Studies on gene expression during flower development in *Brassica napus*

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Contents

Abbreviations

BSA	bovine serum albumin
cpm	counts per minute
d	days
dNTP	deoxyribonucleoside triphosphate
DIG	digoxigenin
DNAase	deoxyribonuclease
EDTA	diaminoethanetetraacetic acid, disodium salt
EtBr	ethidium bromide
GUS	β-glucuronidase
h	hours
HPRI	human placental ribonuclease inhibitor
IPTG	isopropylthiogalactoside
MES	2(N-morpholino)ethanesulphonic acid
min	minutes
MOPS	3-morpholinopropane sulphonic acid
NaOAc	sodium acetate
NaPPi	sodium pyrophosphate
NH_4OAc	ammonium acetate
NTP	ribonucleoside triphosphate
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
psi	pounds per square inch
PVP	polyvinyl pyrrolidine
RNAase	ribonuclease
s	seconds
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

Summary

Floral development is central to the life cycle of the plant. It is the most complex example of tissue differentiation, and as such is appropriate to study in order to gain more knowledge of how a plant develops. In recent years the understanding of flower development has been greatly advanced by molecular and genetic studies of floral mutants of two species, *Arabidopsis thaliana* and *Antirrhinum majus*. In order to obtain a panel of genes which are expressed in the early stages of floral morphogenesis, it was decided to make and differentially screen a cDNA library. However the *Arabidopsis* plant is small and it is difficult to obtain sufficient tissue to allow the production of a floral apex cDNA library. Therefore *Brassica napus*, a closely related member of the Cruciferae, was used to construct a cDNA library from floral buds at an early stage of morphogenesis.

The floral development of *Brassica napus* was investigated by light and scanning electron microscopy. To isolate genes involved in early flower morphogenesis a cDNA library was made to floral buds of *Brassica napus* exhibiting sepal primordia only. Differential screening of this library yielded several cDNAs the genes of which show enhanced expression in immature floral buds.

These cDNAs are not expressed in roots. The transcripts represented by two of the cDNAs, pLF3 and pLF10B, are present at low levels in leaf, seedling, flower tissue and seed pods. The genes corresponding to pLF2 and pLF7 are expressed in vegetative apices and are up-regulated in floral apices. The levels of the pLF2, pLF3, pLF7 and pLF10B transcripts do not increase on wounding, heat-shock or cold treatment of tissue. However, pLF5 shows a strong and rapid induction in both leaves and flower buds when plants are transferred to a low temperature, which does

not occur in wounded or heat-shocked leaves. The up-regulation of this gene in response to cold temperatures is rapid, reaching a maximum within 12 hours.

The genes represented by these cDNAs are present in the *Arabidopsis thaliana* genome at a low copy number, and are expressed in the floral tissue of *Arabidopsis*. The expression of the genes represented by pLF3 and pLF10B is not altered in the *Arabidopsis* homeotic mutants, *apetala-1*, *apetala-2*, *agamous*, *pistillata* and *clavata-2*. It was not possible to investigate expression of the genes represented by pLF2 and pLF7 in the *Arabidopsis* floral mutants by northern analysis.

In situ hybridisation experiments have shown that pLF10B is expressed in vascular tissue of stem and leaves, and hybridisation appears to be confined to the phloem vessels. pLF3 is epidermis-specific and is expressed in stem, flower buds and leaves. pLF2 and pLF7 do not seem to be expressed in a specific location in the floral apex.

Transgenic *Arabidopsis* plants have been generated which contain an antisense copy of pLF2 under control of the CaMV 35S promoter. These plants do not show any visible alteration of phenotype. The sequencing of the cDNAs has been initiated. Preliminary analysis of the LF sequences shows no homology to those in the GenEMBL databases.

Chapter 1: Introduction

1.1 Flower development

While the field of plant molecular biology has progressed rapidly over the previous decade, until recently there have been few advances in understanding the molecular basis of plant development. Most progress has been made in the area of floral morphogenesis where a few model systems such as *Arabidopsis thaliana* and *Antirrhinum majus* have been used to derive genetic models of flowering and allow the testing of these models using molecular techniques (Coen and Meyerowitz, 1991). The development of the flower is central to the life-cycle of the plant and is probably the most complex example of differentiation. Although it occurs as a continuous process, floral development can be divided into two phases; floral induction and flower morphogenesis. Within these two phases three sets of genes can be seen to act; the genes which are involved in floral induction, the genes which control organ positioning and specification within the flower, and floral-specific genes whose expression results in production of the final physical form of each organ. Various methods have been employed to isolate and characterise these genes, and assess their function. Research in the area of floral development will lead to the understanding of how a plant interacts with it's environment as well as how the plant organs signal to each other in order to effect developmental switches. This research may also prove of commercial importance in allowing genetic manipulations to increase the yield of seeds, for example, or production of male-sterile lines.

1.2 Model systems to study flower development

Flower development has been studied in many species but the most progress has

been made with one or two model systems. Floral induction can be best studied in those plants which are induced by a single photoperiod, such as *Sinapis alba* or *Pharbitis nil* (reveiwed in Bernier, 1988). However there are few floral mutants available for these species and they are not suitable for studying morphogenesis using molecular genetics. *Arabidopsis thaliana* has been used extensively for molecular and genetic studies. It has a small genome (70-100,000 kbp), with few repeated sequences which makes it amenable to chromosome walking experiments. The mature plant is small and can be easily grown at high density, and has a short life-cycle of six weeks from seed to seed. There are several floral mutants available, which have been well studied (e.g. Bowman et al., 1989; Schultz and Haughn, 1991) and some of the genes controlling floral morphogenesis have been cloned (e.g. Yanofsky et al., 1990). There are also late flowering mutants of *Arabidopsis* which may provide information about the process of floral induction. *Sinapis* and *Arabidopsis* are both members of the Brassicaceae, and genes isolated from *Arabidopsis* should have corresponding roles in floral induction in *Sinapis*. One disadvantage of *Arabidopsis* is the small size of the plant which makes it difficult to obtain sufficient material for the construction of organ-specific cDNA libraries.

The work on floral morphogenesis in *Arabidopsis* has been complemented by corresponding studies using *Antirrhinum majus* (Coen and Meyerowitz, 1991). *Antirrhinum* has large flowers, which can easily be scored for floral mutations. There are several floral mutants available in *Antirrhinum*, some of which correspond to mutations isolated in *Arabidopsis*. *Antirrhinum* has a family of active transposons present in it's genome and some of the mutants have been isolated from a transposon mutagenesis experiment (Carpenter and Coen, 1990). Genes involved in floral morphogenesis have been cloned using these floral mutants (e.g. *floricaula*; Coen et al., 1990) and the corresponding genes isolated from *Arabidopsis* (Coen and

Meyerowitz, 1991). However *Antirrhinum* cannot yet be transformed and is not as amenable as *Arabidopsis* to molecular genetic studies.

1.3 Ways to isolate genes involved in flower development

1.3.1 Protein-based methods

Proteins are the end products of gene expression, and one way to identify genes involved in flower development is to first isolate proteins specific to the flower. The identification of the genes encoding the pistil-specific self-incompatibility glycoproteins of *Nicotiana alata* occurred initially by purifying an S-locus glycoprotein and obtaining partial amino acid sequence to derive a probe for screening cDNA libraries (Mau et al., 1991). Other approaches have centred on identifying a panel of flower-specific proteins. Evans et al. (1988) have produced a range of monoclonal antibodies which recognise either anther-specific or pistil-specific antigens of the tomato flower. However most of the antibodies recognise carbohydrate epitopes, and are useless for screening cDNA expression libraries. While antibodies recognising glycoproteins are useful as developmental markers (e.g. Pennell et al., 1991) other methods must be adopted to obtain floral-specific proteins to ultimately isolate the corresponding genes. Lifschitz has produced a panel of partially purified proteins present in flowers which can be identified by two-dimensional PAGE, and antibodies have been raised to several of these proteins for use in screening cDNA expression libraries (reviewed in Gasser, 1991).

1.3.2 Differential screening

Differential screening relies on the assumption that certain genes are expressed

more abundantly in one organ type than another. A cDNA library is constructed of mRNA from the tissue of interest, which contains transcripts unique to or highly abundant in that tissue. Replicate plaque filters are hybridised with probes derived from mRNA from the tissue of interest (the plus stage) and mRNA from another tissue which contains transcripts common to both tissues and not those unique to or less abundant than in the tissue of interest (the minus stage) (St. John and Davis, 1979). Transcripts which are more abundant in the tissue of interest can then be detected by their increased hybridisation to the 'plus' probe, compared to the 'minus' probe, upon comparing the filters. This technique can be used to identify genes expressed preferentially in a specific organ, or at a specific stage in development. The library can only be screened at a low plaque density to prevent masking of weak signals by those corresponding to highly abundant transcripts, and therefore this technique can be time-consuming and limited in sensitivity.

Panels of floral specific genes have been isolated using the technique of differential screening by several groups (e.g. Gasser et al., 1989; Scott et al., 1991; Melzer et al., 1990). Olszewski et al. (1989) have used a dual-labelling method to identify gibberellin-regulated genes from tomato flowers. The probe derived from the wild type flower RNA was labelled with ^{35}S and the probe derived from RNA of flowers from gibberellin-deficient mutants was labelled with ^{32}P. Genes with differing expression levels in wild type and mutant flowers were identified using autoradiography/fluorography of filters made from a wild type flower cDNA library. Genomic clones corresponding to differentially expressed genes have also been isolated using a differential screen (Simoens et al., 1988). However a potential disadvantage of this technique could be the masking effect produced by other coding sequences within the genomic clone, which may be highly expressed and/or present in several tissues.

Differential screening is limited in that it can only detect sequences of moderate to high abundance (>0.1% of the total mRNA). To detect genes which are expressed at very low levels several groups have employed the method of subtractive hybridisation. RNA or cDNA derived from two different tissues is incubated at an optimal temperature and salt concentration to allow common sequences to hybridise. Messages which are specific to the tissue of interest can then be purified away from the common sequences. Purification of the single-stranded sequences can be performed by chromatography over a hydroxylapatite column, or by labelling the subtracting population with biotinylated nucleotides. mRNA or cDNA may be labelled using photobiotin and the hybridising common sequences removed by avidin affinity resin (Duguid et al., 1988) or phenol extraction of streptavidin-bound complexes (Sive and St. John, 1988). cDNAs have been subtracted using mRNA as a driver to select against common sequences, but this requires large amounts of starting material. In order to subtract using mRNA when both tissues are limiting Duguid et al. (1988) have developed a method based on hybridisation of single-stranded cDNAs derived from two cDNA libraries. The subtracting population was labelled with biotin, single-stranded copies of two cDNA libraries were hybridised and the common sequences removed by avidin affinity resin chromatography. The resulting single-stranded cDNA was reverse transcribed to yield a subtracted cDNA library. This method can be refined to prevent non-specific hybridisation by directional cloning of the two libraries to select against common transcripts which are complementary to one another (Rubenstein et al., 1990). A method for the subtraction of genomic libraries has been developed which allows the cloning of wild type DNA which has been deleted in a mutant (Straus and Ausubel, 1990). This method also relies on labelling of the mutant DNA with biotin and hybridisation to enrich for the deleted sequences. Most of the mutants studied in *Arabidopsis* are likely to be point mutations and the corresponding genes would be impossible to isolate using genomic

subtraction. Deletion mutants in plants can be generated by mutagenising seed with X-rays or diepoxybutane (Ehrenberg and Hussain, 1981).

A disadvantage of subtractive hybridisation is that small amounts of subtracted material remain after the hybridisation and these can be difficult to clone without substantial losses. The use of the polymerase chain reaction (PCR) has made the cloning of small amounts of cDNA much easier by allowing its amplification (Erlich et al., 1991). It has also allowed for the production of cDNA from very little tissue (e.g. Jepson et al., 1991). For very small amounts of tissue total RNA (of the order of 50-100 μg) can be extracted and used to make cDNA which can then be amplified. The use of oligo(dT) as a primer for reverse transcriptase eliminates much of the background due to ribosomal RNA sequences when using total RNA as a template (Lu and Werner, 1988).

1.3.3 Insertional mutagenesis

The strategy of insertional mutagenesis or gene-tagging relies on the production of a mutant where a recognisable piece of DNA has inserted into the region of the gene to disrupt its function. The tagged gene can then be isolated by cloning the flanking regions around the inserted DNA. This can be done by making a genomic library from the mutant tagged line, isolating a genomic clone which is recognised by the insert DNA, and using the flanking region to isolate the corresponding genomic clone from a wild type genomic library. The flanking regions around the insert DNA can also be identified using inverse PCR (Erlich et al., 1991). The inserted DNA can either be a transposon or T-DNA. Complementation of the mutant with the isolated DNA from the mutant will prove that the gene corresponding to the mutation has indeed been identified.

Species such as maize and *Antirrhinum* have families of endogenous transposons which have been used to isolate genes of interest (e.g. *Knotted-1* gene of maize; Volbrecht et al., 1991; *deficiens* gene of *Antirrhinum*; Sommer et al., 1990). In species which do not have active transposons such as *Arabidopsis* attempts are being made to generate seed populations which contain combinations of maize transposons in order to generate either novel mutations or mutations corresponding to those already available for which the genes cannot easily be cloned (Balcells et al., 1991; Lisjebettens et al., 1991). T-DNA insertional mutagenesis has already been succesful in cloning genes in *Arabidopsis* (e.g. *AGAMOUS*; Yanofsky et al., 1990; *APETALA-2*; Lisjebettens et al., 1991). A population of T-DNA tagged *Arabidopsis* seed has been produced by co-cultivation of imbibed seed with *Agrobacterium tumefaciens*. The *Agrobacterium* is taken up by the plant and infects the plant during some stage of development, and mutants can be detected in the M2 generation (Feldman and Marks, 1987).

1.3.4 Chromosome walking

Chromosome walking in *Arabidopsis* is a feasible method for isolating genes involved in flowering and several groups are trying to walk to late flowering genes (e.g. Putterill et al., 1991). Restriction fragment length polymorphism (RFLP) maps are available in *Arabidopsis* (Chang et al., 1988; Nam et al., 1989) and these are being integrated with the genetic map (Koornneef et al., 1983). The distance between the gene of interest and the nearest RFLP marker is generally 50-250 kb (Grill and Somerville, 1991). Chromosome walking has been facilitated by the construction of yeast artificial chromosome (YAC) libraries. YACs can accomodate large pieces of DNA, and have the advantage of potentially containing sequences that are not

represented in a cosmid library. The YACs which hybridise to the nearest RFLP have inserts of sufficient size to potentially contain the target gene and end-specific riboprobes generated from phage promoters in the YAC vector allow the identification of overlapping sequences to facilitate walking long distances along the chromosome (Grill and Somerville, 1991). A physical map is also being constructed of the *Arabidopsis* genome which can be aligned to the genetic and RFLP maps (Hauge et al., 1991). The physical map will allow identification of a collection of cosmid clones which correspond to a genetically defined region of the genome. However the approach of chromosome walking has yet to be successful in cloning a gene related to flower development.

1.4 Floral initiation and evocation

In 1977 Murfetdescribedthe process of floral induction as follows:

"Flowering is the end-result of physiological processes, biochemical sequences, and gene action, with the whole system responding to the influence of environmental stimuli and the passage of time."

Fifteen years later floral induction cannot be defined in more specific terms. The transition of a plant from a vegetative state to a reproductive state is a complex and little understood process involving both environmental and intrinsic signals. The process of floral induction can be divided into two phases: initiation, which can be defined as the production of a floral signal which is produced in the leaves and transmitted to the meristem which is competent to respond to this stimulus, and evocation, the response of the meristem after the arrival of the floral signal and the commitment to the formation of floral primordia.

Plants will flower in response to the correct environmental signals or will flower

autonomously. For example *Sinapis* is a long day plant (LDP) which can be induced by one inductive cycle (a long day photoperiod) at low irradiance (Bernier, 1988) while tobacco is a day neutral plant (DNP) which flowers when a minimum number of internodes exist between the roots and the shoot apical meristem (McDaniel et al., 1987). The two major environmental factors which influence the production of a competent meristem and the floral signal are temperature and photoperiod. Vernalisation is the term given to the period of exposure of a plant to cold temperatures prior to the production of the floral stimulus and generally results in the promotion of flowering. The length of this period varies between species, and between cultivars of the same species, and vernalisation is not required by all plants. The cold temperature is perceived at the apical meristem and plays a role in producing the condition of meristem competence, though in some species e.g. *Pharbitis* and sugar beet, the leaves may respond to cold treatment also (Bernier, 1988). Photoperiodism can be defined as the response to the change in day length (or length of night) and phytochrome plays an important role in detecting these changes (Rees, 1987). Plants can be categorised by their requirement for long days (LD), short days (SD) or a lack of requirement for a specific photoperiod (DN). The photoperiod is perceived by young leaves which can produce the floral stimulus under inductive conditions. The floral stimulus is thought to be transported via the phloem to the apical meristem (Deitzer, 1987). The term stimulus implies a 'promoter' but equally it could be the absence of an inhibitory signal which triggers floral evocation. This has led to the 'florigen/antiflorigen' hypothesis discussed below.

1.4.1 Models of floral induction

There are several models to explain the process of initiation and evocation of flowering which have been recently reviewed by Bernier (1988). The evidence for the

existence of a floral stimulus, generated by photoinduced leaves, has come from grafting experiments. This promoter was thought to be a hormone and named 'florigen'. No-one has isolated or identified this substance. Further work from grafting experiments has led to the suggestion of the presence of a floral inhibitor, termed 'antiflorigen'. The florigen/antiflorigen concept assumes the presence of two antagonistic compounds present in plants, the equilibrium being shifted in favour of the floral promoter on photoinduction.

Some evidence suggests that the transport of a floral signal occurs too rapidly to account for movement by translocation in the phloem. This has led to the suggestion of an electrical stimulus or wave of membrane depolaristion as the floral stimulus, though no such effect has been found (Bernier, 1988). Francis (1987) suggests that altered sensitivity of the apex to respond to the floral stimulus may be a result of changes in membrane potential at the apex, and that an ionic signal may act as a floral stimulus, perhaps generated by phytochrome action in the leaves.

The transport of the floral stimulus by translocation in the phloem cells implicates photosynthetic assimilates as the floral stimulus. It would be expected that the nutrient status of the apical meristem would change to accomodate the morphogenetic changes. The idea that altered assimilate flow promotes flowering is known as the nutrient diversion hypothesis, which claims that any substance which alters the source-sink relationship in plants so that the meristem receives a higher concentration of assimilates upon induction can be seen as a floral stimulus.

Bernier (1988) has proposed a multifactorial control model for floral induction which combines features of the florigen/antiflorigen concept and the nutrient diversion hypothesis together with the role of plant growth regulators. This model suggests that floral induction will take place only when all the factors necessary for flowering are

delivered to the meristem, in the correct sequence and in the correct concentrations. The model explains all the contradictory evidence available from the species examined and also implies that the floral stimuli or induction mechanism may not be the same for all plants, and thus information gathered from one or two model systems may not be widely applicable.

1.4.2 Mutants which are affected in floral induction

Identification of genes involved in floral induction and their presence or role in other species may be one way of examining common factors of the floral induction process. Several mutants are available which would allow this. *Pisum* has been well studied with regard to the genetics of floral induction. There are six loci identified in pea which affect floral control, *Lf*, *E*, *Sn*, *Dne*, *Hr* and *Veg* (Rees 1987). The *veg* mutant will not flower in any circumstance. *Sn* and *Dne* are thought to produce a floral inhibitor, their effects are magnified by *Hr* and *E* is thought to reduce the inhibition of *Sn* and *Dne*. *Lf* determines the minimun number of nodes for floral initiation and acts to determine the threshold level for the floral stimulus at the meristem. However there are difficulties in using pea as a model system for molecular biological investigation due to genome size and lack of routine transformation procedures. *Arabidopsis thaliana* is the species of choice for this type of molecular analysis and there are a number of late flowering mutants of *Arabidopsis* available, including *co*, *fg*, *fca*, *gi* and *fve*. Koornneef et al. (1990) have isolated *Arabidopsis* late flowering mutants which map to 12 different loci. The loci *fca*, *fe*, *fy* and *fpa* show increasd responsiveness to vernalisation. Martinez-Zapater and Somerville (1990) have examined mutants at these loci and others and have interpreted their results in the light of a multifactorial pathway for the control of floral induction. The authors assume the presence of a constitutive pathway for flowering which may

be influenced by blue light and assimilate availability. This pathway may be depressed in short days or dim light, in which case far red light and cold treatment would have a supplementary effect. The *fg* mutant of *Arabidopsis* will flower as the wild type in SD but has a late flowering phenotype in long days. Putterill et al. (1991) are walking to the *FG* gene, and have begun a transposon tagging strategy in *Arabidopsis* in an attempt to clone this and other flowering genes. There are no mutants available in *Arabidopsis* comparable to the *veg* mutant of pea, which remain vegetative and don't produce an inflorescence structure. However Belachew et al. (1991) have recently isolated an early flowering mutant of *Arabidopsis* which bypasses vegetative development and flowers immediately after germination. This mutation segregates as a single recessive gene and may encode an inhibitor of the vegetative to floral transition.

1.4.3 Molecular approaches to cloning genes involved in the floral transition

Various strategies have been adopted in the attempt to clone genes involved in the floral transition. Several groups have shown that there are changes, as determined by 2D-PAGE, in the *in vitro* translation products of the apical meristem upon photo-induction (reviewed in Bernier, 1988), but these changes are difficult to reproduce and quantify. Other groups are using the *Arabidopsis* late flowering mutants to attempt to walk to genes involved in floral induction in this species, as mentioned above. However, even in a plant with as small a genome as *Arabidopsis*, this is tedious and time-consuming. The most promising approach so far seems to be the differential screening of subtracted cDNA libraries made to poly(A)$^+$ RNA isolated from induced apices. Melzer et al. (1990) made subtracted cDNA libraries from apical tissue at three different stages after *Sinapis alba* plants were transferred to inductive

long days. They isolated two groups of genes, one of which was expressed early in development whereas the other group encoded anther-specific messages expressed later in development. The transcripts of the early expressed group of genes were present in vegetative meristems and up-regulated throughout floral development. One of these genes was expressed in the peripheral cells of the receptacle, and another was expressed in the peripheral zone of the apical meristem and floral buds. In a similar study, Kelly et al. (1990) also differentially screened a subtracted cDNA library made from transition apices of tobacco, using PCR-amplified subtracted probes. Two of the genes identified in this study were expressed in the vegetative apex and up-regulated upon floral induction. They were expressed maximally in petals and stamens. The functions of the genes isolated from these subtracted libraries are not yet known, but sequence data and antisense RNA experiments may yield such information.

1.4.4 *flo* and *lfy*: mutations which affect the transition from inflorescence to floral meristem

Once the floral stimulus has reached the apical meristem, the plant produces either a single terminal flower or a series of flowers upon an inflorescence stem. The inflorescence can be determinate or indeterminate. The production of an inflorescence meristem is distinct from the production of floral primordia, and there are mutations which affect this transition. The *floricaula* (*flo*) mutant of *Antirrhinum majus* produces an indeterminate inflorescence meristem as in the wild type, but instead of producing flowers in the axils of the bracts further inflorescence meristems are produced. The mutant was isolated by transposon tagging and the *FLO* gene has been cloned (Coen et al., 1990). The putative protein sequence shows features similar to transcriptional activators but there is no sequence homology with the other floral

homeotic genes cloned so far e.g. *AG*, *DEF*. *In situ* hybridisation studies have shown that the *FLO* gene is expressed in the bract primordium and in the floral primordium prior to production of a defined sepal. The *FLO* gene is also expressed in petal and carpel primordia and Coen et al. (1990) have suggested that it may play a role in controlling expression of the floral homeotic genes (see section 1.6). The *squamosa* mutant of *Antirrhinum* is another example of an indeterminate inflorescence mutant which does not produce floral meristems (Schwarz-Sommer et al., 1990). The equivalent mutation in *Arabidopsis* to the *flo* mutant is *leafy* (*lfy*). This mutant also produces an indeterminate inflorescence in place of a floral meristem, with sepal-like or carpel-like organs in place of the bract (Schultz and Haughn, 1991). Shannon and Meeks-Wagner (1991) have isolated an early flowering mutant of *Arabidopsis* called *terminal flower* (*tfl*) which produces an inflorescence with one or two lateral flowers and terminates with a compound flower. The *TFL* gene appears to restrict the capacity of the inflorescence meristem for growth and production of flowers without affecting the vegetative or floral meristems. Another mutation in *Antirrhinum* which affects inflorescence development is *centroradialis*, a determinate meristem mutant (Coen, 1991) which causes the inflorescence meristem to convert to a floral meristem producing a terminal flower.

1.5 The role of plant growth regulators in floral induction and morphogenesis

Studies show that the role of plant growth regulators (PGRs) in floral induction is complex. Both cytokinins and auxins have promotive and inhibitory effects on floral induction, depending upon the species, though in general cytokinins tend to promote floral induction and auxins to inhibit it. The role of gibberellins (GA) in floral induction is also controversial as different GAs seem to be involved in the

control of stem elongation and the promotion of flowering. GA has a primary role in the control of floral induction in *Arabidopsis* (Napp-Zinn, 1985), though the GA-insensitive mutants isolated thus far are only affected in the vegetative phase of the life cycle. Bernier (1988) suggests that there may be a rapid alteration of sensitivity to GA during the floral transition though without knowledge of a GA receptor or its mode of action this hypothesis is difficult to test. It is known that several steps of GA biosynthesis are controlled by daylength though how this may relate to the photo-induced production of a floral stimulus is unknown. In *Arabidopsis* promotion of flowering by cytokinins or GA may be through the alteration of the sensitivity of the apex to these PGRs. Stimulation of cell division in the meristem is elicited by exogenous GA, but only in plants grown for at least 3 months in SD (Bernier, 1988). In *Sinapis*, changes in endogenous cytokinin levels seem to occur only after the inductive LD (Bernier, 1988). Other agents which may play a role in floral induction are pectic cell wall fragments. Eberhard et al. (1989) found that polysaccharide fragments will induce the production of flower buds in tobacco thin cell layer explants. Malmberg and co-workers have isolated several tobacco floral mutants which have elevated polyamine levels (Malmberg and Rose, 1987, Trull and Malmberg, 1991) and they suggest that polyamines may influence developmental pathways in tobacco.

The role of PGRs in flower morphogenesis is equally complex. In general GAs are thought to promote stamen development, and auxins and cytokinins to promote the development of carpels. Work on the tomato *stamenless-2* mutant has shown that application of GA rescued the mutant phenotype, and that indoleacetic acid induced the carpellisation of stamens in the mutant (Sawhney and Greyson, 1973). The *pin-formed* mutant of *Arabidopsis*, which produces an inflorescence with either no terminal floral structure or a single pistil-like structure, has been shown to be deficient

in auxin transport (Okada et al., 1991). An auxin-resistant mutant of *Arabidopsis*, *axr1*, is also reported to be altered in its flower development (Lincoln et al., 1990), though the phenotypes of both of these mutants are not restricted to flowers, being also affected in leaf development. Polowick and Sawhney (1991) have found in *Brassica napus* that the cytokinin, benzylaminopurine, is necessary for development of a full complement of floral organs in buds cultured *in vitro*. Both auxins and GAs proved inhibitory to growth of the buds in culture. The authors postulate that cytokinins may not be synthesised in the early stages of flower development in *B. napus*, hence the requirement *in vitro*, and may be transported from other parts of the plant *in vivo*. Auxins do affect cytokinin metabolism which may account for their inhibitory effect on *in vitro* cultured buds.

1.6 Mutants affected in floral morphogenesis

Once the inflorescence structure is formed flowers are produced, generally in a spiral phyllotaxis. Flowers consist of four types of organ: sepal, petal, stamen and carpel. The organs are produced in four concentric whorls, each of which produces a single organ type. Many mutants exist which are affected in floral morphogenesis. The mutants can be divided into three classes; those which cause production of an organ in the inappropriate whorl (homeotic), those which affect the number of organs in a whorl (meristic) and those which affect the symmetry of the flower. The polyamine mutants of tobacco and the *stamenless-2* mutant of tomato have already been mentioned, but most work has been done using two species, *Arabidopsis thaliana* and *Antirrhinum majus*. Wild type *Arabidopsis* has a radially symmetrical flower with four sepals, four petals, six stamens and two carpels, while the wild type *Antirrhinum* flower has bilateral symmetry, consisting of five sepals, five petals, four

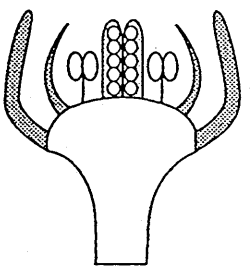
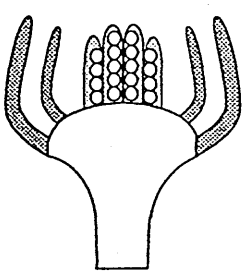
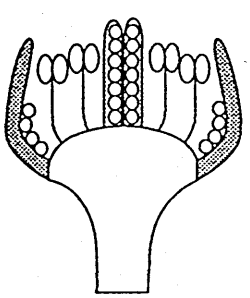
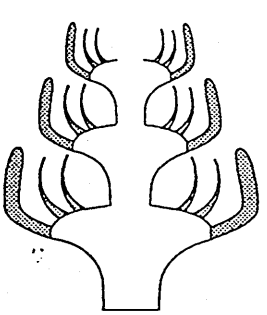
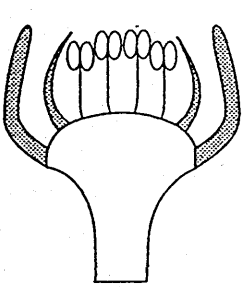
stamens (the fifth is aborted early in development) and two carpels. Comparisons of mutants in both *Arabidopsis* and *Antirrhinum* show that both species have a common developmental pathway for floral morphogenesis, which has been highly conserved during evolution (Coen and Meyerowitz, 1991). The homeotic mutations of both species have been extensively studied and several of the genes cloned. There are three types of homeotic mutant: type 1 controls the identity of the whorl 1 and 2 organs, type 2 the identity of the organs in whorls 2 and 3, and type 3 the identity of the organs in whorls 3 and 4. Mutants of all three types exist in both *Arabidopsis* and *Antirrhinum* and each type will be discussed separately. Fig. 1.1 summarises the phenotypes of the homeotic mutants.

1.6.1 *ap2* and *ovu*: type 1 homeotic mutants

The *apetala-2* (*ap2*) mutation of *Arabidopsis* affects the perianth organs. Several alleles of the *ap2* locus have been identified which show phenotypic variation (Kunst et al., 1989; Bowman et al., 1989; Bowman et al., 1991). The *ap2-1* allele shows the least departure from wild type with four leaves in whorl 1, four petalloid stamens in whorl 2 and normal third and fourth whorls, while the phenotypically most extreme mutant *ap2-2* has carpels or leaf-like organs in the first whorl and second whorl organs are absent. Summation of the phenotypes of all the *ap2* alleles has led to the general description of carpels, stamens, stamens, carpels as the floral structure. Three *ap2* alleles are temperature-sensitive and temperature shift experiments have shown that the *AP2* gene begins to act when the first whorl organ primordia arise (Bowman et al., 1989). The *AP2* gene has been cloned by T-DNA insertional mutagenesis (Jofuku et al., 1990; Lijsebettens et al., 1991) though the function of the gene is not yet known. The *ovulata* (*ovu*) mutant of *Antirrhinum* has a similar phenotype to the *ap2* mutant and has been isolated by transposon mutagenesis

Fig. 1.1 Phenotypes of the *Arabidopsis* homeotic mutants

This figure shows a stylised version of the *Arabidopsis* wild type and homeotic mutant phenotypes. The equivalent *Antirrhinum* mutant is given alongside.
SE, sepal; PE, petal; ST, stamen; CA, carpel; * indicates the presence of further organs within the fourth whorl. Adapted from Bowman et al. (1991).

	Arabidopsis	*Antirrhinum*
	SE, PE, ST, CA	
	wild type	
	SE, SE, CA, CA	
	apetala-3, pistillata	*deficiens, sepaloidea*
	CA, ST, ST, CA	
	apetala-2	*ovulata*
	SE, PE, PE, SE*	
	agamous	*pleniflora*
	SE, PE, ST, ST*	
	superman/flo 10	

(Carpenter and Coen, 1990). The *ovu* mutation is semi-dominant and the most severe phenotype has five carpelloid structures in the first whorl, five petalloid anthers in the second whorl and normal third and fourth whorls. There appeared to be no change in organ number unlike the *ap2* mutants. Schwarz-Sommer et al. (1990) describe a mutant *macho*, in *Antirrhinum*, with a similar phenotype to *ovu*.

1.6.2 *ap3*, *pi* and*def*: type 2 homeotic mutants

Apetala-3 (*ap3*) and *pistillata* (*pi*) are mutations at two different loci of *Arabidopsis* which give the general phenotype sepals, sepals, carpels, carpels. The *ap3* mutant has been described by Bowman et al. (1989). The *ap3-1* allele is temperature-sensitive and forms sepals in the second whorl in place of petals. The third whorl organs range from carpelloid stamens to unfused carpels, depending on temperature and age of the inflorescence. Temperature shift experiments with the *ap3-1* allele show that *AP3* acts later than *AP2* and is effective up to the time of differentiation of the second and third whorl organs. The *pi* mutant has been described by Bowman et al. (1989, 1991) and Hill and Lord (1989). The *pi-1* allele has small sepals in the second whorl, no third whorl organs and an abnormal gynoecium. The *pi* allele described by Hill and Lord (1989) has petalloid sepals in the second whorl. The *pi-2* and *pi-3* alleles (Bowman et al., 1991) are less severe than *pi-1*, the *pi-2* allele having two whorls of sepals and filamentous structures in the third whorl which may be fused to the gynoecium, and *pi-3* having carpels or stamenoid carpels in the third whorl, a phenotype similar to *ap3*.

The *deficiens* (*DEF*) locus of *Antirrinum* has four different alleles, *def-621* (Carpenter and Coen, 1990), *globifera*, *nicotioanoides* and *chlorantha* (Sommer et al., 1991). The general phenotype of this mutant is sepal, sepal, carpel, carpel. There

are also four mutants in *Antirrhinum* at non-allelic loci which exhibit the same phenotype; *globosa*, *femina*, *viridiflora* (Schwarz-Sommer et al., 1990) and *sepaloidea* (Schwarz-Sommer et al., 1990; Carpenter and Coen, 1990). The *def*$^{\text{globifera}}$ (*defA-1*) allele has been cloned by differential screening of a subtracted cDNA library (Sommer et al., 1990). The *DEFA* gene is expressed chiefly in second and third whorl primordia, and is active throughout organ development, as somatic excision of the transposon in the tagged mutant *def-621* produced small clonal patches of petal cells on the second whorl sepals (Carpenter and Coen, 1990). The DEFA protein has features common to transcription factors and contains a region with homology to the DNA binding domains of MCM1 of yeast and the serum response factor (SRF) of mammals. The subsequent cloning of the *agamous* gene of *Arabidopsis* (Yanofsky et al., 1990) showed that the AG protein has the same region of homology which Schwarz-Sommer et al. (1990) have termed the 'MADS' box. Using the *DEFA* gene as a probe Jack et al. have recently cloned the *AP3* gene from *Arabidopsis* (unpublished results, quoted in Coen and Meyerowitz, 1991).

1.6.3 *ag* and *ple*: type 3 homeotic mutants

The type 3 homeotic mutants affect the identity of whorl 3 and 4 organs and examples are *agamous* (*ag*) in *Arabidopsis* (Bowman et al., 1989) and *pleniflora* (*ple*) in *Antirrhinum* (Carpenter and Coen, 1990). The general phenotype of these mutants is sepals, petals, petals and either sepals or petalloid sepals. These mutants are also affected in fourth whorl determinacy as within the fourth whorl there are generally further organs. This gives a 'flower-within-a-flower' phenotype, as the sepal, petal, petal pattern is repeated within the fourth whorl. The *AG* gene has been cloned by T-DNA insertional mutagenesis (Yanofsky et al., 1990) and has a region of similarity to the *DEFA* gene as mentioned above. The *AG* gene product is presumed

to be a DNA-binding protein and is expressed in the third and fourth whorls of wild type flowers (Yanofsky et al., 1990). The *Antirrhinum* gene *PLE* has been cloned and is homologous to the *AG* gene (Bradley et al., unpublished results, quoted in Coen and Meyerowitz, 1991). A second *Arabidopsis* mutant which affects the phenotype of whorl 4 is the *flo10* or *superman* mutant. The morphology of the *flo10* mutant has been described by Schultz et al. (1991). Whorls 1, 2 and 3 organs are as wild type but the fourth whorl consists of two to six stamens or carpelloid stamens.

1.6.4 Meristic mutants

The term meristic is given to mutations which affect the the number of organs without replacing one organ type by another. Examples of meristic mutants in *Arabidopsis* are *apetala-1* (*ap1*) and mutant alleles of the *clavata* locus, *clv-1* and *clv-2*. The *ap1-1* mutation causes the conversion of sepals into bract-like organs and the production of a second flower within the axils of the first whorl organs (Irish and Sussex, 1990). There is a repeat of this developmental pattern in that tertiary buds can form within the secondary flower. Second whorl primordia are never formed and petals are never seen. The third and fourth whorl organs appear to develop as wild type in both the primary and secondary buds. The *ap1* mutant may also be termed a homeotic mutant in that the petals are replaced by another flower bud. However the cells that form the second whorl organs are not recruited for secondary bud development; these buds develop in the axils of first whorl organs, and this mutant is not homeotic in the true sense of the word. The *ag* and *ap2* mutants of *Arabidopsis* may be termed meristic as the number of organs is increased in *ag* due to a reiteration of the developmental pathway and in *ap2* there are often fewer organs than in the wild type flower.

The *clv* mutants of *Arabidopsis* are affected in fourth whorl development (Haughn and Somerville, 1988). The gynoecium is composed of three to four fused carpels and is club-shaped hence the name. Three *clv* alleles have been described by Leyser and Furner (1990) and the mutants show stem fasciation and altered phyllotaxy in addition to extra organs. The organs themselves are wild type. Leyser and Furner (1990) have shown that the apical meristem of the *clv* mutants is enlarged and postulate that this may account for the increased organ number.

1.6.5 Symmetry mutants

Plants may have one or more planes of symmetry in the fully formed flower. An example of a flower with two planes of symmetry (actinomorphic) is *Arabidopsis*, and all the organs in a whorl have similar morphology. Zygomorphic flowers have organs with distinct morphology within a whorl and an example of this is *Antirrhinum*, which has a single plane of symmetry. The *Antirrhinum* flower has been extensively studied with regard to it's symmetry and several mutants have been isolated with radially symmetrical or peloric flowers. Carpenter and Coen (1990) have described two mutants generated by transposon mutagenesis, *cycloidea-608* and *cycloidea-609*, which form two complementation groups. The *cyc-608* mutant is allelic to the extreme *cyc-25* mutant which has radially symmetrical flowers where all members of whorls two and three resemble respectively, the lower petal and stamen morphologies of the wild type flower. In addition the upper middle stamen is not aborted as is seen in wild type flowers. The *cyc* mutations do not give inappropriate expression of an organ type within a whorl but seem to act in concert with the homeotic genes to provide positional information for a particular organ morphology within each whorl. Carpenter and Coen (1990) have suggested a polar coordinate model to explain *CYC* function. They postulate that the *CYC* gene function varies

along the vertical axis of the flower, and the point where the *CYC* gene interacts with a homeotic gene will determine both the type of organ and it's particular morphology at that point. It is tempting to speculate whether the *CYC* gene encodes a receptor which detects a gradient within the flower, or interacts with such a receptor. Hopefully the gene sequence for *CYC* will be available soon to allow this hypothesis to be tested.

