Development of Techniques for the Isolation of Genes Expressed in the Shoot Apex of Sugar Beet (*Beta vulgaris*).

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

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January 1993
Re-submitted February 1994

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Acknowledgements

I must first thank both Dr Gareth Jenkins of the Department of Biochemistry, the University of Glasgow, for his help and support during this project, and Dr Wolfgang Schuch of the Department of Exploratory Plant Sciences, ICI, Jealotts Hill, for providing financial support and allowing me to use the facilities of his department. I would also like to thank Prof. Richard Cogdell for allowing me to use the facilities of the Botany Department and the S.E.R.C. for providing the funding for this project.

In addition I am indebted to Dr Keith Edwards for his patience and advice in development of the PCR library procedure, to all of the members of lab B4a of this department, to those of the Department of Botany who helped to make the course of this study run smooth despite the many setbacks and to NormanTait for photographic work.

Finally I would like to thank my parents for their constant support and encouragement.

List of Abbreviations

A_{260}	Absorbance at a wavelength of 260 nm
A_{280}	Absorbance at a wavelength of 280 nm
A_{600}	Absorbance at a wavelength of 600 nm
ABA	Abscisic acid
AMV	Avian myeloblastosis virus
ATT	Aurintricarboxylic acid
BSA	Bovine serum albumin
CCC	2-Chloroethyltrimethylammonium chloride
cDNA	Complementary deoxyribonucleic acid
COR	Cold regulated
cpm	Counts per minute
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphopsphate
DEPC	Diethyl pyrocarbonate
dGTP	2'-Deoxyguanosine 5'-triphosphate
DN	Day neutral
DNA	Deoxyribonucleic acid
DNP	Day neutral plant
dNTP	2'-Deoxynucleoside 5'-triphosphate
DTT	Dithiothreitol
dTTP	2'-Deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminetetra-acetic acid
5-FU	5-Fluoro uracil

GA	Gibberellic acid
GAs	Gibberellins
GA_n	Specific numbered giberellin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IPTG	Isopropylthio-β-D-galactoside
kb	Kilo base
kbp	Kilo base pairs
kD	Kilo daltons
LD	Long day
LDP	Long day plant
µCi	Microcuries
µg	Microgrammes
µM	Micromolar
M	Molar
mg	Milligrammes
min	Minute
mm	Millimetres
MOPS	3-[N-Morpholino] propane sulphonic acid
mRNA	Messenger ribonucleic acid
NaPP	Sodium pyrophosphate
NB	Night break
ng	Nanogrammes
nm	Nanometres
nM	Nanomolar
PCR	Polymerase chain reaction

Pfu	Plaque forming units
pg	Picogrammes
Poly (A^+) RNA	mRNAs carrying polyadenylated sequences at their 3' termini
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Short day
sddH_2O	Sterile double distilled water
SDP	Short day plant
SDS	Sodium dodecyl sulphate
sec	Second
SLDP	Short long day plant
SM	100 mM NaCl, 10 mM $MgSO_4$,
	50 mM Tris-HCl (pH 7.5), 0.01% gelatin
SSC	Standard saline citrate
SSPE	Standard saline phosphate + EDTA
TCA	Trichloroacetic acid
TE	10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH8.0)
Tris	Tris [hydroxymethyl] aminomethane
2-TU	2-Thio uracil
v/v	Volume per unit volume
w/v	Weight per unit volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Summary

Flowering plants are the most complex and highly evolved organisms within the plant kingdom. The process of flowering is regulated by a combination of intrinsic and environmental factors which signal the switch from vegetative to floral development. The molecular mechanisms involved in this process are poorly understood, and although there have been many models put forward to try to explain the way in which the environment interacts with these intrinsic factors to bring about floral induction at the shoot apex, no one model has been shown to satisfy every case.

The sugar beet plant (*Beta vulgaris*) is commercially important due to its ability to store a large concentration of sucrose in its expanded tap root (the beet). *Beta vulgaris* exists in two growth forms, annual and biennial. Both are obligate long day plants (LDP) but the biennial requires an additional period of cold treatment (vernalization) prior to exposure to the long days. A substantial amount of the sugar beet crop is lost each year due to a phenomenon referred to as bolting. Here the biennial beet flowers in its first growing season as a consequence of exposure to low temperature early in that growing season and, therefore, loses its supply of sucrose to the formation of the flower stalk. There are also several biennial genotypes of sugar beet that require very specific environmental growth conditions for the induction of flowering. Generating seed from these plants, and thus maintaining these lines of biennial beets, can be very difficult. To understand, and be able to control, flowering in sugar beet crops at the molecular level would, therefore, be of advantage to both the sugar beet grower and to the breeder.

It is known that the annual beet plant possesses a gene, B, which confers annuality upon the beet plants. This gene shows almost complete Mendelian dominance over the gene for bienniality. Since the biennial requires vernalization to induce flowering it is possible that

the vernalization process induces changes within the biennial that allow the expression of this B gene, or some other gene specific to the biennial plant. Alternatively the biennial may not contain a functional B gene product. The process of vernalization is known to be perceived at the shoot apex. Hence, in this study the shoot apices of annual sugar beet plants that had experienced non-floral-inductive conditions (short days, 20°C) were isolated to look for genes expressed specifically in the shoot apex, and possibly involved in the process of vernalization.

To do this the Polymerase Chain Reaction (PCR) was employed to amplify cDNA copies of transcripts expressed in such a small amount of tissue. To develop the techniques required for the cloning of PCR amplified cDNA the more abundant leaf RNA was used. Double stranded cDNA was prepared using total RNA, thus eliminating the step of enriching for poly (A^+)RNA. This did not appear to affect the final quality of the cDNA libraries produced, as a ribosomal DNA clone hybridised at a very low frequency to the *Beta vulgaris* cDNA clones studied. The screening of the leaf library with a light harvesting complex chlorophyll *a/b* binding protein (LHCP) cDNA clone demonstrated that the leaf PCR amplified library was of plant tissue, and the LHCP clone was represented at a frequency within the expected order of magnitude. A number (35) of annual apices were isolated and a small amount of RNA extracted from them. This RNA was treated in the same manner as the leaf RNA and a PCR amplified apical meristem cDNA library was successfully prepared from this material. The frequency of LHCP clones within this library was approximately 10 fold less than that of the leaf library. Thus, the apical library did contain clones of plant origin, and the frequency of the LHCP clones was again of the expected order of magnitude. Therefore, the apical library was differentially screened to look for clones representing apex-specific transcripts. On secondary screening a number of clones several putative positives were isolated. However, the most promising of these putative apex-

specific clones was shown to be most highly expressed in leaf tissue, as shown by hybridisation to a northern blot. Subsequently, over 200 'cold' plaques were isolated to explore the possibility that the apex-specific mRNAs may be present at too low a frequency to be detected by differential screening. However, none of the 'cold' plaques isolated was seen to be apex-specific on first screening. It was, therefore, decided to attempt to eliminate all cDNA species common to the apex and leaf cDNA populations using a subtraction procedure. This proved a relatively simple procedure to develop, but it did not prove possible to obtain a subtracted probe that contained sequences hybridising only to an apical cDNA probe. Therefore, using this technique it was not possible to isolate apex-specific cDNA clones. There may be several reasons for this result. It is unlikely that the number of cDNA clones screened was too small for the isolation of apex-specific cDNA clones, but this possibility must not be ignored. The amplified apical cDNA may have contained apex-specific cDNA represented at too low a frequency to be detected by the methods used in this study. They may also not have been present in the small sample of cDNA cloned into the vector, and would therefore never have been isolated. Finally it is possible that the apical tissue of *Beta vulgaris* does not possess any mRNA species specific to itself, and therefore it would never have been possible to isolate a cDNA clone representing such a mRNA from this library.

CHAPTER 1

INTRODUCTION

1.1. General Introduction

It has long been the wish of many agronomists to introduce desirable traits into species of economic importance. Since the earliest farming practices began the realisation that man could manipulate species to his own advantage has given agronomists the incentive to break down the barriers between species, and to select for novel combinations of desired characters.

Conventional breeding programmes, though still essential to the improvement of certain crop species, are dogged by factors such as the long generation times of plants, the limited variability of genetic information, and the fact that recombination is random. Hence, the isolation of new varieties requires selection on a large scale. Therefore, one major problem in using interspecific hybridisation in plant breeding programmes is the low probability of obtaining in one individual the desired genetic information from the parental species. Although sexual hybrids have not been pursued to their limits, e.g. African violet, and the miracle rices IR8 and IR36 (Cocking, 1990), the intervention of more sophisticated genetic engineering techniques whereby novel genetic information is directly introduced into the desired species, does have many advantages over conventional breeding programmes. Genetic engineering offers more direct changes in a shorter period of time. The genetic information transferred may be totally novel, and to a large extent the process is non-random.

The introduction of genes for resistance to viruses, insects, and herbicides, has been successfully achieved using the tools of genetic engineering (Newbury *et al.*, 1989, De Block *et al.*, 1987). These advances have been achieved in a much shorter time, and with much greater efficiency than would have been possible using conventional techniques. As more problems are overcome by the use of molecular biology, greater, and more complex challenges are put before the molecular biologist. The science of genetic engineering to date has focussed on the introduction of monogenic traits. The problem faced by the genetic engineer now is one of introducing multigenic traits into new host plants. One example where advances are being made in this area is that of the nitrogen fixation process. Most commonly this process occurs with the association of leguminous plants and *Rhizobium* bacteria. Genetic information from both plant and bacterium is essential for the development of nitrogen fixing root nodules (Sprent, 1989). A degree of success has been obtained in this field (Al-Mallah *et al.*, 1989), but there is still a long way to go before multigenic traits may be introduced into new hosts as easily as single genes.

One important issue for crop improvement is a better understanding of how environmental stresses (such as light, temperature, and humidity) affect the development of plant species (Hughes and Pearce, 1988). Light and temperature in particular, are known to affect the reproductive development of many flowering plant species and, as breeding programmes are hindered in many cases by a long generation time, the ability to control flowering in plant species is of great importance to the breeder. Therefore the study of flowering in plants plays a key role in the acquisition of knowledge important to the improvement of crop species.

1.2 Flowering

The development of flowering plants depends on meristems, the groups of dividing cells that are the source of new plant structures. Both the root and shoot apical meristems are present in the developing seed as the radicle and plumule respectively. The shoot apical meristem gives rise to a proliferation of vegetative leaf and stem tissue until intrinsic or environmental factors signal the meristem to begin floral development. Although the process of flowering is complex and covered by a large area of research, it can be divided into three major phases:

1) Induction, the exposure to favourable conditions for flowering which result in the production of a floral stimulus.

2) Evocation, synonymous with initiation, and described as the events occurring at the shoot apex as a result of the arrival of the floral stimulus from the leaf, committing it to develop as a floral meristem.

3) Morphogenesis, the development of differentiated floral organs.

The vegetative shoot meristem is organised into several zones containing cells with distinct histological characteristics and fates. By contrast the floral meristem contains only two distinct zones, designated the core and the mantle (Sussex, 1989). A major consequence of floral induction is the loss of vegetative meristem zones, and the concentration of floral meristem cells into the region of the mantle. The molecular and cellular events which occur during this meristematic reorganisation are not clearly understood, and therefore a vast amount of energy is being channelled into research on this subject.

In general floral meristems develop in whorls, or helical rows, from meristematic

cells in a progressive order, i.e. sepals, petals, stamens, and carpels or pistils. Experimental manipulation of the floral meristem has shown that there is a progressive and generally irreversible commitment of the primordia to develop into specific organ systems (Steves and Sussex, 1989). At early stages of flower development the floral meristem is able to give rise to all floral organ systems. However, as flower development proceeds, primordia are committed to differentiate into specific organ systems. The molecular events required for the specification of rows of floral meristem cells into specific organ system primordia are not yet known, but an increasing number of floral homeotic mutants and genes are being isolated and are beginning to provide new insights into this process.

1.3 Factors Affecting Flowering

1.3.1 Photoperiod

The effects of changes in daylength (photoperiod) upon the flowering of plants have been studied from as early as the middle of the nineteenth century. Sachs (1863, 1866, cited in Murneek and Whyte, 1948) showed an interest in the process of floral induction, but it was not until the early twentieth century that a French botanist by the name of Turnois established the association of the relative period of light and dark with the ability of plants to flower. More extensive studies by Garner and Allard (1920) with Maryland Mammoth variety of tobacco (*Nicotiana tobaccum*) and soybean (*Glycine max*) established the need for daylengths of less than a critical maximum number of hours to allow the development of flowering in these species. Plant species with such a

requirement are referred to as short day plants (SDP). Soon after this discovery it became apparent that many plants display a photoperiodic requirement to flower and a number of different categories were formed to accommodate them. Plants requiring more than a critical minimum number of daylight hours to induce flowering (e.g. *Hyoscyamus niger*, *Lolium temulentum*) are referred to as long day plants (LDP). A few species assumed to be SDP were later observed to have a dual photoperiodic requirement in which a period of short days (SD) did not initiate flowering unless it was followed by a period of long days (LD). These species are referred to as short long day plants (SLDP). Conversely it is true that some plant species require exposure to LD prior to exposure to SD for flower development to continue. There are, of course, plant species that will flower irrespective of the photoperiodic conditions. These are known as day neutral plants (DNP). There is a degree of overlap in the photoperiodic requirements of different classes of plants. For example the SDP *Dactylis glomerata* will flower when exposed only to daylengths of 14 h, whereas the LDP *Hyoscyamus niger* will flower in a photoperiod of as little as 12 h.

Most of the plant species already mentioned are of the obligate photoperiodic group of plants, having an absolute requirement for favourable photoperiodic conditions to initiate flowering. Many other plant species will display an enhanced ability to flower under SDs or LDs, but given time they would eventually flower under non-inductive conditions. These are known as facultative photoperiodic plants and include such species as *Digitalis purpurea*, *Dianthus barbatus*, *Cherianthus cheiri*, and *Teucrium scorodonia* (Vince-Prue, 1975). It was soon established that the length of the light period was less critical than the length of the exposure to darkness. For example in the SDP *Pharbitis nil*, induction occurs when a single period of darkness longer than a critical minimum duration is interposed into continuous light, thus establishing that in common with most SDP elapsed time in darkness is the critical factor for floral induction. However,

exposure to light is not unnecessary but is an integral part of the overall mechanism.

1.3.2 Perception of Photoperiod.

Experimental evidence from several plant species indicates that the daylength requirement of photoperiodically induced plants is perceived by the leaf tissue (Vince-Prue, 1975). For many years the sensitivity of leaves to photoperiod was suggested to increase as the leaves expanded until they reached full size, gradually decreasing as they senesce. Work by Moskov (1936) on *Chrysanthemum*; Borthwick and Parker (1938 a) on Biloxi soybean; and Naylor (1941) with *Xanthium* indicated that in these SDPs the first expanded leaf, rather than the mature expanded leaves, showed the greatest sensitivity to photoperiodic induction. This led Khudairi and Hamner (1954) to assess the relative sensitivity of leaves of different ages from the SDP *Xanthium* to photoperiodic induction. They concluded that the sensitivity of the *Xanthium* leaves to photoperiodic induction increases with age of the expanding leaves reaching a maximum when they are half expanded. Sensitivity then decreases with an increase in age of the expanding leaves. This viewpoint was reinforced by Salisbury (1955). However, Zeevart (1958) showed that the LDP *Perilla* retained sensitivity in its leaves for a considerable time after full expansion, and suggested that the position of the leaves on the plant was of greater importance.The LDP *Lolium tumulentum* may also retain sensitivity within its fully expanded leaves as the response of plants with only their basal leaves remaining was similar to that of young plants with only a few young leaves remaining. This observation overlooks the fact that plant age plays a major role in sensitivity of leaves to photoperiod (Borthwick and Parker 1938a; Moskov 1936). Therefore, in considering the response to

photoperiod of leaves of different ages, it is essential to use plants of the same age and not to introduce new variables which may complicate interpretation of the experiment in hand.

The work of Gressel *et al.* (1980) demonstrated using fibre optics that the apical tissues may also perceive inductive photoperiods. However, the light perceived by the apex is believed only to modify the responsiveness of the apex to the arrival of the floral stimulus. Therefore the apical meristem is not believed to influence the production of the floral stimulus under inductive conditions directly, but to alter its responsiveness to the stimulus produced. Similar results have been obtained by Baldev (1959) with exposed apical buds of *Cuscuta*. It is possible, therefore, that tissue containing rapidly dividing cells plays a major role in the perception of photoperiod. This does not rule out all other tissue types as being able to induce flowering in plant species, and Nitsch (cited in Wareing and Phillips, 1978) has shown stem tissue alone is able to induce flowering in cultured explants of *Plumbago indica*. However, this is an exception to the rule, based on extensive evidence, that young unexpanded leaves are the most sensitive to photoinductive conditions.

1.3.3 Evidence From Grafting Experiments for a Model of Photoperiodic Floral Induction.

It was Sachs who, as early as 1882, is reported to have postulated the occurrence of flower producing substances (Blüstoffe) formed in the leaves and translocated to the meristematic regions (cited in Jaffe *et al.*, 1987). Chailakhyan later (1938) obtained evidence that a floral inductive agent was synthesised in the leaves under favourable photoperiodic conditions. He found that leaves from induced *Perilla* plants grafted onto

uninduced plants caused the latter to flower. The agent was assumed to be hormonal in nature and was termed florigen. Gardner and Loomis (1953) obtained evidence for the presence of a floral inductive hormone with their work on the SDP *Dactylis glomerata*, and stated that flowering was induced by a chemical or hormonal differentiation in response to favourable conditions. Because an induced SDP will stimulate a non-induced LDP it has been suggested that the floral stimulus is identical in the two types of plant (Zeevart, 1976). This theory of a flower-inducing hormone led to the belief that inhibitors of flowering may exist, and it is the balance of the agonist and antagonist within the shoot apical meristem which determines the status of the plant, either vegetative or floral (Hamner, 1940). Earlier work by Borthwick and Parker (1938b) on some SDPs had also implied that inhibitors exist within the leaves of the plant. They used plants with two branches of nearly equal size, one was exposed to SDs whilst the other was maintained in LDs. The donor branch was reintroduced to LDs and it was observed that the receptor branch did not flower unless it was defoliated. The belief here is that the presence of the leaves on the receptor stalk supplying photosynthates to the shoot apex dilutes the effect of the stimulus arriving from the donor stalk. Withrow *et al.* (1943) reported that non-induced leaves positioned between the induced leaves and the apex have an inhibitory effect in spinach. Lang *et al.* (1952) reported that *Hyoscyamus niger* and *Chenopodium amaraticolor* will flower in non-inductive conditions if all the leaves are continually removed. Later work by Lang (1965) suggests that an inhibitory graft transmissible agent, termed antiflorigen, is present in a variety of photoperiodic and non-photoperiodic plants, and is not specific in a taxonomic sense as scions from non-induced *Nicotiana sylvestris* and *Hyoscyamus niger* are able to inhibit flowering in *Nicotiana tobaccum*. There is also evidence from work with mutants of pea (*Pisum sativum*) that genes which code for inhibitors of flowering exist in this species (Reid, 1986). To date, however, nobody has

succeeded in isolating or characterising the proposed hormones, and so alternative models have been suggested by several workers to accommodate the requirements of the control of flowering.

1.3.4 Alternative Hypotheses for the Control of Floral Induction.

It has been suggested that the translocation of such a specific hormone as "florigen" by mass flow with the photosynthetic assimilates would be the most inefficient way of having it arrive at the shoot apex, since very few molecules would ever arrive at their destination (McDaniel, 1985). Considering the many different types of plant responses it seems unlikely that a single mechanism will control flowering in all plants. This has led people to look in other directions for a suitable hypothesis. Evidence from work by Sachs and Hackett (1983) on *Xanthium* and *Nicotiana sylvestris* of the need for transport of the photoperiodic stimulus to the roots led to the belief that something more complex was in operation in the stimulation of flowering. Therefore the nutrient diversion hypothesis was put forward postulating that induction of flowering resulted from modifying the source-sink relationship within the plant such that the shoot apical meristem receives a higher concentration of assimilates than under normal conditions. The inhibitory effect of non-induced leaves may be interpreted on the basis that the leaves provide an alternative source of assimilates (Lang, 1965; Zeevart *et al.*, 1977). Non-induced leaves close to the apical bud have been shown to prevent flowering, but removal results in flower formation. The use of $^{14}CO_2$ has shown that assimilates accumulate in the leaves when present (Zeevart, 1976), suggesting that the presence of

the leaves close to the apical meristem may affect the ability of the plant to flower by the retention of necessary assimilates. The fact that starch levels in leaves grown under SDs are 2-3 times higher than under LDs, and that isolated shoot tips or embryos may be induced to flower only when an external supply of sugars is provided (Atherton *et al.*, 1987), indicates the need for assimilates in the developing tissue prior to floral induction.

1.4.2 Application of a variety of chemicals, including plant growth regulators and carbohydrates, promotes flowering. This led Bernier (1988) to propose the multifactorial hypothesis of floral induction. This hypothesis takes into account the fact that several factors are involved in the control of flowering, promotors and inhibitors. Floral induction is believed to take place only when all factors required are present in the shoot apical meristem at the appropriate concentrations and at the right times. Martinez - Zapater and Sommerville (1990) argue that differences in phenotypes of various *Arabidopsis* mutants suggest there are several factors involved in the control of flowering, and the effect of one of these factors can be offset in many cases by the action of others. This is in agreement with the belief of Bernier and his co-workers. Thus, there are several hypotheses available which attempt to explain floral induction, but further experimental evidence is required before it will be clear which is most likely to be correct.

1.4 The Effects of Temperature

1.4.1 Introduction

Photoperiodic responses cannot be considered in isolation from temperature,

because it is rare to find a plant in which the daylength response is wholly independent of temperature. Many plants which are day neutral (DN) at one temperature are sensitive to daylength at another. Therefore the effects of temperature on flowering plants have been studied extensively (Chroboczek, 1934; Murneek and Whyte, 1948; Lang, 1965).

1.4.2 Vernalization.

The requirement for certain varieties of plants to be exposed to cool temperatures to allow the induction or promotion of flowering was termed by Lysenko as vernalization in 1928 (cited in Murneek and Whyte, 1948). This term originates from the Latin for spring (*vernum*) and relates to the action of low temperatures converting winter varieties into spring varieties (Chouard, 1960). As early as 1857 the effects of low temperatures upon the initiation of flowering were noted by Kilppart whilst working on winter wheat (cited in Murneek and Whyte, 1948). He stated that to convert winter wheat to spring wheat you need only allow the winter wheat to germinate slightly and maintain it at near freezing temperatures until it is sown out in the spring. The realisation of the importance of the near freezing temperatures to induce flowering, and not just to suspend the plants in a state of readiness to germinate, was not realised until the early twentieth century. Gassner (1918) exposed plants of Pektus winter rye, and Pektus spring rye to temperatures of 1-2^{o}C, 5-6^{o}C, 12^{o}C and 24^{o}C. He noticed that flowering in the spring rye was not influenced by temperature, whereas the winter rye flowered most successfully if it had been exposed to 1-2^{o}C. Flowering at 5-6^{o}C only occurred if the plants were removed from the constraints of the greenhouse at the end of March during the cold of the early spring, and thus experienced continued cold treatment at temperatures below that to which they had been exposed in the greenhouse. Winter rye

plants grown under conditions in which they never perceived temperatures below 12°C showed little, if any, floral induction. Since that time many other species have been observed to require vernalizing temperatures as an obligatory step before flowering can be initiated (e.g. *Beta vulgaris*, *Hordeum vulgare* and *Hyoscyamus niger*).

The process of vernalization is, like photoinduction, not a simple one. It has been observed that some plant varieties which do not have an obligate requirement for vernalization to flower, will flower in a shorter time if they are exposed to vernalizing conditions. Those plants which show an obligate requirement for vernalization to initiate flowering will flower at a faster rate the longer the cold period and the cooler the temperature. Certain species may posses both facultative and obligate vernalization requiring genotypes within them. Some species may be vernalized after a lengthy period of exposure to temperatures as high as 15°C. Above this temperature they will not flower unless other important factors are favourable (e.g. light, water, and nutrients). In general, however, temperatures between 2-10°C are considered best for vernalization as these low temperatures are not low enough to cause freezing damage to the plant tissue but have been shown to be highly successful in the induction of flowering in many plant species, provided an adequate supply of O_2 and carbohydrates is available at the shoot apex (Chouard, 1960). After vernalization is completed the differentiation of flower initials does not generally occur until the plant is later returned to higher temperatures, and in many cases a particular photoperiodic regime (Vince-Prue, 1975).

1.4.3 Association of Vernalization with Photoperiod

The cold requirement for flower formation is often linked with a particular photoperiodic condition. Most frequently it is the association of LD with a vernalization

requirement (Vince-Prue,1975). However, vernalization in LD is ineffective in inducing flower formation unless the LD are perceived during the latter part of the vernalization period (Magara, 1964). The effective temperature for vernalization under these conditions is increased in species such as sugar beet.

Exposure to SD after vernalization will have little or no effect on the vernalized state of the plant unless the SD conditions are maintained for an extensive period of time. Even as many as 200 SD after vernalization of the LDP *Hyoscyamus niger* has no effect on the vernalized state of the plant (Bernier *et al.*, 1981). Therefore, the temperature effect on the growing tip is a self perpetuating physiological change. All the tillers of vernalized winter rye will flower when exposed to the right photoperiodic conditions, even though they were not formed prior to the cold period (Bernier *et al.*, 1981). However, the tillers must have developed from tissue that was exposed to the cold, and they are, therefore, able to respond to inductive daylength conditions by flowering.

The vernalization requirement of many plants may be replaced, or modified, by exposure to non-inductive photoperiodic conditions. SD may substitute partly or wholly for the low temperatures in some LDP. Wellensiek (1958) showed that certain plants became sensitive to LD one month sooner when exposed to SD than they did when exposed to low temperature. Whilst the minimum cold requirement decreased with age, the SD requirement remained constant. Also fewer LD were necessary in LDP after exposure to SD than to cold (Vince-Prue, 1975). Wellensiek (1958) thus concluded that exposure to low temperatures and SDs are different mechanisms of obtaining the same result.

The partial or total replacement of chilling by LD is exemplified only by certain *Chrysanthemum* species, but not by any other plants (Vince-Prue *et al.*, 1975).

1.4.4 Perception of Cold Temperatures.

In sugar beet, *Chrysanthemum*, celery, cauliflower and olive exposure of the shoot apical meristem to low temperatures results in vernalization. Successful reciprocal graft transfers of shoot tips between vernalized and non vernalized *Hyoscyamus niger*, and *Althea rosea*, and vernalization of excised shoot tips of carrot and cabbage, demonstrate the shoot apex to be the perceptive tissue (Bernier *et al.*, 1981). Curtis and Chang (1930) demonstrated that cooling coils around the shoot tip of celery induced flowering in these plants when the rest of the plant was exposed to temperatures of 20^{o}C. It is generally accepted, therefore, that most cold-requiring plants perceive low temperatures at the shoot tip (Vince-Prue, 1975). However, chilling of the apical bud of carrot after removal of 4/5's of the root had no effect (Chouard, 1960) and the author suggested that the sugar supply to the stem from the roots seems to play some part in the vernalization process, as has also been suggested by Bernier (1988). Vernalization has been reported in detached leaf tissue of *Streptocarpus* and *Lunaria biennsis* (Zeevart, 1963), but later evidence indicated that merisematic tissue at the base of the petiole was responsible. This is suggested to occur because vernalization takes place only when dividing cells are present during cold treatment (Wellensiek, 1964). Apical meristems contain a large proportion of dividing cells, and are therefore likely to give the greatest response to vernalizing temperatures. It is possible, however, that all dividing cells are potential sites for vernalization and experiments with *Lunaria* show the base of the petiole to be vernalizable (Vince-Prue, 1975). It seems to be the case that meristematic activity is important in the process of vernalization, however winter rye is vernalizable at temperatures of -2^{o}C and below and the same is observed in *Cherianthus* seeds. At these temperatures mitosis is most likely to be suspended. Therefore, vernalization only seems

to occur in cells able to divide, or those about to undergo cell division, and is not solely confined to the apical meristem.

1.4.5 Juvenile Phase

In many plant species there is a requirement for the plants to reach a certain age before they are able to respond to vernalizing temperatures. Juvenility is most often observed in obligate vernalization plants, many of which are biennial in habit (Bernier *et al.*, 1981). This rule, like many associated with flowering, has its exceptions, and in certain plant species vernalization of the germinating seed, or even the developing seed on the mother plant, is possible to a degree. However, the younger the plants are when they receive the cold temperatures, the longer is the time to flowering. In the case of seeds developing on the mother plant, in almost every instance a subsequent period of low temperature is required to vernalize the plants completely, but the cold treatment necessary is reduced in length. Therefore, partial vernalization has been attained (Lexander, 1985).

The apical meristem in certain vernalization requiring species with a juvenile phase is reported to be less active in young plants than in those possessing a more mature apical meristem (Williams and Atherton, 1990). This may be an important factor in the determination of juvenile incompetence to perceive low temperatures and may be due to growth elsewhere on the plant exerting priority demands upon the limiting nutrient supply. Evidence from Williams and Atherton (1990) suggests that inadequate carbohydrate availability for juvenile cauliflower plants is a consequence of allocation of assimilates to the leaf sink. This adds to the evidence stated in section 1.3.4.supporting the theory that depletion of assimilates influences competence to respond to floral

inductive conditions.

The duration of the juvenile phase varies from plant to plant, being 10-30 days for *Hyoscyamus niger* (Sakar 1958), and 8 weeks for *Lunaria biensis* (Wellensiek 1958). Sensitivity to cold treatment increases with time after this initial juvenile period up to a threshold (Vince-Prue, 1975). There is no correlation between the size of the seed and the duration of the juvenile phase.

1.4.6 Reversion to Vegetative Growth

Sussex (1989) stated that plants that flower in response to inductive photoperiods can usually be transferred to non-inductive conditions once flowering is initiated, where flower formation continues to completion. The implication here is that floral meristems are stably determined. McDaniel *et al.* (1991) established clearly that the floral stimulus acts within a few hours to make the apex florally determined. The commitment to flowering in plants exposed to inductive daylengths is usually, therefore, only reversible under unusual growth conditions and is dependent on the time at which such unfavourable conditions were experienced. There is extensive evidence that the effect of the vernalizing temperatures experienced by many plants may be reversed if unusually high temperatures are experienced. This process is referred to as devernalization. It was first reported by Gregory and Purvis (1936) where the effect of high temperatures for a few days was to nullify the effect of the cold treatment. For devernalization to occur chilling treatment should be sub-optimal as optimally vernalized species, in general, cannot be devernalized. Winter wheat exposed to temperatures of 35^{o}C for 3 days can be completely devernalized provided the duration of the vernalizing temperatures does not exceed about 12 weeks (Purvis, 1948). Exposing vernalized peas, however, to 35^{o}C for

18 -24 h can completely devernalize them (Chouard, 1960). The temperature at which the peas were vernalized was not reported, however, and so it is possible that vernalization was not optimally achieved. In general heat treatment must immediately follow the chilling treatment. An intervening period of non-devernalizing temperatures stabilises the effect of the chilling (Bernier *et al.*, 1981). One exception to this rule is *Cheiranthus allionii*, which is better devernalized 1-2 months after the chilling period.

Devernalization has also been observed to be caused by CO_2 depletion and SD (Chouard, 1960). SD devernalization is of particular interest as many plants, such as *Beta vulgaris* and *Oenothera* species have demonstrated this phenomenon. The processes involved in SD devernalization and devernalization by high temperatures are probably of different origin. The process of SD devernalization has, however, been shown to be a true photoperiodic response as a night break (NB) has been demonstrated to nullify the effect. Given the range of responses of plant species to temperature a unifying principle underlying devernalization is difficult to obtain (Zeevart, 1963).

1.4.7 Evidence from Grafting Experiments for a Model of Floral Initiation by Low Temperatures

The transmissibility of a floral stimulus produced during low temperature treatment has been demonstrated in grafting experiments with a number of related and unrelated families, between different species and genera, and in plants of different response types (Vince-Prue, 1975). It is suggested that the presence of mature leaves, and light, impair the establishment of the necessary sucrose gradient from the donor to the receptor and, as the stimulus is transmitted with the photosynthates, removal of mature leaves or growth in the dark allows the correct flow of stimulus. In the majority of

grafting experiments most low temperature graft partners were LDP with or without the low temperature requirement, and experiments were usually carried out under LD conditions. Under these conditions the donor plant would always be competent to flower having first perceived a period of low temperature. Therefore, the receptor plant may have been induced by the production of the correct floral stimulus in the donor plant, and as such the receptor plant would have bypassed the need for vernalization. The belief is, therefore, that vernalization may be a strictly localised state that enables the plant to produce a transmissible substance under favourable conditions at a later stage. The question remains as to whether a transmissible substance specific for vernalization, and distinct from that stimulus produced by photoinduction, is produced after low temperature treatment. Evidence for the existence of such a transmissible substance, termed vernalin (Melchers and Lang, 1948) comes from work with the SDP *Nicotiana tobaccum* (Maryland mammoth) grafted to a stock of *Hyoscyamus niger*. The Maryland mammoth, after being exposed to low temperatures, was able to induce flowering in the non-induced stock plant under LD conditions whilst the Maryland mammoth donor remained vegetative. In this case photoinduction of the floral state in the stock was not a possibility as the stock plant has an absolute requirement for low temperature prior to photoinduction. Floral induction has also been observed under SD conditions after grafting annual *Hyoscyamus niger* donor tissue to unvernalized biennial *Hyoscyamus niger* stocks, but only after the removal of the donor. Under these conditions flowering took place 12 h later (Vince-Prue, 1975). Only a few plants have, therefore, been shown to be graft inducible to flower under non-floral inductive conditions.

1.5 Gibberellic Acid

Gibberellins (GAs) are a class of plant hormones which have been shown to induce the flowering of many LDP and cold requiring plants under non-inductive conditions (Zeevart, 1976). Applied GAs are usually ineffective in triggering flowering in SDP but there are a few exceptions such as *Chrysanthemum* (Pharis and King 1985). Nanda *et al.* (1967), however, report that GAs have an inhibitory effect on flowering in some SDP under inductive conditions.

GAs most often stimulate cell elongation. However, there are many different GAs that exist, some of which are more effective at inducing floral development in some plant species than others. GA_7 is active in the majority of plants, but GA_3 has the greatest effect on a small number of plants, including *Beta vulgaris* (Zeevart, 1976). GA_3 was, however, only effective in promoting flowering in *Beta* species at temperatures slightly above the vernalization temperature range, and was ineffective at higher temperatures (Vince-Prue, 1975). This implies that some degree of the low temperature effect may be necessary prior to the application of GA_3 for the initiation of flowering in these plants. GA_3 was also shown to overcome the cold requirement of biennial *Hyoscyamus niger* plants to flower under LD conditions (Chouard, 1960). It was observed, however, that application of GA_3 to *Hyoscyamus niger* induced shooting prior to flower formation, whereas after vernalization the flower primordia develop prior to shooting. Lang (1965) cites many more examples where stem elongation only is observed under non-inductive conditions including *Oenothera biennsis*, *Scrofularia alta*, and *Scabiosa* varieties. GAs, therefore, do not replace totally the cold requirement of these plants to flower.

The ability of plants to be vernalized as imbibed seed has often been reported (Chouard, 1960). Endogenous applications of GA were generally ineffective at these

early stages. This lack of response of seed-vernalizable plants to treatment with GA, together with the work on *Beta* species and *Hyoscyamus niger*, suggests that GA does not directly control, or substitute for vernalization. It is possible that GA is not a direct product of thermoinduction, but the vernalization process may lead to the synthesis of GA, which may be required for processes leading to flowering. This hypothesis may help to explain why only partial replacement of the cold requirement is observed in many plants.

The endogenous concentration of GAs of plants is often found to increase after low temperature treatment (Wareing and Phillips, 1978). In wheat, it is reported that the amount of GA-like substances appears greater in vernalized than in non-vernalized specimens (Vince-Prue, 1975). The GA was not, however, thought to be involved in floral induction, but only in the elongation response to low temperature. Exogenous applications of extracts from *Xanthium* plants, thought to be GA-like in substance, have been reported to promote flowering under non-inductive conditions (Wareing and Phillips, 1978). It would be wrong to assume, however, that GA is the sole causative agent in floral induction as other factors within the extract may also have acted to stimulate floral induction.

Since GA has no stimulatory effect on the flowering of most SDP Chailakyan (1937) suggested that the causative agent in SDP for floral induction was a separate entity termed "Anthesin". This agent was suggested to be active in limiting floral induction in SDP during LD and GA was suggested to be active in limiting floral induction in LDP during SD. If this were the case then a graft union of non-induced LDP and SDP under non inductive conditions should induce flowering in both plant types due to the inductive effect of the non-induced graft partner. This has been demonstrated not to be the case (Zeevart, 1976). It is not now generally accepted that GA is not the sole flower inducing

agent in all plant species. It is suggested that GAs are probably involved in vernalization and may be required as a precursor to a co-factor, or the synthesis of a hormone (Pharis and King, 1985). This is consistent with most evidence to date which suggests that GAs are only one of many factors necessary to induce flowering in LD and cold requiring plants.

