Cloning and characterisation of genes determining pod morphology in pea

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Cloning and Characterisation of Genes Determining Pod Morphology in Pea

by

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ABSTRACT.

Genes expressed in developing pea pods were isolated as cDNAs by differential screening techniques. The cDNAs were characterised by DNA sequencing and expression studies were used to investigate the role of isolated cDNAs in pod development.

A clone isolated from a pea (*Pisum sativum* L.) pod cDNA library was shown to contain the complete coding sequence of a polypeptide with considerable homology to various members of the Rab subfamily of small ras-related GTP-binding proteins. Conserved sequences in the isolated clone include the GTP-binding site, GDP/GTP hydrolysis domain and C-terminal Cys residues involved in membrane attachment. The high percentage amino acid identity suggests that this cDNA may be the product of a gene, designated Psa-*rab*, which is the plant counterpart of Rab7. Rab/Ypt proteins are thought to be involved in intracellular transport from the endoplasmic reticulum to the Golgi apparatus and in vesicular transport. If Psa-*rab* is a functional counterpart of yeast *YPT7* (*Rab7*) it should be able to complement a yeast *YPT7* mutant. An attempt was made to demonstrate that this was the case. Northern analysis showed invariant expression of Psa-*rab* in developing pods with different phenotypes, indicating an essential function for Psa-rab in developing pods. Hybridisation of the Psa-*rab* cDNA to pea genomic DNA showed that this protein is probably encoded by a single gene.

Nearly isogenic pea lines were selected to investigate the genetic basis for lignification of the pea (*Pisum sativum* L.) pod endocarp. The development of the pod endocarp in the normal and mutant pea pod phenotypes was examined by histochemical staining and light microscopy. The effect of plant growth regulators on endocarp development was also investigated.

A pea pod cDNA library representing poly $(A)^+$ RNA purified from L59 pea pods (genotype, PV; phenotype, lignified endocarp) was differentially screened with total cDNA probes prepared from total pod RNA from L59 and L1390 (genotype, pv; phenotype, no lignification of endocarp) pods 4 - 6 days after flowering (DAF). Two clones, designated pLP18 and pLP19, were selected for further characterisation on the basis of hybridisation to the L59 cDNA probe, but not the L1390 cDNA probe.

Northern blotting was used to show that pLP18 represented a mRNA of 0.95 kb. The predicted polypeptide from the LP18 cDNA encoded a putative blue type I copper protein. The expression pattern of LP18 mRNA in pods and tissues of the experimental pea lines was determined using RT-PCR

quantitation. Hybridisation of the cDNA to pea genomic DNA showed that this protein is probably encoded by a single gene.

Clone pLP19 yielded a 1.02 kb cDNA fragment encoding the C-terminal portion of an Hsp70 homologue belonging to a highly conserved family of proteins found in a number of eukaryotic species. Northern analysis of RNA from lignified and unlignified pods showed the presence of differentially expressed LP19 transcripts of varying lengths, which may represent differently processed transcripts. Southern analysis confirmed the presence of a single hybridised band in genomic digests of L59, L58 and L1390. Several mRNA transcripts of the LP19 gene were isolated which differ in the length of their 3' untranslated regions.

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MEMORANDUM.

Parts of the work presented in this thesis have been presented previously in the following publications (see appendix):

Drew, J.E., Bown, D. and Gatehouse, J.A. (1993) Sequence of a novel *ras*-related cDNA from Pisum sativum. Plant Mol Biol, 21, 1195-1199.

Drew, J.E. and Gatehouse, J.A. (1994) Isolation and characterisation of a pea pod cDNA encoding a blue copper protein correlated with lignin deposition. J Exp Bot, 45, 1873-1884.

ABBREVIATIONS.

°C degrees Celsius

% percent

aa amino acid

amp ampicillin

ATP adenosine-5′-triphosphate

6-BAP benzyladenine

bp base pair

BSA bovine serum albumin

CAD cinnamyl alcohol dehydrogenase

cDNA copy DNA

COMT caffeate 3-*O*-methyltransferase

cpm counts per minute

C-terminal carboxy-terminal

DAF days after flowering

dATP deoxyadenosine-5′-triphosphate

dCTP deoxycytosine-5′-triphosphate

ddH_2O deionised distilled water

DEPC diethylpyrocarbonate

dGTP deoxyguanosine-5′-triphosphate

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

DNase deoxyribonuclease

DTT dithiothreitol

dTTP deoxythymidine 5′-triphosphate

EDTA ethylaminediamine tetra acetic acid

EGTA ethylenediamine tetra acetic acid

ER endoplasmic reticulum

FF Feltham First

FPP farnesyl pyrophophate

g gram

GA gibberellic acid

GGPP geranylgeranyl pyrophosphate

GP green podded mutants from the PP line

IAA indole-3-acetic acid

IPTG isopropyl-β-thiogalactopyranoside

kb kilo base

kD kiloDalton

l litre

LP lignified pod

M Molar

µg microgram

µl microlitre

µM micromolar

ml millilitre

mm millimetre

mM millimolar

MOPS 3-(N-morpholino) propane-sulphonic acid

mRNA messenger RNA

mt mitochondria

MVA mevalonic acid

NAA napthol acetic acid

N-terminal amino-terminal

NC nitrocellulose

nm nanaometre

OD_{260} optical density at 260nm

PAL phenylalanine ammonium lyase

PCR polymerase chain reaction

pmol picomole

poly (A) polyadenylic acid

PP purple podded

ppt precipitate

PVP polyvinylpyrrolidone

RNA ribonucleic acid

RNase ribonuclease

rpm revolutions per minute

RT-PCR reverse transcription polymerase chain reaction

SDS sodium dodecyl sulphate

SSC saline sodium citrate

TCA trichloroacetic acid

TE tris-EDTA

Tris tris(hydroxymethyl)methylamine

UTR untranslated region

UV ultra violet light

v/v volume for volume

w/v weight for volume

X-gal 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Single letter abbreviations for amino acids and bases are as specified in: Biochem J (1984) **219**,345-373 and Biochem J (1985) **229**,281-286 respectively.

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Thanks to Dr. Dietrich Scheglmann for kindly supplying the yeast mutant and the helpful advice. Thanks also to Caroline Hartley and Dr. Martin Watson for undertaking the yeast complementation experiment.

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To my partner Susan and our daughter Sarah, thank you both for your faith and for seeing it through.

1. INTRODUCTION.

1.1 General Introduction and Aims of the Project

Peas (*Pisum sativum* L.), belonging to the family Leguminosae, sub-family Faboideae, are one of the most important crop legumes. They consist of a collection of domesticated races originating from human instigated and selected genetic variations with a diploid chromosome number of fourteen. The pea plant is particularly suitable for biological research and has a long history of studies, as a representative of dicotyledonous plants, dating from Mendel's work in the nineteenth century. Pea seed is available throughout the year, and will germinate easily when placed in suitable conditions. Dormancy, hard seed coats and special conditions for germination are not a problem. The life cycle of the pea proceeds rapidly, facilitating studies of any stage of growth within relatively short time periods. Germination is initiated after approximately 25 hours at 20 °C - 25 °C. The appearance of lateral roots usually occurs after about 75 hours and the epicotyl after approximately three to four days. Flower initiation may proceed from as early as the two leaf stage. The first flower may occur after the second up to the thirtyfourth sterile node.

There is also great genetic variation within peas which may be a result of its long history of collection by research workers or a natural phenomenon (translocation and chromosome rearrangements are common in pea). A large number of mutant pea lines has been recorded and the controlling genes identified e.g. seed colour and shape; flower colour; pod dehiscence, shape, size and colour. There are around 1000 cultivars with catalogued variations (Pea Gene Bank Catalogue, 1989). The pea line 110 in the Weibullsholm collection has been chosen to represent the normal chromosome structure. Capitalised symbols indicate a dominant character relative to line 110 and lower case symbols represent a character recessive with respect to line 110. Each mutant is assigned a type line (usually the line in which the mutant was first identified). Loca are then determined by defining the position and effect of a particular gene in its type line. Pea has a relatively large genome size (4.8×10^9 bp per haploid genome), which is

comparable to the human genome. However, it is thought that up to 90% of the pea genome is secondary DNA and, therefore, not essential for basic functions such as coding and gene regulation. Its large genome size has made a pea a less favoured subject for plant molecular biology, despite its advantages as a subject for biochemical and physiological investigation. Nevertheless, extensive molecular characterisation of genes in this species have been carried out.

Pods fulfil a number of important biological functions. They modify and ameliorate the environment of the developing seeds and provide a route for passage of minerals and nutrients to the seeds. The pod is a modified leaf, retaining many leaf-like characteristics, such as the capacity to photosynthesise (Atkins *et al*, 1977). Thus, it provides a carbohydrate reservoir, mobilised in later stages of seed development (Flinn and Pate, 1968). During desiccation of senescing pod tissue protein is mobilised and translocated to the developing seeds (Pate and Flinn, 1977).

Among the catalogued pea cultivars there are twenty-six identified genes determining distinct pod characteristics. Several of the identified pod genes alter pod structure e.g. n, pod wall thickness (Wehner and Gritton, 1981); the P and V loci, lignification of the pod endocarp (Lamprecht, 1948); Pu and Pur, colour (Lamprecht, 1948); and gp, chlorophyll mutant with yellow pods (White, 1917).

Investigation of pod mutants assists in elucidating the mechanisms involved in normal pod function. Possible breeding strategies to improve pod features and increase productivity can be planned. In addition, study of the mechanisms of pod development may have implications and relevance to the whole plant.

This study involves the investigation of the characteristics of the P and V loci, which affect pod endocarp development. The P and V loci are worthy of investigation for a number of reasons. The sclerenchymatous layer, which develops in the pods of peas with genotype PV, has a significant effect on the optical properties of the pod wall. Reflection, transmission and absorption spectra are radically altered as a consequence of the presence, or absence of the

sclerenchymatous layer of the pod endocarp. The layer of sclerenchyma cells has been shown to exhibit a high UV-B absorbance and low transmission of light below 370 nm (Donkin and Price, 1990). UV-B irradiances can have a deleterious effect on the developing seeds. Light scattering due to reflection may also increase light capture by chloroplasts of the inner mesocarp (Donkin and Price, 1990). The lignified tissues in the pericarp of pods remain an effective UV filter as the pod desiccates (Price *et al.*, 1994) and may play a role in pod dehiscence (Fahn and Zohary, 1955; Meakin and Roberts, 1990). The sclerenchymatous layer in the pod also acts as a protective layer preventing attack by insects which may eat the seeds or lay eggs (Walter, 1992).

The effects of mutations at the P and V loci on the process of lignification is another aspect worthy of investigation. Lignin is a very complex polymer, which is of economic importance. It is extremely difficult to degrade chemically and biochemically (Crawford, 1981). The presence of lignin interferes with the release of cellulose from wood during pulp and paper production and its removal by chlorine and chlorine derivatives gives rise to toxic waste constituting an environmental hazard (Eriksson, 1990). The lignified sclerenchymatous layers are indigestible by animals, which do not have the necessary enzymes in their gut to digest such tissue (Goto *et al.*, 1992). Its presence in animal fodder decreases digestibility and nutritional value. At present, although a number of genes encoding enzymes involved in the lignin synthesis pathway are known the process of initiation and regulation is poorly understood. There are only a few mutants in the general phenylpropanoid pathway (Chapple *et al.*, 1992), therefore it is difficult to determine the exact nature of the process whereby cells are induced to differentiate and lignify. Lignin has also been found to have a role in disease resistance and plant structure (Delmer and Stone, 1988; Walter, 1992). Hence, there are a number of potential advantages to genetic manipulation of lignin quality and quantity in crop and forest species.

Finally, the orderly development of the pod requires correct temporal and spatial expression patterns of multiple genes. Molecular mechanisms of plant development may be more fully understood by

isolating and characterising tissue-specific and developmentally expressed genes.

1.2 Pea Lines L59, L58, L1390 and Feltham First

The alleles of interest in L59, L58, and L1390 are P and V (White, 1917), which are located on chromosomes 6 and 4 respectively (Lamprecht, 1948) (Figure 1.1). All of these lines are maintained by the Weibullsholm Plant Breeding Institute, with strict selection maintained to keep the genotype as defined in the Pea Gene Bank Catalogue (1989). The cultivar L58 has the alleles Pv. L59 is a mutant v to V from L58. L1390, a pedigree line derived from a cross between L58 and L966, is doubly recessive and has the alleles pv. Feltham First is a commercially available variety assumed to have the alleles PV, as indicated by histochemical staining. P and V alter the structure of the pod wall. The recessive alleles of the P and V loci individually reduce the parchment layer and in combination remove it altogether (Lamprecht, 1948) (see Table 1.1). Most of the sclerenchymatous membrane is removed from the inner pod wall of lines carrying the v allele. Small patches of sclerenchyma having a slightly shiny appearance on the inside of the pod wall or a thin layer, unevenly distributed, often only towards either end of the pod may be apparent in lines carrying one recessive P or V allele.

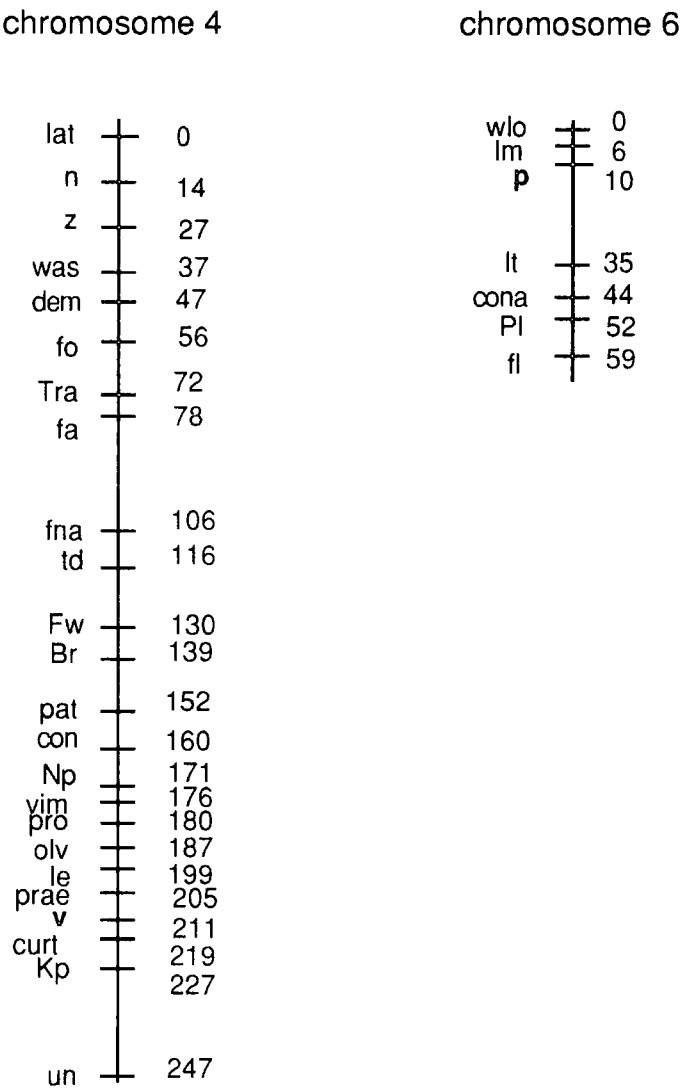


Figure 1.1 The genetic map of chromosomes 4 and 6 of pea showing the location of p and v loci, adapted from Blixt (1974).

Table 1.1 Pod genotypes and corresponding phenotypes (Pea Gene Bank Catalogue, 1989)

Genotype	Phenotype
PV	normal parchmented pod
pV	strip of sclerenchyma
Pv	inner membrane reduced to patches of sclerenchyma
pv	entirely without parchment

Spontaneous mutation rates p to P are 0.05% - 0.2% and correspondingly 0.3% - 3% for v to V (Pea Gene Bank Catalogue, 1989).

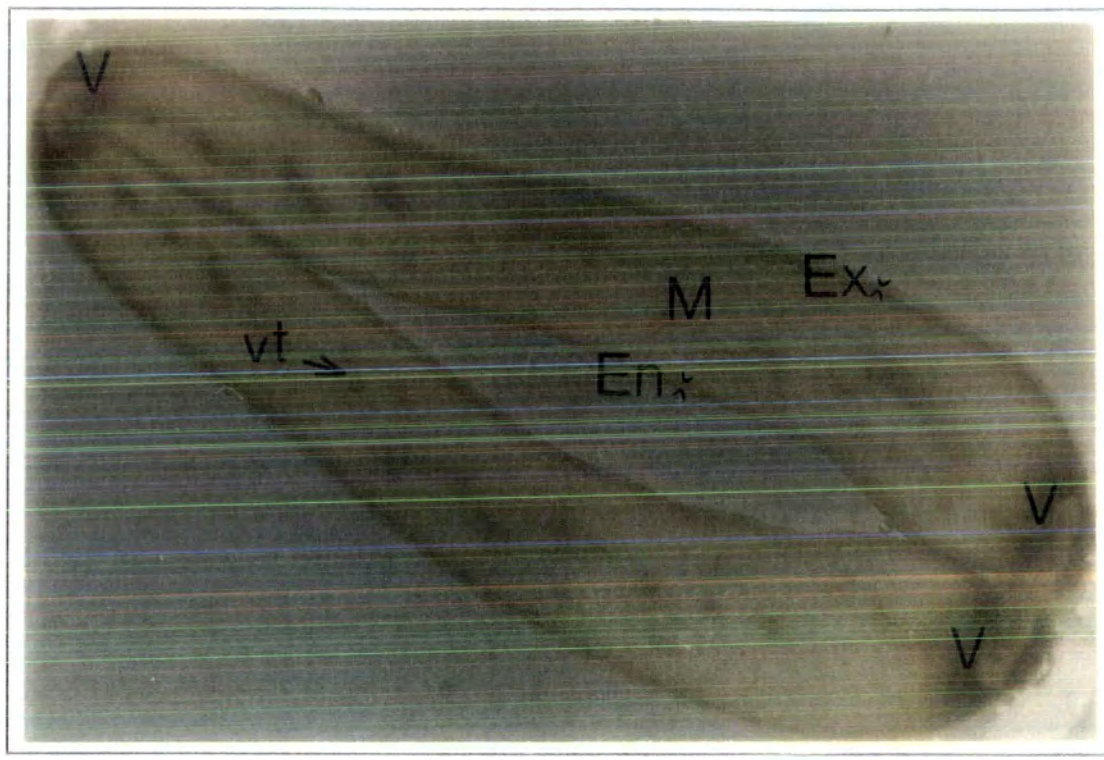
1.3 Purple-podded Pea Lines

Purple-podded pea lines are commercially available (Sutton's Seeds) and were chosen as good growers. They carry the dominant genes Pu on chromosome 3 and Pur on chromosome 1 as indicated by the purple-podded phenotype (both Pu and Pur are necessary for this character). The purple-podded character is variable in expression and unstable. A differential screen of this purple-podded line has already led to the isolation of mRNAs which encode enzymes thought to be involved in the anthocyanin pigment biosynthesis pathway (Bown, 1992). In addition mRNAs encoding pectinesterase (Bown, 1992) have also been isolated and are thought to play a role in pod dehiscence of the purple pods.

1.4 Pod Wall Anatomy

There are three distinct layers of the pea pod wall (pericarp) i.e. the exocarp, mesocarp and the endocarp (Fahn, 1974; Mauseth, 1986) (see Plate 1.1). The exocarp consists of an epidermis, which is about 3 % of the pod wall thickness. The mesocarp consists of thin walled parenchymatous cells (18 - 20 layers of cells), which have large

Plate 1.1 Pod wall anatomy. Ex, exocarp, 3% of the pod wall thickness, layer of epidermal cells; M, mesocarp, approximately 84% of the pod wall thickness, 18 - 20 layers of thin walled parenchymatous cells containing the bulk of the pod chlorophyll, mid to outer mesocarp contains the main vascular network; En, endocarp, 13 % of the pod wall thickness, consists of an outer region of 2 - 3 layers of sclerenchymatous cells which contribute to dehiscense on drying and separated from the epidermis of the endocarp by thin walled parenchyma; V, main veins; vt, vascular tissue.


V
M
Ex
vt
En
V
V

vacuoles. The outer layers of the mesocarp contain the bulk of the pod chlorophyll (Atkins *et al*, 1977), while the inner layers often contain large quantities of starch. The mid to outer mesocarp contains the main vascular network. Calcium rich crystals in the cells of the innermost mesocarp layer may indicate the presence of calcium oxalate, which have a developmental association with fibrous tissue. The mesocarp is approximately 84 % of the pod wall thickness. The endocarp consists of an outer region of sclerenchymatous (fibrous) tissue (commonly three to five layers of cells), which contributes to dehiscense on drying. Thin walled parenchyma (three to four cell layers) may be present between the fibre layers and the epidermis of the endocarp. The endocarp contributes to approximately 13 % of the pod wall thickness. It is probable that fibre layers were thicker in ancestral forms of present day pods, which have been selected for non-shattering characters. Chloroplasts are present in the inner epidermis of *Pisum* and may have a role in assimilation of CO_2 from the ripening seeds. The inner epidermis lacks stomata and has a hydrophilic cuticle in contrast to the outer epidermis (averaging 20 stomata/mm^2) (Pate and Kuo, 1981).

Pea pods have typical legume vasculature (see Plate 1.1). A pair of veins equivalent to the marginal veins of the carpel and lying longitudinally supply nutrients to the seeds. A third vein equivalent to the mid rib of the carpel runs along the pods lower (adaxial) surface. The three veins converge at either end of the pod. A network of minor veins traverse the side walls of the pod, interconnecting with the main longitudinal veins. Xylem vessels are less obvious in the branches supplying the individual seeds.

1.5 Sclerenchyma

Sclerenchymatous tissue consists of hard lignified cells with secondary wall thickening. Between 18 % and 35 % of the secondary wall may be composed of lignin. Sclerenchyma provide a support function similar to collenchyma, but may be distinguished by their absence of protoplasts when mature. The secondary wall (interior to the primary wall) provides elasticity and resiliency.

There are two types of sclerenchyma cells found in plants i.e. sclereids and fibres (Fahn, 1974). Sclereids are usually isodiametric cells associated with xylem and phloem or parenchymatous tissue. They are found singly, or in groups and exhibit great variation in shape. Fibres consist of sclerenchyma cells of varying lengths (usually more elongated than sclereids). They are found in various tissues e.g. root, stem, leaves and fruit. They may be associated with vascular tissue or the parenchymatous tissue of pith or cortex. They may occur singly, but are more commonly found in bundles. A complete layer of sclerenchyma may be present in the pea seed coat (Harris, 1984) and a layer or discrete patches may be present in the pea pod wall endocarp (Lamprecht, 1948).

Sclerenchyma may originate from parenchyma, or collenchyma, and can only be readily identified when differentiation proceeds and they elongate, branch, or form lobed ends. Such cells grow rapidly and branch into neighbouring tissue. Cell wall development in differentiating sclerenchyma is associated with transparent and electron dense vesicles, coated vesicles and a proliferation of polysomes on the ER (Lawton *et al.*, 1979; Harris, 1983). These observations are indicative of the intense activity in differentiating sclerenchyma. The vesicles accumulate at the cell surface and are involved in discharge of materials destined for the cell wall (Harris, 1984). Maturation of the cell coincides with the deposition of a lignified secondary wall, which varies between cells. The majority of mature sclerenchyma cells die post lignification of the secondary cell wall. However, there are a few instances of long lived sclerenchyma, particularly fibres (Fahn and Leshem, 1963). Osmotic pressure and hormonal factors have been implicated in the differentiation of sclereids (Garcia-Martinez and Carbonell, 1980).

1.6 Cytodifferentiation Sequence Leading to Development of a Sclerenchymatous Pod Endocarp

Cytodifferentiation of the pod endocarp follows a sequence of co-ordinated steps from anthesis to maturity. The developmental stages from anthesis to maturity may be designated sequentially as the initial, elongation and maturation phases and have been characterised by

Vercher *et al.*, (1987). The cells which are about to differentiate to form sclerenchyma enlarge initially. Secondary wall monomers (nucleotide diphosphate sugars of the D-glucose series) are produced concomitant with diminished epimerase activity. Epimerase enzyme activity may shift metabolism from production of primary wall monomers to secondary wall monomers during cytodifferentiation. Microtubules, which are often observed to be associated with the cell wall at this stage, may play a role in orientation of cellulose microfibrils. Cellulose and hemicellulose synthesis proceeds, followed by lignin metabolism, involving the activation of enzymes associated with lignin synthesis and synthesis of lignin precursor pools prior to deposition of lignin. The controls for future cell autolysis and hydrolyase activation of the lignified cells will also be set.

The endocarp structure during the initial phase of pod development, two to three DAF, consists of three layers (Vercher *et al.*, 1987): a transition layer of typically parenchymatous cells, smaller and denser than the mesophyll, with thin walls and a large vacuole; a middle zone, which consists of three to four strata of meristematic cells, which later differentiate to form sclerenchyma in pea lines with genotype PV; and the epidermis.

As the initial phase ends the middle zone increases its number of strata by increased cell division. At around four to five DAF the elongation phase is initiated and the endocarp consists of the three layers described above, together with a fourth pre-sclerenchyma layer, which originates from the middle zone (Vercher *et al.*, 1987). The cells of the pre-sclerenchyma layer then characteristically develop large nuclei, large numbers of ribosomes, dense cytoplasm, well developed rough endoplasmic reticulum (RER) and Golgi with electron transparent and electron dense vesicles observed close to the cell wall. The cells of this fourth layer commence differentiation by elongating. The plasmalemma is undulating, vesicles, RER and Golgi continue to be in close proximity to the cell wall.

Five to six DAF the pre-sclerenchyma layer of elongated cells have thickened walls, dense cytoplasm with large numbers of polysomes and highly developed Golgi. The transition layer cells increase in size and

the epidermal cell walls thicken and enlarge perpendicularly to the cell wall. Pods at around nine DAF typically have two to three layers of sclerenchyma.

1.7 The Regulation of Pea Pod Development

The plant hormones, gibberellins, auxins and cytokinins, are thought to play major roles in the pod developmental processes outlined above (Eeuwens and Schawbe, 1975; Carbonell and Garcia-Martinez, 1980; Garcia-Martinez and Carbonell, 1980; Vercher *et al.*, 1987). Cytokinins are thought to play a role in the initial phase of cell division, while auxins and gibberellins are thought to be involved in the elongation and cell wall thickening stages.

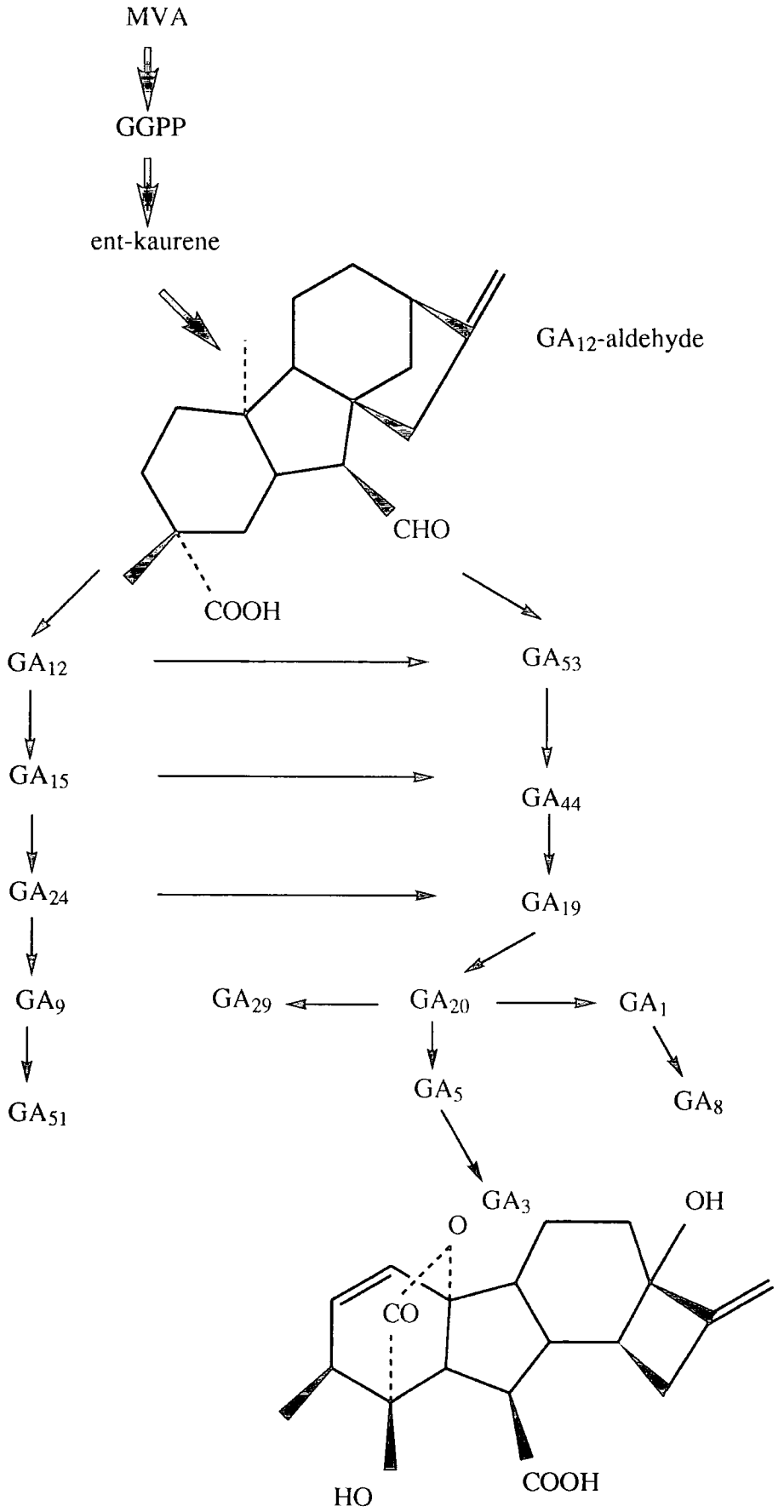
Gibberellins play a central role in regulating pod development and appear to be a major stimulus for the initiation of a series of complex developmental phases controlled by different levels of plant growth regulators. Gibberellins are synthesised in young tissues of the shoot (Pereto *et al.*, 1988) and developing seeds and fruit and in roots (Sponsel, 1988), and may be transported throughout the plant in phloem and xylem. The initiation of pod development from flower buds is thought to result from gibberellin stimulation from pollination and, or, fertilisation. Peas are self fertilised one day before anthesis (Blixt, 1974). The fertilised ovule is thought to supply gibberellins, which then initiate and regulate pod development. High concentrations of GA3 have been observed in ovules (Garcia-Martinez and Carbonell, 1980), and fertilised ovules are a major site of GA1 and GA3 synthesis, with a maximum at the stage of rapid pod elongation (Garcia-Martinez and Carbonell, 1980). Additional gibberellin may be supplied by vegetative plant parts, including tissues of the pod wall at later stages of development (Garcia-Martinez and Carbonell, 1980). Pods at four DAF have been observed to synthesise gibberellins from GA12 and GA20 (Ozga and Brenner, 1990). Gibberellins appear to induce transformation of the unpollinated pea ovary into a developing pod in two significant ways. The mesocarp cells enlarge considerably and the typical differentiated endocarp is formed. If the gibberellin stimulation is not received by the pea ovary a degenerative process is initiated in most pea ovaries. Unpollinated ovaries exhibit a

senescence process which produces degeneration of the endocarp cell layers initially, and ultimately the whole pod (Vercher *et al.*, 1987).

However, some pods may develop parthenocarpically. Parthenocarpic development results from a failure to pollinate, or embryo abortion after fertilisation and may be natural (Haan, 1930) or induced (Gustafson, 1936). Parthenocarpy can occur under certain environmental conditions (Gustafson, 1942). Parthenocarpy has provided a means of identifying the role of plant hormones in the early stages of fruit development (George *et al.*, 1984). Exogenous application of hormones, such as gibberellins, cytokinins, or auxins to the ovary of emasculated flower buds (Garcia-Matinez and Carbonell, 1980), or to the leaf adjacent to the emasculated flower bud (Pereto *et al.*, 1988), are known to induce parthenocarpy. Application of gibberellins, GA1 and GA3, was observed to be the most effective, producing fruit of similar size and morphology to pollinated fruits (Vercher *et al.*, 1984). Threshold levels of gibberellin must be supplied to induce development of fruit in unfertilised ovaries (Talon *et al.*, 1990).

The metabolic pathway for biosynthesis of gibberellins is complex, with much branching (for review, see Graebe, 1987). Different pathways are observed in different plants and in different organs of the same plant (Sponsel, 1988). Gibberellin biosynthetic pathways may be activated and inactivated to regulate normal plant development during the normal life cycle of a plant.

The first stage of biosynthesis of gibberellins is the conversion of mevalonic acid (MVA) via the terpenoid pathway to geranylgeranyl pyrophosphate (GGPP) (Graebe *et al.*, 1965), which is then converted to ent-kaurene (Figure 1.2). Microsomal monooxygenases then catalyse reactions producing GA12-aldehyde from ent-kaurene (Sponsel, 1988). The biosynthetic pathway up to GA12-aldehyde appears to be identical in higher plants, and diverges considerably thereafter (Graebe, 1987; Sponsel, 1988). A number of gibberellin metabolic pathways have been observed in pea (Figure 1.2). The origin of GA3 in plants is not well understood, but studies have shown that labelled GA5 is metabolised to GA3 (Graebe, 1987).

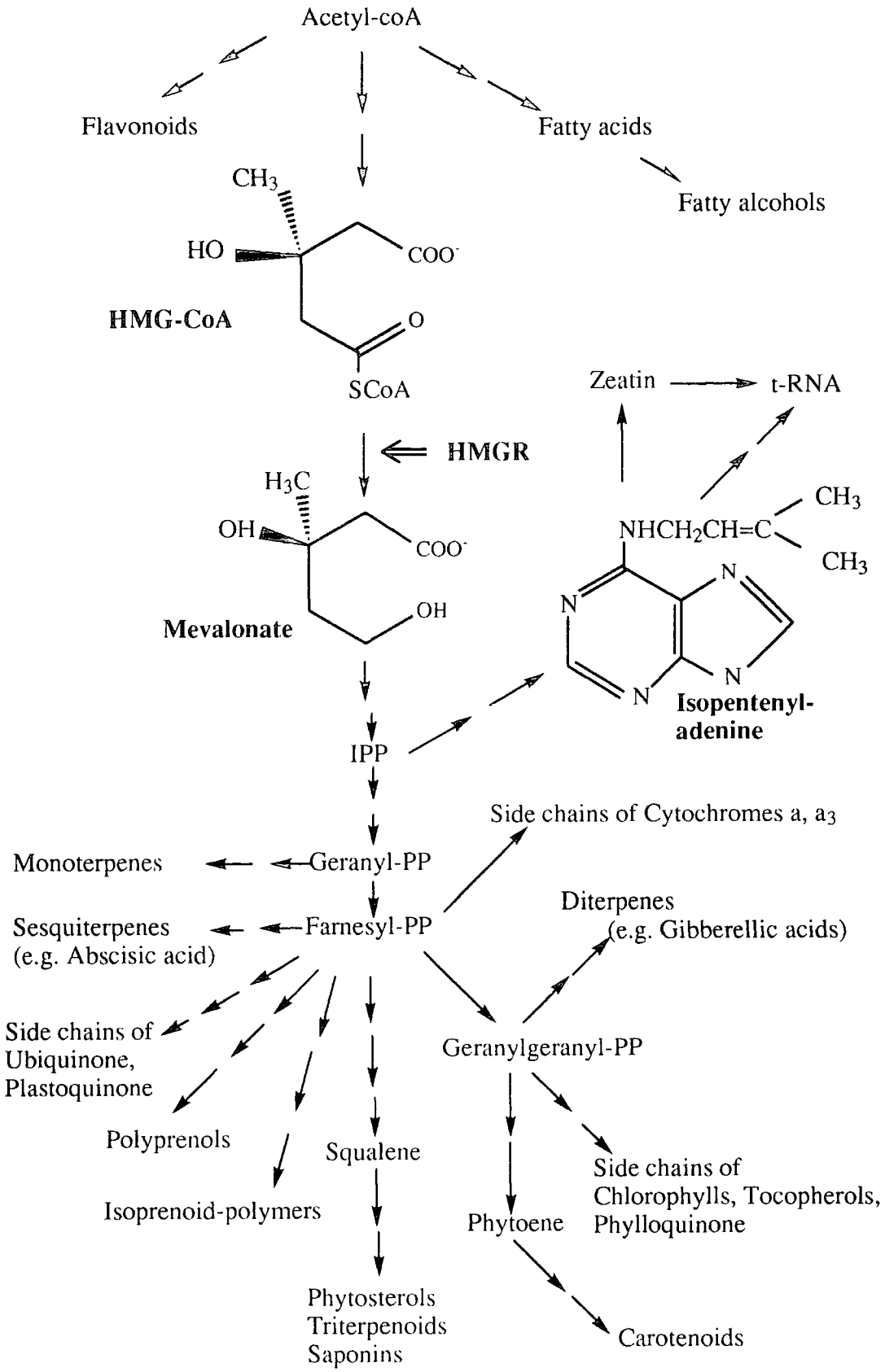

MVA
GGPP
ent-kaurene
GA12-aldehyde
CHO
COOH
GA12
GA53
GA15
GA44
GA24
GA19
GA9
GA29
GA20
GA1
GA5
GA8
GA51
GA3
OH
O
CO
HO
COOH

Mevalonic acid is the precursor of myriad isoprenoid entities, and is essential for cell growth and division (for review see, Beytia and Porter, 1976; Bach, 1987). Mevalonic acid is differentially utilised by major and minor routes of the multi-branched isoprenoid pathway depending on developmental demands of plant organs or tissues (Bach and Lichtenthaler, 1983) (Figure 1.3). Synthesis is catalysed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which is closely regulated by a number of mechanisms including phytohormones (Brooker and Russell, 1979) and feedback mechanisms (Russell and Davidson, 1982).

HMGR is high in tomato fruit during the rapid cell division and membrane expansion phases of early development (Gillaspy *et al.*, 1993) and inhibition leads to a disruption of normal development of tomato fruit. Exogenous application of MVA can be utilised by tomato fruits to sustain cell division and expansion producing mature parthenocarpic fruit, which are phenotypically normal (Gillaspy *et al.*, 1993). High levels of HMGR are also found in young, developing pea tissue and tissues with high levels of gibberellin. Immature pea cotyledons are competent to synthesise GA20 and GA29 from MVA (Kamiya and Graebe, 1983).

Absicic acid (ABA), a sesquiterpene derived from MVA, has been observed to have an inhibitory effect on fruit set and development (Garcia-Martinez and Carbonell, 1980). It is transported from leaves in the phloem (Davies, 1988; Walton, 1988). It is thought that initiation of fruit set and development, or, conversely, degeneration of the ovary, occurs in response to the respective gibberellin/ABA ratio in the ovary. Thus, gibberellin application to ABA treated emasculated ovaries has been observed to counteract the inhibitory effect of ABA. Whilst, removal of the developing leaves of pea, thought to be a source of ABA (Jordan *et al.*, 1975), can induce parthenocarpic fruit set, supporting the role of the developing leaves as sources of ABA, which inhibits fruit set and development (Carbonell and Garcia-Martinez, 1980).

Cytokinins are also associated with pod development, and like gibberellins and ABA are produced via the MVA pathway (Short and Torrey, 1972) (Figure 1.3). Cytokinins are produced by biochemical


Acetyl-coA
Flavonoids
Fatty acids
Fatty alcohols
CH3
HO
COO-
HMG-CoA
O
SCoA
HMGR
Zeatin
t-RNA
H3C
OH
COO-
Mevalonate
OH
NHCH2CH=C
CH3
CH3
N
N
N
N
Isopentenyl-
adenine
IPP
Side chains of Cytochromes a, a3
Monoterpenes
Geranyl-PP
Diterpenes
(e.g. Gibberellic acids)
Sesquiterpenes
(e.g. Abscisic acid)
Farnesyl-PP
Side chains of
Ubiquinone,
Plastoquinone
Geranylgeranyl-PP
Polyprenols
Squalene
Side chains of
Chlorophylls, Tocopherols,
Phylloquinone
Isoprenoid-polymers
Phytoene
Phytosterols
Triterpenoids
Saponins
Carotenoids

modification of adenine. They are produced in root apices and developing seeds and are transported in plants via the xylem from roots to shoots (Forsyth and Van Staden, 1981; McGaw, 1988). Their mode of action is poorly understood, but they are characterised by their ability to stimulate cell division and determine differentiation in combination with auxin. Cytokinin is a cell division factor and can replace the role of MVA in initiating DNA replication. Cytokinins have been demonstrated to play a limiting and regulating role in fibre differentiation in the presence of auxin and GA3, stimulating differentiation of secondary xylem fibres (Aloni, 1982). Fruit set may be achieved with exogenous application of benzyladenine (6-BAP) to emasculated flowers. However, the pods obtained are considerably smaller than normal pods or parthenocarpic pods treated with gibberellin (Garcia-Martinez and Carbonell, 1980).

The application of gibberellins is known to stimulate endogenous levels of auxin-like compounds in various fruits (Sastry and Muir, 1963; Mainland and Eck, 1971). IAA (indole-3-acetic acid) is the major auxin in most plants and is synthesised from tryptophan, primarily in leaf primordia, young leaves and in developing seeds. Transport of IAA proceeds from cell to cell and via the phloem to roots. It is thought that the ovary develops in response to specific levels of gibberellin-stimulated auxin (Gillaspy *et al.*, 1993). Pollen grains are a rich source of auxin, which in some species is sufficient to induce fruit set (Gustafson, 1937). Parthenocarpic fruit have higher gibberellin and auxin levels than normal fruit (Mapelli *et al.*, 1979; Mapelli and Lombardi, 1982). Parthenocarpic fruit development may also be induced by blocking the outward flow of auxin from the ovary (Robinson *et al.*, 1971; Beyer and Quebedeaux, 1974). However, exogenous application of IAA had no effect on fruit set of pea pods, while NAA (napthaleneacetic acid) had a slight effect at high concentrations. The most effective auxin for inducing fruit set and development was observed to be 2,4-D (2, 4-dichlorophenoxyacetic acid). Fruits were of similar length to normal pods, but at higher concentration 2,4-D produced deformed pods (Garcia-Martinez and Carbonell, 1980).

