Studies of a *Brassica napus* gene encoding a putative lipid transfer protein

by

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Summary

The epidermis is an extremely important plant tissue because it is at the interface of the plant with its environment. To isolate genes expressed in the epidermis, a cDNA library constructed from leaves of *Brassica napus* was screened with an unidentified *B. napus* epidermis-specific, partial cDNA clone, pLF3A, which was isolated previously. Several full length cDNA clones were isolated, one of which was fully sequenced. This clone, designated BnLTP, encoded a putative non-specific lipid transfer protein (LTP) of 118 amino acids. Two other closely related cDNA clones, p2A4 and p5A9, were isolated and sequenced partially at the 3' ends. Sequence comparison of the three clones with pLF3A indicated the presence of four closely related but non-identical cDNA species. Based on this observation and previous Southern blot analysis of *B. napus* genomic DNA, it was concluded that BnLTP, p2A4, p5A9 and pLF3A were four members of a small closely related multigene family encoding putative LTPs.

A genomic clone, designated λ3.2A, homologous to BnLTP was isolated. A 4.8 kb *Eco* RI fragment from λ3.2A, which hybridised strongly to the cDNA, was subcloned and sequenced. The fragment contained the full length gene, designated *BnLTP*. *BnLTP* encoded a 118 amino acid putative LTP, which differed at five amino acids from the cDNA. It consisted of two exons of 116 and 2 amino acids, respectively, interrupted by a 269 bp intron. The gene contained a 2.3 kb 5' upstream region that contained a TATA box and several cis-acting elements conserved in other *LTP* genes and genes involved in phenylpropanoid biosynthesis.

B. napus LTP transcripts exhibited an organ-specific pattern of expression. They were expressed at high levels in leaves, stems, and floral tissues but were not detected in roots. In addition, endogenous *LTP* transcripts were induced in response to high white, blue and red light in *B. napus* and *Arabidopsis* leaves but not in response to UV-B light treatment.

The *BnLTP* promoter was regulated in a spatial and temporal manner during development, as demonstrated by histochemical localisation of β-glucuronidase (GUS) in transgenic *Arabidopsis* plants carrying a 2.3 kb *BnLTP* promoter-*GUS* fusion

(*BnLTP-GUS*). GUS was expressed at higher levels in younger developing leaves compared to older leaves. Cross-sections of transgenic leaf and stem tissue indicated that *BnLTP-GUS* was expressed predominantly in the epidermal cells. GUS activity was observed in the trichomes, epidermal pavement cells and guard cells. However, expression was also observed in the vascular bundles (xylem and phloem of leaves) and in the lateral root initials. In floral tissue, GUS was localised in sepals, stigmas, petals and stamens, but as the flower matured, it was expressed only at low levels in the stigma and sepal/petal abscission zone. However, GUS was not detected in mature petals but persisted in the stamens (pollen sacs and the filament).

Expression of *BnLTP-GUS* in transgenic *Arabidopsis* was moderately induced in response to high white, blue and red light but not following UV-B light treatment of leaves. *BnLTP-GUS* was moderately induced in cold treated leaves and upon infection with CaMV but not in response to wounding. *LTP* expression increased in transgenic *Arabidopsis* plants constitutively expressing the CaMV *Gene VI*, which is essential for viral replication and disease symptoms.

In *Arabidopsis* mutants altered in trichome development, *glabrous 1* (*gl1*) and *2* (*gl2*) and *transparent testa glabra* (*ttg*), endogenous *LTP* expression was not altered, and in each case expression was induced in response to high white light similar to wild type plants. However, in the photoregulation mutant *icx1* (*i*ncreased *c*halcone *s*ynthase *e*xpression), which is characterised not only by enhanced light stimulation of the epidermis-specific gene, *CHS*, but also by altered epidermal development, endogenous *LTP* expression was increased. In the double mutant *ttg/icx1*, which has phenotypic characteristics of both parents, endogenous *LTP* expression was similar to *icx1*.

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For my family

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science. He to whom this emotion is a stranger, who can no longer wonder and stand rapt in awe, is as good as dead: his eyes are closed.....To know that what is impenetrable to us really exists, manifesting itself as the highest wisdom and the most radiant beauty which our dull facilities can comprehend only in the most primitive forms- this knowledge, this feeling is at the centre of true religiousness. In this sense, and in this sense only, I belong to the ranks of devoutly religious men."

Albert Einstein,
What I Believe (1930)

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Abbreviations

ABA	Abscisic acid
BSA	Bovine serum albumin
bp	base pairs
cDNA	complementary deoxyribonucleic acid
dATP ; ddATP	deoxy-; dideoxyadenosine triphosphate
DEPC	diethyl pyrocarbonate
dCTP: ddCTP	deoxy-; dideoxycytidine triphosphate
dGTP: ddGTP	deoxy-; dideoxyguanosine triphosphate
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
dNTP	deoxynucleotide triphosphate
dTTP; ddTTP	deoxy-; dideoxythymidine triphosphate
d.p.i	days post inoculation
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylene diaminetetracetate
ER	endoplasmic reticulum
EtBr	ethidium bromide
g	standard acceleration of gravity
GUS	β-glucuronidase
IPTG	isopropyl β-D-thiogalactopyranoside
kD	kiloDalton
kb	kilobase or kilobase pairs
MES	N-morpholino ethanesulfonic acid
M-MLV	Moloney murine leukemia virus
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	messenger RNA
4-MU	4-methylumbelliferone
MUG	4-methylumbelliferyl glucuronide

n	nucleotide(s)
NaOAc	sodium acetate
NaPPi	sodium pyrophosphate
NH_4OAc	ammonium acetate
O.D.	optical density
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidyethanolamine
p.f.u.	plaque-forming units
PI	phosphatidylinositol
PVP	polyvinylpolypyrrolidone
RNAse	ribonuclease
rRNA	ribosomal RNA
RT	reverse transcriptase or room temperature
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TAE	Tris-acetate
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylene diamine
Tris	N-tris(hydroxymethyl)amino-methane
UV	ultraviolet
(v/v)	volume/volume
(w/v)	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactoside
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

Abbreviations for amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Hisitidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1 Introduction

1.1 Study of plant development

The coordinated control of cell division, cell expansion and cell differentiation which leads to many morphologically diverse forms in the plant kingdom, has for many years been a keen interest for plant molecular biologists. Recent studies involving gene transfer techniques for the generation of transgenic plants and isolation of mutants, indicate that the major control point is at the molecular level (Stitte, 1992). Indeed, the fundamental aspect of normal development is the determination of cell fate, and the control of cell fate in a multicellular organism is no doubt complex. Furthermore, in higher plants it is estimated that during plant development, approximately 16,000 to 33,000 genes are expressed depending on the plant species (Gibson and Somerville, 1993). In addition, a high proportion of these are present in multigenic families. A deeper understanding of how selective expression of these genes comes about would greatly enhance our understanding of how a diverse array of plant structures are generated. Although there are many fundamental similarities between the regulation of gene expression in plants and animals, there are clear differences in the cell biology. Plants are sedentary organisms with semi-rigid cell walls and so, in contrast to animal cells, direct membrane-membrane interaction during morphogenesis is limited. Unlike mammalian cells, the fate of plant progenitor cells has been shown for a long time to be determined by their position and the local environment and, a single plant cell taken from cultured cells can be induced to produce a multicellular plant (Henshaw *et al.*, 1982).

1.1.1 Experimental approaches

Plant development has been extensively studied by a combination of histological (Barton and Poethig, 1993; Dolan *et al.*, 1993) and chimera analyses (Satina *et al.*, 1940), genetic mosaics and cell lineage analysis (Hake, 1987), immunofluorescence techniques (Webb and Gunning, 1991), scanning electron microscopy and dye-coupling (Scott *et al.*, 1958; 1991; Duckett *et al.*, 1994), the production of transgenic plants (Vroemen *et al.*, 1996) and mutant analysis (Koornneef, 1991; Marx, 1983). The introduction of easily assayable, tissue-specific

and cell-specific molecular markers into transgenic plants has provided a vital tool for the study of epidermal cell fate (Jefferson *et al.*, 1987; Vroemen *et al.*, 1996).

1.1.2 *Arabidopsis thaliana* : a model in the study of epidermal development

One of the most important model systems to emerge in the study of plant developmental biology in the last twenty years has been the proverbial weed, *Arabidopsis thaliana* (L.) Heynh (Meyerowitz, 1987). The main advantage of its use has been the relative ease with which it can be used in classical and molecular genetic studies. It has a small genome size of approximately 100 Mb, very few repetitive DNA sequences and a rapid rate of reproduction, around 4-6 weeks. Furthermore it has a small size with small seeds which enables a large number of mutagenized seed (around 10,000 per Petri dish) to be screened. A relatively powerful approach in the study of developmental processes has been through the generation of *Arabidopsis* mutants (Koornneef, 1991). A number of mutagenic processes have been employed in the generation of *Arabidopsis* mutants including chemical treatment of seeds with ethyl methyl sulphonate (EMS), by radiation with X-rays and gamma rays (Koornneef, 1982) and by insertional mutatagenesis with T-DNA (Feldmann, 1991) from *Agrobacterium tumefaciens* (Zambryski *et al.*, 1989; Walbot, 1992) or by transposon insertion (Chandler and Hardeman, 1992) such as the Mu or the Ac/Ds transposon from maize (Bancroft *et al.*, 1992). Its remarkable use as a genetic and molecular model in the study of plant development is discussed throughout this review. Indeed several *Arabidopsis* mutants altered in various aspects of epidermal development have already been generated in this way. Some of them will be discussed in greater detail later. By identifying the mutated genes and determining their expression patterns we can begin to build a molecular picture as to how epidermal development is achieved.

A wealth of knowledge exists for the study of developmental processes in *Arabidopsis* organs such as the seed (Jurgens *et al.*, 1991), root (Schiefelbien and Benfey, 1991; Dolan, 1993), shoot meristem (Medford *et al.*, 1992), leaf (Pyke *et al.*, 1991) and flower (Smyth *et al.*, 1990). However, very little definitive information on the development of the plant epidermal layer is available. Its study has many potentially important implications as it forms the protective outer surface of the plant and is

constantly in contact with diverse environmental stresses such as extreme light and temperatures, pathogen attack, physical damage, drought and man-made chemicals. The isolation and characterisation of epidermal-specific genes would be extremely useful in the characterisation of the molecular processes central in epidermal development and epidermal-specific gene expression. By identifying those genes expressed, if not exclusively, but mainly in the epidermis and comparing their common sequence elements, we can begin to build a molecular picture inter-relating epidermal development to gene expression and ultimately to transcription factors. However, one of the main problems in identifying such genes is that they are expressed in a restricted cell type and the transcript or protein level may be extremely low and/or expressed only transiently during development. Although differential screening of cDNA libraries enables the identification of tissue-specific genes, it is often the case that these are only easily detected due to the relatively high abundance of transcripts in a given organ or tissue. Furthermore, in many cases these genes represent structural or metabolic proteins.Conversely, genes that encode regulatory proteins that play a central role in cell differentiation and development, are often expressed at very low levels. Although cDNA and genomic subtractive hybridisation techniques based on a modifications of the methods originally described by Bautz and Reilly (1966), can be employed, it has often proven to be technically difficult. A more powerful approach is that of insertional mutagenesis of genes using T-DNA (Feldmann, 1991). The strategy is relatively simple but extremely informative. Once a gene is disrupted and cannot be complemented by other genes, such as closely related members of a multigenic family, progeny homozygous for the mutation appear abnormal either morphologically or biochemically. Although mutants can be generated by chemical or physical treatment, a distinct advantage of T-DNA insertional mutagenesis is the use of known sequences that are inserted into the target gene enabling the gene to be subsequently cloned.

The engineering of T-DNA to contain selectable and/or screenable markers such as the *nptII* gene, which confers resistance to kanamycin (Reynaerts *et al.*, 1988) and *uidA* (Jefferson *et al.*, 1987), which encodes the bacterial β-glucuronidase (GUS) enzyme, enables the tagged gene to co-segregate with these additional markers in crossing experiments. This greatly improves the selection process. Furthermore, with technical advances in gene transfer techniques, we can begin to identify the regulatory

networks of isolated genes and determine the interaction of their encoded products during plant development. More recently, a number of transcription factors known to be involved in plant growth and development, as well as those known to determine environmental regulation, have been characterised at the molecular and biochemical level (Ramachandran *et al.*, 1994; Batschauer *et al.*, 1994; Terzaghi and Cashmore, 1995).

1.2 The plant epidermis

The epidermis of plants is a single cell layer which covers the entire body of the plant. With the exception of stomatal guard cells, epidermal cells lack chlorophyll and are characterised by anticlinal divisions which give them an elongated appearance. The main function of the epidermis is to protect the plant from biotic and abiotic stresses. These include pathogen (Jenks *et. al.*, 1994a) and insect attack (Eigenbrode and Espelie, 1995), water loss (Hall and Jones, 1961) and cold acclimation (Thomas and Barber, 1974). As a result the outer walls of epidermal cells are often thicker compared to the inner walls. The epidermis also regulates the uptake of gases and water through special pores present on the surface of the epidermis.

The shoot epidermal layer is often covered by a hydrophobic amorphous layer known as the cuticle which plays a central role in the plant's survival. It is composed of insoluble high molecular weight lipid polyesters known as cutins which are formed following intermolecular esterification between the carboxylic and hydroxyl groups of long chain (C16 and C18) polyhydroxylated fatty acids (Kolattukudy, 1975).

The root epidermis (section 1.3.4) on the other hand is involved in the uptake of ions and water and in some cases forms a symbiotic relationship with various fungal and bacterial organisms (Peterson, 1992). It is generally accepted that roots do not synthesize cutin (Buvat, 1989).

1.2.1 Specialised epidermal cells: trichomes, guard cells and root hair cells

Epidermal cells can be differentiated into specialised epidermal cells such as trichomes and guard cells, which are located on the aerial surfaces of the plant, and root-hair cells found on the mature root. Trichomes are single cell hairs that cover the

epidermis of leaves, petioles, sepals and stems. They are between 200 and 500 μm in height with a thickened cell wall, and are generally not essential for viability as mutant plants lacking these stuctures are viable. During early epidermal cell (protoderm) development, a subset of epidermal cells differentiate to produce an enlarged cell that protrudes from the surface of the epidermis. This then develops into a branched hair or trichome. This complex morphogenesis of a relatively simple epidermal cell has made trichome development an attractive model for the study of epidermal cell fate and differentiation in plants (see section 1.3.5). The function of trichomes varies between species but in general their function is protection against environmental stresses. In some desert plants, trichomes are thought to moderate leaf surface temperature as well as photosynthetic and respiration rates (Ehkeringer, 1984). Also, some trichomes located on leaves and stems have glandular properties and are capable of synthesizing secondary metabolites that protect plants from herbivores (Wagner, 1991).

The opening and closing of the stomata or pores on the surface of leaves and stems is regulated by a pair of specialised epidermal cells known as guard cells. They are found on the aerial surface of leaves, stems and reproductive organs. The opening and closing of guard cells is regulated by signals such as turgor pressure, light, abscisic acid and Ca^{2+} treatment (Assmann, 1993). The spacing between the stomata (and trichomes) is often regular, suggesting a tightly controlled developmental pattern. Very little is known about the mechanism of stomatal patterning and guard cell differentiation. Furthermore, genes that control these mechanisms are unknown.

The relative simplicity of epidermal root cells has made them an ideal model in the study of epidermal development (Schiefelbien and Benfey, 1991; Dolan *et al.*, 1993). The root epidermis consists of two cell types: root-hair cells or trichoblasts and hairless cells or atrichoblasts. The main function of root hairs is to aid in anchoring roots in soil to interact with soil micro-organisms and to increase the surface area of roots which aids water and nutrient uptake (Cormack, 1962; Clarkson, 1985).

1.3 Epidermal development

1.3.1 Origins of the shoot and root epidermis

The main part of the plant body plan of flowering plants such as *Arabidopsis* is laid down during embryogenesis (Steeves and Sussex, 1989; Jurgens *et al.*, 1991),

which is one of the most complex developmental processes that takes place in higher plants. A large number of genes, up to 4000, are thought to be involved in the embryogenesis process (Jurgens *et al.*, 1991; Meinke, 1991). Following fertilisation, the diploid zygote within the embryo sac of the flower begins a number of cell divisions until a 16 cell stage is reached. The first cell division of the fertilised zygote gives rise to an apical-basal pattern of development which is clearly visible in the developing zygote which elongates three-fold in the apical direction. The asymmetry generates a small upper apical cell which is entirely surrounded by the dermatogen and a lower basal cell. The apical cell develops into the embryo shoot meristem, cotyledons, hypocotyl and part of the embryonic root. The larger basal cell forms the suspensor and the root meristem (West and Harada, 1993; Goldberg *et al.*, 1994; Jurgens, 1995).

The radial pattern of development is characterised by three concentric layers of tissue of the apical cell when the eight cell stage is reached (Satina *et al.*, 1940; Irish, 1991; Jurgens *et al.*, 1991). The outer layer known as the protoderm gives rise to the epidermal cell line and is characterised by anticlinal divisions, the next layer gives rise to the main body of the plant and the central layer gives rise to the vasculature (West and Harada, 1993; Goldberg *et al.*, 1994; Jürgens, 1995). Further divisions of the embryo then lead to the globular stage of embryogenesis which is followed by the torpedo and finally the heart stage in which the two cotyledons begin to form. The mature embryo is encased in the seed until germination is stimulated.

Upon seed germination, the shoot and root meristems maintain embryonic characteristics to produce postembryonic shoot and root systems of the adult plant (Sussex, 1989). Schmidt (1924) first observed that shoot apical meristems had two distinct regions, the *tunica* and the *corpus*. The *tunica* region was characterised by anticlinal divisions that increased the apex length whereas the cells in the *corpus* region had irregular divisions that increased the volume of the shoot apex. The shoot meristem was subsequently described as having three layers, the upper L1 layer from which the epidermis is derived, a sublayer known as the L2 from which the ground tissue was derived and finally the central L3 layer which gave rise to the vasculature (Satina *et al.*, 1940). However, in *Arabidopsis* the shoot apical meristem consists of two *tunica* layers equivalent to the L1 and L2 covering a *corpus* layer (Medford *et al.*, 1992). An analysis of several pattern mutants in *Arabidopsis* (see section 1.3.2) have

suggested that the development of the shoot and root meristems are under different mechanistic controls. For example in the *shoot meristemless* mutant, *stm* (Barton and Poethig, 1993), root growth is normal whereas the shoot meristem is absent.

1.3.2 Embryo pattern mutants altered in epidermal development

The isolation and characterisation of genes involved in embryogenesis, in particular during differentiation of epidermal cells, has been technically difficult due to the lack of sufficient tissue which is embedded in floral embryo sacs. However, several *Arabidopsis* embryo mutants altered in various aspects of epidermal development have been characterised (Meinke, 1986; Jurgens *et al.*, 1994). Genetic analysis of *Arabidopsis* embryo development showed they consisted of independently patterned regions along the apical-basal and radial axes (Mayer *et al.*, 1991). Three main classes of mutant phenotypes were identified: those altered in cotyledon number, in apical-basal patterning and in radial patterning. Several *Arabidopsis* mutants altered in the radial pattern of morphology have been isolated and between 15 and 50 genes have been estimated to control pattern formation in *Arabidopsis* embryos (Jurgens *et al.*, 1991).

1.3.2.1 *keule, knolle, knopf, mickey*: mutants altered in early epidermal patterning

The *keule* (*keu*) mutant embryos at the globular stage of development have enlarged, bloated epidermal cells but relatively normal inner cells (Mayer *et al.*, 1991, 1993; Jurgens, 1991). The *keule* gene appears to control both epidermal cell differentiation and growth since both ground tissue and vasculature are normal. The *knolle* (*kn*) mutant embryos on the other hand have enlarged cells from the point when the epidermal primodium would normally be established. At the globular stage of *knolle* mutants there is no clear difference between the outer cell layer destined to become the epidermis and the inner cells. The product of the *KNOLLE* (*KN*) gene resembles a secretory pathway protein like the animal and yeast syntaxins (Lukowitz *et al.*, 1996) which are thought to act as transport vesicle receptors. To determine

whether the enlarged outer layer cells in the *kn* mutants were of epidermal origin, Lukowitz *et al.* (1996) used the *fusca* (*fus*) mutant (Misera *et al.*, 1994), which normally accumulates anthocyanins in the inner cell layers but not in the epidermal cells, and genetically crossed it with a *kn* mutant. In *kn/fus* double mutants only a fraction of the outer cells in the embryos accumulated anthocyanins which suggested *KNOLLE* normally determines cell identity along the radial axis. Furthermore, Vroemen *et al.* (1996) observed that a lipid transfer protein (section 1.4.3) that is normally expressed only in the protoderm of young developing embryos was expressed only in internal cells but not in the abnormal epidermal progenitor cells of the *kn* mutant.

Other mutants altered in the radial pattern of epidermal development include the *knopf* and *mickey* (Mayer *et al.*, 1991) mutants. The *knopf* mutants contain tightly stacked epidermal cells in the hypocotyl whereas the *mickey* mutants have very large, disc-shaped cotyledons with enlarged epidermal cells. Analysis of these mutant phenotypes amongst others suggested that pattern formation was independent of cell shape but that the cell *position* in the young embryo was a major factor in determining cell fate (Mayer *et al.*, 1991).

1.3.3 Homeotic genes and epidermal cell fate

Cell type characteristics in plants are acquired in a progressive process involving positional cues, environmental signals and internal genetic programs. Several plant homeobox genes have been implicated in determining cell fate, in positional morphogenetic cues and pattern formation in the shoot apical meristem and embryo (Gebring *et al.*, 1994; Lawerence and Morata, 1994). Homeobox genes are characterised by a common 180 bp sequence, known as the homeobox, that encodes a 60 amino acid homeodomain which comprises the DNA-binding domain (Kornberg, 1993). In plants, four different types of homeodomain proteins have been identified: the *Arabidopsis* homeodomain protein *GLABRA2* (*GL2*) (Rerie *et al.*, 1994), homeodomain zipper proteins that posses a leucine zipper adjacent to the homeodomain (Ruberti *et al.*, 1991; Mattson *et al.*, 1992; Schena and Davis, 1992, 1994), plant homeodomain finger proteins that share a conserved cysteine-rich motif

(Korfhage *et al.*, 1994) and *KNOTTED1* (*KN1*) and related proteins (Volbrecht *et al.*, 1991; Lincoln *et al.*, 1994; Ma *et al.*, 1994).

The *GL2* gene in *Arabidopsis* is required for normal trichome and root development (see section 1.3.5.1). The *SHOOT MERISTEMLESS* (*STM*) gene, a *KN1*-like gene in *Arabidopsis*, and the *KN1* gene in maize are thought to be involved in shoot apical meristem formation in the embryo (Smith *et al.*, 1995; Long *et al.*, 1996). Plants that are mutated in the *STM* locus failed to develop a shoot apical meristem during embryogenesis (Long *et al.*, 1996). In maize shoot apical meristems, *KN1* mRNA was expressed in the L2 and L3 *corpus* layers but not in the L1 layer suggesting a role in establishing a boundary for the meristem from the rest of the shoot and thus enabling the meristem to remain in an undetermined state (Jackson *et al.*, 1994).

1.3.3.1 *ATML1* may determine epidermal-specificity of genes

An L1 layer-specific gene in *Arabidopsis* that expressed a new class of homeodomain protein designated *ATML1* (*Arabidopsis thaliana* meristem L1 layer) was isolated by Lu *et al.* (1996). This protein was shown to be highly similar to the *Arabidopsis GL2* homeodomain protein and the Phalaenopsis O39 (Nadeau *et al.*, 1996) proteins within and outside the homeodomain regions. This protein was therefore classed as a plant homeodomain-containing protein (HD-GL2). *ATML1* mRNA was restricted to the protoderm layer. However, at the torpedo stage of embryogenesis, *ATML1* was no longer expressed but reappeared in the L1 layer of the shoot apical meristem. Following germination, the L1-specific pattern of expression was maintained in the vegetative shoot apical meristem as well as the floral meristem, inflorescence and in young floral organ primodia. However, *ATML1* accumulated only in the undifferentiated actively dividing cells and not in the mature epidermal cells. Unlike other L1-specific genes such as those that encode lipid transfer proteins (see section 1.5.3), tomato polyphenoloxidase (Shahar *et al.*, 1992) and several unknown sequences isolated from *Pachyphytum* (Clark *et al.*, 1992), *ATML1* was not expressed in mature epidermal cells. Therefore, *ATML1* has been implicated as a transcriptional regulator determining the epidermal-specific expression pattern of downstream target genes such as those mentioned above.

1.3.4 Root epidermal development

The simplicity of the root epidermis (Schiefelbien and Benfy, 1991; Dolan *et al.*, 1993) makes it an ideal model to study cell differentiation in higher plants as differentiation of specific epidermal cells into root hair cells can be easily observed. The development of the root epidermis has been studied histologically (Dolan *et al.*, 1993), by clonal analysis (Scheres *et al.*, 1994), in mutants altered in various aspects of root development (Baskin *et al.*, 1992; Benfey *et al.*, 1993) and by dye-coupling (Duckett, *et al.*, 1994).

The *Arabidopsis* root has a simple, radial structure with four distinct cell layers; the epidermis, the cortex, endodermis and pericycle consisting each of a single layer of cells with the vascular tissue at the centre. The developing epidermal cells are derived from a set of 16 initials that are located in a ring. Each of the 16 cells gives rise to clones of cells that are arranged into columns or files along the length of the root. The growing root can be divided into three distinct zones: the meristematic zone, the elongation zone and the cell differentiation zone. Newly formed epidermal cells are located near the root meristem whereas more advanced ones are located further from the tip. Duckett *et al.* (1994) used a number of dye-loading methods to show that undifferentiated, meristematic cells and cells in the elongation zone are symplastically connected. However, cells in the differentiated zone become symplastically isolated, and as the root hair begins to grow, the cells of the epidermis are completely isolated. This process is similar to the pattern of gap-junction connections observed in animal systems where cells are coupled during cell patterning but gradually become isolated as they differentiate. As mentioned previously, the root epidermis differentiates into two cell types: root-hair bearing cells (trichoblasts) and hairless cells (atrichoblasts) (Bünning, 1951; Cutter, 1978). Eight of the files develop as root hairs and between 8 and 13 differentiate into hairless cells. Hair cells are always located between two underlying cortical cells while the hairless cells are located next to a single cortical cell file.

Sussex (1995) identified more than 40 genes whose mRNA expression levels were increased in the lateral root primodium during lateral root development. Around 14 pericycle cells are founder cells of lateral root primodia in *Arabidopsis*. The rate of lateral root formation could be increased by exposure to auxin and the main class of

genes which displayed increased expression upon exposure to auxin were ribosomal proteins. By *in situ* hybridisation, one protein, RPL16A (Sussex, 1995) was shown to be expressed transiently in pericycle cells that are involved in the initiation of lateral root primodia. Keller *et al.* (1994) identified a cell wall protein expressed specifically in lateral root initials.

1.3.4.1 Differentiation of root hair and hairless cells

The production of root hair and hairless cells in the *Arabidopsis* root has been shown to be affected by five loci: *TRANSPARENT TESTA GLABRA* (*TTG*), *GLABRA2* (*GL2*), *ROOT HAIR DEFECTIVE6* (*RHD6*), *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*) and *AUXIN RESISTANT2* (*AXR2*), as well as the two plant hormones, ethylene and auxin. By a combination of reporter gene studies and the epistatic interaction of the genes in double mutants, Masucci and Schiefelbein, (1996) demonstrated that *TTG* and *GL2*, which established hairless cells, act early to negatively regulate the ethylene and auxin pathways. Furthermore, auxin and ethylene pathways promoted root hair outgrowth once cell-type identity had been established. Masucci and Schiefelbein (1996) proposed a model where patterning of root-epidermal cells in *Arabidopsis* is regulated by the cell position-dependent action of the TTG/GL2 pathway, and the auxin and ethylene pathways promote hair outgrowth at relatively later stages of cell differentiation.

In pea, Mylona *et al.* (1994) revealed the existence of at least ten proteins expressed at high levels in root hairs by two-dimensional gel electrophoresis of root and root hair proteins. However, these proteins have not been fully characterised. Furthermore, *in situ* hybridisation revealed the existence of a root epidermis-specific gene, *RH2* that had 95 % amino acid identity to the pea pathogenesis-related gene, I49a (Mylona *et al.*, 1994).

1.3.4.2 Mutants altered in root epidermal morphology: *reb*, *rhd*, *ttg*

Several *Arabidopsis* mutants altered in root development have been characterised. Baskin *et al.* (1992) isolated a mutant with altered epidermal cells. The *r*oot *e*pidermal *b*ulger (*reb1*) plants were characterised by epidermal cells that bulged

and became large and inflated. These larger cells were observed only at the region where cells were expanding. In addition, root hair development was not repressed. Another mutant allele, *reb1-2*, was temperature-sensitive, displaying a similar mutant phenotype at 33°C. This mutation, unlike the *ttg* mutation (see below), was specific to the root epidermis and did not affect the shoot epidermal development. Baskin *et al.* (1992) speculated that a change in the arrangement of the cortical microtubules may be the cause of bulged epidermal cells.

Several *Arabidopsis* mutants altered in specialised epidermal root hair cell differentiation have also been isolated (Schiefelbein and Sommerville, 1990). Four different genes were identified as playing a role in root hair development. These genes were termed *RHD* for *r*oot *h*air *d*evelopment. The *rhd1* mutant had normal root hairs but a bulged base. The *rhd2* mutant produced shorter root hairs whereas the *rhd3* mutants produced short root hairs with a wavy and sometimes branched appearance. The *rhd3* mutants were also much smaller and had shorter roots. Conversely, the *rhd4* mutants produced slightly shorter root hairs with bulges along the length of the root hair. Both the *rhd3* and *rhd4* mutants are thought to result from aberrant deposition of cell wall components. Epistatic analysis of several double mutants indicated *RHD1* was involved early in root hair cell differentiation by softening and pushing out the root hair. All double mutants of *rhd1* had bulbous root hair bases. *RHD2* appeared to be involved in activating root hair growth whereas *RHD3* and *RHD4* were involved in controlling the hair tip growth.

In *Arabidopsis ttg* (*transparent testa glabra*) mutants that lacked trichomes, anthocyanins and seed coat mucilage, root epidermal cells in all positions differentiated into root-hair cells. This mutant phenotype could however be rescued by the maize *R* gene (Lloyd *et al.*, 1992). Also, over-expression of *R* driven by the CaMV35S promoter in transgenic *Arabidopsis* plants produced plants completely devoid of root-hairs. Therefore, the normal function of the *TTG* product was to respond to positional signals and hence, to cause neighbouring cells to adopt different fates, either becoming root-hair producing or hairless cells (Galway *et al.*, 1994). The *Arabidopsis icx1* (Jackson *et al.*, 1995, see section 1.3.5.2) mutant also showed abnormal root growth. The roots were shorter in length and had root hair development in adjacent files.

1.3.5 Trichome development

1.3.5.1 *Arabidopsis* trichome mutants: *gl1*, *gl2* and *ttg*

The first stage of wild type trichome development is characterised by swelling of the progenitor cell which comes about as a result of three rounds of endoreplication of the nuclear DNA (Larkin *et al.*,1994). The young trichome then protrudes from the leaf epidermal base and forms the branched structures that require a final round of endoreplication. In *Arabidopsis*, more than 70 trichome mutants have been isolated and characterised representing 21 genes (Koornneef *et al.*, 1982; Marks and Feldmann 1989; Hülskamp *et al.*, 1994). Trichome initiation has been shown to be controlled by two genes, *TRANSPARENT TESTA GLABRA* (*TTG*) and *GLABRA1* (*GL1*) (Oppenheimer *et al.*, 1991; Larkin *et al.*, 1994), since recessive mutations in these genes block the initiation of trichomes. Mutations at the *TTG* locus are pleiotrophic; mutant plants lack not only trichomes but also seed coat mucilage and seed coat colour, and are also affected in root development (section 1.3.4.1). Mutations at the *GL1* locus on the other hand affect only trichome development. *GL1* encodes a myb-like transcripitional factor which is expressed at a low level throughout the developing epidermis (protoderm) of leaves and stems and is upregulated as trichomes begin to develop (Larkin *et al.*,1994). *Arabidopsis* mutants that lack the *GL1* gene do not produce any trichomes. Transgenic *Arabidopsis* plants expressing *GL1* constitutively under the control of the CaMV35S promoter showed a decrease in trichome density and number compared to wild type. Over-expression also resulted in ectopic expression of trichomes on the abaxial surface of the cotyledon and of the first leaf, locations where trichome development normally do not occur. It was clear from these experiments that *GL1* expression alone was not enough to commit all epidermal cells to form trichomes.

Like *gl1*, *ttg* mutants that lack the *TTG* gene are devoid of trichomes. However, they can be complemented with the maize *R* gene (Bodeau and Walbot, 1992; Goff *et al.*, 1992) under the constitutive control of the CaMV35S promoter which suggested *TTG* in *Arabidopsis* either encoded an *R* homologue or a factor that activates the *R* gene homologue (Larkin *et al.*, 1994). The authors also generated transgenic *Arabidopsis* plants that constitutively expressed the *GL1* gene but in a *ttg* background. In this way it was possible to determine their relative times of expression

during development. These plants did not form any trichomes suggesting that *GL1* did not act downstream of *TTG*. Also, Larkin *et al.* (1994) generated transgenic *gl1* plants expressing the *R* gene constitutively and showed that these plants did not form any trichomes. Collectively, these results suggested that *TTG* was not downstream of *GL1* and that both genes acted independently but at the same position in the trichome development pathway. Therefore, Larkin *et al.* (1994) suggested that *TTG* may be inhibiting differentiation of neighbouring cells into trichomes by possibly acting as a diffusible signalling component. For example, if one epidermal cell becomes determined to become a trichome, it can signal to the neighbouring cell to inhibit trichome differentiation. This hypothesis was supported by experiments where *Arabidopsis* plants hemizygous for the *35S-GL1* construct and heterozygous for the *TTG* locus, ie, *35S-GL1/-;+/ttg*, produced clustered trichomes. Another mutation implicated in lateral inhibition of trichome development was found at the *TRY* locus (Hülskamp *et al.*, 1994) since *try* (*triptychon*) mutants displayed clusters of trichomes.

The *gl2* (*glabra2*) mutant (Rerie *et al.*, 1994) is characterised by the production of very few or underdeveloped trichomes that still undergo initial rounds of endoreplication. The *GL2* transcript was shown by *in situ* hybridisation to be expressed in the epidermal progenitor cells and at stages associated with trichome development. Mutations in the *GL2* gene seem to interfere specifically with the local outgrowth of the trichome cell. However, in addition to *GL2* expression, the size of the trichome cell also influences the local outgrowth. In general, a smaller than normal trichome cell size influences the *gl2* phenotype. The gene encodes a homeodomain-containing protein required for transcription of genes necessary for normal trichome development in *Arabidopsis* (Rerie *et al.*, 1994).

1.3.5.2 *icx 1* (increased chalcone synthase expression)

The *icx1* mutant was derived from transgenic *Arabidopsis thaliana* ecotype Landsberg *erecta* plants carrying the *Sinapsis alba* (white mustard) *CHS* promoter-*GUS* transgene (Batschauer *et al.*, 1991) in an attempt to isolate mutants altered in light signal transduction (Jackson *et al.*, 1995). The *CHS-GUS* transgene was regulated by UV-B, UV-A, blue and high intensity white light treatment in *Arabidopsis*, but was barely active in low white light and red light, which is similar to

the endogenous *CHS* gene (Jackson *et al.*, 1995; Fuglevand *et al.*, 1996). Seed from these plants were mutagenized by EMS treatment and then screened for mutants with altered light-induction of the transgene by growing the seedlings under low white light conditions. Several mutant lines were selected and one, A12, was selected which had elevated levels of not only *GUS* activity but also endogenous *CHS* expression. This implied that A12 was a true mutant altered in the regulation of the *CHS* promoter. The A12 line was designated *icx1* (increased chalcone synthase expression) as it had enhanced light-stimulation of *CHS-GUS* expression. The mutant, however, behaved in a similar manner to wild type plants in the dark and under low intensity white light conditions. As well as increased chalcone synthase (*CHS)* expression, chalcone isomerase (*CHI*) and dihydroflavonol reductase (*DFR*) gene expression were enhanced in the mutant, which correlated with a 2-3-fold increase in anthocyanin production. Interestingly, the mutant was also altered pleiotropically in several aspects of epidermal development. It had fewer trichomes, smaller seed, narrow leaves, shorter roots with disorganised root hairs and was generally smaller than wild type plants (Jackson *et al.*, 1995; J. A. Jackson and G. I. Jenkins, unpublished). A closer look at the epidermal layer by SEM indicated the epidermal pavement cells were abnormal in shape. From these observations it was concluded that the *ICX1* gene product normally functioned as a repressor of the *CHS*, *CHI* and *DFR* genes and that it was also involved in the development of the epidermis.

1.3.6 Guard cell development

As described previously in section 1.2.1, guard cell pairs control the pore size of stomata. The guard cells are derived from epidermal cells known as 'meristemoids' (Bünning and Sagromsky, 1948; Bünning, 1953). In the *Brassicaceae* family, which includes *Arabidopsis*, stomatal development is dependent on meristemoid formation and activity (Pant and Kidwai, 1967). The meristemoid is formed during aysmmetric division of the protodermal cell. The larger of the two daughter cells gives rise to a non-stomatal epidermal cell whereas the smaller cell gives rise to the meristemoid. The latter then undergoes several asymmetric divisions whereupon the larger ones differentiate into epidermal cells. After the last epidermal cell is produced the meristemoid is converted into a guard mother cell which undergoes symmetric division

to produce two guard cells at which point cell division is arrested. A striking feature of stomata formed in this way is the tightly ordered spacing between each stoma (Sachs, 1991).

1.3.6.1 Stomata mutants *tmm* and *flp*

Unlike trichome and root hair development, where more than 30 different loci have been identified by mutation, very few mutants affected in stomata development have been identified. Zeiger and Stebbins (1972) described a stomata-defective mutant in barley, *ecerifcrum-g* which has double and triple adjacent stomatal complexes. More recently, Yang and Sack (1995) isolated two *Arabidopsis* mutants, *tmm* (*too many mouths*) and *flp* (*four lips*) which contained extra adjacent stomata, by screening cotyledons from ethylmethanesulfonate-mutagenized seed by light microscopy. The *tmm* mutation resulted in stomatal clustering and increased meristemoid cell formation whereas the *flp* mutation was charcterised by several paired stomata and a few unpaired guard cells in the cotyledons. Furthermore, the double mutant *tmm/flp* displayed characteristics from both mutants. However, the genes involved in these processes have not been identified.

1.4 Epidermal-specific genes

A number of genes have been reported to be expressed in the epidermis of plants. These include anti-fungal proteins such as thionins (Florack and Stiekema, 1994), cell wall proteins such as hybrid proline-rich proteins (Wyatt *et al.*, 1992), the tomato polyphenoloxidase gene (Shahar *et al.*, 1992) and the carrot *EP1* glycoprotein gene which shows homology to the *Brassica napus* S-locus glycoprotein (Van Engelen *et al.*, 1993). Mylona *et al.* (1994) identified a root epidermal-specific gene, *RH2* which is 95 % identical to the pea pathogenesis-related (PR) gene, I49a. The gene was switched on during post-embryonic development after the transition of protoderm into epidermis. Also, Clark *et al.* (1992) described several unidentified epidermal-specific transcripts in *Pachyphytum* in an attempt to characterise epidermis-specific gene expression. Furthermore, the *GL1* and *GL2* (sections 1.3.5 and 1.3.4.1) genes that control trichome development were expressed at low levels in the epidermis but were upregulated in regions of trichome differentiation. Other epidermal-specific genes

discussed in this review include those involved in the epicuticular wax biosynthetic pathway (section 1.4.1), genes involved in flavonoid biosyhthesis (section 1.4.2) and genes encoding plant lipid transfer proteins (section 1.4.3).

1.4.1 Epicuticular waxes

The plant epidermis is almost always covered with a waxy layer known as the epicuticular waxy layer and, as would be expected, is the main site of wax biosynthesis (Kolattukudy, 1968; Lessire, 1982). The waxes interact with a relatively insoluble lipid polymer of hydroxy and epoxy fatty acids known as cutin which is the main structural component of the cuticular membrane and, as a result, form a continuous amorphous covering on the outer surface of the plant. In underground tissues and regions of wound healing waxes are associated with the suberin matrix which is another polymer related to cutin. Suberin has both aliphatic and aromatic domains located between the plasmalemma and walls of the outermost one or two cell layers. The epidermal cell walls are linked together and to the cuticle by pectic cuticular components that attach to the outer cell. Furthermore, the outer cell wall is linked to a polymeric carbohydrate layer. It is on these carbohydrate fibres that the cuticular and epicuticular waxes are deposited. Plants with this layer usually have a glaucous or grey appearance (Kolattukudy, 1975).

Epicuticular waxes are found on leaves, stems, fruit, seeds, seed coat, flowers, bark and husks. The composition of waxes varies considerably not only between plants of the same and different species, but also within the same plant such that organ-specific and tissue-specific waxes exist. For example, in *Arabidopsis*, the leaf (Jenks *et al*, 1995), stem (Hannoufa *et al.*, 1993), pollen (Preuss *et al.*, 1993) and silique (Koorneef *et al.*, 1989) have different wax compositions and contents. The major classes of wax components in different plant species vary considerably. For example, the *Cruciferae* family which includes the *Brassica napus* and *Arabidopsis thaliana* species contain acids, alcohols, aldehydes, β-diketones, esters and hydrocarbons as the major classes of wax components, whereas others such as the *Gramineae* family which includes species such as *O. sativa*, *Z. mays* and *S. vulgare* contain in addition to the compounds already mentioned, alkenes, phytosterols, polycyclic triterpines and triglycerides. Wax composition also varies considerably during development. For

example, in young maize leaves primary alcohols predominate (63%) whereas older leaves have mainly wax esters (42%) (Avato *et al.*, 1990; Bianchi *et al.*, 1979).

The main function of the epicuticular waxy layer is to prevent water loss through the epidermis which contributes to drought tolerance (Jordan *et al.*, 1984). Waxes also protect plants from pathogen and insect attack (Eigenbrode and Espelie, 1995). Some plant epicuticular lipids can also contribute to insect resistance (Eigenbrode *et al.*, 1991). Furthermore, leaf epicuticular waxes have been implicated in protection against mechanical damage (Eglinton and Hamilton, 1967) and agricultural chemical sprays (Schreiber and Schonherr, 1992). Also, Preuss *et al.* (1993) found wax composition and structure are important factors in pollen/pistil interaction.

1.4.1.1 Biosynthesis of epicuticular waxes

Cuticular wax biosynthesis occurs almost exclusively in epidermal tissue (Lessire, 1982) despite the fact that the wax precursors which are fatty acids are derived from *de novo* synthesis in plastids which occurs throughout the plant. Fatty acid synthesis is catalysed by a number of enzymatic steps known as the fatty acid synthase (FAS) (Post-Beittenmiller, 1996). Acetyl-CoA carboxylase (ACCase) catalyses the first committed step in fatty acid synthesis, namely the formation of malonyl-CoA. Two forms of the enzyme, the prokaryotic and eukaryotic forms, have been found in plants (Sasaki *et al.*, 1993; Konishi and Sasaki, 1994; Sasaki, *et al.*, 1995; Alban *et al.*, 1994). The prokaryotic form is composed of several subunits and is located in plastids whereas the eukaryotic ACCase, which is composed of a single multifunctional polypeptide, is located in the cytosol. Malonyl-CoA is also used for flavonoid biosynthesis (see section 1.4.2). The first step in fatty acid synthesis is the condensation of malonyl-acyl carrier protein (ACP) with acetyl-CoA (Jaworski *et al.*, 1993), followed by the sequential reduction of 3-ketoacyl-ACP, the dehydration of 3-hydroxyacyl-ACP and the reduction of trans-delta^2-enoyl-ACP. The fatty acyl primer remains esterified to the ACP cofactor and is further extended, two carbons at a time, by the donor, malonyl-ACP. For each two carbon addition, there is a sequential round of condensation, reduction, dehydration, and second-reduction steps. NAD(P)H serves as a reductant for the two reductases. The long chain products (C16, C18) are subsequently processed by hydrolases including stearoyl-ACP desaturase, plastidial

acyl-transferases and acyl-ACP thioesterases. The fatty acids produced are then partitioned to synthesise glycerolipids, waxes or cutin and suberin depending on the tissue types and the particular developmental stage.

1.4.1.2 Wax biosynthesis is characterised by elongase activity

Following *de novo* fatty acid biosynthesis, partitioning occurs that delivers C16:0 and C18:1 hydroxy fatty acids to the glycerolipid or cutin/suberin biosynthetic pathways and the C18:0 to wax biosynthetic pathways in the ER (Post-Beittenmiller, 1996). This partitioning mechanism is not clearly understood, but it is likely that the mechanism is dependent either on precursor availability and elongase specificity or on the relative fatty acid pool levels in individual cells. Several experimental data support the idea that epicuticular plant waxes are derived from stearate and that elongases are involved in lengthening the chain followed by several reactions responsible for making other derivatives such as aldehydes, ketones and alkane wax esters (Liu *et al.*,1995). The main enzyme activity characterising wax biosynthesis is fatty acid elongation. The majority of wax components are derived from very long chain fatty acids (VLCFAs) which are 20-32 carbons in length. Also, fatty acids esterified to alcohols can be 40-60 carbons in length (von Wettstein-Knowles, 1995). The VLCFAs are derived from the C16 or C18 fatty acids that are elongated extraplastidially by microsomal enzymes similar to *de novo* fatty acid biosynthesis. The acyl chains undergo four basic reactions for every two carbon addition. These are, condensation, reduction, dehydration and second reduction. Together, these four activities are known as elongases (von Wettstein-Knowles, 1992). Multiple elongation systems are present in wax biosynthesis that produce a variety of lipid classes. These can be split into three different pathways: decarbonylation, acyl-reduction and β-ketoacyl-elongation all of which are found in epidermal cell layer (Cheesbrough and Kolattukudy, 1984). The different pathways were elucidated by a combination of inhibitor studies (for example, Agrawal *et al.*, 1984) and isolation of wax-deficient mutants (see section 1.4.5). The decarbonylation pathway gives rise to aldehydes, odd-chain alkanes, secondary alcohols and ketones whereas the acyl-reduction pathway is characterised by the production of aldehydes, primary esters and wax esters which are produced following esterification of elongated fatty acids and primary alcohols. The β-ketoacyl-elongation

pathway gives rise to β-diketones and their derivatives. However, different organs of the same plant and different species utililize the components of these pathways in different ways. As in the FAS reaction, NAD(P)H provides the reducing power for the elongase associated reduction processes (Post-Beittenmiller, 1996). The main differences between the FAS and elongase processes are that for the elongase process, VLCFA elongation is ATP dependent (Cassagne *et al.*, 1987; Evenson *et al.*, 1995), it does not occur on ACP and manoyl-CoA (not malonyl-ACP) provides the carbon source. In addition, elongases are membrane-associated and extra-plastidial rather than stromal and soluble (Cassagne *et al.*, 1987). However, the regulation of this elongating activity, the specific elongase substrates and cofactors involved in the process and the number of elongases catalysing VLCFA biosynthesis are poorly understood. Although an epidermis-specific elongase has been isolated from leek (*Allium porrum* L.) (Bessoule *et. al.*, 1989), characterisation of elongases has been a slow process mainly due to the low enzyme activity, enzyme instability in detergents and the problem of isolating elongases from membrane (Lessire *et. al.*, 1985).

Cutin is the major component of plant aerial surfaces and is made exclusively in the epidermis whereas suberin is a polymer associated with roots and wound sites in the suberin pathway. Both cutin and suberin form the initial barrier to the environment and are part of the cuticle. Both are polymers of C16:0 and C18:1 hydroxy fatty acids (Kolattukudy, 1987). They are produced by a family of oxidases which have similar activities to those of the decarbonylation and β-diketoacyl-elongation pathways for wax biosynthesis (von Wettstein-Knowles, 1995).

1.4.1.3 Movement of cuticular waxes to the outer surface

The mechanism by which lipids are transported from their main site of synthesis, the endoplasmic reticulum, and the chloroplast to other cellular organelles is unknown. Furthermore, there is no evidence of vesicular trafficking between the ER and the chloroplast. Although extensive research has been carried out to identify the components of the wax biosynthetic pathway, the mechanism by which the wax products are transferred to the surface of the plants is still not clearly understood. Early SEM work carried out by Jefree *et al.* (1976) suggested general diffusion of wax components through small spaces in the cell wall, based on the observation of an

amorphous coating of wax throughout the cuticle. This also suggested that the cuticular wax was deposited at regular intervals or continuously. Furthermore, epicuticular wax is secreted to the plant surface after cell-wall and cuticle layers are well developbed (Jenks *et al.*, 1994b). Another proposed hypothesis was that the transport of wax components may occur through well defined pores in the cell wall, which are found on the surface of some plants (von Wettstein-Knowles, 1995). However, a particular epicuticular crystalline structure probably arises from the physical and chemical properties of the wax as well as external, environmental influences, rather than the nature of the transport process. For example, in recrystallisation studies of wax on spruce needles that were mechanically damaged the wax structures reformed on the original crystalline structure even on dead needles, in the absence of new synthesis or export of wax components (Bermadinger-Stabentheiner, 1995).

Several people have shown that lipid transfer proteins (LTPs) can enter the secretory pathway and are located extracellularly (Mundy and Rogers, 1986; Sterk *et al.*, 1991) (discussed in section 1.4.3). An *Arabidopsis LTP1* (Thomas *et al.*, 1993) and barley and maize LTPs (Molina *et al.*, 1993; Sossountzov *et al.*, 1991) were shown to be located in epidermal cell walls and a *Brassica oleracea* LTP (WAX9D) was found to be the major protein in the surface wax of broccoli leaves (Pyee and Kolattukudy, 1994). Furthermore, a *Brassica napus* LTP was able to bind acyl-CoA *in vitro* (Østergaard *et al.*, 1995). This led to the hypothesis that LTPs may be involved in transporting cuticular lipid components such as wax and cutin or suberin monomers to the surface. However, the LTP:lipid ratio does not correlate with this postulated role of LTPs (Molina and Garcia-Olmeda, 1993). Moreover, it has not been demonstrated whether LTPs can recycle for additional lipids from the ER lumen. This would clearly limit the number of lipids that could be transported to the surface.

1.4.1.4 Environmental regulation of wax biosynthesis

Wax biosynthesis has been shown to be controlled by light and photoperiod (Reicosky and Hanover, 1978), humidity, chilling, seasonal variation (Sutter, 1982), and developmental regulation (Rhee *et al.*, 1997). Analysis of wax biosynthesis in leek leaves indicated a differential accumulation of total wax along the length of the leaf

(Rhee *et al.*, 1997). A C31 ketone was used as a marker to monitor wax content during leaf development since it was found to be the major component in the leaf wax. Furthermore, the presence of the C31 ketone indicated that the decarbonylation pathway was the dominant pathway in leek. Light microscopic analysis indicated that initiation of wax accumulation in leek leaves occurred after cell elongation was complete. The lower section of the leaf had very little wax compared to the upper portion, which suggested that rapidly expanding tissue generated more wax than older tissue.

1.4.1.5 Mutants altered in wax biosynthesis

The main site of cuticular wax biosynthesis is the epidermis (Kolattukudy, 1968; Lessire *et al.*, 1982). It is generally difficult to isolate and characterise genes controlling the wax biosynthetic pathway as plant epidermal tissue is difficult to obtain in large quantities (Kolattukudy *et al.*, 1976). However, mutant plants with altered wax composition and wax ultrastructure have been isolated from *Brassica napus* and *Pisum sativum* (Holloway *et al.*, 1977), *Brassica oleracea* (Eigenbrode *et al.*, 1991), and *Zea mays* (Bianchi, 1979) and with the recent isolation of *Arabidopsis eceriferum* (*cer*) and maize *glossy* (*gl*) mutants obtained either by T-DNA or transposon tagging (Koornneef *et al.*, 1989; Walbot, 1992), the isolation of genes that regulate wax biosynthesis has been made relatively easy. In both cases the normal accumulation of cuticular waxes was altered. The mutants isolated include the *cer-cqu*, cer-yy, *cer-n* from barley, *gl1* , *gl2*, *gl4*, *gl6*, *gl3*, *gl5*, *gl6*, *gl7*, *gl8*, *gl9*, *gl11*, *gl12*, *gl15* from maize, *bm22* from *Sorghum* and from *Arabidopsis*, *cer1* to *cer 21* and *it5* (all reviewed in Post-Beittenmiller, 1996). Mutants completely devoid of waxes have not been reported probably due to the essential role waxes play in protection against the environment. Furthermore, to date only the *Glossy15* (section 1.4.1.5.2) and *CER2* (section 1.4.1.5.1) genes have been reported to be epidermal-specific. Moreover, *Glossy1* (section 1.4.1.5.2) from maize showed strong homology to an epidermal-specific cDNA from *Kleinia odora* which encodes a receptor-like integral membrane protein.

1.4.1.5.1 *Arabidopsis* wax mutants: *cer1*, *cer2*, *cer3* and *cer 6*

In *Arabidopsis thaliana* 22 gene products (*CER1* to *CER21*) and *TT5* have been shown to be involved in wax biosynthesis (Koornneef *et al.*, 1989). Mutations in these loci were identified by their bright, dark green stems when compared to their more glaucous wild type counterparts. Several of the *cer* mutants also exhibited reduced fertility which was thought to be due to a lack of wax deposits on the pollen surface resulting in abnormal pollen-stigma interaction (Preuss *et al.*, 1993).

CER1 was predicted to encode an aldehyde decarbonylase (Aarts *et al.*, 1995), an enzyme required for the production of the alkane fraction of the epicuticular waxes specific to *Arabidopsis*. Mutations in the *CER1* locus (*cer1*) caused enrichment of aldehydes and a depletion of the alkanes and alkane-derived ketones and secondary alcohols. *cer2* was defective in the elongation of C28 to C30 fatty acids (Hannoufa *et al.*, 1993). In two mutant T-DNA insertion lines, BRL5 and BRL9, T-DNA was found to be inserted in the second exon of the *CER2* gene and in the intron of the gene, respectively, and in both cases *CER2* was not expressed (Negruk *et al.*, 1996). *CER2* encoded a 47.3 kD polypeptide and transcripts were differentially expressed in the flowers and stems and specifically accumulated in the epidermis. A separate *CER* gene in *Arabidopsis*, *CER6* also appeared to be involved in the elongation of 28 carbon saturated fatty acids (Lemieux *et al.*, 1994). Both *cer2* and *cer6* appeared to be defective in the synthesis of pollen lipids and a number of mutant alleles of the *cer6* locus have a reduced fertility because of reduced lipid concentration (Preuss *et al.*, 1993). Interestingly, fertility could be rescued under humid conditions (Koornneef *et al.*, 1989). Jenks *et al.* (1995) suggested *cer2* may have a regulatory role based on differences in the wax composition between leaf and stem, although this can also be attributed to differential expression patterns of *CER2*.

Hannoufa *et al.* (1996) cloned the *CER3* gene through the isolation of a T-DNA insertion mutant line designated BRL1. They showed the gene was composed of nine introns and 10 exons and encoded a 90 kD protein. However, the second exon of *CER3* encoded a nuclear localisation sequence (NLS). The gene did not share extensive homology with any other genes in the database. *CER3* transcripts were shown to be expressed in leaves, stems, flowers, roots, and apical meristems but were not present in the insertion mutant BRL1 line. *cer3*, unlike most of the other *cer*

mutants was found to have significantly lower levels of leaf wax compared to wild type plants (Jenks *et al.*, 1995). The mutant also had a lower level of free fatty acids and long chain length alkanes on the stems compared to the wild type plants (McNevin *et al.*, 1993). These observations led to the hypothesis that the *CER3* product may be involved in the hydrolysis of fatty acyl-CoA into free fatty acids and CoA (Jenks *et al.*, 1995). However, *CER3* did not display any sequence homology with fatty acyl-ACP thioesterases (Dormann *et al.*, 1995). It was more likely therefore that *CER3* encoded a factor that regulated acyl-CoA thioesterase activity or a protein that transported acyl-CoAs to a cytoplasmic thioesterase. The gene has also been implicated in pollen-stigma recognition in *Arabidopsis*.