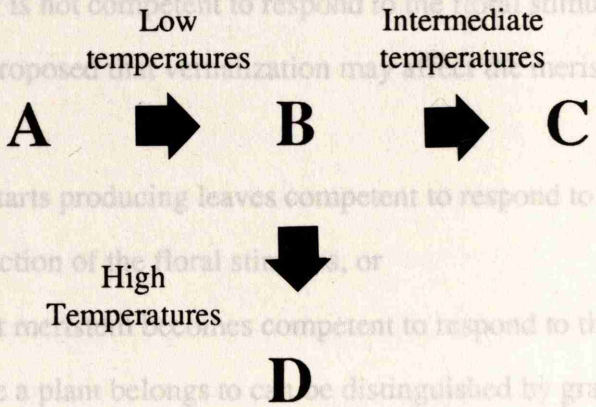
1.6 Models for the Combined Affects of Vernalization and Photoperiod

Salisbury (1963) and Lang (1965) proposed that low temperatures could act by suppressing the action of a flowering inhibitor relatively more than the activity of a promoter. This could occur via a differential effect on the synthesis of the promotor/inhibitor substances due to different temperature coefficients of enzymes involved in their synthesis, such that production of the inhibitor is suppressed at lower temperatures. Over the years various models have been constructed to try to account for the observed effects on vernalization of different environmental stimuli. Lang and Melchers (cited in Chouard, 1960) formulated a model (see Fig 1.1) to account for the observations they made on *Hyoscyamus niger*. The model incorporates a precursor (A) used in a reaction at low temperatures to give rise to a thermolabile substance (B) which is lost at high temperatures, but which at intermediate temperatures can be used to form a floral stimulus (C). Later models were fashioned along the same lines but involved more intermediates.

Alternatively the physiological effects of growth at low temperatures, for instance the depression of leaf growth, and associated altered assimilate levels at the shoot apex may result in vernalization. The accumulation of carbohydrates at the shoot apex being a

Fig 1.1 **Model to try to Account for the Observed Effects on Vernalization of Different Temperatures** Taken from Gregory and Purvis, 1936

A is used in a reaction at low temperatures which gives rise to B. B is thermolabile. At high temperatures it is used in a reaction to produce D. At intermediate temperatures it is used to produce the floral stimulus C.


Low
temperatures
Intermediate
temperatures
A
B
C
High
Temperatures
D

requirement for floral meristem formation could also account for the effect of other factors which alter assimilate availability being able to substitute for low temperatures (e.g. SD, high light intensity, and high pressure) (Williams and Atherton, 1990).

McDaniel (1985) discussed the effects of vernalization in terms of the competence of tissues to respond to inductive conditions. In photoperiodic plants with a vernalization requirement, where daylength is perceived by the leaves, a floral stimulus of some sort is produced in response to the inductive daylength. The lack of response of non-vernalized plants must mean that either the leaves are not competent to respond to the daylength or the apex is not competent to respond to the floral stimulus.

Thus McDaniel proposed that vernalization may affect the meristem in either of two ways:

1) the meristem starts producing leaves competent to respond to the inductive daylength with the production of the floral stimulus, or

2) an incompetent meristem becomes competent to respond to the floral stimulus.

The response type a plant belongs to can be distinguished by grafting experiments (see Fig 1.2). Fig 1.2(a) shows the accepted scheme for the way in which low temperatures and inductive photoperiods act to bring about floral induction in many flowering plants. Those requiring low temperatures must be exposed to such conditions prior to exposure to the inductive photoperiod for a flowering response to be observed.

1.7 Events at the Apical Meristem

One of the major problems in the study of flowering is to discover the underlying cause of the relatively sudden transition of a vegetative growing point from the production of leafy organs to the formation of floral organs. There are several distinct

Fig 1.2 **Model to Explain the Differences Between Different Vernalization Response Types**

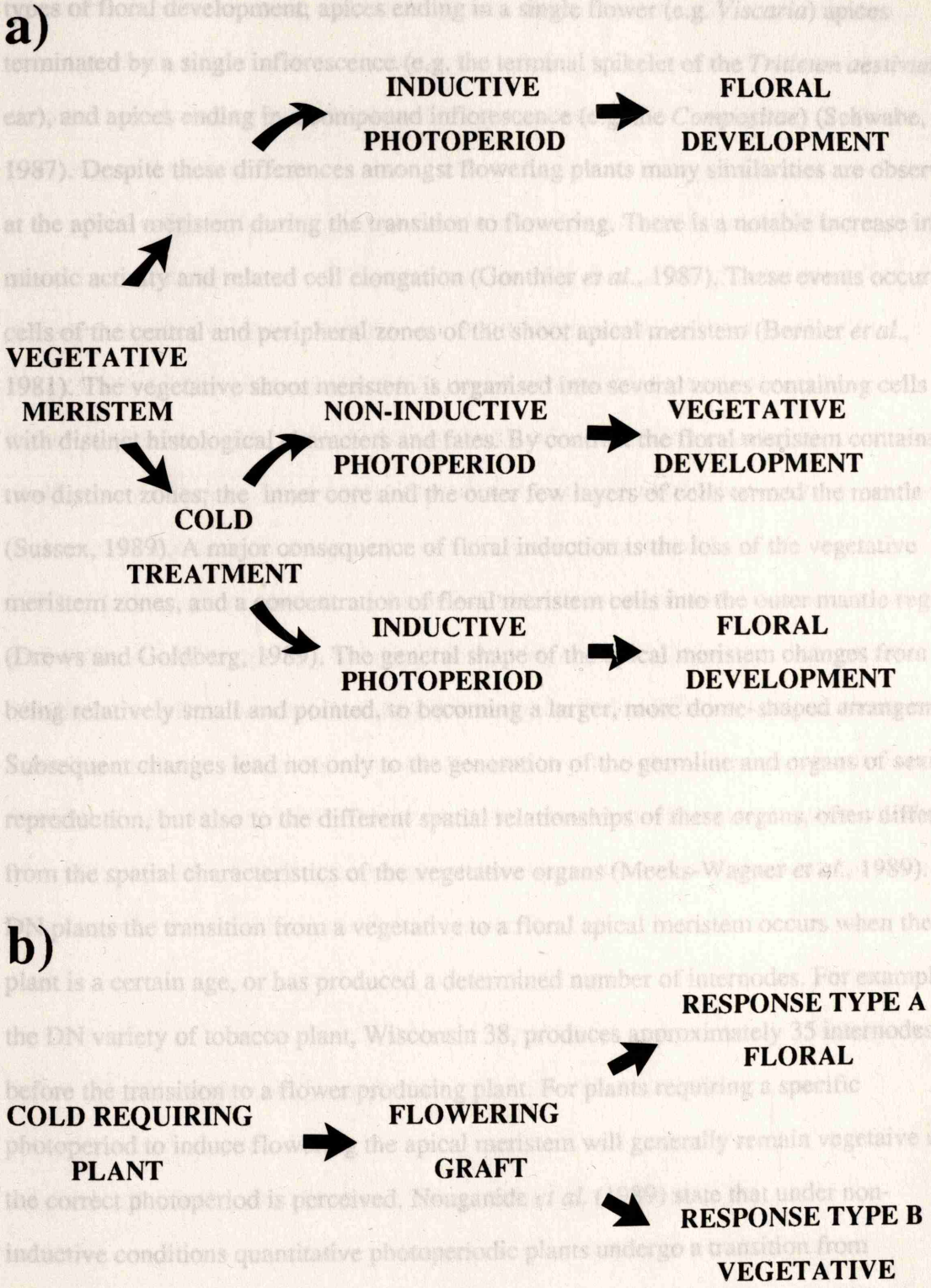
In a photoperiodic plant which requires vernalization flowering will only occur when both the cold and daylength requirements have been met.

a) **Distinction Between Cold Requiring and Non-Cold Requiring Genotypes.**

Plants not requiring vernalization will flower in an inductive photoperiod without prior cold treatment (upper line). Plants which require vernalization (lower lines) will only flower after cold treatment followed by the appropriate daylength

b) **Hypothetical Vernalization Response Types**

In plants with response type A the cold requirement is eliminated by grafting on a flowering shoot. In this type of plant the unvernalized meristem is evidently able to respond to the floral stimulus translocated from the graft, but the leaves are unable to produce it. In plants with response type B grafting on a flowering shoot does not induce flowering. In this type of plant the unvernalized meristem is unable to respond to the floral stimulus


a)
INDUCTIVE PHOTOPERIOD
FLORAL DEVELOPMENT
VEGETATIVE MERISTEM
NON-INDUCTIVE PHOTOPERIOD
VEGETATIVE DEVELOPMENT
COLD TREATMENT
INDUCTIVE PHOTOPERIOD
FLORAL DEVELOPMENT
b)
RESPONSE TYPE A
FLORAL
COLD REQUIRING PLANT
FLOWERING GRAFT
RESPONSE TYPE B
VEGETATIVE

types of floral development; apices ending in a single flower (e.g. *Viscaria*) apices terminated by a single inflorescence (e.g. the terminal spikelet of the *Triticum aestivum* ear), and apices ending in a compound inflorescence (e.g. the *Compositae*) (Schwabe, 1987). Despite these differences amongst flowering plants many similarities are observed at the apical meristem during the transition to flowering. There is a notable increase in mitotic activity and related cell elongation (Gonthier *et al.*, 1987). These events occur in cells of the central and peripheral zones of the shoot apical meristem (Bernier *et al.*, 1981). The vegetative shoot meristem is organised into several zones containing cells with distinct histological characters and fates. By contrast the floral meristem contains two distinct zones; the inner core and the outer few layers of cells termed the mantle (Sussex, 1989). A major consequence of floral induction is the loss of the vegetative meristem zones, and a concentration of floral meristem cells into the outer mantle region (Drews and Goldberg, 1989). The general shape of the apical meristem changes from being relatively small and pointed, to becoming a larger, more dome-shaped arrangement. Subsequent changes lead not only to the generation of the germline and organs of sexual reproduction, but also to the different spatial relationships of these organs, often differing from the spatial characteristics of the vegetative organs (Meeks-Wagner *et al.*, 1989). In DN plants the transition from a vegetative to a floral apical meristem occurs when the plant is a certain age, or has produced a determined number of internodes. For example the DN variety of tobacco plant, Wisconsin 38, produces approximately 35 internodes before the transition to a flower producing plant. For plants requiring a specific photoperiod to induce flowering the apical meristem will generally remain vegetaive until the correct photoperiod is perceived. Nougaréde *et al.* (1989) state that under non-inductive conditions quantitative photoperiodic plants undergo a transition from vegetative to intermediate before achieving a pre-floral state. They interpret this

intermediate phase as being a preparatory step, and noted that obligate photoperiodic plants generally miss out this intermediary phase. It may be the case that when the correct photoperiod is perceived by such plants they pass through this preparatory step to the pre-floral stage.

One effect of low temperature on the apical meristem of vernalization requiring plants is to alter the rate of cell metabolism, and this change is suggested not to be directly related to vernalization (Zeevart, 1976). Most evidence for the role of low temperature on floral induction points to an improvement of the supply of assimilates to the apex. (Bernier *et al.*, 1981; Salisbury, 1963). Under warm non-inductive conditions rapid growth of young leaves could deprive the shoot apices of certain growth factors such as carbohydrates limiting further development of the apex (Williams and Atherton, 1990). Chilling could, therefore, inhibit leaf growth and increase the availability of growth factors to the apex by reducing the requirement for a supply to the leaves. Hussey (1963) showed that there was a decrease in the growth rate of leaf initials in tomatoes at low temperatures whilst there was an increase in dry weight of the apex and accelerated flowering. Atherton *et al.* (1987) indicate that carbohydrates accumulate in the shoot tips of cauliflower during chilling and suggest that suppression of leaf development in mature plants may be essential for vernalization to occur.

1.8 Molecular Events Upon Induction

1.8.1 Early Biochemical Studies of Floral Induction

Floral induction and subsequent development is considered to be mediated

through the control of gene expression. The nature of the changes which take place in the transition to the vernalized state is unknown, but may involve the expression of previously inactive genes or the inhibition of previously active genetic information (Vince-Prue, 1975). Tran Thahn Van *et al.* (1985) give evidence that oligosaccharide fragments from plant cell walls play a role in flowering, although since that time they have been unable to confirm or deny this. Many inhibitors of flowering have been found in the search for that one molecule which may bring about the switch from vegetative to floral development. Actinomycin D is an inhibitor of RNA synthesis which is known to suppress flowering in *Pharbitis nil* cv violet when applied to the cotyledons prior to a single inductive long night (Arzee *et al.*, 1970). The inhibitor of protein synthesis, cycloheximide, has been shown to inhibit floral induction in *Xanthium* (Ross, 1970), whilst Zeevart (1962) has demonstrated that the process inhibited by 5- fluro uracil (5-FU) in the bud of *Xanthium* plants during the first part of the inductive dark period is RNA synthesis. Zeevart also demonstrated that 5-FU inhibited flowering in *Pharbitis nil* by suppressing DNA replication in the shoot apex. The ability to develop flowers was restored by the addition of thymidine, thymidylic acid, deoxycytidine, and 5-methyl deoxycytidine, all of which are precursors of DNA (Zeevart, 1962). Hess found that 2-thio uracil (2-TU) inhibits RNA synthesis and flower formation (cited in Zeevart, 1963). 2-TU incorporated into RNA also inhibits floral initiation in *Streptocarpus wendlandii* (Vince-Prue, 1975). More recent work with mutants of *Pisum sativum* has demonstrated that two loci, *Sn* and *Dne*, confer the ability of *Pisum sativum* plants to respond to photoperiod (Murfet and Reid, 1987). Reid (1986) has demonstrated by grafting experiments that the gene combination *Sn*, *Dne* acts by regulating the production of an inhibitor which delays flower initiation. Using the evidence from such experiments with inhibitors it was suggested that nucleic acid synthesis was involved in the control of floral

induction. Direct observations of differences in protein and nucleic acid synthesis of cells of induced and non-induced plants have shown promising results. Cold treated *Arabidopsis* of a variety requiring vernalization showed an increase in RNA content whilst devernalization brought about a decrease in RNA synthesis by 40% (Zeevart, 1976). Under the same conditions, varieties of *Arabidopsis* not requiring vernalization did not show these results. Embryos of seeds of winter wheat have also been shown to contain increased RNA contents upon exposure to cold, but so too have embryos of spring wheat, although to a lesser extent (Vince-Prue, 1975). Finch and Carr (1956, cited in Zeevart, 1963), however, found no differential expression of nucleic acids between vernalized and unvernalized winter wheat. This is contradicted by Danyluk and Sarhan (1990) who indicate that low temperature induces differential gene expression in winter wheat, and suggest that protein kinase is involved in the regulation of transcription. Meza-Basso *et al.* (1986) showed preferential protein synthesis under cold temperature conditions in *Brassica napus*, of which 14 new proteins were shown to accumulate after 6 h. Numerous reports have therefore clearly established that plants exposed to low temperature synthesise a new set of proteins (Zeevart, 1976; Meza Basso *et al.*, 1986; Danyluk and Sarhan, 1990). Terekota (cited in Vince-Prue, 1975) reported that in winter wheat the histone pattern is much more complex than in spring wheat. Furthermore, it becomes less complicated on exposure to low temperatures eventually resembling the simpler pattern found in spring wheat. A histone mediated suppression of genetic information in unvernalized winter wheat was therefore suggested.

Warm (1984) showed there to be qualitative and quantitative changes in the mRNA population in leaves of the LDP *Hyoscyamus niger* on exposure to LD. However it is not possible to distinguish between changes associated with floral induction and those caused by a change in photoperiod. It must be established whether the increase in

RNA synthesis is due to an increase in synthesis of RNA species already present, or the production of new RNA species. Lay-Yee *et al.* (1987) showed substantial quantitative and qualitative differences in RNA translation products from cotyledons of *Pharbitis nil* maintained under different photoperiodic conditions. The ability of red light to inhibit flowering in *Pharbitis* when given in the middle of the dark period was utilised in their experiments, and the effect of a NB yielded one specific 28 kD polypeptide difference compared to that of a SD induced cotyledon. The more specific effect of the NB on floral induction indicates the observed change is likely to be specifically linked to floral induction, and demonstrates that induction of flowering may involve changes in gene expression. The fact that only one protein change was detectable with the system used by Lay-Yee *et al.* indicates, also, that very few genes may be involved in floral induction in *Pharbitis nil*, and this may also be the case in other species.

With the above information in mind McDaniel (1991) posed the question " Does an inductive photoperiod lead to the production of new mRNAs in the apical meristem that are related to the transmission of the floral stimulus from the leaves to the apex ?" The inaccessibility of the shoot apical meristem and the small amount of material retrieved makes such a study very difficult. Studies on the protein composition of vegetative and floral apices of *Pharbitis nil* and *Xanthium* show no consistent differences in types of proteins (Kohli *et al.*, 1980), which is probably due to the difficulty of reproducing data with such small amounts of material. The introduction of the Polymerase Chain Reaction (PCR) to the field of molecular biology has meant that it may now be possible to work with minute amounts of tissue (e.g. a single cell) and look for changes in gene expression within that tissue. Therefore, it appears that the inaccessibility of the shoot apical meristem of many plant species, due to its small size, can be overcome. Kelly *et al.* (1990) and Meltzer *et al.* (1990) have used PCR to identify

differences correlated with the onset of floral induction in the apical meristem.

Meeks-Wagner *et al.* (1989) note that the majority of mRNAs from tobacco floral structures are common to leaf mRNAs. To isolate genes involved in the transition to flowering differential screening (as described in section 1.10) of cDNA libraries is one option. This still requires large amounts of starting material, but using the property of tobacco that its cells can be grown in liquid culture as thin cell layers, and that these cells may be induced to floral or vegetative growth, means that large amounts of meristematic tissue is available for the study of gene expression during the transition to flowering in tobacco plants. Using differential screening Meeks-Wagner *et al.* (1989) isolated many families of cDNAs showing specificity to tissue induced to floral growth. Two transcripts corresponding to cDNAs from one family (PB 7-1 and PB 7-2) were shown to be present in pre-floral and floral apices, but not in juvenile apices. The levels of RNA corresponding to these two, and one other cDNA clone (PB 7-5) from the same family, was high in the roots of plants possessing immature influoresences, but in the roots of seedlings the level was very low, again indicating the importance of the roots in the transition to flowering.

1.8.2 Genetic Control of Flowering

Genetic control of photoperiod insensitivity has been cited as being directed by a single gene in Maryland mammoth tobacco (Murfet, 1977) and the *Ld* mutant of *Arabidopsis* (Réide, 1975) or by several genes, as in *Sorghum* (Poa and Morgan, 1986) and *Triticum aestivum* (Law, 1987). In *Sorghum* the response to daylength is dominant over insensitivity, whereas in tobacco and wheat the opposite is found. The most extensive advances in this area of study have been with the work on mutants of Pea

(*Pisum sativum*). Seven major loci have been identified that confer or modulate the ability to perceive or respond to daylength changes (Murfet, 1989). Of these genes, two (*Sn* and *Dne*) acting together confer sensitivity to daylength and the end product of their combined effect is a graft transmissible inhibitor (Murfet, 1977). Two other genes (*Lf* and *Veg*) confer sensitivity of the apex to the floral stimulus, and the most recent finding is that of the locus (*G*) which confers synthesis of the floral stimulus. The joint presence of dominant alleles of *Sn* and *Dne* confer a requirement for either LD or vernalization, which here act on the same process (Bernier, 1988). However it is generally accepted that the photoperiod (*Ppd*) and vernalization (*Vrn*) genes have proved to be separate and independent genes, probably controlling different component processes (Bernier 1988; Law, 1987). Purvis (1939) makes the assumption that the allele for winter habit of Petkus rye is dominant, but the progeny of F2 crosses between winter and spring rye do not follow the normal Mendelian pattern of inheritance (Pollock and Eagles, 1988). This is also true for *Hyoscyamus niger*, of which there are two races, annual and biennial. The biennial includes two forms, one with an obligate and one with a facultative vernalization requirement. This shows that the dominance of the biennial habit is incomplete. Genes controlling the response of plants to vernalization and photoperiod act independently in wheat suggesting that they may influence different component processes (Law, 1987). However there appears to be a degree of diversity amongst the known examples of plant genes produced in response to vernalization and photoperiod making a single unifying hypothesis for the effect of vernalization and photoperiod upon genes of flowering plants difficult.

Besides the role of genes involved in the perception of light and low temperature there is a great deal of interest in general in those genes which govern pattern formation and cell differentiation in the meristematic tissue of flowering plants. Of particular

interest are genes that play a role in the reorganisation of the vegetative meristem into the floral meristem and those required for establishing morphological patterns of petals, sepals, stamens and pistils. This is now developing into a vast topic of interest, and is beyond the scope of this report. However, it should be mentioned that the isolation of homeotic mutants (plants that transform one of the floral organ systems into another), has been successful in *Arabidopsis* (Schultz *et al.*, 1991) and *Antirrhinum majus* (Sommer *et al.*, 1990). Those cDNA clones isolated to date correspond to transcripts which are involved in determination of the identity of floral organ systems. One exception is the *leafy* mutant gene of *Arabidopsis* which is similar to the *floricula* gene isolated from *Antirrhinum*. They are expressed in the young flower primordia, but not in the inflorescence meristem, consistent with the proposal that they are involved in floral meristem identity.

1.9 Sugar Beet

1.9.1. Introduction

Sugar beet (*Beta vulgaris*, shown in Fig 1.3) is of particular interest commercially and physiologically because of its ability to store sugar at high concentrations, mainly as sucrose with a small concentration of reducing sugars (Terry, 1968). Before the seventeenth century all sugar came from sugar cane, but today half of the world production is refined from sugar beet. Cultivated beets are derived from the genus *Beta* which possesses useful characteristics such as disease resistance (Campbell, 1953).

Fig 1.3 Mature Annual *Beta vulgaris* Plant

A 24 week old annual *Beta vulgaris* plant grown under short day conditions at a temperature of 20^{o}C. The expanded tap root indicates that an excess of sugars is being stored in the root of this plant

1.9.2 Effects of Photoperiod and Temperature

Sugar beet plants exist in two growth forms, annual and biennial. Both are obligate LDP but the biennial has, in addition, a vernalization requirement prior to exposure to LD for floral induction. Therefore, all sugar beet varieties may be maintained as vegetative rosette plants indefinitely if they are grown in SDs at warm temperatures (Stout, 1945). The cultivated sugar beet plant is biennial in growth habit (Lexander, 1985), and it is in the cold period of the winter months that reserves built up during the first growing season are stored in the expanded tap root (referred to as the beet). It is the expanded tap root that is harvested, and the sugar then extracted. However, different genotypes have different requirements for daylength and temperature (Lexander, 1985). The most susceptible biennial genotypes do not need very long days, or very long periods of low temperature exposure, to undergo floral initiation. They may, therefore, be induced to develop inflorescence stalks in their first growing season. This behaviour is very undesirable for the sugar producer as the tap roots of plants that have undergone this bolting process are very thin and highly lignified, making it difficult to retrieve a significant amount of sugar. It is, therefore, desirable for the sugar producer to suppress this bolting tendency. For the seed producer the biennial habit of the cultivated beet is, however, an obstacle to the rapid improvement of certain genotypes as they often have very specific requirements for daylength and temperature (Lexander, 1987).

The daylength is known to be perceived by the leaves of the sugar beet plant (Stout, 1945). The optimum light conditions required to induce flowering depend on the quality of the light (wavelength), the quantity of the light (fluence rate), and the periodicity (photoperiod). For sugar beet continuous illumination gives rise to flowering earliest, although some beet plants have been seen to flower in as little as 12 h of light

(Lexander, 1985). In general 16 h of light in a 24 h photoperiod induces flowering in the majority of sugar beet genotypes. The fluence range of light is required to be between 1000-2000 pmol m^{-2} s^{-1} for optimum flowering (Lasa and Péres-Peña, 1978). As for the wavelength requirement, it is only necessary that the appropriate amount or R/FR light is perceived at the right time to influence the action of phytochrome (Lane *et al.*, 1965). The extension of the daylength with fluorescent white light (Heide, 1973), or even low intensity red light (Lexander, 1975), was demonstrated to increase the bolting tendency of sugar beet plants. The intensity of light used to extend the daylength is, therefore, not required to be very strong to have an inductive effect, but the higher the light intensity, and the longer the daylength, the earlier the bolting of the sugar beet plant (Lexander, 1987).

The low temperature requirement of the biennial sugar beet plant has been demonstrated to be perceived by the shoot apex (Curth, 1960). After induction by LDs the floral stimulus produced is transported to the shoot apex. The apex of the biennial beet is only competent to respond to the floral stimulus once it has been vernalized. The belief is that the leaves produced by the unvernalized biennial plant are not competent to respond to the LDs and, therefore, do not produce the floral stimulus in LDs. It is only after vernalization that leaves competent to respond to LDs are produced at the apex, and in subsequent LD conditions the leaves will respond by producing the floral stimulus, which is perceived by the apex (Bernier *et al.*, 1981). Some biennial genotypes of sugar beet require as little as a few weeks of very mild temperatures for floral induction to occur, but, in general, plants of 4-8 weeks old at the start of the vernalization period require 10-14 weeks of vernalization at temperatures between 2-10^{o}C for a total commitment to floral development (Lexander, 1985). Some sugar beet genotypes, after exposure to a period of low temperatures, will flower even in SDs (Vince -Prue, 1975).

However, the time to flowering is dramatically decreased in LDs compared with SDs, and this phenomenon is only observed in a few bolting sensitive genotypes (Lexander, 1985). Fife and Price (1953) observed that certain genotypes of sugar beet bolted in continuous darkness after vernalization, provided they possessed a large amount of reserves in their tap root. This indicates that thermal induction may be more important than photoinduction in the initiation of reproductive growth in sugar beet. However, the importance of daylength in bringing seed formation to completion was demonstrated in their work, as seed set was only successful after vernalization and LDs. In the majority of genotypes the vernalization effect may be reversed if a sufficiently long period of SDs, or a period of high temperatures is perceived immediately after the vernalization period (Lexander, 1985). High temperature devernalization has been observed at lower temperatures in SDs than in LDs (perhaps due to the additive devernalizing effect of SDs). SD devernalization has been observed at temperatures above 18^{o}C (Magara 1960) and at 24^{o}C and above some devernalization also occurs in LDs, particularly if the plants were chilled in SD. Subsequent bolting is arrested until a further low temperature treatment is supplied (Heide, 1973).

The flowering of the cultivated genotypes of sugar beet plants, therefore, requires low temperatures prior to LDs, and can be promoted by artificial light used to extend the daylength, and counteracted by high temperatures and SDs prior to vernalization. There are also many different genotypes of cultivated sugar beet plants, all of which have specific individual requirements for daylength and temperature.

1.9.3 Juvenile Phase

Sugar beet possesses no juvenile phase (Chouard, 1960; Heide, 1973; Lexander,

1985). The responsiveness of the plant to environmental factors does increase with age but vernalization, or at least partial vernalization, is possible whilst the seeds are developing on the mother plant. For complete successful vernalization the tap root has to have developed to supply the nutrients required for vernalization (Chouard, 1960), therefore the observed vernalization of sugar beet embryos is incomplete and requires a further period of low temperature treatment to induce flowering. This partial vernalization means that plants originating from these seed are more easily induced to flower by treatment at low temperatures (Lexander, 1987). Therefore, seed produced in colder climates will yield a higher percentage of undesirable bolters than those produced in warmer climates. Also, if the seed is planted early in the spring and a late frost is experienced, a large percentage of the crop may be lost to bolting. Therefore, it is seen as a great disadvantage to the sugar beet industry that vernalization may be induced at any age.

1.9.4 Genetics of Sugar Beet Annuality

Cultivated sugar beet varieties show a wide range of genetic variability in respect of the annual/biennial growth habit. In 1876 Rimpau stated that the annual habit is dominant (cited in Abegg, 1936). In controlled crosses between annual wild beet (*Beta patula*) and pollen from biennial commercial stock all progeny proved to be annual. Munerati (cited in Abegg, 1936) contributed the first significant advance in the genetics of bolting character in 1931. Crossing uniformly annual and biennial strains all the F1 progeny were shown to be annual, although the time required from germination to flowering was slightly longer than in the annual parent. The F2 progeny showed the typical 3:1 ratio of annuals to biennials indicating that annuality was controlled by a

simple Mendelian factor. The non-requirement for a low temperature period to induce flowering in annual sugar beet plants has therefore been assigned to a gene B (for bolting) which is nearly completely dominant over b. In addition the existence of the gene B' is supposed which would confer the requirement for a short period of vernalization to induce flowering. Cultivated sugar beet plants do not contain the gene B for annuality, but by hybridisation with wild beets it can be introduced and cause unwanted bolting (Lexander, 1985).

The hypothesis for incomplete dominance is that the heterozygotes (Bb) are slower at inflorescence stalk development than the homozygotes (BB). Detailed examinations of various hybrids has shown that dominance is not complete (Abegg, 1936). The annual habit is, however, strongly dominant and the F2 ratios are always approximately 3:1 annual:biennial. Although the annual habit is determined by this single dominant gene (B) several genes must be involved in the bolting susceptibility of biennial sugar beets. The implication from the relationship between characteristics of non-induced plants and bolting-susceptible plants is that some genes are active before bolting inducing conditions occur (Lexander, 1987).

1.9.5 Events at the apex

The vegetative shoot apical meristem of sugar beet produces leaf primordia close to the terminal point of the apex. Consequently the axial zone is short and the apical dome is flat. After floral induction the apex elongates and the leaf primordia arise at greater distances from each other. When the apical meristem begins divisions leading to flower primordia and their bracts, these structures originate more superficially at the apex than do the vegetative primordia. Bracts are formed by the tunica cells, and the floral

primordia are produced by the outer corpus layer. The axial portion of the apex proliferates and the rib meristem gives rise to longitudinal rows of cells, thus initiating the inflorescence axis. The flower primordia and bracts are formed successively closer to the tip, and finally a terminal flower primordium is laid down. Buds of the second order are produced at the sides of each flower primordium. The second tunica layer gives rise to the floral parts. Flowering proceeds from the base to the top in the main stem before the bracts. Within a cluster the bud in the middle is the first to open (Lexander, 1985).

1.9.6 Mechanisms involved in the Regulation of Vernalization

Lexander (1975) suggested that membrane proteins were involved in the regulation of bolting susceptibility on the basis of observations of the sulphydryl content of the membrane proteins under low temperature conditions. Many exceptions were observed during this work and so to state that membrane permeability is affected in all sugar beet genotypes under vernalizing conditions is not possible. Sugars have also been observed to be limiting for flower initiation and an optimal concentration of sugars at the apex is possibly one requirement for flowering in this species as sucrose utilisation accompanies flower formation in sugar beet. Although many factors may be optimal for bolting, the requirement for one or more additional factors could be lacking as it is believed that several genes must be involved in bolting susceptibility in sugar beet (Lexander, 1975.) Some genes are assumed to be active before bolting induction occurs, but whether others exist that are activated only when such conditions are experienced is not known.

The theory of a hormone, referred to as vernalin, produced under low temperature conditions was abandoned since it was only possible to demonstrate transfer of a floral

stimulus, under non-photoinductive conditions, after vernalization in very few species (Vince-Prue, 1975). Flowering can be induced in non-vernalized scions of sugar beet by grafting them to vernalized stocks. This implies that a transmissible substance produced in the stocks under vernalizing conditions is transported to the scion, but the plants used as the donor in these grafts had been induced to flower by exposure to LDs. Therefore, it is probable that the unvernalized receptor plant receives the floral stimulus from the vernalized, and flowering, donor through the production of a photoinduced stimulus by the donor (Lexander, 1987).

GA_3 has been shown to replace part of the cold requirement for extension of the inflorescence stalk of many plants, including sugar beet (Magara, 1964; Zeevart, 1976; Lexander, 1985). It is therefore assumed that GA is involved in the process of vernalization (Pharis and King, 1985). It is improbable that GA constitutes the flowering hormone, however, as in all but a few exceptional cases exogenous application of several different GAs only results in stem elongation and not flowering (Lexander, 1985). However, it is reported that the seeds of these exceptional individuals may have previously experienced a period of low temperature treatment (Gaskill, 1957; Stout, 1959). Application of GA does not, however, substitute for the LD requirement of sugar beet as such photoperiodic conditions are still required by plants after application of GA (Snyder and Wittwer, 1959). An increase in the endogenous concentration of GA has been observed in sugar beet plants after a period of vernalization (Lexander, 1985), but the quantity extracted from bolting and vegetative plants appeared to be the same according to Magara (1960). This was contradicted by Radley (1975) who suggested that there is an increase in the concentration of endogenous GA during the start of bolting in sugar beet.

Some sugar beet genotypes could be highly sensitive to GA even before the cold

period, whereas others attain a high degree of sensitivity when experiencing low temperatures. The degree of sensitivity may be a function of the state of the cell membrane, or the abundance of the receptors (Lexander, 1975). Many chemicals have been shown to inhibit unwanted bolting in sugar beet (Lexander, 1985). The most interesting have been those that decrease the endogenous concentration of GA, such as 2-chloroethyltrimethylammonium chloride (CCC) and Ethrel. However, in most cases flowering was not inhibited, only the process of stem elongation (Magara, 1967; cited in Lexander, 1985). This indicates that the major role of GA is in the control of stem elongation during floral development, and the production of floral organs is not dependent on the presence of GA. This was emphasised by the work of Reid (1986) on dwarf mutants of pea that are lacking in GA biosynthesis, in which the process of flowering is not inhibited.

The process of vernalization is believed to consist of two phases, and it is during the second phase that plants become sensitive to daylength and GA (Magara, 1964). This is in agreement with the suggestion of Vince-Prue (1975) and Bernier (1988) that low temperature makes the plant sensitive to other factors which, in turn, induce flowering. There is, therefore, evidence for many factors associated with the induction of flowering in sugar beet, all of which may be equally important, and none that have an effect on flowering in the absence of one or more other factors.

1.9.7 Grafting Experiments

Curtis and Hornsey (1964) showed that unvernalized biennial scions of sugar beet grafted onto unvernalized and flowering stocks were induced to flower. This was also demonstrated by Magara (1960). However, it was noted that defoliation of the scion may

be necessary for floral induction. Naylor (1941) grafted annual and biennial sugar beet plants but failed to provide conditions necessary for translocation between them. Therefore the annual donor plant bolted whilst the biennial remained vegetative.

From grafting experiments concerned with the photoperiod alone it was shown that 3-6 weeks of continuous light are required to produce the floral stimulus in the leaves, translocate this to the apical bud, and induce the formation of the seed stalk (Stout, 1945). It was demonstrated in this work that vegetatively growing plants do not produce any substance antagonistic to reproductive development in sugar beet, but merely lack the substance in sufficient amounts that causes flowering.

1.10 Aims

Since the low temperature requirement of the biennial *Beta vulgaris* plant has been demonstrated to be perceived at the shoot apex (Curth, 1960), and since the annual *Beta vulgaris* plant is known to possess a gene which confers annuality upon the plant (Lexander, 1985), the principle aim of this study was to develop techniques for the isolation of cDNA clones corresponding to genes expressed in the shoot apex of the annual *Beta vulgaris* plant grown under non-floral-inductive conditions (short days, 20°C). Once such techniques were developed the aim was to characterise apex-specific cDNA clones to investigate whether any of these might be connected with vernalization by studying expression in the annual and biennial genotypes.

