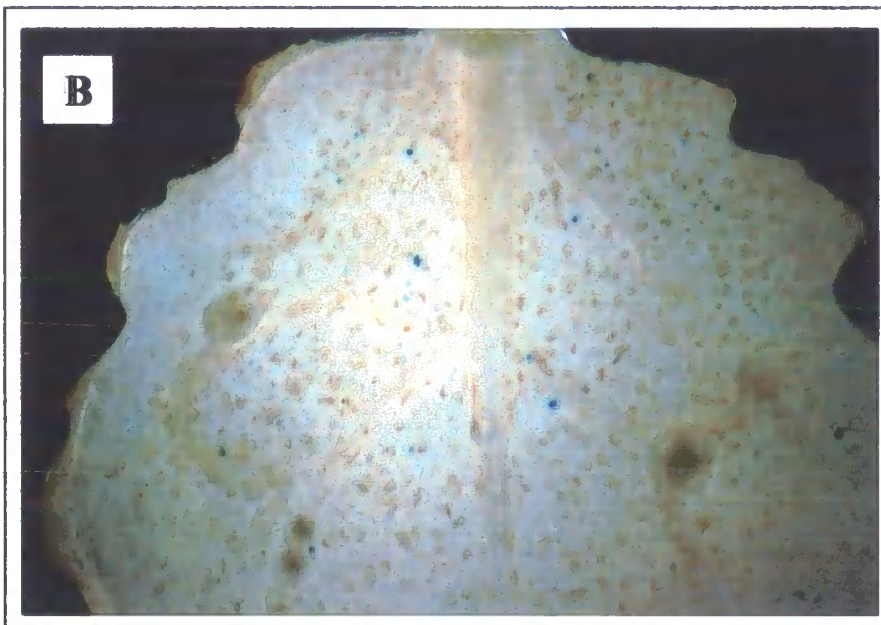
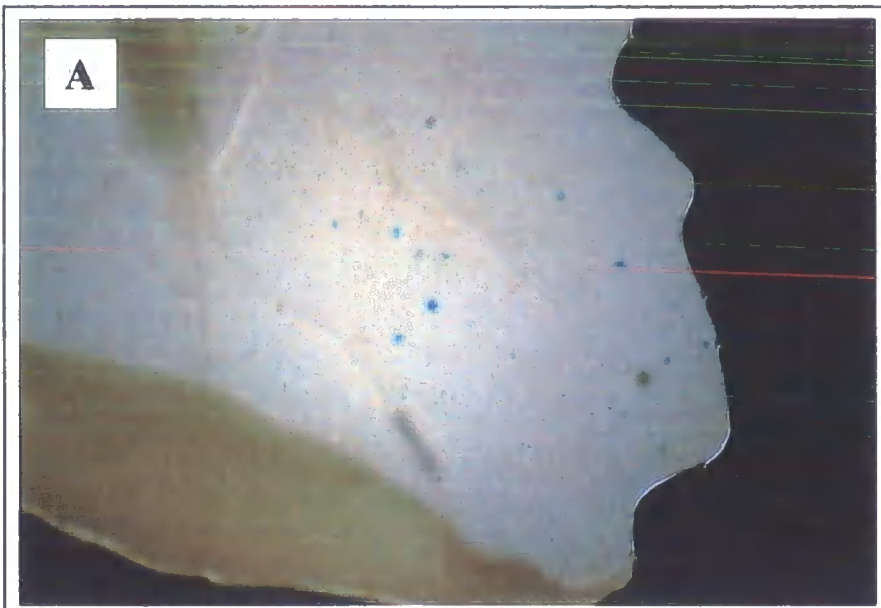
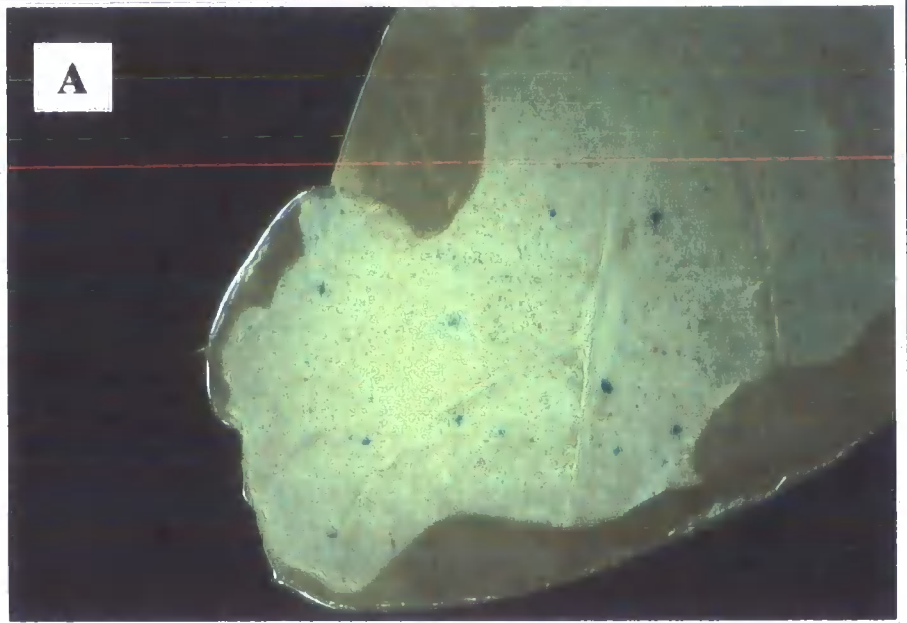


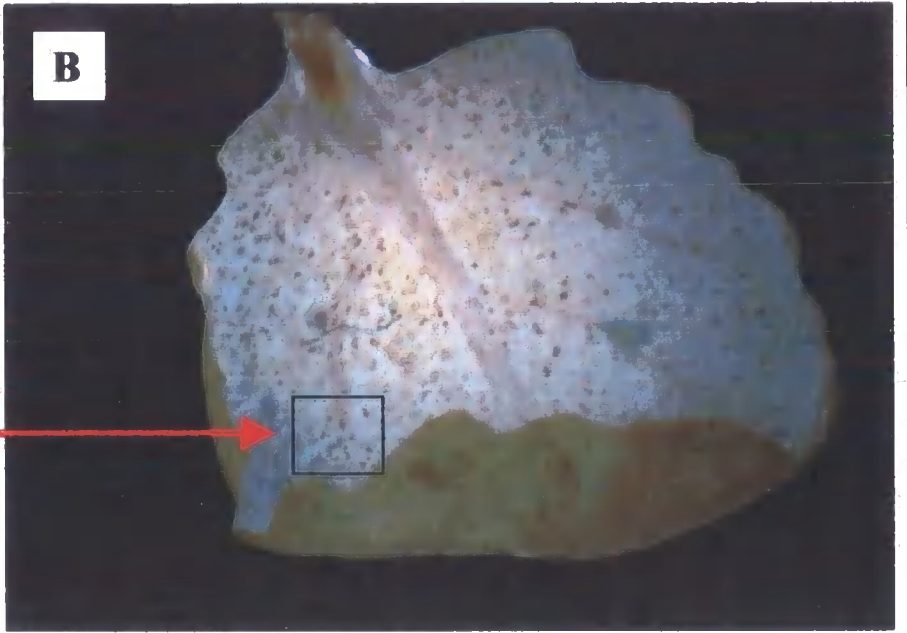
Figure 5.17 GUS Staining within Leaves for Constructs Kr4 & Kr5

- A) Kr5 showed similar GUS staining to Kr6 and Kr7 and the positive CaMV35S control promoter.
- B) There was a noticeable decrease in GUS staining apparent upon the deletion from Kr5 to Kr4.