1.4.1.5.2 Maize wax mutants: *gl1*, *gl8* and *gl15*

Hansen *et al.* (1997) cloned the *Glossy1* (*GL1*) locus of maize by insertional mutagenesis by the Mutator1 (Mu1) transposon which resulted in the reduction of waxes on the surface of maize seedlings to about one-fourth that of wild-type levels (Bianchi *et al.*, 1989). The GL1 protein showed sequence homology to the epidermis-specific EPI23 protein from *Senecio odora* (*Kleinia odora*). The two proteins also showed sequence similarilarity to the *Arabidopsis* EST 178C3T7 and rice EST RICS2751A. Aarts *et al.* (1995) have shown that the *Arabidopsis* EST 178C3T7 is the product of the *CER1* locus, a gene that affects cuticular wax biosynthesis. Comparison of the NH_2-termini of the four proteins indicated strong seqence conservation whereas the COOH-termini were less conserved. Furthermore, 40 amino acids of the NH_2-terminus of the proteins had features similar to membrane-targeted signal peptides. An interesting feature of the predicted secondary structure of GL1 was its distinct similarity to the mammalian superfamily of β-chemokine receptors (Raport *et al.*, 1996) including the HIV co-receptor fusin (CXCR4) (Loetscher *et al.*, 1994), all of which consist of seven transmembrane helices similar to *CER1*, EP123 and RICS2751A. However, GL1 was smaller, which suggested it possibly had five transmembrane helices. Based on hydrophobicity profiles, the COOH-terminus of all these proteins had a globular, water-soluble domain located on the cytosolic side of the membrane. In mammals such receptors bind ligands at their extracellular domains and transduce signals via G-protein interactions with their cytosolic-oriented domains.

Despite strong homology with *CER1*, it is unlikely that *GL1* encodes an aldehyde decarbonylase, based on the observation that only 1 % of alkanes were found in cuticular waxes of wild type maize seedlings. Furthermore, mutations in the *Glossy1* locus affected fatty aldehydes, alcohols and fatty esters of cuticular waxes.

The *Glossy8* gene was also isolated by transposon tagging with the Mu1 transposon (Hansen *et al.*, 1997). The 327 amino acid GL8 protein had a predicted molecular mass of 36 kD and showed homology to β-ketoacyl reductases suggesting that it is part of the membrane-bound fatty acid elongase system which generates the VLCFAs required for cuticular wax biosynthesis. Conversely, the *Glossy15* gene encodes an *APETALA2*-like gene that regulates leaf epidermal cell identity (Moose and Sisco, 1996). In maize the transition from young to adult shoot development affects several epidermal traits including epicuticular waxes, leaf hairs and cell wall characteristics, and GL15 is required for the expression of young epidermal cell traits after the development of the second leaf.

1.4.2 Genes involved in flavonoid biosynthesis

1.4.2.1 Flavonoids accumulate epidermal cells

Flavonoids are a large family of secondary metabolites found in all higher plants. These are phenolic compounds which include the flavonoids such as flavonols, flavonones, isoflavonoids and anthocyanins. Synthesis of flavonoids initiates with the condensation of one molecule of 4-coumaroyl CoA and three molecules of malonyl-CoA resulting in naringenin chalcone. This reaction is carried out by chalcone synthase (*CHS*). Coumaroyl-CoA is derived from the general phenylproponoid pathway where it is synthesised from the amino acid phenylalanine by the action of three enzymes, phenylalanine ammonialyase (*PAL*), cinnamate 4-hydroxylase (*C4H*) and 4-coumaroyl-coenzyme A ligase (*4CL*). The general phenylpropanoid pathway is so called due to the formation of a myraid of phenylpropane-based structures used to synthesise compounds such as lignin, coumarins, stilbenes and flavonoids. Malonyl-CoA is synthesised by the carboxylation of acetyl-CoA and is also required in fatty acid metabolism (section 1.4.1). Chalcone is further isomerised by the enzyme chalcone flavone isomerase (*CHI*) to generate flavonone and subsequent actions by

dihydroflavonol 4-reductase (*DFR*), isoflavonoid synthase (*IFS*) and flavanone 3β-hydroxylase (*F3H*), amongst others, generate a myraid of flavonoids.

Flavonoids have been proposed to have a number of different functions, including protection against harmful effects of UV-B radiation as they are potent absorbers of UV wavelengths. In response to UV-B irradiation the flavonoid biosynthetic genes are induced and consequently flavovoids accumulate in the vacuoles of epidermal cells (Schmelzer *et al.*, 1988). Other functions of these compounds include protection against pathogen invasion and fungal elicitors, and roles in sexual reproduction and pollination. In addition they have been shown to play a structural role (Dixon and Paiva, 1995). The genes are regulated in a tissue-specific manner during plant development at least in part at the level of transcription.

1.4.2.2 *CHS, PAL* and *4CL* are expressed in epidermal cells.

By a combination of *in situ* hybridisation and immunolocalization studies, flavonoids, *CHS*, *PAL* and *4CL* transcripts and the *CHS* enzyme were shown to accumulate in epidermal cells following UV-irradiation in parsley (Schmelzer *et al.*, 1988; Wu and Hahlbrock, 1992). However, whereas *CHS* was exclusively epidermal-specific, *PAL* and *4CL* were expressed in the vascular bundles of leaves, although *4CL* was present at a much lower level. In addition, levels of all three transcripts were greatly enhanced in response to light treatment. None of the transcripts were detected in root apical meristem but all three were expressed at high levels in root caps and regions where root hairs emerge (Yang *et al.*, 1992; Wu and Hahlbrock, 1992). Furthermore, *CHS* mRNAs were most abundant in the epidermal cells of young leaves and petioles. Similarly, in *Arabidopsis*, flavonoids such as kaempferol and phytoalexins also accumulated in the epidermis in response to stress (Li *et al.*, 1993). In peas, flavonoids and *CHS* are present in the epidermis and *CHI* was distributed in epidermal and parenchyma tissue (Hrazdina *et al.*, 1982).

The flavonoid biosynthetic pathway in *Arabidopsis* was well characterised by a combination of genetic, biochemical and molecular studies (Shirley *et al.*, 1995). The flavonoid group can be further subdivided into other compounds such as tannins and anthocyanins that are responsible for the red, brown and purple pigments of flowers,

fruit and seeds (Dixon and Paiva, 1995). Flavonoid regulatory genes have been isolated from maize and *Antirrhinum* and shown to have homology to the mammalian transcription regulators, myc and myb (Goff *et al.*, 1992; Paz-Ares *et al.*, 1987; Sablowski *et al.*, 1994). A similar mechanism of flavonoid biosynthetic gene regulation is thought to occur in *Arabidopsis* (Mol *et al.*, 1996).

1.4.3 Lipid transfer proteins (LTPs)

Plant lipid transfer proteins (LTPs) have been shown by a number of people to be expressed predominantly in the epidermal cells of leaf, stem and floral tissue in a number of species. LTPs are small (90 to 93 amino acids), soluble, basic proteins with a pI of around 9 or 10 and were first isolated by Kader *et al.* (1975). Their *in vivo* function is as yet unknown and they were so called due to their ability to transfer phospholipids between membranes *in vitro* as well as having the ability to bind acyl-CoA esters and fatty acids (Kadar *et al.*, 1984; Arondel and Kader, 1990). Based on these observations, LTPs were thought to participate in membrane biogenesis and regulation of intracellular fatty acid pools. However, nearly all plant LTPs described to date possess a signal peptide sequence which suggests a membrane-bound and/or extracellular location. This led a number of people to believe that their biological role was something other than the transfer of lipids between membranes. Because they lack the ER retention signal H/KDEL (Munroe and Pelham, 1987), LTPs are thought to enter the secretory pathway. With the finding that they were located in the cell wall of *Arabidopsis* (Thoma *et al.*, 1994) and waxy layer of broccoli leaves (Pyee and Kolattukudy, 1994, 1995) it was speculated their function may be that of cutin formation (Sterk *et al.*, 1991). Furthermore, their innate anti-pathogenic properties suggested a possible role in defence against invading pathogens (Terras *et al.*, 1992; Cammue *et al.*, 1995; Garcia-Olmedo *et al.*, 1995; Molina and Garcia-Olmedo, 1993; Molina *et al.*, 1993, 1996; Segura *et al.*, 1993; Pyee and Kolattkudy, 1994, 1995).

1.4.3.1 Lipid binding and transfer assays of LTPs

In order to class a protein as a lipid transfer protein, it must have the ability to transfer lipids between membranes. The principal assay devised was the monitoring of the transfer of radiolabelled (Yamada *et al.*, 1978), spin-labelled (Nishida *et al.*, 1985)

or fluorescent (Moreau *et al.*, 1994) lipids from donor to acceptor membranes. Acceptor membranes were either natural membranes such as the plasma membrane or those of mitochondria, chloroplasts and microsomes, or artificial membranes such as liposomes or lipid vesicles. Donor membranes are either natural membranes or liposomes (Kader *et al.*, 1984). In assays involving radioactively labelled substrates, the donor membranes are prepared from ^{3}H-phosphatidylcholine (PC), the lipid to be transferred, and a separate non-transferrable compound such as ^{14}C-trioleoylglycerol. The acceptor and donor membranes are mixed and incubated in the presence of purified lipid transfer proteins. The extent of ^{3}H-PC transfer can be estimated from the ^{3}H/^{14}C ratio.

Plant LTPs have been purified by gel-filtration, cation exchange chromatography and reverse-phase HPLC (Douady *et al.*, 1986; Kader *et al.*, 1984; Neumann *et al.*, 1995; Terras *et al.*, 1992). Several peaks of LTP activity were detected suggesting the presence of several isoforms. However, Pyee and Kolattukudy (1995) extracted an LTP from broccoli leaves following a brief wash in chloroform. Subsequent purification showed that at least 90 % of total protein in the surface wax was LTPs. Analysis of LTPs by gel-filtration or SDS-PAGE indicated a molecular mass of 9 or 10 kDa, although several LTPs with a lower molecular mass have also been isolated (Kalla *et al.*, 1994).

Plant LTPs are able to transfer phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) from liposomes to mitochondria but the specificity can vary between different LTPs and even between different isoforms. The binding of fatty acids or acyl-CoA esters to LTPs was determined by gel filtration or by temperature dependent ligand affinity (Tsuboi *et al.*, 1992). LTPs isolated from sunflower and spinach had affinity for oleoyl-CoA (Arondel and Kader, 1990; Rickers *et al.*, 1985). A carrot LTP was able to bind not only oleoyl-CoA but also had affinity for palmitic acid (Meijer *et al.*, 1993). Furthermore, LTPs from castor bean (Tsuboi *et al.*, 1992) were able to bind acyl-CoA. Several LTP isoforms from oilseed rape separated by cation exchange chromatography had the ability to transfer PC and bind oleoyl-CoA (Østergaard *et al.*, 1995). In contrast, mammalian acyl-CoA binding proteins are unable to transfer phospholipids (Hills *et. al.*, 1994).

Guerbette *et al.* (1997) compared the binding of lyso-PC and acyl-CoA to a maize LTP isoform. After incubating the maize LTP with lyso-PC and separating on a Sephadex G100 column, the ^{3}H radioactivity corresponding to lyso-PC was observed in LTP- containing fractions detected by ELISA. 58 % of the initial radioactivity of lyso-PC was found associated with LTP. The elution peak was divided into two where the LTP:lyso-PC complex was more retained than free LTP. This pattern of elution was also observed with LTP:PC complexes (Grosbois *et al.*, 1993). However, PC transfer activity was impaired by 83 % following pretreatment by lyso-PC. This suggested that lyso-PC:LTP complexes were more stable than LTP:PC complexes. In another study only 33 % of the initial radioactivity of oleoyl-CoA was found associated with the maize LTP and only 25 % inhibition of PC transfer activity was observed. In contrast a rapeseed LTP was unable to transfer PC after oleoyl-CoA binding (Østergaard, *et al.*, 1995). Taken together, these results suggest that different LTPs display different binding affinities for lipid chains and may therefore perform different functions. Although lipid transfer proteins are in some cases classed as non-specific lipid transfer proteins, these observations suggest some degree of specificity.

1.4.3.2 Structure and mode of action of LTPs

Non-specific lipid transfer proteins have been isolated from maize (Douady *et al.*, 1982), spinach (Kadar *et al.*, 1984), castor bean (Takishima *et al.*, 1986), barley (Breu *et al.*, 1989; Mundy and Rogers, 1986), wheat (Désormeaux *et al.*, 1992), oilseed rape (Østergaard *et al.*, 1995) and broccoli (Pyee and Kolattukudy, 1995). They exhibit strong structural homologies with each other. For example, they all contain eight conserved cysteine residues that form four disulphide bridges as determined by tryptic digestion of castor bean LTP (Takishima *et al.*, 1986). The conformation of a wheat LTP was determined by infra-red and Raman spectroscopy (Desormeaux *et al.*, 1992). The protein was made up of 41 % α-helices and 19 % β-sheets and 40 % of the structure was undefined or composed of turns. The importance of the disulphide bonds was demonstrated by the reduction of a maize LTP with DTT which led to loss of lipid-transfer activity (Grosbois *et al.*, 1993) and a 15 % reduction of the α-helices and a 13 % increase of β-sheets. Furthermore, the transfer process of LTPs can be disrupted in the presence of ions which indicates the importance of

electrostatic interaction between LTPs and membranes (Désormeaux et al., 1992). NMR and X-ray crystallography have shown that the proteins are comprised of a single compact domain with four alpha-helices and a long COOH-terminus. They posses a hydrophobic cavity which is capable of binding acyl chains.

1.4.3.3 Isolation of *LTP* genes

The first LTP cDNA to be cloned was from maize (Tchang, 1988). A cDNA library was constructed from mRNA isolated from maize seedlings, which was then screened with a polyclonal antibody made from purified maize LTPs. Furthermore, a barley protein previously identified by Mundy and Rogers (1986) as a probable amylase/protease inhibitor was found to have strong homology to the maize LTP. To date, over 40 cDNAs and gene sequences encoding plant LTPs have been published. Several of the sequences were identified as a result of partial sequencing of anonymous cDNA clones and expressed sequence tags (ESTs) from amongst others *Arabidopsis*, castor bean, maize, tobacco, and oilseed rape (Kader, 1996).

One prominent feature of LTP genes is that almost all that have been identified exist as small multigenic families with up to four or five closely related members. For example, cDNAs isolated from castor bean (Weig and Komor, 1992), barley (Molina and Garcia-Olmedo, 1993), broccoli (Pyee and Kolattkudy, 1995) and *Arabidopsis* (Thoma *et al.*, 1994) showed very strong sequence similarities. Hybridisation to genomic Southern blots revealed the presence of several gene copies, for example, four copies in broccoli (Pyee and Kolattukudy, 1995) and two in tomato (Torres-Schumann *et al.*, 1992). However, in carrot (Sterk *et. al.*, 1991) and spinach (Bernhard *et al.*, 1991) only one gene was detected. This was attributed to high stringeny washing of the Southern blot.

1.4.3.4 Organ, cell and tissue-specific gene expression of LTPs

Several experimental approaches have been adopted in the study of lipid transfer protein expression. These include, northern blot analysis, *in situ* hybridisation and expression of a reporter gene such as *GUS* driven by an LTP promoter. Several of the LTP genes studied were developmentally regulated in a temporal and spatial manner. Expression was predominantly in the epidermal cells of aerial organs of the

plant (leaves, stems, shoot meristem and floral tissue) with very little or no expression in the roots in spinach, tobacco, barley, cotton fibres, broccoli, rapeseed, tomato, castor bean, winter barley and *Arabidopsis* (Bernhard *et al.*, 1991; Fleming *et. al.*, 1993; Gausing *et al.*, 1994; Ma *et al.*, 1995; Molina *et al.*, 1993; Pyee and Kolattukudy, 1995; Soufleri *et al.*, 1996; Torres-Schumann *et al.*, 1992; Weig and Komor, 1992; White *et al.*, 1994; Thoma *et al.*, 1994). Also, expression of the LTP genes was found to be higher in young rapidly developing tissue compared to older tissues (Fleming *et al.*, 1992). Three cDNAs, (*ltp1, ltp2 and ltp3*) isolated from germinating *Brassica napus* cotyledons by rapid amplification of cDNA ends (3' RACE) (Soufleri *et al.*, 1996), exhibited an expression pattern indicative of strong developmental, hormonal and environmental regulation. The corresponding transcripts were expressed only in the cotyledons and hypocotyls of germinating seedlings and were induced upon treatment with abscisic acid (ABA) and NaCl. However, they were not detected in mature leaves, stems or flowers. *ltp2* and *ltp3* started to accumulate on the second day of germination reaching maximum expression levels on the third day and began to fall after this. *ltp1* expression however, was very low throughout, increasing on the third day. *ltp3* was equally expressed in cotyledons and hypocotyls whereas *ltp2* was expressed at a higher level in cotyledons. None were expressed in roots. LTP genes were also shown to be expressed in developing embryos in *Arabidopsis* (Thoma *et al.*, 1994) and carrot (Sterk *et al.*, 1991). However, Canevascini *et al.* (1996) reported that a tobacco LTP, *Ntltp1* was expressed at high levels in the root hairs and root epidermis at the stage where the first true leaves were forming. In cross-sections of roots of transgenic plants expressing the *Ntltp1* promoter-GUS gene, Canevascini *et al.* (1996) showed that GUS was expressed predominantly in the root epidermal cells. These observations conflicted with earlier data showing that *Ntltp1* was expressed at low levels in root tissue. (Fleming *et al.*, 1992).

A different pattern of LTP expression was observed in other species. For example, in castor bean two LTP genes, *LTPA* and *LTPC* were found exclusively in the cotyledons, whereas *LTPB* was expressed in the endosperm and *LTPD* was only expressed in the axis (Watanabe and Yamada, 1986; Tsuboi *et al.*, 1991). In barley, at least six LTPs were identified (Molina and Garcia-Olmedo, 1993). Expression of three,

Ltp2, *Ltp3* and *Ltp4* was detected in stem, shoot, apex, and kernel but not in the endosperm. Very little expression was detected in the roots. However, *Ltp1*, was expressed only in the aleurone cell layer (Mundy and Rogers, 1986). Similarly, Kalla *et al.* (1994) described an aleurone-specific gene, *Ltp2*, from barley expressed shortly after aleurone cell differentiation. The transcript was not detected in the embryonic and vegetative tissue unlike those mentioned previously. Analysis of the *Ltp2* promoter demonstrated the presence of myb (CAACTA) and myc (CAGCTG) binding sites previously identified in the promoter of the maize Bz1 gene (Roth *et al.*, 1991). One possible role of aleurone-specific LTPs in barley and wheat could be the formation of an amorphous waxy layer on the outside of the testa in wheat grains.

During flower development LTP genes were expressed at high levels in inflorescences (Fleming *et al.*, 1992; Pyee and Kolattukudy, 1995; Sterk *et al.*, 1991), in the sepals of unopened flowers in *Arabidopsis* (Thoma *et al.*, 1994), in flower buds of broccoli (Pyee and Kolattukudy, 1995), in the microspores and tapetum of rapeseed flowers (Foster *et al.*, 1992) and the tapetum layer of the tobacco anther (Koltunow *et al.*, 1990).

LTPs exhibit strong epidermal cell specificity. A maize LTP was expressed in the epidermis of coleoptiles (Sossountzov *et al.*, 1991) and in the epidermis of the tobacco shoot apical meristem (Fleming *et al.*, 1992). LTP expression was also detected in the epidermal cells of floral organs in *Arabidopsis* (Thoma *et al.*, 1994) and *G. hybrida* (Kotilainen *et al.*, 1994). In carrot (Sterk *et al.*, 1991) and *Arabidopsis* (Thoma *et al.*, 1994) embryos, an LTP was expressed in protodermal cells which give rise to the epidermis. In adult *Arabidopsis* plants, an LTP gene was shown to be expressed in the leaf and stem epidermal cells, guard cells and flowers (Thoma *et al.*, 1994).

1.4.3.5 LTPs are localised in the cell wall

With the exception of castor bean (Tsuboi *et al.*, 1992), all LTP cDNAs isolated to date possess a leader sequence. In a spinach LTP, the signal peptide is able to insert co-translationally into the lumen of the ER *in vitro* (Bernhard *et al.*, 1991). However, the proteins do not contain an ER retention signal, K/HDEL and are thought therefore to be secreted (Sterk *et al.*, 1991; Mundy and Rogers, 1986). The

extracellular location of LTPs was confirmed by immunocytochemical studies using polyclonal antibodies. For example, the *Arabidopsis* LTP protein was shown to be located in the cell wall of epidermal cells (Thoma *et al.*, 1994). Also, in maize coleoptiles, LTP-immunogold complexes were observed in the cell walls of epidermal cells (Sossountzov *et al.*, 1991). The broccoli WAX9D protein was also located predominantly in the epidermal and mesophyll cell walls (Pyee and Kolattukudy, 1994). Another LTP gene was found to be expressed during tracheary element differentiation in *Z. elegans* (Ye *et al.*, 1993) which is related to cell wall thickening.

1.4.3.6 Environmental regulation of LTP expression

Lipid transfer proteins show a diverse and complicated mode of expression. They are regulated by environmental stimuli as well as in a spatial and temporal manner during development. A number of LTP genes have been shown to be regulated by cold treatment, drought, salt and mannitol (osmotic stress), abscisic acid (ABA), high temperature and following pathogen attack (see section 1.4.3.7). Transcripts corresponding to three germination-specific cDNAs (*ltp1, ltp2 and ltp3*) isolated from *B. napus* (Soufleri *et. al.*, 1996) were induced upon treatment with ABA and NaCl. The expression of a different germination-specific LTP gene from tomato (TSW12) was also shown to be induced upon NaCl, mannitol, high temperatures and ABA treatment (Torres-Schumann *et al.*, 1992). A cold-induced barley LTP gene, *blt4.1*, was also induced following treatment with ABA (White *et al.*, 1994). A putative ABA response element (ABRE) GTACGTGG and a putative low-temperature response element ACACGTCA were found in some barley LTP genes (White *et al.*, 1994). An ABRE was common in genes responding to low-temperature and drought (Hughes *et. al.*, 1992). Interestingly, White *et. al.* (1994) observed a winter barley LTP gene to be induced in roots and stems following drought-stress. As previously mentioned, very few LTPs were shown to be expressed in roots.

1.4.3.7 LTPs implicated as plant defence proteins

LTPs have been shown to have potent anti-fungal and anti-bacterial activities (Garcia-Olmedo *et al.*, 1995; Cammue *et al.*, 1995; Molina and Garcia-Olmedo, 1993; Terras *et al.*, 1992; Molina *et al.*, 1993, 1996; Segura *et al.*, 1993; Pyee and Kolattukudy, 1994). Transgenic plants expressing LTPs show enhanced tolerance to a variety of pathogens (Molina *et al.*, 1996) and LTP-sensitive mutants of bacterial pathogens were shown to be avirulent (Molina *et al.*, 1996). The ability of the waxy layer to protect against environmental stresses such as pathogen attack may therefore be aided by the presence of LTPs (Garcia-Olmedo *et al.*, 1995). The ability of plant lipid transfer proteins to inhibit fungal and bacterial growth *in vitro* was reported by Terras *et al.* (1992), Molina *et al.* (1993) and Cammue *et al.* (1995). Four proteins from barley leaves (LTP2-LTP5) were active against the bacterial pathogen *Clacibacter michiganansis* subsp. *sepedonicus* (Molina *et al.*, 1993). LTPs isolated from radish (Terras *et al.*, 1992), onion (Cammue *et al.*, 1995) and broccoli (Pyee *et al.*, 1994) all exhibited antibiotic activity. However, the relative activities of the different LTPs varied between pathogens indicating some specificity (Molina *et al.*, 1993). They were also shown to be more active than thionins (Florack and Stiekema, 1994) (another large group of plant-defence proteins) against *C. michiganensis* subsp. *sepedonicus*. However, when both proteins were tested together, an additive response was observed. Interestingly, thionins were more active than LTPs against a different fungus, *Fusarium solani* but when the two proteins were used in concert, a synergistic response was observed.

It was postulated that one mechanism of anti-pathogenic activity was the binding of essential cations such as Ca^{2+} required in bacterial and fungal activities. Indeed, $CaCl_2$ addition has been shown to antagonize LTP activity (Terras *et al.*, 1992). This, however, would not explain the difference in activity of a given LTP to different strains of a pathogen as well as to different pathogens. The high concentration of LTPs at the surface of organs (Molina and Garcia-Olmeda, 1993) probably enables the formation of a re-inforced protective barrier against invading pathogens rather than responding exclusively as anti-fungal components.

LTP genes have been shown to respond to pathogen infection in a complex manner. The expression of three barley LTPs, *Ltp2*, *Ltp3* and *Ltp4*, was studied in

response to several pathogens (Molina *et al.*, 1993, 1996; Garcia-Olmedo *et al.*, 1995) and compared to the response to a thionin gene and the barley pathogenesis-related protein *PRHv-1* (Garcia-Olmedo *et al.*, 1995) expression. The results confirmed that expression of LTP genes was either induced considerably above the basal level, or switched off, and that each member of the LTP gene family responded differently depending on the cultivar and pathogen strain. Following infection of barley cv. Atlas46, with the fungal pathogen *Rhynchosporium secalis*, which is an incompatible interaction, *Ltp4* expression is greatly induced concomitant with the *PrHv-1* gene. *Ltp2* and thionin expression was also induced although at a lower level. The barley cv. Atlas46 contains the *Rh3* gene which confers resistance to *R. secalis*. A nearly isogenic line, Atlas which lacks the *Rh3* gene does not display the same response. Instead only the thionins were induced at low levels. These results suggest that *Ltp4*, *Ltp3* and *PrHv-1* are regulated by the *Rh3* gene and are involved in conferring resistance to the fungus. They also indicate multiple elicitation pathways. Furthermore, a successful compatible infection of the barley Bomi cultivar with *Pseudomonas syringae* pv. *japonica* switched off the expression of all three LTP genes whereas both thionin and *PrHv-1* genes were induced (Garcia-Olmeda *et al.*, 1995). In each of the pathogen interactions studied, the pattern of *Ltp4* expression suggested a role in plant defence. Furthermore, the expression and distribution of *Ltp4* resembles the *LTP1* gene from *Arabidopsis* (Thoma *et al.*, 1993, 1994) and the WAX9D from broccoli (Pyee *et al.*, 1994, 1995). However, LTP expression in response to viral pathogens has not been reported.

1.5 Aims of the project

The overall aim of this project was the investigation of epidermal-specific expression of genes and their possible role in epidermal cell differentiation in higher plants. At the time the project was initiated, very few epidermal-specific genes had been identified and very little definitive information on the process of epidermal development was available. The isolation of epidermal-specific genes would provide valuable additional markers to monitor epidermal development. In addition, by identification of common regulatory elements in the promoter regions of different

epidermally-expressed genes we can begin to functionally define the molecular processes central to epidermal development.

1.5.1 Isolation and characterisation of an epidermis-specific gene from *Brassica napus*

The initial objective was to screen a *Brassica napus* cDNA library with an unknown partial cDNA clone previously shown to be expressed at high levels in the epidermal cells of *Brassica napus* leaf, stem and floral tissue (Pallas, 1992) in order to isolate the full length clone. The full length clone was fully sequenced and compared to other sequences in the data base. The expression of transcripts corresponding to the full length cDNA clone was studied using northern blot analysis to define their organ-specificity. The corresponding *Brassica napus* gene was cloned and sequenced. Its expression pattern was examined by generating transgenic *Arabidopsis* plants expressing the gene promoter-*GUS* fusion. The tissue- and cell-specific expression pattern of the gene was examined by histochemically localising GUS activity. Furthermore expression of the gene in response to a number of environmental stimuli was examined by a combination of northern blot analysis and fluorometric GUS assays.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All general chemicals were purchased from BDH (Analar® grade), Poole, Dorset unless otherwise stated.

2.1.2 Radiochemicals

Radiochemicals were purchased from Amersham International plc., Amersham, UK.

2.1.3 Restriction enzymes

All restriction enzymes were purchased from Promega Ltd., Southampton, UK.

2.1.4 Antibiotics, IPTG and X-Gal

All antibiotics were purchased from Sigma Chemical Co., Dorset, UK.

Stock solutions were prepared according to Sambrook *et al.* (1989). Ampicillin, kanamycin and gentamycin stock solutions were prepared in distilled H_2O at 25, 20, and 10 mg/ml, respectively, and were used at a final concentration of 50 μg/ml. Tetracycline was prepared in 50% EtOH at 12.5 mg/ml and used at a final concentration of 12.5 μg/ml. Rifampicin was prepared in MeOH at 20 mg/ml and used at a final concentration of 100 μg/ml. All stock solutions were filter sterilised and stored at -20°C. Isopropyl thiogalactoside (IPTG, Life Technologies, Gaithersburg, USA) was prepared in H_2O as a 1 mM stock and used at a final concentration of 0.1 mM. 5-bromo-4-chloro-3-indolyl β-D-galactoside, (X-Gal, Life Technologies) was made as a 20 mg/ml stock in dimethylformamide (DMF) and used at a final concentration of 50 μg/ml.

2.1.5 Bacterial strains

E. coli XL-1 Blue (Bullock *et al.*, 1987) and DH5α cells were used to maintain and amplify plasmids (purchased from Stratagene, Cambridge, UK) and for plating the

cDNA library. *E. coli* SOLR™ (Hay and Short, 1992, Stratagene) cells were used for the rescue and *in vivo* excision of cDNA clones from the Uni-Zap™XR cDNA library (Stratagene). DL491 cells (supplied by Mr. J. Jardine, Division of Biochemistry and Molecular Biology, University of Glasgow) were used for plating the genomic library and subsequent amplification of isolated clones.

Agrobacterium tumefaciens GV3101 (Koncz and Schell, 1986) containing the helper plasmid pMP90 ($Rif^{R}Gn^{R}Kan^{S}$), was provided by Dr. Mark Hooks, Division of Biochemistry and Molecular Biology, University of Glasgow, and was used for plant transformations.

2.1.6 Plasmids and DNA probes

The pBluescript® II SK (-) phagemid (Short *et al.*, 1988, Stratagene) was used to clone DNA fragments for sequencing and restriction digest analysis. The pBI101.1 binary vector (Jefferson *et al.*, 1987) which contains the promoterless bacterial reporter gene, *β-glucuronidase* (GUS) and the *nos* 3' terminator, was used to construct the promoter-GUS fusion. The *B. napus* partial cDNA probe, pLF3A (Pallas, 1993), was provided by Dr. Jackie A. Pallas, University of Leeds. The *Arabidopsis LTP1* cDNA probe (CD3-11) (Thoma *et al.*, 1994) was obtained from the *Arabidopsis* Biological Resource Centre at Ohio State University (Columbus, OH). The *Arabidopsis CHS* cDNA probe (Feinbaum and Ausubel, 1988; Trezzini *et al.*, 1993) was provided by Dr. Geeta Fuglevand, University of Glasgow. A *Phaseolus vulgaris* cDNA fragment encoding *H1* was obtained from Dr. John M. Christie, University of Glasgow.

2.1.7 Plant virus strains

Cauliflower Mosaic Virus (CaMV) isolate BARI (Covey, 1991) was obtained from Dr. Edi Cecchini and Dr. Joel J. Milner, Division of Biochemistry and Molecular Biology, University of Glasgow.

2.1.8 Libraries

The *Brassica napus* cDNA library was constructed by Dr. William Goodwin, University of Glasgow, in the bacteriophage vector Uni-ZAP™XR (

Stratagene). The *Brassica napus* genomic library constructed in the EMBL-3 vector (Clontech, California, U.S.A) was provided by Dr. A. Greenland, Zeneca Seeds, Bracknell, Berkshire.

2.1.9 Seeds

Brassica napus L. cv 'Cobra' (winter oilseed rape) seeds were purchased from Booker Seeds, Sleaford, Lincolnshire. *Arabidopsis thaliana* Landsberg *erecta* wild type and the mutants *ttg* (Koornneef, 1981), *gl1* (Marks and Feldmann, 1989) and *gl2* (Rerie *et al.*, 1994) were obtained from the Nottingham *Arabidopsis* Stock Centre. The *icx1/ttg* seed was provided by Dr. Jennie A. Jackson, University of Glasgow.

2.1.10 Potting compost

Potting compost (S. A. I. Horticulture Ltd., Stirling), was sterilised by autoclaving in individual autoclave bags.

2.1.11 Transgenic plants

NM4 plants were obtained from Jackson *et al.* (1995). These were constructed by transforming the *Sinapsis alba* (white mustard) *SA-CHS* promoter-*GUS* construct (Batschauer *et al.*, 1991; Frohnmeyer *et al.*, 1992) into wild type *Arabidopsis thaliana* Landsberg *erecta* plants by root transformation with *Agrobacterium tumefaciens* based on the method described by Valvekens *et al.* (1988). *35S*-GUS plants (Jackson *et al.*, 1995) were generated by transforming *Arabidopsis thaliana* Landsberg *erecta* plants with pBI121 (Jefferson *et al.*, 1987), a binary vector containing the *GUS* reporter gene under constitutive control of the cauliflower mosaic virus 35S promoter and the *nos* terminator. These plants were used as positive controls in fluorometric MUG assays and during histochemical localisation of GUS activity.

A12 plants were derived from EMS mutagenized NM4 seeds and were selected for increased *CHS-GUS* expression (Jackson *et al.*, 1995). These were designated *icx 1* (*i*ncreased *c*halchone *s*ynthase e*x*pression). Transgenic plants, Baji-A1, Bari-B6 and Bji-C3 which constitutively expressed the CaMV *Gene VI* were produced by transforming *gl1* (Rerie *et al.*, 1994) plants with the *35S-Gene VI* transgene (obtained from Dr. Edi Cecchini and Dr. Joel Milner, University of Glasgow).

2.2 General experimental procedures

2.2.1 Glassware

Glassware for RNA work was sterilised by baking at 180° C for at least four hours.

2.2.2 Autoclaving

Sterilisation of solutions, bottles and plasticware was achieved by autoclaving at 15 psi for 15 min.

2.2.3 Filter sterilisation

Heat-labile solutions were sterilised by passing through a Nalgene® syringe filter (pore size 0.2 μm) into a sterile container.

2.2.4 pH measurement

Solutions were measured for the correct pH using a Corning pH meter 220 and combination electrode. The pH of phenol was determined by Whatman Narrow Range pH paper (pH 6.0 - pH 8.0).

2.2.5 Silicon-coating of sequencing glass plates

One of the two sequencing plates was siliconised with dimethyldichlorosilane solution (BDH, AnalaR® grade). A few drops of the solution were sprinkled onto the plate in a fume hood and spread evenly over the surface of the plate with a paper towel. The plates were then washed thoroughly with sterile H_2O.

2.3 Buffers

All routinely used buffers were prepared as a 10 X stock solution essentially as described in Sambrook *et al.* (1989). The buffers were diluted to the desired concentration with sterile H_2O. 1 X TBE (90 mM Tris-borate, 2 mM EDTA) was used to resolve DNA agarose and acrylamide sequencing gels. 1 X TAE (40 mM Tris HCl, 2 mM EDTA, pH to 8 with acetic acid) was used to run agarose gels prior to gel-purification of DNA fragments. 1 X MOPS (3-[N-Morpholino]propanesulphonic acid)

(20 mM MOPS, 5 mM NaOAc, 1 mM EDTA pH 7.0) was used to resolve denaturing formaldehyde RNA gels. Restriction digestion buffers were provided with the restriction enzyme. DNA and RNA sample buffers were prepared essentially as described in Sambroock *et al.* (1989).

2.4 Preparation of phenol and extraction of nucleic acids

Phenol was prepared as described in Sambrook *et al.* (1989).

Essentially, 500 g of solid phenol (BDH, Analar® grade) was heated at 68°C until it dissolved fully after which 0.1% 8-hydroxyquinoline (w/v) was added which acts as an RNase inhibitor, antioxidant and metal ion chelator. An equal volume of 0.5 M Tris-HCl, pH 8.0 was added and mixed thoroughly. The aqueous and organic phases were allowed to separate and the upper aqueous phase removed via aspiration. This was then repeated with the addition of an equal volume of 0.1 M Tris-HCl pH 8.0 until the pH was > 7.8. The phenol was then stored under 1 X TE (100 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 4° C in a light-tight container for up to 1 month.

2.4.1 Phenol/chloroform extractions

An equal volume of phenol was added to the nucleic acid solution and the whole mixture was mixed by inverting the tube a few times. Organic and aqueous phases were separated by brief microcentrifugation at high speed. The aqueous supernatant was transferred to a fresh tube and the extraction repeated with an equal volume of phenol supplemented with 48% chloroform (v/v) and 2% isoamyl alcohol (v/v) (phenol/$CHCl_3$/IAA). The aqueous nucleic acid phase was transferred to a fresh tube and extracted with an equal volume of chloroform to remove any residual phenol. The nucleic acid was EtOH precipitated as described in section 2.9.

2.5 Culture media

The media were prepared essentially as described in Sambrook *et al.* (1989). Bacto-tryptone, yeast extract and bacto-agar were purchased from Difco Laboratoraties, Michigan, USA. NZ amine, casamino acids and maltose were purchased from Sigma, Dorset, UK.

2.5.1 Liquid media

L-Broth (1% (w/v) bacto-tryptone, 1% (w/v) NaCl and 0.5% (w/v) yeast extract, pH 7.3 in H_2O) supplemented with antibiotic(s) (where appropriate) was used to culture *E. coli* in the preparation of competent cells and for amplification of plasmid DNA. L-Maltose (L-Broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$) was used for plating the cDNA library. NZCYM broth (1% NZ amine (w/v), 0.1% casamino acids (w/v), 0.5% yeast extract (w/v), 0.5% NaCl (w/v), 10 mM $MgSO_4$) supplemented with 0.2% (w/v) maltose was used to culture DL491 cells for plating the genomic library. 2 X YT media (1.6% (w/v) bacto-tryptone, 1% (w/v) yeast extract and 1% (w/v) NaCl in H_2O was used to culture SOLR® cells to rescue the recombinant pBluescript® II SK (-) phagemid from the Uni-Zap® XR vector. YEP (1% bactotryptone, 1% yeast extract, 0.5% NaCl) media was used to culture *Agrobacterium.*

2.5.2 Solid media

L-agar (1.5% (w/v) bacto-agar in L-Broth or NZCYM) was used to culture *E. coli*. Top agarose was prepared by adding agarose at an appropriate concentration (0.5-1%) and 10 mM $MgSO_4$ to L-Broth or NZCYM media. *Agrobacterium tumefaciens* was grown on YEP-agar (1.5% agar (w/v) (Difco) in YEP media).

2.6 Preparation of competent bacteria

2.6.1 Preparation of competent *E.coli*

A single colony of bacteria was grown overnight at 37°C in 5 ml L-Broth with continuous shaking in a 10 ml bijou. This was used to inoculate 100 ml of L-Broth contained in a 500 ml conical flask and grown at 37°C with continuous shaking for 1.5-2 h until the $OD_{550} = 0.48$. The cells were chilled on ice for 5 min and then split into 2 x 50 ml Falcon tubes and spun at 3000 x g for 10 min at 4°C. The L-Broth was decanted off and each pellet was resuspended in 20 ml ice-cold buffer 1 (30 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH 5.8). The cells were chilled on ice and spun again as before. Each pellet was resuspended in ice-cold buffer 2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, pH 6.5).

The cells were chilled on ice for 15 min and then immediately dispensed into pre-cooled Eppendorf® tubes in 100 µl aliquots. The cells were flash frozen in liquid nitrogen and stored at -80°C.

2.6.2 Preparation of competent *Agrobacterium*

This method was based on that of Holsters *et al.* (1978).

A single colony of *Agrobacterium* containing the appropriate helper Ti plasmid was grown overnight in 5 ml of YEP media at 28°C. 2 ml of this culture was added to 50 ml of YEP media in a 250 ml flask and shaken vigourously at 28°C until the OD_{600} was between 0.5 and 1.0. The culture was chilled on ice and then centrifuged at 3000 x g for 5 min at 4°C. The resulting pellet was resuspended in 1 ml of ice-cold 20 mM $CaCl_2$ and 100 µl aliquots dispensed immediately into pre-chilled Eppendorf® tubes. The cells were either flash-frozen in liquid nitrogen and stored at -70°C or used immediately to transform the appropriate plasmid (see section 2.7).

2.7 Transformation of bacteria with plasmid DNA

2.7.1 Transformation of *E.coli*

100 µl of competent cells described in section 2.6.1 were allowed to thaw on ice and 25-250 ng of DNA in a total volume of 5 µl was added. The cells were mixed gently by flicking the bottom of the tube. The mixture was incubated on ice for 15-30 min after which they were heat-shocked at 42°C for 90 s and returned immediately to ice for a further 2 min. 200 µl of L-Broth was added to the cell/DNA mixture and incubated at 37°C with shaking for 30 min to allow expression of the antibiotic resistance gene. The cells were then spun briefly in a microcentrifuge and the pellet resuspended in 100 µl of warm L-Broth. Aliquots of 1, 5, 20 and 50 µl of the cell suspension were plated onto L-agar containing the appropriate antibiotic. The plates were incubated at 37°C overnight to allow the transformed bacteria to grow. A control transformation containing no DNA was also carried out.

2.7.2 Transformation of *Agrobacterium*

The method used was as described by Holsters *et al.* (1978).

Essentially, 1 µg of plasmid DNA in a total volume of 5 µl was added to 100 µl of competent *Agrobacterium* (see section 2.6.2) in an Eppendorf® tube. If the cells were already frozen the cells/DNA mixture was thawed by incubating at 37°C for 5 min. If the cells were freshly prepared, the cells/DNA mixture was quick frozen in liquid nitrogen prior to thawing at 37°C. 1 ml of YEP medium was then added to the mixture and incubated at 28°C for a further 2-4 h with gentle shaking to allow the bacteria to express the antibiotic resistance genes. The tube was then microcentrifuged for 30 s and the supernatant discarded. The pelleted cells were resuspended in 100 µl of YEP medium and spread onto YEP-agar plates containing the appropriate antibiotics and incubated at 28°C for 2-3 d to allow the transformed colonies to appear.

2.8 Isolation of DNA from bacteria and plants

2.8.1 Small scale plasmid preparation

The Wizard™ *Plus* Minipreps DNA Purification kit from Promega (Madison, USA) was used.

A single colony of bacteria was picked and used to inoculate 5 ml of L-Broth supplemented with the appropriate antibiotic in a 10 ml sterile bijou. The culture was grown overnight at 37°C with continous shaking. 3-5 ml of cells was transferred to a 1.5 ml Eppendorf® tube and pelleted by centrifugation at 10,000 x g for 10 min. The supernatant was discarded and the pellet resuspended in 300 µl of cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A, provided in ≥ 30 units/mg, [Boehringer Mannheim]). The cells were lysed with the addition of 300 µl of cell lysis solution (0.2 M NaOH, 1% SDS) and mixed by inverting the tube 4 times. Complete lysis resulted in a clear solution. 300 µl of neutralisation solution (1.32 M KOAc, pH 4.8) was then added and the tube mixed by inversion. After centrifugation at 10,000 x g for 5 min the supernatant was transferred to a disposable Wizard™ Miniprep column which consisted of a 1 ml syringe barrel, containing 1 ml of DNA purification resin, attached to the Luer-Lok® extension of a minicolumn. The column was attached to a vacuum manifold and a vacuum applied to allow the DNA/resin

slurry to pass through the column. The vacuum was broken after which 2 ml of wash solution (55% EtOH, 80 mM KOAc, 8.3 mM Tris-HCl, pH 7.5, 40 μM EDTA) was added and the vacuum reapplied. The resin was dried by continuing to draw the vacuum for a further 30 s. The syringe barrel was detached from the minicolumn and discarded. The minicolumn was then attached to a 1.5 ml Eppendorf® tube and centrifuged at 10,000 x g for 2 min to remove any residual column wash solution. The minicolumn was then transferred to a fresh 1.5 ml Eppendorf® tube and DNA was eluted by the addition of 50 μl of sterile H_2O for 1 min followed by centrifugation at 10,000 x g for 20 s. The minicolumn was discarded and the plasmid DNA stored at 4 or -20°C.

2.8.2 Large scale plasmid preparation

A single colony of bacteria harbouring the appropriate plasmid was inoculated into 5 ml of L-Broth supplemented with the appropriate antibiotic. This was then used to inoculate 100-500 ml of L-Broth/antibiotic and grown overnight at 37°C with continuous shaking. The cells were then pelleted by centrifugation at 5000 x g for 10 min at 22°C in 250 ml centrifuge tubes (Beckman). The supernatant was discarded and the pellet resuspended in 15 ml of cell resuspension buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNase (provided in kit). After complete resuspension, 15 ml of cell lysis solution (0.2 M NaOH, 1% SDS) was added and the cells were mixed gently by inverting the tube several times. Once the solution became clear and viscous, 15 ml of neutralisation solution (1.32 M KOAc, pH 4.8) was added and the contents of the tube were mixed again by inverting several times. The solution was centrifuged at 14,000 x g for 15 min at 22°C after which the clear supernatant was filtered through a Whatman® #1 filter paper to remove any residual white precipitate. Plasmid DNA was precipitated by the addition of 0.5 volumes of isopropanol and mixing by inversion. Plasmid DNA was then recovered by centrifugation at 14,000 x g at 22°C for 10 min, the supernatant was discarded and the DNA-containing pellet resuspended in 2 ml of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). 10 ml of Wizard™ Maxipreps DNA Purification Resin was added to the DNA solution and, the whole solution was mixed by swirling. The DNA/resin mix was then transferred to a Wizard™ Maxicolumn which had been attached to a vacuum manifold. The vacuum was applied and the

DNA/resin mix drawn into the maxicolumn. The column was washed sequentially with 13 and 12 ml of column wash (85 mM NaCl, 8 mM Tris-HCl pH 7.5, 2 mM EDTA, 55% EtOH). The column was then desalted by washing with 80% EtOH. Once the EtOH had passed through, the vacuum was drawn for a further 1 min to dry the column. The maxicolumn was then inserted into a 50 ml Falcon tube and centrifuged in a benchtop swinging rotor at 1,300 x g for 5 min to remove any residual EtOH. The column was then reattached to the vacuum manifold and the vacuum drawn for a further 5 min to ensure that the column was completely dry. It was then placed into a fresh Falcon tube, 1.5 ml of pre-heated H_2O applied and the DNA eluted after 1 min by centrifuging the maxicolumn/Falcon tube at 1,300 x g for 5 min in a Beckman benchtop centrifuge with a swinging rotor. The plasmid DNA concentration was determined as described in section 2.9. The plasmid solution was stored at 4 or -20°C.

2.8.3 Isolation of plasmid DNA from *Agrobacterium*

This method was based on that described by Birnboim *et al.* (1979).

A single colony of *Agrobacterium* was grown overnight in 1 ml of YEP medium containing the appropriate antibiotic in a 5 ml sterile bijou at 28°C with vigorous shaking. The culture was then transferred to a 1.5 ml Eppendorf® tube and centrifuged for 30 s at 10,000 x g. The supernatant was discarded and the pellet resuspended in 0.1 ml of ice-cold solution I (25 mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM EDTA, 4 mg/ml lysozyme). The cells were incubated at room temperature for 10 min after which 0.2 ml of solution II (0.2 M NaOH, 1% SDS) was added in order to lyse the cells. After a 10 min incubation at room temperature, 30 µl of phenol equilibrated with two volumes of solution II, was added and the cells vortexed gently for a few seconds. Chromosomal DNA was then precipitated by the addition of 0.15 ml of 0.3 M NaOAc pH 4.0 and recovered by centrifugation for 3 min. Plasmid DNA was recovered from the supernatant by precipitation with 95% EtOH. After a 15 min incubation at -80°C the tube was centrifuged for 3 min, the supernatant discarded and the pellet resuspended in 0.5 ml 0.3 M NaOAc, pH 7.0. The EtOH precipitation step was repeated and the resulting pellet was washed in 70% EtOH. The plasmid DNA pellet was resuspended in 50 µl of TE and restriction digestion of the DNA was carried

out essentially as described in section 2.10 except for the addition of 100 µg/ml of RNase A (≥ 30 units/mg, Boehringer Mannheim) in the reaction mix.

2.8.4 Isolation of plant genomic DNA

0.1 - 1.0 g of plant tissue was ground under liquid nitrogen into a fine powder with a pestle and mortar and transferred to 15 ml of DNA extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl) in a 50 ml Falcon tube. 1 ml of 20% SDS was then added and the mixture vortexed for 20 s. The tube was incubated at 65°C for a minimum of 1 h after which it was cooled on ice for 2-5 min. 5 ml of 5 M NH_4OAc was added and the tube shaken gently to mix. The solution was centrifuged for 20 min at 3000 x g in a benchtop centrifuge, the supernatant was removed into a fresh Falcon tube containing 14 ml of iso-propanol and mixed several times. The tube was then centrifuged at 3000 x g for 20 min and the supernatant discarded. The pellet was desalted by washing in 70% EtOH and centrifuged as before. It was then air-dried for a minimum of 15 min. 500 µl of sterile H_2O was added to the pellet and incubated at 4°C overnight to aid resuspension. The DNA was RNase-treated by the addition of 2 µl of DNAse-free RNase A (10 mg/ml, ≥ 30 units/mg, Boehringer Mannheim) per 100 µl of DNA solution at 37°C for 1 h. The RNase A was denatured by heating at 65°C for 10 min. The DNA was EtOH precipitated as described in section 2.9 and stored at 4°C.

2.8.5 Purification of oligonucleotides

200 µl of oligonucleotide in 0.88 M NH_4OH was lyophilised then resuspended in 200 µl of sterile H_2O. The DNA was phenol/chloroform extracted, and ethanol precipitated as described in sections 2.4.1 and 2.9. The oligonucleotide was resuspended in 20 µl of sterile H_2O and the concentration determined as described in section 2.9. It was then diluted to give a final concentration of 0.5 pmol/µl for sequencing, 20 pmol/µl for primer extension or 100 pmol/µl for PCR.

2.9 Ethanol precipitation and quantification of nucleic acids

Nucleic acids were precipitated by the addition of 0.1 vol. of 3 M NaOAc, pH 5.2 for plasmid DNA and oligonucleotide preparations or 5 M NH_4OAc for genomic DNA and 2.5 vol. of ice-cold EtOH. The mixture was incubated at -20°C for a minimum of 20 min or at -80°C for a minimum of 15 min. DNA was recovered by centrifugation at 10,000 x g for 10 min. The pellet was then washed in ice-cold 70% EtOH, briefly centrifuged, air-dried and resuspended in H_2O.

The concentration of nucleic acids was determined by measuring the absorbance at 260 and 280 nm in a 1 ml quartz cuvette. An absorbance unit of 1 at 260 nm was taken to be equivalent to 50 µg/ml double-stranded DNA, 40 µg/ml single-stranded DNA and RNA and 20 µg/ml oligonucleotides. Nucleic acid purity was assessed by the 260/280 ratio (Sambrook *et al.*, 1989).

2.10 Restriction digestion of DNA

DNA was digested in a total volume of 10-80 µl containing 1 X the appropriate restriction digestion buffer and 1-20 units of restriction enzyme. The reactions were incubated for 2 to 24 h at 37°C unless otherwise stated. A small aliquot was then subjected to agarose gel electrophoresis as described in section 2.11.

2.11 Agarose gel electrophoresis

2.11.1 Electrophoresis of DNA

Agarose (*ultra*PURE™) was purchased from Life Technologies, Gaithersburg, USA.

0.7-2% (w/v) agarose was boiled in 1 X TBE or TAE buffer until it was completely dissolved. The agarose solution was allowed to cool to 50-60°C. Ethidium bromide (EtBr) was added to a final concentration of 0.5 µg/ml before the solution was poured into a horizontal gel mould and allowed to set for 20-30 min. After the addition of one-tenth volume of loading buffer (0.25% (w/v) xylene cyanol FF, 0.25% (w/v) bromophenol blue, 1 mM EDTA pH 8.0, 50% glycerol) the DNA sample was subjected to electrophoresis in the appropriate 1 X running buffer at 5 V/cm. DNA was visualised using a UV transilluminator.

2.11.2 Denaturing formaldehyde-gel electrophoresis of RNA

This method was based on that described by Sambrook *et al.* (1989).

1.5 g of agarose was dissolved in 73 ml of H_2O by boiling for 3 min after which 10 ml of 10 X MOPS buffer (200 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0) was added. Once the agarose solution had cooled to 55° C, 17 ml of formaldehyde (37%, v/v; Sigma) was added and the mixture swirled around ensuring no bubbles were formed. The agarose solution was poured into a horizontal gel mould and allowed to set for a period of 30 min. The gel was placed into a gel tank containing enough 1 X MOPS running buffer to cover the gel. RNA samples were then prepared in a total volume of 20 µl containing 10 µg of total RNA, 50% (v/v) formamide (Fluka Biochemicals, Gillingham, UK), 1 X MOPS, 5.92% (v/v) formaldehyde and 2.5 µg/ml EtBr. The RNA was heated at 65° C for 15 min then placed immediately on ice, after which 0.1 vol. of RNA loading buffer (50% glycerol (v/v), 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) was added. The sample was loaded onto the gel and electrophoresed at 5 V/cm until the dye front migrated approximately three quarters of the way down the gel. The gel was then washed in distilled H_2O to remove the formaldehyde and the RNA was visualised under UV light.

2.12 Gel purification of DNA

2.12.1 Phenol/$CHCl_3$ method

The DNA fragment of interest was visualised briefly under UV light, sliced out of a 1% low-melting point, TAE-run agarose gel and fragmented into small pieces using a clean scalpel blade and then placed into a sterile Eppendorf® tube containing 500 µl of phenol. The tube was vortexed for 2 min until the mixture became cloudy and then placed into liquid nitrogen for 10 s and spun immediately at 14,000 x g for 10 min in a microcentrifuge. The aqueous layer was transferred to a fresh tube and an equal volume of phenol/$CHCl_3$ mixture added. After vortexing for a few seconds and centrifugation at 10,000 x g for 2 min the aqueous layer was transferred to a fresh tube and the DNA was EtOH precipitated as described in section 2.9. The pellet was resuspended in 20 µl of sterile H_2O and the concentration determined as described in

section 2.9. The DNA was diluted to a final concentration of 25 ng/µl for ligations and probes.

2.12.2 GENECLEAN II®

The GENECLEAN® kit was purchased from Stratech Scientific, Luton, UK.

The DNA fragment of interest was excised out of a TAE-run gel (1 g of gel slice was approximately 1 ml) and placed into an Eppendorf® tube containing 3 volumes of 6 M NaI. The DNA/gel-slice was incubated at 45-55°C for 10 min until the agarose dissolved after which 5 µl of a silica matrix, GLASSMILK®, was added per 5 µg of DNA, mixed and incubated at room temperature for 5 min. The GLASSMILK®/DNA mix was then centrifuged at 10,000 x g for 5 s, the supernatant discarded and the pellet washed 3 times with 1 ml of ice-cold NEW wash(NaCl/ethanol/H_2O mix provided in the kit). After centrifuging briefly, the pellet was air-dried and the DNA eluted by resuspending the pellet in 10-50 µl of H_2O and incubating at 55°C for 3 min. After a brief centrifugation the supernatant containing the DNA was transferred to a new Eppendorf® tube. Approximately 60-80% of the applied DNA was recovered by this method.

2.13 Ligation of restriction fragment into plasmid

Ligations were carried out for 16 h at 15°C or 2 to 4 h at room temperature. Plasmid DNA and insert DNA, with compatible cohesive ends or blunt ends, were mixed together at a 3:1 insert:plasmid ratio in a reaction volume of 20 µl containing 1 X ligation buffer (66 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ 10 mM DTT, 0.3 mM ATP, 1 mM spermidine), and 1 unit T4 DNA ligase (Promega). Control ligations of vector DNA alone with and without ligase were also set up with every ligation. The efficiency of ligation was monitored by transforming 1-5 µl of the ligation reaction into competent bacteria essentially as described in section 2.7.1. The ligations were stored at -20°C.