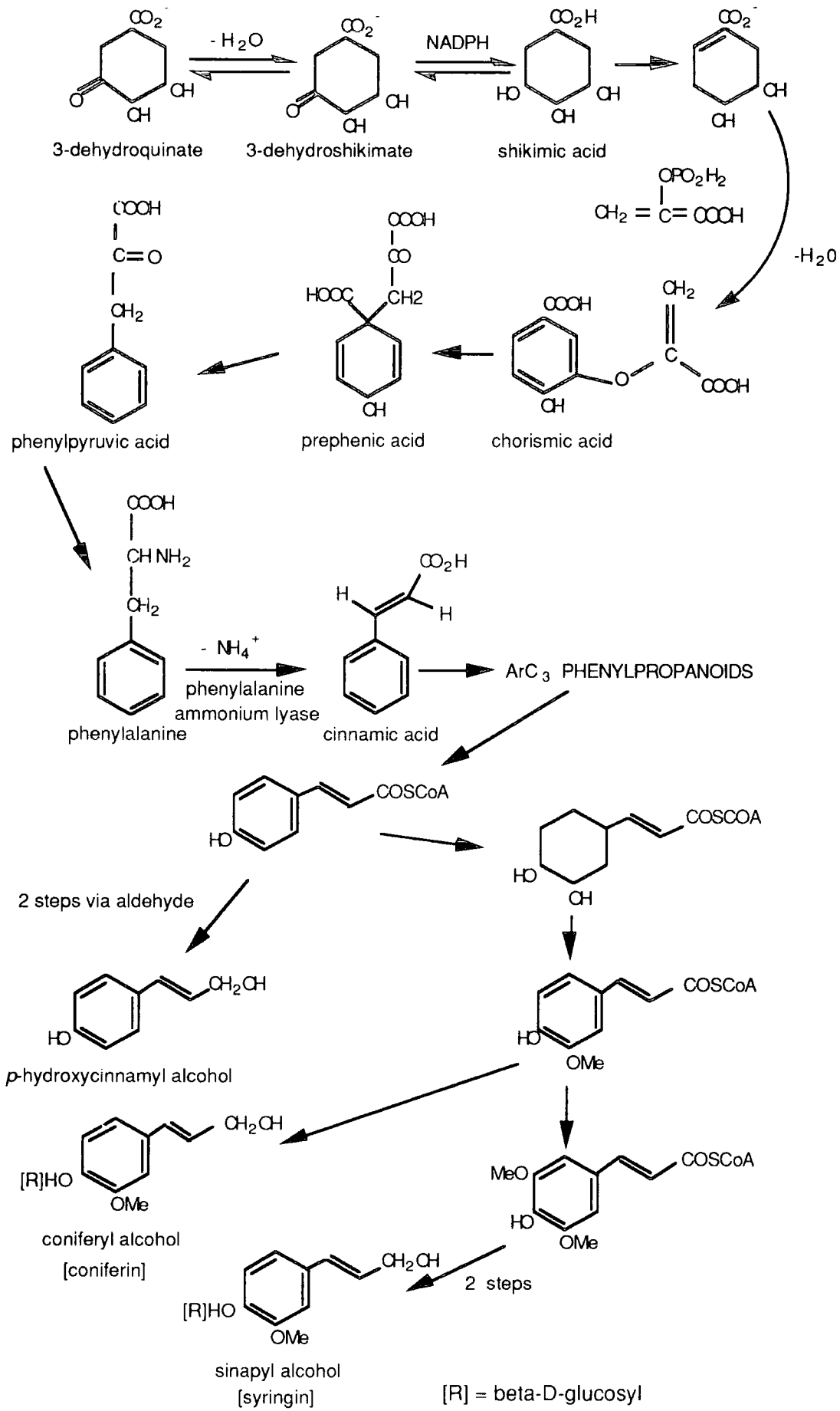
The sterol intermediates of the MVA pathway, FPP (farnesyl pyrophosphate) and GGPP, are required for modification and biological activity of signal transduction proteins, the receptor-coupled G-proteins, and the proteins which control secretory functions (Schafer and Rine, 1992). It has been suggested that these prenylated GTP-binding proteins may be induced by plant hormones (Zaina *et al.*, 1990). The Rab/Ypt proteins, a sub-family of the ras superfamily, have recently been identified as having a regulatory role in vesicular trafficking through the exocytic and endocytic pathways of eukaryotic cells (Wichmann *et al.*, 1992; Cheon *et al.*, 1993; Bednarek *et al.*, 1994). Isoprenylation is necessary for biological function of the Rab/Ypt proteins (Molenaar *et al.*, 1988). Mevalonate starvation prevents isoprenylation and membrane binding of Rab proteins (Khosravi-Far *et al.*, 1991; Kinsella and Maltese, 1992). A number of Rab proteins have now been isolated from plants (Dallman *et al.*, 1992; Palme *et al.*, 1992; Drew *et al.*, 1993; Nagano *et al.*, 1993). It has been speculated that the Rab proteins may play a role in transport of cell wall proteins, carbohydrates and enzymes involved in synthesis of new cell wall materials. High expression of one of the Rab family members has been observed in the early, but not the late phase of tomato fruit development (Gillaspy *et al.*, 1993). Expression has also been observed in a number of actively growing pea pods from different pea lines (Drew *et al.*, 1993).

1.8 Lignin Biosynthesis

Lignins are natural polymers derived from phenylpropane units linked in a 3D network. The lignin polymers formed are often associated with polysaccharides in cell walls and sometimes with phenolic acids. Biosynthesis of lignin occurs via the shikimic acid pathway (Figure 1.4).

The first committed step in phenylpropanoid metabolism is the reaction catalysed by the homotrimeric enzyme, L-phenylalanine ammonium lyase (PAL; EC 4.3.1.5), which results in the conversion of L-phenylalanine to trans-cinnamic acid with the loss of NH^{4+}. Cinnamic acid is the parent phenylpropane from which phenylpropane derivatives, such as lignin, are obtained. Several PAL genes have been isolated and characterised (Liang *et al*, 1989a).

Figure 1.4 Lignin biosynthesis via the shikimic acid pathway, adapted from Mann (1987) and Harbourne (1980).


3-dehydroquinate
- H2O
3-dehydroshikimate
NADPH
shikimic acid
-H2O
phenylpyruvic acid
prephenic acid
chorismic acid
- NH4+
phenylalanine ammonium lyase
phenylalanine
cinnamic acid
ArC3 PHENYLPROPANOIDS
2 steps via aldehyde
p-hydroxycinnamyl alcohol
coniferyl alcohol
[coniferin]
2 steps
sinapyl alcohol
[syringin]
[R] = beta-D-glucosyl

PAL is highly regulated during development associated with cell-type specific synthesis of lignin. PAL activity is also stimulated by elicitor molecules from fungi (Farmer, 1985), UV (Chapell and Hahlbrock, 1984; Kuhn *et al*, 1984) and wounding and infection (Lawton and Lamb, 1987). Induction of mRNAs which encode PAL occurs de novo immediately tissues are exposed to the stimulants mentioned above (Ishizuka *et al*, 1991). A family of PAL genes have been found in plants, with each gene encoding distinct polypeptide isoforms. This selective synthesis of functional variants may indicate different biological functions e.g. wound-protectant cinnamic acid esters; lignin synthesis as a stress induced barrier or as a structural polymer in sclerenchyma and vascular tissue (Bevan *et al.*, 1989); UV protectants; or antimicrobial phytoalexin synthesis (Bolwell *et al.*, 1985).

Other lignin biosynthesis enzymes which have been investigated are cinnamyl-alcohol dehydrogenase (CAD) (Kutsuki *et al.*, 1982); caffeic acid 3-O-methyltransferase (COMT; EC 2.1.1.6) (Kuroda *et al.*, 1975); hydroxycinnamate:CoA ligase (Luderitz *et al.*, 1982); and cinnamoyl:CoA reductase (Luderitz and Grisebach, 1981) . CAD is a molecular marker specific for lignin biosynthesis (Walter *et al.*, 1988). The branch pathway specific for the production of lignin monomers involves two reductive steps catalysed by cinnamoyl:CoA reductase and cinnamyl-alcohol dehydrogenase giving lignin precursor alcohols. CAD isoenzymes from angiosperms utilise coniferaldehyde and sinapaldehyde, whereas CAD isolated from gymnosperms can only reduce coniferaldehyde substrate (Kutsuki *et al.*, 1982). Cinnamoyl:CoA reductase and CAD from soybean use both sinapoyl and feruloyl CoAs as substrate. COMT is also a lignin specific enzyme involved in production of lignin monomers and may also be induced by exposure to fungal pathogens and yeast (Dalton *et al.*, 1990). The very rapid induction of CAD and COMT transcripts, together with the production of dehydrodiconiferyl glucosides, which exhibit cytokinin like activity, may indicate a role in the generation of secondary messengers and a role in signal production, as well as lignification (Binns *et al*, 1987).

Expression of PAL and COMT has been observed to occur preferentially in roots, stems and petioles (Gowri *et al.*, 1991). This observation

perhaps reflects the extent of lignification in these tissues. Petioles undergo sclerification as the plant ages (Fahn, 1982), while stems and roots undergo differentiation of tissue to form supporting and conducting tissue which is lignified. Reduced expression of COMT is observed in root nodules, which do not have large amounts of vascular tissue and do not exhibit the wound response. Expression of the PAL and COMT genes has been investigated using gene fusion in transgenic plants (Bevan *et al.*, 1989; Liang *et al*, 1989b; Peleman *et al*, 1989). COMT-GUS fusion genes correlate the presence of COMT in vascular tissue with the demand for methylation of hydroxycinnamic acids in lignin biosynthesis. Many of the enzymes discussed are thought to play a regulatory role in the formation of guaiacyl and syringyl lignins (Shimada *et al.*, 1973; Kutsuki *et al* 1982; Luderitz *et al.*, 1982).

There is evidence that some enzymes involved in lignin biosynthesis are cytochrome-P-450-dependent. PAL and cinnamic acid hydroxylase activity was observed to increase on irradiation with red light (Russell, 1971). Suspension cultures of parsley showed induction of shikimic acid pathway enzymes on illumination (McCue and Conn, 1990). Grand (1984) showed cytochrome P-450 dependent enzyme activity for ferulic acid 5-hydroxylase and cinnamic acid 4-hydroxylase, which are involved in synthesis of sinapic acid.

The lignin precursor alcohols p-hydroxycinnamyl, coniferyl and sinapyl are synthesised in the protoplast and accumulate as β-D-glucosides. The β-D-glucosides are later hydrolysed to monomeric species which cross the plasmalemma and permeate the cell wall where they are oxidatively polymerised forming C-C and C-O linkages (Figure 1.5). The mode of oxidative polymerisation is not known, however, it is thought to be a free radical or an ionic process (Marcinowski *et al.*, 1979; Burmeister and Hosel 1981). Lignin is then deposited within the cellulose framework of the lamellae. Progressive lignification then occurs from this region and between adjacent cells, until finally, the secondary wall becomes lignified.

Dicotyledons have an abundance of synapyl alcohols and the corresponding polymer in dicots is termed syringyl lignin. Lignin

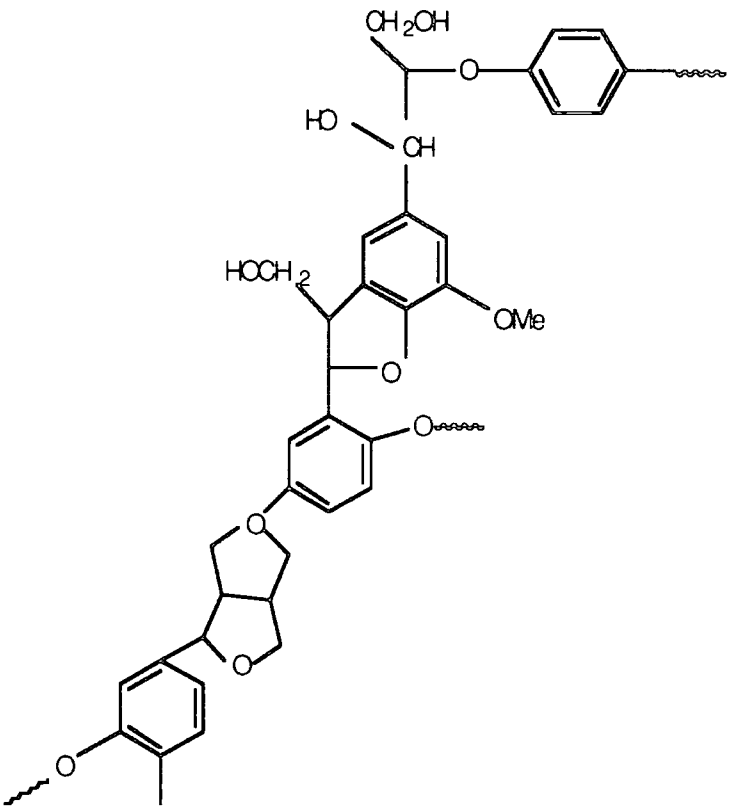


Figure 1.5 Representation of an idealised structure of polymerised lignin, adapted from Mann (1987).

monomer composition is also dependent on its site of deposition in various tissues. Sclerenchyma have a higher percentage of syringyl lignin compared to xylem (Grand *et al.*, 1983). It is thought that various isoenzymes may play a role in controlling the monomeric composition of lignin. Two forms of hydroxycinnamate:CoA ligase from poplar were found to have distinct patterns of location in xylem and sclerenchyma. Form I was essentially located in xylem (Grand *et al.*, 1983). Low amounts of form II could be a contributing factor to low percentages of guaiacyl units in this tissue. Isoenzymes of 4-coumarate:CoA ligase from soybean cultures have also shown substrate specificity. One isoenzyme activates sinapic acid to form lignin, while another is thought to be involved in the flavonoid pathway (Luderitz *et al.*, 1982).

The last enzymatic step in lignin biosynthesis involves oxidative polymerisation of free radicals catalysed by cell wall peroxidases (Williams, 1988; McDougall, 1991; O'Malley *et al.*, 1993; Sato *et al.*, 1993). Extracellular peroxidases are known to be involved in polymerisation of lignin and suberin precursors, IAA catabolism and regulation of the tightening of cell walls by modulation of cross-linking of extensin and polysaccharide-bound phenols (Fry, 1986). The peroxidases exist as multiple isoenzymes. Four isoenzymes of peroxidase (EC 1.11.1.7) have been found in cell walls of pea (Gibson and Lui, 1981). Peroxidase is a heme containing enzyme which catalyses oxidative polymerisation of monomeric precursors to form lignin complexes in the cell wall (Stafford, 1974).

It is thought that phenolic compounds may be electron donors for the inactive peroxidase intermediates. On activation the active peroxidase proceeds to oxidise NADH (nicotinamide-adenine dinucleotide phosphate, reduced form) (Mader and Fussl, 1982). NADH oxidation by peroxidase isoenzymes gives rise to H_2O_2. *In vitro* studies on the peroxidatic oxidation of NADH, giving rise to H_2O_2, followed by the peroxidatic polymerisation of cinnamyl alcohols to produce a lignin-like substance may indicate dual roles for peroxidases in lignin production *in vivo* (Gross *et al.*, 1977). Further support for the role of peroxidase in lignification was illustrated by histochemical techniques by Hepler *et al.* (1972). Histochemical staining localised peroxidase in

primary and secondary cell walls and in the dictyosomes. Peroxidase and lignin were found together in secondary thickenings and areas of the primary wall where the secondary wall attaches. A fine textured stain was also observed throughout the secondary wall (Hepler *et al.*, 1970). Hepler *et al.* (1970) also observed that peroxidase staining was greatest in the recently formed part of the cell wall. The positive staining for peroxidase in dictyosomes agrees with the observation that acidic peroxidases are transported as secretory proteins, possibly as glycosides, to the cell wall (Schlob *et al.*, 1987). Peroxidase activity significantly increases in prelignifying tissue (Bowling and Crowden, 1973). However, the precise relationship between lignification and peroxidases is obscure. Pea lines carrying the alleles pV, PV and Pv show an initial increase in wall-bound peroxidase activity during pod development followed by a decline as the pods mature. The actual maximum varies between each genotype. Lines carrying pv exhibit continued increase in wall bound peroxidase activity during development. Cells undergoing lignification in lines carrying the alleles pV and Pv show more intense staining for peroxidase activity.

A number of other oxidases have been associated with lignification in plants (Dean and Eriksson, 1992; O'Malley *et al.*, 1993). Laccase, a copper oxidase, has been demonstrated to induce polymerisation of lignin precursor molecules *in vitro* (Freudenberg, 1965; Sterjiades *et al.*, 1992). Laccase and laccase like activity has been observed in lignifying tissues of a number of plant species (Driouich *et al.*, 1992; Sterjiades *et al.*, 1993). Ascorbate oxidase is a copper enzyme related to laccase and exists primarily in cell walls (Ohkawa *et al.*, 1989). Polyamine oxidase and diamine oxidase have also been localised in cell walls of lignifying tissue (Slocum and Furey, 1990).

1.9 Blue Type I Copper Proteins

Copper, an essential element for plant growth, is known to be an important factor in several plant biochemical processes (Walker and Webb, 1981; Williams, 1988; Hay, 1992; Kendrick *et al* ., 1992). The function of some of the plant copper proteins, such as cytochrome oxidase, laccase, plastocyanin, ascorbate oxidase, and copper amine oxidase are now known to be involved in a diverse range of

biochemical processes including respiration, photosynthesis, lignification, cellular defence mechanisms and hormone metabolism (Bussler, 1981; O'Malley *et al* ., 1993). A number of blue copper proteins have, as yet, no known biological function e.g. stellacyanin (Sc) (Bergman *et al.*, 1977), cucumber basic blue protein (CBP) (Murata *et al.*, 1982) and cucumber peeling cupredoxin (CBC) (Mann *et al.*, 1992). These are known as blue type I copper proteins and belong to the family of blue copper proteins.

Blue type I copper proteins are around 10 - 22 kDa in size and have a "type I" bound copper atom characterised by unique spectroscopic features with an intense absorption band at 600 nm in the oxidised CuII form (Solomon *et al.*, 1992; Malmstrom, 1994). The CuI/CuII couple has a useful redox potential and the blue type I copper proteins are thought to be involved in electron transfer. They may accept electrons to generate free radicals in polymerisation reactions. Most of the copper proteins are found in association with cell walls or cell membranes rather than the cytoplasm (Frausto da Silva and Williams, 1991).

The copper ion in blue type I copper proteins has a tetrahedral configuration, as determined by electrochemical and spectroscopic studies. Crystallographic studies confirmed that the geometry was that of a distorted tetrahedron facilitating electron transfer. The Cu^{2+}-methionine bond distance is extended in comparison to the other three Cu^{2+} - ligand bond lengths. CuII and CuI have an almost identical position in blue copper proteins. This results in a reduction in structural changes of the protein and minimal geometric change during electron transfer. Proposed structures for the blue single copper proteins stellacyanin and cusacyanin consist of a β barrel with a single domain and possible reaction sites either side for substrates (Murata *et al.*, 1982; Fields *et al.*, 1991). The four ligands involved in binding copper are two His, one Cys and one Met (Adman, 1985).

It has been observed that the monocopper proteins exhibit enhanced oxidation in the presence of multicopper oxidases, such as ascorbate oxidase and laccase. Stigbrand (1971) demonstrated an increase in velocity of reoxidation of umecyanin, a single copper blue protein from

horseradish peelings, in the presence of multicopper oxidases. The single copper blue proteins may function as electron carriers in connection with other oxidases. Blue copper proteins have been isolated in crude extracts with other oxidases. Umecyanin was isolated in crude extracts containing laccase, peroxidase and ascorbate oxidase (Stigbrand, 1971). Crude extracts of mavicyanin, a single blue copper protein from the skin of green squash, also contained ascorbate oxidase and peroxidase (Marchesini *et al.*, 1979).

1.10 Directing Proteins to the Cell Wall or Membranes

Signal sequences are highly hydrophobic, N-terminal sequences (15 - 30 residues), which are essential for targeting nascent proteins destined for secretion via the endoplasmic reticulum (ER) (for reviews, see Jones and Robinson, 1989; Chrispeels, 1991). Furthermore, they play a role in translocation of the nascent protein across the ER membrane (Dorel *et al.*, 1988). Signal sequences do not exhibit conserved primary sequence homology, but may be characterised by the common distribution of different types of residues in the signal sequence (von Heijne, 1983). There are three distinct regions of the signal peptide; the n-region, at the N-terminus; the h-region, the hydrophobic core; and the c-region, at the carboxy-terminus. The n-region, located at the N-terminus, is highly variable in length and composition, but has a net positive charge (approximately + 1.7). The h-region is rich in Leu, Ala, Met, Val, Ile, Phe and Trp (and occasionally Pro, Gly, Ser or Thr) and characterises the hydrophobic core (von Heijne, 1985). The length of the hydrophobic core, 10 $\pm$ 3 residues, distinguishes it from membrane spanning sequences, 24 $\pm$ 2 residues, and from hydrophobic segments of globular proteins, 6 - 8 residues (Gierasch, 1989). The so-called c-region (5 - 7 residues), has a high polarity compared to the h-region immediately N-terminal to the c-region. The c-region cleavage site conforms to the (-1, -3) rule. The residues -1 and -3 of the cleavage site are small and neutral, Ala, Ser, Gly, Cys, Thr (occasionally Glu, Pro or Leu) and Ala, Ser, Gly, Cys, Thr, Ile, Leu, or Val, respectively. Aromatic, charged, or large polar residues, are absent in the -1 and -3 positions (von Heijne, 1983). The residues common to position -2 are often large and bulky (von Heijne, 1983). The hydrophobic core is thought to be essential for

its function in translocating nascent proteins. Deletions or additions to the hydrophobic core can prevent translocation (Benson *et al.*, 1985).

Targeting of the signal sequence is thought to be achieved by binding to signal recognition particle (SRP) (Prehn *et al.*, 1987; for review, see Vitale *et al.*, 1993). SRP binds to putative ER receptors (Prehn *et al.*, 1987). Despite the lack of primary sequence homology, it is thought that there is only one SRP, which has the ability to recognise different signal sequences. Synthesis and translocation of plant mRNAs has been observed to proceed in animal (Gallili *et al.*, 1987) and fungal systems (Rothstein *et al.*, 1987). Signal peptidase cleaves the signal on the lumenal side of the ER where the polypeptide undergoes various post-translational modifications and secretion to its destined cellular location (for reviews, see Jones and Robinson, 1989; Chrispeels, 1991).

1.11 The Heat Shock Protein Gene Family

The heat shock response has been found to be conserved in a diverse range of eukaryotes and prokaryotes (for review, see Lindquist, 1986) and is characterised by the synthesis of a set of proteins, termed heat shock proteins (Hsps), in response to increased temperature. Further investigation led to the discovery of constitutively expressed *hsps*, shown to be essential under normal growth conditions (Lindquist, 1986; Gething and Sambrook, 1992) and a number of *hsps* which were developmentally induced (Kurtz *et al.*, 1986; Winter and Sinibaldi, 1991), or induced in response to a variety of stresses e.g. arsenite (Edelman *et al.*, 1988), heavy metals (Winter *et al.*, 1988).

There are several classes of Hsps, designated by their molecular weights in kDa: Hsp110, Hsp90, Hsp70, Hsp60 and low molecular weight (LMW) Hsps (15 - 30 kDa), which are structurally related, but functionally distinct. The direct involvement of Hsps in protein biogenesis, which has become apparent over the past few years, has led to the alternative term, molecular chaperone (Ellis and van der Vies, 1991).

The molecular chaperone activities of the Hsp70s have been investigated by a number of researchers (for reviews, see Hendrick and

Hartl, 1993; Becker and Craig, 1994). Subsequently, the Hsp70 family have been implicated in stabilisation of unfolded precursor proteins prior to assembly into multimolecular complexes in the cytosol; translocation into cell organelles, such as the endoplasmic reticulum, the mitochondria and chloroplasts; maintenance of translocated proteins in an unfolded state before folding and assembly in organelles; rearrangement of protein oligomers; resolution of protein aggregates; and modulation of receptor activities.

The *hsp*70 genes have been found to exist as multi-gene families in a number of eukaryotes and are expressed in response to various physiological conditions. The Hsp70s may be found in mitochondria (mt) and chloroplasts (Amir-Shapira *et al.*, 1990), the cytosol (Wu *et al.*, 1988), endoplasmic reticulum (Munro and Pelham, 1986; Normington et al., 1989; Rose et al., 1989) and the nucleus. The *hsp*70 genes are highly homologous with at least 50% similarity at the amino acid level; greatest homology is observed in the N-terminal two thirds of the predicted protein sequence. The Hsp70s exhibit very similar biochemical properties. They all bind unfolded proteins and ATP, and have a weak ATPase activity, stimulated by binding unfolded proteins and synthetic peptides (Rothman, 1989). ATP-binding activity is located in the N-terminal two thirds of the polypeptide, while peptide binding is effected by the C-terminal third (Chappell *et al.*, 1987). The structure of the ATP-binding domain has been determined (Flaherty *et al.*, 1990) and consists of two lobes forming an ATP-binding cleft, structurally similar to the ATP-binding domains of G-actin and hexokinase (Flaherty *et al.*, 1991). Release of bound peptides is dependent on ATP-binding and hydrolysis. BiP is the single Hsp70 family member of the ER (Haas and Wahl, 1983). Hsp70s located in the ER are known to associate with newly synthesised proteins imported into the ER (Haas and Wahl, 1983), and are induced by the accumulation of misfolded proteins in the ER (Rose *et al.*, 1989). The C-terminal of BiP is thought to contain seven amino acids involved in peptide binding. The 7-mer has an enriched aliphatic content with no polar un-charged amino acids (Flynn *et al.*, 1991). It is thought that peptide binding and ATP hydrolysis is accompanied by a conformational change (Liberek *et al.*, 1991), resulting in release of bound peptides in a manner thought to be similar to the G protein

model (Palleros *et al.*, 1993). The characteristic ability of Hsp70s to bind and release hydrophobic stretches of partially unfolded proteins enables a functional role for these proteins in numerous essential intracellular activities, such as protein synthesis, protein folding and oligomerisation and protein transport.

A number of mitochondrial Hsp70s have been isolated with a characterisitic N-terminal leader sequence for targeting to the mitochondria (Craig *et al.*, 1989; Watts *et al.*, 1992). Analysis has shown that the mt Hsp70s are necessary for the import of translocated precursor proteins (Voos *et al.*, 1993). They bind precursor proteins on penetration of the mt membrane in a manner comparable with the role of BiP in the ER. The translocated precursor is stabilised in a partially folded state until ATP hydrolysis permits release and further folding.

Cytosolic hsp70s are closely associated with protein synthesis, folding and secretion. The accumulation of unfolded proteins (Anathan, 1986) or secretory precursors (Normington *et al.*, 1989) in the cytosol has been observed to induce *hsp*70 expression. Studies of yeast cytosolic Hsp70s has revealed two groups, SSA and SSB which are functionally distinct. The two groups are also transcriptionally regulated in different ways. The SSA genes are induced by heat shock. Conversely, SSB genes are switched off (Werner-Washburne *et al.*, 1989). The SSA genes are essential for cell viability. Studies of transport of precursor protein in yeast and mammalian cells support the role of cytosolic Hsps in translocation of unfolded secretory proteins and transport to cell organelles (Chirico *et al.*, 1988; Zimmerman *et al.*, 1988). Constitutively expressed mammalian *hsp*70s, termed *hsc*70s, encode proteins which catalyse the ATP-dependent disassembly of clathrin-coated vesicles *in vitro* (Chappell, *et al.*, 1987; De Luca-Flaherty *et al.*, 1990). Some cytosolic Hsp70s appear to bind signal sequences in peptides destined for degradation (Chiang *et al.*, 1989). It is thought that a similar mechanism may facilitate binding of Hsp70s to nuclear localisation signals for import into the nucleus (Dingwall and Laskey, 1992). They may also play a role in a signal recognition particle (SRP)-independent protein translocation pathway. SRP is known to bind signal sequences of proteins destined for secretion via the ER.

Inactivation of the genes encoding SRP does not produce a lethal phenotype. Thus, it has been speculated that Hsp70s provide an alternative pathway for translocation to the ER (Hann and Walter, 1991). Several plant cytosolic Hsp70s in *Arabidopsis* (Wu *et al.*, 1988), petunia (Winter *et al.*, 1988) and maize (Rochester *et al.*, 1986) have been characterised, which are similar to the yeast SSA family and human cytosolic Hsp70 genes. It is thought that plants may contain multiple genes encoding cytoplasmic Hsp70s. Variations in structure and regulation have yet to be characterised. The tomato *hsp70* (Duck *et al.*, 1989) is regulated in a complex tissue specific pattern and is not heat inducible. *In situ* hybridisation has located expression of the constitutively expressed tomato *hsp70* in the vascular system of the ovary, inner integument of developing seeds and the lateral root tips. A number of cognate *hsp70* genes have been demonstrated to be developmentally expressed in plants (Duck *et al.*, 1989; Zimmerman *et al.*, 1989; De Rocher *et al.*, 1990; Kruse *et al.*, 1993).

In addition to evidence for the role of Hsp70s in protein folding, they are also implicated in dissociation of folded protein complexes. The *E. coli* Hsp70 homologue, DnaK, converts inactive dimeric forms of RepA to active monomers in conjunction with other proteins (Wickner *et al.*, 1992). It is thought that the Hsp70 recognises specific peptide sequences resembling those exposed in partially unfolded proteins. Modification of Hsp70 function has been shown to be induced by complex interactions with other proteins leading to increased protein binding and stimulation of ATPase activity in the case of DnaK (Liberek *et al.*, 1991; Langer *et al.*, 1992).

Hsp60s have similar biochemical properties to Hsp70 proteins, but appear to act sequentially in a common pathway facilitating different steps in protein folding and assembly. Evidence indicates that Hsp70s bind the peptide backbone in its extended conformation, while Hsp60 binds the partially folded intermediate (Langer *et al.*, 1992; Landry and Gierasch, 1991). Hsp70 proteins, together with Hsp90 and Hsp56 have also been found associated with mammalian steroid receptor heterocomplexes (for review, see Pratt, 1993). Hsp70 binds to the hormone binding domain of steroid receptors (Schowalter *et al.*, 1991; Scherrer *et al.*, 1993), and is required for binding of Hsp90 to the

receptor (Hutchison *et al.*, 1992). The resulting heterocomplex formed by Hsp70, Hsp90, Hsp56 and an unknown factor present in rabbit reticulocyte lysate (Pratt, 1993), and steroid receptors is thought to be involved in receptor trafficking into and out of the nucleus through the cytoplasm via microtubules (Dalman *et al.*, 1989).

The regulation, intracellular location and mode of peptide recognition of the numerous heat induced and constitutive and developmentally specific Hsps will need further examination to determine the precise significance of this complex gene family in plants. The large number of *hsp*70 genes and the complex expression patterns, constitutive and induced, lead to significant difficulties in characterisation of this gene family. Study of these genes is further complicated by the observation that in mammalian systems, differential processing at the 3' end of mRNAs is a major factor in control of expression (Petersen and Lindquist, 1988).

1.12 Modification of the 3′ Termini of mRNA

Formation of mature eukaryotic mRNAs requires a transcriptional unit encoding information, which can be transcribed into precursor mRNAs (pre-mRNAs) by polymerase II. The primary transcript transcribed by polymerase II in eukaryotes then undergoes a number of complex physical modifications to produce a mature mRNA. These include capping, cleavage to form new 3′ termini, polyadenylation, splicing, base methylation and transport to the cytoplasm from the site of transcription in the nucleus (Nevins, 1983). Each maturation step provides a means of regulating mRNA formation and function.

Nearly all eukaryotic mRNA primary transcripts undergo endonucleolytic cleavage to generate a new 3′ terminus. Endonucleolytic cleavage of the pre-mRNA is followed by the addition of up to 250 adenylate residues (the poly (A) tail). The 3′ terminus may be cleaved at different sites in different cell types or at different stages of development (Capetanaki *et al.*, 1983; Dean *et al.*, 1986; Hernandez-Lucas *et al.*, 1986; for reviews see Breinbart *et al.*, 1987; Green, 1991). Attempts to determine conserved sequences for termination of transcription in the 3′ untranslated region of mRNA have proved less

straightforward than determination of site of initiation of transcription at the 5′ termini. Although a number of cis-acting signals and trans-acting factors are now characterised in mammalian systems (for review see Wahle and Keller, 1992), much less is known of the factors determining 3′ end formation of plant mRNAs.

The conserved AAUAAA sequence 10 - 35 bases upstream from the cleavage site, was finally established as a polyadenylation signal for correct processing and polyadenylation in mammalian systems (Proudfoot and Brownlee, 1976). A second GU- or U-rich element situated downstream of the poly (A) addition site was also discovered to be required for efficiency of 3′ end processing (McDevitt *et al.*, 1986). In contrast the cis-acting sequences for plant mRNA processing do not possess the downstream element (DSE), and in many cases do not have the conserved AAUAAA sequence. Even when present the AAUAAA motif may not act as part of the polyadenylation signal (Sanfacon, 1994). Other similar consensus motifs have since been discovered in plants, which serve as polyadenylation signals with varying degrees of efficiency (Wu *et al.*, 1993; Joshi, 1987).

Plant polyadenylation signals appear to be much more complex than in mammalian systems. Plant mRNAs appear to have cis-acting signals upstream of the cleavage site, as opposed to the DSE in mammalian systems (Sanfacon, 1994; Wu *et al.*, 1993; Sanfacon *et al.*, 1991). Sequences close to the cleavage site (near upstream elements, NUEs), and regions further upstream (far upstream elements, FUEs), appear to play a role in polyadenylation and regulation of processing efficiency respectively (Mogen *et al.*, 1992). In the pea rubisco gene, polyadenylation sequences upstream and downstream of the poly (A) site are required for polyadenylation (Hunt and MacDonald, 1989). There is a high degree of variability in positioning of poly (A) tails and multiple polyadenylation sites have been observed for a number of plant genes such as small subunit ribulose bisphosphate carboxylase (rbcS) genes of petunia , the bronze gene of *Zea mays* and chlorophyll a/b binding protein (Cab) (Dean *et al.*, 1986). Studies on adenovirus transcription indicate that initiation of transcription by different promoters may lead to utilisation of different poly (A) sites (Nevins and Wilson, 1981). The possibility exists for regulating expression of a

transcriptional unit by selecting transcriptional termination of RNA, poly (A) addition and RNA-chain cleavage (Ingelbrecht *et al.*, 1989).

Poly (A) addition is an early event in RNA formation, occurring prior to splicing (Nevins and Darnell, 1978). The subsequent splicing of the mRNA proceeds with the involvement of small nuclear RNAs. Antibodies to ribonucleoprotein particles containing snRNAs prevent splicing of viral RNAs in adenovirus-infected cells (Yang *et al.*, 1981). The ability to achieve alternative splicing provides another means of regulating gene expression. Various coding sequence assemblies processed via alternative mRNA splicing can alter gene output.

The processed mRNAs are transported from the nucleus to the cytoplasm for translation. This provides the cell with another means of regulating expression. The level of functional mRNA available for protein formation is dependent on the rate of delivery and stability in the cytoplasm. In addition, rapid degradation is necessary when a given gene is switched off. Several studies suggest that the poly (A) tail plays a role in mRNA stability (Gallie *et al.*, 1989). Enzymatic removal of the poly (A) tail of globin mRNA was shown to result in rapid degradation of the mRNA and loss of globin synthesis (Marbaix *et al.*, 1975). Histone mRNA which naturally lacks a poly (A) tail was stabilised by poly (A) addition (Huez *et al.*, 1978). Sequences in the 3′ UTR of *hsp70* mRNAs have been found to play a role in directing turnover of the transcript. Sequences in the 3′ UTR of an *hsp70* mRNA were able to alter stability in consequence to variations in temperature (Petersen and Lindquist, 1988). Post-transcriptional regulation may then be achieved through changes in cell physiology which alter mRNA degradation depending on 3′ sequences (Simcox *et al.*, 1985). Regulatory elements downstream of the polyadenylation sites can alter mRNA processing and stability. Increased expression levels have been achieved using the PI-II terminator (An *et al.*, 1989). It is thought that various terminators may bind to terminator sequences of a gene and direct 3′ cleavage.

1.13 GTP-binding Proteins and Vesicular Transport

GTP-binding proteins are the gene products of various gene families found in a diverse range of species. There are two groups of GTP-binding proteins found in eukaryotic cells. The first is the α-subunit of the trimeric G proteins (Gilman, 1987; Lochrie and Simon, 1988) and the latter is the ras superfamily. Members of the ras superfamily are monomeric proteins of 21 - 25 kDa in size and include the Ras, Rho, Ral and Rab/Ypt subfamilies (for reviews, see Barbacid, 1987; Downward, 1990; Hall, 1990; Valencia *et al*, 1991; Terryn *et al.*, 1993). All the members of this group share at least 30 % homology to Ras proteins. The GTP-binding proteins all have characteristic biological and biochemical properties. These include GDP/GTP binding activity; intrinsic GTPase activity; Ras proteins and the α-subunit of G proteins seem to be encoded by small families of homologous genes; they appear to function by interaction with various receptor and effector systems; and they are all associated with membranes, and indeed, localisation at cell membranes appears to be essential for function.

The G proteins are perhaps the best characterised group and are known to be involved in signal transduction utilising cyclic AMP (for reviews, see Gilman, 1987; Lochrie and Simon, 1988). It is believed that Ras proteins have a similar role in regulating cell metabolism through signal transduction involving coupling cell growth and cell division to external stimuli (Barbacid, 1987).

Other members of the Ras superfamily have been implicated in intracellular transport. Mammalian Rab proteins (Touchot *et al.*, 1987; Bucci *et al.*, 1988) and their yeast counterparts SEC (Goud *et al*, 1988; Walworth *et al*, 1989) and Ypt (Segev *et al*, 1988) proteins, have been implicated in intracellular trafficking of vesicles to their appropriate subcellular locations (for review, see Novick and Brenwald, 1993). A number of plant rab homologues have now been isolated and characterised (Matsui *et al*, 1989; Anuntalabhochai *et al*, 1991; Sano and Youssefian, 1991; Terryn *et al*, 1992; Drew *et al.*, 1993; Nagano *et al.*, 1993; Palme *et al.*, 1993). The secretory pathway of eukaryotic cells utilises a vesicular-mediated transport system to transport proteins to their various intra- and extra-cellular locations (reviews, Balch, 1989;

Steinman *et al.*, 1984). Secreted proteins are transported from the ER via the Golgi complex. Further transport through the Golgi complex is facilitated by budded vesicles and finally in vesicular endosomes to the appropriate target membrane. The vesicular intermediates required for each step in this transport pathway must be regulated to ensure, directionality, targeting and fusion. GTP is implicated directly or indirectly in regulation of vesicular transport (review, Balch, 1990). Rab proteins are thought to undergo a transformational change upon hydrolysis of GTP, thus acting as a molecular switch determining directionality and specificity of membrane trafficking. On receiving a particular stimulus cell membrane receptors are thought to interact with Rab proteins resulting in GTP binding. The resulting conformational change allows interaction of GTP bound Rab with its effector system. Once the interaction has taken place, GTPase activity proceeds and GTP is replaced by GDP resulting in a conformational change, which leads to dissociation of Rab and its effector system.

Different members of the Rab family have been localised to specific exocytic and endocytic compartments of each transport step from endoplasmic reticulum to late endosomes (Balch, 1990; Chavrier *et al*, 1990; Goud *et al.*, 1990; Plutner *et al*, 1990; Kinsella and Maltese, 1991; van der Sluis *et al.*, 1992). In addition, any given organelle in vesicular transport may bear more than one Rab protein, each having a different biological function (Lombardi *et al.*, 1993).

A number of accessory proteins have been implicated in efficient functioning of the Rab proteins. GAP (GTPase activating protein), has a stimulatory effect on GTP hydrolysis (Novick and Brennwald, 1993), GEF (guanine nucleotide exchange factor), stimulates the rate of GTP hydrolysis (Moya *et al.*, 1993), and GDI (guanine dissociation inhibitor) acts to inhibit dissociation of GDP from Rab proteins (Novick and Brennwald, 1992).

Evidence of a soluble pool in cytosol fractions was found by Goud *et al.* (1988), and it is thought that GTP-binding proteins involved in vesicular transport may be recycled between membranes (van der Sluijs *et al.*, 1992). Cell free systems reconstituting vesicular transport

have been utilised to identify the cytosolic and membrane components involved (Goda and Pfeffer, 1988).

The conserved terminal Cys residues of Ras proteins undergo modification by isoprenyl groups of the isoprenoid precursor MVA (Farnsworth, 1991; Kinsella and Maltese, 1991). Either one or both of the terminal Cys residues have been implicated in post translational modification (Willumsen *et al.*, 1984a; Willumsen *et al.*, 1984b; Molenaar *et al.*, 1988; Khosravi-far *et al.*, 1991). It has been observed that Rab proteins with a CXC (where X is alanine, serine or glycine) or CC motif are substrates for addition of geranylgeranyl moieties via a thioether linkage to either one, or both, of the conserved terminal Cys residues (Khosravi-far *et al.*, 1991; Kinsella and Maltese, 1992). A mammalian Rab GG transferase has been isolated (Andres *et al.*, 1993; Seabra *et al.*, 1992). Alternatively, Rab proteins may have C-terminal motifs similar to the CAAX box found in most ras proteins (where A is an aliphatic residue) (Palme *et al.*, 1993). It is also suggested that domains upstream of the conserved terminal Cys residues are required for prenylation (Andres *et al.*, 1993; Wilson and Maltese, 1993). Some members of the Ras, Rho and Ral subfamilies have a basic sequence upstream of the Cys residues (Hall, 1990). This basic sequence is thought to be an additional signal for palmitoylation. The CXC carboxyl terminal motif together with residues upstream (which exhibit greatest amino acid divergence between members of the Rab family members) may determine specific localisation of each Rab protein in membranes involved in endocytic and exocytic pathways.

The high degree of homology shared by the mammalian and plant Rab proteins with their yeast counterparts, and the results of yeast complementation experiments suggest conserved mechanisms for regulation of vesicular transport in eukaryotes. A number of vps (vacuole protein targeting) mutants (Banta *et al.*, 1988; Horazdovsky *et al.*, 1994) have provided a means of identifying the precise step of vesicular transport regulated by a particular Ypt family member (Segev *et al.*, 1988; Wichman *et al*, 1992). Functional complementation of yeast strains with mutations of specific Ypt proteins has been achieved with plant (Cheon *et al.*, 1993; Bednarek *et al.*, 1994) and mammalian (Haubruck *et al.*, 1989; Hengst *et al.*, 1990) Rab/Ypt homologues. A

minimal requirement for successful complementation appears to be an identical effector domain (Haubruck *et al.*, 1989; Hengst *et al.*, 1990). The Rab proteins have a conserved effector domain, which is required for regulation of GTP hydrolysis (Plutner *et al.*, 1990). The effector domain is proposed to bind a putative effector protein GAP (GTPase activating protein), which stimulates GTP hydrolysis. Other strategies adopted to investigate the function of plant Rab homologues include, overexpression in plants (Kamada *et al.*, 1992) and expression analysis (Palme *et al.*, 1992).

1.14 Specific Aims of the Project

Initial work in the project aimed to characterise a cDNA selected from a differential screen of purple- and green-podded pea lines. It was hoped to investigate its role in developing pods by examining expression in pods with different phenotype/genotype.

The major aim of the project was the investigation of the genetic basis for phenotypic differences between pods with or without lignified sclerenchyma in the pod endocarp. It was planned to construct a cDNA library from pods with lignified sclerenchyma and perform differential screening to isolate clones containing cDNAs of differentially expressed genes. It was intended to correlate expression of differentially expressed genes with lignification by investigating the physiology of pod development and the expression pattern of differentially expressed genes in pods and other plant tissues.

2. MATERIALS AND METHODS.

2.1 Materials

2.1.1 Chemical Reagents and Equipment Suppliers

All reagents were obtained from BDH-Merck Ltd., Lutterworth, Leics., Oxoid Ltd., Basingstoke, Apin Chemicals Ltd., Oxon, or Sigma Chemical Co., Poole, Dorset, except those listed below. The reagents used were of analytical grade or the best available grade.