The amount of RNA available from small amounts of tissue such as the apical meristem of plants is limiting. Therefore, the intention was to develop techniques involving the Polymerase Chain Reaction (PCR). Since its introduction in 1985 (Saiki *et*

al., 1985) PCR has transformed the way DNA analysis is carried out, and nucleic acid amplification has rapidly become one of the most important tools for the molecular biologist. The reaction is based on repeated thermal denaturation of double stranded DNA and extension by a thermostable DNA polymerase (from the bacterium *Thermus aquaticus*) of two primers, each annealed to a complementary template, to achieve amplification of greater than one million fold. PCR has recently lent itself to the field of cDNA cloning where small amounts of starting material are to be cloned. Using defined DNA adaptors with known restriction sites small amounts of cDNA may be amplified via PCR to build up enough material to allow cloning of that material into a suitable vector. This has been successfully demonstrated by Akowitz and Manuelidis (1989), Belyavsky *et al.* (1989), Timblin *et al.*(1990) and Domec *et al.* (1990). In all but the last reference cited here the cloning procedure involved the enrichment for poly(A^+)RNA. Since the amount of RNA available for this cloning procedure was very small it was decided to eliminate this step, and develop a technique for producing cDNA libraries from small amounts of total RNA. A major disadvantages of using PCR is that amplification is prone to mismatching where a nucleotide substitution occurs. Therefore, the sequence of the cDNA clones produced by PCR may not represent the true sequence of the original mRNA. There is also the possibility that in a population of cDNA sequences PCR will preferentially amplify cDNAs of small size giving rise to a cDNA library which does not truly represent the population of cDNA clones that would have been produced using conventional cloning methods. Therefore, the representitiveness of any cDNA libraries produced was ascertained using several cDNA clones representing known plant mRNAs. The ultimate aim of this study was then to isolate cDNA clones representing mRNAs specific to the annual *Beta vulgaris* apical meristem tissue conferring annuality upon the genotype, and also involved in the process of vernalization. Therefore, the intention was

to prepare cDNA libraries from annual *Beta vulgaris* apical meristem tissue which had not been exposed to floral inductive conditions. These plants must, therefore, be maintained at warm temperatures, and exposed to short days only. On successful production of a PCR amplified cDNA library from such annual, warm, short day apical tissue it was hoped that clones specific to the apical meristem may be isolated using the techniques of differential screening. Here cDNA is prepared from mRNA isolated from two distinct cell types, plus and minus. cDNA clones specific to the plus cell type can be identified using a plus-minus hybridisation technique. Typically, duplicate nylon filters carrying cDNA clones derived from plus cells are screened with radiolabelled cDNA from plus and minus cells respectively. Clones shown to hybridise to the plus and not the minus cDNA probe are selected and characterised (Boll *et al.*, 1986). This procedure requires vast amounts of cDNA. However, since the intention was to use PCR to prepare the cDNA for making the apical library, and since leaf tissue was used to develop the techniques involved in the PCR library production, an ample supply of cDNA should be available for this purpose. Differential screening does not, however, lend itself to the isolation of cDNA clones representing mRNAs present at a low abundance (<0.1%).

Subtractive hybridisation is a technique recently employed to try to overcome just such a problem. This process removes cDNA species common to both the plus and minus cDNA populations used in the differential screen by the use of an agent such as biotin, which is bound to the minus cDNA species present at a 1000 fold excess over the plus cDNA species. The two populations of cDNA are denatured and then incubated together in the presence of a high salt concentration at a temperature that will facilitate annealing of common cDNA species but not those specific to one cDNA population. The minus cDNA, whether it is single stranded, or double stranded, or a double stranded hybrid of plus and minus cDNA, is removed using avidin, a protein with a high affinity for the

biotin moiety. The remaining cDNA should be specific to the plus population, and after several rounds of PCR amplification and subtraction low abundance cDNA species specific to the plus population of cDNA should be represented to a degree at which they are easily detectable. Such subtractive screening and library construction is possible without using PCR, but when starting material is limiting this procedure is aided by the amplification of plus and minus species. This procedure has recently aided the identification of many novel and important genes (Sive and St John, 1988. Timblin *et al.*, 1990).

CHAPTER 2

MATERIALS AND METHODS

2.1 General Laboratory Methods

General molecular techniques were carried out essentially as described in Sambrook *et al.* (1988).

2.2 Sources of General Laboratory Reagents

All general laboratory reagents were of Analar purity and obtained from either Sigma or BDH unless otherwise indicated.

2.3 Sources of Enzymes and Other Related Products

All restriction and modification enzymes were obtained from BRL and used according to their instructions, unless otherwise indicated. Deoxynucleoside triphosphates and Sephadex G-50 were obtained from Pharmacia UK. Bio-gel A 150 was obtained from Bio-Rad. *Taq* Polymerase was obtained from Promega. All radiochemicals were obtained from Amersham International.

2.4 Growth and Maintenance of Bacterial and Phage Stocks

The bacterial strains used in this study were grown in solutions of Luria broth or Luria broth solutions containing agar or agarose to give a solid support to the growth media.

Luria broth
1% (w/v) Bacto-Tryptone (Difco),
0.5% (w/v) Yeast extract (Difco)
1% (w/v) NaCl
pH 7.2

L-agar contained the above plus 1.5% (w/v) Bactoagar (Difco).

Top agarose contained the above plus 0.6% (w/v) agarose (Gibco/BRL)

Bacterial strains were stored at -70°C as a frozen stock, prepared by mixing 1ml of an overnight culture with an equal volume of sterile glycerol. Stocks of bacteriophage were stored at 4°C in SM phage buffer containing 1% (v/v) chloroform.

SM Buffer;

100 mM NaCl
10 mM $MgSO_4$
50 mM Tris-HCl (pH 7.5)
0.01% gelatin

2.5 *E.coli* Strains Used in this Study

Strain	Relevant Genotype	Source
XL-1 Blue	*supE*, *hsdR*, *lac*$^-$ *F'*, *proAB*$^+$, *lac*^{q}, *lacZΔM15*	I Jepson, ICI Seeds, Bracknell.
DP-50	*supE*, *supF*, *hsdS*	Stratagene
HB101	*hsdRk*$^-$, *hsdSk*$^-$, *recA13*, *thi*$^-$, *supE44*.	Gibco/BRL

E.coli strains were routinely cultured at 37^{o}C for between 18 and 20 h. Where bacteria containing plasmids were cultured the appropriate antibiotic was included at the following concentrations:

Ampicillin	150 μg/ml final concentration
Tetracycline	50 μg/ml final concentration

All antibiotics were stored as filter sterilised concentrate at -20^{o}C.

2.6 Bacteriophage Strain Used in this Study

Strain	Relevant Genotype	Source
λ ZAP II	λ*sbh*Iλ1^o *chi*A131 *sr*Iλ3^o c*Its*857 *sr*Iλ4 *nin*5 *sr*Iλ5^o Sam100	Stratagene

2.7 Maintenance of Plants

2.7.1 Light and Temperature Regimes

Warm, short day growth conditions.

Temperature at plant height, 20-23^{o}C; 8 h fluorescent light (Osram 45W warm white light); light intensity 100-120 µmol $m^{-2}s^{-1}$, (400-700 nm) as measured with a Li COR LI 185b quantum sensor.

2.7.2 Growth of Plants

Both diploid annual (7S 31660) and tetraploid biennial (5P 606) sugar beet (*Beta vulgaris*) seeds were supplied by Dr Wolfgang Schuch, ICI Seeds, Bracknell, UK. The seeds were soaked overnight in distilled water to leach out germination inhibitors, then sown in flat trays containing autoclaved compost and left to germinate in the warm SD growth room. When the first pair of true leaves emerged (after approximately 2 weeks) the seedlings were potted into 4 inch pots and grown to at least the 6 leaf stage.

2.7.3 Sampling of Plant Tissue

Samples of leaf and apical tissue for the isolation of RNA were dissected as described below and then frozen in liquid nitrogen (BOC). Samples of the apical tissue were used immediately. Samples of leaf tissue were stored at -70^{o}C for later use.

For the dissection of apical tissue the large leaves were removed from the region of the shoot apex. The remaining leaves were removed with fine microdissection scissors under

a dissecting microscope. When the apical meristem was visible it was dissected out and placed in a 1.5 ml microcentrifuge tube immersed in liquid nitrogen.

2.7.4 Microscopy of Apical Tissue

Apices were fixed in 3% (w/v) glutaraldehyde (Aldrich Chemical Company Ltd. Dorset) for a minimum of 48 h. Mr Eoin Robertson, Department of Chemistry, University of Glasgow, embedded, sectioned and stained the tissue. Briefly, after fixing in the glutaraldehyde the tissue was rinsed in distilled water and dehydrated through immersion in solutions of increasing concentration of ethanol. The tissue was then cleared in xylene and after decanting most of the xylene, covered in wax pellets and placed in a 60^{o}C incubator overnight. This was repeated twice more, and the tissue was then embedded in wax in preshaped plastic containers.

Sections of 10 μm thickness were cut on a rotary microtome, separated on water and collected on standard glass slides. The sections were dewaxed in xylene, hydrated with water and mordanted in 1% (w/v) ferric ammonium sulphate for 40 min, rinsed in water and then transferred to 0.5% (w/v) haematoxylin and left overnight. Slides were regressively destained in 1% (w/v) ferric ammonium sulphate and the destaining process was stopped by a 1 h wash in tap water. Finally, the sections were dehydrated through an ethanol series and counterstained in 0.5% (w/v) Fast Green in xylene and mounted in DPX mounting medium.

2.8 General Molecular Biology Methods

2.8.1 Estimation of Nucleic Acid Concentration

DNA and RNA concentrations were calculated by measuring the absorbance (A) of a solution at 260 nm and 280 nm in a Pye Unicam Model SPB-400 spectrophotometer, using a quartz cuvette with a 1 cm path length. A DNA solution with an A_{260} of 1.0 was taken to have a concentration of 50 µg/ml. An oligonucleotide with the same A_{260} was taken to have a concentration of 20 µg/ml. An RNA solution with the same A_{260} was taken to have a concentration of 40 µg/ml (Sambrook *et al.*, 1988).

2.8.2 Estimation of RNA Purity

The A_{260}/A_{280} ratio was used to assess the purity of a given RNA sample, with an acceptable ratio being within the range of 1.8-2.0. Samples having a ratio outside this range were subjected to a further round of phenol/chloroform extraction to remove proteins, carbohydrates, and unwanted salts. All RNA samples were also examined on a 1.5% (w/v) denaturing agarose gel to establish the level of degradation.

2.8.3 Phenol Extraction

After the various enzymatic treatments that DNA was subjected to, proteins and other contaminants which could inhibit further reactions were removed by phenol extraction using an equal volume of ultra pure phenol (BRL) containing 0.1% (w/v) 8-hydroxyquinoline (BDH Chemicals Limited). Phenol-extracted DNA was further extracted with phenol

saturated with chloroform, and finally twice with chloroform to remove residual phenol before ethanol precipitation. RNA was extracted using the protocol described in Jepson *et al.* (1991).

2.8.4 Ethanol Precipitation

DNA in aqueous solution was precipitated in the presence of 0.15 M ammonium acetate with 2.5 volumes of ethanol which had been stored at -20°C. This solution was kept at -20°C for at least 1 h before recovering the DNA by centrifugation at 5,000 xg for 15 min in a cold room at 4°C. After removal of the supernatant the pelleted DNA was vacuum dried (or left to air dry for 1 h) before being resuspended in either 1xTE (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) or sddH_2O.

2.8.5 Agarose Gel Electrophoresis

DNA was separated in agarose gels (1.0 - 2.5% (w/v)) using a Tris borate buffer as described (Sambrook *et al.*, 1988: pp 6.9-6.13). Before loading on to the gel, the DNA was mixed with 0.2 volumes of 5x loading buffer (80% (v/v) glycerol, 0.01% (w/v) bromophenol blue in 1x TE) (Sambrook *et al.*, 1988).

RNA was separated in agarose gels containing 1.5% (w/v) agarose in a formaldehyde gel buffer consisting of the following;

2.2 M formaldehyde

20 mM MOPS (pH 7.5)

8 mM Na Acetate (pH 5.5)

1 mM EDTA (pH 8.0)

Final pH: 7.0

All the solutions were added and heated to 65°C before adding the formaldehyde. The gel was then poured and allowed to set for 30-60 min in a fume hood. RNA in solution was made up to a concentration of 10-30 µg in 4.5 µl of DEPC treated sddH_2O. To the RNA samples the above solutions were added to the same final concentrations with the addition of 12.5 M formamide to maintain the RNA in a denatured form at low temperatures (37-42°C). The samples were then denatured at 65°C for 15 min. Immediately 2 µl of formaldehyde gel loading buffer was added to each sample before loading onto the gel.

Formaldehyde Gel Loading Buffer

50% (v/v) glycerol

1 mM EDTA (pH 8.0)

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

Once loaded the gel was run at 25 V overnight.

2.8.6 Isolation of DNA from Agarose Gels

DNA was isolated from agarose gels by microcentrifugation of the gel slice through siliconised glass wool (Heery *et al.*, 1990). A hole was pierced in a 1.5 ml microcentrifuge

tube with a needle before adding the glass wool and the gel slice. This tube was inserted into a second 1.5 ml microcentrifuge tube to collect the solution containing the DNA. Both tubes were centrifuged at 5,000 xg for 20 min and the liquid collected was sequentially extracted with phenol and chloroform before being ethanol precipitated as described in section 2.8.4.

2.8.7 Denaturing Polyacrylamide Gel Electrophoresis

Denaturing polyacrylamide gel electrophoresis was set up according to the described method utilising urea as a denaturing agent (Sambrook *et al.*, 1988: pp 6.49). These gels were used for purifying oligonucleotides and monitoring ligation reactions. The usual running conditions were as follows:- 17 watts (approximately 350 V, 40 mA) for 2 h on an Atto preparative gel apparatus.

2.9 Preparation and Purification of Oligonucleotides

Oligonucleotides for use as PCR primers were synthesised on an Applied Biosystems 380b Synthesiser. Oligonucleotides were routinely purified by denaturing polyacrylamide gel electrophoresis on a 20% (w/v) gel as previously described. Oligonucleotides were first deblocked by incubation in 40% (v/v) ammonium hydroxide at 55^{o}C for 7 h before being concentrated by freeze drying. Approximately 100 µg of crude deblocked oligonucleotide in 50 µl of 1X TE was mixed with 10 µl of 5X gel loading buffer and run on a 20% (w/v) gel. Oligonucleotides were visualised by UV shadowing using a short wave UV light (260nm) and a TLC plate with fluorescent indicator (Kodak Eastman 13252 TLC plate with fluorescent indicator N^{o} 6063). The product was then excised from the gel with a sterile

scalpel and placed into a 1.5 ml microcentifuge tube. The oligonucleotide was eluted from the gel by crushing the gel with a sterile disposable inoculation loop and incubating at 65°C with 500 µl of 1X TE for between 5 and 16 h. The contents of the tube were transferred to a fresh microcentrifuge tube which had been prepared by piercing a hole in it with a sterile needle. This tube was packed with siliconised glass wool and placed in to a second microcentrifuge tube. This was centrifuged for ten sec at 5,000 xg, which was sufficient to force the liquid through the glass wool but retain the acrylamide. The lower microcentrifuge tube was recentrifuged to remove the final traces of polyacrylamide particles. The oligonucleotide solution was made up to 10 mM MgCl, 0.1 M NaCl and ethanol precipitated. Oligonucleotides were resuspended in 1X TE at a final concentration of 100 µg/ml.

2.10 Oligonucleotides Used in This Study

Two major Oligonucleotides were used in this study for the purpose of PCR amplification of double stranded cDNA. These were as follows:

Primer 1 (JB1): (5'-ATGCTTAGGAATTCCGATTTAGCCTCATA-3')

Primer 2 (JB2): (3'-AAATCGGAGTAT-5')

Two further oligonucleotides were used in this study to PCR amplify cDNA inserts from purified bacteriophage plaques. These were as follows:

Primer 3 (PUC1): (5'-ATGCGGATCCGTTTTCACACAGCAAACAGCTATGAC-3')

Primer 4 (PUC2): (5'-TGGGGATTCGTGCGACGTTGTAAAACGACGGCCAGT-3')

Primer 1 (JB1) was extensively used in the PCR amplification of double stranded cDNA ligated to annealed primers 1 and 2, as well as for the PCR amplification of cDNA inserts from selected plaques to show the number of inserts present in a single recombinant.

2.11 End Labelling of Double Stranded DNA

Double stranded cDNA was end labelled with ^{32}P dCTP to ensure that all cDNA was blunt-ended prior to ligation to annealed primer 1 and primer 2 oligonucleotides. The end labelling reaction (50 µl) contained:

10 µg DNA

125 nM each of dATP, dTTP, dGTP

50 mM NaH_2PO_4 (pH 7.5)

3 mM $MgCl_2$

1 mM 2-mercaptoethanol

5 µCi [α-^{32}P] dCTP (3000 Ci/mmol)

50 units Klenow fragment

H_2O to 50 µl

The reaction mixture was incubated at room temperature (22^{o}C) for 20 min. Finally cold dCTP was added to a final concentration of 125 nM to ensure that dCTP was not limiting, enabling the reaction to go to completion. This was left at room temperature for 10 min before extracting sequentially with phenol and chloroform (as described in section 2.8.3) and being passed down a Sephadex G-50 column.

2.12 Radiolabelling of DNA Fragments

In almost every case cDNA fragments were radiolabelled using an Amersham random priming kit according to their instructions. After labelling the unincorporated

nucleoside triphosphate was removed by chromatography through a Sephadex G-50 column. The column was a Pasteur pipette plugged with siliconised glass wool. Two column volumes of 1X TE was passed down the column prior to loading the radiolabelled DNA sample. 200 µl fractions were collected and the radioactivity within them estimated using a scintillation counter. The void peak was pooled (fractions 3-6 in most instances) and stored on ice before use. When labelled DNA was to be used in a hybridisation experiment it was first heated in a boiling water bath for 2-3 min to denature the DNA duplex, before adding it to the hybridisation solution as quickly as possible.

2.13 Non-Radioactive Labelling of DNA

For the purpose of producing subtracted probes, cDNA from sugar beet leaf material was labelled with biotin by nick translation using the BRL kit (cat No 8160SB) containing biotin-7-dATP. Unincorporated biotin was removed by chromatography on a Sephadex G-50 column as previously described (section 2.12). 5 µl aliquots from each of the twenty 200 µl fractions were analysed for biotin incorporation by dot blotting and visualising with the BRL blue GENE non-radioactive nucleic acid detection system.

2.14 Hybridisation of Radiolabelled Probes

2.14.1 Plaque Hybridisation (Benton and Davies, 1977)

Plaque hybridisation was carried out by placing 10 cm^2 nylon membrane filters onto

agar plates containing the phage plaques. These plates were placed at 4°C for at least 1 h prior to applying the filters. The filters were allowed to contact the plate for 30 sec (60 sec, 120 sec, and 180 sec for lifts 2, 3, and 4 respectively). After orientating the filters with India ink, they were placed plaque side up on 3MM paper soaked in the following solutions;

i) 0.5 M NaOH, 1.5 M NaCl for 5 min

ii) 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl for 5 min

iii) 2X SSC pH 7.5 for 5 min

The filters were allowed to air dry for 10 min before being baked at 80°C in a vacuum oven for 1-2 h. The filters were then pre-hybridised for at least 4 h in the following buffer;

6X SSPE pH 7.5

0.25% (w/v) milk powder (Marvel)

0.1% (w/v) SDS

In general 10 ml of pre-hybridisation buffer were used for every filter being pre-hybridised. The pre-hybridisation took place in 10 cm^2 sandwich boxes capable of holding 200 ml of solution. For the hybridisation procedure a maximum of 10 ml of pre-hybridisation solution was added to a 10 cm^2 petri dish to which was added the radiolabelled probe DNA. Each filter to be treated was added individually to eliminate air bubbles, and ensure all filters were exposed to the probe. The petri dish lid was held on with two lengths of Nesco film bound tightly around the edge of the dish. The petri dish containing the filters and the probe was placed inside a 20 cm^2 petri dish, acting as a spill tray, and incubated at 65°C for 16-20 h. The filters were removed from the incubator and washed in 3X SSC pH 7.5, 0.1% (w/v) SDS, at 65°C for 90 min, with three changes of wash solution during that time, unless

otherwise indicated. Autoradiography was carried out at -70°C for 24-72 h as indicated, with or without an intensifying screen.

2.14.2 Southern Analysis of DNA Fragments (Southern, 1975)

Complementary strand DNA and DNA produced by PCR amplification were subjected to electrophoresis on agarose gels at 25-30 V overnight. The gel was then placed into 1 l of 0.5 M NaOH, 1.5 M NaCl for 45 min , followed by 5 min in 1 l of 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl. The gel was then placed onto the blotting apparatus as described (Sambrook *et al.*, 1988: pp 7.46-7.48) and blotted overnight on to a nylon membrane. After blotting the membrane was placed into 2X SSC for 5 min, after which time it was allowed to air dry for 10 min before being baked at 80°C for 1-2 h. The membrane was then used immediately, or stored at 4°C in prehybridisation solution for up to 12 months. Hybridisation and autoradiography were carried out essentially as previously described.

2.14.3 Dot Blot Analysis of DNA Fragments

Aliquots of DNA in solution were heated to 95°C for 2-3 min and immediately chilled on ice. An equal volume of ice-cold 20X SSC was added to the samples. A section of Hybond-N (Amersham) filter was cut to 120 x 80 mm and immersed in distilled water for 5 min, followed by 10X SSC for 20 min. The filter was then set up in a BRL HYBRI-DOT 96-hole manifold apparatus (No. 580-1050 MM) and suction sufficient to pull 1 ml of water up a 2 ml glass pipette applied. Each well was washed through with 200 µl of 10X SSC and the required number of samples were loaded into the appropriate wells. Following loading of DNA solutions, the wells were washed through with two further applications of 200 µl of

10X SSC. The manifold apparatus was then dismantled and the filter subjected to denaturing conditions and baking as previously described (section 2.14.1).

2.14.4 Northern Analysis of RNA Samples

Denatured RNA samples were subjected to electrophoresis through 1.5% (w/v) agarose gels containing 2.2 M formaldehyde at 25 V overnight. Before blotting the lane containing the RNA markers was excised with a sterile scalpel. The remainder of the gel was blotted as previously described. Once blotted the filter was baked at 80^{o}C for 1-2 h before pre-hybridising for 4-6 h at 65^{o}C in 6X SSC, 2X Denhardts solution (0.04% (w/v) Polyvinylpyrolidone, 0.04% (w/v) Ficoll (type 400, Pharmacia), 0.04% (w/v) Bovine Serum Albumin (fraction V, Sigma)), 0.1% (w/v) SDS.

The filter could be probed immediately, or stored at 4^{o}C for up to 12 months. Denatured radiolabelled probe was added to a new hybridisation bag containing the filter to be probed. The denatured DNA probe was added and the bag was sealed, removing all air bubbles in the process. The filter was then incubated at the recommended temperature for 16-20 h in a shaking water bath before washing in a range of salt washes at 65^{o}C depending on the size and relative homology of the probe being used.

2.15 Polymerase Chain Reaction

For the duration of this study two separate PCR amplification protocols were used. For the PCR amplification of double stranded cDNA a protocol devised by Keith Edwards

and colleagues (at ICI Research Station, Jealotts Hill) was used. PCR reactions were set up to contain the following in a final volume of 50 µl

10 mM Tris-HCl (pH 8.3)

50 mM KCl

1.5 mM $MgCl_2$

0.1% (w/v) gelatin

200 µM of each of the four dNTP's

600 ng of the primer JB1

Template DNA

2.5 units of *Taq* DNA polymerase

The protocol for PCR amplification using primer 1 (JB 1) was as follows;

i) The reaction mixture was heated to 72°C for 2 min to allow the smaller primer (JB2) to melt off, and *Taq* polymerase enzyme filled in the overlap remaining.

ii) The DNA was then denatured at 94°C for 1 min.

iii) The primer (JB1) was allowed to anneal to its complementary DNA by cooling to 68°C for 0.8 min.

iv) *Taq* polymerase was allowed to extend the product by heating to 72°C for 3 min. Steps ii), iii) and iv) were repeated 35 times.

v) *Taq* polymerase was allowed to extend all unfinished DNA products by maintaining the temperature at 72°C for a further 10 min.

The cycling process was carried out on a Techne PHC-1 machine pre-set to complete the desired cycle.

In the second protocol primers PUC 1 and PUC 2 were used instead of the single JB 1 primer, and steps iii) and iv) were altered as follows;

iii) The primers (PUC 1 and PUC 2) were allowed to anneal to their complementary DNA strands by cooling to 65^{o}C for 1 min.

iv) *Taq* Polymerase was allowed to extend the product by heating to 72^{o}C for 1.2 min.

2.16 Preparation of Total Plant RNA

2.16.1 Large Scale RNA Prep

All plasticware used throughout this procedure was autoclaved at 20 psi for 15 min and glassware was treated with 0.1% (v/v) diethylpyrocarbonate (DEPC) before baking for 16-24 h at 200^{o}C. Solutions other than those containing Tris were rendered RNAse free by treating them overnight with 0.1% (v/v) DEPC and autoclaving as for the plasticware. Stock solutions of Tris-HCl were filter sterilised before use.

Between 5 and 10 g of plant tissue was frozen in liquid nitrogen and then ground to a fine powder using a mortar and pestle which had been sterilised by autoclaving. Once a fine powder was obtained 25 ml of phenol-cresol solution was added along with 50 ml of homogenising buffer.

Homogenising Buffer

400 mM NaCl

50 mM Tris-HCl (pH 9.0)

5 mM EDTA

1% (w/v) SDS

10 mM Dithiothreitol (DTT)

1 mM Aurintricarboxylic acid (ATT)

10 units Heparin

Phenol-Cresol Solution

100 g phenol

0.1 g 8-hydroxyquinoline

10 ml Cresol

Homogenising buffer added to saturation.

Phenol-Chloroform Solution

100 g phenol

0.1 g 8-hydroxyquinoline

50 ml chloroform

sdd H_2O added to saturation

The plant material and solutions within the mortar were allowed to thaw whilst continuing to grind until a paste formed. The paste was then transferred to 30 ml Corex tubes and centrifuged at 22,000 xg, 4°C for 15 minutes. The supernatant was transferred to 50 ml Falcon tubes (Elkay Industries Ltd) containing an equal volume of phenol-chloroform and

centrifuged at 5,000 xg, 4°C for 30 min. This last step was repeated before adding 12 M LiCl to the supernatant to a final concentration of 2 M and incubating the tubes at 4°C overnight. The tubes were then centrifuged at 5,000 xg, 4°C for 30 min as before. The supernatant was discarded and the pellet resuspended in a minimal volume of 5 mM Tris-HCl (pH 7.5) and precipitated with LiCl as previously described. The tubes were centrifuged once more at 5,000 xg and the pellet remaining was washed in ice cold 70% ethanol and left on ice for 10 min. The tubes were centrifuged once more and the pellet dried under vacuum before being resuspended in a small volume (100-500 µl) of DEPC treated sdd H_2O. The concentration of RNA was then determined as described in section 2.8.1.

2.16.2 Small Scale RNA Prep

To isolate RNA from apical meristem tissue from sugar beet it was necessary to scale down the above procedure to accommodate such small amounts of tissue. The volumes used in the large scale procedure were reduced 100 fold to allow the extraction of apical meristem RNA to be performed in 1.5 ml microcentrifuge tubes. It was assumed that the weight of the 35 sugar beet apical meristems would be approximately 50 µg which is at least 100 fold less than the amount of starting material used in the large scale RNA prep. Measuring the weight of such a small amount of tissue without thawing that material proved to be extremely difficult and so the assumption was made by measuring the weight of a similar volume of small sized pieces of sugar beet young leaves. The small amount of tissue was ground to a fine powder under liquid nitrogen in a microcentrifuge tube using a small glass rod. 250 µl of phenol-cresol solution was then added along with 500 µl of homogenising buffer. The plant material and solutions within the microcentrifuge tube were allowed to thaw whilst continuing to grind. The resultant paste was centrifuged for 15 min at 5,000 xg and the

supernatant transferred to a second microcentrifuge tube. To the solution remaining in the first microcentrifuge tube a further 250 µl of homogenising buffer was added to ensure that as much RNA as possible was extracted from the small amount of starting material. This first microcentrifuge tube was shaken by hand for 10 min before being centrifuged as before. The supernatant from this second centrifugation was added to the original supernatant in the second microcentrifuge tube and an equal volume of phenol-chloroform was added before centrifugation as before. This last step was repeated before adding 12 M LiCl to the supernatant at a final concentration of 2 M and incubating the microcentrifuge tube at $4^{o}C$ overnight. Essentially from here on the small scale RNA prep followed the method of the large scale RNA prep except that microcentrifuge tubes were used and the final pellet of RNA was resuspended in approximately 10 µl of DEPC-treated sdd H_2O.

2.17 Synthesis of Double Stranded cDNA (Gubler and Hoffman, 1983; Lu and Werner, 1988)

cDNA was synthesised in all cases using an Amersham cDNA synthesis kit. The method used is a development of the method of Gubler and Hoffman (1983). In brief, the first strand cDNA is primed with oligo dT and synthesised using AMV (avian myeloblastosis virus) reverse transcriptase. Second strand cDNA synthesis is performed using the mRNA/cDNA hybrid as a substrate as follows;

1) *E.coli* ribonuclease H (an endoribonuclease which will digest an RNA/DNA hybrid only) is utilised to produce nicks and gaps in the mRNA strand.

2) This provides efficient primers with 3'OH for *E.coli* DNA polymerase I to replace the mRNA strand by a nick translation type reaction

3) T4 DNA polymerase is used to remove any small 3'-overhangs on the first strand

cDNA

A variety of different RNA concentrations were used to establish the best conditions for cDNA synthesis using the Amersham cDNA synthesis kit. Since the eventual intention was to prepare cDNA from small quantities of apical meristem RNA it was decided to use total RNA and not to enrich for poly (A^+) RNA. The sample of RNA to be used was dissolved in a total volume of 10 ml of DEPC treated sdd H_2O. The first strand cDNA synthesis was set up using the following;-

4µl first strand buffer (contents unknown)

1 µl Na pyrophosphate (concentration unknown)

1 µl human placental RNAse inhibitor (20 units/µl)

2 µl dNTPs (10 mM dATP, dGTP and dTTP, 5 mM dCTP)

1 µl oligo dT (1.6 µg/µl)

5 µCi [α-^{32}P] dCTP (3000 Ci/mmol)

Xµg of RNA * denatured at 65^{o}C for 5 min

sdd H_2O to 20 µl

* The amount of RNA used was dependent on the purity of the RNA. 100 X more RNA was used than would have been if poly (A^+) RNA and not total RNA was used.

The reactants were added in the order shown above and mixed gently. 20 units of AMV reverse transcriptase were added and the solution mixed and centrifuged for 3 sec. The reactants were then incubated at 42^{o}C for 40 min.

A 2 µl aliquot was removed from the first strand reaction mixture to be subjected to denaturing agarose gel electrophoresis. The remainder of the first strand mixture was

combined with the following:

37.5 µl second strand buffer (contents unknown)

20 µCi [α-^{32}P] dCTP (3000 Ci/mmol)

1 µl *E.coli* ribonuclease H (0.8 units/µl)

6.5 µl *E.coli* DNA polymerase (3.5 units/µl)

H_2O to 100µl

The reactants were added in that order, mixed quickly and centrifuged for 3 sec before incubating at:-

12°C for 60 min

22°C for 60 min

70°C for 10 min

At this point 4.0 units of T4 DNA polymerase I were added and the reactants incubated at 37°C for 10-15 min. The reaction was stopped by adding EDTA (pH. 8.0) to a final concentration of 1 mM. A 10 µl sample of the second strand reaction mixture was removed to be subjected to denaturing agarose gel electrophoresis along with the first strand mixture and a sample of radiolabelled *Hind* III digested lambda DNA. The gel was washed in 7% (w/v) trichloroacetic acid for 30 min and then blotted according to the procedure described in section 2.14.2.

The double stranded cDNA was size fractioned on a Pasteur pipette column containing Sephacryl 300 (Pharmacia) added to 0.5 cm below the top of the pipette. The column was washed with 2 column volumes of 1X TE before the radiolabelled cDNA was added. A further 100 µl of 1X TE was added before collecting 200 µl fractions from the column. A 10 µl aliquot was taken from every fraction containing any radioactivity. These

samples were subjected to non denaturing agarose gel electrophoresis and blotted as previously described (section 2.14.2). Those fractions containing unincorporated radioactivity were discarded and the remaining fractions were pooled together and ethanol precipitated. The resulting pellet was resuspended in a small volume (10-20 µl) of 1X TE. To ensure that the cDNA was blunt ended prior to ligation of the linkers the Klenow fragment of DNA polymerase 1 was used as described in section 2.11.

2.18 Preparation of Sugar Beet cDNA Libraries

2.18.1 PCR Amplified cDNA

To establish the feasibility of PCR amplification of blunt ended cDNA using primers JB 1 and JB 2 λ DNA digested with *Hind* III was used as a control. Primers JB 1 and JB 2 were annealed by incubating 10 µg of JB 1 with 5 µg of JB 2 in 5 µl of 1X TE containing 100 mM NaCl at 70°C and allowing them to cool, over a period of 4-5 h, to room temperature. The annealed oligonucleotide primers were subjected to ligating conditions with a sample of λ DNA digested with *Hind* III (λ/*Hind* III DNA) which had been blunt-ended using the Klenow fragment of DNA polymerase I. Several control ligations using λ/*Hind* III DNA were set up as follows;

Non Self Ligation Control

i) λ/*Hind* III DNA incubated overnight at 12°C in ligation buffer but in the absence of any ligase enzyme

ii) λ/*Hind* III DNA incubated overnight at 12°C in ligation buffer in the presence of

T4 DNA ligase.

iii) λ/*Hind* III DNA which had been blunt-ended using the Klenow fragment of DNA polymerase I was incubated overnight at 12°C in ligation buffer in the presence of T4 DNA ligase

iv) λ/*Hind* III DNA which had been blunt-ended using the Klenow fragment of DNA polymerase I was incubated overnight at 12°C in ligation buffer in the presence of T4 DNA ligase and the annealed JB 1 and JB 2 primers.

PCR Amplification Control

λ DNA digested with *Hind* III and ligated to the annealed primers was subjected to agarose gel electrophoresis and individual bands were removed from the gel with a sterile scalpel. The DNA from each gel slice was eluted by centrifugation at 5,000 xg for 20 min as described in section 2.8.6. PCR amplification of the DNA using the cycling procedure designed for use with the JB 1 primer, described in section 2.15, was performed on an undiluted sample eluted from the gel and a 1:10 dilution of this sample.

Leaf cDNA was next used to confirm that the amplification procedure would be successful with a sample of multiple mixed cDNA species. Blunt-ended leaf cDNA was ligated to the annealed primers and passed down a BIO-GEL A-150 column, prepared in a similar manner to the sephadex G-50 column described in section 2.12. A 1 µl aliquot was removed from all fractions off the column containing any radioactivity and subjected to PCR amplification directly. A further 10 µl aliquot was removed from each fraction and subjected to agarose gel electrophoresis and blotted as described in section 2.14.2. The remainder of the fractions of interest were pooled together and ethanol precipitated. This sample of cDNA was subjected to electrophoresis through a 1.5% (w/v) agarose gel for a short time (20 min at

40 mA) and several slices of agarose were removed at 4 mm intervals down the gel, and the DNA eluted as previously described (section 2.8.6). Each sample was diluted and PCR amplified. The PCR amplified products were size fractioned on a 1.5% (w/v) agarose gel by removing all DNA above 200-300 base pairs. The larger DNA was eluted from the gel as previously described (section 2.8.6) and digested with *Eco* RI. Once the digestion was completed the PCR amplified cDNA was ethanol precipitated and resuspended in 10 to 20 µl of 1X TE. The concentration of the cDNA was estimated as described in section 2.8.1. To establish that the cDNA had been digested with the *Eco* RI enzyme it was incubated under ligating conditions and an increase in size of the cDNA, when subjected to electrophoresis through a 1.5% (w/v) agarose gel, demonstrated that the *Eco* RI digestion was successful. The DNA concentration of the remainder of PCR amplified *Eco* RI digested leaf cDNA was estimated as previously described (section 2.8.1) and 50 to 250 ng of the cDNA was ligated to 1 µg of λ ZAP II DNA before packaging.

2.18.2 Preparation of Plating Cells

Cells used to plate lambda phage were prepared freshly as required by inoculating 20 ml of L-broth containing 0.2% (w/v) maltose and 100 mM MgCl, with 200 µl of an overnight culture of the desired strain. The cells were grown to an A_{600} of 0.2 and centrifuged at 3000 xg. for 20 min. The cells were resuspended in 8 to 10 ml of ice cold 10 mM $MgSO_4$ and stored on ice until required.

2.18.3 Packaging of Cloned cDNA into Bacteriophage Lambda

Between 50 and 250 ng of cDNA was ligated to 1 µg of λ ZAP II DNA as previously described (section 2.18.1). The ligated DNA was then packaged into λ bacteriophage from

the invitrogen kit supplied by Stratagene, following their instructions. The packaged DNA was diluted with 500 µl of SM phage buffer (described in section 2.4) and 10 µl of chloroform was added. Serial dilutions were made from 10 µl of this stock and incubated with 200 µl of plating cells. The inoculated cells were plated onto L-agar with top agarose containing 40 µg/ml IPTG and 40 µg/ml X-Gal and incubated at 37°C for 16 to 24 hours.

2.18.4 Estimation of Phage Concentration

The number of phage within a sample was determined by preparing serial ten-fold dilutions of the phage in SM buffer (described in section 2.4). 20 µl aliquots of these dilutions were spotted onto a lawn of the appropriate host strain of bacterium, prepared by mixing the 200 µl of plating cells with 3 ml of top agarose. The spots were allowed to dry for 20 min at room temperature, before being incubated at 37°C for 10-12 hours. The titre of the phage was then determined.