2.14 Shotgun cloning of restriction fragments

The DNA of interest was digested with an appropriate restriction enzyme as described in section 2.10. A small volume of the digest was run on an agarose gel to check for complete digestion. The digestion mix was then phenol/$CHCl_3$/IAA extracted (see section 2.4), EtOH precipitated (see section 2.9) and the DNA resuspended in sterile H_2O. The concentration of the DNA fragments was estimated by electrophoresing in a 0.8-1%(w/v) agarose gel in 1 X TAE buffer. Ligations were set up as described in section 2.13 and the clone of interest selected after restriction digestion analysis.

2.15 Radio-labelling DNA

The DNA fragment to be labelled was purified as described in section 2.12. DNA (25 ng) was made up to a total volume of 45 µl with sterile H_2O. After boiling for 5 min the DNA was snap-cooled on ice for a few minutes and added to the Rediprime labelling mix (Amersham International plc). 5µl of [α^{32}P] dCTP (3000 Ci/mmol, 10 mCi/ml) was added and the mixture incubated at 37°C for 10 min. The reaction was terminated by the addition of 5 µl of 0.2 M EDTA, pH 8.0. Unincorporated label was removed by spinning the probe through a glass bead/sephadex G50 column as described in section 2.15.1. The specific activity was determined by TCA precipitation as described in section 2.15.2. Prior to use, the probe was denatured by boiling for 5 min and then snap-cooled on ice for five min.

2.15.1 Removal of unincorporated radio-nucleotides

A sterile 0.5 ml Eppendorf® tube was pierced at the base with a syringe needle. The tube was then plugged with sterile glass beads and filled with Sephadex G50 resin (Pharmacia, Milton-Keynes, UK) equilibrated in TESN (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl, 0.05% (w/v) SDS). The tube was placed into a 1.5 ml Eppendorf® which had also been pierced at the base and centrifuged at 2000 x g for 4 min at room temperature. The tube was filled again with Sephadex G50 and respun until the packed resin volume was 0.5 ml. 200 µl of 1 X TE was loaded onto the column and was centrifuged again at 2000 x g for a further 4 min. The labelled

DNA was made up to a 50 µl volume with 1 X TE and loaded onto the column which was placed into a fresh 1.5 ml Eppendorf® and centrifuged again at 2000 x g for 4 min. The unincorporated label remained in the Sephadex beads whilst the labelled DNA eluted into the fresh tube. The labelled DNA was denatured and added to the pre-hybridisation solution or stored at -20°C.

2.15.2 Measurement of incorporation of radiolabel into DNA probes by TCA precipitation

This method was essentially as described in Sambrook *et al.* (1982).

Briefly, 1 to 2 µl of labelled sample was spotted onto 2 x 25 mm Whatmann GF/C filter discs and allowed to dry. One filter was placed in 20 ml of ice-cold 10% TCA and chilled on ice for 10-15 min. It was then washed twice in 20 ml 10% TCA and twice in 20 ml 95% ethanol and was dried thoroughly. The unwashed (total; unincorporated plus incorporated label) and washed (incorporated label) filters were then placed in separate scintillation vials and counted in the Cherenkov channel. Label incorporation was then expressed as a% of total cpm.

2.16 DNA Sequencing

DNA was sequenced using either the Sequenase® Version 2.0 sequencing kit (Amersham International plc., Amersham, UK) or the Hot *Tub* DNA sequencing system (Amersham International plc.). DNA was prepared as described in section 2.8.1.

2.16.1 Dideoxy sequencing of double stranded DNA

2.16.1.1 Annealing of primer to DNA template

Template DNA (3-5 µg) in a total volume of 18 µl was denatured in 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37°C. The DNA was EtOH precipitated as described in section 2.9. The pellet was air-dried and resuspended in 7 µl H_2O, 2 µl Sequenase® reaction buffer (0.2 M Tris-HCl pH 7.5, 0.1 M $MgCl_2$, 0.25 M NaCl) and 1 µl (0.5 pmol) primer. The primer and DNA were annealed by heating at 65°C for 2

min then cooled to 30°C over 15-30 min. The tube was incubated on ice (for up to 2 h) before proceeding with the labelling reaction.

2.16.1.2 Labelling and termination reactions

To the annealed DNA, 1.0 µl 0.1 M DTT, 2.0 µl diluted (1:5) dGTP mix (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 0.5 µl [α-^{35}S]dATP (10 µCi/µl, 1000 Ci/mmol) and 2 µl of diluted (1:8, enzyme:Sequenase® dilution buffer) Sequenase® 2.0 were added. The mixture was incubated at room temperature for 2-5 min after which 3.5 µl was transferred to four separate tubes each containing 2.5 µl of the appropriate dideoxynucleotide termination mix (ddA, ddC, ddG and ddT) preheated to 37° C. The termination mix was incubated at 37°C for 5 min. The reaction was terminated by the addition of 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF). The samples were heated at 75°C for 5 min prior to loading on an 8% (w/v) acrylamide sequencing gel as described in section 2.16.3.

2.16.2 Hot *Tub* DNA sequencing for double-stranded DNA template

Regions of DNA that had gross secondary structures or compressions were sequenced using the Hot *Tub* DNA sequencing system (Amersham International plc.). The Hot *Tub* DNA polymerase is extremely thermostable with a half life of 90 min at 95°C. It was therefore possible to carry out sequencing reactions at higher temperatures. Also, the labelling mix and termination mixes contained the analog, 7 - deaza 2'deoxyguanosine triphosphate, instead of dGTP which also aided in eliminating the compression zones (Mizusawa *et al.*, 1986).

2.16.2.1 Annealing

To a 1.5 ml Eppendorf® tube 4.5 µl of reaction buffer (260 mM Tris-HCl, pH 8.8, 26 mM $MgCl_2$, 67% (v/v) formamide), 2 pmol of double-stranded template DNA, 10 pmol of primer and sterile distilled H_2O to 13 µl were added. The contents were mixed and centrifuged briefly. The reaction mix was boiled for 5 min to denature the

DNA and placed immediately on an ice-water slurry for 30 min. Meanwhile, four separate tubes containing 2.5 µl of the appropriate termination mix, ddA, ddC, ddG or ddT were prepared.

2.16.2.2 Labelling and termination reactions

The annealed mix was centrifuged briefly after which 1 µl of labelling mix (7.5 µM dCTP, 7.5 µM 7-deaza dGTP, 7.5 µM dTTP and 15% (v/v) formamide) and 1 µl of [^{35}S]dATPαS (10 µCi/µl, 1000 Ci/mmmol) were added. The mixture was vortexed and centrifuged briefly after which 2 units of Hot *Tub* DNA polymerase were added. The reactions were incubated at room temperature for 5 min. 3.5 µl of the labelled mix was then transferred to each of the tubes containing the termination mix, mixed well and incubated at 65°C for 5 min. The reactions were terminated by the addition of 4 µl of formamide stop buffer (19 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF, 95% (v/v) formamide). The terminated reactions were either stored overnight at -20°C or denatured at 95°C for 5 min and 3 µl loaded onto an 8% (w/v) denaturing polyacrylamide/urea sequencing gel as described in section 2.16.3.

2.16.3 Sequencing gel electrophoresis

Sequencing gel plates which measured 20 cm x 40 cm were washed with detergent and one of the two plates was siliconised as described previously in section 2.2.5. 40 cm spacers with a 4 mm thickness were placed between the plates, which were then taped together. 50 ml of de-gassed sequencing solution (6% acrylamide:bisacrylamide, (38:2 w/v), 7 M urea, 1 X TBE) supplemented with 300 µl of 10% ammonium persulphate (w/v) (Sigma) and 50 µl of TEMED (Sigma) was poured between the plates. After placing a 24-well comb in place, the gel was allowed to set at room temperature for a minimum of 2 h. The gels were then pre-run for a minimum of 30 min at 35 W in 1 X TBE buffer. Aluminium plates were fixed to the sequencing plates to ensure even heat distribution. When the gel reached 55 to 60°C samples were loaded onto pre-washed wells. The gel was electrophoresed for the appropriate time period (usually 2, 4 or 6 h) at a constant wattage of 35 to 40 W. After electrophoresis, the plates were prised apart carefully ensuring that the gel remained

stuck to the unsiliconised plate. The plate/gel was placed into fixing solution containing 10% acetic acid (v/v), 10% MeOH (v/v) for a minimum of 20 min. The gel was carefully drained and transferred onto Whatman 3MM paper, covered with SaranWrap™ and dried on a slab-drier at 80°C for 2 h. After removing the SaranWrap™, the gels were autoradiographed for 24 h to 72 h at room temperature.

2.17 Isolation of RNA from plants

In a fume hood, 0.5 g of plant tissue was frozen in liquid N_2 in a mortar and ground to a fine powder and transferred to 5 ml of RNA extraction buffer (guanidine thiocyanate, N-lauryl sarcosine, sodium citrate and β-mercaptoethanol [added fresh each time] in a DEPC-treated 8 ml polycarbonate tube (Beckman). The tube was inverted several times to mix, after which 0.5 ml of 2 M NaOAc, pH 4, 5 ml of phenol, pH 8 and 3 ml of $CHCl_3$ were added and the contents mixed thoroughly by inverting several times. The tubes were centrifuged at 6000 x g for 30 min at 15°C and the aqueous layer transferred to a fresh tube containing 5 ml of phenol and 3 ml of $CHCl_3$. After mixing briefly and centrifuging at 6000 x g for 10 min at 15°C, the aqueous layer was transferred to a fresh tube and extracted with an equal volume of $CHCl_3$. The tubes were centrifuged again as before and the supernatant transferred to 15 ml Corex® tubes containing an equal volume of ice-cold isopropanol. The tubes were inverted once and the RNA allowed to precipitate for 30 min to 2 h at -20°C. RNA was recovered by centrifugation at 7000 x g for 20 min at 4°C. The RNA was washed by swirling gently in 80% (v/v) EtOH : 20% (v/v) DEPC-treated H_2O and air-dried for 15 min. The RNA pellet was resuspended in 50 µl of DEPC-treated H_2O, transferred to an Eppendorf® tube and stored at -70°C.

2.18 Blotting of nucleic acid onto nylon filter

2.18.1 Northern blotting

RNA was electrophoresed in a formaldehyde denaturing gel as described in section 2.11.2. The gel was washed briefly in distilled H_2O and then placed well side down on top of a support, which consisted of a wick of Whatman 3 MM paper soaked in a reservoir of 20 X SSC (3M NaCl, 0.3 M trisodium citrate). Air bubbles were

rolled out using a sterile plastic pipette. A small volume of 20 X SSC was then poured onto the gel to create a film of liquid. A piece of nylon membrane (Hybond N, Amersham) the exact size of the gel was then placed onto the gel ensuring again that no air bubbles formed between the gel and membrane. Two pieces of Whatman 3 MM paper the same size as the gel were soaked in 20 X SSC and placed on top of the nylon membrane. The wick area surrounding the gel was covered with cling film to prevent a short circuit and a quantity of absorbant paper towels placed on top of the 3 MM papers. A glass plate was placed on top of the towels and finally a 250 g weight on top of the glass plate. The gel was blotted for a minimum of 16 h after which the nylon membrane was rinsed briefly in 2 X SSC and air-dried for a few minutes. The RNA was fixed onto the membrane by a UV cross-linker for a 10-15 s.

2.18.2 Southern blotting

DNA was separated on an agarose gel as described in section 2.11.1. The gel was placed for 15 min in 0.25 N HCl to depurinate the DNA fragments. This would enable larger fragments of DNA to be transferred more efficiently. The gel was then rinsed briefly in distilled H_2O and placed in an excess volume of denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 45 min. The gel was then placed in neutralisation buffer (1 M Tris-HCl pH 8, 1.5 M NaCl) for a further 45 min. After rinsing briefly in distilled H_2O the gel was blotted and fixed essentially as described above except that Hybond N+ (Amersham) was used rather than of Hybond N.

2.19 Hybridisation analysis

2.19.1 Northern hybridisation

This technique is based on the method described by Denhardt (1966).

Nylon filters blotted as described in section 2.18 were pre-hybridised for a minimum of 4 h in a sealed plastic bag containing 1 ml per cm^2 of filter of pre-hybridisation buffer (0.5 M sodium phosphate, pH 7.2, 7% SDS (v/v), 10 mg/ml BSA for homologous probes or 5 X SSC, 50% formamide (v/v), 10 X Denhardt's solution [0.1% Ficoll 400 (w/v), 0.1% PVP 360 (w/v), 0.1% SDS, (w/v), 0.1% BSA (w/v), 50 µg/ml denatured herring sperm DNA (Boehringer Mannheim), 0.1% SDS (w/v)] for heterologous probes) and placed in a shaking H_2O bath inside a plastic tupperware

box. The probe was prepared as described in section 2.15, denatured by boiling for 5 min, snap-cooled on ice and added to the pre-hybridisation buffer. Hybridisation was carried out for 16 h at 55°C and at 37°C to 42°C for homologous and heterologous probes, respectively.

2.19.2 Washing Northern blots

After hybridisation the filters were washed in 2 X SSC:0.1% (w/v) SDS at 55°C or 60°C for 5 min. The washing was then repeated in 1 X SSC:0.1% SDS (w/v) for 5-10 min. Both washes were in 200-300 ml volumes. The filters were then wrapped in SaranWrap™ and autoradiographed for up to 24 h at -70°C. The filters were then developed in an X-OMAT (Kodak) automatic developer.

2.19.3 Southern hybridisation

Nylon filters blotted as described in section 2.18, were pre-hybridised for a minimum of 4 h in a sealed plastic bag or H2O tight box containing 1 ml per cm^2 of filter of pre-hybridation buffer (4 X SET [0.6 M NaCl, 4 mM EDTA, 80 mM Tris-HCl pH 8.0], 0.5% NaPPi (w/v), 0.1% SDS (w/v), 100 μg/ml denatured, sonicated herring sperm DNA (Boehringer Mannheim), 10 X Denhardt's solution [0.1% Ficoll 400 (w/v), 0.1% PVP 360 (w/v), 0.1% BSA (w/v) (all Sigma)]). After pre-hybridisation, the denatured probe (2 x 10^8 cpm) was added and hybridisation was carried out for a further 16 h. All filters were pre-hybridised and hybridised at 55°C in a shaking water bath unless otherwise stated.

2.19.4 Washing of Southern blots

The filters were washed based on the stability of the DNA/DNA hybrids (Anderson and Young, 1985). They were washed once for 1 min in 2 X SSC:0.1% SDS at room temperature, twice at 65°C in 2 X SSC:0.1% SDS, for 10 min each, once at 65°C in 0.5 X SSC:0.1% SDS for 10 min and finally once at 65°C in 0.1 X SSC:0.1% SDS for 10 min. The filters were air dried for a few minutes before autoradiography.

2.19.5 Autoradiography

Washed filters were wrapped in cling film and exposed to Fuji X-ray film type RX in an autoradiography cassette with an intensifying screen for the appropriate time period at -80°C for ^{32}P-radiolabelled probes. For sequencing gels autoradiography was carried out at room temperature without intensifying screens. The films were developed in an X-OMAT (Kodak) automatic developer.

2.19.6 Stripping filters bound with radiolabelled probes

Probes bound to nylon filters were removed by pouring a boiling solution of 0.5% (w/v) SDS onto the filters and allowing them to cool to room temperature. The filters were checked with a Geiger counter for any residual radioactivity and if required the process was repeated. The filters were then checked by autoradiography to ensure none of the probe remained. This procedure allowed the filter to be reprobed for up to five times.

2.20 Primer extension analysis

2.20.1 Primer labelling with [γ-^{32}P] ATP

The 5' transcriptional start site was determined using the AMV Reverse Transcriptase Primer Extension System (Promega).

The primer to be used for the primer extension reaction was phosphorylated at the 5' end in a reaction mix containing 2 µl (10 pmol/µl) of primer, 1 µl of 10 X Forward Exchange buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermine), 30 µCi [γ-^{32}P]ATP (10 Ci/ml, 3000 Ci/mmol,), 1 µl T4 polynucleotide kinase (8-10 U/µl) and DEPC-treated H_2O to a final volume of 10 µl. The reaction mix was incubated at 37°C for 10 min, after which the T4 polynucleotide kinase was inactivated by heating to 90°C for 2 min. The end-labelled primer was diluted to 100 fmol/µl by the addition of an appropriate volume of DEPC-treated H_2O. The percentage incorporation of radiolabel into primer was determined as described in section 2.15.2. The labelled primer was stored at -20°C.

2.20.2 Primer extension reaction

1 µl (100 fmol/µl) of ^{32}P-end-labelled primer was added to Eppendorf® tubes each containing 1, 2, 5 and 10 µg of total RNA in 5 µl aliquots. In addition a 'no RNA' control tube was also set up containing 5 µl of DEPC-treated H_2O. To these, 5 µl of 2 X AMV Primer Extension (PE) Buffer (100 mM Tris-HCl pH 8.3, 100 mM KCl, 20 mM $MgCl_2$, 20 mM DTT, 2 mM each of dATP, dCTP, cGTP, dTTP and 1 mM spermine) was added. The components were mixed thoroughly. The primer was allowed to anneal to the RNA by incubating the reaction mix at the appropriate temperature (Tm of the primer minus 5°C) for 1-2 h. 9 µl of the extension mix containing 5 µl of AMV PE 2 X buffer, 1.4 µl (40 mM) NaPPi, 1 µl AMV reverse transcriptase (5 U/µl) and DEPC-treated H_2O to volume was added to the annealed primer/RNA. After incubating the reaction mix at 42°C for 30 min, 20 µl of loading dye was added, the samples heated to 90°C for 10 min and loaded onto a sequencing gel. The gel was run for 1-2 h until the dye-front reached 3 cm from the bottom of the 20 cm x 40 cm gel. The gel was dried and autoradiographed for up to 72 h at -80° C.

2.21 Screening libraries

2.21.1 Preparation of plating cells

The same protocol was used for screening both the cDNA and genomic libraries. A single colony of XL1-Blue cells (for cDNA library) or DL491 cells (for genomic library) was used to inoculate 5 ml of L-Broth and NZCYM media respectively, containing 12.5 µg/ml of tetracycline. The culture was grown overnight at 37°C in a shaking incubator. 1ml of the culture was then used to inoculate 100 ml of the appropriate medium and grown for approximately 2 h until the OD_{600} was 0.5. The cells were centrifuged at 3000 x g for 5 min, and the pellet resuspended in 10 ml of ice-cold 10 mM $MgSO_4$. The cells were split into 100 µl or 200 µl aliquots in pre-cooled Eppendorf® tubes, flash-frozen in liquid nitrogen and stored at -80° C.

2.21.2 Titration of phage library

A serial logarithmic dilution of phage was prepared in SM buffer (0.1 M NaCl, 2% gelatine, 50 mM Tris-HCl, pH 8.0 and 8 mM $MgSO_4$); 10 µl of phage stock was

added to 990 µl of SM buffer to give a 10^{-2} dilution. Serial dilutions were then made up to 10^{-10} in SM buffer and 100 µl of each phage dilution was added to 100 µl of plating cells and incubated at room temperature or 37°C for 20 min to allow the phage to adsorb to the cells. 5 to 8 ml of Top-agarose (kept warm at 55°C) was added to the phage/cells mix and immediately poured onto thoroughly dried L-agar plates. The Top agarose was allowed to set and the plates placed upside down in a 37°C incubator overnight to allow plaque formation.

2.21.3 Plating the phage library

Petri dishes with a diameter of 150 mm were used in the primary screen and 90 mm diameter plates were used for subsequent secondary and tertiary rounds of screening. 200 µl of the appropriate phage dilution (approx. 2 x 10^8 pfu per plate for the primary screen and 50-500 pfu for subsequent rounds) was added to 200 µl of plating cells (prepared as described in section 2.21.2). The phage was allowed to adsorb to the cells for 20 min at room temperature after which 8 ml of Top agarose (pre-warmed to 55°C) was added to the tube, and was briefly mixed and poured immediately onto dried agar plates. The plates were placed upside down at 37°C overnight to allow plaques to form. The next day the plates were placed at 4°C for three days to enable the Top agarose to harden. Plaque lifts were carried out as described in section 2.21.4

2.21.4 Plaque lifts

This method was based on that of Benton and Davis (1977).

Hybond N filters (Amersham International plc., Amersham, UK) were placed for 30 s onto the plates and the filters marked asymmetrically at three positions with a syringe needle to orientate the plaques. The plates were also marked at the same positions with a marker pen. After removing carefully with forceps the filter was placed immediately, DNA side up, into denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 4 min. The filter was briefly dried on Whatman 3MM paper and then immersed twice for 3 min each in neutralisation buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 8) prior to washing in 0.1% SDS:2 X SSC. The filter was dried on Whatman 3MM

paper prior to baking for 2 h at 80°C. The plaque lift was duplicated by placing a second filter on the plate for 60 s and processing as the first filter.

2.21.5 Selection of positive plaques

The filters from each round of screening were hybridised to a radiolabelled probe and washed as described for Southern blots in section 2.19.3 and 2.19.4. Following each round of screening the resulting autoradiograph was placed onto the plate and the pattern of dots aligned with the plaques. The orientation of the plaqes was determined by aligning the asymmetric marks on the filters and plates produced by the syringe needle. The plaques of interest were cored out of the agar using the wide end of a pasteur pipette and the agar/agarose/plaque(s) were placed into a 1.5 ml Eppendorf® tube containing 1 ml of SM buffer and 50 µl of chloroform. The tube was vortexed briefly and incubated at room temperature for 1 h or at 4°C overnight in order to allow the phage to diffuse out of the agarose into the buffer. The phage was then titered as described in section 2.21.2. The phage was then plated out at a lower density and the screening process repeated until plaque purity was achieved. This normally took up to three rounds of screening.

2.22 *In vivo* excision of recombinant pBluescript® from Uni-Zap XR

This was carried out essentially as described in the manufacturer's manual.

The plaque of interest was cored and placed into a sterile Eppendorf® tube containing 1 ml of SM buffer and 50 µl of chloroform and vortexed to release the phage. The phage was allowed to diffuse into the buffer for 1 to 2 h at room temperature. In a 50 ml conical flask 200 µl of XL1-Blue plating cells [OD_{600} = 1], 100 µl of phage (containing approximately 1 x 10^6 phage particles) and 1 µl of ExAssist helper phage (Stratagene) (containing > 1 x 10^6 pfu/ml) were combined and the mixture incubated at 37°C for 15 min. 3 ml of 2 X YT medium was then added and the mixture incubated for a further 2 to 2.5 h with continuous shaking. The tube was heated at 70°C for 20 min and centrifuged in a bench top centrifuge for 15 min at 4000

x g. The supernatant which contains the Bluescript® II SK (-) phagemid packaged as a filamentous phage particle was decanted into a sterile Falcon tube and stored at 4° C.

1 µl and 50 µl of the supernatant was mixed with 200 µl of SOLR® cells [OD_{600} = 1] in 2 x 1.5 ml sterile Eppendorf® tubes and incubated for 15 min at 37°C. 100 µl of phage/SOLR® mix from each tube were plated onto L-agar plates containing 50 µg/ml of ampicillin and incubated at 37°C overnight. Single bacterial colonies appearing on the plates the next day were grown in L-Broth/ampicillin and the phagemid purified using the Promega Wizard™ miniprep kit (section 2.8.1).

2.23 Isolation of Lambda DNA from genomic clones

2.23.1 Preparation of plate lysates

The Wizard™ Lambda preps DNA purification system (Promega) was used.

For each genomic clone 7 x 90 mm NZCYM plates were prepared. 10^5 pfu of phage stock was mixed with 100 µl of competent DL491 cells and allowed to adsorb at 37°C for 20 min. 2 to 3 ml of NZCYM Top agarose was heated to 45°C, added to the phage/cell mixture and plated immediately onto previously prepared NZCYM plates. The Top agarose was allowed to set and the plates incubated upside down at 37°C overnight. The next day 3 ml of SM buffer was added to each plate. The Top agarose was carefully scraped off with a sterile spatula and transferred to a high speed 50 ml centrifuge tube. The agarose was broken up and the tubes incubated at RT for 30 min with regular shaking. The agarose was centrifuged at 10,000 x g for 10 min at 4°C and the supernatant transferred to a 50 ml Falcon tube to which was added 0.3% of $CHCl_3$. The phage lysates were either stored at 4°C or used immediately as described in the next section.

2.23.2 Removal of Phage Coat

10 ml of lysate was transferred to a 30 ml Corex® tube to which was added 40 µl of nuclease mix (250 µg/ml each of RNase A and DNase I solution, 150 mM NaCl, 50% glycerol, supplied in kit) and the mixture was incubated at 37°C for 15 min. The lambda DNA was precipitated with the addition of 4 ml of Phage Precipitant (33%

(w/v) polyethylene glycol (PEG-8000), 3.3 M NaCl) and incubated on ice for 30 min. The phage DNA was pelleted by centrifugation at 10,000 x g for 10 min. The supernatant was decanted off and the pellet resuspended in 500 µl of Phage Buffer (150 mM NaCl, 40 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$). The resuspended lambda DNA was proteinase K (Boehringer Mannheim) treated at 37°C for 5 min at a final enzyme concentration of 0.5 mg/ml (20 units/mg, Boehringer Mannheim) to remove any residual nuclease mix. The DNA was then transferred to a 1.5 ml Eppendorf® tube, centrifuged at 12,000 x g for 10 s to remove insoluble debris and the supernatant transferred to a fresh Eppendorf® tube containing 1 ml of Purification Resin. The DNA/resin was mixed by inverting the tube and then transferred to a disposable Wizard™ Minicolumn which consisted of a 3 ml syringe barrel attached to the Luer-Lok® extension of the minicolumn attached to a vacuum manifold. The vacuum was applied and the DNA/resin mix drawn into the minicolumn. The vacuum was broken and the column washed with 2 ml of 80%(v/v) iso-propanol. The vacuum was reapplied and the column wash drawn through. The minicolumn was dried thoroughly by drawing the vacuum for a further 2 min. The syringe barrel was discarded, the minicolumn attached to a 1.5 ml Eppendorf® tube and centrifuged at 12,000 x g for 20 s to discard any residual isopropanol. The minicolumn was attached to a new Eppendorf® tube, 100 µl of H_20, preheated to 80°C, applied and the lambda DNA eluted by centrifugation at 12,000 x g for 20 s.

2.24 Polymerase chain reaction (PCR)

Up to 200 ng of *in vivo* excised cDNA phagemid, genomic clones or genomic DNA were placed into a 0.5 ml Eppendorf® containing 5 µl of 10 X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9, 1% Triton X-100), 8 µl of dNTP mix (Pharmacia, 2.5 mM each of dATP, dCTP, dGTP, dTTP), 5 µl of 2.5 mM $MgCl_2$, 1 µl each of 100 pmol/µl of T3 and T7 or gene-specific primer (see Tables 3.1 and 4.1) and 0.5 µl of *Taq* DNA polymerase (Promega, 2.5 U/µl). The reaction was made up to 50 µl with sterile H_20, mixed thoroughly and overlayed with 50 µl of light mineral oil (Sigma). The reaction was placed in a thermal cycler (Omnigene, Hybaid Ltd., Middlesex, UK) under the following conditions: 95°C for 1 min (denaturation), 50°C for 2 min (primer

annealing) and 72°C for 2 min (extension), cycled 30 times. The reactions were left to extend at 72°C for a further 7.5 min at the end of the cycle.

The promoter region of the *BnLTP* gene was amplified using *Pfu* polymerase (Stratagene) in a reaction mix containing the same reagents as described above except using a different 10 X buffer (200 mM Tris-HCl pH 8.2, 100 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1 mg/ml BSA, 1% Triton X-100 (v/v)) and cycled as above except the annealing and extenstion times were increased to 5 min. *Agrobacterium* transformed with the binary vector containing the *BnLTP-GUS* construct was boiled for a few minutes in sterile H_2O before being subjected to PCR with *Taq* DNA polymerase. The PCR conditions were the same as those described for amplification of genomic clones.

2.25 Surface sterilisation and germination of *Arabidopsis* seed

Germination medium (GM) containing 1 X Murashige and Skoog salt mixture (Flow Labs, Irvine, Scotland, UK), 1% (w/v) sucrose, 100 mg/l inositol, 1 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid and 0.5 g/l N-morpholino ethanesulfonic acid (MES) in H_20 was adjusted to pH 5.7 with 1 M KOH. The medium was autoclaved and allowed to cool to 55°C before pouring into Petri dishes.

Seed was placed onto small Whatman 3MM filter paper, folded twice and fixed into place with paper clips. These were then soaked in 70 % ethanol for 2 min then placed immediately in 10 % (v/v) hypochlorite solution (BDH, AnalaR® grade, Poole, Dorset) containing 1 % (v/v) Triton X-100 (Sigma-Aldrich) for 20 min. Under sterile conditions (in a flow hood), the seed was washed thoroughly in sterile H_20 to remove the bleach. It was then dried thoroughly under sterile conditions for a minimum of 4 h. The seeds were sprinkled evenly onto germination medium in Petri dishes under sterile conditions, sealed with surgical tape, cooled at 4°C for 2 days then placed under the appropriate light conditions.

2.26 Transformation of *Arabidopsis* by Vacuum Infiltration

2.26.1 Preparation of *Arabidopsis* plants

Arabidopsis thaliana ecotype Landsberg *erecta* were grown under continuous white light to a stage where bolts were just emerging. Approximately 10 plants per 3.5 inch pot were prepared. The emerging bolts were clipped four to eight days prior to infiltration in order to encourage multiple bolting. The soil was also kept dry in order to maximise infiltration (usually from the point when the bolts were clipped).

Two days prior to transformation, a single colony of *Agrobacterium* GV3101 transformed with the appropriate construct was inoculated into a 25 ml culture of YEP medium containing 50 μg/ml of kanamycin and 25 μg/ml of gentamycin. The culture was grown overnight at 30°C with continuous shaking. The next day the 25 ml culture was added to 400 ml of YEP/kanamycin/gentamycin in a 1 litre conical flask and grown with continuous shaking at 30°C until the OD_{600} was > 2.0. The bacteria were pelleted by centrifuging at 5000 x g for 10 min at room temperature and resuspended in 3 volumes of vacuum infiltration medium (0.5 X Murashige & Skoog salts, 1 X B5 vitamins, 5 % sucrose, 0.044 μM benzylamino purine) until the OD_{600} was approx. 0.8. A 400 ml culture was enough to infiltrate six pots. 200 ml of *Agrobacterium* resuspended in infiltration medium was placed into a 1 litre beaker. The plants were inverted into the medium ensuring that all the bolts, leaves and soil were fully submerged. The beaker was placed into a bell jar attached to a vacuum pump and vacuum drawn until bubbles formed around the submerged leaf, stem and bolts. After 2 to 3 min the vacuum was released very rapidly ensuring the medium was completely absorbed by the soil. At this stage the leaves had a very dark green appearance. The entire plant and pot was covered with cling film to maintain humidity and was placed on its side. The next day the cling film was removed and the pots placed upright and grown for four weeks until siliques were fully developed and completely dry. Seed was harvested from individual plants and screened for putative transformants.

2.26.2 Analysis of progeny from transformed plants

Seed was harvested from vacuum infiltrated plants, surface sterilised and plated out on germination media supplemented with 50 μg/ml kanamycin. Wild type and NM4 seed were plated as negative and positive controls, respectively. Seed were

vernalised for a few days at 4°C and placed under continuous white light. Within seven to ten days, seedlings that appeared dark green with fully expanded cotyledons with emerging secondary leaves were selected. Kanamycin-sensitive seedlings bleached and died within seven days. Seedlings were transferred to soil and transformed plants (T1 generation) were allowed to grow and self fertilise. Seed were harvested and the segregation ratio (kanamycin resistant:kanamycin sensitive) of the progeny was calculated. A segregation ratio of 3:1 in the T2 generation indicated that the transgene was located at a single locus.

2.27 Electroporation of *Brassica napus* stem sections with GUS constructs

Three to four week old *B. napus* stems were cut into 1-2 mm sections and washed thoroughly in sterile H_2O on ice for 1 h. 6-10 sections were placed into a plastic cuvette (Bio-Rad, Gene Pulser cuvette) with a 0.4 cm electrode gap containing 300 µl of sterile H_2O (everything was kept on ice). 25 µg of plasmid DNA to be electroporated was added to the sections and mixed thoroughly by inverting the cuvette several times. Plasmid containing the *35S-GUS* fusion and 'no DNA' controls were also set up. The stem sections were electroporated at different conditions using the Bio-Rad Gene Zapper (see Chapter 5, section 5.3.3, Table 5.1). The stem sections were then immersed in 500 µl of a solution containing 400 mM mannitol, 8 mM $CaCl_2$, 5 mM MES pH 5.6, and mixed thoroughly and incubated at room temperature under high intensity white light for 24 h. The next day the stem sections were washed several times in 50 mM sodium phosphate and incubated for 24 h at 37°C in 1 mg/ml of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc; BioGene Ltd., Bedfordshire, UK) in 50 mM sodium phosphate. The stems were cleared in 80% EtOH and observed for GUS activity (formation of a blue precipitate).

2.28 Plant growth conditions

2.28.1 General growth conditions

Seeds were sown onto moist, sterile potting compost, covered with cling film and vernalised at 4°C for two to five days prior to germination. Plants were grown

under continuous low intensity white (LW) light (35 ± 5 μmol/m^2 /s) or high intensity white (HW) light (100-200 μmol/m^2/s) using 45 Watt warm white fluorescent tubes (Osram, Munich, Germany) at 22°C unless otherwise stated. Plants to be infected with CaMV were grown in a 10 h light (45 μmol/m^2/s)/ 14 h dark photoperiod until the first two leaves emerged.

2.28.2 Light treatments

Light qualities (intensity and wavelengths) were measured by a spectroradiometer (model SR9910; Macam Photometrics, Livingston, UK). UV-B was obtained from TL 40W/12 UV fluorescent tubes (Philips, London, UK) covered with cellulose acetate, which was changed every 24 h. Blue light was provided by 40W T12 blue fluorescent tubes (GTE Sylvania, Shipley, UK.) with a λmax 430 nm covered with a UV226 filter (Lee Filters, Andover, UK.) which removes wavelengths < 400 nm. Red light was obtained by covering white fluorescent tubes with orange cinemoid; no emisson <500 nm was detected. Plants were grown under LW light for 3 weeks and then transferred for 24 h to either HW (100-200 μmol/m^2/s), UV-B (3 μmol/m^2/s; fluence rate between 280 and 320 nm), blue (80-90 μmol/m^2/s) or red (80-90 μmol/m^2/s) light conditions at 22°C. The plants were then either harvested into liquid nitrogen and used for DNA or RNA extractions or used for fluorometric GUS assays.

2.28.3 Cold treatment and wounding of leaves

For cold treatments, 21 day old plants LW-grown at 22°C were placed at 4°C for the appropriate time period under the same light conditions. Plants were then harvested for RNA isolation or GUS assays.

Plants to be wounded were grown under LW light conditions for three weeks. The fifth or sixth leaf was then wounded by piercing with a sterile syringe needle numerous times or by cutting the leaf into several sections with a blade. The wounded and control plants (unwounded) were then placed for a further 3 days under LW light prior to GUS assays.

2.28.4 Viral infection

The cauliflower mosaic virus (CaMV) isolate BARI was used to infect plants. Plants were grown in a dark/light photoperiod (10 h light (45 μmol/m²/s) and 14 h dark) until the first two leaves began to emerge. The virus was mixed with a small amount of abrasive powder and 100 ng of virus applied to the surface of each leaf with a small glass spectre. The abrasive enabled the surface of the leaf to be scratched slightly to allow the virus to enter. Inoculated plants were placed back into the growth chamber. After the appropriate time period the first inoculated leaf and/or a secondary leaf were assayed for GUS activity or used to extract RNA or DNA.

2.29 Analysis of GUS activity

2.29.1 Histochemical localisation of GUS expression

Pieces of tissue were placed in fixative solution (0.3% formaldehyde, 10 mM MES, pH 5.6 (Sigma), 0.3 M D-mannitol for 1 min under vacuum. The fixative was replaced and the tissue fixed for a further 45 min at room temperature after which it was washed thoroughly in 50 mM sodium phosphate pH 7.0. The tissue was then incubated in 1 mg/ml X-Gluc in 50 mM sodium phosphate pH 7.0 for 24 h at 37°C. The next day the tissue was washed several times in 50 mM sodium phosphate, cleared in EtOH and photographed or embedded in OCT compound (BDH, Gurr®) and sectioned at -20°C using a cryostat microtome (Bright Instrument Company Ltd., UK). The 10-12 μm sections were placed onto polylysine coated slides and photographed under a light microscope (Axiophot Zeiss).

2.29.2 Fluorometric GUS assays

GUS activities were assayed quantitatively essentially as described by Jefferson *et al.* (1987). After the appropriate treatment 1 or 2 leaves were homogenised in 50 μl of GUS extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) sarkosyl, 0.07% (v/v) β-mercaptoethanol) in a 1.5 ml Eppendorf® tube. Eight to ten plants were harvested for each treatment or time point. The homogenate was placed on ice and 450 μl of 1 mg/ml 4-methylumbelliferyl glucuronide (MUG; BioGene Ltd.) in GUS extraction buffer was added. The

homogenate/buffer was mixed by inverting the tube four times and then incubated at 37°C for 15 min (T15). After the 15 min incubation period GUS activity was terminated by placing 100 µl of the assay mixture into 900 µl of 0.2 M Na_2CO_3. The remaining mixture was incubated for a further 45 min (T45) at 37°C after which another 100 µl aliquot was added to 900 µl of Na_2CO_3 (T60). The T15 and T60 samples were measured for production of the fluorescent product 4-methylumbelliferone (4-MU). The fluorescence was measured in a Perkin-Elmer LS5 luminometer (excitation 365 nm, emission 455 nm) against a series of known 4-MU concentration standards. The remaining 300 µl of the assay mixture was used to determine protein concentrations using the Bradford (1976) protein assay as described in Sambrook *et al.* (1989). GUS activity is expressed as the number of nmols of 4-MU produced per min per mg of protein.

Chapter 3 Isolation of a full length *Brassica napus* epidermis-specific cDNA

3.1 Introduction

Previously, an epidermis-specific partial cDNA clone, designated pLF3A (Pallas, 1992), was isolated following a differential screen of a *Brassica napus* cv. Cobra cDNA library. This library was constructed from poly (A)+ RNA isolated from shoot apices with floral buds and sepal primodia. The aim was to isolate genes expressed early during floral morphogenesis. The cDNA library was constructed in the vector, λNM1149 (Murray, 1983), using a method previously described by Dorssers and Postmes (1987). Essentially, the cDNAs were ligated to linkers containing an internal *EcoR* I site and four base pairs of a *Hind* III site, which resulted in the formation of a unique *Hind* III site adjacent to the poly (A) tail. The library was screened differentially using cDNA probes from two distinct developmental stages, vegetative and floral buds with sepal primodia only. λLF3 was one of several clones isolated. pLF3A was derived from λLF3, which upon digestion with *EcoR* I and *Hind* III generated two insert fragments. The larger of the two fragments of 376 bp was subcloned into pBluescript® II SK (-) and was designated pLF3A.

3.1.1 Characteristics of pLF3A

It had already been shown by Pallas (1992) that pLF3A hybridised to a single transcript of approximately 700 nucleotides from total RNA isolated from *Brassica napus* cv. Cobra leaf, stem and floral tissue. Expression was not evident in root tissue. In Southern blots of *Brassica napus* genomic DNA digested with *EcoR* I, *Hind* III and *Bam* HI, pLF3A hybridised to at least four bands, suggesting the presence of several copies of closely related genes. The cDNA also cross-hybridised to *Arabidopsis thaliana* genomic DNA. Transcripts complementary to pLF3A were localised in the epidermal cells of leaf and floral tissue using *in situ* hybridisation experiments. Transcripts hybridising to pLF3A were not affected upon wounding, cold treatment or heat shock (Pallas, 1992). Preliminary sequence analysis of pLF3A did not reveal a characteristic poly (A) tail which is present at the 3' termini of most eukaryotic genes.

This implied that the clone probably represented the 5' region of the λLF3 cDNA. Preliminary sequence comparison of the cDNA to other sequences in the database did not reveal homology to any other plant genes.

The initial aim of the project was to use pLF3A as a probe to isolate the full length clone. This chapter describes the isolation of a cDNA clone from a *Brassica napus* cDNA library constructed in the Uni-Zap™ XR vector where the inserts were cloned directionally into the *Xho* I and *Eco* RI sites.

3.2 Screening of a cDNA library

Goodwin *et al.* (1996) used the ZAP-cDNA® synthesis kit (Stratagene) to construct the cDNA library as described in the manufacturer's manual. Poly (A)+ RNA was isolated from cold-treated *Brassica napus* cv. Cobra leaf tissue and reverse transcribed. The resulting cDNAs were then cloned directionally into the *Xho* I and *EcoR* I sites of the Uni-ZAP™ XR vector.

The first strand cDNA synthesis was primed using a 50 base oligonucleotide containing a "GAGA" repeat sequence at the 5' terminus followed by a *Xho* I restriction site and an 18 base poly dT sequence. The GAGA sequence protected the *Xho* I restriction site. The poly dT was annealed to the poly (A) tail on the mRNA template then reverse transcribed with the Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MuLVRT; Zeef, 1987). The *Xho* I restriction site at the 3' end allowed the completed cDNA to be cloned into the Lamda Zap® vector (Short, 1988) in a sense orientation in relation to the *Lac* Z promoter. During the first strand synthesis, 5-methyl-dCTP was used in the nucleotide mix instead of dCTP, whereas during the second strand synthesis with DNA polymerase I, dCTP was incorporated which reduced the probability of 5-methyl dCTP being incorporated in the second strand. This enabled only the restriction sites in the primer-linker to be susceptible to restriction enzyme digestion. The termini of the cDNAs were blunt-ended with T4 DNA polymerase and ligated to the *Eco* RI adapters. Subsequent *Xho* I digestion of the cDNA-adapters released the *Eco* RI adapter. The cDNAs were then size fractionated and ligated directionally into the Uni-ZAP™ XR vector arms. The library was then packaged in a high efficiency Gigapack® II packaging extract which was

mcrA-, *mcrB*- and *mrr*- (Kertz *et al.*, 1989) and plated on the *E. coli* cell line SURE™ which was also *mcrA*-, *mcrB*- and *mrr*-. Use of this bacterial strain was preferred at this stage because it did not destroy the hemi-methylated cDNAs. After the initial plating on SURE™ cells the library was subsequently plated on other bacterial strains such as XL1-Blue.

The cDNA library was titred to estimate the number of plaque forming units (p.f.u.) required for plating. Basically the number of plaques that appear on a lawn of bacteria is directly proportional to the amount of phage. This can be represented by the equation: $n = y/vx$, where y represents the number of plaques produced by plating a volume v (ml) of a dilution x of phage giving a titre n of p.f.u. per ml.

The library was diluted in SM buffer and added to XL1-Blue plating cells in a total volume of 200 µl. The plates were confluent at 10^{-4} to 10^{-1} dilution. At dilutions of 10^{-5}, 10^{-6} and 10^{-7}, 240, 26 and 2 plaques were formed resulting in titres of 2.4 x 10^8, 2.6 x 10^8 and 2 x 10^8 p.f.u./ml, respectively.

3.2.1 Primary, secondary and tertiary screening of the cDNA library

The pLF3A cDNA clone was excised from the pBluescript® II SK (-) phagemid by restriction digestion with *EcoR* I and *Hind* III, purified and used as a probe to screen the cDNA library. In the primary screen the library was plated at 10^{-3} dilution (> 2 x 10^8p.f.u./ml) on six, 150 mm plates which enabled the screening of >1.2 x 10^9 plaques. As *B. napus* is a close relative of *Arabidopsis*, which is estimated to have approximately 20,000 genes (Gibson and Sommerville, 1993), this number of plaques was considered sufficient in order to isolate at least one of the four putative pLF3A gene homologues. Duplicate filters were hybridised to the radio-labelled pLF3A cDNA. A total of 30 putative positive plaques (four positives from plate 1 (1A1 to 1A4), fifteen plaques from plate 2 (2A1 to 2A15), two plaques from plate 4 (4A1 and 4A2) and finally nine plaques from plate 5 (5A1, 5A2, 5A3, 5A4, 5A5, 5A6, 5A7, 5A8 and 5A9) were observed. There was no significant difference in the intensity of the radioactive signal among the putative positives. After secondary and tertiary

rounds of screening at lower plaque densities (described in section 2.21), sixteen of the thirty putative positives were plaque purified and characterised further by PCR and sequencing.

3.3 Amplification of inserts with the T3 and T7 primers by PCR

The approximate size of inserts in the sixteen putative positive plaques was estimated by PCR using the primer pair T3 and T7 that flank the multiple cloning site of the pBluescript® II SK (-) phagemid contained within the linear Lamda Zap® vector. Essentially, each plaque was boiled for a few minutes in a total volume of 20 µl of sterile water, centrifuged briefly at 10,000 x g and the supernatant was subjected to PCR, the conditions for which are detailed in section 2.24. The products were resolved on a 1% agarose gel along with DNA size markers (Fig. 3.1).

Nine clones, λ2A4, λ2A6, λ2A7, λ2A8, λ2A15, λ5A6, λ5A4, λ5A8 and λ5A9 were positive, amplifying a strong band of approximately 700 bp. One clone, λ2A11 amplified a smaller band of around 600 bp. A faint band of around 700 bp was detected for the three clones, λ2A1, λ2A10 and λ5A7, whereas three clones, λ2A3, λ2A5 and λ2A9 were negative. The ten clones that amplified a high intensity band were rerun on a 1% agarose gel, Southern blotted onto Hybond N+ nylon and hybridised to pLF3A (Fig. 3.2). pLF3A was included as a positive control. The conditions for pre-hybridisation and hybridisation were essentially the same as that described for Southern blots (described in 2.19.3 and 2.19.4). Only four clones, λ2A4, λ2A6, λ2A7 and λ2A8, hybridised to pLF3A. A faint band was detected for λ2A11 and λ2A15, whereas clones λ5A4, λ5A6, λ5A8 and λ5A9 were negative.

3.4 *In vivo* excision of the recombinant pBluescript® II SK (-) phagemid

The Uni-ZAP™ XR vector is designed so that the clone of interest can be isolated easily by a method known as *in vivo* excision, which bypasses the restriction digestion and subcloning of the insert into a new vector. The helper phage, f1, was

Fig. 3.1 Amplification of putative positives by PCR using primers T3 and T7

The inserts from putative positive clones from the tertiary screen were amplified by PCR using the primers T3 and T7 which flank the multiple cloning site of the Uni-ZAP™ XR vector. The PCR products were resolved on a 1% (w/v) agarose gel. Lanes 1-16 correspond to the 16 positive clones taken from the tertiary screen. The size of the inserts was estimated by comparing to the 1 kb DNA size markers (lane m).

Lane 1	λ2A1	Lane 9	λ2A10
Lane 2	λ2A3	Lane 10	λ2A11
Lane 3	λ2A4	Lane 11	λ2A15
Lane 4	λ2A5	Lane 12	λ5A6
Lane 5	λ2A6	Lane 13	λ5A4
Lane 6	λ2A7	Lane 14	λ5A7
Lane 7	λ2A8	Lane 15	λ5A8
Lane 8	λ2A9	Lane 16	λ5A9

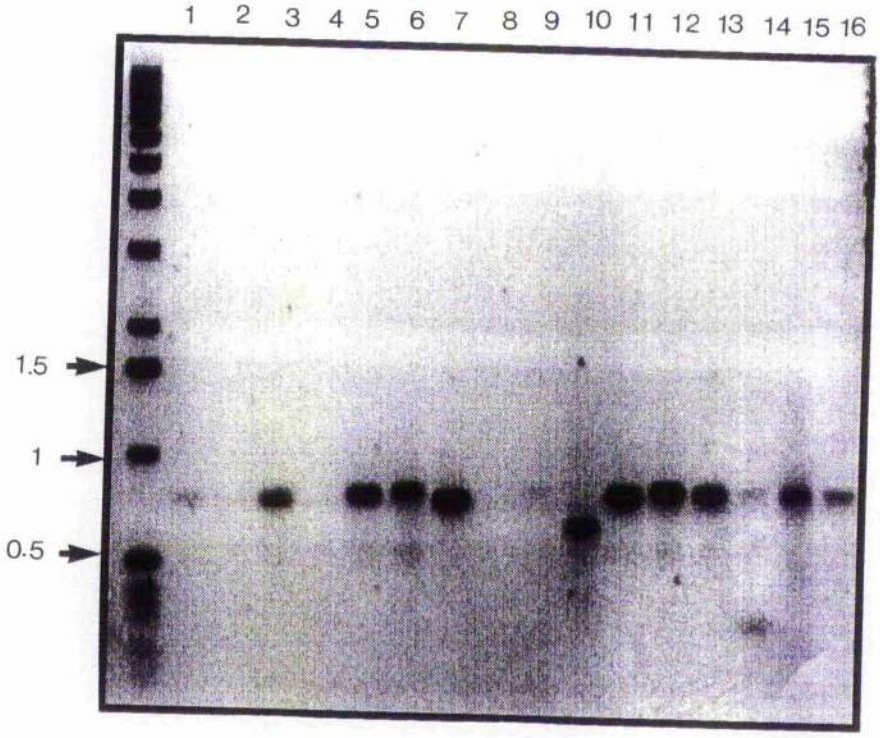
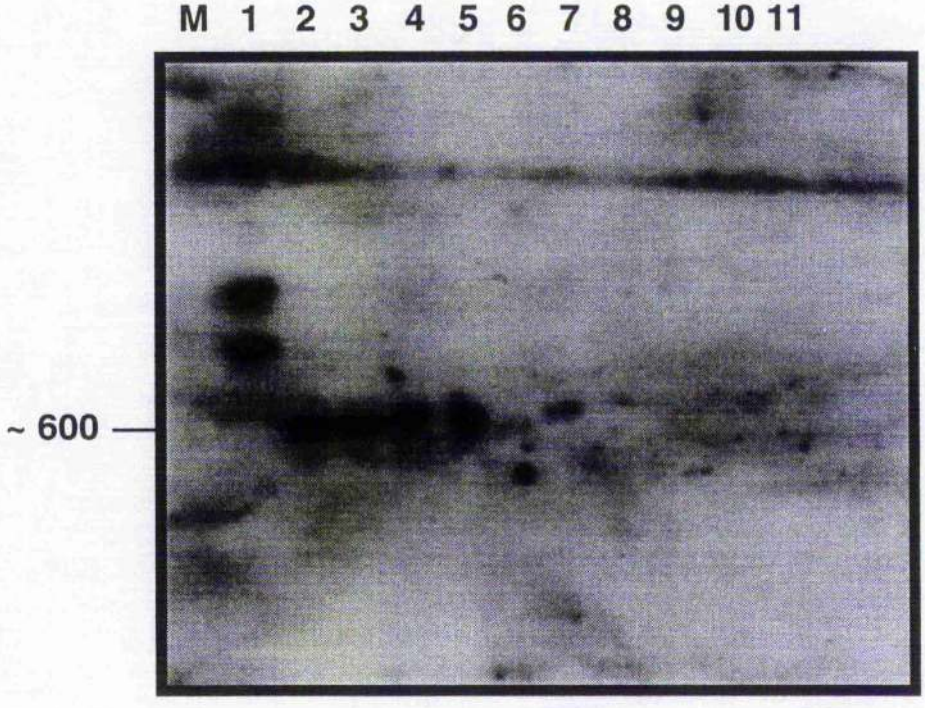

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
1.5
1
0.5

Fig. 3.2 Southern blot analysis of PCR-amplified putative positive clones

Ten of the positive clones that amplified a strong band described in Fig. 3.1 were rerun on a 1% (w/v) agarose gel (top panel), Southern blotted onto Hybond N+ and hybridised to radioactively labelled pLF3A (botton panel). pLF3A plasmid was run as a positive control. The size of the fragments were estimated by comparing with the 1 kb DNA size markers (Lane M).

Lane 1	uncut pLF3A	Lane 7	λ2A15
Lane 2	λ2A4	Lane 8	λ5A4
Lane 3	λ2A6	Lane 9	λ5A6
Lane 4	λ2A7	Lane 10	λ5A8
Lane 5	λ2A8	Lane 11	λ5A9
Lane 6	λ2A11		


M 1 2 3 4 5 6 7 8 9 10 11
4.074
3.054
2.036
1.636
1kb
506
396
344
298


M 1 2 3 4 5 6 7 8 9 10 11
~ 600

required for the *in vivo* excision process. Essentially, the phage and the Uni-ZAP™ vector which contained the fragment of interest were co-transfected into bacterial cells where the f1 phage expressed helper proteins that bound to sites on the Uni-ZAP™ vector that enabled DNA synthesis of the positive strand to begin. These proteins also recognised a terminator sequence further downstream where the synthesis of the new strand was completed. The newly synthesised single-stranded DNA which contained the pBluescript® II SK (-) phagemid and the insert, if one was present, was then circularised by the gene II product of the f1 helper phage. The recombinant phagemid was then packaged using signals contained within the terminator sequence, secreted from the bacteria and subsequently rescued when mixed with fresh bacteria. The phagemid/bacteria mixture was plated immediately onto selective L-Broth agar plates containing 50 μg/ml ampicillin.

The nine clones (with the exception of p2A11) described in Fig 3.2 were *in vivo* excised. Approximately 100 to 200 putative positive colonies were observed for each of the rescued recombinant phagemids. The colonies were inoculated into L-Broth supplemented with ampicillin and the phagemid was isolated using the Promega plasmid DNA isolation kit. The recircularized, rescued phagemids were digested with *Eco* RI and *Xho* I to excise the insert and the products were separated on a 1% agarose gel along with 1 kb size markers (Fig. 3.3). All nine clones contained an insert of approximately 650 bp. Interestingly those clones that did not hybridise to pLF3A in the Southern blot described in Fig. 3.2 also contained the right sized insert. The clones that did hybridise to pLF3A were considered to be the full length clones based on previous northern blots of total *B. napus* RNA, where pLF3A hybridised to a single transcript of approximately the same size (Pallas, 1992).