DNA size markers and restriction and DNA modifying enzymes were obtained from Northumbria Biochemicals Ltd., Cramlington, Northumberland, Promega, Southampton, Boehringer Mannheim UK Ltd., Lewes, E. Sussex, or Stratagene Ltd., Cambridge.

Agarose was supplied by GibcoBRL Life Technologies Ltd., Paisley, Scotland.

A Zap-cDNA synthesis kit was purchased from Stratagene Ltd., Cambridge. An *in vitro* transcription kit, RNase free RQ1 DNase and murine moloney reverse transcriptase were purchased from Promega, Southampton.

A DIG Labelling Kit was purchased from Boehringer Mannheim UK Ltd., Lewes, E. Sussex.

Alkaline phosphatase conjugated polyclonal sheep anti-digoxygenin antibody was supplied by Sigma Chemical Co., Poole, Dorset.

Microscope slides and chemicals for microscopy were purchased from TAAB Laboratories Equipment Ltd., Reading, Berks., except National Diagnostics Histo-clear, which was supplied by Fisons Scientific Equipment, Loughborough.

DNA synthesiser and sequencers were supplied by Applied Biosystems Inc., Warrington, Ches.

Bacto-agar was obtained from Difco Laboratories, W. Molesey, Surrey. Oxoid yeast extract was supplied by Unipath Ltd., Basingstoke and trypticase-peptone was obtained from Becton Dickinson, Cowley, Oxon.

Radiochemicals and nylon filters were purchased from Amersham International plc, Aylesbury, Bucks.

Nitrocellulose filters, Schleicher and Schuell, grade BA-85, were supplied by Anderman and Co. Ltd., Kingston-upon Thames, Surrey.

National Diagnostics "Ecoscint" scintillation fluid was supplied by B.S. & S. (Scotland Ltd.), Edinburgh.

X-ray cassettes and X-ray film were supplied by Genetic Research Instrumentation Ltd., Dunmow, Essex.

Fixer, Kodak Unifix, was supplied by Phase Separations Ltd., Deeside, Clwd. Developer, Ilford Phenisol, was supplied by Ilford Ltd., Mobberly, Ches.

3 MM filter paper was supplied by Whatman Labsales Ltd., Maidstone, Kent.

Disposable pipette tips and Eppendorfs were supplied by Greiner Labortechnik Ltd., Dursley, Glos.

Microtitre plates and petri dishes were supplied by Bibby Sterilin Ltd., Stone, Staffs.

2.1.2 Plant Material

Pea seed, L59, L58 and L1390 were kindly supplied by the Weibullsholm Institute, Sweden, and Feltham First (FF) and Purple podded (PP) cultivars were purchased from Sutton's Seeds, Torquay, Devon. L59 have a lignified parchment layer (genotype, PV), L58 has a partially lignified layer (genotype, Pv) and the lignified parchment layer is absent in L1390 (genotype, pv) (Pea Gene Bank Catalogue, 1989). Subsequent

seed supplies were raised from plants grown under laboratory conditions at Durham University.

2.1.3 Growth of Plant Material

Pea seeds were germinated on damp tissue paper in darkness at 25 °C. Desiccation of the seeds was prevented by intermittent spraying with water. The germinated seeds were routinely planted at a density of 2 seedlings/5 inch pot or 3 seedlings/7 inch pot in John Innes No. 1 compost and placed on a polythene lined tray in a growth room. The compost, canes and pots were sterilised prior to use. Growing plants were routinely maintained in a heated growth room, or alternatively, plants were grown in glasshouses at the Durham University Botanic Gardens during the summer months between May and September. Temperature fluctuated, but extremes were never higher than 28 °C or lower than 12 °C. Extremes of temperature, greater than 28 °C or lower than 12 °C, caused various physiological problems, such as bud abortion, flower inhibition and poor pod development. Plants were abandoned if temperature extremes occurred during the flowering period.

Consequently, plants grown for the physiology experiment, involving exogenous application of plant growth regulators to emasculated flower buds to determine their effect on endocarp development, were removed to a growth cabinet in order to provide a more regulated environment. Plants grown for the physiology experiment were planted at a density of 1 seedling/5 inch pot and were maintained in a growth cabinet at 25 °C with 12 hours full light and at 22 °C with 4 hours half light and 8 hours darkness. Humidity was maintained at 65 %. Temperature fluctuations ranged from 18 °C - 26 °C, during the course of the experiment, as measured with a maximum/minimum thermometer.

2.1.4 Bacterial Strains and Plasmid and Bacteriophage Vectors

M13 and pUC vectors, DH5α and JM101 were purchased from Northumbria Biologicals Ltd., Cramlington, or Pharmacia Biotech Ltd., St. Albans. Bacterial strains, plasmid vectors and bacteriophage used in constructing the cDNA library, DNA screening and *in vivo* excision of inserts were purchased from Stratagene Ltd., Cambridge. The

Invitrogen pYES2 yeast expression vector and Top 10 F′ were supplied by R & D Systems Europe Ltd., Abingdon.

Competent SURE and Top 10 F′ were prepared as described (2.2.13)

The bacterial strains, plasmids and bacteriophage vectors are listed below (Table 2.1).

Table 2.1 Bacterial strains, plasmids and bacteriophage vectors

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Bacterial Strains

DH5α (BRL): F⁻, *end*A1, *hsd*R17 (r_K^-,m_K^+), *sup*E44, *thi-1*, λ⁻, *rec*A1, *gyr*A96, *rel*A1, (*arg*F-*lac*zya)U169, ϕ80d*lac*ZΔM15.

JM101 (Yannisch-Perron *et al.* (1985) or BRL): *sup*E,*thi*Δ(*lacpro*AB)/F' *tra*D36, *pro*A,B, (r_K^+, m_K^+), *aq*I^{q}z Δ M15

XL1-Blue (Stratagene): *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [F' *proAB*, *lacI*^{q}ZΔM15,Tn*10*(tet^r)]

SURE strain (Stratagene): e14⁻(*mcrA*), Δ(*mcrCB-hsdSMR-mrr)171*, *sbcC*, *recB*, *recJ*,*umuC*:Tn5 (kan^r), *uvrC*, *supE44*, *lac*, gyrA96, *relA1*, *thi-1*, *endA1*, [F' *proAB*, *lacI*^{q}Z*ΔM15*, Tn*10*,(tet^r)]

SOLR Strain (Stratagene): e14-(*mcr*), Δ(*mcrCB-hsdSMR-mrr)171*,*sbcC*, *recB*, *recJ*,*umuC*:*Tn*5, (kan^r), *uvrC*, *lac*, *gyrA96*, *relA1*, *thi-1*, *endA1*, λR, (F' *proAB*, *lacI*^{q}Z*ΔM15*) Su⁻ (non-suppressing)

Top 10 F' (Invitrogen): F'(tet^r)*mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80 ΔlacΔM15*lac*X74*deo*R*recA1ara*D139Δ(*ara,leu*)7679*gal*U*gal*Kλ*ps*L(Str^r)*end*A1*nup*G

Plasmids

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pUC18 (Pharmacia): cloning vector

pUC19 (Yannisch-Perron *et al.* (1985) or (Pharmacia): cloning vector

pBluescript (Short *et al.* (1988) or Stratagene): cloning vector

pYES2 (Invitrogen): yeast expression and cloning vector

Bacteriophage

M13mp19 (Yannisch- Perron *et al.* (1985) or Boehringer Mannheim): multiple cloning site

M13mp18 (Yannisch-Perron *et al.* (1985) or Boehringer Mannheim): multiple cloning site as for M13mp19

Lambda ZAPII (Short *et al.* (1988) or Stratagene): multiple cloning site

R408 helper phage (Stratagene): *in vivo* excision

EXAssist helper phage (Stratagene): *in vivo* excision

2.1.5 Growth Media for Bacterial and Bacteriophage Cultures

Media routinely used for growth of bacterial and bacteriophage cultures are listed below (Table 2.2).

Table 2.2 Media preparations

Liquid Media

LB medium: 10 g NaCl, 10 g bacto-tryptone, 5 g yeast extract, per litre.

LB/Maltose/$MgSO_4$: To autoclaved LB medium , add filter sterilised maltose solution to 0.2 % and $MgSO_4$ solution to 10 mM.

NZY medium: 5 g NaCl, 2 g $MgSO_4.7H_2O$, 5 g yeast extract, 10 g casein hydrolysate, per litre, pH 7.5 with NaOH.

Terrific Broth: 12 g bacto-tryptone, 24 g yeast extract, 4 ml glycerol in 900 ml, autoclave. Then add 100 ml of filter sterilised 0.17 M KH_2PO_4/0.72 M K_2HPO_4.

2 XL: 20 g bacto-tryptone, 10 g yeast extract, 1 g NaCl, per litre, pH 7.0 with 1 M NaOH. Add 10 ml of filter sterilised 20 % glucose after autoclaving.

YT medium: 8 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, per litre.

2 x YT medium: 16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl, per litre.

Solid Media

M9 minimal agar plates: Prepare 10 x M9 salts: 60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, per litre. Add 20 ml of 10 x M9 salts to 3 g of agar in 175 ml of distilled water. Autoclave and cool to 55 °C - 60 °C before adding the following filter sterilised solutions: 0.2 ml of 1 M $MgSO_4$, 2 ml of 20 % (w/v) glucose, 0.2 ml of thiamine 10 mg/ml. Adjust the volume to 200 ml with sterile water and pour plates. The plates can be stored up to 3 months at 4 °C.

LB, YT and NZY Plates: Add 15 g of agar per litre of medium. Autoclave and pour plates while media is still molten.

Top Agar: 10 g bacto-tryptone, 5 g NaCl, 8 g bacto-agar, per litre.

NZY Top Agar: Add agarose to 0.7 % to NZY broth.

Additives

Antibiotics: ampicillin 50 µg/ml of media
kanamycin 50 µg/ml of media
tetracycline 12 µg/ml of media

X-Gal: 50 µl of 2 % X-Gal (in dimethylformamide) to 2 - 3 ml of top agar.

IPTG: 15 µl of 0.1 M or 0.5 M IPTG (for plating out bacteria containing pBluescript) to 2 - 3 ml of top agar.

2.1.6 Maintenance of Bacterial Strains

Bacterial strains for cloning experiments were maintained by mixing 1 ml of stationary phase culture with 1 ml of sterile 80 % glycerol. The culture was then stored at -80 °C. Revival was brought about by streaking aliquots on the appropriate selective media and incubating overnight at 37 °C. Short term working strain stocks were maintained as bacterial streaks on appropriate media and stored at 4 °C.

2.1.7 Frequently Used Buffers and Solutions

10 x Column buffer: 1.5 M NaCl, 0.1 M EDTA, 1 % SDS , 0.5 M tris, pH 7.5.

Chloroform/iso amyl alcohol: 24:1, saturated with tris buffer (pH 7.5), stored at 4 °C

Denaturing solution: 1.5 M NaCl, 0.5 M NaOH, 1 mM EDTA, filtered and stored frozen at -20 °C.

50 x Denhardt's solution: 1 % Ficoll, 1 % PVP, 1 % BSA.

Ethidium bromide: 10 mg/ml of deionised distilled water.

Freshly deionised formamide: 1 g of amberlite resin MB1/10 ml of formamide, stirred for 1 hour, filtered and stored at -20 °C.

10 x MOPS: 0.5 M MOPS, 0.01 M EDTA, pH 7.0.

Neutralising solution: 3 M NaCl, 0.5 M tris, 1 mM EDTA, pH 7.0.

Orange G: 3.125 ml glycerol, 2 ml 50 mM tris.HCl, pH 8.0, 0.2 M EDTA, 4.375 ml deionised distilled water, 10 mg fast orange G. Sterilised by autoclaving. Aliquots stored at -20 °C.

Phenol: redistilled, saturated with tris buffer (pH 7.5), stored frozen at -20 °C.

SM buffer: 5.8 g NaCL, 2.0 g $MgSO_4.7H_2O$, 50 ml 1 M tris.HCl (pH 7.5), 5 ml 2 % gelatin, sterilised by autoclaving.

SOC Buffer: 2 % bacto-tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, sterilised by autoclaving. Cool, then add filter sterilised glucose to 20 mM.

20 x SSC: 175 g NaCl, 88 g tri-sodium citrate, pH 7.0.

15 x SSPE: 1.5 M NaCl, 0.1 M NaH_2PO_4, 0.01 M EDTA, pH 7.7.

TAE buffer: 40 mM tris, 10 mM EDTA, pH 7.7.

TE buffer: 10 mM tris, 1 mM EDTA, pH 8.0

Tris buffer: 1 M tris(hydroxymethyl)methylamine, titrated to required pH with HCl

2.2 Methods

2.2.1 Glass and Plasticware

Glass and plasticware used in experimental work was washed thoroughly with teepol, rinsed with tap water and given three rinses with distilled water prior to use. Eppendorfs, pipette tips and vessels in contact with DNA or RNA were autoclaved. Glass vessels used in contact with DNA or RNA were siliconised by immersing in dichlorodimethylsilane, drying at room temperature, rinsing with deionised distilled water to remove traces of HCl formed during the treatment, followed by autoclaving. Sterile disposable plasticware was used for RNA transfer and storage without further treatment. To ensure RNase free glassware for experimental work involving RNA, glassware was baked overnight at 180 °C, or immersed in 0.1 % diethylpyrocarbonate overnight followed by autoclaving. Gloves were worn throughout manipulations involving RNA and DNA to avoid nuclease contamination from the hands.

2.2.2 Solutions

Aqueous solutions were prepared with deionised distilled water and sterilised by autoclaving. Solutions for use in minipreps, transformations and restriction digests were stored at 4 °C.

Solutions for work involving RNA manipulations were prepared using baked glassware (see 2.2.1). Aqueous solutions were prepared with 0.1 % diethylpyrocarbonate treated water (0.1% diethylpyrocarbonate was added to the water and allowed to incubate overnight at room temperature followed by autoclaving). (Diethylpyrocarbonate was not used in solutions containing tris, as tris decomposes diethylpyrocarbonate producing ethanol and carbon dioxide.)

2.2.3 Pod Anatomy and Development Studies

2.2.3.1 Preparation of Microscope Slides

Microscope slides were immersed in 2 % TESPA (2 g of 3-aminopropyltriethoxysilane dissolved in 100 ml of acetone) for 20 seconds, followed by two rinses in acetone and a final rinse in deionised distilled water (DEPC treated water was used for slides to be used for *in situ* hybridisation). The slides were then dried in an oven at 45 °C. Treated slides were stored in foil prior to use.

2.2.3.2 Preparation of Wax Embedded Pod Sections

Transverse pod sections (2 - 3 mm thick) were immersed in 3 % paraformaldehyde, 1.25 % gluteraldehyde in 1 x PBS (phosphate buffer saline: 76.5 g NaCl, 7.2 g Na_2HPO_4, 2.1 g KH_2PO_4, pH 7.4 - 7.6). Fixation was allowed to proceed on a rotating wheel at room temperature overnight, or at 4 °C if sections were to be used for *in situ* hybridisation.

The tissues were then dehydrated in a series of ethanol solutions of 12.5 %, 25 %, 50 %, 75 %, 95 % and finally 100 % ethanol at room temperature for 60 minutes each (ethanol solutions were prepared with DEPC treated water for dehydrating tissues being prepared for *in situ* hybridisation). The tissues were then incubated in ethanol/Histoclear (50:50), overnight at room temperature.

This mixture was then replaced by Histoclear for at least the next 24 hours, with regular changes every 6 - 12 hours, to remove the ethanol. The tissue sections were then immersed in wax/Histoclear (50:50) overnight at 57 °C, before infiltrating with wax over a period of 48 hours with regular changes of molten wax to ensure removal of the Histoclear. The tissue sections were then blocked out in moulded trays.

Transverse sections, 10 µm thick, were cut from the pod sections embedded in the wax blocks on a microtome (Leitz 1512). The ribbons were floated in a waterbath at 45 °C (DEPC treated water for sections used for *in situ* hybridisation), then transferred to TESPA treated slides. The sections were baked onto the slides at 40 °C overnight. Special

precautions were taken in preparing wax embedded pod sections for *in situ* hybridisation with regard to solutions, glassware and wearing of gloves (see 2.1.1 and 2.2.2).

2.2.3.3 Preparation of Resin Embedded Pod Sections

Transverse pod sections (2 - 3 mm thick) were fixed and dehydrated as above (2.2.3.2), then incubated overnight in ethanol/L.R. white resin (50:50). The sections were then infiltrated with resin over a period of 48 hours at room temperature, with regular changes of resin. The pod sections were then baked individually in polypropylene tubes filled with L.R. white resin at 60 °C for 24 hours. Transverse resin embedded pod sections, 1 µm thick, were cut on a microtome (OmU3, C. Reichert, Austria).

2.2.3.4 Phloroglucinol Staining of Fresh Pod Sections

Razor cut transverse pod sections were cut as thin as possible, placed on microscope slides and a few drops of phloroglucinol solution (10 g phloroglucinol in 95 ml ethanol) added. After 1 - 3 minutes, 1 - 2 drops of concentrated hydrochloric acid were added. The sections were rinsed with deionised distilled water and examined under a light microscope. Lignin is stained red by phloroglucinol (Gahan, 1984).

2.2.3.5 Anatomical Analysis of the Pod Endocarp

Transverse wax embedded pod sections (10 µm thick) were dewaxed in histoclear for 2 minutes, rinsed in 100 % ethanol and rehydrated through ethanol/water (50:50), then water and stained with toluidine blue (0.1 % toluidine blue in 1 % boric acid, filtered prior to use) for approximately 5 minutes. The sections were rinsed with distilled water and mounted with DPX before examining under a light microscope.

Transverse resin embedded pod sections (1 µm thick) were stained with toluidine blue for approximately 5 minutes, rinsed with distilled water and mounted with DPX before examining under a light microscope.

2.2.3.6 Induction of Parthenocarpic Fruit Set

The emasculated ovaries of pea plants (grown as described above, 2.1.3), were supplied with exogenous applications of various plant growth regulators. Three ovaries were treated per plant. The stamens were removed 2 days before anthesis (day -2). At day 0 (anthesis), 20 µl of the appropriate plant growth regulator in a 0.1 % (v/v) tween 20 aqueous solution was added to the emasculated ovaries. The compounds used were GA3 (gibberellic acid), 6-BAP (benzyladenine) and MVA (mevalonic acid). Stock solutions of 2 mg/ml of GA3 and MVA were dissolved in distilled water. A 6-BAP stock was prepared by boiling 500 µg/ml in water to dissolve. The plant growth regulator solutions were applied at a concentration of 100 µg/ml by diluting aliquots of the stock solutions. The working solutions were stored at 4 °C and used throughout the experiment. Where combinations of plant growth regulators were applied they were prepared by diluting aliquots of stock solutions. Appropriate controls were obtained by applying 0.1 % tween 20 in aqueous solution to emasculated ovaries, or alternatively, allowing pea pods to develop normally. The length of the ovaries were noted at day 6, and transverse sections (2 - 3 mm) were cut from the pod mid-section, fixed and embedded in L.R. white resin for microscopical examination of endocarp development as described above 2.2.3.3. and 2.2.3.5

2.2.3.7 Photomicroscopy

Tissue sections were photographed using a Nikon AFX Optiphot-2 microscope fitted with a Nikon FX-35 camera and 400 ASA colour print film (Fuji, Holland).

2.2.4 Minipreps of DNA

2.2.4.1 Protocol for Alkaline Lysis Plasmid Miniprep

This method, based on that of Birnboim and Doly (1979), provides plasmid DNA, which is a good substrate for the action of restriction enzymes. However, the DNA is not of pure quality for sequencing, and

RNase must be added to restriction digests of DNA obtained by this method.

Cells from the required recombinant colony, were picked off with a sterile cocktail stick, and placed in 10 ml of the appropriate selective media in a McCartney bottle and grown overnight at 37 °C. The culture was then spun at 3500 rpm for 10 minutes in a centrifuge (Mistral 3000). The supernatant was poured off and the McCartney bottle was inverted over absorbent paper for a few minutes. The pellet was then resuspended in 200 µl of a solution containing 2 mg/ml lysosyme, 50 mM glucose, 10 mM EDTA and 25 mM tris.HCl pH 8.0 and incubated on ice for 30 minutes. 600 µl of a NaOH/SDS solution (200 µl of 1 M NaOH, 50 µl of 20 % SDS and 750 µl of sterile water) was added to the resuspended pellet. The mixture was transferred to an Eppendorf, vortexed gently and maintained on ice for 5 minutes. A 3 M sodium acetate solution (450 µl), pH 4.8, was added and mixed periodically over a 60 minute period of storage at 0 °C.

1100 µl of the clear supernatant obtained after microfugation for 5 minutes was removed to a fresh Eppendorf and 500 µl of isopropanol was added. The tube was incubated at -20 °C for 30 minutes. The resultant precipitate was collected by microfugation for 5 minutes. The supernatant was poured off and the pellet was dissolved in 400 µl of 0.1 M sodium acetate/0.05 M tris.HCl pH 9.0 and reprecipitated with 1 ml of ethanol at -20 °C for 10 minutes. Care was taken to ensure complete solvation of the pellet. The precipitate was collected as before and resuspended in 0.01 M sodium acetate/0.05 M tris.HCl pH 9.0 and ethanol precipitated as above. The pellet was then dried under vacuum and resuspended in 50 µl - 100 µl of sterile distilled water.

2.2.4.2 Plasmid Miniprep Alkaline Sequencing Quality

DNA for sequencing was prepared by a further modification of the above method (2.2.4.1). *E. coli* containing recombinant plasmids were grown up overnight and spun in a centrifuge as above (2.2.4.1). The bacterial pellet was then resuspended in 200 µl of 50 mM glucose, 10 mM EDTA, and 25 mM tris.HCl, pH 8.0 and stored on ice for 30 minutes. 400 µl of a freshly prepared NaOH/SDS solution (see above 2.2.4.1) was added to

the bacterial suspension and mixed gently, stored on ice for 5 minutes and then transferred to a 1.5 ml Eppendorf. Ice cold 5 M acid potassium acetate (600 µl of 5 M potassium acetate, 115 µl of glacial acetic acid and 285 µl of ddH_20), 300 µl, was added and mixed by gently inverting. The sample was then stored on ice for 20 minutes before centrifuging for 30 minutes. 0.7 ml of the clear supernatant was then pipetted off into a fresh Eppendorf and 2 µl of 10 mg/ml RNase (DNase free) added. The mixture was incubated at 37 °C for 20 minutes.

The mixture was then extracted with phenol/chloroform/isoamyl alcohol to remove proteins followed by ethanol precipitation (see 2.2.5). The pellet was then resuspended in 16.8 µl of sterile water and 3.2 µl of 5 M sodium chloride and 20 µl of 13 % PEG 8000 were added, with mixing on addition of each solution. The suspension mixture was incubated on ice for 20 minutes, before microfugation for 10 minutes. The supernatant was pipetted off carefully. The pellet was rinsed with 70 % ethanol and the DNA was redissolved in 15 µl of sterile water.

2.2.4.3 Preparation of M13 Template for Sequencing

The desired recombinant plaque was picked off a plate of selective media with a sterile cocktail stick and placed in a McCartney bottle containing 1.8 ml of 25 ml of 2 x YT media inoculated with 20 µl of JM101 exponentially growing cells. The culture was incubated on a rotating wheel at 37 °C for 6 - 7 hours.

Cultures were then transferred to an Eppendorf and spun for 10 minutes at 3500 rpm in a centrifuge. The supernatant was transferred to a second tube and spun for a further 10 minutes to isolate the M13 particles. The pellets were labelled and stored for up to one month at 4 °C should regrowth be required. (The supernatant may also be stored at this stage for up to one month at 4 °C.) 1.25 ml of each supernatant was then transferred to a fresh Eppendorf and suspended in 125 µl of 40 % PEG and 125 µl of 5 M sodium chloride. The suspension was left at room temperature for 20 minutes.

The PEG/phage suspension was then spun for 5 minutes, before removing the PEG via a pipette, taking care to avoid removing the

precipitate. Each tube was then spun briefly and the excess PEG removed by pipette. Removal of the PEG and salt was critical to avoid inhibition of extension reactions. Each pellet was then resuspended in 200 µl of TE buffer.

Isolation of the single stranded DNA from the M13 suspension was facilitated by extracting with phenol/chloroform/isoamyl alcohol (2.2.5). The resulting aqueous phase was extracted with 200 µl of water saturated ether (to remove traces of phenol, which may affect dye performance) and vortex mixed until the cloudiness disappeared. The emulsion was spun for 1 minute to separate the phases, and the upper organic layer was removed to a waste container. Any remaining ether was then removed by air drying under a fume hood for 10 minutes. The DNA was then precipitated with ethanol (2.2.5) and resuspended in 20 µl of TE pH 8.0.

2.2.5 Phenol Extraction and Ethanol Precipitation of Nucleic Acids

DNA solutions were extracted with an equal volume of tris saturated phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was vortex mixed and centrifuged for 3 minutes to separate the phases. The upper aqueous phase was removed by pipette to a fresh Eppendorf, and extracted once more with an equal volume of tris saturated chloroform/isoamyl alcohol (24:1). The aqueous phase was collected by centrifugation and ethanol precipitated with the following: 1 µl glycogen solution, 5 M ammonium acetate (4 µl/100 µl) and 2 - 2.5 volumes of ethanol. This mixture was incubated for at least an hour at -20 °C before spinning at 4 °C for 20 minutes. The supernatant was then poured off carefully and the pellet rinsed with 1 ml of 70 % ethanol. The mixture was spun for 5 minutes at 4 °C and the supernatant again poured off. The pellet was then vac dried for 5 minutes. The DNA was resuspended in sterile water and stored at -20 °C.

2.2.6 Restriction Endonuclease Digestion

2.2.6.1 Plasmids and Bacteriophage DNA

DNA samples, up to 1 µg, were cleaved in an Eppendorf in a total volume of 30 µl, with an excess of the appropriate restriction enzyme in a volume of 1 µl, and 3 µl of the appropriate 10 x buffer (supplied with the enzyme). Restriction digests were allowed to proceed for 2 hours at 37 °C.

2.2.6.2 Genomic digests

10 - 20 µg quantities of genomic DNA were digested with a five times excess of the appropriate enzyme (in a volume of 2 - 5 µl) and 5 µl of the appropriate 10 x buffer (supplied with the enzyme). 1 µl of RNase (10 mg/ml) was added and the volume made up to 50 µl with sterile water. The mixture was tapped and spun before incubating in a water bath at 37 °C. After 15 minutes the digest was placed on a shaker at 37 °C for 4 hours. During this period the digest was twice removed from the shaker, tapped and spun, and replaced on the shaker. On the second occasion the digest was vortex mixed.

2.2.7 Agarose Gel Electrophoresis

2.2.7.1 Agarose gels

Agarose gels, 18.5 cm x 15.0 cm, were used to separate and, or, isolate DNA fragments. The required amount of agarose (0.6 - 0.8 %, w/v) was dissolved in 180 ml of water by warming in a microwave. The molten agarose was cooled to 60 °C and 20 ml of 10 x TAE buffer and 20 µl of ethidium bromide solution (10 mg/ml) were added. The gel was then cast in a perspex frame mounted on a base consisting of a glass plate and sealed with silicone grease. A well former was suspended approximately 1 mm above the glass base. The gel was placed in a horizontal submarine electrophoresis tank when set and 1 x TAE buffer was added to the tank to a level sufficient to just cover the gel. Samples were loaded into the wells with 10 µl of orange G and electrophoresed for

4 hours at 100 V, or overnight at 25 V. Lambda Pst was routinely used as a size marker.

Minigels, 50 ml of 0.7 % (single-stranded DNA), or 0.5 % (double-stranded DNA) agarose, were used to determine the quantity and quality of miniprep DNA and to identify the presence of inserts of interest. DNA samples were loaded with 5 µl of orange G and electrophoresed in a Pharmacia minigel apparatus at 50 V for 1 - 2 hours in 1 x TAE buffer.

2.2.7.2 Alkaline Agarose Gels

Alkaline agarose gels were prepared for the analysis of labelled first and second strand cDNA synthesised from mRNA. 1.6 g of agarose was dissolved in 180 ml of water by warming in a microwave. The molten agarose was cooled to less than 60 °C, before adding 20 ml of 10 x alkaline buffer (0.3 M NaOH, 0.01 M EDTA) and casting the gel as above (2.2.7.1). 1 x alkaline buffer was used as the electrolyte solution. Samples were loaded into the wells with an equal volume of 2 x alkaline loading buffer (200 µl glycerol, 730 µl water, 46 µl saturated bromophenol blue, 25 µl 1 M NaOH) and electrophoresed overnight at 100 mA.

2.2.7.3 Formaldehyde Agarose Gels

Formaldehyde gel electrophoresis was used to separate small molecular weight RNAs under denaturing conditions according to a method based on that of Miller (1987). A 18.5 cm x 15.0 cm gel was prepared by dissolving 1.4 g of agarose in 67 ml of water. The molten agarose was cooled to less than 60 °C and 9.3 ml of 10 x MOPS buffer (see 2.1.7) and 17 ml of formaldehyde (37 % solution) were added. The gel was allowed to set for 1 hour in a fume hood prior to use.

The following buffers were prepared: buffer A (294 µl of 10 x MOPS, 706 µl of DEPC treated water), formaldehyde/formamide (89 µl of formaldehyde, 250 µl of formamide, freshly deionised), dyes (322 µl of buffer A, 5 mg of xylenol cyanol, 5 mg bromophenol blue, 400 mg sucrose), gel loading buffer (2 µl formaldehyde, 5 µl of formamide, 7 µl dyes) and electrophoresis buffer (1 x MOPS).

Samples of RNA in a volume of 1 µl (dilute samples of RNA were concentrated using a Uniscience Speed-Vac) were prepared by adding the following: 4.4 µl of buffer A and 11.6 µl of formaldehyde/formamide. The samples were heated to 70 °C for 10 minutes, then chilled on ice. 1.5 µl of gel loading buffer was added to the samples, mixed, and loaded in the wells of the formaldehyde agarose gel, which was pre-electrophoresed at 60 V for 3 minutes. The gel was electrophoresed at 100 V for 4 hours in circulating buffer.

Formaldehyde minigels, used for routine qualitative analysis of total RNA preparations, were prepared by dissolving 0.37 g of agarose in 43 ml of water, followed by 2.47 ml of 10 x MOPS buffer and 4.44 ml of formaldehyde. Formaldehyde minigels were electrophoresed at 30 - 40 V for 2 hours.

Ribosomal RNA, 5 - 10 µg, from pea and *E. coli* were routinely used as size markers, giving the following size bands: pea ribosomal RNA, 3.65 kb and 2.09 kb; *E. coli* ribosomal RNA 3.15 kb and 1.56 kb. Formaldehyde gels were stained with ethidium bromide solution (5 µg/ml) for 5 minutes, then destained in DEPC treated water until the ribosomal bands were visible.

2.2.8 Visualisation and Photography of Ethidium Bromide Stained Gels

Gels were drained and photographed with a UVP gel documentation system using incident illumination with minimum handling. Illumination was achieved by placing the gel on a transilluminator, wavelength 254 nm for DNA/RNA visualisation, thus effecting fluorescence of the ethidium bromide-DNA/RNA complex. It was necessary to have a minimum of 0.1 µg for visualisation of the DNA/RNA by this method.

UV can be harmful to eyes and skin if exposure is prolonged, therefore, goggles were worn and care was taken to avoid prolonged exposure to skin. Gloves were worn while handling gels and solutions containing ethidium bromide, which is a powerful carcinogen.

2.2.9 Determination of Nucleic Acid Concentration

2.2.9.1 Spectrophotometric Determination of Nucleic acid Concentration

Spectrophotometric measurement of UV absorbance at 260 nm was used as a simple and accurate measurement for quantifying nucleic acid concentration of relatively pure samples. A Pye Unicam SP8-150 UV/VIS dual beam spectrophotometer was used to measure UV absorbance in a 1 cm path length cuvette. Absorbance readings of 1 µl of nucleic acid solution in 1 ml of sterilised water (DEPC water for RNA samples) were obtained at A260, A235 and A280. The spectrophotometric measurement of absorbance at A 280 and A 235 was measured to determine the presence of protein or carbohydrate contamination, respectively. An A 260/A 280 of less than 1.7 - 1.8 indicates probable protein contamination, and an A 260/A 235 of less than 1.8 - 2.0 indicates probable carbohydrate contamination. A 1 mg/ml solution of DNA was assumed to give an OD_{260} of 20, and a 1 mg/ml solution of RNA an OD_{260} of 25.

2.2.9.2 Determination of DNA Concentration by DABA Assay

The DABA (diamine benzoic acid) determination of DNA concentration is based on that of Thompson and Farquhar (1978) and was used to ascertain accurate determinations of DNA concentration in the presence of contaminating RNA. Duplicate samples of standard amounts of DNA from 0.1 - 10 µg were ethanol precipitated and vacuum dried. 20 µl of freshly prepared DABA solution (400 mg/ml of sterile water) was added to each of the standard DNA samples together with two blanks containing no DNA, vortex mixed and incubated at 60 °C for 30 minutes. The samples were cooled on ice and 1 ml of 1 M hydrochloric acid was added. Fluorescence was measured with a Baird-Atomic Fluoripoint spectrofluorimeter set at 405 nm excitation and 505 nm emission in a 1 cm path length cuvette, with the addition of another 1 ml of 1 M hydrochloric acid.

A standard curve was plotted of fluorescence against amount of DNA (µg). Aliquots of solutions of DNA of unknown concentration were

assayed, as above, and concentration determined from the standard curve.

2.2.10 DNA Sequencing

The method used was developed from the dideoxy chain termination method (Sanger *et al*, 1977) for single stranded phage M13 vectors. The protocol followed for sequencing of the clone pPP406 was that outlined in the Amersham booklet "M13 Cloning and Sequencing Handbook" (P1/129/84/10) using an Applied Biosystems model 370A DNA sequencer. Subsequent sequencing was performed using the Applied Biosystems PRISM Ready Reaction Dye Primer Cycle Sequencing Kit (Part Number 401386) using an Applied Biosystems model 370A. Sequencing work was performed by Ms. J. Bryden.

2.2.11 Synthesis of Oligo Nucleotide Primers

Synthetic oligo deoxynucleotide primers, P1 and P2 (Figure 2.1), for DNA sequencing and RT-PCR were synthesised on an Applied Biosystems 381A DNA synthesiser by Mr. J.S. Gilroy.

2.2.12 Orientation Test (C - Test) of Inserted DNA

The C-test (Messing, 1983) enabled identification of insert orientation in M13 transformants. Minipreped DNA (1 µl) from a pair of clones to be C tested was mixed with 7.5 µl of 1 M sodium chloride and 5 µl of dye mix (3 % SDS, 0.1 % bromophenol blue, 60 % deionized formamide, 25 mM EDTA). The sample was incubated for 1 hour at 65 °C, before electrophoresing on a 0.7 % agarose gel, with a sample of the original single stranded prepped DNA. Inserts of opposite orientation are complimentary, producing a hybridised molecule with reduced mobility in agarose gels compared to single circular transformed M13. If annealing has occurred the insert fragments must be the opposite strand of the same insert.

Figure 2.1 The nucleotide sequence of the synthetic oligonucleotide primers used for LP18 sequencing and RT-PCR.

P1 sense primer

5′ CAAATCATGCACATCTGG 3′

P2 antisense primer

5′ GCTGAAGGTTCATTTTGC 3′

2.2.13 Preparation of Competent *Escherichia coli* Cells

Transformation competent cells were prepared based on a method by Alexander *et al.* (1984). An aliquot (1 ml) of a 5 ml culture of *E. coli* grown up overnight at 30 °C, was used to inoculate 100 ml of 2XL media, prewarmed to 30 °C in a 500 ml flask. The culture was incubated at 30 °C on a shaker platform. At OD_{600} = approximately 0.2 (performed using a micro-titre plate reader), sterile 2 M $MgCl_2$ was added to 20 mM. The culture was re-incubated until OD_{600} = 0.5 (0.45 - 0.55), then placed in ice-water for 2 hours. Approximately 50 ml aliquots were spun down in sterile Falcon tubes at 3000 rpm in a centrifuge (Mistral 3000). The supernatant was poured off and the pellets resuspended, with gentle shaking, in one half the original culture volume of ice-cold Ca/Mg medium (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM NaAc, pH 5.5). The Ca/Mg medium was prepared fresh, starting from a sodium acetate solution of pH 7.0 and adjusting the pH down. The cells were incubated on ice for 1 hour and collected by centrifuging at 3000 rpm for 5 minutes. The pellet was resuspended in 1/20 the original culture volume with Ca/Mn solution, containing 15 % (v/v) glycerol. Aliquots of 50 µl were collected in 1.5 ml Eppendorfs and frozen in liquid nitrogen. The competent cells were stored at -80 °C until required.

2.2.14 Cloning cDNA

2.2.14.1 Ligation Procedure

Vector DNA (0.1 - 0.2 µg) and cDNA to be ligated were mixed in 1:1 or 2:1 ratios with 1 µl of T4 DNA ligase and 1 µl of 10 x ligation buffer (supplied with the enzyme) in a total volume of 10 µl. Ligations were performed at 15 °C for 48 hours. Ligation mixtures were stored at -20 °C until required.

2.2.14.2 Transformation of Competent Cells with Plasmid Vectors

An aliquot of ligated plasmid was added to 20 - 50 µl of competent cells (freshly thawed), mixed and incubated for 20 minutes on ice. The cells were then heat shocked for 2 minutes at 45 °C to facilitate uptake of the plasmid DNA by the cells. SOC buffer , 80 µl, was added and the cells

incubated for 1 hour at 37 °C. Aliquots of the transformed cells were plated out on appropriate selective media and incubated overnight at 37 °C.

2.2.14.3 Transformation of Competent Cells with M13 Vectors

An aliquot of the ligated M13 was added to 50 µl of commercially available DH5α competent cells and incubated on ice for 40 minutes. The incubated cells and M13 were heat shocked for 2 minutes at 45 °C to facilitate uptake of the recombinant M13. The cells were cooled and mixed with 200 µl of exponentially growing JM101 cells. The cells were then plated out on the appropriate selective media with 3 ml of TB agar, 10 µl of 0.1 M IPTG and 50 µl of 2 % X-Gal. The plate was incubated at 37 °C overnight. Colourless plaques contain recombinant M13 particles.

2.2.15 Elution of DNA from Agarose Gel

Electrophoresed ethidium bromide stained DNA was cut from gels in as small a gel slice as possible. The gel slice was inserted into a piece of dialysis tubing filled with electrolyte buffer (1 x TAE) and clamped at both ends. The dialysis tube was placed horizontally to the flow of electric current in a Pharmacia minigel apparatus containing electrolyte buffer (1 x TAE). Electrophoresis was carried out for 15 minutes at 50 mA. The gel in the tubing was then viewed using a transilluminator to determine whether the DNA had been eluted from the gel. The buffer containing the dialysed DNA was pipetted into an Eppendorf. The gel slice in the dialysis tube was given a rinse with 200 µl of TE buffer. The DNA solution was phenol extracted and ethanol precipitated (as described 2.2.5).

2.2.16 Random Primed Labelling of DNA Inserts

Approximately 100 ng of DNA for labelling (prepared by cleavage from plasmid and electroelution) was labelled with 50 µCi of [α^{32}P] dCTP >400 Ci/mmol (or >3000 Ci/mmol for genomic blots) by the random primed method (Feinberg and Vogelstein, 1983). Labelling was allowed to proceed overnight at room temperature to a specific activity >10^7 cpm/µg (>10^8 cpm/µg for genomic blots).

The reaction was stopped by the addition of 20 % SDS (5 µl/100 µl of probe) and the radiolabelled probe was separated from unincorporated [α^{32}P] dCTP by gel filtration, using a 5 ml Sephadex G50 column washed with 1 x column buffer. The movement of the radiolabelled probe was followed with a mini monitor and the appropriate fractions collected as they were eluted from the column.

Aliquots of labelled probes were counted with Ecoscint scintillation fluid. DNA probes were boiled for 5 minutes prior to use to render single-stranded DNA for hybridisation experiments.

2.2.17 Preparation of Genomic DNA

Genomic DNA was prepared as specified by Ellis *et al.* (1984). 1 g of frozen plant tissue was ground in a mortar cooled in dry ice. 5 ml of JI extraction buffer (0.45 M NaCl, 0.045 M tri-sodium citrate, 0.1 M sodium diethyl dithiocarbonate, 0.1 M EDTA, pH 8.9, filter sterilised) was added and mixed. On thawing, 100 µl of 20 % SDS was added, mixed, and the mixture extracted with 10 ml of chloroform/isoamyl alcohol 24:1 in a 30 ml Corex tube, and centrifuged at 400 rpm for 10 minutes.

The aqueous phase was transferred to a siliconised 100 ml beaker and 20 ml of ethanol at room temperature was slowly pipetted onto the surface. The DNA was then spooled out with a sterile pipette tip into an Eppendorf and dried under a flow of N_2 from a pressurised gas cylinder. The DNA was resuspended overnight in 500 µl of TE buffer, pH 8.0, on a rotating wheel at 4 °C. The resuspended genomic DNA was then extracted with an equal volume of phenol. The aqueous phase, collected after microfugation for 3 minutes, was transferred to a fresh Eppendorf. 1 ml of ethanol was added and the DNA clot was removed with a sterile pipette tip, dried with N_2 and resuspended in 500 µl of TE buffer as before. Genomic DNA preparations were stored at -80 °C.

Genomic DNA was quantitatively and qualitatively analysed by cleaving 10 µl of the genomic DNA solution with restriction enzymes, EcoRI or HindIII, followed by electrophoresis. Accurate determination of concentration was then obtained by DABA assay (as described 2.2.9.2).

2.2.18 Southern Analysis of DNA

2.2.18.1 Southern Blotting of DNA by Alkaline Transfer

Plasmid DNA and RT-PCR amplification products were Southern blotted by alkaline transfer onto nylon filters using a vacuum blotter. The gel containing DNA to be transferred was rinsed in 0.4 M NaOH, 2 x 30 minutes. The DNA was then blotted onto nylon filters using the Appligene vacuum blotter according to the manufacturers instructions, using 0.4 M NaOH as the transfer buffer and a vacuum of 50 mbar for 1 hour. The well positions were marked with a pencil and the filter was rinsed briefly in 2 x SSC, air dried for 30 minutes and baked at 80 °C for 1 - 2 hours. The blotted gel was re-stained with ethidium bromide (2.2.7.3) to ensure that DNA transfer was complete.

2.2.18.2 Southern Hybridisation Using Hybsol

Southern blot filters were prehybridised in a hybridisation tube at 65 °C for 4 - 6 hours in sufficient Hybsol hybridisation solution (Yang *et al.*, 1993) to cover the filters. The Hybsol solution consisted of the following: 1.5 x SSPE, 3 % PEG, 7 % SDS, 250 µg/ml heparin and 100 µg/ml denatured, sonnicated herring sperm DNA. Hybridisation was performed for 16 - 18 hours at 65 °C in fresh Hybsol solution containing 2 - 10 ng/ml of [α^{32}P] dCTP labelled DNA probe. The filters were washed 2 x 30 minutes with 2 x SSC/0.1 % SDS and 2 x 30 minutes with 1 x SSC/0.1 % SDS. Washing to a final stringency of 0.1 x SSC/0.1 % SDS at 65 °C was achieved by washing for a period of time sufficient to remove background (changing the wash solution if necessary after 30 minutes) as monitored with a minimonitor. The filter was radiographed before drying out completely (see 2.2.19).