A



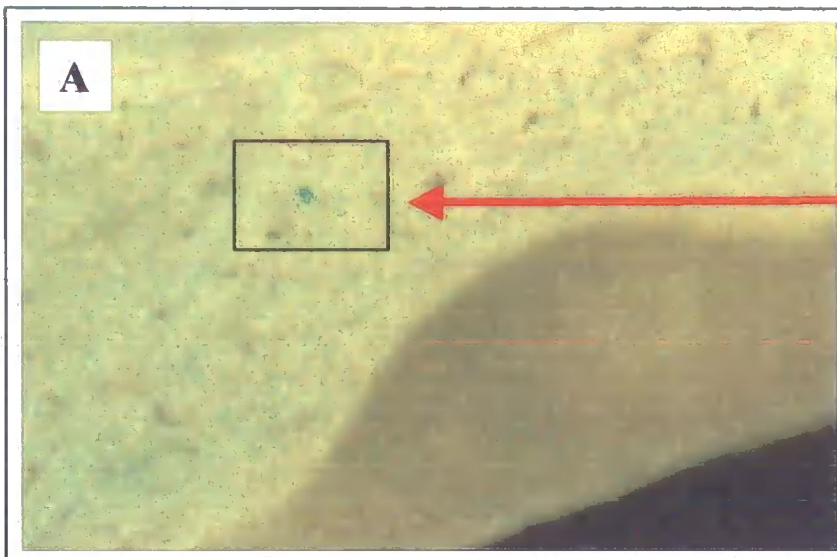
B



GUS
Expression

Figure 5.18 GUS Staining within Leaves for Constructs Kr1, Kr2 & Kr3

- A) Construct Kr3 – very small areas of GUS staining were observed in comparison to the positive control.
- B) Construct Kr2 – very small areas of GUS staining were observed with only one or two spots on each leaf. Therefore Kr4, Kr3 and Kr2 appeared to show the smallest GUS staining of all the constructs observed.
- C) Construct Kr1 - No GUS staining was observed.



GUS
Expression



GUS
Expression

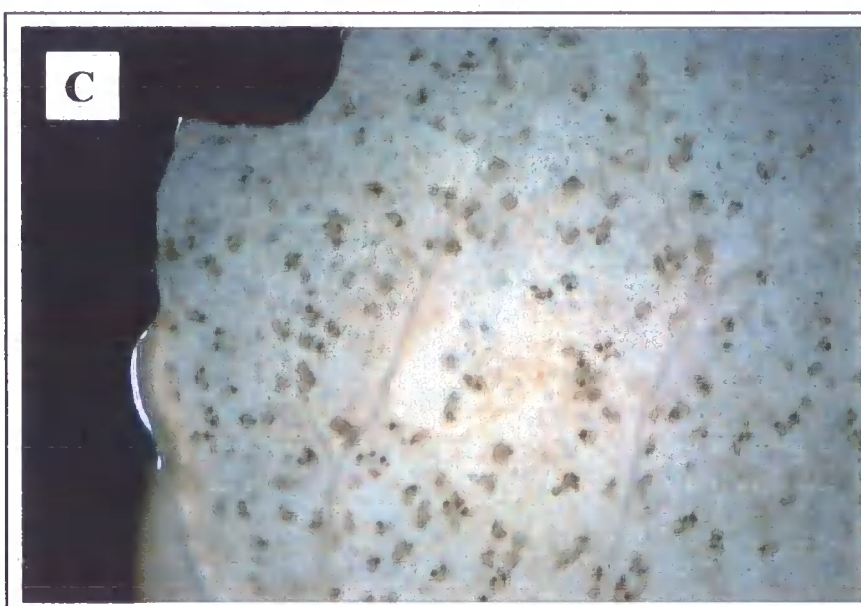


Figure 5.19 GUS Staining within Leaves for Construct Kr2

- A) GUS staining within leaves for construct Kr2.
- B) Closer examination of the leaf shows the area of GUS staining and allows the visualisation of where the gold beads have entered the tissue (brown spots) and the region of GUS staining surrounding the gold beads (blue spots).