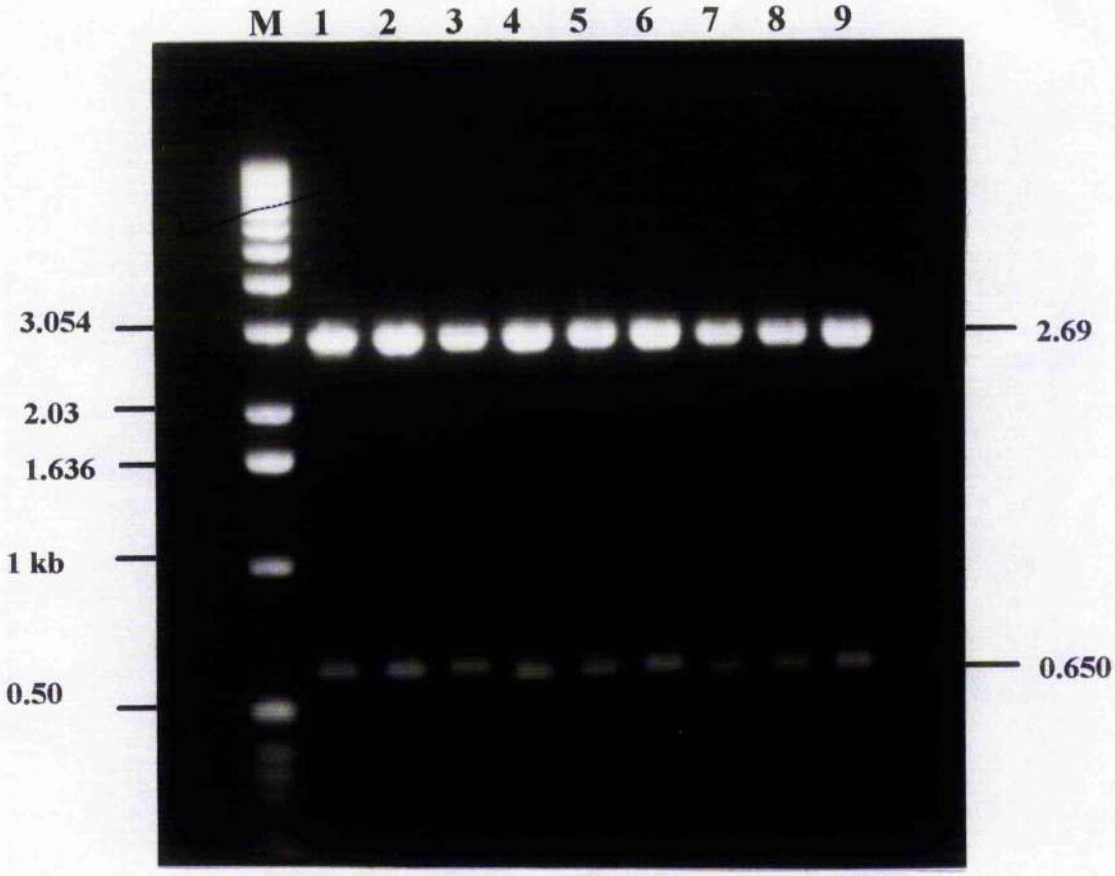
3.5 Sequencing 3' termini with T7 primer

The *in vivo* excised, recombinant pBluescript® II SK (-) clones were designated p2A4, p2A6, p2A7, p2A8, p2A15, p5A6A, p5A4, p5A8 and p5A9. They were sequenced using the dideoxynucleotide chain termination method of Sanger *et al.* (1977). Initial sequencing reactions were carried out using primer T7. As previously mentioned, the cDNAs were cloned directionally into the Uni-ZAP™ XR vector such

Fig.3.3 ***In vivo*** **excision and restriction digestion of recombinant pBluescript® II SK (-)**

Phage from the positive plaques from the tertiary screen that amplified the correct sized band was used to infect XL1-Blue cells. These cells were then superinfected with the ExAssist helper phage which excised the pBluescript® SK II (-) phagemid from the Uni-ZAP™ XR vector. The excised phagemid was rescued into SOLR™ cells and transformed bacteria were selected on LB/amp agar plates. Phagemid minipreps were carried out with the Promega DNA miniprep kit. The inserts were excised by restriction digestion with *Eco* RI and *Xho* I and sized on a 1% (w/v) agarose gel. (p:phagemid)

Lane M	1 kb ladder
Lane 1	p2A4
Lane 2	p2A6
Lane 3	p2A7
Lane 4	p2A8
Lane 5	p2A15
Lane 6	p5A4
Lane 7	p5A6
Lane 8	p5A8
Lane 9	p5A9


M 1 2 3 4 5 6 7 8 9
3.054
2.03
1.636
1 kb
0.50
2.69
0.650

that the 5' termini of the cloned cDNAs were adjacent to the T3 promoter and the 3' termini were adjacent to the T7 promoter. Therefore, it was relatively easy to determine the orientation of the clones and the coding strand. Subsequent sequencing reactions were carried out using synthetic primers complementary to the newly derived sequences (Table 3.1).

A characteristic poly (A) tail was observed in all of the clones confirming they represented expressed genes. The length of poly (A) tail as well as the sequences immediately flanking the poly (A) tail differed in the clones. However, due to substantial secondary structure which produced compression zones in these regions, it was not possible to fully sequence all the clones. Similarly, clones sequenced at the 5' termini with the T3 primer also contained several compression zones. Those clones that contained differences in the regions flanking the compression zones were selected and sequenced further. Both DNA strands of one clone, p2A7 were fully sequenced using the dideoxynucleotide chain termination method of Sanger *et al.* (1977), and regions with compression zones were sequenced using the *Hot Tub* sequencing method (Amersham). The remaining clones were sequenced only partially from the 3' termini with T7.

3.6 Analysis of the p2A7 sequence

The primers used to sequence p2A7, their sequences and the region in the cDNA where they hybridised are described in Table 3.1. The complete sequence of p2A7 is shown in Fig. 3.4. The cDNA was 651 bp in length. The *EcoRI* and *Xho* I restriction sites at the 5' and 3' termini, respectively, are shown as well as the relative positions and orientations of the T3 and T7 primers. The clone contained one open reading frame of 118 amino acids which is shown below the putative codons along with the the TGA stop signal designated by an asterix (*). A putative translation start site (ATG) with the consensus sequence AAAC/ATG/GC (Joshi, 1987) is shown at position 69. The 5' untranslated region upstream of the ATG start site was 62 bp in length. The length of the 3' untranslated region prior to the first adenine of the poly (A) tail was 201 bp. A putative polyadenylation signal, AAATAA, was located at nucleotides 488 to 493 in the 3' untranslated region. An internal *Hind* III site, AAGCTT, is shown at nucleotides 286 to 291. Positions of the internal sequencing and

Table 3.1 Primers used to sequence the cDNA p2A7

The synthetic oligonucletides used to sequence the cDNA clone p2A7 are shown along with the target sequences. The clone was initially sequenced with primers T3 and T7 that hybridised to pBluescript® II SK (-) at target sequences that flank the multi-cloning site. Subsequent primers were then designed from the newly derived sequences. The cDNA nucleotide positions where several of the primers hybridised are also shown. F and R refer to forward and reverse primers, respectively.

Primer	Sequence 5'- 3'	Template	Nucleotide
T3	*AATTAACCCTCACTAAAGGG*	pBluescript®	(F)
T7	*GTAATACGACTCACTATAGGGC*	pBluescript®	(R)
SK	*CGCTCTAGAACTAGTGGATC*	pBluescript®	(F)
1919	*ACGTCTGCATTA*	BnLTP	444 to 455 (R)
1689	*TGGTCTTGGCCTGCATGAT*	BnLTP	97 to 115 (F)
2014	*GCGGCGTTACTAGTCTAAAC*	BnLTP	235 to 254 (F)
2207	*GTGCTGCTGGACTTCCTAAG*	BnLTP	343 to 362 (F)
2240	*GTTTAGACTAGTAACGCCGC*	BnLTP	235 to 254 (R)

Fig. 3.4 Nucleotide and predicted amino acid sequence of p2A7

The 651 bp nucleotide sequence of the p2A7 cDNA is shown. The 118 amino acid polypeptide sequence is shown below the putative codons. The *Eco* RI (GAATTC) and *Xho* I (CTCGAG) sites at the 5' and 3' termini, respectively, are underlined. The putative translation start site with the consensus sequence AAAC/ATG/GC (Joshi, 1987) is shown in bold at position 69. The asterisk (*) indicates the stop codon (TGA). An internal *Hind* III (AAGCTT) site is underlined (nucleotide 286-291). The orientations and relative positions of the T3, T7, 1689 and 1919 sequencing and PCR primers are indicated by an arrow. A putative polyadenylation signal, AAATAA (shown in bold) is located 62 bp from the stop codon at nucleotides 488 to 493 in the 201 bp 3' untranslated region. The poly (A) tail initiates at nucleotide 627. The arrow following the alanine residue at amino acid 25 depicts the putative signal cleavage site.

T3→

Eco RI

```
                GA ATT CGG CAC GAG CTC GTG CCG AAT TCG    29
GCA CGA GAT TTG CAA ACA AAA AAA ATC TAA GAG AGA AAT    68

                                          1689 →
ATG GCT GCT CTA ATG AAG TTG GCA TGC TTG GTC TTG GCC   107
 M   A   G   L   M   K   L   A   C   L   V   L   A    13

TGC ATG ATC GTG GCC GGT CCA ATC ACA TCG AAC GCG GCT   146
 C   M   I   V   A   G   P   I   T   S   N   A   A    26
                                                  ↑
CTG AGC TGT GGC ACC GTT AGC GGC TAC GTG GCA CCG TGC   185
 L   S   C   G   T   V   S   G   Y   V   A   P   C    39

ATT GGC TAC CTG GCC CAG GGT GCG CCG GCC CTT CCC AGA   224
 I   G   Y   L   A   Q   G   A   P   A   L   P   R    52

GCG TGC TGC AGC GGC GTT ACT AGT CTA AAC AAC CTG GCC   263
 A   C   C   S   G   V   T   S   L   N   N   L   A    65
                    Hind III
CGT ACA ACC CCA GAC CGT CAG CAA GCT TGC CGT TGC CTT   302
 R   T   T   P   D   R   Q   Q   A   C   R   C   L    78

GTA GCA GCC GCT AAC GCC TTC CCT ACT CTC AAC GCT GCC   341
 V   G   A   A   N   A   F   P   T   L   N   A   A    91

CGT GCT GCT GGA CTT CCT AAG GCA TGT GGA GTC AAC ATT   380
 R   A   A   G   L   P   K   A   C   G   V   N   I   104

CCT TAC AAG ATC AGC AAA ACC ACC AAC TGC AAC AGT GTG   419
 P   Y   K   I   S   K   T   T   N   C   N   S   V   117
                                          ←1919
AAA TGA GCG GCG GTC GGA TGA AGC TAA TGC AGA CGT TCA   458
 K   *                                           118

AGT ATT ATG TAT TGG ATG AGC GAA TAC TAA AAT AAG ATG   497
TTC CAT GGT TGT TGT TTT TAG AGT TTT TCA GTT TCC TGT   536
CTT TTA TGT TGT GAC GTT CCT ATT ACT TTG GTC GTC TGT   575
ACT ATG TTC ACA ATC AAC GTT ATA TGA ATT TCA GAT CTA   614
AGA TTT ACG TTT AAA AAA AAA AAA AAA AAA CCT CGA G     651
                                          Xho I
```

←T7

PCR primers, 1689 (upstream, nucleotides 97 to 115) and 1919 (downstream, nucleotides 444 to 455) are also shown. Arrows indicate the direction of sequencing and PCR.

Restriction digestion of p2A7 with *Eco* RI and *Hind* III yielded two fragments, of approximately 290 bp and 3.3 kb (Fig. 3.5). The 290 bp *Eco* RI/*Hind* III fragment most probably corresponded to the 5' terminus of the cDNA clone and the 3.3 kb fragment corresponded to the 2.69 kb pBluescript® II SK (-) plus the remaining 3' terminus of the cDNA clone.

3.7 p2A7 encodes a putative non-specific lipid transfer protein

The nucleotide and deduced protein sequences of cDNA p2A7 were compared to known sequences in the EMBL database using the UWGCG programs, FASTA and tFASTA, respectively. The putative protein encoded by p2A7 was highly similar to a large class of small molecular weight plant proteins known as non-specific lipid transfer proteins (nsLTPs) (Fig. 3.6A) and was therefore designated a *Brassica napus* lipid transfer protein (BNLTP). Comparison of the deduced protein sequence to other LTPs indicated a high degree of similarity that ranged from 40% to 95%. The highest degree of similarity (94.8%) was observed with a 118 amino acid *Brassica oleracea* cv. *italica* lipid transfer protein designated WAX9D (Pyee, *et al.*, 1994, 1995). WAX9D was found to be the major protein associated with the surface wax of broccoli leaves. The amino acid sequence was also highly similar to three *Brassica napus* germination-specific (gs) cDNAs which also encoded putative lipid transfer proteins, designated gs*ltp*1 (91%), gs*ltp*2 (93%) and gs*ltp*3 (92%) isolated from young *Brassica napus* cv Darmar germinating seedlings (Soufleri *et al.*, 1996). BNLTP was 70% identical to the *Arabidopsis LTP1* cDNA (Thoma *et al.*, 1994), 52% identical to a tobacco LTP (Fleming *et al.*, 1992), 47% identical to a tomato LTP (Torres-Schumann *et al.*, 1992), 47% identical to a maize LTP (Tchang *et al.*, 1988), 52% identical to a spinach LTP (Bernhard *et al.*, 1991) and only 42% identical to another *Brassica napus* LTP, E2 isolated from developing microspores (Foster *et al.*, 1992). A common feature amongst all LTPs is the conservation of eight cysteines (Fig. 3.6B).

Fig. 3.5 Restriction digestion of p2A7

p2A7 was digested with *Eco* RI, *Bam* HI, *Xho* I, *Sal* I and *Hind* III and resolved on a 1% (w/v) agarose gel along with 1 kb size markers (M).

Lane 1. *Eco* RI

Lane 2 *Bam* HI

Lane 3. *Sal* I

Lane 4. *Xho* I

Lane 5. *Eco* RI/*Hind* III

Lane 6. *Eco* RI/*Bam* HI

Lane 7. *Eco* RI/*Sal* I

Lane 8. *Bam* HI/*Sal* I

Lane 9. *Xho* I/*Sal* I

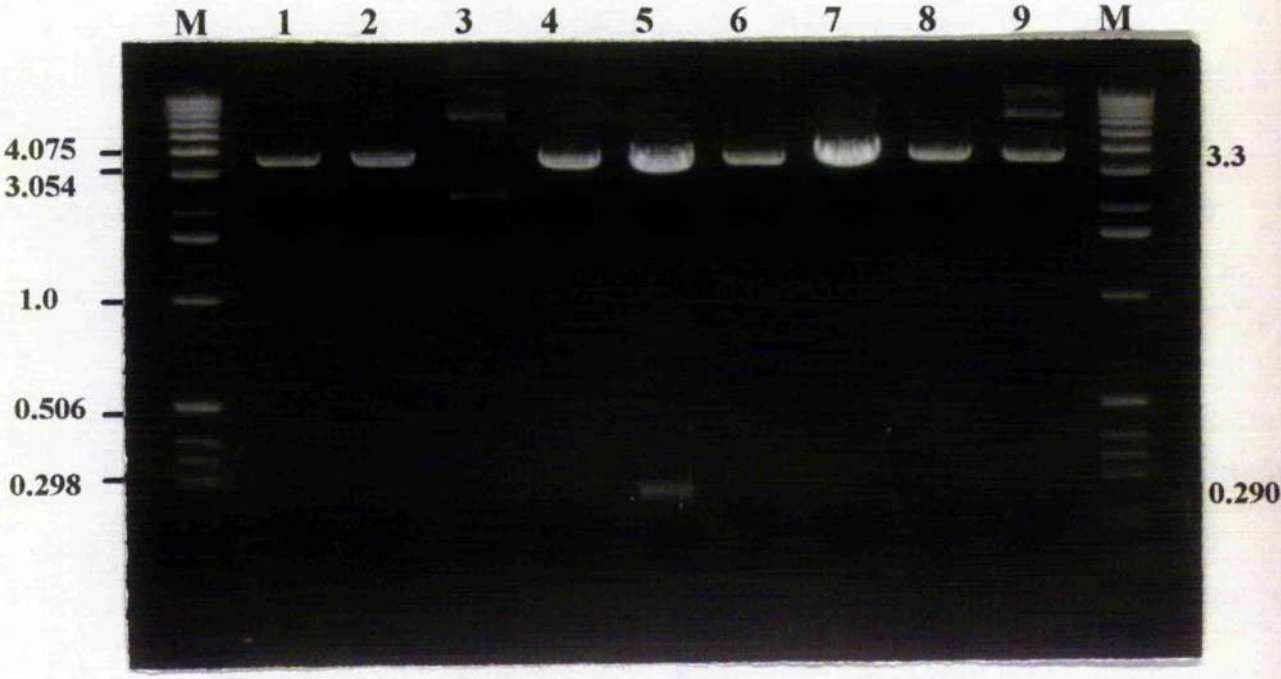

M
1
2
3
4
5
6
7
8
9
M
4.075
3.054
1.0
0.506
0.298
3.3
0.290

Fig. 3.6 Comparison of BNLTP with other plants LTPs

A. The protein sequences were aligned using the UWGCG PILEUP program. The small dots (.) indicate gaps. The star at the end of the sequence indicates the stop codon. The conserved cysteine residues are shown below the sequence. The line depicts the putative signal peptide. Accession numbers are indicated in brackets: *Brassica napus* AB/LTP II (Østergaard *et al.*, 1995); broccoli LTP, WAX9D (L33907) (Pyee and Kolattkudy, 1995); oilseed rape, gsltp1 (U22174) (Soufleri *et. al*, 1996); *Arabidopsis*, LTP1 (M80567) (Thoma *et. al*, 1994); tobacco, Ltp1 (X62395) (Fleming *et al.*, 1992); tomato, TW12 (X56040) (Torres-Schumann *et. al*, 1992); spinach, PW132 (M58635) (Bernhard *et. al*, 1991); barley, LTP4 (X68656) (Molina and Garcia-Olmeda, 1993); maize, 9c2 (J04176) (Tchang *et. al*, 1988), oilseed rape, E2 (Foster *et al.*, 1992). BNLTP is 100% identical to AB/LTP II, 96.6% to WAX9D, 88.9% to gsltp1, 70% to *Arabidopsis* LTP1, 52.1% to tobacco Ltp1, 47.4% to TW12, 52.6% to PW132, 40.3% identical to LTP4, 47% to 9c2 and 42.2% identical to E2. B. Alignment of cysteine residues in BNLTP and the *Brassica napus* cold-inducible hybrid proline-rich protein, BNPRP (Goodwin *et al.*, 1996) and the *Medicago sativa* salt-inducible chimeric proline-rich cell wall protein, MsPRP2 (Deutch and Winicov, 1995).

A

```
                1                                                    50
      BNLTP   ~~~MAGLMKL ACLVLACMIV AGPITSNAAL SCGTV.SGYV APCIGYLAQG
   AB/LTPII                                  AAL SCGTV.SGYV APCIGYLAQG
      WAX9D   ~~~MAGLMKL ACLIFACMIV AGPITSNAAL SCGTV.SGYV APCIGYLAQN
     gsltp1   ~~~MAGLMKL ACLVLACMIV AGPITSNAAL SCGTV.SGYV APCIGYLTQN
Arabidopsis   ~~~~~~~~~~ ~MLALGLHDC GRSNTSNAAL SCGSVNSSNL AACIGYVLQG
    Tobacco ~~~MEIAGKI ACFVVLCMVV AAP.CAEA.I TCGQVTSN.L APCLAYLRNT
     Tomato ~~~MEMVSKI ACFVLLCMVV VAP.HAEA.L TCGQVTAG.L APCLPYLQGR
    Spinach ~MASSAVIKL ACAVLLCIVV AAP.YAEAGI TCGMVSSK.L APCIGYLKGG
     Barley MARAAATQLV LVAMVAAMLL VA...TDAAI SCGQVSSA.L SPCISYARGN
      Maize MARTQQLAVV ATAVVALVLL AAA.TSEAAI SCGQVASA.I APCISYARGQ
         E2 ~~MAFASKII TCLLILTIYI AAP..TESHI TCGTVTS.TM TQCISYLTNG
                                             C-------- --C-------

                51     **                       *  *                100
        BNLTP  APALPRACCS GVTSLNNLAR TTPDRQQACR CLVGAANAFP T.LNAARAAG
     AB/LTPII  APALPRACCS GVTSLNNLAR TTPDRQQACR CLVGAANAFP T.LNAARAAG
     Broccoli  APAVPTACCS GVTSLNNMAR TTPDRQQACR CLVGAANALP T.INVARAAG
       gsltp1  GP.LPRGCCT GVTNLNNMAR TTPDRQQACR CLVGAANSFP T.LNAARAAG
  Arabidopsis  G.VIPPACCS GVKNLNSIAK TTPDRQQACN CIQGAARALG SGLNAGRAAG
      Tobacco  G..PLGRCCG GVKALVNSAR TTEDRQIACT CLKSAAGAI. SGINLGKAAG
       Tomato  G..PLGGCCG GVKNLLGSAK TTADRKTACT CLKSAANAI. KGIDLNKAAG
      Spinach  P..LGGGCCG GIKALNAAAA TTPDRKTACN CLKSAANAI. KGINYGKAAG
       Barley  GAKPPVACCS GVKRLAGAAQ STADKQAACR CLKSLATSI. KGINMGKVSG
        Maize  GSGPSAGCCS GVRSLNNAAR TTADRRAACN CLKNAAAGV. SGLNAGNAAS
           E2  GP.LPSSCCV AVKSLNQMAQ TTPDRRQVCE CLKSAGKEI. KGLNIDLVAA
               -------CC- ---------- --------C- C--------- ----------

               101                          127
        BNLTP   LPKACGVNIP YKISKTTNCN SVK*~~~
     ABLTP/II   LPKACGVNIP YKISKTTNCN SVK*~~~
     Broccoli   LPKACGVNIP YKISKTTNCN SVK*~~~
       gsltp1   LPKACGVNIP YKISKSTNCN SVR*~~~
  Arabidopsis   IPKACGVNIP YKISTSTNCK TVR*~~~
      Tobacco   LPSTCGVNIP YKISPSTDCS KVQ*~~~
       Tomato   IPSVCKVNIP YKISPSTDCS TVQ*~~~
      Spinach   LPGMCGVHIP YAISPSTNCN AVH*~~~
       Barley   VPGKCGVSVP FPISMSTDCN KVH*~~~
        Maize   IPSKCGVSIP YTISTSTDCS RVN*~~~
           E2   LPTTCGVSLS YPIGFNTNCD SISIAV*
               ----C----- --------C- ---
```

B

```
  BNLTP     CGTVSGYVAPCIGYLAQGAPALPRA******CCSGVTSLNNLARTTPDRQQACRCLV
  BNPRP     CPIDTLKLGACVDVLGGLIHIGLGGSSAKKECCPVLGGLVDLDAAV******CLCTT
 MsPRP2     CSIDALKLGACVDVLGGLIHIGIGGSAKQT*CCPLLQGLVDLDAAI******CLCTT

  BNLTP     GAANAFPTLNAARAAGLPKA*CGVNIPYKISKTTNCNS
  BNPRP     IKAKLLIVDLIIPIALELLIDCGKTPPPGFK****CPS
 MsPRP2     IRLKLLNINLVIPLALQVLIDCGKTPPEGFK****CPA
```

Consensus

```
LTPs  --C---(7-9)--C-----(12-15)----CC----(8-19)------C-C-------(21-22)--------C-----(6-13)--------C--
PRPs  --C---(9)----C------(19-20)---CC----(13)--------C-C-------(23)---------- C-----(9)-----------C--
```

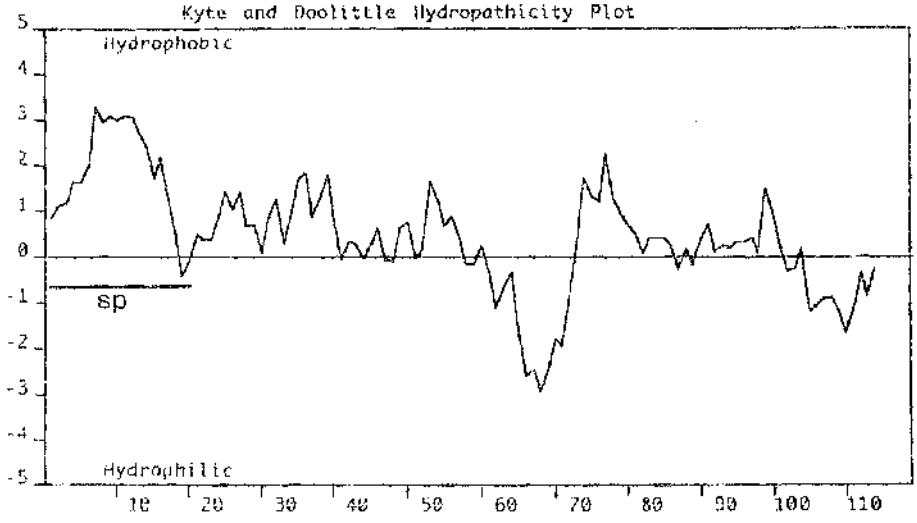
3.8 Analysis of the BNLTP protein

The BNLTP protein of 118 amino acids was high in alanine content comprising 16% of the protein; 10% of the residues were cysteines, whereas histidine, tryptophan and glutamine residues were absent from the protein, a feature observed in other LTPs (Østergaard *et al.*, 1995). The predicted amino acid sequence of BNLTP was analysed using the Kyte and Doolittle programme (Kyte and Doolittle, 1982) which measures the average hydrophobicity of the amino acids (Fig. 3.7). The first 25 amino acids were extremely hydrophobic and were followed by a region in the middle of the protein that comprised a high proportion of hydrophilic amino acids. This profile is comparable to proteins that are either membrane associated or secreted, suggesting the first 25 amino acids probably represented a signal peptide sequence. Furthermore, BNLTP lacked the carboxy-terminal H/KDEL endoplasmic reticulum retention signal (Munroe and Pelham, 1987). Indeed, the sequence conformed to the '-3, -1 rule' typical of naturally occuring cleavage sites (von Heijne, 1988). Generally the -1 and -3 positions (upstream of the cleavage site) must be small and uncharged residues such as Ala, Gly, Cys and Thr whereas the -2 position is a large bulky and charged residue. The BNLTP putative signal peptide cleavage (arrow in Fig. 3.4) had the sequence Ser-Asn-Ala↓Ala where the serine residue was at position -3 and the alanine residue was at position -1. After removal of the putative signal peptide BNLTP was 100% identical to one of the three *Brassica napus* acyl-binding / lipid transfer proteins (AB/LTP II) of 93 amino acids (Mr of 9408) purified from germinated *Brassica napus* seedlings by HPLC (Østergaard *et al.*, 1995) (data not shown). The AB/LTP II protein sequence was determined by automated Edman degradation of intact proteins and peptides, which suggested that AB/LTP II was purified as the mature protein where the signal peptide had already been cleaved. Two other LTP isoforms, AB/LTP I and AB/LTP III were also isolated but were not completely sequenced.

An interesting feature of all LTPs isolated to date (reviewed in Kader, 1996) including BNLTP, is the spacing between the conserved cysteine residues which is almost identical to the cysteine residues located at the COOH-terminus of plant cell wall hybrid proline-rich proteins (hyPRPs). These include the *B. napus* cv. Bridger hyPRP, BNPRP, (Goodwin *et al.*, 1996) and the *Medicago sativa*

Fig. 3.7 Hydropathy profile of the deduced BNLTP sequence

The hydropathy value of each amino acid was calculated by the Kyte and Doolittle (1982) programme. Values above the horizontal line indicate the hydrophobic regions and values below the horizontal line indicate the hydrophilic regions. The region marked with SP denotes the putative signal peptide sequence.


Kyte and Doolittle Hydropathicity Plot
Hydrophobic
Hydrophilic
sp
5
4
3
2
1
0
-1
-2
-3
-4
-5
10
20
30
40
50
60
70
80
90
100
110

hyPRP, MsPRP2, (Deutch and Winicov, 1995) shown in Fig. 3.6B. This region of the PRPs is thought to represent three membrane spanning domains (Goodwin *et al.*, 1996).

3.9 Comparison of BnLTP with pLF3A

The DNA sequences of pLF3A and BnLTP (p2A7) were compared using the UWGCG BESTFIT programme (Fig.3.8). The clones were 99% similar over a region of 241 bp covering nucleotides 38 to 277 of BnLTP (5'-terminus) and nucleotides 136 to 372 of pLF3A, which represented most of the coding regions in both clones. The previous prediction that pLF3A represented the 5' terminus of the clone was shown to be true. The sequence alignment introduced five gaps. Bases 38 to 68 of BnLTP, which represented the putative untranslated region, aligned with bases 136 to 164 of pLF3A after the introduction of two gaps in pLF3A. A putative translation start site was located at position 165 in pLF3A which corresponded to the ATG in BnLTP at position 69. A single base difference (C to G) is indicated at position 263 of BnLTP. The 5' untranslated region represented by bases 7 to 37 of BnLTP diverged considerably from the putative 5' untranslated region represented by bases 1 to 135 of pLF3A (data not shown). These observations demonstrated that BnLTP was closely related but not identical to pLF3A.

3.10 Comparison of BnLTP, p2A4 and p5A9 DNA sequences

The 3'-untranslated regions of cDNAs BnLTP, p2A4 and p5A9 were aligned and compared using the UWGCG programm PILEUP (Fig. 3.9). The BnLTP nucleotide sequence was 93% (10 gaps) and 95% (1 gap) identical to p2A4 and p5A9, respectively, over a region of 390 bp. The clones differed at several bases indicated by (*). The partially sequenced coding regions flanking the 3' untranslated region were compared for any amino acid differences. Four amino acid substitutions were observed: an alanine (BNLTP) to serine (p5A9) at amino acid 84 (codon at position 318-320; GCC to TCC), a phenylalanine (BNLTP) to a leucine (p2A4) at amino acid 85 (codon at position 321-323; TTC to TTG), a leucine (BNLTP) to an isoleucine (p2A4) at amino acid 88 (codon 330-332; CTC to ATC) and an alanine (BNLTP) to a valine (p2A4) at amino acid 90 (codon 336-338; GCT to GTT). All

Fig 3.8 Comparison of BnLTP cDNA sequence with pLF3A

The DNA sequences of the cDNA clones pLF3A and BnLTP (shown in bold) were aligned using the UWGCG program BESTFIT. The clones were 99% identical over a region of 241 bp. Bases shown in lower case correspond to the 5' untranslated region of BnLTP. The putative 5' untranslated regions corresponding to the first 135 bp of pLF3A and the first 37 bp of BnLTP diverged considerably and therefore were not aligned. 5 gaps were introduced for the alignment. The putative translational start sites (ATG) in both clones are underlined.

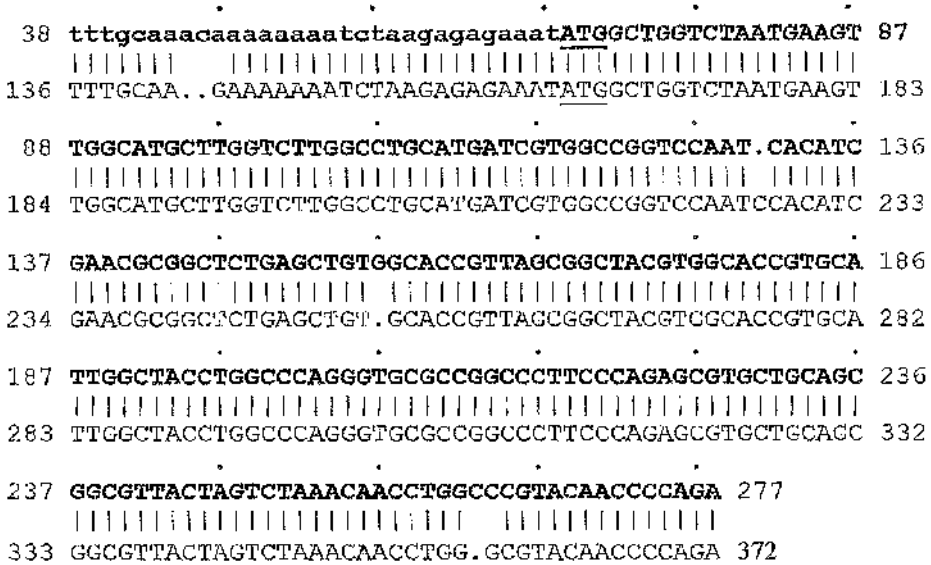

38 tttgcaaacaaaaaaaatctaagagagaaatATGGCTGGTCTAATGAAGT 87
136 TTTGCAA..GAAAAAAATCTAAGAGAGAAATATGGCTGGTCTAATGAAGT 183
88 TGGCATGCTTGGTCTTGGCCTGCATGATCGTGGCCGGTCCAAT.CACATC 136
184 TGGCATGCTTGGTCTTGGCCTGCATGATCGTGGCCGGTCCAATCCACATC 233
137 GAACGCGGCTCTGAGCTGTGGCACCGTTAGCGGCTACGTGGCACCGTGCA 186
234 GAACGCGGCTCTGAGCTGT.GCACCGTTAGCGGCTACGTGGCACCGTGCA 282
187 TTGGCTACCTGGCCCAGGGTGCGCCGGCCCTTCCCAGAGCGTGCTGCAGC 236
283 TTGGCTACCTGGCCCAGGGTGCGCCGGCCCTTCCCAGAGCGTGCTGCAGC 332
237 GGCGTTACTAGTCTAAACAACCTGGCCCGTACAACCCCAGA 277
333 GGCGTTACTAGTCTAAACAACCTGG.GCGTACAACCCCAGA 372

Fig. 3.9 Sequence comparison of the 3' ends of *BnLTP*, p5A9 and p2A4

The partially sequenced 3' ends of p5A9 and p2A4 were aligned with *BnLTP* (p2A7) using the UWGCG PILEUP programme. The amino acid substitutions are indicated. The putative polyadenylation signal, AAATAA, is underlined and the TGA stop signal is shown. Base differences are indicated by (*).

```
          307                                                        356
BnLTP     GAGCCGCTAA CGCCTTCCCT ACTCTCAACG CTGCCCGTGC TGCTGGACTT
p5a9      GAGCCGGTAA CTCCTTCCCT ACTCTCAACG CTGCCCGTGC AGCTGGACTT
p2a4      GAGCCGCTAA CGCATTGCCT ACTATCAACG TTGCCCGTGC AGCTGGACTT
                    A>S F>L        L>I     A>V

          357                                                        406
BnLTP     CCTAAGGCAT GTGGAGTCAA CATTCCTTAC AAGATCAGCA AAACCACCAA
p5a9      CCTAAGGCAT GTGGAGTCAA CATTCCTTAC AAGATCAGCA AAACCACCAA
p2a4      CCTAAGGCAT GTGGAGTCAA CATTCCTTAC AAGATCAGGA AAACCACCAA


          407                                                        456
BnLTP     CTGCAACAGT GTGAAATGAg cggcggtcgg atgaagctaa tgcagacgtt
p5a9      CTGCAACAGT GTGAAATGAg cggcggtcgg atgaagctaa tgcagagctt
p2a4      CTGCAACAGT GTGAAATGAg cggcggtcgg atgaagctaa tgcagacgtt
                             stop

          457                                                        506
BnLTP     caagtattat gtattggatg agcgaatact .aaaataaga tgttcc.atg
p5a9      caagtattat gtattggatg agcgaatact .gaaataaga tgttcc.atg
p2a4      caagtattat gtaatggatg agcgaataat aaaaataaga tgctccaatg
                       *                 *  *                 *

          507                                                        556
BnLTP     gttgttgttt ttagagtttt tcagtttcct gtcttttatg ttgtgacgtt
p5a9      gtggttgttt ttagagtttt tcagtttcct gtcttttatg ttgtgacgtt
p2a4      gttgttgttt ttagagtttt tcaattt.ct gtcttttatg ttgtgacgtt
            *                          *   *

          557                                                        606
BnLTP     cctattactt tggtcgtctg tactatgttc acaatc.aac gttatatgaa
p5a9      cgtattagtt tggtcgtatg tactatgttc acaatcaaac gttatatgaa
p2a4      cttattactt tggtcgtttg tagtatgttc aaa...tcaa gttatatgaa
           *     *          *     *          * ***** *

          607                                                  646
BnLTP     tttcagatct aagatttacg tttaaaaaaa aaaaaaaaaa
p5a9      tttcagatct aagattt... .......... ..........
p2a4      tttcagatgt aagatttacg tttg...... ..........
                 *                 *
```

three cDNA used TGA as the putative stop signal. The AATAAA polyadenylation signal was located at identical positions in all three clones. Several nucleotide substitutions and insertions were observed in the 3' non-coding region (denoted with a *). There was not enough sequence overlap between clones p5A9 and p2A4 and pLF3A for a reasonable DNA and amino acid sequence comparison. However, in view of the differences observed between the four cDNAs it is likely that sequences of at least four of the possible five or six members of the *B. napus* cv Cobra multigene family encoding putative non-specific lipid transfer proteins were isolated.

3.11 Discussion

A partial cDNA clone designated pLF3A isolated from a *Brassica napus* cDNA library had previously been shown to be expressed specifically in the epidermis of leaf, stem and floral tissue using *in situ* hybridisation. The clone lacked a poly (A) tract and it hybridised to a single transcript of 700 nt. It was therefore thought to represent the 5' end of the original full length cDNA. This chapter describes the isolation and characterisation of a full length cDNA homologous to pLF3A. A *Brassica napus* cv. Cobra cDNA library constructed in the Uni-Zap™ XR vector (Goodwin *et al.*, 1996) was screened with the partial clone.

A total of 30 positive clones were observed in the primary screen, sixteen of which were plaque purified following subsequent rounds of screening. PCR of each clone with the T3/T7 primer pair that flanked the insert in the Uni-Zap™ XR vector amplified a fragment ranging from 600 to 700 bp (Fig. 3.1) which was the expected fragment size based on previous northern blot analysis. In Southern blots of the PCR products, only four clones hybridised at high stringency to pLF3A (Fig. 3.2) which suggested some of the PCR products were either artefactual or represented fragments with less homology to pLF3A. However, a 600-650 bp fragment was produced in nine clones following *Eco* RI and *Xho* I digestion of the *in vivo* excised Uni-Zap™ XR vector including those that did not hybridise to pLF3A in Southern blots (Fig. 3.3).

Sequence analysis with T7 revealed a characteristic poly (A) tract in all nine clones which suggested that expressed genes were isolated. By using a combination of manual sequencing using the dideoxynucleotide chain termination method of Sanger *et*

al. (1977) and the *Hot Tub* Sequencing method which enabled sequencing of regions with compression zones, one clone p2A7 was fully sequenced (Fig. 3.4).

The 651 bp cDNA contained one putative open reading. of 118 amino acids with an ATG start site at position 69 in the consensus sequence AAAC/ATG/GC (Joshi, 1987) and a stop signal (TGA) at position 423. The length of the 3' untranslated region prior to the first adenine of the poly (A) tail was 201 bp. A putative polyadenlyation signal AAATAA, was present, which is the same as that found in both animal and viral pre-mRNAs (Nevins, 1983). Comparison of the deduced protein sequences with others in the database indicated that p2A7 encoded a putative non-specific lipid transfer protein and was designated a *Brassica napus* lipid transfer protein, BNLTP (Fig. 3.6A).

Lipid transfer proteins were first defined by their ability to facilitate the transfer of phospholipids between membranes *in vitro* (Kader, 1975). However, an *in vivo* function has not been assigned. BNLTP was high in alanine residues (16%), and 10% of the residues were cysteines; it lacked tryptophan, histidine and glutamine residues which was similar to other LTPs (Østergaard *et al.*, 1995). A Kyte-Doolittle hydropathy profile of the protein sequence indicated that the first 25 amino acids were highly hydrophobic and had characteristics of a signal peptide sequence (von Heijne, 1988). Signal peptides are typically 15 to 30 amino acids in length and generally have three distinct regions, the first, n-region, is typically 1 to 20 amino acids in length with a net positive charge (von Heijne, 1988). The next region (h-region) is rich in apolar residues such as Phe, Ile, Leu, Met, Val and Trp and generally ranges from 7 to 16 amino acids in length (von Heijne, 1988). The final c-region which is close to the cleavage site has a distinct pattern of amino acids. Nearly all cleavage sites follow the -3, -1 rule where the -1 and -3 regions are small uncharged amino acids and the -2 region is characterised by a large, bulky, charged amino acid. The putative BNLTP signal sequence conformed to this rule; the -3, -2 and -1 residues were Ser-Asn-Ala, respectively. Furthermore, the protein lacked the carboxy-terminal K/HDEL sequence associated with proteins retained in the lumen of the endoplasmic reticulum (Munroe and Pelham, 1987). The hydropathy profile of BNLTP was similar to the carrot LTP, EP2 (Sterk *et al.*, 1991), *Arabidopsis* LTP1 (Thoma *et al.*, 1995) and broccoli LTP (Pyee and Kolattukudy, 1995) all of which are located extracellularly (Fig. 3.6A).

There is good evidence that LTPs are expressed as high molecular weight precursors with a signal peptide sequence at the N-terminus which participates in co-translational membrane transport. For example, Bernhard *et al.* (1991) showed that a spinach (*Spinacia oleracea*) LTP cDNA when translated *in vitro* in pancreatic microsomes or microsomes from cultured maize endosperm cells, produced a preprotein which was cotranslationally inserted into microsomal membranes. This protein also lacked the K/HDEL ER retention signal.

Sequence alignment of BNLTP to other LTPs confirmed the presence of eight conserved cysteine residues at positions 29, 39, 54, 55, 75, 77, 100 and 114 (Fig. 3.6A). All LTPs isolated to date contain eight conserved cysteine residues. NMR and X-ray crystallography studies have shown the cysteines to form four disulphide bonds in wheat (Dèsormeaux *et al.*, 1992). Furthermore, the proteins are thought to comprise a compact domain with four alpha-helices and a long COOH-terminus. This structure leads to a tunnel-like hydrophobic cavity which is thought to bind one of the two fatty acid acyl chains (see section 1.4.3.2). Dèsormeaux *et. al.* (1992) suggested that the central part of the wheat LTP protein from Ala-50 to Leu-65 is involved in binding of phospholipids. They hypothesised that Arg-47 and Lys-55 which are conserved in the sequences are possible candidates for interaction with the phosphate negative charges. However, in the BNLTP protein, after the signal peptide is cleaved the Lys-55 is substituted with a Val-55 (Fig. 3.6A). Whether this suggests an alternative function for BNLTP remains to be seen. Interestingly, the distance between the conserved cysteines is almost identical to that in the COOH-terminus of cell wall hybrid-proline-rich proteins (PRPs) from *Brassica napus* BNPRP; Goodwin *et al.* (1995) and *M. sativa* MsPRP; Deutch and Winicov, (1995) (Fig. 3.6B). This region of PRPs is thought to form three plasma membrane spanning domains where some of the cysteine residues are exposed for electrostatic bonding with other cell wall proteins. Whether this has a functional significance in LTPs remains to be seen.

UWGCG BESTFIT and GAP analysis of BNLTP indicated that it was highly similar (97.5%) to the broccoli WAX9D LTP (Pyee and Kolattukudy, 1995). Moreover, it was 100% identical to the AB/LTP II after the putative signal peptide was removed (Østergaard et al., 1995). It was also highly similar to three germination-specific LTPs, *gsltp1*, *gsltp2* and *gsltp3* (91%, 93% and 92%, respectively) isolated

from *Brassica napus* cv. Darmar which suggests that BNLTP has a similar function to these proteins. It was postulated that WAX9D, which is the major surface wax protein in broccoli possibly functions in the transport of wax precursors to the surface (Pyee and Kolattukudy, 1994, 1995; Sterk *et al.*, 1991). It is very likely BNLTP is located in the surface wax of *Brassica napus* leaves and may also be involved in the biosynthesis of the waxy layer.

A further two cDNA clones, p2A4 and p5A9, were sequenced partially from the 3'-termini. Comparison of the 3' untranslated regions of the three clones suggested that they were closely related, with sequence identities of 93% and 95% between BnLTP and p2A4 and p5A9, respectively (Fig. 3.9). These results demonstrated that three closely related cDNAs, which probably represented members of a small multigene family, were isolated. Indeed, previous Southern blot analysis of *B. napus* genomic DNA indicated that pLF3A probably represented one of four or five members of a small multigene family (Pallas, 1992). Furthermore, *B. napus* is an allotetraploid, which means that the genome may contain more copies, perhaps up to six, of LTP genes. Several LTPs isolated from different plant species are encoded by small multigene families (review, Kadar, 1996). For example, four closely related cDNAs encoding putative LTPs designated WAX9A, WAX9B, WAX9C and WAX9A were isolated from broccoli (*Brassica oleracea*) (Pyee and Kolattukudy, 1995) and four members of a lipid transfer protein gene family, *blt*4.1, *blt*4.2, *blt*4.6 and *blt*4.9 (Hughes *et al.*, 1992; White *et al.*, 1994) were isolated from the winter barley cv. Igris. Multigene families encoding lipid transfer proteins have also been reported in castor bean (*Ricinus communis* L.) (Tsuboi *et al.*, 1991) and *Arabidopsis thaliana* (Thoma *et al.*, 1994).

Comparison of the BnLTP and pLF3A DNA sequence also indicated two very closely related but not identical sequences (Fig.3.8). The putative coding regions were highly conserved with an amino acid sequence similarity of 99%. BnLTP contained an internal *Hind* III site also present in λLF3. Restriction digestion of p2A7 generated two fragments, the larger represented the 5' end of the clone and probably corresponded to pLF3A (Fig. 3.5). However, the 5' untranslated regions diverged quite considerably, suggesting that pLF3A and BnLTP probably represented two separate members of the multigene family. Whether pLF3A represented a fourth

member was not possible to determine with certainty, as sufficient overlap between p2A4 and p5A9 with pLF3A sequences was not achieved.

Chapter 4 Isolation and characterisation of the genomic clone gBnLTP

4.1 Introduction

The identification of a *Brassica napus* multigene family which encoded a putative non-specific lipid transfer protein described in chapter three prompted the questions as to how the genes were regulated, where the translated products were located and what possible role they played *in vivo* during development. It is well recognised that plant genes contain promoter sequences at the 5' flanking region that contain information for correct transcriptional initiation as well as information that determines tissue- and organ- specificity and environmental regulation during growth and development. A number of transcription factors and their respective target sequences that control various aspects of plant gene expression during growth and development have been identified (Ramachandran *et al.*, 1994). Downstream of the promoter is the coding region which is often composed of exons interrupted by non-coding regions or introns followed by a non-coding 3' region that contains signals for polyadenylation and transcript stability (Green, 1993). By identifying at least some of these features it may be possible to ascertain how a gene is regulated.

This chapter describes the isolation and characterisation of a genomic clone homologous to the BnLTP cDNA.

4.2 Preliminary experiments

Previously, a *Brassica napus* cv. Bridger genomic library constructed in the replacement bacteriophage λ vector, EMBL-3 (Clontech, California, U.S.A.), was screened with pLF3A to isolate the corresponding genomic clone (J. Jardine and G. I. Jenkins, 1993, unpublished). The EMBL-3 vector consists of a central or "stuffer" fragment flanked by two arms which are essential for phage propagation. The left arm which is 20 kb in length encodes virion proteins and the right arm which is 8.8 kb in length contains the origin of replication, promoters and a number of essential genes. The "stuffer" fragment is flanked by polylinkers that are in reverse orientation (*Sal* I-

Bam HI-*Eco* RI---stuffer---*Eco* RI-*Bam* HI-*Sal* I). All three restriction sites can therefore be used for cloning foreign DNA ranging from 9 to 22 kb.

The genomic library was constructed by ligating partially digested *Sau* 3AI *Brassica napus* cv. Bridger genomic DNA into *Bam* HI digested vector arms. Although the digested fragments and the vector have compatible ends, the *Bam* HI site is recreated only 25% of the time. Digestion of the recombinant vector with *Bam* HI often yields partially digested, high molecular weight fragments which leads to difficulty in interpreting restriction digestion patterns on agarose gels. Also the *Eco* RI sites are destroyed as a result of cloning into the *Bam* HI site leaving only the *Sal* I site in the vector arm intact. The complexity of the library was approximately 3 x 10^6. It was plated at a density of 1 x 10^6, which was more than the necessary 3.48 x 10^5 to 3.79 x 10^5 recombinants required to represent 99% of all DNA sequences in the genome (Clarke and Carbon, 1976). Eight EMBL-3 genomic clones designated λ1.1A, λ1.7B, λ3.1A, λ3.1B, λ3.2A, λ3.2B, λ3.3A and λ15.3, were plaque purified (J. Jardine, 1993 unpublished data).

4.2.1 Preliminary sequence analysis of genomic clone 1.7B

Clone λ1.7B which previously hybridised strongly to pLF3A in Southern blots was selected for further analysis by sequencing (J. Jardine, 1993 unpublished data) of subclones generated by directed deletions using the Stratagene *Exo* II deletion kit. However, subsequent sequence comparison of each of the λ1.7B subclones with pLF3A and BnLTP did not reveal sufficient homology. It was therefore concluded that λ1.7B either did not contain the corresponding gene or that the deletion series did not represent the entire clone. Instead of generating additional deletion subclones, it was decided that the eight genomic clones should be rescreened with the BnLTP cDNA so that a restriction fragment could be subcloned and analysed.

4.3 Rescreening of the 8 genomic clones with BnLTP

It was not necessary to amplify the clones as most amplified phage libraries could maintain a relatively high titre for a number of years. The clones were plated at 100-200 plaques per 8.8 cm Petri dish and duplicate plaque lifts were probed with the radiolabelled BnLTP cDNA clone. In the primary screen, around 40% of the plaques in

each case were positive. A second and final round of screening at a lower plaque density (approximately 50 plaques per 8.8 cm Petri dish) was carried out and plaque purity was achieved. Individual plaques were picked at random, placed into SM/CCl_3 buffer and allowed to diffuse overnight at 4°C. The resuspended phage was titred and plated at 10^5 p.f.u./ml to isolate phage DNA (described in section 2.23).

4.4 Restriction digestion and Southern blot analysis of the genomic clones

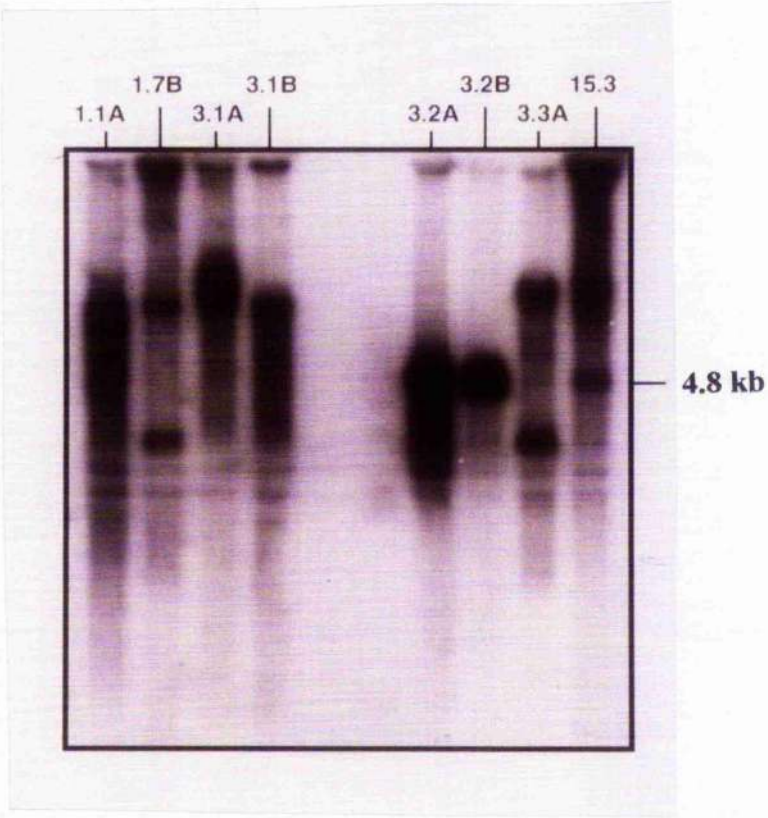
Ten micrograms of phage DNA isolated from each genomic clone was digested with *Eco* RI, *Bam* HI and *Sal* I. The fragments were resolved on a 0.8% (w/v) agarose gel along with DNA size markers (data not shown). As expected, *Bam* HI digestion yielded very large fragments that did not resolve efficiently in low percentage agarose and it was therefore difficult to estimate the size of the fragments (data not shown). However, *Sal* I digestion of each clone yielded three fragments ranging from approximately 10 to 30 kb. *Eco* RI digestion on the other hand proved to be the most informative enzyme as fragments ranging from 0.5 kb to 30 kb were generated. The *Eco* RI restricted fragments were Southern blotted onto Hybond N+ and hybridised to the radiolabelled BnLTP cDNA probe (Fig. 4.1).

Clones λ1.7B and λ3.3A had an identical restriction pattern. A 2.8 kb *Eco* RI fragment and a larger fragment which co-migrated with the 20 kb lambda arm strongly hybridised to the probe as did clones λ3.2A and λ3.2B which generated a single *Eco* RI fragment of 4.8 kb which hybridised strongly to the BnLTP cDNA probe. Clone λ15.3 also yielded a 4.8 kb *Eco* RI restriction fragment but it hybridised at a much lower intensity. Another band which co-migrated in the 20-30 kb range was also observed which probably represented an incompletely digested fragment from which the 4.8 kb fragment was derived. Conversely, clones λ3.2A and λ3.2B were digested to completion and so did not contain the larger 20-30 kb fragments. Clones λ1.1A, λ3.1A and λ3.1B were also partially digested and yielded a band in the 20-30 kb region, which hybridised strongly to the cDNA probe.

Fig. 4.1 Southern blot analysis of *Eco* RI digested genomic clones

DNA was isolated from the eight plaque-purified genomic clones and 10 μg was digested with *Eco* RI. The restriction fragments were resolved on a 0.8% (w/v) TAE agarose gel, Southern blotted onto Hybond N+ and hybridised to ^{32}P-radioactively labelled *BnLTP*. The blots were hybridised and washed as described previously in sections 2.19.3 and 2.19.4.

The lanes containing the clones, λ1.1A, λ1.7B, λ3.1A, λ3.1B, λ3.2A, λ3.2B, λ3.3A and λ15.3 are labelled accordingly. The 4.8 kb *Eco* RI restriction fragment which hybridised to the cDNA probe is indicated.


1.1A
1.7B
3.1A
3.1B
3.2A
3.2B
3.3A
15.3
4.8 kb

4.5 PCR of genomic clones using primers 1689/1919

To determine whether the genomic clones contained the full length or part of the cDNA sequence, PCR reactions were performed with primers designed from the BnLTP DNA sequence (shown in Fig.3.4). The upstream primer (forward), 1689, is located in the 5' coding region (nucleotides 97 to 115) and the downstream (reverse) primer, 1919, is located in 3' non-coding region (nucleotides 444 to 455) of the cDNA. Positive controls were also set up: one containing the BnLTP cDNA template cloned into pBluescript® II SK (-) (p2A7) along with primer pairs 1919/1689 and T3/T7. The expected PCR products of the latter two reactions were 359 bp and 769 bp, respectively. The T3/T7 amplified fragment included an extra 138 bp of the vector sequence where the primers hybridised 84 bp and 42 bp upstream of the *Eco* RI and *Xho* I sites, respectively. The PCR conditions were denaturation at 95°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 3 min, cycled 30 times. A further extension at 72°C for 7.5 min was included. The amplified products were resolved on a 1.2% (w/v) agarose gel along with 1 kb molecular weight markers (Fig 4.2).

Two genomic clones, λ3.2A and λ15.3 were positive. Both amplified a single fragment of 650 bp. As expected the 359 bp and 769 bp fragments were amplified in reactions containing the BnLTP cDNA template. The intensity of the 359 bp fragment was relatively low compared to the 650 bp and 769 bp fragments possibly due to non-optimal PCR conditions. The amplification of a 650 bp fragment in the reactions containing the genomic clones λ3.2A and λ15.3 with primer pair 1689/1919 compared to the 359 bp fragment produced in the amplification of the cDNA clone suggested either non-specific annealing or a sequence insertion in the genomic clone of approximately 200 bp. The remaining six genomic clones, λ1.1A, λ1.7B, λ3.1A, λ3.1B, λ3.2B and λ3.3A were negative.

4.6 Restriction mapping of clone λ3.2A

Based on Southern blot and PCR analyses, λ3.2A was characterised further. A restriction map was generated based on restriction digestion with *Eco* RI, *Sal* I and *Bam* HI. Digests in each case were terminated at 1 min, 10 min, 30 min, 1 h and 2 h time points to generate an array of partial and complete digest fragments (Fig. 4.3A).

Fig. 4.2 Amplification of purified genomic clones with 1689 and 1919

Genomic clones isolated from the tertiary screen of a *Brassica napus* EMBL-3 genomic library with the *BnLTP* cDNA clone were subjected to PCR with primers 1689 and 1919 that hybridised to the cDNA clone at the 5'-translated and 3'-untranslated regions, respectively (shown in see Fig.3.3). The PCR conditions were: denaturation at 95°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 3 min, cycled 30 times. A further final extension at 72°C for 7.5 min was included. The amplified fragments were resolved on a 1.2% (w/v) TBE agarose gel along with 1 kb size markers. As positive controls the *BnLTP* cDNA was amplified with either primer pairs 1689/1919 or T3/T7 which amplified a 359 bp and 769 bp band, respectively.