2.2.18.3 Genomic Blotting and Hybridisation

Digests of genomic DNA for analysis by genomic blotting were loaded onto 0.6 % - 0.7 % agarose gels and electrophoresed overnight at 25 - 30 V (see 2.2.7.1). Cleaved plasmid DNA was diluted to give samples of various gene copy numbers using the following formula:

$$\text{1 gene copy equivalent} = \frac{\text{plasmid size (bp) x genomic DNA on gel (g)}}{\text{size of pea genome } (4.8 \times 10^9)}$$

Prior to blotting, the gel was treated as follows: 2 x 30 minutes in 0.25 M hydrochloric acid, followed by 2 x 30 minutes in denaturing solution, and finally, 2 x 30 minutes in neutralising solution.

The washed gel was then transferred to NC filters by capillary blotting essentially as described by Sambrook *et al.* (1989). Blotting was allowed to proceed for 16 - 18 hours with 20 x SSC.

The positions of the wells were marked on the NC filter with a pencil and the filter was air dried for 30 minutes, then baked in a vacuum oven at 80 °C for 1 to 2 hours. The blotted gel was re-stained with ethidium bromide (2.2.7.3) to ensure DNA transfer was complete.

Genomic blot filters were prehybridised in a heat sealed polythene bag at 65 °C for 4 - 6 hours in sufficient hybridisation solution to cover the filter. The hybridisation solution consisted of 6 x SSC, 5 x Denhardt's solution, 0.5 % SDS, 100 µg/ml sonnicated herring sperm DNA. Following prehybridisation the filters were hybridised at 65 °C for 16 - 18 hours in fresh hybridisation solution containing 5 - 10 ng/ml of α^{32}P dCTP labelled probe. The filters were washed as described above (2.2.18.2) and radiographed (see 2.2.19).

2.2.19 Autoradiography

Filters hybridised with radiolabelled DNA were placed on cling film wrapped 3 MM paper and overwrapped with cling film while still damp. The filter orientation was marked with radioactive ink (writing ink with a small quantity of ^{32}P added). This assembly was then placed in a film cassette fitted with intensifying screens and a pre-flashed film and radiographed at -80 °C.

2.2.20 Removal of Radiolabelled Probes from Filters

Radiolabelled probes were removed from Southern blotted nylon filters by washing the filter in a hybridisation oven with 50 ml of 0.4 M NaOH for 30 minutes at 45 °C, followed by washing with 50 ml of 0.1 x SSC, 0.1 % SDS, 0.2 M tris.HCl pH 7.5 for 30 minutes at 65 °C. Nitrocellulose filters were stripped of radiolabelled probes by immersing in 0.1 % SDS at 90 °C for 10 minutes for northern blot filters and 0.5 % SDS for Southern blot filters. Washing was repeated if necessary after checking removal of the probe was complete by radiography. Filters were stored in 3 MM paper until required for re-hybridisation.

2.2.21 Identification of Inserts in Transformed Bacteria by Hybridisation

Bacterial colonies transformed with recombinant plasmids were screened by hybridisation. Sterile cocktail sticks were used to transfer individual transformed colonies plated out on selective media in a grid pattern onto two agar plates with a nitrocellulose filter laid on the surface of the agar. An untransformed bacterial colony was also transferred as a negative control. The plates and filter were marked with a sterile needle to allow orientation of the bacterial streaks. The plates were incubated 16 - 18 hours at 37 °C.

The master plate was sealed and stored at 4 °C. The bacterial colonies on the nitrocellulose filter from the second plate were lysed and bound to the nitrocellulose by placing the filter on 3 MM paper soaked in 10 % SDS for 3 minutes. The filter was then transferred to a second sheet of 3 MM paper saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, followed by neutralisation for 5 minutes on 3 MM paper saturated with neutralising solution (1.5 M NaCl, 0.5 M tris.HCl, pH 7.4). A final 5 minute rinse with 2 x SSC was performed, before air drying the filter, and baking for 1 - 2 hours at 80 °C in a vacuum oven. The immobilised DNA was then hybridised, as for Southern hybridisation using Hybsol, with ^{32}P labelled insert cDNA (see 2.2.18.2).

2.2.22 Preparation and Analysis of RNA

Plant cells contain a variety of RNA species. The RNA content of plant cells comprises mainly ribosomal RNA together with low molecular weight species (transfer RNA, small nuclear RNA etc.) and mRNA. MessengerRNA is heterogeneous in size and sequence, but can be isolated from total RNA extracts by means of the poly (A) tail attached to the majority of mRNAs. The mRNA thus obtained collectively encodes the cell polypeptides. The procedures outlined in 2.2.1 and 2.2.2 were followed in order to obtain good preparations of plant RNA, minimising ribonuclease activity and avoiding the introduction of traces of ribonucleases from solutions and glassware. Plant material for RNA extraction was harvested, frozen immediately in liquid nitrogen, and stored at -80 °C until required.

2.2.22.1 Preparation of Total RNA by the Hot SDS Method

Total RNA was extracted by the hot SDS method based on that of Hall *et al.* (1978). 10 g of frozen plant material, wrapped in foil, was placed on ice for 10 minutes before transferring to a polytron tube and adding 26 ml of RNA extraction buffer (0.2 M boric acid, 1 % SDS, pH 9.0. DTT to 5 mM and EGTA to 30 mM added after autoclaving), warmed to 100 °C. Nescofilm was wrapped around the tube and polytron blade to prevent splashing. The plant material was homogenised with the polytron for 20 seconds. The homogenised plant material was then transferred to a 50 ml Oakridge tube with the addition of isoamyl alcohol (as little as required) to reduce foaming, and cooled to 40 °C. 8 mg of proteinase K was added and the mixture was incubated at 37 °C, with occasional mixing, for 1 hour.

2 ml of 2 M potassium chloride was added and the mixture was cooled on ice for 30 - 40 minutes to precipitate the protein-KDS (potassium dodecyl sulphate) complex. The precipitate of KDS was collected by spinning at 10 K (Sorvall) for 10 minutes at 4 °C. The suspension was then transferred to a fresh Oakridge tube and solid lithium chloride added to 2 M (0.085 g/ml). The tube was shaken over ice to dissolve the lithium chloride. The mixture was then incubated overnight at 4 °C.

The supernatant of a 10 K spin for 10 minutes at 4 °C was poured off and the pellet washed twice with an equal volume of 2 M lithium chloride. The resulting pellet was resuspended in 3 ml of 0.2 M potassium acetate pH 5.5 and spun at 10 K for 10 minutes at 4 °C. The supernatant was poured into a Corex tube and precipitated overnight with 2.5 volumes of ethanol at -20 °C.

The ethanol precipitate was collected by spinning at 10 K for 10 minutes at 4 °C and resuspending in 2 ml of tris buffer, pH 7.5. The sample was then extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). The resulting aqueous phase was precipitated with 5 M ammonium acetate (4 µl/100 µl sample volume) and 2.5 volumes of ethanol at -20 °C overnight.

The ethanol precipitate was collected by spinning at 10 K for 10 minutes at 4 °C, rinsed with 70 % ethanol and dried under vacuum for 5 minutes. The mRNA was then resuspended in 250 - 500 µl of DEPC treated water. An aliquot, 1 µl, was taken for spectrophotometric determination of concentration (see 2.2.9.1) and an aliquot containing 10 µg of total RNA was taken for qualitative analysis by formaldehyde gel electrophoresis (see 2.2.7.3).

2.2.22.2 Preparation of Total RNA by the Guanidium Thiocyanate Method

Total RNA was extracted with guanidium thiocyanate based on a method by Logeman *et al.*. (1987). This method was used for isolation of high yields of total RNA from small amounts of tissue. Frozen tissue, 1 g, was ground with a mortar and pestle, before adding 5 ml of GuHCl extraction buffer (8 M HCl, 20 mM Na_2EDTA, pH 7.0 with NaOH, filtered and stored in a dark bottle, 2-mercaptoethanol to 50 mM was added fresh prior to use). The tissue and extraction buffer were ground together and the mixture was added to a 15 ml Corex tube and spun in a centrifuge at 10 K for 10 minutes at 4 °C. The supernatant was filtered through miracloth into a fresh Corex tube and extracted with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1). The layers were separated by centrifugation at 10 K for 45 minutes at room temperature. The aqueous phase was removed to a fresh Corex tube and 0.7 volumes

of ethanol and 0.2 volumes of 1 M acetic acid were added. The RNA was precipitated at -20 °C overnight. The RNA was then pelleted by centrifugation at 10 K for 30 minutes and given a final rinse with 70 % ethanol and dissolved in 100 µl of DEPC treated water.

The RNA solution was then treated with DNase to remove traces of genomic DNA. RNase free DNase (2 U) was added to the RNA solution, together with 1 µl of 2 M MgCl. The mixture was then incubated for 15 minutes at 37 °C. The RNA solution was then extracted with equal volumes of phenol/chloroform /isoamyl alcohol (25:24:1), followed by chloroform/isoamyl alcohol (24:1), and precipitated with 0.5 volumes of 7.5 M ammonium acetate and 3 volumes of ethanol at -80 °C for 30 minutes. The RNA was then pelleted in a microcentrifuge and re-precipitated with 100 µl of 1 M ammonium acetate and 3 volumes of ethanol at -80 °C overnight. The RNA pellet was obtained by microfugation for 30 minutes and resuspended in 50 - 100 µl of DEPC treated water. An aliquot, 1 µl, was taken for spectrophotometric determination of concentration (see 2.2.9.1) and an aliquot containing 5 µg of total RNA was taken for qualitative analysis by formaldehyde gel electrophoresis (see 2.2. 7.3).

2.2.22.3 Isolation of Poly (A)$^+$ RNA

Isolation of poly (A)$^+$ RNA was performed using a PolyATtract mRNA Isolation System III (Promega) according to the manufacturers instructions. This system utilises a biotinylated oligo(dT) primer to hybridise at high efficiency to the poly (A) tail of mRNAs in total RNA samples. The biotin-oligo(dT) primers bind to the poly (A) tail of mRNAs in a sample of total RNA. Streptavidin coupled to paramagnetic particles (SA-PMPs) are added to the total RNA sample hybridised with biotin-oligo (dT) primers. The biotin-oligo (dT) hybridised mRNAs bind to the SA-PMPs and are then magnetised to a magnetic separation stand. The SA-PMPs are washed to high stringency to remove the aqueous phase components, and the mRNAs are eluted from the solid phase with ribonuclease-free deionized water.

Total RNA samples (0.1 - 1.0 mg), extracted as described above (2.2.22.1), were made up to a volume of 500 µl with RNase-free water in a 1.5 ml

Eppendorf. The RNA sample was then placed at 65 °C for 10 minutes prior to adding 3 µl of biotinylated-oligo(dT) primer (50 pmol/µl) and 13 µl of 20 x SSC. The solution was mixed gently, and after cooling to room temperature, was added to a tube containing 0.5 x SSC washed SA-PMPs. After incubating for 10 minutes at room temperature the SA-PMPs were captured using a magnetic stand. The supernatant was then removed without disturbing the SA-PMP pellet. The particles were washed four times with 0.1 x SSC by resuspending the SA-PMP pellet each time and then collecting the SA-PMP pellet using a magnetic stand.

Finally the mRNAs were eluted by resuspending in 100 µl of RNase-free water. The eluted mRNA aqueous phase was removed to a sterile Eppendorf and the SA-PMPs eluted with an additional 150 µl of sterile RNase free water. The two eluates were pooled to give 250 µl of mRNA solution. The yield of mRNA was insufficient for qualitative analysis, but was estimated to be approximately 1 % of the total RNA, as predicted by Promega. The mRNA solutions were stored at -80 °C until required.

2.2.22.4 Analysis of RNA by Northern Blotting

Total poly RNAs, 10 µg, were electrophoresed in formaldehyde gels (see 2.2.7.3). Ribosomal RNA size markers run in the outside lane of the gel were excised after electrophoresis, stained with ethidium bromide (5 µg/ml), destained overnight in DEPC water and photographed with a ruler alongside.

The RNA in formaldehyde gels was transferred to NC or nylon filters by capillary blotting, essentially as described by Sambrook *et al.* (1989). Blotting was allowed to proceed for 16 - 18 hours with 20 x SSC.

The positions of the wells were marked on the filter with a pencil and the filter was air dried for 30 minutes, then baked in a vacuum oven at 80 °C for 1 to 2 hours. The blotted gel was re-stained with ethidium bromide, as above, to ensure RNA transfer was complete.

2.2.22.5 Northern Hybridisation Using Formamide

Northern blot filters were prehybridised in a hybridisation tube or a heat-sealed polythene bag at 42 °C for 4 - 6 hours in sufficient hybridisation solution to cover the filter. The prehybridisation solution consisted of the following: 50 % formamide (freshly deionised), 5 x Denhardt's, 5 x SSC, 0.1 % SDS and 100 µg/ml herring sperm DNA. Following prehybridisation filters were hybridised at 42 °C for 16 - 18 hours in hybridisation solution containing 50 % formamide (freshly deionised), 2 x Denhardt's, 5 x SSC, 0.1 % SDS and 100 µg/ml herring sperm DNA and 2 - 10 ng/ml of [α^{32}P] dCTP labelled probe. The filters were washed to a final stringency of 0.1 x SSC at 65 °C and radiographed (as described 2.2.19).

2.2.22.6 Northern Hybridisation Using Hybsol

Northern hybridisation using Hybsol was performed as described above (2.2.18.2).

2.2.22.7 Analysis of RNA by Dot Blotting

Total RNA, 5 - 10 µg, in a total volume of 10 µl was incubated at 68 °C for 5 minutes in 2 volumes of formamide (freshly deionised), 7 µl of formaldehyde (37 % solution) and 2 µl of 10 x MOPS buffer. The samples were chilled on ice and 2 volumes of 20 x SSC added. Samples were then dot blotted onto a NC filter using a Hybri Dot Manifold (Bethesda Research Laboratories, Scotland) according to the manufacturers instructions. The filter was dried at room temperature for 30 minutes, then baked at 80 °C in a vacuum oven for 1 to 2 hours. Filters were hybridised using Hybsol (as described above 2.2.18.2).

2.2.23 Construction of a L59 Lignified Pod cDNA Library

Total RNA was extracted from L59 pea pods (phenotype, lignified endocarp; genotype PV) harvested 4 - 6 DAF. The embryos and main veins were excised prior to RNA extraction by the hot SDS method (see 2.2.21.1). Isolation of poly $(A)^+$ RNA was performed using the PolyATtract mRNA Isolation System III (as described 2.2.22.3).

A Stratagene ZAP-cDNA Synthesis Kit was used for construction of a lignified pod cDNA library using the L59 4 - 6 DAF pod mRNAs as a template for reverse transcription. The prepared cDNAs were ligated into Uni-ZAP XR vector and cloned in SURE cells according to the manufacturers instructions. The L59 cDNA library was then amplified in *E. coli* strain XL1-Blue .

2.2.23.1 Synthesis of First and Second Strand L59 Total Pod cDNA

The mRNAs extracted from 4- 6 DAF L59 pea pods (approximately 7 µg, based on Promega PolyATract mRNA Isolation System III recovery prediction), were dried down in a Uniscience Speed-Vac and rehydrated in 31.5 µl of DEPC treated water. First and second strand synthesis was then performed according to the manufacturers instructions.

The 50 base oligo shown below was used as a primer for reverse transcription with M-MuLVRT (Moloney-Murine Leukemia Virus Reverse Transcriptase):

Poly dT

5' GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTT 3'

"GAGA" sequence XhoI

The "GAGA" sequence serves as protection for the XhoI restriction enzyme recognition sequence. The XhoI restriction site allows the subsequent synthesised cDNA to be ligated into the Uni-ZAP XR vector in a sense orientation (EcoRI - XhoI) with respect to the *LacZ* promotor. The 18 base poly(dT) sequence bound to the 3' poly (A) region of messenger RNA templates allows reverse transcription by the M-MuLVRT to proceed. The Stratagene cDNA synthesis kit utilises a dNTP mixture which includes 5-methyl dCTP for first strand synthesis. The incorporation of methylated cytosine bases protects the cDNA from the restriction enzymes used in subsequent cloning steps.

Second strand synthesis was achieved with RNase H and DNA Polymerase I. RNase H nicks the RNA template producing multiple fragments, which serve as primers for DNA Polymerase I synthesis of

second strand cDNA. The use of unmethylated dNTPs in the second strand nucleotide mixture ensures that the linker-primer can be cleaved by restriction enzymes.

Aliquots of the L59 pod cDNA first and second strand reactions were removed for later analysis (as recommended by the manufacturer) to assess quantity and quality of the synthesised cDNA, as described below (2.2.23.5). A 5 µl aliquot of the first strand reaction mixture was pipetted into a separate tube containing 0.5 µl of [α^{32}P] dATP (5 µCi) as a control, allowing analysis of first strand synthesis. An aliquot, 4.5 µl, of the second strand reaction was removed and frozen at -20 °C for later analysis. The cDNA termini of the remaining 39 µl of second strand cDNAs were blunt ended using T4 DNA polymerase according to the manufacturers instructions.

2.2.23.2 Ligation of L59 Pod Total cDNA to EcoRI Adaptors

The blunt ended L59 pod total cDNA was phenol/chloroform /isoamyl alcohol (25:24:1) extracted and pelleted by microfugation, followed by ligation to EcoRI adaptors according to the manufacturers instructions. The ligation was allowed to proceed for 2 days at 8 °C. The ligase was then heat inactivated in a waterbath at 70 °C for 30 minutes.

The EcoRI adaptor molecules consisted of the following 9-mer and 13-mer oligos:

```
5' AATTCGGCACGAG 3'
    3' GCCGTGCTC 5'
```

The 9-mer oligo is kinased to facilitate blunt-ended ligation to cDNA termini. The adaptor 13-mer oligo remains dephosphorylated to prevent ligation of cohesive ends. It is possible that the adaptor blunt termini may ligate.

2.2.23.3 Kinasing the EcoRI Ends of L59 Pod cDNA:EcoRI Adaptor Molecules

The ligated L59 pod cDNA:EcoRI adaptor molecules were then kinased with T4 polynucleotide kinase, according to the manufacturers

instructions, in preparation for ligation to the dephosphorylated vector arms. The kinase was then heat inactivated for 30 minutes at 70 °C, before cooling to room temperature.

2.2.23.4 XhoI Digestion and Precipitation of L59 Pod cDNA

XhoI restriction enzyme was used to cleave the EcoRI adaptors and residual linker-primers from the 3' end of the L59 pod total cDNAs and the resulting fragments were separated on a Sephacryl spin column, prepared from a 1 ml plastic syringe, spun at 600 g in a table top centrifuge according to the manufacturers instructions.

Three L59 pod total cDNA fractions were collected from the Sephacryl spin column. The fractions were pooled and extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by precipitation with 2 volumes of ethanol overnight at -20 °C. The cDNAs were then pelleted by microfugation for 60 minutes at 4 °C, rinsed with an equal volume of 80 % ethanol and vacuum dried for 5 minutes. The L59 pod total cDNA pellet was resuspended in 30 µl of water.

2.2.23.5 Analysis of First and Second Strand cDNAs in Alkaline Agarose Gels

The first and second strand cDNAs extracted from the above synthesis of first and second strand L59 pod total cDNA (2.2.23.1) were electrophoresed on an alkaline agarose gel (see 2.2.7.2).

The size marker lane was excised from the gel containing radiolabelled first and second strand cDNAs, neutralised by washing twice with neutralising solution, stained with ethidium bromide and photographed with a ruler alongside. The gel containing the first and second strand cDNAs was then immersed in 7 % trichloroacetic acid for 30 minutes and dried for 8 hours at room temperature using a Bio-Rad model 483 slab dryer. The gel was then placed on a glass plate and wrapped in cling film. The well position was marked with radioactive ink and the gel was exposed to sensitised film and an intensifying screen at room temperature.

2.2.23.6 Trial Ligation of L59 Pod cDNA into Uni-ZAP XR Vector Arms

An initial trial ligation of the prepared L59 pod total cDNAs with Uni-ZAP XR vector was performed. A 2.5 µl aliquot of the prepared L59 pod total cDNAs was ligated according to the manufacturers instructions for 2 days at 4 °C in a total volume of 5 µl.

2.2.23.7 Packaging the Recombinant Uni-ZAP XR

A 1 µl aliquot of the L59 pod cDNA/Uni-ZAP XR trial ligation was packaged into Gigapack II Gold packaging extract (Stratagene) according to the packaging instructions supplied by the manufacturer. 500 µl of SM buffer and 1 µl of chloroform were added to the packaged phage and stored at 4 °C.

2.2.23.8 Plating and Titering the L59 Lignified Pod cDNA Library

The titer of the packaged phage mixture was ascertained before packaging the remaining 4 µl of ligation mixture as above. Serial dilutions of the packaged phage , 10^{-1} - 10^{-6}, were cloned in SURE cells according to the manufacturers instructions. SURE is an mcrA-, mcrB-strain, preventing digestion of the methylated C containing DNA. Each serial dilution was pre-incubated with 200 µl of SURE cells at OD_{600} = 0.5 (performed using a micro-titre plate reader), for 20 minutes prior to plating out with 3 ml of NZY top agar containing 15 µl of 0.5 M IPTG and 50 µl of X-gal (250 mg/ml) onto NZY plates. The plates were incubated at 39 °C overnight. The number of recombinant plaques (colourless plaques) counted on the serial diluted plates was used to estimate the titer of the original phage mixture.

2.2.23.9 Amplification of the L59 Lignified Pod cDNA Library

One round of amplification of the Uni-ZAP XR L59 lignified pod cDNA library was performed to make a large, stable quantity, of high titer stock. Aliquots of the packaged recombinant phage, containing approximately 1.3×10^5 plaques, were mixed with 600 µl of exponentially growing SURE cells. The phage and host cells were pre-incubated for 20 minutes at 37

°C prior to plating out on a 200 mm^2 NZY plate with 50 ml of NZY top agar. The top agar was allowed to set and the plate was then incubated at 39 °C for 6 - 8 hours until plaques of approximately 1 - 2 mm were visible.

Each plate was overlaid with 100 ml of SM buffer and stored overnight at 4 °C. The next day the bacteriophage suspension was poured from each plate into sterile polypropylene tubes. The plate was rinsed with an additional 10 ml of SM buffer and pooled with the bacteriophage suspension in the polypropylene tubes. The bacteriophage suspension was then spun for 10 minutes at 2000 g. The supernatant was transferred to sterile medical flats and 5 µl of chloroform was added to each container. This stock was stored at 4 °C. The titer of the library was checked by plating out 10^{-1} - 10^{-6} dilutions with XL1-Blue on NZY plates as described (2.2.23.8).

Aliquots of the Uni-ZAP XR L59 cDNA library were stored frozen with the addition of DMSO [dimethyl sulphoxide] to a final concentration of 7 % . Each aliquot was plunged into liquid nitrogen and transferred to a freezer at -80 °C for long term storage (Sambrook *et al.*, 1989).

2.2.23.10 *In Vivo* Excision of cDNA Inserts

Recombinant phage were cored from plates and inserted into an Eppendorf containing 500 µl of SM buffer and 1µl of chloroform. Cored plaques were incubated at room temperature for 4 hours or overnight at 4 °C before taking a 200 µl aliquot and mixing with 200 µl of XL1-Blue (OD_{600}=1.0) and 1 µl of R408 helper phage or Ex-assist helper phage if using the EXAssist/SOLR system. This bacteria/phage mixture was then incubated for 20 minutes at 37 °C. A control consisting of 1µl of helper phage and 200 µl of XL1-Blue was performed. The bacteria/phage mixture was then added to 3 ml of 2 x YT in McCartneys and incubated at 37 °C for 2 - 2.5 hours. The cultures were then incubated at 70 °C for 20 minutes before spinning for 5 minutes at 2500 rpm in a centrifuge (Mistral 3000). 1 ml of the resultant supernatant was transferred to a sterile Eppendorf. The rescued phagemid could be stored at 4 °C for 1 - 2 months at this stage. A 50 µl aliquot of the rescued phagemid was pre-incubated with 200 µl of XL1-Blue

(OD_{600}=1.0), or SOLR if using the EXAssist/SOLR system, at 37 °C for 20 minutes. The rescued phagemid were then spread on LB/amp plates and incubated overnight at 37 °C. The colonies on the LB/amp plates contain double stranded pBluescript containing the cloned DNA insert.

2.2.23.11 *In Vivo* Excision of Trial Inserts from L59 Lignified Pod cDNA Library

Six plaques were cored from the recombinant phage plated out from the test ligation to check for the presence of inserts. The trial rescued plasmids were grown up in LB/amp and minipreped. The rescued plasmids containing L59 lignified pod cDNAs were XhoI cleaved and electrophoresed on a minigel.

2.2.23.12 cDNA Library Screening Protocol

The library was titered to determine concentration and 4 x 10^4 - 5 x 10^5 plaques were pre-incubated with 600 µl of XL1-Blue OD_{600} = 0.5 cells for 20 minutes before plating out on 200 mm^2 plates of NZY agar with 50 ml of NZY top agar. The plates were incubated at 37 °C for 6 - 8 hours, until the plaques, 1 - 2 mm, were visible. The plates were then cooled at 4 °C for at least 2 hours prior to transfer of the plaques to NC filters. Duplicate plaque lifts were performed by placing NC filters on the agar surface, with the first lift performed for 2 minutes and the second for 4 minutes. During transfer the filter was pricked with a needle through the agar for orientation. The filters were blotted briefly on 3 MM paper to reduce diffusion and blurring of the plaques during subsequent treatments. The filters were then placed plaque side up in a tray containing 3 layers of 3 MM paper soaked with denaturing solution for 2 minutes. This was followed by similar treatment with neutralising solution for 5 minutes, and finally rinsing solution (0.2 M tris-HCl pH 7.5, 2 x SSC) for 30 seconds. The filters were air dried for at least 30 minutes prior to baking in a vacuum oven at 80 °C for 2 hours. The transferred agar stock plates were then stored at 4 °C for use after screening.

The filters were prehybridised in a hybridisation tube at 65 °C for 4 hours with 20 ml of 5 x SSC, 5 x Denhardt's, 0.1 % SDS, 200 µg/ml denatured, sonnicated herring sperm DNA. Hybridisation was performed for 16 -

18 hours at 65 °C in 20 ml of 5 x SSC, 5 x Denhardt's, 0.1 % SDS, 100 µg/ml denatured, sonnicated, herring sperm DNA and 2 - 10 ng of [α^{32}P] labelled probe. The filters were washed to a final stringency of 0.1 x SSC at 65 °C (as described 2.2.18.2) and radiographed (as described 2.2.19). Alternatively, hybridisation was performed using Hybsol (as described 2.2.18.2).

After radiography the film was marked with a pen corresponding to the needle pricks orientating the filter. A plug was cored from the transferred stock plate where strongly hybridised putative positive clones lined up with the orientated film and placed in 1 ml of SM buffer in an Eppendorf. Putative positive clones were titered by plating out serial dilutions (as described 2.2.23.8) to give approximately 50 - 200 plaques on a petri dish of NZY agar. The plates were incubated overnight at 37 °C. Plaque lifts were transferred onto NC filters as before, prehybridised and hybridised as before.

Isolates were then cored from the secondary screen plates. If positive plaques were too close to background plaques, a tertiary screen was performed as for the secondary screen. Cored isolates were stored at 4 °C in SM buffer until required for excision to obtain the insert-containing pBluescript vector.

2.2.23.13 Trial Screen of L59 Lignified Pod cDNA Library

A trial screen was performed to check that the L59 lignified pod cDNA library contained full length cDNA representative of developing pods. The L59 lignified pod cDNA library was plated out at a density of 5 x 10^5 plaques/200 mm^2 plate and screened as described above (2.2.23.12) using [α^{32}P] dCTP labelled PP406 cDNA. The PP406 cDNA encodes a GTP-binding protein, Psa-rab, which was found to be expressed in developing pods from different pea lines (Drew *et al.*, 1993).

2.2.24 Differential Screen of the L59 Lignified Pod cDNA Library

A differential screen of the L59 lignified pod cDNA library was performed according to a method based on that of Olszewski *et al.* (1989), using [α^{35}S] dCTP and [α^{32}P] dCTP labelled total cDNAs synthesised

from 4 - 6 DAF pod mRNAs from L59 and L1390 respectively. Secondary and tertiary screens were performed using [α^{32}P] dCTP labelled cDNA probes from both lines. The differential screening strategy is shown (Figure 2.2).

2.2.24.1 Primary Differential Screen of L59 Lignified Pod cDNA Library

The L59 lignified pod cDNA library was plated out on two 200 mm^2 plates (4 x 10^4 plaques/plate). The plaques were transferred, in duplicate, onto NC filters and treated as described above (2.2.23.12). One filter was hybridised with 100 ng of L59 pod total cDNAs labelled with [α^{32}S] dCTP and the duplicate filter was hybridised with 100 ng of L1390 pod total cDNAs labelled with [α^{32}P] dCTP as described above (2.2.23.12). The pod total cDNAs from L59 and L1390 were prepared as for pod total cDNAs for construction of the L59 lignified pod cDNA library. The filters were radiographed as shown in Figure 2.3.

2.2.24.2 Secondary and Tertiary Differential Screen of L59 Lignified cDNA Library

Putative positive plaques were cored from the primary screen stock plates on the basis of observed hybridisation with the [α^{35}S] labelled L59 pod total cDNA, but not to the [α^{32}P] labelled L1390 pod total cDNA as described above (2.2.23.12). Secondary and tertiary rounds of screening were performed by hybridising duplicate lifts of 50 - 100 plaques on NC filters as described above (2.2.23.12) with either [α^{32}P] dCTP labelled L59 pod total cDNA or [α^{32}P] dCTP labelled L1390 pod total cDNA.

Putative differentially expressed clones were selected on the basis of consistent hybridisation to radiolabelled L59 pod total cDNAs through three rounds of differential screening.

2.2.25 *In situ* Hybridisation

Wax embedded transverse pod sections, 10 µm thick, were dewaxed in histoclear 5 - 10 minutes, rinsed with ethanol 1 - 2 minutes, rinsed with ethanol/DEPC treated water (50:50), followed by two rinses with DEPC

Figure 2.2 Strategy for isolating differentially expressed genes as cDNAs representing the lignified endocarp phenotype.

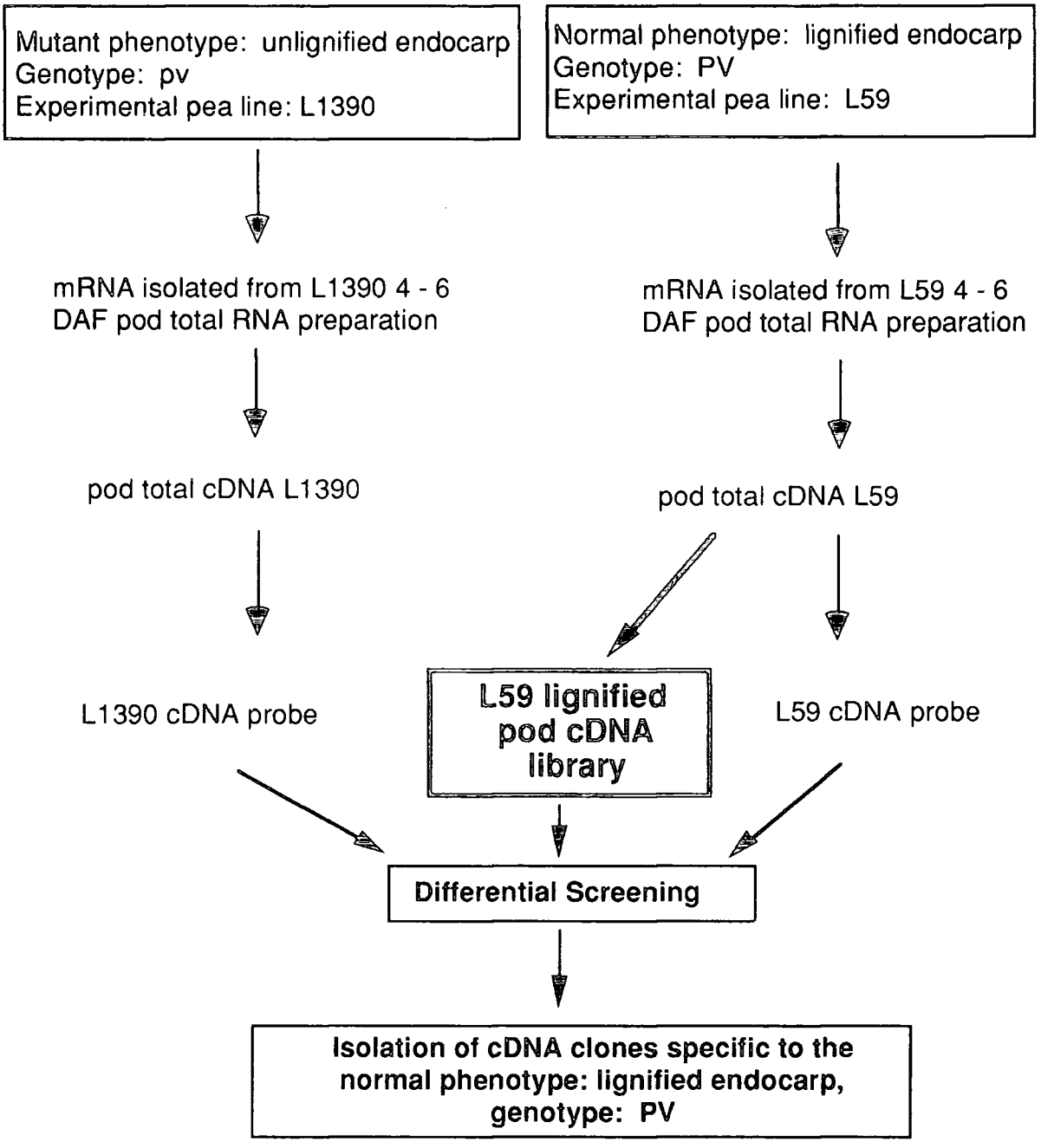
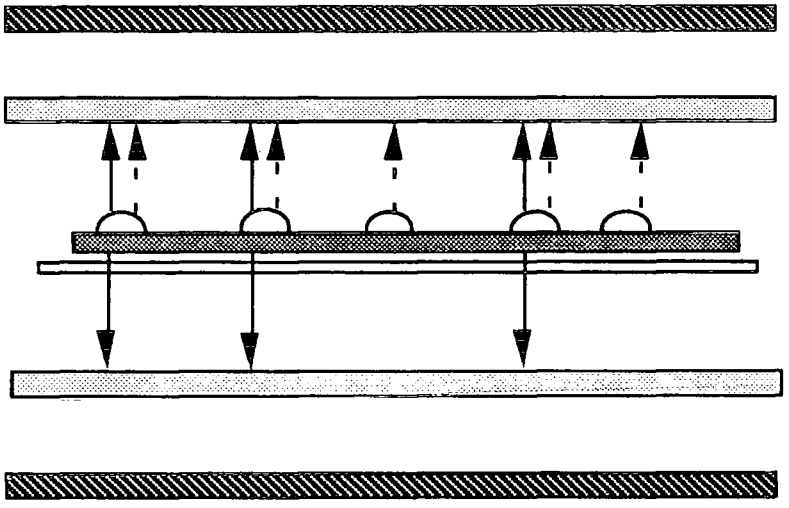

Mutant phenotype: unlignified endocarp
Genotype: pv
Experimental pea line: L1390
Normal phenotype: lignified endocarp
Genotype: PV
Experimental pea line: L59
mRNA isolated from L1390 4 - 6 DAF pod total RNA preparation
mRNA isolated from L59 4 - 6 DAF pod total RNA preparation
pod total cDNA L1390
pod total cDNA L59
L1390 cDNA probe
L59 lignified pod cDNA library
L59 cDNA probe
Differential Screening
Isolation of cDNA clones specific to the normal phenotype: lignified endocarp, genotype: PV

Figure 2.3 Autoradiography of L59 lignified pod cDNA library primary differential screen filters.



- film cassette, with intensifying screens blocked

- sensitised film

- NC filter containing plaques from L59 lignified pod cDNA library hybridised to radiolabelled total cDNA probes from L59 and L1390 4 - 6 DAF pods.

- 3 MM paper

- $\alpha^{32}P$ labelled L1390 4 -6 DAF pod total cDNA

- $\alpha^{35}S$ labelled L59 4 - 6 DAF pod total cDNA

treated water. Excess water was drained from the slide and 50 µl of hybridisation solution was added to each pod section containing 50 % formamide, 4 x SSC, 1 x Denhardt's, 1 g dextran sulphate, 500 ng/ml herring sperm DNA and digoxygenin labelled probe (1µg/ml). The probe was prepared using a digoxygenin (DIG) DNA labelling kit (Boehringer Mannheim) according to the manufacturers instructions. Parafilm was placed on top of the sections to ensure close contact of the hybridisation solution with the pod sections and placed in a moist chamber at 42 °C for 16 - 18 hours.

An alternative procedure involved a proteinase K treatment prior to hybridisation and an RNase treated control. Sections were dewaxed as above and treated with proteinase K (10 µg/ml in TE buffer, pH 7.5) for 10 minutes at 37 °C. RNase treated controls were also dewaxed as above, and treated with RNase (10 µg/ml) for 10 minutes at 37 °C. The treated sections were then rinsed in water, followed by 1 x PBS, followed by 10 minutes in fixing solution. The sections were then rinsed for 10 minutes in 1 x PBS, followed by water and hybridised as above.

Hybridised sections were rinsed with 2 x SSC/0.1 % SDS, twice, at room temperature, followed by two rinses in 2 x SSC/0.1% SDS at 42 °C. The sections were then dehydrated through a series of ethanol solutions buffered with 300 mM ammonium acetate, then allowed to air dry.

The sections were then incubated in 2 % sheep serum in buffer A (100 mM tris.HCl pH 8.0, 150 mM NaCl) for 30 minutes, followed by incubation in alkaline phosphatase conjugated polyclonal sheep anti-digoxygenin antibody (1:500 dilution in buffer A) for 2 hours at room temperature. The sections were then rinsed in buffer A for 1 hour with fresh changes of buffer A every 15 minutes. Freshly prepared substrate solution containing 0.5 mM napthol AS-MX phosphate and 2.0 mM fast red TR in tris buffer pH 8.0 was added to each section. Colour development was allowed to proceed in darkness at room temperature with monitoring by microscopic examination. Colour development was arrested by rinsing with distilled water, followed by air drying.

2.2.26 Semi-Quantitative RT-PCR for Expression of LP18 mRNA

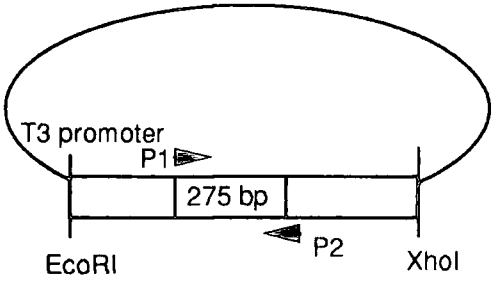
Total RNA was prepared from four, five and six DAF pods from each of the experimental pea lines, L59, L58, L1390 and Feltham First by the acid guanidium thiocyanate method (as described 2.2.22.2). The RNA concentration of each total RNA sample was then determined by spectrophotometry. Qualitative analysis of each total RNA sample was ascertained by comparison of ethidium bromide stained ribosomal bands after formaldehyde gel electrophoresis.

Semi-quantitative RT-PCR for expression of LP18 mRNA was performed according to the method described by Chang *et al.* (1993) (see Figure 2.4). A synthetic RNA species (for use as an internal standard) was prepared by ligating an NdeI cleaved lambda fragment of 556 bp into NdeI cleaved pLP18 to produce a synthetic cDNA clone pRLP18 (Figure 2.4). Synthetic RNA was reverse transcribed with T3 polymerase from rRLP18 using an *in vitro* transcription kit (Promega). The concentration of rRLP18 was determined by spectrophotometry.

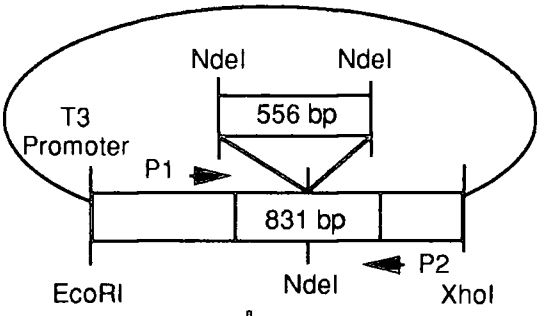
The optimal concentration of rRLP18 to be incorporated in each reaction tube was determined according to the procedure outlined by Chang *et al* . (1993). A fixed amount of L59 6 DAF total RNA (1 µg) was selected to perform reverse transcription, followed by PCR amplification with increasing concentrations of internal standard, rRLP18. A serial dilution of rRLP18 was prepared and a series of reverse transcription reactions were performed with various amounts of rRLP18, 0.005 ng - 3.1 ng. To each rRLP18 standard, 1 µg of L59 6 DAF total pod RNA and 100 ng of P2 3'-end antisense primer was added. The volume of each sample was adjusted to 42 µl and incubated at 65 °C for 15 minutes. The samples were cooled on ice and the following added: 5 µl of 5 x reverse transcription buffer (Promega), 4 U of Promega RNase inhibitor, RNasin, 0.5 mM 4 dNTPs, 200 U of Mu-LVRT and DEPC treated water to a total volume of 50 µl. First strand cDNAs were reverse transcribed from the RNA samples for 1 hour at 37 °C. The reactions were stopped by the addition of 150 µl of 100 mM tris.HCl/100 mM EDTA, pH 7.5, and phenol/chloroform/isoamyl alcohol (25:24:1) extracted. The reverse transcription products were then obtained by ethanol precipitation overnight at -80 °C.

Figure 2.4 Schematic representation of RT-PCR for expression of the putative blue copper protein cDNA, LP18, in 4, 5 and 6 DAF pea pods. [A] The pLP18 clone encoding target mRNA. The location and orientation of the oligonucleotide primers used for reverse transcription and PCR amplification are indicated. [B] Outline of procedure for construction of synthetic gene, RLP18, and the quantitative RT-PCR protocol.

[A] Clone pLP18, containing the EcoRI - XhoI located LP18 cDNA insert.