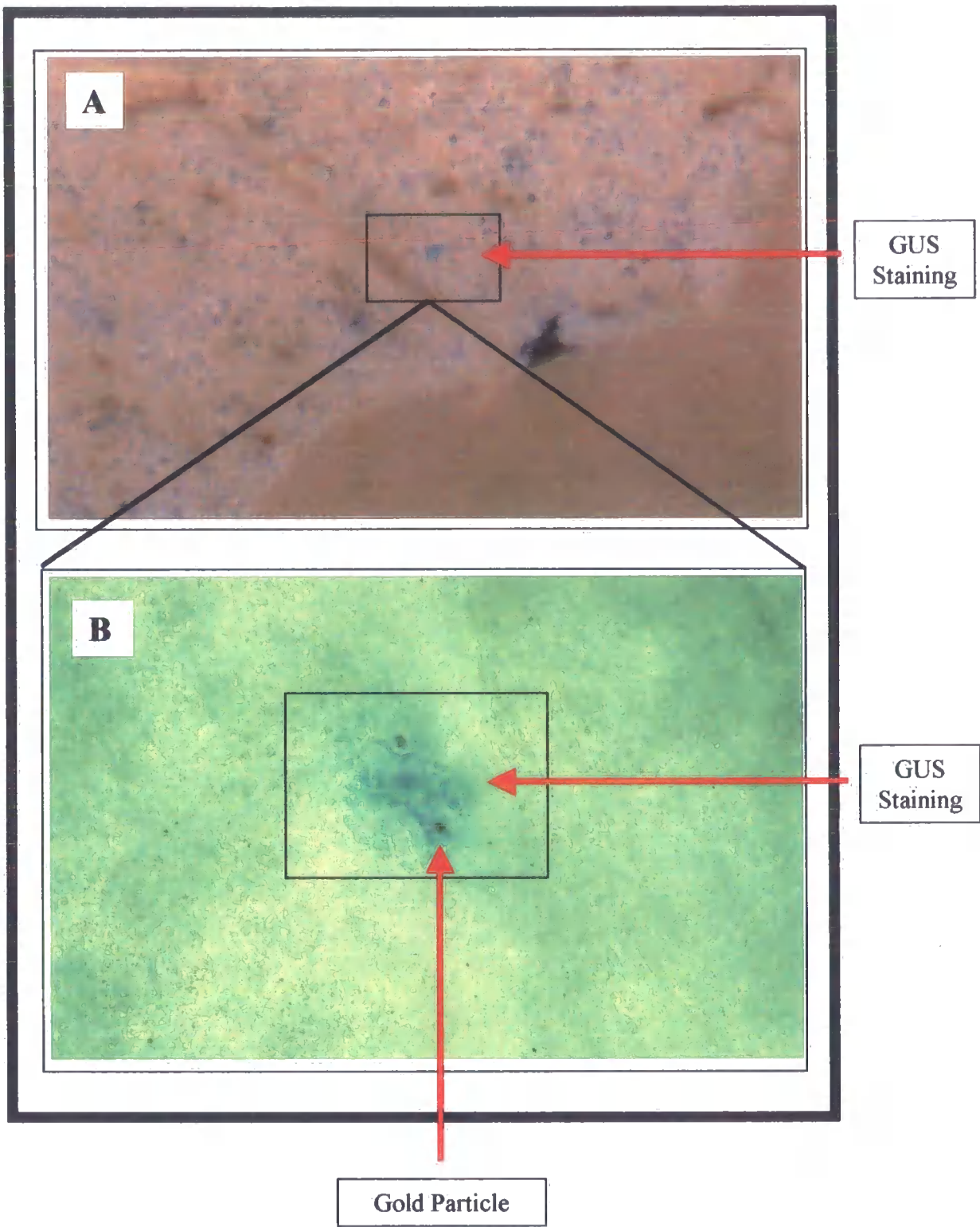
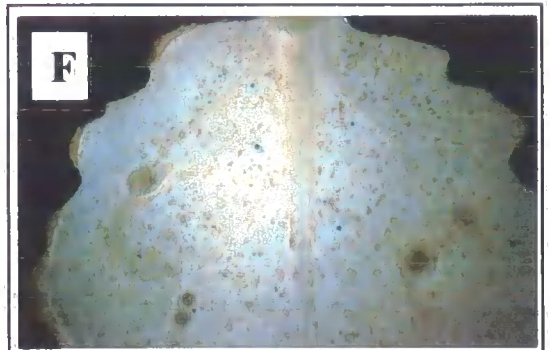
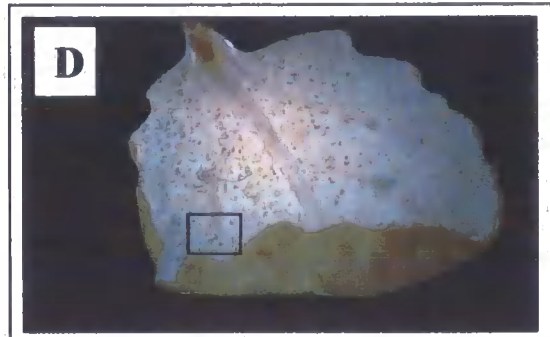
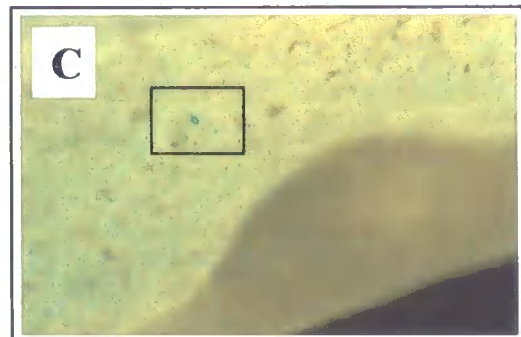
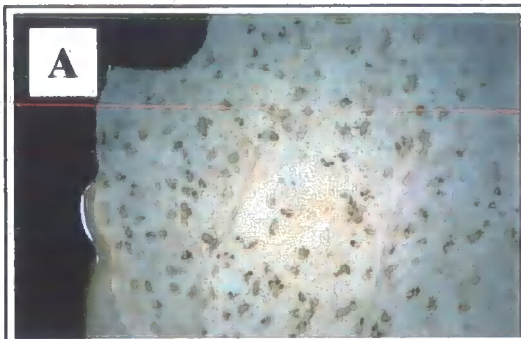


Figure 5.20 Comparison of GUS Staining within Leaves for Constructs Kr1-Kr6

The GUS staining between the different constructs is shown. There is a noticeable reduction in GUS staining between constructs Kr5 and Kr4. Kr2-Kr4 appear to show the smallest GUS staining observed and Kr5-Kr7 seem to exhibit the highest levels of GUS staining similar to the positive controls.

- A) Kr1
- B) Kr2
- C) Kr3
- D) Kr4
- E) Kr5
- F) Kr6



5.4.1 Discussion

These transient expression experiments were carried out to examine the minimal β -Kr1 promoter that would direct transcription. They were not undertaken as a quantitative study. However upon examination of the results, differences were apparent that seemed to be more than a coincidence. The constructs showed differences in GUS staining, and because the experiment was repeated four times for each construct and new dilutions made up each time it is unlikely that this has occurred by chance. Therefore these differences in GUS staining observed are discussed and examined with regards to the position of the putative *cis*-element identified. This however, is only an indication of the expression pattern of the β -Kr1 promoter and stable transformations would be required for a quantitative approach to examine the differences in intensity of expression. These experiments are currently underway within the laboratory (Biogemma Ltd., Cambridge) using the constructs prepared for this study. Therefore quantitative results should be obtained and the difference in intensity of GUS expression will be examined using the same constructs as used within this study. The affect of the deletions on GUS expression will be examined and the quantitative levels of GUS expression will be analysed. The importance of this *cis*-acting element within different tissues will therefore be examined further.

The results show that the *B.napus* β -Kr 1 promoter can be deleted down to 132bp (Kr1) and still direct transcription within embryos, and to 259bp (Kr2) within leaves. This however does not necessarily contain the specific *cis*-acting elements that upregulate the basal levels of transcription, as suggested by the differences in GUS staining between the different promoter constructs. Enhancer elements have shown to be present in promoters that upregulate the basal levels of transcription, therefore without these sequences transcription can still occur. However this is minimal transcription and the *cis*-acting elements act to upregulate this basal level of transcription. The results show that the smallest promoter fragment was sufficient to direct transcription within embryos (132bp). Therefore the general transcriptional

machinery for expression would be expected to be located within this region (the TATA box and general transcription factor binding sites). The TATA box would be required for minimal expression of the gene, as this is where the transcription initiation complex assembles. Therefore this implies that the putative TATA boxes previously identified within this promoter are not the actual ones, and the functional one must be located within this 132bp region. The 132bp sequence was examined for a putative TATA box, and an AT rich region identified that was located within this sequence as shown in figure 5.21.