Lane M	1 kb ladder
Lane 1	*BnLTP* cDNA T3/T7
Lane 2	*BnLTP* cDNA 1689/1919
Lane 3	1.7B
Lane 4	3.1A
Lane 5	3.1B
Lane 6	3.2A
Lane 7	3.2B
Lane 8	3.3B
Lane 9	15.3

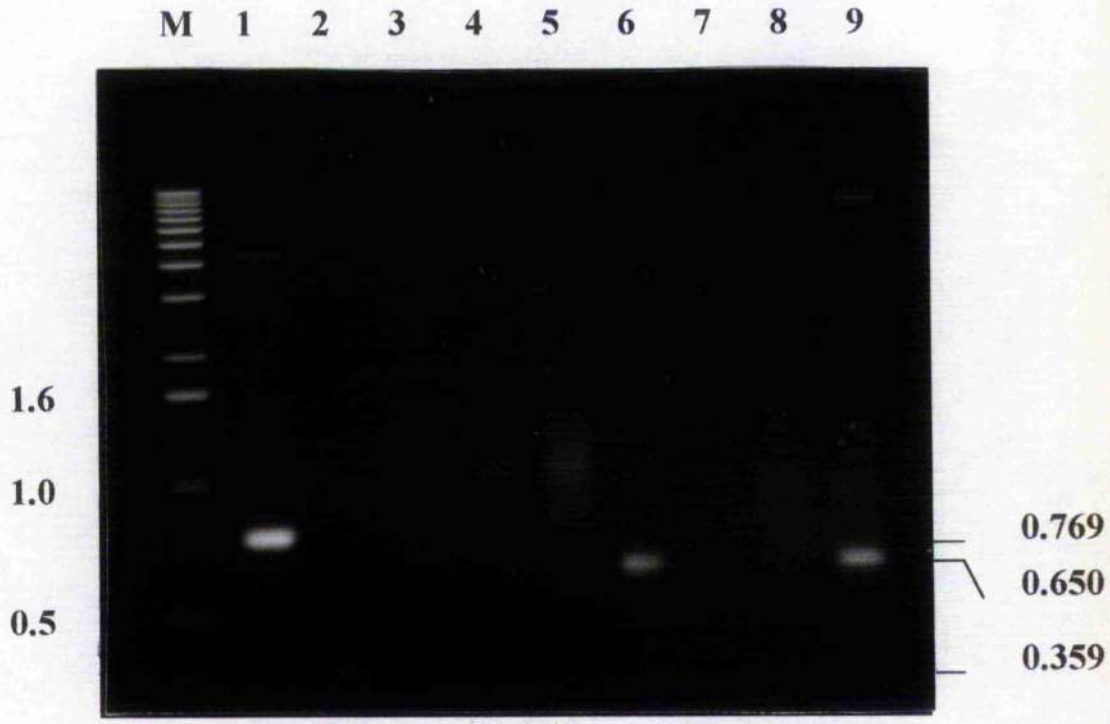
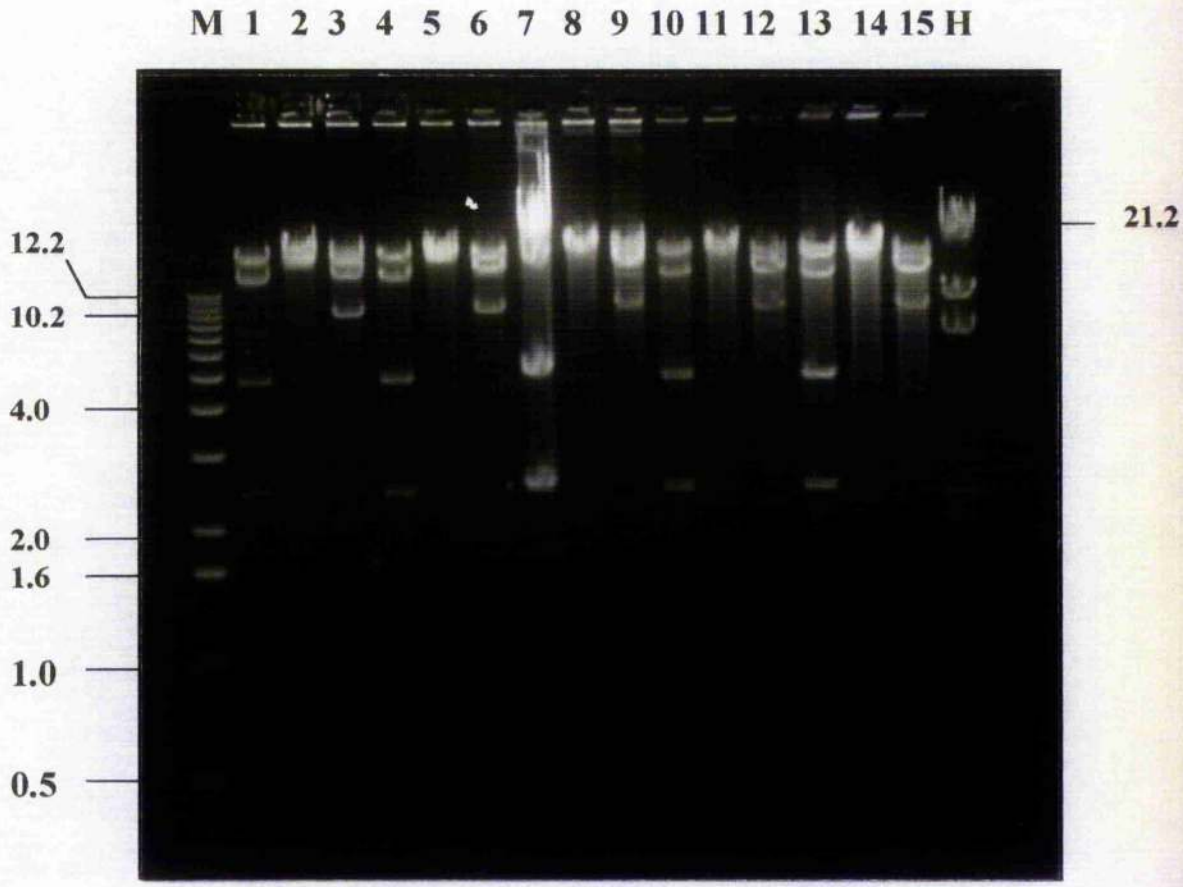

M 1 2 3 4 5 6 7 8 9
1.6
1.0
0.5
0.769
0.650
0.359

Fig. 4.3A Restriction digest analysis of genomic clone λ3.2A

5 μg of DNA isolated from genomic clone λ3.2A was digested with *Eco* RI, *Bam* HI and *Sal* I for 1 min, 10 min, 30 min, 1 h and 2 h.. The restriction fragments were resolved on a 0.8% (w/v) agarose gel along with DNA markers (lanes M and H).

Lane M	1 kb ladder
Lane 1	3.2A digested with *Eco* RI for 1 min
Lane 2	3.2A digested with *Bam* HI for 1 min
Lane 3	3.2A digested with *Sal* I for 1 min
Lane 4	3.2A digested with *Eco* RI for 10 min
Lane 5	3.2A digested with *Bam* HI for 10 min
Lane 6	3.2A digested with *Sal* I for 10 min
Lane 7	3.2A digested with *Eco* RI for 30 min
Lane 8	3.2A digested with *Bam* HI for 30 min
Lane 9	3.2A digested with *Sal* I for 30 min
Lane 10	3.2A digested with *Eco* RI for 1 h
Lane 11	3.2A digested with *Bam* HI for 1 h
Lane 12	3.2A digested with *Sal* I for 1 h
Lane 13	3.2A digested with *Eco* RI for 2 h
Lane 14	3.2A digested with *Bam* HI for 2 h
Lane 15	3.2A digested with *Sal* I for 2 h
Lane H	Lamda *Hind* III markers


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 H
12.2
10.2
4.0
2.0
1.6
1.0
0.5
21.2

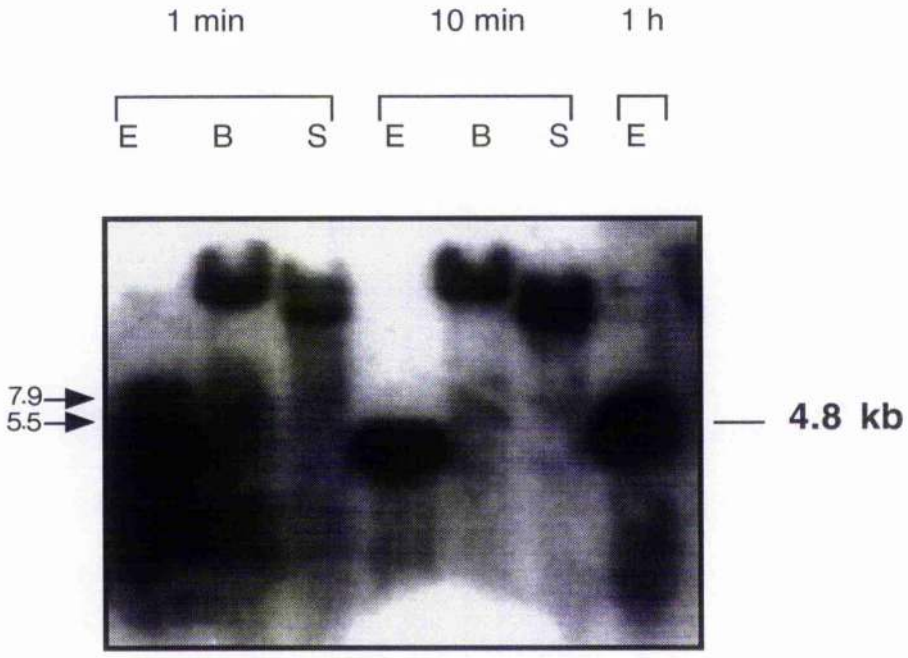
The fragments were resolved on a 0.8% (w/v) agarose gel, blotted onto Hybond N+ and probed with the BnLTP cDNA (Fig. 4.3B). Only a Southern blot of clones digested for 1 min, 10 min and 1 h are shown as the 2 h and 16 h digestion fragments were the same as those following 10 min and 1 h digestion.

Bam HI digests were extremely inefficient. Only one fragment of around 20-30 kb was produced which hybridised to the cDNA probe. *Sal* I partial and complete digestion products consisted of three very large fragments (Fig. 4.3A, lanes 3, 6, 9, 12 and 15) whose exact size could not be discerned. However, the largest fragment of 20-30 kb, which most probably corresponded to the left arm of the EMBL-3 vector and the incompletely digested clone, migrated the slowest. As expected only the incompletely digested fragment hybridised to the BnLTP cDNA probe (Fig. 4.3B, lane S, upper band). Below that a 14-15 kb fragment which most probably corresponded to the insert, as confirmed by hybridisation to the cDNA probe, was generated (Fig. 4.3B, lane S, lower band). A smaller fragment of around 10 kb which probably represented the vector right arm did not hybridise to the BnLTP cDNA (Fig. 4.3B, lane S).

Eco RI partial digestion produced seven restriction fragments, 20 kb, 16 kb, 7.9 kb, 5.5 kb, 4.8 kb, 2.4 kb and 0.7 kb (Fig. 4.3A, lane 1). Of the seven restriction fragments produced, only the 7.9 kb, 5.5 kb and 4.8 kb fragments hybridised to the cDNA probe (Fig. 4.3B, lane E, 1 min). After complete digestion with *Eco* RI (Fig. 4.3A, lanes 4, 7, 10 and 13) the 7.9 kb and 5.5 kb fragments disappeared and five fragments of 0.7 kb, 2.4 kb, 4.8 kb, 16 kb and 20 kb were produced of which only the 4.8 kb fragment hybridised to the BnLTP cDNA (Fig. 4.3B, lane E, 10 min and 1 h digestion). These results indicated that the 4.8 kb fragment was derived from the 5.5 kb fragment which itself was derived from the 7.9 kb fragment. The 20 kb fragment most probably corresponded to the vector left arm. However, a fragment corresponding to the 8.8 kb right arm was not produced. This was not surprising as the *Eco* RI restriction sites in the polylinker were lost following ligation of the *Sau* 3AI digested fragments into the *Bam* HI site leaving only the *Sal* I and *Bam* HI sites. The 16 kb band most probably corresponed to the lambda right arm plus an additional 7.2 kb of the insert suggesting one of the *Eco* R1 sites was at least 7 kb from the 8.8 kb right arm. The presence of five *Eco* RI fragments suggested that the insert contained at least four *Eco* RI sites, one of which was located very close to the 20 kb left arm.

Fig. 4.3B Southern blot analysis of clone λ3.2A

5 μg of DNA isolated from clone λ3.2A was digested with *Eco* RI (E), *Bam* HI (B) or *Sal* I (S) for the appropriate time period, resolved on a 0.8% (w/v) agarose gel and blotted onto nylon filters. The fragments were hybridised to ^{32}P-radioactively labelled *BnLTP* cDNA. The blots were hybridised and washed as described previously in sections 2.19.3 and 2.19.4. The partially digested 7.9 kb and 5.5 kb *Eco* RI fragments are indicated. The 4.8 kb *Eco* RI fragment was subcloned into pBluescript® II SK (-) (see Fig. 4.6).


1 min
10 min
1 h
E
B
S
E
B
S
E
7.9
5.5
4.8 kb

Double digests of λ3.2A with *Eco* RI and *Sal* I produced seven restriction fragments of 20-30 kb, 8.8 kb, 5.2 kb, 4.8 kb, 2.5 kb, 2 kb and 0.7 kb (Fig.4.4). Three fragments, 4.8 kb, 2.5 kb and 0.7 kb were also present in the single *Eco* RI digests. The 0.7 kb fragment was not visible on the gel. However, *Sal* I digestion of the 16 kb fragment which was produced in the *Eco* RI single digests, yielded three restriction fragments of 8.8 kb, which corresponded to the vector right arm, 5.2 kb and 2 kb. This suggested that the 16 kb fragment contained two internal *Sal* I sites. However, *Sal* I single digests did not produce the 2 kb fragment, probably due to incomplete digestion. The approximately 10 kb *Sal* I fragment described previously probably represented the 8.8 kb plus the 2 kb fragment. Several high molecular weight products in the 20 to 30 kb range most probably represented the lambda left arm plus the uncut clone as well as some partially digested fragments. Based on restriction digestion and Southern blot analyses, a restriction map of the genomic clone λ3.2A was determined as described in Fig. 4.5.

4.7 Subcloning of *Eco* RI fragments of genomic clone λ3.2A into pBluescript® II SK (-)

Clone λ3.2A digested to completion with *Eco* RI produced the restriction fragments shown in Fig.4.3A. After phenol/chloroform extraction, the restriction fragments were shot-gun cloned into *Eco* RI digested phagemid pBluescript® II SK (-). *E. coli* DH5α cells were transformed with the putative ligations and selected on LB/Amp plates containing X-gal and IPTG for β-galactosidase (blue/white colonies) selection. Putative transformants (white colonies) were grown overnight in L-Broth containing ampicillin and plasmid DNA was isolated. The subclones were digested with *Eco* RI and products of digestion were resolved on a 0.8% (w/v) agarose gel (Fig. 4.6).

Four colonies, 5, 6, 9 and 15 contained the desired 4.8 kb *Eco* RI fragment. These were designated subclones $p3.2A_5$, $p3.2A_6$, $p3.2A_9$ and $p3.2A_{15}$, respectively. Clone $p3.2A_9$ also contained an additional fragment of 8.8 kb which was probably due to contamination with the lambda right arm. Colonies 1, 3 and 11 contained an unaccounted for 3.9 kb fragment and colony 10 contained an unaccounted for 2 kb

Fig. 4.4 Restriction digestion of clone λ3.2A with *Eco* RI and *Sal* I

Clone λ3.2A was double digested with *Eco* RI and *Sal* I. The digests were terminated after 30 s (lane 1), 1 min (lane 2), 5 min (lane 3) and 30 min (lane 4). The restriction fragments were resolved on a 0.8% (w/v) TAE agarose gel along with DNA size markers (lanes H and M).

Lane H	Lambda *Hind* III markers
Lane 1	3.2A digested with *Eco* RI and *Sal* I for 30 s
Lane 2	3.2A digested for 1 min
Lane 3	3.2A digested for 5 min
Lane 4	3.2A digested for 30 min
Lane M	1 kb ladder

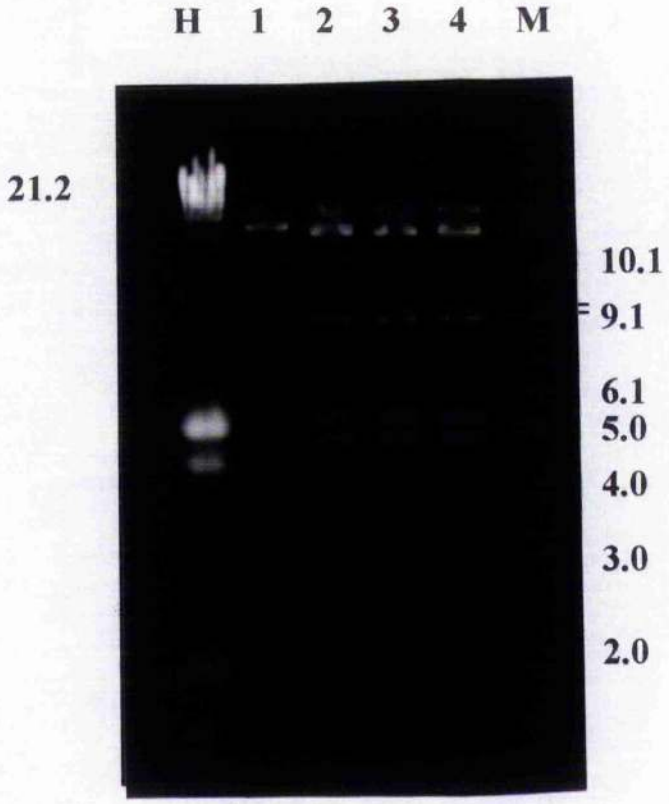

H
1
2
3
4
M
21.2
10.1
9.1
6.1
5.0
4.0
3.0
2.0

Fig. 4.5 Restriction map of genomic clone λ3.2A

Based on restriction digestion (Figs. 4.3A and 4.4) and Southern blot analyses (Figs. 4.1 and 4.3B), a restriction map of the clone was determined. The orientation of the insert within the EMBL-3 genomic clone was not known for certain, but the most likely one is shown. The 20 kb and 8.8 kb fragments represent the lambda left and right arms, respectively. The size of the fragment represented by the dark coloured region adjacent to the 20 kb left arm was not determined. The partial fragments, 5.5 kb, 7.9 kb and 16 kb are represented by the arrowed lines.

E and S represent the *Eco* RI and *Sal* I sites, respectively.

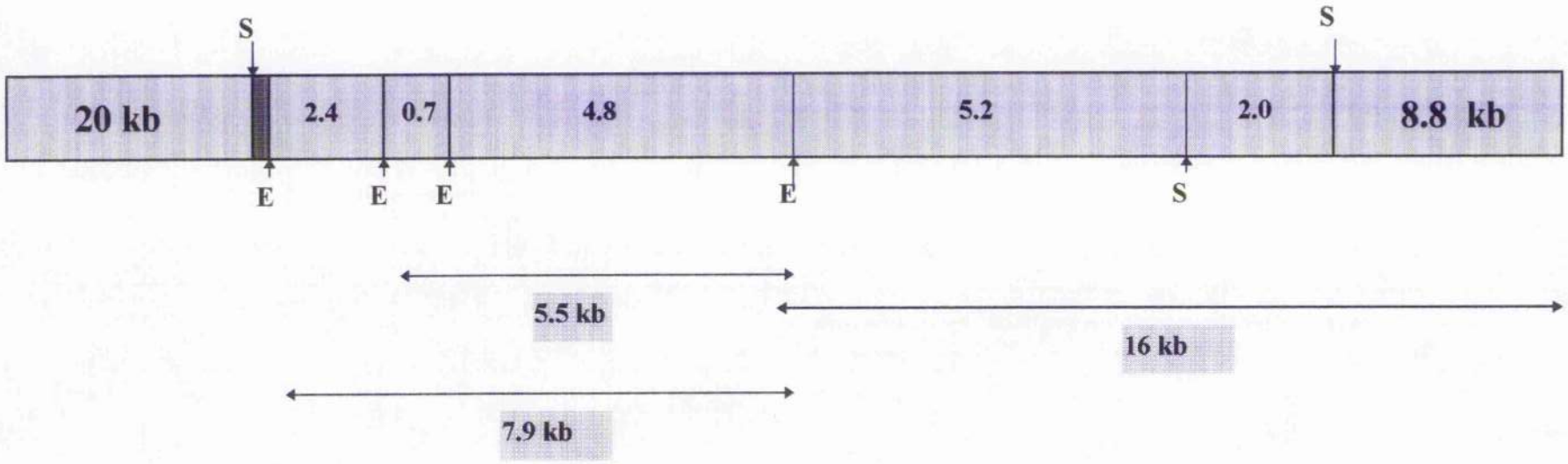
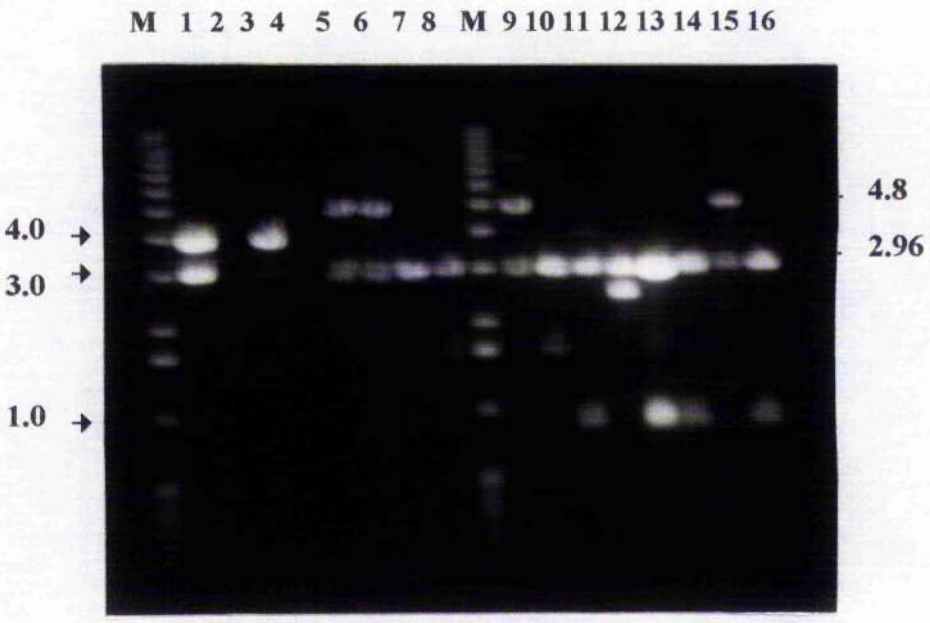

S
S
20 kb
2.4
0.7
4.8
5.2
2.0
8.8 kb
E
E
E
E
S
5.5 kb
16 kb
7.9 kb

Fig. 4.6 Subcloning of λ3.2A *Eco* RI-digested fragments into pBluescript® II SK (-)

Clone λ3.2A was digested with *Eco* RI for several hours. The resulting fragments were subcloned into the corresponding site in pBluescript® II SK (-). Plasmid DNA was isolated from several putative positive colonies and 1 μg of putative recombinant plasmid DNA was digested with *Eco* RI and resolved on a 0.8% (w/v) agarose gel along with DNA size markers (M).

Lane M	1 kb ladder	Lane M	1 kb ladder
Lane 1	Colony 1	Lane 9	Colony 9*
Lane 2	Colony 2	Lane 10	Colony 10
Lane 3	Colony 3	Lane 11	Colony 11
Lane 4	Colony 4	Lane 12	Colony 12
Lane 5	Colony 5*	Lane 13	Colony 13
Lane 6	Colony 6*	Lane 14	Colony 14
Lane 7	Colony 7	Lane 15	Colony 15*
Lane 8	Colony 8	Lane 16	Colony 16

The 2.96 kb band represents the pBlusecript® II SK (-) plasmid. Four colonies labelled with (*) contained the 4.8 kb *Eco* RI fragment. These were designated p3.2A_5, p3.2A_6, p3.2A_9 and p3.2A_{15}.


M 1 2 3 4 5 6 7 8 M 9 10 11 12 13 14 15 16
4.0
3.0
1.0
4.8
2.96

fragment. Colonies 2 and 4 contained no DNA. Four subclones from colonies 11, 13, 14 and 16 contained the small 0.8 kb fragment whilst colony 12 contained the 2.4 kb fragment. The fragments were blotted onto Hybond N+ and hybridised to the BnLTP cDNA (data not shown). As expected only subclones from colonies 5, 6, 9 and 15 hybridised to the BnLTP cDNA probe.

4.8 Analysis of p3.2A$_{15}$

4.8.1 Restriction digestion and PCR

Subclone p3.2A$_{15}$ was digested with several restriction enzymes and the products were resolved on a 1% (w/v) agarose gel (Fig. 4.7). Single digests with *Eco* RI (E), *Hind* III (H), *Sal* I (S) and *Xho* I (X), and double digests with E/H, E/S, E/X, H/S and H/X and S/X were carried out. The clone contained an internal *Hind* III site. Three fragments of approximately 2.3 kb, 3 kb and 3.2 kb were produced with E/H digestion whereas *Sal* I and *Xho* I digestion produced a single fragment of approximately 8 kb. As large molecular weight fragment sizes are only approximations from agarose gels, the 2.3 kb and 3 kb (total 5.3 kb) fragments most probably represented the previously estimated 4.8 kb *Eco* RI fragment from the λ3.2A genomic clone.

In order to estimate the approximate sizes of the putative 5' and 3' untranslated regions of the clone, PCR with primer pairs T3/1919 and T7/1919 were set up as T3 and T7 flanked the 4.8 kb fragment cloned in pBluescript® II SK (-) and primer 1919 was orientated towards the 5' untranslated region in the BnLTP cDNA (described in Fig. 3.4). The PCR products were resolved on a 1% (w/v) agarose gel along with 1 kb molecular weight markers (data not shown). The T3/1919 primers amplified a fragment of approximately 3 kb whereas the T7/1919 reaction was negative. These results confirmed that the 5' untranslated region of the putative *BnLTP* gene was adjacent to the T3 region in pBluescript® II SK (-) and that it was approximately 2 kb in length. They also suggested that the 3' untranslated region was around 2-3 kb. Both these observations confirmed that the entire gene was present within this 4.8 kb insert and that it was located approximately in the middle of the clone.

Fig. 4.7 Restriction digestion analysis of clone p3.2A$_{15}$

Subclone p3.2A$_{15}$ was digested with several restriction enzymes and the products were resolved on a 1% (w/v) agarose gel along with DNA markers (Lane M). Single digests with *Eco* RI (E), *Hind* III (H), *Sal* I (S) and *Xho* I (X), and double digests with E/H, E/S, E/X, H/S and H/X and S/X were carried out.

Lane 1 *Eco* RI

Lane 2 *Hind* III

Lane 3 *Sal* I

Lane 4 *Xho* I

Lane 5 *Eco* RI/*Hind* III

Lane 6 *Eco* RI/*Sal* I

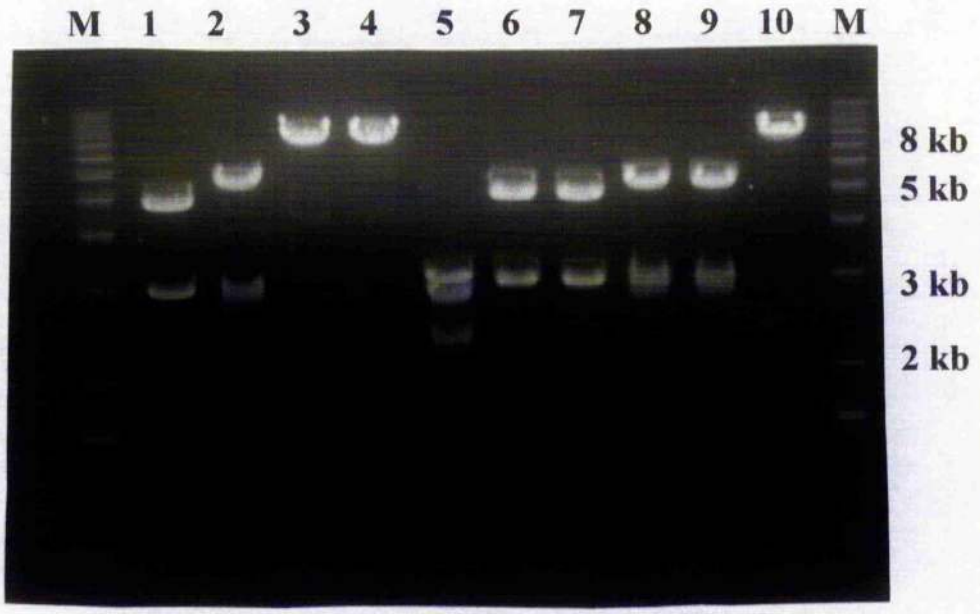
Lane 7 *Eco* RI/*Xho* I

Lane 8 *Hind* III/*Sal* I

Lane 9 *Hind* III/*Xho* I

Lane 10 *Sal* I/*Xho* I

Lane M 1 kb ladder


M 1 2 3 4 5 6 7 8 9 10 M
8 kb
5 kb
3 kb
2 kb

Clone p3.2A$_{15}$ amplified a fragment of 650 bp with the primer pair 1689/1919 as described previously (Fig. 4.2) (data not shown). A positive control containing the BnLTP cDNA clone was also set up with primer pairs 1689/1919 and T3/T7. The resulting amplified fragments were the same size as those produced previously with λ3.2A (section 4.5 and Fig. 4.2).

4.8.2 Sequencing of genomic clone p3.2A$_{15}$

Clone p3.2A$_{15}$ was sequenced using the Sanger dideoxynucleotide chain termination method (Sanger, 1977). Primers 1919 and 1689 were used in the initial sequencing reactions. New synthetic oligonucleotides were then designed from the newly derived sequence. Primers were designed, depending on the available sequence, at least 50 bp from the end of the previously sequenced region to ensure no gaps were present. The primers, their sequence and the region to which they hybridised are shown in Table 4.1. A schematic diagram showing the relative positions and direction of the primers used for each sequencing reaction is shown in Fig. 4.8.

4.8.3 Analysis of the *BnLTP* gene sequence

Clone p3.2A$_{15}$ contained the full length gene with high sequence similarity to the BnLTP cDNA. 1,750 bp of the 4.8 kb insert was sequenced which included 905 bp of the 5' non-coding region from the putative translation initiation codon, ATG. Similarly, 269 bp of the 3' non-coding region was sequenced. The nucleotide and the deduced amino acid sequences are shown in Fig. 4.9. The gene, designated *BnLTP* contained one open reading frame encoding a polypeptide of 118 amino. The intron was integrated after the second base of codon 116. The intron was 72% AT-rich which was within the 42 to 89% range found in most dicots (Simpson *et al.*, 1993). The AT-richness has been shown to be required for intron processing (Goodall *et al.*, 1989). Also, the GT-AG rule applied to the intron boundaries (Padgett *et al.*, 1986). The transcription initiation site was determined by primer extension analysis (see section 4.8.4 and Fig. 4.10). Two putative transcription initiation sites were located 56 and 59 bp, respectively, upstream of the ATG start codon. Comparison of the 5' untranslated

Table 4.1 Primers used for sequencing and PCR of genomic clones

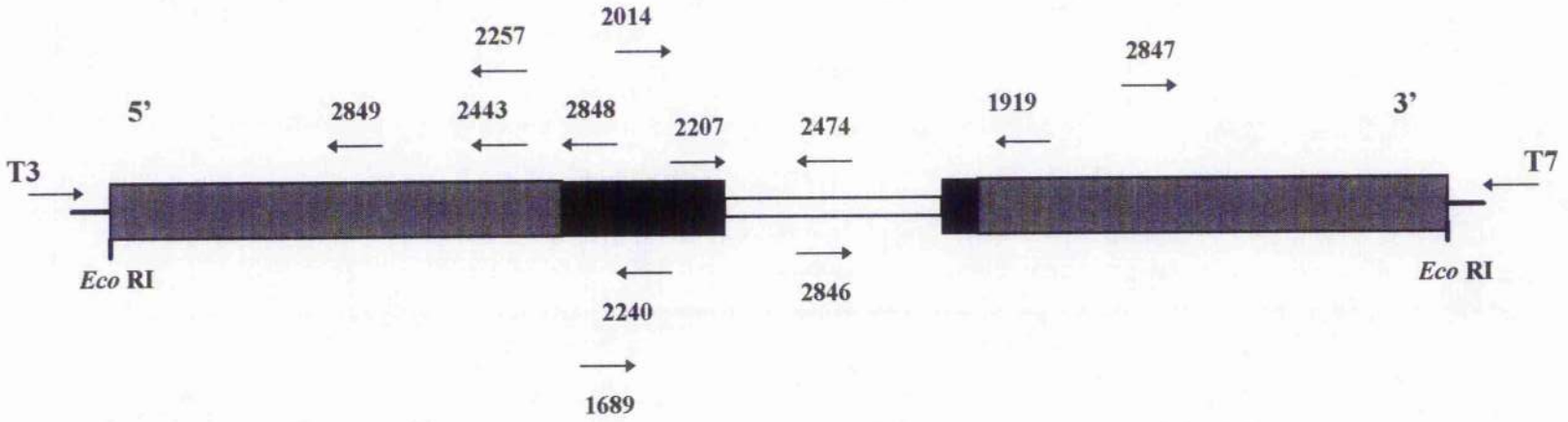
The primer sequences are shown in the 5' to 3' orientation. The target templates are indicated as well as the region of hybridisation. Primers marked with a (*) were also used to sequence the *BnLTP* cDNA.

Primer	Sequence 5'-3'	Template	Nucleotide
T3*	*AATTAACCCTCACTAAAGGG*	pBluescript®	
T7*	*GTAATACGACTCACTATAGGGC*	pBluescript®	
SK	*CGCTCTAGAACTAGTGGATC*	pBluescript®	
1919*	*ACGTCTGCATTA*	g*BnLTP*	704-715
1689*	*TGGTCTTGGCCTGCATGAT*	g*BnLTP*	79-106
2014*	*GCGGCGTTACTAGTCTAAAC*	g*BnLTP*	226-244
2207*	*GTGCAGCTGGACTTCCTAAG*	g*BnLTP*	334-353
2240*	*GTTTAGACTAGTAACGCCGC*	g*BnLTP*	226-244
2443	*GTGATGAAGAGAGTAGGG*	g*BnLTP*	-13 - +51
2474	*GGATAATACCGGTC*	g*BnLTP*	578-591
2846	*GTTTAACCATATCCCGATGG*	g*BnLTP*	560-579
2847	*GTATTGGATGAGCGAATAC*	g*BnLTP*	722-740
2848	*CAAGCATGCCAACTTCATTAGACCAGCC*	g*BnLTP*	62-88
2849	*CTACAACCTACGTTCTTG*	g*BnLTP*	-185--168

Fig. 4.8 Schematic diagram depicting the strategy used to sequence the genomic clone p3. $2A_{15}$

A 4.8 kb *Eco* RI fragment from the genomic clone λ3.2A was subcloned into pBluescript® II SK (-) (clone $p3.2A_{15}$) and sequenced. The *Eco* RI restriction sites which flank the T3 and T7 primers on the pBluescript® II SK (-) vector are shown. The black rectangles represent the exons separated by the intron (narrow white rectangle) and the 5' and 3' flanking regions are represented by the grey rectangles. The arrows represent the synthetic oligonucleotide primers used to sequence the clone as well as the direction of sequencing. The primer sequences and targets to which they hybridise are described in Table 4.1.

The diagram is not drawn to scale.


2257
2014
2847
5'
2849
2443
2848
2207
2474
1919
3'
T3
T7
Eco RI
Eco RI
2240
2846
1689

4.8 kb

Fig. 4.9 Nucleotide and deduced amino acid sequence of the *BnLTP* gene

The amino acids are shown below the putative codons. The coding region is composed of two exons, 116 and 2 amino acids, respectively, separated by a 269 bp intron. Exon 1 contains 115 complete codons and only the first two bases of codon 116. Exon 2 begins with the third base of codon 116. The asterisk indicates the translation stop codon. The putative TATA-box in the 5' flanking region is shown in bold at nucleotides -29 to -25 and three putative polyadenylation signals (AAATAA) in the 3' untranslated region at nucleotides +748 to +753, +903 to +908 and +920 to +924 are underlined. The +1 denotes the transcription start site at an adenine in the context TTCATCAC, 59 bp from the ATG of the deduced *BnLTP* mRNA. The arrow between the alanine residues indicates the putative cleavage site of the signal peptide. Amino acid differences between the BnLTP cDNA and the *BnLTP* gene are shown in bold. An internal *Hind* III restriction site, CAAGCTT is shown at nucleotides +376 to +382.

```
TTATTCTTCTGGGTTCGGACCCCCCGGCCATATGTTTTTAACCCAAGGAC  -797

CTGGACCCAAGCCGGGATAAGCCTTTTGGGGTTCACAGGGTATTCGAACT  -747

CGGGTCCGTATTGGGGTTTAATTTTTTAGACCACTAGCCCACCACACCGT  -697

AGTTAGTCATATATTGGACACACGTTGTGTTAATCAAATATCTTTTTTTT  -647

ACTAGTGATTATAAACACTAGTGTTAATGATTATAAACATCGGATGTGTT  -597

TCCAAAAAAAAGTGTTTCAAAAAAAATAATTATAAACATCGGCAGTTCCTA  -547

CAACTTAGTTGGAGTTTGTTTGGGAAAGACAATGATAAATATGAGAGTTT  -597

GAAACTCGTAGTGTTCTTTCTTGTTTTGTAAATTGAGAGTGAAACATTTT  -447

TTTTTGTACTCAAACACGTACATCTTGTAGTCTTAGACTGAACTGAGAGA  -397

GAGGAGCACTGCCGGTAATCAAGCTACGTCATGCATGATTCTAATTATAT  -347

AAAAGAGATTGAACATAATGTTAAGATTAAAATAGATAATTAATGATAGT  -297

TGTATGTACAATAACTATTTCTACCATCCATATCTGCATTTCCCGTTTTG  -247

AGTTTTCAGTATTGCATCTATTAAAATTAAAAGTCATTTTACATGATGCA  -197

ATAGCAAAAACAAGAACGTAGGTTGTAGAAATTTCAAACGAAGTAGACGT  -147

TCATGAGGTACTACATAATTAGCTAGGAACCTCTACATTAAATGAAACCA  -97

ACAACCAACCAACTACTCCTTTCCTCTAAATCGCATTCACATTCATAACA  -47
                                                  +1
TAAACTCTCTTTGTCTATATAAACACTCACTTCCCCTACTCTCTTCATCA  +4

CTCATCATTCCACACACATCTCATTTTGCAAACAAAAAAAATCTAAGAGA  54

GAAATATGGCTGGTCTAATGAAGTTGGCATGCTTGATCTTCGCCTGCATG  104
      M  A  G  L  M  K  L  A  C  L  I  F  A  C  M   15

ATCGTGGCCGGTCCAATCACATCGAACGCGGCTCTGAGTTGTGGCACCGT  154
 I  V  A  G  P  I  T  S  N  A  A  L  S  C  G  T  V   32
                         ↑
TAGCGGCTACGTGGCACCGTGCATTGGCTACCTGGCCCAGAATGCGCCGG  204
  S  G  Y  V  A  P  C  I  G  Y  L  A  Q  N  A  P     48

CCCTTCCCAGAGCGTGCTGCAGCGGCGTTACTAGTCTAAACAACCTGGCC  254
A  L  P  R  A  C  C  S  G  V  T  S  L  N  N  L  A   65
                Hind III
CGTACAACCCCAGACCGTCAGCAAGCTTGCCGTTGCCTTGTAGGAGCCGC  304
 R  T  T  P  D  R  Q  Q  A  C  R  C  L  V  G  A  A   82

TAACGCCTTGCCTACTATCAACGCTGCCCGTGCAGCTGGACTTCCTAAGG  354
  N  A  L  P  T  I  N  A  A  R  A  A  G  L  P  K    98

CATGTGGAGTCAACATTCCTTACAAGATCAGCAAAACCACCAACTGCAAC  404
A  C  G  V  N  I  P  Y  K  I  S  K  T  T  N  C  N   115

AGgtatatatgtcttaatctgtctctttctctctctgcggtttttttaaa  454
 S                                                   116
caagaagtagataatatgaagattatttttgttaaatgaaaacttgagtt  504

tttatattaacagtttagtgttttagtaaagactgtattaacgatttgaa  554
```

gttctgtttaaccatatcccgatggataataccggtcaacaagtatatac 604

taaaaaaatttttaatggagatttgagtattttattataacggtgttatg 654

tttatttgtatgtggttgcagTGTGAAATGAgcggcggtcggatgaagct 704
V K *
aatgcagacgttcaagtattatgtaatggatgagcgaatactaaaataag 754

atgctccaatggttgttgttttagagtttttcaatttcctgtcttttat 804

gttgtgacgttcctattactttggtcgtttgtactatgttcacaatcaac 854

gttatatgaatttcagatcataaaatatacgcatatgaatttttatttaa 904

taaatattctttcgaaataatatatatatatatatatatatatatatata 954

regions of a number of genes indicate that transcription initiates at an adenine in the context CTCATCAA (Joshi, 1988), which closely resembles the adenine located 59 bp from the ATG, in the sequence context TTCATCAC. This adenine was therefore designated as the transcription start site. A TATA-box (positions -29 to -25 relative to the transcription start site) was located in the 5' flanking region and three putative poly (A) signals, AAATAA (positions +748 to +753, +902 to +907 and +919 to +924) (Wahle *et al.*, 1992), were identified at the 3' untranslated region. An internal *Hind* III restriction site is shown at position +376 to +382. Several putative cis-acting regulatory elements were also identified in the promoter region. These are discussed in more detail in chapter six.

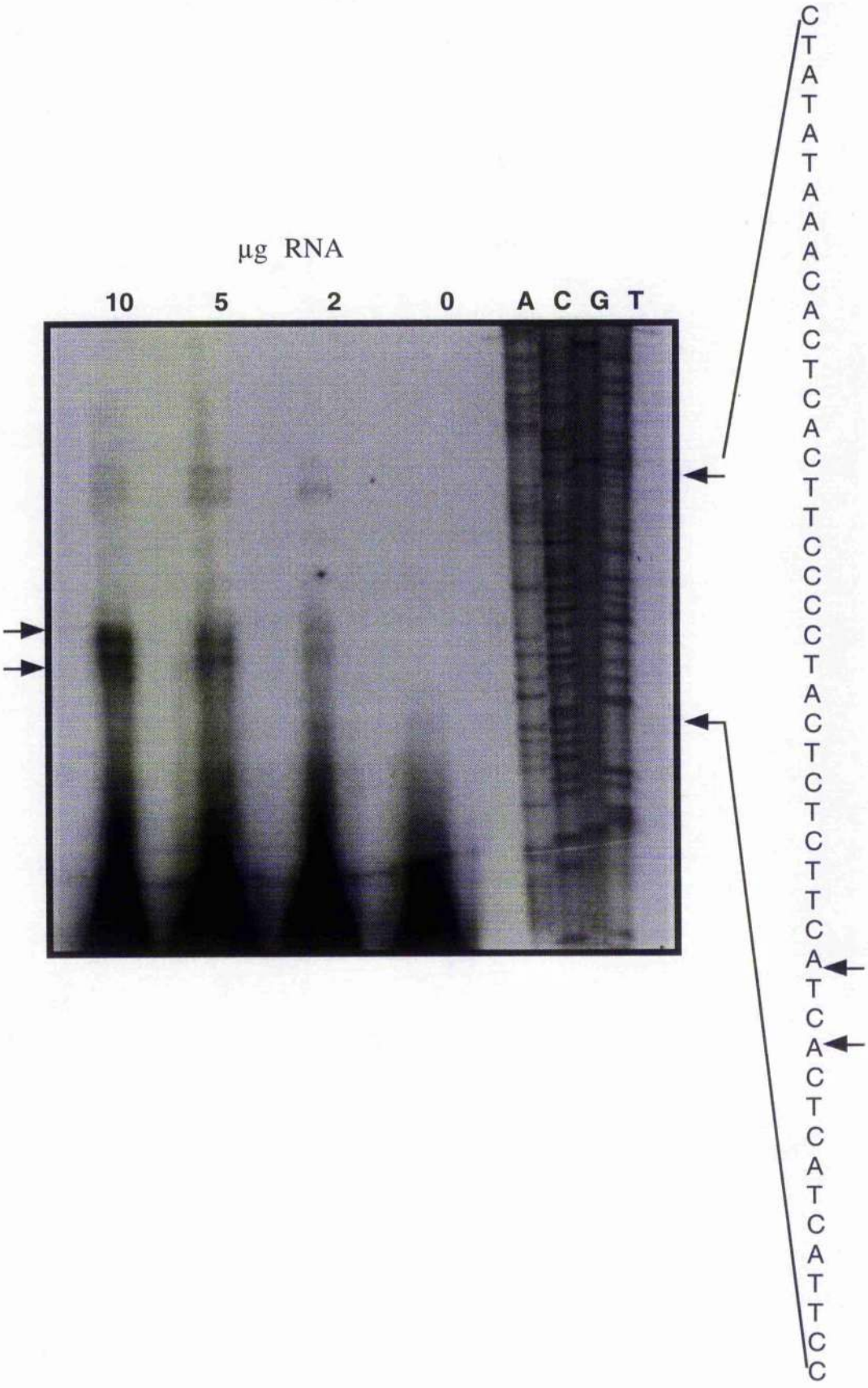
4.8.4 Determination of transcription initiation in the *BnLTP* gene by primer extension

The transcription initiation site of the *BnLTP* gene was determined by primer extension analysis (Fig. 4.10) (Ausubel *et al.*, 1987). A 27 bp synthetic oligonucleotide (2848, see Table 4.1 and Fig. 4.9) that corresponded to the putative translated region starting from the ATG codon (position 62) up to the tenth codon (position 88) was used in the primer extension reaction. Leaf tissue from *Brassica napus* cv. Cobra grown under continuous high intensity white light was used to isolate total RNA. The same primer was used to sequence the gBnLTP clone. The sequencing primer was not end-labelled with γ^{32}P-ATP prior to the sequencing reaction. Instead, ^{35}S-dATP was used in the sequencing extension reaction by using the standard sequencing protocol. This should have minimal effect on the mobility of fragments greater than 50 bp in length (Trapper and Clayton, 1981).

There were two major primer extension products both of equal intensity generated in the analysis of the *BnLTP* mRNA. The intensity of the bands increased as the corresponding mRNA concentration was increased. Two larger products which corresponded to a cytosine nucleotide, located above each of the smaller doublets, were also produced, but the intensity of these bands did not increase with increasing amounts of RNA eliminating these bands as possible transcription start sites. These bands, however, served as an internal control rendering the smaller bands as true initiation start sites. As expected, no primer extension product was observed in the "no

Fig. 4.10 Mapping of the transcription start site of the *BnLTP* mRNA by primer extension

A 27-nucleotide primer (2848: see Table 4.1) was annealed to increasing amounts (0, 2, 5 and 10 μg, respectively) of total RNA extracted from *Brassica napus* cv. Cobra leaf tissue and extended with reverse transcriptase. The products indicated by the arrows were resolved on an 8% (w/v) denaturing polyacrylamide sequencing gel. The same primer was used to sequence the *BnLTP* gene. The sequencing ladder was run adjacent to the primer extension products. The coding strand DNA sequence corresponding to the region flanking the extended product (position -35 to +11 in Fig. 4.9) is shown to the right of the ladder. The putative transcription start sites at adenine residues are indicated by an arrow.


µg RNA
10
5
2
0
A C G T
CTATATAAACACTCACTTCCCCCTACTCTCTTCATCACTCATCATTCC

RNA" control. The two primer extension products corresponded to adenine residues in the sequence context 5'-TTCATCA-3' located 56 and 59 nucleotides upstream of the ATG start codon. Comparison of this sequence to the transcription start sites of several plant genes closely resembled the CTCATCA consensus sequence (Joshi, 1987). Approximately 85% of genes studied initiated transcription from an adenine (bold) in this context. The adenine located 59 bases from the ATG was therefore considered as the start site and marked (+1) (Fig. 4.9). Taking into consideration the transcription start site and the first polyadenylation signal, the *BnLTP* mRNA was calculated to be 600 bases long, which is in agreement with the transcript size observed in previous northern blots (Pallas, 1992).

4.9 Comparison of the *BnLTP* gene and cDNA nucleotide and amino acid sequences

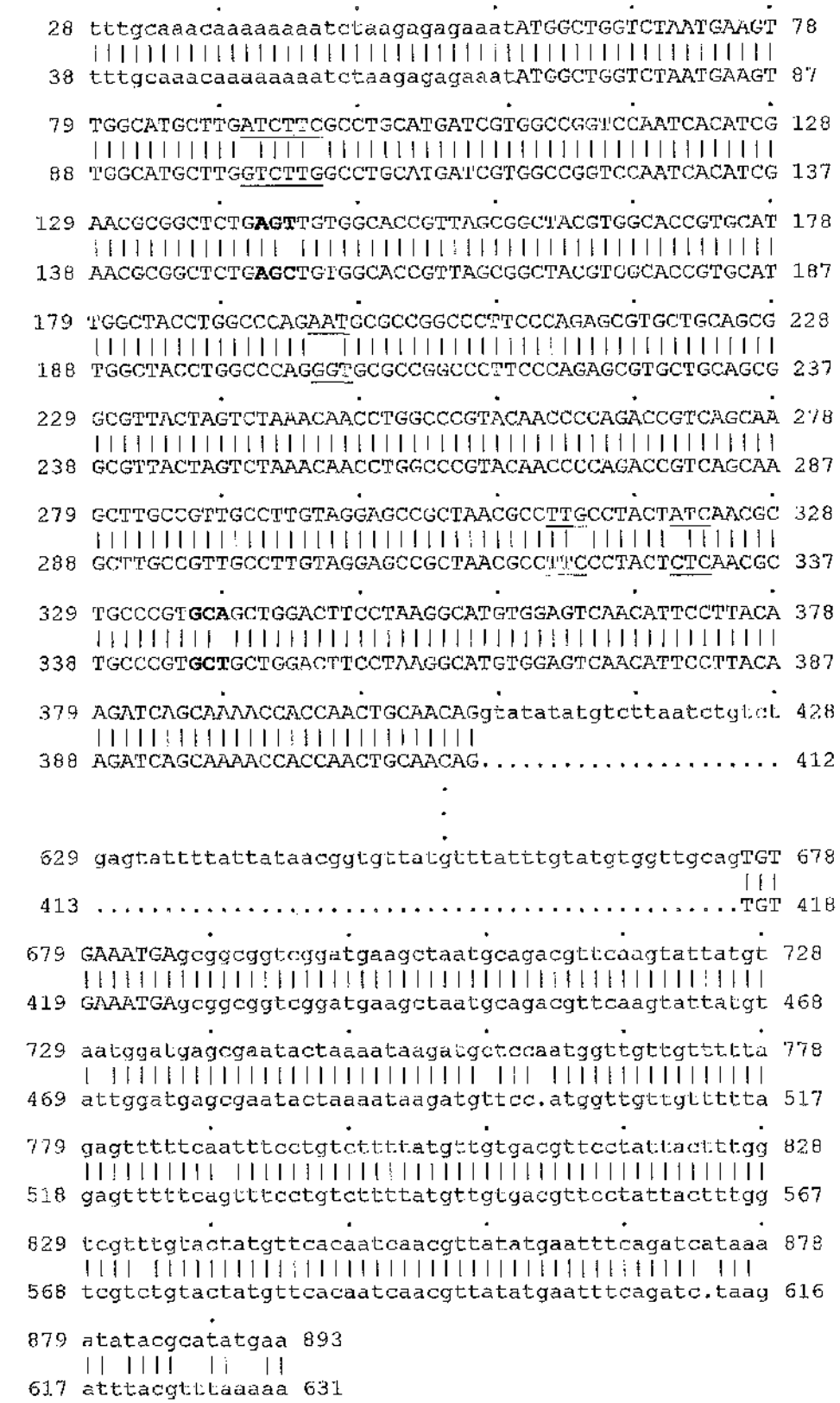
The nucleotide and amino acid sequences of gBnLTP were compared with those of BnLTP cDNA described in chapter three (Fig. 4.11A and Fig. 4.11B). The gene and cDNA both encoded proteins of 118 amino acids and were highly similar (98%) at the amino acid level. Five amino acid substitutions were observed at amino acids 11 (I to V), 12 (F to L), 46 (N to G), 85 (L to F) and 88 (I to L) (Fig. 4.11B). The putative protein sequence of *gBnLTP* had a high alanine content (comprising 16%) and 10% of the residues were cysteines, which was identical to BNLTP. Similarly, the deduced gBNLTP protein lacked glutamate, histidine and tryptophan residues. A Kyte and Doolittle (1982) hydropathy profile of the predicted amino acid sequence revealed a 25 amino acid hydrophobic region at the N-terminus with characteristics of a signal sequence (von Heijne, 1986) very similar to BNLTP. The potential cleavage site following an alanine residue at position 25 (Fig. 4.11B) was identical in both sequences. The gene contained three putative polyadenylation signals with the consensus AAATAA, 63, 218, and 234 bp downstream of the stop codon whereas the cDNA sequence contained a single polyadenylation signal 62 bp from the stop codon. However, it is unlikely that the first polyadenylation signal in the gene is used in preference to the other two based on the size of the transcript in northern blots. The 3' untranslated region also differed at a number of bases. These results confirmed that the *BnLTP* gene and cDNA sequences were not homologous but highly similar.

Fig. 4.11 Comparison of the *BnLTP* gene and cDNA nucleotide and amino acid sequences

The nucleotide (A) and amino acid (B) sequences of the *BnLTP* gene and cDNA were aligned using the UWGCG PILEUP program. The five codon differences between the two sequences are shown (underlined); codon 90-92, ATC (gene)→GTC (cDNA) (residue 11, I to V); codon 93-95, TTC (gene)→TTG (cDNA) (residue 12, F to L); codon 195-197, AAT (gene)→ GGT (cDNA) (residue 46, N to G), codon 321-323, TTG (gene)→ TTC (cDNA) (residue 85, L to F) and at codon 328-330, ATC (gene)→CTC (cDNA) (residue 88, I to L). Several nucleotide changes were observed in the 3' untranslated region. Two degenerate codons were also present at nucleotide 141-143, AGT (gene) to AGC (cDNA) (Ser) and at nucleotide 336-338 GCA (gene) to GCT (cDNA) (Ala).

A

***BnLTP* gene (top) x BnLTP cDNA**



B

BNLTP gene (top) x BNLTP cDNA

```
          .         .         .         .         .
  1 MAGIMKLACLIFACMIVAGPITSNAALSCGTVSGYVAPCIGYLAQNAPAL 50
    ||||||||||::|||||||||||||||||||||||||||||||||:||||
  1 MAGLMKLACLVLACMIVAGPITSNAALSCGTVSGYVAPCIGYLAQGAPAL 50
          .         .         .         .         .
 51 PRACCSGVTSLNNLARTTPDRQQACRCLVGAANALPTINAARAAGLPKAC 100
    ||||||||||||||||||||||||||||||||||:||:|||||||||||
 51 PRACCSGVTSLNNLARTTPDRQQACRCLVGAANAFPTLNAARAAGLPKAC 100
          .
101 GVNIPYKISKTTNCNSVK* 118
    ||||||||||||||||||
101 GVNIPYKISKTTNCNSVK* 118
```

However, analysis of a number of *LTP* gene sequences, including those members of the same multigene family (see for example Molina *et al.* (1996), has shown that non-coding regions have diverged considerably during evolution compared to coding regions.

4.10 Discussion

Eight *B. napus* EMBL-3 genomic clones designated λ1.1A, λ1.7B, λ3.1A, λ3.1B, λ3.2A, λ3.2B, λ3.3A and λ15.3, isolated from a previous screen with pLF3A were rescreened with the BnLTP cDNA. The clones were characterised further by a combination of restriction digestion, hybridisation to Southern blots and PCR.

Bam HI and *Sal* I digestions of all eight clones were extremely inefficient yielding very large molecular weight fragments that did not resolve well in agarose gels. These results suggested that the insert fragments did not contain any *Bam* HI and *Sal* I sites. The insert size in all eight clones ranged from 10 kb to 20 kb (data not shown).