2.19 Determination of the Status of the PCR Amplified Libraries

2.19.1 Examination of Random Inserts

Plugs of phage plaques were taken at random from plates showing the greatest number of recombinant clones, using the narrow end of a Pasteur pipette. The plugs of agar containing the phage were suspended in 200 µl of SM phage buffer (described in section 2.4) and incubated at room temperature for 60 min. They were then stored at 4°C for 12 to 18 months, or until needed. The inserts from these plaques were obtained by 1 of 2 ways;

i) The insert was PCR amplified directly from the SM buffer solution containing the phage plaque using primer 1 (JB 1) in a PCR reaction containing 28 µl of the phage in solution.

ii) The above was repeated using primer 3 (PUC 1) and primer 4 (PUC 2).

2.19.2 Screening with Specific cDNAs

Several cDNA clones were used to establish whether the PCR amplified cDNA libraries were representative of standard, non PCR amplified cDNA libraries. The cDNA clones used for screening were chosen because of their various relative abundances, and are listed below;

cDNA Clone	Source
LHCP (Light harvesting complex chlorophyll *a/b* binding protein): 1.1 kbp cDNA fragment from *Zea mays* cloned into pWreck vector	Keith Edwards
Gpal 2 (Phenylalanine ammonia-lyase): 1.8 kbp cDNA fragment from *Zea mays* cloned into PUC 8	Keith Edwards
α-Tubulin: 500 bp cDNA fragment from *Zea mays* cloned into pBR 322	Keith Edwards
ALS (Acetolactate synthase): 600 bp fragment from *Pisum sativum* cloned into λ ZAP	Ian Jepson
PTA 71: 700 bp genomic DNA fragment of wheat rDNA	Simon Brown

Phage plaques from the PCR amplified cDNA libraries were plated as previously described (section 2.18.4), to a density of 250 plaque forming units (pfu) per plate, and probed as described in section 2.14.1 with the different cDNA clones. Plaques which showed

a positive signal were removed using a Pasteur pipette and replated to a lower density until a single plaque could be identified as a positive clone. Several purified positive clones screened with a single cDNA probe were PCR amplified using primer 1 (JB 1) and subjected to agarose gel electrophoresis.

2.19.3 Screening with Non-PCR amplified cDNA

To establish that the PCR amplified cDNA libraries contained cDNA clones representing transcripts found within plant tissue single stranded sugar beet apical meristem cDNA was prepared as described in section 2.17 and used to probe the PCR amplified cDNA libraries in the manner described in section 2.14.

2.20 Isolation of Apex-Specific cDNA Clones

2.20.1 Differential Screening

The PCR amplified cDNA libraries were titred as previously described (section 2.18.4) and phage plaques were plated on L-agar in 0.7% (w/v) top agarose (without IPTG and X-Gal) to a relatively low density (1000 pfu per plate) and incubated for 8-10 h. After this time plaques had begun to form but were not overlapping. Four separate lifts were taken from each plate as described in section 2.14.1. After incubation in the pre-hybridisation solution for 2-4 h two of the filters from each plate were incubated with PCR amplified leaf cDNA probe, and the other two were incubated with PCR amplified apical meristem cDNA. The specific activity of each probe was measured using a scintillation counter and an

approximately equal number of cpm of each probe were used to screen the appropriate filters. The filters were incubated and washed as previously described (section 2.14.1). Once the filters were no longer giving more than 10 cps, as measured by a Geiger-Müller counter, they were exposed to Kodak autoradiography film for the time indicated.

Any plaques showing an enhanced positive signal with the PCR amplified apical meristem probe were removed with the sharp end of a Pasteur pipette and stored in 500 µl of SM phage buffer (described in section 2.4) containing 1% (v/v) chloroform. This solution was left at room temperature for 1 h after which a 3 µl aliquot was removed and dispensed onto a lawn of the appropriate strain of *E.coli*. The spots were allowed to dry for 30 min before being incubated at 37°C for 8-10 h. After this time, in the area where the aliquot of phage buffer was dispensed, a "giant plaque" could be seen, brought about by the presence of many phage particles concentrated into a relatively small area. This procedure was also performed with a number of plaques which initially did not show a signal with either probe. This procedure is referred to as "cold plaquing" and is described below.

2.20.2 Screening of 'Cold' Plaques (Scott *et al.*, 1991)

It has been demonstrated that a percentage of phage plaques not showing a positive result with either probe may contain sequences represented at a very low level in the positive probe. For this reason a number of random 'cold' plaques were examined after first amplifying the inserts by producing "giant plaques" as described above. This amplified DNA was then screened as before. Any clones which displayed a positive result with the apical meristem probe were used to probe a northern blot as previously described (section 2.14.4).

2.20.3 Production of a Subtracted Probe

The use of differential probes produces a high percentage of background positive plaques common to both species of probe. To eliminate these common clones a subtractive procedure was used incorporating biotin and avidin (a protein with four high affinity binding sites for biotin). Both the avidin and biotin were purchased from Gibco/BRL, UK Limited. A population of cDNA labelled with biotin, as previously described, was subjected to the following subtraction procedure.

The biotin labelled leaf cDNA was resuspended in H_2O to a concentration of 0.5 µg/µl. 2.5 µg of this leaf cDNA was mixed with 25 ng of apical meristem PCR amplified cDNA (trace labelled with [α-^{32}P] dCTP) in 5 µl of HS solution:

1.5 M NaCl

50 mM HEPES (pH 7.5)

10 µM EDTA (pH 8.0)

0.2% (w/v) SDS

The cDNA in the HS solution was overlaid with 50 µl of paraffin oil and incubated at 95°C for 2 min. The denatured DNA solution was then incubated at 65°C for at least 20 h. After this latter incubation the renatured cDNA mixture was diluted with 50 µl of HE solution (HS solution minus the NaCl and SDS) and incubated at 55°C for 20 min to allow cDNA species to anneal completely. NaCl (2 M) was then added to a final concentration of 0.5 M to aid the binding of the avidin molecules to the biotin labelled cDNA. 5 µg of avidin was then added and the solution was incubated at room temperature for 20 min. After this final incubation the solution was phenol extracted and ethanol precipitated as described in sections 2.8.3/2.8.4. The radiolabelled precipitated cDNA remaining was air dried and

resuspended in 5 µl of HS solution. The above procedure was repeated up to six times. After every second round of subtraction a 1 µl aliquot of precipitated cDNA was removed and subjected to 35 rounds of PCR amplification with primer 1 as described in section 2.15.

CHAPTER 3

RESULTS

3.1 Development of Methods to Produce PCR Amplified cDNA Libraries from *Beta vulgaris*.

As described in section 1.10 the initial objective of this study was to make cDNA libraries representing genes expressed in the shoot apex of *Beta vulgaris*. Because of the very small amount of RNA likely to be obtained from this tissue PCR was employed to amplify cDNA prior to cloning. This section, therefore, describes the development of the techniques employed in the construction of PCR amplified cDNA libraries from small amounts of *Beta vulgaris* tissue. To establish the conditions necessary for such library construction experiments were initially performed with the more abundant leaf RNA.

3.1.1 RNA

It is essential when synthesising cDNA that the RNA sample used is clean, and not degraded. Most commonly an absorption spectrum is used to demonstrate that the peak of absorbance is at a value of 260 nm and that the A260/280 ratio is within the expected range (between 1.8 and 2.0). To establish the degree of degradation of the RNA separation of the RNA sample on a denaturing agarose gel alongside RNA size markers is more useful.

Fig 3.1 shows a 10 μg sample of leaf RNA separated on such a gel alongside 0.24-9.5 kb ladder RNA markers (Gibco/BRL). From the gel in Fig 3.1 it is apparent that the 18S and 25S rRNA bands have a molecular size of 1.6-1.7 Kb and 2.8-3.0 Kb respectively. These values are within the expected size range and, therefore, indicate that substantial degradation of the leaf RNA sample shown has not occurred.

3.1.2 cDNA Synthesis

Having established that the RNA was sufficiently intact, it was possible to proceed with the production of double stranded cDNA using total RNA as indicated in section 2.17. It was decided not to enrich for poly (A^+) RNA here since that would have involved one further step in the method which could have led to loss of material. As the eventual aim of this study was to use small amounts of apical meristem RNA this step was seen as undesirable. The use of total RNA was not expected to present a problem since the cDNA synthesis procedure incorporated oligo dT as a primer for synthesis of the first strand of the cDNA, selecting for poly (A^+) RNA. Samples containing 20, 50 and 100 μg of total RNA were, therefore, used to establish the optimum RNA concentration for cDNA synthesis using the cDNA synthesis kit supplied by Amersham. The values chosen were based on data from Lu and Werner (1988) who stated that approximately 70 μg of total RNA gave optimum cDNA synthesis in their system.

Fig 3.2 shows an increase in radiolabelled cDNA synthesised with an increase in concentration of total leaf RNA from 20-50 μg, but 100 μg of total RNA gave no further increase in cDNA synthesised, and actually appeared to inhibit the process. Therefore, in all subsequent experiments 50 μg of total RNA was used in the synthesis of cDNA.

Fig 3.1

A comparison of the size of the *Beta vulgaris* leaf RNA (lane 1) with 0.24-9.5 kb RNA ladder markers (Gibco/BRL). The ladder is a series of six synthetic poly (A) tailed RNAs, five of which are shown here (lane M).

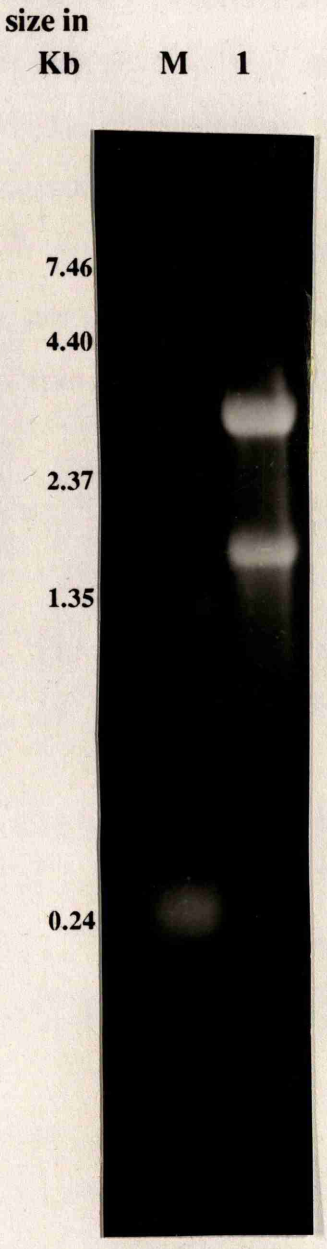

size in
Kb
M
1
7.46
4.40
2.37
1.35
0.24

Fig 3.2 ***Beta vulgaris*** **Leaf cDNA Synthesised from Total Leaf RNA**

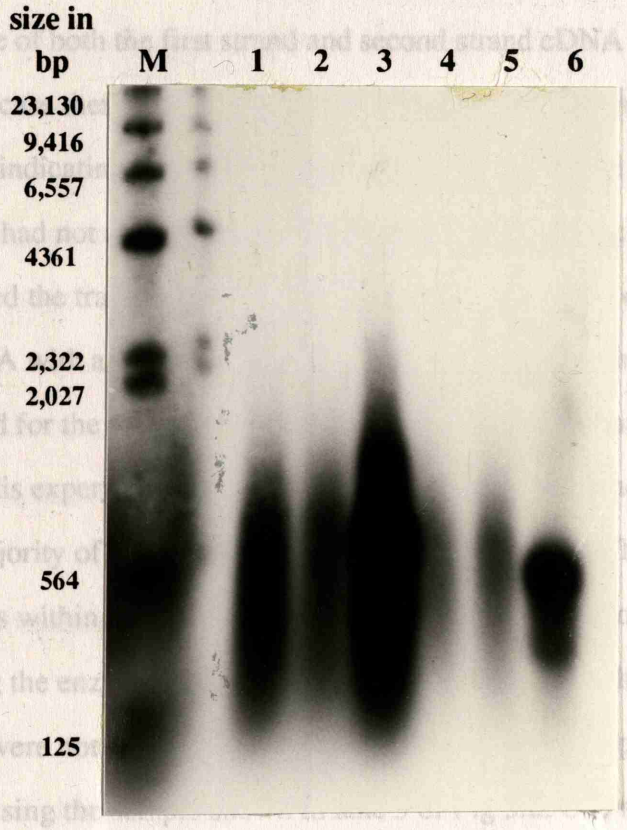
Autoradiograph of a blot of samples of single and double stranded cDNA (internally radio-labelled with ^{32}P) synthesised from increasingly higher quantities of total RNA from *Beta vulgaris* leaf tissue (as described in section 2.17). The samples were separated on a 1.5% (w/v) denaturing agarose gel then transferred by blotting onto Hybond-N nylon membrane (Amersham) before being exposed to X-ray film for 48 h. The resultant autoradiograph is represented here.

Lane M, λ DNA, digested with *Hind* III and end labelled with [α-^{32}P] dCTP

Lanes 1&2, double and single stranded cDNA respectively, prepared from 20 μg of *Beta vulgaris* leaf RNA.

Lanes 3&4, double and single stranded cDNA respectively, prepared from 50 μg of *Beta vulgaris* leaf RNA

Lanes 5&6, double and single stranded cDNA respectively, prepared from 100 μg of *Beta vulgaris* leaf RNA


size in bp
M
1
2
3
4
5
6
23,130
9,416
6,557
4361
2,322
2,027
564
125

The size of the cDNA synthesised is another important factor in the production of representative cDNA libraries. In general the cDNA synthesised should range in size from a few hundred nucleotide pairs to 8-10 kbp, the bulk of which should lie between 1 and 2 kbp. Fig 3.2 shows the size of both the first strand and second strand cDNA from all three samples of leaf RNA. In each case there was no significant difference between the size of the first and second strand cDNA indicating that during synthesis of the first strand cDNA the reverse transcriptase enzyme had not continued on after the full length of the cDNA had been synthesised and started the transcription of a "snapped back" segment of DNA. This would have resulted in cDNA with a hairpin loop at one end, and would not have facilitated ligation of the linkers required for the cloning procedure. Fig 3.2 also shows that the double stranded cDNA produced in this experiment ranged in size from less than a hundred nucleotide pairs to 2 kbp, with the majority of the material in the range of 0.5 - 1 kbp. This range was smaller than expected but was within the right order of magnitude. Attempts to increase the size of the cDNA by altering the enzyme concentration and the timing of each of the steps in the synthesis procedure were not successful. Therefore, it was decided to proceed with the library construction using the sample shown in lane 3 of Fig 3.2. One major factor in this decision was the fact that the size of cDNA produced by PCR is limited to 3 to 4 kb.

3.1.3 Fractionation of Leaf cDNA on a Sephadex G-50 Column

Since the process of PCR is known to preferentially amplify DNA of small size (a few base pairs up to 500 bp), the leaf cDNA was passed down a Sephadex G-50 column which would also serve to remove unincorporated nucleotides from the synthesis reaction. A 10 µl aliquot from each of the 200 µl fractions passed through the column which were

observed to be radioactive (as measured by scintillation counting) was removed and analysed by electrophoresis through a 1.5% (w/v) agarose gel. The gel was subsequently dried in a Bio-Rad gel dryer at 60^{o}C and exposed to X-ray film. Fig 3.3 shows the proportion of cDNA to unincorporated radiolabel within each of these aliquots. Fractions 5, 6 and 7 (lanes 1,2, and 3 of Fig 3.3) contained no unincorporated radiolabel and a large proportion of cDNA species of a size greater than 500 bp. The remaining fractions contained unincorporated nucleotides and a high proportion of very small cDNAs. Therefore the fractions shown in lanes 1,2, and 3 were pooled together and prepared for the ligation of the appropriate linkers.

3.1.4 Development of the techniques for PCR amplification of cDNA Suitable for Cloning.

3.1.4.1 Oligonucleotide Linkers

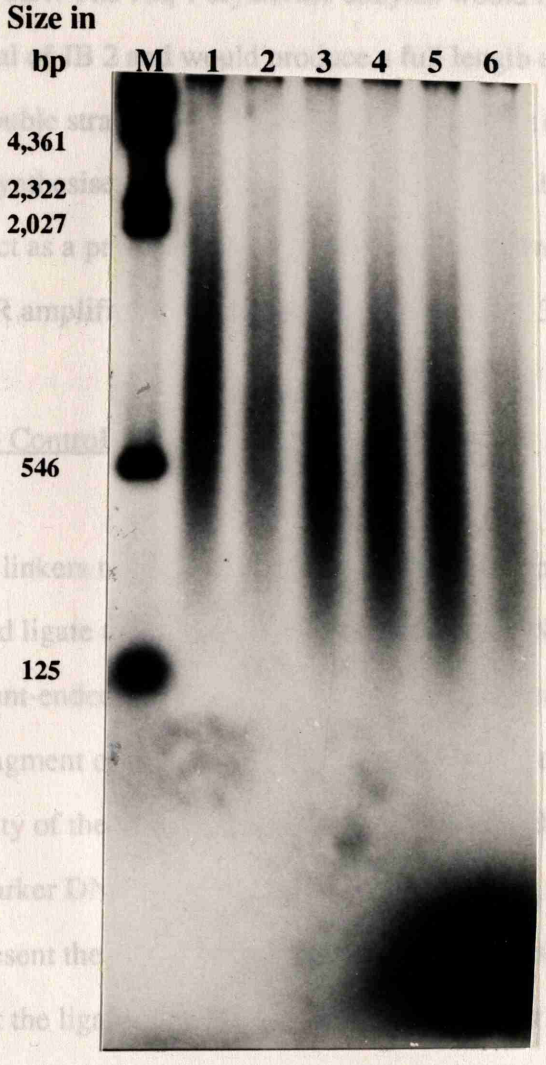
Linkers were designed (Fig 3.4a) which would enable PCR amplification of the cDNA ligated to those linkers. The oligonucleotides JB1 and JB2 were annealed to produce the required linkers. JB1 contained an *Eco* RI restriction site within its 29 nucleotide sequence to facilitate subsequent cloning of the cDNA into the chosen vector (λ ZAP II). The smaller oligonucleotide (JB2), was not phosphorylated at its 5' end, and would therefore not ligate to the 3' end of the double stranded cDNA. However, when annealed to JB1 the smaller 12-mer oligonucleotide was held in place by its complementary base pairing. JB1 would ligate to the 5' end of the double stranded cDNA since this cDNA was phosphorylated in the process of blunt-ending using the Klenow fragment of DNA polymerase I. The fact that

Fig 3.3 Size Fractionation of Leaf cDNA on a Sephadex G-50 column.

A dried gel of 10 µl aliquots from 200 µl fractions of leaf cDNA from lane 3 in Fig 3.2 after being passed through a Sephadex G-50 column. The gel was dried at 80^{o}C under vacuum after precipitation of the nucleotides on the gel with 7% TCA and then exposed to X-ray film for 24 h.

Lane M, λ DNA, digested with *Hind* III and end labelled with [α-^{32}P] dCTP

Lanes 1-6, Fractions 5-10 from the Sephadex G-50 column.


Size in bp
M
1
2
3
4
5
6
4,361
2,322
2,027
546
125
Unincorporated
Radiolabelled dCTP

JB2 was not ligated to the cDNA meant that at 72°C this smaller oligonucleotide would melt away from its complement in JB1. The *Taq* Polymerase enzyme would fill in the single strand remaining after removal of JB 2 and would produce a full length complement to JB1. On denaturing at 94°C the double stranded cDNA would melt apart. At one end of each strand of cDNA would be a synthesised sequence complementary to JB1. Therefore, the JB1 oligonucleotide was able to act as a primer for each of the strands of denatured cDNA. The sequence of events in the PCR amplification procedure is shown in Fig 3.4b.

3.1.4.2 Ligation of Linkers to Control DNA

Before ligation of the linkers to the leaf cDNA it was essential to show that the linkers described above would ligate to blunt-ended double stranded DNA. Using λ/*Hind* III marker DNA that was not blunt-ended, and some that had been subjected to a blunt-ending reaction using the Klenow fragment of DNA polymerase I, four separate treatments were performed to test the suitability of the ligase enzyme, and to show that the oligonucleotide linkers would ligate to the marker DNA. The results are shown in Fig 3.5 and demonstrate that when no linkers were present the ligase was able to ligate the non-blunt-ended marker DNA. This demonstrated that the ligase enzyme was working. However, the efficiency of ligation of the blunt-ended marker DNA was lower than that for the non-blunt-ended DNA. The most interesting finding from Fig 3.5 was that in the presence of the JB linkers self ligation of the marker DNA was reduced substantially. This could have been caused by the linkers being ligated to the marker DNA and inhibiting self ligation. Due to the non-complementary arrangement of the overhang left by JB1, when annealed to JB2, ligation of the linkers to themselves should not occur, as is demonstrated in lane 4 of Fig 3.5.

Fig 3.4 **Oligonucleotide Linker Sequence and PCR Protocol Employed in this Study**

a) shows the sequence of the two oligonucleotides used in this study and the position of the *Eco* RI restriction site

b) shows the sequence of events involved in the PCR protocol employed in this study. **3.4 i)**. Once the oligonucleotide linkers have been ligated to the double stranded cDNA of interest the PCR reaction is heated to 72^{o}C. This allows the smaller linker (JB 2) to melt off and *Taq* polymerase fills in the gap left by this linker.

3.4 ii) The double stranded cDNA is then denatured at 94^{o}C

3.4 iii) &iv) The larger oligonucleotide JB 1 acts as a primer for *Taq* polymerase to synthesise a new complementary strand of cDNA

a) *Eco* RI site

JB1 5' ATGCCTAGG/AATTCCGATTTAGCCTCATA 3'

JB2 3' AAATCGGAGTAT 5'

b)

JB1 Double Stranded cDNA JB2

5'--------3' 5'---------- 3' 5'---- 3'

3'---- 5' 3'---------- 5' 3'--------5'

JB2 JB1

3.4i Heat to 72°C for 5 min

Taq polymerase fills in

JB1 Double stranded cDNA where JB2 melts off

5'--------3' 5'---------- 3' 5'-------- 3'

3'--------5' 3'---------- 5' 3'-------- 5'

3.4ii Denature at 94°C

JB1 single stranded cDNA Complement to JB1

5'--------3' 5'---------- 3' 5'--------3'

3.4.iii Cool to 68°C for 1 min

JB1 single stranded cDNA JB1 primer

3'-------5'

5'--------3' 5'---------- 3' 5'-------3'

The JB1 oligonucleotide primer anneals to the JB1 complement produced in step 3.4i by the melting off of JB2 and filling in of the overhang by *Taq* polymerase

Heat to 72°C for 1.1 min

3.4 iv Complement to JB1 double stranded cDNA JB1 primer

3'--------5' 3'---------- 5' 3'--------5'

5'--------3' 5'---------- 3' 5'--------3'

JB1 complement to JB1

Repeat steps 3.4 ii) iii) and iv)for 35 cycles before finally heating to 72°C for 10 min

Fig 3.5 Ligation of Linkers to Control DNA

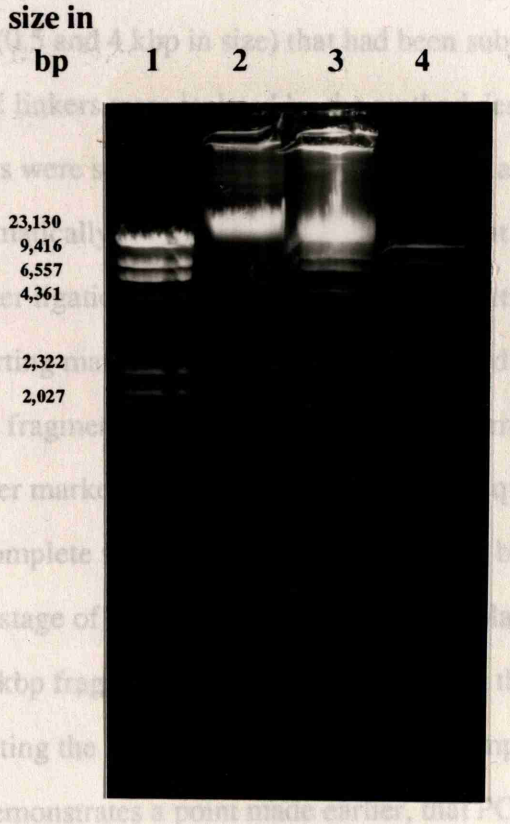
Photograph of an ethidium bromide stained 1.5 % (w/v) agarose gel of ligation reactions using λ/*Hind* III DNA (λ DNA digested with *Hind* III) treated as follows;

Lane 1, untreated control λ/Hind III. i.e. λ DNA digested with *Hind* III.

Lane 2, λ DNA digested with *Hind* III and then ligated.

Lane 3, λ DNA digested with *Hind* III, blunt-ended and then ligated.

Lane 4, λ DNA digested *Hind* III, blunt-ended and ligated in the presence of an excess of JB1 and JB2 oligonucleotide linkers


size in bp
1
2
3
4
23,130
9,416
6,557
4,361
2,322
2,027

3.1.4.3 PCR Amplification of λ *Hind* III marker DNA, Blunt-Ended and Ligated to the JB Linkers

The results described in section 3.1.4.2 above indicate that the JB linkers were successfully ligated to the marker DNA, but as a definitive method of demonstrating this fact two marker DNA fragments (0.5 and 4 kbp in size) that had been subjected to ligation in the presence of the JB 1 and JB 2 linkers were isolated by the method described in section 2.8.6. Aliquots of these two samples were subjected to PCR using JB 1 as a primer, as described in section 2.18 and shown schematically in Fig 3.4b. Fig 3.6 shows that the 0.5 kbp DNA fragment (actually 622 bp after ligation of the primers) was preferentially amplified in both samples, even though the starting material should not have contained this lower sized marker when the 4 kbp marker DNA fragment was subjected to PCR. This may have been caused by the contamination of the larger marker DNA fragment with a small quantity of the 0.5 kbp fragment of DNA due to incomplete separation of the marker DNA by electrophoresis, or contamination at some other stage of the experiment. The latter explanation seems more likely since it is only the 0.5 kbp fragment of marker DNA, and not the 2.0 kbp nor 2.3 kbp fragment which is contaminating the sample produced after PCR amplifying the 4 kbp marker DNA sample. This demonstrates a point made earlier, that PCR preferentially amplifies small sized DNA, and any small sized DNA present in a sample would not be representatively amplified. Besides emphasising this important point, the experiment shown in Fig 3.6 also demonstrates that the system described in Fig 3.4 was feasible, since amplification of both of the marker DNA fragments isolated was observed.

Fig 3.6 **PCR Amplification of Control Marker DNA with JB1 and JB2 Oligonucleotide Linkers Ligated.**

Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel showing the products of 35 rounds of PCR amplification of aliquots of samples containing 0.5 and 4 kbp fragments of λ DNA digested with *Hind* III.

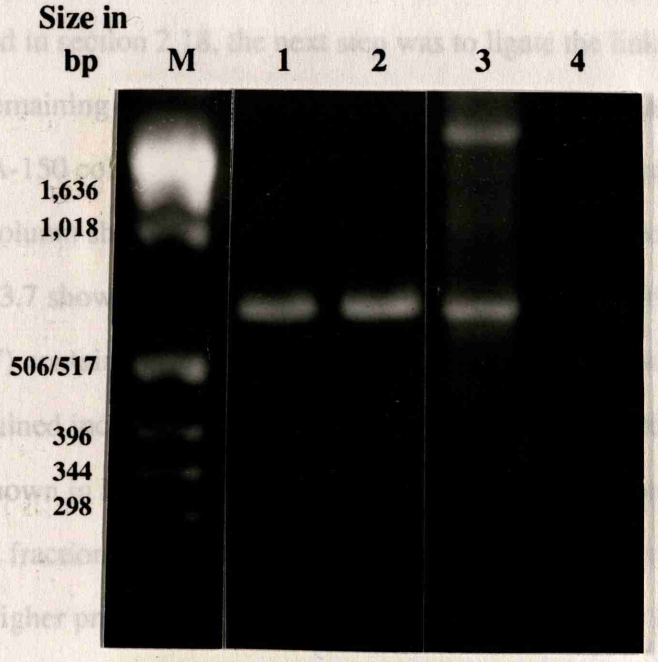
Lane M, 1 kbp "ladder" marker DNA (Gibco/BRL). The bands of the ladder each contain 1-12 repeats of a 1,018 bp DNA fragment. In addition the ladder also contains additional DNA fragments that range from 75-1,636 bp

Lane 1, DNA produced after PCR amplification of a 2.5 µl aliquot of 0.5 kbp marker DNA with the JB 1 and JB 2 linkers ligated

Lane 2, DNA produced after PCR amplification of a 2.5 µl aliquot of a 1:10 dilution of the 0.5 kbp marker DNA with the JB 1 and JB 2 linkers ligated

Lane 3, DNA produced after PCR amplification of a 2.5 µl aliquot of 4 kbp marker DNA with the JB 1 and JB 2 linkers ligated

Lane 4, DNA produced after PCR amplification of a 2.5 µl aliquot of a 1:10 dilution of the 4 kbp marker DNA with the JB 1 and JB 2 linkers ligated


Size in bp
M
1
2
3
4
1,636
1,018
506/517
396
344
298

3.1.4.4 Ligation of Linkers to the cDNA

Having established that the linker ligation was successful with the control marker DNA, and that the linkers could be successfully used for the amplification of DNA by PCR using the protocol described in section 2.18, the next step was to ligate the linkers to the leaf cDNA. To eliminate any remaining cDNAs of a small size, the leaf cDNA ligation mix was passed through a Bio Gel A-150 column. A small aliquot was removed from each of the 100 µl fractions from the column shown to be radioactive and subjected to electrophoresis and Southern blotting. Fig 3.7 shows that the DNA from fractions 6,7, and 8 from the column (lanes 1,2, and 3, in Fig 3.7) contained cDNA of an acceptable size distribution (150 bp - 6 kbp). Other fractions contained increasingly smaller cDNA. A further 2.5 µl aliquot was taken from each fraction shown in Fig 3.7 and amplified using the same protocol as for the control marker DNA. Each fraction contained amplifiable material, as shown in Fig 3.8, with a general trend towards a higher proportion of small cDNA species within the fractions that came off the column later. The range of the size of the amplified cDNA was from a few hundred bp to over 2 kbp (as measured against 1kbp "ladder" marker DNA). The range and size of the cDNA in Figs 3.7 and 3.8 are very similar indicating that the amplification was accurate. Therefore the samples shown to contain a small proportion of small cDNA species (lanes 1,2 and 3 from Fig 3.8) were pooled and used for cloning into λ ZAP II. The remaining 87.5 µl of leaf cDNA material from each of these 3 fractions was stored for later amplification as required. Therefore, the results from Fig 3.8 demonstrate that from 50 µg of total leaf RNA an unlimited supply of cDNA was available.

Fig 3.7 Ligation of Linkers to the Leaf cDNA

An autoradiograph of a blot of leaf cDNA that had been size fractionated through a Bio Gel A-150 column after ligation of the JB1 and JB2 oligonucleotide linkers. From each 100 µl fraction collected from the column and shown to be radioactive (as measured using a Geiger-Müller counter), a 10 µl aliquot was removed and separated on a 1.5% (w/v) agarose gel. The gel was blotted onto Hybond-N nylon and exposed to X-ray film for 48 h.

Lane M, λ DNA, digested with *Hind* III and end labelled with [α-^{32}P] dCTP

Lanes 1-11, Fractions 6-16 from the Bio Gel A- 150 column

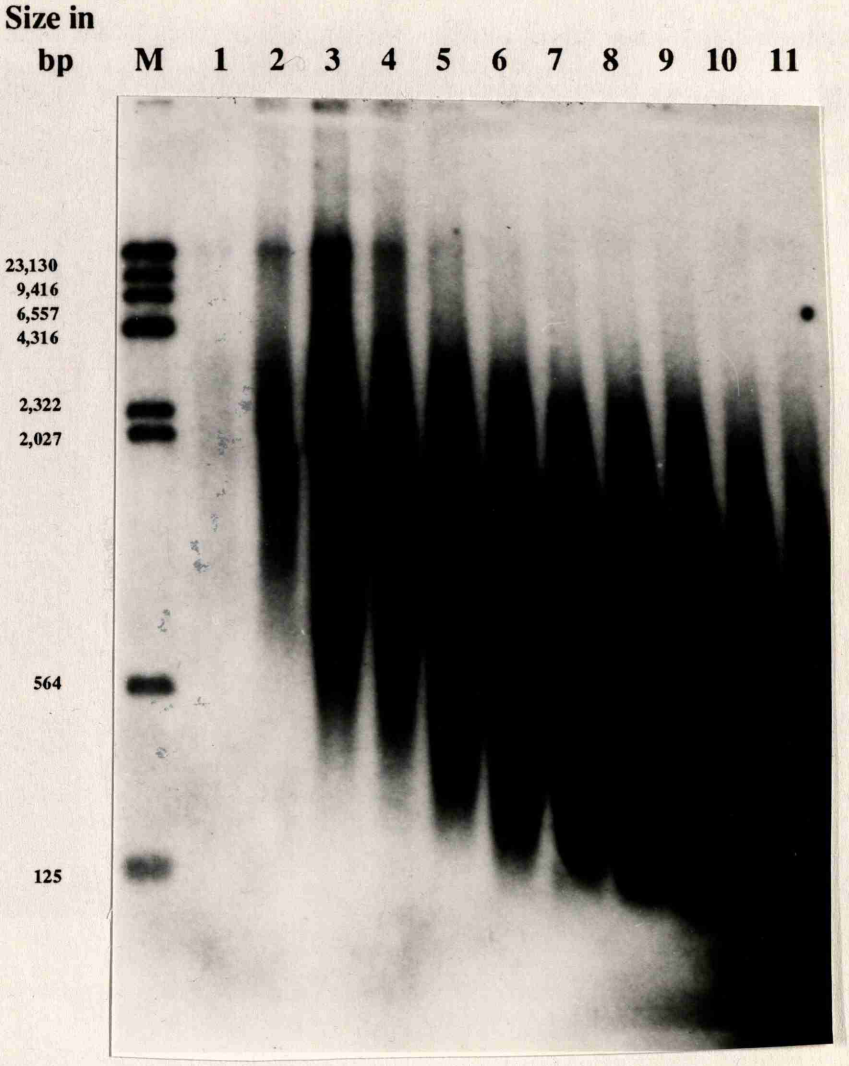
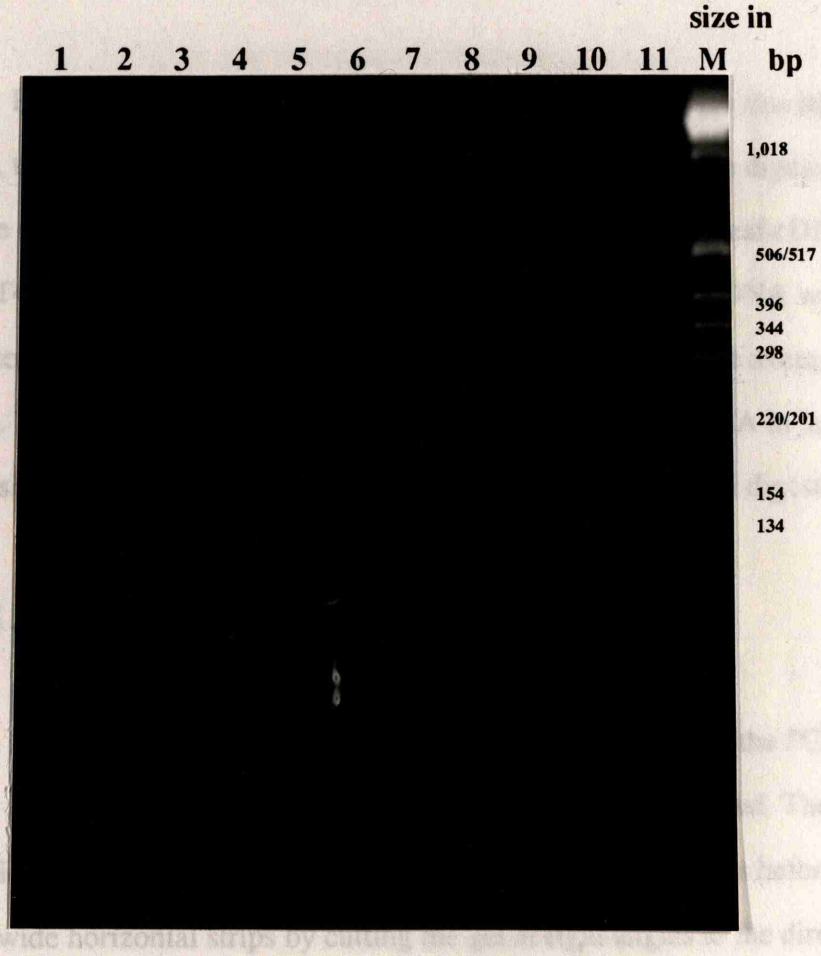

Size in bp
M
1
2
3
4
5
6
7
8
9
10
11
23,130
9,416
6,557
4,316
2,322
2,027
564
125

Fig 3.8 PCR Amplification of Leaf cDNA

Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel containing the products of PCR amplification of 2.5 µl aliquots of fractions 6-16 from the Bio-Gel A-150 column containing linker-ligated leaf cDNA. The aliquots of leaf cDNA were subjected to 35 cycles of PCR using the JB 1 oligonucleotide primer and 10 µl aliquots of these products were separated on an agarose gel.