A - β -Kr - *B.napus*

TGGATACTACTACAAACTGGGAAAACCTTTAAAATCAGTTCGGTCCGGTC
 CGGTACGGTTCAATCTAGCCTCCTTCCAACCTCTCTCGTCTGGCCGTCTAA
 ATTCAACCGAAGCTCCAACCTACTGACTACCAACTAAACACTGTACAACCT
 CGTCCGTATCGCTTTTCGAATTAAATATTCACACCTTTTTCCTCTCTCTC
 TCTCTCTCTCTCTCTCTCTACACTGTGTCGATCTTACGAGGTTCACTAG
 CTCGGATCCCCTTCTCCACCTCCTCCTCCGTTTCGATGGCAACC

 = Pr27 (Kr1)

 = Pr26

Figure 5.21 Putative TATA Box within the β -Kr1 Gene

- A) The 132bp promoter fragment shown to direct transcription within embryos is shown. This is thought to contain the general transcriptional machinery and therefore this sequence was examined for a putative TATA box. An AT rich sequence was identified (in bold) that may contain the functional TATA box.

5.4.2 Discussion of GUS Staining within Embryos

Within embryos the promoter could be deleted down to 132bp (Kr1), the smallest of the promoter fragments and still direct transcription. This showed very small areas of GUS staining in comparison to the positive controls, and therefore may be the basal level of transcription. The two largest promoter sequences: Kr7 and Kr6 both appeared to direct GUS staining similar to the positive napin control. The napin control was taken as strong expression as this is a highly seed specific promoter. Therefore if this were true then Kr7 and Kr6 would contain specific *cis*-acting elements that upregulate the basal levels of transcription. Two apparent decreases in GUS staining were observed, the first between constructs Kr6 and Kr4 and the second between Kr4 and Kr3. Between both of these, one of the GMSA boxes previously identified within Chapter 4 is located (figure 5.22). However it is unsure whether the first decrease observed is a result of the deletion between Kr6 and Kr5 (GMSA box present) or between Kr4 and Kr5 (no GMSA box). If the decrease was shown to occur between Kr6 and Kr5 then this may be suggestive of the importance of the GMSA box in upregulating the basal amount of transcription. It may have a synergistic effect as the first box between constructs Kr3 and Kr4 may increase the basal level of transcription and the second box may act to increase this level further. Figure 5.22 shows a representation of the apparent staining patterns observed. The position of the TATA boxes and GMSA elements in relation to the GUS staining can be seen.

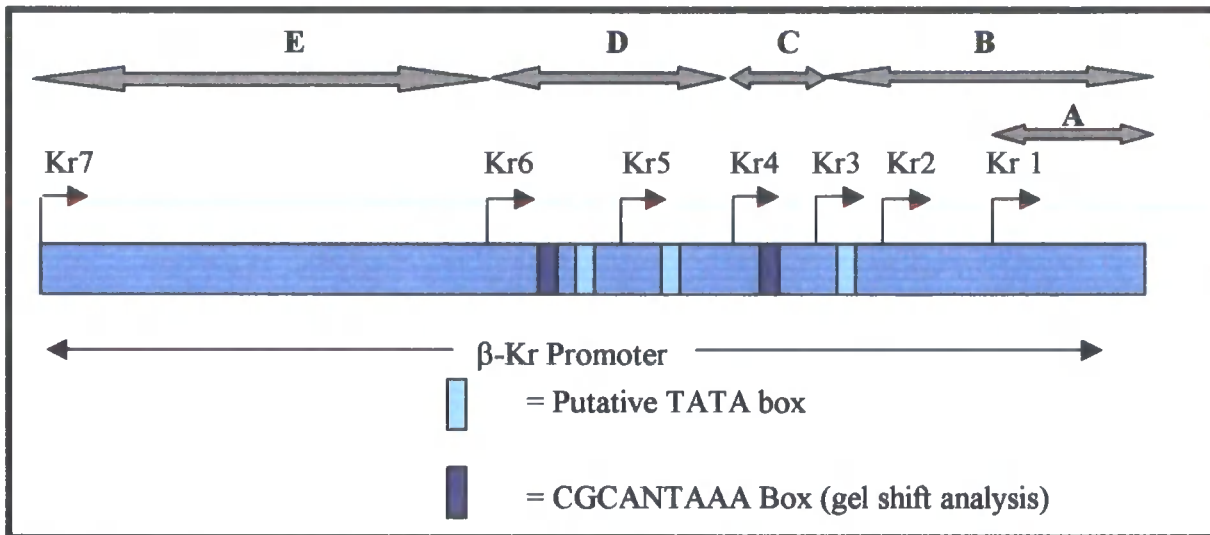


Figure 5.22 Diagrammatic Representation of the GUS Staining found within Embryos for the β -Kr Promoter Constructs (Kr1-Kr7)

- A) Region A was the smallest of the promoter fragments (132bp) and was shown to direct transcription. This region therefore should contain the basal transcriptional machinery (TATA box).
- B) Region B containing fragments Kr1-Kr3 showed the smallest areas of GUS staining observed for all of the fragments. If this is the case then it suggests that no specific *cis*-acting elements are located within this region involved in upregulating the basal levels of transcription.
- C) Within region C there appears to be an increase in GUS staining between Kr3 and Kr4, this could be suggestive of the presence of a specific *cis*-acting element within this region. A GMSA box is located here.
- D) There appeared to be an increase in GUS staining within region D. It is unsure whether this increase is between Kr4 and Kr5 or Kr5 and Kr6. However the Kr6 construct showed similar GUS staining to the positive controls. Therefore if this is the case it would suggest the presence of a *cis*-element that upregulates the basal levels of transcription.
- E) Region E does not appear to show any further increase in GUS staining from that found with Kr6. Showing similar GUS staining to the positive controls.