However, *Eco* RI digestion produced fragments ranging from 0.5 kb to 30 kb and as the *Eco* RI restriction site was lost during the construction of the library, these results indicated that the restriction sites were all present in the insert fragment. *Eco* RI digested fragments of all eight clones hybridised strongly to the BnLTP cDNA (Fig. 4.1, section 4.4), indicating that the genomic clones were closely related to the BnLTP cDNA. However, PCR analysis of the clones with primers designed from the 5' untranslated (primer 1689) and 3' untranslated (primer 1919) regions of the BnLTP DNA indicated that only two clones, λ3.2A and λ15.3 were positive (Fig. 4.2), which suggested that these two clones were more similar to the cDNA than the other six clones. A larger than expected PCR product was amplified compared to the cDNA. This suggested that clones λ3.2A and λ15.3 contained an extra region between the primer annealing regions, compared to the cDNA and that they were identical. In agreement with the PCR data, it was observed that the two clones had an identical *Eco* RI restriction map (Fig. 4.1). However, clone λ3.2B, which also had the same *Eco* RI restriction pattern did not amplify a PCR fragment, which suggested that it may only differ in the nucleotides of the primer-annealing regions or that optimal PCR conditions were not achieved. It therefore appears that amongst the eight genomic clones, λ3.2A

and λ15.3 were identical and most closely related to the BnLTP cDNA and clone λ3.2B, compared to the remaining five clones.

Based on the above results, the 4.8 kb *Eco* RI fragment from clone λ3.2A was subcloned into pBluescript® II (SK (-) and sequenced. The clone contained the full length gene with high sequence similarity to the BnLTP cDNA (Fig. 4.11). The *BnLTP* gene contained a single open reading frame encoding a protein of 118 amino acids, which consisted of two exons, comprising 116 and 2 amino acids, respectively, interrupted by a 269 bp intron (Fig. 4.9). This was consistent with the PCR results (Fig. 4.2) where a larger than expected PCR fragment was amplified. The extra 269 bp corresponded to the intron region. Genes isolated from other species including *Arabidopsis* (Thoma *et al.*, 1994) tobacco (Fleming *et al.*, 1992) and broccoli (Pyee and Koluttukudy, 1995), all contain an intron in the region corresponding to the C-terminus of the protein, two codons from the stop signal. The intron is in the same location in the *BnLTP* gene. Whether the conservation of this gene structure has a functional significance remains to be elucidated. However, the intron length varies between different genes (Thoma *et al.*, 1994; Pyee and Koluttukudy, 1995; Fleming *et al.*, 1992).

There were only five amino acid differences between the deduced *BnLTP* gene and cDNA amino acid sequences (V to I at position 11, L to F, 12, G to N, 46, F to L, 85 and L to I, 88) (Fig. 4.11B). The putative polypeptide was high in alanine and cysteine residues, comprising 17% and 10%, respectively, and lacked histidine, tryptophan and glutamate residues. In addition, the eight cysteine residues were at conserved positions, identical to the BnLTP cDNA amino acid sequence.

The 4.8 kb subclone contained a 2.3 kb region 5' of the first ATG codon, 905 bp of which was sequenced. Primer extension analysis revealed the presence of two putative transcription start sites (Fig. 4.10), in the sequence context TTCATCAC. Comparison of the 5'untranslated regions of a number plant genes indicates that transcription initiates at an adenine in the sequence context CTCATCAA (Joshi, 1987), which closely resembles the adenine located in the sequence context, TTCATCA. This adenine was therefore designated the transcription start site (+1)

(Fig. 4.9). A TATA box at positions -29 to -25, relative to the transcription start site, was located in the 5' flanking region and three putative polyadenylation signals (AAATAA) were observed in the 3' untranslated region at nucleotides +748 to +753, +903 to +908 and +920 to +924.

A Kyte and Doolittle (1982) hydropathy profile of the predicted protein revealed a hydrophobic N-terminal region of 25 amino acids, which had the characteristics of a signal peptide sequence (von Heijne, 1986). In addition, the protein lacks an ER retention signal, K/HDEL (Vitale *et al.*, 1993), which indicates that the protein probably enters the secretory pathway. Both these characteristics were found in the deduced amino acid sequence of the BnLTP cDNA.

In summary, a gene encoding a putative lipid transfer protein was isolated, which was highly similar but not identical to the BnLTP cDNA. Although a number of characteristics were common to both proteins (discussed above), it is clear that the two represent different members of the *B. napus LTP* gene family. It is possible that members of the gene family are regulated differently.

Chapter 5 Generation and characterisation of *Arabidopsis* transgenic plants expressing the *BnLTP* promoter-*GUS* fusion

5.1 Introduction

To be able to use the *BnLTP* gene as a molecular marker for the study of cell fate it was necessary to develop a simple and quick method of determining the cellular location of the gene product. Although conventional methods such as northern blotting and *in situ* hybridisation are useful in determining temporal and spatial expression patterns of genes, they have some disadvantages. Total RNA isolated from an organ or tissue contains a myriad of transcripts expressed in each of the cell types which make up the organ or tissue such as epidermal, mesophyll and parenchyma cells, which results in difficulty in determining expression at the cellular level. *In situ* hybridisation requires gene-specific probes to distinguish between transcripts of closely related genes such as members of a multigene family. As described in chapter 3, *BnLTP* may represent a small multigenic family with several closely related genes. This would require sequencing the entire gene family and designing gene-specific probes in order to distinguish between different gene members. The method, although extremely informative, is very time consuming. Also, conventional histological techniques often distort the tissue and therefore it is difficult to distinguish between the different cell types.

The putative *BnLTP* promoter (described in chapter 3) was therefore used to drive the expression of an easily quantifiable reporter gene, namely the bacterial enzyme, β-glucuronidase (GUS) (Jefferson *et al.*, 1987). Transgenic host plants expressing the *BnLTP-GUS* transgene would enable the promoter activity to be characterised *in vivo*. As GUS activity is easily quantifiable by a simple and extremely sensitive fluorometric assay using MUG as a substrate, small changes in promoter activity under different environmental conditions could easily be detected. Moreover, it would serve as a molecular 'marker' to follow the spatial and temporal expression patterns of the *BnLTP* gene throughout development. The fate of those cells

expressing GUS could be visualised easily by histochemical analysis. On addition of the substrate X-Gluc, a blue precipitate is formed in cells expressing GUS. In this way the tissue specificity of the *BnLTP* promoter would be established. By correlating the expression patterns with the known sequence elements present in the promoter region we can begin to functionally define these elements in the promoter.

This chapter describes the generation and characterisation of transgenic *Arabidopsis thaliana* expressing the *BnLTP* promoter-*GUS* fusion.

5.2 Isolation of the putative promoter

The *BnLTP* promoter contains a well characterised and highly conserved TATA-box at position -29 (described in Fig. 4.9), which determines transcriptional initiation and in some cases tissue-specificity in a number of eukaryotic genes (Aso *et al.*, 1994). Also primer extension analysis of the gene revealed a putative transcription initiation site at an adenine residue located 59 bp from the first ATG of the coding region (see Fig. 4.9 and 4.10). The *BnLTP* promoter was isolated from the 4.8 kb *Eco* RI fragment cloned in pBluescript® II SK (-) (clone $p3.2A_{15}$) by PCR. The 5' non-coding region of the gene was approximately 2 to 3 kb based on PCR with T3 and 1919 primers described in section 4.8.1. The 5' end of the clone was adjacent to the T3 region of pBluescript® II SK (-) and was therefore partially sequenced with this primer. The newly derived sequence was used to design the upstream (forward) primer designated 2285, (5'gggttG TCG ACC CCA CTA ACT TCC GCG ATA ATC ATG-3'), designed so that it introduced a unique *Sal* I site (underlined) at the 5' end of the amplified fragment. Previous restriction digestion analysis of the clone indicated that the 4.8 kb fragment did not possess *Sal* I and *Bam* HI sites. The downstream (reverse) primer, 2257 (5' gggccGG ATC CTG TTT GCA AAA ATG AGA TGT GTG TGG 3') was designed from the 5' untranslated region 21 bp from the ATG start site at positions +14 to +38 (see gene sequence, Fig.4.9). A unique *Bam* HI site (underlined) adjacent to the GC-overhang was introduced at the 3' end of the PCR-amplified fragment. The gggtt and gggcc overhangs adjacent to the *Sal* I and *Bam* HI sites, respectively, were included as many restriction enzymes do not cut restriction sites that are present at the end of a PCR product, but will cut when a few extra bases are added

(Kaufman and Evans, 1990). A PCR reaction was set up with primers 2285 and 2257 and plasmid $p3.2A_{15}$ as the template. The putative promoter region was amplified by PCR with the *Pfu* DNA Polymerase (Stratagene, Lundberg *et al.*, 1991). The PCR product containing the putative promoter was approximately 2.3 kb. The fragment was gel purified, digested with *Sal* I and *Bam* HI and cloned upstream of *GUS* contained in the vector pBI101.1 (Fig. 5.1).

5.3 Generation of *Arabidopsis* plants expressing the heterologous *BnLTP-GUS* transgene

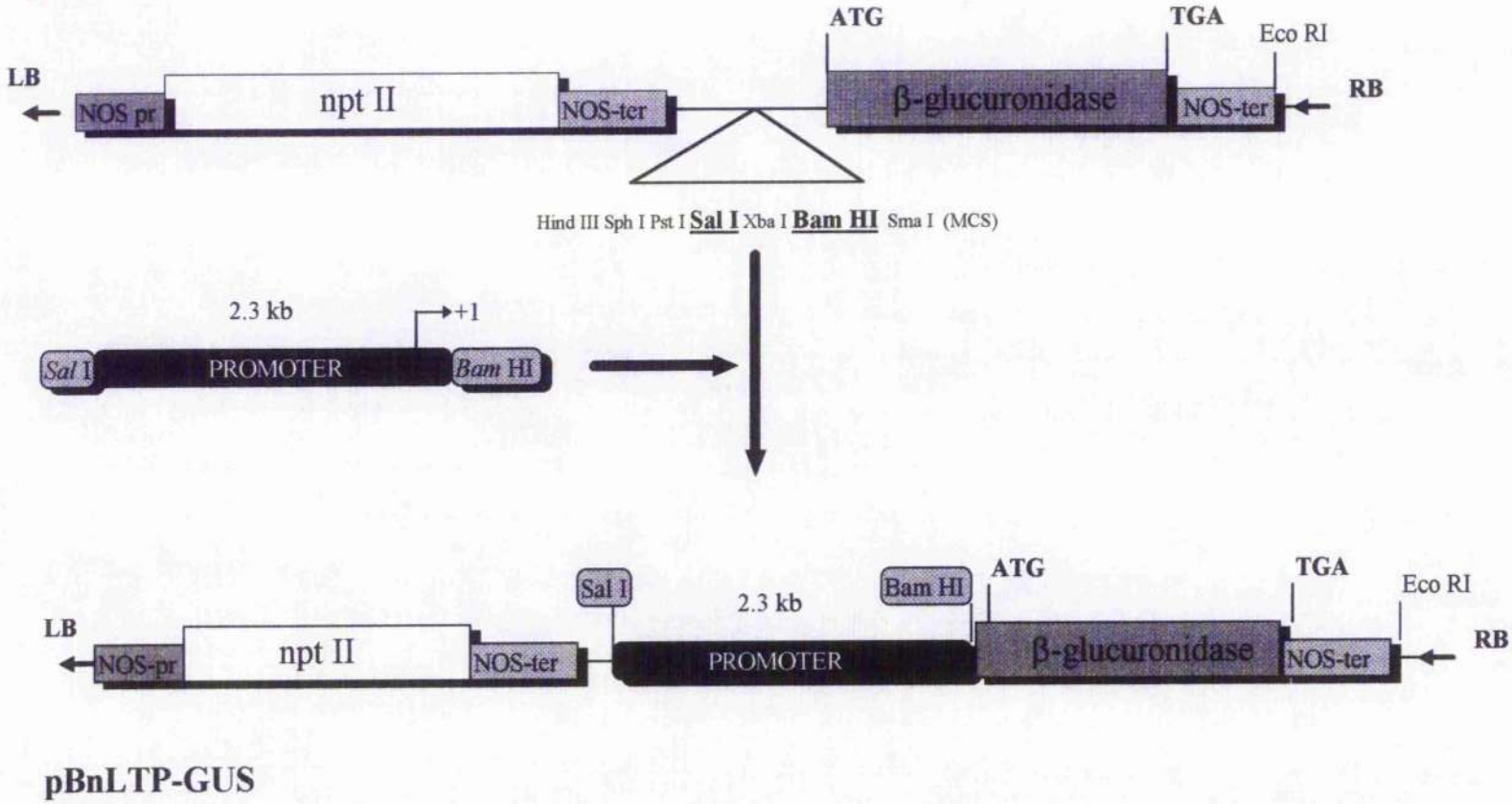
5.3.1 *BnLTP* promoter-*GUS* construct formation

The 2.3 kb putative promoter described above was cloned into the *Bam* HI and *Sal* I sites in the multiple cloning site (MCS) upstream of the *GUS* gene in the vector pBI101.1 (Fig. 5.1). The 'promoterless', 1.87- kb *GUS* 'cassette' in pBI101.1 was cloned in the *Agrobacterium* binary vector pBIN 19 (Jefferson *et al.*, 1987). Downstream of the *GUS* gene was a 260 bp fragment which contained the polyadenylation signal from the nopaline synthase gene (NOS-ter) of the *Agrobacterium* Ti plasmid (Zambryski *et al.*, 1989). Upstream of *GUS* is the MCS where fragments can be inserted directionally to produce transcriptional *GUS* gene fusions. The vector is kanamycin resistant due to the *npt II* coding sequence cloned upstream of the MCS. The gene is driven by the NOS promoter and terminated by NOS terminator. The product of *npt-II* confers kanamycin resistance to transformed plant cells. However, a separate *npt II* gene contained within the pBIN 19 vector (not shown in Fig. 5.1) enabled selection of kanamycin resistance in bacterial cells. The pBI101.1 vector is 12.2 kb. The distal ends of the cassette contained the left (LB) and right (RB) borders derived from the T-DNA (Walden *et al.*, 1991). Breaks in the chromosomal DNA enable insertion of the T-DNA strand, initially at the right border region.

The single 2.3 kb PCR product was excised from the agarose gel, purified and restriction digested with *Sal* I and *Bam* HI for a minimum of 4 h. The digested PCR product was phenol/chloroform extracted and ligated to the corresponding restriction sites in pBI101.1 (Fig. 5.1). The putative ligations were transformed into XL-1 blue cells and the transformants were selected on L-Broth agar plates containing 50 μg/ml

Fig. 5.1 Schematic diagram of the construction of the *BnLTP* promoter-*GUS* transcriptional fusion

A putative promoter region of 2.3 kb upstream of the start codon in the *gBnLTP* gene was amplified with primers tagged with restriction enzyme sites for *Sal* I and *Bam* HI at the 5' and 3' ends, respectively, and cloned into the corresponding sites upstream of the promoterless β-glucuronidase (*GUS*) reporter gene in pBI101.1. The ATG start and TGA stop codons of GUS are indicated. Downstream of *GUS* is a 260 bp fragment which contains the polyadenylation signal from the nopaline synthase gene (NOS-ter) of the *Agrobacterium* Ti plasmid. Upstream of the *GUS* gene is a multiple cloning site (MCS) where fragments can be inserted directionally to produce transcriptional *GUS* gene fusions. The vector is kanamycin resistant due to the *npt II* coding sequence cloned upstream of the MCS. This gene is driven by the NOS promoter (NOS-pr) and terminated by NOS terminator (NOS-ter). LB and RB refer to the left and right borders, respectively. Arrows indicate the direction of integration. The *Sal* I, *Bam* HI and *Eco* RI restriction sites are shown. The resulting plasmid construct is designated pBnLTP-GUS.


pBI101.1
LB
NOS pr
npt II
NOS-ter
ATG
TGA
Eco RI
β-glucuronidase
NOS-ter
RB
Hind III Sph I Pst I Sal I Xba I Bam HI Sma I (MCS)
2.3 kb
+1
Sal I
PROMOTER
Bam HI
LB
NOS-pr
npt II
NOS-ter
Sal I
2.3 kb
Bam HI
ATG
TGA
Eco RI
PROMOTER
β-glucuronidase
NOS-ter
RB
pBnLTP-GUS

kanamycin. Appropriate controls were also set up. Plasmid preparations were carried out from the positive colonies. The plasmids were digested with *Sal* I and *Bam* HI and the resulting fragments were resolved by agarose gel electrophoresis. Two colonies generated the correct sized fragments, a 12.2 kb fragment which corresponded to the pBI101.1 vector and a 2.3 kb fragment which represented the putative *BnLTP* promoter (Fig. 5.2).

An *Eco* RI site was situated downstream of the *GUS* gene as shown in Fig.5.1. *Sal* I and *Eco* RI digestion of the positive clones produced two fragments (data not shown), a 4.17 kb fragment which represented the *BnLTP*-promoter-*GUS* fusion (i.e. 2.3 kb promoter plus the 1.87 *GUS* cassette) and the 8 kb vector. These results confirmed the presence of a single copy of the putative promoter in the *GUS* cassette. The two plasmid constructs were designated pBnLTP-GUS1 and pBnLTP-GUS2.

5.3.2 Subcloning the *BnLTP-GUS* cassette into pBluescript® II SK (-)

It was necessary to sequence the construct to check for any sequence changes in the promoter region following the PCR reaction and also to check the promoter-*GUS* junction. The pBIN19 vector contains a low copy number RK2 origin of replication which results in low concentrations of the pBnLTP-GUS plasmids. A higher concentration of DNA was required for sequencing. The *Sal I/Eco* RI fragment described previously was therefore excised from pBnLTP-GUS2 and subcloned into a high copy number vector, pBluescript® II SK (-). The ligations were transformed into DH5α cells and selected on L-Broth agar plates containing 50 μg/ml ampicillin. A number of colonies were picked for plasmid purification which were then checked by restriction digestion with *Eco* RI and *Sal* I shown in Fig. 5.3. Out of thirteen plasmids, two displayed the correct restriction pattern. Clones pBS4 and pBS11 contained the 4.17 kb *BnLTP-GUS Sal I/Eco* RI fragment. These constructs were named pBS-LTP-GUS4 and pBS-LTP-GUS11. The pBS-LTP-GUS4 plasmid was sequenced with a 22-nucleotide primer GUS1 (5' GAT TTC ACG GGT TGG GTT TCT 3') which was complementary to the GUS coding strand and terminated 14 bp downstream of the A of the initiator ATG. It was also sequenced with primers 2443 and 2849 that spanned

Fig. 5.2 Isolation and restriction digestion of the putative *BnLTP-GUS* fusions

The putative *BnLTP-GUS* construct described in Fig. 5.1 was transformed into XL1 blue cells, selected on YEP-agar plates supplemented with kanamycin and several putative positive clones were amplified for plasmid purification. Plasmids were digested with *Sal* I and *Bam* HI and resolved on a 0.8% (w/v) agarose gel along with 1 kb molecular weight markers (lane M). Two positive clones are shown (lanes 1 and 2). The 12.2 kb and 2.3 kb fragments refer to the pBI101.1 vector and putative promoter, respectively.

Lane M	1 kb marker
Lane 1	Construct pBnLTP-GUS1
Lane 2	Construct pBnLTP-GUS2

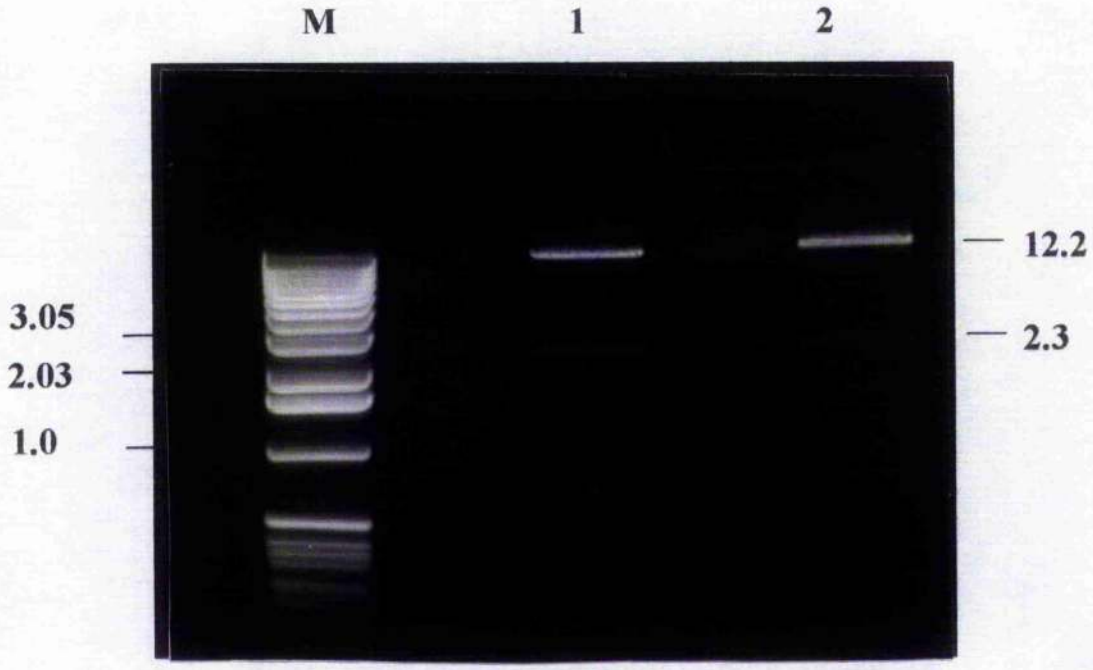
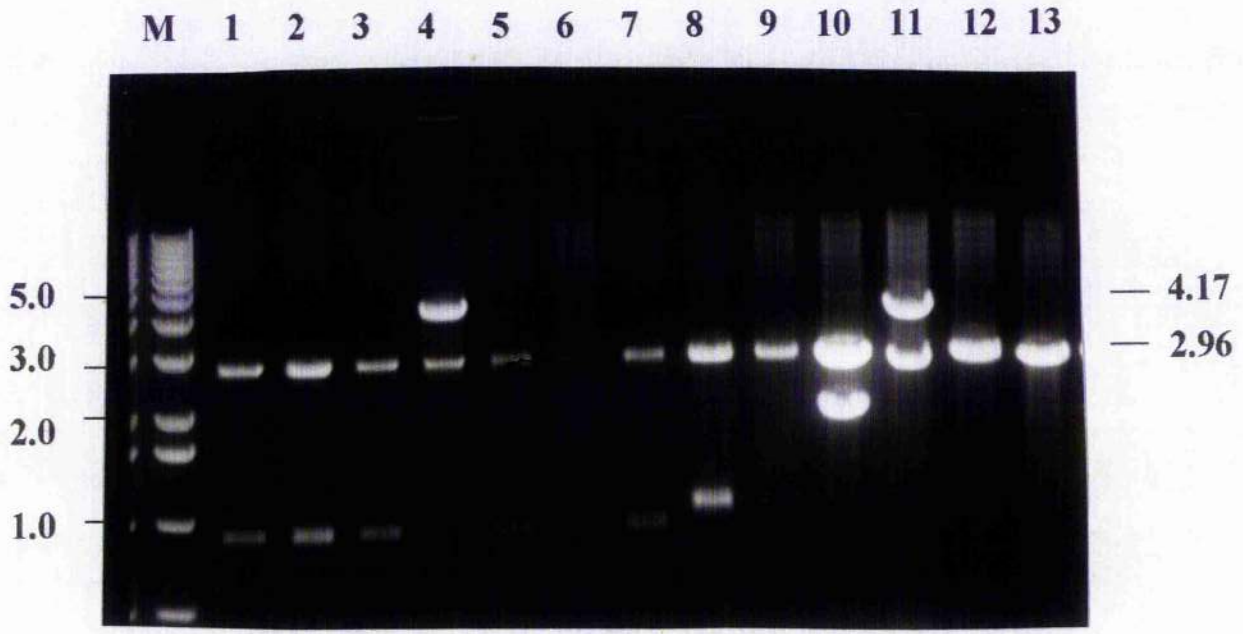

M
1
2
3.05
2.03
1.0
12.2
2.3

Fig. 5.3 Subcloning of the *BnLTP-GUS* fusion from pBnLTP-GUS2 into pBluescript® II SK(-)

A 4.17 kb *Sal* I/*Eco* RI fragment from pBnLTP-GUS2 (see Figs. 5.1 and 5.2) was subcloned into the corresponding sites in the pBluescript® II SK (-) MCS and transformed into DH5α cells. Putative positive colonies (pBS) were selected on L-Broth agar plates supplemented with ampicillin. Plasmid DNA was isolated and checked by restriction digestion with *Sal* I and *Eco* RI. Plasmids from two colonies pBS4 and pBS11 contained the desired 4.17 kb fragment. The 2.96 kb fragment indicates the pBluescript® II SK (-) plasmid.

Lane M	1 kb ladder
Lane 1	Colony pBS1
Lane 2	Colony pBS2
Lane 3	Colony pBS3
Lane 4	Colony pBS4 (pBS-LTP-GUS4)
Lane 5	Colony pBS5
Lane 6	Colony pBS6
Lane 7	Colony pBS7
Lane 8	Colony pBS8
Lane 9	Colony pBS9
Lane 10	Colony pBS10
Lane 11	Colony pBS11 (pBS-LTP-GUS11)
Lane 12	Colony pBS12
Lane 13	Colony pBS13


M 1 2 3 4 5 6 7 8 9 10 11 12 13
5.0
3.0
2.0
1.0
4.17
2.96

the promoter region as shown in Fig.4.9. The sequences were compared with those of $p3.2A_{15}$ and no differences were detected.

5.3.3 Electroporation of pBS-LTP-GUS4 into *B. napus* stem sections

Transformation of plants with promoter-GUS constructs is a process that requires several months' work before promoter activity can be detected. A relatively quicker method to determine whether the promoter is active is transformation of the construct of interest into *B. napus* tissue sections by electroporation (Fromm *et al.*, 1985). A small amount of *B. napus* tissue and the plasmid of interest were mixed together and an electric charge was applied across them using the Bio-Rad Gene Pulser® for a few seconds to allow the plasmid to enter the cell. The process was quick, taking less than a few seconds and required a minimum amount of tissue, about five or six 2 mm tissue sections. After electroporation the sections were washed thoroughly in phosphate buffer and incubated overnight in the appropriate buffer containing X-Gluc. *GUS* activity was detected by the presence of a blue-indigo precipitate.

Table 5.1 details the conditions and resulting relative *GUS* expression following electroporation of the plasmids into *B. napus* tissue sections. The expression level was estimated by the relative intensity of the blue precipitate. pBS-LTP-GUS4 was used in the electroporations. A positive control plasmid, pBI121 which expressed *GUS* constitutively under the control of the CaMV *35S* promoter (*35S-GUS*) was also set up. Under all the electroporation conditions used the 'no DNA' control was negative. Similarly, GUS activity in *35S-GUS* and pBS-LTP-GUS4 was not present in the leaf sections under all the conditions used. However, under condition A where a voltage, resistance and capacitance of 1.8 kV, 1000 ohms and 250 μF, respectively, were applied, only the 35S-GUS plasmid was active in *B. napus* stem sections whereas pBS-LTP-GUS4 was negative. However, following electroporation of stem sections under condition B, where the voltage was altered from 1.8 kV to 1.6 kV, pBS-LTP-GUS4 activity was extremely high estimated by the level of blue precipitate. *35S-GUS* on the other hand was negative under these conditions. Under condition C (voltage: 1.8 kV, resistance: 1000 ohms, capacitance: 7 μF) GUS activity was detected for both

Table 5.1 Electroporation of *Brassica napus* leaf and stem tissue with the pBS-LTP-GUS4 and 35S-GUS plasmid constructs

Young *Brassica napus* leaves and stems were sliced into small 1-2 mm sections with a sterile blade and washed thoroughly in ice-cold sterile water for 1 h. Several sections were placed into a Bio-Rad Gene Pulser cuvette in a total volume of 300 µl of ice-cold water and 25 µg of the plasmid to be electroporated was mixed with the tissue sections ensuring everything was kept on ice. The *35S-GUS* plasmid constitutively expresses the *GUS* gene driven by the CaMV *35S* promoter and is used as the positive control. The sections were electroporated using the conditions shown. Electroporated sections were washed thoroughly as described in section 2.27 and incubated in X-Gluc to monitor GUS activity for up to 24 h. GUS activity was estimated qualitatively by the level of blue precipitate. The voltage (kV), resistance (ohms) and capacitance (µF) values are indicated.

(-) refers to no GUS activity (no blue precipitate)
(+) refers to low GUS expression (faint blue precipitate)
(+++) refers to strong GUS activity (strong blue preciptate)

Electroporation Conditions	**35S GUS**		**pBS-LTP-GUS4**		**No DNA**	
kV : ohms : μF	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**
1.8 : 1000 : 250	–	+++	–	–	–	–
1.6 : 1000 : 250	–	–	–	+++	–	–
1.8 : 1000 : 7	–	+	–	+	–	–
1.6 : 1000 : 14	–	+	–	–	–	–

plasmids but was very low, whereas under condition D (1.6 kV, 1000 ohms, 14 μF) only *35S-GUS* was active. These experiments indicated that the *BnLTP* promoter-*GUS* fusion contained within the pBS-LTP-GUS4 plasmid was functional.

5.3.4 Transformation of *Agrobacterium tumefaciens* with the *BnLTP-GUS* fusion

The pBnLTP-GUS2 plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 by direct freeze-thaw as described in section 2.7.2. Essentially, 1 μg of plasmid was mixed with competent *Agrobacterium* prepared as described in section 2.6.2. The *Agrobacteria*/DNA mixture was flash-frozen in liquid nitrogen and then immediately incubated at 37°C for 5 min. Warm YEP media was added to the whole mixture was incubated at 28°C for 2 to 4 h with gentle shaking to allow the antibiotic resistance genes to be expressed. The cells were then plated onto YEP agar plates supplemented with gentamycin (*Agrobacterium* selection) and kanamycin (plasmid selection). The plates were incubated for 2 to 4 days at 28°C. A total of ten putative transformants were picked. Prior to plasmid preparations each colony was subjected to PCR analysis with primers specific to the *BnLTP* 2.3 kb putative promoter and the GUS coding region.

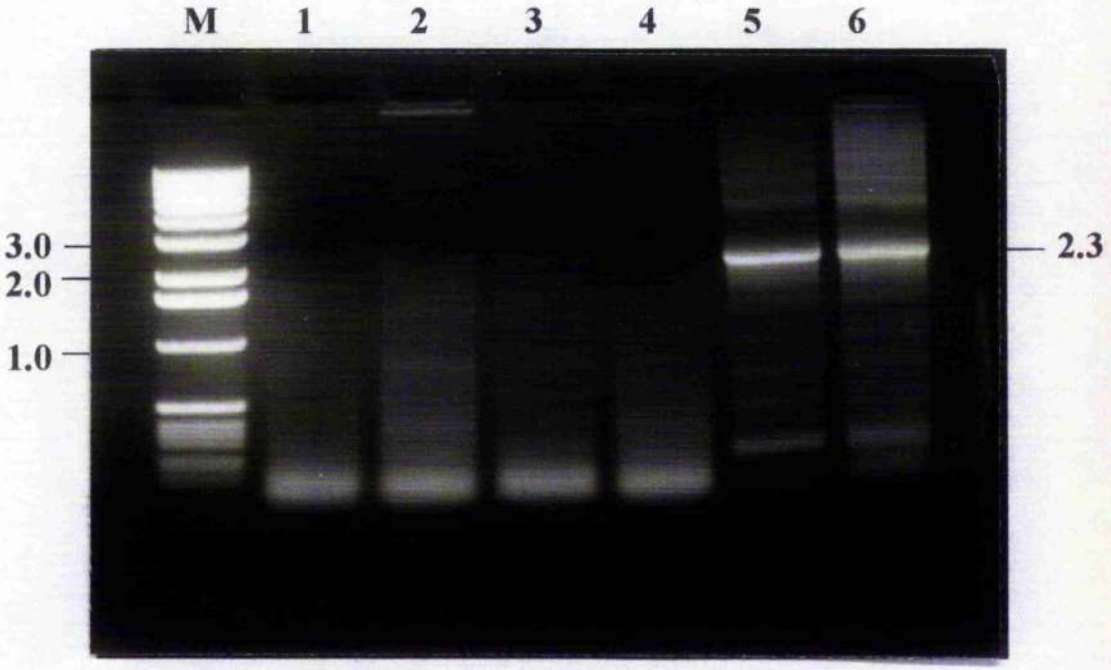
5.4 PCR with primer pairs 2285/2257 and 2285/GUS1

To check whether the transformed *Agrobacterium tumafaciens* contained the binary vector pBnLTP-GUS2, the colony of interest was directly subjected to PCR with primers that hybridised to the promoter region and part of the *GUS* coding sequence. The primer pairs used were 2257/2285 that hybrisised to the promoter region, and 2285/GUS1 that spanned the promoter region and the first 14 bp of the *GUS* coding strand. The colony of interest was placed into a microfuge tube containing 20 μl of distilled water, boiled for a few minutes and the crude supernatant extract was subjected to the PCR reaction. In this case *Taq* DNA polymerase was used instead of *Pfu*. Appropriate control PCR reactions containing the pBnLTP-GUS2 plasmid, $p3.2A_{15}$ which contained the original 4.8 kb genomic fragment along with untransformed *Agrobacterium* were also set up. The amplified fragments were resolved on an agarose gel along with the 1 kb size markers (Fig. 5.4). Colonies

Fig. 5.4 **PCR of putative *Agrobacterium tumefaciens* transformants of the pBnLTP-GUS2 plasmid with *BnLTP* promoter-specific primers, 2285 and 2257**

Putative positive colonies were directly subjected to PCR with primers 2285 and 2257 that amplified a 2.3 kb 5' upstream region of the *BnLTP* promoter. The PCR conditions were denaturation 95°C, 1 min; annealing 50°C, 5 min; extension, 72°C, 5 min, cycled 35 times (see section 2.24 and 5.4). The PCR products were resolved in 0.8% (w/v) agarose gels along with DNA size markers.

Lane M	1 kb ladder
Lane 1	Untransformed *Agrobacterium* (negative control)
Lane 2	Putative positive Colony A
Lane 3	Putative positive Colony B
Lane 4	Putative positive Colony C
Lane 5	Plasmid pBnLTP-GUS2 (positive control)
Lane 6	Plasmid p3.2A_{15} (positive control)


M
1
2
3
4
5
6
3.0
2.0
1.0
2.3

A and B (lanes 2 and 3, respectively) amplified a faint band of 2.3 kb with primers 2285 and 2257. Untransformed *Agrobacterium*, as expected, did not amplify a band (Lane 1). Conversely, Colony C (Lane 4) either did not amplify the appropriate sized fragment or produced a fainter band than that produced in Colonies A and B. This may be attributed to a difference in copy number of plasmid in these bacteria. pBnLTP-GUS2 (Lane 5) and $p3.2A_{15}$ (Lane 6) both amplified a relatively stronger band of 2.3 kb.

A band of approximately 2.3 kb amplified with primers 2285 and GUS1 is shown in Fig. 5.5. Lane 1 contained plasmid pBnLTP-GUS2, Lane 2 contained *Argobacterium* Colony A transformed with pBnLTP-GUS2, Lane 3 contained untransformed *Agrobacterium*, Lane 4 contained plasmid $p3.2A_{15}$ and Lane 5 contained a 2.3 kb 2285/2257 amplified fragment from pBnLTP-GUS2. As expected the untransformed *Agrobacterium* and $p3.2A_{15}$ did not amplify the appropriate band. The latter did however amplify non-specific bands, ranging from 1.0 to 3 kb. These results confirmed that the pBnLTP-GUS2 plasmid was present in *Agrobacterium* Colony A.

5.5 Restriction digestion of pBnLTP-GUS isolated from *Agrobacterium*

Plasmid preparations were also carried out from pBnLTP-GUS2 transformed *Agrobacterium* Colony A. However, the concentration of the isolated plasmid was extremely low and it was difficult to interpret the restriction digestion pattern. The *Agrobacterium*-isolated pBnLTP-GU2 plasmid was therefore retransformed into DH5α cells, amplified and the plasmid isolated. Although plasmid concentrations were still relatively low owing to the low copy number origin of replication, RK2, enough was isolated for restriction digestion. 0.5 to 1 μg of plasmid DNA was digested with *Hind* III, *Eco* RI, *Bgl* II and *Sau* 3A (Fig. 5.6). The restriction pattern of the transformed plasmid was identical to the original untransformed plasmid. The PCR and restriction digestion analyses therefore confirmed that the pBnLTP-GUS2 plasmid transformed into *Agrobacterium* had not undergone any gross sequence rearrangements.

Fig. 5.5 PCR of putative *Agrobacterium* transformants of plasmid pBnLTP-GUS2 with primers 2285 and GUS1

The PCR reactions described in Fig. 5.4 were repeated using primers 2285 (upstream) and GUS1 (5'GAT TTC ACG GGT TGG GTT TCT3') which is complementary to the *GUS* coding strand and terminates 14 bp from the adenine residue of the initiator ATG. The PCR conditions were denaturation 95°C, 1 min; annealing 50°C, 5 min; extension, 72°C, 5 min, cycled 35 times (see sections 2.24 and 5.4). The PCR products were resolved in 0.8% (w/v) agarose gels along with DNA size markers.

Lane M	1 kb ladder
Lane 1	Plasmid pBnLTP-GUS2 (positive control)
Lane 2	Putative postive Colony A
Lane 3	Untransformed *Agrobacterium* (negative control)
Lane4	Plasmid $p3.2A_{15}$ (negative control)
Lane 5	2285/2257 amplified fragment (from PCR reaction in lane 5, Fig. 5.4)

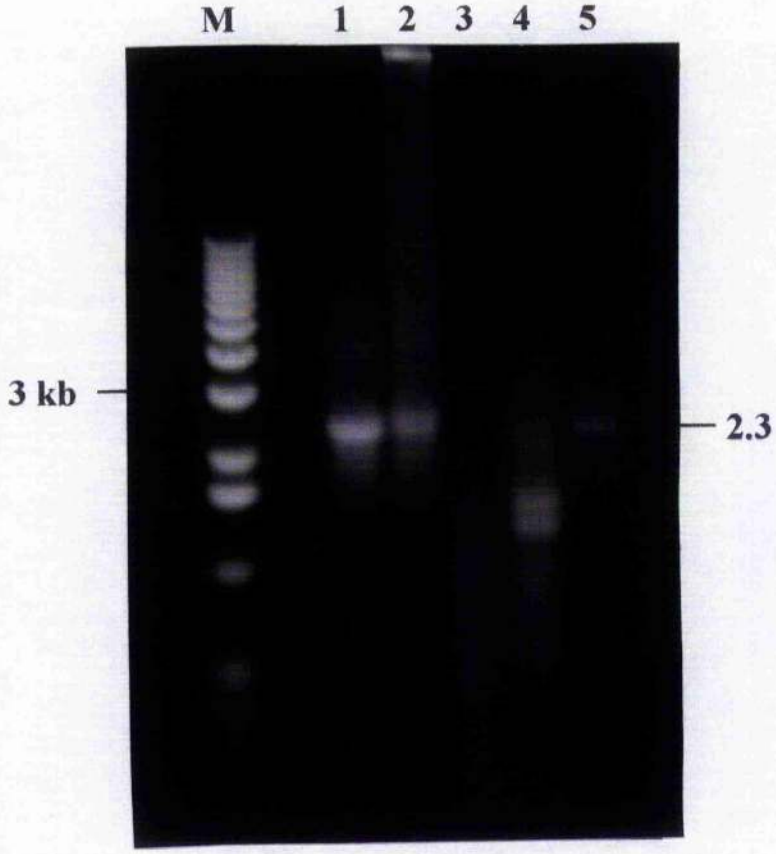

M
1
2
3
4
5
3 kb
2.3

Fig. 5.6 Comparison of restriction digestion patterns of pBnLTP-GUS2 before and after *Agrobacterium tumifaciens* transformation

Plasmid pBnLTP-GUS2 was isolated from *Agrobacterium* Colony A using the method described in section 2.8.3. The plasmid was then re-transformed into DH5α cells, amplified and isolated using the Promega Wizard™ *Plus* Minipreps plasmid purification kit (see section 2.8.1). 0.5 to 1 μg of purified pBnLTP-GUS2 and the original untransformed plasmid were digested with *Hind* III, *Eco* RI, *Bgl* II, and *Sau* 3A and resolved in a 0.8% (w/v) agarose gel along with 1 kb markers (Lane M).

Lane 1 Purified pBnLTP-GUS2 cut with *Hind* III

Lane 2 Untransformed pBnLTP-GUS2 cut with *Hind* III

Lane 3 Purified pBnLTP-GUS2 cut with *Eco* RI

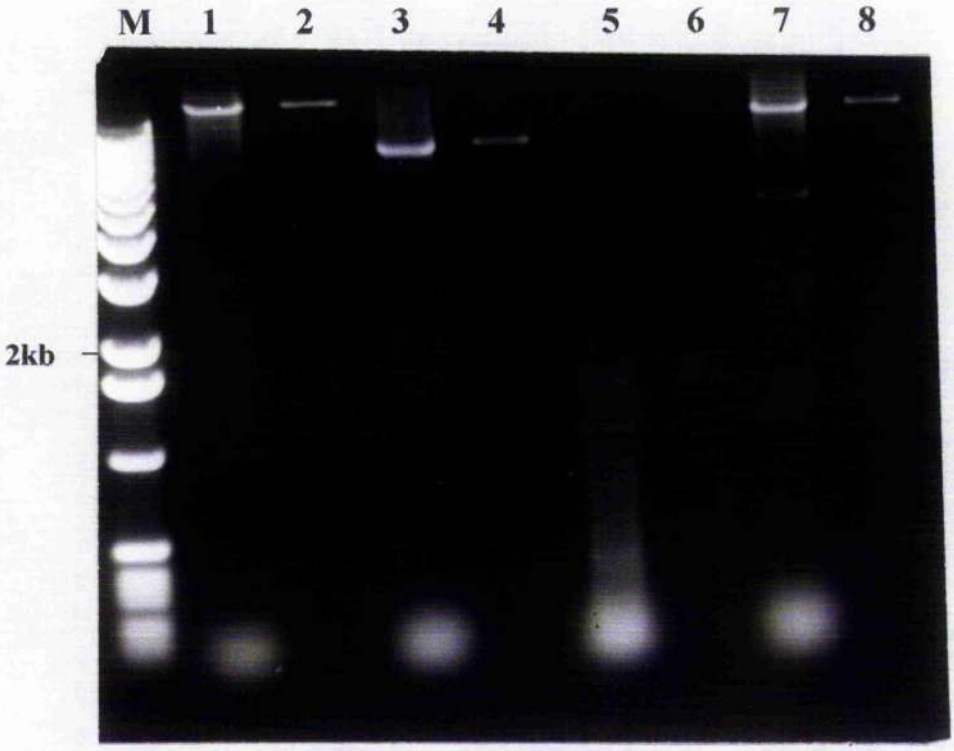
Lane 4 Untransformed pBnLTP-GUS2 cut with *Eco* RI

Lane 5 Purified pBnLTP-GUS2 cut with *Bgl* II

Lane 6 Untransformed pBnLTP-GUS2 cut with *Bgl* II

Lane 7 Purified pBnLTP-GUS2 cut with *Sau* 3A

Lane 8 Untransformed pBnLTP-GUS2 cut with *Sau* 3A


M
1
2
3
4
5
6
7
8
2kb

5.6 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* with pBnLTP-GUS2 by vacuum infiltration

Wild type *Arabidopsis thaliana* ecotype Landsberg *erecta* (T_0) were transformed with the *Agrobacterium* Colony A containing the pBnLTP-GUS2 plasmid using the vacuum infiltration method described by Bechtold *et al.* (1993). The bacteria were allowed to penetrate the developing embryos, normally at the stage where the flower buds were just beginning to open. This plant cell and bacterium interaction was achieved by immersing the floral buds in a solution of the *Agrobacterium* (Colony A) and a vacuum was applied for several minutes. The rapid release of the vacuum ensured that the plant tissue was penetrated with the bacterium. Seed was harvested from each transformed plant and selected for kanamycin resistance on germination media containing 50 μg/ml kanamycin.

5.7 Selection and analysis of transformants

Seven kanamycin resistant plants (T1) were selected in the primary screen. Seed from T2 and T3 generations were analysed further with respect to segregation of the kanamycin-resistance phenotype. Results of the segregation analysis are presented in Table 5.2. Wild type and NM4 (*CHS* promoter-*GUS*) seed were also sown as negative and positive controls, respectively. After ten days on selective germination media, 100% of the wild type seed were completely bleached and died within a few days whereas 100% of the NM_4 seed were dark green and had started to produce secondary leaves. Around 40% of the pBnLTP-GUS2 transformed seedlings were dark green, some of which had started to produce secondary leaves. They were transferred to soil and T2 seed was collected.

T2 seedlings that produced a segregation ratio of 3:1, kanamycin resistant: kanamycin sensitive were selected. T3 seedlings that did not show a 3:1 segregation pattern and were 100 % kanamycin-resistant were considered to be homozygous for the transgene. Three putative T3 homozygous lines, line 1C, line 2D and line 3A were selected for further molecular analysis.

Table 5.2 Analysis of progeny of *Arabidopsis thaliana* transformants

Primary transformed plants were selfed and seed collected. The seeds were grown on germination medium supplemented with 50 μg/ml kanamycin. The number of kanamycin-resistant (KnR) and kanamycin-sensitive (KnS) plants were counted and the KanR:KanS ratio was calculated. Seed was collected from each KnR plant (T2 generation) and replated on selective media and the seedlings (T3 generation) analysed for kanamycin resistance. Wild type and NM4 seed were plated as negative and positive controls, respectively.

Plant T2	Kn^R	Kn^S	Kn^R:Kn^S	Phenotype
Line 1	70	26	2.69:1	normal
Line 2	28	6	3.11:1	normal
Line 3	41	18	2.28:1	Early flowering, seedling and mature plant smaller than normal
Line 4	62	77	0.8:1	normal
Line 5	38	39	0.9:1	normal
Line 6	20	7	2.85:1	normal
Line 7	47	19	2.47:1	normal
Plant T3				
Line 1A	82	26	3.15:1	
Line 1B	41	7	5.86:1	
Line 1C	106	0		
Line 2A	44	14	3.14:1	
Line 2B	41	17	2.14:1	
Line 2C	38	14	2.71:1	
Line 2D	112	0		
Line 3A	98	0		
Line 3B	80	26	3.07:1	

5.8 Molecular characterisation of Lines 1C, 2D and 3A

5.8.1 PCR with primers 2285/GUS1

Genomic DNA was isolated from each of the three lines and subjected to PCR with primers 2285 and GUS1 that spanned the entire *BnLTP* promoter and the 5' coding region of the *GUS* gene. DNA from wild type untransformed *Agrobacterium* and pBnLTP-GUS2 was also amplified. In each case *Taq* DNA polymerase was used. The fragments were resolved on agarose gel along with DNA molecular weight markers as shown in Fig.5.7. A band was not amplified when genomic DNA from untransformed plants was used (Lane 1). However, lines 1C, 2D and 3A (Lanes 2, 3 and 4, respectively) all amplified the predicted 2.3 kb band corresponding to the *BnLTP* promoter along with two other bands of approximately 1.4 kb and 1.2 kb, respectively. As expected, pBnLTP-GUS2 amplified a single band of 2.3 kb (Lane 5). Lane 6 contained the 2.3 kb fragment amplified by PCR with primers 2285 and 2257 of $p3.2A_{15}$.

5.8.2 Determination of transgene copy number by Southern blot analysis

Transgene insertion sites and therefore copy number were revealed by Southern hybridisation with the full length *GUS* DNA probe. Genomic DNA isolated from the T3 transgenic lines was digested with *Bam* HI and *Hind* III, both of which did not digest within the *GUS* coding region and the resulting fragments were hybridised to the full length *GUS* DNA probe. Untransformed *Arabidopsis* DNA and transgenic plants carrying the *35S-GUS* transgene were used as controls (Fig. 5.8A).

Hind III digestion of line 1B produced three hybridising bands of approximately 10 kb, 8 kb and 6kb (L1). Line 2D (L2) also produced three bands of approximately 12 kb, 10 kb and 7 kb. Four *Hind* III fragments of approximately 12.2 kb, 10 kb, 8 kb and 6 kb hybridised to *GUS* in Line 3A (L3) whereas a single *Hind* III fragment of 3.2 kb was produced from *35S-GUS* genomic DNA and as expected no bands were detected in untransformed wild-type genomic DNA (WT). The site of integration of the transgene was different in each case, as evident from the different restriction patterns produced. These results also suggested that multiple copies of the

Fig. 5.7 PCR of genomic DNA isolated from transgenic lines 1C, 2D and 3A with primers 2285 and GUS1

Genomic DNA was isolated from untransformed *Arabidopsis* and putative transgenic lines 1C, 2D and 3A using the method described in section 2.8.4 and used in PCR reactions with primers 2285 and GUS1. The PCR conditions were denaturation 95°C, 1 min; annealing 50°C, 5 min; extension, 72°C, 5 min, cycled 35 times (see section 2.24 and 5.4). The PCR products were resolved on 0.8% (w/v) agarose gels along with a DNA size marker.

Lane M	1 kb ladder
Lane 1	Untransformed *Arabidopsis*
Lane 2	Transgenic line 1C
Lane 3	Transgenic line 2D
Lane 4	Transgenic line 3A
Lane 5	Plasmid pBnLTP-GUS2
Lane 6	2285/2257 amplified fragment (from PCR reaction in lane 5, Fig. 5.4)

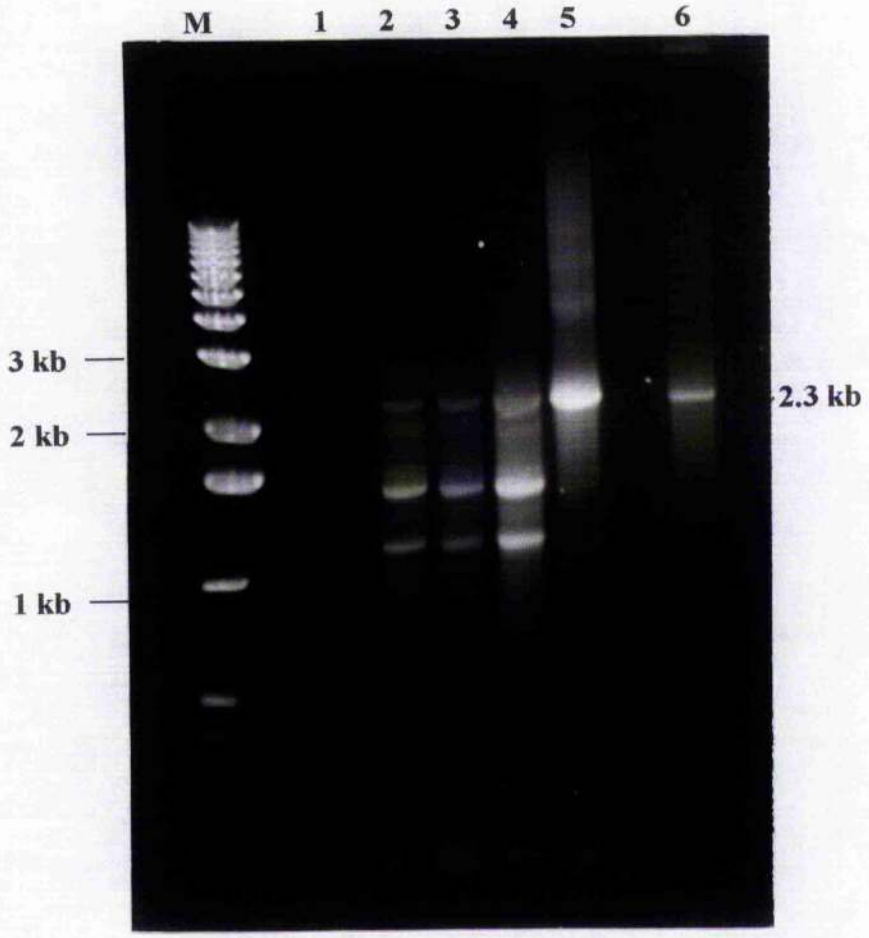
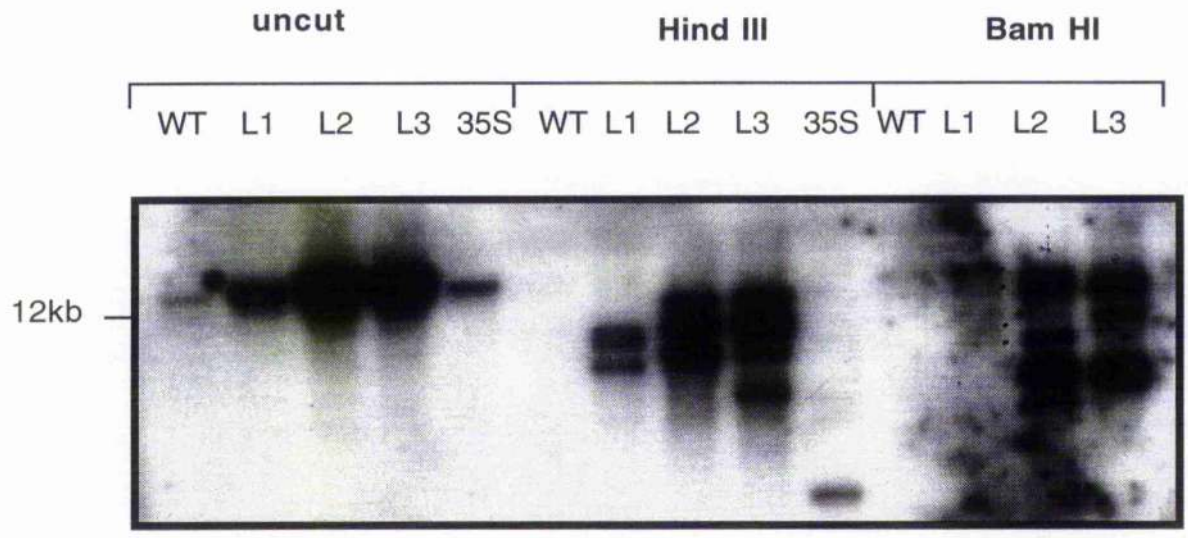

M
1
2
3
4
5
6
3 kb
2 kb
1 kb
2.3 kb

Fig. 5.8A Southern blot analysis of transgenic lines 1C, 2D and 3A

Genomic DNA (5-10 μg) was digested with *Hind* III or *Bam* HI, resolved on a 0.8% (w/v) agarose gel along with uncut genomic DNA and blotted onto Hybond N+ nylon filter. The blot was hybridised with ^{32}P-labelled *GUS* DNA. Transgenic plants carrying a single copy of the CaMV *35S-GUS* transgene were used as a positive control.

WT, untransformed, transgenic *Arabidopsis*; L1, line 1C; L2, line 2D; L3, line 3A; 35S, CaMV 35S-GUS transgenic plants.


uncut
Hind III
Bam HI
WT L1 L2 L3 35S WT L1 L2 L3 35S WT L1 L2 L3
12kb

transgene were present in the three lines (3 copies in line 1B and 2D and 4 copies in line 3A) and did not correlate with the previous segregation analysis (3:1), which indicated that the transgene was present at a single locus in each line. However, there was a strong possibility that the transgene was fragmented during the integration event. Southern hybridisation with the 2.3 kb 2285/GUS1 PCR-amplified fragment from pBnLTP-GUS2 which spanned the entire *BnLTP* promoter and *GUS* junction produced relatively fewer bands (Fig. 5.8B). A single *Hind* III fragment in line 1B and two in line 2D and three bands from line 3A hybridised to this PCR probe. The upper band in lines 2D and 3A represented undigested DNA. Based on these results it was concluded that a single full length transgene was present in lines 1B and 2D and that two full length copies were present in line 3A. The multiple bands produced in the *GUS*-probed Southern (Fig.5.8A) were probably due to fragmented transgene copies.

5.9 *GUS* expression driven by the *BnLTP* promoter

Phenotypically, lines 1B and 2D were like wild type untransformed *Arabidopsis* in terms of growth and development. Line 3A however, appeared to be relatively smaller. It had a shorter and thinner stem and the leaves were rounder and smaller. Also, it flowered one week earlier than lines 1B and 2D. In order to test whether the *BnLTP* promoter was active in these plants, 2 to 3 week old seedlings from each of the three T3 lines were incubated in phosphate buffer containing the GUS substrate, X-Gluc. The seedlings were incubated between 2 to 24 h at 37°C. As shown in Fig.5.9 all three seedlings produced the blue precipitate in the leaves, stems, petioles and flower buds which was indicative of *GUS* activity. Younger leaves had higher GUS activity compared to older leaves. The level of background GUS expression was estimated by incubating wild type untransformed *Arabidopsis* seedlings in X-Gluc. Although GUS appeared to be expressed in all organs it was not clear whether it was expressed in a cell-specific manner.