Lanes 1-11, PCR amplified cDNA products obtained on subjecting 2.5 µl aliquots of solution from lanes 1-11 in Fig 3.7 to 35 cycles of PCR

Lane M, 1 kb "ladder" marker DNA (Gibco/BRL)


size in
1 2 3 4 5 6 7 8 9 10 11 M bp
1,018
506/517
396
344
298
220/201
154
134

3.1.5 Development of the Cloning Procedure for PCR Amplified cDNA.

3.1.5.1 *Eco* R1 Digestion of the Leaf cDNA

For the PCR amplified leaf cDNA to be inserted into the *Eco* RI digested λ ZAP II vector, the *Eco* RI restriction site within the JB linkers had to be digested. To demonstrate that the digestion had been successful digested PCR amplified leaf cDNA was self ligated using T4 DNA ligase. If the digestion was successful the leaf cDNA would have been able to ligate to itself, and thus there would have been an increase in the average size of the leaf cDNA. This is shown to have occurred in Fig 3.9. The leaf cDNA in lane 3 is of an overall larger size than that in lane 2 which was not subjected to *Eco* RI digestion.

3.1.5.2 Removal of Small Sized cDNA Prior to Cloning

In a final attempt to eliminate small cDNA species from the PCR amplified leaf cDNA sample a final size fractionation experiment was performed. The *Eco* RI digested PCR amplified leaf cDNA was subjected to electrophoresis for 20 min before being sectioned into 4 mm wide horizontal strips by cutting the gel at right angles to the direction of migration, and the DNA eluted from these strips according to the method described in section 2.8.6. An aliquot of each fraction from the gel was examined on a second 1.5% (w/v) agarose gel. Fig 3.10 shows the size of the cDNA contained within each of the five strips of gel measured against 1 kbp DNA "ladder" markers. There is an obvious decrease in the size of the cDNA from fraction 2-4. There was no detectable DNA in fraction 1. The cDNA in fraction 2 (lane 2 of Fig 3.10) contained cDNA of a size greater than 300 bp extending to 1 kbp.

Fig 3.9 *Eco* RI Digestion of PCR Amplified Leaf cDNA

Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel containing a sample of leaf cDNA digested with *Eco* RI restriction enzyme and subjected to ligation with T4 DNA ligase compared with leaf cDNA not digested with this *Eco* RI but subjected to the same ligation conditions.

Lane 1, λ DNA digested with *Hind* III

Lane 2, Leaf cDNA not subjected to *Eco* RI digestion prior to incubation with T4 DNA ligase

Lane 3, Leaf cDNA subjected to *Eco* RI digestion prior to incubation with T4 DNA ligase

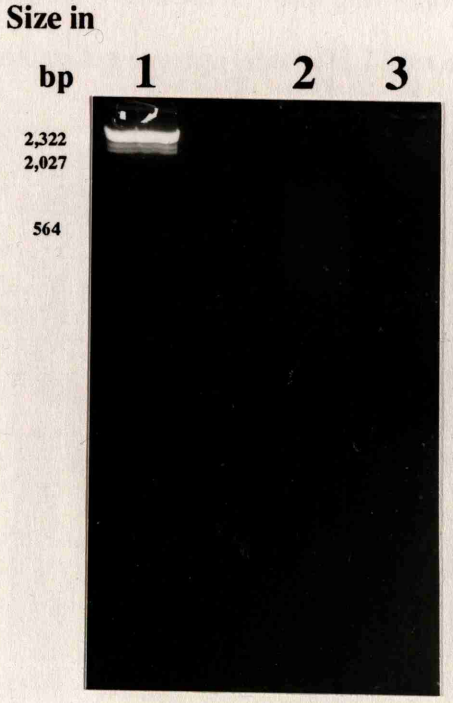
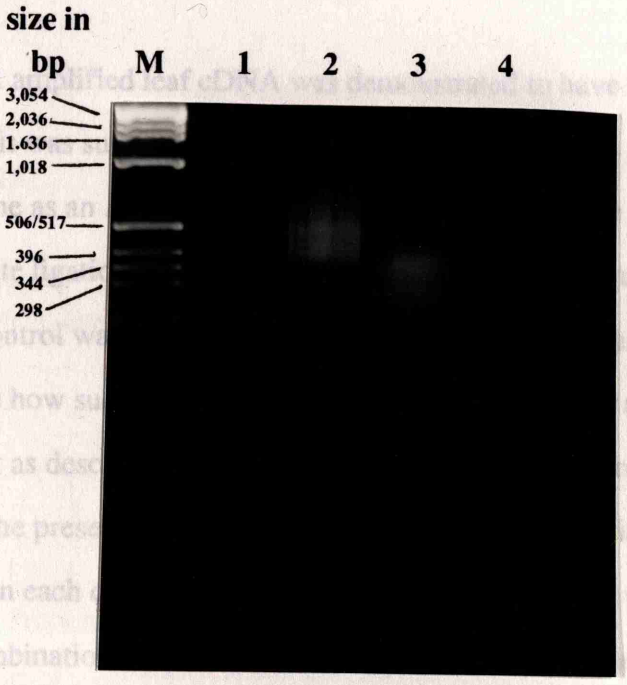

Size in
bp
1
2
3
2,322
2,027
564

Fig 3.10 Removal of Small Sized Leaf cDNA Prior to Cloning

Eco RI digested PCR amplified leaf cDNA was subjected to size fractionation by separation through a 1.5% (w/v) agarose gel and then sectioning of that gel into 4mm strips at right angles to the direction of migration of the DNA. Aliquots of the DNA isolated from these strips were run on a second 1.5% (w/v) agarose gel stained with ethidium bromide, shown here.

Lane M, 1kb "ladder" marker DNA(Gibco/BRL)

Lanes 1-4, DNA products isolated from sequential 4mm strips from the first agarose gel


size in bp
M
1
2
3
4
3,054
2,036
1.636
1,018
506/517
396
344
298

This fraction was thought to be most suitable for the subsequent cloning procedure and was, therefore, used for ligation to the λ ZAP II vector.

3.1.5.3 Ligation of PCR Amplified cDNA to λ ZAP II Phage Vector

Since the PCR amplified leaf cDNA was demonstrated to have successfully been digested with *Eco* RI it was subjected to ligation in the presence of the phage vector λ ZAP II (supplied by Stratagene as an *Eco* RI digested stock). According to the recommendation of Stratagene two separate ligations were performed with the leaf cDNA using 50 and 250 ng of DNA. The relevant control was also performed to ensure the vector was able to accept a test insert of DNA. To see how successful the ligation was, the vector was next packaged into its necessary protein coat as described in section 2.18.3. Each packaging reaction was diluted before plating out in the presence of X-Gal and IPTG to establish the titre of the library. Table 3.1 shows that in each case the ligation and packaging reactions were successful and that the greatest recombination frequency and titre were observed in the library produced with 250 ng of leaf PCR amplified cDNA with 80% recombinant clones and a titre of of $1x\ 10^6$ clones within the library.

This series of experiments, therefore, demonstrated that the techniques used here were successful in producing a PCR amplified cDNA library from small quantities of *Beta vulgaris* leaf total RNA.

3.1.6 Characterisation of the Leaf PCR Amplified cDNA Library

Since thermal cycling is able to produce large amounts of DNA from very small

Table 3.1

Amount of DNA packaged	No. of clones obtained	Recombination frequency
250 µg leaf PCR amplified cDNA	$1x10^6$	80%
50 µg leaf PCR amplified cDNA	$1.5x10^5$	60%
100 µg test insert supplied by Strategene	$1x10^8$	95%

Table 3.1 Properties of cDNA libraries in λ ZAP II prepared from different amounts of PCR amplified leaf cDNA compared with a test insert. No clones were obtained in a control packaging reaction with no insert present.

amounts of starting material (less than a few pg), and the possibility of contamination with foreign DNA cannot be dismissed (despite the rigorous controls used), it was essential to establish that the material cloned into the phage vector was PCR amplified *Beta vulgaris* leaf cDNA. It was also necessary to establish if the cDNA library was representative of transcripts present in the leaf RNA. For instance, it is possible that the library might be enriched in clones representing small transcripts because the PCR reaction favours small DNA fragments. Moreover, it is possible that PCR amplification distorts the relative abundance of cDNAs in the population. Hence, the library was screened with probes for known genes whose transcripts were assumed to be of different size and abundance in the leaf RNA.

3.1.6.1 Examination of Random Plaques

To show that the PCR amplified leaf cDNA had inserted into the vector, as indicated by the blue/white selection, 10 plaques were taken at random (including 2 blue plaques as a control) and amplified using oligos PUC 1 and PUC 2 (as described in section 2.15) designed to amplify inserts from any vector related to the pUC plasmid vector. Fig 3.11 shows the DNA that was amplified from each of these random plaques. The blue plaques produced a DNA product of about 200 bp which is caused by the fact that the PUC 1 and PUC 2 primer oligonucleotides flank the multiple cloning site of pUC related vectors leaving 100 bp of vector DNA available to be amplified. Taking this into account the average size of the insert DNA from the remaining 8 plaques was between 500 and 600 bp and ranged up to 1.3 kbp, as measured against 1 kbp "ladder" DNA markers. Therefore the procedure used here was shown to produce inserts of a reasonable size distribution.

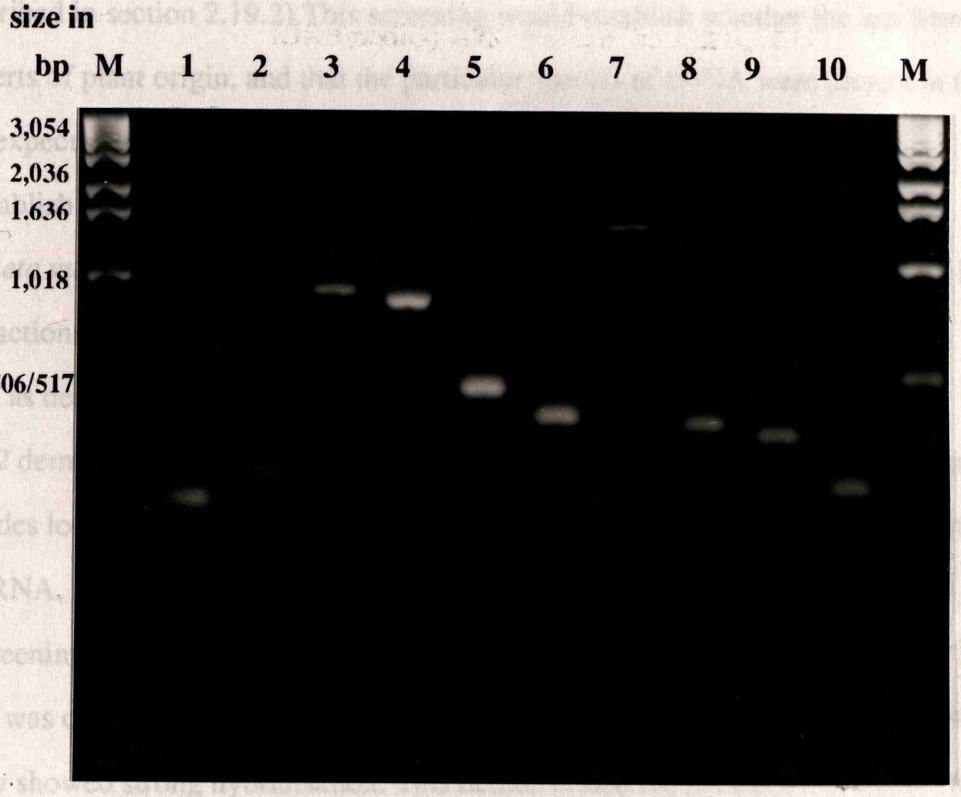
Fig 3.11 Examination of Inserts from Random Plaques of the Leaf cDNA Library.

28 µl aliquots from the SM storage buffer of 10 random clones were subjected to PCR amplification using the PUC1 and PUC 2 oligonucleotides. 10 µl aliquots of these products were separated on a 1.5% (w/v) agarose gel stained with ethidium bromide.

Lanes M, 1 kbp "ladder" marker DNA (Gibco/BRL)

Lanes 1&10, DNA products isolated from plaques showing a blue colour when plated out in the presence of X-Gal and IPTG and subjected to PCR amplification with PUC 1 and PUC 2 oligonucleotide primers

Lanes 2-9, DNA products isolated from random plaques showing a white colour when plated out in the presence of X-Gal and IPTG and subjected to PCR amplification with PUC1 and PUC 2 oligonucleotide primers


size in
bp M 1 2 3 4 5 6 7 8 9 10 M
3,054
2,036
1.636
1,018
506/517

3.1.6.2 Screening of the Leaf Library with Known DNA Probes

To show that the leaf cDNA was representatively amplified by PCR, and as such was representative of the transcripts present in the leaf RNA, three clones of plant origin were used to screen the leaf library. These were the LHCP clone, the α-tubulin clone and clone PTA 71 (described in section 2.19.2).This screening would establish whether the leaf library contained inserts of plant origin, and that the particular species of cDNA were present in the library at the expected frequency.

To establish the frequency of gene transcripts complementary to the LHCP DNA probe in the *Beta vulgaris* leaf tissue, RNA isolated from the leaf tissue used to make the leaf library was fractionated on a formaldehyde-containing agarose gel (described in section 2.8.5), blotted as described in section 2.14.4 and hybridised to the LHCP cDNA of *Zea mays*. Fig 3.12 demonstrates that the *Zea mays* LHCP cDNA hybridises to a single transcript 1400 nucleotides long (as determined using RNA markers supplied by Gibco BRL) in *Beta vulgaris* leaf RNA, and is not detectable in the root RNA.

On screening 1,000 plaques from the leaf cDNA library with the *Zea mays* LHCP DNA probe it was observed (Fig. 3.13) that approximately 5% of the cDNA clones within the leaf library showed strong hybridisation. This demonstrated the presence of cDNA clones within the leaf library that were hybridising to a clone of leaf origin, and therefore, were likely to be of leaf origin themselves. They were also present at a relatively high frequency which was expected from the nature of the clone and the data from Cuming and Bennett (1981) who reported that LHCP mRNA was approximately 2% of the total translatable poly (A^+) RNA in illuminated pea leaves. To estimate the number of true LHCP clones within the leaf library 14 random putative positive LHCP clones were plated out individually at a lower

Fig 3.12 Northern Blot of *Beta vulgaris* Leaf and Root RNA Probed with the LHCP cDNA Probe

10 μg of *Beta vulgaris* leaf RNA and 30 μg of *Beta vulgaris* root RNA were separated on a 1.5% (w/v) denaturing agarose gel. The gel was blotted onto Hybon-N nylon membrane (Amersham) and hybridised with the radiolabelled *Zea mays* LHCP cDNA clone at 65°C. The nylon membrane was washed three times in 2X SSC, 0.1% (w/v) SDS at 65°C for 20 min before being exposed to X-ray film for 48 h. Lanes M,1 and 2 show the ethidium bromide stained gel prior to blotting and lanes 3 and 4 show the autoradiograph produced from the blotted gel after hybridisation to the LHCP cDNA clone of *Zea mays*.

Lane M, 0.24-9.5 kb RNA ladder markers (Gibco/BRL). The ladder is a series of six synthetic poly (A) tailed RNAs, three of which are shown here

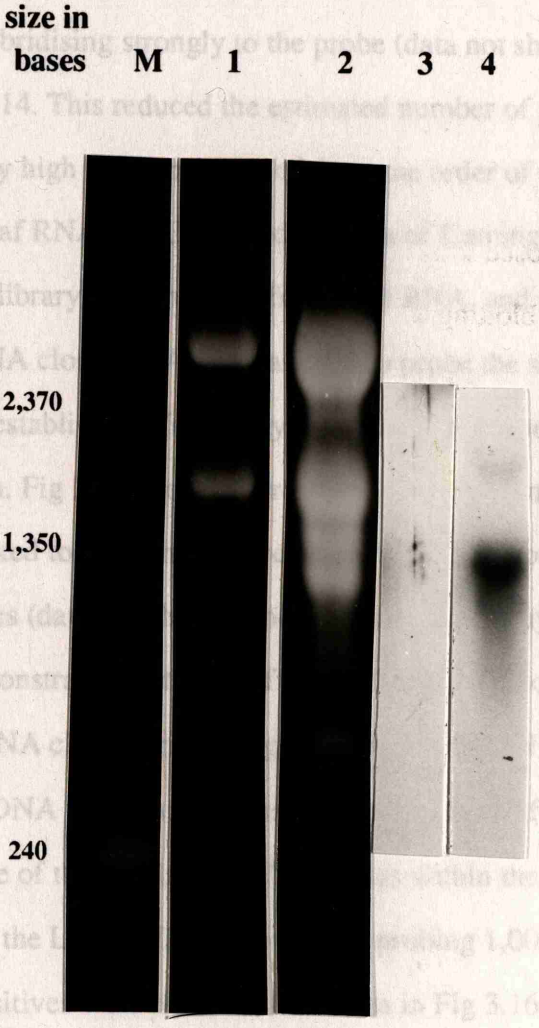
Lane 1, 30 μg of annual *Beta vulgaris* root RNA

Lane 2, 10 μg of annual *Beta vulgaris* leaf RNA

Lane 3 transcript of root RNA hybridising to the LHCP cDNA clone of *Zea mays*

Lane 4, transcript of annual leaf RNA hybridising to the LHCP cDNA clone of *Zea mays*

The transcript hybridising to the LHCP clone in lane 3 is of 1.4 kb in size. The fact that no visible hybridisation of the LHCP clone is observed in the root RNA is not surprising since what was assumed to be 30 μg of root RNA is clearly less than 10 μg when compared with the leaf RNA. This is a common problem experienced with sugar beet root RNA and subsequent experiments should have been performed using greater quantities of root RNA. However, the lack of a signal in the lane containing the root RNA after hybridisation with the LHCP probe is indicative of the lack of transcripts encoding LHCP in the roots of *Beta vulgaris*.


size in bases
M
1
2
3
4
2,370
1,350
240

density than before. These 14 clones were subjected to a second screen with the LHCP DNA probe and Fig 3.14 shows that 12 of the 14 putative positives gave a positive signal after a second screen. However after a third screen using the LHCP DNA probe, 8 of the putative positives were still hybridising strongly to the probe (data not shown). The 8 positive clones are indicated in Fig 3.14. This reduced the estimated number of positive clones to around 3%. This is still a relatively high frequency and of the same order of magnitude as expected from the hybridisation to leaf RNA (Fig 3.12) and the data of Cuming and Bennett (1981).

Since the leaf library was prepared from total RNA, and not poly (A^+)- enriched RNA, a ribosomal DNA clone (PTA 71) was used to probe the same 1,000 clones used in the LHCP experiment to establish the frequency of clones within the leaf library likely to be of ribosomal RNA origin. Fig 3.15 shows the result of this experiment, and it was observed that a number of areas looked to contain putative positive clones. However, second screening of several random plaques (data not shown) showed non-specific hybridisation to the ribosomal DNA clone. This demonstrated that the leaf cDNA library did not contain an unacceptably high proportion of cDNA clones originating from ribosomal RNA.

An α-tubulin DNA probe was also used to probe the leaf library to investigate whether the abundance of the α-tubulin cDNA clones within the library was higher or lower than that observed for the LHCP cDNA clones. On probing 1,000 cDNA clones from the leaf library 12 putative positives were observed as shown in Fig 3.16. Subjecting 4 of these putative positive clones to a second round of screening with the α-tubulin cDNA probe showed that all 4 were positive α-tubulin cDNA clones (Fig 3.17). Therefore, the frequency of this cDNA clone within the leaf library is roughly 1%. This is lower than for the LHCP clone, which is to be expected. The above evidence suggests that the leaf library contains cDNA representative of the transcripts present in leaf RNA.

Fig 3.13 **Screening of the PCR Amplified Leaf Library with the LHCP cDNA Clone from *Zea mays***

1000 clones from the PCR amplified leaf cDNA library were bound to Hybond-N nylon membrane filters (Amersham) and hybridised to the radiolabelled LHCP cDNA clone from *Zea mays* at 65^{o}C for 24 h. The nylon membrane filters were washed three times in 2X SSC 0.1% (w/v) SDS for 20 min and exposed to X-ray film for 24 h. The resultant autoradiograph is shown here.

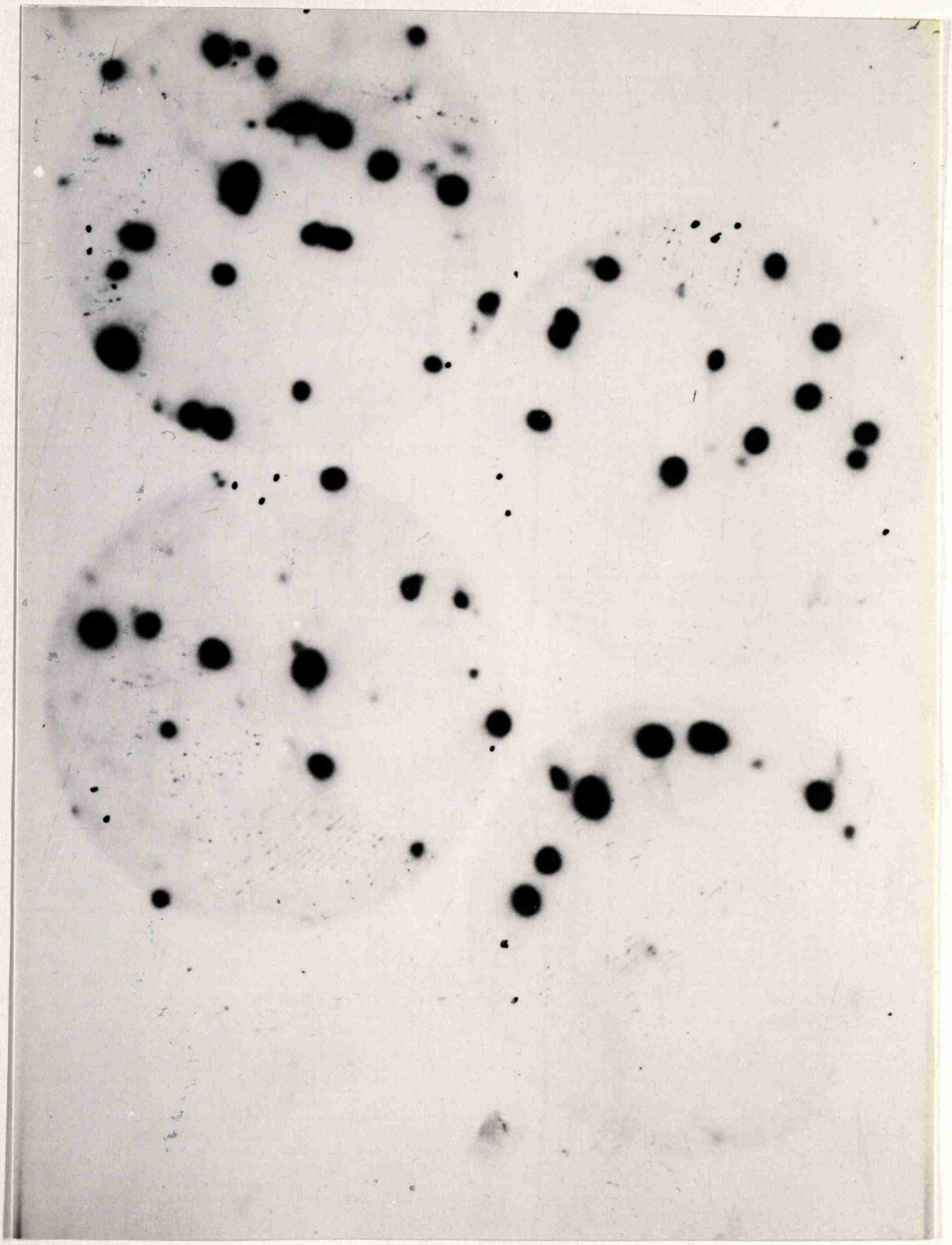


Fig 3.14 Secondary Screening of Putative Positive LHCP Clones.

14 Separate Hybond-N nylon membrane filters (Amersham) were bound to plaques from 14 putative positive LHCP cDNA clones plated out at a relatively low density. The filters were probed with the radiolabelled LHCP cDNA clone from *Zea mays* at 65^{o}C for 24 h. The filters were washed three times in 2X SSC 0.1% (w/v) SDS for 20 min before exposure to X-ray film for 24 h. The resultant autoradiograph is shown here. Those clones which were seen to be hybridising to the LHCP cDNA probe after a third round of screening are indicated with arrows.

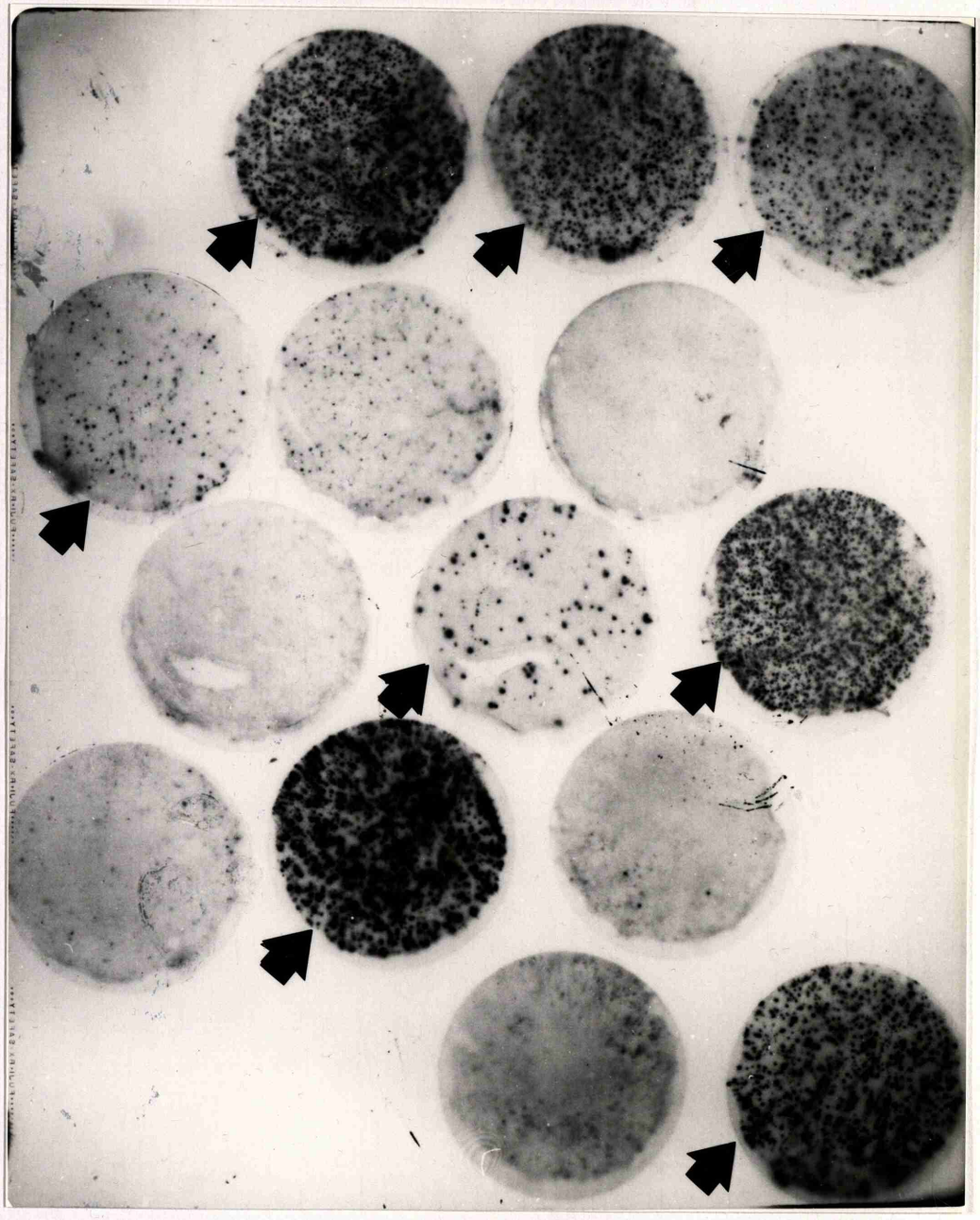


Fig 3.15 Screening of the Leaf cDNA Library with a Ribosomal DNA probe

1000 leaf PCR amplified cDNA clones were bound to Hybond-N nylon membrane (Amersham) and hybridised with the radiolabelled wheat ribosomal DNA probe PTA 71 at 65^{o}C for 24 h. The nylon membrane filters were washed three times in 2X SSC 0.1% (w/v) SDS for 20 min before exposure to X-ray film for 24 h. The resultant autoradiograph is shown here.

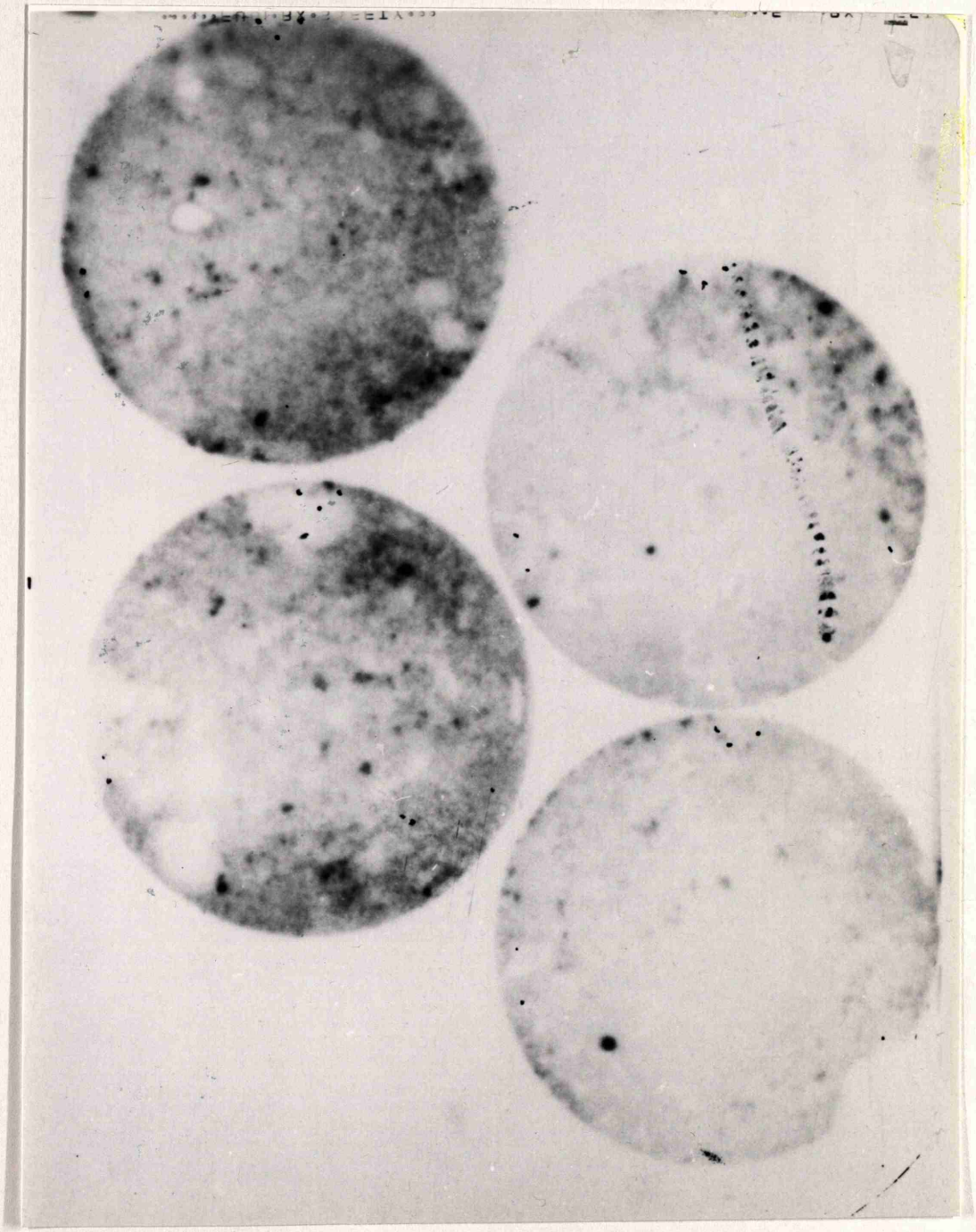


Fig 3.16 Screening of the Leaf cDNA Library with an α-Tubulin cDNA probe

1000 leaf cDNA clones were bound to Hybond-N nylon membrane filters (Amersham) and hybridised to the radiolabelled α-tubulin cDNA probe at 65°C. The nylon membrane filers were washed three times in 2X SSC, 0.1% (w/v) SDS at 65°C for 20 min before exposure to X-ray film for 48 h. The resultant autoradiograph is shown here.