5.4.3 Discussion of GUS Staining within Leaves

The *B.napus* leaves seemed to exhibit a different GUS staining pattern in comparison to embryos, suggesting that there may be different *cis*-acting elements involved in their transcription. The minimal sequence to show GUS expression within the leaves was 259bp (Kr2). The strongest GUS staining was observed for constructs Kr5, Kr6 and Kr7, which showed similar levels to the CaMV35S positive control. There appeared to be one main decrease in staining which was observed upon the deletion of Kr5 to Kr4. Construct Kr4 showed similar GUS staining to Kr3 and Kr2, which appeared to be very minimal in comparison to the positive controls. Therefore within leaves there appears to be only one increase in GUS staining; between Kr4 and Kr5. This could contain a specific leaf-acting element that upregulates the basal levels of transcription, as within this region there is no GMSA box. The other possibility could be that the embryo may be increasing in this region but is not as noticeable as within the leaf and therefore the element is the same for leaves and embryos. However there was no noticeable increase between Kr4 and Kr5 observed within embryos and the leaves did not show the same increase in staining between Kr3 and Kr4 as seen within embryos. Therefore suggesting different *cis*-acting elements may be present. Construct Kr2 appeared to be the minimal promoter to direct transcription however it may be the case that sequence could be deleted down to 132bp (Kr1) and still show expression but that it was too small to be observed. Within Embryos only very small amounts of staining were observed for Kr1 that were difficult to visualise. Only four leaves were used in comparison to 200 embryos and only a few of these embryos showed staining therefore if more leaves were used staining may have been observed. The promoter is likely to have the same general transcriptional machinery (TATA box) for embryos and leaves, therefore unless leaf expression needs a specific element that is not required for embryos to initiate transcription, this sequence is likely to direct transcription if it does within embryos. However other studies have shown that different tissues may require different elements to direct transcription (de Boer *et al.*, 1999).

Figure 5.23 shows a representation of the differences observed in GUS staining for leaves, showing where these occur in relation to the putative *cis*-elements identified.

Leaf GUS Expression

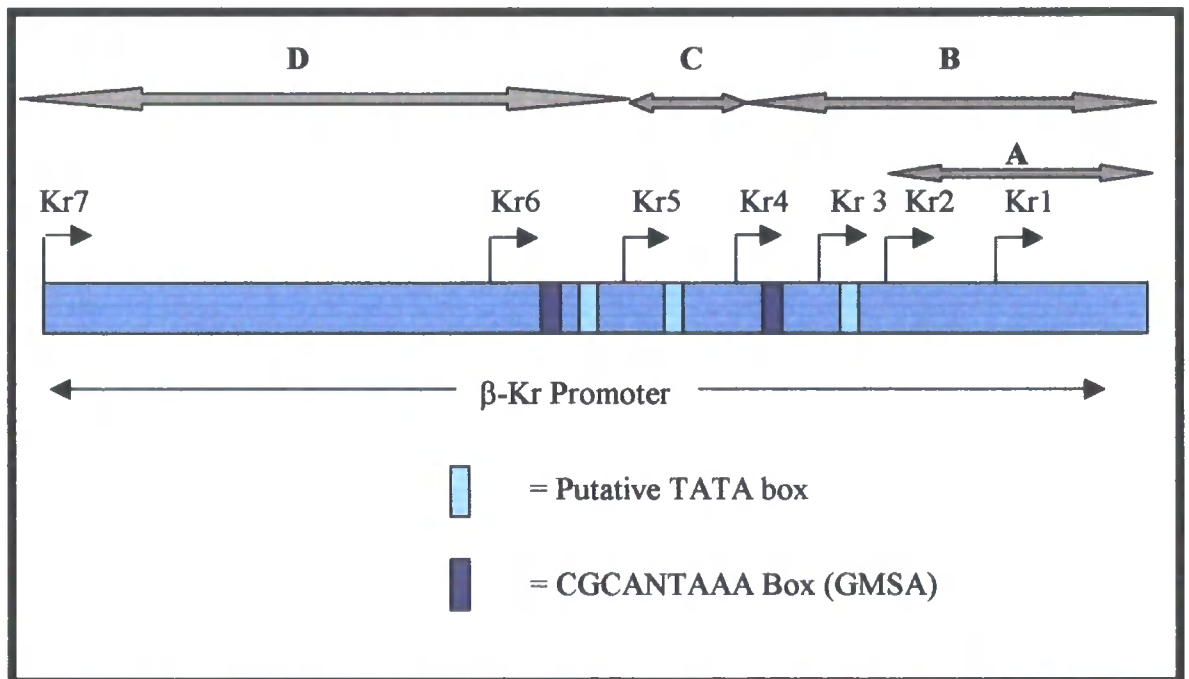


Figure 5.23 Diagrammatic Representation of the GUS Staining found within Leaves for the β -Kr Promoter Constructs (Kr1-Kr7)

- A) The minimal promoter sequence shown to direct transcription.
- B) The largest promoter sequence showing the smallest GUS staining (Kr1-Kr4)
- C) Kr5 is the smallest sequence to show the GUS staining similar to the positive control. Upon deletion to Kr4 there is a noticeable decrease in GUS staining. This could be suggestive of a *cis*-acting element within region C that may be involved in upregulating transcription within the leaves.
- D) There is no apparent increase in staining between these two constructs. They appear to show similar levels of GUS staining to Kr5 and to the positive control.

The pattern of GUS staining within leaves for the transient expression experiments is inconsistent with that obtained for the stable transformations, which showed GUS staining only within the cotyledons, but not however within the true leaves. The reason for this difference between the stable transformations within *Arabidopsis* and the transient expression experiments within *B.napus* is not clear however possible explanations will be discussed later (section 6.4.2).

5.4.4 GUS Expression within Roots

GUS expression would have been expected within the roots as a high expression was observed in the *Arabidopsis* stable transformants. However no results were obtained for the examination of root material. This may be due to the difficulty of the particle penetration into the root material. This has shown to be the case within leaves, as the upper surface does not allow the penetration of the gold particles into the cells. Subsequently the lower surface is used for particle bombardment. This may be the reason within roots as upon closer examination no gold particles could be observed within the root cells. Thereby suggesting the particles have not been able to penetrate into the root cells and therefore no GUS expression was observed.

5.4.5 Final Discussion

The results appear to suggest differences in the staining patterns observed between the embryos and leaves. The GMSA box appears to have no apparent affect on the level of expression within leaves. This is suggested upon examination of the sequence implicated in the increase in GUS staining within leaves, within this sequence no GMSA box is present. However the results for the embryos suggest that this GMSA may be involved in the upregulation of the minimal expression of the β -Kr1 promoter. This was suggested because between Kr3 and Kr4 there appeared to be an increase in staining, this sequence is only an increase of 36bp, and within this is located the 9bp GMSA box. The second increase is not so conclusive however, as it

is unsure whether this occurs between Kr4 and Kr5 (no GMSA present) or Kr5 and Kr6 (GMSA box present). If it is the case that it occurs between Kr4 and Kr5 where the second GMSA box is located then it could be indicative of the importance of this GMSA box identified. This would be interesting as this study is aiming to examine an embryo specific element that is responsible for the upregulation of transcription within the embryo. The box would therefore appear to have a synergistic affect with an increase in expression with the first box and a further increase with the presence of the second box. This synergistic affect has been observed in other promoters where the presence of more than one copy of the *cis*-acting element can work synergistically together to increase transcriptional rates. Multiple *cis*-acting elements have been shown to be present in a number of promoters that are involved in expression. An example is the AVP1 gene from *Arabidopsis* where it was shown that upon subsequent deletions of the promoter from -281 to -286 to -243 showed successive reductions in expression and therefore it was concluded that expression of the gene is in some way regulated by at least three *cis*-acting elements. Certain genes have shown that there are different elements responsible for expression and other elements responsible for increasing the basal levels of expression. The pea legumin gene is an example of such a promoter (Shirsat *et al.*, 1989). Here elements from -97 to -549 were responsible for gene expression and the sequence between -549 and -1203bp were responsible for an increase in expression.