[B] Preparation of a synthetic RNA, rRLP18, by ligating an NdeI lambda fragment into the internal NdeI site of the LP18 insert between the synthetic oligo primers P1 ➤ and ◄ P2.



in vitro transcription of pRLP18 with ligated 556 bp lambda fragment using T3 RNA polymerase

spectrophotometric determination of concentration of synthetic RNA, rRLP18

Total RNA from 4, 5 or 6 DAF pods from either L59, L58 L1390 or FF

Synthetic RNA rRLP18

reverse transcription of specific Ist strand cDNA with ◄ P2 synthetic 3' antisense oligo primer

cDNA of target LP18 + cDNA of synthetic RNA RLP18

PCR amplification with synthetic oligo primers P1 ➤ and ◄ P2

275 bp, LP18 target PCR product + 831 bp, RLP18 internal standard PCR product

aliquot of PCR products Southern blotted and hybridised with radiolabelled LP18

- hybridised bands excised from filter and scint counted to determine cpm

Quantification of target mRNA, LP18, using the ratios of the two species of PCR products as a Quantitative Index:

$$\frac{\text{internal standard cpm}}{\text{target cpm}} = \frac{\text{amount of internal standard rRLP18 ng}}{\text{amount of target LP18 ng}}$$

Hence, Quantitative Index = $\frac{\text{amount of internal standard, rRLP18 x target cpm}}{\text{internal standard cpm}}$

The first strand cDNAs were resuspended in 40 µl of water. Aliquots, 10 µl, of each first strand reverse transcription reaction were taken for PCR amplification. The PCR amplification mixture contained 1 x *Taq* polymerase buffer (supplied with the enzyme), 0.2 mM 4 dNTPs, 1 µM each of 5′ and 3′ primers, P1 and P2 respectively (see Figure 2.1), in a total volume of 100 µl. The reaction mixtures were overlaid with 100 µl of mineral oil and heated to 95 °C for 3 minutes. The reaction mixtures were then chilled on ice prior to adding 2 U of *Taq* DNA polymerase and amplifying with a Techne PHC-3 thermal cycler. The PCR profile involved denaturation at 94 °C for 30 seconds, primer annealing at 47 °C for 30 seconds (based on the following calculation of annealing temperature: 4(G + C) + 2(A + T)) and extension at 72 °C for 1 minute. Amplification was carried to 21 cycles.

Analysis of the PCR amplification products was performed by electrophoresis of a 30 µl aliquot through 0.8 % agarose gel. The gel was stained with ethidium bromide and photographed. The DNA was transferred to nylon filters by the alkaline transfer method using a vacuum blotter (as described 2.2.18.1). The Southern blots were hybridised with [α^{32}P] dCTP labelled LP18 insert in 15 ml of Hybsol (as described 2.2.18.2). The hybridised target and internal standard bands were then excised from the filters and scintillation counted in Ecoscint after radiography. The ratio of internal standard cpm to target RNA cpm determined by scintillation counting the hybridised PCR amplification products was plotted against internal standard (µg) to ascertain the limit of the linear response, and thus, a suitable amount of internal standard to be added to the RT-PCR reactions.

Subsequent RT-PCR reactions using 1 µg of total pod RNAs from L59, L58 and L1390 at four, five and six days after flowering were achieved by the reverse transcription and PCR amplification procedure outlined above, with a fixed amount of internal standard, rRLP18. Semi-quantitative analysis for the presence of LP18 mRNA transcripts in the pod total RNA samples was achieved by hybridising Southern blots of the electrophoresed PCR amplification products with [α^{32}P] dCTP labelled LP18. The hybridised target and internal standard bands were then excised from the filters and scintillation counted after radiography. A quantitative index was calculated (see Figure 2.4) to give a comparison

of the level of LP18 mRNA expression relative to the amount of internal standard added. Expression of LP18 mRNA was also determined by RT-PCR, using 1 μg of total RNA from root, leaf and stem tissue.

All RT-PCR reactions were performed in duplicate. Control PCR reactions were performed with each set of RT-PCR reactions consisting of a blank containing water only, rRLP18 only, and L59 6 DAF total pod RNA only. Filters were re-exposed to film after excision of hybridised PCR amplification products for scintillation counting, to determine that the correct area of the filter had been removed.

3. RESULTS.

3.1 INVESTIGATION OF A cDNA CLONE, pPP406, SELECTED FROM A DIFFERENTIAL SCREEN OF PURPLE- AND GREEN-PODDED PEA LINES

A cDNA library was constructed in the vector pUC18, using mRNA isolated from 5 DAF purple pea pods (with embryos removed) (Bown, 1992). A differential screen of the PP (purple pod) cDNA library was then performed by Bown (1992) in an attempt to isolate clones associated with the purple-podded phenotype. A clone, designated pPP406, was isolated from the differential screen on the basis of appearing to give a greater degree of hybridisation to pod total cDNA prepared from the PP pea line, compared to hybridisation to pod total cDNA from the green-podded pea line (FF).

3.1.1 Subcloning and Sequencing of PP406

The insert from pPP406 was fully sequenced on both strands by utilising restriction fragments to make a series of subclones; both double-stranded plasmid template and single-stranded templates (after subcloning into M13) were used. A restriction map of the PP406 cDNA and the sequencing strategy are shown in Figure 3.1.

The sequenced insert comprised a cDNA of 915 bp, which contained an open reading frame of 621 bp, encoding a putative polypeptide of 206 amino acid residues from an initiation codon at nucleotides 72 - 74 and a termination codon at nucleotides 690 - 692 (Figure 3.2). The putative 3' non-coding region of 223 base pairs contained a single motif corresponding exactly to the consensus eukaryotic polyadenylation signal, AATAAA, at nucleotides 719 - 724 (Proudfoot and Brownlee, 1976). The molecular mass of the native translation product would be 23 kDa. The nucleotide sequence is available from the EMBL database, accession number X65650.

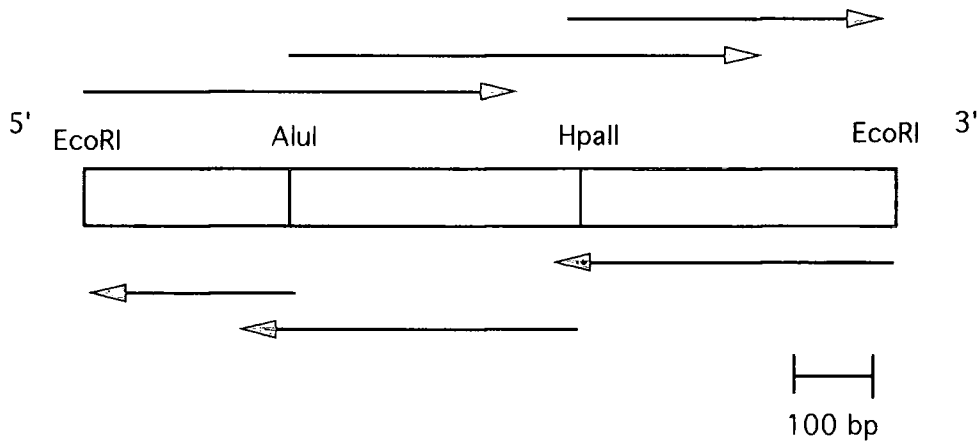


Figure 3.1 The sequencing strategy for the cDNA insert, PP406, showing restriction enzyme sites for subcloning. The sequenced fragments and the direction of sequencing are indicated by the arrows.

Figure 3.2 Psa-*rab* nucleotide sequence and predicted polypeptide. The amino acids underlined indicate conserved domains in ras-related GTP-binding proteins. The polyadenylation sequence is in bold.

```
         10         20         30         40         50
CTAGTTGAAG TAAAAAAAGA TCATCAAACA CAAGCAAACA ACAGTTTCTT

         60         70         80         90        100
CTTCTTCCAC CGATCCGTAC TATGCCTTCT CGCAGAAGAA CTCTCTTAAA
                       M  P  S  R  R  R  T  L  L  K
        110        120        130        140        150
GGTCATCATT CTCGGTGACA GCGGTGTGGG GAAGACGTCT TTGATGAACC
 V  I  I  L  G  D  S  G  V  G  K  T  S  L  M  N  Q
        160        170        180        190        200
AATATGTGAA TAAGAAGTTT AGTAATCAGT ACAAGGCAAC CATTGGAGCG
  Y  V  N  K  K  F  S  N  Q  Y  K  A  T  I  G  A
        210        220        230        240        250
GATTTCTTAA CCAAAGAAGT GCAATTTGAA GATAGGCTTT TCACCTTACA
D  F  L  T  T  E  V  Q  F  E  D  R  L  F  T  L  Q
        260        270        280        290        300
GATTTGGGAT ACAGCTGGCC AGGAGAGATT CCAAAGCCTA GGAGTTGCTT
 I  W  D  T  A  G  Q  E  R  F  Q  S  L  G  V  A  F
        310        320        330        340        350
TCTATCGTGG TGCTGATTGC TGTGTTCTTG TATATGATGT TAATTCAGTG
  Y  R  G  A  D  C  C  V  L  V  Y  D  V  N  S  V
        360        370        380        390        400
AAGTCATTTG ACAACCTTAA TAACTGGAGG GAAGAGTTTC TCATTCAAGC
K  S  F  D  N  L  N  N  W  R  E  E  F  L  I  Q  A
        410        420        430        440        450
AAATCCTTCT GATCCAGAGA ATTTTCCCTT TGTCGTTATA GGAAACAAGA
 N  P  S  D  P  E  N  F  D  F  V  V  I  G  N  K  I
        460        470        480        490        500
TAGATATTGA TGGTGGAAAC AGTAGAGTGG TTTCTGAAAA GAAGGCTCGG
  D  I  D  G  G  N  S  R  V  V  S  E  K  K  A  R
        510        520        530        540        550
GCATGGTGTG CAGCAAAAGG AAATATCCCA TATTTTGAGA CATCTGCTAA
A  W  C  A  A  K  G  N  I  P  Y  F  E  T  S  A  K
        560        570        580        590        600
AGAAGGTATT AATGTTGAAG AAGCATTCCA AACCATAGCA AAGGATGCCC
 E  G  I  N  V  E  E  A  F  Q  T  I  A  K  D  A  L
        610        620        630        640        650
TGAAAAGTGG GGAAGAGGAA GAATTATACC TGCCGGACAC AATTGATGTT
  K  S  G  E  E  E  E  L  Y  L  P  D  T  I  D  V
        660        670        680        690        700
GGAAACAGCA GTCAGCCAAG GTCAACAGGA TGTGAGTGCT GAACATATAG
G  N  S  S  Q  P  R  S  T  G  C  E  C  *
        710        720        730        740        750
ATTTTGTTCT CAATACAAAA TAAAGTATAT TATTTAAAAA TCATTTTGGC

        760        770        780        790        800
ATGTCTAGCC ATTGCTGTCT ATGAGGTTTT ATTGTACATT TATGTTTGAT

        810        820        830        840        850
CAAGTGCGAT CTGTTGGGTG CTTGTTTGGC TTGTGTTAAT CGATCATGTT

        860        870        880        890        900
GTTCTCCTTG TATGCTATTC CAACATTGTG AAAAAAACAG CAAAGGATCA

        910
TTCTGAAGTT ATTTC
```

3.1.2 Amino Acid Sequence Comparison of the Polypeptide Encoded by Clone pPP406

A homology search of the polypeptide predicted by clone pPP406 against Gene Bank data revealed highest homologies with the polypeptide products of BRL-ras from rat liver (Bucci *et al.*, 1988) and canine Rab7 (Chavrier *et al.*, 1990) (70% and 67% respectively). The polypeptide predicted by pPP406 was therefore designated Psa-rab. The function of the BRL-*ras* gene product is not known; however the product of the canine gene *Rab7* has been found in association with late endosomes (Chavrier *et al.*, 1990), and may be involved in linking late endosomes to lysosomes, or to the trans Golgi network (Goda and Pfeffer, 1988). A similar role for Psa-rab is possible on the basis of sequence similarity. The predicted amino acid sequence also showed considerable, but lower homology (30 - 37%) with *ras*-related gene products from a range of eukaryotic species (Table 3.1)

The predicted polypeptide has the following domains, which are conserved in GTP-binding proteins (Pai *et al.*, 1989) (see Figure 3.2):

(i) GDSGVGK (nucleotides 114 - 134), involved in Mg^{2+} and phosphate binding;
(ii) TIGADF (nucleotides 189 - 206), effector residues;
(iii) DTAG (nucleotides 258 - 269), in the highly conserved WDTAGE motif shared by Rab/Ypt and ras proteins, which interacts with the γ-phosphate of GTP;
(iv) YRG (nucleotides 303 - 311), a highly conserved motif shared by the Rab/Ypt proteins;
(v) NKXD (nucleotides 444 - 455), the guanine specificity region;
(vi) ETSA (nucleotides 537 - 548), which interacts with the D residue (nucleotides 453 - 455);
(vii) CXC (nucleotides 681 - 689), the C-terminal motif shared by Rab proteins and a substrate for geranylgeranyl-moieties.

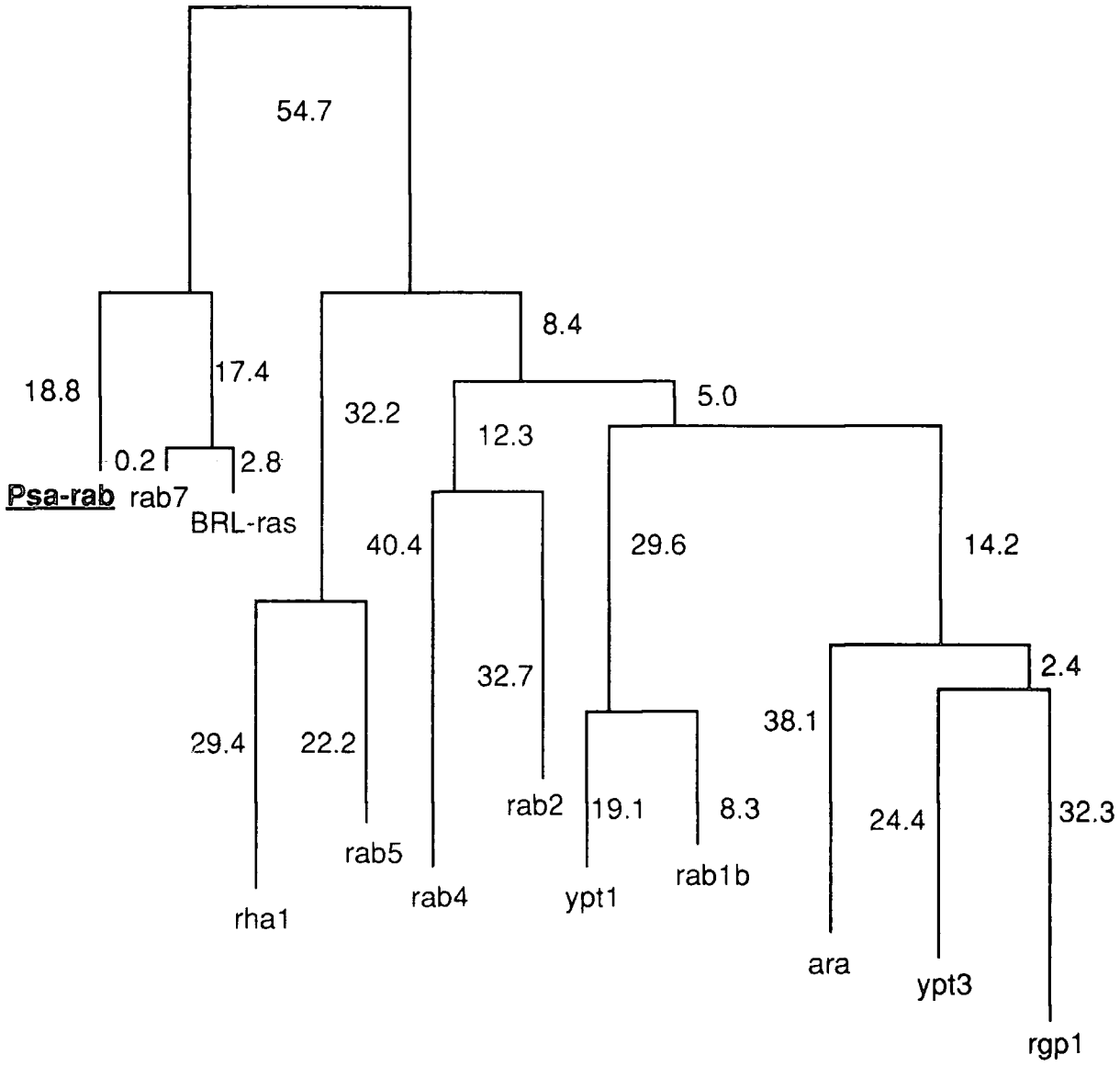
Analysis using the CLUSTAL computer package (Higgins and Sharp, 1988) was performed to compare the Psa-rab predicted polypeptide with several Rab/Ypt amino acid sequences and several plant GTP-binding proteins. The dendogram constructed (Figure 3.3), based on the

Table 3.1 Amino acid homology of Psa-rab with various ras-related GTP-binding proteins.

Organism	Protein	% identity with Psa-rab protein[a]
rat	BRL-ras	70%
dog	Rab7	67%
slime-mould	SAS1	39%
slime-mould	SAS2	38%
rat	Rab1B	36%
yeast	ras	36%
human	Rab2	35%
A. thaliana	ara	35%

[a] Sequence homology between Psa-rab and other related amino acid sequences was searched using the FASTA programme package (Pearson and Lipman, 1988).

Figure 3.3 A dendogram of predicted amino acid sequences from 12 ras-related GTP-binding proteins produced using CLUSTAL (Higgins and Sharp, 1988). The figures along each branch are percentage divergence along that particular branch of molecular evolution. The tree was rooted half way along the longest branch (54.7%). The dendogram was constructed by calculating crude similarity scores by the Wilbur and Lipman (1983) method and using these calculations to generate a preliminary dendogram by the UPGMA method of Sneath and Sokal (1973). The preliminary dendogram is then used to dictate the order of sequence alignment for the final multiple alignment. Percent divergence figures were then calculated between all pairs of sequence after multiple alignment, generating a tree by the Neighbour Joining method of Saitou and Nei (1987). Bootstrapping techniques were then used to confirm the significance of the groupings obtained. The input sequences were derived from the following sources: rab1b (Vielh *et al.*, 1989), rab2 and rab4 (Touchot *et al.*, 1987), rab5 (Zahroui *et al.*, 1989), rab7 (Chavrier *et al.*, 1990) and BRL-ras (Bucci *et al.*, 1988) from rat, canine and human cDNA libraries; ara (Matsui *et al.*, 1989) and rha1 (Anuntalabhochai *et al.*, 1991) from Arabidopsis thaliana, rgp1 (Kamada *et al.*, 1992) from rice; and ypt1 (Wichmann *et al.*, 1989) and ypt3 (Miyake *et al.*, 1990) from yeast.


54.7
18.8
17.4
8.4
32.2
12.3
5.0
0.2
2.8
Psa-rab
rab7
BRL-ras
40.4
29.6
14.2
32.7
2.4
29.4
22.2
38.1
rab2
19.1
8.3
24.4
32.3
rab5
rab1b
rha1
rab4
ypt1
ara
ypt3
rgp1

method for reconstructing phylogenetic trees by Saitou and Nei (1987), showed that the pea sequence is more closely related to the BRL-ras and Rab7 sequences than to other plant ras-related proteins previously isolated. The BRL-ras, Rab7 and Psa-rab polypeptides show a considerable degree of divergence from the other Rab/Ypt and plant ras-related amino acid sequences. This cluster of three sequences forms a separate sub-family distinct from the other Rab/Ypt family members, which include the other plant ras-related proteins.

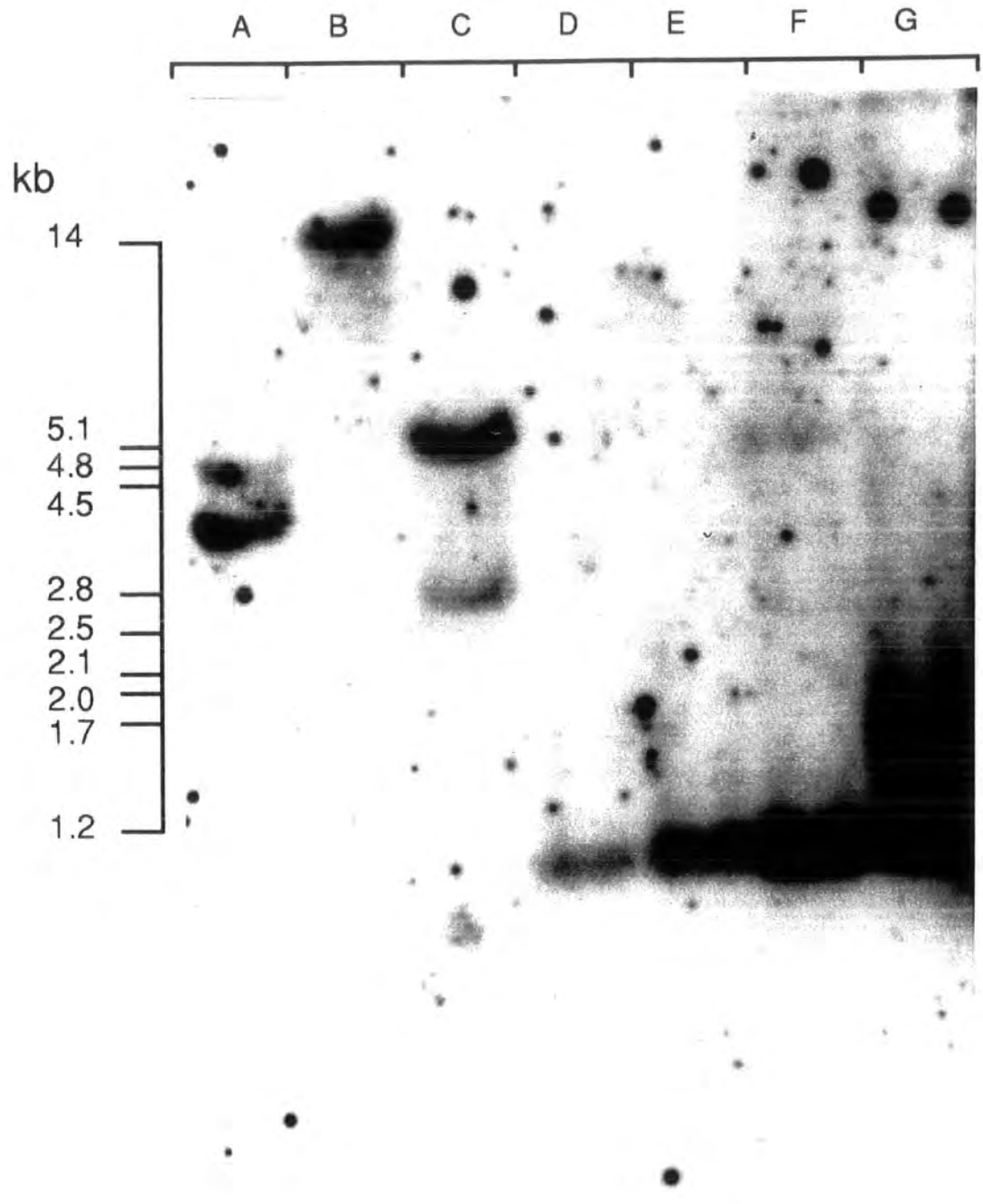
3.1.3 Genomic Analysis of PP406

The Psa-*rab* cDNA was hybridised to pea genomic DNA in Southern blotting experiments to investigate the number of Psa-*rab* genes in *Pisum sativum* (Figure 3.4). PP genomic DNA was cleaved with EcoRI, BamHI or HindIII and blotted onto an NC filter with Psa-*rab* gene copy equivalents and hybridised with the entire PP406 cDNA sequence (as described 2.2.18.3). The filter was washed to high stringency (0.1 x SSC/0.1 % SDS at 65 °C). Different hybridisation patterns were observed in each digest (Figure 3.4). One strongly hybridised band was observed in all three digests and one other more weakly hybridising band was observed in the HindIII genomic digest and possibly the EcoRI digest. As the PP406 cDNA does not contain EcoRI or HindIII restriction enzyme sites, the Southern data indicate that there are one to two genes encoding the Psa-rab protein in pea. It is possible that Psa-*rab* contains an intron with these restriction sites, which could result in the more weakly hybridising fragment in the HindIII and EcoRI genomic digests. Alternatively, the more weakly hybridising band may represent a related sequence belonging to the Rab sub-family of ras-related proteins.

3.1.4 Expression Analysis of PP406 in Pods from Different Pea Lines

3.1.4.1 Expression of PP406 in PP, FF and GP Pods

Expression analysis of PP406 in PP, GP and FF pods was performed by Dr. David Bown (Durham University) to investigate possible differential expression in the purple-pod phenotype. The PP406 insert from clone pPP406 was used to probe northern blots containing total


A
B
C
D
E
F
G
kb
14
5.1
4.8
4.5
2.8
2.5
2.1
2.0
1.7
1.2

RNA from PP, FF and GP (mutant green pod from the PP line). The PP406 insert hybridised with similar intensity to a 1.1 kb mRNA species in all of these lines (Figure 3.5). Background hybridisation to ribosomal RNA bands was also observed, as indicated by RNA size markers, even when the blot was washed to high stringency.

3.1.4.2 Expression of PP406 in L59, L58 and L1390 Pods

It has been observed that ER, Golgi and cytoplasmic vesicles are numerous and predominant in close proximity to the wall of cells undergoing differentiation and formation of lignified sclerenchyma cells (Lawton and Harris, 1979; Harris, 1984; Vercher *et al.*, 1987). The ER, Golgi and the electron dense and electron transparent vesicles are presumed to be involved in intracellular transport of wall materials and enzymes required for construction of new cell walls in differentiating sclerenchyma and for lignin formation. It was observed from microscopic examination of PP, GP and FF transverse pod sections that these lines all have a differentiated endocarp. Considering that Psa-rab may play a role in intracellular transport, based on sequence similarity with other Rab proteins, and the strong and invariant expression in the PP, GP and FF pods, it was thought that Psa-rab expression may be associated with the intense intracellular transport activity associated with the differentiating pod endocarp present in all of these experimental pea lines.

The endocarp development of experimental pea lines, L59, L58 and L1390 has been characterised as a result of investigation of the genetic basis for lignification of the pod endocarp (described below 3.2.1 and 3.2.2). Expression of PP406 in total pod RNA from L59, L58 and L1390 was investigated in order to determine any variation in expression, which may be associated with the distinct differences in pod endocarp development in these lines.

Hybridisation of the Psa-*rab* cDNA to total pod RNA from L59, L58 and L1390 in a northern blotting experiment showed a similar degree of hybridisation to a mRNA species of approximately 1.1 kb in all lines (Figure 3.6). It does not, therefore, seem likely that PP406 expression is associated specifically with vesicular transport in the differentiating

Figure 3.5 Northern blot hybridisation of total pod RNA (10 ug) of three varieties of pea - purple-podded (PP), Feltham First (FF) and green-podded (GP) - probed with Psa-*rab* cDNA ^{32}P-labelled by random priming. The pods were harvested 5 days after flowering and the seeds were excised. Hybridisation was performed for 14 hours at 42 °C in 50 ml of a solution containing: 5 x SSC, 2 x Denhardt's reagent, 50 % formamide and 200 µg/ ml salmon sperm DNA. The blots were washed to a stringency of 0.1 x SSC, 0.1 % SDS at 50 °C for 2 x 20 minutes.

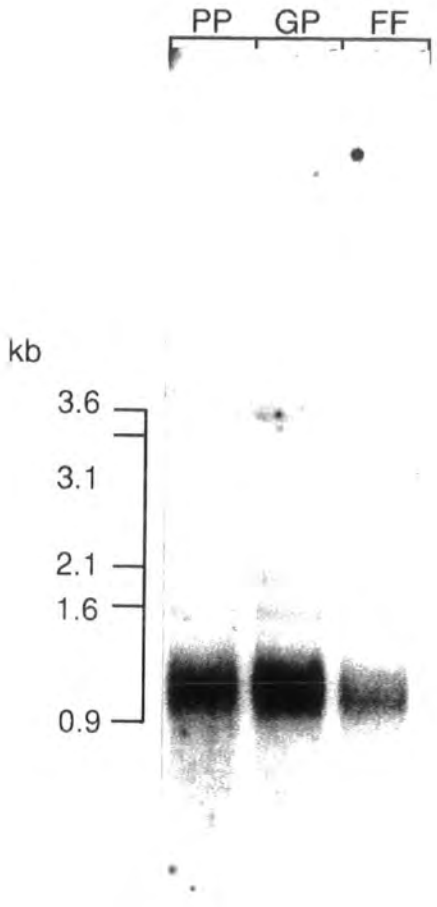
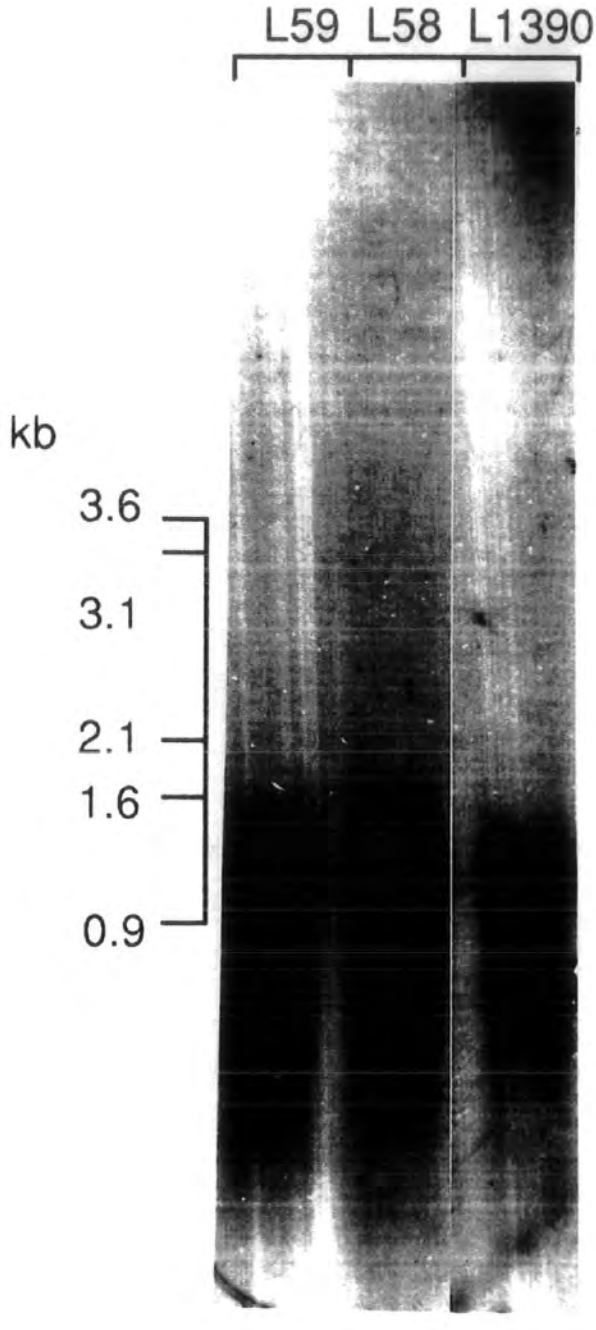

PP
GP
FF
kb
3.6
3.1
2.1
1.6
0.9

Figure 3.6 Northern blot hybridisation of total pod RNA (10 ug) of three pea lines - L59 (phenotype: lignified endocarp), L58 (phenotype: partially lignified endocarp) and L1390 (phenotype: unlignified endocarp) - probed with Psa-*rab* cDNA ^{32}P-labelled by random priming. The pods were harvested 4 - 6 days after flowering and the seeds and main veins were excised. Hybridisation was performed for 14 hours at 42 °C in 50 ml of a solution containing: 5 x SSC, 2 x Denhardt's reagent, 50 % formamide and 200 µg/ ml salmon sperm DNA. The blots were washed to a stringency of 0.1 x SSC, 0.1 % SDS at 50 °C for 2 x 20 minutes.


L59
L58
L1390
kb
3.6
3.1
2.1
1.6
0.9

endocarp, which is present in L59, reduced in L58 and absent in L1390 (see Plate 3.3).

3.1.5 Complementation of the Yeast *YPT7* Mutant with PP406

A *YPT7* null mutant was observed to have an altered phenotype, characterised by abnormally fragmented vacuoles (Wichmann *et al.*, 1992). The *YPT7* null mutant carries a defective *YPT7* gene, which is a homologue of the mammalian *Rab7*. In contrast, the wild type has a prominent vacuoler compartment, which may be observed by fluorescence microscopy (Makarow, 1985).

The *YPT7* null mutant, Y7L1:Mata ura3leu2 his4 lys2 bar1 ypt7::LEU2) was obtained from Dr. Dietrich Scheglman (Max-Planck-Institute for Biophysical Chemistry, Gottingen). It was hoped to attempt to complement the fragmented vacuole phenotype of the *YPT7* null mutant with Psa-*rab*, which is 55.5 % homologous to *YPT7* and has an identical effector domain, thought to be necessary for successful complementation in yeast (see Figure 3.7).

3.1.5.1 Construction of a Yeast Expression Vector Containing the PP406 cDNA

EcoRI cleaved PP406 insert was ligated into an EcoRI cleaved pYES2 vector. The pYES2 vector has an *S. cerevisiae GAL1* gene for inducible transcription from the T7 promoter. The ligated products were used to transform DH5α and plated out on LB/ampicillin plates. The pYES2 vector does not allow blue/white colour selection. Hence, 20 colonies were selected from 100 - 200 transformants at random and DNA prepared for analysis. None of the plasmids contained the PP406 insert as determined by cleavage with restriction enzymes. In addition, electrophoresis of prepped DNA was of poor quality with diffuse bands observed. Hence, it was decided to plate out the ligations with competent TOP 10 F' (as recommended by Invitrogen) and the presence of inserts was identified by hybridisation to lysed bacterial streaks (as described 2.2.21).

Figure 3.7 Alignment of the Psa-rab amino acid sequence with mammalian RAB7 and yeast YPT7 homologues. The identical effector domains are underlined. The colons (:) represent identical residues and the periods (.) represent residues defined as "similar' in the mutation matrix using the FASTA protein sequence comparison programme (Pearson and Lipman, 1988). Gaps have been introduced for maximal alignment.

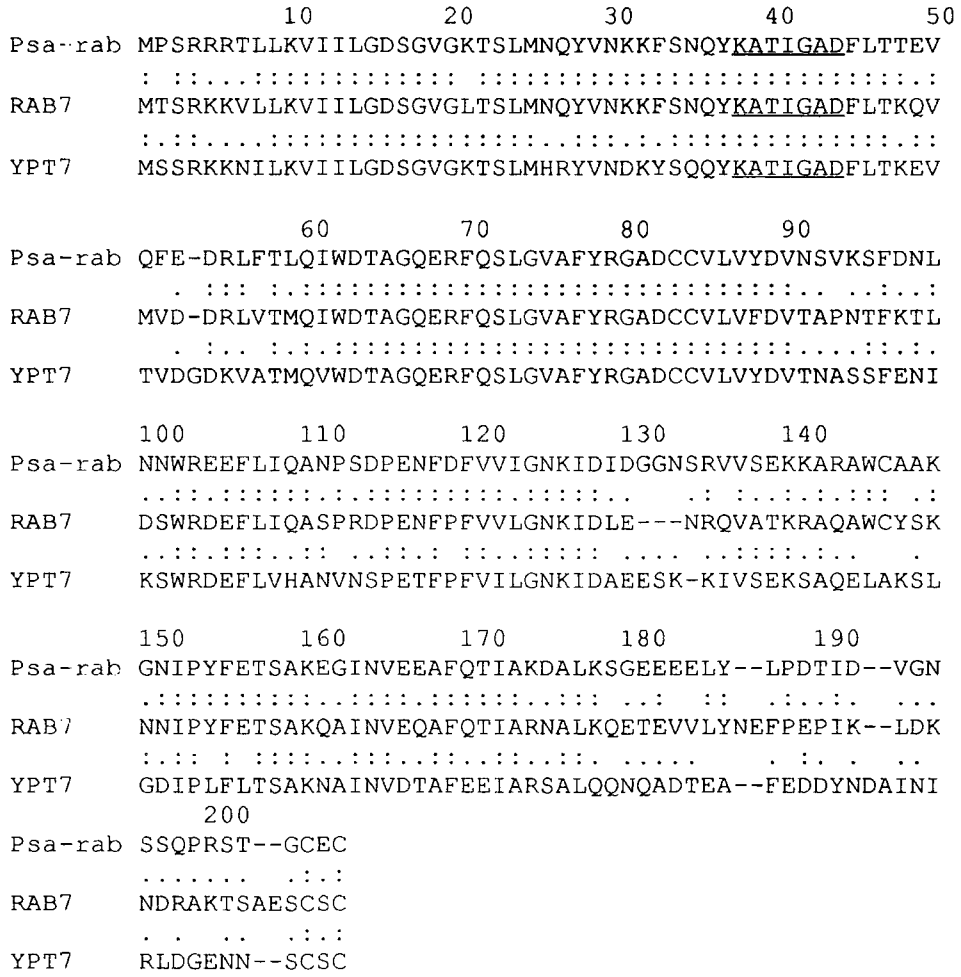
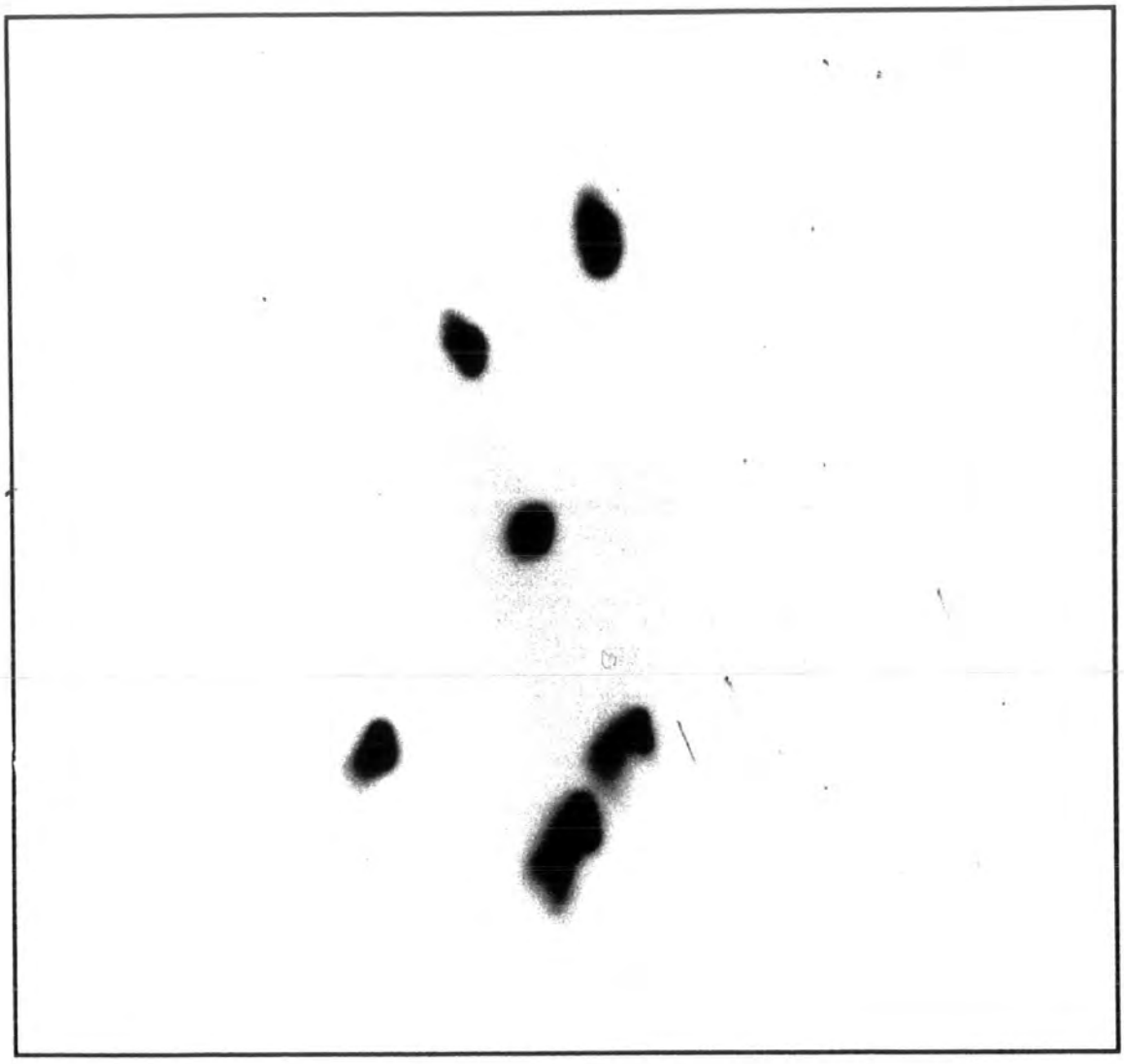

10 20 30 40 50
Psa-rab MPSRRRTLLKVIILGDSGVGKTSLMNQYVNKKFSNQYKATIGADFLTTEV
RAB7 MTSRKKVLLKVIILGDSGVGLTSLMNQYVNKKFSNQYKATIGADFLTKQV
YPT7 MSSRKKNILKVIILGDSGVGKTSLMHRYVNDKYSQQYKATIGADFLTKEV
60 70 80 90
Psa-rab QFE-DRLFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSVKSFDNL
RAB7 MVD-DRLVTMQIWDTAGQERFQSLGVAFYRGADCCVLVFDVTAPNTFKTL
YPT7 TVDGDKVATMQVWDTAGQERFQSLGVAFYRGADCCVLVYDVTNASSFENI
100 110 120 130 140
Psa-rab NNWREEFLIQANPSDPENFDFVVIGNKIDIDGGNSRVVSEKKARAWCAAK
RAB7 DSWRDEFLIQASPRDPENFPFVVLGNKIDLE---NRQVATKRAQAWCYSK
YPT7 KSWRDEFLVHANVNSPETFPFVILGNKIDAEESK-KIVSEKSAQELAKSL
150 160 170 180 190
Psa-rab GNIPYFETSAKEGINVEEAFQTIAKDALKSGEEEELY--LPDTID--VGN
RAB7 NNIPYFETSAKQAINVEQAFQTIARNALKQETEVVLYNEFPEPIK--LDK
YPT7 GDIPLFLTSAKNAINVDTAFEEIARSALQQNQADTEA--FEDDYNDAINI
200
Psa-rab SSQPRST--GCEC
RAB7 NDRAKTSAESCSC
YPT7 RLDGENN--SCSC

Figure 3.8 Hybridisation of ^{32}P-labelled PP406 to bacteria transformed with the yeast expression vector, pYES2, containing the PP406 cDNA.



A selection of 169 lysed transformed bacteria were hybridised with $\alpha^{32}P$ labelled PP406. Two bacterial streaks of TOP 10 F' transformed with pYES2 were used as controls to determine background hybridisation levels. Five putative positive transformed bacteria, designated pYP52, pYP69, pYP87, pYP92, pYP143 and pYP145, were identified (Figure 3.8). DNA prepared from the five putative positives picked of the replica stock plate for restriction enzyme analysis confirmed the presence of an insert of approximately 900 bp. Restriction enzyme analysis with HincII and BamHI or HincII and XhoI was performed on the five colonies and confirmed that the clones designated pYP52, pYP92 and pYP145 contained PP406 inserts in the correct orientation with respect to the T7 promotor (Figure 3.9).

DNA for sequencing was prepared from pYP52. Sequencing from the T7 site confirmed the identification of the PP406 insert in the correct orientation for inducible transcription.