1.7 A genetic model for flower morphogenesis

The study of the phenotypes of the various floral mutants has led Haughn and Somerville (1988) and Bowman et al. (1989, 1991) to propose a model to explain floral organogenesis in *Arabidopsis*. The model assumes that there are three 'fields' of gene expression. The first field comprises whorls one and two, the second field whorls two and three and the third field whorls three and four. The genes controlling field determination are, respectively, *AP2*, *AP3/PI* and *FLO10/AG*. Expression of the *AP2* gene in whorl one results in the formation of sepals. The *AP2* gene product, together with the products of the *AP3* and *PI* genes, determines petal formation in whorl two. As the model assumes that the *AP2* and *AG* gene products are antagonistic, *AP2* is switched off in whorl three and *AG* switched on. Thus in the third whorl, the presence of *AG* together with *AP3* and *PI* determines that stamens will form. In whorl four, *FLO10* switches off expression of *AP3* and *PI*, and expression of *AG* alone results in carpel formation and termination of further organ development. Thus in an *ap2* mutant there is expression of *AG* in all four whorls and the order of organ formation would be carpels, stamens, stamens, carpels, the *AP3* and *PI* functions being unaffected. Similarly in an *ap3* mutant whorls one and two would contain *AP2* alone (*PI* being ineffective in the absence of *AP3*) and whorls three and four *AG* alone. This mutant would be expected to show the

phenotype sepals, sepals, carpels, carpels, which it does (see Fig. 1.1).

In addition to this model Irish and Sussex (1990) have proposed a role for *AP1* acting in concert with *AP2* to initiate organ development and determine the field position of the first and second whorls. The authors also propose an additional function for *AG* as a mapping gene for the third and fourth whorls, suggesting that *AP3* and *PI* have no mapping functions but respond to positional information provided by *AP1*, *AP2* and *AG*. Presumably *FLO10* is also responding to information provided by *AG*, by acting to prevent *AP3/PI* expression in whorl four. This model is simplistic; obvious anomalies are the absence of organs in strong *ap2* alleles, and the role of *AG* in preventing indeterminate growth of the flower. One explanation for the altered organ number of the *ap2* alleles is that lack of *AP2* function allows *AG* to be expressed in the first and second whorls, suppressing organ formation in these whorls (Meyerowitz et al., 1991).

A corresponding model has been proposed by Carpenter and Coen (1990) for floral organogenesis in *Antirrhinum*. This also assumes the presence of three organ determination genes, named *a*, *b* and *c*, which act over two consecutive whorls. Studies of the *Antirrhinum* floral mutants suggest that the *a* function is encoded by *OVU*, the *b* function by *DEFA* and the *c* function by the *PLE* gene. The cloning of the *floricaula* gene and studies on the pattern of *FLO* gene expression have allowed Coen et al. (1990) to suggest that *FLO* may act to control the pattern of expression of the homeotic genes in *Antirrhinum*. *FLO* can control the switch from inflorescence to floral meristem and therefore acts early in floral development. It is also expressed in the bracts, sepals, petals and carpels of the flower. Coen et al. (1990) suggest that the presence of *FLO* early in floral development will activate the *a* gene in whorls one and two, but the presence of *b* and *c* in whorl three later in

floral development would inhibit the expression of *FLO* in this whorl (therefore no expression is seen in stamens). In whorl four *FLO* may inactivate the *b* function but be unable to activate expression of *a* due to the establishment of the *c* function. This model has yet to be tested in the *Antirrhinum* homeotic mutants.

1.7.1 The genetic model predicts the structure of double mutants

The model proposed by Meyerowitz and co-workers (Bowman et al., 1989; Meyerowitz et al., 1991) can be used to suggest phenotypes for double mutants of homeotic function in *Arabidopsis* and this is one way to test the model. Double and triple mutants of the floral homeotic genes have been constructed in *Arabidopsis* by Bowman et al. (1989, 1991). The double mutants which included *ag* all showed additive phenotypes as predicted by the model; *ap3 ag* and *pi ag* both had phenotypes which consisted of many whorls of sepals. The *ap3 pi* mutant did not have a phenotype altered from the singly mutant strains, consistent with the hypothesis that these genes have a similar function. The phenotype of the double mutant *ap2 ag* is more difficult to predict as there is an absence of homeotic genes in whorls one and four, and the genes *AP3* and *PI* require the presence of *AP2* and *AG* respectively, to determine petal and stamen formation. The actual phenotype seen in these mutants is leaves, stamenoid petals, stamenoid petals, with a reiteration of this pattern within the fourth whorl due to lack of *AG* function. This suggests that in the absence of the homeotic genes organ primordia will form the ground state organ, the leaf. However the triple mutants *ap2 ap3 ag* and *ap2 pi ag* have many whorls of leaf-like organs or carpelloid leaves, indicating that the ground state for floral development is not entirely vegetative as would be expected.

1.7.2 Molecular studies of the homeotic genes support the genetic

model for floral morphogenesis

Another way of testing the models proposed by Bowman et al. (1989) and Carpenter and Coen (1990) is by cloning the genes involved in organ patterning and formation and examining the patterns of their expression in the flower. As mentioned above several of these genes have now been isolated from either *Arabidopsis* or *Antirrhinum*. The *AG* gene of *Arabidopsis* was cloned from a T-DNA insertional mutant and is expressed in whorls three and four in wild type flowers (Yanofsky et al., 1990). Drews et al. (1991) examined the expression of the *AG* gene in the mutants *ap2*, *ap3* and *pi* by *in situ* hybridisation. In *ap3* and *pi* the *AG* transcript is localised in the third and fourth whorl carpels as in wild type, but in an *ap2* background the *AG* transcript is present as the first whorl primordia develop and is expressed throughout first and second whorl organ development as well as in the third and fourth whorls. This is the pattern of expression predicted by the model. The *apetala2* gene of *Arabidopsis* has been cloned by T-DNA insertional mutagenesis though the sequence is not yet available (Jofuku et al., 1990; Lijsebettens et al., 1991). It will be interesting to know if the expression of *AP2* is restricted to the first and second whorls and how soon it is expressed after inflorescence formation in its function as a mapping gene.

The *DEFA* gene of *Antirrhinum* has been cloned by subtractive hybridisation of wild type flowers using the *def*^{globifera} mutant, which is a strong allele generated by a Tam 7 insert within the coding sequence of the *DEFA* gene (Sommer et al., 1990). The expression of the *DEFA* gene is not restricted to the second and third whorls as would be expected by the model; the transcript is found also in sepals and carpels (Sommer et al., 1991). The initiation of *DEFA* expression and the up-regulation of expression in petals and stamens appear to be controlled in different ways. Schwarz-Sommer et al. (1992) have suggested that *DEFA* can autoregulate

it's own expression in concert with the *globosa* (*GLO*) gene. The *glo* mutant is homeotic, having a similar phenotype to the *def* mutant, and the *GLO* gene was cloned from a flower cDNA library using the MADS box region of *DEFA* as a probe (Sommer et al., 1991). Gel-shift experiments have shown that a DEFA/GLO heterodimer can bind the *STE6* promoter, the binding site for another MADS box protein, MCM1 from yeast (Schwarz-Sommer et al., 1992), while DEFA and GLO homodimers do not appear to bind this promoter region. The DEFA protein is a phosphorylated nuclear protein and the MADS box region of DEFA contains a potential phosphorylation site for calmodulin-dependent protein kinases (Sommer et al., 1991). This raises the possibility of post-translational modifications and protein-protein interactions playing a role in the organ-specific expression of *DEFA*.

Screening of a cDNA library with the *DEFA* conserved domain has led to the isolation of a further nine MADS box genes from *Antirrhinum* (Sommer et al., 1991). The genes are expressed in flowers and some are also expressed in vegetative organs. One of these cDNAs corresponds to the *Antirrhinum* homeotic gene *SQUAMOSA* (*SQUA*). The *squamosa* mutant has a similar phenotype to the *flo* mutant and the *SQUA* gene has a role in controlling the transition from inflorescence to floral meristem. Using the *AG* gene as a probe Ma et al. (1991) have cloned a family of *AG*-like genes (*AGL1-6*) from *Arabidopsis* which are expressed preferentially in flowers and immature siliques. The *AGL* family contain MADS box motifs as well as a second conserved region, the K box, which has homolgy to the keratin protein. Potential phosphorylation and glycosylation sites exist in these genes, suggesting similar means of control of their function to the *DEFA* gene. The roles of members of the *AG*-like genes and the *DEFA*-like genes have yet to be elucidated.

The homeotic genes discussed thus far are probably not the only factors

involved in organ specification. Jackson et al. (1991) have recently cloned a family of *myb* genes from *Antirrhinum*, three of which show specific expression in floral tissue, and another two members which are expressed in all organs. There is yet no evidence that the *myb* genes are involved in organ specification in the flower. The *C1* gene of maize, which has homology to the *myb* proto-oncogene in mammals, is involved in regulation of anthocyanin biosynthesis, and the *myb* genes from *Antirrhinum* may have a similar function (Jackson et al., 1991). There are also homeobox genes, similar to those involved in embryo development in *Drosophila* (Ingham, 1988), which are present in plants and these genes may play a role in controlling aspects of floral development. A gene which has homology to human and yeast homeodomains, *Knotted-1*, has been isolated from maize (Volbrecht et al., 1991) which is involved in leaf development. Corresponding genes containing homeodomains have been isolated from *Arabidopsis* using *Knotted-1* as a probe (Serikawa et al., 1991). It may prove that floral development is much more complex than the simple genetic models suggest, involving cascades of DNA-binding proteins controlling the temporal and spatial expression of flower-specifc genes.

1.8 Genes specific to floral organs

Genes involved in floral development may show organ-specific expression without having a function in controlling development. Such genes include, for example, those for floral pigment biosynthesis enzymes, enzymes involved in pollen grain wall formation and the self-incompatibility glycoproteins. RNA-DNA hybridisation experiments in tobacco have estimated that appoximately 40% of the mRNAs in the anther (about 11, 000) are anther-specific, for example, and that there are approximately 10, 000 ovary-specific messages (Kamalay and Goldberg, 1984). Therefore there must be many genes as yet unidentified which are involved in organ

specification. Methods which have been used to identify such genes include identification of specific proteins and subsequent cloning from partial protein sequencing (e.g. S-locus glycoprotein; Mau et al., 1991), transposon tagging (e.g. chalcone synthase; Sommer and Saedler, 1986) and differential screening of organ-specific cDNA libraries (e.g. Gasser et al., 1989). Three classes of genes will be discussed in turn, petal-specific, anther-specific and pistil-specific genes, though in the case of some genes e.g. chalcone synthase, they may also be expressed in other floral or vegetative organs. Sepal-specific genes have not been studied as such, as sepals are modified leaves and probably share many homologous sequences.

1.8.1 Petal-specific genes

The petal is a modified leaf-like organ which can be highly pigmented. The major class of genes identified as petal-specific are involved in the production of the flower pigments. The floral pigments are end products of the flavonoid pathway, a side branch of phenylpropanoid metabolism, and the first committed step of flavonoid synthesis is catalysed by chalcone synthase (CHS). The CHS genes from several species have been cloned (Mol et al., 1988) and these genes are wound- and UV-inducible as well as showing flower-specific expression (e.g. Schmid et al., 1990). The CHS genes are not exclusively expressed in petals but are also expressed in anthers and pistils though to a much lower extent. Dissection of a CHS promoter from *Petunia hybrida* has shown that a small upstream element directs flower-specific expression (van der Meer et al., 1990). Associated members of the flavonoid pathway are also expressed in petals, for example, chalcone isomerase and dihydroflavenol reductase (Mol et al., 1989). Others enzymes in the phenylpropanoid pathway are present predominantly in petals, and the best studied are phenylalanine ammonia lyase (PAL) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).

PAL controls the first step of phenylpropanoid synthesis, catalyzing the deamination of phenylalanine. The PAL genes exist as a multi-gene family in *Phaseolus vulgaris*, of which only one member, PAL2, is expressed in flowers (Liang et al., 1989). The EPSPS enzyme is part of the shikimate pathway, which produces aromatic amino acids. The EPSPS promoter directs reporter gene expression in petals in petunia (Benfey et al., 1990b) and a DNA-binding protein has been cloned from petunia which binds to the EPSPS promoter, and is present in petals (Takatsuji et al., 1992).

Alterations in the expression of the CHS genes can alter flower colour. van der Krol et al. (1988a) have repressed CHS expression using an antisense copy of the CHS gene and produced flowers with different pigmentation patterns from wild type flowers in petunia. The pigmentation of the flower can also be altered by expressing a gene involved in flavonoid synthesis, which is not normally found in the host plant, using a petal-specific promoter. For example, Benfey et al. (1990b) have used the EPSPS promoter to express a maize dihydroquercetin 4-reductase gene in a mutant, unpigmented petunia line, which subsequently produced pink flowers.

1.8.2 Anther-specific genes

The stamen consists of an anther which is borne upon a filament. The anther consists of a tetra-lobed structure in which the pollen is formed. In the immature anther, the tapetum is important in providing nutrition for developing pollen grains and is both metabolically and transcriptionally highly active. The mature pollen grain also has a store of mRNAs which are utilised upon pollen germination. Anther-specific sequences have been cloned from several species mostly by differential screening of cDNA libraries (e.g. Scott et al., 1991; Goldberg, 1986; McCormick et al., 1991). Scott et al. (1991) have identified three tapetal-specific cDNAs from *B.*

napus, and further tapetal sequences have been identified in tobacco (Koltunow et al., 1990). One tobacco sequence, TA29, is glycine-rich and may form part of the pollen wall. Another anther-specific sequence identified by Kultunow et al. (1990) has homology to a thiol endopeptidase. It is expressed in the connective tissue and may play a role in anther dehiscence. Two anther-specific genes isolated from tomato are present in the mature pollen grain at high levels and encode proteins with homology to pectate lyase (Ursin et al., 1989). These genes are not expressed in male sterile lines of tomato. A gene from *B. napus* which shows microspore-specific expression, is also not expressed in male-sterile lines of *B. napus* (Roberts et al., 1991).

The generation of male sterile crops is of commercial importance, allowing the production of hybrids with increased yield. Mariani et al. (1990) have genetically engineered male sterile lines of tobacco and *B. napus* by transformation with an RNAase gene under control of the TA29 promoter. The promoter directed specific expression of the RNAase in the tapetum, causing the destruction of the tapetal layer. The transgenic plants did not produce viable pollen, though in general the plant was unaffected.

1.8.3 Pistil-specific genes

The pistil is the 'female' reproductive structure of the flower. It consists of an ovary of one or more carpels, containing the ovules or female gametophytes. The pistil has a stigma upon which the pollen grains arrive, and a style through which the pollen tubes grow to reach the ovules. The best studied genes of the pistil are those involved in the self-incompatibility (SI) interactions. There are two types of SI; gametophytic, where if the pollen carries the same allele as one of the two in the pistil, the pollen does not germinate, and sporophytic, where the pollen SI factor is specified by the parent tissue and interacts with the pistil SI factors to determine an incompatible

cross. The two systems best studied are *Nicotiana alata* (Mau et al., 1991), which has a gametophytic SI, and *Brassica oleracea* (Thorsness et al., 1991), with a sporophytic SI. In *N. alata* several of the S-locus proteins have been cloned and comparison of the sequences for these alleles show that there are hypervariable regions which may determine S-allelic specificity (Mau et al., 1991). The proteins are glycosylated and are found as extracellular proteins of the stigma and transmitting tract. The S-glycoproteins have sequence similarity to RNAases, although it is unknown whether this RNAase activity is important in the incompatibility interaction (Mau et al., 1991). The S-locus glycoproteins (SLG) of *B. oleracea* have no sequence similarity to the *N. alata* S-glycoproteins, suggesting that the two incompatibility systems have evolved separately (Haring et al., 1990). The SLGs identified have a higher sequence homology between alleles than seen among the *N. alata* S-glycoproteins, and are secreted from the stigmatic papillar cells. The promoter sequence from an SLG gene can direct reporter gene expression in the tapetum as well as in the stigma of transgenic *Arabidopsis*, indicating that the components of sporophytic SI exist both in the anther and the pistil (Toriyama et al., 1991). There is also a family of SLG-related genes in *B. oleracea*, which are highly conserved and don't appear to play any role in determining allelic specificity, but may be involved in the incompatibility mechanism (Haring et al., 1990).

Gasser et al. (1989) have isolated several pistil-specific genes from tomato using a differential screening approach. Some of the cDNAs isolated also showed low levels of expression in stamens. One of the genes has been characterised further, and encodes a protein with a secretory signal sequence and potential glycosylation sites (Gasser, 1991). The transcript is present in the transmitting tract tissue, and the protein may be one of the glycoproteins found in the extracellular mucilage of the transmitting tract.

Pathogenesis-related (PR) proteins have also been found to have flower-specific expression. An antibody to a β-(1,3)-glucanase recognised a stylar glycoprotein in tobacco (Lotan et al., 1989) and Meeks-Wagner and co-workers identified several floral-specific cDNAs from tobacco thin cell layer explants, which were subsequently found to encode a range of PR proteins (Neale et al., 1990). In addition, a pollen allergen from birch has sequence homology with PR proteins from bean (Walter et al., 1990). These data would suggest that, like chalcone synthase, the PR proteins have a role to play in flower development outside their roles as defence genes.

1.9 Aims of the project

The primary aim of this project was the isolation of genes involved in the early stages of floral morphogenesis. At the time the project was initiated there was little indication that techniques such as insertional mutagenesis would result in such rapid progress towards the cloning of genes controlling flower development, whereas differential screening of cDNA libraries had already led to the isolation of floral organ-specific genes. The strategy adopted was therefore to make a cDNA library to floral apices at the earliest possible stage of development, which could be differentially screened to isolate genes expressed at a specific developmental stage within the flower. This strategy would hopefully lead to the isolation of novel genes which showed specific developmental and temporal expression during the early stages of floral morphogenesis, rather than genes expressed in the floral organs.

Arabidopsis thaliana is a particularly suitable species for developmental studies, and because of the availability of floral mutants, the possibility existed to look for altered expression of the isolated genes in these mutants. However, *Arabidopsis* is

a poor choice for directly cloning flower-specific genes because the very small size of the flowers would mean that very large numbers of plants would be needed to obtain sufficient amounts of poly(A)$^+$ RNA for cDNA cloning. Moreover, to increase the chances of obtaining genes that are involved in controlling development, it would be important to use flowers at an early stage of development, which further limits the availability of tissue. Subtractive cloning techniques and the use of PCR were not widely applied to plants at the time of initiation of this project. It was therefore proposed to use *Arabidopsis* in combination with *Brassica napus* (oilseed rape) to obtain floral stage-specific genes. *B. napus* and *Arabidopsis* are very closely related and their flower structures are very similar, so genes in one species are likely to have a corresponding role in the other, and should be sufficiently homologous to be used as probes in cross-hybridisation. This has been shown subsequently to be true as Yanofsky et al. (1990) have isolated two *AG* homologues from *B. napus* using the *AG* gene of *Arabidopsis*. *B. napus* is grown extensively as a crop plant and so tissue at the appropriate developmental stage would be available in abundance. Moreover, the flowers and floral buds of *B. napus* are much larger enabling a sufficient yield of tissue to construct cDNA libraries.

1.9.1 Flower development in *Brassica napus*

Brassica napus is an important crop which is widely grown in the U.K. In the field flowers are initiated in winter rape varieties after a cold treatment (Friend, 1985). The flowers start to develop during winter and become visible in early spring. The flowers are subsequently borne on an elongated stalk and usually open in May or June. A growth stage key for *B. napus* has been developed (Sylvester-Bradley and Makepeace, 1984) and this has been correlated with floral organ development (Smith and Scarisbrick, 1990). Flower development in *Brassica napus* has been extensively

characterised and the pattern of floral development is markedly similar to *Arabidopsis* (Smyth et al., 1990). Polowick and Sawhney (1986) have studied the development of the *B. napus* flower using scanning electron microscopy. The flower buds arise in a spiral pattern of phyllotaxis. The four sepal primordia arise first, followed by initiation of the four long stamens. This is not the typical acropetal sequence seen in flowering plants, but appearance of the stamen primordia prior to petal initiation appears to be characteristic of crucifers. However, in *Arabidopsis* Smyth et al. (1990) were unable to differentiate the timing of petal initiation from that of stamen initiation. In *B. napus* the sepals grow to cover the developing floral organs, which remain enclosed throughout development. The initial growth of the petals is slow and they do not begin to enlarge until just prior to anthesis when growth is rapid. The two short stamens are initiated after the four long stamens. Scott et al. (1991) have correlated bud length with stamen length, and hence the growth stage of the anthers. The gynoecium develops from the remainder of the apex, initially as an indentation, and forms as two fused carpels (Polowick and Sawhney, 1986). Remains of this invagination can still be seen in the papillate head of the stigma, giving it a slightly bilobed appearance. This is not seen in *Arabidopsis* (Smyth et al., 1990) though the gynoecium develops in a similar manner.

1.9.2 Isolation of flower-specific genes of *B. napus* and study of their function

The first aim of the project was to isolate genes from *B. napus* which are specific to early stages of floral development. This was attempted by the construction of a cDNA library to floral apices, and differential screening of the library using probes derived from floral and vegetative apices. The expression of the flower-specific clones thus isolated was studied using RNA dot blot and northern analysis to

define their organ-specificity and temporal expression. Further analysis of the expression of these cDNAs was carried out by *in situ* hybridisation. Sequencing was initiated to obtain information about the potential function of the gene products. A more speculative method to determine the function of genes isolated in this study was the transformation of *Arabidopsis* with an antisense copy of the *B. napus* cDNAs. The phenotype of the transformed plants may yield clues to the function of the gene. This method has subsequently proved successful in tomato (Hamilton et al., 1990) and was attempted in this study.

Chapter 2: Materials and Methods

2.1 Materials

Most chemicals were obtained from BDH (AnalaR® grade; Poole, Dorset) or Fisons (PrimaR® grade; distributed by FSA Laboratory Supplies). Unless otherwise indicated, fine chemicals were obtained from Sigma (Poole, Dorset). Radiochemicals were obtained from Amersham International plc (Amersham, U.K.).

2.2 General experimental procedures

2.2.1 Autoclaving

Equipment and solutions were sterilised in a Lab Thermal Equipment 225E autoclave at 15 psi for 20 min, or in a Prestige Hi-Dome pressure cooker.

2.2.2 Filter-sterilisation

Heat-labile solutions were sterilised by passing through a Corning syringe filter (pore diameter 0.2μm) into a sterile receptacle.

2.2.3 Glassware

Glassware was sterilised by baking at 180°C in a dry oven for 12 h.

2.2.4 pH measurement

The pH of solutions was determined using a Corning pH meter 220 and combination electrode.

2.2.5 Equipment and solutions for RNA work

All solutions for RNA work were treated with 0.1% diethyl pyrocarbonate overnight at room temperature and autoclaved for 20 min at 15 psi. Solutions containing Tris were made in DEPC-treated autoclaved H_2O. Glassware was baked for 12 h at 180°C.

2.2.6 Silicon-coating of tubes

Glass and plastic tubes were siliconised by immersing in Repelcote (2% dimethyldichlorosilane in 1,1,1-trichloroethane) for 15 s in a fume hood. The tubes were washed several times with distilled H_2O before sterilisation.

2.3 Growth media

All media were sterilised by autoclaving at 15 psi for 20 min.

2.3.1 Liquid media

L-Broth (1% bacto-tryptone (supplied by Difco Laboratories, Michigan, USA), 1% NaCl, 0.5% yeast extract (Difco), pH 7.2) was used for culture of *E. coli*, supplemented with the appropriate antibiotic. For preparation of plating cells (*E. coli* NM621), L-Broth was supplemented with 0.4% maltose. For preparation of competent cells *E. coli* XL-1 Blue were cultured in φ-broth (2% bacto-tryptone

(Difco), 0.5% yeast extract (Difco), 0.4% $MgSO_4$, 10 mM KCl. For preparation of plasmid DNA for sequencing, *E. coli* XL-1 Blue was cultured in Terrific broth (1.2% bacto-tryptone (Difco), 2.4% yeast extract (Difco), 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4).

2.3.2 Solid media

For plating out *E. coli*, L-agar was prepared by adding 1.5 g/l agar (Difco) to L-Broth prior to sterilisation. L-agarose plates were prepared by adding 1.5 g/l agarose (Sigma, type I-A) to L-Broth. Top agarose was prepared by adding agarose to an appropriate concentration (0.5-1.0%) and $MgSO_4$ to 2.5 g/l to L-Broth. *Agrobacterium tumefaciens* was grown on AB medium which was prepared by combining solution I (x 20, 60 g/l K_2HPO_4, 20 g/l NaH_2PO_4) and solution II (x 20, 20 g/l NH_4Cl, 6 g/l $MgSO_4.7H_2O$, 3 g/l KCl, 3 g/l $CaCl_2$, 50 mg/l $FeSO_4.7H_2O$) to a final concentration of x1, and adding glucose to 0.5% and Oxoid agar to 1.5%.

2.3.3 Antibiotics

All antibiotics used were supplied by Sigma Chemical Co. except vancomycin which was supplied by Eli Lilley and Co. (Basingstoke). Ampicillin, 25 mg/ml in H_2O, and kanamycin, 10 mg/ml in H_2O, were filter-sterilised and added to a final concentration of 50 μg/ml to sterile media which had been allowed to cool to 50°C. Tetracyclin, 12.5 mg/ml in 50% ethanol, was used at a final concentration of 12.5 μg/ml. Rifampicin, 20 mg/ml in methanol, was used at a final concentration of 100 μg/ml. Isopropyl thiogalactoside (IPTG; Life Technologies, Gaithersburg, USA) was used at a final concentration of 0.1 mM. X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside; Life Technologies) was made as a 20 mg/ml stock in dimethylformamide

and used at a final concentration of 50 μg/ml.

2.4 Plasmids and strains

Plasmids used are listed in Table 2.1. The cDNA library was constructed in the bacteriophage vector λNM1149 (Murray, 1983). cDNA inserts were sub-cloned into pBluescript SK(-) (Stratagene, Cambridge, U.K.). λ DNA for markers was supplied by Gibco-BRL (Paisley, Scotland). *E. coli* NM621 was used as a host strain for the cDNA library. cDNA sub-clones were maintained in *E. coli* XL-1 Blue from which plasmid DNA was regularly prepared. The strain used for plant transformation was *Agrobacterium tumefaciens* C58C1 Rif^R containing pGV2260. This strain was grown at 28°C to maintain the plasmid. The helper plasmid pRK2013 was maintained in *E. coli* DH5α.

2.5 Growth and harvest of plants

Brassica napus L. cv 'Cobra' seeds (winter oilseed rape) were obtained from Booker Seeds, Sleaford, Lincs. *Arabidopsis thaliana* Landsberg erecta wild type and mutant seed were obtained from the Nottingham *Arabidopsis* Stock Centre (University of Nottingham). Mutants used are described in Table 2.2. Seeds were stored at 4°C and germinated on ICI potting compost. Plants were grown in continuous light (150 μmol/m^2/s; Osram 45W 'warm white' fluorescent tubes supplemented with tungsten bulbs) at a temperature of 23°C. Cold-induction took place at 4-6°C in continuous light. Plants were wounded by pricking the leaves 2-3 times per cm^2 with a sterile needle and harvested after 8-12 h. Plants were heat-shocked by cutting the leaf rosette away from the base of the stem and immersing in distilled H_2O in a water-bath for 3 h at 42°C (Vierling et al., 1989). For isolation of

DNA and RNA tissue was harvested directly in liquid N_2. For field-grown tissue, the whole plant was harvested and stored at 4°C until the apex was dissected out (typically 1-3 h).

2.6 Isolation of DNA

2.6.1 Small scale plasmid DNA preparation

This method is as described by Birnboim and Doly (1979) and modified by Maniatis et al. (1982).

Overnight cultures of *E. coli* were dispensed into 1.5 ml aliquots and centrifuged in a microfuge for 5 min. The supernatants were discarded and the pellet resuspended in 100 µl ice-cold GET (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10mM EDTA) containing 2 mg/ml lysozyme. The tubes were left on ice for 5 min and 200 µl of 0.2 M NaOH, 0.1% SDS was added. The contents were mixed and the tubes left on ice for 10 min. 150 µl of 3 M NaOAc pH 4.5 was added to the tubes which were vortexed and left on ice for a further 10 min. The tubes were centrifuged for 10 min and the supernatants were transferred to fresh tubes containing 200 µl phenol and 200 µl chloroform. The tubes were vortexed, centrifuged for 3 min and the aqueous phase re-extracted with 400 µl of chloroform. The aqueous phase was then transferred to a fresh tube and the plasmid DNA precipitated with 2 vol ethanol at -20°C for 1 h. The tubes were centrifuged for 10 min and the pellets washed with 70% ethanol (ice-cold). Residual ethanol was removed using a drawn-out pasteur pipette and the pellets allowed to air dry before resuspending in 1 x TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Table 2.1

PLASMID	DESCRIPTION	REFERENCE
pBluescript SK(-)	Vector with multiple cloning sites used for subcloning of cDNA inserts	Stratagene
pLF1-10	cDNA inserts sub-cloned into pBluescript SK(-)	Chapter 3
pUC9	Used for preparation of DNA size markers	Viera and Messing (1982)
pTA71	9 kb wheat rRNA in pUC 9	Gerlach and Bedbrook (1979)
pJR1	CaMV 35S promoter and nos 3' terminator in Bin 19	gifted by ICI seeds, Bracknell
p2AS, p2S	Antisense and sense constructs of pLF2 cloned into pJR1	Chapter 5

Table 2.2. *Arabidopsis thaliana* flowering mutants

This table describes the phenotype and source of the *Arabidopsis* flowering mutants used during this work. The code refers to the Wageningen stock of seed. Stocks used in this project were obtained from the Nottingham Seed Stock Centre, University of Nottingham. EMS, ethyl methane sulphonate, is the mutagen used to produce the mutants. The mutants were produced in the ecotype Landsberg *erecta*.

LOCUS	NAME	PHENOTYPE	MUTAGEN	CODE
ap1	*apetala-1*	without petals	EMS	W28
ap2	*apetala-2*	without petals	EMS	W29
ag	*agamous*	double flowering	EMS	W25
clv2	*clavata-2*	club-like siliques	EMS	W46
pi	*pistillata*	without petals and anthers	EMS	W77

2.6.2 Mini-prep of plasmid DNA for sequencing template

This is a modification of the alkaline lysis procedure, developed by Jones and Schofield (1990).

A single colony was picked with a sterile loop and used to inoculate 2 ml of Terrific broth (TB) plus antibiotic. The culture was incubated overnight with shaking at 37°C. 1.5 ml of the culture was decanted into a 1.5 ml Eppendorf® tube and centrifuged for 1 min in a microfuge. The supernatant was discarded and the bacterial pellet resuspended in 200 µl GET, 2 mg/ml lysozyme. 400 µl of 0.2 M NaOH, 1% SDS was added to the tube, inverted to mix and incubated on ice for 5 min. The contents were neutralised by addititon of 300 µl 3M KOAc pH 4.8, inverted to mix and replaced on ice for 5 min before centrifuging for 5 min. The supernatant was removed into a 2 ml Eppendorf® tube containing 900 µl ethanol and centrifuged immediately for 5 min. The DNA pellet was washed in 0.5 ml 70% ethanol, dried and resuspended in 40 µl 1 x TE.

2.6.3 Large scale plasmid DNA preparation

This method is a modification of that of Birnboim and Doly as described in Maniatis et al. (1982).

750 ml of L-Broth was inoculated with an overnight culture of *E. coli* and incubated at 37°C, with shaking, for approximately 24 h until the A_{550} of the culture reached 0.8. The culture was then poured into sterile 250 ml screw-cap bottles and centrifuged for 15 min at 3 000 rpm (approximately 5 000 x *g*) in an MSE High Speed 18 centrifuge at 4°C. The supernatant was discarded and the pellet

resuspended in 3.2 ml ice-cold GET containing 2 mg/ml lysozyme. The suspension was transferred to a sterile 50 ml polypropylene tube and left on ice for 20 min. 6.6 ml of 0.2 M NaOH, 0.1% SDS was added and the contents mixed and left on ice for 15 min. To this mixture was added 5 ml of 3 M NaOAc pH 4.5, and the tube vortexed. After a further 15 min on ice the tubes were centrifuged at 10 000 rpm (approximately 15 000 x *g*) for 15 min at 4°C. The supernatant was transferred to a 30 ml Corex® tube containing 12 ml isopropanol, the contents mixed and allowed to stand for 15 min at room temperature. The tubes were centrifuged as above for 10 min and the pellets washed in 70% ethanol. The plasmid DNA pellet was dried gently under vacuum and resuspended in 7.1ml 1 x TE. This solution was added to an ultracentrifuge tube containing 6.72 g of CsCl and the tube inverted to mix. 425 µl of 10 mg/ml EtBr was added and tubes balanced in pairs to within 0.2 g. The tubes were centrifuged in a Beckman Ti 70.1 rotor in a Beckman L7-55 Ultracentrifuge for 36 h at 20°C at 53 000 rpm. The plasmid band was removed using a 250 µl Hamilton syringe into a 15 ml Corex® tube containing 2 vol of water-saturated butan-1-ol. The two phases were mixed and allowed to separate. The upper phase was removed and the lower aqueous phase re-extracted with butan-1-ol until no pink colour remained. The aqueous phase was made up to 4 ml with 1 x TE and 400 µl of 3 M NaOAc pH 6.0, and 10 ml of ethanol were added. The DNA was precipitated at -20°C and pelleted by centrifugation at 10 000 rpm for 10 min. The pellet was washed with 70% ethanol, dried *in vacuo* and resuspended in 1 x TE.

2.6.4 Small scale preparation of phage DNA

This method is that of Maniatis et al. (1982) with modifications.

50-100 µl of a phage suspension were added to 100 µl of *E. coli* NM621

plating cells (see section 2.22.1) and incubated at 37°C for 15 min in a sterile bijou. 2.5 ml of molten top agarose was added, mixed and poured onto the surface of a freshly-poured L-agarose plate. The plates were incubated (not inverted) at 37°C overnight. The phage were eluted into 5 ml of phage diluent (10 mM Tris-HCl pH 7.5, 10 mM $MgSO_4$) with gentle shaking for 1-2 h. The phage solution was transferred to a 13 ml centrifuge tube and the debris pelleted by centrifugation at 4 000 rpm for 10 min in a Beckman TJ-6 centrifuge. The supernatant was transferred into a 50 ml polypropylene tube and DNAase I and RNAase A added to a final concentration of 1 µg/ml. The tube was incubated at 37°C and the phage precipitated by addition of an equal vol of 20% PEG 8000, 2 M NaCl in phage diluent and incubation for 1 h in ice-water. The phage were pelleted by centrifugation at 10 000 rpm (approximately 15 000 x *g*) for 20min at 4°C in an MSE 18 High Speed centrifuge. The supernatant was removed by aspiration and the phage redissolved in 0.5 ml of phage diluent. The tube was centrifuged as above for 2 min and the supernatant removed into a microfuge tube. 5 µl of 10% SDS and 5 µl of 0.5 M EDTA were added and the tube incubated at 68°C for 15 min. Following addition of 10 µl 5 M NaCl, the solution was extracted once with an equal volume of phenol/chloroform (1:1) and once with an equal volume of chloroform. To the final aqueous phase was added an equal volume of isopropanol and the DNA precipitated at -20°C for 20 min. The tube was centrifuged for 10 min as above, the DNA pellet washed in 70 % ethanol, dried and dissolved in 50 µl of 1 x TE.

2.6.5 Isolation of plasmid DNA from *Agrobacterium tumefaciens*

Plasmid DNA was isolated from *A. tumefaciens* according to the method of An et al. (1988) using a technique based on the alkaline lysis procedure.

Agrobacterium tumefaciens was grown for 24-36 h in 2 ml L-broth plus antibiotic at 28°C. The culture was transferred to a microfuge tube and the cells pelleted in a microfuge for 30s. The supernatant was discarded, the cells resuspended in ice-cold GET, 4 mg/ml lysozyme and incubated at room temperature for 10 min. 200 µl of freshly prepared 1% SDS, 0.2 M NaOH was added to the tube which was shaken to mix. The tube was incubated at room temperature for 10 min, 30 µl of phenol (equilibrated with 1% SDS, 0.2 M NaOH) added and the tube vortexed gently. The contents were neutralised by addition of 150 µl 3 M NaOAc pH 4.8 and the tube incubated at -20°C for 15 min. The tube was centrifuged for 3 min as above and the supernatant transferred to a fresh tube containing ice-cold 95% ethanol. The contents were mixed by inversion and stored at -80°C for 15 min. The DNA was pelleted by centrifugation for 3 min as above and the supernatant discarded. The pellet was resuspended in 0.5 ml 0.3 M NaOAc pH 7.0, the tube filled with ice-cold 95% ethanol and mixed by inversion. The tube was stored at -80°C for 15 min before centrifuging for 3 min as above. The supernatant was discarded and the pellet washed with 1 ml ice-cold 70% ethanol. The pellet was dried and resuspended in 50 µl 1 x TE.

2.6.6 Preparation of plant genomic DNA

The method is based on that of Dellaporta *et al.* (1983).

1 g of frozen plant tissue was added to a pre-cooled mortar and ground in liquid N_2 into a fine powder. The tissue was transferred to a 50 ml polypropylene tube containing 15 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.5 M NaCl, 10 mM mercaptoethanol). The contents were mixed, 1 ml 20% SDS added, the contents mixed again and placed at 65°C for 10 min. 5 ml of 5 MKOAcwere

added to the tube which was shaken vigorously and incubated at 0°C for 20 min. The tube was centrifuged at 10 000 rpm (approximately 15 000 x *g*) in an MSE High Speed 18 centrifuge and the supernatant poured through two layers of muslin into a polypropylene tube containing 10 ml isopropanol. The contents of the tube were mixed and incubated at -20°C for 30 min. The DNA was pelleted by centrifugation as above and the tube inverted to drain. The pellet was dissolved in 0.7 ml of 50 mM Tris-HCl pH 8.0, 10 mM EDTA and transferred to an Eppendorf® tube. The tube was incubated at 65°C for 5 min before centrifuging for 10 min in a microfuge. The supernatant was transferred to a fresh Eppendorf® tube containing 75 µl 3M NaOAc pH 5.2 and 500 µl isopropanol. The contents were mixed and centrifuged for 30 s. The DNA pellet was washed with 70% ethanol, dried and allowed to dissolve in 1 x TE overnight at 4°C.

2.7 Agarose gel electrophoresis

Agarose (*ultra*PURE™) was supplied by Life Technologies.

2.7.1 Electrophoresis of DNA

Agarose gels of appropriate concentration (0.5-2.0%) were made by adding the correct amount of agarose to 1 x TBE (0.09 M Tris-borate, 2 mM EDTA) and heating in a microwave oven until the solution just reached boiling point. The melted agarose solution was allowed to cool to 60°C and EtBr added to a final concentration of 0.5 µg/ml. The solution was poured into the electrophoresis apparatus and allowed to set for approximately 30 min. Electrophoresis running buffer was 1 x TBE. Prior to loading, one-tenth volume of loading buffer (125 mM EDTA, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue) was added to the

DNA samples and the solutions mixed. Samples were loaded with an automatic pipette. Gels were run at 40-100 mA until the bromophenol blue reached approximately $^2/_3$ down the gel. If the DNA fragments were to be isolated using the GeneClean® kit, the electrophoresis buffer used was 1 x TAE (0.04 M Tris-acetate, 1 mM EDTA).