If these observations are a true reflection of GUS expression and the GMSA box is shown to be an important *cis*-acting element then the results suggest it may be embryo specific. It appears to have no affect on leaf expression, as an increase in GUS staining does not correlate with the location of the GMSA box. This regulation of fatty acid genes by different *cis*-acting elements for differential expression within tissues has previously been shown for the ACP promoter from *Arabidopsis* (Baerson., *et al* 1994). It would have been desirable to carry out stable transformations to analyse these constructs, however these transient expression experiments were carried out due to time constraints. Stable transformants have the advantage that the expression pattern of the gene can be examined and also a quantitative analysis can

be undertaken. Therefore these constructs have been used to make reporter constructs for stable transformations into *B.napus* (Biogemma Ltd, Cambridge). Upon examination of these transgenic plants and quantitative analysis the importance of this GMSA box within *B.napus* tissue will be examined further. If similar results to this study are obtained then future work could involve mutagenesis of the 9bp sequence to examine the affect on transcription, and examination of the trans-factor that binds to this sequence.

Discussion

Discussion

This study involved the analysis of the *B.napus* β -Keto-ACP reductase 1 promoter for potential regulatory *cis*-elements. The gene is a member of a family of four and was shown to be the most highly expressed of the isoforms (Fraser *et al.*, in preparation). The study was carried out to identify sequences important for the temporal and tissue specific regulation of β -Keto-ACP reductase gene. This isoform was chosen as it was shown to be the most highly expressed within tissues including the seed. The investigation analysed the 5' sequence of the gene to examine if it would function as a promoter. Stable transformations and transient expression experiments were used to examine the expression pattern of the gene and the affect of a deletion series on transcription. Finally the binding of proteins to the 5' region was analysed to identify protein-DNA complexes using the gel mobility shift assay (GMSA). Competitors were used to look at the promoter fragments specificity of binding, to each other and other promoters (using both a fatty acid specific and non-specific gene promoter).

The fatty acid genes appear to show both temporal and tissue specific regulation. This has been shown by a number of the genes exhibiting the same expression pattern (Fawcett *et al.*, 1994 & 2002 and Elborough *et al.*, 1996). Therefore it is thought that these genes may in some way be co-ordinately regulated, however little is currently known about their regulation. Metabolic pathways have previously been shown to be upregulated by the overexpression of a single transcription factor (Kasuga *et al.*, 1999). Therefore if an overall control factor does exist then the identification of this protein and the over expression of its cDNA within plants could potentially upregulate the pathway and increase the amount of oil produced. If a common coordinate regulator exists then it would be expected to bind to all of the fatty acid genes and therefore all of these gene promoters should contain the *cis*-

acting element to which the protein binds. Sequence comparison of the fatty acid genes to date has not revealed any element conserved in all of the lipid biosynthetic gene promoters. However the problem is complicated by the variations in the *cis*-acting elements that the same transcription factor can recognise and bind. Often it is just a few of the bases to which the transcription factor will recognise and bind. Therefore the bases in between these may vary, thereby making searching for a similar sequence in other promoters difficult. There is also the consideration that the transcription factor may recognise and bind to the 3-Dimensional structure of the DNA, and therefore the actual sequence would not be important in recognition for binding and therefore an element specific for the lipid biosynthetic genes would not be present and therefore not be identified.

6.1 Transcription Factors and Lipid Biosynthesis

To date little has been published about transcription factors involved in fatty acid biosynthesis within plants, however within other systems more information is available. Two transcription factors involved in the regulation of fatty acid synthesis have been identified from *E.coli* and *Drosophila*. FadR from *E.coli* has been identified as a transcription factor involved in the regulation of the fatty acid genes. FadR was shown to bind to a number of the fatty acid genes and inhibition of FadR was shown by long chain acyl CoA thioesterases (DiRusso *et al.*, 1998). The second transcription factor is the SREBP (Steroyl Regulatory Element-Binding Protein) from *Drosophila* that was shown to be involved in controlling the enzymes involved in membrane lipid biosynthesis (Dobrosotskaya *et al.*, 2002). SREBP is thought to monitor the cell membrane lipids and regulate their synthesis. In *Drosophila* the SREBP is regulated by feedback inhibition by phosphatidylethanolamine, the end phospholipid product. To become active the SREBP is transported to the Golgi and two proteases cleave the protein to produce the basic leucine zipper domains, which then enter the nucleus and initiate transcription.

The majority of research on the transcriptional control of the fatty acid enzymes in plants has focused on the analysis of the promoter sequences to identify potential regulatory *cis*-acting elements (de Boer *et al.*, 1999 and Baerson *et al.*, 1994). Deletion analysis of the promoter allows the sequences involved in the upregulation or initiation of transcription, both temporally and spatially, to be identified. Upon identification of a region of DNA shown to be important for transcriptional control this can be used to isolate and purify the trans-acting factor(s), which binds. The aim of this study was to examine the β -Kr1 promoter for potential regulatory *cis*-elements, and therefore upon identification of these potential regulatory trans-factors could be isolated that bind to this promoter.

6.2 *Arabidopsis* Transformations

The β -Keto-ACP reductase 1 gene had previously been cloned, however the 5' sequence of the gene had not been proven to act as a functional promoter. Previous studies have shown that usually the 5' sequence directly upstream of the gene contains the functional promoter, however certain genes contain an intron within this region for example the enoyl-ACP reductase promoter from *Arabidopsis*. Therefore the β -Keto-ACP reductase sequence was examined and shown that no intron was apparent within this region. Stable transformations within transgenic *Arabidopsis* plants revealed that both the 2Kb and 1Kb sequence upstream of the translational start codon could initiate transcription and act as a promoter. There was no apparent difference in GUS expression between KrI and KrII, therefore suggesting that the 1Kb sequence contains all the transcriptional machinery necessary to direct transcription. It was this 5' region that was used as a promoter for the basis of this investigation to identify potential *cis*-acting elements involved in the regulation of the *B.napus* β -Keto-ACP reductase 1 gene.