3.1.5.2 Complementation of the Yeast *YPT7* Mutant with PP406

Transformation of the yeast strain, Y7L1 (Mata ura3leu2 his4 lys2 bar1 ypt7::LEU2), with the pYP52 vector and analysis of the complemented yeast strain was performed by Ms. Caroline Hartley and Dr. Martin Watson (Durham University). The transformed yeast strain, designated Y7l1YP52, was induced by incubation in a glucose minus media containing galactose and the resulting phenotype was examined by fluorescence microscopy. Results to date (October, 1994) have been inconclusive due to poor growth of the yeast strain; this work is continuing.

3.2 PHYSIOLOGY OF LIGNIFICATION OF THE PEA POD ENDOCARP

3.2.1 Histochemical Staining for Lignin in the Pea Pod Endocarp

Transverse pod sections cut from fresh pods of L59, L58, L1390 and FF were stained with phloroglucinol (as described 2.2.3.4) and photographed (2.2.3.7) (Plate 3.1). Lignification of the pod endocarp of L59 (genotype, PV) and Feltham First was observed to occur at around

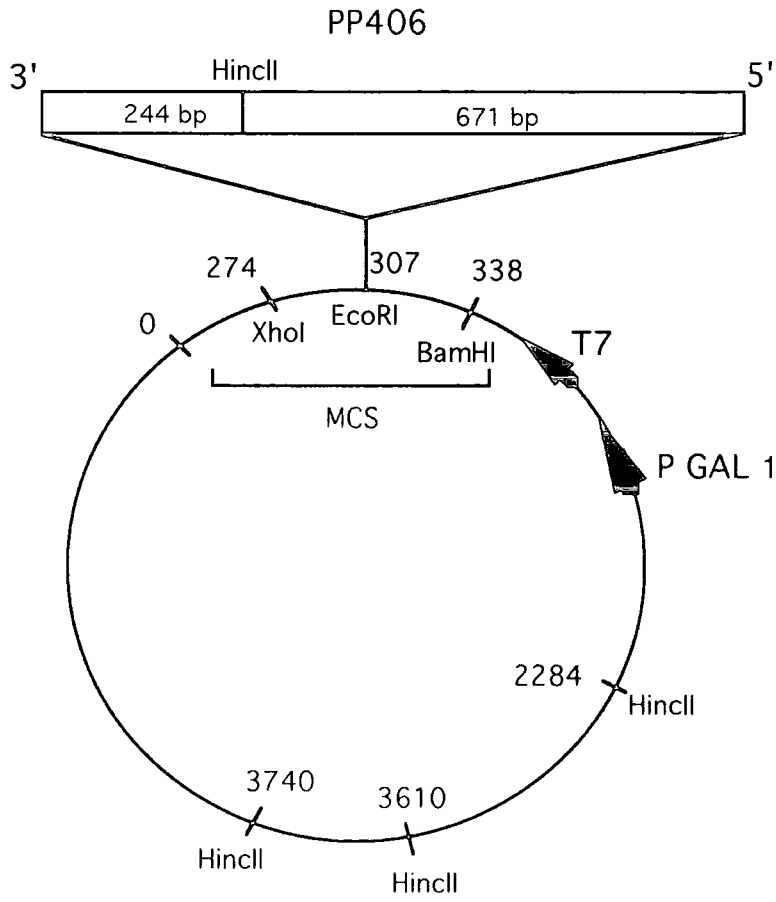
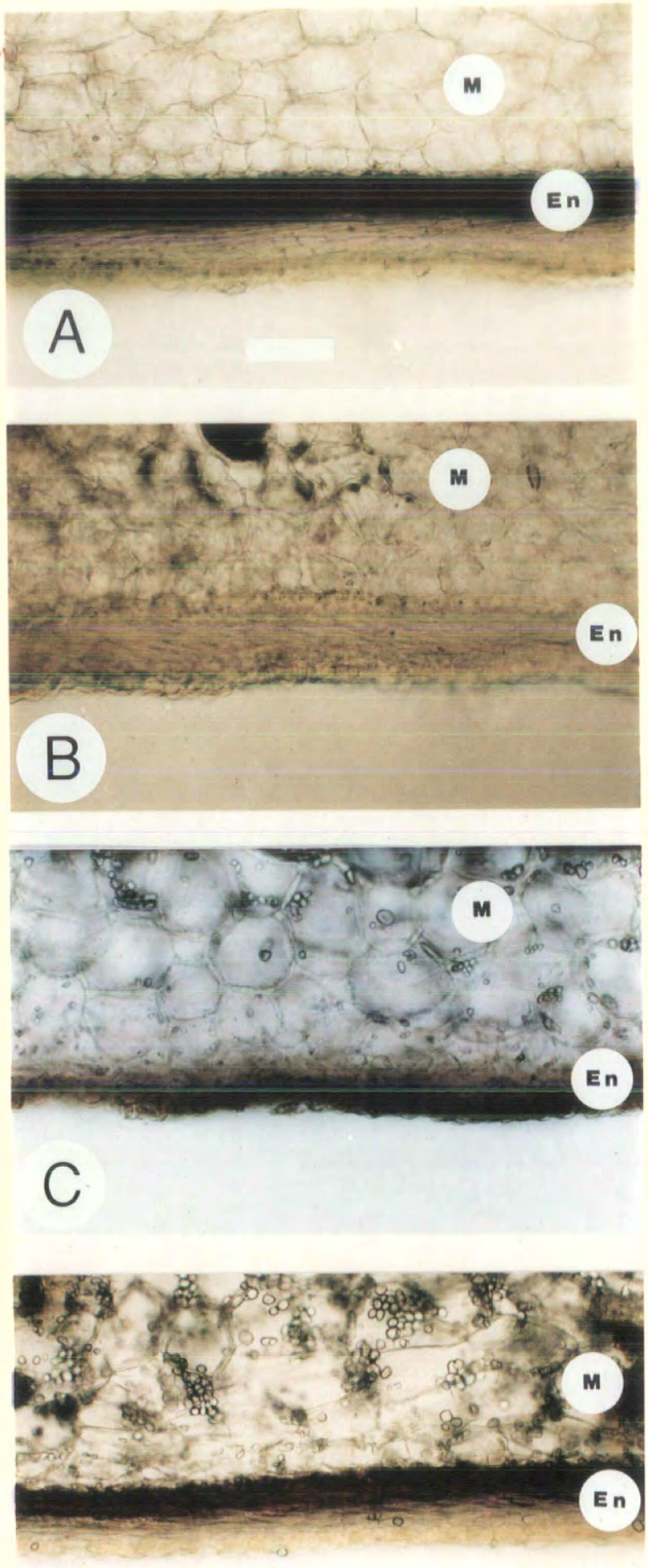


Figure 3.9 Schematic representation of the yeast expression vector, designated pYP52, containing the PP406 cDNA nucleotide sequence. The restriction enzyme sites used to determine insert orientation are shown. MCS indicates the multi-purpose cloning site and the location of the upstream activating promotor sequences from the *S. cerevisiae GAL1* gene and the T7 primer site are shown. The numbering corresponds to nucleotide positions in the pYES2 vector, which is 5.9 kb in size.

Plate 3.1 Histochemical staining for lignin in the pea pod endocarp. Light micrographs of transverse pod sections (fresh tissue) stained with phloroglucinol. Phloroglucinol staining produces a red coloration on reaction with lignin (Monties, 1989). A, L59 6 DAF; B, L58 6 DAF; C, L1390 6 DAF; D, FF 6 DAF. En, endocarp; M, mesocarp. All figures at the same magnification, bar = 100 μm


M
En
A
M
En
B
M
En
C
M
En
D

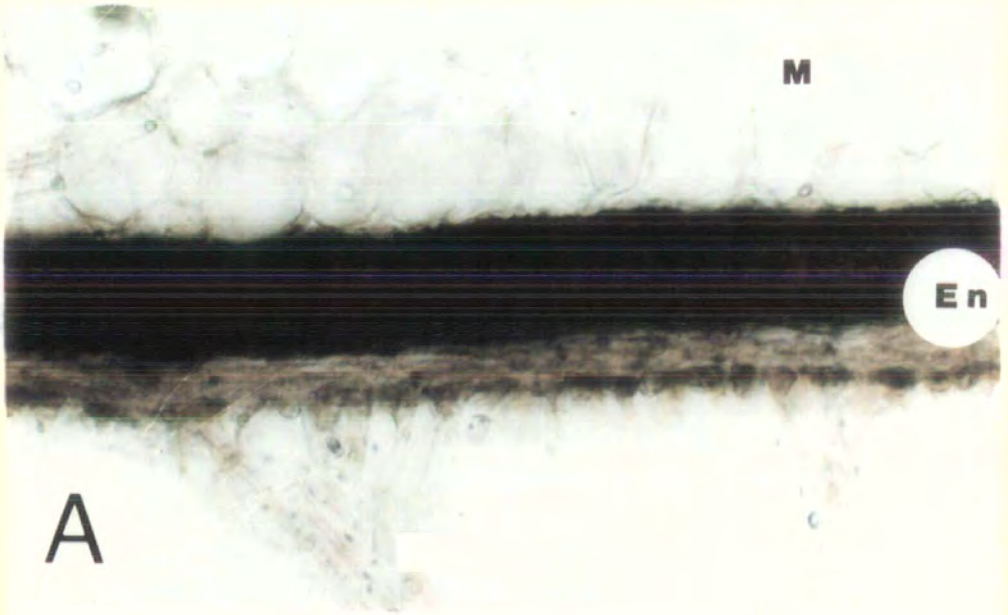
six DAF, indicated by the presence of red staining in the endocarp of the phloroglucinol stained pod sections (Plates 3.1A and 3.1D). The positive staining in pods of FF 6 DAF confirmed the PV genotype in FF. The extent of endocarp lignification in mature pods at 12 DAF is also shown (Plate 3.2A). There was no positive staining for lignin with phloroglucinol in L58 (genotype, Pv) pods at 6 DAF (Plate 3.1B). However patches of sclerenchyma became apparent in mature pods of L58 (Plate 3.2B). The absence of lignification in the endocarp of L1390 (genotype, pv) at six DAF was verified (Plate 3.1C). No lignification of the L1390 pod endocarp was apparent in mature pods at 12 DAF (Plate 3.2C), confirming the L1390 genotype, pv.

3.2.2 Pod Endocarp Development in L59, L58 and L1390

Microscopic examination of the fresh transverse pod sections revealed possible differences in the morphology of the endocarp in L1390 compared to L59 and L58 (Plates 3.1 and 3.2). Hence, it was decided to prepare resin embedded pod sections from L59, L58 and L1390 for a more detailed examination of the pod endocarp. Transverse pod sections, 1 μm thick, were prepared and stained with toluidine blue (as described 2.2.3.3 and 2.2.3.5).

Examination of the structural development of resin embedded sections of the pod endocarp from four, five and six DAF pods of L59, L58 and L1390 revealed distinct morphological differences in development (see Plate. 3.3). At four DAF, L59 exhibits the typical four layered endocarp as described by Vercher *et al.* (1987) (Plate 3.3A). The endocarp consists of a layer of endodermal cells, a middle zone consisting of three to four strata of meristematic cells, the transition layer consisting of a layer of cells similar to parenchymatous mesocarp cells, but smaller, and a layer of elongating cells situated between the middle zone and the transition layer. At five DAF the elongating cell layer adjacent to the transition layer has begun to differentiate to form sclerenchyma (Plate 3.3B). At six DAF the elongating cells have differentiated to form sclerenchyma which have commenced lignification (as indicated by staining with toluidine blue) (Plate 3.3C). At 12 DAF there are three layers of lignified sclerenchyma in the pod endocarp of L59 (Plate 3.4A).

Plate 3.2 Histochemical staining for lignin in the pea pod endocarp at 12 DAF. Light micrographs of transverse pod sections (fresh tissue) stained with phloroglucinol. Phloroglucinol staining produces a red coloration on reaction with lignin (Monties, 1989). A, L59 12 DAF; B, L58 12 DAF; C, L1390 12 DAF. En, endocarp; M, mesocarp. All figures at the same magnification, bar = 100 μm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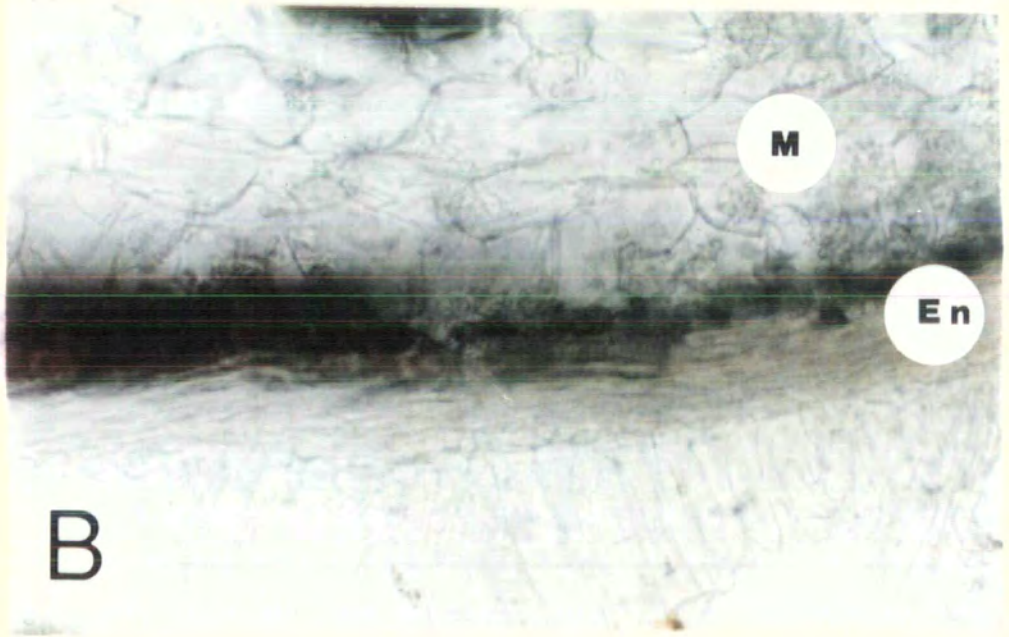
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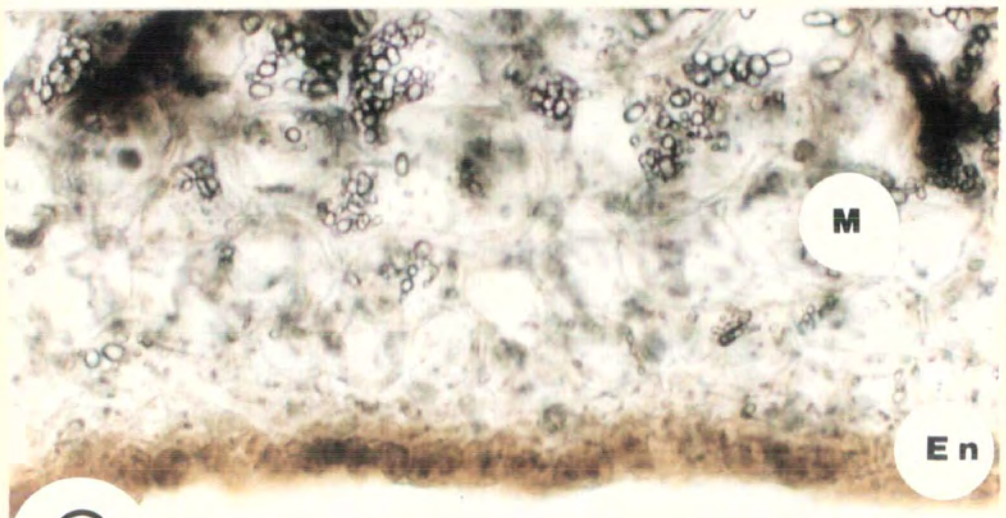
M
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Plate 3.3 Development of the endocarp of L59, L58 and L1390 from four to six days after flowering. Light micrographs of 1 μm thick transverse pod sections stained with toluidine blue. A, L59 4 DAF; B, L59 5 DAF; C, L59 6 DAF; D, L58 4 DAF; E, L58 5 DAF; F, L58, 6 DAF; G, L1390 4 DAF; H, L1390 5 DAF; I, L1390 6 DAF. a, endodermal layer; b, middle zone; c, pre-sclerenchyma layer; d, transition layer; Sc, sclerenchyma; En, endocarp; M, mesocarp. All figures at the same magnification, bar = 50 μm.

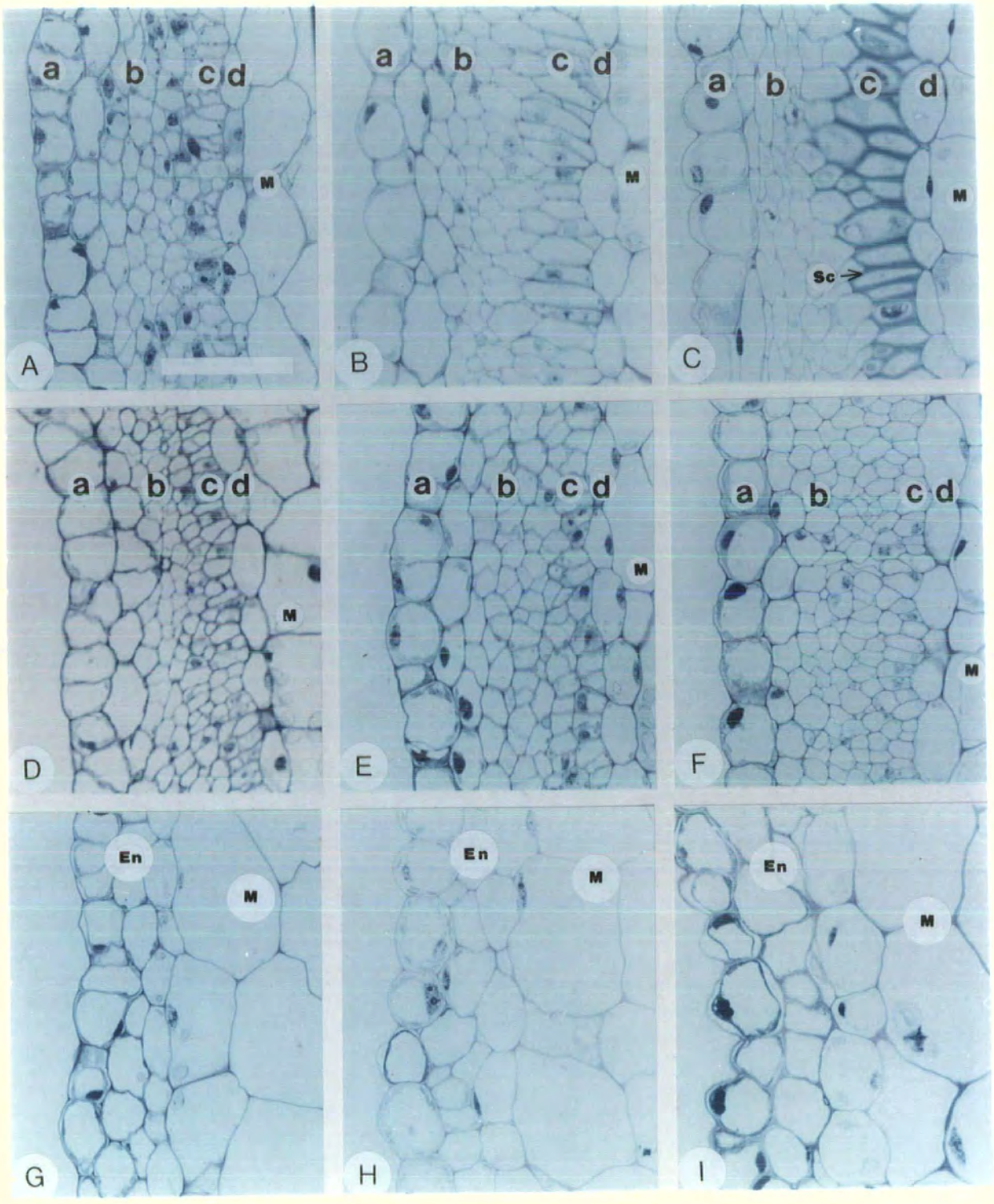
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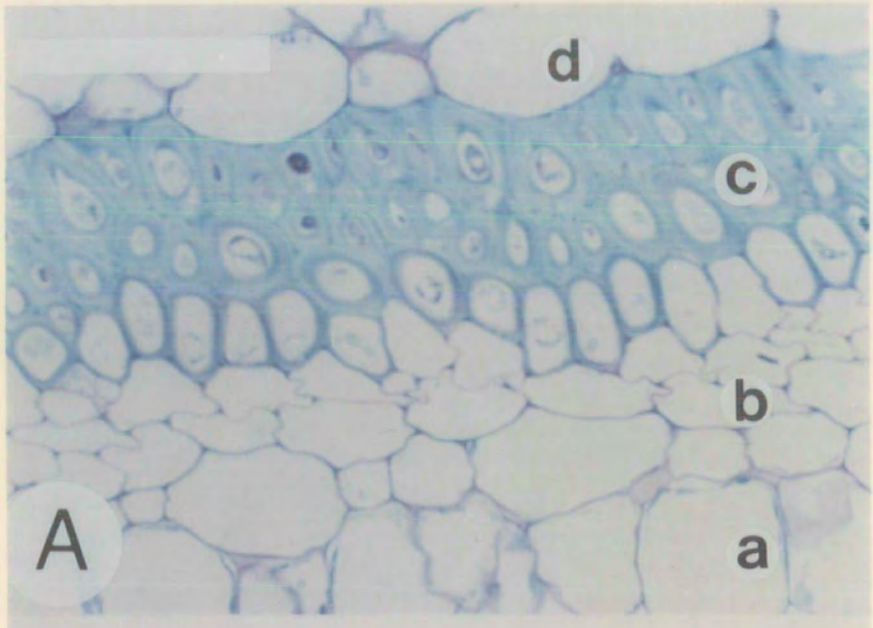
Plate 3.4 Development of the endocarp of L59, L58 and L1390 at 12 DAF. Light micrographs of 1 μm thick transverse pod sections stained with toluidine blue. A, L59 12 DAF; B, L58 12 DAF; C, L1390 12 DAF. a, endodermal layer; b, middle zone; c, pre-sclerenchyma layer; d, transition layer; Sc, sclerenchyma; En, endocarp; M, mesocarp. All figures at the same magnification, bar = 50 μm.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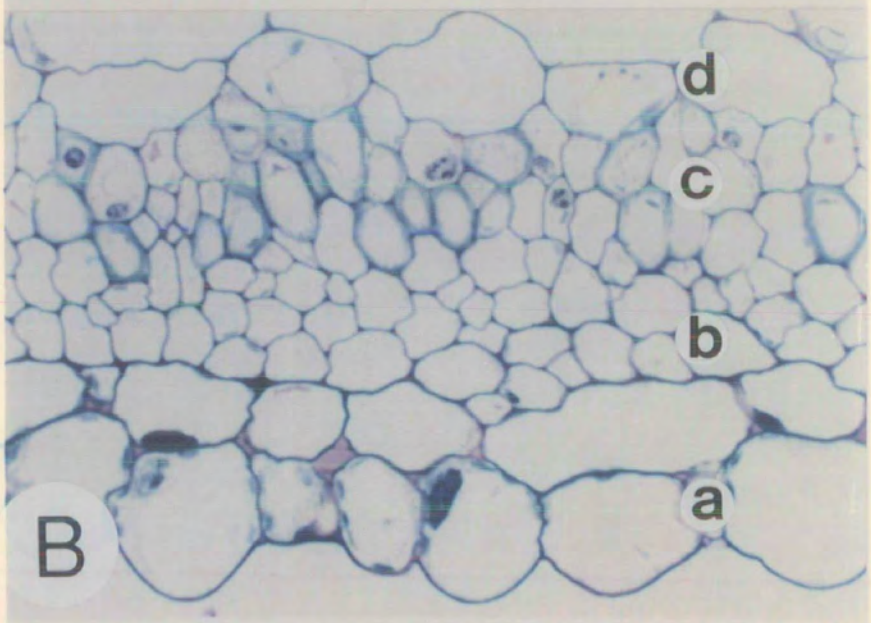
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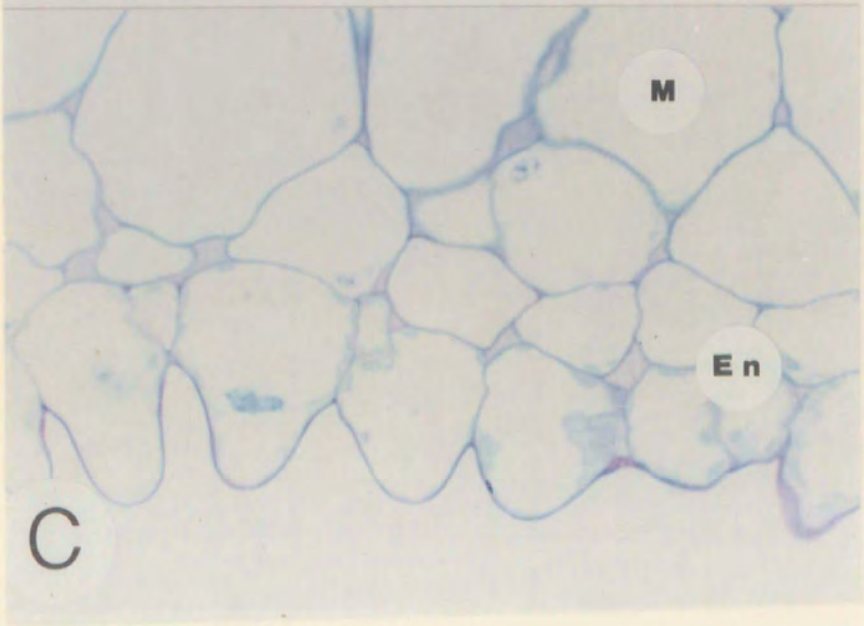
M
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C

Pods of L58 at four, five and six DAF have similar morphology to the L59 pods in that they have the characteristic endodermal layer, a middle zone of meristematic cells, a transition layer and an elongating cell layer (Plates 3.3D, 3.3E and 3.3F). However, there is no differentiation to produce lignified sclerenchyma in the elongating cell layer of L58. Mature pods of L58 show patches of lignified sclerenchyma in the endocarp (Plate 3.4B). Hence, differentiation of some of the cells of the elongating cell layer does occur, but at a later stage of development compared to pods of L59.

L1390 revealed none of the morphological changes in the endocarp exhibited by L58 and L59. The characterisitic four layered endocarp is absent. The endocarp of L1390 at four, five and six DAF consists of a layer of endodermal cells, with one to two layers of small parenchyma-like cells adjacent to the larger mesocarp parenchyma cells (Plates 3.3G, 3.3H and 3.3I). Microscopic examination of 12 DAF pods revealed no further structural development of the endocarp in L1390 (Plate 3.4C).

3.2.3 The Effect of Plant Growth Regulators on Endocarp Development

Fertilised ovules are normally necessary for normal pod development and growth of the pod wall (see 1.7). Furthermore, it has been observed that plant hormones supplied by the fertilised ovule provide a major stimulus for ovary development (Eeuwens and Schwabe, 1975; Garcia-Martinez and Carbonell, 1980; Vercher *et al.*, 1984).

Plant growth regulators were applied to emasculated ovaries (as described 2.2.3.6) to determine whether the various treatments could alter the course of endocarp development in the normal, or mutant pod phenotypes of L59, L58 and L1390. The pea flowers were emasculated at day -2 (prior to flower opening) to prevent pollination and fertilisation, thus preventing input signals for development from the fertilised ovule (see 1.7). The exogenous application of plant growth regulators to ovaries at day 0 (corresponding to day at which fertilisation would occur in seedy pods), circumvented the ovule as a route for the transfer of hormonal stimuli, and ensured that all pods received the signal to develop at a similar stage of development. The pods were harvested at day 6, the pod length was measured (Table 3.2)

Table 3.2 Parthenocarpic pod development in response to exogenous application of plant growth regulator treatments.

Treatment[a]	Pod Length (mm)[b]		
	L59	L58	L1390
MVA	27.5±8.5	28.0±6.0	36.5±7.5
MVA + GA3	62.0±3.0	61.5±3.5	53.5±2.5
6-BAP	36.5±6.5	31.0±6.0	44.5±2.5
6-BAP + GA3	62.5±4.5	64.5±2.5	63.0±7.0
GA3	66.0±4.0	61.0±6.0	53.0±5.0
Tween	20.5±7.5	36.5±7.5	41.5±8.5
Self-pollinated	58.0±5.0	63.5±6.5	66.0±6.0

[a] In each case the first three flowers on each plant were treated with one application at day 0.

[b] The value for pod length is the mean value of the length of the three pods treated per plant.

and the endocarp development of the harvested pods was observed by microscopic examination of resin embedded sections (prepared as described 2.2.3.3 and 2.2.3.5) (see Plates 3.5, 3.6 and 3.7).

As shown in Table 3.2 parthenocarpic fruit set and development was induced to a varying degree in all of the treated pods. GA3 was the most effective treatment in inducing fruit set and development in L59. The L59 pods treated with GA3 were longer than those of seedy pods and did not show any deformity. Endocarp development was enhanced with a greater degree of lignification than the corresponding normal fertilised pod (Plates 3.5A and 3.5F). In contrast, the L58 and L1390 pods treated with GA3 were smaller than the normal fertilised pods. It would appear that the L1390 pods have a reduced response to exogenous application of GA3 compared to L59. L58 pods treated with GA3 are also smaller than normal fertilised pods, but exhibit enhanced endocarp development in comparison to L58 normal fertilised pods (Plates 3.6A and 3.6F).

L59 pods appear to show the greatest response to the cytokinin, 6-BAP, when compared with L58 and L1390, with a slightly increased growth compared to the respective tween treated control. Cytokinin appears to have significantly enhanced endocarp development in L59 pods (Plate 3.5D). The pods are considerably smaller than fertilised pods (being only slightly larger than the tween treated control (Plate 3.5G), but endocarp development is considerable, with differentiated, lignified sclerenchyma observed (Plate 3.5D). L1390 pods treated with 6-BAP were also slightly larger in size compared to the tween treated controls for L1390. L58 pods treated with 6-BAP were slightly smaller in size compared to the tween treated controls for L58. The endocarp of 6-BAP treated L58 pods (Plate 3.6D) is also less well developed than the fertilised pod (Plate 3.6A).

The mixed applications of 6-BAP and GA3 to emasculated flowers of L59 produced pods smaller than single applications of GA3, but significantly larger than single applications of 6-BAP. Correspondingly, the 6-BAP/GA3 treated pods have a less well developed endocarp (Plate 3.5E) compared to GA3 treated pods of L59 (Plate 3.5F). The mixed application of 6-BAP/GA3 to emasculated

Plate 3.5 Endocarp development in parthenocarpic pods of L59 treated with plant growth regulators. Light micrographs of 1 μm thick transverse pod sections stained with toluidine blue. A, normal fertilised pod, 6 DAF; B, MVA treated pod, 6 DAF; C, MVA/GA3 treated pod, 6 DAF; D, 6-BAP treated pod, 6 DAF; E, 6-BAP/GA3 treated pod, 6 DAF; F, GA3 treated pod, 6 DAF; G, tween treated control pod, 6 DAF. a, endodermal layer; b, middle zone; c, pre-sclerenchyma layer; d, transition layer; Sc, sclerenchyma; En, endocarp; M, mesocarp. All figures at the same magnification, bar = 50 μm.

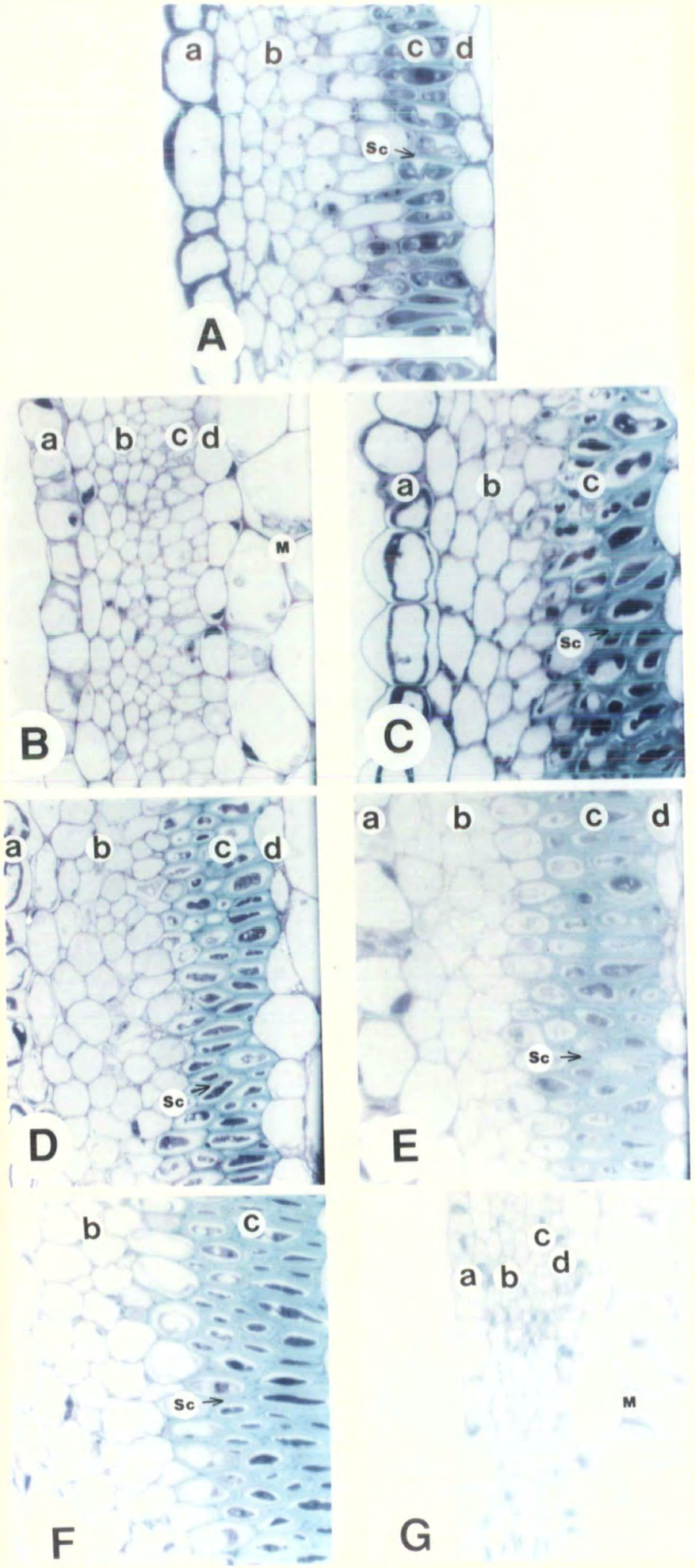
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Plate 3.6 Endocarp development in parthenocarpic pods of L58 treated with plant growth regulators. Light micrographs of 1 μm thick transverse pod sections stained with toluidine blue. A, normal fertilised pod, 6 DAF; B, MVA treated pod, 6 DAF; C, MVA/GA3 treated pod, 6 DAF; D, 6-BAP treated pod, 6 DAF; E, 6-BAP/GA3 treated pod, 6 DAF; F, GA3 treated pod, 6 DAF; G, tween treated control pod, 6 DAF. a, endodermal layer; b, middle zone; c, pre-sclerenchyma layer; d, transition layer; Sc, sclerenchyma; En, endocarp; M, mesocarp. All figures at the same magnification, bar = 50 μm.

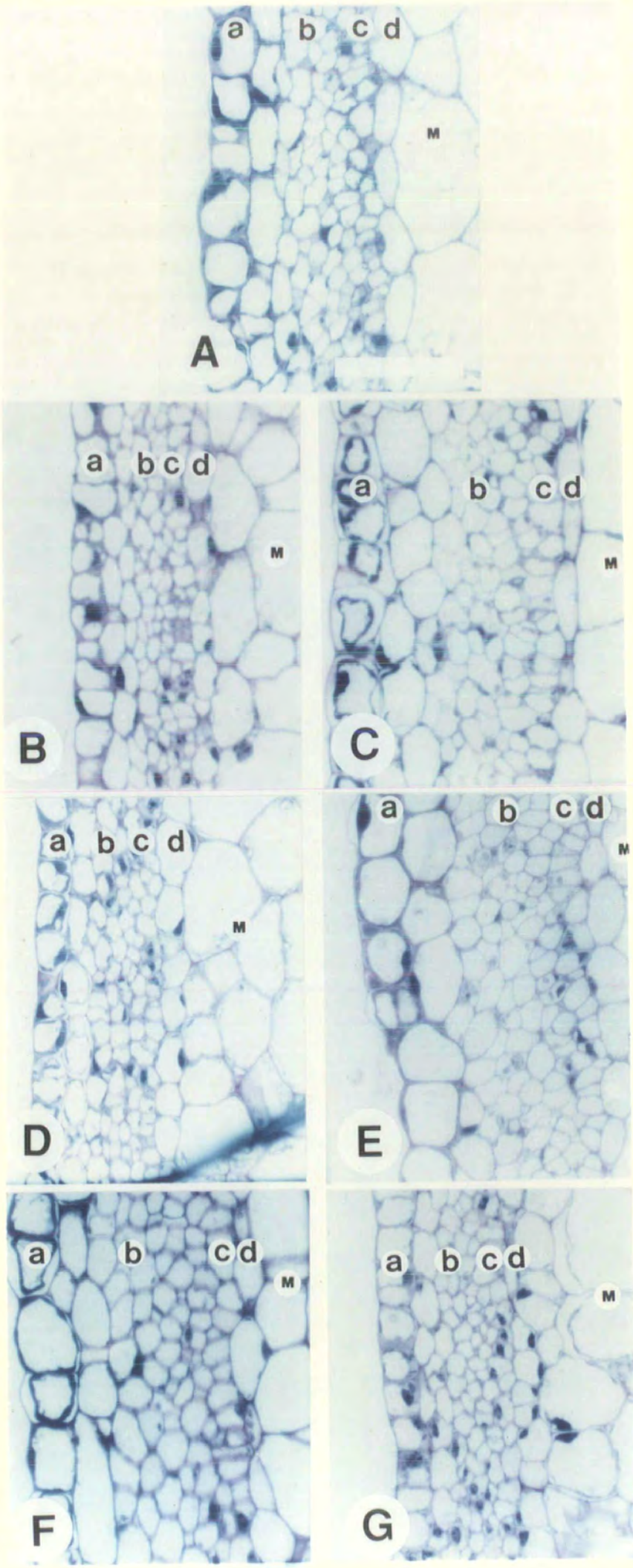
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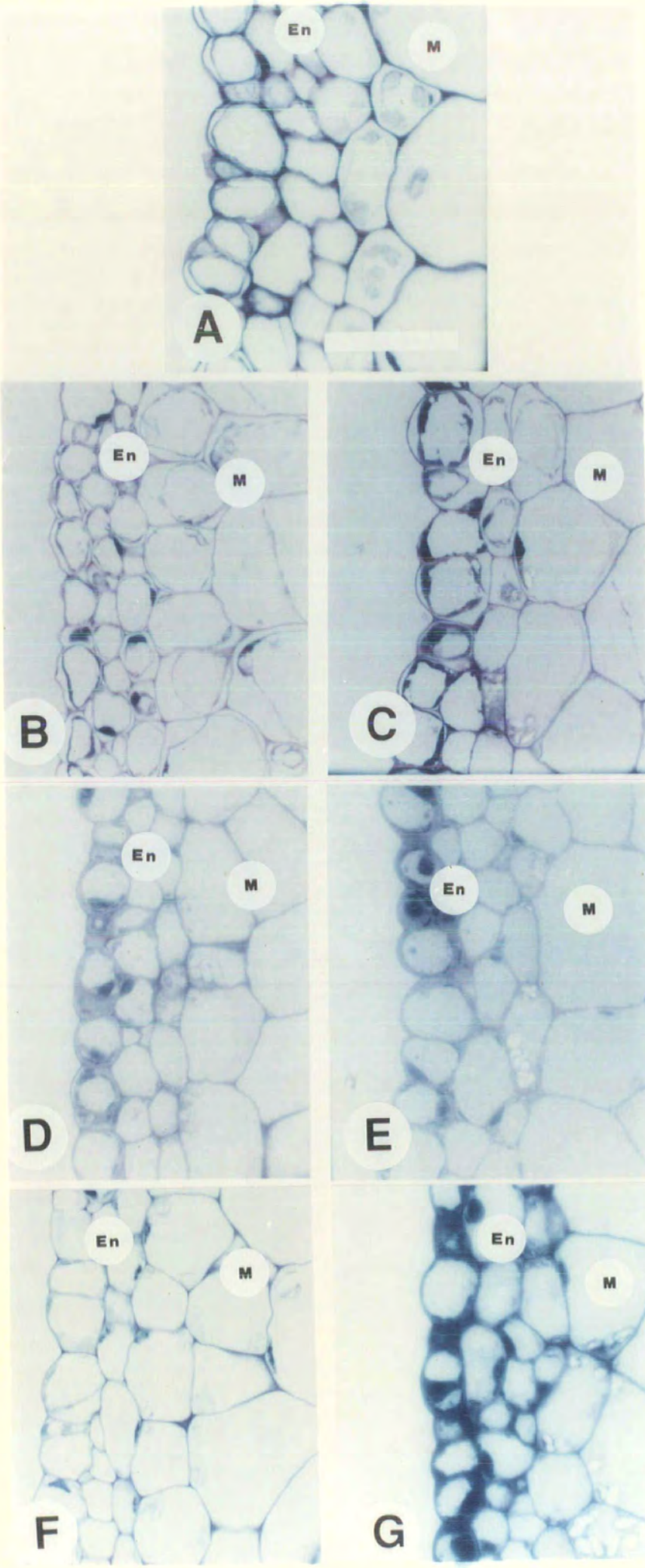
Plate 3.7 Endocarp development in parthenocarpic pods of L1390 treated with plant growth regulators. Light micrographs of 1 μm thick transverse pod sections stained with toluidine blue. A, normal fertilised pod, 6 DAF; B, MVA treated pod, 6 DAF; C, MVA/GA3 treated pod, 6 DAF; D, 6-BAP treated pod, 6 DAF; E, 6-BAP/GA3 treated pod, 6 DAF; F, GA3 treated pod, 6 DAF; G, tween treated control pod, 6 DAF. En, endocarp; M, mesocarp. All figures at the same magnification, bar = 50 μm.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flowers of L58 and L1390 produced pods larger than single applications of either, GA3 or 6-BAP. The mixed applications of 6-BAP and GA3 to emasculated flowers of L1390 produced pods nearest in size to L1390 normal fertilised pods. The endocarp of L58 pods treated with mixed applications of 6-BAP/GA3 (Plate 3.6E) is of greater width than the single applications of 6-BAP (Plate 3.6D) and the normal fertilised pods Plate 3.6A).

L59 pods treated with MVA showed a slight increase in growth compared to the tween treated controls, whereas L58 and L1390 pods treated with MVA appeared to be slightly smaller than the respective tween treated controls. The endocarp development of MVA treated L58 pods and the tween treated controls are very similar (Plates 3.6B, and 3.6G); whereas the endocarp of the MVA treated L59 pods has increased in size compared to the tween treated control (Plate 3.5B and 3.5G). Mixed applications of MVA and GA3 to emasculated flowers of L59 produced pods slightly smaller than single applications of GA3, but significantly larger than single applications of MVA. Correspondingly, the endocarp development was considerably greater than the MVA treated L59 pods (Plate 3.5B), but less than the GA3 treated pod (3.5F). Mixed applications of MVA and GA3 to emasculated flowers of L58 produced pods and a pod endocarp (Plate 3.6C), which were similar in size to pods in these lines treated with single applications of GA3 (Plate 3.6F), and significantly larger than single applications of MVA (Plate 3.6B).