2.7.2 Alkaline agarose gel electrophoresis

Agarose was prepared in 50 mM NaCl, 1 mM EDTA and the gel allowed to set. The gel was soaked in alkaline electrophoresis buffer (30 mM NaOH, 1 mM EDTA) for 30 min before loading of samples. Loading buffer used was 50 mM NaOH, 1 mM EDTA, 2.5% (w/v) Ficoll, 0.025% (w/v) bromophenol blue.

2.8 Digestion of DNA with restriction endonucleases

Restriction enzymes and buffers were supplied by Gibco-BRL. DNA to be digested was incubated for 1-6 h at 37°C in 1 x appropriate restriction buffer, 0.25 mg/ml RNAase (if necessary) and 1-20 U/ µg DNA of restriction endonuclease. For digestion of plant genomic DNA, spermidine was added to a final concentration of 1 mg/ml.

2.9 Isolation of DNA fragments using GeneClean®

The GeneClean® kit was supplied by BIO 101 Inc. (La Jolla, USA) and was used according to the manufacturers instructions.

The DNA band required was located by illumination of the gel with long-

wave UV light and excised with a scalpel blade. The amount of agarose was estimated by weighing (1 g = 1 ml) and 2.5 vol of 6 M NaI (supplied with kit) added. The agarose was dissolved by incubation at 55°C for 10 min. The required amount of Glassmilk® (5 µl for up to 5 µg DNA) was added, the contents mixed and allowed to stand at room and the supernatant removed. The pellet was washed three times with 0.5 ml ice-cold NEW wash and resuspended in 5-20 µl 1 x TE. The DNA was eluted by incubation at 55°C for 3 min, transferred to a clean Eppendorf® tube and the pellet extracted once more with 1 x TE. This procedure yielded 50-80% recovery of DNA.

2.10 Transformation of *E. coli*

This method is that of Armitage et al. (1988).

2.10.1 Production of competent cells

A single colony of *E. coli* XL-1 Blue was resuspended in 5 ml of φ-broth and incubated at 37°C for 2-3 h. The starter culture was used to inoculate 100 ml of pre-warmed φ-broth, which was shaken at 37°C until the $O.D._{550}$ reached 0.35. The culture was transferred to two 50 ml sterile centrifuge tubes and stored on ice for 15 min. The culture was centrifuged for 5 min at 2 500 rpm in a Beckman TJ-6 centrifuge and the supernatant decanted. The cells were resuspended with gentle vortexing in 10.5 ml ice-cold TFB1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% glycerol, pH 5.8), pooled and incubated on ice for 90 min. The cells were centrifuged as above, the supernatant decanted and the cells resuspended in 2.8 ml ice-cold TFB2 (10 mM MOPS pH 7.0, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol) and dispensed into 200 µl aliqouts in chilled microfuge tubes. Aliquots were

flash-frozen in liquid N_2 and stored at -80°C until use.

2.10.2 Transformation of competent cells

Competent cells were thawed at room temperature and stored on ice for 10 min. Up to 10 ng of DNA (<10 µl vol) was added to a 200 µl aliquot, the cells mixed gently and incubated on ice for 20 min. The cells were heat-shocked for 60 s at 37°C and returned to ice for a further 2 min. 800 µl of φ-broth was added and the cells incubated at 37°C for 50 min. The cells were pelleted in a microfuge for 30 s and resuspended in 100 µl φ-broth. Two 10-fold serial dilutions of the cell suspensions were made and the contents of the tube were spread onto L-agar plates containing antibiotics and X-GAL and IPTG where required. Control plates of uncut plasmid, restricted plasmid and restricted, re-ligated plasmid were also set up. The plates were dried and incubated upside-down overnight at 37°C.

2.11 Ligation of insert DNA to vector

Vector DNA was digested to completion with the appropriate restriction enzyme(s) and dephosphorylated to prevent re-ligation to self. Alkaline phosphatase (Northumbria Biologicals Ltd., Cramlington, U.K.) was used at a concentration of 0.1 U/µg vector DNA and the reaction took place at 37°C for 30 min. The phosphatase was inactivated by incubation at 70°C for 10 min. The vector DNA was extracted once with phenol/chloroform (1:1), once with chloroform and ethanol precipitated. Insert DNA was isolated from agarose gels using the GeneClean® kit. A ratio of insert to vector DNA of 2:1 (picomole ends) was used in a vol of 10 µl containing 1 U T4 DNA ligase (Gibco-BRL) and 2 µl 5 x ligation buffer (supplied with enzyme). The reaction was incubated overnight at 14°C. The reaction was

stopped by heating at 65°C for 5 min.

2.12 Isolation of RNA from plant tissue

2.12.1 Isolation of total RNA

This method is adapted from Ismail (1988).

A pre-weighed amount of frozen tissue was added to a pre-chilled mortar and ground to a fine powder in liquid N_2. The powder was transferred to a baked Corex® tube containing 5 ml of phenol/chloroform (1:1) and 5 ml of extraction buffer (0.05 M NaCl, 5 mM NaOAc, 2.5 mM EDTA, 5% SDS). Tubes were thawed at 60-65°C and vortexed before centrifuging at 3 000 rpm (approximately 2 500 x *g*) for 15 min at 10°C in an MSE Mistral 2L centrifuge with a swing-out rotor. The aqueous phase was stored on ice and the phenol phase re-extracted with a further 5 ml of extraction buffer. The aqueous phases were pooled and extracted once with 10 ml of phenol/chloroform and once with 10 ml of chloroform before precipitating the RNA with 1.0 ml of 3 M NaOAc pH 5.2 and 20 ml of ethanol at -20°C for at least 2 h. The RNA was pelleted by centrifugation in an MSE 18 High Speed centrifuge at 10 000 rpm (approximately 15 000 x *g*) for 15 min at 4°C. The pellet was washed with 80% ethanol, dried *in vacuo* and dissolved in H_2O. The purity of the preparation was estimated by measuring the O.D. at 260 nm and 280 nm. A pure preparation was taken as having an A_{260}/A_{280} ratio of 1.8-2.0. The integrity of the RNA was examined on a 2% agarose mini-gel. All RNA preparations were stored at -80°C.

2.12.2 Isolation of poly(A)$^+$ RNA

Poly(A)$^+$ RNA was isolated according to the method of Maniatis et al. (1982) with modifications.

Oligo-dT cellulose type 7 (Pharmacia, Milton Keynes, U.K.) was suspended in loading buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 0.1% sodium lauryl sarcosine) and the suspension poured into a disposable plastic column to give a column volume of approximately 1 ml. The column was washed with 1 vol H_2O, 1 vol 0.1 M NaOH, 5 mM EDTA, and again with H_2O until the pH of the column effluent was less than 8.0. The column was then re-equilibrated with loading buffer. Total RNA was heated to 65°C for 5 min and chilled on ice. An equal vol of two-fold concentrated loading buffer was added to the RNA which was then loaded on the column. The column effluent containing unbound RNA was heated to 65°C, chilled on ice and reapplied to the column. The column was washed with loading buffer until the A_{260} of the column effluent reached zero. Poly(A)$^+$ RNA was eluted from the column into a siliconised Corex® tube with elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.05% sodium lauryl sarcosine) at 65°C. The RNA was precipitated with 0.1 vol 3 M NaOAc pH 5.2 and 2.5 vol ethanol. After pelleting the poly(A)$^+$ RNA, the pellet was washed with 70% ethanol and dried. The RNA was resuspended in H_2O containing 50 U of human ribonuclease inhibitor (HPRI; Amersham) and stored at -80°C.

2.13 Electrophoresis of RNA in formaldehyde gels

This was according to Maniatis et al. (1982). Molecular size markers were a 0.24-9.5 Kb RNA ladder and a 0.16-1.77 Kb RNA ladder, both supplied by Gibco BRL.

Agarose gels (1.2-2.0%) were prepared in 1 x MOPS buffer (20 mm MOPS pH 7.0, 8 mM NaOAc, 5 mM EDTA) and 2.2 M formaldehyde. The samples were prepared by adding to the RNA formamide to a final concentration of 50%, formaldehyde to 2.2 M and MOPS buffer to x 1. The samples were then denatured by heating to 65°C for 5 min and chilling on ice. 0.1 vol of RNA loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue) was added to the sample prior to loading. The gel was electrophoresed in 1 x MOPS buffer at 30-50 V for 4-6 h until the dye reached $^2/_3$ of the way down the gel. RNA was visualised by adding 0.5 µl of EtBr (10 mg/ml) to the sample before loading on the gel and examining under UV light after electrophoresis.

2.14 *In vitro* translation of total or poly(A)$^+$ RNA using wheat germ extract

5 µg of total RNA or 1 µg of poly(A)$^+$ RNA was added in a volume of 7.5 µl to a mixture containing 2 µl of 1 mµ amino acid mixture minus methionine, 25 µCi (2.5 µl) of ^{35}S-L-methionine, 15 µl of wheat germ extract (Amersham) and 3 µl of 1 M KAc. The reaction mixture was incubated at 25°C for 90 min. The incorporated activity was estimated by TCA precipitation of an aliquot of the translation reaction. Translation products were visualised by one-dimensional SDS-PAGE by loading 2 x 10^5 cpm per track.

2.15 One-dimensional SDS-PAGE

Electrophoresis was carried out according to previously described protocols (Laemmli, 1970).

A 12% resolving gel was prepared by combining 16 ml of acrylamide solution (30% acrylamide, 0.8% bis-acrylamide (w/v)), 12 ml of solution I (1.5 M Tris-HCl pH 8.8, 0.4% SDS), 3.2 ml 50% glycerol, 16.4 ml H2O, 16 µl TEMED and 180 µl 10% (w/v) ammonium persulphate. The solution was mixed, de-gassed under vacuum for 5 min and poured into a set of glass plates with 1.5 mm spacers. 1 ml of butanol was applied to the top of the solution to ensure a level surface. Once the gel had polymerised (1-2 h) the stacking gel was prepared by combining 1.5 ml of acrylamide solution, 3.75 ml of solution II (0.5 M Tris-HCl pH 6.8, 0.4% SDS), 9.75 ml H2O, 8 µl TEMED and 150 µl of 10% ammonium persulphate. After mixing the stacking gel was poured on top of the resolving gel and the sample well former set in place. Samples were boiled for 2 min and applied in loading buffer (50 mM Tris-HCl pH 8.0, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.1% bromophenol blue). One lane typically contained 14C labelled protein molecular weight markers (Amersham). Running buffer (25 mM Tris-HCl, 0.192 M glycine, 0.1% SDS) was placed in the upper and lower reservoirs and electrophoresis was conducted at 65 mA for ~2.5 h (until the dye front reached the bottom of the gel). Gels were removed from the plates and fixed in 7% acetic acid for at least one hour.

2.15.1 Fluorography and autoradiography of SDS-PAGE gels

After fixing gels were incubated with EN^{3}HANCE™ (NEN-Dupont, Stevenage, U.K.) for 1 h at room temperature with gentle agitation. The EN^{3}HANCE solution was removed and the gels were rinsed under water for 30 min. Gels were dried under vacuum and then placed in a film cassette with Fuji X-Ray film type RX. No intensifying screens were necessary.

2.16 Transfer of nucleic acids onto nylon filters

2.16.1 Southern blotting

The method is essentially as described by Southern (1975) with modifications.

Restricted genomic or plasmid DNA was separated on an agarose gel containing 0.5 µg/ml EtBr and the gel photographed. The DNA was denatured by washing in 1.5 M NaCl, 0.5 M NaOH for 30 min and neutralised in 1.5 M NaCl, 1 M Tris-HCl pH 8.0 for 30 min. The gel was rinsed in 10 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate) for 10 min. The gel was placed upside-down on a wick of 2 layers of Whatman 3MM paper wet with 10 x SSC and air-bubbles removed. Cling film was placed around the wick to the edge of the gel. A Hybond™ N nylon filter (Amersham) was cut to the size of the gel and placed on top of the gel followed by four pieces of Whatman 3MM paper. Absorbent tissue was placed over the gel to a height of 5-6 cm and this was weighed down with a 500 ml bottle. The blotting apparatus was left for at least 6 h, dismantled and the gel examined under UV to check efficiency of transfer. The filter was wrapped in SaranWrap and DNA was fixed to the filter by cross-linking under UV light for 1-2 min.

2.16.2 Northern blotting

RNA from agarose-formaldehyde gels was blotted onto nylon filters as described above. The gel was not pre-treated prior to blotting.

2.16.3 RNA dot-blotting

Aliquots of RNA were denatured in 3 vol of buffer (65.6% formamide, 7.87% formaldehyde, 1.31 x MOPS buffer; see Section 2.11) at 65°C for 5 min and chilled on ice. An equal vol of ice-cold 20 x SSC was added and the samples mixed and stored on ice. A nylon filter (12 x 8 cm) was set up in a BRL HYBRI-DOT 96-hole manifold apparatus and gentle suction applied. Each well was washed with 200 µl 10 x SSC and the samples loaded in separate wells in a volume of 100-200 µl. The wells were flushed with 2 applications of 10 x SSC. The manifold was dismantled and the RNA cross-linked to the filter by exposure to UV light.

2.17 Hybridisation of nucleic acids

Nylon filters containing bound DNA or RNA were pre-hybridised in hybridisation solution (5-6 x SSC, 50% formamide, 5 x Denhardt's solution (0.1% Ficoll 400, 0.1% PVP 360, 0.1% BSA), 100-200 µg/ml denatured, sonicated salmon sperm DNA, 0.1% SDS) in sealed bags for 4 h at the appropriate temperature. DNA or RNA probes were denatured by heating and chilled on ice. The hybridisation solution in the bag was replaced and the probe added before re-sealing the bag. Care was taken to exclude all air-bubbles. Hybridisation was carried out at the appropriate temperature for 16 h. The filters were washed at the appropriate stringency in an excess of wash solution in plastic boxes. The filters were wrapped in SaranWrap and autoradiographed.

2.18 Radio-labelling of DNA

2.18.1 Preparation of cDNA probes for differential screening

First strand cDNA was synthesised from 5 µg of poly(A)+ RNA in the presence of α-^{32}P-dCTP using the Amersham cDNA Synthesis Plus kit.

To a siliconised microfuge tube was added: 10 µl first strand reaction buffer, 2.5 µl sodium pyrophosphate, 5 µl nucleotide mix (10 mM dGTP, 10 mM dATP, 10 mM dTTP), 2.5 µl oligo dT, 500 µCi α-^{32}P-dCTP (>3000 Ci/mmol, dried *in vacuo* and reconstituted in 5 µl H_2O), 5 µg poly(A)$^+$ RNA and 100 U AMV reverse transcriptase in a final vol of 50 µl. The tube was incubated for 60 min at 42°C after which time 5 µl 10 mM dCTP was added and the reaction continued for 30 min. Template RNA was degraded by addition of an equal vol of 0.6 M NaOH, 20 mM EDTA and incubation at 60°C for 30 min. Labelled cDNA was precipitated with an equal vol of 4 M NH_4OAc and 2 vol ethanol at -20°C for 2 h, washed with 70% ethanol and resuspended in 50 µl 1 x TE.

2.18.2 Random priming of dsDNA

DNA was labelled withα-^{32}P-dCTP using the Multiprime DNA labelling kit supplied by Amersham.

DNA was digested and electrophoresed in a low melting point agarose gel containing 0.5 µg/ml EtBr. The concentration of the desired band was estimated by comparison with standards. The band was excised and transferred to a pre-weighed Eppendorf® tube. H_2O was added at a ratio of 3 ml per gram of gel and the tube

boiled for 7 min to melt the agarose and denature the DNA. The tube was transferred to a 37°C water bath for at least 10 min. To a siliconised screw-cap Eppendorf® was added: 25 ng of DNA, 10 µl of reaction buffer containing unlabelled dTTP, dGTP and dATP, 5 µl of primer solution containing random hexanucleotides, 30-50 µCi α-^{32}P-dCTP (>3000 Ci/mmol), 2 µl enzyme solution (2 U DNA polymerase I 'Klenow' fragment) and H_2O to 50 µl. The contents were mixed and incubated at room temperature overnight. The reaction was stopped by adding 2 µl 10% SDS and 2 µl 0.5 M EDTA. The labelled DNA was precipitated by the addition of an equal vol of 2 M NH_4OAc and 2 vol of ethanol in the presence of 10 µg carrier DNA (sonicated salmon sperm DNA) at -20°C for 30-90 min. The pellet was washed twice with ice-cold 70% ethanol and resuspended in 50 µl 1 x TE. A 1 µl aliquot was Cerenkov counted to estimate the extent of labelling.

2.18.3 End-labelling of DNA markers

DNA markers were prepared by digestion to completion of λ DNA with Eco RI and Hind III or pUC 9 DNA with Hpa II. The markers were prepared by addition of 2 µCi of α-^{32}P-dNTP and 1 U of *E. coli* DNA polymerase, Klenow fragment to 20 µl of restricted DNA and incubation for 10 min at room temperature. The reaction was stopped by the addition of 1 µl 0.5 M EDTA. λ markers were labelled with α-^{32}P-dATP and pUC9 markers with α-^{32}P-dCTP.

2.19 Labelling of *in vitro* transcribed RNA

In vitro transcribed RNA was synthesised from cDNA inserts cloned into pBluescript SK(-) vector (Stratagene). RNA was synthesised using the RNA Transcription kit supplied by Stratagene.

2.19.1 Preparation of plasmid templates

Plasmid templates were prepared by digestion to completion with a restriction enzyme which cleaves downstream of the insert in the multiple cloning site. Enzymes were chosen which produced 5' protruding termini to prevent non-specific initiation. The DNA was treated with 50 µg/ml proteinase K for 30 min at 37°C, extracted once with phenol/chloroform (1:1), once with chloroform and ethanol precipitated. The DNA was resuspended in RNAase-free 1 x TE at a concentration of 1 µg/µl.

2.19.2 ^{35}S-labelled RNA transcripts for *in situ* hybridisation

To a screw-cap Eppendorf® tube was added in the following order: 5 µl 5 x transcription buffer (200 mM Tris-HCl pH 8.0, 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl), 1 µg plasmid template, 1 µl each of 10 mM ATP, 10 mM GTP, 10 mM CTP and 0.4 mM UTP, 1 µl 0.75 M DTT, 5 U HPRI (Amersham), 100 µCi α-^{35}S-rUTP (>1000 Ci/mmol), 10 U T3 or T7 RNA polymerase and H_2O to 25 µl. The reaction was incubated at 37°C for 30-60 min. The template was removed by addition of 2 U DNAase I (RNAase-free; Worthington, Freehold, NJ, USA) and 1 µl 0.75 M DTT, followed by incubation at 37°C for 10 min. The volume was made up to 100 µl with 10 mM DTT and 5 µl removed for analysis. 2.5 µg of yeast tRNA (type X-SA, Sigma) was added and the RNA extracted once with phenol/chloroform (1:1), once with chloroform and precipitated with 0.1 vol 3 M NaOAc pH 5.2 and 2 vol of ethanol. The RNA was resuspended in 75 µl of 10 mM DTT. The probes were hydrolysed to a mean length of 150 bp by adding 75 µl of 2 x carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2) and incubating for the required time at

60°C. The hydrolysis time was calculated from the equation:

$$t = \frac{Li - Lf}{K.\ Li.\ Lf}$$

where t = time (min), K is the rate constant (= 0.11 kb/min), Li is the initial length (kb) and Lf the final length (kb). After hydrolysis the reaction was neutralised by adding 7.5 µl 10% acetic acid and the RNA precipitated with 0.1 vol 3 M NaOAc pH 5.2 and 2.5 vol ethanol. The RNA was resuspended in 10 mM DTT, 50% formamide.

2.19.3 Analysis of ^{35}S-labelled RNA transcripts

The % incorporation of label in the reaction was calculated by TCA precipitation (see section 2.20.1). The size of the probe before and after hydrolysis was estimated by running on a 3% acrylamide denaturing gel (see section 2.25.3) and comparing to ^{32}P-labelled markers (Hpa II digest of pUC 9).

2.19.4 ^{32}P-labelling of RNA transcripts

In vitro transcribed RNA labelled with α-^{32}P-UTP was used for probing blots of genomic DNA and RNA from transgenic plants. The reaction was essentially as described above (see section 2.19.2) with the inclusion of 50 µCi of α-^{32}P-UTP (400-800 Ci/mmol) in place of the ^{35}S. The reaction was not supplemented with cold UTP to allow generation of high specific activity probes. After incubation at 37°C for 30-60 min a 2 µl aliquot was removed to calculate % incorporation. The transcripts were separated from unincorporated nucleotide on an RNAase-free Sephadex G-50 column (Maniatis et al., 1982). A 1 ml sterile syringe was plugged with siliconised, baked glass wool and a column of Sephadex G-50 (0.9 ml vol, equilibrated in 0.1 M

NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA (STE), 0.1 % SDS) prepared. The column was inserted in a glass centrifuge tube and centrifuged at 3000 rpm for 4 min. Sephadex was added until the column volume reached 0.9 ml. Two applications of 100 µl of STE, 0.1% SDS were added to the column which was centrifuged as above. The RNA sample was made up to 100 µl in STE and applied to the column. The column was centrifuged as above, the eluent being collected in an Eppendorf® tube. Unincorporated nucleotide remained in the column which was discarded appropriately. The probe was stored at -80°C until use.

2.19.5 RNA labelling with digoxigenin-UTP

In vitro transcribed RNA was synthesised in the presence of digoxigenin-11-UTP and used to probe tissue sections in situ. DIG RNA labelling mix was supplied by Boehringer Mannheim Biochemica (Lewes, U.K.) and RNA synthesised using the RNA transcription kit (Stratagene).

The following were added to an RNAase-free Eppendorf® tube: 4 µl RNA transcription buffer, 2 µl DIG RNA labelling mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM digoxigenin-11-UTP), 1 µg of plasmid template, 4 U HPRI (Amersham), 10 U T3 or T7 RNA polymerase and H_2O to 20 µl. The reaction was incubated at 37°C for 2 h and template DNA removed by incubation with 2 U DNAase I (RNAase-free ,Worthington) for 10 min at 37°C. The reaction was stopped by the addition of 4 µl 0.1 M EDTA. An aliqout of 2 µl was removed for analysis and the RNA precipitated with 0.1 vol 4 M LiCl and 3 vol of ethanol at -20°C for 2 h. The RNA pellet was resuspended in 50 µl H_2O. The RNA was analysed on a 1.5% agarose-TBE gel containing 0.5 µg/ml EtBr.

2.20 Estimation of incorporation of radio-nucleotides into

probes

2.20.1 By TCA precipitation

This method was essentially as in Maniatis et al. (1982).

A known vol (typically 1 µl) of sample was spotted onto two 25 mm Whatman GF/C filter discs and allowed to dry. One filter was transferred to a beaker containing >10 ml of ice-cold 10% TCA and chilled on ice for 15 min. The filter was washed twice with 10 ml 10% TCA and twice with 10 ml 95% ethanol. The filter was dried and both filters placed in plastic vials for counting. ^{32}P-labelled probes were counted on the Cerenkov channel and ^{35}S-labelled probes by the addition of 4 ml Ecoscint prior to counting. The % incorporation was estimated by:

$$\frac{\text{cpm of washed filter}}{\text{total cpm}} \times \text{dilution factor}$$

2.20.2 By binding to Whatman DE81 paper

Up to 5 µl of labelled DNA solution was spotted onto two 23 mm discs of Whatman DE81 ion-exchange paper. One disc was washed with 0.5 M Na_2HPO_4 six times for 5 min each time, twice for 1 min with H_2O and twice with 95% ethanol for 1 min each time. The discs were allowed to dry before transferring to scintillation vials for counting. The % incorporation was estimated as above.

2.21 Construction of cDNA library

2.21.1 cDNA synthesis

cDNA was synthesised using the Amersham cDNA Synthesis Plus kit.

5 µg of poly(A)$^+$ RNA was heated at 68°C for 5 min and chilled on ice. To a siliconised Eppendorf® tube was added in order: 10 µl 5 x first strand buffer, 2.5 µl NaPPi, 2.5 µl (50 U) HPRI, 5 µl dNTPs (10 mM dATP, 10 mM dGTP, 10 mM dTTP, 5 mM dCTP), 2.5 µl (4.0 µg) oligo(dT), 1 µl (10 µCi) α-^{32}P-dCTP (800 Ci/mmol), 5 µl (5 µg) poly(A)$^+$ RNA, H_20 to 45 µl, 100 U (5µl) AMV reverse transcriptase. The contents of the tube were mixed, centrifuged briefly in a microfuge and incubated at 42°C for 60-90 min. After the reaction was complete 2 µl was removed for analysis and to the remainder was added; 93.5 µl second strand buffer, 5 µl (50 µCi) α-^{32}P-dCTP (800 Ci/mmol), 4 U (5 µl) *E. coli* RNAase H, 115 U (33 µl) *E. coli* DNA polymerase I, and 66 µl H_2O. The contents were mixed, centrifuged and incubated sequentially at 12-16°C for 60 min, 22°C for 60 min and 70°C for 10 min. 10 U (2.5 µl) of T4 DNA polymerase was added to the tube which was then incubated at 37°C for 15 min. 10 µl of reaction mix was removed for analysis and the reaction stopped by the addition of 15 µl 0.5 M EDTA and 25 µl 10% SDS. The cDNA was extracted twice with an equal volume of phenol/chloroform, the pooled phenol phases back-extracted with 50 µl 1 x TE, and the combined aqueous phases extracted once with ether. NH_4OAc was added to a final concentration of 2 M followed by 2 vol ethanol and the cDNA precipitated on ice for 20 min. The cDNA was pelleted by spinning in a microfuge for 10 min, the supernatant removed with a drawn out pasteur pipette and the pellet resuspended in 60 µl TE. The cDNA was precipitated a second time as above and the final pellet

left at -20°C overnight in 70% ethanol.

2.21.2 Methylation of cDNA and ligation of linkers

The cDNA was cloned directionally using the method of Dorssers and Postmes (1987). Linkers contained the last four bases of the Hind III recognition sequence (GCTT) and an internal Eco RI site. The 3' end of the double-stranded cDNA supplied the first two bases of the Hind III sequence (AA). Alu I methylase which methylates the C residue of the sequence AGCT was used to protect Hind III sites in the cDNA due to the unavailability of Hind III methylase. Kinased linkers were kindly donated by Dr. Kim Kaiser (Dept. of Genetics, University of Glasgow).

The cDNA was resuspended in 36 µl H_2O and to this was added: 5 µl 10 x Alu I methylase buffer, 25 U (5µl) Alu I methylase (New England Biolabs, Beverley, MA, USA) and 4 µl 1.3 µM S-adenosyl methionine (SAM) and the tube was incubated at 37°C for 60 min. The Eco RI sites were subsequently methylated by adding to the tube; 5 µl Eco RI methylase buffer, 5 µl Eco RI methylase (New England Biolabs), 4 µl 1.3 µM SAM and 46 µl H_2O, and incubating for 60 min at 37°C. The methylated cDNA was phenol/chloroform extracted, the phenol phase back extracted and the pooled aqueous phases extracted with ether. The cDNA was precipitated with 0.1 vol 3 M NaOAc pH 5.2, and 2 vol ethanol on ice for 20 min. The pellet was washed and air-dried before resuspending in the following; 5 µl kinased linkers (Huynh et al., 1985), 2 µl 5 x ligation buffer, 1 µl 10 mM ATP, 1 µl T4 ligase (Gibco-BRL) and 1 µl H_2O. The ligation reaction was allowed to proceed overnight at 14°C. The reaction was terminated by heating to 70°C for 10 min and the excess linkers digested sequentially with 120 U Hind III and 120 U

Eco RI to a final volume of 300 µl. The cDNA was precipitated with 200 µl 5 M NaOAc and 1 ml ethanol, pelleted and resuspended in 20 µl column buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM EDTA). The linkers were separated from the cDNA by running the sample over a Sepharose CL4B column (bed volume approximately 10 ml). Fractions of 100 µl were collected from the column and Cerenkov counted. Appropriate fractions were run on a 1% alkaline agarose mini-gel.

2.21.3 Ligation of vector arms and packaging of library

The linkered cDNA was cloned into the vector λNM1149 (Murray, 1983). Vector DNA which had been digested with Eco RI and Hind III and phosphatased, was kindly donated by Dr. Kim Kaiser.

The fractions from the column which contained cDNA were pooled and resuspended in a smaller volume (50 µl) following ethanol precipitation. The concentration of the vector (λNM1149) and cDNA was estimated by spotting onto a 1% agarose plate, together with standards, and flooding the plate with EtBr before photographing it. 5 µg of vector arms and 0.1 µg of cDNA were co-precipitated with ethanol. The DNA was resuspended in 8 µl 10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$ and heated to 42°C for 15 min. The arms were ligated onto the cDNA by adding 1 µl 10 mM ATP, 1 µl 0.1 M DTT and 1µl T4 DNA ligase, and placing at 14°C overnight. The ligated DNA was packaged using the Gigapack Gold kit supplied by Stratagene.

2.22 Screening of the cDNA library

2.22.1 Preparation of plating cells

A single colony of *E. coli* NM621 was used to inoculate 50 ml of sterile L-broth plus 0.4% maltose in a flask which was incubated overnight in a shaking incubator at 37°C. The cells were transferred to a sterile tube and centrifuged at 3 000 rpm in a Beckman model TJ-6 centrifuge for 10 min at 4°C. Cells were resuspended in ice-cold 10 mM $MgSO_4$ to an O.D. of 2.0. Cells were used within 3 days of preparation.

2.22.2 Infection of plating cells and growth of plaques

150 µl of plating cells were mixed with 100 µl of diluted phage in SM buffer in a sterile bijou and placed at 37°C for 15 min. 6 ml of top agarose (1%) at 45°C was added to the bijou and the contents mixed and poured onto a 10 cm x 10 cm L-agarose plate. The plates were incubated upside down at 37°C for 7 to 12 h until plaques had grown.

2.22.3 Preparation of filters for hybridisation

Filters were prepared by cutting Hybond™ N (Amersham) into 9.5 cm squares for plaque lifts. A plaque lift was taken by placing a nylon filter on top of an L-agarose plate and allowing contact for 30 s. Each subsequent filter was left in contact for an additional 30 s to a maximum of 6 filters per plate. The filters were placed plaque side up onto Whatman 3MM filter paper soaked with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min. The filters were then transferred to neutralising

solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 min and washed in 2 x SSC for a further 5 min. The filters were air-dried and baked at 80°C for 2 h.

2.22.4 Hybridisation procedure for differential screening

Prior to hybridisation the filters were immersed in 1% (v/v) Triton X-100 for 5 min and washed in 5 x SSC for 15 min. The filters were then placed in plastic food storage boxes containing 50 ml of hybridisation solution (6 x SSC, 10 x Denhardt's solution, 100 µg/ml sonicated denatured salmon sperm DNA, 15 µg/ml polyadenylic acid (Pharmacia), 0.1% SDS, 0.005% sodium pyrophosphate) for 16 h at 68°C. Prehybridisation fluid was discarded and 50 ml of fresh hybridisation solution containing 5-10 x 10^7 cpm of cDNA probe (see Section 2.18.1) was added to the boxes. The filters were hybridised at 68°C for 36-48 h. Membranes were washed in 2 x SSC at room temperature for 30 min; in 2 x SSC, 0.1% SDS, 0.1 % sodium pyrophosphate at 68°C for 30 min; in 0.1 x SSC, 0.1% SDS, 0.1 % sodium pyrophosphate at 68°C for 30 min. The filters were wrapped in SaranWrap aand autoradiographed overnight with two intensifyng screens at -80°C.

2.22.5 Secondary screening of putative positive plaques

Putative differential signals on the autoradiographs were aligned with the original plates and plaques corresponding to these signals were picked into 1 ml of SM buffer. An appropriate volume of NM621 cells in 10 mM $MgSO_4$ was mixed with 6.5 ml of 1% agarose-LB and poured onto prewarmed 1.5% agarose-LB plates. The plates were placed on a 10 cm by 10 cm grid and 1µl of undiluted lysate was spotted, in duplicate, onto the surface of the plate. The plates were then inverted and placed at 37°C for 8-12 h to form 'giant plaques' 7-10 mm in diameter. Nylon filters

were prepared from these plates and rescreened. Lysates which showed a differential signal as giant plaques were screened a third time by preparing a plate containing an identical population of plaques. Filters were then prepared and rescreened with the differential probes as in section 2.22.4.

2.23 *In situ* hybridisation

Tissue was fixed in four different types of fixative to examine the effect of the fixative on *in situ* hybridisation. The fixatives used were: 4% formaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4); 3% formaldehyde, 1.25% glutaraldehyde in 50 mM phosphate buffer pH 7.0; 1% glutaraldehyde in 50 mM phosphate buffer pH 7.5 and 3.7% formaldehyde, 5% acetic acid, 50% ethanol (FAA). Solutions to contain formaldehyde were prepared, treated with DEPC and autoclaved before addition of formaldehyde. Paraformaldehyde was added to the appropriate concentration and dissolved at 70°C for 30 min. The tissue was dehydrated using three different methods depending on the fixative used as described below.

2.23.1 Fixation of tissue in 4% formaldehyde

This method follows that of Jackson (1989).

Tissue was harvested and placed directly into 5 ml of 4% formaldehyde in 50 mM phosphate-buffered saline in a sterile bijou. The tissue was vacuum infiltrated for 10 min, the fixative replaced and the sample placed at 4°C overnight. The fixative was replaced with ice-cold 0.85% NaCl and placed on ice for 30 min. The tissue was dehydrated through a series of de-gassed ethanols; 50% ethanol, 0.85%

NaCl for 90 min on ice, 70% ethanol, 0.85% NaCl for 90 min on ice, 85% ethanol, 0.85% NaCl for 90 min at 4°C, 95% ethanol, 0.85% NaCl for 90 min at 4°C and 100% ethanol for 90 min at 4°C. The tissue was placed in fresh 100% ethanol and left overnight at 4°C.

2.23.2 Fixation of tissue using formaldehyde/glutaraldehyde

This method follows that of Cox and Goldberg (1989) with modifications.

Tissue was harvested directly into 5 ml of fixative (3% formaldehyde, 1.25% glutaraldehyde in 50 mM phosphate buffer pH 7.0 or 1% glutaraldehyde in 50 mM phosphate buffer pH 7.5) in a sterile bijou. The tissue was vacuum infiltrated for 10 min, the fixative replaced and the sample left overnight at 4°C. The tissue was dehydrated in the following ethanol series at room temperature under vacuum for 30 min in each ethanol concentration; 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 95%, 100%. The tissue was transferred to fresh ethanol and left overnight at 4°C.

2.23.3 Fixation of tissue in FAA

This method is that of Cox and Goldberg (1989).

Tissue was harvested directly into 5 ml of FAA in a sterile bijou and vacuum infiltrated for 10 min. The fixative was replaced and the tissue left at 4°C overnight. The tissue was dehydrated in an ethanol series; 50%, 60%, 70%, 80%, 95%, 100%. The tissue was left in each ethanol concentration for 30 min at room temperature under vacuum. Fresh 100% ethanol was added to the tissue which was

left at 4°C overnight.

2.23.4 Wax-embedding of fixed tissue

This method follows that of Cox and Goldberg (1989).

The tissue was transferred to fresh 100% ethanol at room temperature for 1 hr. The tissue was then placed in 5 ml of the following solutions for 30 min each at room temperature; 25% xylene in ethanol, 50% xylene in ethanol, 75% xylene in ethanol, 100% xylene (three times). Wax chippings were added to the final xylene change to about equal volume and the tissue left at 45-50°C overnight (on top of the 60°C oven). The wax/xylene was poured off and molten wax added to the tissue which was placed at 60°C. The wax was changed every 12 h for 3 days. The tissue was then transferred to a plastic mould containing a small amount of wax. More wax was added until the tissue was covered and the wax allowed to solidify by floating on a cool water bath. Once the wax had set, the block was removed from the mould and cut into a trapezoid shape around the tissue. The tissue block was mounted onto a 3 cm x 3 cm x 1 cm piece of plastic or wood using melted wax. The tissue blocks were stored at 4°C until sectioned.

2.23.5 Preparation of slides for *in situ* hybridisation

Slides were washed in 5% Decon 90 and rinsed in several changes of distilled H_2O before drying. The slides were coated by dipping in 2% 3-aminopropyl triethoxysilane (TESPA; Sigma) in acetone for 5-10 s as recommended by Angerer and Angerer (1989). The slides were washed in two changes of acetone for 5 min each and two changes of H_2O for 5 min each. The slides were then baked

overnight at 180°C and stored in a dust-free container until use.

2.23.6 Sectioning of tissue

The tissue blocks were sectioned using a rotary microtome. Ribbons of sections were cut at 5-10 μm thickness and floated on a water-bath containing sterile distilled H_2O at 40-44°C. Once the ribbon had flattened out, 2-4 sections were transferred onto a TESPA-coated, baked slide. The slides were placed on a hot plate at 37°C overnight to adhere the tissue to the slide.

2.23.7 Tissue pretreatments

The tissue was de-waxed by treatment with two changes of xylene (at least 10 ml per slide) for 5 min each followed by removal of the xylene by passing the sections through 100% ethanol, 85% ethanol, 70% ethanol and H_2O (5 min each, 10 ml per slide). The rehydrated tissue sections were placed in PBS for 5 min and then treated with proteinase K (1 μg/ml in PBS) for 15 min at 37°C. Slides to be probed with digoxigenin-labelled probes were given a blocking pretreatment of 1% BSA in PBS for 30 min prior to proteinase digestion. The slides were rinsed in PBS and the sections re-fixed with 4% formaldehyde in PBS for 5 min at room temperature. The slides were incubated in 0.1 M triethanolamine (TEA; pH 8.0) for 1 min at room temperature and treated with 0.25% acetic anhydride in TEA for 10 min with constant stirring. This acetylates the tissue to prevent non-specific binding of the probe. The slides were washed in 5 x SSC for 5 min and dehydrated in 70% ethanol, 85% ethanol and 100% ethanol for 2 min each. The slides were then stored in a dust-free container containing dessicant until hybridisation.

2.23.8 Hybridisation and washing of slides

Pretreated tissue sections were hybridised with antisense or sense RNA probes which had been labelled with either ^{35}S-UTP or DIG-11-UTP (see sections 2.17.4 and 2.17.5). Denatured probe was added to hybridisation solution (50% formamide, 0.3 M NaCl, 10 mM Tris-HCl pH 7.5, 10 mM sodium phosphate pH 6.8, 5 mM EDTA, 1 x Denhardt's reagent, 0.75 mg/ml yeast tRNA (Sigma), 10 mM DTT) and 50 µl applied per slide. Siliconised, baked coverslips were placed over the slides which were placed on a polystyrene support. The slides were hybridised in a plastic box containing tissue soaked in 5 x SSC, 50% formamide for 16 h at 52°C. After hybridisation, the slides were placed in a stainless steel rack and transferred to 4 x SSC at room temperature for 10 min to loosen coverslips. Once coverslips had been removed, the slides were washed in 4 x SSC for 1 h at room temperature and in 2 x SSC, 50% formamide for 1 h at 50°C. The slides were treated with RNAase A (Sigma) to reduce background by washing briefly with NTE (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and incubating in 20 µg/ml RNAase A in NTE for 30 min at 37°C. The slides were given a further wash of 0.5 x SSC at 50°C for 1h. All wash solutions used for ^{35}S-UTP-probed sections contained 10 mM DTT to keep the probe reduced. Sections which were probed with ^{35}S-UTP probes were then dehydrated through: 70% ethanol, 0.3 M NH4OAc; 90% ethanol, 0.3 M NH4OAc, 100% ethanol and stored in a dust-free container containing dessicant. Slides which had been probed with DIG-UTP-labelled probes were stored in 130 mM NaCl, 10 mM sodium phosphate (pH 7.0) at 4°C for 16-48 h.