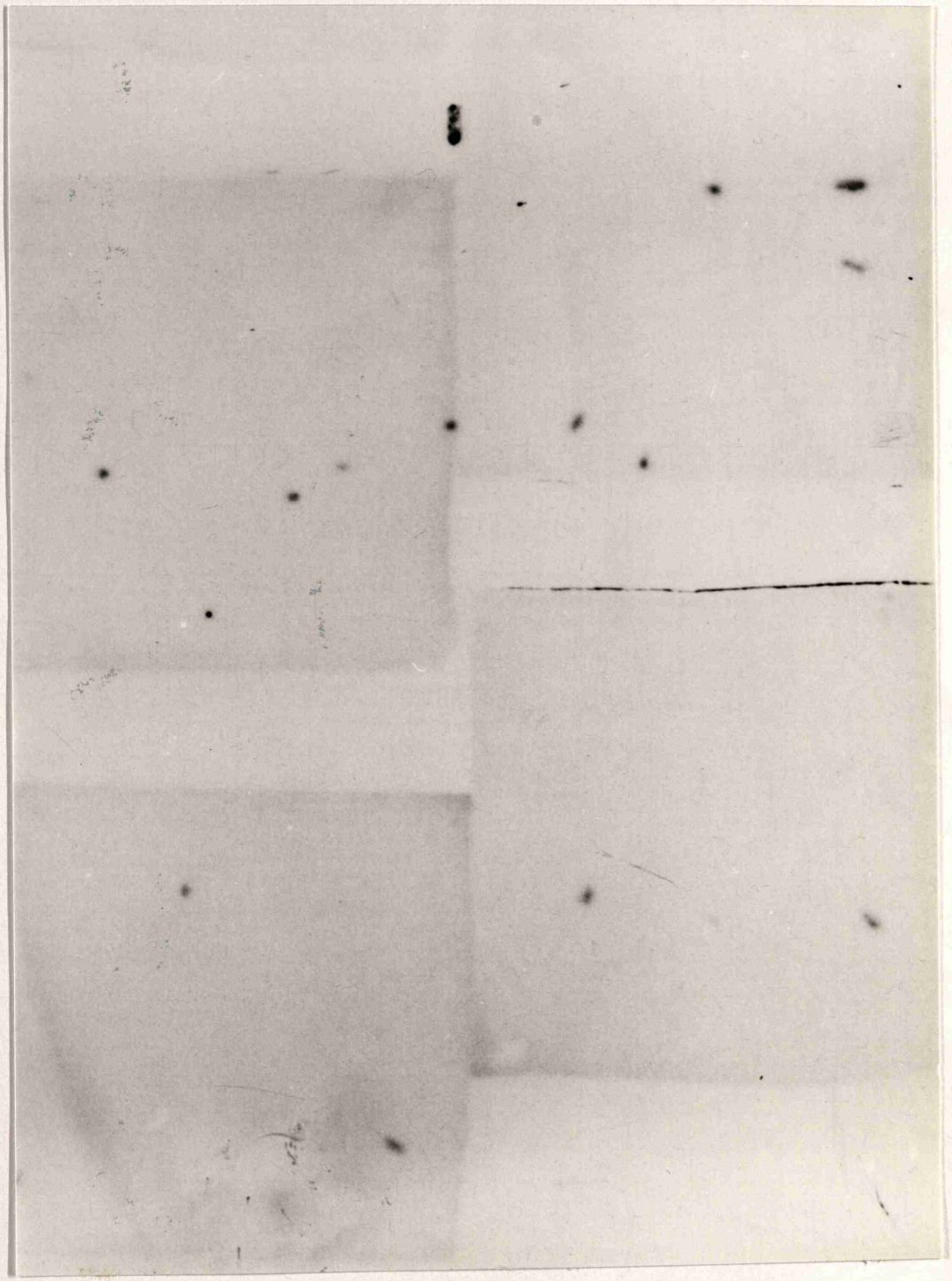
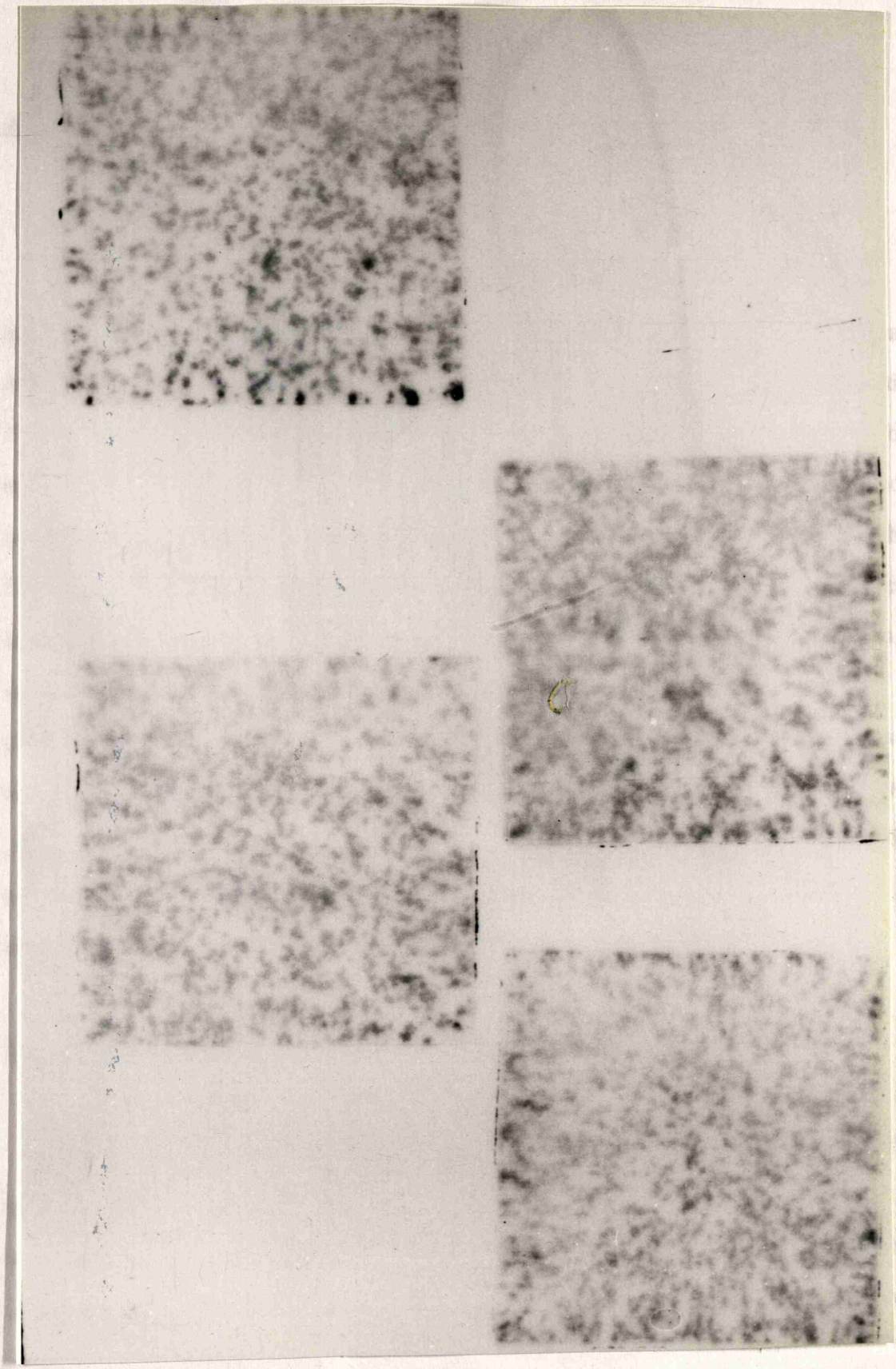


Fig 3.17 Secondary Screening of Putative Positive α-Tubulin Clones.

Plaques from 4 putative positive α-tubulin clones from the PCR amplified leaf cDNA library were plated out at a relatively low density and bound to Hybond-N nylon membrane (Amersham). The nylon membrane filters were hybridised to the radiolabelled α -tubulin cDNA probe at 65^{o}C for 24 h. The filters were washed three times in 2X SSC, 0.1% (w/v) SDS at 65^{o}C for 20 min before exposure to X-ray film for 24 h. The resultant autoradiograph is shown here.



3.2 Preparation of a PCR Amplified cDNA Library from Shoot Apical Meristem Tissue

Having developed a method of PCR amplification and cloning to produce what appears to be a representative cDNA library from 50 µg of *Beta vulgaris* leaf RNA it was decided to proceed with the production of a similar library from shoot apical meristem tissue. Taking annual *Beta vulgaris* plants grown in SD conditions at a temperature of 20°C (for the reasons explained in section 1.10) 35 shoot apical meristems were successfully dissected for small scale RNA extraction (See section 2.16 and Jepson *et al.* 1991). To demonstrate that the material isolated did contain apical meristem tissue a further two samples were dissected and embedded in wax ready for sectioning as described in section 2.7.4. Fig 3.18 shows a sample of one of these sections examined under the light microscope. The material did contain apical meristem tissue, as well as a proportion of leaf primordium and stem pith tissue. This, however, was due to the need for a larger sample for sectioning than was used to isolate RNA. Therefore, the tissue used to isolate the RNA would have contained very little contaminating tissue although non-meristematic tissue would not necessarily be absent. This tissue would, however, be enriched in RNA from apical meristems.

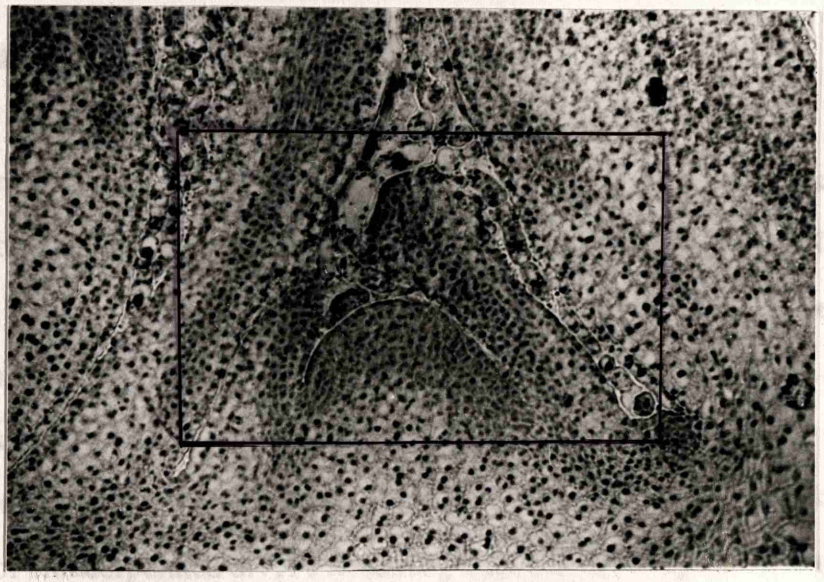
3.2.1 Isolation of RNA from Annual *Beta vulgaris* Shoot Apical Meristem Tissue

The data presented in Fig 3.2 demonstrate that 50 µg of total RNA was required for optimum cDNA synthesis. Shoot apical meristems were isolated from as many healthy, eight week old, annual, *Beta vulgaris* plants, that had experienced the desired growth conditions, as was possible at the one time. From the fifty plants chosen only 37 apical meristems were

Fig 3.18 **Examination of a Section of Annual *Beta vulgaris* Shoot Apical Meristem Tissue by Light Microscopy**

The tissue was dissected from the sugar beet plant and treated as described in section 2.7.4. The photograph shows the dissected apical meristem tissue at a magnification of x40. The inner box indicates the tissue dissected for use in isolating RNA.

Scale _____1 mm_____



successfully isolated, two of which were sectioned for Fig 3.18. A small aliquot (5%) of the RNA isolated from this apical meristem tissue was run on a 1.5% (w/v) agarose gel to establish the presence of RNA, and to determine whether this apical RNA was degraded or not. Fig 3.19 shows that RNA was successfully isolated from this tissue, and that it did not appear degraded. The visible bands are those of rRNA.

3.2.2 Preparation of Annual Shoot Apical Meristem cDNA

The remaining 95% of the RNA extracted from the dissected apices was used immediately for the preparation of double stranded cDNA. Aliquots of the cDNA produced were analysed in the same manner as for the leaf cDNA, and Fig 3.20 shows the cDNA produced from the apical tissue to be smaller than the expected size range. The majority of the cDNA was within the range 0.2-2 kbp but, as was shown in section 3.1, the small size of the cDNA should not pose a problem. The dried gel in this figure shows that there was a large amount of unincorporated dNTPs remaining after the cDNA synthesis reaction was completed.This indicates that less than 50 μg of total RNA was isolated from the apical meristem tissue.

3.2.3 Removal of Unincorporated Radiolabelled Nucleotides

As was described for the leaf cDNA library (section 3.1.2) the unincorporated radiolabelled nucleotides were removed from the cDNA reaction mix by passing it through a Sephadex G-50 column. An autoradiograph of the dried gel of 10 μl aliquots taken from 200 μl fractions run through the column is shown in Fig 3.21. Fractions 6,7, and 8 from the

Fig 3.19 **RNA Isolated from Annual *Beta vulgaris* Shoot Apical Meristems Exposed to Non-Floral Inductive Conditions (Short Days and Warm Temperatures)**

The RNA from 35 annual *Beta vulgaris* warm, short day treated apical meristems was isolated as described in section 2.16 and Jepson *et al.* (1991). Of the 10 µl of RNA solution obtained 0.5 µl was run on a 1.5% (w/v) agarose gel. The bands visible in the figure are those of the 25S and 18S ribosomal RNA.


25S RNA
18S RNA

Fig 3.20 **Annual *Beta vulgaris* Apical Meristem cDNA**

An autoradiograph of a 1.5% (w/v) agarose gel dried under vacuum at 80^{o}C after TCA precipitation of the radiolabelled polynucleotides and exposure to X-ray film for 24 h. The dried gel represents the single and double stranded cDNA synthesised from an aliquot of 95% of the RNA isolated from 35 dissected apical meristems of annual *Beta vulgaris* plants grown under warm short day conditions.

Lane M, λ DNA, digested with *Hind* III and end labelled with [α-^{32}P] dCTP

Lane 1, single stranded apical meristem cDNA

Lane 2, double stranded apical meristem cDNA

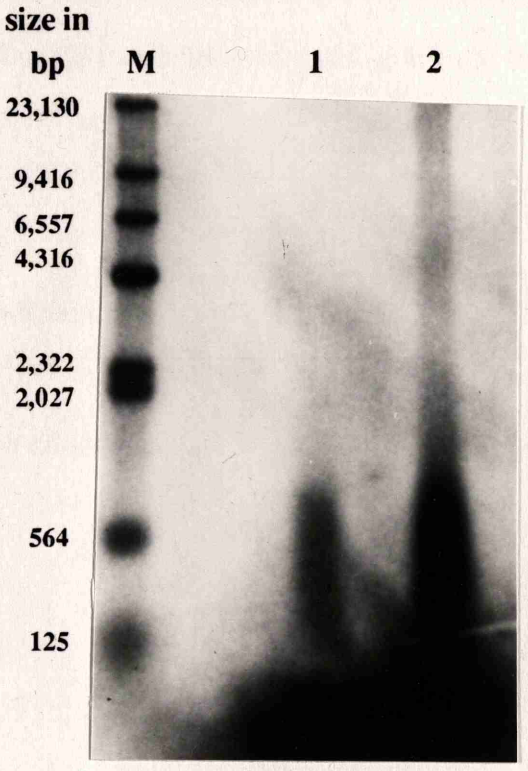
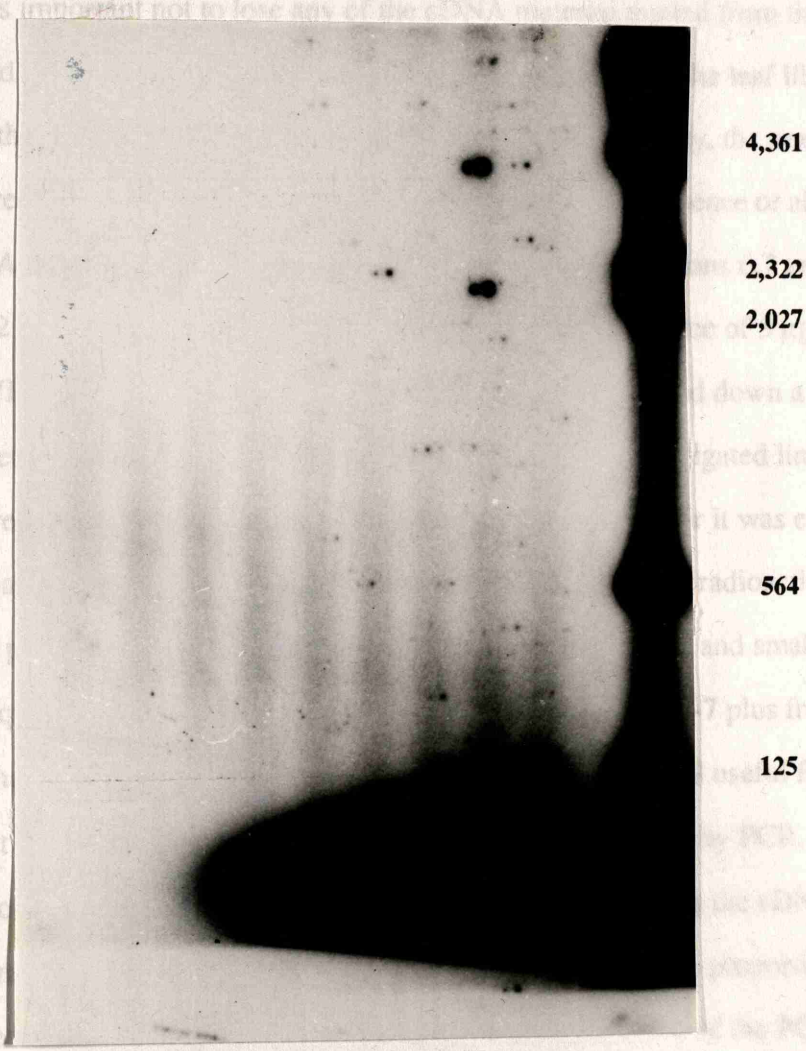

size in
bp
M
1
2
23,130
9,416
6,557
4,316
2,322
2,027
564
125
unincorporated
radiolabelled dCTP

Fig 3.21 **Size Fractionation of Apical Meristem cDNA through a Sephadex G-50 Column.**

An autoradiograph of a 1.5% (w/v) agarose gel dried under vacuum at 80^{o}C after TCA precipitation of the radiolabelled nucleotides and exposure to X-ray film for 24 h. 10 µl aliquots of cDNA from each of the 200 µl fractions passed through a Sephadex G-50 column which were shown to be radioactive (as measured using a Geiger-Müller counter) were separated through the agarose gel.

Lanes 1-9, 10 µl aliquots from 200 µl fractions 6-14 taken from the Sephadex G-50 column.

Lane M, λ DNA digested with *Hind* III and end labelled with [α-^{32}P] dCTP


size in
1 2 3 4 5 6 7 8 9 M bp
4,361
2,322
2,027
564
125
unincorporated
radiolabelled dCTP

column (lanes 1,2, and 3 of Fig. 3.21) contained cDNA with no contaminating unincorporated radiolabel, and the least small sized cDNA. These three fractions were, therefore, pooled for ligation to the JB linkers.

3.2.4 Amplification of Apical Meristem cDNA After Ligation of Linkers

Since it was important not to lose any of the cDNA material pooled from the three fractions mentioned in the previous section, and since the preparation of the leaf library had demonstrated that the method employed in this study worked satisfactorily, the next few steps were monitored using a Geiger -Müller counter to establish the presence or absence of radiolabelled cDNA, thereby retaining as much cDNA as possible. Fractions 6,7 and 8 (lanes 1,2,and 3 of Fig 3.21) were subjected to ligation conditions in the presence of 5 μg of annealed JB1 and JB2. The material from this ligation reaction was passed down a Bio Gel A-150 column to remove the unincorporated radionucleotides and the unligated linkers which may interfere with the PCR process. Using a Geiger-Müller counter it was established that fractions 4-7 passed through the Bio-Gel column contained a peak of radioactivity consistent with the presence of cDNA free from contaminating radiolabel and small sized cDNA. A small aliquot (2.5 μl) was removed from each of the fractions 4-7 plus fractions 3 and 8, which may have contained a small amount of apical cDNA material useful for cloning. Fig 3.22 shows that each of the 6 fractions contained material amplifiable by PCR. Therefore the ligation was shown to be successful in this experiment. Fig 3.22 shows the cDNA amplified in all 6 fractions to be of the right size range, with an acceptable proportion of small cDNA species. Lane 1 of Fig 3.22 demonstrates that contamination of the PCR reagents with foreign DNA had not occurred since when no apical cDNA was present, no

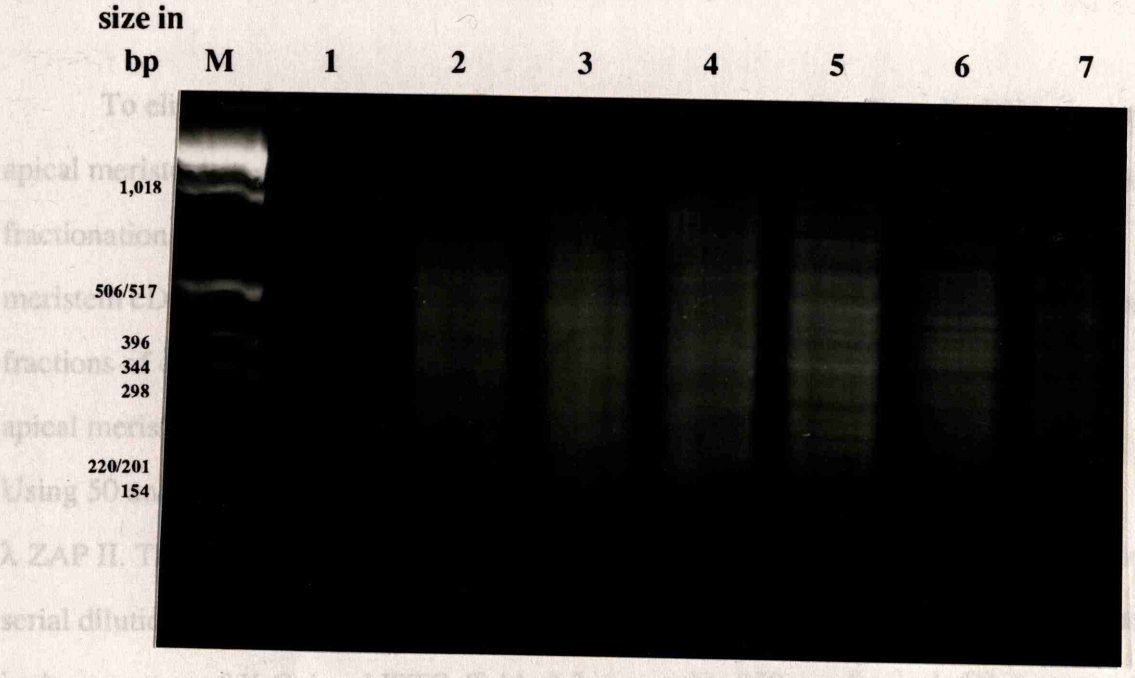
Fig 3.22 PCR Amplification of Apical Meristem cDNA

Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel of apical meristem cDNA. Fractions 6,7 and 8 from the Sephadex G-50 column from Fig 3.21 were subjected to ligation conditions and passed down a Bio-Gel A-150 column to remove unincorporated radionucleotides and the unligated linkers. 2.5 µl aliquots from fractions 3-8 passed through the Bio-Gel A-150 column (described in section 3.2.4) were subjected to 35 cycles of PCR using the JB1 oligonucleotide primers and an aliquot of the products of these reactions was run on a 1.5% (w/v) agarose gel.

Lane M, 1kb "ladder" marker DNA (Gibco/BRL)

Lane 1, The product of 35 cycles of PCR in the absence of any apical cDNA

Lanes 2-7, The product of PCR amplification of 2.5 µl aliquots from fractions 3-8 of the Bio-Gel A-150 column


size in
bp
M
1
2
3
4
5
6
7
1,018
506/517
396
344
298
220/201
154

detectable amount of cDNA was amplified. The pooled PCR amplified apical cDNA from fractions 3-7 (lanes 2-6 of Fig 3.22) was digested with *Eco* RI.

3.2.5 Removal of Small Sized cDNA Prior to Cloning

To eliminate the possibility of including a large proportion of small cDNAs in the apical meristem library, fractions 3-7 (lanes 2-6 of Fig 3.22) were subjected to a further size fractionation as described in section 3.1.5.2. Fig 3.23 shows the separation of the apical meristem cDNA by this method (described in section 3.1.5.2) to have successfully produced fractions of cDNA containing progressively smaller sized material. The *Eco* RI digested apical meristem cDNA shown in lane 2 of Fig 3.23 was cloned into the λ ZAP II vector. Using 50 and 250 ng of this digested cDNA ligations were performed in the presence of λ ZAP II. The ligated material was packaged, as previously explained in section 2.18.3, and serial dilutions of the packaged material were spread on to a lawn of X-L1 Blue *E. coli* cells in the presence of X-Gal and IPTG. Table 3.2 shows that 250 ng of apical cDNA gave the best recombination frequency, of approximately 80%. The library prepared from this material was also shown to contain 1.4×10^6 recombinant clones.

3.2.6 Amplification of Inserts from the Apical Meristem cDNA Library

To show that the PCR amplified apical meristem cDNA library prepared from 250 ng of cDNA contained inserts of an acceptable size distribution 8 random plaques (7 white and 1 blue) were amplified using PUC 1 and 2 as described in section 2.15. Fig 3.24 shows that the average size of these inserts was 600 bp with a range from less than 100 bp to 1.6 kbp.

Fig 3.23 Removal of Small Sized Apical Meristem cDNA Prior to Cloning

Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel of PCR amplified apical meristem cDNA from fractions 4-7 of the Bio-Gel A-150 column after being pooled and digested with *Eco* RI and then size fractioned as follows: The pooled digested apical meristem cDNA was subjected to electrophoresis and sectioned into four sequential 4mm strips at right angles to the direction of migration of the DNA. Aliquots of the cDNA recovered from these strips were run on a 1.5% (w/v) agarose gel

Lane M, 1 kb "ladder" marker DNA (Gibco/BRL)

Lanes 1-4, The DNA isolated from the first four strips of the 1.5% (w/v) agarose gel used to size fractionate the cDNA

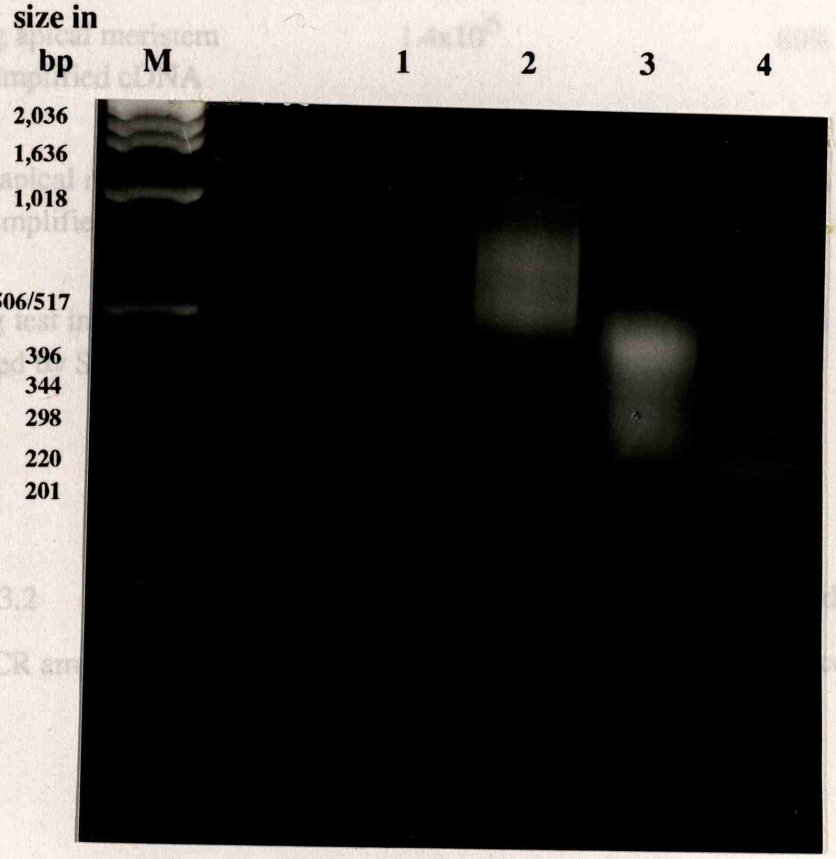

size in bp
M
1
2
3
4
2,036
1,636
1,018
506/517
396
344
298
220
201

Table 3.2

Amount of DNA packaged	No. of clones obtained	Recombination frequency
250 ng apical meristem PCR amplified cDNA	$1.4x10^6$	80%
50 ng apical meristem PCR amplified cDNA	$2.3x10^3$	45%
100 ng test insert supplied by Strategene	$3.4x10^7$	92%

Table 3.2 Properties of cDNA libraries in λ ZAP II prepared from different amounts of PCR amplified apical meristem cDNA compared with a test insert control.

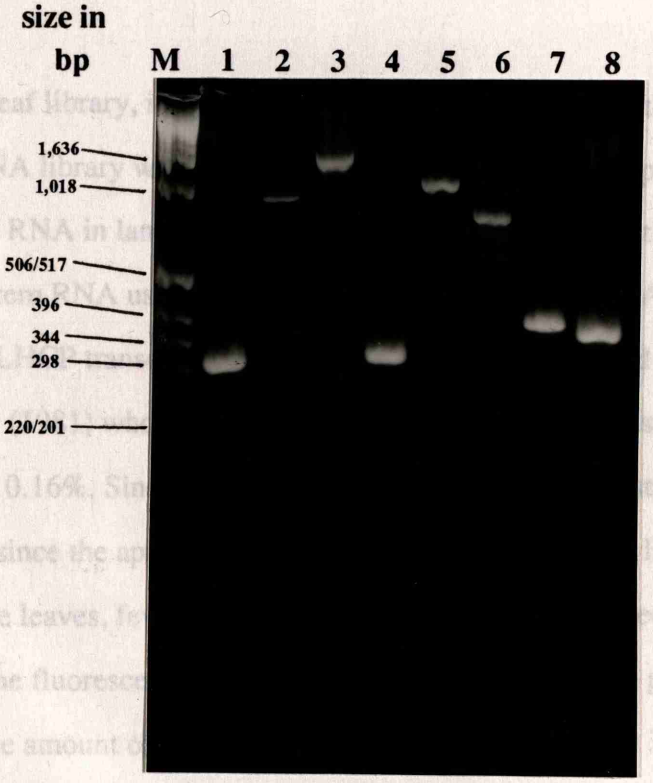
Fig 3.24 Isolation of Random Inserts from the Apical Meristem cDNA Library.

Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel of the inserts from 8 random apical meristem cDNA clones (1 blue and 7 white). 28 µl aliquots of SM phage buffer (described in section 2.4) containing 8 random plaques from the apical meristem cDNA library were subjected to 35 cycles of PCR using the PUC 1 and PUC 2 oligonucleotide primers.The DNA products of PCR amplification of these aliquots are shown here.

Lane M, 1 kb "ladder" marker DNA (Gibco/BRL)

Lane 1, The DNA product of 35 rounds of PCR amplification using oligonucleotides PUC 1 and PUC 2 of a plaque showing a blue colour when plated out in the presence of X-Gal and IPTG

Lanes 2-8, The DNA product of 35 rounds of PCR amplification using oligonucleotides PUC 1 and PUC 2 of random plaques showing a white colour when plated out in the presence of X-Gal and IPTG


size in bp
M
1
2
3
4
5
6
7
8
1,636
1,018
506/517
396
344
298
220/201

Having successfully cloned PCR amplified cDNA prepared from *Beta vulgaris* apical meristem RNA it was next necessary to show that the cloned inserts were of plant origin.

3.2.7 Screening the Apical Meristem cDNA Library with Known cDNA Probes

As with the leaf library, it was important to investigate whether the PCR amplified apical meristem cDNA library was representative of transcripts in the apical meristem RNA. If the amount of leaf RNA in lane 8 of Fig 3.25 is assumed to be 10 μg then it would appear that the apical meristem RNA used to prepare the apical meristem cDNA library contains a lower proportion of LHCP transcripts than the leaf RNA. This is backed up by the work of Cuming and Bennett (1981) who state that the level of LHCP transcripts in pea leaf not exposed to light was 0.16%. Since LHCP is involved in the light harvesting process in photosynthesis, and since the apical meristem tissue is shaded from the light by the leaf primordia and mature leaves, fewer transcripts for LHCP would be expected in the apical tissue. However, if the fluorescence from the ethidium bromide stained gel from Fig 3.25 is to be believed and the amount of leaf RNA present is actually nearer to 30 μg then the northern blot in Fig 3.25 shows there to be little difference between the level of LHCP transcripts in leaf and apical meristem RNA.

One thousand clones from the apical meristem cDNA library were probed with the LHCP cDNA from *Zea mays*. It was found that the clones hybridising to the LHCP cDNA probe were present at a lower abundance in the apical meristem library than in the leaf library. Fig 3.26 shows that of 1,000 clones screened only four of the clones from this sample of the apical library were giving a positive signal. These four clones were plaque purified and screened a second time. Fig 3.27 shows three of the four (all but the bottom left panel in Fig

Fig 3.25 **Northern Blot of Warm, Short Day *Beta vulgaris* Annual andBiennial Apical RNA from Meristem Tissue compared with RNA Isolated from Annual Mature Leaf and Root Tissue Probed with the LHCP cDNA Probe of *Zea mays*.**

a) Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel containing 2.2 M formaldehyde. The gel contains differing amounts of annual and biennial apical meristem RNA alongside 10 μg* of annual leaf RNA and 30 μg (see Fig 3.12) of annual root RNA. All RNA was isolated from plant tissue grown under short day conditions at 20°C. The 25S and 18S rRNA are indicated.

Lanes 1-3, 2, 10 and 5 μg of biennial apical RNA respectively

Lane 4 , 30 μg of annual root RNA

Lanes 5-7, 10, 5 and 2 μg of annual apical RNA respectively

Lane 8, 10 μg* of annual leaf RNA

b) An autoradiograph showing the resultant hybridisation of the *Zea mays* LHCP cDNA probe to the RNA from the above figure after transfer to Hybond-N nylon membrane.

Lanes 1-3, Transcripts from 2, 10 and 5 μg of biennial RNA hybridising to the radiolabelled LHCP cDNA clone of *Zea mays*

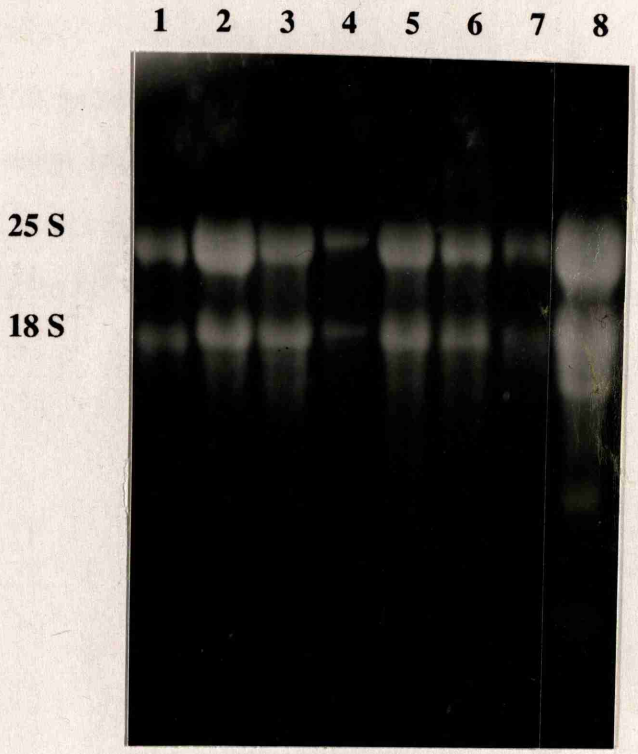
Lane 4, Transcript from 30 μg of root RNA hybridising to the radiolabelled LHCP clone from *Zea mays*

Lanes 5-7, Transcripts from 10, 5 and 2 μg of annual apical RNA hybridising to the radiolabelled LHCP clone from *Zea mays*

Lane 8, Transcript from 10 μg* of annual leaf RNA hybridising to the radiolabelled LHCP clone from *Zea mays*

*The amount of leaf RNA in lane 8 was determined as 10 μg according to the A_{260} reading but the fluorescence from the ethidium bromide stained gel indicates the amount to be closer to 30μg.

a)



b)

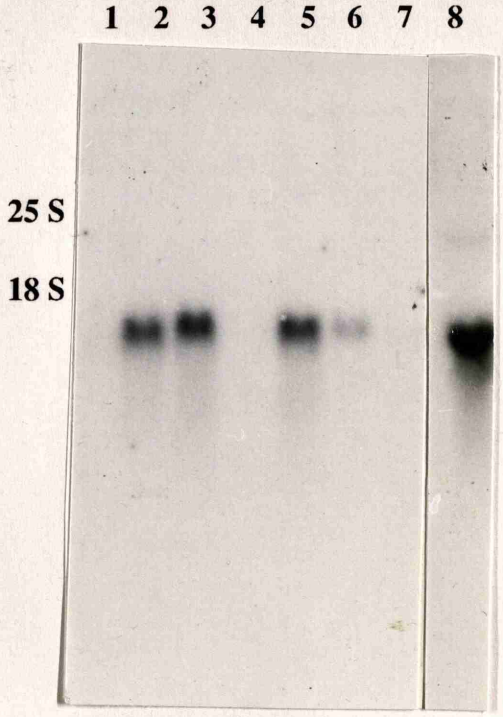


Fig 3.26 Screening the Apical Meristem Library with the LHCP Probe

Autoradiograph of 1,000 cDNA clones from the PCR amplified apical meristem cDNA library plated out and bound to Hybond-N nylon membrane. The nylon membrane filters were hybridised with the radiolabelled LHCP cDNA clone from *Zea mays* at 65^{o}C for 24 h. The filters were washed three times in 2X SSC, 0.1% (w/v) SDS at 65^{o}C for 20 min before being exposed to X-ray film for 24 h.

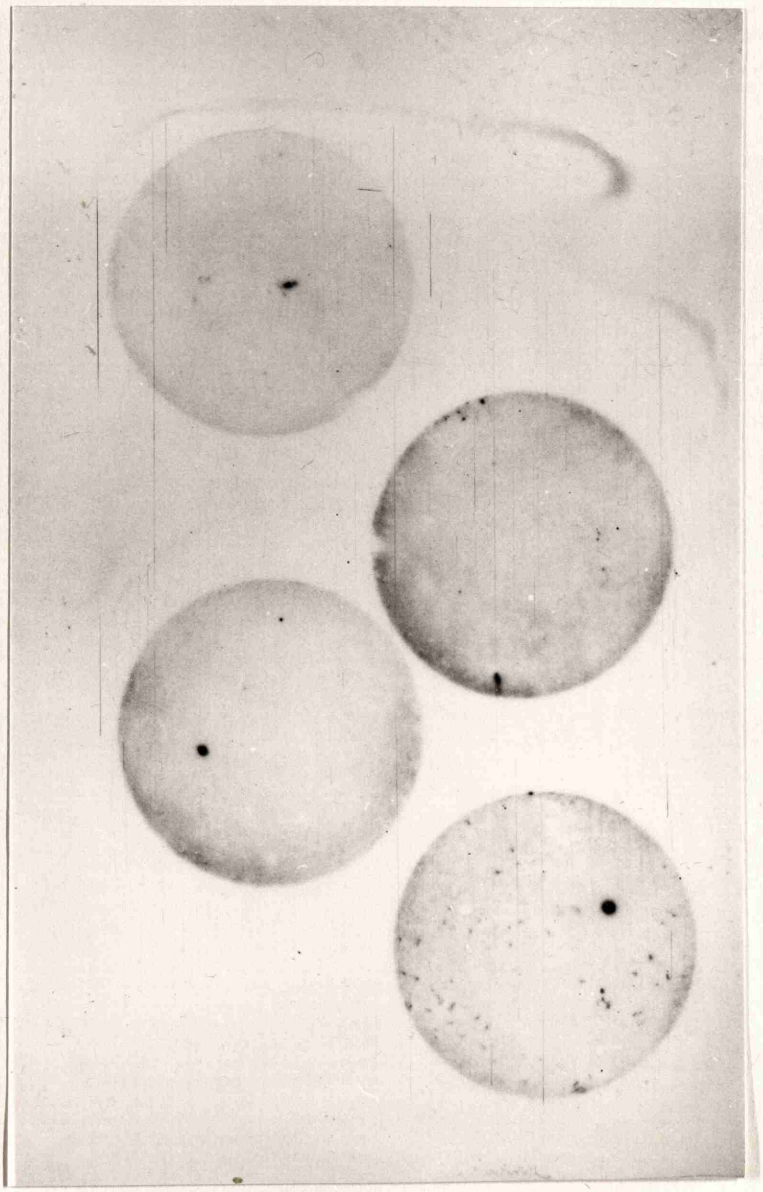


Fig 3.27 Secondary Screening of Putative LHCP Clones from the Apical Meristem cDNA Library.