Parthenocarpic development of tween treated controls was observed to a varying degree in L59, L58 and L1390. Parthenocarpic development in tween treated control pods was greatest in L1390, followed by L58, with L59 tween treated controls producing the smallest pods. This is reflected in the development of the endocarp in the tween treated control pods compared to the corresponding normal fertilised pods (Plate 3.5A and 3.5G). The length of normal fertilised pods in each experimental line exhibits a similar pattern in length variation, with L1390 producing the longest seedy pods, followed by L58, with L59 producing the smallest seedy pods. However, the ratio of the length of seedy pod to the length of tween treated control in each line, indicates that parthenocarpic development of tween treated controls occurs to a

greater degree in L1390 (ratio = 1.59), followed by L58 (ratio = 1.7), with parthenocarpic development least in L59 (ratio = 2.9) (as measured by pod length).

3.3 L59 LIGNIFIED POD cDNA LIBRARY

3.3.1 Construction of the L59 Lignified Pod cDNA Library

Histochemical staining for lignin and subsequent microscopical examination of pod endocarp development revealed that the onset of lignification occurred at around 6 DAF (see 3.2.1 and 3.2.2). The pod endocarp development studies further revealed that differentiation of cells ultimately forming lignified sclerenchyma in the endocarp occurred during the period of pod growth 4 - 6 DAF (see 3.2.2). Hence, it was decided to use pods from 4 - 6 DAF to construct the L59 lignified pod cDNA library for the investigation of the genetic basis of lignification in the pea pod endocarp.

Total RNA was extracted from L59 pea pods (phenotype, lignified endocarp; genotype PV) harvested 4 - 6 DAF. The embryos and main veins were excised prior to RNA extraction by the hot SDS method (as described 2.2.22.1). Isolation of poly $(A)^+$ RNA was performed using the PolyATtract mRNA Isolation System III (as described 2.2.22.3) .

A Stratagene ZAP-cDNA Synthesis Kit was used for construction of a lignified pod cDNA library using the L59 4 - 6 DAF pod mRNAs as a template for reverse transcription. First and second strand cDNAs were synthesised (as described 2.2.23.1). Electrophoresis analysis on an alkaline agarose gel (as described 2.2.23.5) indicated the presence of second strand L59 4 - 6 DAF pod total cDNAs of approximately 0.5 - 2.8 kb.

3.3.2 Trial Ligation of the L59 Lignified Pod cDNA into Uni-ZAP XR Vector Arms

The initial trial ligation of the prepared L59 total pod cDNAs with Uni-ZAP XR vector was performed as described, 2.2.23.6. The packaged reaction contained 2.1 x 10^4 recombinant phage. The

remainder of the test ligation was packaged (as described 2.2.23.7) and the titer was ascertained to be 4.0 x 10^5 recombinant phage. The L59 lignified pod cDNA library was then amplified in *E. coli* strain XL1-Blue (as described 2.2.23.9).

3.3.3 *In Vivo* Excision of Trial Inserts from the L59 Lignified Pod cDNA Library

Analysis of the XhoI linearised trial rescued plasmids by minigel electrophoresis (as described 2.2.7.1) revealed the presence of pBluescript carrying inserts from approximately 0.5 - 2 kb. Due to problems with helper phage co-infection, the alternative ExAssist/SOLR *in vivo* excision protocol suggested by Stratagene, was followed. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a non-suppressing *E. coli* host strain, SOLR.

3.3.4 Trial Screen of the L59 Lignified Pod cDNA Library

The PP406 mRNA was previously shown to be expressed in developing pods from different pea lines at 4 - 6 DAF (see Figures 3.5 and 3.6). The PP406 cDNA was therefore used in a trial screen as representing a gene expressed in developing pea pods at 4 - 6 DAF. It was also hoped to establish the presence of full-length cDNAs. The trial screen of 5 x 10^5 plaques of the amplified L59 lignified pod cDNA library using α^{32}P labelled PP406 insert as a probe (see 2.2.23.12), yielded fourteen putative positives from the primary screen. The putative positives were isolated and subjected to secondary and tertiary screening. Three clones, designated LP4, LP12 and LP13 were isolated from tertiary screening on the basis of strong hybridisation to the PP406 probe on duplicate filters (Figure 3.10). The cDNA inserts from clones LP4, LP12 and LP13 were plasmid rescued and the DNA inserts purified to produce DNA for sequencing. Partial sequencing with forward and reverse primers revealed three full-length clones which were identical to pPP406 over the regions sequenced, apart from extra sequence at the 5' end (nucleotides 0 to -23) and the 3' end (nucleotides 916 to 1024) (Figure 3.11).

Figure 3.10 Tertiary screen of putative positive clones selected from the 14 putative positive clones isolated from the primary trial screen. A, LP4; B, LP12; and C, LP13. Duplicate plaque lifts of the putative positive clones were hybridised with ^{32}P-labelled PP406. The filters were exposed to sensitised film at -80 °C for 48 hours.

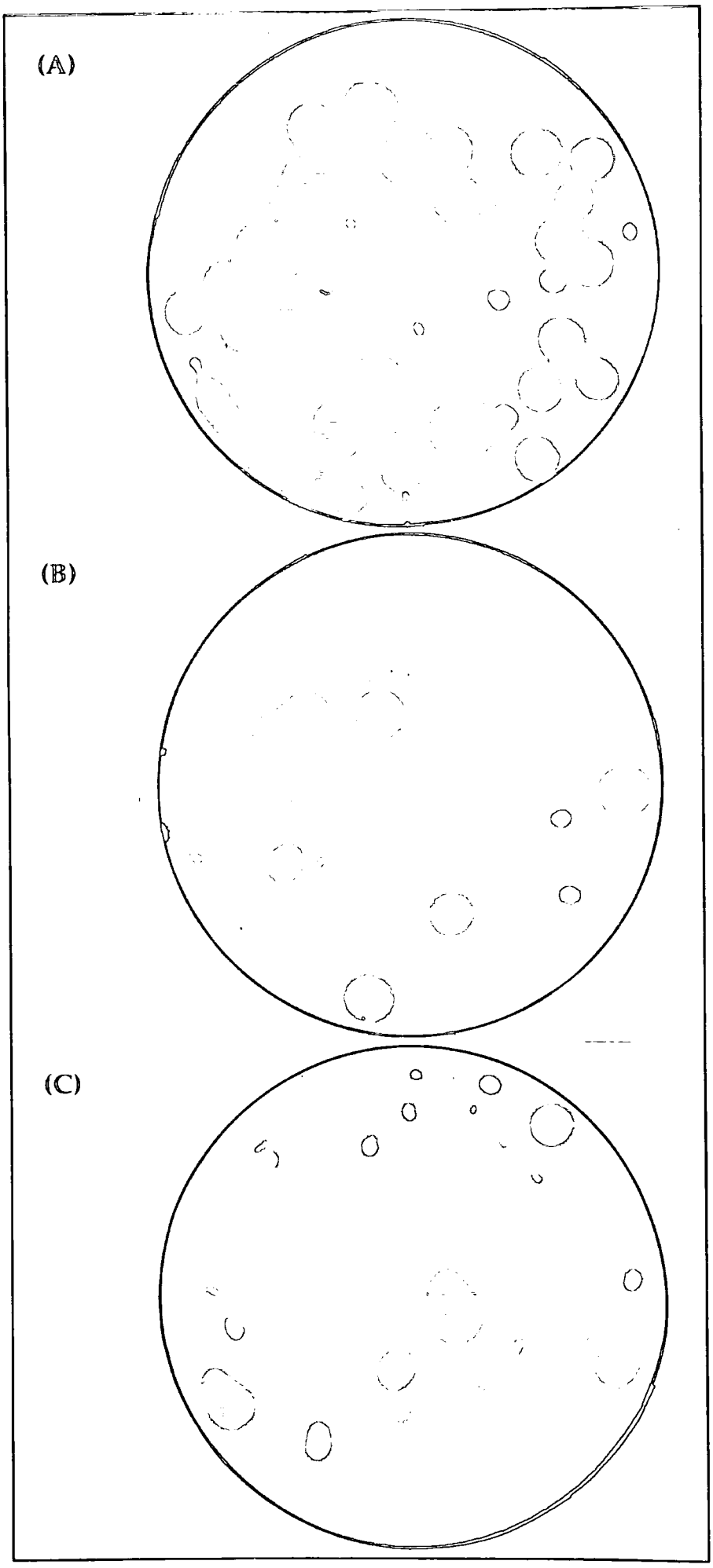
(A)
(B)
(C)

Figure 3.11 Alignment of the PP406 nucleotide sequence with nucleotide sequences obtained from partial sequencing of inserts LP4, LP12 and LP13 from the homologous clones isolated from the trial screen of the L59 lignified pod cDNA library. Colons (:) represent identical nucleotides. The insert, LP4, was sequenced from clone pLP4 with forward and reverse primers and inserts, LP12 and LP13, were sequenced from clones pLP12 and pLP13 respectively, with forward primer. The numbering corresponds to the PP406 cDNA.

```
                                                -20        -10
LP4                                           TGA AGACGGTGTG AGAGAGAGTC
                    10         20         30         40         50
PP406       CTAGTTGAAG TAAAAAAAGA TCATCAAACA CAAGCAAACA ACAGTTTCTT
LP4         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
                    60         70         80         90        100
PP406       CTTCTTCCAC CGATCCGTAC TATGCCTTCT CGCAGAAGAA CTCTCTTAAA
LP4         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
                   110        120        130        140        150
PP406       GGTCATCATT CTCGGTGACA GCGGTGTGGG GAAGACGTCT TTGATGAACC
LP4         :::::::::: :::::::::: :::::::::: ::::::::::
                   160        170        180        190        200
PP406       AATATGTGAA TAAGAAGTTT AGTAATCAGT ACAAGGCAAC CATTGGAGCG
                   210        220        230        240        250
PP406       GATTTCTTAA CCAAAGAAGT GCAATTTGAA GATAGGCTTT TCACCTTACA
                   260        270        280        290        300
PP406       GATTTGGGAT ACAGCTGGCC AGGAGAGATT CCAAAGCCTA GGAGTTGCTT
                   310        320        330        340        350
PP406       TCTATCGTGG TGCTGATTGC TGTGTTCTTG TATATGATGT TAATTCAGTG
                   360        370        380        390        400
PP406       AAGTCATTTG ACAACCTTAA TAACTGGAGG GAAGAGTTTC TCATTCAAGC
                   410        420        430        440        450
PP406       AAATCCTTCT GATCCAGAGA ATTTTCCCTT TGTCGTTATA GGAAACAAGA
                   460        47         480        490        500
PP406       TAGATATTGA TGGTGGAAAC AGTAGAGTGG TTTCTGAAAA GAAGGCTCGG
                   510        520        530        540        550
PP406       GCATGGTGTG CAGCAAAAGG AAATATCCCA TATTTTGAGA CATCTGCTAA
                   560        570        580        590        600
PP406       AGAAGGTATT AATGTTGAAG AAGCATTCCA AACCATAGCA AAGGATGCCC
                   610        620        630        640        650
PP406       TGAAAAGTGG GGAAGAGGAA GAATTATACC TGCCGGACAC AATTGATGTT
LP12                                                             ::
LP13                                                             ::
                   660        670        680        690        700
PP406       GGAAACAGCA GTCAGCCAAG GTCAACAGGA TGTGAGTGCT GAACATATAG
LP4         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP12        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP13        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
                   710        720        730        740        750
PP406       ATTTTGTTCT CAATACAAAA TAAAGTATAT TATTTAAAAA TCATTTTGGC
LP4         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP12        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP13        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
                   760        770        780        790        800
PP406       ATGTCTAGCC ATTGCTGTCT ATGAGGTTTT ATTGTACATT TATGTTTGAT
LP4         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP12        .::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP13        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
                   810        820        830        840        850
PP406       CAAGTGCGAT CTGTTGGGTG CTTGTTTGGC TTGTGTTAAT CGATCATGTT
LP4         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP12        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP13        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
                   860        870        880        890        900
PP406       GTTCTCCTTG TATGCTATTC CAACATTGTG AAAAAAACAG CAAAGGATCA
LP4         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP12        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP13        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
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                  910        920        930        940        950
PP406       TTCTGAAGTT ATTTC
LP4         :::::::::: :::::ATTAG TTATTTTTCT TTCCCGTACA AAATATTGTA
LP13        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP12        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

                  960        970        980        990        1000
LP4         ATCAATGGTG ATTGGTGGTT GTGGTGATGC TTTGAGTTAT TAAATTTGTT
LP13        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
LP12        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
                  1010       1020
LP4         TGAACAAAAA AAAAAAAAAA AAAA
LP12        :::::::::: :::::::::: ::::
LP13        :::::::::: :::::::::: ::::
```

3.4 DIFFERENTIAL SCREEN OF THE L59 LIGNIFIED POD cDNA LIBRARY

A differential screen of the amplified L59 lignified pod cDNA library was performed (as described 2.2.24) using [α^{35}S] dCTP and [α^{32}P] dCTP labelled total cDNAs synthesised from 4 - 6 DAF pod mRNAs from L59 (phenotype, lignified endocarp; genotype, PV) and L1390 (phenotype, unlignified endocarp, genotype, pv) respectively, in an attempt to isolate cDNAs involved in differentiation and lignification of the pea pod endocarp (see Figure 2.2). The secondary and tertiary screens were performed using [α^{32}P] dCTP labelled cDNA probes from both lines. It was decided to use α^{32}P labelled probes in the secondary and tertiary screen, as the positives were more readily identified when screening the relatively fewer numbers of clones in the secondary and tertiary screens. In addition, exposure time for radiographs was minimised with α^{32}P dCTP labelled probes.

3.4.1 Preparation of the cDNA Probes for the Differential Screen

L59 pod total cDNA prepared for construction of the L59 lignified pod cDNA library was used for preparing radiolabelled probe for the differential screen. L1390 pod total cDNA was prepared as for L59 total pod cDNA (see 2.2.23.1). Aliquots containing 100 ng of L59 and L1390 pod total cDNA were used to prepare [α^{35}S] and [α^{32}P] dCTP labelled probes respectively (as described 2.2.16).

3.4.2 Primary Differential Screen of the L59 Lignified Pod cDNA Library

A primary differential screen of 8×10^4 plaques (as described 2.2.24.1) yielded 16 positives on the basis of observed hybridisation to the ^{35}S labelled L59 pod total cDNAs, but not to the ^{32}P labelled L1390 pod total cDNAs.

3.4.3 Secondary and Tertiary Differential Screen of the L59 Lignified cDNA Library

The putative positive plaques cored from the primary screen stock plates were subjected to secondary and tertiary rounds of screening by

hybridising duplicate lifts of 50 - 100 plaques on NC filters (as described 2.2.24.2) with either [α^{32}P] dCTP labelled L59 pod total cDNA (100 ng) or [α^{32}P] dCTP labelled L1390 pod total cDNA (100 ng).

Putative differentially expressed clones, designated pLP18, pLP19 and pLP28, were selected on the basis of consistent hybridisation to a total cDNA probe from the lignifying pods of L59, but not to a total cDNA probe from pods of L1390, which do not lignify, through three rounds of differential screening (Figures 3.12, 3.13 and 3.14). The other clones selected from the primary screen failed to give consistent preferential hybridisation to the total cDNA probe from the lignified pods of L59 compared to the total cDNA probe from pods of L1390.

3.4.4 Clone pLP28 Isolated from the Differential Screen of the L59 Lignified Pod cDNA Library

A clone, designated pLP28, was isolated from the differential screen of the L59 cDNA library representing poly $(A)^+$ RNA from developing pea pods (4 - 6 DAF) which have a lignified endocarp (see 3.4.2 and 3.4.3). Selection was on the basis of hybridisation to a total cDNA probe from the lignifying pods of L59, but not to a total cDNA probe from pods of L1390, which do not lignify. Subsequent selection of the putatively differentially expressed clone, pLP28, was made following a secondary and tertiary screen (see Figure 3.14).

The cDNA insert from clone pLP28 was plasmid rescued. Cleavage with EcoRI/XhoI yielded a restriction enzyme fragment of approximately 500 bp. Partial sequencing of the clone pLP28 using reverse primer yielded a cDNA fragment of 225 bp encoding a polypeptide fragment of 75 amino acid residues (Figure 3.15).

A homology search of the polypeptide fragment predicted by clone pLP28, using the FASTA programme package (Pearson and Lipman, 1988) revealed no significant homology with database sequences. It was considered that further characterisation of clone pLP28 would be inappropriate considering the small size of the LP28 cDNA and the failure to identify homology with database sequences. Hence, further experimental work on clones isolated from the differential screen of

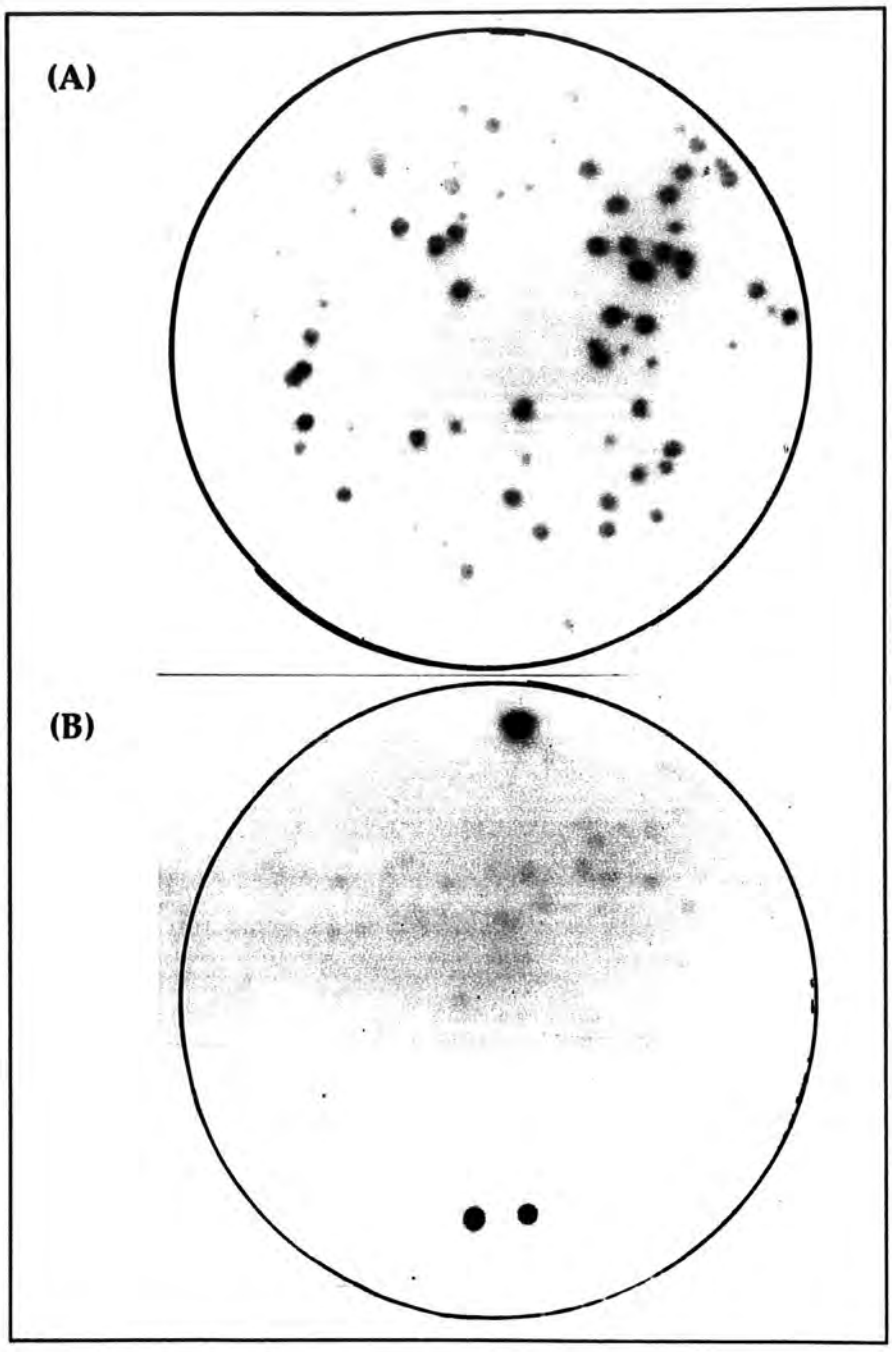

(A)
(B)

Figure 3.13 Tertiary screen of clone pLP19. Duplicate plaque lifts of pLP19 hybridised with (A) ^{32}P-labelled L59 pod total cDNAs and with (B) ^{32}P-labelled L1390 pod total cDNAs. The filters were exposed to sensitised film at -80 °C for 7 days.

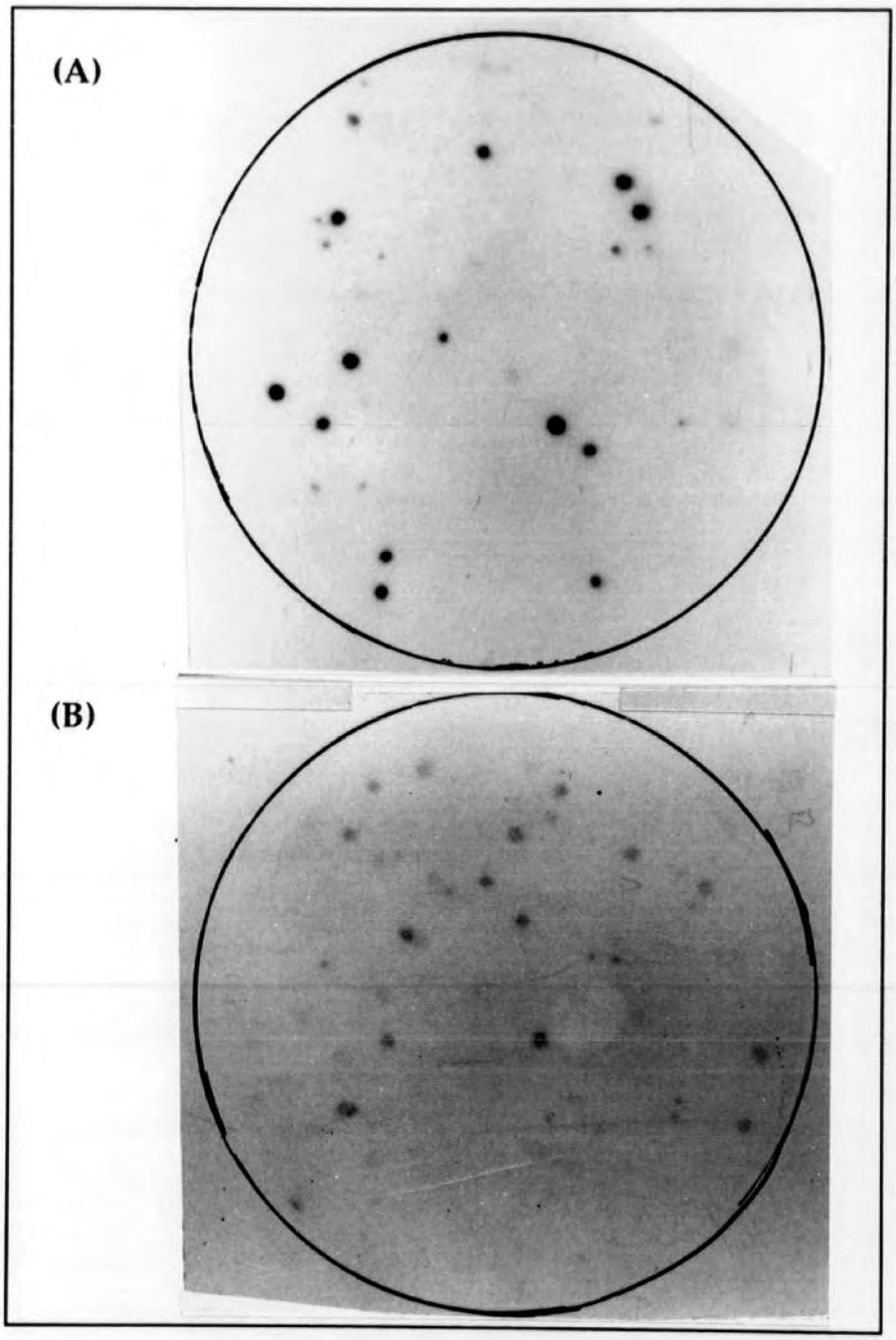
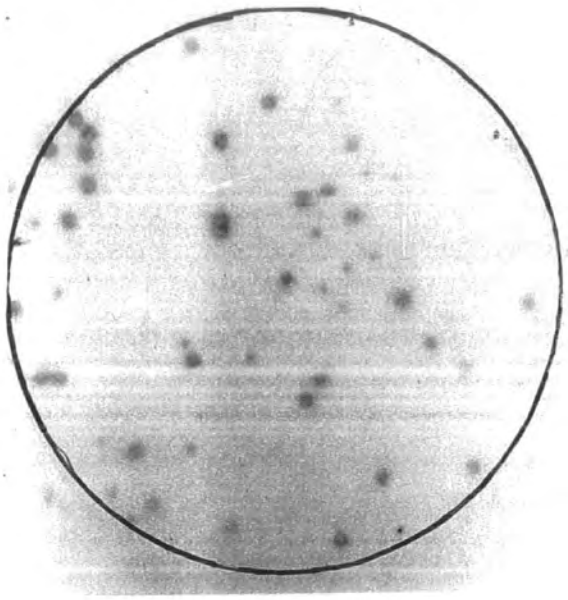

(A)
(B)

Figure 3.14 Tertiary screen of clone pLP28. Duplicate plaque lifts of pLP28 hybridised with (A) ^{32}P-labelled L59 pod total cDNAs and with (B) ^{32}P-labelled L1390 pod total cDNAs. The filters were exposed to sensitised film at -80 °C for 7 days.

(A)



(B)

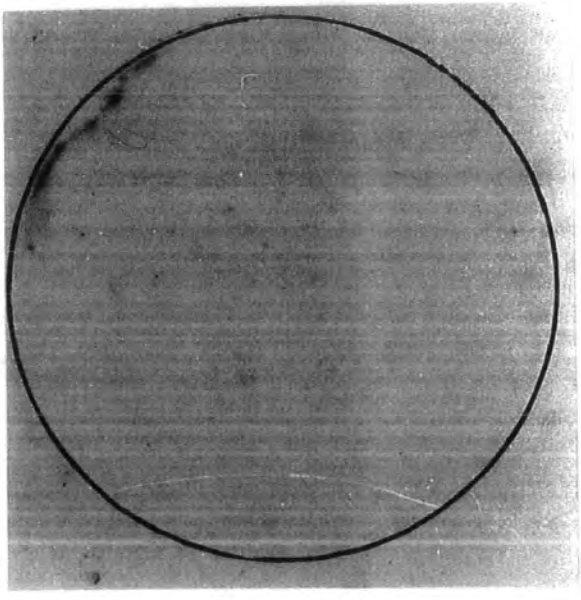


Figure 3.15 Partial nucleotide sequence of LP28 and predicted polypeptide.

```
1/1                                                 31/11
GCA CGA GCG GCA CGA GCT GCG GGC TCC CTC GTA TTA AAA GAT GCC CCT CCC CGC
A   R   A   A   R   A   A   G   S   L   V   L   K   D   A   P   P   R

        61/21                                               91/31
AGC ATT GTG GCA GGA ATA CCG GCA AAA GTT ATT GGT GGT CTA AGG GAG CAT GAC
S   I   V   A   G   I   P   A   K   V   I   G   G   L   R   E   H   D

                121/41                                              151/51
CCG GCT TTA ACC ATG AAA CAT GAT GCT ACA AGA CCA TTT TTC ACT GAT GTA GCT
P   A   L   T   M   K   H   D   A   T   R   P   F   F   T   D   V   A

                        181/61                                          211/71
GTT AAC ATT ATA GAT GAA AAA TTC AGT GGA GGA AAG AAT CAA GAC AAA AAG GAA
V   N   I   I   D   E   K   F   S   G   G   K   N   Q   D   K   K   E

GCA AAC ACT
A   N   T
```

the L59 lignified pod cDNA library was concentrated on clones pLP18 (see 3.5) and pLP19 (see 3.6).

3.5 CLONE, pLP18, ISOLATED FROM THE DIFFERENTIAL SCREEN OF THE L59 LIGNIFIED POD cDNA LIBRARY

The clone designated pLP18 was isolated from the differential screen of the L59 cDNA library representing poly $(A)^+$ RNA from developing pea pods (4 - 6 DAF) which have a lignified endocarp (see 3.4). Selection was on the basis of hybridisation to a total cDNA probe from the lignifying pods of L59, but not to a total cDNA probe from pods of L1390, which do not lignify. Subsequent selection of the putatively differentially expressed clone, pLP18, was made following a secondary and tertiary screen (see Figure 3.12).

3.5.1 Sequencing of LP18

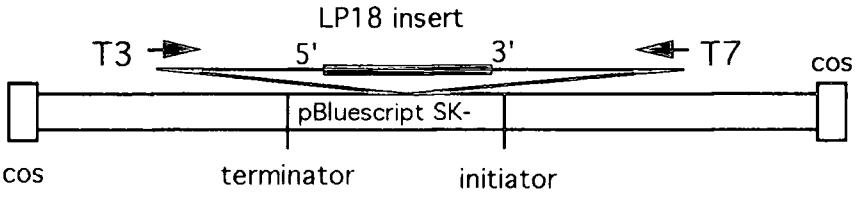
The insert in pLP18 was characterised by DNA sequencing using M13 primers and synthetic oligo primers (Figure 3.16). The cDNA was 790 bp in length, plus a poly (A) tail of 18 bp and contained an open reading frame of 570 bp, encoding a putative polypeptide of 189 residues from an initiation codon at nucleotides 21 - 23 to a termination codon at nucleotides 588 - 590 (Figure 3.17). The 3' non-coding region of 218 bp contained a single motif, AATATA, corresponding to the consensus eukaryotic polyadenylation signal, AATAAA, at nucleotides 770 - 775 (Proudfoot and Brownlee, 1977). The nucleotide sequence is available from the EMBL database, accession number Z25471.

3.5.2 Amino Acid Sequence Comparison of the Polypeptide Encoded by LP18

A homology search of the whole polypeptide sequence predicted by clone pLP18, using the FASTA programme package (Pearson and Lipman, 1988) revealed significant homology with blue type I copper proteins. The highest similarities were with the polypeptide products of stellacyanin (Sc) (47.7 %) from the latex of lacquer tree (Engeseth, *et al.*, 1984), cucumber basic blue protein (CBP) (36.1 %) (Murata *et al.*, 1982), cucumber peeling cupredoxin (CPC) (36.8 %) (Mann *et al.*, 1992)

Figure 3.16 (A) The cloned cDNA insert, LP18, in pBluescript SK- within the Lambda ZAP II vector. (B) The sequencing strategy for the cDNA insert, LP18, excised in the pBluescript plasmid by co-infection with helper phage. P1 and P2 are synthetic oligo nucleotide primer sites. The direction of sequencing is indicated by the arrows.

(A)



(not to scale)

(B)

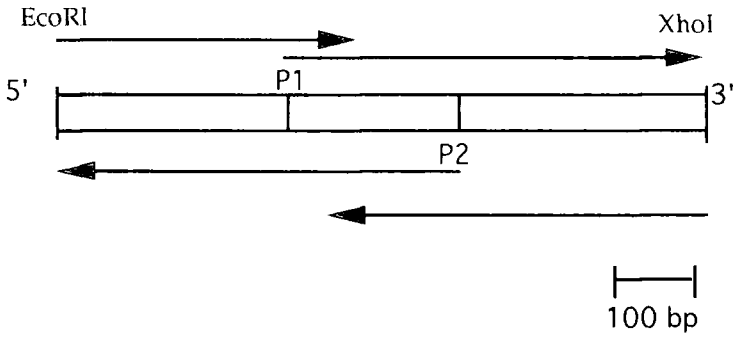


Figure 3.17 Nucleotide sequence of LP18 cDNA and predicted polypeptide, PBP. The polyadenylation signal is underlined.

```
1                                                   30/4
 TC TCC TCA GTA TAT CAT CCA ATG GCA TTC TCT AAT GCT TTG GTT TTG TGC TTC CTT TTA
                            M   A   F   S   N   A   L   V   L   C   F   L   L
60/14                                               90/24
GCA ATC ATC AAC ATG GCA CTT CCA TCC CTT GCA ACT GTC TAC ACT GTT GGA GAT ACT TCA
A   I   I   N   M   A   L   P   S   L   A   T   V   Y   T   V   G   D   T   S
120/34                                              150/44
GGT TGG GTG ATT GGT GGT GAT TAT AGC ACA TGG GCT AGT GAC AAA ACC TTT GCA GTT GGT
G   W   V   I   G   G   D   Y   S   T   W   A   S   D   K   T   F   A   V   G
180/54                                              210/64
GAT AGC CTC GTG TTC AAC TAT GGA GCT GGT GCG CAC ACT GTG GAT GAA GTT AAA GAA AGT
D   S   L   V   F   N   Y   G   A   G   A   H   T   V   D   E   V   K   E   S
240/74                                              270/84
GAC TAC AAA TCA TGC ACA TCT GGA AAT TCA ATT AGT ACA GAC AGT ACT GGT GCG ACA ACC
D   Y   K   S   C   T   S   G   N   S   I   S   T   D   S   T   G   A   T   T
300/94                                              330/104
ATT CCT CTT AAG AAA GCA GGC AAA CAT TAC TTC ATA TGT GGT GTT CCG GGA CAT TGT ACC
I   P   L   K   K   A   G   K   H   Y   F   I   C   G   V   P   G   H   C   T
360/114                                             390/124
GGT GGC ATG AAA CTT TCT ATT AAG GTT AAG GCC TCT TCT GGT TCT TCT GCT GCT CCT TCT
G   G   M   K   L   S   I   K   V   K   A   S   S   G   S   S   A   A   P   S
420/134                                             450/144
GCA ACA CCA TCT TCA TCG GGA AAA GGT TCA CCT TCT TCT GAT GAT ACC CCC GCC GCA ACT
A   T   P   S   S   S   G   K   G   S   P   S   S   D   D   T   P   A   A   T
480/154                                             510/164
ACC ACC ACT ACT ACT CCA ACT AAG CAA AAT GAA TCT TCA GCT ACT AGT CTC TCA CCA ATT
T   T   T   T   T   P   T   K   Q   N   E   S   S   A   T   S   L   S   P   I
540/174                                             570/184
GTT GCT TTG TTT TTT ACT GTT TCA TGG ATC TGT AGC TAT GTT TTG GTA TGA TGA GTT TCC
V   A   L   L   F   T   V   S   W   I   C   S   Y   V   L   V   *
600/201                                             630/211
CTG AGG TAT AGT CAG GAC AGA GGC AGT GCT TCC ATG GTT TTC TGT TCA TTT TTC ATA TTA
660/221                                             690/231
TGA TTA TTA TTA TTA TTA TCA TTG ATT TCA TTT CTT GTT TCA TCT ATT CAC ATT TAT GGA
720/241                                             750/251
GAT TCT AAT CTA GGA AAT CTT GTA TTG TTA TTA TGT TTC TGT CTT TTT TTA ATA TAT TGA
780/261
ATG ATT ATA AGA AAA AAA AAA AAA AAA AA
```

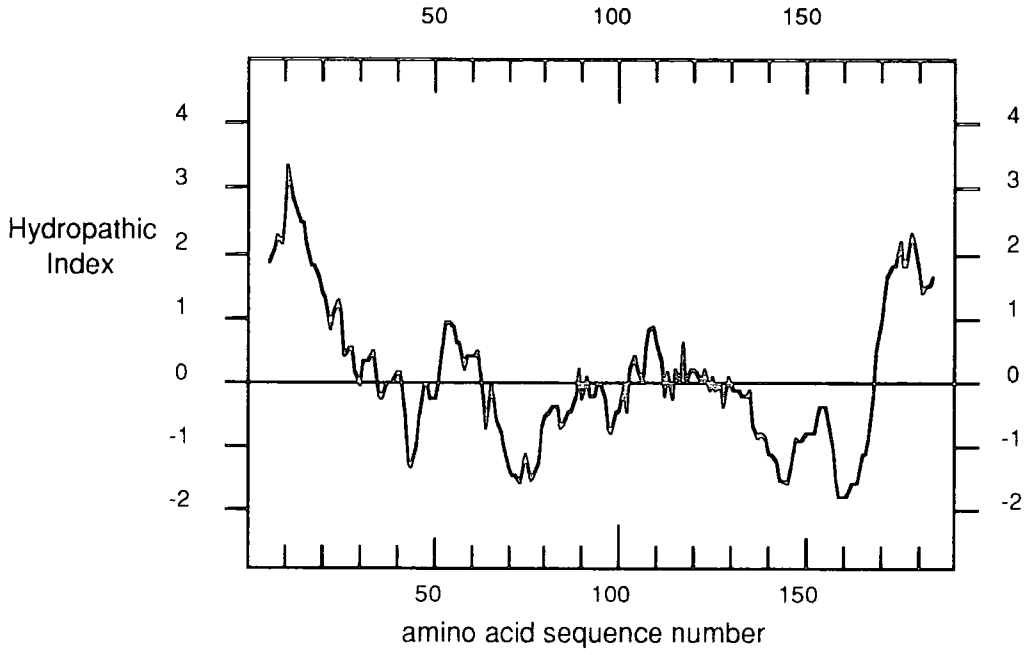
and a blue copper binding protein (BCB) (31.1 %) from *Arabidopsis thaliana* (Van Gysel *et al.*, 1993) (Figure 3.18). These polypeptides are known as blue type I copper proteins, which are thought to be involved in electron transfer reactions. Clone pLP18 was therefore designated PBP (putative blue copper protein). The molecular mass of the translated polypeptide would be 19.4 kDa. The polypeptide predicted by clone pLP18 has four distinguishable domains: a hydrophobic N-terminal region of 24 residues (1 - 24); a central domain (25 - 126); a domain rich in serine and threonine (127 - 161); and a hydrophobic C-terminal domain (162 - 189). A hydropathy plot shows a hydrophobicity profile of the PBP amino acid sequence (Figure 3.19) (Kyte and Doolittle, 1982). The hydrophobic N-terminal and the hydrophobic C-terminal are easily identified. Comparison of the predicted polypeptide of LP18 with the CBP and Sc amino acid sequences suggest that the N-terminal region may be a signal peptide with a cleavage site between residues A24 and T25. Comparison with von Heijnes's data (1983) also support a signal cleavage site at A24/T25. The central Cu^{2+} binding domain is C-terminally flanked by a region rich in threonine and serine (residues 127- 161). The corresponding serine, threonine and hydroxyproline rich region in BCB is thought to play a structural role in anchoring and targeting of the blue copper protein to membranes (Van Gysel *et al.*, 1993). The C-terminal hydrophobic region of PBP and the threonine and serine rich region shows no significant homology when compared with database sequences.

The central region of PBP is most similar to the copper binding regions of the other blue type I copper proteins stellacyanin, CBP, CPC and BCB (Figure 3.18). The central region contains the conserved copper binding ligands and two conserved cysteine residues (Figure 3.18). The three dimensional structure of CBP and Sc as determined by X-ray diffraction has shown the single copper atom to be bound by His39, Cys79, His84 and Met89 (Guss *et al.*, 1988) and His46, Cys87, His90 and Glu97, respectively (Vangaard, 1972). These Cu binding residues are conserved in a number of type I blue copper proteins such as azurin, plastocyanin and rusticyanin. Comparison with the Cu binding sites of CBP and Sc suggest the following Cu binding sites in LP18: His67, Cys111, His116 and Met121. Besides the conserved Cu binding ligands

Figure 3.18 Comparison of PBP amino acid sequence with blue type I copper proteins BCB (blue copper binding protein), Sc (stellacyanin), CPC (cucumber peeling cupredoxin) and CBP (cucumber basic blue protein). The colons (:) represent identical residues and the periods (.) represent residues defined as "similar", using the mutation matrix in the FASTA protein sequence comparison programme (Pearson and Lipman, 1988). Gaps have been introduced for maximal alignment. The conserved copper binding residues are in bold print. The putative cysteine residues forming a disulphide bridge in Sc and CBP and the corresponding cysteine residues in PBP are underlined.


10 20 30 40 50 60
PBP MAFSNALVLCFLLAIINMALPSLATVYTVGDTSGWV--I----GG-D----YS-TWASDKTFAVGDSLVFNYGAGAHT
BCB MA--GVFKTVTFLVLVFAAVVVFAEDYDVGDDTEWT--R----PM-DP-EFYT-TWATGKTFRVGDELEFDFAAGRHD
CPC ESTVHIVGDNTGWS--V----PS-SPNF-YS-QWAAGKTFRVGDSLQFNFPANAHN
Sc TVYTVGDSAGWK--VPFFGDV-D----YDWKWASNKTFHIGDVLVFKYDRRFHN
CBP AVYVVGGSGGWT--F----NT-E-------SWPKGKRFRAGDILLFNYNPXMHN
70 80 90 100 110 120 130 140
PBP VDEVKESD-YKSCTSGNSIS-TDSTGATTIPLKKAGKHYFICGVPGHCTGGMKLSIKVKASSGSSAAPSATPSSSG
BCB VAVVSEAA-FENCEKEKPIS-HMTVPPVKIMLNTTGPQYFICTVGDHCRFGQKLSITVAAGATGGATPGAGATPAP
CPC VHEMETKQSFDACNFVNSDNDVERTSPVIERLDELGMHYFVCTVGTHCSNGQKLSINVVAANATVSMPPPSSSPPS
Sc VDKVTQKN-YQSCNDTTPIA-SYNTGBBRINLKTVGQKYYICGVPKHCDLGQKVHINVTVRS
CBP VVVVNQGG-FSTCNTPAGAK-VYTSGRDQIKLPK-GQSYFICNFPGHCQSGMKIAVNAL
150 160 170 180
PBP KGSPSSDDTPAATTTTTTPTKQNESSATSLSPIVALFFTVSWICSYV-----LV
BCB GSTPSTGGTTPPTAGGTTTPSGSSGTTTPAGNAASSLGGATFLVAFVSAVVALF
CPC SVMPPPVMPPPSPS

Figure 3.19 A Kyte and Doolittle (1982) hydropathy plot of the PBP amino acid sequence determined using a DNA Strider programme package (Marck, 1988). The profile indicates the putative hydrophobic N-terminal signal peptide and the hydrophobic C-terminal.


50
100
150
4
3
2
1
0
-1
-2
Hydropathic Index
50
100
150
amino acid sequence number

(Engeseth *et al.*, 1984) and CBP (Guss *et al.*, 1988) to form a disulphide bridge.

3.5.3 Genomic Analysis of LP18

The PBP cDNA was hybridised to pea genomic DNA from L59 in a Southern blotting experiment (Figure 3.20). Washing to high stringency (0.1 x SSC, 65 °C) showed the PBP probe hybridised to a single band in each of three genomic digests with different restriction enzymes, suggesting that a single gene probably encodes this polypeptide.