5.10 Discussion

Transformation of *Arabidopsis thaliana* using *Agrobacterium tumefaciens* can be achieved in a number of ways: inoculation of cotyledons and leaf tissue, inoculation of roots, seeds and wounded inflorescence meristems and a relatively new and quicker

Fig. 5.8B Southern blot analysis of transgenic lines 1C, 2D and 3A with the *BnLTP-GUS* fragment

Genomic DNA (5-10 μg) was digested with *Hind* III, resolved on a 0.8% (w/v) agarose gel and blotted onto Hybond N+ nylon filter. The blot was hybridised to the *BnLTP-GUS* PCR amplified fragment (see section 5.8.2).
Untransformed plants, WT; L1, transgenic line 1C; L2, transgenic line 2D; L3, transgenic line 3A.

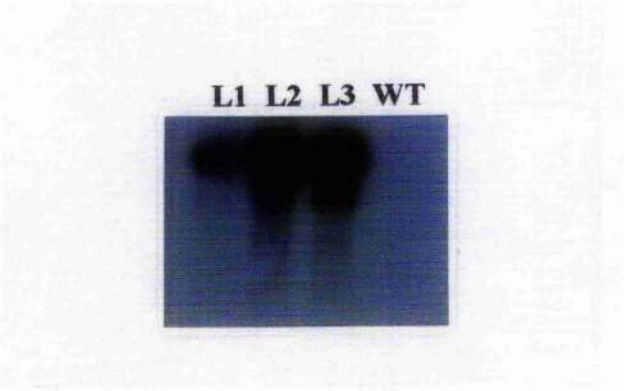

L1 L2 L3 WT

Fig. 5.9 GUS expression in transgenic lines 1B, 2D and 3C

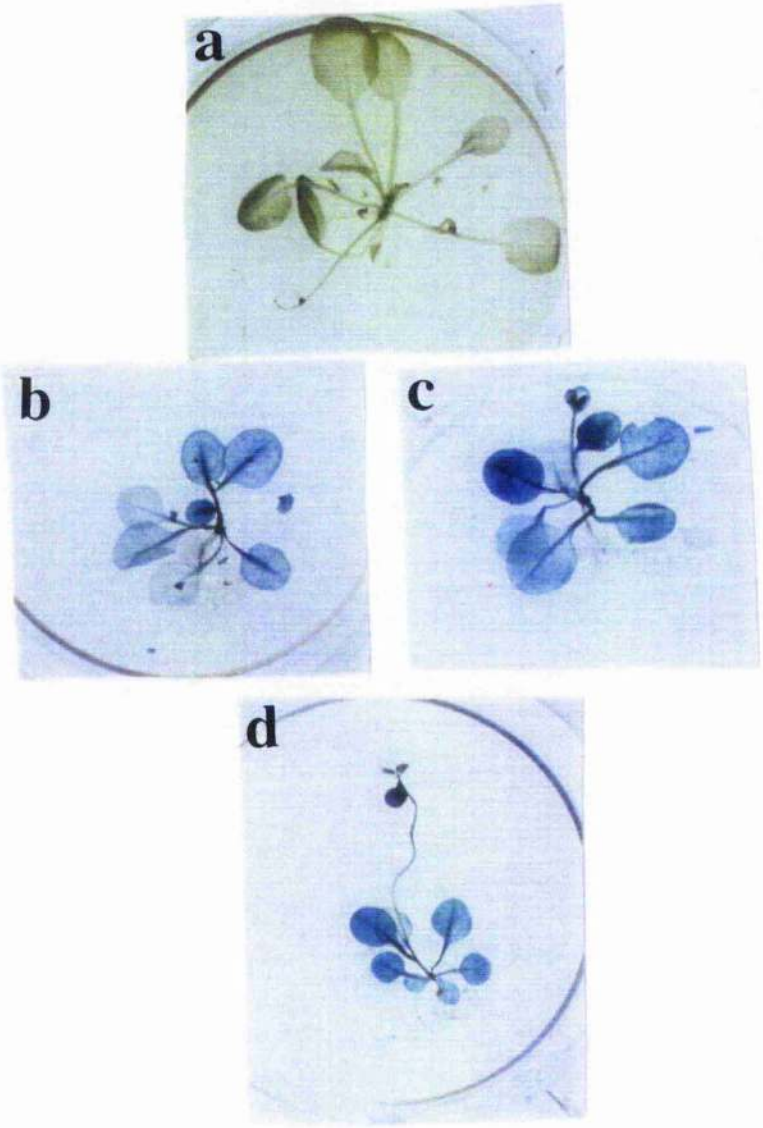
Four week old seedlings were incubated in 50 mM sodium phosphate, pH 7.0 containing 1 mg/ml X-Gluc at 37°C for up to 24 h until a blue precipitate was formed. Background GUS activity was estimated by incubating non-transformed *Arabidopsis* plants in X-Gluc. Plants were washed thoroughly in 50 mM sodium phosphate, cleared in EtOH and viewed using a dissecting microscope. Magnification X 1.5

a. Non transformed *Arabidopsis*

b. Transgenic line 1B

c. Transgenic line 2D

d. Transgenic line 3C


a
b
c
d

method of vacuum infiltration of flowering plants in a liquid suspension of *Agrobacterium* (Schmidt and Willmitzer, 1988; Valvekens *et al.*, 1988; Feldmann and Marks, 1987; Chang *et al.*, 1994; Betchold *et al.*, 1993). This chapter describes the transformation by vacuum infiltration and characterisation of *Arabidopsis* plants with a promoter-*GUS* fusion.

A 2.3 kb upstream region of the *BnLTP* gene was amplified by PCR with a high fidelity DNA polymerase, *Pfu*, and cloned upstream of the *GUS* reporter gene in the binary vector pBI101.1 to produce the *BnLTP*-promoter-*GUS* fusion. *Pfu* polymerase was used in preference to *Taq* poylmerase since it has a lower mutation rate during replication (approximately 1 in 100,000) due to its proofreading ability (Lundberg *et al.*, 1991; Eckert and Kunkel, 1991). This property of the enzyme was important especially when amplifying larger regions of DNA (> 1000 bp), and in particular in the amplification of putative promoter regions. Any mutations in the promoter elements required for transcription may lead to altered promoter activity.

The promoter-*GUS* fusion was introduced into *Arabidopsis* plants via *Agrobacterium*-mediated transformation by vacuum infiltration in an attempt to study the spatial and temporal expression pattern of the gene. Although this system involved the introduction of a heterologous *B. napus* promoter into *Arabidopsis* it was considered a suitable approach. First of all, Kowalski *et al.* (1994) have shown that *Arabidopsis* and *Brassica* have similar developmental processes and high sequence homology of several genes. Moreover, *Arabidopsis* is an ideal model for studying a myriad of developmental processes (discussed in section 1.1.2). In addition the successful use of reporter genes driven by heterologous promoters has been described by a number of authors (for example, Pyee and Kolattukudy, 1995). Although homologous systems are ideal in the studies of foreign gene expression, heterologous systems have some advantages. For example, there is often the danger of gene silencing by DNA methylation and a phenomenon known as repeat sequence induced point mutations (RIP) (Matzke and Matzke, 1993; Finnegan and McElroy, 1994) in plants carrying several homologous gene copies. However, it has to be stressed that these phenomena have been reported for coding rather than promoter sequences (Matzke and Matzke, 1993).

The 2.3 kb putative PCR-amplified promoter fragment was sequenced and no sequence differences were observed when compared to the original genomic clone, $p3.2A_{15}$. The putative promoter was cloned upstream of the promoterless GUS gene in pBI101.1. Prior to tranformation of host plants the *BnLTP-GUS* cassette was subcloned into pBluescript® II SK (-) and electroporated into *Brassica napus* stem sections, a process that takes a few seconds. The stems were then incubated overnight in X-Gluc to monitor GUS activity. This was a quick and simple method for testing whether the promoter was active prior to plant transformation. Several of the stem sections under different electroporation conditions produced a blue precipitate suggesting that the 2.3 kb PCR-amplified fragment was driving *GUS* expression.

The pBnLTP-GUS2 plasmid was transformed into *Agrobacterium* using the quick freeze-thaw method. To check for any rearrangement of the construct during the transformation process, pBnLTP-GUS2 was isolated from *Agrobacterium* and analysed by restriction digestion. No gross sequence rearrangements were observed. The fusion was then transformed into *Arabidopsis* developing flowers by vacuum infiltration. Around 40% of the primary transformed seed tested were kanamycin resistant, which suggested the method was extremely efficient and relatively quicker than other methods described in the literature.

Several putative positive lines were selected and those that segregated in a typical 3:1 (Kn^R:Kn^S) ratio in the T2 generation were selected. This observation suggests that the *BnLTP-GUS* transgene was present at a single locus and segregated in a true Mendelian fashion. It was therefore expected that one third of the T2 resistant lines from one segregation event would be homozygous for the transgene, whereas two thirds would be heterozygous. The putative homozygotes and heterozygotes appeared to be equally resistant to kanamycin based on their rates of development on kanamycin selective media.

Three T3 lines, 1B, 2D and 3A were selected and analysed further by PCR with promoter-*GUS* gene specific primers and by Southern blot analysis. In Southern blots of *Hind* III and *Bam* HI digested genomic DNA isolated from each of the putative transgenic lines, several bands hybridising to *GUS* were observed (Fig. 5.8A), which suggested several integration events. At least three copies of the transgene were

present in line 1C, three in line 2D and four in line 3A, which contradicted the 3:1 (KnR:KnS) segregation pattern of the transgene. However, hybridisation of the same fragments with the *BnLTP-GUS* PCR-amplified fragment that spanned the entire promoter and part of the *GUS* gene at the 5' end reduced the number of hybridising fragments in *Bam* HI digested lines (Fig. 5.8B); one in line 1C, one in line 2D and two in line 3A, which indicated that some of the *GUS*-hybridising bands were fragmented transgenes. This may also explain the presence of smaller bands when the promoter region was amplified with primers 2285 and GUS1 (Fig. 5.7). It is therefore likely that some of the transgene copies were non-functional, accounting for the presence of a single, functional locus in the segregation analysis.

The level of GUS expression did not vary considerably between the different lines (Fig. 5.9). Line 3A had a slightly higher level of GUS expression compared to the other two lines. This variation in transgene expression may be attributed to positional effects, copy number, transgene configuration (Hobbs *et al.*, 1993), various gene silencing (epigenetic) phenomena (Matzke and Matzke, 1993; Finnegan and McElroy, 1994) and transgene rearrangements or deletions induced in the host during the integration process (Gheysen *et al.*, 1987). It is inevitable that genetic transformation of plants leads to a large and random variation in the expression of the newly introduced transgene between individual transformants (Nap *et. al.*, 1993). This observation is most likely due to different integration sites of the transgene, the expression of which can be influenced by the surrounding chromatin. It is often the case that transgenes can integrate in tandem arrays at a single or multiple loci, become inverted or integrate as direct repeats (Spielmann and Simpson, 1986). Indeed, the three transgenic lines described in this work had different restriction digest patterns indicating different integration events. In addition, Line 3A had a different phenotype from the other two lines which indicates the difference in transgene integration in this line. Mlynarova *et al.* (1995) overcame this variability in transgene expression by including chromatin boundary elements at the borders of the *Agrobacterium* T-DNA. They introduced the chicken lysozyme matrix-associated region (MAR) or the A element at the T-DNA borders which is thought to protect the transgenes from the influences of the surrounding chromatin (Laemmli *et al.*, 1992) or to prevent

mislocalization of the genes in the heterochromatin (Dorer and Henikoff, 1994) by virtue of their affinity for the nuclear matrix.

Chapter 6 Analysis of *LTP* expression

6.1 Introduction

In order to study tissue-specific and environmental regulation of *BnLTP* expression, a 2.3 kb 5' flanking region directly upstream of the translation start site of the gene was fused to the *GUS* reporter gene and introduced into *Arabidopsis* plants (described in Chapter 5). A closer analysis of the promoter region (Fig. 6.1, section 6.2) indicated that it contained a number of putative regulatory elements implicated in tissue-specific and environmental regulation. Expression of the *BnLTP* promoter- *GUS* fusion in transgenic plants was examined throughout development by a combination of histochemical localisation of GUS in whole organs and in tissue cross-sections. GUS expression was also quantified by a fluorometric assay in response to a variety of environmental stimuli. In parallel experiments, endogenous *LTP* transcript levels were examined by northern blot analysis in wild type *B. napus* and *Arabidopsis* plants and in epidermally altered *Arabidopsis* mutants.

6.2 Analysis of the *BnLTP* promoter

The *BnLTP* promoter region was analysed and several putative transcriptional regulatory cis-acting elements were identified (Fig. 6.1). A putative cis-acting element, designated box D1, implicated in epidermal-specific expression of the tobacco *LTP* gene (*Ntltp1*) (Canevascini *et al.*, 1996) was observed at nucleotide -129 in the *BnLTP* promoter. The sequence (ATAGCTAG) differed at a single nucleotide (C→A substitution) from the eight-nucleotide palindromic sequence CTAGCTAG located in the *Ntltp1* leader sequence (Canevascini *et al.*, 1996). Deletion analysis of the *Ntltp1* promoter from its 5' end indicated that the 148 bp preceding the translation start site was sufficient for epidermal-specific expression. This element is conserved in other *LTP* genes (Fleming *et al.*, 1992; Pelese-Siebenburg *et al.*, 1994; Kalla *et al.*, 1994).

Several sequences with the core ACGT (or TGCA) were identified. This core sequence is common to several light and abscisic acid (ABA) response elements in a number of genes including the *Arabidopsis CHS* and *PAL* genes, cold and ABA-induced barley *LTP* genes, *gbltp4.2*, *gbltp4.6* and *gbltp4.9* (White *et al.*, 1994) and the

Fig. 6.1 Sequence analysis of the *BnLTP* promoter

The putative ATG translation start site (ATG:met) is underlined. The TATA-box, D1 element, I-box, GT-1 motifs and the MYB-like elements are shown in bold. The ACGT and TGCA elements are underlined. The +1 above the adenine residue (in bold) refers to the transcription start site. The numbers shown on the left hand side of the sequence indicate the nucleotide position relative to the transcription start site. Other putative cis-acting regulatory elements are discussed in section 6.1.

-846 TTATTCTTCTGGGTTCGGACCCCCCGGCCATATGTTTTTAACCCAAGGAC

I-box

-796 CTGGACCCAAGCCGG**GATAA**GCCTTTTGGGGTTCACAGGGTATTCGAACT

-746 CGGGTCCGTATTGGGGTTTAATTTTTTAGACCACTAGCCCACCACACCGT

-696 AGTTAGTCATATATTGGACACACGTTGTGTTAATCAAATATCTTTTTTTT

-646 ACTAGTGATTATAAACACTAGTGTTAATGATTATAAACATCGGATGTGTT

-596 TCCAAAAAAAGTGTTTCAAAAAAAATAATTATAAACATCGGCAGTTCCTA

MYB-like GT-1

-546 **CAACTT**AGTTGGAGTTTGTTTGGGAAAGACAAT**GATAAA**TATGAGAGTTT

-496 GAAACTCGTAGTGTTCTTTCTTGTTTTGTAAATTGAGAGTGAAACATTTT

-446 TTTTTGTACTCAAACACGTACATCTTGTAGTCTTAGACTGAACTGAGAGA

-396 GAGGAGCACTGCCGGTAATCAAGCTACGTCATGCATGATTCTAATTATAT

GT-1

-346 AAAAGAGATTGAACATAATCTTAAGATTAAAATA**GATAAT**TAATGATAGT

-296 TGTATGTACAATAACTATTTCTACCATCCATATCTGCATTTCCCGTTTTG

-246 AGTTTTCAGTATTGCATCTATTAAAATTAAAAGTCATTTTACATGATGCA

-196 ATAGCAAAAACAAGAACGTAGGTTGTAGAAATTTCAAACGAAGTAGACGT

D1-like

-146 TCATGAGGTACTACATA**ATAGCTAG**GAACCTCTACATTAAATGAAACCAA

MYB-LIKE

-96 ACAACCAAC**CAACTAC**TCCTTTCCTCTAAATCGCATTCACATTCATAACA

TATA-box +1

-46 TAAACTCTCTTTGTCTA**TATAA**ACACTCACTTCCCCTACTCTCTTC**A**TCA

5 CTCATCATTCCACACACATCTCATTTTGCAAACAAAAAAAATCTAAGAGA

55 GAAATATGGC

Met

rice *rab16A* (GTACGTGG) (Mundy *et al.*, 1990) gene. A putative low temperature responsive (LTRE) element with the core CCGAC element found in the winter *B. napus* cold-induced gene *BN115* (Jiang *et al.*, 1996) and the barley *LTPs* (White *et al.*, 1994; Hughes *et al.*, 1992) was not observed in the *BnLTP* promoter.

Five cis-acting elements located in the four WAX9 genes from broccoli (Pyee and Kolattukudy, 1995) were also conserved in the *BnLTP* promoter at positions -116 (box I; TCTACATTAA), -96 (ACAACCAACCAACTA, box II), -68 (AATCGCATTCACATTC; box III), -31 (TATATAAACACTCACT; box IV) and +29 (TTTGCA; box V). Furthermore, sequence elements of the type CAAA at positions +33, +37, -162, -192, -186, -594, -580 and -662 were observed. These sequences are associated with a number of root-specific genes including, *HRGPnt3* (Keller and Lamb, 1989) and *TobR7* (Yamamoto *et al.*, 1991).

A light and elicitor-inducible cis-acting element designated box 2 (consensus C/TCAACA/CAACCA/CC) which is located in the parsley and bean *PAL* and *CHS* as well as the *Arabidopsis LTP1* and *PAL* promoters (Ohl *et al.*, 1990; Thoma *et al.*, 1994; Lois *et al.*, 1989) was located in the *BnLTP* promoter at position -92 to -81. The sequence contained a myb-like element CAACTA which is also present in a barley *LTP* gene (Kalla *et al.*, 1994) and in the broccoli *WAX9D* (box 4) promoter (Pyee and Kolattukudy, 1995). Adjacent to this was located a 9 bp AC-rich element with the consensus AACCAACAT (-102 to -93) designated box 3 in the *Arabidopsis LTP1* and *PAL* as well as the bean *CHS* promoters (Ohl *et al.*, 1990; Lois *et al.*, 1989). Another imperfect, myb-like element CAACTT was located at nucleotide -546.

Elements found consistently in light-regulated genes include the GT-1 site (consensus GPu(T/A)AA(A/A), I-box (GATAA) and related motifs and A/T rich elements (Terzaghi and Cashmore, 1995). These elements are located in the *BnLTP* promoter.

A 10 bp element (TCATCTTCTT) implicated in salicylic acid-inducible binding in several stress-inducible genes in tobacco (Goldsbrough *et al.*, 1993), which was also present in the *Arabidopsis LTP1* promoter (Thoma *et al.*, 1994), was not observed in the *BnLTP* promoter.

6.3 Organ-specific expression of *BnLTP*

Spatial regulation was initially studied by hybridising the BnLTP cDNA to blots of total RNA isolated from *Brassica napus* organs. The probe hybridised to a 700 nt transcript present in mature leaves, stems, petals, carpels, sepals and stamens, but not detectable in roots (Fig. 6.2). This pattern of *LTP* expression was in part verified by histochemical staining of GUS activity in transgenic *Arabidopsis* seedlings expressing the *BnLTP-GUS* fusion (Figs. 6.3 and 6.4D). GUS expression was detected in leaf and stem tissue. Younger developing leaves expressed GUS at a higher level compared to mature leaves in four week old plants (Fig. 6.3A). GUS activity was localised in the lignified tip of cotyledons (Fig. 6.3A). In floral tissue (Fig. 6.3B), GUS expression was present only in stigmas, stamens and in the sepal-petal abscission zone, and was not detected in the mature petals. Although *LTP* transcripts were not detected in root tissue on RNA blots (Fig. 6.2) GUS activity was clearly present in lateral root initials (Fig. 6.3C) and the root tip (data not shown) in the transgenic plants. As the lateral root developed GUS expression remained at the tip (data not shown). GUS expression was not evident in the root hairs (Fig. 6.3C). The same pattern of expression was observed in the transgenic lines 1C and 3A (see Chapter 5, sections 5.7 and 5.8). The Figures shown are for line 2D.

6.4 *BnLTP* expression is largely confined to the epidermis

Previously, single-stranded pLF3A anti-sense probes were used for *in situ* hybridisation of the corresponding mRNAs in tissue cross-sections of *Brassica napus* organs. Transcripts were detected predominantly in epidermal cells of leaf, stem and floral buds (Pallas, 1992). A distinct disadvantage of using anti-sense probes for *in situ* hybridisations is the problem with cross-hybridisation with closely related transcripts. Furthermore, as described previously, BnLTP and pLF3A probably represent two of several closely related members of a small multigene family of lipid transfer protcins in oilseed rape and it was not possible to distinguish between the different gene members in relation to spatial expression patterns. However with *LTP-GUS* fusions it is possible to follow the expression pattern of a single gene.

In tissue cross-sections of four week old BnLTP-GUS2 (line 2D) transgenic plants, GUS activity was present predominantly in epidermal cells of the leaf blade (Fig

Fig. 6.2 Organ-specific expression of *BnLTP*

10 μg of total RNA from *Brassica napus* cv. Cobra leaf (L), stem (S), petals (P), carpels (C), sepals (Sp), stamen (St) and roots (R) was separated on a 1.5% (w/v) formaldehyde gel and blotted onto Hybond N nylon filter. The filter was hybridised with ^{32}P- radioactively labelled BnLTP cDNA probe at 55°C for 16 h in hybridisation buffer containing 0.5 M sodium phosphate, pH 7.2, 10 mg/ml BSA, 7% (w/v) SDS. Equal loading of RNA was determined by re-hybridization the with *H1* DNA probe. The filters were given a stringent wash in 1 X SSC, 0.1 % SSC at 65°C and autoradiographed for 24 h at -70°C.

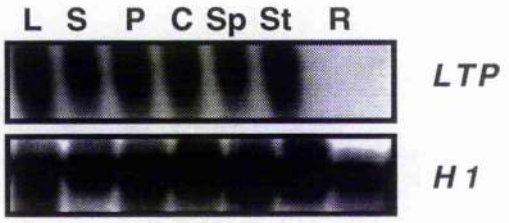

L S P C Sp St R
LTP
H1

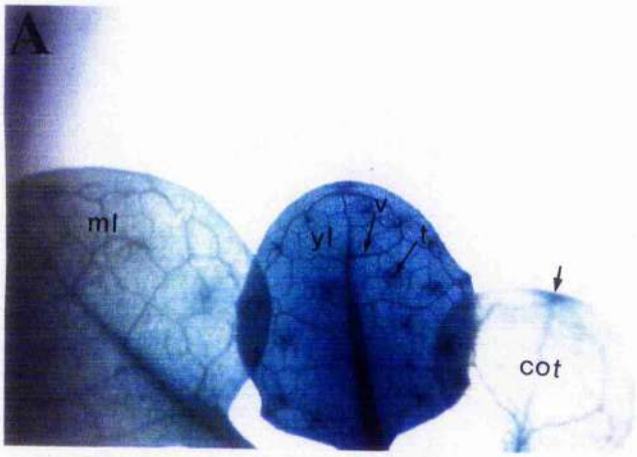
Fig. 6.3 Spatial regulation of *BnLTP-GUS* expression in transgenic *Arabidopsis*

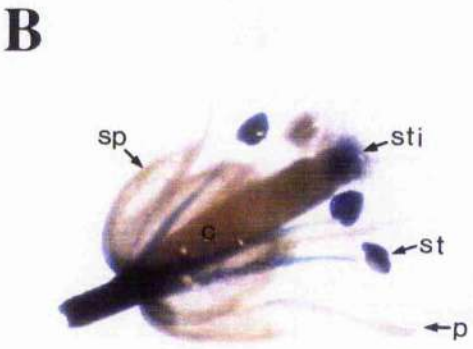
Plants were harvested, incubated in fixing solution (see section 2.29.1) containing 0.3% (v/v) formaldehyde, 0.3 M D-mannitol, 10 mM MES, pH 5.6 for up to 1 h at RT. The tissue was washed several times in 50 mM sodium phosphate and incubated for 24 h in the same buffer containing 1 mg/ml X-Gluc at 37°C. The tissue was then washed several times in phosphate buffer, cleared in EtOH and photographed under an Axiophot Zeiss or SZ-PT OLMYMPUS dissecting microscope.

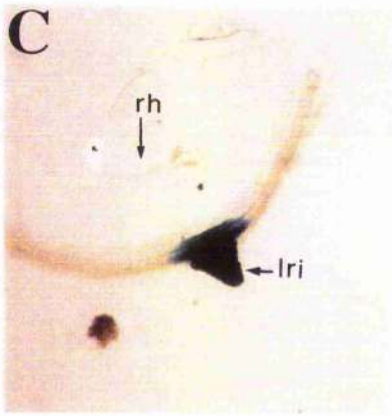
A. Four week old plants; ml, mature leaf; yl, young leaf; cot, cotyledon; v, veins; t, trichomes. Arrow indicates lignified tip of cotyledon. Magnification X 10

B. Flower; sti, stigma; p, petal; sp, sepal; st, stamen, c, carpel. Magnification X 10; The average length of an *Arabidopsis* flower is 0.5 cm

C. Root; lri, lateral root initial; rh, root hair. Magnification X 27; the average diameter of a mature *Arabidopsis* root is 125-150 μm


A
ml
yl
v
t
cot


B
sp
sti
c
st
p


C
rh
Iri

6.4A), leaf midvein (Fig. 6.4C) and stem (Fig. 6.4D). However, GUS activity was also detected in the vascular tissues, including the vascular bundles in leaf blades (Fig. 6.4A), in the xylem, and phloem of the leaf midvein (Fig. 6.4C) and in the sclerified parenchyma of the stem (p, Fig. 6.4D). However, GUS was not detected in the xylem, phloem or cortex (Fig. 6D) of stems. GUS activity in lower epidermal strips of three to four week old leaves was localised in guard and pavement cells (Fig. 6.4G). GUS activity was very strong in the accessory socket cells of trichome cross-sections (Fig. 6.4H), although GUS expression in the trichome itself varied between plants and between the leaves of a single plant (6.4I and J). No background GUS activity was detected in any tissue in untransformed *Arabidopsis* plants treated with X-Gluc (Fig. 6.4B, E, and F). Similar spatial expression of GUS was observed in the transgenic lines 1C and 3A (data not shown).

6.5 The *BnLTP* promoter is moderately light-induced

There is some evidence that *LTP* transcripts are regulated by environmental stimuli such as low temperature (Dunn *et al.*, 1991, 1994; Hughes *et al.*, 1992; White *et al.*, 1994; Molina *et al.*, 1996), drought, ABA and salt stress (Torres-Schumann *et al.*, 1992; Kahn *et al.*, 1993) and pathogen attack (Molina *et al.*, 1996) in some species. However, no such experiments have been carried out with stably transformed promoter-GUS fusions in plants nor has a range of factors been examined for any one gene. Furthermore, analysis of the *BnLTP* promoter sequence indicated the presence of several putative cis-acting elements implicated in light, stress and pathogen attack detailed in section 6.1.

To investigate light-responsiveness of *LTP* expression, *B. napus* and wild type *Arabidopsis* plants were grown in a fluence rate of 30 ±5 μmol m^{-2} s^{-1} of white light for three weeks, then transferred to 90 ± 5 μmol m^{-2} s^{-1} blue or red light or 3 μmol m^{-2} s^{-1} UV-B light for 24 h. Using the BnLTP cDNA as a probe, *LTP* transcripts were found to increase in blue and red light treated *B. napus* plants, whereas no comparable increase was observed in UV-B treated plants (Fig. 6.5A). The expression of chalcone synthase (*CHS*), which is known to be light regulated in *Arabidopsis* (Kubasek *et al.*, 1992; Fuglevand *et al.*, 1996), was used as a control. *CHS* expression in the same plants was induced upon blue and UV-B treatment but was not significantly increased

Fig. 6.4 Expression of GUS is restricted mainly to epidermal cells

Three to four week old transgenic *Arabidopsis* plants expressing the *BnLTP-GUS* fusion were harvested and fixed according to section 2.29.1. The tissue was then incubated in X-Gluc overnight at 37°C, washed and embedded in OCT and sectioned using a freezing microtome. The 20 μm sections were photographed using a Zeiss Axiophot microscope.

A. Cross-section of leaf blade of transgenic plants. ue, upper epidermis; m, mesophyll cell; v, vascular, bundle. Bar = 72 μm.

B. Cross-section of leaf blade of wild type, untransformed *Arabidopsis* (control). e, epidermis. Bar = 84 μm

C. Cross-section of leaf midvein of transgenic plants. ue, upper epidermis; m, mesophyll cell; le, lower epidermis; xy, xylem; ph, phloem. Bar = 42 μm

D. Cross-section of transgenic stem. e, epidermis; xy, xylem; ph, phloem; cor, cortex; p, parenchyma; pi, pith. Bar = 24 μm

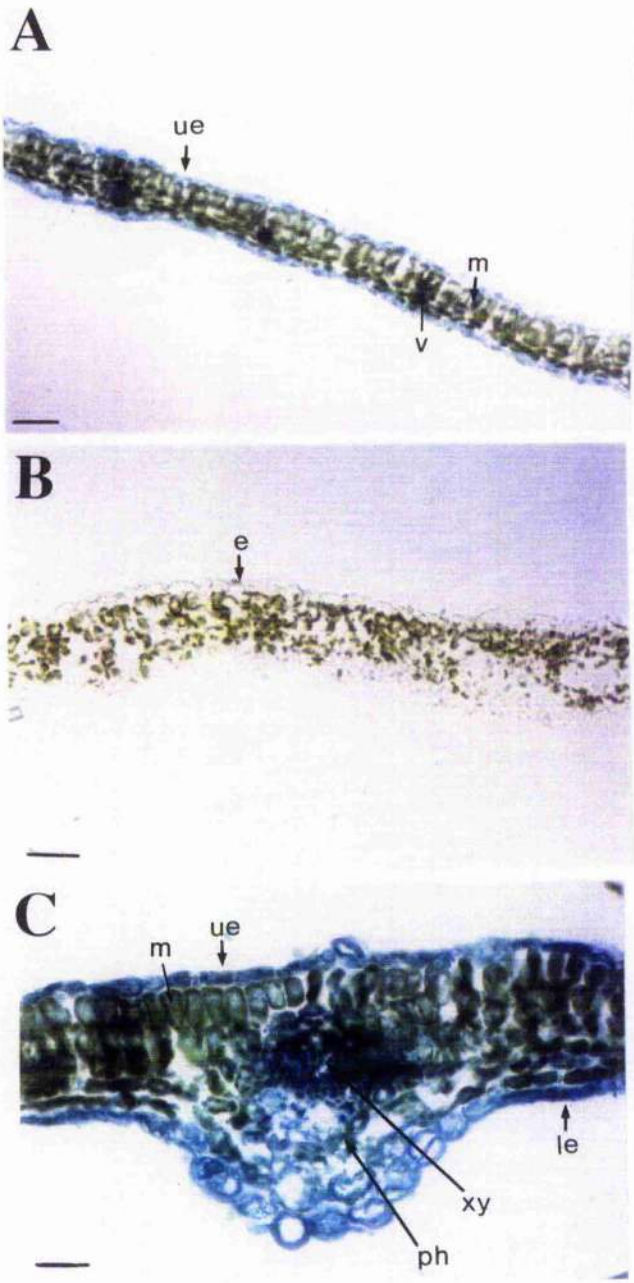
E. Cross-section of untransformed *Arabidopsis* stem (control). Bar = 24 μm

F. Abaxial epidermal strip of wild type, untransformed leaf. gc, guard cell; pc, pavement cell. Bar = 1.7 μm.

G. Abaxial epidermal strip of transgenic leaf. gc, guard cell; pc pavement cell. Bar = 1.7 μm.

H. Cross-section through trichome of transgenic leaves. ue, upper epidermis; t, trichome; sc, socket cell. Bar = 84 μm.

I. and J. Trichomes from two different leaves from the same transgenic plant. Bar = 21 μm.


A
ue
m
v
B
e
C
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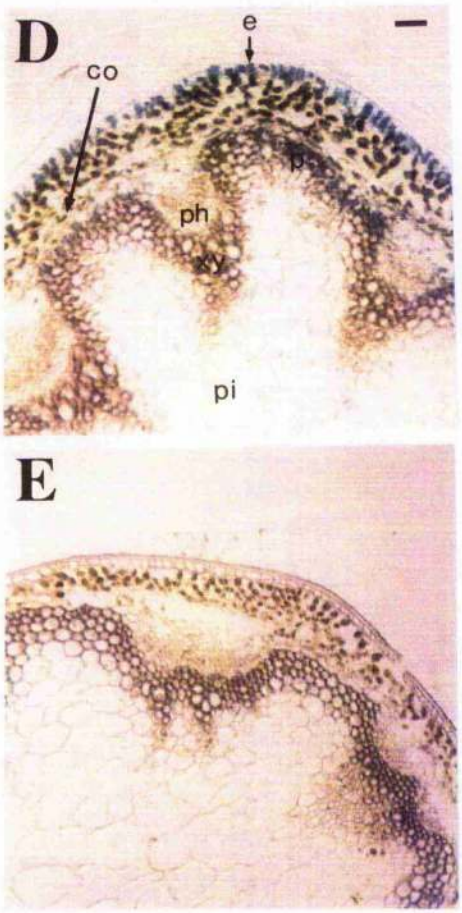
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co
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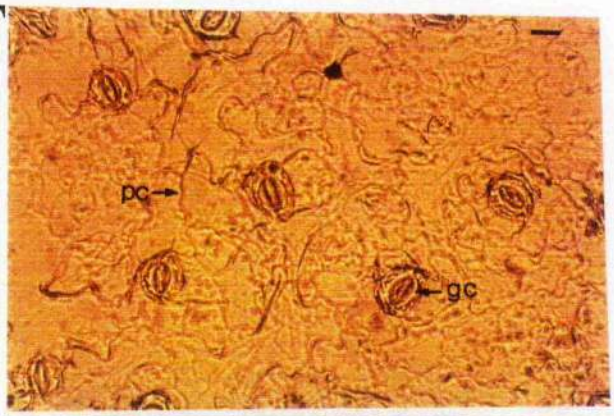
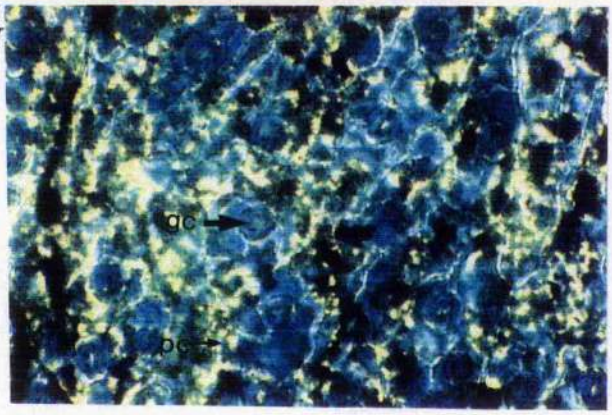
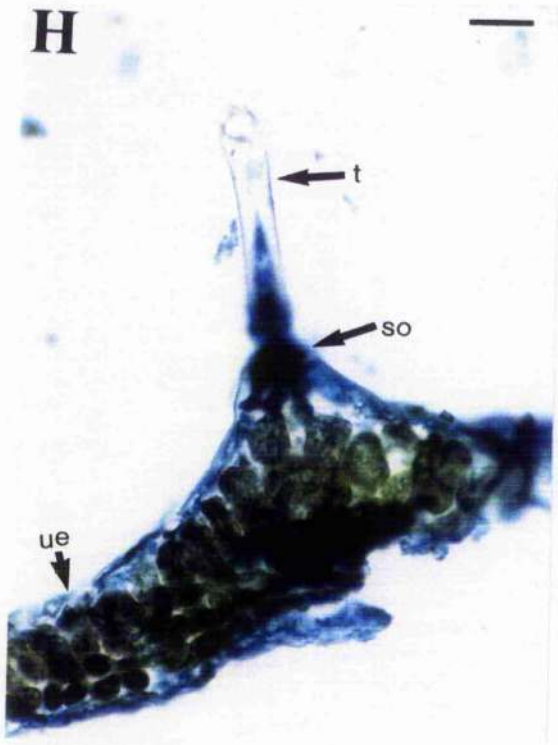
xy
pi
E

F



G




H
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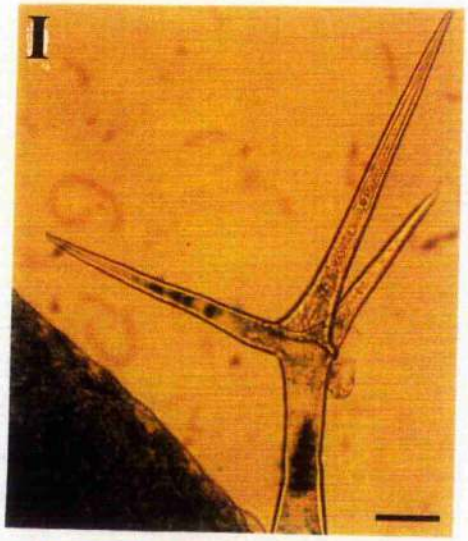

I

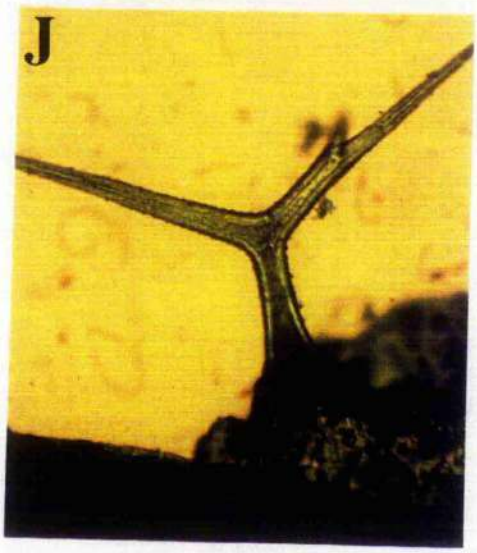
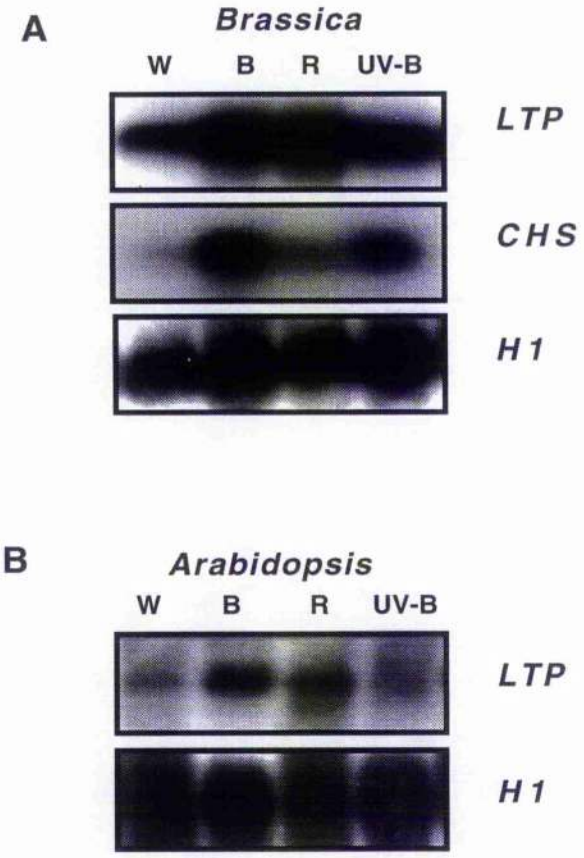

J

Fig. 6.5 Light induction of *LTP* transcripts

Brassica napus (A) and *Arabidopsis* (B) plants were grown in a low fluence rate of 30 ± 5 μmol m^{-2} s^{-1} of white light (W) for three weeks, then transferred to 90 ± 5 μmol.m^2 s^{-1} blue (B) or red (R) light or 3 μmol.m^{-2} s^{-1} UV-B light for 24 h. Plants were harvested and total RNA was extracted from leaves 10 μg of which was resolved on a gel, blotted onto nylon filters and hybridised to the *BnLTP* (A) or the *Arabidopsis LTP1* (B) cDNA. The blots were washed after autoradiography and rehybridised to the *Arabidopsis CHS* and *P. vulgaris H1* cDNAs.


A
Brassica
W
B
R
UV-B
LTP
CHS
H1
B
Arabidopsis
W
B
R
UV-B
LTP
H1

in red light treated plants, as reported previously for mature *Arabidopsis* leaf tissue (Jackson *et al.*, 1995; Fuglevand *et al.*, 1996). Expression of control *H1* transcripts (Lawton and Lamb, 1987) was unaltered under each condition. A similar pattern of expression was seen in *Arabidopsis* (Fig. 6.5B). The endogenous *LTP* transcript levels, measured by hybridisation to the homologous *LTP1* probe, CD3-11 (Thoma *et al.*, 1994), increased following blue and red light treatment, but not UV-B.

To determine whether induction of *LTP* expression by light was mediated by transcription, transgenic *Arabidopsis* plants carrying the *BnLTP-GUS* fusion were given the same light treatments as described above. Transgenic *Arabidopsis* plants containing the *CHS* promoter-*GUS* fusion (line NM4; Jackson *et al.*, 1995) were treated similarly and used as a control. Following blue and red light treatments, but not UV-B, *BnLTP-GUS* was moderately induced (Fig. 6.6A). Induction of *CHS-GUS* in response to blue and UV-B light was greater compared to *BnLTP-GUS*. In transgenic *Arabidopsis* plants carrying the homologous *Arabidopsis LTP1* promoter-*GUS* fusion (LTP-GUS 1A; Thoma *et al.*, 1994), GUS activity increased only slightly following blue light treatment whereas the increase in response to red light was not evident (Fig. 6.6B). A similar response was observed in the transgenic lines 1C and 3A.

6.6 Expression of *BnLTP* and *BnLTP-GUS* in response to cold and wounding

Fig. 6.7A shows the endogenous *LTP* transcript levels following a 72 h low-temperature treatment of *Brassica napus* leaf and root tissue. Cold treatment was at 4°C under 30±5 μmol m^{-2} s^{-1} white light. Control plants were placed at 22°C for the same time period under identical light conditions. Expression levels in leaves were unaltered by the low temperature treatment and the transcript was at the limit of detection in root tissue. Similarly, *LTP* transcripts levels were unaltered following a 24 h and 48 h cold treatment (data not shown).

Under the same conditions, the expression of the *BnLTP-GUS* fusion in transgenic *Arabidopsis* was moderately induced in leaf tissue following a 72 h cold treatment (Fig. 6.7B). There was a small, significant increase in *CHS-GUS* expression in control plants, consistent with previous studies of *CHS* expression (Leyva *et al.*,

Fig. 6.6 **Effect of light on the expression of *LTP* promoter-*GUS* fusions in transgenic *Arabidopsis***

Transgenic plants containing either the *BnLTP* promoter-*GUS* fusion (A, dark columns), the *Arabidopsis LTP1* promoter-*GUS* fusion (B, dark columns) or the *CHS* promoter-*GUS* fusion (A and B, light columns) were grown and illuminated as in Figure 6.5. GUS activity was measured in leaf tissue using MUG as a substrate in a fluorimetric assay and is expressed as units of 4-MU produced per unit leaf protein as described in section 2.29.2. Error bars indicate the standard error of the mean of at least three experiments; the absence of bars on some columns indicates that the error was very small.

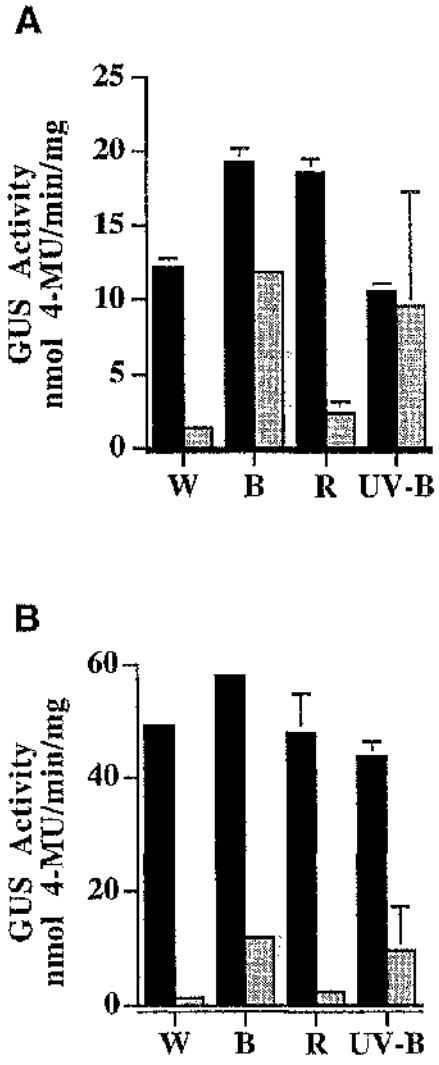
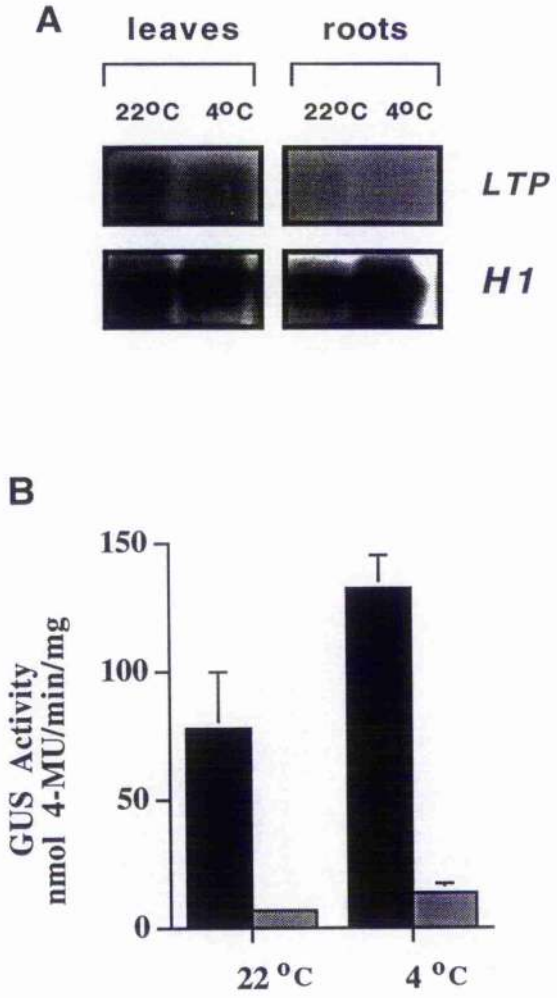

A
GUS Activity
nmol 4-MU/min/mg
25
20
15
10
5
0
W
B
R
UV-B
B
GUS Activity
nmol 4-MU/min/mg
60
40
20
0
W
B
R
UV-B

Fig. 6.7 Effect of low temperature on levels of *LTP* transcripts in *B. napus* and *BnLTP-GUS* expression in transgenic *Arabidopsis*

A. *B. napus* plants grown for three weeks in 30 ± 5 μmol m^2 s^{-1} white light were transferred to 4°C or kept at 22°C for 72 h. Total RNA was isolated from leaves and root tissue, resolved on a gel, blotted onto nylon filters and hybridised to the *BnLTP* cDNA. To ensure equal loading of RNA the blot was washed and rehybridised to the *P. vulgaris H1* cDNA. B. Transgenic *Arabidopsis* plants carrying the *BnLTP* promoter-*GUS* fusion (dark columns) or the *CHS* promoter-*GUS* fusion (light columns) were grown and treated as in A. GUS activity was measured in leaf tissue as in Fig. 6.6 (see section 2.29.2). Error bars represent the standard error of the mean of three experiments.


A
leaves
roots
22°C 4°C
22°C 4°C
LTP
H1
B
GUS Activity
nmol 4-MU/min/mg
150
100
50
0
22 °C
4 °C

1995). Transgenic plants cold-treated for 24 and 48 h were not altered in transgene expression (data not shown).

The *BnLTP-GUS* transgene was not induced upon wounding and neither was *CHS-GUS* (Fig. 6.8). Three week old leaves were pierced numerous times with a syringe needle and incubated for a further three days under 30±5 μmol m^{-2} s^{-1} white light and GUS expression was quantified in each wounded leaf. In addition, there was no evidence of local expression around the wound site (data not shown). Previously, endogenous transcripts isolated from *B. napus* leaf, stem and floral tissue, hybridised to the pLF3A cDNA, showed no effect in response to wounding and cold treatment (Pallas, 1992).

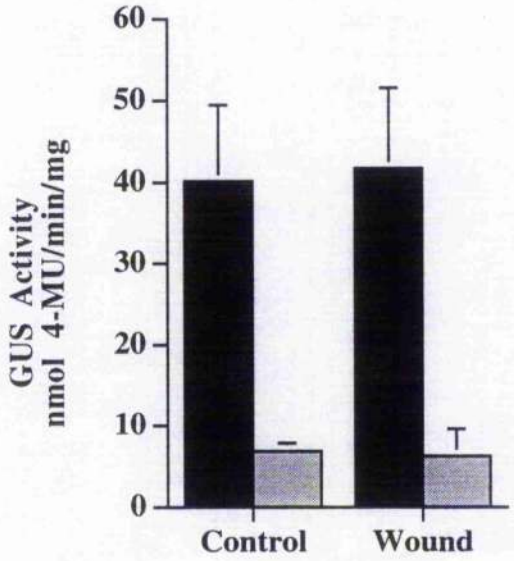
6.7 The *BnLTP* promoter is induced upon viral infection

There is growing speculation that LTPs play an important role in plant defence against invading pathogens. Indeed, there have been several reports of *LTP* transcript accumulation following bacterial and fungal infection (Garcia-Olmeda *et al.*, 1996; Pyee and Kollattukudy, 1995). However, no such experiments have been undertaken following viral infection. To determine whether *LTP* expression was affected following invasion by a virus, the cauliflower mosaic virus (CaMV) BARI isolate (Stratford *et al.*, 1988) was used. This isolate establishes a compatible interaction with members of the *Cruciferae* (Covey, 1991) characterised by local lesions and chlorosis on inoculated leaves, and vein clearing, enhanced greening of secondary leaves and stunted growth following systemic infection (Covey, 1991). However, in contrast to some isolates (Stratford *et al.*, 1988), BARI produces very mild symptoms. BARI infected plants are also slower in developing vein clearing symptoms compared to some severe isolates. There is no evidence of CaMV viral resistance in the ecotype Landsberg *erecta*.

The first and second emerging leaves of BnLTP-GUS2 (line 2D) transgenic *Arabidopsis* plants grown in a 10 h light, 14 h dark photoperiod, were inoculated with BARI. GUS assays were carried out on the inoculated leaf (leaf 1 or 2) plus a secondary leaf (leaf 3 or 4) after 0, 3, 7 and 14 days post inoculation (d.p.i). On 0, 3 and 7 d.p.i., GUS activity in the mock and virally infected leaves was unaltered. However, on day 14, when the virus is thought to start replicating in the systemically

Fig. 6.8 *BnLTP-GUS* expression in not affected by wounding

Transgenic *Arabidopsis* plants containing either the *BnLTP* promoter-*GUS* fusion (dark columns) or the *CHS* promoter-*GUS* fusion (light columns) were grown as in Figure 6.5 and leaves were wounded numerous times with a needle. GUS activity was measured in wounded and control leaf tissue after 72 h as in Figure 6.6 and section 2.29.2. Error bars represent the standard error of the mean of three experiments.


GUS Activity
nmol 4-MU/min/mg
60
50
40
30
20
10
0
Control
Wound

infected leaves (Covey, 1991; J. J. Milner, personal communication), GUS expression in leaves was elevated approximately 2 fold (Fig. 6.9). GUS expression in control NM4 plants was unaltered. The presence of virus in infected plants was demonstrated by PCR of genomic DNA isolated from virally infected leaves with virus specific primers (data not shown).

The virally and mock infected transgenic plants were also incubated with X-Gluc (Fig. 6.10). GUS activity in virally infected plants was enhanced in all the leaves and was activated in the root hairs (6.10A), where previously GUS expression was absent (6.10B). *CHS-GUS* expression in NM4 plants on the other hand was unaffected in both leaves and roots (data not shown).

6.7.1 *LTP* expression in *Arabidopsis* plants constitutively expressing the CaMV *Gene VI*

Although *LTP* expression was enhanced in response to viral infection it was not clear whether this was due to a specific interaction between viral components and the transgene. It has previously been shown that the 62 kD CaMV Gene VI product (PGVI) is a multifunctional polypeptide, which plays a central role in viral protein synthesis and DNA replication. The protein acts as a translational activator and contains an RNA-binding domain and a zinc finger domain (Turner *et al.*, 1996). Its expression is essential for symptom expression; this is supported by the observation that transgenic *Arabidopsis* plants that constitutively express *Gene VI* develop symptoms similar to plants infected with the native virus.

To determine whether *LTP* expression was enhanced as a direct result of CaMV Gene VI expression and therefore directly correlated with disease symptoms, endogenous *LTP* expression was examined in transgenic *Arabidopsis* plants expressing *Gene VI*. Three lines that constitutively expressed *Gene VI* at different levels, Baji-A1, Bari-B6 and Bji-C were examined. *LTP* expression was compared with that in untransformed plants. Phenotypically Bari-B6 exhibited very severe symptoms characterised by stunted growth and chlorosis of leaves (yellow leaves as a result of chloroplast bleaching). Bji-C3 and Baji-A1 on the other hand had less severe symptoms, the latter having very mild symptoms with a phenotype similar to non-transformed plants. Severe symptoms were correlated with enhanced *Gene VI*

Fig. 6.9 Induction of *BnLTP-GUS* expression in transgenic *Arabidopsis* by viral infection.

Transgenic *Arabidopsis* plants containing either the *BnLTP* promoter-*GUS* fusion (dark columns) or the *CHS* promoter-*GUS* fusion (light columns) were grown in a light/dark cycle as described in sections 2.28 and 2.28.4 and leaves were inoculated with the CaMV BARI isolate. GUS activity was measured as described in Figure 6.6 in leaves of BARI-and mock- inoculated plants, 7 and 14 days post inoculation (d.p.i.). Error bars represent the standard error of the mean of three experiments.

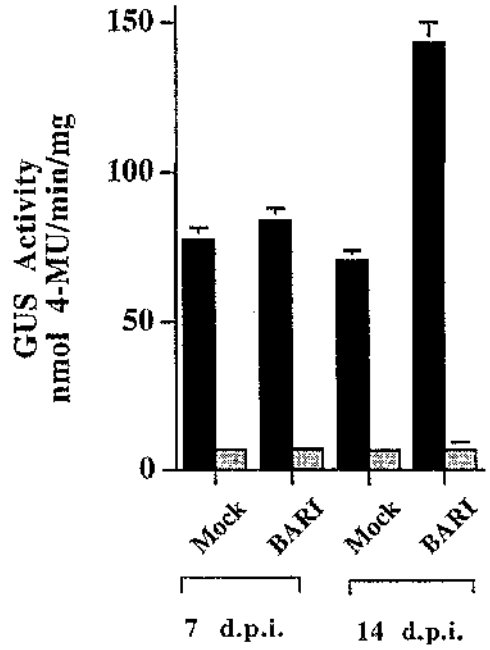
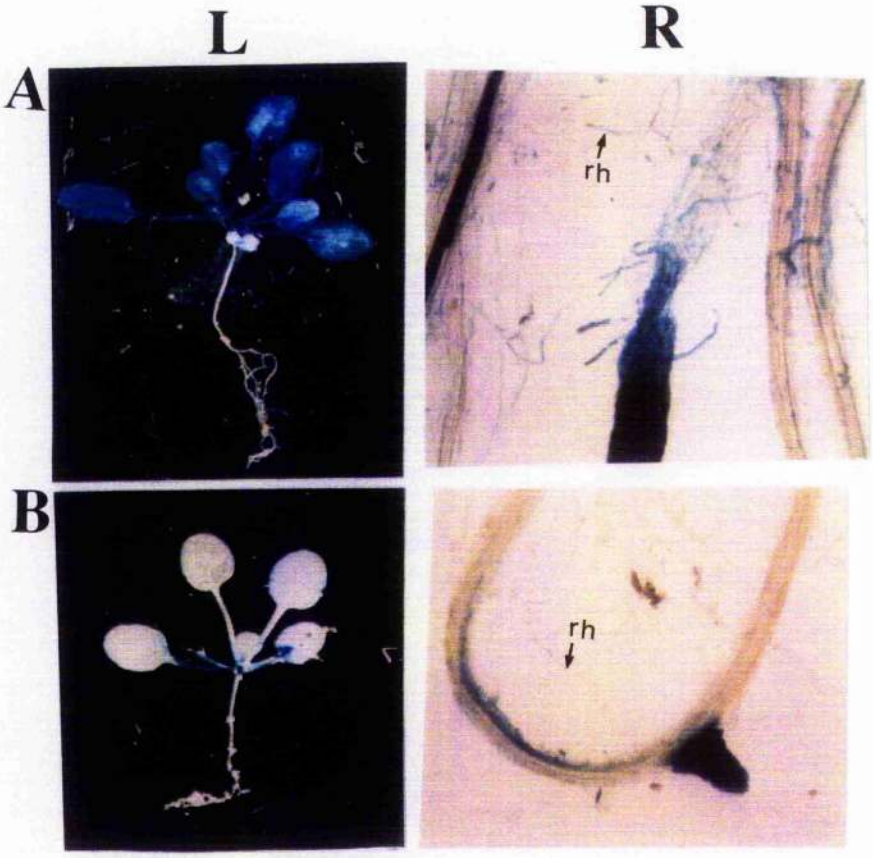

GUS Activity
nmol 4-MU/min/mg
150
100
50
0
Mock
BARI
Mock
BARI
7 d.p.i.
14 d.p.i.