2.23.9 Autoradiography of ^{35}S-labelled slides

All procedures were carried out in a dark room fitted with a safety light

(Kodak type GBX-2). Amersham emulsion LM-1 was melted in a water bath at 45°C for 30 min. About 12 ml of melted emulsion was poured slowly into a plastic slide mailer to prevent bubbles forming. The slide mailer was placed at 45°C. Several blank slides were dipped in emulsion to remove any bubbles present. Each slide was then dipped in emulsion for 5 s, drained vertically for 5 s and the back of the slide wiped free of emulsion with tissues. The slides were placed on an ice-cold stainless steel tray for 10 min before air-drying the slides for 2 h. The slides were stored in a light-tight box (double-wrapped with aluminium foil) containing dessicant at 4°C for the required exposure time (1-5 d). Development of slides was at room temperature. Before developing, the slides were allowed to warm up for at least 1 h. All reagents were at room temperature before use. The slides were placed in a plastic rack and developed for 2-4 min in Ilford Phenisol developer (diluted $^1/_5$ with H_2O). The slides were transferred to stop solution (0.5% acetic acid) for 1 min and agitated gently. The slides were fixed with 30% sodium thiosulphate for 4 min, agitating gently, after which time the lights were switched on. The slides remained in fix for a further 4 min before rinsing with distilled H_2O. The slides were washed in gently running cold water for 15 min. Sections were lightly stained with 0.05% toluidine blue in H_2O for 1 min before drying through a graded ethanol series. The sections were mounted under coverslips using either D.P.X. mountant (BDH) or 90% glycerol, 0.1 x PBS.

2.23.10 Detection of digoxigenin-labelled probes

This protocol is that recommended by Boehringer Mannheim using the reagents in the nucleic acid detection kit, with modifications suggested by Coen et al. (1990). All procedures were carried out at room temperature.

The slides were rinsed in buffer A (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl) and incubated in 1% blocking agent (supplied with kit) in buffer A for 1 h. The slides were transferred to 1% BSA, 0.3% Triton X-100 for 1 h before incubating with antiDIG-alkaline phosphatase conjugate (1:5000 dilution in 1% BSA, 0.3% Triton X-100) for 1 h. The slides were washed in 4 changes of buffer A for 20 min each and rinsed in buffer C (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$). The slides were then incubated with 100 µl of colour reagent (4.5 µl of 75 mg/ml nitroblue tetrazolium salt and 3.5 µl of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 1 ml of buffer C). The colour development took place in the dark in a plastic box with moist tissue to prevent drying out of slides. The colour development was halted after 6-24 h by transferring the slides to 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Sections were mounted under coverslips in either D.P.X. mountant or 90% glycerol, 0.1 x PBS.

2.24 Microscopy of plant tissue

2.24.1 Scanning electron microscopy

Plant material was harvested and fixed in 3.25% formaldehyde/1.25% glutaraldehyde at 4°C overnight. The material was rinsed in 0.05 M cacodylate buffer pH 7.0 and postfixed in 1% osmium tetroxide in the same buffer for 2 h. The material was rinsed with glass distilled water and dehydrated, on ice, in a graded ethanol series. The material was critical-point dried in liquid carbon dioxide. Individual inflorescences were mounted on SEM stubs for further microdissection and coated with gold in an Edwards S150 B sputter-coater. The buds were observed on a JEOL T100 scanning electron microscope at an accelerating voltage of 15 kV and the images photographed on Ilford FP4 film.

2.24.2 Light microscopy

Plant material was harvested and fixed in 3.25% formaldehyde/1.25% glutaraldehyde at 4oC overnight. The plant material was fixed, wax-embedded and sectioned as in sections 2.23.1, 2.23.4 and 2.23.6. After de-waxing, the sections were stained with 0.1% fast green in clove oil (BDH) and photographed using a Zeiss Photomicroscope II.

2.25 Dideoxy DNA sequencing

DNA was sequenced using the Sequenase® Version 2.0 enzyme and kit obtained from United States Biochemicals (Cleveland, Ohio, USA). Double-stranded template DNA was prepared as in section 2.6.2.

2.25.1 Annealing of template and primer

The mini-prep template DNA (3-5 µg) was denatured in 0.2 M NaOH, 0.2 mM EDTA for 15 min at room temperature. Denatured DNA was precipitated by addition of 0.1 vol 2 M NH_4OAc pH 4.0 and 2 vol ethanol for 10 min on ice. The washed DNA pellet was resuspended in 7 µl H_2O, 2 µl reaction buffer (0.2 M Tris-HCl pH 7.5, 0.1 M $MgCl_2$, 0.25 M NaCl, supplied with kit) and 1 µl (2.5 ng) primer. Annealing took place at 37°C for 20 min after which time the tube was stored on ice.

2.25.2 Labelling and termination reactions

To the annealed template-primer was added: 1.0 µl 0.1 M DTT, 2.0 µl diluted labelling mix (usually dGTP mix; 1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 0.5 µl (5 µCi) α-^{35}S-dATP (1000 Ci/mmol), 2.0 µl diluted Sequenase® 2.0. The contents were mixed and incubated for 2-5 min at room temperature. Four microfuge tubes were labelled G, A, T and C. Into each tube was placed 2.5 µl of the appropriate termination mix and the tubes prewarmed for 1 min at 37°C. On completion of the labelling reaction, 3.5 µl of the reaction mix was placed in each of the four tubes. Termination reactions proceeded for 3-5 min at 37°C after which time the tubes were stored at -20°C prior to electrophoresis. Samples were denatured by heating to 75-80°C for 2 min before loading on the gel.

2.25.3 Sequencing gel electrophoresis

Gels of dimension 20 cm x 40 cm x 0.4 mm were prepared using 50 ml of de-gassed linear sequencing mix (6% acrylamide:bisacrylamide (38:2), 7 M urea, 1 x TBE), to which was added 300 µl 10% ammonium persulphate and 50 µl TEMED. Aluminium plates were placed either side of the gel to ensure even heat distribution during electrophoresis. The gels were pre-run for 20 min at a constant power of 40-45 W before loading the samples. Gels were run for periods of 2 and 5 h for reading the maximum number of nucleotides. After electrophoresis the gel plates were removed from the sequencing apparatus and separated. The gel was fixed in 10% acetic acid, 10% methanol for at least 15 min. The gel was transferred onto Whatman 3MM paper and dried on a slab gel-drier at 80°C for 30 min. Gels were autoradiographed for 16-48 h without intensifying screens.

2.26 Transformation of *Agrobacterium tumefaciens*

A. tumefaciens was transformed using the method of Armitage et al. (1988).

Cultures of *E. coli* XL-1 Blue containing p2AS and p2S constructs were grown overnight along with overnight cultures of *E. coli* containing pRK2013 and *A. tumefaciens* C58C1. 0.1 ml of each culture was mixed on an L-agar plate (no selection) as follows;

p2AS	+ C58C1	+ pRK2013		p2AS	+ pRK2013
p2S	+ C58C1	+ pRK2013		p2S	+ pRK2013
p2AS	+ C58C1			C58C1	+ pRK2013
p2S	+ C58C1				

The plates were incubated at 28°C overnight and streaks from across each plate were spread on plates containing AB medium plus 100 µg/ml rifampicin and 50 µg/ml kanamycin. The plates were incubated at 28°C for 3 d and single colonies restreaked onto AB plates and grown for 3 d. Single colonies were used to prepare cultures for mini-preps and glycerol stocks.

2.27 Transformation of *Arabidopsis thaliana*

2.27.1 Surface-sterilisation of seed

Seeds were placed on Whatman no. 1 filter paper (9 cm diameter) which was then folded into quarters and folded once more to form a packet. The packet was secured with a plastic-coated paper-clip and labelled in pencil. The packet was

immersed in 70% ethanol in a Magenta pot for 2 min and allowed to drain before transferring to a Magenta pot containing 10% sodium hypochlorite (1.4% (w/v) available chlorine), 0.5% SDS, 0.02% Triton X-100 for 15 min. From this point manipulations were carried out in a sterile flow hood. The packet was placed in a Magenta pot containing sterile distilled H_2O for 5 min to remove any bleach. This was repeated 5 times. The packet of seed was dried on a Magenta jar lid in the flow hood for at least 3 h. The seeds were stored in a petri dish sealed with Micropore® tape (3M Health Care, Loughborough).

2.27.2 Growth of plants

Surface-sterilised seed was sown onto petri dishes containing germination medium (GM; 1 x Murashige and Skoog salt mixture (Flow Labs, Irvine, Scotland), 2% sucrose, 100 mg/l inositol, 1 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 0.5 g/l MES pH 5.7, 0.8% agar). The seeds were grown in continuous white light for approximately three weeks and roots harvested before the plants flowered.

2.27.3 Transformation of roots

The transformation procedure used is based on that of Valvekens et al. (1988) with modifications suggested by Dean et al. (1991). All manipulations were carried out in a flow hood.

Roots were cut off plants, pulled apart, then roots were placed on solid callus inducing medium (CIM; 1 x Gamborg's B5 medium (Sigma), 2% glucose, 0.5 g/l MES pH 5.7, 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.05 mg/l kinetin) ensuring

that all roots were in contact with the medium. The plates were incubated for 3 d in low white light (15 μmol/m^2/s). The roots were stacked in an empty petri dish and cut into 0.5 cm explants. The explants were placed in a sieve and washed in 20 ml liquid CIM in a petri dish. 0.5 ml of an overnight culture of *A. tumefaciens*was added and the explants left to co-cultivate for 2 min. The sieve was drained and excess medium removed by blotting the root explants on sterile Whatman no. 1 filter paper. The root explants were transferred in small clumps to a petri dish containing solid CIM . The plates were incubated in a growth room at 24°C for 2 d to allow co-cultivation of the *A. tumefaciens*. After incubation, the root explants were washed in 20-25 ml of liquid shoot-inducing medium (SIM; 1 x Gamborg's B5 medium, 2% glucose, 0.5 g/l MES pH 5.7, 5 mg/l N6-(2-isopentenyl)adenine, 0.15 mg/l indole-3-acetic acid). The root explants were blotted on sterile filter paper and transferred to solid SIM plus 750 mg/l vancomycin, 50 mg/l kanamycin (SIM V750 K50). Root explants which had not been co-cultivated with *A. tumefaciens* were placed on control plates of SIM plus 750 mg/l vancomycin (SIM V750) (positive control) and SIM V750 K50 (negative control). The plates were placed in continuous white light to allow callus formation. After approximately 10 d callus can be seen on the positive controls and callus appears on the non-control plates after about three weeks. The explants were transferred to fresh SIM V750 K50 plates every 10 d until shoots appeared. The shoots were excised, placed on GM plus 500 mg/l vancomycin and allowed to flower. On formation of flower buds plants were transferred to individual Magenta pots containing GM with the lids loosely attached with Micropore® tape to allow the anthers to dehisce. Seeds were harvested when the pods were a yellowish-brown colour.

2.27.4 Analysis of progeny from transformed plants

Seed collected from transgenic plants was surface-sterilised and plated out on GM plus 50 μg/ml kanamycin. Wild-type seed was included as a control. The plates were placed in continuous white light. Seedlings were examined after expansion of the cotyledons for expression of kanamycin resistance. Kanamycin-sensitive seedlings are bleached and do not develop leaves. The segregation ratio (kanamycin resistant: kanamycin sensitive) was calculated. Plants were transferred to compost on formation of flower buds and seed collected.

2.28 Histochemical localisation of β-glucuronidase

Arabidopsis thaliana transformed with the construct pBI101 (35S CaMV promoter, β-glucuronidase (GUS) reporter gene and nos 3' terminator; Jefferson et al., 1987) was supplied by Dr. J.A. Jackson (Dept. of Botany, University of Glasgow). The histochemical assay for GUS expression was performed according to Scott et al. (1988).

Small pieces of plant tissue were fixed in 0.3% formaldehyde, 10 mM MES pH 5.6, 0.3 M mannitol for 1 min under vacuum. The fixative was replaced and the tissue fixed for a further 45 min at room temperature. The tissue was then washed several times with 50 mM sodium phosphate pH 7.0. The tissue was incubated in 100 μl of 1 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) in 50 mm sodium phosphate pH 7.0 for 8-24 h at 37°C. The tissue was then cleared in 70% ethanol and mounted onto slides for microscopy.

Chapter 3: Isolation of cDNAs from a floral apex specific cDNA library

3.1 Introduction

Attempts to isolate genes expressed during flower development have involved a variety of different approaches. The approach adopted in this project involved the differential screening of a cDNA library made to a specific stage of floral development. It was decided initially to use *Brassica napus* to isolate cDNAs expressed during the early stages of flowering. The development of flowers has been extensively studied in *B. napus* (e.g. Polowick and Sawhney, 1986). Furthermore tissue is available in large amounts from field-grown plants. This chapter describes the construction of a cDNA library to an early stage in flower development of *B. napus* and isolation of floral apex-enriched clones by differential screening.

3.2 Floral development of *Brassica napus*

The floral development of *Brassica napus* cv. 'Cobra' (winter oilseed rape) was examined by both light microscopy and scanning electron microscopy of field-grown tissue. Plants were harvested from a local field at various times during the growing period from 21-10-88 to 31-5-89. Fig. 3.1 shows light micrographs of vegetative and floral apices of *B. napus*. The plants were assigned a growth stage according to the key developed by Sylvester-Bradley and Makepeace (1984). The stage at which the plants initiated flower development in the field was compared to plants grown in a continuous light growth room (see Table 3.1). The field-grown plants exhibited a domed apex and emerging floral bud primordia at the 1.10 stage (10 exposed leaves) and produced buds with sepal primordia at stage 1.12, 2.2 (extension of 2 stem internodes) as can be seen in the scanning electron micrograph in Fig. 3.2. Plants

Table 3.1 Floral development of *Brassica napus* under field and growth room conditions

The stage of development indicated is based on the growth stage key as described by Sylvester-Bradley and Makepeace (1984). Plants grown in the field were harvested on the dates indicated, the apices or floral parts dissected out and placed in a glutaraldehyde/formaldehyde fixative for microscopy. Plants were grown in the growth room under continuous white light at 23°C. The age refers to the number of days from sowing of the seed.

STAGE OF DEVELOPMENT			DESCRIPTION	
FIELD-GROWN PLANTS				
DATE	LEAF	STEM	FLOWER	
21-10-88	1.06	2.0	3.0	vegetative, rosette
07-11-88	1.08	2.0	3.0	vegetative, rosette
25-11-88	1.10	2.0	3.0	domed apex, floral primordia
22-12-88	1.12	2.2	3.0	6 floral buds with 4 sepal primordia, stem extension
05-01-89	1.12	2.2	3.1	20 floral buds, stamens developing
03-02-89	1.18	2.5	3.3	flower buds exposed
GROWTH ROOM PLANTS				
AGE (d)	LEAF	STEM	FLOWER	
21	1.04	2.0	3.0	vegetative, rosette
26	1.06	2.0	3.0	domed apex, floral primordia
32	1.08	2.1	3.0	floral buds with sepal primordia
44	1.12	2.4	3.1	stamen primordia seen in flower buds
50	1.14	2.6	3.3	flower buds exposed

Fig. 3.1 Light micrographs of *B. napus* apices

Field grown tissue was fixed in glutaraldehyde/formaldehyde, wax-embedded and sectioned. 10 μm sections were stained with 0.1% fast green in clove oil (BDH) and photographed using a Zeiss Photomicroscope II. Magnification x 110.
A: apex, L: leaf primordium, Se: sepal primordium, St: stamen primordium, F: flower bud primordium

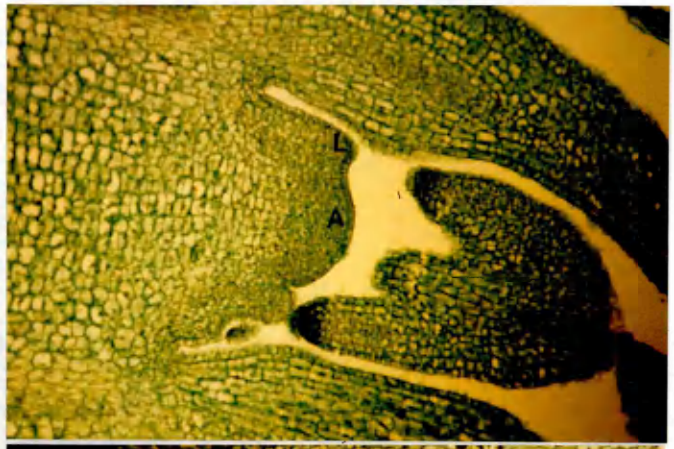
a. Vegetative apex, harvested 21-10-88

b.Vegetative apex, harvested 07-11-88

c. Floral apex , harvested 22-12-88

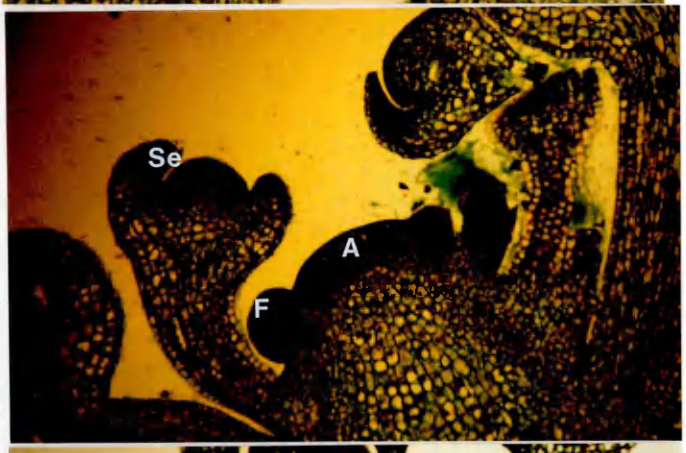
d. Floral apex, harvested 5-01-89

a



b

c



d

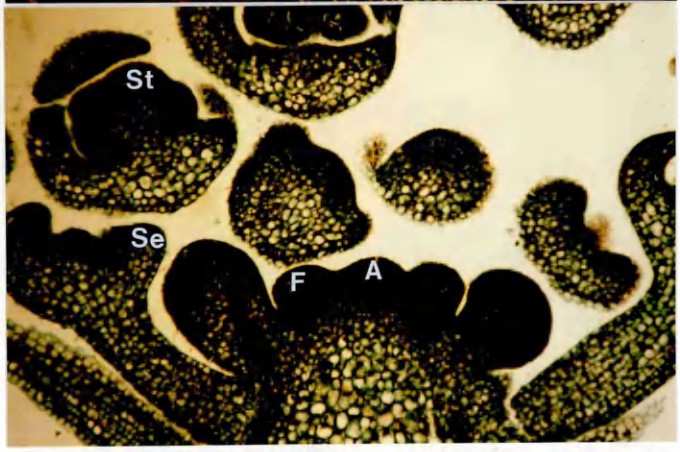
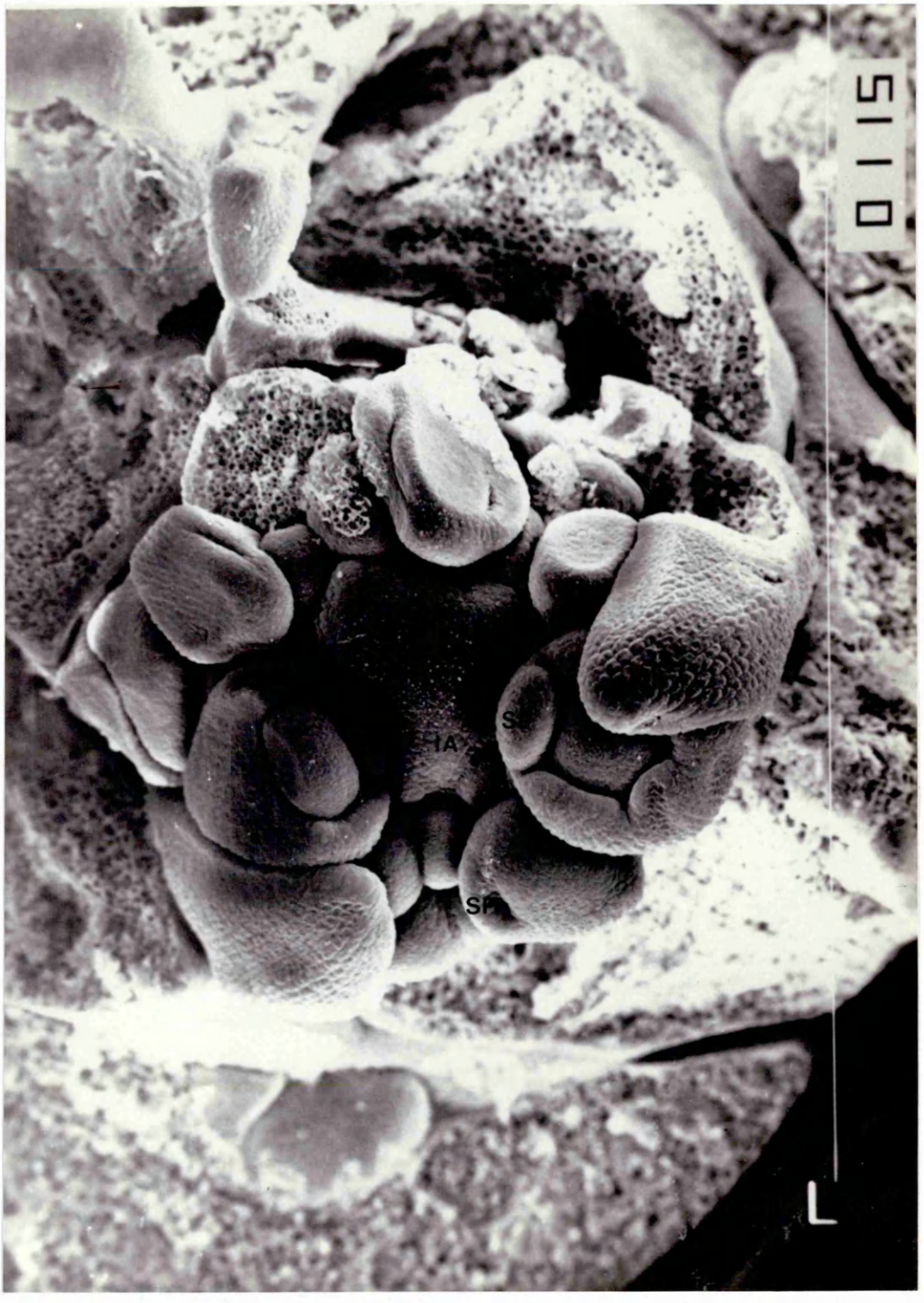


Fig. 3.2 Scanning electron micrograph of floral apex from *B. napus*

Plant material was harvested 22-12-88 and fixed in formaldehyde/glutaraldehyde. The material was potfixed in 1% osmium tetroxide, dehydrated and critical-point dried in liquid carbon dioxide. A single inflorescence was mounted and coated with gold in an Edwards S150 B sputter-coater. The inflorescence was observed on a JEOL T100 scanning electron microscope at an accelerating voltage of 15 kV and the image photographed on Ilford FP4 film. Magnification x 150.

IA: inflorescence apex; SP: sepal primordia


S1 I D
IA
S
SP
L

grown in continuous light at a constant temperature of 23°C reached an equivalent stage of development (production of floral buds) after the production of 7-8 exposed leaves. Floral buds which showed defined sepal primordia could be seen on these plants by the time 10 leaves were exposed. At this point stem extension had also begun. Leaf number was used as an indication of the developmental stage of floral buds of the plant both in the field and the growth room as it proved less variable than other parameters such as age, height of the plant and stem width with regard to early bud development (data not shown). The development of floral organs correlated well with the study of Polowick and Sawhney (1986) who described this process in detail in the cultivar 'Westar'.

3.3 Analysis of *in vitro* translation products from apical tissue

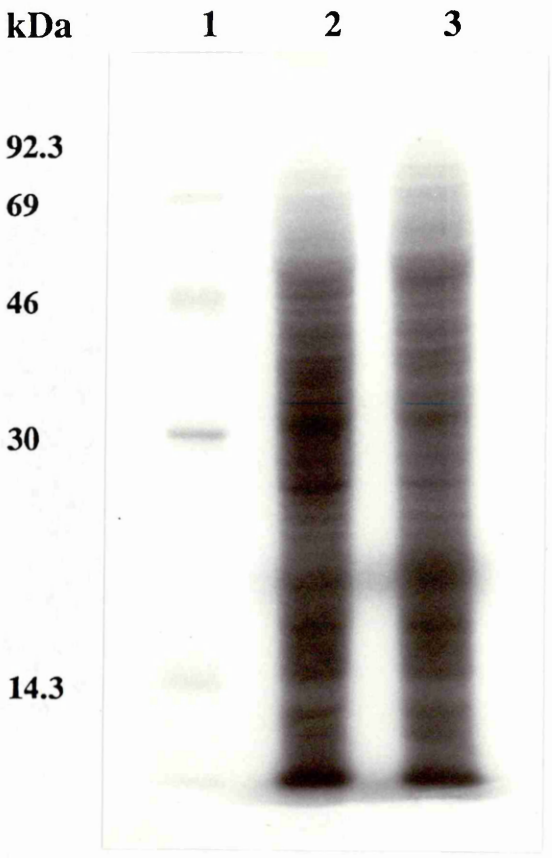
In order to isolate genes expressed during the initiation of the floral organs it was decided to extract RNA from apices with the most advanced flower buds on the developing inflorescence showing sepal primordia only. These plants were considered to be at a sufficiently early stage in flower development potentially to yield clones involved in the determination of floral organs. Total RNA wasisolated from both vegetative and floral apices and *in vitro* translated. The protein products examined by one-dimensional SDS-PAGE. No significant differences between the translation products of RNA from these tissues were seen (see Fig. 3.3).

3.4 Construction of the cDNA library

Polyadenylated RNA was isolated from apices at two stages, vegetative and floral buds showing sepal primordia only. The integrity of the poly(A)$^+$ RNA was tested by translation *in vitro*. cDNA was synthesised from 5 μg of poly(A)$^+$ RNA

Fig. 3.3 *In vitro* translation products of *B. napus* total RNA

Total RNA was isolated from vegetative (lane 2) and floral (lane 3) apices of *B. napus* and translated in a wheat germ translation system. Labelled products were resolved on a 12% SDS-polyacrylamide gel (2 x 10^5 cpm/well) which was exposed to X-Ray film for 48 h after fluorography and drying. Markers are ^{14}C-labelled proteins supplied by Amersham International plc (lane 1) with molecular size indicated in kDa.


kDa
1
2
3
92.3
69
46
30
14.3

derived from floral apices with buds showing sepal primordia only. ^{32}P-dCTP was added to both first and second strand synthesis reactions to monitor the efficiency of each reaction and facilitate the calculation of the amount of cDNA produced. The % of poly(A)$^+$ RNA transcribed into first strand cDNA was within the expected range (15-30%) and the total amount of cDNA synthesised was 569 ng.

It was decided to clone the cDNA directionally into the vector λNM1149 (Murray, 1983). The method used was that of Dorssers and Postmes (1987) who designed linkers containing an internal Eco RI site and four base pairs of a Hind III site. This resulted in the creation of a unique Hind III site adjacent to the poly(A) tail (see Fig. 3.4). The cDNA produced was in the size range 0.5-3.5 kb as examined by alkaline agarose gel electrophoresis (Fig. 3.5). The cDNA was ligated into the vector in a molar ratio of 2:1 (vector:cDNA) and packaged using a commercial packaging extract. The resulting library was titred and the estimated size calculated to be 8 x 10^5 p.f.u. Plaques were randomly picked from an L-agarose plate and DNA isolated. The DNA was digested with Eco RI and Hind III and the inserts examined on an agarose gel (data not shown); the total insert size varied between 100 and 3500 bp. All the clones examined contained inserts and several had internal sites for either Eco RI or Hind III.

3.5 Screening of the cDNA library

3.5.1 Preparation of first strand cDNA probe

The probes for the differential screen were synthesised from poly(A)$^+$ RNA from two different stages of development. The 'plus' stage was taken to be apices

Fig. 3.4 Dorssers and Postmes (1987) directional cloning method (adapted from Kaiser, 1990a).

1. cDNA is synthesised from mRNA using oligo(dT) as a primer.
2. Internal Eco RI and Hind III (Alu I) sites are methylated using Eco RI and Alu I methylases.
3. Linkers containing 4 bp of the Hind III recognition site and an internal Eco RI site are added to the double-stranded cDNA.
4. The linkered cDNA is sequentially cleaved with Hind III and Eco RI producing an Eco RI site at the 5' end and a Hind III site at the 3' end of the cDNA.

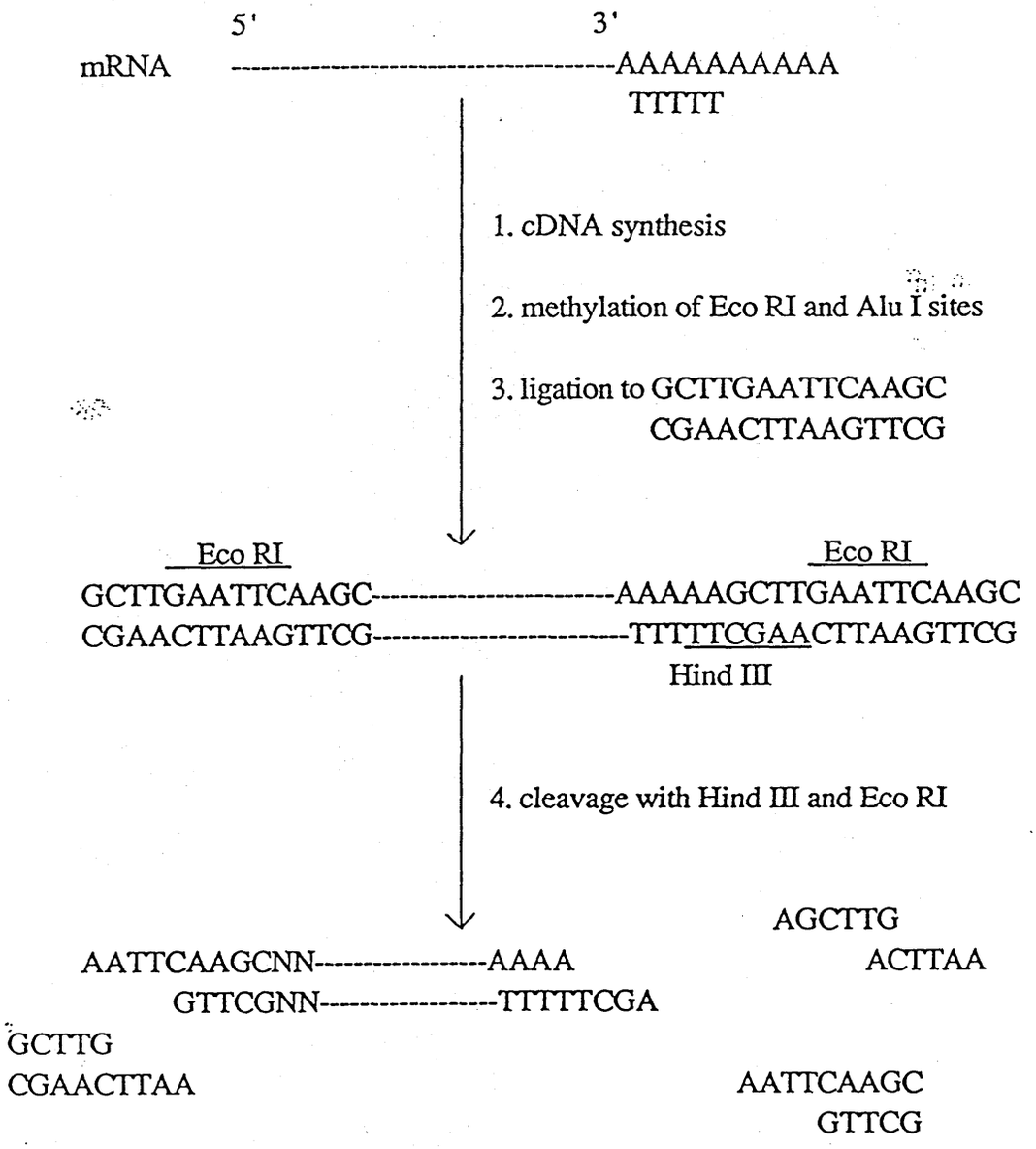
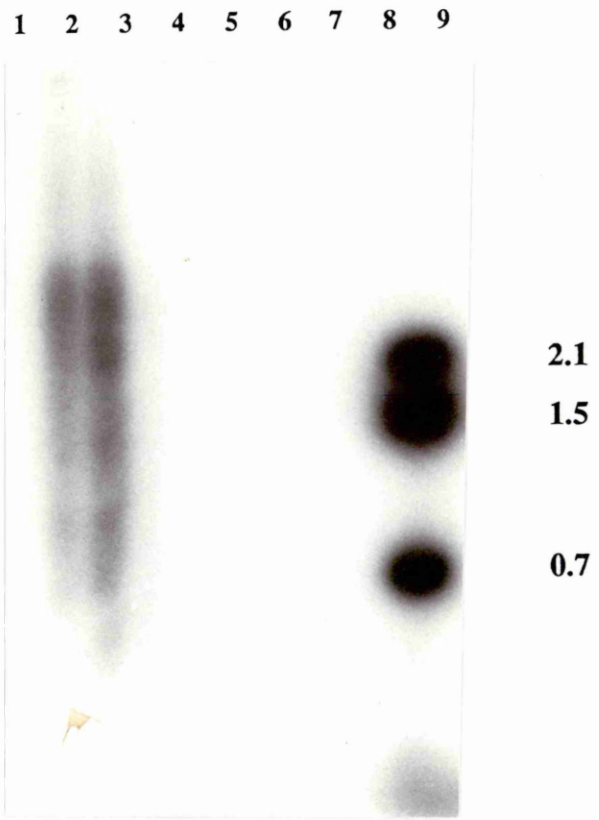

5'
3'
mRNA
AAAAAAAAAA
TTTTT
1. cDNA synthesis
2. methylation of Eco RI and Alu I sites
3. ligation to GCTTGAATTCAAGC
CGAACTTAAGTTCG
Eco RI
Eco RI
GCTTGAATTCAAGC
AAAAAGCTTGAATTCAAGC
CGAACTTAAGTTCG
TTTTTCGAACTTAAGTTCG
Hind III
4. cleavage with Hind III and Eco RI
AGCTTG
AATTCAAGCNN
AAAA
ACTTAA
GTTCGNN
TTTTTCGA
GCTTG
CGAACTTAA
AATTCAAGC
GTTCG

Fig. 3.5 cDNA synthesis products analysed by alkaline agarose gel electrophoresis

Linkered cDNA synthesis products were separated from free linker by column chromatography. Aliquots of selected column fractions (lanes 1-8) were separated on a 1% alkaline agarose gel, which was dried and autoradiographed overnight. Size markers (indicated in kbp) were end-labelled fragments of pBR322 produced by digestion with Rsa I (lane 9).


1 2 3 4 5 6 7 8 9
2.1
1.5
0.7

with floral buds showing sepal primordia only and the 'minus' stage to be vegetative apex. The probes were synthesised using 5 μg of poly(A)$^+$ RNA in the presence of 500 μCi of α-^{32}P-dCTP. This reaction produced sufficient labelled single stranded cDNA to screen up to 20 10 cm x 10 cm filters per probe. The probes were routinely checked on alkaline agarose gels prior to use to determine the size range of the products. Priming with oligo(dT) produced slightly longer probes (see Fig. 3.6) than priming with random hexanucleotides.

3.5.2 Differential screening of the library

The library was differentially screened using first strand cDNA probes synthesised as described above (section 3.3.1). For the primary screen the library was plated out at a low density (approx. 1000 p.f.u per 100 cm^2) to allow identification of a signal to a single plaque. Duplicate filters were hybridised with each of the two probes. Autoradiographs of a typical primary screen are shown in Fig. 3.7. The initial screen was of 22 000 p.f.u. of which 111 plaques showed a positive differential signal. These plaques were rescreened as 'giant plaques'. This allowed rapid screening of many clones (see Fig. 3.8). Significant differences in the hybridisation of the two probes to individual plaques could be seen easily on these filters. However in some cases it could be seen that the lysate contained more than one population of p.f.u. and selected clones were rescreened a third time as whole plate lysates. The third screen consisted of 30 clones, 10 of which still exhibited significant positive differential hybridisation. These 10 clones were selected for further study and named λLF1-10.

3.6 Analysis of cDNA clones

Fig. 3.6 First strand cDNA probes used for differential screen

First strand cDNA was synthesised from poly $(A)^+$ RNA isolated from (a) floral and (b) vegetative apices, using as primers either (1) random hexanucleotides or (2) oligo (dT). 10^3 cpm per track was loaded onto a 1% alkaline agarose gel. After electrophoresis the DNA was fixed with 7% TCA, the gel dried and exposed to X-ray film for 4 h. The markers are ^{32}P-labelled fragments of an Eco RI/Hind III digest of λ DNA (size indicated in kbp).