6.2.2 Expression Pattern of the β -Kr Gene

These stable transformations allowed the expression pattern of the promoter to be examined. The study was looking to identify factors involved in regulation of fatty acid biosynthesis within seed, as it is here that the major form of triacylglycerols are stored as reserves. Therefore it was important to establish that this isoform was expressed within embryos. Three main areas of GUS expression were observed within the transgenic *Arabidopsis*; embryos, roots and cotyledons. The fact that the cotyledons showed expression is interesting as they are found within the seed. Therefore this isoform appears to be expressed within seeds (embryos and cotyledons). The absence of expression within the true leaves suggests that one of the other isoforms may be responsible for expression in leaves. Within roots the most apparent GUS expression was observed in the youngest part of the roots, but was absent from the root tip. The GUS expression also appeared to become more restricted with age. Therefore it is suggestive that the GUS expression is highest in the youngest parts where the cells are expanding and are therefore laying down lipid for the production of cell membranes. Therefore this β -Keto-ACP reductase isoform appears to be specific to the embryos, roots and cotyledons, but does not appear to be leaf specific. Examination of one of the other isoforms of the β -Keto-ACP reductase genes may identify an isoform more specific for leaf expression. The expression pattern of the β -Keto-ACP reductase 1 gene would correlate with that expected for a fatty acid gene, as it appears to be expressed where there is high demand for lipid production (embryos, cotyledons and expanding roots).

6.3 Identification of DNA/Protein Complexes

Upon establishing that the 1Kb sequence could function as a promoter, gel mobility shift analysis was performed to try and identify trans-acting factors that bind to this β -Keto-ACP reductase promoter. Six overlapping promoter fragments surrounding the putative TATA boxes appeared to show the formation of protein-DNA

complexes upon the addition of embryo protein extract. Competition analysis on four of the fragments using the lipid transfer protein (LTP) and the enoyl-ACP reductase promoter sequences suggested the trans-acting factor appears to share a binding site within the enoyl-ACP reductase promoter, but not the LTP protein. If this is the case then this is important as the overall control factor would be expected to bind to other fatty acid gene promoters. Competition analysis using the other β -Kr promoter fragments also suggested that the factor binds to a number of regions along the promoter. Sequence analysis was carried out on the fragments to try and identify a common element to which the factor was binding. A 9bp sequence located within four of the six fragments was identified (CGCANTAAA), this sequence only appeared twice within the overlapping fragments, however both times it was present within the overlapping regions and therefore appeared in four of the fragments.

The binding of transcription factors to multiple sites within a promoter has been shown for a number of transcription factors (Schaffner *et al.*, 1988). They have been shown to be important in enhancing transcription by the presence of more than one protein binding element. Multiple repeats of the same sequence has been shown for the B subunit of the chloroplast glyceraldehydes-3-phosphate dehydrogenase (GADPH) gene (GAPB) from *Arabidopsis*, which contains four direct repeats of the Gap box (important for light activation). These repeats were located within the region -237 and -181 of the promoter (Chan *et al.*, 2001). A number of soybean genes have also been shown to contain multiple binding regions (Jenson *et al.*, 1988) for example the soybean heat shock genes are regulated by multiple heat shock elements (Baumann *et al.*, 1987). Three GT-1 binding sites (light responsive sites) must be present for significant binding of the GT-1 protein (Green *et al.*, 1989), and the SEF 3 and 4 transcription factors interact with multiple regions within the soybean β -Conglycinin promoter (Allen *et al.*, 1989). Other studies have also revealed the importance of multiple *cis*-acting elements involved in tissue specific expression. For example within the rice glutelin promoter four different motifs were shown to be required for endosperm specific expression (Wu *et al.* 2000). It was shown that 1 of the GCN4 motifs is responsible and essential for endosperm specific

activity, whereas the other three are responsible in combination for quantitative expression of the gene. Another example is CycB1 cyclin *CYM* promoter from *C.roseus* (Ito et al 1998). Deletions of the promoter were carried out and attached to the Luciferase gene, this was then stably transformed tobacco. Mutational analysis was carried out on one of the constructs shown to direct expression and a 9bp sequence was shown to be important in regulation of expression. The promoter was analysed and three other elements that showed high similarity with this 9bp element were identified. However these sequences were in opposite direction to the original. Therefore within the promoter region (-203 and -80) there were four elements important for expression that appeared to be similar.

The reason that the exact 9bp sequence was only found in four of the six fragments, but all showed binding, could be due to the transcription factor being able to recognise and bind to slightly different may be related motifs. This was shown to be the case with the rice RITA-1 bZIP transcription factor that was shown to bind to A, C and G boxes, which are comprised of similar sequences (Izawa *et al.*, 1994). The DREB proteins are thought to specifically interact with certain bases, 4th and 7th C and the 5th G (Sakuma *et al.*, 2002), and appear to recognise slightly different analogues of the core motif. The 9bp sequence (GMSA box) identified within this study of the β -Keto-ACP reductase promoter contains a variable residue within the centre of the sequence (CGCANTAAA). This has also been found in other *cis*-acting elements for example the *Arabidopsis* homeodomain leucine zipper transcription factor ATHB5 was shown to bind to a 9bp DNA binding site containing a variable residue: CAATNATTG (Johannesson *et al.*, 2001). Therefore when searching for related sequences within the other fatty acid biosynthetic gene promoters the fact that the protein may only recognise a certain number of the 9bp residues makes sequence comparisons more difficult.

6.4 Transient Expression Experiments of the β -Kr promoter

Transient expression studies of the *B.napus* β -Keto-ACP reductase promoter using *B.napus* tissue (embryo and leaf) were carried out to identify the smallest promoter fragment that could initiate transcription. The deletions were constructed to sequentially delete part of the promoter containing either a putative TATA box or one of the GMSA boxes identified. It appeared that the smallest fragment of 132bp could direct transcription within embryos. Although the technique is not quantitative there were apparent differences in the areas of GUS staining. At least one of the apparent decreases in GUS expression correlated with a deletion of one of the GMSA boxes. The results were also suggestive that the expression within leaves and embryos may be differentially regulated, as the expression within leaves appeared not to be affected by the deletion of the GMSA box. Other fatty acid biosynthetic promoters have been shown to contain different *cis*-acting elements responsible for regulation of the gene within different tissues for example the *Arabidopsis* enoyl-ACP reductase promoter (de Boer *et al.*, 1999).