Autoradiograph of 4 separate putative LHCP clones from the apical meristem cDNA library plated out at a relatively low density and bound to separate Hybond-N nylon membrane filters. The nylon membrane filters were hybridised to the radiolabelled *Zea mays* LHCP cDNA probe at 65^{o}C for 24 h. The filters were washed three times in 2X SSC, 0.1% (w/v) SDS at 65^{o}C for 20 min before being exposed to X-ray film for 24 h.

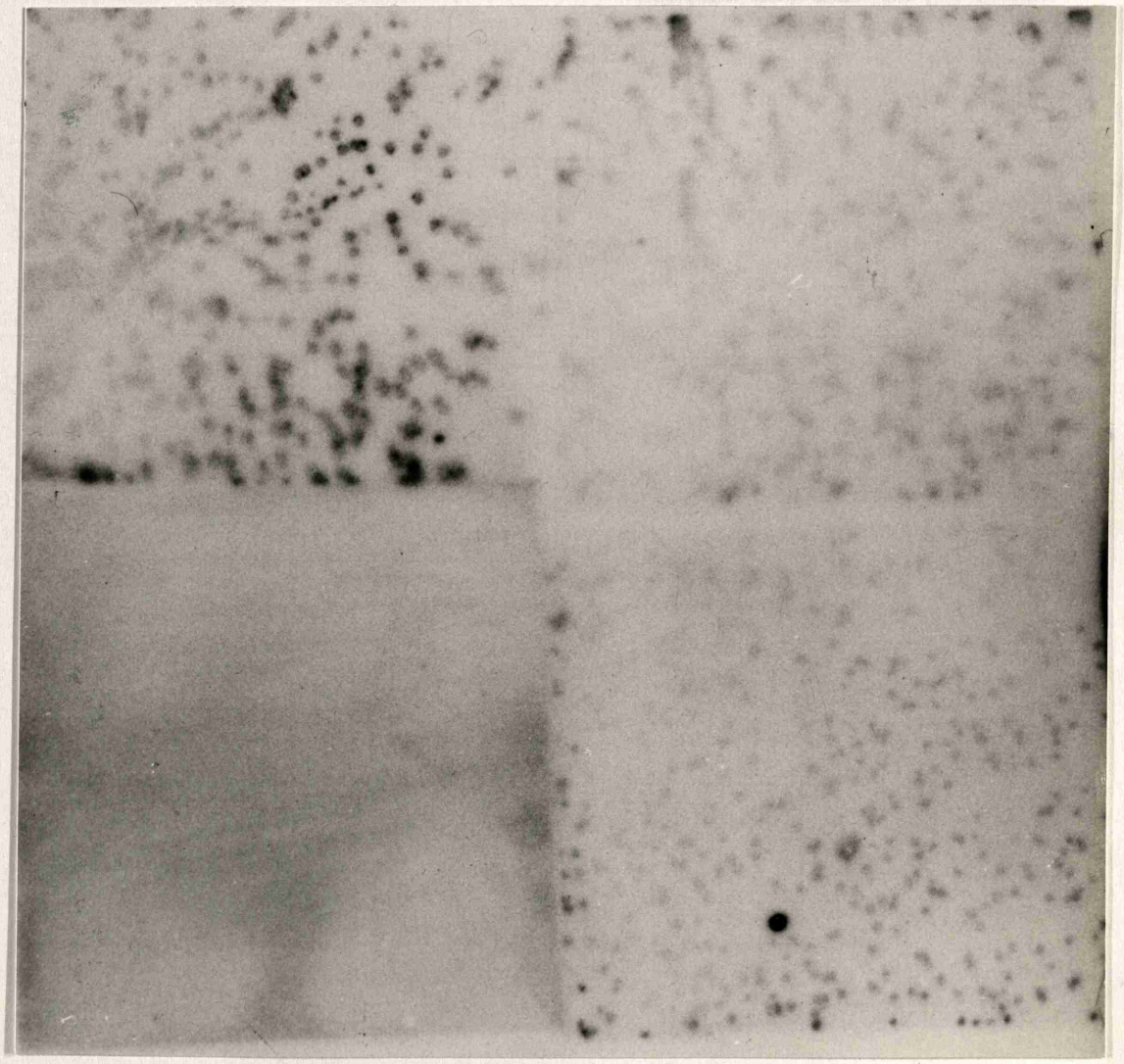


Table 3.3

cDNA probe	% of clones hybridising to the probe under stringent hybridisation conditions
LHCP	0.3%
α-tubulin	0.7%
G PAL-2	<0.1%
ALS	<0.1%

Table 3.3 Frequency of clones in the PCR amplified apical meristem cDNA library hybridising to several DNA probes

3.27) to be true positive clones for LHCP under the hybridisation conditions employed in this study.

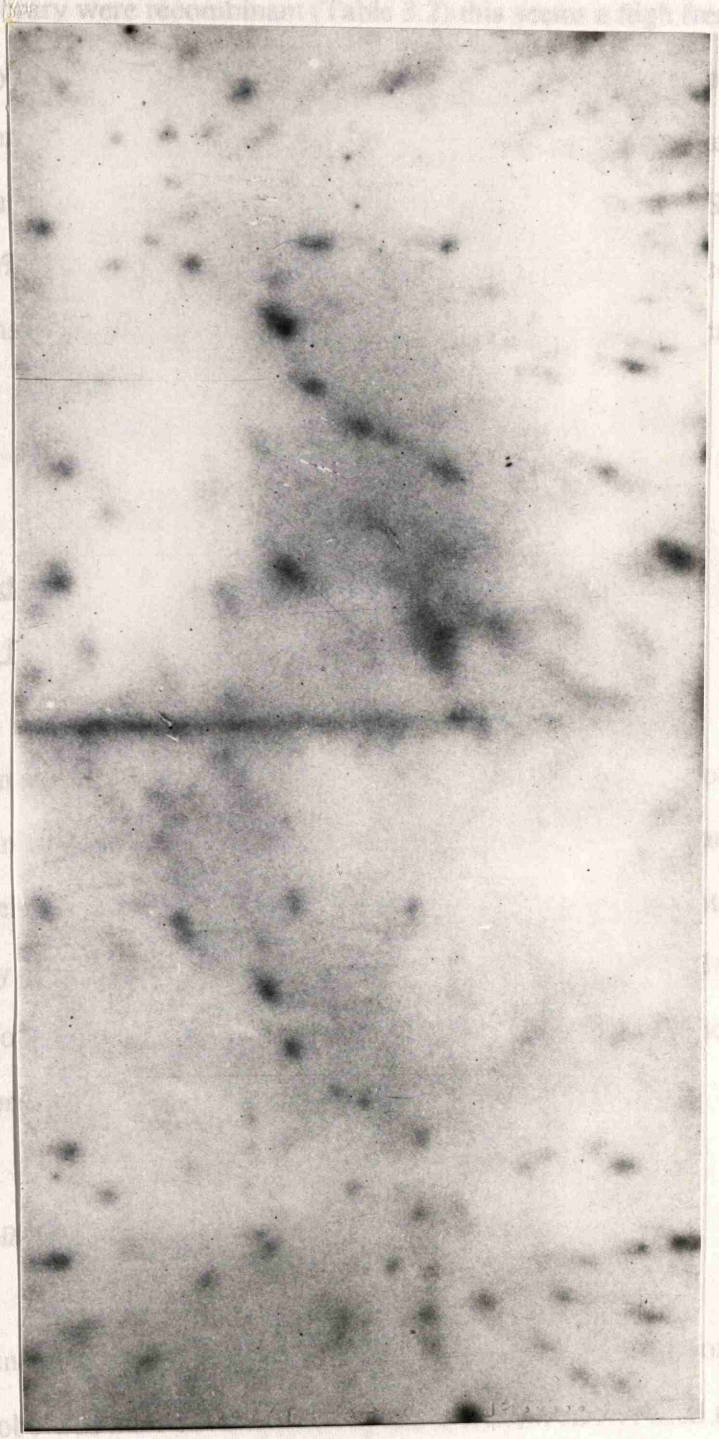
Using the α-tubulin, G-PAL 2 and ALS cDNA clones (described in section 2.19.2) as examples of clones encoding different sized mRNAs which are assumed to be expressed at different levels in the RNA of apical tissue from sugar beet, 1,000 apical meristem cDNA clones were probed with these three cDNA probes. Table 3 shows the results of these probing experiments and demonstrates that after a second round of screening with all three probes levels of representitiveness ranged from less than 0.1% to 0.7% of clones in the library. These data suggest that the corresponding transcripts would be present at relativelylow abundance in apical RNA.

3.2.8 Screening the Apical Meristem cDNA Library with Single Stranded Apical Meristem cDNA

Although the apical meristem cDNA library was found to contain clones which hybridised to cDNA probes of plant origin, it was important to establish that the cloned inserts within the apical meristem cDNA library were of *Beta vulgaris* apical meristem origin. A small sample of apical meristem cDNA clones was, therefore, hybridised to single stranded cDNA prepared from apical meristem RNA as described in section 2.17. These clones from the apical meristem cDNA library were plated out at a low density (50-60 clones per plate) to enable an accurate assessment of the hybridisation frequency. Fig 3.28 shows hybridisation of these clones to the apical meristem probe. Approximately 75% of the clones screened from the apical meristem library with the apical meristem single stranded cDNA, hybridised to that probe under relatively stringent washing conditions. Since only 80% of the

Fig 3.28 Screening of the Apical Meristem cDNA Library with Single Stranded Apical Meristem cDNA

Autoradiograph of two Hybond-N nylon membrane filters of plaque lifts from two separate plates, each containing 50-60 plaques, after hybridisation to radiolabelled single stranded apical meristem cDNA. The filters were hybridised with the single stranded cDNA at 65^{o}C for 24 h and then washed three times at 65^{o}C in 2X SSC, 0.1% (w/v) SDS for 20 min before being exposed to X-ray film for 48 h.



clones in the library were recombinant (Table 3.2) this seems a high frequency of complementary sequences. Furthermore, it is important to note that a proportion of the clones could have been represented at too low a frequency in the apical meristem cDNA probe for them to show a signal under these conditions (so called 'cold' plaques, Scott *et al.*, 1991).Thus 75% was considered an acceptable figure indicating the presence of a high frequency of inserts derived from apical meristem RNA within the apical meristem cDNA library.

3.3. Methods of Isolating Apex-Specific cDNA clones from the Apical Meristem cDNA Library.

The annual apical meristem cDNA library has been shown to contain inserts of varying size range and to represent certain specific cDNA clones of plant origin at an acceptable level. It has also been shown to contain a substantial amount of inserts complementary to single stranded cDNA prepared from the tissue used to produce the library. Therefore, it was assumed that the library derived from this apical meristem material was suitable for screening to isolate apex-specific cDNA clones.

3.3.1 Differential screening

The principles of differential screening were explained in section 1.10. In this study the positive probe was the PCR amplified apical meristem cDNA used to prepare the apical meristem cDNA library. The negative probe was the PCR amplified cDNA used in the

preparation of the leaf cDNA library. Both of these were available in large amounts and so many thousands of clones from the apical meristem cDNA library could be screened for the presence of apex-specific cDNA clones.

Initially 100,000 clones of the apical meristem cDNA library were plated out and duplicate plaque lifts were taken from each plate as described in section 2.14.1. Fig 3.29 shows the result of a typical differential screen from this study. The arrows indicate putative apex-specific clones. Since only 38 putative apex-specific clones were isolated from this initial study a further 100,000 clones were differentially screened in the same way. A total of 64 putative apex-specific clones were chosen for further study from the 200,000 clones screened. This was a disappointingly low number of clones isolated from such a large number of clones screened.

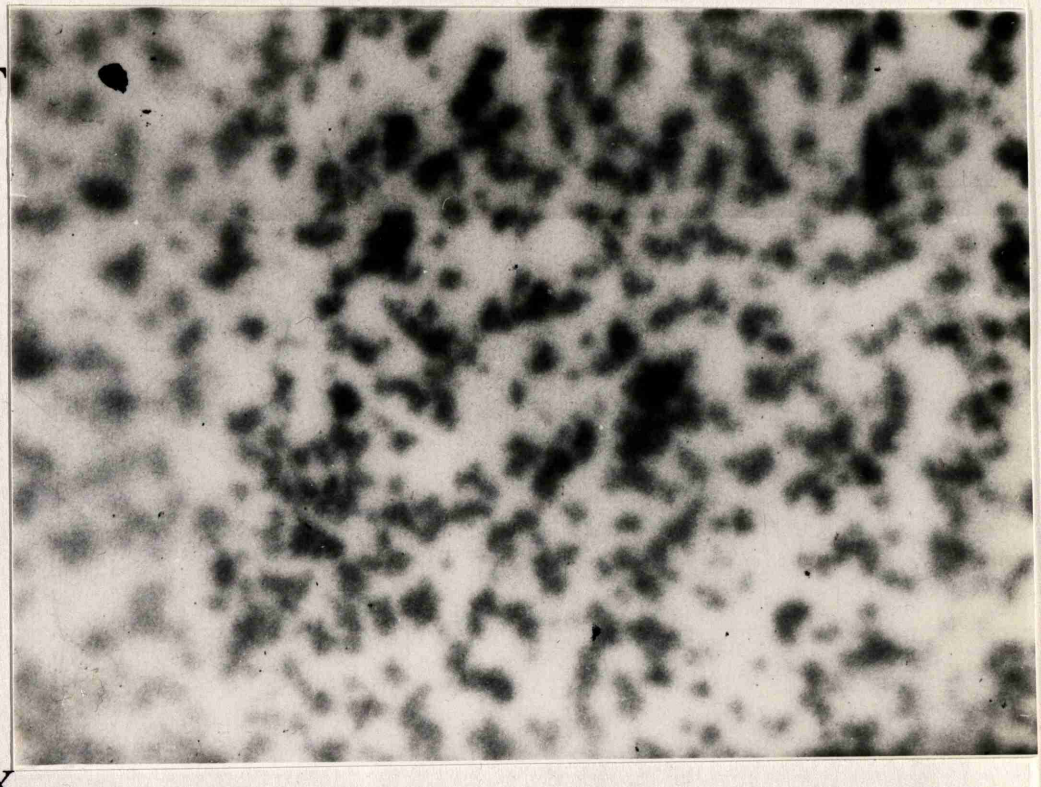
3.3.2 Secondary Screening of Putative Apex-Specific cDNA Clones

Of the 64 clones subjected to a secondary screen, by the "giant plaque" method described in section 2.20.1, only 8 of them appeared to be hybridising more strongly to the apical cDNA probe than the leaf cDNA probe (Fig 3.30). None of these putative positive clones were hybridising specifically to the apical cDNA probe. This, again was a disappointing result since what was initially only a very few promising clones suddenly diminished to none that were apex-specific, and only 8 that could possibly be enhanced in the apical meristem cDNA probe.

Fig 3.29 Differential Screening of the PCR Amplified Apical Meristem cDNA Library

Autoradiograph of duplicate Hybond-N nylon membrane filters prepared from a typical sample of apical meristem cDNA clones plated out at a density of approximately 500 plaques per plate. One filter from each plate was hybridised to annual leaf PCR amplified cDNA radiolabelled to a specific activity of 10^8 cpm per μg of DNA and the other filter from the same plate was hybridised to annual apical PCR amplified cDNA radiolabelled to a specific activity of 10^8 cpm per μg of DNA. All filters were hybridised at 65°C for 24 h before being washed three times in 2X SSC, 0.1%(w/v) SDS at 65°C and exposed to X-ray film for 24 h. The arrows in b) indicate clones that were putatively apex-specific.

a) LEAF



b) APEX

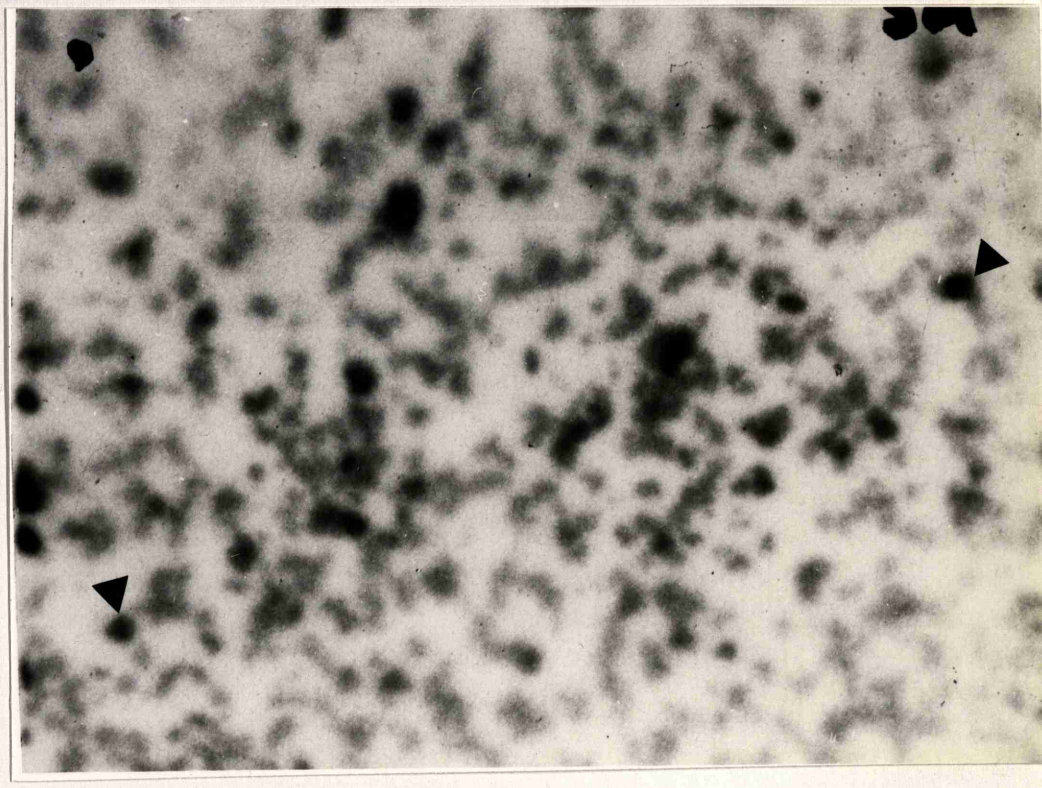
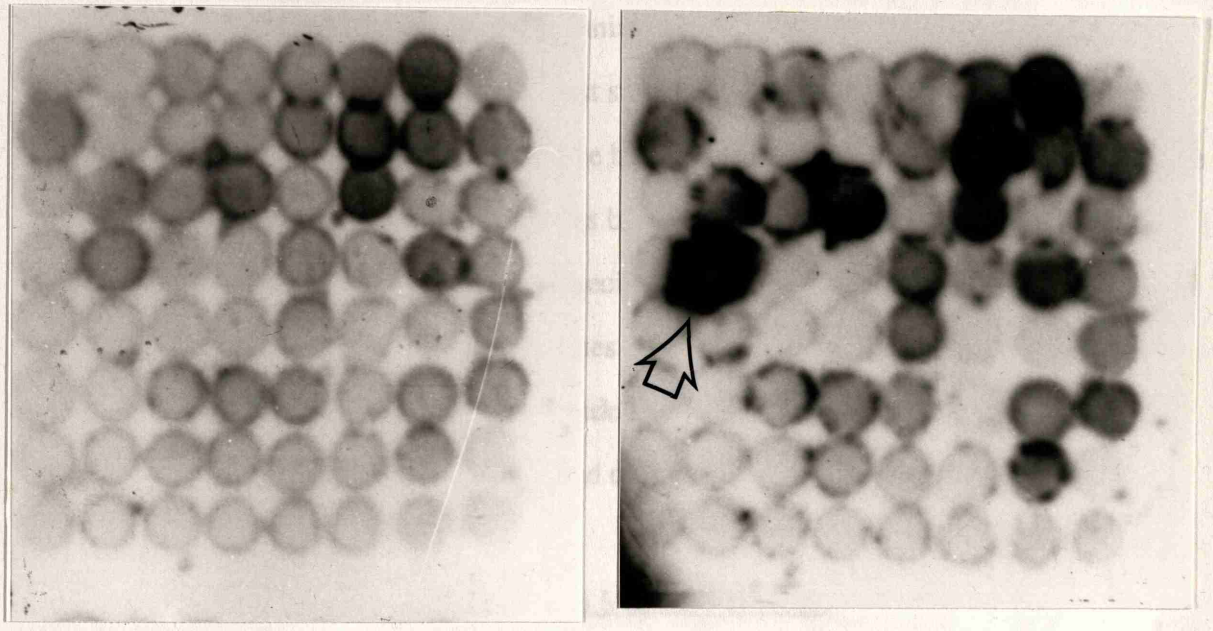


Fig 3.30 Secondary Screening of Putative Apex-Specific cDNA Clones.

Autoradiograph of duplicate Hybond-N nylon filters containing 64 putative apex-specific "giant plaques". The plaques were prepared by spotting an aliquot of SM phage buffer, (described in section 2.4) containing an agar plug within which was hopefully a single bacteriophage plaque, onto a lawn of *E.coli* cells and incubating at 37^{o}C for 24 h. One filter was hybridised to the radiolabelled PCR amplified annual leaf cDNA whilst the other filter was hybridised to the radiolabelled PCR amplified apical cDNA. Both hybridisations were performed at 65^{o}C and in each case the probes used were labelled to a specific activity of 10^{8} cpm per μg of DNA. The filters were then washed three times at 65^{o}C in 2X SSC, 0.1% (w/v) SDS for 20 min before being exposed to X-ray film for 24 h.

The arrow indicates the most likely apex-specific cDNA clone (number 26)

a) LEAF **b) APEX**



3.3.3 Investigation of Putative Positive Clones to Establish the Abundance of their Transcripts Within Leaf and Apical Meristem RNA

The most promising of the 8 clones still giving a differential signal when hybridised to leaf and apical cDNA (clone 26) was used to probe a northern blot of RNA of *Beta vulgaris* tissue isolated from annual and biennial apical meristems and mature leaves. Fig 3.31 shows that clone 26 hybridised most strongly to mature leaf RNA (apparently to rRNA) and gave an almost equal signal in the biennial and annual apex RNA. Although there is evidence of degradation of the RNA in this blot (Fig 3.31) the hybridisation of the leaf RNA indicates that the cDNA is not apex-specific. Hybridisation of similar northern blots to inserts of several other putative positive clones gave no indication of apex-specificity (data not shown). These results provide further evidence indicating that the small number of putative apex-specific clones initially isolated did not contain any true apex-specific clones.

3.3.4 'Cold' Plaque Screening of the Apical Meristem Library.

The previous results were not very promising, as they indicated that of the 200,000 clones screened none were apex specific. Of the few which were putatively apex-enhanced in expression after secondary screening, the most promising clone (clone 26) turned out to be expressed most strongly in the leaf tissue. It was decided, therefore, to use the cold plaque method of Scott *et al.* (1991) to see if apex-specific clones could be obtained from the PCR amplified apical meristem cDNA library. A significant number of clones (200) from this library, which did not hybridise to either the leaf or apical meristem cDNA probes, were screened by the "giant plaque" method described in section 2.20.1. Fig 3.32 shows that of the

Fig 3.31 Northern Blot of Annual and Biennial Apical Meristem RNA and Annual Leaf RNA Probed with Clone 26

a) Photograph of an ethidium bromide stained 1.5% (w/v) agarose gel containing 2.2 M formaldehyde. The gel contains 10 μg of annual and biennial apical meristem RNA alongside 5μg of annual leaf RNA.

Lane 1, 10 μg of biennial apical meristem RNA

Lane 2, 10 μg of annual apical meristem RNA

Lane 3, 5 μg of annual leaf RNA

b) Autoradiograph showing hybridisation of the cDNA insert of clone 26 to the RNA from the formaldehyde gel in a) after blotting onto a Hybond-N nylon membrane filter. The membrane was hybridised to the radiolabelled clone 26 cDNA probe at 65^{o}C and then washed at 65^{o}C in 2X SSC, 0.1% (w/v) SDS; 0.2X SSC, 0.1% (w/v) SDS; and 0.1X SSC, 0.1% (w/v) SDS, each wash was for a duration of 20 min. The filter was exposed to X-ray film for 48 h.

Lane 4, Transcripts from a 10 μg sample of biennial apical meristem RNA hybridising to the radiolabelled clone 26 cDNA probe

Lane 5, Transcripts from a 10 μg sample of annual apical meristem RNA hybridising to the radiolabelled clone 26 cDNA probe

Lane 6, Transcript from a 5 μg sample of annual leaf RNA hybridising to the radiolabelled clone 26 cDNA probe

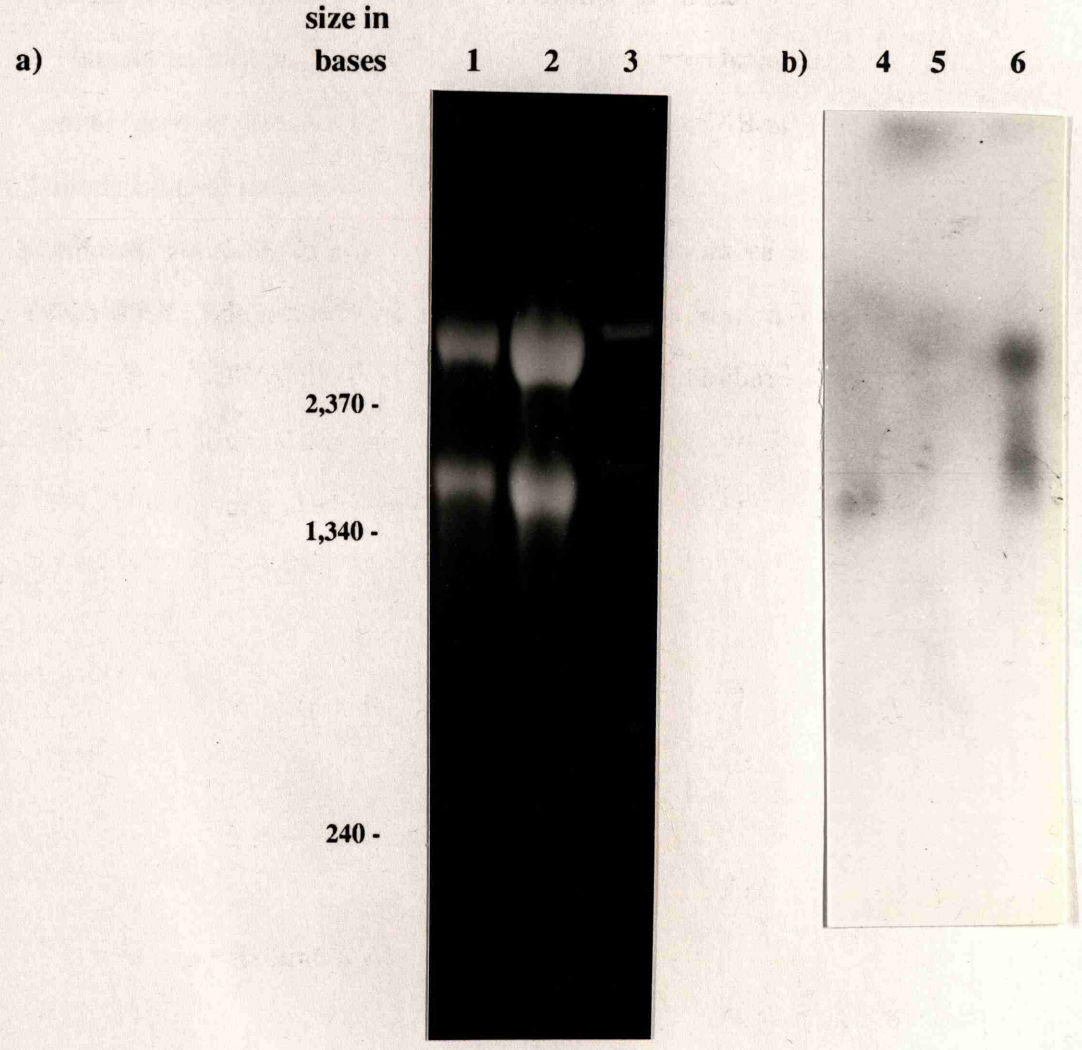
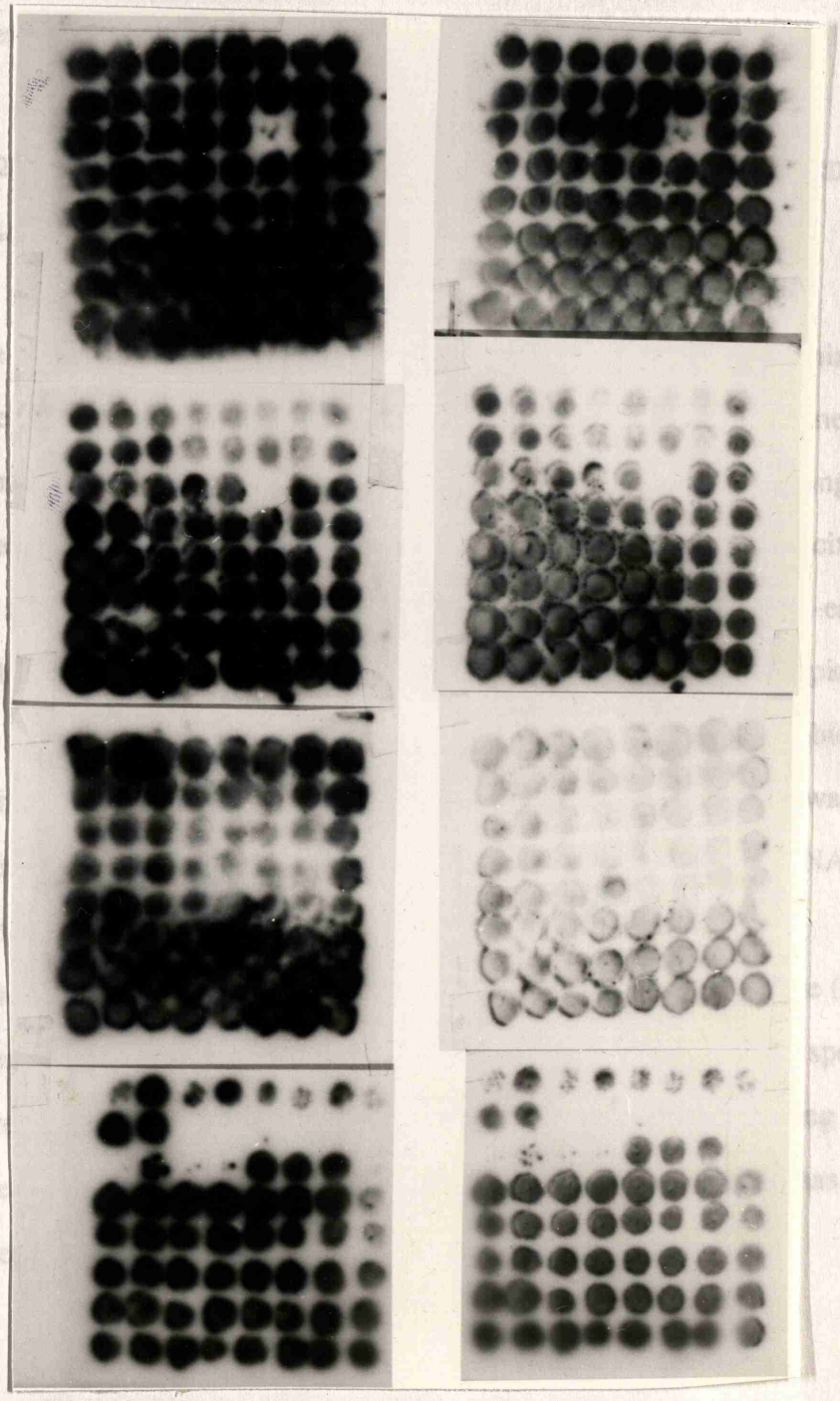

a)
size in bases
1
2
3
2,370 -
1,340 -
240 -
b)
4
5
6

Fig 3.32 'Cold' Plaque Screening of the Apical Meristem cDNA Library

Autoradiograph of duplicate Hybond-N nylon membrane filters of over 200 plaques which were white in the presence of X-Gal and IPTG but which were seen not to hybridise to either the apical meristem or the leaf cDNA probes. Aliquots of the SM phage buffer (described in section 2.4) from over 200 of these 'cold' plaques were spotted onto a lawn of *E.coli* cells and incubated for 24 h. Duplicate plaque lifts were made of each of four plates. One copy of the plaque lifts from each plate was hybridised to the PCR amplified annual *Beta vulgaris* leaf cDNA probe whilst the other copy from each plate was hybridised to the PCR amplified annual apical meristem cDNA probe. Both cDNA probes were radiolabelled to a specific activity of 10^8 cpm per μg of DNA and were hybridised to the nylon membrane filters at 65°C for 24 h. The filters were washed three times at 65°C in 2X SSC, 0.1% (w/v) SDS for 20 min before being exposed to X-ray film for 48 h.


LEAF
APEX

200 'cold' plaques isolated none hybridised more strongly to the apical meristem cDNA probe. Hence, there was no evidence that the 'cold' plaque method would reveal any significant number of apex-enhanced (let alone apex-specific) cDNA clones, and so this method was therefore, not pursued.

3.3.5 Development of Methods for Subtractive Screening of the Apical Meristem cDNA Library. (based on the method of Sive and St John, 1988)

Because of the lack of encouraging results obtained by differential screening, described above, it was decided that a more positive set of results could be obtained using a subtractive approach (see section 1.10 and 2. 20.3). The hope was that in removing as many cDNA species as possible common to both the apical and leaf cDNAs, apex-specific cDNA would be more abundant.This cDNA enriched for apex-specific, or at least apex-enhanced, sequences would then facilitate the isolation of corresponding clones from the apical library. The subtractive procedure employed in this study involved the incorporation of biotin labelled dATP into PCR amplified leaf cDNA (driver cDNA). This leaf cDNA was incubated with 100 fold less PCR amplified apical meristem cDNA before removal of cDNA labelled with biotin, or bound to a biotin labelled cDNA fragment, using streptavidin.

It is important when attempting this procedure to ensure that the negative (or driver) DNA is sufficiently biotin labelled to enable the subtraction of common cDNA species when bound to the streptavidin protein. This protein body has four high affinity binding sites for biotin and, therefore, any cDNA that is labelled with the biotin moiety, or that has hybridised to cDNA labelled with biotin, should be removed by this step.

3.3.5.1 Incorporation of Biotin Labelled dATP into PCR Amplified Leaf cDNA

Two methods of biotin labelling were available to use in this study;

1) nick translation of the driver cDNA incorporating biotin labelled dATP into the reaction mixture;

2) photolabelling of the driver cDNA with photoactive biotin using a high intensity ultra-violet light source.