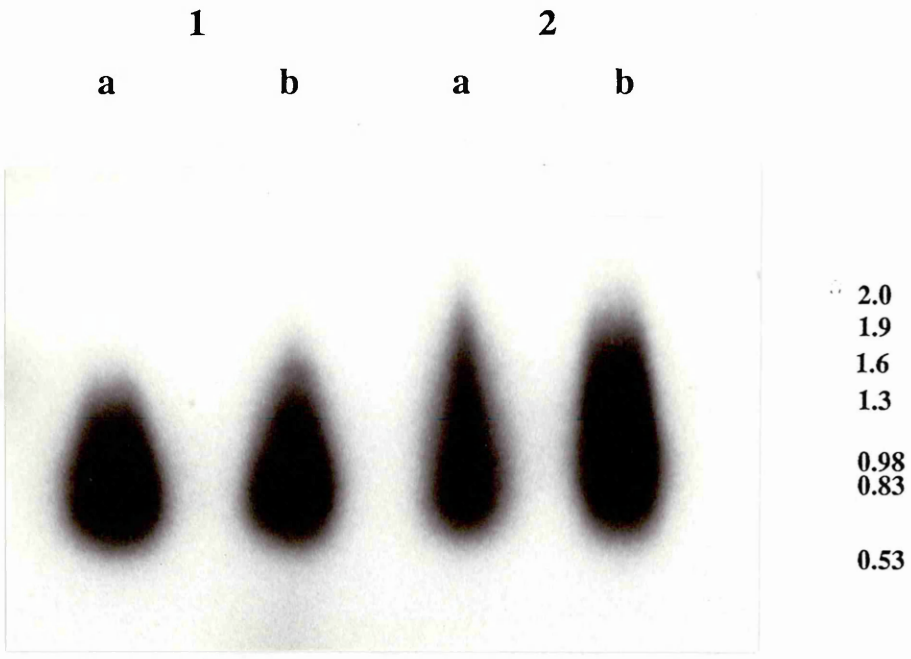
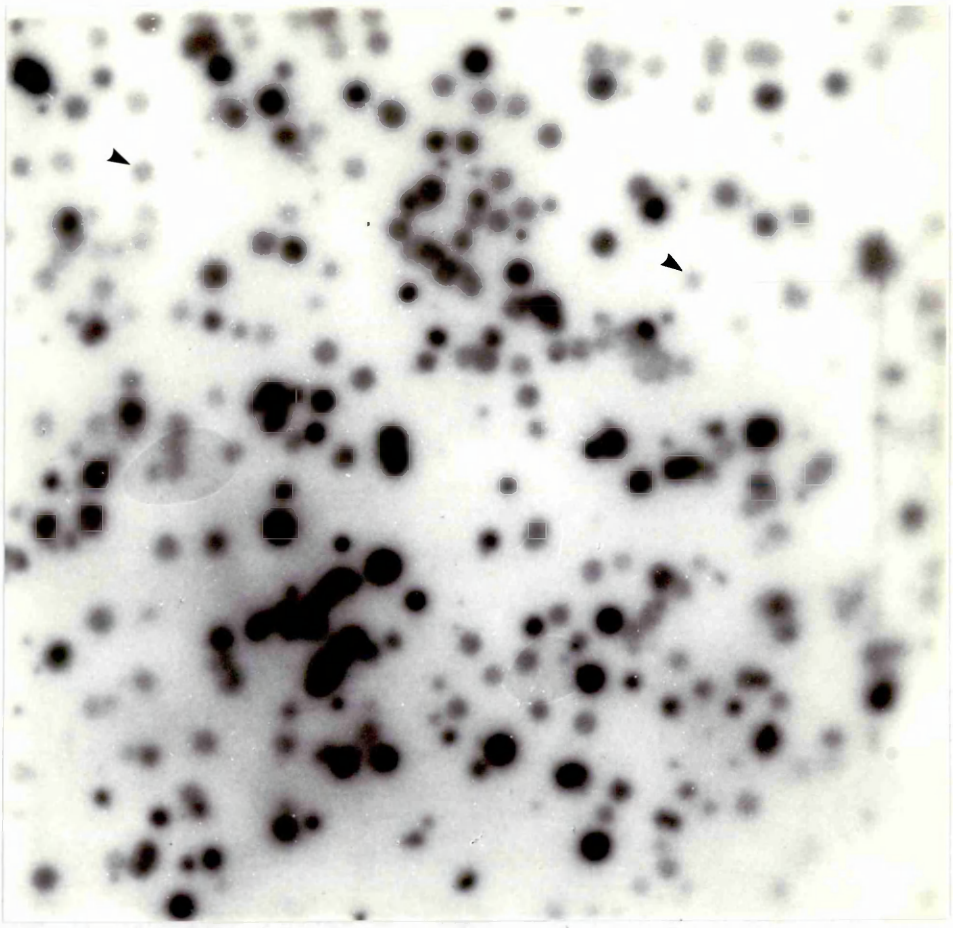

1
2
a
b
a
b
2.0
1.9
1.6
1.3
0.98
0.83
0.53

Fig. 3.7 A typical example of a filter produced from the initial screen of the library.

1000 p.f.u. were plated out on a 10 x 10 cm plate and 4 plaque lifts taken. Filters were probed in duplicate with first strand cDNA probes made from (a) vegetative or (b) floral apices. After hybridisation and washing the filters were autoradiograhed overnight. Positively differential signals were identified (examples indicated with arrows) and the plaques picked for secondary screening.

a



b

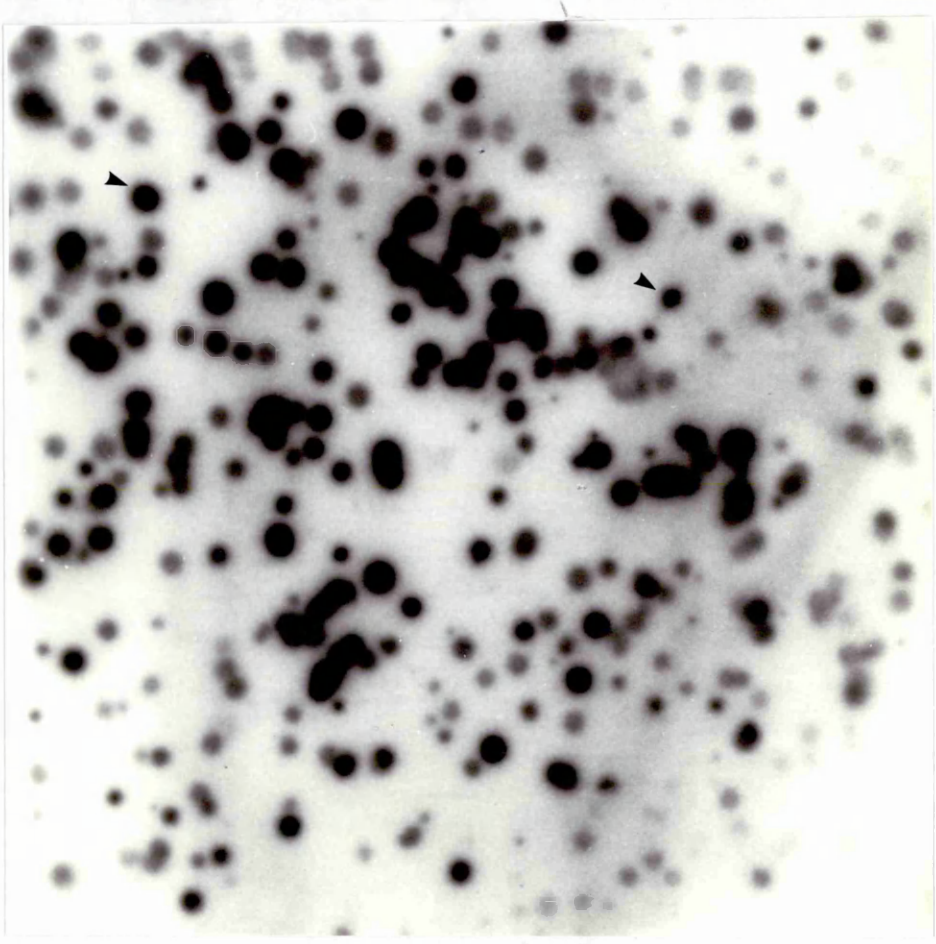
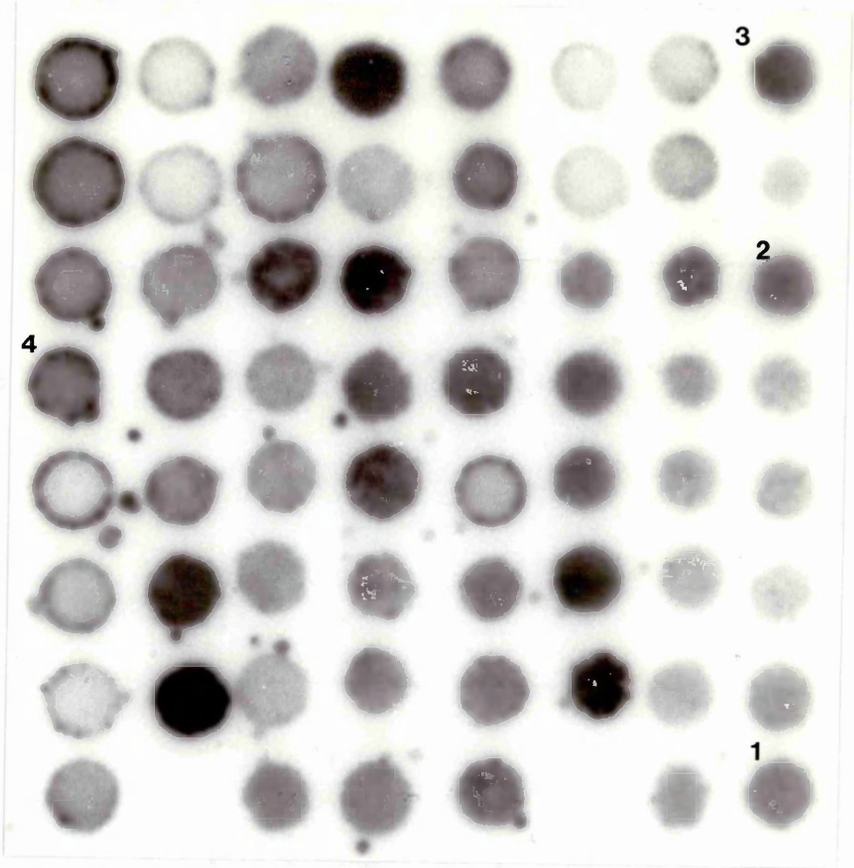


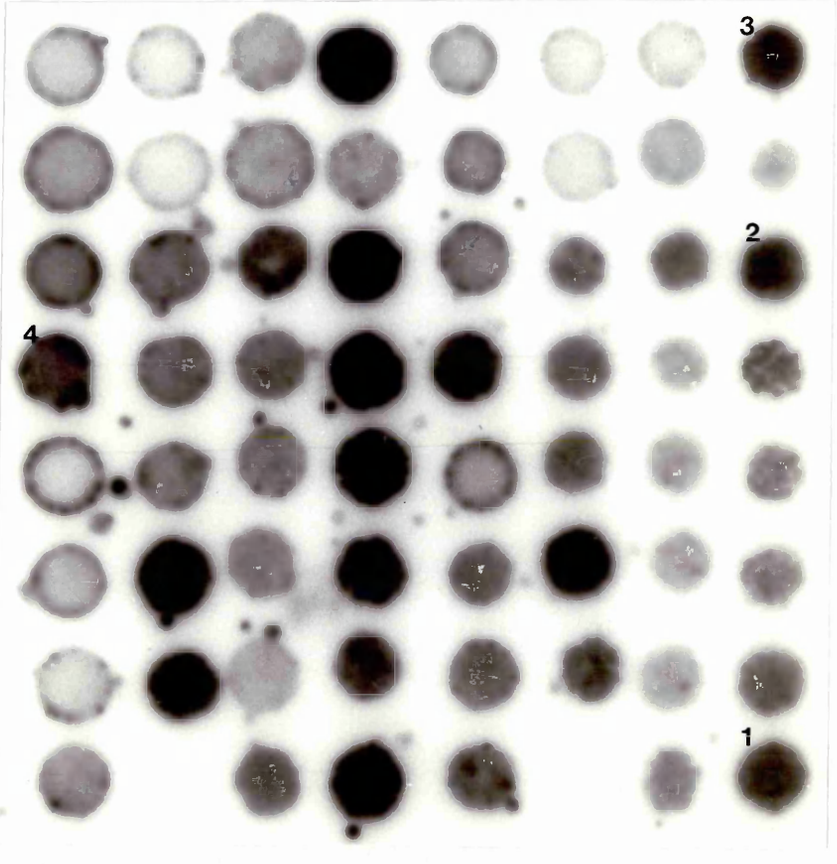
Fig. 3.8 Secondary screening of 'giant plaques'

Plaques showing a positively differential signal were rescreened as 'giant plaques'. Plaques were spotted in duplicate and filters prepared and screened as in Fig. 3.7 using cDNA probes from (a) vegetative and (b) floral apices. The plaques indicated correspond to the cDNAs (1) λLF1, (2) λLF2, (3) λLF3, (4) λLF4.

a



b



DNA was prepared from the 10 cDNAs selected for further study and digested with Eco RI and Hind III. The fragments obtained are shown in Fig. 3.9. The cDNA λLF3 has an internal Hind III site within the cDNA insert. The cDNA λLF7 produced three fragments on digestion with Eco RI and Hind III and has a single site for each enzyme within the cDNA insert. The inserts derived from the cDNAs were cross-hybridised to blots of the digested phage DNA from λ1-10 (Fig. 3.10). The clones λLF2, λLF3, λLF5, λLF7 and λLF8 were unique. However the lower band from clone λLF10 hybridised to the clones λLF1, 4, 6, 9 at high stringency and it was concluded that these cDNAs were of closely related or identical sequence. This fragment of the cDNA λLF10 was used for further investigation, the upper band being an artefact isolated during preparation of DNA from the phage lysate, and is referred to as pLF10B after subcloning. The fragments generated by Eco RI/Hind III digestion of λLF1-10 were sub-cloned into the Eco RI and Hind III sites of the plasmid vector pBluescript SK(-) and are subsequently referred to as pLF1-10. In the case of λLF3 and λLF7 the subclones derived from the larger fragments were labelled pLF3A and pLF7A respectively.

3.7 Discussion

The development of *Brassica napus* flowers has been extensively characterised (e.g. Polowick and Swahney, 1986) and the work presented in this thesis correlates well with that already published. As *Brassica* species are generally quantitative long day plants, they are not as amenable to the study of the floral transition as, for example, *Sinapis*, which has an absolute (qualitative) long day photoperiod requirement. However *B. napus* is a good system for studying floral morphogenesis, as a single inflorescence bears flowers and siliques at all different stages of development. For this study it was important to be able to identify the stages of development of the apex to allow harvest of tissue for construction and

Fig. 3.9 Eco RI/Hind III digests of cDNA clones λLF1-10

DNA was prepared from clones λLF1-10 and digested with Eco RI and Hind III. The DNA was run on a 1.5% agarose gel and photographed. Lanes 2-11 correspond to λLF1-10 respectively. Insert size was estimated by comparison to DNA markers (Eco RI/Hind III digest of λ DNA, lane 1).

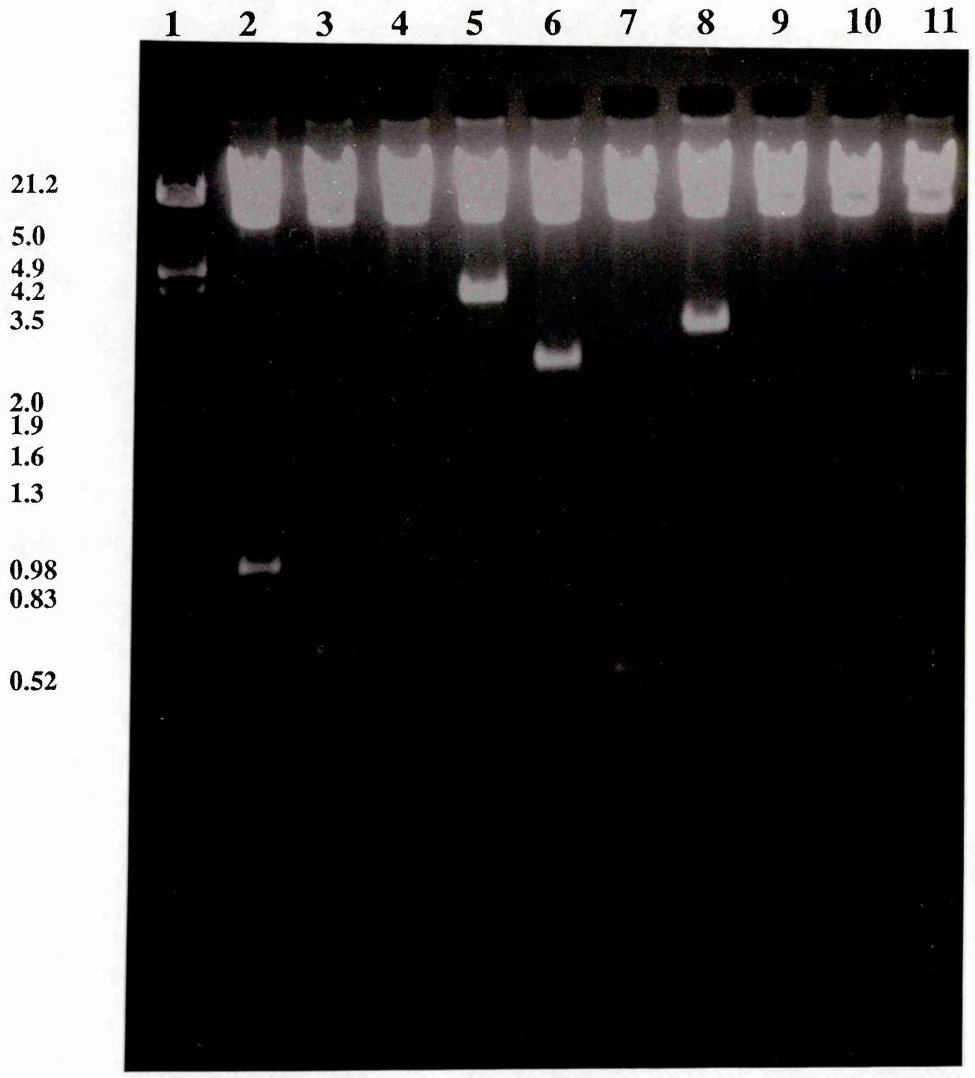
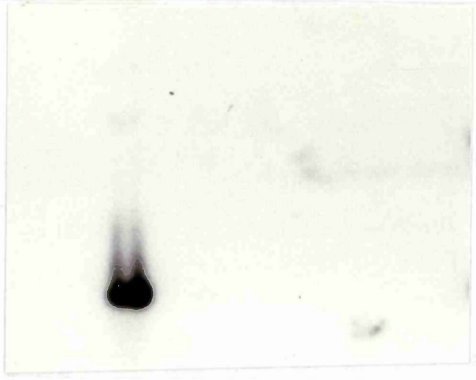

1
2
3
4
5
6
7
8
9
10
11
21.2
5.0
4.9
4.2
3.5
2.0
1.9
1.6
1.3
0.98
0.83
0.52

Fig. 3.10 Cross-hybridisation of cDNA probes

Eco RI/Hind III digests of clones λLF1-10 (lanes 1-10 respectively) were run on 1.5% agarose gels and blotted onto nylon filters. The filters were hybridised at 42°C in hybridisation solution containing 5 x SSC, 50% formamide with ^{32}P-labelled inserts from the appropriate individual clones (2.5 x 10^5 cpm/ml). The filters were washed to a final stringency of 0.2 x SSC, 0.1% SDS at 65°C and exposed overnight to X-ray film. (A) λLF2, (B) λLF10.

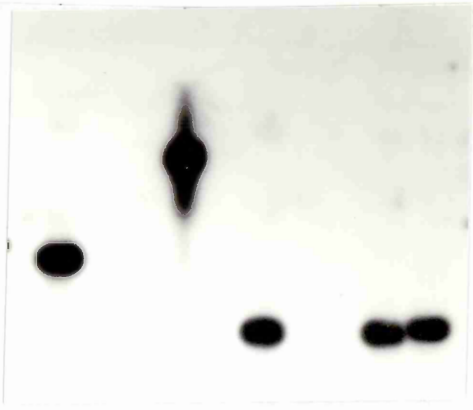
λLF2

1 2 3 4 5 6 7 8 9 10



λLF10B

1 2 3 4 5 6 7 8 9 10



screening of the cDNA library. This was possible due to the presence of a detailed growth stage key (Sylvester-Bradley and Makepeace, 1984), and the developmental stage of plants grown in both the field and the growth room was easily comparable.

Comparing two different stages of flower development one would expect to find significant differences at a molecular level. No differences were seen on a one-dimensional gel between the *in vitro* translation products of floral and vegetative apices. However it would be expected that differences would be seen using two-dimensional PAGE. It was decided not to pursue this approach in *Brassica napus* due to the potential irreproducibility of this technique. It was thought that any transcripts showing enhanced expression in the floral apex could be isolated and examined by construction and differential screening of a cDNA library.

The tissue used for isolation of polyadenylated RNA did include some sub-apical tissue, therefore the relevent floral apex-specific sequences in the library may be diluted out. It is important to minimise the amount of non-essential tissue and it has subsequently been shown that it is possible to dissect out small numbers of apices and isolate sufficient RNA to make cDNA (Jepson et al., 1991). It is also possible to make amplified and/or subtracted libraries from small numbers of cells (for a review see Kaiser, 1990b). However when this work was initiated the techniques for the construction and amplification of subtracted cDNA libraries were not in routine use. This work shows that it is possible to isolate cDNAs from a floral apex at an early stage of morphogenesis using a conventional differential screen. The use of a directional cloning strategy decreased significantly the amount of background present among the population of cDNAs due to vector self-ligation and cloning of linker multimers (discussed in Kaiser, 1990a).

The choice of tissue for construction and differential screening of a cDNA library is obviously very important. Several similar studies (e.g. Gasser et al., 1989; Scott et al., 1991) have used seedlings with developed leaves and roots as the 'minus' stage to screen organ-specific libraries. However the aim of this project was not to isolate apex-specific cDNAs but to isolate those cDNAs which were up-regulated at an early stage of floral morphogenesis. Therefore vegetative apical tissue was chosen as the 'minus' stage for the differential screen rather than whole seedlings. This was to maximise the chance of isolating transcripts enriched in the floral apex. Vegetative apical-specific mRNAs would be present at a lower percentage in a population of poly(A)$^+$ RNA derived from seedlings and could lead to the isolation of apex-specific clones which have a limited role in floral development. The screening process could have been improved by incorporating a second 'minus' control of a cDNA probe derived from seedlings. This would aid differentiation between floral apex-specific cDNAs and those cDNAs which are more prevalent in the population of mRNA isolated from the floral apex compared to vegetative apex but are unrelated to flowering.

The technique of differential screening is limited by the amount of hybridisation which has to occur to a single plaque to register that plaque as a positive signal therefore the library can only be screened at a low density. This can be time-consuming and the use of giant plaques as a second screen makes the process of screening many putative positives more rapid and less tedious. Differential screening however is also limited in that only those cDNAs corresponding to genes expressed at moderate to high abundance (>0.1% of total mRNA) can be detected and the cDNAs isolated from the library discussed in this study were of moderate abundance (0.2-1% of total mRNA; data not shown). Olszewski et al. (1988) report that their dual labelling technique has greater sensitivity, detecting sequences as rare as 0.02% of the

total mRNA population. The use of subtractive hybridisation and PCR allows the construction of cDNA libraries in which rare sequences are represented and this must be the method of choice for further studies of the kind represented in this thesis.

Chapter 4: Temporal and spatial expression of the genes corresponding to pLF1-10

4.1 Introduction

Differential screening of a cDNA library will produce a panel of cDNAs whose expression is up-regulated in the tissue of interest but it is possible that the genes are also expressed in other tissues. A rapid and easy way of demonstrating the presence of transcripts in a particular tissue type or developmental stage is by northern blot analysis. However this technique cannot give information about the cell-specific expression. *In situ* hybridisation is a widely used technique which shows whether the expression of a gene is restricted to a particular cell type and it may also yield clues as to the physiological role of the gene. A panel of cDNAs was isolated from a floral apical cDNA library derived from *Brassica napus* and the characterisation of the genes represented by these cDNAs was undertaken. Their temporal and spatial expression in *B. napus* and *Arabidopsis thaliana*, as determined by northern blot analysis, is discussed. The spatial expression of these genes was also examined by *in situ* hybridisation to various tissues of *B. napus*.

4.2 Expression of the genes represented by pLF1-10 in *Brassica napus*

As discussed in section 3.6, the cDNA pLF10B cross-hybridised with several other clones, and this cDNA was used to represent the cDNAs λLF1, λLF4, λLF6 and λLF9 in all the expression studies described. Initial examination of the expression patterns of the pLF cDNAs by RNA dot blot analysis showed that pLF8 did not show differential expression in vegetative and florally induced apices, and was

expressed at consistently high levels throughout flower development (see Fig. 4.1). It was felt that the expression pattern of the gene corresponding to pLF8 was such that it did not warrant further study.

4.2.1 Expression in apical and floral tissues

The expression of the genes corresponding to the cDNAs pLF1-10 was studied using northern blot analysis. Figure 4.2 shows the relative expression of transcripts corresponding to pLF3, pLF7B and pLF10B in vegetative and floral apices. The autoradiographs were scanned using a Bio-Rad Model 620 Densitometer. The transcripts corresponding to cDNAs pLF3, pLF7B and pLF10B show increased expression in floral apices with sepal primordia with ratios of floral to vegetative apical tissue of 1.7, 3.4 and 5.7 respectively. The genes represented by cDNAs pLF2 and pLF5 also show expression in vegetative apices which is not significantly different to that in floral apices with sepal promordia.

The expression of the genes corresponding to the cDNAs was examined in floral buds, flowers and seed pods (Fig. 4.3). The levels of the transcripts corresponding to pLF3 and pLF10B remain constant throughout flower development and the genes are also expressed in mature flowers and immature seed pods. Alterations in the level of the signal on the northern blots are due to loading differences, which were detected by probing the blots with a ribosomal RNA probe. In contrast, the expression of the pLF2 transcript is restricted to floral apices and very young flower buds. This pattern of expression also occurs with the genes corresponding to clones pLF5 and pLF7, though the abundance of the pLF7 transcript is much lower than that of either pLF2 or pLF5.

Fig. 4.1 Expression of the cDNA pLF8 in vegetative and floral apices, and floral tissue

RNA was dot-blotted onto a nylon filter which was hybridised for 16 h at 42°C in hybridisation solution containing 6 x SSC, 50% formamide and 1 x 10^6 cpm of labelled insert. The filter was washed to a final stringency of 0.1 x SSC, 0.1% SDS at 68°C. The filter was autoradiographed overnight with two intensifying screens. Lanes 1 and 2 contain 1 μg of poly(A)$^+$ RNA and lanes 3-8 contain 10 μg of total RNA.

1. Vegetative apex, harvested 21-10-88
2. Floral apex, harvested 22-12-88
3. Floral apices with sepal primordia only
4. Floral buds >2 mm
5. Green buds 2-4 mm
6. Yellow buds <6 mm
7. Mature flowers
8. Immature siliques

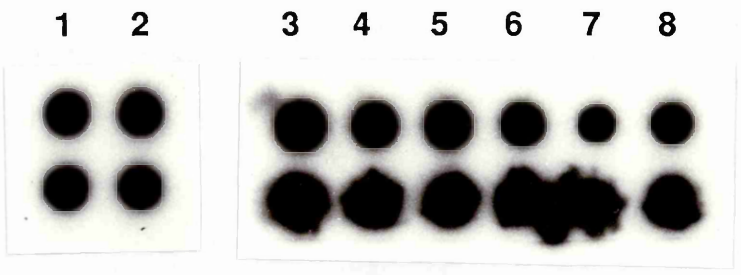
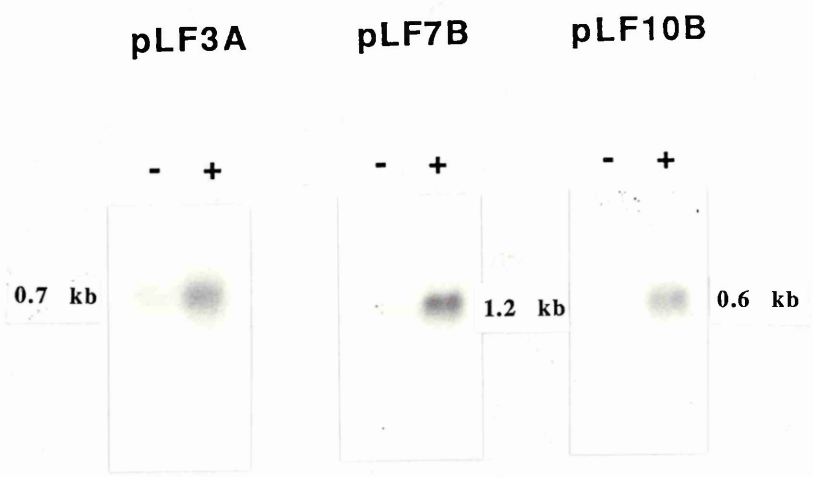

1 2 3 4 5 6 7 8

Fig. 4.2 Expression of cDNAs in vegetative and floral apices

Poly(A)$^+$ RNA from vegetative (-; harvested 21-10-88) and floral (+; harvested 22-12-88) apices was separated on a 1.5% agarose/formaldehyde gel (2 μg per track) and blotted onto nylon filters. The filters were hybridised in hybridisation solution containing 6 x SSC, 50% formamide for 16 h with 1 x 10^6 cpm/ml of the appropriate labelled cDNA insert and washed to a final stringency of 0.1 x SSC, 0.1% SDS at 68°C. The filters were autoradiographed overnight with two intensifying screens.


pLF3A
pLF7B
pLF10B
- +
- +
- +
0.7 kb
1.2 kb
0.6 kb

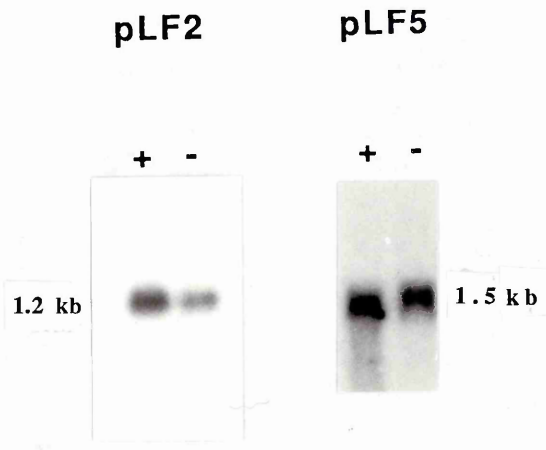
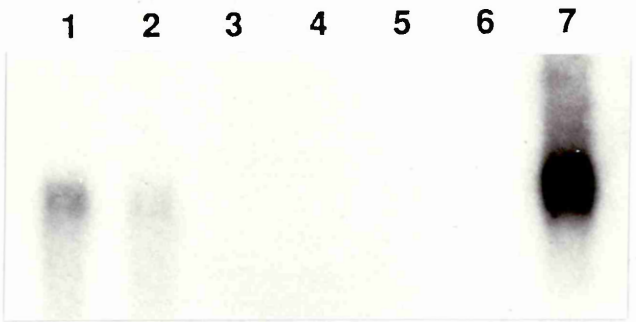

pLF2
pLF5
+ -
+ -
1.2 kb
1.5 kb

Fig. 4.3 Expression of cDNAs in floral tissue

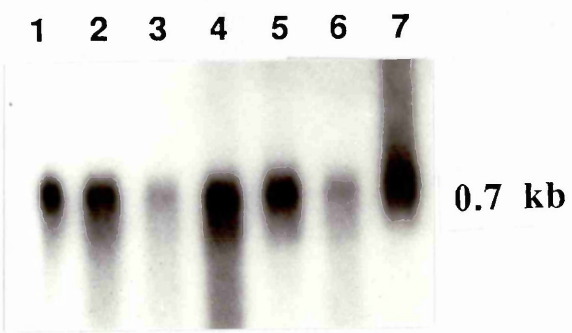
Total RNA was isolated from various floral tissues and separated on 1.5% agarose/formaldehyde gels (10 μg per track). The gels were blotted onto a nylon filter which was hybridised for 16 h with the appropriate labelled insert (4 x 10^5 cpm/ml) at 40°C in hybridisation solution containing 6 x SSC, 50% formamide. The filters were washed to a final stringency of 0.2 x SSC, 0.1% SDS at 40°C. The filters were autoradiographed overnight with two intensifying screens. Lane 7 contains 1 μg of poly(A)$^+$ RNA from floral apices as a control.

1. Floral apices with sepal primordia only
2. Floral buds >2 mm
3. Green buds 2-4 mm
4. Yellow buds <6 mm
5. Mature flowers
6. Immature siliques

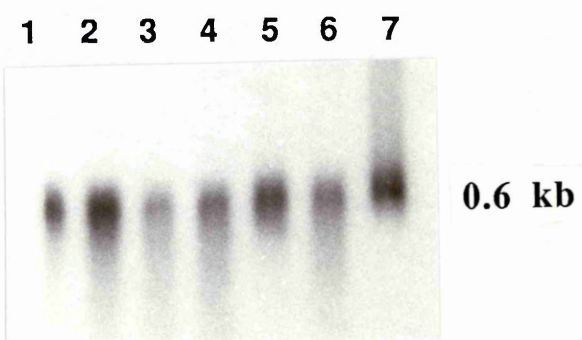
pLF2



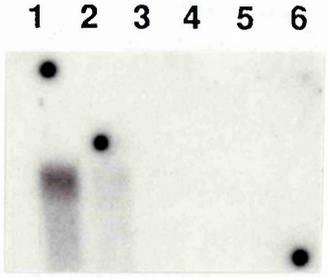
pLF3A



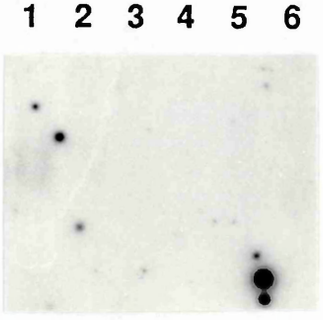
pLF10B



pLF5



pLF7



rRNA

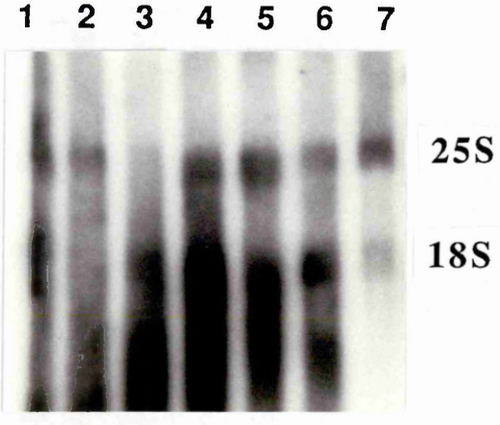


Figure 4.4 shows that the genes are expressed in an organ-specific manner. No expression could be detected in root tissue for any of the cDNAs. Low levels of expression could be seen in leaves and seedlings (with two leaves, stem and root system) for pLF3 and pLF10B. Expression of mRNA representing pLF2 and pLF7 in leaves and seedlings could not be detected at this level.

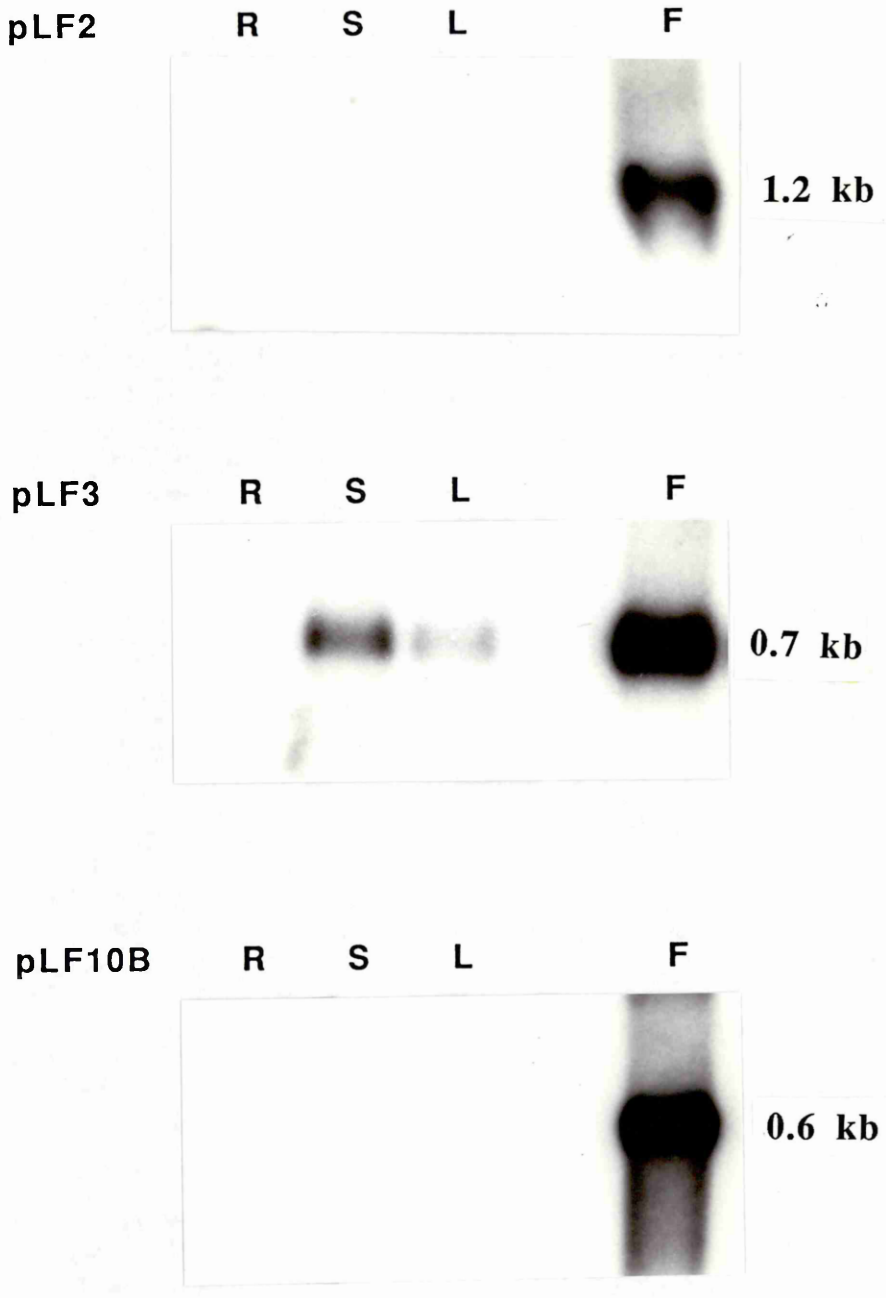
The sizes of the mRNAs recognised by the pLF cDNAs are as follows; pLF2, 1.2 kb, pLF3, 0.7 kb, pLF5, 1.5 kb, pLF7, 1.2 kb and pLF10B, 0.6kb, as determined by comparison with RNA markers.

4.2.2 Expression in wounded, cold-induced and heat-shocked plants

Because the plants used to isolate RNA for construction and screening of the cDNA library were harvested from field material, it was thought possible that the cDNAs isolated may derive from transcripts induced in response to environmental stress. The plants are grown in the field and may cold-acclimate in response to winter temperatures. At the time of harvest, the plants may induce wounding related mRNAs in response to the physical stress of up-rooting the plant and dissecting out apical tissue. There was also a time lapse of 1-3 h between harvest of the plant and placing the apex in liquid nitrogen which involved transfer to a warmer temperature (20°C c.f. a field temperature of 5-12°C). Therefore the levels of the pLF transcripts were examined in wounded, heat-shocked and cold-induced plants to see if the genes were up-regulated in response to any of these stresses. The transcripts represented by pLF2, pLF3, pLF7 and pLF10B did not show any change in expression in response to these stresses (data not shown). However the pLF5 transcript was significantly up-regulated in response to low temperature (see section 4.4).

Fig. 4.4 Organ-specific expression of cDNAs

1 μg of poly(A)$^+$ RNA from roots (R), seedling (S), leaf (L) and floral apices (F) was separated on a 1.5% agarose/formaldehyde gel and blotted onto a nylon filter. Identical filters were hybridised with 4 x 10^5 cpm/ml of the appropriate labelled cDNA insert at 37°C for 16 h in hybridisation solution containing 5 x SSC, 40% formamide. The filters were washed to a final stringency of 0.2 x SSC, 0.1% SDS at 42°C. The filters were autoradiographed for 24 h with two intensifying screens.


pLF2
R
S
L
F
1.2 kb
pLF3
R
S
L
F
0.7 kb
pLF10B
R
S
L
F
0.6 kb

4.3 Further subcloning and analysis of the cDNA inserts

The transcripts corresponding to cDNAs pLF5 and pLF7 were found to have a smaller size, as compared to RNA markers on a northern blot, than the total size of the cDNA insert originally isolated. These anomalies were also found in other cDNAs examined from this cDNA library (H. Argo, J.A. Pallas and G.I. Jenkins, unpublished). The DNA fragments from pLF5 and pLF7A were digested with various restriction enzymes. The pLF5 cDNA has three sites for BamHI and digestion with Bam HI and Hind III produces four fragments of sizes 980, 780, 500 and 430 bp. Each fragment was subcloned into pBluescript and the subclones were numbered pLF5B1, 2, 3 and 4 based on decreasing size of the insert. Each subclone was used to probe RNA blots of cold-acclimated tissue to see which fragment contained the cDNA corresponding to the cold-induced transcript. The insert of the subclone pLF5B1 hybridised strongly to a cold-induced transcript of a similar size to that which the pLF5 insert recognised. The 3 kb insert of pLF7A has three sites for Pst I and the fragments produced by Pst I digestion were also subcloned and numbered pLF7P1, 2 and 3 based on decreasing size. The inserts of the subclones were used to probe blots of vegetative and floral apical RNA. The insert of pLF7P2 (1.0 kbp) hybridised to a similar sized transcript to that recognised by pLF7A and 7B.