De Boer studied the *Arabidopsis* enoyl reductase promoter to identify *cis*-acting elements involved in the regulation of the gene. The 1470bp region upstream of the ATG was fused to the GUS reporter gene. The minimal sequence to direct transcription was identified by sequential deletion of the promoter and analysis of GUS expression. The study identified different *cis*-acting elements within the promoter regions required for the spatial expression of the gene. The results showed that different *cis* acting elements were required for expression within different parts of the plants (leaves, roots and seeds). Two sequences were shown to be required for expression within leaves, the first was located between -329 and -201 relative to the transcription start site and the second was contained within the intron in the UTR. Root expression was shown to be unaffected until deletions down to -19 where root expression was diminished. Removal of a region within the intron contained within the UTR was shown to increase root expression. Finally seed expression was shown to still be observed until deletion to -19bp of the transcriptional start site, this was

shown to be the minimal promoter. This study of the *B.napus* β -Kr gene promoter was also carried out to examine potential regulatory cis-acting elements by sequential deletions of the promoter and analysis of GUS expression. The stable transformations identified the spatial expression pattern of the gene and similar to the *Arabidopsis* enoyl reductase expression the gene was highly expressed within embryos. De Boer showed GUS expression within the youngest leaves in *Arabidopsis* however this disappeared with age, this study of the *B.napus* β -Kr showed little or no expression with the leaves. This study however unlike the *Arabidopsis* identified very strong expression within the roots. The transient expression experiments of the *B.napus* β -Kr gene also suggested that there may be different cis-acting elements responsible for spatial expression of the gene as in *Arabidopsis*, however stable transformations will be required to confirm this. Despite the similarities between the expression pattern between the two genes, there are differences. These differences may be explained partly by the fact that the *Arabidopsis* enoyl reductase is encoded by a single housekeeping gene and therefore is responsible for the expression of the gene in all tissues and at all times. However the β -Kr is a member of a gene family and therefore different isoforms may be responsible for either temporal or spatial expression of the gene. Therefore the enoyl reductase *Arabidopsis* must contain within its promoter all the cis-acting elements required for expression in every tissue, whereas the β -Kr1 from *B.napus* may be a tissue specific isoform.

The transient expression experiments reported in this study showed that the putative TATA box previously identified by PLACE did not appear to be the functional one as it was not present within the smallest fragment, which was shown to direct transcription. Therefore the β -Keto-ACP reductase promoter sequence of the smallest fragment was examined and an A/T rich sequence was identified that contained the reverse sequence of the TATA box identified within the enoyl-ACP reductase promoter from *Arabidopsis*.

6.4.2 Differences between Stable and Transient Transformations

Differences between the transient expression within *B.napus* and the stable transformations with *Arabidopsis* were observed. This however is not unusual and has been observed in other transgenic plants both with expression across different species and between transient expression and stable transformation methods. In this study both the stable and transient studies showed expression within embryos, however expression within the true leaves was only observed for the transient experiments and not for the stable transformations. Differences like these have been shown in other studies, it is not known why these differences occur, a number of possible suggestions have been put forward to explain them. The first could be the difference in the transcriptional machinery, as the two types of transformations were carried out in different species. The stable transformations were carried out in *Arabidopsis* and the transient expression experiments in *B.napus*. However these two species are members of the same brassicae family, therefore it is unlikely that the machinery is that different. Another possibility to account for these differences is that the bombardment of tissues may not reflect the *in vivo* physiological conditions of the plants, as the concentration of the DNA bombarded into the tissue will be higher than normal.

The analysis of the Isocitrate lyase promoter using both transient expression and stable expression showed similar discrepancies (Zhang *et al.*, 1996). The transient expressions were carried out in *B.napus* and the stable transformations in *Arabidopsis* as with this study. It was shown that one of the constructs showed GUS expression within leaf for the bombarded material but not for the stable transformations. There was also variability in the expression of GUS between the *Arabidopsis* and the *B.napus*. Differences were also observed between the expression of the GABP gene in different plant species; tobacco and *Arabidopsis* (Chan *et al.*, 2001). As with all experiments to confirm any result the experiment needs to be repeated and shown to be reproducible.

6.5 Advances in Technology to Identify Transcription Factors

The technology available for identification of proteins in recent years has advanced considerably with the increase of interest into proteomics, thereby increasing the potential for the identification of transcription factors. Previous identification of transcription factors has involved a laborious task of purification procedures to identify the DNA binding protein, often involving various DNA affinity column purifications. The problem challenging their purification is the low concentration of these transcription factors. However within the past year or so recent advances in such techniques as DNA array and MALDI will help enormously in the future identification of transcription factors.

Two recent articles examine the use of MALDI to identify the transcription factor of interest. The first involves using paramagnetic particles to immobilise the DNA, which contains the sequence element specific for the protein (Nordoff *et al.*, 1999). This technique has the advantage that only very small amounts of proteins are required. The proteins are then analysed by MALDI directly from the particles. A second paper using MALDI-TOF has recently been published employing the use of two-dimensional gels (Woo *et al.*, 2002). The molecular mass and pI of the protein is estimated and then run out on a 2D gel. Using the predicted PI and molecular mass the protein is located from the gel, eluted, checked for activity and analysed by MALDI. Therefore with the advent of these new technologies and the increase in precision of mass spectrometry, the purification and identification of transcription factors will be made easier. This will no doubt increase our knowledge of transcription factors and how they regulate genes. At the beginning of this study these technologies were not available and therefore future studies using these techniques should help to increase the knowledge of transcription factors at a much quicker rate. The results of this study on the *B.napus* β -Keto-ACP reductase promoter could be used in conjunction with the MALDI technology to identify potential trans-acting factors that bind to this promoter.

Mutational analysis is also an important tool used to study regulatory proteins. The screening of mutants phenotypically allows the function of that gene to be examined. Transposon and T-DNA mutagenesis are two of the main techniques that have been used to identify gene function. T-DNA mutagenesis has been widely used within *Arabidopsis*. T-DNA (transfer DNA) comes from the *Agrobacterium tumefaciens* and is inserted at random into the plant's genome. A number of transcriptional regulators have been identified using this technique, examples include GL1 a transcription factor involved in trichome formation (Oppenheimer *et al* 1991) and COP1 another transcriptional regulator (Deng *et al* 1992). Mutational analysis been used to identify the regulatory proteins that control flower pattern and colour. Mutations have been identified where either anthocyanin production has been either reduced or arrested. These mutations have been studied and shown to be either due to a mutation in one of the enzymes in the biosynthetic pathway or due to a mutation in one of the regulatory proteins. The regulatory proteins responsible were shown to be members of either the MYB family or bHLH transcription factor families. It was shown that these two families were responsible for regulating the whole of the anthocyanin biosynthetic pathway. These transcription factors were shown to induce anthocyanin production in unpigmented maize tissue by their ectopic expression. (Goff *et al* 1990). However more recently gene entrapment and activation tagging techniques have been used in the analysis of gene identification. Gene entrapment uses the genes regulatory elements to activate the transgene whereas activation tagging involves the transgene being used to drive the overexpression of the native genes (Lindsey *et al* 1999). These techniques are relative new, however this type of mutational analysis will be very useful in identifying regulatory factors involved in metabolic pathways.

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