Both procedures were equally efficient at biotin labelling the driver cDNA, but the nick translation process was cheaper, safer to the user and any fellow workers, and allowed the incorporation of radiolabelled nucleotides into the cDNA. This last point is important because it enables the user to monitor the presence of the driver cDNA whilst establishing the necessary conditions for subtraction. The leaf cDNA nick translated in the presence of both radiolabelled dCTP and biotin labelled dATP was passed through a Sephadex G-50 column to eliminate unincorporated biotin labelled dATP (which would hinder the removal of the driver cDNA by competing for the binding sites of the streptavidin). 10 µl aliquots were removed from each of the 200 µl fractions passed through the column containing traces of radiolabel (as measured with a Geiger-Müller counter) and run on a 1.5 % (w/v) agarose gel. The gel was Southern blotted and first exposed to X-ray film before being stained (using the blue-GENE kit supplied by Gibco-BRL) to test for the presence of biotin. Fig 3.33 b) shows that the nick translation procedure efficiently incorporated radiolabelled dCTP into fractions 4-8 from the G-50 column. Very little unincorporated dCTP was present in any of the fractions from this column (as measured using a Geiger-Müller counter) indicating that the incorporation of biotin labelled dATP was also likely to be efficient. Fig 3.33 a) shows that those fractions which appeared to contain radiolabelled cDNA also contained biotin-

Fig 3.33 Incorporation of Biotin Label in to the PCR Amplified Leaf cDNA

A blot of 10 µl aliquots from 200 µl fractions of PCR amplified leaf cDNA passed through a Sephadex G-50 column. The cDNA was labelled with both biotin-7-dATP and [α-^{32}P] dCTP by nick translation. The leaf cDNA was separated on a 1.5% (w/v) agarose gel and transferred to a Hybond-N nylon membrane filter. The filter was stained for the presence of biotin using the blue-GENE non-radioactive detection system before being exposed to X-ray film for 24 h.

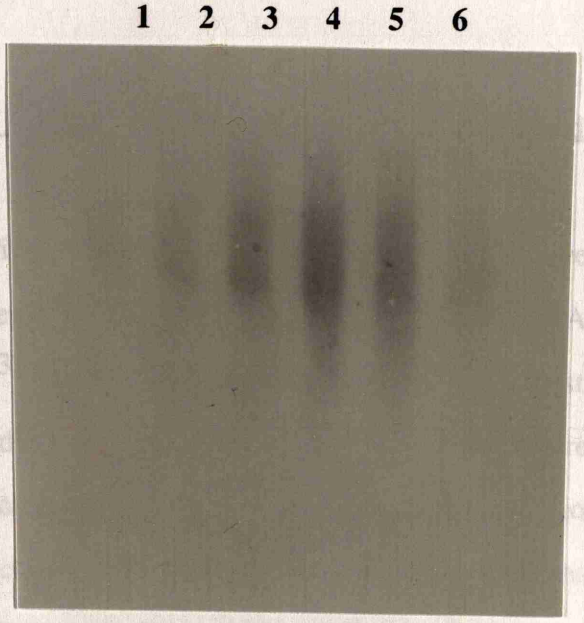
a) Shows the incorporation of biotin into the leaf cDNA after staining with the blue-GENE kit from Gibco/BRL.

Lanes 1-6, Biotin-7-dATP labelled PCR amplified leaf cDNA from fractions 3-8 of the Sephadex G-50 column.

b) Shows the incorporation of [α-^{32}P] dCTP into the leaf cDNA after exposure of the blot to X-ray film for 24 hours

Lanes 1-6, Radiolabelled PCR amplified leaf cDNA from fractions 3-8 of the Sephadex G-50 column

a)



b)

labelled cDNA. Therefore, it was decided that fractions 3-8 from the G-50 column (lanes 1-6 in Figs 3.33a and b) should be used to establish the efficiency of removal of such DNA by streptavidin.

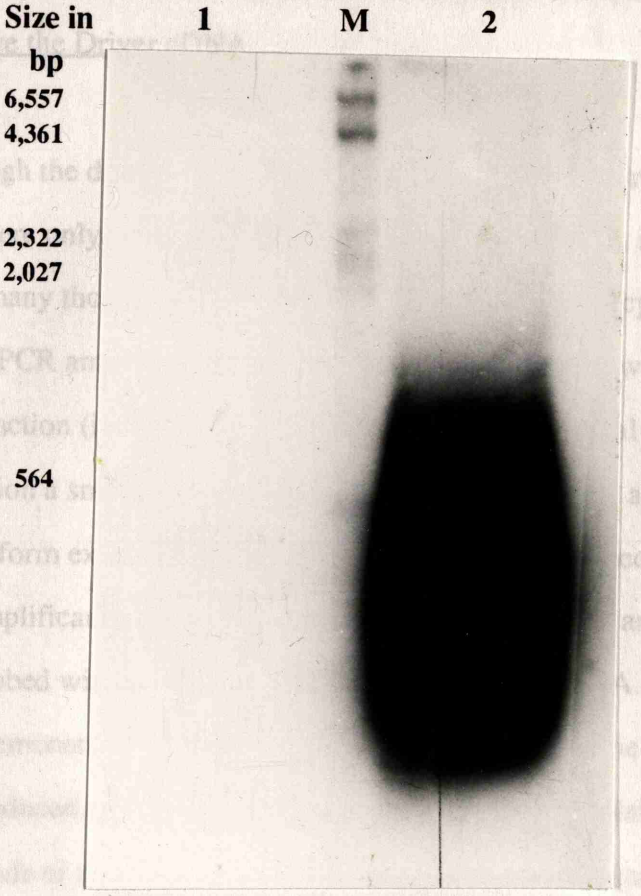
3.3.5.2 Removal of Biotin Labelled Leaf cDNA Using Streptavidin

Since the driver cDNA was trace labelled with radioactive dCTP it was not difficult to show that the streptavidin was able to remove the driver cDNA very efficiently. The biotin-labelled, [α-^{32}P] labelled cDNA was incubated with the streptavidin under the conditions described in section 2.20.3. After this incubation the reaction mixture was subjected to a phenol/chloroform extraction as described in section 2.8.3. Since streptavidin is a protein, and since phenol is able to remove protein from an aqueous solution, the streptavidin would have been taken up by the phenol, along with any cDNA bound to the streptavidin. The aqueous phase from this phenol/chloroform extraction was removed and run on a 1.5 % (w/v) agarose gel along with radiolabelled λ/*Hind* III marker DNA and an equivalent amount of biotin-labelled driver cDNA as was used in this experiment. This gel was Southern blotted. Fig 3.34 shows the resultant blot after exposure to X-ray film and indicates that no detectable radiolabelled driver cDNA (and therefore, presumably no biotin labelled driver cDNA) remained after binding with the streptavidin. This demonstrates that the conditions used to remove biotin-labelled cDNA were very efficient.

Fig 3.34 Removal of Biotin Labelled Leaf cDNA using Streptavidin

Autoradiograph of a blot of biotin labelled, [α-^{32} P] dCTP labelled cDNA remaining after binding to steptavidin compared with an equivalent amount of biotin labelled, [α-^{32} P] dCTP labelled leaf cDNA not incubated in the presence of streptavidin.

Leaf cDNA internally labelled by nick translation using [α-^{32}P] dCTP and biotin-7-dATP was incubated in the presence (lane 1) or absence (lane 2) of streptavidin in 0.5 M NaCl at room temperature for 20 min. After phenol/chloroform extraction the aqueous phase was removed and run on a 1.5% (w/v) agarose gel. The size markers (lane M) were λ DNA digested with Hind III and end labelled with [α-^{32}P] dCTP


Size in bp
1
M
2
6,557
4,361
2,322
2,027
564

3.3.5.3 Establishing the Number of Rounds of Subtraction Required to Remove the Driver cDNA

Although the data in Fig 3.34 show there was no visible radiolabelled cDNA after subtraction, there only needs to be a very small amount of DNA available for PCR to amplify that material many thousand fold. Therefore, to test the efficiency of the subtraction procedure the PCR amplified biotin-labelled leaf driver cDNA was subjected to four rounds of mock-subtraction (i.e. subtraction in the absence of any apical cDNA). After each round of mock-subtraction a small aliquot (10 µl) was removed from the aqueous phase left after phenol/ chloroform extraction, and subjected to PCR as described in section 2.15. The products of amplification were barely visible on an agarose gel and so the agarose gel was blotted and probed with radiolabelled PCR amplified leaf cDNA. Fig 3.35 shows the result of this blot and demonstrates that amplification of samples from the first three rounds of mock subtraction produced enough cDNA to be detected with a radiolabelled leaf cDNA probe. After four rounds of mock-subtraction PCR amplification of an aliquot of the supernatant remaining after phenol /chloroform extraction did not give rise to a detectable amount of amplifiable cDNA. Therefore, in all subsequent experiments at least four rounds of subtraction were performed, in the hope that this would remove enough driver cDNA to allow the isolation of apex-specific cDNA.

3.3.5.4 Subtraction of Leaf Driver cDNA with Apical Meristem cDNA

Since the mock-subtraction procedure was shown to be successful in the previous experiments subtraction with apical meristem cDNA present was performed. Using 100 fold

Fig 3.35 Establishing the Number of Rounds of Subtraction Required for Efficient Removal of the Driver cDNA

Autoradiograph of a Southern blot of cDNA prepared by PCR amplification of 10 µl aliquots taken from the aqueous phase remaining after each round of mock-subtraction (using leaf cDNA in the absence of any apical meristem cDNA). Samples taken from up to four rounds of mock-subtraction were amplified by 35 cycles of PCR and the products were separated on a 1.5% (w/v) agarose gel and transferred to Hybond-N nylon membrane. The nylon membrane was hybridised with [α-^{32}P] dCTP labelled PCR amplified leaf cDNA at 65^{o}C for 24 h. The nylon membrane was washed three times in 2X SSC, 0.1% (w/v) SDS for 20 min before exposure to X-ray film for 24 h.

Lane 1-4, Hybridisation of the radiolabelled leaf cDNA probe to DNA prepared by PCR amplification of aliquots of cDNA taken from the 1st to 4th rounds of mock-subtraction respectively.

Lane 5, Hybridisation of the radiolabelled leaf cDNA probe to 25 ng of PCR amplified leaf cDNA as a control

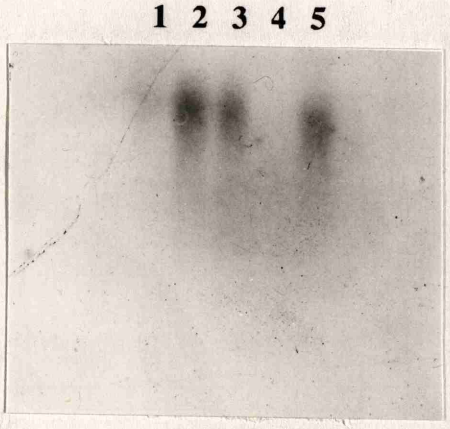

1 2 3 4 5

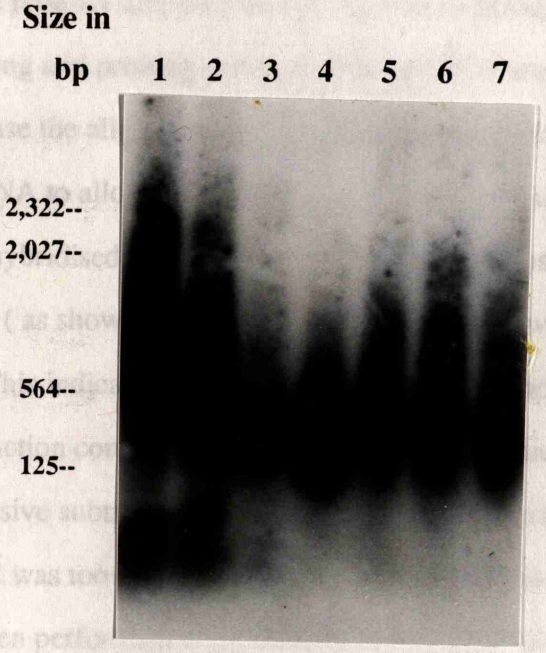
Fig 3.36 Subtraction of Leaf Driver cDNA with Apical Meristem cDNA

Autoradiograph of a Southern blot of PCR amplified cDNA prepared by subtraction of apical meristem cDNA with 100 fold excess of driver PCR amplified leaf cDNA. The apical meristem cDNA was subtracted against PCR amplified leaf cDNA for five successive rounds. An aliquot of the cDNA remaining after each round of subtraction was subjected to 35 rounds of PCR amplification. A sample of each of these amplified products was run on a 1.5% (w/v) agarose gel and blotted onto Hybond-N nylon membrane. The nylon membrane was hybridised at 65°C with [α-^{32}P] dCTP labelled PCR amplified leaf cDNA for 24 h and washed three times in 2X SSC, 0.1% (w/v) SDS for 20 min before being exposed to X-ray film for 48 h. The position of λ/*Hind* III DNA markers (taken from the ethidium bromide stained gel prior to blotting and probing) are indicated.

Lane 1, resultant hybridisation of the radiolabelled leaf cDNA probe to 100 ng of PCR amplified apical meristem cDNA

Lane 2, resultant hybridisation of the radiolabelled leaf cDNA probe to 100 ng of PCR amplified leaf cDNA

Lane 3-7, resultant hybridisation of the radiolabelled leaf cDNA probe to the cDNA synthesised after 35 rounds of PCR amplification from aliquots of cDNA remaining after 1-5 rounds of subtraction with 100 fold excess of driver leaf cDNA.


Size in
bp
1
2
3
4
5
6
7
2,322--
2,027--
564--
125--

more driver cDNA than apical cDNA five rounds of subtraction were performed as described in section 2.20.3. An aliquot of the aqueous phase from each of these rounds of subtraction was amplified and the product subjected to electrophoresis through a 1.5% (w/v) agarose gel before Southern blotting and probing with radiolabelled PCR amplified leaf cDNA. Fig 3.36 shows that in every case the aliquot of aqueous phase removed from the subtraction mix contained enough cDNA to allow amplification of that cDNA. Also the product from each round of subtraction hybridised to the driver leaf cDNA, when used to probe the Southern blot of these products (as shown in Fig 3.36). This experiment was repeated and the same result was obtained. This indicates that, under the conditions employed, it was not possible to obtain a subtracted fraction containing purely apex-specific sequences. Any sequences that did remain after extensive subtraction still showed hybridisation to leaf cDNA. It is likely that 35 rounds of PCR was too stringent for the detection of apex-specific cDNA and fewer cycles should have been performed. It is possible then that the cDNA produced from the aqueous phase of the subtracted cDNA fractions after fewer cycles of PCR would have been enhanced for apex-specific cDNA, and as such would have proved more useful in the isolation of such cDNA clones.

CHAPTER 4

DISCUSSION

4.1 Development of the PCR Cloning Technique

The ultimate aim of this study was to establish the nature of the agent responsible for floral induction upon exposure to low temperature treatment in sugar beet (*Beta vulgaris*). Since it is known that the annual sugar beet plant possesses a dominant gene (B) conferring annuality upon the beet plants it was assumed that the agent responsible for induction of flowering upon exposure to low temperatures may be the B gene, or some product thereof. As vernalization is perceived by the shoot apical meristem it seemed reasonable to investigate the genes of the annual sugar beet shoot apical meristem and look for apex-specific cDNA clones. Any cDNA clones isolated would be further characterised to see if any may be involved in the low temperature induction of flowering, and could possibly represent the B gene. The isolation of apex-specific cDNAs would prove a useful tool in the study of meristematic development since very little is known of the molecular mechanisms involved in the transition to flowering. The introduction of molecular biological techniques has enabled scientists to look at differences in the genomes of various organisms, and this has led to the isolation of many important genes.

The results from the previous section show that from small amounts of sugar beet total RNA representative cDNA libraries can be prepared using the protocol shown in Fig 3.4. This protocol provides a simple and rapid procedure for the construction of such

libraries. The fact that good quality RNA, suitable for efficient cDNA synthesis, can be produced from very small amounts of plant tissue is significant. This contrasts with the PCR cloning protocols designed for the isolation of genes expressed in mammalian systems which require large amounts of poly (A^+)RNA (Timblin *et al.*, 1990). Furthermore, this procedure is straightforward when compared with other systems in that precipitation steps are kept to a minimum, thus no carriers are required. Complex cloning strategies are also minimised (Belyavsky *et al.*, 1989). The work of Domec *et al.* (1990) demonstrates that it is possible to clone cDNA synthesised from total RNA by PCR but more complex amplification procedures are involved since the linkers used to flank each end of the double stranded cDNA are different from each other; therefore, two PCR primers are required for the amplification of cDNA using this protocol.

The cDNA prepared from the sugar beet tissue was smaller than would have been expected, and several attempts to increase the size of the cDNA were unsuccessful. Therefore, it was decided to use this cDNA for cloning. Since PCR amplification is limited to a size of 3 to 4 kbp (Erlich *et al.*, 1991) any gain in cDNA size may have been lost subsequently due to the PCR amplification of that cDNA. If the full sequence of any gene was desired a clone could have been isolated from a sugar beet genomic library probed with the cDNA clone of interest. The small size of the starting cDNA is represented in the size of the inserts isolated from random recombinant cDNA clones from both the apical meristem and leaf cDNA libraries. For the reasons stated above, however, this was not seen as a problem.

On screening the leaf library with the LHCP cDNA clone (Fig 3.13) it was observed after secondary screening that the number of clones hybridising to the probe was of the expected order of magnitude. The work of Cuming and Bennett (1981) on the expression of

LHCP in *Pisum sativum* demonstrated that LHCP mRNA is present at an abundance of 2% of the poly (A^+) RNA in photosynthetically active tissue and 0.16% in etiolated leaf tissue. Since the apical meristem of sugar beet is shaded from the light by the surrounding leaves this tissue may be assumed to be less photosynthetically active than the leaf tissue, and as such resemble etiolated leaves. The data in the previous chapter (section 3.1.6.2) on this subject show that after secondary screening LHCP clones were represented at a level of 3% in the leaf cDNA library and 0.3% in the apical cDNA library (section 3.2.7). This is within the expected order of magnitude and is considered to be a good indication that the two libraries were representative of the RNA transcripts used to make the double stranded cDNA, and as such representative of cDNA libraries prepared by conventional techniques (Huynh *et al.*, 1985).

Since both libraries were prepared from total RNA, which removes one very wasteful step from the cloning strategy, a ribosomal DNA clone was used to demonstrate the lack of an excessively high number of cDNA clones originating from ribosomal RNA (section 3.1.6.2; section 3.2.7). For both libraries it was observed that eliminating the step of enriching for poly (A^+)RNA had not been to the detriment of the cDNA libraries. Using this evidence, and further data from screening the apical meristem cDNA library with α-tubulin, G-PAL 2, and ALS cDNA probes, it was assumed that the leaf and apical meristem cDNA libraries prepared by this PCR cloning technique were representative of the initial RNA transcripts used in their construction. They were, therefore, considered to be significantly different from each other to indicate that the cDNA amplified from the leaf and apical meristem cDNA species were not contaminated with cDNA species from each other.

4.2 Techniques for the Isolation of Apex-Specific cDNA Clones

Using PCR amplified cDNA for the differential screening of the apical meristem cDNA library led to the isolation of several putative positive apex-specific cDNA clones. This process was very labour intensive and eventually, from differentially screening 200,000 apical meristem cDNA clones only 64 appeared to give an indication that they may be more highly expressed in the apical meristem cDNA probe. On secondary screening of these 64 putative positive clones 8 still appeared to be more highly represented in the plus (apical meristem cDNA) probe than in the minus (leaf cDNA) probe. However, no single cDNA clone stood out as a true apex-specific clone, being represented only in the plus cDNA probe.

Although the cloning procedure used here has overcome the need for large quantities of RNA as the starting material for cloning, there is still a need for large amounts of RNA to be available for the characterisation of interesting cDNA clones. It may be the case that the PCR amplification procedure misrepresents the cDNA species in a population enough to disrupt the analysis of cDNA libraries prepared from that material. If a low abundance cDNA species is represented at twice its usual level in the plus probe this may lead to that cDNA species being represented at the same level in the plus and minus differential probes. For this reason PCR is not recommended as the best method for preparing cDNA probes that are to be used in differential screening. The data in Chapter 3 indicate that misrepresentation should not be a problem for this study, but the possibility must not be ruled out.

Subsequent characterisation of clones of interest was hindered by a lack of annual, warm, short day apical RNA available from sugar beet plants of the desired age (8-10 weeks). For enough RNA for one northern blot at least 100 apices must be isolated and the

RNA successfully retrieved from them. This compares with the 35 apices isolated for the cloning procedure. When using the more abundant leaf RNA for northern analysis of cDNA clones of interest there is a good chance of success since large quantities of RNA (mg amounts) may be isolated at any one time from a small number of leaves. One of the major stumbling blocks in this study was the apparent degradation of apical meristem RNA used in the analysis of the 8 putative positive cDNA clones (data not shown). This is most likely to be caused by the action of a ribonuclease so RNA must be prepared under conditions where the ribonuclease activity is eliminated. Equipment used for the isolation and analysis of RNA was therefore sterilised, gloves were worn and DEPC was used to inhibit the enzymes action. Several attempts were made to isolate non-degraded apical meristem RNA. On only two occasions was apical RNA that appeared to be non-degraded successfully isolated (Fig 3.25 and 3.31). However, on probing the northern blot in Fig 3.31 with the radiolabelled clone 26 cDNA probe the apparently intact RNA gave what appeared to be a non-specific signal. This could not have been due to the radiolabelled probe hybridising to a multigene family since there appeared to be no distinct bands of RNA transcripts hybridising to the probe but a smeared signal. However, it is more likely that a degree of degradation had occurred in the transfer of the RNA to the nylon membrane. Therefore, in this study it was not possible to show that all of the putative positive clones were false positives but one which appeared most likely to be an apex-specific clone was seen to be expressed more highly in the leaf RNA. Although several repeat northern blots were performed they all gave the same smeared result and so were not included in the results section. To repeat this northern blotting procedure with apical meristem RNA would require growing annual sugar beet plants under warm, short day conditions for 8-10 weeks. A maximum of 150 plants may be grown up at any one time, and usually 1/3 of them fall foul to pest infestation or waterlogging. From the

remaining healthy plants 80-90% of the apical meristems were usually successfully isolated. Since this operation is very tedious and painstaking, not surprisingly some of the apices were lost. From these apices it was possible to isolate enough RNA for 1-2 northern blots. If this apical meristem RNA was degraded by the ribonuclease enzyme, or was not precipitated in the presence of 2 M LiCl then a further 8 weeks must pass for the experiment to be repeated. Therefore, the conclusion here is that the difficulty of the experiments has meant that characterising a large number of putative positive apex-specific cDNA clones has not been possible. The limited amount of data obtained during this study on the characterisation of putative positive cDNA clones did, however, indicate very strongly that none of the putative positive clones isolated from the apical meristem library was enhanced in the apical meristem and, as shown by secondary differential screening, none was apex-specific.

As previously mentioned the two cDNA libraries appear to represent different populations of cDNA species yet no apex-specific cDNA clones were isolated. This may be because the cDNA used for differential screening was prepared by PCR amplification after the initial amplification of the cDNA used to make the libraries. PCR is a very sensitive procedure so a small amount of contaminating cDNA would be amplified a million fold, leading to results which may be misrepresentative of the original population. To try to eliminate contamination as a problem for this study separate equipment was used when handling PCR amplified cDNA material, solutions used in the PCR reaction were aliquotted into small amounts so that carryover from previously used solutions did not occur, and all reactions were carried out using sterile equipment. Therefore, contamination should have been eliminated but cannot be ruled out.

The susceptibility of the PCR reaction to contamination may explain to a degree the reason for the subtraction of apex-specific cDNA species with the leaf driver cDNA being

unsuccessful. In the absence of any apical meristem cDNA 4 rounds of mock-subtraction were seen to reduce the PCR amplified cDNA present in the final aqueous phase to an amount undetectable via Southern blotting and probing with a radiolabelled leaf cDNA probe. Therefore, the subtraction procedure was, in essence, working satisfactorily. However, upon addition of apical cDNA at a concentration 100 X lower than the driver DNA, even after 5 rounds of subtraction, sufficient cDNA remained to hybridise to a leaf cDNA probe (Fig 3.36) after PCR amplification. There are several reasons why this may have occurred. First of all, contaminating cDNA from either the leaf or apical meristem cDNA populations may have entered into the subtraction system at any point in the procedure. If this was the case several rounds of subtraction should have removed the contaminating cDNA species. Secondly, the hybridisation conditions used during subtraction may have been too stringent for common cDNA species to hybridise. This should not have been the case since the hybridisation conditions used during subtraction were the same as those used in differential screening of the apical meristem cDNA library. Since both the apical meristem and leaf cDNA species hybridised to the apical meristem cDNA clones studied under these conditions, it would be expected that any common cDNA species from the apical and leaf cDNA probes in the subtraction system would have hybridised to each other, if not after the first round of subtraction then after the second or third. The use of excess driver leaf cDNA in the subtraction procedure could have been a problem in that a majority of common cDNA species would not have bound to their complement in the apical cDNA population. Since after each round of subtraction the remaining cDNA is amplified, and since some of the leaf cDNA species should have been removed from the subtraction set-up, the number of apex-specific cDNA species within the subtraction should gradually increase and out number the common cDNA species in the subtracted cDNA population. The third possibility would be

that the streptavidin protein was not binding to the driver cDNA. This was demonstrated not to be the case in Fig 3.34 and 3.35. Everything, therefore, points to contamination of the PCR amplifiable subtracted cDNA samples with either leaf or apical cDNA species. This material could have been introduced into the subtraction procedure after binding of the driver cDNA to the streptavidin and, as such, would not have been exposed to any form of subtraction. If this contamination was repeated at the same point after each round of subtraction then a subtracted apex-specific cDNA probe would never have been produced by this method.

Considering all of these drawbacks with the characterisation of cDNA clones isolated from the apical meristem cDNA library it cannot be said that the apical meristem cDNA library did not contain clones specific to the apical meristem but, using the methods employed in this study, it was not possible to isolate cDNA clones specific to the apical meristem. Using similar PCR protocols Meeks-Wagner *et al.* (1989), and Medford *et al.* (1991) have utilised plant systems that give rise to large quantities of shoot meristem material to isolate genes expressed in the shoot tips of tobacco and cauliflower respectively. For the study of tobacco a thin cell layer system developed by Tran Thanh Van *et al.* (1985) was used to isolate large quantities of bud tissue from plant material induced and not induced to flower. This work has enabled the isolation of genes expressed upon the induction of flowering in the cells of buds induced to flower in an *in vitro* cell system. Therefore, this study did not concentrate on purely meristematic tissue but involved the whole of the developing floral bud. The work of Medford *et al.* (1991) utilised the unusual growth habit of cauliflower, a member of the *Brassica* family. This plant is unusual in that the head of the cauliflower is a proliferation of shoot apical meristems. This allows for the isolation of large quantities of apical meristem tissue for cloning and subsequent characterisation of any clones of interest. This was of great advantage to Medford *et al.* (1991) in that cDNA libraries were

constructed by the conventional methods (Huynh *et al.*, 1985) which meant that they could be sure that their libraries were representative of the transcripts contained within the starting RNA material. PCR was only used to increase the quantity of cDNA used for differential screening. Several genes shown by northern blotting to be highly expressed in the shoot tips of cauliflower were introduced into *Arabidopsis* plants to study their expression (using the GUS assay) in a plant which does not develop in the unusual manner of the cauliflower plant. None of these genes was shown to be truly specific to the apical meristem but were also expressed in the shoots, roots, seedlings or flowers. Medford *et al.* (1991) also observed that one gene, termed meri-5, was expressed in different areas of the transformed *Arabidopsis* plant as the plant aged. Therefore, it may be the case that the apical tissue used in this study of sugar beet should have been of a younger age to allow the isolation of genes specific to the apical meristem. The isolation of meristematic genes by Medford *et al.* (1991) will enable future workers to assess the possibility that plants of a younger age may be more useful in the study of genes expressed specifically in the apical meristem tissue of flowering plants.

4.3 Concluding Remarks

The introduction of molecular biological techniques to the process of understanding the mechanisms of flowering has advanced our knowledge of the genes involved in the morphology of flowering considerably. We do not know the mechanistic basis for fating of cells or tissues; however, in the flower organ formation we are beginning to understand how fate is acquired (Coen and Meyerowitz, 1991). Homeotic mutants have allowed the

identification of gene products that appear to be transcriptional regulatory factors that directly, or indirectly instruct the cells of an organ primordium as to what organ to form. Whether or not this type of molecular mechanism is employed in the initiation of floral development is not known (McDaniel, 1992).

There are still those who believe that the florigen/antiflorigen theory holds true. The difficulty many scientists have in accepting this theory is that it assumes one process is acting in the same manner in all flowering plants. The hypothesis that the same factor(s) is (are) involved in the initiation of flowering in all flowering plant species has been criticised by many workers (reviewed in Bernier, 1988) who have observed that graft transmissible substances do not initiate flower development in all non-induced graft partners. It is not possible to obtain graft partnerships between every species of flowering plant and so only a limited number of plant species has been studied for the effects of graft transmission from induced to non-induced graft partner. However, of those species studied a minority have demonstrated the ability of non-related induced flowering plants to influence non-induced graft partners to flower. Therefore this unifying theory can only be applied to a select few species. The possibility that the concentration of nutrients in the apex increasing in low temperatures has something to do with the initiation of flowering is an assumption that many workers are considering (Salisbury, 1963; Atherton *et al.*, 1987; Bernier, 1988). Since a substantial amount of data indicates that an increase in sugar content of low temperature treated apical meristems does occur, and under these conditions floral induction is observed, there is reason for investigating this hypothesis further. The need for an increase in nutrients is understandable if the apical meristem is to divide rapidly, and differentiation is to occur. For this to be the only factor involved in the switch from vegetative to floral induction appears improbable since these data only apply to flowering plants requiring vernalization

and the process of flowering is known to be influenced by many different factors (intrinsic and extrinsic). Many inhibitors of nucleic acid synthesis have been shown to inhibit flowering, which implies that this process is in some way linked to the regulation of flowering. It may be that the inhibition of nucleic acid synthesis disrupts housekeeping processes within the plant, and as such inhibits all further development of the plant. The belief from these studies is that floral induction requires the synthesis of new mRNAs specific to the process of flowering. Several workers have shown that under conditions necessary for floral induction in many plants nucleic acid, and protein synthesis is altered (reviewed by Bernier, 1988). Very few, however, have demonstrated that the new products are related to the development of flowers, and not related to other processes within the plant which change under inductive conditions. For example, under conditions of low temperature most species synthesise proteins associated with cold tolerance. These may play some part in the ability of plants to flower under the correct, subsequent, photoperiodic conditions, but serve a more general purpose in permitting the growth of the plant concerned under very low temperature conditions. Nucleic acid species known to be synthesised in LDP under LD conditions may well be associated with initiation of flowering, but to date it has not been possible to state that any one gene is associated solely with the induction of flowering, and not with some other associated process, such as an increased rate of photosynthesis. The association of a NB with the induction of flowering under SD conditions has been studied and shows that there are proteins synthesised during SDs which are not simply associated with the change in daylength, but are involved in the perception of photoperiodic induction of flowering (Lay-Yee *et al.*, 1987). Therefore the implication that simple nutrient shifts are responsible for the change from vegetative to floral development does not take into account all of the evidence to date. There appears to be a lot more involved in this developmental

change than a hormonal, or nutrient shift. One explanation of the molecular mechanisms involved in vernalization and its effects on flowering may be that induction by low temperatures brings about changes in membrane permeability to a hormone previously synthesised under non-inductive conditions overcoming some barrier to the flowering process. However, at the same time the concentration of nutrients within the apical meristem must be sufficient for the increased cell division that is about to take place to commence. This would explain the observation of several workers that the removal of root tissue is detrimental to the flowering process. Alternatively, it may be the case that vernalization acts to allow the phosphorylation of a protein already present in the apical meristem cells of the plant and this phosphorylation alters the properties of the protein significantly to allow its action to be perceived by the apical meristem cells and induce the change from vegetative to floral development. This is all speculation as, to date, nobody has succeeded in isolating a gene associated with floral induction, neither has anybody succeeded in isolating the hormone(s) proposed by so many workers so long ago to be involved in the process of floral induction.

The idea that GA is involved in the induction of flowering is an old one, and it has been demonstrated that external applications of GA in some form or other do cause stem elongation in many flowering plants. However, for GA to be the hormone implicated in the florigen/antiflorigen theory is not acceptable since GA is only effective when applied to rosette LDP and a few other LD species. SDP, in general, do not respond to applications of GA. Again, however, there are exceptions as *Chrysanthemum morifolium* is a SDP which is affected by application of GA causing elongation of its stem without exposure to SD conditions. The general consensus is that GA plays a role in stem elongation, and can account for part of the vernalization requirement of many flowering plants, but GA alone is

not the agent responsible for the transition from vegetative to floral development.

Although the methods used in this study have not proved to be successful in isolating any apex-specific cDNA clones they have raised some interesting questions. Does the apical meristem of sugar beet possess apex-specific mRNAs ? The data of Medford *et al.* (1991) indicates that this should be the case although few other workers have yet managed to demonstrate this in other plant species. Work with *Pharbitis nil* (Keith Edwards, personal communication) has proved fruitless in the search for apex-specific cDNA clones, adding to the evidence that the isolation of apex-specific cDNA clones is a difficult process when tissue is limiting. The introduction of PCR has increased the success of attempts to clone cDNA from small amounts of both plant and animal tissue, but the characterisation of cDNA clones from libraries produced in this way still requires the isolation of large quantities of RNA from such tissues. Therefore, should methods of enhancing for apex-specific cDNA clones be explored? Subtractive screening and cloning have proved useful in the isolation of cDNA clones specific to certain tissues (Sive and St John, 1988) but were not successful in this study, possibly due to the sensitivity of PCR. It is hoped that the work of Medford *et al.* (1991) will lead to a better understanding of the expression of genes in the apical meristem of plant shoots, allowing the field of floral induction to be studied with a greater in depth knowledge of the subject.

Over the past decade advances in the field of plant molecular biology indicate that isolating a gene (or genes) for a process whereby the gene product is not known may be best accomplished using a method of gene-tagging. The most extensively studied method is that of transposon-tagging which is a process originally described for work with *Drosophila* (Bingham *et al.*, 1981). A few years later gene tagging experiments were reported in plants, the first of which was the successful cloning of the *bz* locus of *Zea mays* using a transposable

element known as *Ac* (*Activator*) (Fedoroff *et al.*, 1984).

Transposable elements, or transposons, are mobile segments of DNA which inactivate, or alter gene expression by insertion into the genetic locus. Transposon-induced mutations have been described in a few plant species and a few plant transposable elements have been cloned (mostly from *Zea mays*). All of the early transposable elements isolated from *Zea mays* were associated with characteristics of the *Zea mays* kernel which is easily scored. This is an important factor in the isolation of genes by gene-tagging.

In *Zea mays* some elements are capable of self encoded excision and transposition. Such elements are termed autonomous. *Ac* is an autonomous transposable element isolated from *Zea mays* and is capable of catalysing its own excision and transposition. Another element, *Ds*, (which resembles the terminal sequence of the *Ac* element) is non-autonomous and requires an active *Ac* element in the genome before it will move. As has been demonstrated for *Zea mays* it is useful to choose a target gene that can be recognised at the seed or seedling level. In a genetic cross to attempt to isolate a gene of interest one parent plant would carry an active transposon and be homologous for the wild type of the target gene while the other carries the homozygous stable recessive allele of the target gene. Thus, in the F1 progeny a wild type phenotype would be expected. If the sample size is large enough a few exceptional progeny not exhibiting the wild type phenotype may be found, suggesting that something has occurred to alter the wild type allele of the heterozygote. This phenotype may reflect the insertion of the active transposon into the wild type allele of the target gene.

To establish that a transposable element has caused a mutation of the target gene the transposon must be used as a hybridisation probe to detect pieces of DNA harbouring the transposon.

It is also possible to introduce transposable elements into plant species by transformation as a viable alternative to isolating endogenous transposons. This would require the ability to stably transform the plant species of interest which, to date, has not proved possible for sugar beet, but such species as tobacco (Baker *et al.*, 1986), *Arabidopsis* and carrot (Van Sluys *et al.*, 1987), have been stably transformed with transposable elements and interesting genes isolated by gene-tagging. Recently a male sterility gene of *Arabidopsis* was isolated using the *Zea mays Enhancer-inhibitor* transposable element system (Aarts *et al.*, 1993) and a *Petunia* flower colour gene was isolated with the use of the *Zea mays* transposable element *Ac* (Chuck *et al.*, 1993).

This all bodes well for the field of plant molecular biology as it seems to be that many new and interesting genes are being isolated by gene-tagging but the procedure is limited to those few species known to contain transposable elements, or easily transformed with such elements. To apply this methodology to sugar beet would require stable transformation. Also, since we would be looking for a phenotypic change involving flowering the progeny of any experiment would need to be grown up for a long period of time, taking up a large amount of space, to isolate a few putative mutants tagged by the transposon at the gene of interest. Using a model system like *Arabidopsis* to look for genes involved in the induction of flowering using gene-tagging may be more appropriate and successful since this plant species is easily transformable with transposable elements and has a rapid generation time. It is quite probable that genes involved in the induction of flowering in *Arabidopsis* are also involved in the same process in sugar beet. If gene-tagging can prove successful in isolating such genes from some other plant species these genes may be used to probe a sugar beet library provided the conservation of floral inductive genes is high between highly unrelated plant species.

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