Fig. 6.10 GUS expression in virally infected leaf and root tissue

Plants infected with virus for 14 days were incubated in X-Gluc to estimate overall GUS expression in BARI- (A, top panel) and mock- (B, bottom panel) infected leaves and roots.

L, leaves; R, roots; rh, root hair.


L
R
A
rh
B
rh

expression as indicated by increased Gene VI protein levels in ELISAs (E. Cecchini and J. J. Milner, unpublished data).

LTP expression was greatly enhanced in Bari-B6 (high *Gene VI* expressor) compared to Bji-C3 and Baji-A1, both of which have relatively lower levels of *Gene VI* expression (Figs. 6.11A and B). However, *LTP* expression in the latter plants was higher than that in non-transformed plants. It can be clearly seen that *LTP* and *Gene VI* expression levels show a direct correlation in that they are increased in plants exhibiting severe symptoms (Fig. 11B).

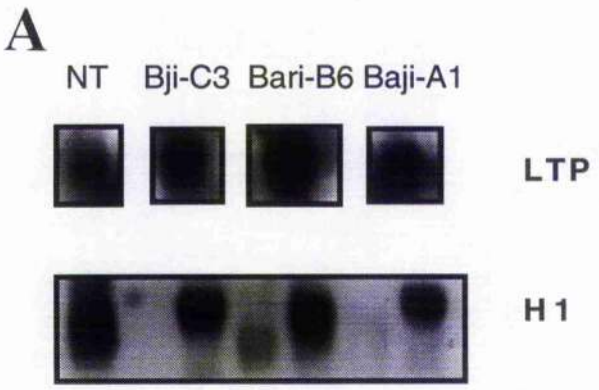
6.8 *LTP* expression in epidermally altered mutants, *gl1*, *gl2*, *ttg*, *icx1* and *icx1/ttg*

The glabrous *gl1* and *gl2* mutants (Koorneef *et al.*, 1983) are altered in trichome development. *gl1* is completely devoid of trichomes whereas *gl2* has very few trichomes. The *ttg* mutant not only lacks trichomes but is also altered in anthocyanin production (seeds are yellow), seed mucilage production and lacks *DFR* expression (Shirley *et al.*, 1995). The photoregulation mutant, *icx1* (see section 1.3.5.2, Jackson *et al.*, 1995), is altered not only in epidermal development but also in the light-regulation of *CHS* expression, which is an epidermal-specific gene. The gene products altered in these mutants are therefore concerned with the regulation of gene expression and development in the epidermis. Since *BnLTP* is expressed predominantly in epidermal tissue, as described in Fig. 6.4A, endogenous *LTP* expression was examined in these mutants.

Wild-type *Arabidopsis* and each of the mutants *icx1*, *ttg*, *gl1* and *gl2*, were grown at a fluence rate of 30 ±5 μmol m^{-2} s^{-1} white light (low white light, LW) for three weeks, then transferred to 100 ± 5 μmol m^{-2} s^{-1} white light (high white light, HW) for 24 h. Total RNA isolated from leaf tissue was hybridised to the *Arabidopsis LTP1* cDNA probe. *LTP* expression in *gl1*, *gl2* and *ttg* was identical to wild type plants under LW light and HW light grown plants, whereas *icx1* showed a considerable increase in *LTP* expression in response to HW (Fig. 6.12). Endogenous *CHS* expression was barely detectable in wild type and each of the mutants, including *icx1*, in LW light, whereas following 100 ± 5 μmol m^{-2} s^{-1} light treatment, expression was

Fig. 6.11 ***LTP*** **expression in transgenic** ***Arabidopsis*** **plants expressing the CaMV** ***35S*** **promoter-*Gene VI* fusion**

A. Non-transformed *Arabidopsis* (NT), Bari-B6, Bji-C3 and Baji-A1 were grown under 50 μmol m^{-2} s^{-1} white light for three weeks. Total RNA was isolated from leaves, resolved on a gel, blotted onto nylon and hybridised to the *Arabidopsis LTP1* cDNA. The blot was washed after autoradiography and rehybridised to the *P. vulgaris H1* cDNA. B. Relative levels of the endogenous *Gene VI* and *LTP* transcripts in non-transgenic and each of the transgenic lines, Bji-C3, Bari-B6 and Baji-A1 normalised for differences in RNA loading.


A
NT
Bji-C3
Bari-B6
Baji-A1
LTP
H1

B

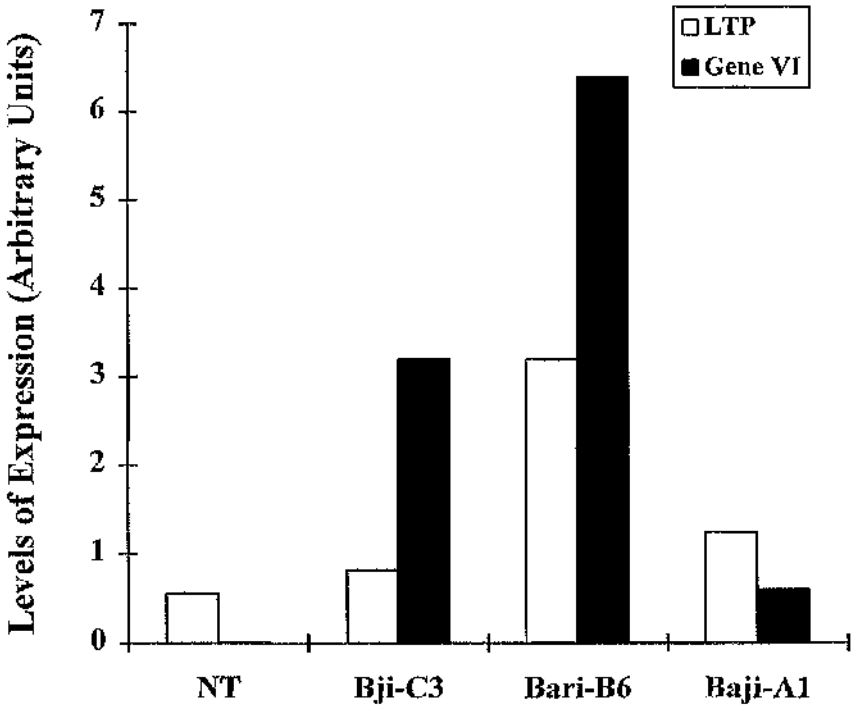
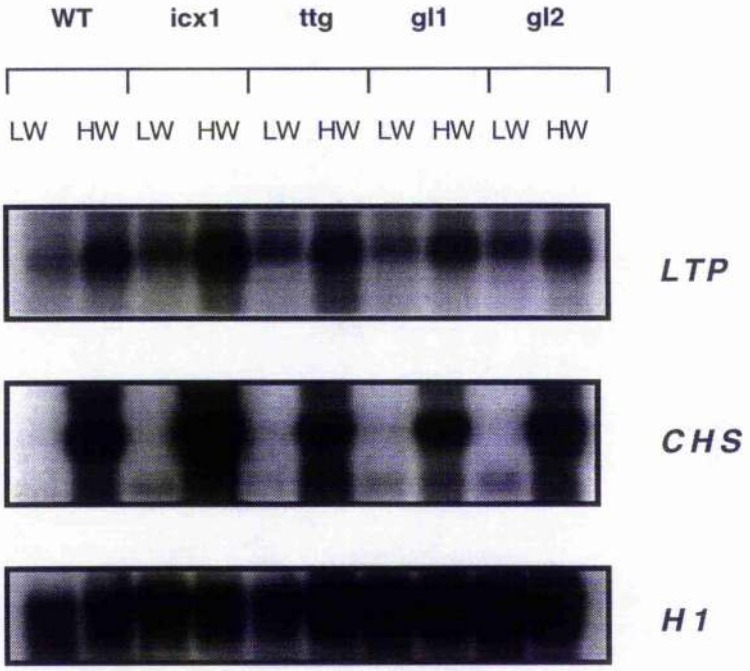

Levels of Expression (Arbitrary Units)
0
1
2
3
4
5
6
7
LTP
Gene VI
NT
Bji-C3
Bari-B6
Baji-A1

Fig. 6.12 ***LTP*** **expression in the** ***Arabidopsis*** **epidermally altered mutants** ***icx1, ttg, gl1*** **and** ***gl2***

Plants were grown in a low fluence rate of 30 ± 5 μmol m^{-2} s^{-1} white light (LW) for three weeks, then transferred to 100 ± 5 μmol m^{-2} s^{-1} white light (HW) for 24 h. Plants were harvested and total RNA was extracted from leaves, resolved on a gel, blotted onto nylon filters and hybridised to the *Arabidopsis LTP1* cDNA. The blot was washed after autoradiography and rehybridised to the *Arabidopsis CHS* and *P. vulgaris H1* cDNAs (WT, wild type).


WT
icx1
ttg
gl1
gl2
LW HW LW HW LW HW LW HW LW HW
LTP
CHS
H1

considerably induced. Again, *icx1* displayed a higher induction of *CHS* expression which was similar to previous observations (Jackson *et al.*, 1995).

An important observation was that *icx1* behaved like wild type plants in 30 ±5 μmol m^{-2} s^{-1} white and red light (data not shown), which suggested that the *ICX1* product affected only the light regulation of the genes discussed. Also as discussed previously (section 1.3.5.2), *icx1* is not only characterised by enhanced expression of genes regulating the flavonoid biosynthetic pathway including *CHS*, *DFR* and *CHI*, it is also altered in epidermal development. Analysis of *icx1* leaf epidermal strips by light-microscopy indicated the epidermal pavement cells were altered in shape (data not shown). They have an elongated, more disorganized appearance. Similarly, analysis of the epidermis by SEM displayed a rougher appearance (J. A. Jackson and G. I. Jenkins, personal communication). It is therefore likely that the normal function of *ICX1* is the regulation of epidermal-specific genes, some of which are subject to light regulation and some of which are concerned with epidermal development.

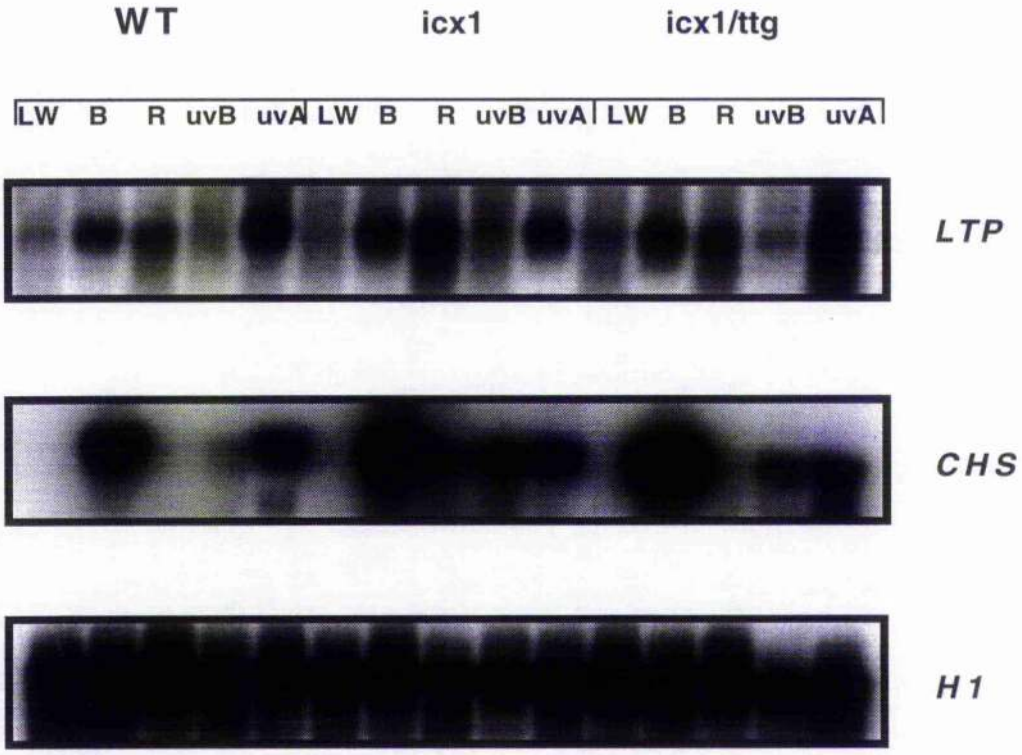
The *icx1/ttg* double mutant, which has phenotypic characteristics of both parents, was also examined for endogenous *LTP* expression. In wild type *Arabidopsis*, *icx1* and the *icx1/ttg* double mutant, *LTP* and *CHS* expression was determined in response to blue, red, UV-A and UV-B light (Fig. 6.13). In response to each light treatment with the exception of UV-B, *LTP* expression was induced. A striking observation was that *LTP* expression in the *icx1* and *icx1/ttg* double mutant was enhanced in response to blue, red and UV-A treatment; this was similar to the 100 ± 5 μmol m^{-2} s^{-1} white light results described previously (Fig. 6.12). In 30 ±5 μmol m^{-2} s^{-1} white and red light treated plants, *CHS* transcripts were barely present, whereas in response to blue and UV-A, *CHS* expression was enhanced considerably. The level of induction was higher than that observed for *LTP*. Expression of *CHS* was moderately induced in response to UV-B although it was not as high as observed in previous experiments (Fig. 6.5; Fuglevand *et al.*, 1996).

6.9 Discussion

There is some evidence that *LTP* expression is regulated in a spatial and temporal manner during development (Fleming *et al.*, 1993) and by environmental stimuli such as low temperature (Dunn *et al.*, 1991, 1994; Hughes *et al.*, 1992; White

Fig. 6.13 ***LTP*** **expression in** ***Arabidopsis*** **wild type,** ***icx1*** **and** ***icx1/ttg*** **mutants in response to blue, red, UV-B and UV-A light treatment**

Plants were grown in a low fluence rate 30 ± 5 μmol m^{-2} s^{-1} of white light (LW) for three weeks, then transferred to 90 ± 5 μmol.m^2 s^{-1} blue (B), red (R), and UV-A light or 3 μmol.m^{-2} s^{-1} UV-B light for 24 h. Plants were harvested and total RNA was extracted from leaves, resolved on a gel, blotted onto nylon filters and hybridised to the *Arabidopsis LTP1* cDNA. The blot was washed after autoradiography and rehybridised to the *Arabidopsis CHS* and *P. vulgaris H1* cDNAs. (WT, wild type plants).


WT
icx1
icx1/ttg
LW B R uvB uvA LW B R uvB uvA LW B R uvB uvA
LTP
CHS
H1

et al., 1994; Molina *et al.*, 1996), drought, ABA and salt stress (Plant *et al.*, 1991; Torres-Schumann *et al.*, 1992; Kahn *et al.*, 1993) and in response to bacterial and fungal invasion (Terras *et al.*, 1992; Molina *et al.*, 1993, 1996; Segura *et al.*, 1993). However, no such studies have been conducted with stably transformed promoter-GUS fusions nor has the expression of *LTP* genes been studied in response to light treatment and viral infection.

6.9.1 Analysis of the *BnLTP* promoter

Analysis of the *BnLTP* promoter sequence (Fig. 6.1) indicated the presence of a previously identified cis-acting element designated box 2 (consensus C/TCAACA/CAACCA/CC) implicated in light and stress inducibility of the bean and parsley *PAL* and the *Arabidopsis PAL* and *LTP1* promoters (Ohl *et al.*, 1990; Lois *et al.*, 1989; Thoma *et al.*, 1994). In addition, several sequence elements with the core sequence, ACGT (or TGCA) were identified. This core sequence is common to several light and ABA response elements in several genes including the *Arabidopsis PAL* and *CHS* genes, the barley *LTP* genes, *gbltp4.2*, *gbltp4.6* and *gbltp4.9* (White *et al.*, 1994) and the rice *rab16A* gene (Mundy *et al.*, 1990). Furthermore, in contrast to the parsley *PAL* gene which is stimulated by light and fungal elicitors, *CHS* expression is strongly induced by light and following light induction is strongly repressed by elicitor (Lozoya *et al.*, 1991). Such *CHS* responses are thought to be controlled by a promoter region designated Unit 1, which contains an ACGT element (Box II) (Lozoya *et al.*, 1991) and a myb recognition element (Feldbrügge *et al.*, 1997). A putative transcription factor, CPRF, which has high affinity for Box II and several other ACGT-containing elements, was shown to regulate the *CHS* gene (Feldbrügge *et al.*, 1994; Weißhaar *et al.*, 1991). It is possible that this factor may also regulate expression of *LTP* genes. However, while sequence similarities are interesting to note and may be functionally significant, no definitive conclusions can be drawn until detailed promoter analyses have been undertaken to identify critical functional elements.

6.9.2 Spatial regulation of *BnLTP* expression

Endogenous *B. napus LTP* transcripts were highly regulated and responded to organ-specific signals (Fig. 6.2). In all aerial organs including leaf, stem and floral

tissue (sepals, petals, carpels, stamens), *LTP* transcripts were expressed at high levels, but no expression was detected in roots. This pattern of expression was similar to other *LTP* genes, including those of *Arabidopsis* (Thoma *et al.*, 1993, 1994), broccoli (Pyee *et al.*, 1995) spinach (Bernhard *et al.*, 1991), barley (Gausing, 1994; White *et al.*, 1994; Molina *et al.*, 1993) cotton (Ma *et al.*, 1995) tomato (Torres-Schumann *et al.*, 1993) and castor bean (Weig and Komor, 1992). An exception is the tobacco *LTP* gene, *Ntltp1* described by Canevascini *et al.* (1996), where expression was detected in young root and root hairs. Sequence elements of the type, CAAA, which have been found in several root-specific promoters including the *HRGPnt3* (Keller and Lamb, 1989) and *TobR7* (Yamamoto *et al.*, 1991) genes, were observed in several regions of the *BnLTP* promoter (see Fig. 6.1 and section 6.1).

To determine the tissue and cellular location of expression, transgenic *Arabidopsis* plants expressing the *BnLTP* promoter-*GUS* fusion were examined. GUS was expressed from early germination in developing cotyledons, young leaves, stems and flower buds. As the plant matured, GUS expression decreased in mature leaves and cotyledons and became confined to the tips (Fig. 6.3A). In mature flowers GUS expression was very low in the sepals and no expression was detected in the petals (Fig. 6.3B). It was however present in the stamens and pollen. A flower-specific expression pattern was demonstrated for the *Brassica napus* E2 *LTP* (Foster *et al.*, 1992) which was shown to be expressed in developing microspores. E2 was expressed specifically in the tapetal cells. Foster *et al.* (1992) proposed that E2 may be involved in the transfer of lipids in both the tapetum and developing microspores of *B. napus*, functioning in the formation of new membranes and storage lipids. A similar suggestion was made by Sossountzov *et al.* (1991). However, amino acid sequence comparison of E2 with BNLTP indicates only a 40% identity which suggests BNLTP may have a different function in floral tissue. These observations clearly indicate that the *BnLTP* promoter is developmentally regulated being highly expressed in young developing tissues and less so in mature leaves.

In tissue cross-sections, GUS activity was shown to be predominantly epidermis-specific in mature leaves and stems (Fig. 6.4). This was similar to previous *in situ* experiments where pLF3A transcripts were detected predominantly in the epidermal cells of leaf, stem and sepal in *Brassica napus* plants (Pallas, 1992).

Interestingly, analysis of the *BnLTP* promoter indicated the presence of a putative sequence element (ATAGCTAG) implicated in epidermal-specific expression of a tobacco *LTP*, *Ntltp1* (Canevascini *et al.*, 1996). However, whether this element is functionally important in *BnLTP* remains to be determined. Epidermal-specific expression of *LTPs* in mature leaves and stems was also detected in other species including maize (Sossountzov *et al.* 1991), tobacco (Fleming *et al.*, 1993; Canevascini *et al.*, 1996) and barley (Molina and Garcia-Olmedo, 1993) and in other *Cruciferae* species including *Arabidopsis* (Thoma *et al.*, 1994) and broccoli (Pyee and Kolattukudy, 1994, 1995). However, epidermal specificity of *BnLTP-GUS* expression was not exclusive. Strong expression was observed in the vascular tissue (in the xylem and phoem) in leaves and in the cortical cells of the stem, which was similar to the *Arabidopsis LTP1* (Thoma *et al.*, 1994). Very faint expression was also observed in the spongy mesophyll and palisade cells, similar to the broccoli WAX9D (Pyee and Kolattukudy, 1995). This may however be attributed to physical damage of the tissue when sectioning was carried out. In addition, the specialised epidermal cells, trichomes, had particularly strong GUS expression suggesting that transcription factors that control trichome differentiation (Koorneef *et al.*, 1983; Hülskamp *et al.*, 1994) may act as positive regulators of the *BnLTP* promoter.

In contrast to the northern blot results (Fig. 6.2), GUS activity was also observed in the main root tip and the lateral root initials and remained confined therein as the initial extended. Failure to detect transcripts in root tissue in northern blots may reflect the small area in which the *LTP* gene was expressed. Also, in northern blots, *LTP* transcripts were highly abundant in petals whereas *BnLTP-GUS* expression was absent. This implies that other closely related members of the *Brassica napus LTP* gene family are upregulated in petals whereas the *BnLTP* promoter is not. This is not surprising, as a common characteristic of closely related genes is that their non-coding regions diverge extensively during evolution compared to coding regions, exemplified by the *HvLTP4* gene from barley (Molina *et al.*, 1996), which suggests that their expression patterns may be different. It seems that the *BnLTP* promoter is subject to organ-specific expression resulting from positively acting elements in young and green organs (leaves, stems, sepals) or from a negatively acting element leading to transcriptional repression in non-green organs (petals and roots). The above

observations are consistent with the hypothesis that LTPs are involved in wax/cuticle biosynthesis (Sterk *et al.*, 1991) as younger, developing organs (which express GUS at a much higher level than mature organs) synthesise more wax than mature organs (Bianchi *et al.*, 1985; Moose and Sisco, 1990). In addition, wax biosynthesis is predominantly epidermal-specific (section 1.4.1), similar to *BnLTP* expression, and the BNLTP protein is highly similar to the broccoli WAX9D protein, which was found to be the major surface wax-associated protein in broccoli leaves. Furthermore, expression of *BnLTP-GUS* in stigmas may be related to the deposition of surface lipids other than cuticular waxes (Thoma *et al.*, 1994).

LTP expression was not observed in the *Arabidopsis* cell cultures (data not shown) as these cells do not possess an epicuticular waxy layer, although Sterk *et al.* (1991) did observe expression of an *LTP* gene, *EP1*, in carrot cell culture. However, *LTP* expression is observed in non-epidermal cells (e.g. vascular tissue) and strongly in root initials, neither of which are likely to be highly active in wax biosynthesis. The purpose of this expression is less obvious.

6.9.3 Environmental regulation of *BnLTP* expression

Light plays an important role in the environmental regulation of plant development and gene expression (Jenkins, 1991; Chory, 1991). Light also influences the development and morphology of plant surfaces including the epicuticular waxy layer and leaf thickness (Caldwell *et al.*, 1983). Furthermore, with the isolation of several light-response mutants it is clear that many important aspects of growth and development are regulated by a range of photoreceptors including the well-characterised phytochrome family which absorb red and far-red parts of the spectrum (Furuya, 1993), the UV-A/blue photoreceptors (cryptochromes) one of which has been recently cloned (Ahmad and Cashmore, 1993) and the elusive UV-B-absorbing receptor(s) (Britt *et al.*, 1993; Jenkins *et al.*, 1995).

BnLTP-GUS expression and endogenous *LTP* transcripts in *Arabidopsis* and *B. napus* were induced in response to increased white light, blue and red light treatment but not UV-B. This suggests that the gene may be regulated by phytochrome and cryptochrome but not by the UV-B photoreceptor. The lack of induction of *LTP* expression by UV-B light was surprising as it is quite clear from a number of reports

(Reicosky and Hanover, 1978) that cuticular waxes play an important protective role against UV radiation and that LTPs are implicated very strongly in wax biosynthesis (Sterk *et al.*, 1991; Pyee and Kolattukudy, 1995; Thoma *et al.*, 1994). Therefore, one may have expected *LTP* expression to be induced by UV-B light. The low level *LTP* and *BnLTP-GUS* in response to UV-B light may be due to the damaging effect of UV-B to the plants.

The increase in *LTP* expression in response to light possibly reflects a general increase in metabolism and gene expression, including *LTP* gene expression, since the increase in light, specifically red and blue light, could stimulate photosynthesis. The consequent increase in energy and carbon skeletons for biosynthesis, including cuticular wax formation, may promote *LTP* expression. On the other hand, application of light in excess to plants grown in low light may saturate photosynthesis and act as a stress factor. One consequence of light-stress is the formation of free-radicals and oxidative stress which is harmful to chloroplasts (Aro *et al.*, 1992). In addition, *BnLTP* is expressed in the dark (data not shown), albeit at lower levels, which suggests it is not a truly light regulated gene, and high expression is associated with green, photosynthetically active tissues compared to non-green tissues as discussed previously. Analysis of the *BnLTP* promoter indicates the presence of light and stress inducible elements which are also present in the promoter of the light, stress and elicitor inducible gene *PAL* (Lois *et al.*, 1989; Ohl *et al.*, 1990). Moreover, in contrast to *CHS* and *CHS-GUS* expression, which are regulated by blue, UV-A and UV-B light but not by red light, and are not expressed in the dark or under low white light conditions (Jackson *et al.*, 1995; Fuglevand *et al.*, 1996), *BnLTP* and *BnLTP-GUS* were induced by red light.

Both *CHS* and *BnLTP* are epidermal-specific, suggesting that factors which determine epidermal-specificity recognise common sequence elements in their respective promoters despite the difference in their light regulation. Although a putative sequence element (CTAGCTAG) implicated in epidermal-specific expression of a tobacco *LTP*, *Ntltp1* (Canevascini *et al.*, 1996) was observed in the *BnLTP* promoter (ATAGCTAG), this elment was not observed in the *Araidopsis CHS* promoter suggesting additional elements may be required for epidermal-specificity.

The low level of *BnLTP-GUS* and *CHS-GUS* transgene induction in GUS assays (Figs. 6.6A and 6.6B) compared to endogenous *LTP* and *CHS* transcripts (Figs. 6.5A and 6.5B) may reflect greater stability of the GUS transcript compared to the *LTP* and *CHS* transcripts. However, this may also be due post-transcriptional regulation of the genes. That is to say, the level of induction of the promoters may not be the same as the level of transcript accumulation. Also, the regulation of the *BnLTP* gene may be different from the other genes that give rise to the *LTP* transcript level. Moreover, the *BnLTP* promoter activity may differ in *B. napus* and in the transgenic *Arabidopsis* plants. Interestingly, *BnLTP-GUS* expression in *Arabidopsis*, which is a heterologous system, showed a similar developmental pattern of expression to the homologous *Arabidopsis LTP1-GUS* transgene system described by Thoma *et al.* (1994), which suggests that both *Arabidopsis* and *Brassica napus* express transcription factors that recognize common sequence elements. The *Arabidopsis LTP1* promoter appears to be more active than *BnLTP* under 30 ±5 μmol m^{-2} s^{-1} white light (Figs. 6.6A and 6.6B) which may reflect positional effects on the transgene.

Unlike light induced expression of both endogenous *LTP* transcripts and the *BnLTP-GUS* fusion in transgenic plants, a different response was observed following cold treatment. *BnLTP-GUS* was induced in transgenic leaves whereas endogenous *LTP* transcripts did not show a response under the same conditions. This may be attributed to post-transcriptional regulation of the endogenous transcripts that alter transcript accumulation regardless of the level of promoter induction. However, a moderate increase of endogenous *LTP* expression was observed in *Brassica napus* stems (data not shown). This may be attributed to the accumulation of transcripts of other gene family members or due to differential post-transcriptional regulation of the transcript in stems. The increase in *BnLTP-GUS* expression observed (Fig. 6.7B) supports the hypothesis that LTPs are involved in the synthesis of the cuticle (Sterk *et al.*, 1991) as the waxy layer is important in protection against water loss.

Cell walls also play an important role in plant protection against cold temperatures. They play an important role in excluding ice from cells, which is very much dependent on cell-wall pores (Burke *et al.*, 1976). These pores have an important function in the movement of water from cells during freezing temperatures (Wisniewski *et al.*, 1987). In addition, the lipid composition of plant cell membranes is

altered in response to cold treatment (Johnson-Flanagan and Singh, 1987). As LTPs have been shown to be located in the epidermal cell wall (Thoma *et al.*, 1994) and the epicuticular waxy layer (Pyee and Kolattukudy, 1995), their induction in response to cold treatment may reflect their possible *in vivo* role in forming a protective barrier from dehydration in response to cold treatment. As LTPs transfer lipids between membranes *in vitro* (Arondel and Kader, 1990), the increase in *LTP* expression may reflect the alteration in membrane lipid composition in response to cold treatment.

There are several lines of evidence suggesting that ABA levels rise in response to cold treatment (Zeevaart and Creelman, 1988). Also, White *et al.* (1994) reported the induction of a barley *LTP* gene family in response not only to cold but also to ABA treatment. Two putative low-temperature response elements (LTRE) (ACCGACA) and an ABA-response element (ABRE) (GTACGTGG) with an ACGT-core sequence were located in the barley *gblt4.9* promoter (White *et al.*, 1994). Several sequence elements with the core ACGT or TGCA sequences were observed in the *BnLTP* promoter. These sequences are also located in the promoter region of two barley cold- and pathogen-induced *LTP* genes, *HvLTP4.2* and *HvLTP4.3* (Molina *et al.*, 1996). The sequences flanking the ACGT core in each case differed, which is possibly the mechanism for altering the specificity of transcription factor binding in response to different stimuli. The *CHS-GUS* expression (Fig. 6.7B) and endogenous *CHS* transcripts (data not shown) were also induced in response to cold treatment, which may reflect the presence of common regulatory elements in the two promoters that mediate cold perception.

Both the *Arabidopsis LTP1-GUS* and *B. napus BnLTP-GUS* transgene expression were not affected upon wounding (Fig. 6.8). This observation was unexpected as one would have expected an increase in *LTP* expression and increased extracellular wax deposition in the regions of wound repair. However, one cannot exclude the possibilty that the expression of other members of the *LTP* gene family may be increased in response to wounding.

6.9.4 Viral induction of *BnLTP* expression

The induction of *BnLTP-GUS* expression in response to viral infection (Fig. 6.9) supports the previously postulated theory (Garcia-Olmedo *et al.*, 1995) that LTPs

are involved in plant defence against pathogen attack. The first contact between pathogens and plants is with the epidermis. How this interaction affects host gene expression has many potential implications with particular emphasis on protection of economically important plants. Blocking the initial interaction of pathogen at the epidermis would require additional knowledge of epidermal-specific gene expression in response to viral invasion.

The host range of CaMV is mainly the *Cruciferae* family, which includes *Arabidopsis* and the *Brassicas*. A compatible infection is characterised by local lesions in the inoculated leaf and subsequent systemic infection. 2 to 3 weeks post inoculation infection is observed (depending on the strain) in secondary leaves, characterised by vein clearing and dark green, curly leaves (Covey, 1991). The virus is thought to move to the apical meristem where it infects cells of the leaf primodia. It is also thought to spread by cell-cell interaction via plasmodesmata. As plasmadesmata formation requires an increase in lipid metabolism (Lucas and Wolf, 1993), *BnLTP-GUS* expression induction in response to CaMV infection may reflect this phenomenon. Under optimal infection conditions, that are probably dependent on environmental conditions, the virus is thought to start replicating 14 days post inoculation (dpi). Non-replicating viral minichromosomes are also detected in the roots (E. Cecchini and J. J. Milner, unpublished data). In agreement with these observations, *BnLTP-GUS* expression was induced in CaMV infected plants 14 days post infection which suggests that viral replication and viral gene expression affects *LTP* gene expression.

It has been shown that the CaMV *Gene VI* product plays a central role in viral replication and symptom formation (Covey, 1991). Several *Arabidopsis* transgenic plants constitutively expressing the CaMV *Gene VI* display disease-like symptoms similar to plants infected with the native virus. More severe symptoms were correlated with elevated *Gene VI* expression. The Gene VI protein is a 62 kD translational activator, which has an RNA binding domain and a zinc finger domain (Turner *et al.*, 1996). Whether *Gene VI* expression directly affected *LTP* expression was examined by determining endogenous *LTP* expression in these plants. The high *Gene VI* expressers had higher *LTP* expression compared to low expressers (Fig. 6.11A and 6.11B). These results suggest that Gene VI may act as a translational activator of the *BnLTP-GUS* transgene. However, it is also possible that the *Gene VI* product regulates a different

component in the cell that affects *BnLTP-GUS* expression. It is also possible that factors which control *Gene VI* expression also control *LTP* expression.

These results support the previous hypothesis that LTPs may play an active role in plant defence against pathogens because the increase in *BnLTP-GUS* and endogenous *LTP* expression is correlated with *Gene VI* expression and therefore with disease symptoms. In addition, the high *Gene VI* expressers were also associated with severe symptoms, including chloroplast bleaching. These results suggest that the *Gene VI* product interferes with the normal chloroplast development pathway (possibly chlorophyll synthesis pathway).

The induction of *BnLTP-GUS* in virally infected root hairs (Fig. 6.10A) may reflect an osmotic stress response in the presence of viral minichromosomes in the root. Interestingly, White *et al.* (1994) also observed that a barley *LTP* whose expression was normally absent in roots was induced in response to drought, ABA and salt stress. Whether the virus minichromosomes located in the roots cause some form of osmotic stress remains to be seen. It has been postulated that a pea root epidermis-specific gene *RH2* that has 95 % homology to the pea PR protein 149a (Mylona *et al.*, 1996) contributes to a constitutive defense barrier in the root epidermis. A similar role has been proposed for chitinase pathogenesis-related proteins that are constitutively expressed in certain epidermal tissues (Mylona *et al.*, 1994). Also, PR expression in the pea root was shown to be induced in response to CaMV infection. It is possible that *LTP* expression utilizes the same defence pathway. The CAAA-like elements in the *BnLTP* promoter could mediate expression in roots in the presence of additional stimuli such as pathogen attack or osmotic stress.

Cramer *et al.* (1996) have shown that in *Arabidopsis* roots, both NaCl and ABA treatments influence internal Ca^{2+} levels. By using the Ca^{2+}-sensitive dye, Fura-2, and ratiometric techniques, Ca^{2+} levels were measured in cells of the root meristematic region. Results showed that by increasing external NaCl and ABA concentrations, Ca^{2+} levels in the meristem decreased. In addition, an *Arabidopsis* root-specific kinase homolog, ARSK1, was shown to be induced by dehydration, ABA and NaCl (Hwang and Goodman, 1995). This protein was shown to be expressed predominantly in the root epidermal cells and has been proposed to have a role in the signal transduction of osmotic stress. It is possible that the induction of *BnLTP-GUS* in root hairs in response

to CaMV infection may be mediated by Ca^{2+} and/or ARSK1. Related to this, Neumann *et al.* (1993), showed that several LTP-like proteins present in wheat and barley, were substrates of wheat Ca^{2+}-dependent protein kinases. They possessed several phosphorylation sites.

6.9.5 *LTP* expression in epidermally altered *Arabidopsis* mutants

A major problem in epidermal development studies is the lack of sufficient tissue for the isolation of genes expressed in the epidermis. With such a restricted cell type, the relative level of transcripts and protein products would be extremely small. Although differential and subtractive screening methods are useful, both depend on abundantly expressed genes which is certainly the case for many structural genes including *LTP*. However, regulatory genes are often expressed at low abundance and their isolation therefore requires a different experimental approach. A more informative approach is the isolation of genetically altered mutants. Several *Arabidopsis* mutants altered in various aspects of epidermal development have been isolated. An indication of altered expression in these mutants would not only enable us to correlate *LTP* expression with epidermal development but also enable us to isolate/characterise other genes (both regulatory and structural) implicated in epidermal cell differentiation. Amongst the five epidermally altered mutants studied (Fig. 6.12 and 6.13), only two, *icx1* and *icx1/ttg* showed an altered *LTP* expression pattern. The *icx1* mutation is characterised by enhanced *CHS* expression in white light (Jackson *et al.*, 1995). In addition, the *icx1* mutation affects epidermal development, including trichome development. Because *CHS* is expressed predominantly in the epidermis, this suggests that *ICX1* controls not only epidermal development but also epidermal-specific gene expression. Both *icx1* and *icx1/ttg* mutants were enhanced in endogenous *LTP* expression. The level of expression was even more elevated in response to blue and red light compared to wild type plants. Since *LTP* is an epidermally expressed gene, it is possible that *ICX1* encodes a factor that recognises a common sequence element in the *CHS* and *LTP* promoters that determines epidermal specificity. *ICX1* may function as a transcriptional repressor because the expression of other genes, including *DFR* and *CHI*, also showed enhanced expression in response to high white light in the *icx1* mutant (Jackson *et al.*, 1995). However, the expression of these genes is similar to

wild type under low intensity white light, suggesting that *ICX1* expression may be dependent on light. It seems that the normal function of *ICX1* is repression of *LTP* gene expression in response to high white, blue, red and UV-A light and, blue, UV-A and high white light in the case of *CHS* expression.

The similarity between the *icx1* and *icx1/ttg* in *LTP* and *CHS* expression suggests that the *ICX1* and *TTG* gene products do not interact in affecting the expression of these genes. The double mutant has phenotypic characteristics of both parents, suggesting that the two genes function independently and possibly at the same time during development.

Chapter 7 General Discussion

LTPs have been shown to be expressed predominantly in the epidermal layer of a number of plant species including tobacco (Fleming *et al.*, 1992; Canevascini *et al.*, 1996), *Arabidopsis* (Thoma *et al.*, 1993, 1994), tomato (Fleming *et al.*, 1993) and broccoli (Pyee and Kolattukudy, 1994, 1995). The data presented in this thesis strongly supports the above observations, in that a *Brassica napus* gene encoding a putative non-specific lipid transfer protein, BNLTP, is active predominantly but not exclusively in the epidermal cells of leaf and stem tissue. The recent finding that *LTPs* are expressed very early on in the protoderm (progenitor of epidermal cells) during embryogenesis, and throughout development in mature epidermal cells (Sterk *et al.*, 1991; Thoma *et al.*, 1994), makes them ideal molecular markers for the study of epidermal cell differentiation and patterning as demonstrated by Vroemen *et al.* (1996).

7.1 *BnLTP* represents a member of a small multigene family encoding a putative LTP

Analysis of the BnLTP cDNA, (Fig.3.6), showed that it encodes a putative LTP. It represented one member of a multigene family consisting of at least four genes. This conclusion was based on sequence analysis of the 3' non-coding regions of several full length cDNA clones (Fig. 3.9). This conclusion was also supported by previous Southern blot analysis (Pallas, 1992) where several *Brassica napus* genomic fragments hybridised to pLF3A. *LTP* gene families have been reported in other species including castor bean (Tsuboi *et al.*, 1991), broccoli, (Pyee and Kolattukudy, 1995), tobacco (Fleming *et al.*, 1992) and *Arabidopsis* (Thoma *et al.*, 1994).

The BNLTP protein satisfied all the structural features of a typical LTP (see chapter 3). It is likely that BNLTP is either membrane associated or secreted. The protein contained the characteristic eight conserved cysteine residues, which are thought to form four disulphide bonds (Désormaux *et al.*, 1992). The predicted tertiary structure of LTPs is thought to be that of a compact domain with four α-helices and a long COOH-terminus and a hydrophobic pocket that is able to accommodate acyl chains of lipid substrates (Østergaard *et al.*, 1995; section 1.4.3.2). Further understanding of the structure of LTPs and the role of the cysteines is likely to come from detailed X-ray crystallographic studies of the proteins. The conservation of

the cysteines in BNLTP indicates that it is functionally important. Hybrid-proline-rich cell wall proteins, (hyPRPs), some of which are also expressed in the epidermis (Wyatt *et al.*, 1992) and possess a signal peptide, also contain eight conserved cysteine residues in the carboxy terminus. Curiously, the distances between these are almost identical to those in LTPs (Fig. 3.6), which suggests a common function. The carboxy terminus of a *Brassica napus* hyPRP (Goodwin *et al.*, 1996) contains three membrane-spanning domains, which suggests that the protein is not fully secreted but is anchored in the plasma membrane. According to the hyPRP function model described by Goodwin *et al.* (1996), some of the cysteine residues of the protein would be exposed at the surface of cells having the potential to form disulphide bonds with other cysteine residues in the carboxy terminus or indeed with proteins in the vicinity. Whether LTPs anchored in the cell wall function in interacting with other cell wall components extracellularly is not yet known but may indicate a structural role for LTPs in protection against environmental stresses.

In order to isolate the promoter region of the *BnLTP* gene, several genomic clones were isolated from a *Brassica napus* cv. Bridger genomic library using the BnLTP cDNA as a probe (described in chapter 4). A 4.8 kb *Eco* RI fragment from one clone, 3.2A, contained the full length gene (Fig. 4.9). The structure of the gene was similar to that of other *LTP* genes, including in *Arabidopsis* (Thoma *et al.*, 1994). The deduced protein sequence of the gene differed at five amino acids from that encoded by the BnLTP cDNA (Fig.4.11B).

Primer extension analysis of the *BnLTP* promoter indicated a transcription start site at an adenine residue in the context CTCATCA, 59 bp from the TATA box (Fig.10). Most importantly the gene contained a 2.3 kb 5' upstream region which contained several elements that were conserved in the broccoli *LTP* genes and several elements found in light and stress induced genes (Fig.6.1; section 6.1). This region was cloned upstream of the *GUS* gene and the fusion was introduced into *Arabidopsis* plants (described in Chapter 5). The localisation of GUS would inevitably provide clues to the possible function of BNLTP *in vivo*.

7.2 *BnLTP* expression is subject to strong developmental and spatial regulation

The *in vivo* function of plant LTPs is open to debate. In animals at least three types of lipid transfer proteins are present. The first two classes have specific lipid binding properties whereas the third group has the ability to bind a wide range of lipids (Wirtz, 1991). This latter property is common to all plant LTPs analysed to date. They appear to have very little substrate specificity and are able to transfer a broad range of lipids substrates between membranes *in vitro* (Kader *et al.*, 1984). There is very little sequence homology between plant and mammalian LTPs.

In *BnLTP* promoter-*GUS* transformants, expression of GUS was regulated in an organ-specific manner (Fig. 6.3A), where younger developing organs had a higher GUS activity than mature organs. During flower development (Fig. 6.3B), *BnLTP-GUS* displayed differential expression, being associated with organs active in lipid biosynthesis (for example the anthers and stigmas). In contrast to indications from northern blots (Fig. 6.2), GUS activity was not observed in petals (6.3B) and was present in root tissues (Fig. 6.3C). These differences may be attributed to differential expression among *LTP* gene family members in *B. napus*. A more accurate approach in determining the spatial regulation and expression of individual gene family members would be to use gene-specific oligonucleotides and perform RT-PCR using RNA from different organs (Simpson *et al.*, 1992). The method requires minimum amounts of tissue, and the organ-specifc expression of each gene member can be determined. Furthermore, the cell specific expression of each family member could be determined by isolating the individual gene promoters and locating GUS activity of each promoter-GUS fusion.

One clear difference between the *in situ* hybridisation and the histochemical localisation of GUS expression was the failure to detect *LTP* transcripts in the vascular tissue during *in situ* hybridisation experiments (Pallas, 1992). This can be attributed to the fundamental difference in the two techniques. *In situ* hybridisation determines mRNA distribution at a given time whereas GUS histochemistry reveals the accumulation of the GUS protein over a period of time. Furthermore, *GUS* transcripts have a relatively longer half life, of around 60 h, and will therefore remain longer in a

given cell compared to most endogenous transcripts. It is possible that the *BnLTP* transcripts in the vascular tissue are not very stable and therefore were not detected during the *in situ* hybridisation experiments. It is also likely that the two different transcripts may be subject to different post-transcriptional regulation. A similar argument can be applied, in part, to the lack of detection of *LTP* transcripts in roots (Fig. 6.2). Also, during *in situ* hybridisation it is difficult to distinguish between closely related, highly similar transcripts as would be the case for the *LTP* gene family, whereas analysis of promoter-*GUS* fusions focuses on a single promoter.

The high level of GUS in the epidermal cells of leaf (Fig. 6.4A and 6.4C) and stem tissue (6.4D) is consistent with the speculation that BNLTP is involved in the transport of cuticular components to the surface (Sterk *et al.*, 1991; Thoma *et al.*, 1994). This is supported by the observation that BNLTP is highly similar to a broccoli leaf major surface protein, WAX9D (Pyee and Kolattukudy, 1995) (see chapter 3, Fig. 3.6), which suggests that BNLTP has a similar function to WAX9D. Pyee and Kolattukudy (1995) hypothesised that the WAX9D protein functions in the transport of cuticular monomers to the surface of the plant. They also demonstrated that WAX9D was present in the cell wall of epidermal and mesophyll cells as well as in the phloem. In addition, Thoma *et al.* (1993, 1994) and Sossountzov *et al.* (1991) demonstrated the location of LTPs in the cell wall.

LTPs appear to be important components in the formation of a "normal" epidermal cell wall. For example, Vroemen *et al.* (1996) demonstrated that the *Arabidopsis LTP* displayed a position-specific expression pattern in the epidermal layer of *Arabidopsis* embryos. However, this spatial expression was altered in the *Arabidopsis* mutant *knolle* (see section 1.3.2.1), which is altered in epidermal cell development. *KNOLLE* (Lukowitz *et al.*, 1996) encodes a protein similar to syntaxins, which are involved in vesicular trafficking. Furthermore, *knolle* mutants contained many incomplete cell walls and walls with small holes, which suggested that *KNOLLE* controls the transport of a variety of cell wall components to the surface. In view of these observations, it is unlikely that LTPs are involved in the intracelluar transfer of lipids between membranes as previously thought, as this would require a predominantly cytoplasmic location. The expression of *BnLTP-GUS* in the stigmas (Fig.6.3B) is

consistent with the idea that LTPs are involved in lipid mobilization such as secretion of stigma lipids (Thoma *et al.*, 1994).

BnLTP-GUS expression, like *WAX9D-GUS*, was observed at low levels in mesophyll cells of leaves and stems (Fig. 6.3A, 6.3C and 6.3D) whereas strong expression was observed in the vascular tissue of leaves and stems (Fig. 6.4C and 6.4D) and in the lignified tips of cotyledons (Fig. 6.3A). The role of BNLTP and WAX9D in the latter two tissues is less obvious. However, these observations suggest that transcription factors which control genes involved in the lignin biosynthesis may also regulate the *BnLTP* gene by recognizing specific elements in the promoter region.

Lignification is a process that occurs through a series of enzymatic steps initiated by a PAL-catalysed reaction to produce lignin precursors, the cinnamyl alcohols, which are derived from phenylalanine. The lignin monomers are polymerised into a complex phenolic polymer that forms a matrix in plant cell walls particularly in the vascular bundles (Lewis and Yamato, 1990). *PAL* and *4CL* are expressed in lignified xylem and in the vascular bundles (see section 1.4.2.2). If indeed *BnLTP* expression is coordinately expressed with the lignin biosynthetic pathway, which is a branch of the general phenylpropanoid biosynthetic pathway, it would be expected that genes such *PAL* and *4CL*, which are key enzymes in the lignin biosynthesis, would show a similar spatial expression pattern to *BnLTP*. Indeed both *PAL* and *4CL* transcripts are readily detected in the epidermal tissue (Wu and Hahlbrock, 1992; Jahnen and Hahlbrock, 1988), and *BnLTP-GUS* expression is observed in the xylem and vascular bundles. It is likely that several common elements that determine tissue-specificity may be identified in these promoters.

Canevascini *et al.* (1996) identified an element designated the D1 box (CTAGCTAG) in the promoter region of several *LTP* genes including those of tobacco (Canevascini *et al.*, 1996), *H. vulgare* cv. Bomi (Linnestad *et al.*, 1991; Kalla *et al.*, 1994) and S. *vulgare* (Pelese-Siebenburg *et al.*, 1994). This element is implicated in epidermal-specific expression. In the *BnLTP* promoter, an imperfect element with the sequence ATAGCTAG was identified at position -191 (Fig. 6.1), which may be involved in epidermal-specificity. Whether this element determines epidermal specificity of *BnLTP* expression could be tested by generating promoter deletions of

the transgene and expressing them in transgenic plants. How the expression of these genes is maintained throughout epidermal development and how the expression patterns in young and mature tissue are differentiated is not known, although differential screening and the isolation of mutants altered in candidate gene expression, may point us in the right direction. Also, protein extracts from young and old epidermal tissue can be used in gel-retardation experiments as an attempt at isolating developmental- and tissue-specific transcription factors and identifying the corresponding target sequences of the *BnLTP* promoter.

BnLTP expression appears to be associated with young, rapidly dividing cells and is demonstrated by the high level of *BnLTP-GUS* expression in developing trichome cells (Fig. 6.4H), although the expression of GUS in mature trichomes varied considerably (Fig. 6.4I and J). These observations suggest that trichome progenitor cells contain transcriptional activators that upregulate *BnLTP-GUS* expression. The *Arabidopsis GL1* and *GL2* genes are known to be upregulated in progenitor cells (see section 1.3.5.1) that are committed to form trichomes and in the meristematic region of the root (Galway *et al.*, 1994). *GL1* and *TTG* are necessary for trichome formation (Hülskamp *et al.*, 1994). The *GL1* gene product encodes a member of the myb family of transcription factors (Oppenheimer *et al.*, 1991) and like several other plant myb proteins contains two repeats of the myb DNA-binding domain at the NH_2-terminus and an acidic COOH-terminus with no similarity to other myb proteins. The *GL2* gene encodes a homeodomain protein (see section 1.3.5.1). The *TTG* gene has not been fully characterised, although its expression is essential for trichome development. The *Arabidopsis ttg* mutants, which lack trichomes can be complemented by the maize *R* gene which encodes a myc-like transcription factor (Lloyd *et al.*, 1992). Therefore, *TTG* may encode an *R* gene homolog. It is possible that as *TTG* and *GL1* promote trichome development, they may also promote *BnLTP-GUS* expression. Moreover, two myb-binding elements CAACTA, (-146 to -141) and CAACTT (-610 to -605), are located in the *BnLTP* promoter (Fig. 4.9). However, in the *Arabidopsis* mutants *gl1 gl2* and *ttg*, all of which lack trichomes, endogenous *LTP* expression was similar to that in wild type plants, which suggests that other transcription factors are responsible for the basal level of *LTP* expression.

7.3 *BnLTP* expression displays a complex response to environmental stresses

It is clear from the above discussion and from Chapter 6, Figs. 6.2, 6.3 and 6.4, that *BnLTP* gene expression is under tissue- and cell-specific developmental regulation. However, data presented in Figs. 6.5 to 6.13, demonstrates that there is also complex environmental regulation of the *BnLTP* gene.

The gene is induced in response to light, in particular high white, red, blue and UV-A, but not in response to UV-B (Figs. 6.5 and 6.6). The transgene, but not endogenous *LTP* transcripts, was induced in response to cold treatment (Fig. 6.7). Moreover, GUS activity was enhanced in response to viral infection in both leaf tissue (Figs. 6.9 and 6.10A) and root hairs (Fig. 6.10B), but not in response to wounding (Fig. 6.8). Although several sequence elements implicated in stress responses are present in the *BnLTP* promoter (Fig. 6.1), additional experiments are required to elucidate the cis-acting elements implicated in the different responses. To do this, *BnLTP* promoter deletion constructs can be expressed transiently in cell cultures or stably in transgenic plants. Furthermore, expression could be assayed in the presence of pharamacological inhibitors that are known to inhibit various components of the signalling pathways (see for example, Christie and Jenkins, 1996). In this way, we can begin to functionally define biochemically and molecularly the various components of the signalling pathway during *LTP* expression.

The function of BNLTP in response to light stresses is not immediately obvious, although responses such as thickening of the leaf and the development of a more glaucous appearance (possibly due to increased wax biosynthesis) may indicate a role in the formation of a protective waxy layer. In addition, the *icx1* mutant (section 1.3.5.2), which displays an altered epidermal surface, is altered in *LTP* expression in response to light (Fig. 6.10). In order to investigate the reason for this alteration, *icx1* plants expressing the *BnLTP-GUS* transgene can be generated by genetically crossing *icx1* with the BnLTP-GUS2 transgenic plants, and following GUS activity histochemically. For example, the increase in *LTP* expression in *icx1* may be due to other cell types, such as the spongy and palisade mesophylls, displaying an increase in *LTP* expression. Similarly, protein extracts taken from low and high light treated *icx1* and wild type plants, can be used in gel-retardation experiments to evaluate the differences in binding activities between the two extracts to the *BnLTP* promoter.

Although *BnLTP* expression was not induced in response to cold, some *LTPs* are clearly induced in response to low temperature (Hughes *et al.*, 1992). Freezing can induce dehydration, which can result in the collapse of the protoplasm (Pearce and Ashworth, 1990). As the waxy layer is important in reducing water loss and LTPs have been implicated in formation of the cuticle, it is possible that in response to cold treatment, LTPs function in reinforcing the waxy layer. It is also possible that LTPs strengthen the epidermal cell wall by physical interaction with each other and with other cell wall proteins expressed in the epidermis, possibly similar to hyPRPs (Goodwin *et al.*, 1996). This could be investigated by the constitutive over-expression of LTPs in transgenic plants and measuring whether the plants with this phenotype become more cold tolerant. Indeed, similar experiments can be carried out to investigate tolerance to other environmental stresses. Related to this, Molina *et al.* (1996) demonstrated an increased resistance to pathogens of plants constitutively expressing LTPs.

In view of their ability to inhibit bacterial and fungal pathogens *in vitro* and their distributions at high concentrations on aerial, exposed surfaces and in the vascular tissue, LTPs may play an active role in plant defence (see section 1.4.3.6). The data presented in this work also supports this view, demonstrated by the induction of *BnLTP-GUS* expression in response to infection with the CaMV BARI isolate (Fig. 6.9). The mechanism of anti-pathogenic activity *in vivo*, if any, is not known. However, direct assays of the effects of purified LTPs on pathogen growth and metabolism would be informative in this respect.

In summary, the isolation of *BnLTP* and its expression studies described in this thesis are a step forward in understanding the *in vivo* function of a gene implicated in a myriad of complex functions including, cuticle formation, wax biosynthesis, lipid mobilization and protection against environmental stresses. Furthermore, the early expression of some *LTP* genes during embryogenesis in the protoderm and during later stages of epidermal development, render them ideal candidates as molecular markers in the study of epidermal cell fate of aerial organs. However, their use in the study of root epidermal cell fate is questionable due to the lack or low level of expression observed in this tissue.

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