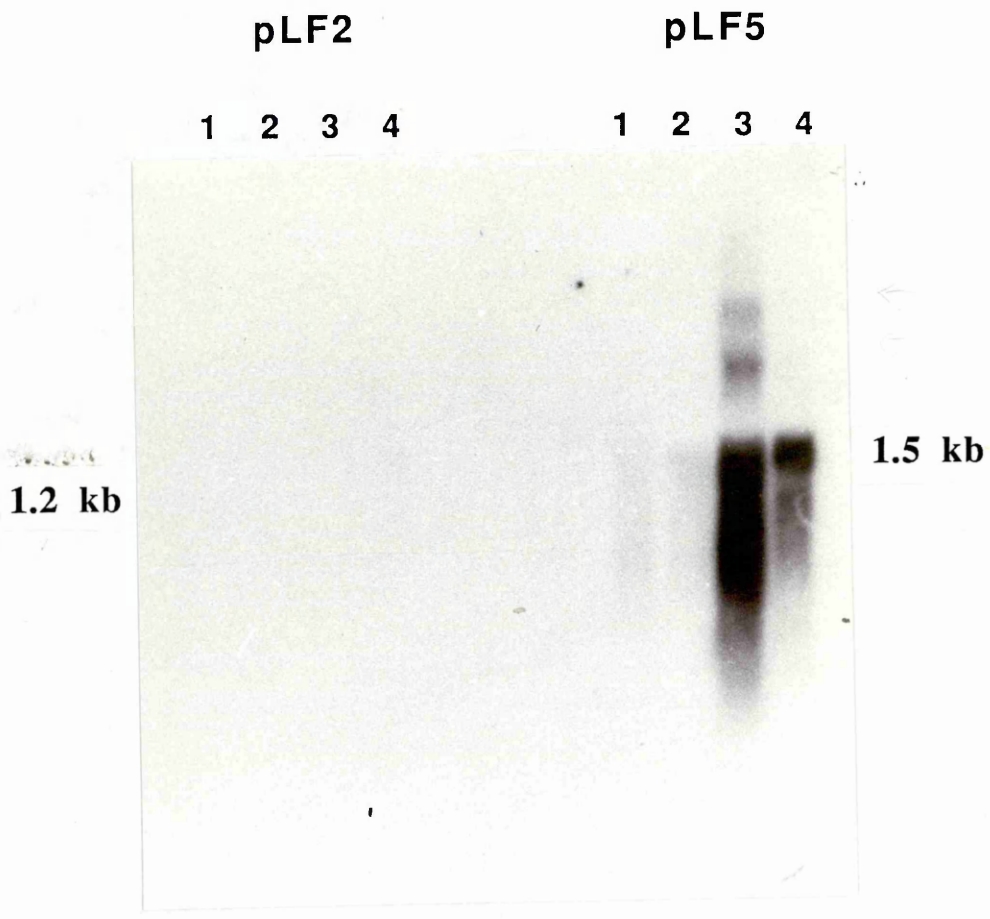
4.4 pLF5 is up-regulated in response to cold temperatures

The up-regulation of expression of the pLF5 transcript was examined in both flower buds and leaves to see if this response was tissue-specific. Figure 4.5 shows that expression of pLF5 can be induced to a high degree in both leaves and flower buds from plants which have been transferred to the cold for 48 h. This was

Fig. 4.5 The expression of the pLF5 transcript is up-regulated upon cold-treatment

Total RNA was isolated from leaves and green buds of plants which had been grown at 23°C and from plants which had been grown at 23°C and transferred to 5°C for 48 h. 10 µg of RNA per track was separated on a 1.5% agarose/formaldehyde gel and blotted onto a nylon filter. Duplicate filters were hybridised with 5 x 10^5 cpm/ml of labelled insert from pLF5 or pLF2 for 16 h in hybridisation solution containing 5 x SSC, 50% formamide at 42°C. The filters were washed to a final stringency of 0.1 x SSC, 0.1% SDS at 65°C. The filter was autoradiographed for 3 d with two intensifying screens

1. RNA isolated from leaves of plants at 23°C
2. RNA isolated from flower buds of plants at 23°C
3. RNA isolated from leaves of plants at 5°C
4. RNA isolated from flower buds of plants at 5°C


pLF2
pLF5
1 2 3 4
1 2 3 4
1.2 kb
1.5 kb

compared to the gene represented by pLF2 which shows no increase in expression upon cold treatment. To examine the cold-regulation of this gene further plants were placed at a low temperature (5-7°C) for varying times prior to harvest of leaf material. Figure 4.6 shows that the pLF5 transcript starts to accumulate rapidly, within 4 hours, and remains elevated while the plants remain at a low temperature. Upon transferring plants which have been cold-acclimated for 3 days back to a temperature of 23°C the level of transcript falls also within 8 hours to basal level. The development of cold acclimation and drought tolerance in plants is thought to have a common mechanism in which the plant growth regulator abscisic acid (ABA) may play a role. Plants were therefore deprived of water for several days until the leaves were visibly wilted, and RNA isolated from the leaves. The transcript corresponding to pLF5B1 shows no up-regulation in wilted leaves or in wounded leaves (Fig. 4.6).

4.5 The genes represented by pLF1-10 are expressed in *Arabidopsis thaliana*

The purpose of the project was to use *Brassica napus* to isolate cDNAs which were floral-specific so that these cDNAs could subsequently be used as probes to isolate homologous genes in *Arabidopsis thaliana*. The expression of the genes represented by the pLF cDNAs was therefore examined in floral buds of wild-type *Arabidopsis* and several homeotic flower mutants; *agamous*, *apetala-1*, *apetala-2*, *pistillata* and *clavata-2*. Figure 4.7 shows that the expression of the gene corresponding to pLF2 occurs in young floral buds of *Arabidopsis* and that the expression is lower in more mature buds and flowers. As significant expression can only be detected in *Arabidopsis* flower buds which are smaller than 0.5 mm, then it is difficult to obtain sufficient tissue to examine the temporal expression of pLF2 and pLF7 by northern blot analysis. However pLF3 and pLF10B are expressed throughout flowering and it was possible to compare their expression in buds from

Fig. 4.6 Time course of cold-induction of pLF5

Plants were grown at 23°C until they had 4-5 exposed leaves. The plants were then transferred to 5°C for varying lengths of time and RNA isolated from young expanded leaves. Plants which had been cold-acclimated for 3 d were transferred back to 23°C for various times and RNA isolated. RNA was also isolated from the leaves of wounded plants and plants which had been deprived of water for several days (visibly wilted). 10 µg per track of RNA was separated on a 1.5% agarose/formaldehyde gel and blotted onto a nylon filter. The filter was hybridised with labelled insert from pLF5B1 (1 x 10^6 cpm/ml) for 16 h in hybridisation solution containing 5 x SSC, 50% formamide at 42°C and washed to a final stringency of 0.1 x SSC, 0.1% SDS at 65°C. The filter was autoradiographed for 16 h with two intensifying screens.

1. 0 h cold	5. 12 h cold	9. 0 h warm
2. 1 h cold	6. 24 h cold	10. 1 h warm
3. 4h cold	7. 3 d cold	11. 4 h warm
4. 8h cold	8. 7 d cold	12. 8 h warm
13. Wilted	14. Wounded	

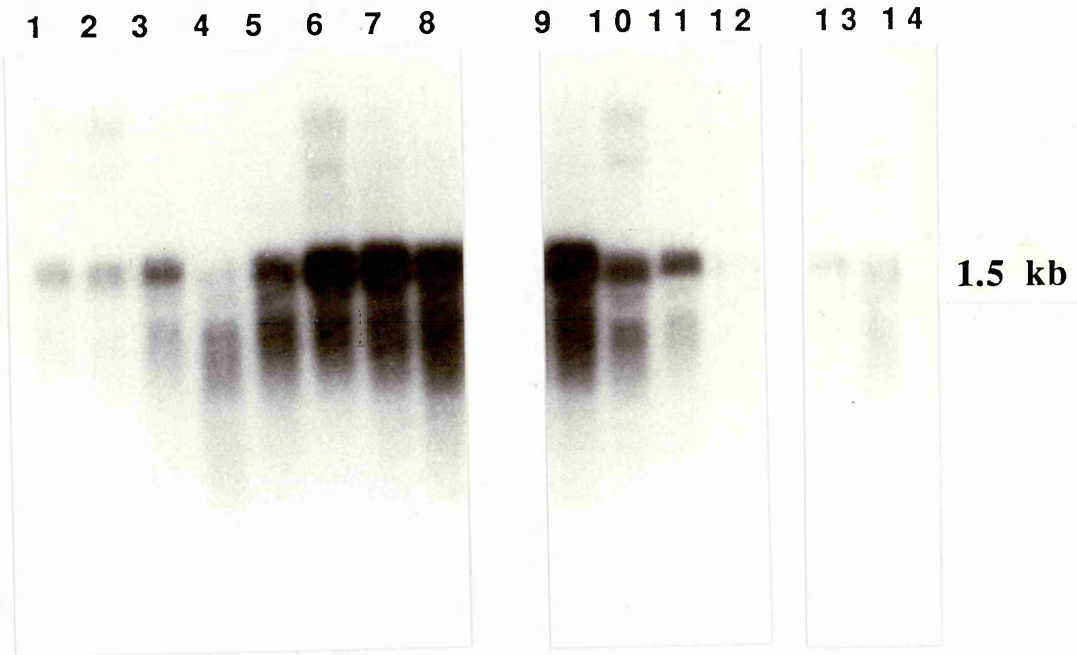

1 2 3 4 5 6 7 8
9 10 11 12
13 14
1.5 kb

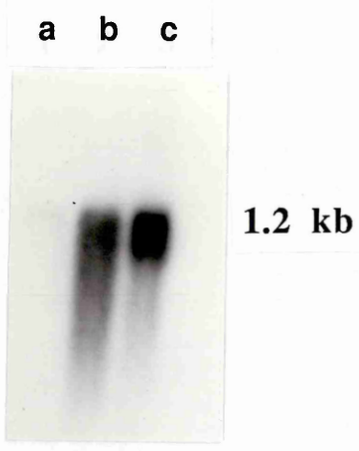
Fig. 4.7 Expression of the cDNA pLF2 in floral apices of *Arabidopsis*

Floral tissue was harvested from *Arabidopsis* and RNA isolated. Total RNA was separated on a 1.5% agarose/formaldehyde gel, and blotted onto a nylon filter. The filter was hybridised in hybridisation solution containing 6 x SSC, 50% formamide and 2 x 10^6 cpm/ml of labelled insert from pLF2, for 16 h at 42°C. The filter was washed to a final stringency of 0.2 x SSC, 0.1% SDS at 42°C, and autoradiographed for 2 d. Lanes 1 and 2 contain 20 µg of total RNA, lane 3 contains 10 µg of total RNA.

a. Floral buds (> 1 mm) and flowers of *Arabidopsis*

b. Floral buds (<1 mm) of *Arabidopsis*

c. Floral buds showing sepal primordia from *B. napus*


a b c
1.2 kb

both wild type plants and the floral mutants. Figure 4.8 shows that transcripts corresponding to both cDNAs are present in all mutants examined as well as wild-type flower buds. Little variation is seen in the level of transcript expression between the wild type plants and the floral mutants, the differences being due to the amount of RNA loaded onto the gel as estimated by reprobing the filter with a ribosomal RNA probe.

4.6 Sequences complementary to cDNAs pLF1-10 are present in the genomes of *Brassica napus* and *Arabidopsis thaliana*

Genomic DNA blots prepared from *Brassica napus* and *Arabidopsis thaliana* were probed with labelled insert from each of the clones pLF2, pLF3, pLF5 and pLF10B. The autoradiographs of these genomic blots are shown in Fig. 4.9. Each of the genes represented by the pLF cDNAs appears to be present in the *B. napus* genome at a low copy number (3-6). Homologous sequences are found in the *A. thaliana* genome, also at a low copy number (1-2 copies).

4.7 Spatial expression of the pLF transcripts as examined by *in situ* hybridisation

4.7.1 Probe preparation

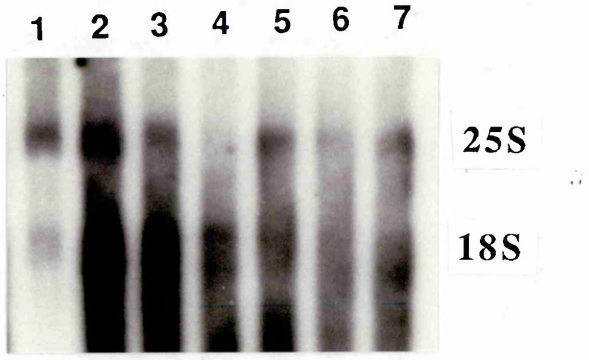
Initial attempts at detecting *in situ* transcripts corresponding to the pLF cDNAs proved unsuccesful. A variety of methods for preparing probes for tissue hybridisation were attempted including biotin and digoxigenin labelling of cDNA inserts. Extensive labelling of tissue sections was seen with a biotin-labelled cDNA

Fig. 4.8 Expression of the cDNAs pLF3 and pLF10B in the floral homeotic mutants of *Arabidopsis*

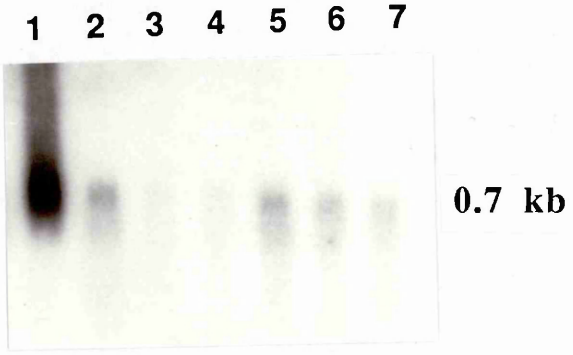
Flower buds (>1 mm) were harvested from wild-type and mutant *Arabidopsis* plants and total RNA isolated. 10 μg per track of RNA was separated on 1.5% agarose/formaldehyde gels and blotted onto nylon filters. Duplicate filters were hybridised with labelled insert from either pLF3A or pLF10B (4 x 10^5 cpm/ml) for 16 h at 40°C in hybridisation solution containing 6 x SSC, 50% formamide. The filters were washed to a final stringency of 0.2 x SSC, 0.1% SDS at 40°C. The filters were autoradiographed overnight with two intensifying screens. Lane 1 contains 1 μg of poly(A)$^+$ RNA from *B. napus* floral apices as a control.

2. wild-type
3. *agamous*
4. *apetala-1*
5. *apetala-2*
6. *pistillata*
7. *clavata-2*

rRNA



pLF3A



pLF10B

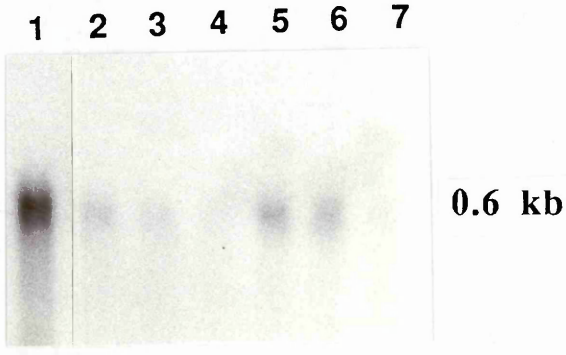
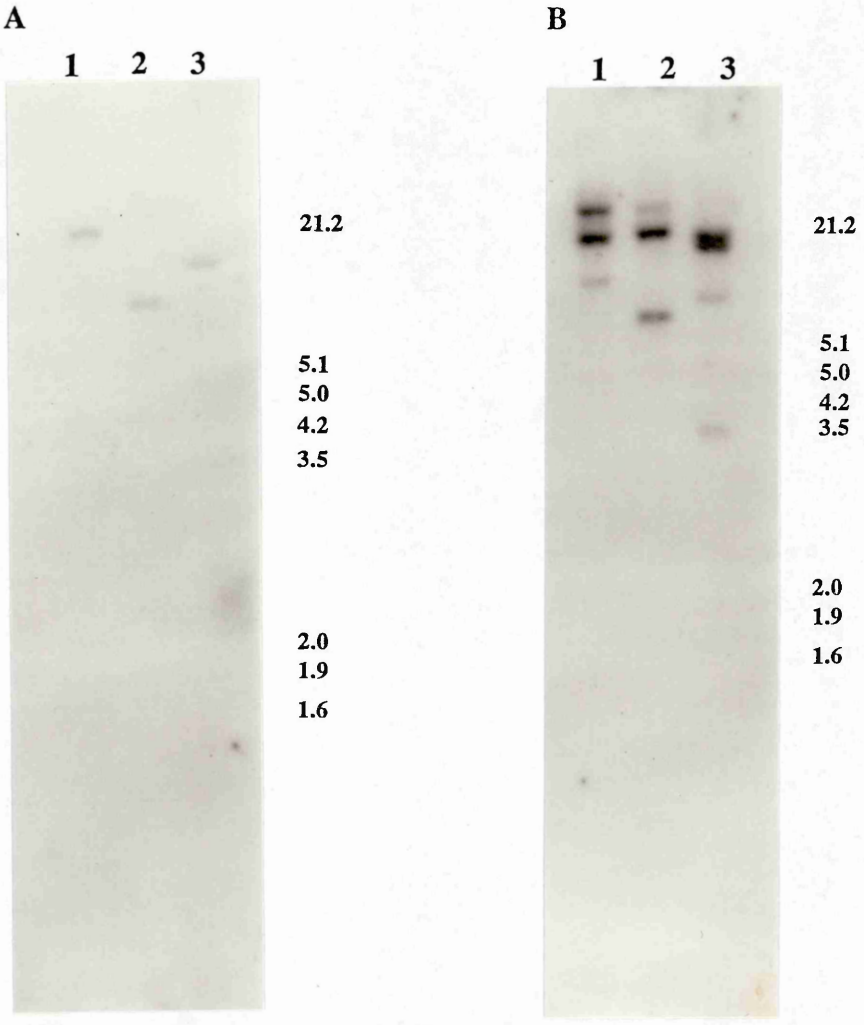


Fig. 4.9 Genomic blots of *Arabidopsis thaliana* and *Brassica napus* hybridised to the pLF cDNAs

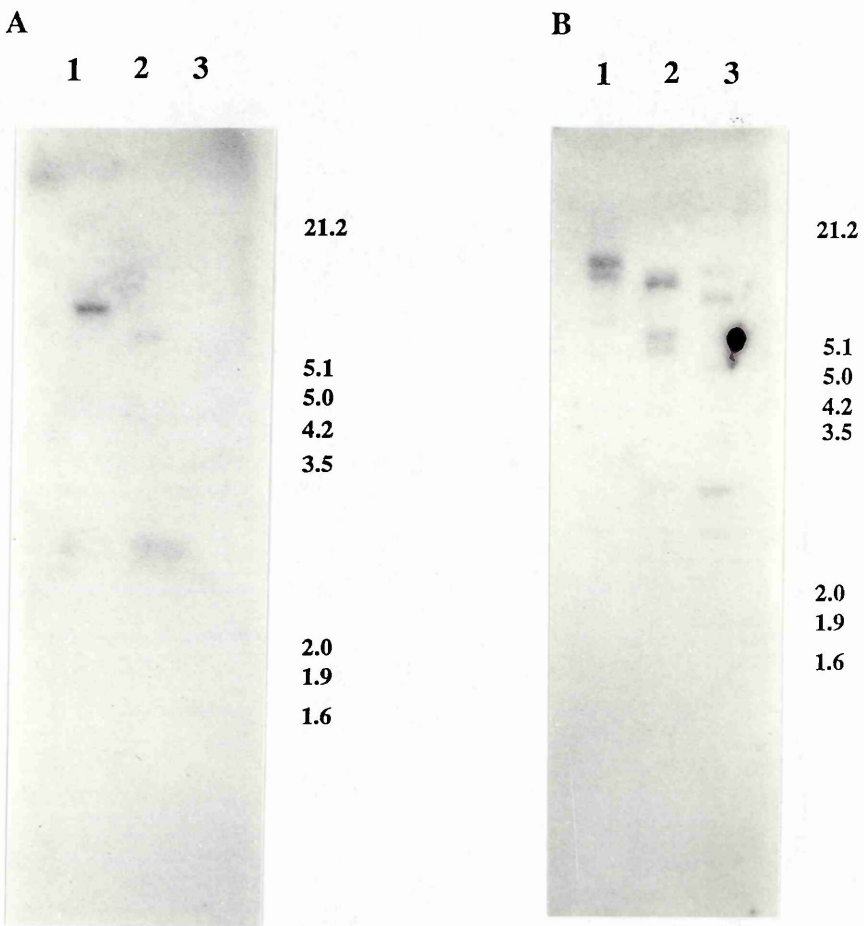
Genomic DNA was isolated from *A. thaliana* (A) and *B. napus* (B) and digested with various restriction enzymes for 3 h at 37°C. The DNA was separated on 0.7% agarose gels and blotted onto nylon filters. Identical filters were hybridised with the appropriate labelled insert from pLF2 (2 x 10^6 cpm/ml), pLF3A (1 x 10^6 cpm/ml), pLF5 (2 x 106 cpm/ml) or pLF10B (2 x 10^6 cpm/ml) for 16 h in hybridisation solution containing 5 x SSC, 50% formamide at 42°C. The filters were washed to a final stringency of 0.2 x SSC/0.1% SDS at 42°C. The filters were autoradiographed for 4 d with two intensifying screens

1. Bam HI
2. Eco RI
3. Hind III

pLF2

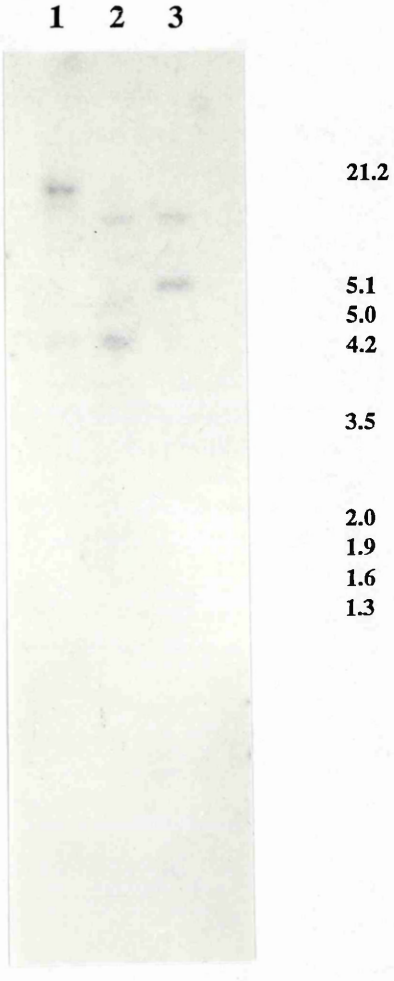


pLF3A

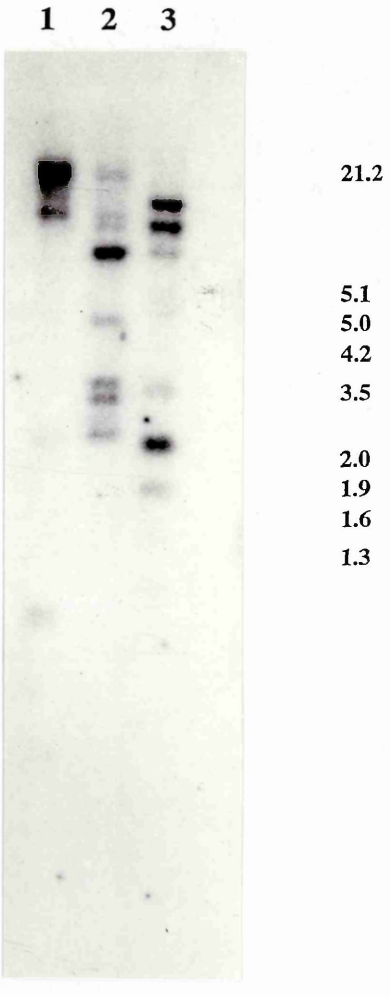


pLF5

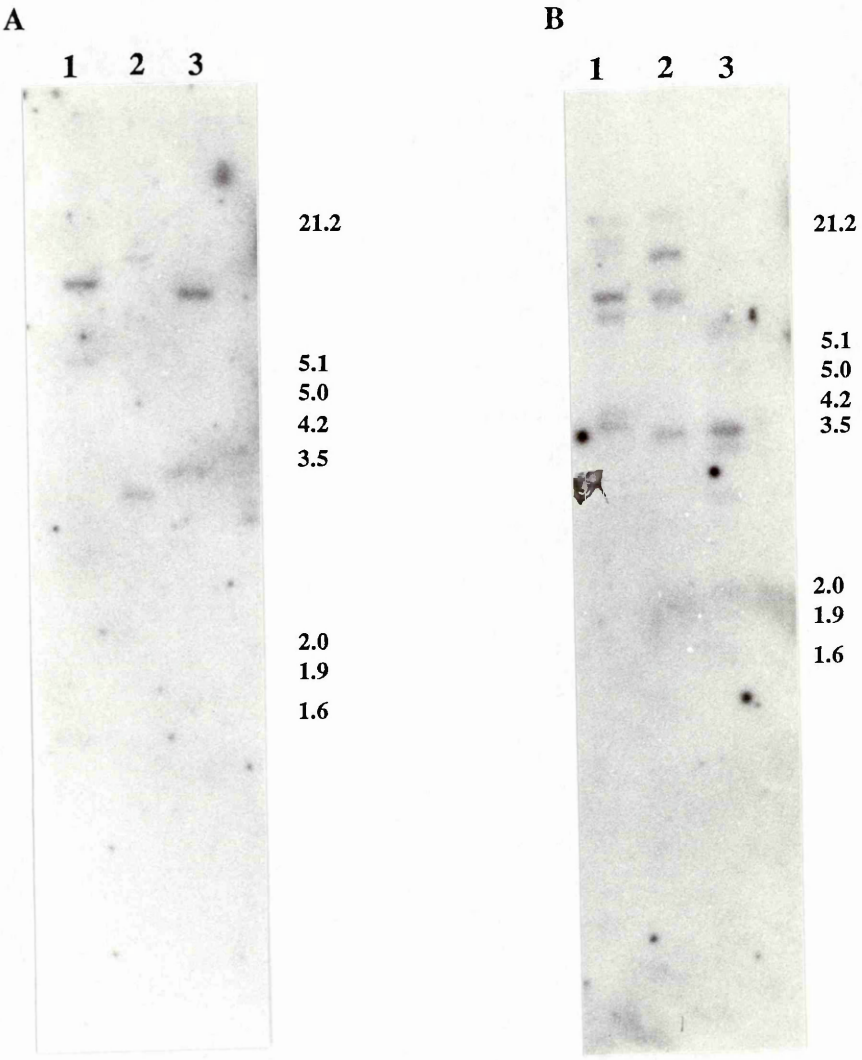
A



B



pLF10B



insert corresponding to a wheat ribosomal RNA, but no specific hybridisation was seen using the pLF cDNAs. Hybridisation of RNA probes labelled with ^{35}S-UTP to tissue sections initially proved unsuccesful due to the high degree of background seen using both sense and antisense probes. To examine whether the low signal to noise ratio was due to the probes, unlabelled RNA was *in vitro* transcribed from plasmid templates and examined on agarose gels. The majority of the transcripts produced from the T3 RNA polymerase promoter were of the expected size but the reaction also produced an RNA transcript of a larger size. It is possible that non-specific initiation of transcription was occurring or that the T3 RNA polymerase was utilising the T7 promoter. Fig. 4.10 shows labelled RNA transcripts synthesised at two different NaCl concentrations, 50 mM and 100 mM. Increasing the NaCl concentration in the reaction to 100 mM decreased the amount of non-specific or inappropriate initiation but lowered the efficiency of the reaction. This was thought to be an acceptable compromise as probes prepared in the presence of 100 mM NaCl produced lower background on tissue sections when used for hybridisation. To prevent possible read-through of the polymerase into the vector in incompletely digested template, the insert and promoter regions were isolated from agarose gels after digestion with Pvu II. The promoter/insert-containing fragments were subsequently used as templates for *in vitro* RNA transcription. There was no effect on the relative degree of hybridisation to the sections due to the length of the probes. Full length probes gave a specific signal with no significant increase in background which was comparable to that produced with probes which were hydrolysed to a mean length of 150 bp.

4.7.2 Fixation of tissue

The most important parameter in determining the signal to noise ratio was the fixative used. Initial attempts at *in situ* localisation of transcripts used tissue sections

Fig. 4.10 ^{35}S-labelled *in vitro* transcribed RNA

In vitro transcribed RNA was synthesised at two different NaCl concentrations in the presence of 10 μCi α-^{35}S-UTP in a total reaction volume of 10 μl. 2 μl of the reaction was loaded per track on a 4% sequencing gel and electrophoresed for 1.5 h at 45 W. The gel was dried and autoradiographed overnight. Lane 5 contains ^{32}P-labelled pUC 9 digested with Hpa II.

Lanes 1-4, 6-9; 50 mM NaCl

Lanes 10-17; 100 mM NaCl

Plasmid templates used:

Lanes 1,10; Eco RI digest of pLF2

Lanes 2, 11; Eco RI digest of pLF3A

Lanes 3, 12; Bam HI digest of pLF7P2

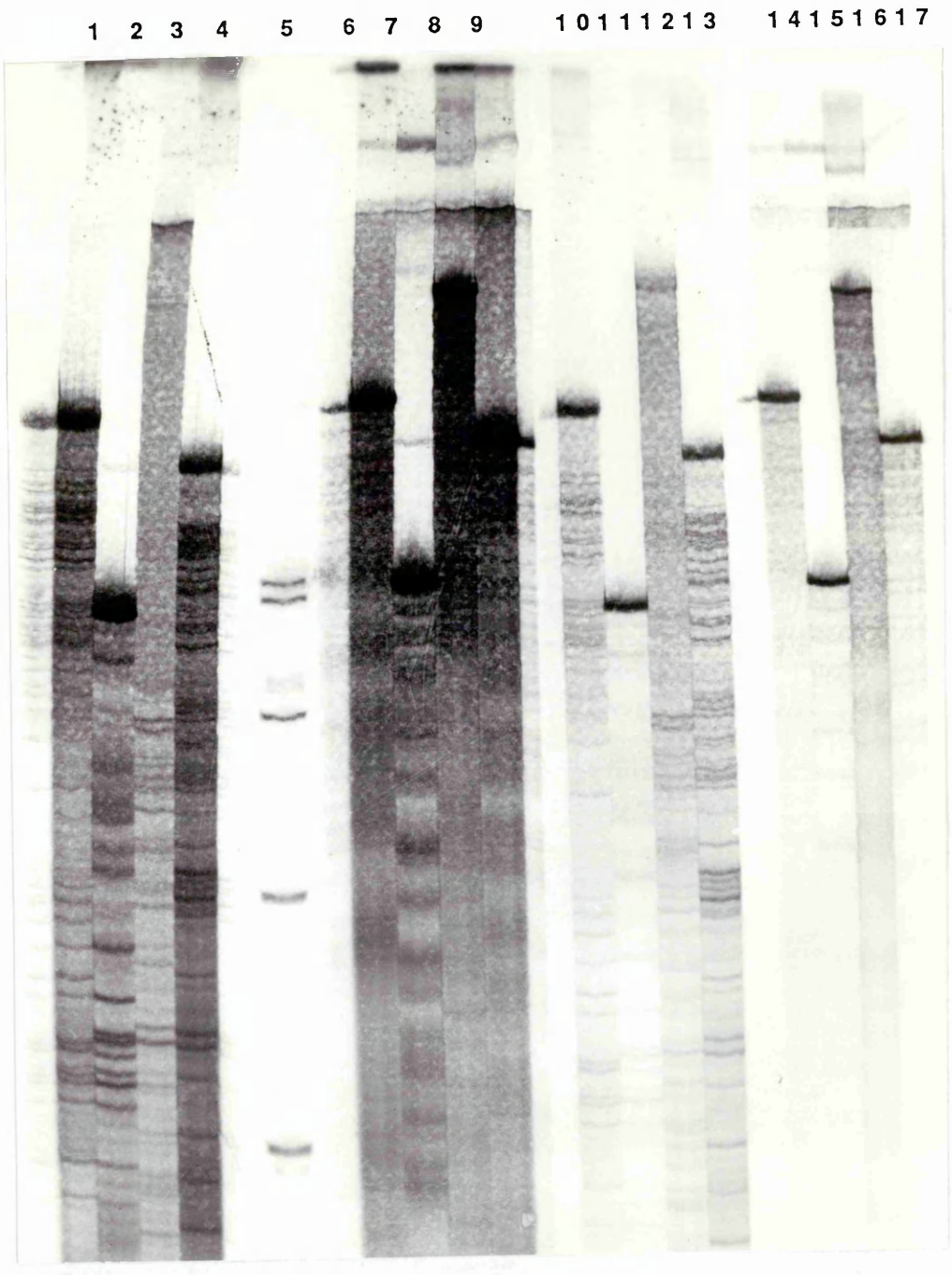
Lanes 4, 13; Eco RI digest of pLF10B

Lanes 6, 14; Hind III digest of pLF2

Lanes 7, 15; Hind III digest of pLF3A

Lanes 8, 16; Eco RI digest of pLF7P2

Lanes 9, 17; Hind III digest of pLF10B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

which had been fixed in 3.25% formaldehyde/1.25 glutaraldehyde as recommended by Dr. N. Harris (Dept. of Botany, University of Durham). However due to persistent problems with background it was decided to evaluate other fixatives. Floral apices were fixed in four different fixatives: 3.25% formaldehyde/1.25 glutaraldehyde, 4% formaldehyde, 1% glutaraldehyde and FAA (formaldehyde, ethanol, acetic acid). The fixed tissue was dehydrated following recommended methods and the tissue was embedded in wax and sectioned following an identical protocol for each sample. The sections were each hybridised initially to sense and antisense RNA probes from pLF2 (see Figures 4.11 and 4.12). All the sections probed with the antisense RNA probe showed hybridisation all over the section. However the sense probes showed little or no hybridisation to the sections prepared from formaldehyde or FAA fixed tissue. High levels of background were seen on tissue sections fixed in the presence of glutaraldehyde (see Fig. 4.12). Increasing the proteinase K digestion time to 30 min did not improve the signal to noise ratio of glutaraldehyde-fixed tissue (data not shown).

4.7.3 Detection of cell-specific expression using digoxigenin-labelled RNA probes

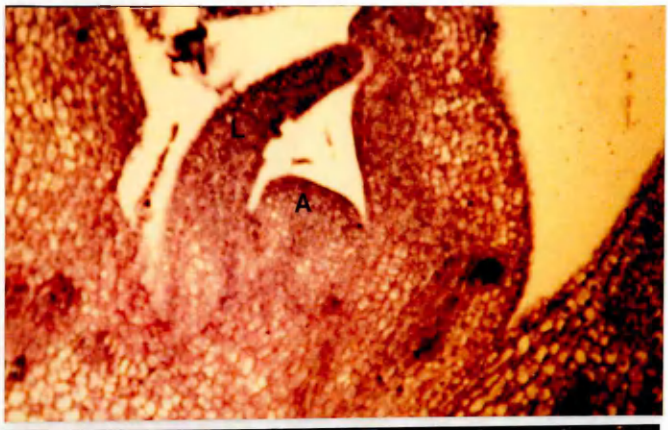
Initial attempts to show specific hybridisation using digoxigenin-labelled (DIG) RNA probes were unsuccessful due to a high degree of background. Pre-incubation of the tissue sections with BSA prior to the acetylation step as well as incorporation of steps suggested by Coen et al. (1990) for increased 'blocking' of the sections prior to incubation with the antibody greatly increased the specificity of the hybridisation. Fig. 4.13 shows a cross-section of an inflorescence apex which has been hybridised with sense and antisense RNA probes to the transcript corresponding to pLF10B. The antisense probe hybridises to vascular tissue as discussed in the next section.

Fig. 4.11 *In situ* hybridisation of ^{35}S-labelled pLF2 RNA to apical tissue fixed using FAA

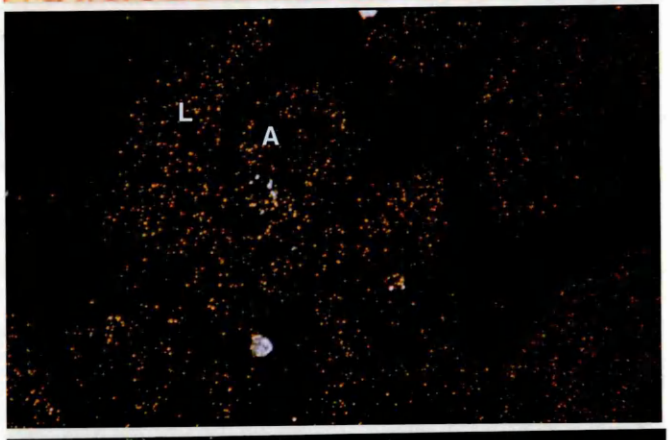
Floral apical tissue at the stage prior to initiation of floral primordia was fixed in FAA (section 2.23.3). The tissue was wax-embedded, sectioned and pretreated as discussed in sections 2.23.4, 6, 7. Each section was hybridised with 1.14 x 10^6 cpm of ^{35}S-labelled either antisense RNA (b and c), or sense RNA (d) (see section 2.23.8) transcribed from the pLF2 cDNA. After washing the slides were coated in emulsion and exposed for 36 h or 4 d at 4°C. After development, the slides were mounted in D.P.X. and photographed using a Zeiss Photomicroscope II. Magnification x 110.
A: apex, L: leaf

a. Toluidine blue-stained section of apex
b. Apical section probed with antisense probe, exposed 36 h.
c. Apical section probed with antisense probe, exposed 4 d.
d. Apical section probed with sense probe, exposed 36 h.

a



b



c

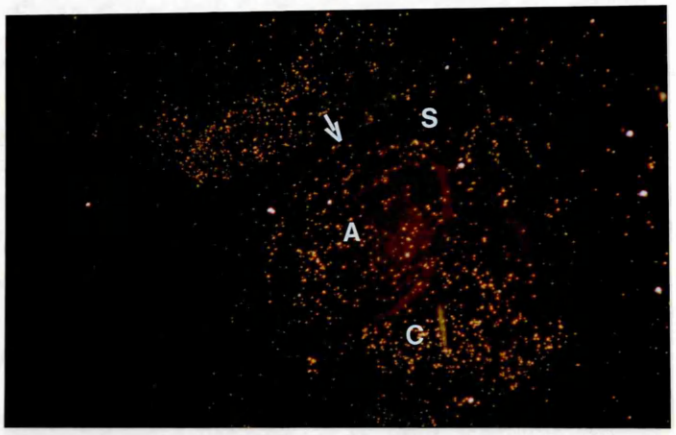
d

Fig. 4.12 *In situ* hybridisation of ^{35}S-labelled RNA from pLF2 to floral tissue fixed using 1% glutaraldehyde

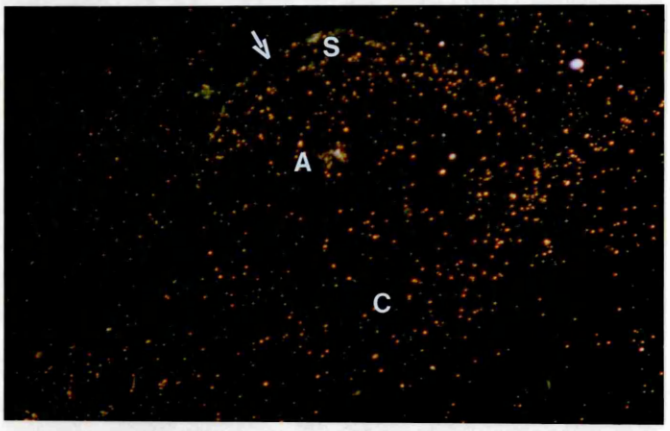
Floral apical tissue was fixed in 1% glutaraldehyde (section 2.23.2). The tissue was wax-embedded, sectioned and pretreated as discussed in sections 2.23.4, 6, 7. Each section was hybridised with 1.14 x 10^6 cpm of ^{35}S-labelled (a) antisense RNA, and (b) sense RNA transcribed from the pLF2 cDNA (see section 2.23.8) or (c) no probe. After washing the slides were coated in emulsion and exposed for 36 h at 4°C. After development, the slides were mounted in D.P.X. and photographed using a Zeiss Photomicroscope II. Magnification x 110. Arrows indicate the non-specific signal seen at the edge of sepals of flower buds

S: sepal, A: anther, C: carpel primordium

a



b



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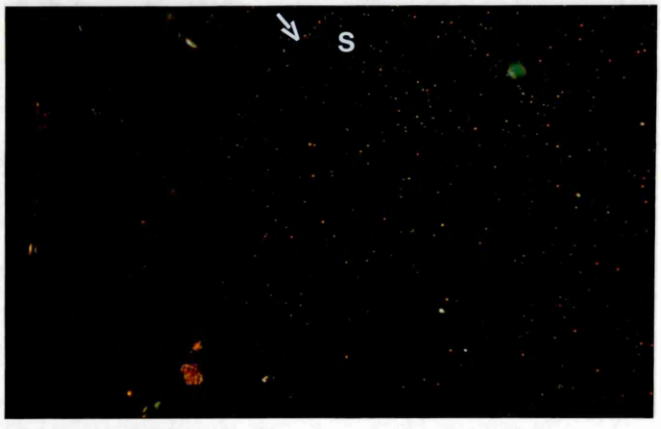
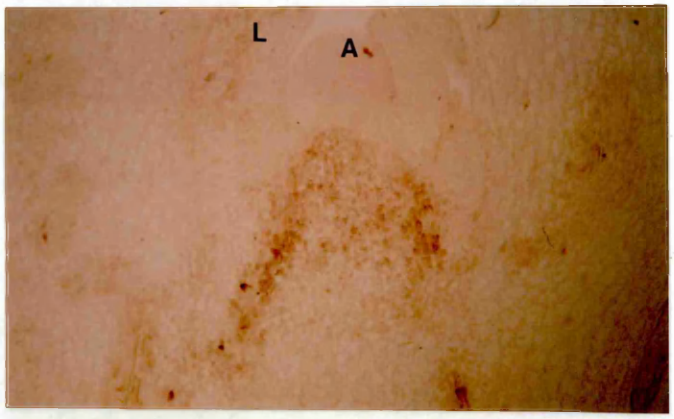


Fig. 4.13 Digoxigenin-labelled RNA from pLF10B hybridised *in situ* to apical tissue

Floral apical tissue was fixed in 4% formaldehyde (section 2.23.1). The tissue was wax-embedded, sectioned and pretreated as discussed in sections 2.23.4, 6, 7. The sections were hybridised with 0.6 µl (of a total vol of 25 µl) of digoxigenin-labelled (a) antisense and (b) sense RNA transcribed from the pLF10B cDNA. After washing the hybridised RNA was detected as in section 2.23.10. The colour reaction was terminated after 6 h. The slides were mounted in D.P.X. and photographed using a Zeiss Photomicroscope II. Magnification x 110.

A: apex, L: leaf

a



b

4.7.4 Cell-specific expression of the pLF transcripts

Figures 4.11 to 4.16 show hybridisation of labelled RNA transcipts derived from the cDNAs pLF2, pLF3, pLF7 and pLF10B to various tissues. Figures 4.11 and 4.14 show the hybridisation of pLF2 and pLF7, respectively, to floral apical tissue. Neither probe showed hybridisation to a specific region of the apex or floral buds although a distinct signal specific to the antisense probe was evident. In contrast RNA transcripts corresponding to the cDNAs pLF3 and pLF10B show highly cell-specific patterns of expression. Fig. 4.13 shows a longitudinal section of an inflorescence apex. The DIG-labelled antisense RNA hybridises to vascular tissue supplying the apex. The large area of hybridisation seen in the apparent region of the pith could be due to oblique sectioning of the tissue as no hybridisation of labelled RNA transcripts in this region was seen in sections derived from other tissue sections. Fig. 4.15 shows a cross-section of an inflorescence stem. A ^{35}S-labelled antisense RNA probe derived from pLF10B appears to hybridise to phloem elements. The metaxylem elements can be detected by their autofluorescence but do not show specific hybridisation. The gene corresponding to pLF3 is expressed in the epidermal layer of flower buds, stems and leaves (see Fig. 4.16).

4.8 Discussion

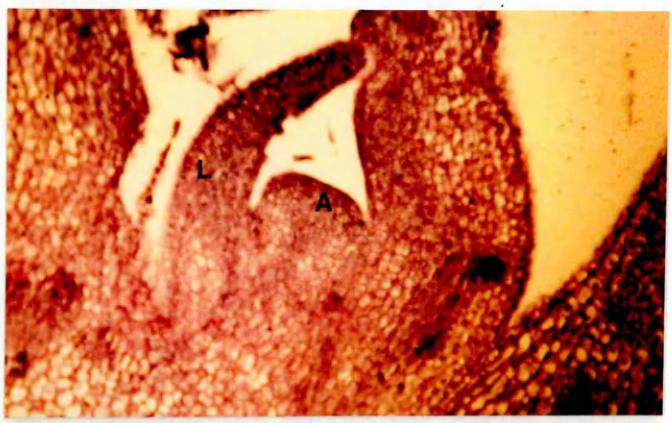
Figures 4.2-4.4 show that several of the genes represented by pLF1-10 are controlled in a organ-specific and developmental manner, the exception being pLF8 which shows a high level of expression in all of the floral tissues examined. Table 4.1 summarises the patterns of expression for each gene as determined by northern blot analysis. On this basis the cDNAs can be divided into two classes: pLF3 and

Fig. 4.14 *In situ* hybridisation of pLF7 RNA transcripts to apical tissue

Floral apical tissue was fixed in 4% formaldehyde (section 2.23.1). The tissue was wax-embedded, sectioned and pretreated as discussed in sections 2.23.4, 6, 7. Each section was hybridised with 0.5 x 10^6 cpm of ^{35}S-labelled (b and c) antisense RNA, and (d) sense RNA transcribed from the pLF7P2 cDNA (see section 2.23.8). After washing the slides were coated in emulsion and exposed for either 3 d (b) or 8 d (c) and (d) at 4°C. After development, the slides were mounted in D.P.X. and photographed using a Zeiss Photomicroscope II. (a) Section of apical tissue stained with 0.1% toluidine blue. Magnification x 110

A: apex, L: leaf

a



b

c

d

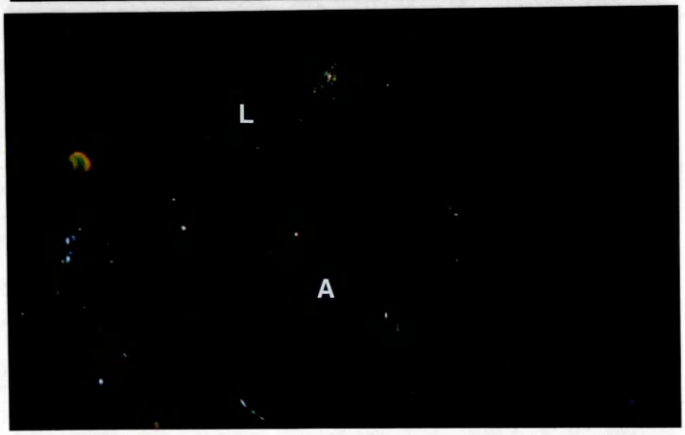


Fig. 4.15 The transcript represented by pLF10B is expressed in the phloem cells of the stem

Inflorescence stem tissue was fixed in 4% formaldehyde (section 2.23.1). The tissue was wax-embedded, sectioned and pretreated as discussed in sections 2.23.4, 6, 7. Each section was hybridised with 1.14 x 10^6 cpm of ^{35}S-labelled antisense RNA or sense RNA (see section 2.23.8) derived from pLF10B. After washing the slides were coated in emulsion and exposed for 3 d at 4°C. After development, the slides were mounted in D.P.X. and photographed using a Zeiss Photomicroscope II. Magnification x 110.

E: epidermis, X: xylem, P: phloem, C: cortex

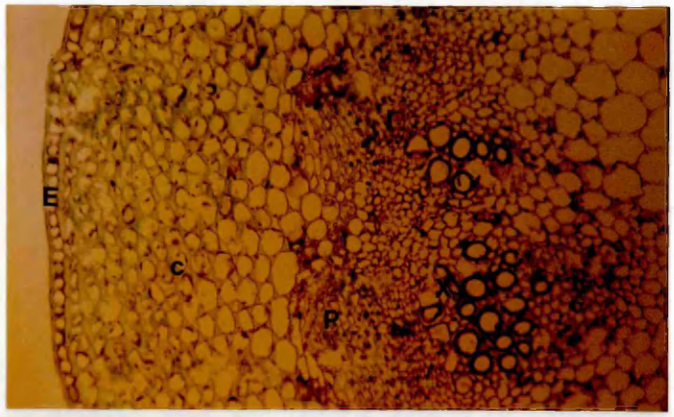
a. Transverse section (TS) stem stained with toluidine blue

b. Dark field picture of TS stem hybridised with antisense probe

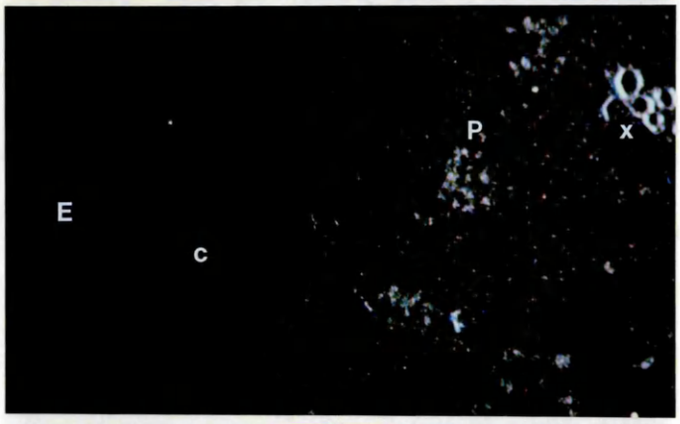
c. Bright field picture of TS stem hybridised with antisense probe

d. Dark field picture of TS stem hybridised with sense probe

a



b



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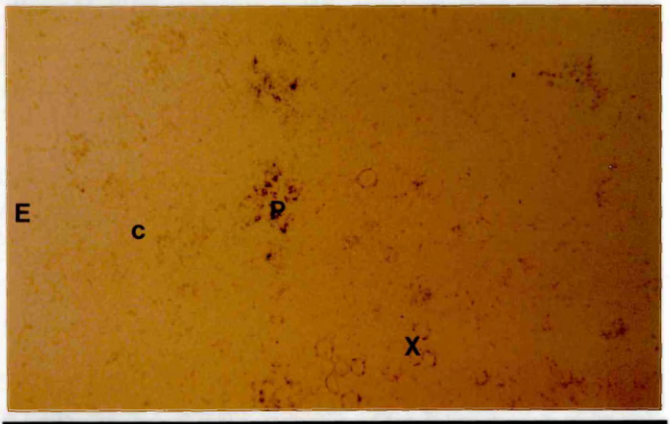


d

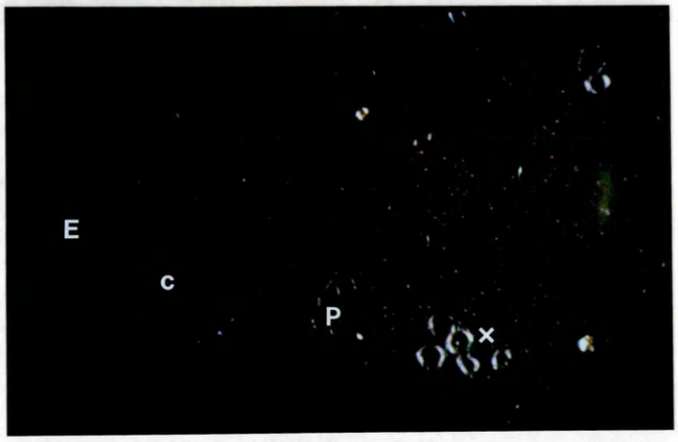


Fig. 4.16 Expression of pLF3 is restricted to epidermal cells

Inflorescence stem and leaf tissue was fixed in 4% formaldehyde (section 2.23.1). The tissue was wax-embedded, sectioned and pretreated as discussed in sections 2.23.4, 6, 7. Each section was hybridised with 0.9 x 10^6 cpm of ^{35}S-labelled antisense RNA or sense RNA (see section 2.23.8) derived from pLF3. After washing the slides were coated in emulsion and exposed for 3 d at 4°C. After development, the slides were mounted in D.P.X. and photographed using a Zeiss Photomicroscope II. Magnification as indicated.

E: epidermis, X: xylem, P: phloem, C: cortex

a. Dark field picture of TS leaf hybridised with antisense probe, x 170

b. Dark field picture of TS stem hybridised with antisense probe, x 170

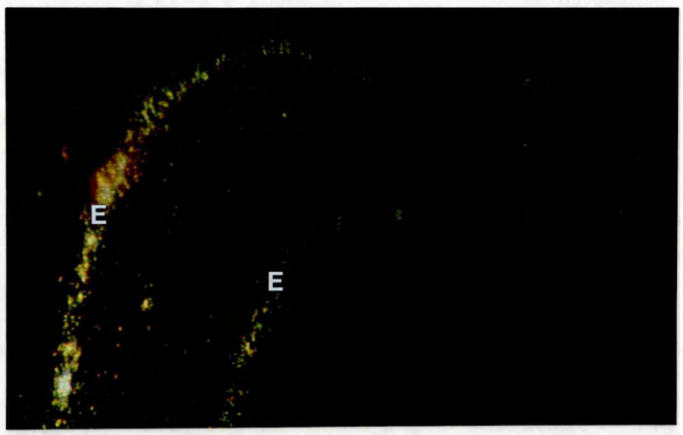
c. Dark field picture of TS stem hybridised with antisense probe, x 450

d. Bright field picture of TS stem hybridised with antisense probe, x 450

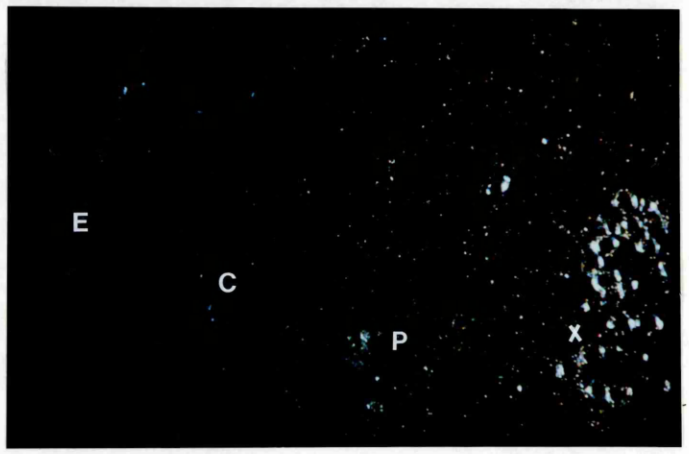
e. Bright field picture of TS stem hybridised with antisense probe, x 450

f. Dark field picture of TS stem hybridised with sense probe, x 450

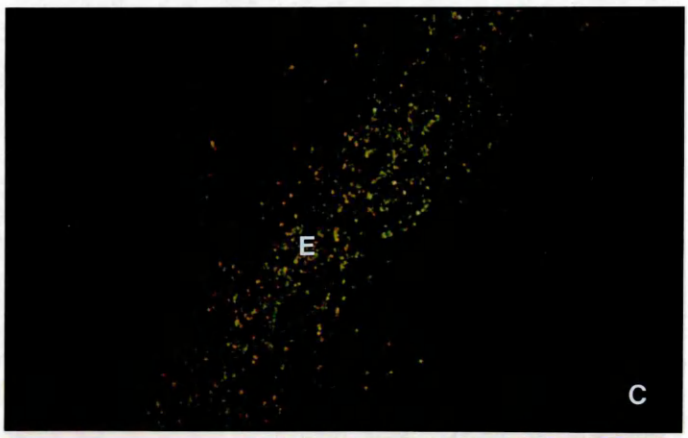
a



b



c



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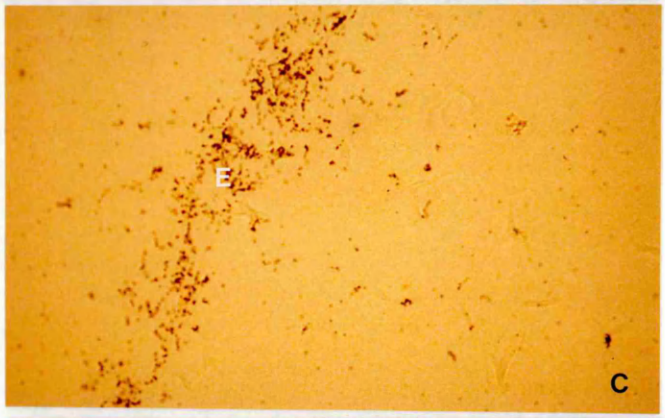
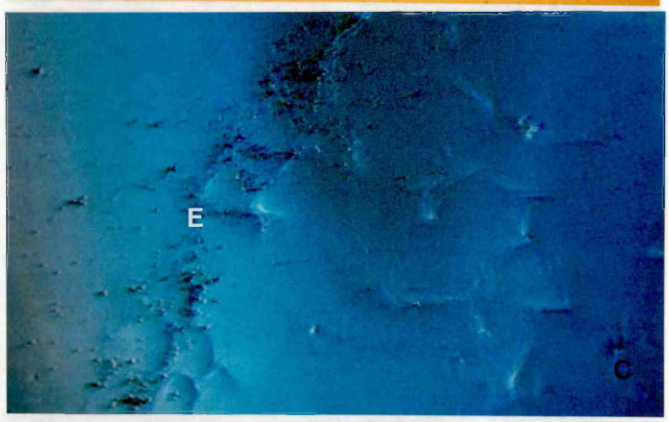


e



f

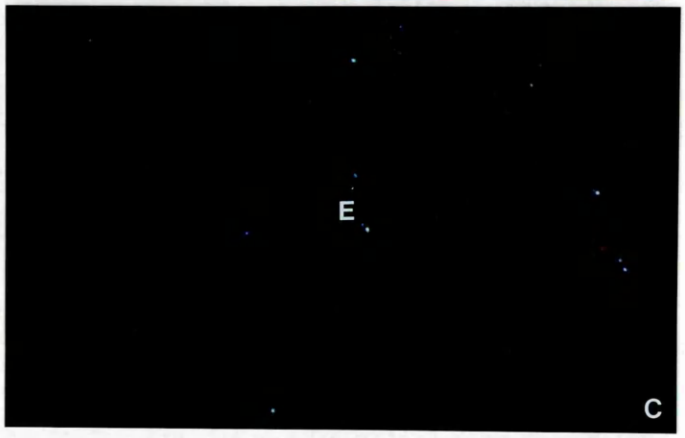


Table 4.1 Temporal and organ-specfic expression of selected cDNAs as analysed by northern blots

This table summarises the organ-specific and temporal expression of the genes represented by the cDNAs pLF2, pLF3, pLF5, pLF7 and pLF10B in various tissues of *B. napus* and *A. thaliana* wild-type and homeotic mutants. All northern blots were carried out according to standard procedures (see sections 2.13 and legends to Figs. 4.2-4.7). The '+' sign indicates the presence of the corresponding transcript. The '?' sign indicates the possible presence of a transcript in these tissues. N.D. (not determined) indicates that these tissues were not examined for the presence of the corresponding transcript.

TISSUE	pLF2	pLF3	pLF5	pLF7	pLF10
Brassica napus					
vegetative apex	+	+	+	+	+
floral apex	+	+	+	+	+
floral buds > 2 mm	+	+	+	+	+
green buds 2-4 mm		+			+
yellow buds < 6 mm		+			+
flowers		+			+
seed pods		+			+
root					
leaf		+	+		+
stem		+			+
seedling		+			+
cold-induced leaf			+		
heat-shocked leaf					
wounded leaf					
wilted leaf	N.D.	N.D.		N.D.	N.D.
Arabidopsis thaliana					
wild-type flower buds (< 1 mm)	+	+	N.D.	+	+
agamous flower buds	?	+	N.D.	?	+
apetala-1 flower buds	?	+	N.D.	?	+
apetala-2 flower buds	?	+	N.D.	?	+
pistillata flower buds	?	+	N.D.	?	+
clavata-2 flower buds (all buds > 1 mm)	?	+	N.D.	?	+

10B, which are expressed in several tissues and throughout flower development; and pLF2, pLF5 and pLF7, which are expressed in vegetative apex and up-regulated during the floral transition. In a similar study to the one discussed here, Melzer et al. (1990) exploited the day length dependence of flower formation in *Sinapis alba* to produce apical-specific cDNA libraries. Differential screening of these libraries produced two classes of cDNAs; one group of transcripts was present in vegetative apex and up-regulated after perception of the floral stimulus, and the second class was all expressed exclusively in the tapetum. The expression of the group I genes declined 15-20 days after the inductive light period when the floral organs had fully developed. The transcripts represented by pLF2, pLF5 and pLF7 show a similar pattern of expression in *B. napus* i.e present in vegetative apex, up-regulated on response to a presumed floral stimulus and returning to basal levels once formation of the organ primordia of the flowers is complete. The transcript represented by pLF5 additionally shows a marked increase in response to low temperatures. Many genes that are induced by environmental stress also show developmental regulation, an example being chalcone synthase (e.g. Schmid et al., 1990). Meeks-Wagner and co-workers found that several clones isolated from a cDNA library made from tobacco thin cell layer explants exhibiting floral buds encoded pathogenesis-related proteins. The genes isolated in this study (Neale et al., 1990) included a chitinase, a β-1,3-glucanase and osmotin, which is induced by water stress. None of the pLF transcripts isolated were found to be up-regulated by wounding or heat-shock.

The expression of the cDNA pLF5 is up-regulated on transfer of plants to the cold. The kinetics of transcript accumulation follow a similar pattern to the cold-regulated (*cor*) genes isolated from *Arabidopsis thaliana* (Hajela et al., 1990). The *cor* genes, together with another cold-inducible gene from *Arabidopsis*, *kin1* (Kurkela and Franck, 1990), are all ABA-inducible in the absence of cold temperatures. Nordin et al. (1991) report that a cold-inducible gene from

Arabidopsis, *lti140*, is also induced by water stress and application of exogenous ABA. By studying the expression of this gene in the ABA-insensitive mutant of *Arabidopsis*, *abi-1*, and by the use of an ABA biosynthesis inhibitor, the authors concluded that three separate but ultimately convergent signalling pathways exist to control expression of the *lti140* gene. Subsequent work in our laboratory has suggested that the increase in the level of transcript corresponding to pLF5B1 seen in cold temperatures cannot be mimicked by application of exogenous ABA (J. Jardine and G.I. Jenkins, unpublished results). If pLF5 is proved to be induced by low temperatures but not water stress or ABA, this would point to a separate pathway in *B. napus* unique to cold-acclimation, but independent of ABA induction.

The genes corresponding to the cDNAs pLF2, pLF3, pLF7 and pLF10B are all expressed in the wild type floral buds of *Arabidopsis thaliana.* It proved impossible to conclusively show that the transcripts represented by pLF2 and pLF7 were present in the *Arabidopsis* floral mutants. If the temporal expression of these genes in *Arabidopsis* is similar to that seen in *Brassica napus* then the genes would be switched off by the time the buds were visible to the naked eye. It would then be tedious and time-consuming to collect sufficient material from the mutants to isolate RNA for northern blots. This difficulty is compounded in the mutant *agamous*. These plants are sterile and propagated as a heterozygous population. The homozygotes cannot be detected until the plants begin to flower. One possibility is to examine the expression of pLF2 and pLF7 in the floral mutants by *in situ* hybridisation. However this technique is only qualitative and not quantitative, and alterations in the level of expression of the genes corresponding to these cDNAs could not be detected. As the homeotic genes of *Arabidopsis* isolated thus far are all DNA-binding proteins it is possible that these genes may modulate expression of the genes corresponding to pLF2 and pLF7. It is unlikely that the homeotic genes initiate

expression of the LF2 and LF7 genes. LF2 and LF7 are already expressed in the vegetative apex prior to the initiation of expression of the homeotic genes, although the homeotic genes may act to switch off the LF2 and LF7 genes during development of the flower.

Brassica napus is an allotetraploid (n=19), being derived from *B. oleraceae* (n=9) and *B. campestris* (n=10) and containing copies of the genomes from both species (Glimelius et al., 1991). The genes represented by the pLF cDNAs are present in 2-6 copies in the *B. napus* genome. This will presumably reflect the inheritance of the corresponding genes from the parent *Brassica* species. Complementary sequences are present in the *Arabidopsis* genome and are present in 1-2 copies.

In situ hybridisation has been used extensively to localise transcripts of specific genes to certain cell types (e.g. Smith et al., 1987). The protocols used for fixation of plant tissue and prehybridisation treatments of tissue sections vary depending upon the type of tissue and probe used. The fixative used for *Brassica napus* tissue was found to have an effect on the final signal to noise ratio obtained. The presence of glutaraldehyde in the fixative gave sections with much higher background which could not be reduced by extending the time of proteinase K digestion. This was also found in chick embryonic muscle cells by Lawrence and Singer (1985) who suggested that the effect may be due to more extensive crosslinking of the cellular matrix by glutaraldehyde than that found using formaldehyde alone. Incubation of *B. napus* tissue sections with RNAase A prior to hybridisation still produced a significant background over the tissue section which would suggest probe retention in glutaraldehyde-fixed tissue. This was not seen with tissue sections fixed with formaldehyde (data not shown). The pretreatments for plant tissue sections suggested

by Cox and Goldberg (1988) include incubation with BSA and acetylation of the sections with acetic anhydride. Using the tissues and probes described in this thesis the acetylation step gave a slight reduction in background. Pre-incubation with BSA significantly reduced the background which resulted from probing sections with DIG-labelled probes as did the modifications after the post-hybridisation washes suggested by Coen et al. (1990). The tissue sections were routinely dehydrated prior to the pretreatments and this was not found to increase the background significantly, as had been suggested by Lawrence and Singer (1985). Pretreating the sections with hybridisation solution minus probe did not have a significant effect on reducing background unlike conventional filter hybridisation. Drews et al. (1991) report a background signal present at the outer edge of the sepals in *Arabidopsis* flower buds which had initiated carpel development. A similar signal was seen in *Brassica* floral buds at a comparable stage of development. Silver grains were localised at this point even in sections which had been hybridised, washed and autoradiographed in the absence of probe. Strand-specific RNA probes were much superior to DNA probes both in background reduction and signal localisation. The *in vitro* transcription reaction was optimised to produce RNA probes which gave the highest signal to noise ratio. Probe length did not seem to be an important parameter as a similar signal was seen with full length transcripts and those which had been hydrolysed to a mean length of 150 bp.

The transcripts corresponding to pLF2 and pLF7 could not be localised to a specific area in the floral buds. This could be due to the presence of transcripts corresponding to these genes in all cell types. However it is also possible that the *in situ* hybridisation protocol used here would not show cell-specific expression, and that the protocol must be optimised for each specific cDNA although hybridisation was seen preferentially with the antisense probe and not with the sense probe. The

cell-specific expression of these genes could be verified by the isolation of the respective promoters and histochemical localisation of a promoter-reporter gene fusion in transgenic plants. β-glucuronidase (GUS) is the most widely used reporter gene in this context (Jefferson et al., 1987). The luciferase gene can also be used as a reporter gene though the expense of the substrate and the requirement for sophisticated equipment to detect low levels of enzyme activity make it less attractive than GUS for routine use. That pLF2 and pLF7 have a narrow temporal but non-specific spatial expression suggests that they are unlikely to have a role in determining the fate of specific cell types. Nevertheless, the specificity of the temporal expression of these cDNAs implies that they may function at a particular stage in floral apex development, though this function is, as yet, unclear.

The gene corresponding to pLF3 was shown by *in situ* hybridisation to be expressed in epidermal cells. The transcript is present in the epidermal layer of apical tissue, floral buds, stem and leaf. Petal and root tissue was also examined for the presence of the transcript by *in situ* hybridisation but this experiment was not successful. Chalcone synthase is expressed in the epidermal cell layer of parsley leaves (Schemlzer et al., 1988) and it is possible that pLF3 encodes an enzyme related to the flavonoid biosynthesis pathway. However the pLF3 transcript is not up-regulated by wounding and is not expressed in roots, as examined by northern blots, in contrast to chalcone synthase (Schmid et al., 1990). Several epidermal-specific cDNAs have recently been isolated from *Sedum* (Clark et al., 1991) and the corresponding genes are being isolated from *Arabidopsis*.

The gene represented by the cDNA pLF10B also shows tissue-specific expression. This gene appears to be expressed in the phloem cells as seen in *in situ* hybridisations of cross-sections of stem tissue. The up-regulation of the gene

represented by pLF10B seen in the floral apex compared to the vegetative apex may reflect the amount of sub-apical tissue isolated in the original tissue harvests, or the presence of vascular elements in the developing flower buds. However it could also represent a role for LF10B in transport of nutrients or 'floral signals' to the apex during and after evocation. Other phloem-specific sequences have been isolated. Goldberg (1988) reports the isolation of a phloem-specific cDNA from tobacco, which hybridises to the inner and outer layers of phloem cells in the tobacco stem and a phloem-specific promoter was isolated by a 'promoter-trapping' experiment in *Arabidopsis* (Kertbundit et al., 1991). The isolated 3 kb promoter sequence directed GUS expression in the phloem of leaves, stems, roots and flowers of *Arabidopsis* and tobacco. The sequences of these phloem-specific genes are not yet available and the function of the genes is unknown.

Chapter 5: Analysis of the function of the genes corresponding to pLF1-10

5.1 Introduction

Differential screening is a useful way to clone genes which are expressed in particular organs or cell-types. However the function of such cloned genes must be addressed. Sequencing of the cDNA and comparison of this sequence to known sequences in the databases is useful and can yield information on the putative nature of the gene product. Another way of assessing the function of a gene is to create a mutant where the expression of the gene is suppressed. Analysis of the mutant phenotype could give clues to the action of the gene product. One means of carrying out this 'reverse-genetics' approach is to transform plants with an antisense copy of the gene of interest (Schuch, 1991). Antisense RNA technology has been used, for example, to produce novel flower colour patterns in petunia (van der Krol et al., 1988a) and tomato fruit with delayed softening and increased shelf-life (Smith et al., 1988). This chapter describes the use of an antisense approach to investigate the function of a cryptic gene corresponding to the *B. napus* cDNA pLF2 in *Arabidopsis thaliana*. In addition, sequencing of the pLF cDNAs was initiated.

5.2 *Arabidopsis* plants transformed with antisense constructs of pLF2

5.2.1 Description of the constructs

The plasmid pJR1 was kindly donated by ICI Seeds, Bracknell. This construct contains 530 bp of the cauliflower mosaic virus (CaMV) 35S promoter and 250 bp of

nopaline synthase 3' region, separated by a multiple cloning region in Bin 19 (Smith et al., 1988). The 35S promoter contains an initiation codon 2 bp from the 3' end. Fig. 5.1 describes the construction of p2S and p2AS. These constructs contain pLF2 inserts cloned 3' to the 35S promoter in sense and antisense orientations. The sense construct p2S was produced by double digestion of pJR1 and pLF2 with Xba I and Sal I, and ligation of the pLF2 insert into pJR1. pLF2 was digested with Kpn I and Bam HI, the insert isolated and cloned into pJR1 to give the antisense construct p2AS. The orientation of the inserts in these contructs was verified by digestion with Eco RI or Hind III.

5.2.2 Transformation of *Agrobacterium tumefaciens*

The vectors p2S and p2AS were transferred from *E. coli* XL-1 Blue to *Agrobacterium tumefaciens* strain C58C1 by triparental mating with pRK2013 in *E. coli* DH5α. Two separate colonies each of *E. coli* XL-1 Blue containing either p2S or p2AS were used. Two streaks were taken from each of the four plates containing the three strains. These were sub-cultured to give four individual colonies of transformed *A. tumefaciens*; 2AS1-4, containing the antisense construct, and 2S1-4, containing the sense construct. Plasmid DNA was prepared from each of these colonies and Southern blots of the DNA show the presence of the pLF2 insert in each colony (see Fig. 5.2).

5.2.3 Transformation of *Arabidopsis thaliana*

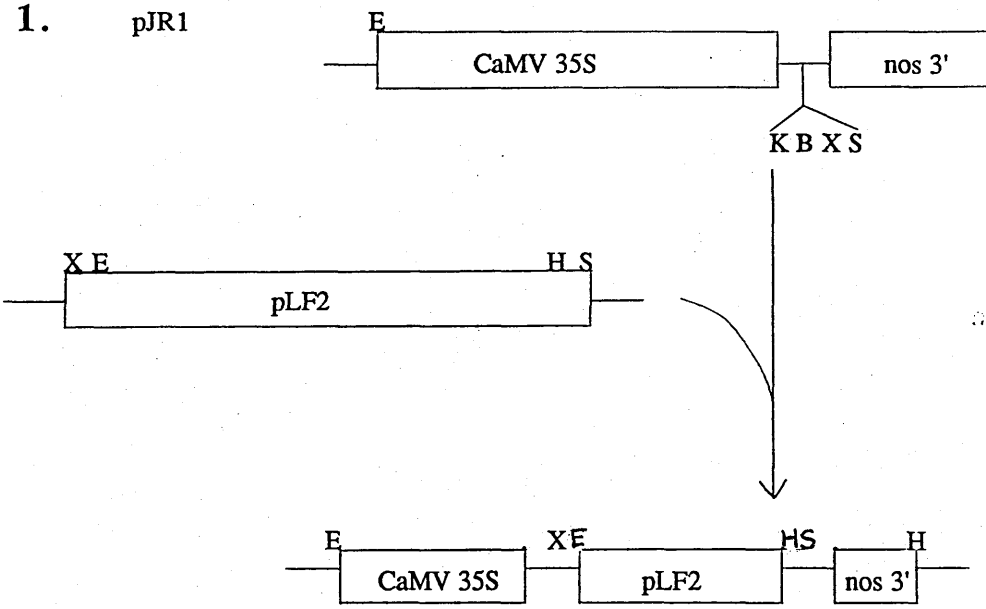
Root explants of *Arabidopsis thaliana* ecotype Landsberg erecta were transformed using the method of Valvekens et al. (1988). Three isolates of *A. tumefaciens* containing p2AS were used to transform root explants. Six primary

Fig. 5.1 Construction of the plasmids p2S and p2AS

1. The Xba I/Sal I fragment of pLF2 was cloned into pJR1 which had been digested with Xba I and Sal I to give the sense construct p2S.
2. The Kpn I/Bam HI fragment of pLF2 was cloned into the Kpn I and Bam HI sites of pJR1 to give the antisense construct p2AS.

B = Bam HI, E = Eco RI, H = Hind III, K = Kpn I, S = Sal I, X = Xba I

1. pJR1


E
H
CaMV 35S
nos 3'
K B X S
X E
H S
pLF2
E
X E
H S
H
CaMV 35S
pLF2
nos 3'

2. pJR1

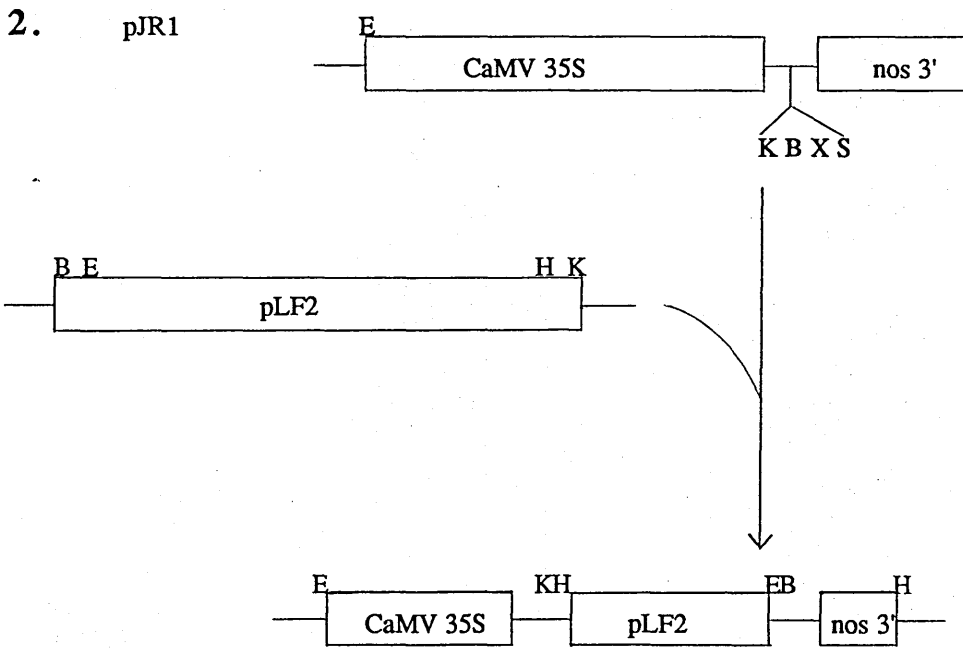
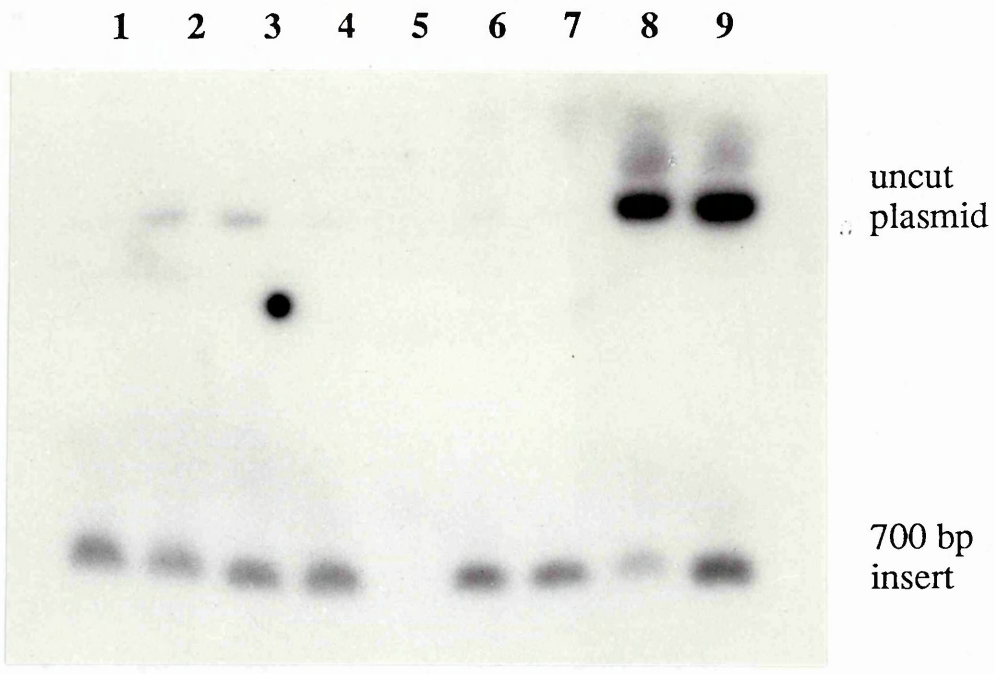

E
H
CaMV 35S
nos 3'
K B X S
B E
H K
pLF2
E
KH
EB
H
CaMV 35S
pLF2
nos 3'

Fig. 5.2 *Agrobacterium tumefaciens* transformed with p2AS and p2S

Plasmid DNA was prepared from cultures derived from 4 individual colonies each of *A. tumefaciens* transformed with p2AS or p2S. The DNA was digested with Kpn I/Bam HI (p2AS; lanes 1-4) or Xba I/Sal I (p2S; lanes 6-9) and separated on a 0.8% agarose gel. After denaturing and neutralising the gel, the DNA was blotted onto a Hybond N filter (Amersham). The filter was hybridised for 16 h with 1 x 10^5 cpm/ml of ^{32}P-labelled insert from pLF2 in 6 x SSC, 50% formamide at 42°C. The filter was washed to a final stringency of 0.1 x SSC, 0.1% SDS at 65°C and exposed overnight to X-Ray film with one intensifying screen. Lane 5 contains DNA size markers obtained from an Eco RI/Hind III digest of λ DNA.


1 2 3 4 5 6 7 8 9
uncut
plasmid
700 bp
insert

transformants were obtained, five being derived from roots co-cultivated with 2AS-1. Attempts were made to transform *Arabidopsis* roots with *A. tumefaciens* containing the sense consruct p2S. Only one plant could be regenerated and this produced very few seeds. Further attempts to produce plants containing the sense construct of pLF2 were unsuccessful and there was no further analysis of the p2S construct or transformant. Transformed plants containing the 2AS transgene were selfed and the seedlings (T2 generation) analysed. Seed was collected from individual T2 plants which had self-fertilised and this formed the T3 generation.

5.3 Expression of a reporter gene β-glucuronidase under the control of the CaMV 35S promoter in transformed *Arabidopsis* plants

Arabidopsis thaliana transformed with the plasmid pBI121.1 (Jefferson et al., 1987) was supplied by Dr. J.A. Jackson (Dept. of Botany, University of Glasgow). The pBI121.1 construct contains the β-glucuronidase gene cloned into the binary plasmid vector pBIN 19 behind an 800 bp fragment of the CaMV 35S promoter. *Arabidopsis* plants transformed with the 35S-GUS fusion were examined for GUS expression in the apex and floral buds. Histochemical localisation showed the presence of the enzyme in the stem and all the floral organs of the plant (see Fig. 5.3).

5.4 Analysis of progeny of antisense transformants

Seed from the T2 and T3 generations was grown on germination medium plus 50 μg/ml kanamycin. Table 5.1 details the phenotype of the T2 and T3 generations of transformants with respect to kanamycin resistance. Of the six transformants obtained, all but two plants did not show segregation of progeny into kanamycin

Fig. 5.3 Localisation of GUS in floral tissue

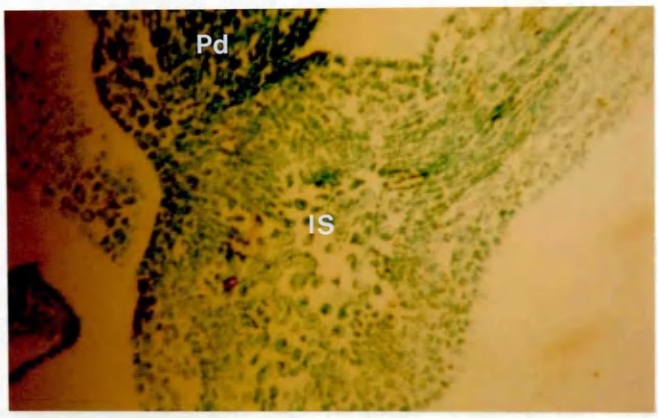
Arabidopsis thaliana transformed with a copy of pBI101.1, a construct containing the GUS gene with the CaMV 35S promoter, were harvested and fixed according to section 2.28. Floral buds and inflorescence stem were incubated for 16 h with a solution of X-Gluc at 37°C. The tissue was then washed, wax-embedded and sectioned according to sections 2.23.4 and 2.23.6. The sections were photographed using a Zeiss Photomicroscope II.

S: sepal, P: petal, A: anther, C: carpel, IS: inflorescence stem, Pd: pedicel

a. Transverse section (TS) through the inflorescence in the area of the lowest flowers

b. TS section through a flower bud

a



b

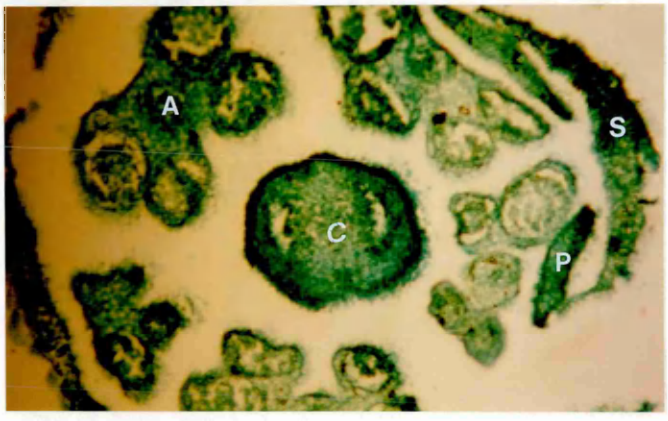


Table 5.1 Progeny of transformants

Primary transformed plants were selfed and the seeds collected. These seeds were plated out on germination medium plus 50 μg/ml kanamycin (GM K50). The number of kanamycin-resistant (Kan^R; healthy, green) and kanamycin-sensitive (Kan^S; pale, did not develop leaves) seedlings were counted and the Kan^R:Kan^S ratio calculated. Seed was collected from individual selfed plants of the T2 generation, plated out on GM K50 and the seedlings (T3 generation) analysed for kanamycin resistance in the same manner. Wild type seed was included on each plate as a control.

CONSTRUCT	PLANT	Kan^R	Kan^S	Kan^R:Kan^S
T2 generation				
2AS-1	1	ALL		
2AS-1	2	ALL		
2AS-1	3	39	8	4.9
2AS-1	4	ALL		
2AS-1	6	18	7	2.6
2AS-2	1	ALL		
T3 generation				
2AS-1	2/1	36	14	2.6
2AS-1	2/2	ALL		
2AS-1	2/3	60	17	3.5
2AS-1	2/4	60	22	2.7
2AS-1	2/5	35	12	2.9
2AS-1	2/6	54	21	2.6
2AS-1	2/7	ALL		
2AS-1	2/8	ALL		
2AS-1	2/9	ALL		
2AS-1	2/10	ALL		
2AS-1	3/1	28	10	2.8
2AS-1	3/2	11	1	11
2AS-1	3/3	11	3	3.7
2AS-1	3/4	ALL		
2AS-2	1/2	ALL		
2AS-2	1/3	ALL		

resistant and sensitive phenotypes. The progeny of the transformant 2AS-1/2 were all kanamycin resistant in the T2 generation but of seed collected from individual T2 plants, 5 out of 10 gave Kan^R:Kan^S ratios of about 3. The T2 progeny of the transformant 2AS-1/3 showed segregation of kanamycin resistance and 2 out of 4 T3 plants gave Kan^R:Kan^S ratios of about 3. The seedlings were examined visually during vegetative and floral development for abnormal organs or changes in the number or position of the floral organs. No visible phenotype could be seen among kanamycin resistant seedlings in either generation.

An attempt was made to determine whether the p2AS transgene was being expressed by isolating RNA from the leaves of 10 different transformed kanamycin resistant T3 plants. Genomic DNA was also prepared from seven T3 plants and digested with either Bam HI or Eco RI. No antisense RNA transcript could be detected using a strand-specific RNA probe transcribed from pLF2 and a band of spurious size was seen on the genomic blot using the same probe. It is thought that an incorrect fragment of the plasmid pLF2 was labelled during the production of the probe. Unfortunately there was insufficient time to repeat these experiments.

5.5 Sequencing of the cDNAs pLF2, pLF3A, pLF7P2 and pLF10B

Sequencing of the cDNAs was initiated using the primers which bind to regions flanking the multiple cloning site of pBluescript (see Fig. 5.4). Unfortunately there was insufficient time to complete the sequencing of these cDNAs.

5.5.1 Sequence of the cDNA pLF2

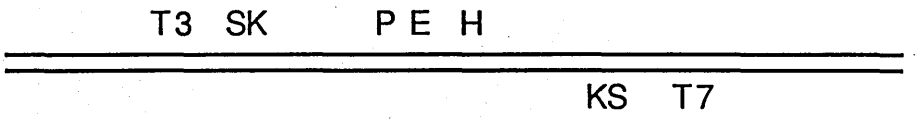
Fig. 5.4 Sequences available for the pLF cDNAs

This diagram shows the inserts of the cDNAs, pLF2, pLF3A, pLF7P2 and pLF10B. The length of each cDNA is given in base pairs and the restriction enzymes used to subclone each insert into pBluescript are indicated. For each cDNA the arrows indicate the region of DNA sequenced, the primer from which the sequence was initiated and the length in base pairs of the sequence obtained. The orientation of the primers and restriction enzyme sites in the multiple cloning region of pBluescript SK(-) is also shown.

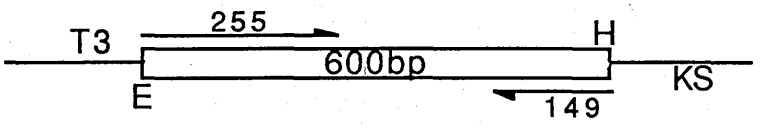
E: Eco RI, H: HindIII, P: Pst I

T3, T7, KS and SK are all sequencing primers for the pBluescript plasmids.

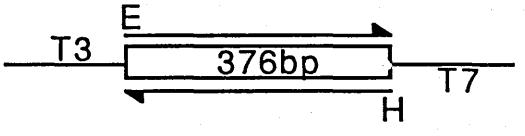
pBluescript



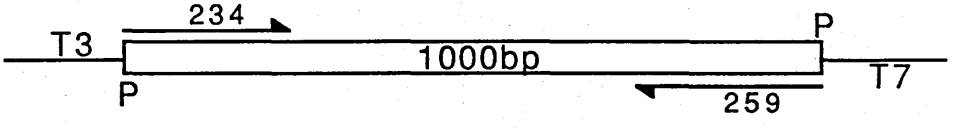
pLF2



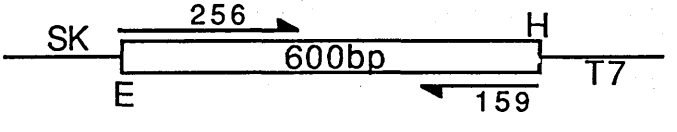
pLF3A



pLF7P2



pLF10B



The insert of pLF2 is approximately 600 bp as estimated on an agarose gel. The cDNA is not full length as it corresponds to an mRNA of 1.2 kb. The insert is cloned into the Eco RI and Hind III sites of pBluescript SK(-). Sequence was obtained from the 5' region of the insert for 255 bp which is shown in Fig. 5.5. Using the KS primer to initiate the sequence reaction, 149 bp of sequence was obtained from the 3' region which included a poly(A) tail of 24 residues.

5.5.2 Sequence of the cDNA pLF3A

The insert of the cDNA pLF3A is approximately 400 bp as estimated on an agarose gel and is cloned into the Eco RI and Hind III sites of pBluescript SK(-). Using the T3 and T7 primers both strands of the pLF3A insert were sequenced. The insert was found to be 376 bp. The insert did not contain a poly(A) tail. This was expected as the original phage clone, λLF3, contained an internal Hind III site within the insert, and pLF3A corresponds to the 5' region from this clone. A further subclone was obtained from λLF3, pLF3B, which was cloned into the Hind III site of pBluescript SK(-). It is presumed that pLF3B contains the 3' portion of λLF3, though this subclone has not been sequenced.

5.5.3 Sequence of the cDNA pLF7P2

The insert from pLF7P2 is a 1.0 kbp fragment from the 5' region of pLF7A, subcloned into the Pst I site of pBluescriptSK(-). The insert from pLF7P2 recognises a 1.2 kb RNA transcript. Using the T7 and T3 primers 259 bp and 234 bp of sequence was obtained. From *in situ* hybridisation experiments it is known that the sense strand of the pLF7P2 insert is generated from the T7 primer (shown in Fig. 5.5) and, therefore, sequence generated from this primer should correspond to the 5' region of the cDNA. The sequence from the 3' end of the insert did not contain a

Fig. 5.5 Partial DNA sequences of pLF2 and pLF7P2

The DNA sequences obtained from pLF2 and pLF7P2 are shown. The pLF2 sequence corresponds to the 5' region of the insert and was initiated using the T3 primer. The pLF7P2 sequence given shows the sense strand corresponding to the 5' region of the cDNA and was initiated using the T7 primer.

The pLF3 sequence is the subject of a patent application and is not shown here for that reason.

pLF2/T3

5' TTCAAGCCCG GTTTACCCGC CAAAATTCAA CAGAAGCCTA ATCGCCGTTC 50

AGGGTACNGT CTTCTGCAAA AGCTGCCAGT ACGCTTCTTC CGATTCACTC 100

ATTGGCGCCA AACCCGTTGA AGGTGCTGTT GTGAGACTAC TGTGTAAGAG 150

CAAGAAGAAC GTAGTCGCGA GACCAAGACG GACAAGAACG GATACTTCTT 200

GTTGTTNGGC CCGAAGACGG TGACAAACTA CGGTTCAGAG GTATCGTGTG 250

TATCT

pLF7P2/T7

5' CTGCAGTTGT CAGAACAAAG GATGTTTGCC TGAAAGCACT CACAGTACTT 50

CTTGAGACAT CCAGATTTCT TGCAGTGACA GCCTTTGTGG TGCTTCCCTA 100

ACATTACAAC TCCACCACCT CCCTGTTATC TCGTGGGTCG TGTGGACTGC 150

TAGCGATTTT AGGCCTGAAG GCATTAGGAT TGCGTTCGAG AGTTGATTCA 200

ATGGCTTCTC GTCTGGCGGG TTCATTGTCA ACGTTCGATT GAAACAGTTT 250

AAACAGTTC

poly(A) tail.

5.5.4 Sequence of the cDNA pLF10B

The insert from pLF10B is cloned into the Eco RI and Hind III sites of pBluescriptSK (-) and is approximately 600 bp as estimated on an agarose gel. Using the SK primer 256 bp of sequence was obtained and 159 bp of sequence was generated from the T7 primer, including a poly(A) tail of 20 residues. A putative polyadenylation signal was found 35 bp upstream from the poly(A) tail.

5.5.5 Analysis of the pLF sequences

The partial DNA sequences obtained from the pLF cDNAs were analysed using the UWGCG software. The DNA sequences were compared to the available data in the EMBL database using the WORDSEARCH program. No significant homology was found for the cDNAs pLF2, pLF3A and pLF10B. However a region of pLF7P2 showed some homology to a cotton late embryogenesis abundant (LEA) gene and an *Arabidopsis* chitinase gene. This homology was further investigated using the program BESTFIT and the sequence alignments are shown in Fig. 5.6. The amino acid sequences from the sequenced regions of pLF7P2, were derived using the programs MAP and EXTRACT. Comparison of the deduced pLF7P2 protein sequences with the Swissprot database yielded no significant homologies.

5.6 Discussion

The cDNA pLF2 was cloned into the expression vector pJR1 in an antisense orientation in an attempt to inhibit the action of the pLF2 gene product in *Arabidopsis*

Fig. 5.6 Comparison of the pLF7P2 sequence to that of a cotton Lea gene

The diagram shows the comparison of the partial sequence of pLF7P2, derived using the T3 primer, with the DNA sequence of a cotton 5B late emryogenesis abundant (LEA) gene for the seed protein B-11. Using the GCG software program BESTFIT homology was found over only 35 bases, with 63% identity. Sequence 1 is pLF7P2/T3 and sequence 2 is the cotton LEA B 11 gene.

```
1    62 TCCCACACCA TTATATTATT  TCATCCTTCT  AATTC 96
        |  |||| |   ||   |  | ||   ||   ||   ||  | |  ||    |
2   163 TGCCACTACA CTTTATATTT  TCTCCCCTAT  AAAAC 197
```

thaliana and so gain an idea of its function *in vivo*. The successful use of an antisense strategy to determine the function of a cryptic gene was reported by Hamilton et al. (1990). The synthesis of a transcript corresponding to the cDNA pTOM13 correlated with ethylene synthesis in ripening tomato fruit but the function of the protein product was unknown. Transformation of tomato plants with an antisense copy of pTOM13 gave plants whose ethylene synthesis was reduced in a gene dosage-dependent manner. Analysis of the transformants suggested the pTOM13 gene product was a subunit of ACC-oxidase.

The antisense experiment discussed in this chapter was unsuccessful. No visible phenotype could be seen in several kanamycin resistant transformants. The kanamycin resistant: kanamycin sensitive ratio of some of the primary transformants was greater than 3:1 indicating that there may be more than one transgene inserted in the genome. Some seedlings from the T3 generation did segregate with a 3:1 ratio but again not all. An initial attempt to analyse the number of transgenes in these transformants proved unsuccessful. This experiment should be repeated using several probes derived from different regions of the plasmid p2AS to determine the number of inserts and any possible rearrangements that may have occured during the integration process. The possibility has to be addressed that the down-regulation of the gene corresponding to pLF2 in *Arabidopsis* would not adversely affect the growth and development of the plant and not confer a mutant phenotype on the transformant.

The antisense copy of the pLF2 cDNA was placed under the control of the 35S promoter from the cauliflower mosaic virus. This promoter has been used to generate antisense transcripts of the polygalacturonase (PG) gene in tomato (Smith et al., 1988). While subdomains of the 35S promoter show tissue-specific expression, the domain used in the experiment described here should give expression in most tissues

including the shoot apex (Benfey et al., 1990). *Arabidopsis* plants transformed with a 35S-GUS fusion showed expression of GUS in the apex and floral buds (Fig. 5.3). While the exact site of transcription of the gene represented by pLF2 could not be determined by *in situ* hybridisation (see Ch. 4) the antisense transcripts should be present in the same cell-types as those expressing the pLF2 sense transcript. It is thought that the presence of the antisense transcript in the cells prior to the transcription of the target gene is important, although Mol et al. (1990) used a tissue-specific promoter has been used to give co-ordinate expression of an antisense chalcone synthase (CHS) gene with the endogenous CHS gene.

The experiment described here relied on the premise that there is sufficient homology between the *Brassica* and *Arabidopsis* genes to give an antisense effect. van der Krol et al. (1990) found that an antisense petunia CHS-A gene transformed into petunia gave down-regulation of both the CHS-A and CHS-J transcripts (86% homology). They also showed that tobacco plants containing the same construct as the petunia transformants exhibited down-regulation of chalcone synthase in the petals (van der Krol et al., 1988a). Hence, if sufficient homology is present between the LF2 genes of *A. thaliana* and *B. napus*, down-regulation could have been observed in the present experiment. That *Arabidopsis* and *Brassica* species have similar developmental processes at a genetic level has been demonstrated by Yanofsky et al. (1990) who have isolated 2 *agamous* homologues from *B. napus* and Medford et al. (1991) who isolated meristem-specific cDNAs from cauliflower and used these to isolate the corresponding genomic clones from *Arabidopsis*. The *meri* clones show similar patterns of expression in both species.

The pLF2 insert is not a full-length cDNA and corresponds to 600 bp of the 3' end of the transcript of length 1.2 kbp. There seems to be substantial variation in the

regions of genes which are most effective in producing an antisense effect. Mol et al. (1990) found that an antisense construct containing the 3' end of the CHS gene proved most effective in giving down-regulation of CHS transcript accumulation, while in tomato the 5' end of the polygalacturonase gene used in an antisense construct gave 99% inhibition of PG transcription (Smith et al. 1988). It has to be considered that the 3' portion of the pLF2 cDNA in an antisense construct may not be effective in reducing the transcription of the endogenous gene in *Arabidopsis*. It is also possible that the antisense transcript is unstable and is being rapidly degraded. There is evidence to suggest that some antisense templates are less stable than others (reviewed in van der Krol et al., 1988b) and that this instability may be overcome by using chimaeric constructs of coding regions and antisense sequences. An initial attempt to detect the antisense message in leaf tissue of transformed, kanamycin resistant plants generated from the study described in this thesis was unsuccessful.

With the exception of a single transformant, transformed plants could not be generated from root explants co-cultivated with *A. tumefaciens* containing the sense construct p2S. It is possible that this is due to the over-expression of pLF2 inhibiting the regeneration process in some way. A similar situation has been seen with regeneration of callus transformed with the *FLO* gene under the control of the CaMV 35S promoter (Elliot et al., 1991) where the presence of the *FLO* gene prevented regeneration of *Arabidopsis* plants from root explants. This problem can be surmounted by placing the target gene under the control of an inducible promoter such as a heat-shock promoter. The single transformant obtained was not further studied as any phenotypic effect which may be seen could not be considered representative.

Sequence analysis of several of the pLF cDNAs was initiated. None of the cDNAs discussed here is likely to be full length. However it was thought that, due

to the anomalies present in the cDNA library, full length clones would be difficult to obtain. Initial attempts at obtaining longer length cDNAs for pLF2 and pLF3 resulted in the isolation of phage clones whose inserts were greater than the estimated size of the RNA transcript. As the ultimate goal of the project was to obtain the genes corresponding to the pLF cDNAs from *Arabidopsis*, a further *B. napus* cDNA library was not constructed. Unfortunately there was insufficient time to complete the sequences of the partial cDNAs obtained. Analysis of the pLF2, pLF3A, and pLF10B sequences using the GCG software showed no significant homology between these sequences and those DNA sequences in the EMBL database. Although these pLF cDNAs showed open reading frames, it was thought premature to compare the presumed amino acid sequences derived from the partial DNA sequences of the pLF cDNAs, to those in the database, as the full coding region had not been obtained and any homology seen may not be significant. However, the partial sequence obtained for the cDNA pLF7P2 showed homology to both a cotton late embryogenesis abundant (LEA) gene and an *Arabidopsis* chitinase gene. This homology was at the DNA level and further examination using the BESTFIT program showed that the homology was limited to a short region of DNA. The amino acid sequences derived from the DNA sequence obtained showed no homology to either the *Arabidopsis* or cotton genes, when compared using the BESTFIT program. It would be more profitable to continue the sequence comparisons after the whole cDNA sequence is available.

Chapter 6: General discussion

A cDNA library was constructed from *Brassica napus* apical tissue with flower buds at an early stage of development. Several cDNAs were isolated from this library by differential screening, three of which (pLF2, pLF5 and pLF7) show specific temporal expression at the early stages of bud development. The gene corresponding to one of these cDNAs, pLF5, is also highly up-regulated upon cold treatment. Two further cDNAs (pLF3 and pLF10B) were isolated which were present at a high level in the mRNA population isolated from floral apical tissue, both of which show tissue-specific expression. The transcript represented by pLF3 is present in the epidermal cell layer of leaves, stems and sepals, and the LF10B transcript is restricted to the phloem tissue of the stem.

The LF5 gene is unique in that it is up-regulated during cold-induction as well as showing a specific pattern of developmental expression. The cultivar of *B. napus* used in this study was a winter variety which produces flower buds during cold temperatures, and induction of this gene occurs in the leaves and floral organs of *B. napus* when exposed to low temperatures. Many of the cold-regulated genes isolated to date show homology to the *rab*/dehydrin family of genes, which encode highly hydrophilic proteins. These proteins are believed to act as cryoprotectants in plants in times of water stress, high salt stress, seed desiccation and in freezing temperatures, when formation of ice crystals in cells reduces the available water (Thomashow, 1990). It is hard to envisage a role for a water stress protein in early flower development. The LF5 gene does not appear to be regulated by ABA in leaves (J. Jardine and G.I. Jenkins, unpublised results), so it is unlikely to be induced by this PGR as part of the floral induction process. The LF5 gene is also down-regulated by the time of silique development, so it could not have a role in seed desiccation. The

sequence of pLF5 is not yet available, so it is not known whether this gene belongs to the *rab*/dehydrin family. A complementary transcript is present in *Arabidopsis thaliana*, which shows increased expression in cold-induced tissue (J. Jardine and G.I. Jenkins, unpublised results), though the expression of this gene during flower development has not been examined.

An attempt to discern the role of pLF2 in *Arabidopsis thaliana* was made by generating transformed plants containing an antisense copy of the cDNA pLF2. This experiment was unsuccessful in that no visible phenotypic changes could be seen in these transgenic plants. This could be for a number of reasons which were discussed in section 5.5. A successful antisense RNA strategy relies on a number of factors; the stability of the RNA transcript, it's expression in the same cell-type as the sense transcript and at the same developmental stage, use of the correct region of the gene to give maximum inhibition of expression and generation of a detectable phenotype caused by down-regulation of this gene. One must also consider possible pleiotropic effects seen when using an antisense gene under a 'constitutive' promoter such as the CaMV 35S promoter. If the gene of interest is one of a multi-gene family of similar sequences, the antisense message may produce down-regulation of other members of the family giving phenotypic changes throughout the plant. Another reverse-genetics approach includes the design of ribozymes to specific RNA sequences to cause degradation of the mRNA (Haseloff and Gerlach, 1988). To my knowledge this approach has not yet been successfully adopted in a plant species.

Few genes have yet been isolated which have a specific role in flower development. The homeotic genes of *Arabidopsis* and *Antirrhinum* are comparatively well-studied (Coen and Meyerowitz, 1991), but these are obviously not the only controlling factors in flower development. Many genes have been isolated

which show specific expression in one of the floral organs. The flavonoid biosynthetic genes are an example, and the promoter sequence from one of these genes, encoding EPSPS, has been used to identify a DNA-binding protein which may act to modulate organ-specific and temporal expression of this gene (Takatsuji et al, 1992). However, the interaction between the homeotic genes and the genes which control expression of organ-specifc genes is unknown. Cell signalling systems in plants are poorly understood, in comparison to most animal systems, especially with regard to plant growth regulators. The study of floral mutants whose phenotype can be modulated by application of exogenous PGRs (e.g. the *stamenless-2* mutant of tomato; Sawhney and Greyson, 1973) implies a role for PGRs in floral organ specificity. However, this is difficult to examine using molecular genetics as little is known about how the PGRs act at a molecular level.

The genes discussed in this study, LF2, LF5 and LF7, and those isolated from *Sinapis* (Melzer et al., 1990), are the only examples presented thus far, which act during the floral transition and throughout early flower bud development, though their functions are as yet unknown. These genes are unlikely to be controlled by the homeotic genes, as they are expressed in the apex and up-regulated after floral induction. Two genes which were isolated from floral transition apices of tobacco were found to be expressed in the floral organs, chiefly in the petals and stamens (Kelly et al., 1990) and other genes isolated from the *Sinapis* floral transition library were expressed exclusively in the tapetum (Melzer et al., 1990). Studies such as these illustrate the importance of choosing the right tissue to make and screen cDNA libraries to obtain clones which show very specific temporal and spatial expression.

Differential screening is a very useful approach to isolate panels of cDNAs and hence genes, which show expression in a specific tissue or developmental stage.

Sequencing of the cDNAs and reverse genetics approaches can yield information about the function of the genes thus obtained. Differential screening may select for genes which have important roles in development but for which no mutant line exists. However if the estimates of the number of organ-specific mRNAs in tobacco are true for all plants (e.g. 11 000 anther-specific mRNAs; Kamalay and Goldberg, 1984) then most mRNAs will be of low abundance and these are unlikely to be isolated by a differential screen. Techniques such as 'cold-plaquing' (Scott et al., 1991) are useful to isolate organ-specific mRNAs of low abundance when the ratio of organ-specific mRNA to total mRNA is high. However when two tissues which are similar but at different stages in a developmental process, are being compared for differential gene expression it is more difficult to isolate low abundance sequences and it may be more profitable to make subtractive libraries. The disadvantage with this technique is that common sequences to the two differential stages are removed from the library, and thus genes which are present in, for example, the vegetative apex and up-regulated in response to the floral stimulus, will not be present in the resulting subtracted cDNA library. The use of PCR has allowed the construction of such libraries from very small amounts of tissue which may allow cloning of genes from floral apices, and an *Arabidopsis* apex-specific cDNA library is no longer an impossibility as it seemed at the outset of this project.

In 1987 a review by Meyerowitz suggested that *Arabidopsis thaliana* was a model organism for molecular biological studies. In particular it's small genome, lack of repeated seqences and high amount of single copy sequence make this plant an ideal species for chromosome walking to genes of interest. RFLP maps are available (Chang et al., 1988; Nam et al., 1989) and a physical map of the *Arabidopsis* genome is being constructed (Hauge et al., 1991). As yet there has been no report of a successful walk though several groups, working with *Arabidopsis*, are reported to

be near their particular genes e.g. *DET* 1 (Delaney et al., 1991), a gene controlling the de-etiolation response, *FG* (Putterill et al., 1991), a flowering time gene, and *ms1* (Fuller et al., 1991), a gene involved in male sterility. The construction of genomic libraries in yeast artificial cloning (YAC) vectors has made the process of chromosome walking easier as large fragments of DNA can be accommodated in these vectors (Grill and Somerville, 1991). However, progress using this approach has not proved as rapid as originally expected.

Of the floral mutants isolated and characterised by 1988 (Haughn and Somerville), only a few of the corresponding genes have been cloned. The genes isolated thus far have been isolated by either a T-DNA tagging (*AG*; Yanofsky et al., 1990) or a transposon tagging strategy (*FLO*; Coen et al., 1990). The progress of cloning genes involved in floral organ formation has been advanced by exploiting the conserved nature of this process between *Arabidopsis* and *Antirrhinum* where heterologous probing has led to the isolation of such genes as *AP3* from the *Antirrhinum* equivalent *DEF*, and *PLE* from the *Arabidopsis* equivalent *AG* (Coen and Meyerowitz, 1991). However many of the *Arabidopsis* floral mutants were generated by mutagenesis with ethane methylsulphonate which causes point mutations (e.g. *leafy* , Schultz and Haughn, 1991; *tfl-1*, Shannon and Meeks-Wagner, 1991) and the only way to obtain the corresponding genes is by chromosome walking. Therefore new strategies are being developed which allow the mutated gene to be easily isolated. These include creating deletion mutants in *Arabidopsis*, equivalent to the existing mutant lines, which allow the gene to be obtained by genomic subtraction (Straus and Ausubel, 1990), or generating mutant lines of *Arabidopsis* by DNA insertional mutagenesis. Tagged lines of *Arabidopsis thaliana* have been generated by Feldmann and Marks (1989) by transformation of seed with *Agrobacterium tumefaciens*. This procedure yields progeny with an average of 1-4 tandem T-DNAs inserted at random in the genome. Several of the mutants generated by the seed

infection/transformation process have been examined to determine the tagged gene (e.g.*agamous*, Yanofsky et al., 1990; *glabrous*, Marks and Feldman, 1989). However some of the mutants examined segregate with a ratio of less than 3:1 in the T3 population, which reduces the probability that the T-DNA insert will cosegregate with the mutation. Work on these lines suggests the presence of a somaclonal mutation in these transformants (Feldmann et al., 1991) and this has reduced the usefulness of this seed population. Several groups are developing transposon-tagging strategies in *Arabidopsis* (Balcells and Coupland, 1991; Lawson et al., 1991) and other species (e.g. Cardon et al., 1991). *Arabidopsis* has no active transposons in it's genome and the maize transposable elements *Activator* (*Ac*) and *Dissociator* (*Ds*) have been adapted as a two component system for use with this species by Coupland and co-workers, and several other groups (reviewed in Balcells et al., 1991). Transposon tagging systems have the advantage over T-DNA tagging strategies in that the genome contains only one insert, and avoids tissue-culture-induced mutations.

Successful tagging strategies rely on good screening procedures to detect genes of interest. One can not always rely on the production of a visible phenotype and the design of a screening strategy must take into account the ability to screen large numbers of plants easily, quickly and cheaply. The screening procedure must also be non-lethal, or allow the rescue of selected plants. In the case of plant hormone mutants King (1988) points out that the function of the hormone must be known in order to design effective screening procedures. Developmental mutants are relatively easy to detect, as the defects are often visible e.g. abnormal inflorescence (*pin-formed*, Okada et al., 1991; *leafy*, Schultz and Haughn, 1991) or increased number of flower parts (e.g. *agamous*, Yanofsky et al., 1990; *clavata*, Leyser and Furner, 1990). However in the case of metabolism mutants the screening procedure may

involve assaying many thousands of visibly similar plants for a mutation. For example, 12 mutations affecting lipid metabolism in *Arabidopsis* were isolated by measuring the fatty acid composition of 10 000 individual plants by gas chromatography (Somerville and Browse, 1991). Mutations have been identified in *Arabidopsis* which cover all aspects of the plant's lifecycle (Meyerowitz, 1989). These mutants can be used to further understand aspects of development which can be applied to other species, including crop plants.

The manipulation of floral induction and flower development has important commercial applications. The end product of the floral process, the seed, is often the harvested part of the plant and a better understanding of how and when a plant flowers may yield crop species with improved yield (Evans, 1987). In addition there is a large world market for cut flowers and there is a great potential for genetically manipulating flower colour and organ number to produce new and unusual phenotypes (Mol et al., 1989). Alterations in the control of floral induction could lead to the production of crop species with changed requirements for photoperiod, an important consideration in permitting the spread of crops to new regions and environments (Evans, 1987). For example, genetic manipulation of photoperiod in a species such as sugar cane may prevent the use of a costly light break system during the period of floral induction, which prevents flowering and increases the sugar yield (Evans, 1987).

Increased understanding of the process of flower development may lead to the application of this knowledge to the production of novel flowers for the ornamental and cut flower market. Classical breeding techniques have the disadvantage of a limited gene pool for a single species. Molecular 'flower breeding' can be used to engineer novel flower colour, alter plant morphology, and improve vase life and productivity (Mol et al., 1989). Advances have already been made in engineering

flower colour, for example, by placing an antisense copy of the flower pigmentation gene, chalcone synthase, into petunia to generate novel pigmentation patterns in the petals (van der Krol et al., 1988a). An alternative approach to producing a new flower colour includes alterations or additions to the flavonoid biosynthetic pathway, an example of which is the insertion of a maize dihydroflavonol 4-reductase into petunia to produce brick red flowers through the production of pelargonidin, a pigment not found in wild type petunia (Meyer et al., 1990). The cloning of floral homeotic genes in *Antirrhinum* and *Arabidopsis* makes the engineering of novel flower morphology a possibility. The *AGAMOUS* gene controls determinacy of the floral meristem (Yanofsky et al., 1990), and alterations in the function of this gene could produce flowers with increased numbers of organs. Delayed senescence in cut flowers is also a desirable characteristic. This process is known to involve the production of ethylene at the onset of senescence for example, in carnation (Cornish et al., 1991) and by the cloning of the genes of the ethylene biosynthetic pathway (e.g. aminocyclopropane carboxylic acid synthase; van der Straeten et al., 1990) it may be possible to extend the vase life of cut flowers by engineering lower levels of ethylene production, or flowers which do not respond to the ethylene stimulus.

Although desired characteristics may be transferred into the plant by molecular techniques, the grower also requires high yields. Improvements in the productivity of crop plants can be envisaged using molecular genetic techniques, although progress has been hindered by the inability to transform monocot plants such as wheat and maize using *Agrobacterium tumefaciens*, and alternative techniques have been developed such as microinjection of DNA, and bombardment of plant cells with DNA-coated microprojectiles (Cocking, 1990). Improvements in the productivity of crop species include resistance to pathogen attack and weed control by engineering herbicide resistance. Examples of this include transformation of plants with coat

protein genes from viruses to protect against viral infection (Nelson et al., 1990), and creating herbicide resistant plants by over-expression of EPSPS to produce glyphosate resistance (Klee et al., 1987), or introduction of bacterial genes into crop species which break down 2,4-dichlorophenoxyacetic acid (Llewellyn et al., 1990). Crops engineered in such a way are already undergoing field trials and should be marketed within this decade (Fraley, 1991).

Other methods of improving crop produtivity and usefulness involve engineering the plant to produce novels products in the seed or harvestable organ. A good example of this is *Brassica napus* (oilseed rape) where a number of desirable characteristics could be introduced into the genome to give an improved crop variety . These include altering the lipid profile of the seed, and producing smaller plants with a shorter flowering time to increase ease of harvest. *B. napus* produces a combination of oils in the seed including a high proportion of erucic acid, which is thought to increase the incidence of myocarditis (Somerville and Browse, 1991). Varieties have been produced by conventional breeding which have a lower level of erucic acid, and these are currently in use (e.g. the variety 'Cobra' used in this study), however the potential remains to further lower the level of, or eliminate, the erucic acid content of the oil by a molecular approach. The possibility also exists to produce novel types of lipid for use as industrial lubricants, detergents or plastics. Collectively plants have the potential to produce a lipid of any chain length with any type of modification. Several mutations in fatty acid metabolism have been identified in *Arabidopsis thaliana* and cloning of the genes at these loci will facilitate attempts to produce new lipid products in oilseed crops (Somerville and Browse, 1991). The oil produced by the seed of *B. napus* is not the only commercial end product. The remains of the seed after the oil has been extracted are used for animal feed. However this feed contains a high level of glucosinolate which can be toxic to animals when

broken down by the enzyme myrosinase present in the seed (Kimber, 1981). Varieties of oilseed rape with low glucosinolate levels are available but these are less resistant to pathogen attack, and an engineered variety with low or no myrosinase activity but resistant to pathogens would be more profitable to farmers. Other desirable seed characteristics which could be introduced into *B. napus* include a thinner seed coat and less tannin to improve the colour and taste of the oil, and a pod which is less resistant to shattering at harvest (Ward et al., 1985). A seed pod with an increased number of valves would be more resistant to shatter, and this trait could be introduced by a mutation similar to the *clavata* mutation of *Arabidopsis*.

Altered floral structure could also be beneficial in *B. napus*. The highly coloured flowers of *B. napus* reflect or absorb up to 60% of the light reaching the plant and this restricts photosynthesis in the leaves, leading to lower yields (Ward et al., 1985). A variety of *B. napus* with fewer or no petals would increase the amount of light reaching the leaves and ultimately the yield of seed from the plant, and a variety of spring oilseed rape, *apetalous*, has already been bred which lacks petals (Thompson and Hughes, 1986). Further requirements which would lead to an improved crop are a shorter flowering period for easy harvesting and a smaller plant to allow better light penetration to the leaves and easier harvesting (Ward et al., 1985). However control of plant height and flowering time are complex traits involving several genes, and these may not easily be altered without affecting other aspects of development. Obtaining promoters from genes which show organ-specific or temporal expression in *B. napus* is an important step in attempting to introduce desirable traits into this species. For example, the epidermal-specific promoter from the LF3 gene isolated in this project could be used, for example, to target expression of defence response to leaves.

The molecular techniques discussed in this thesis can thus be used in the

development of a wide range of crop species with improved commercial characteristics. However this will not be possible without further knowledge of the fundamental processes of plant development. Model plants such as *Arabidopsis thaliana* will allow significant advances to be made in our understanding of how a plant develops and interacts with the environment, and these studies will increase both our understanding of flower development and the application of this knowledge to crop species such as *B. napus*.

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