3.5.4 Northern Analysis of LP18

Northern hybridisation of PBP cDNA to 10 µg of total pod RNA from different pea lines showed hybridisation to a mRNA species of approximately 0.95 kb in lines L59 and Feltham First, and possibly L58 (Figure 3.21). No hybridisation was observed in L1390. Background hybridisation to ribosomal RNA bands was also observed, as indicated by RNA size markers, even when the blot was washed to high stringency.

3.5.5 *In situ* Hybridisation of LP18 mRNA in Pod

A series of trial *in situ* hybridisation experiments were performed with the intention of attempting to localise LP18 gene expression in L59 pods. The first *in situ* hybridisation experiments were performed on L59 5 DAF transverse pod sections, without proteinase K treatment (as described 2.2.25). Pod sections were hybridised with digoxigenin labelled LP18 cDNA. A digoxigenin labelled PP406 cDNA was used as a positive control (PP406 mRNA expression was known to occur in developing pea pods (see 3.1.4 and 3.1.5) and a digoxigenin labelled leg4.4 cDNA (a seed specific gene) (Thompson, 1989) was used as a negative control. No colour development was observed after incubation with substrate solution for one hour.

It was considered that the lack of positive hybridisation signals may be due in part to low, or a lack of expression in the 5 DAF L59 pods chosen

Figure 3.20 Southern blot hybridisation of total pea leaf DNA from L59 with LP18 cDNA probe. DNA (10 μg) was digested with BamHI (lane A), EcoRI (lane B) or HindIII (lane C), electrophoresed on 0.6 % agarose gel, denatured and transferred to nitrocellulose filters. Filters were hybridised with a radio-labelled LP18 cDNA. The filter was washed to a final stringency of 0.1 x SSC at 65 °C and exposed to sensitised film for 4 weeks at -80 °C.

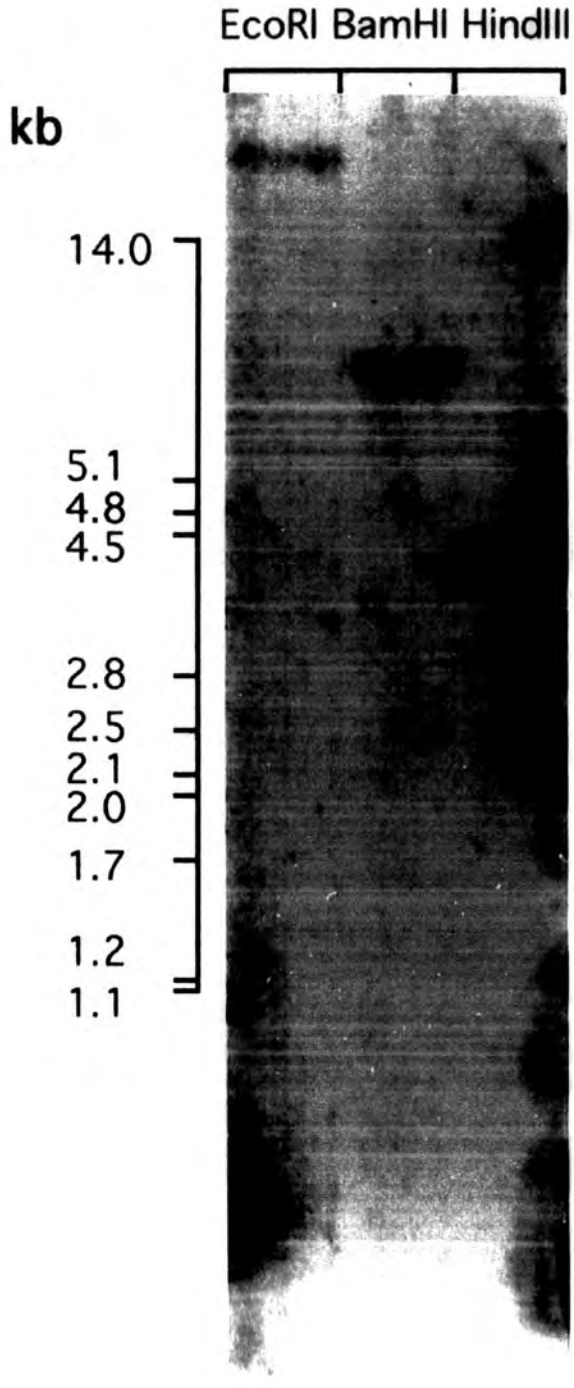
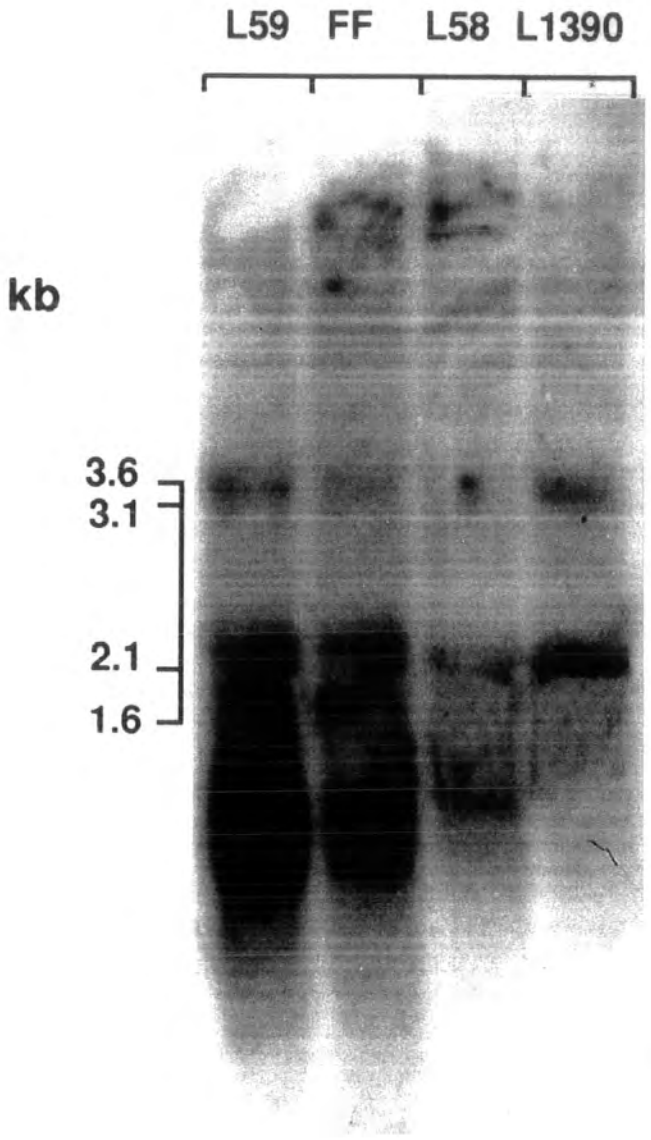

EcoRI BamHI HindIII
kb
14.0
5.1
4.8
4.5
2.8
2.5
2.1
2.0
1.7
1.2
1.1

Figure 3.21 Northern blot hybridisation of 10 µg of total pod RNA, 4 - 6 DAF, prepared from four pea lines - L59, Feltham First (FF), L58 and L1390 - probed with radio-labelled LP18 cDNA, isolated from a differential screen of a cDNA library constructed from 4 - 6 DAF L59 pods. The filter was exposed to sensitised film for 3 weeks at -80 °C.


L59
FF
L58
L1390
kb
3.6
3.1
2.1
1.6

for *in situ* hybridisation. Previous northern expression analysis of L59 pod total RNA revealed expression of the putative positive probes, PP406 and LP18 in 4 - 6 DAF pods (see 3.1.5). Hence, it was not known at which day after flowering the PP406 was most highly expressed in L59. Northern analysis performed on 5 DAF PP pod total RNA revealed fairly strong expression of PP406. Hence, *in situ* hybridisation was repeated using PP 5 DAF transverse pod sections and digoxigenin labelled PP406 probe. Digoxigenin labelled PP406 was hybridised to proteinase K treated pod sections, an RNase treated control, and an untreated pod section (as described, 2.2.25). The proteinase K treatment was performed in an attempt to increase the level of exposed RNA strands available for hybridisation in the pod sections. An RNase treated control was used to simplify the *in situ* hybridisation procedures. Microscopic examination after treatment with substrate solution revealed an absence of colour development in any of the pod sections.

It was decided that the technique would require further trials to produce conclusive and reproducible results. The substantial time necessary to complete such experiments was considered excessive with regard to the time limits for completion of this project and the necessity to complete other experimental work in progress.

3.5.6 Semi-Quantitative RT-PCR for LP18 mRNA Expression

The failure to detect mRNA expression in pod tissues by *in situ* hybridisation strategies led to the adoption of RT-PCR strategies in an attempt to correlate LP18 gene expression with the lignified phenotype.

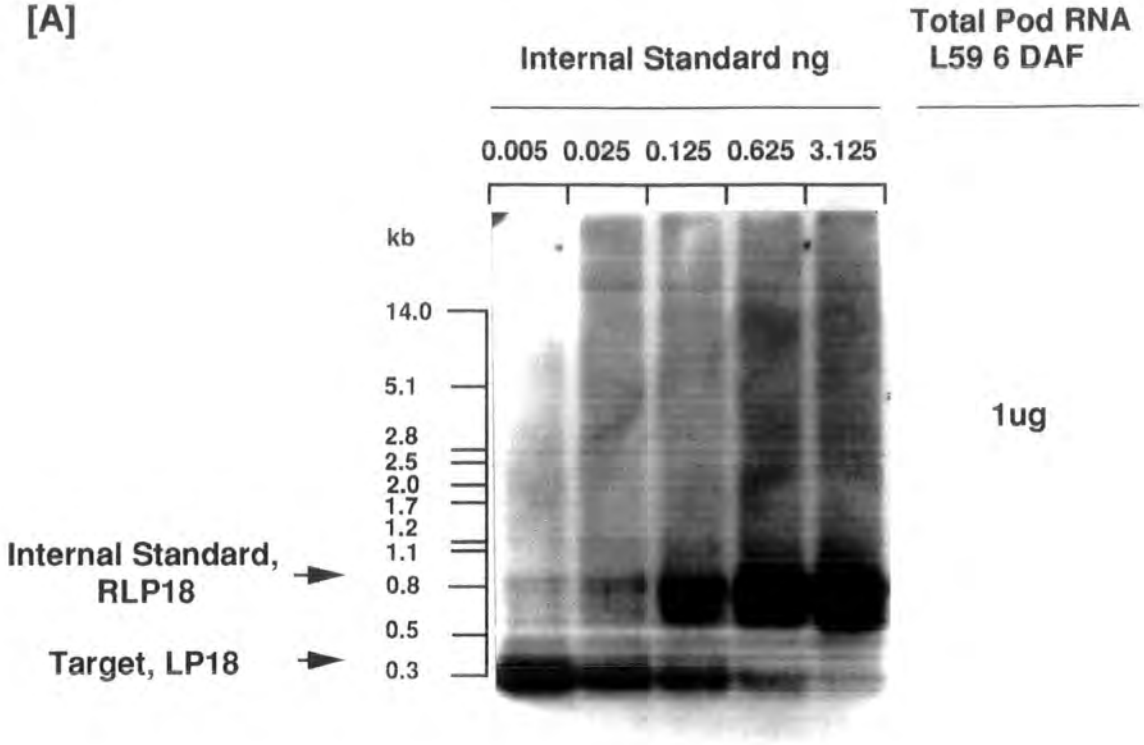
3.5.6.1 Determination of the Optimal Concentration of Internal Standard RNA to be Used for Semi-quantitative RT-PCR

A synthetic RNA species, rRLP18, was prepared and used to determine the optimal concentration required for use in semi-quantitative RT-PCR for LP18 mRNA expression (as described 2.2.26).

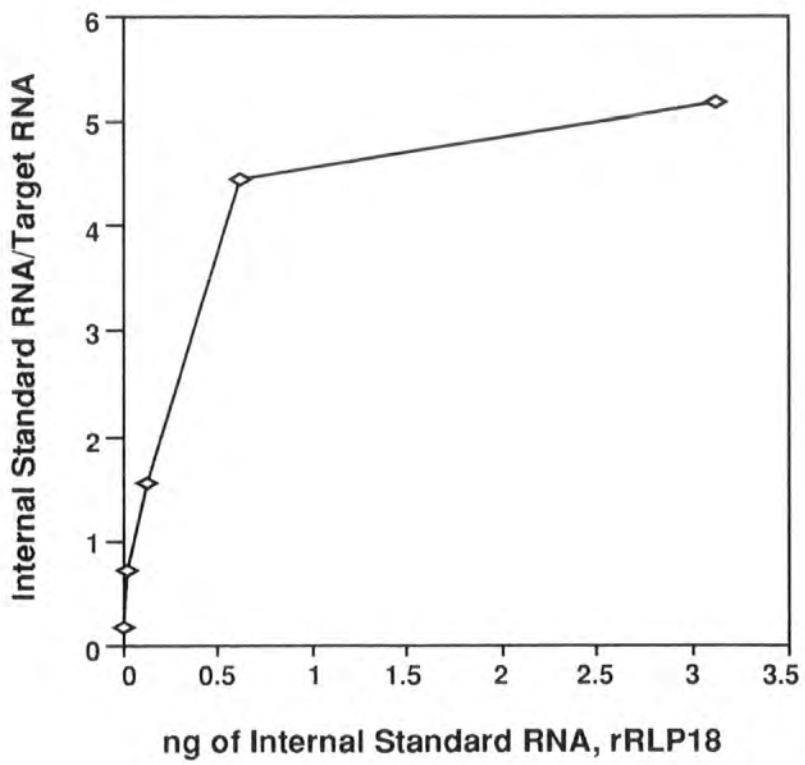
Figure 3.22 shows that with a fixed amount of 1 μg of total RNA (containing the target mRNA), increasing the concentration of internal

Figure 3.22 Competitive RT-PCR with serial dilution of internal standard, rRLP18 and 1 μg of L59 total pod RNA. [A] Autoradiograph of Southern blots of RT-PCR products hybridised with radio-labelled LP18 probe. [B] Hybridised bands in [A] were cut from the filter and scint counted. The ratio of radioactivity [cpm] of the RT-PCR products of internal standard synthetic RNA, rRLP18, to that of target mRNAs, LP18, were plotted against the amount of original internal standard RNA incorporated in each reaction.

[A]


Internal Standard ng
Total Pod RNA
L59 6 DAF
0.005 0.025 0.125 0.625 3.125
kb
14.0
5.1
2.8
2.5
2.0
1.7
1.2
1.1
0.8
0.5
0.3
Internal Standard, RLP18
Target, LP18
1ug

[B]


Internal Standard RNA/Target RNA
0
1
2
3
4
5
6
0
0.5
1
1.5
2
2.5
3
3.5
ng of Internal Standard RNA, rRLP18

standard increases the ratio of the signal of the internal standard to target RNA. The ratio is linear up to approximately 0.125 ng of internal standard RNA and begins to plateau above 0.625 ng. Therefore, 0.025 ng was selected as the fixed amount of internal standard to be incorporated in all samples for reverse transcription and subsequent PCR, as this concentration lies close to the region giving a linear response.

3.5.6.2 Semi-quantitative RT-PCR for LP18 mRNA Expression in Pea Pods

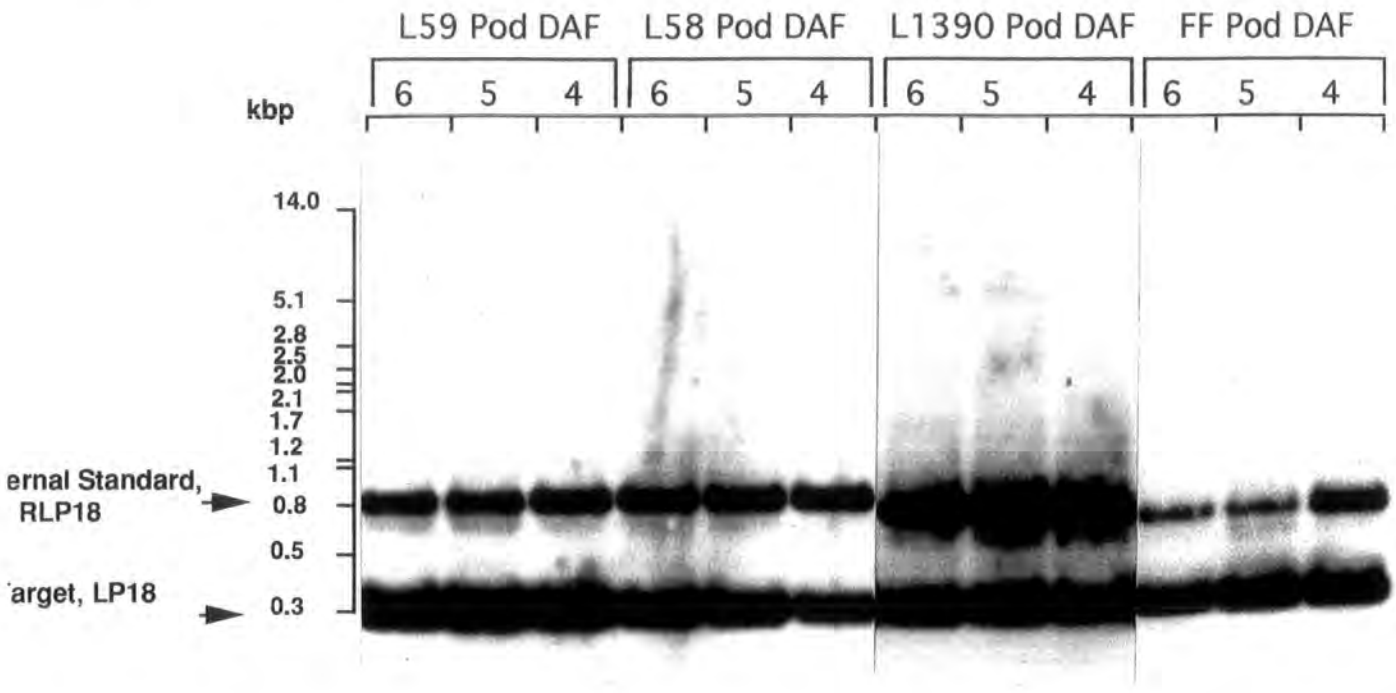
The results of RT-PCR of L59, L58 and L1390 pods are shown in Figure 3.23. It confirms differential expression in the different pea lines and the pattern of LP18 gene expression in the pods at four, five and six days after flowering. The results of RT-PCR of pods for LP18 gene expression show that transcription occurs in varying degrees in all of the pod lines being investigated. LP18 transcripts are most abundant in L59 and FF and expression of LP18 mRNA appears to increase from four through to six DAF. L58 has lower levels of LP18 mRNA than L59 and FF. However, in similarity with L59 and FF, the expression levels do increase from four through to six DAF. L1390 has the lowest levels of LP18 transcripts. The levels of LP18 mRNA during the period of pod development from four to six days after flowering appear to remain stable at around 0.01 - 0.02. These results are consistent with a role for the blue copper protein predicted by LP18 in endocarp development.

3.5.6.3 Semi-quantitative RT-PCR for LP18 mRNA Expression in Pea Tissue

If the blue copper protein encoded by LP18 is involved in lignification in the pods in forming the sclerenchymatous layer where present, it should also be present in lignifying vascular tissue, accounting for the low level of expression in L1390. On this basis, it should also be expressed throughout the plant in developing vascular tissue. Hence, RT-PCR was performed on root, leaf and stem tissue from L59, L58 and L1390 at 4 weeks after planting out seedlings. The main stems were harvested and the leaf tissue was stripped from the main vein and

Figure 3.23 Semi-quantitative RT-PCR for LP18 gene expression in four, five and six DAF pea pods with different phenotype and genotype. For RT-PCR, 0.025 ng of internal standard RNA, rRLP18 was added to 1 μg of total pod RNA from each pod. [A] Autoradiographs of Southern blots of RT-PCR products hybridised with radio-labelled LP18 probe. [B] Bands shown in [A] were excised and radioactivity was determined by liquid scintillation counting. A quantitative index was calculated giving the amount of target, LP18 mRNA, relative to the 0.025 ng of internal standard RNA, RLP18, incorporated in each RT-PCR reaction. An average and standard deviations were calculated from duplicate experiments.

[A]



[B]

Experimental Line	Quantitative index for LP18 mRNA [relative to 0.025 ng of internal standard, RLP18], from 1 ug of total RNA from pea pods at:		
	4 DAF	5 DAF	6 DAF
L59	0.051 ± 0.011	0.055 ± 0.005	0.107 ± 0.001
L58	0.028 ± 0.000	0.039 ± 0.001	0.052 ± 0.002
L1390	0.017 ± 0.004	0.016 ± 0.004	0.016 ± 0.004
Feltham First	0.049 ± 0.009	0.076 ± 0.004	0.090 ± 0.025

petiole. The results show that LP18 mRNA is indeed expressed throughout the plant (Figure 3.24). Comparable levels of LP18 mRNA expression were observed in each tissue type for the three pea lines. Highest expression of LP18 mRNA was observed in the stem for the three pea lines. This is consistent with a role for the blue copper protein encoded by LP18 in lignification, which is required in the growing stem for vascular formation and support.

3.6 CLONE, pLP19, ISOLATED FROM THE DIFFERENTIAL SCREEN OF THE L59 LIGNIFIED POD cDNA LIBRARY

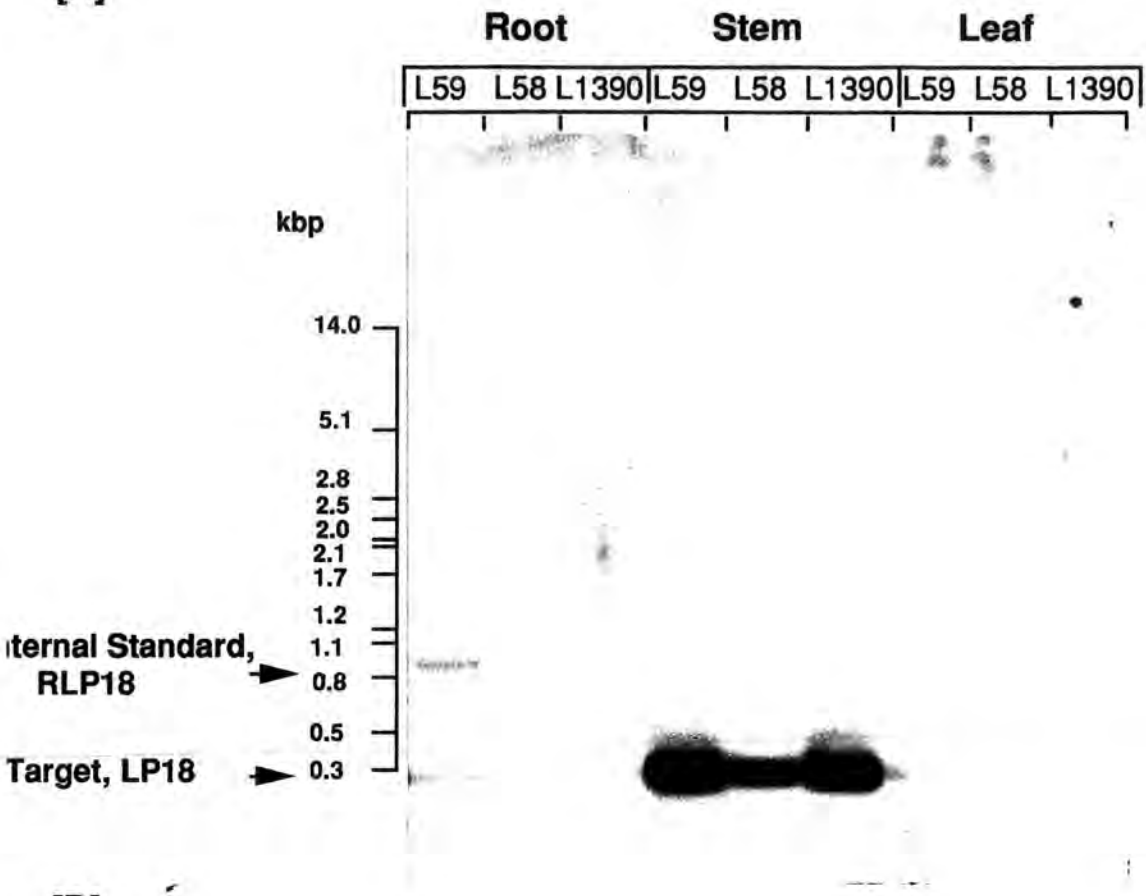
The clone, designated pLP19, was isolated from the differential screen of the L59 lignified pod cDNA library representing poly $(A)^+$ RNA from developing pea pods (4 - 6 DAF), which have a lignified endocarp (see 3.4). Selection was on the basis of hybridisation to a total cDNA probe from the lignifying pods of L59, but not to a total cDNA probe from pods of L1390, which do not lignify. Subsequent selection of the putatively differentially expressed clone, pLP19, was made following a secondary and tertiary screen (see Figure 3.13).

3.6.1 Subcloning and Sequencing of LP19

The insert in pLP19 was characterised by DNA sequencing. A restriction map of the LP19 cDNA and the sequencing strategy are shown (Figure 3.25). The cDNA fragment was 1072 bp in length, including a 3' non-coding region of 256 bp, plus a poly (A) tail of 18 bp. The cDNA fragment encoded 271 residues of the C-terminal of a putative polypeptide (Figure 3.26). A single motif, AATAAA, at nucleotides 1068 - 1073, corresponds exactly to the consensus eukaryotic polyadenylation signal. However, the motif is not usually situated immediately adjacent to the poly (A) tail. Eukaryotic poly (A) signals are usually situated 10 - 35 amino acids upstream of the poly (A) addition site (Proudfoot and Brownlee, 1976). Subsequent analysis of LP19 transcripts with heterogeneous 3' UTRs suggest that this motif is not a polyadenylation signal (see 3.6.4).

Figure 3.24 Semi-quantitative RT-PCR for LP18 gene expression in root, leaf and stem tissue from the experimental pea lines, L59, L58 and L1390. For RT-PCR, 0.025 ng of internal standard RNA, rRLP18 was added to 1 μg of total RNA from each tissue. [A] Autoradiographs of Southern blots of RT-PCR products hybridised with radio-labelled LP18 probe. [B] Bands shown in [A] were excised and radioactivity was determined by liquid scintillation counting. A quantitative index was calculated giving the amount of target, LP18 mRNA, relative to the 0.025 ng of internal standard RNA, rRLP18, incorporated in each RT-PCR reaction. An average and standard deviations were calculated from duplicate experiments.

[A]

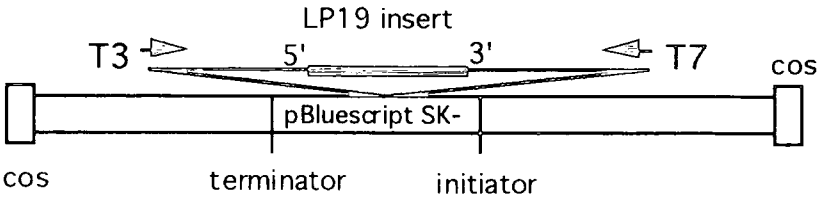


[B]

Experimental Line	Quantitative index for LP18 mRNA [relative to 0.025 ng of internal standard, RLP18], from 1ug of total RNA from pea plant tissues:		
	Root	Stem	Leaf
L59	0.026 ± 0.006	0.242 ± 0.000	0.043 ± 0.003
L58	0.034 ± 0.009	0.124 ± 0.022	0.036 ± 0.010
L1390	0.042 ± 0.012	0.208 ± 0.051	0.053 ± 0.013

Figure 3.25 (A) The cloned cDNA insert, LP19, in pBluescript SK- within the Lambda ZAP II vector. (B) The sequencing strategy for the cDNA insert, LP19, excised in the pBluescript plasmid by co-infection with helper phage. The restriction enzyme sites for subcloning are indicated. The sequenced fragments and the direction of sequencing are indicated by the arrows.

(A)



(not to scale)

(B)

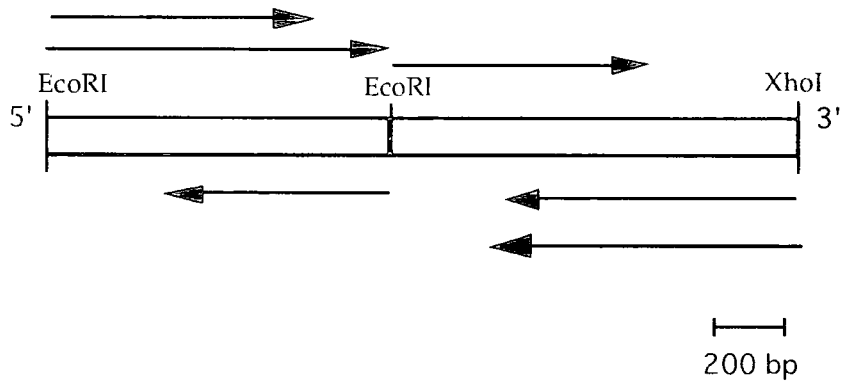


Figure 3.26 Nucleotide sequence of the LP19 cDNA and predicted polypeptide encoding the carboxy-terminal fragment of an Hsp70 homologue.

```
1/1                                                 31/11
GCT GCT GTT CAA GCC GCG ATT TTG ACT GGT GAA GGC GAT GAA AAG GTT CAA GAT CTT TTG
A   A   V   Q   A   A   I   L   T   G   E   G   D   E   K   V   Q   D   L   L
61/21                                               91/31
TTG CTT GAT GTT ACT CCT CTT AGC TTG GGT CTA GAA ACT GCC GGT GGT GTT ATG ACG GTT
L   L   D   V   T   P   L   S   L   G   L   E   T   A   G   G   V   M   T   V
121/41                                              151/51
TTG ATT CCG AGG AAC ACG ACG ATT CCG ACT AAG AAG GAG CAG ATT TTT TCG ACT TAT TCA
L   I   P   R   N   T   T   I   P   T   K   K   E   Q   I   F   S   T   Y   S
181/61                                              211/71
GAT AAT CAA CCT GGT GTT TTG ATT CAA GTT TTT GAA GGT GAA CGT GCG AGA ACA AAG GAT
D   N   Q   P   G   V   L   I   Q   V   F   E   G   E   R   A   R   T   K   D
241/81                                              271/91
AAT AAT CTT CTT GGG AAA TTT GAA CTC ACT GGT ATA CCA CCA GCT CCG AGA GGT GTG CCA
N   N   L   L   G   K   F   E   L   T   G   I   P   P   A   P   R   G   V   P
301/101                                             331/111
CAG GTT AAT GTT TGT TTT GAT ATT GAT GCG AAT GGG ATA TTG AAT GTT TCT GCG GAA GAT
Q   V   N   V   C   F   D   I   D   A   N   G   I   L   N   V   S   A   E   D
361/121                                             391/131
AAA ACA GCT GGC GTG AAG AAC AAG ATA ACT ATA ACA AAT GAT AAA GGG AGG TTG AGT AAG
K   T   A   G   V   K   N   K   I   T   I   T   N   D   K   G   R   L   S   K
421/141                                             451/151
GAA GAG ATA GAG AAG ATG GTG AAA GAT GCA GAG AAG TAT AAG GCA GAG GAT GAA GAG GTG
E   E   I   E   K   M   V   K   D   A   E   K   Y   K   A   E   D   E   E   V
481/161                                             511/171
AAG AGG AAA GTG GAA GCT AAG AAT TCG CTT GAG AAT TAT GCT TAC AAT ATG AGG AAT ACT
K   R   K   V   E   A   K   N   S   L   E   N   Y   A   Y   N   M   R   N   T
541/181                                             571/191
ATT AAG GAT GAC AAG ATT GGT GGG AAG TTG AGT AAT GAT GAT AGA GAG AAG ATT GAG AAG
I   K   D   D   K   I   G   G   K   L   S   N   D   D   R   E   K   I   E   K
601/201                                             631/211
GCT GTG GAG GAG GCT ATT CAG TGG TTG GAA GGG AAT CAA TTG GGT GAA GTG GAG GAG TTT
A   V   E   E   A   I   Q   W   L   E   G   N   Q   L   G   E   V   E   E   F
661/221                                             691/231
GAG GAT AAG CAG AAG GAG TTG GAA GGG GTT TGT AAT CCT ATT ATT GCT AAG ATG TAT CAA
E   D   K   Q   K   E   L   E   G   V   C   N   P   I   I   A   K   M   Y   Q
721/241                                             751/251
GGT GGT GCT GGT GGA GAT GTG CCT ATG GGA GAT GGT ATG CCT GGT GGT CGT TCT AAT GGA
G   G   A   G   G   D   V   P   M   G   D   G   M   P   G   G   R   S   N   G
781/261                                             811/271
TCT GGA CCT GGT CCT AAG ATT GAA GAG GTT GAC TAA AGA AGC CAT ACC AGG GCT AGG GCC
S   G   P   G   P   K   I   E   E   V   D   *
841/281                                             871/291
TAG GGG CAT GTC TGT TTT TAA GAC CTT GGT TGG GTC TAT GGA GTT ACT ATG AAA GTG TCT
901/301                                             931/311
TAT AAC TTG TGT ATG TTA TGT TAT AGA ATG TAG GTT TGG TAA AGT GGG TTT GTT GAG TCT
961/321                                             991/331
TTA TGT TTT TTA AGC TTT TTG TGA ACT ATG TAT TTT GTT TTT TTA TCA AGT ACT TAT GAA
1021/341                                            1051/351
TAG TGT AAT TTC GAG AGA TGA AGA TGA ATG TAA TTT AAT TTT CTT TAT CAA TAA AAA AAA
1081/361
AAA AAA AAA A
```

3.6.2 Amino Acid Sequence Comparison of the Polypeptide Fragment Encoded by LP19

A homology search of the putative C-terminal of the polypeptide predicted by clone pLP19 against Gene Bank data using the FASTA programme package (Lipman and Pearson, 1988) revealed similarity with the C-terminal of the Hsp70 family of proteins. Highest homology was with the plant Hsp70s from soybean (89.7 %) (Roberts and Key, 1991), tomato (83.2 %) (Lin *et al.*, 1991a), petunia (81.3 %) (Winter *et al.*, 1988), maize (79.9 %) (Rochester *et al.*, 1986) and carrot (79.2%) (Lin *et al.*, 1991b) (Figure 3.27). The high degree of homology indicates that LP19 encodes the C-terminal of a pea Hsp70 protein. The Hsp70 family of proteins have been implicated in protein biogenesis, exhibiting a diverse range of biological functions (see 1.11).

3.6.3 Expression Analysis of LP19 in 4 - 6 DAF Pods

Dot blot analysis of total RNA from L59, L58, L1390 and Feltham First, hybridised with ^{32}P labelled LP19 confirmed differential expression of LP19 mRNA (Figure 3.28). Strong hybridisation was observed with L59, L58 and FF. Hybridisation to L1390 total RNA was not above background hybridisation to *E. coli* ribosomal RNA.

Northern hybridisation of the ^{32}P labelled LP19 insert to 10 µg of total pod RNA from the experimental pea lines showed hybridisation to a number of mRNA transcripts of varying molecular weight. Hybridisation to a major transcript of 2.6 kb was observed in L59, L58 and FF. Hybridisation to the major 2.6 kb transcript was greatest in L59 and FF, with a significantly lower degree of hybridisation to the 2.6 kb transcript in L58. Hybridisation to minor transcripts of 1.85 kb and 1.0 kb in L59 and 1.0 kb in FF were observed. No hybridisation was observed in L1390.

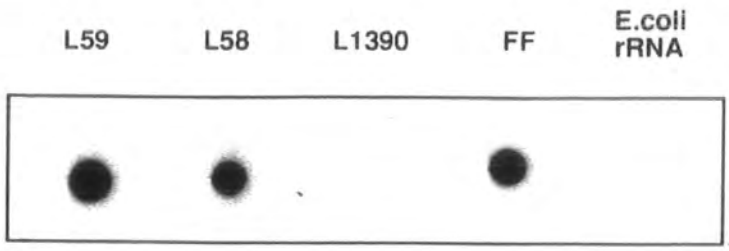
The major 2.6 kb transcript possibly represents unprocessed LP19 mRNA, while the 1.85 kb transcript in L59 may represent processed LP19 mRNA. Similar northern banding patterns have been observed with the maize and petunia *Hsp70s*. The petunia and maize *Hsp70s* have an intron, producing a transcript of 2.8 kb and 2.6 kb respectively

Figure 3.27 Comparison of the carboxy terminal fragment encoded by LP19 with the carboxy terminals of the soybean Hsp70 (Roberts and Key, 1991), maize Hsp70 (Rochester *et al.*, 1986) and the petunia Hsp70 (Winter *et al.*, 1988) amino acid sequences. The numbering corresponds to the soybean Hsp70 amino acid sequence. The colons (:) represent identical sequences and the periods (.) represent residues defined as "similar" in the mutation matrix using the FASTA protein sequence comparison programme (Pearson and Lipman, 1988).

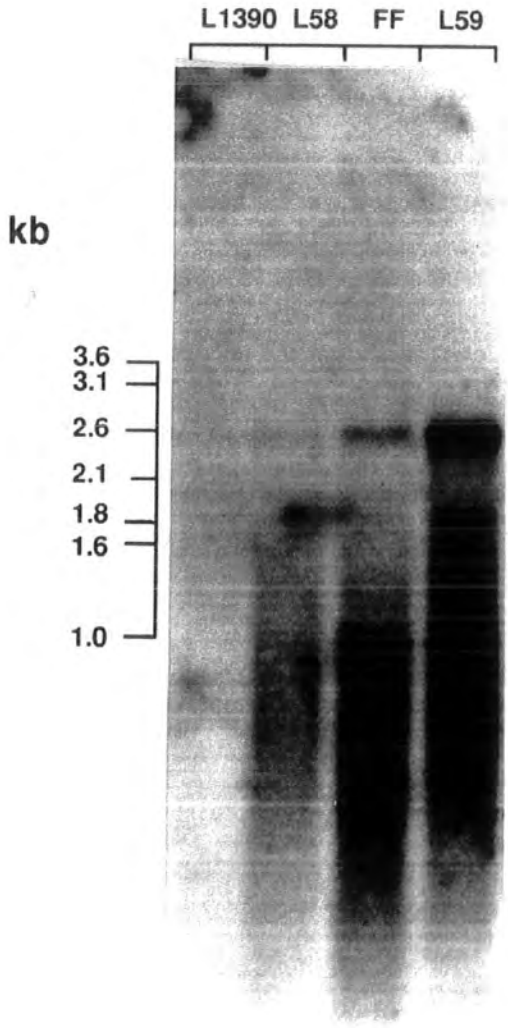
```
                 380        390        400        410        420
LP19             AAVQAAILTGEGDEKVQDLLLLDVTPLSLGLETAGGVMTVLIPRNTTIPT
                 :::::::::.:.:::::::::::::::::::::::::::::::::::::
Soybean Hsp70    AAVQAAILSGQGDEKVQDLLLLDVTPLSLGLETAGGVMTVLIPRNTTIPT
                 :::::::::.:::.:::::::::::::::::::::: ::::::::::::
Maize Hsp70      AAVQAAILSGEGNEKVQDLLLLDVTPLSLGLETAGGGMTVLIPRNTTIPT
                 :::::::::.:::.:.  ::::::::::::::::::::::::::::::::
Petunia Hsp70    AAVQAAILSGEGNERS-DLLLLDVTPLSLGLETAGGVMTVLIPRNTTIPT

                 430        440        450        460        470
LP19             KKEQIFSTYSDNQPGVLIQVFEGERARTKDNNLLGKFELTGIPPAPRGVP
                 ::::::::::::::::::::::::::::::::::::::::::::::::::
Soybean Hsp70    KKEQIFSTYSDNQPGVLIQVFEGERARTKDNNLLGKFELTGIPPAPRGVP
                 ::::.:::::::::::::::::.:::::::::::::::::::.:::::::::
Maize Hsp70      KKEQVFSTYSDNQPGVLIQVYEGERARTKDNNLLGKFELSGIPPAPRGVP
                 ::::.:::::::::::::::::.:::::::::::::::::::.:::::::::
Petunia Hsp70    KKEQVFSTYSDNQPGVLIQVYEGERARTKDNNLLGKFELSGIPPAPRGVP

                 480        490        500        510        520
LP19             QVNVCFDIDANGILNVSAEDKTAGVKNKITITNDKGRLSKEEIEKMVKDA
                 ::::::::::::::::::::::::::::::::::::::::::::::::::
Soybean Hsp70    QVNVCFDIDANGILNVSAEDKTAGVKNKITITNDKGRLSKEEIEKMVKDA
                 :..:::::::::::::::::::.: ::::::::::::::::::::.::..:
Maize Hsp70      QITVCFDIDANGILNVSAEDKTTGQKNKITITNDKGRLSKEEIERMVQEA
                 :..: :::::.:::::::::::.: ::::::::::::::::::::::..:
Petunia Hsp70    QITVTFDIDVNNILNVSAEDKTTG-KNKITITNDKGRLSKEEIEKMVQEA

                 530        540        550        560        570
LP19             EKYKAEDEEVKRKVEAKNSLENYAYNMRNTIKDDKIGGKLSNDDREKIEK
                 :.:::::::::.:::::::::::::::::::::.::::::: :...::::
Soybean Hsp70    ERYKAEDEEVKKKVEAKNSLENYAYNMRNTIKDEKIGGKLSPDEKQKIEK
                 ::::.::::.:.:::::.:::::::::::::::::..::..:...::.
Maize Hsp70      EKYKSEDEELKKKVEAKNALENYAYNMRNTIKDDKINSQLSAADKKRIED
                 ::::::::::::.::.:::.:::::::::::::::..::...:..:::.
Petunia Hsp70    EKYKAEDEEVKKKVDAKNALENYAYNMRNTIKDDKIASKLPAEDKKKIED

                 580        590        600        610        620
LP19             AVEEAIQWLEGNQLGEVEEFEDKQKELEGVCNPIIAKMYQG--GAGGDVP
                 :::.::::::::..::.:::::::::::.:::::::::::  :.::::::
Soybean Hsp70    AVEDAIQWLEGNQMAEVDEFEDKQKELEGICNPIIAKMYQGAAGPGGDVP
                 ::..:: ::..:::.:::::::: :::::.:::::::::.:  ...:  .
Maize   Hsp70    AVDGAISWLDSNQLAEVEEFEDKMKELEGICNPIIAKMYXG--EGAGMGA
                 :..:::.::..:::.:..:::::: :::::..:::::::::::::::..
Petunia Hsp70    AIDEAIKWLDNNQLAEADEFEDKMKELESICNPIIAKMYQG--GAGGATM

                 630                640
LP19             MGDGMPGGRSNGS--GPGPKIEEVD
                 ::..::      ..  :.:::::::::
Soybean Hsp70    MGADMP-----AA--GAGPKIEEVD
                 ..    ...:.::  :.:::::::::
Maize Hsp70      AAGMDEDAPSGGS--GAGPKIEEVD
                 : .   ...:.::  :.:::::::::
Petunia Hsp70    AGMD-EDAPSGGS--GAGPKIEEVD
```

Figure 3.28 Dot blot (A) and northern blot (B) hybridisation of 5 μg and 10 μg, respectively, of total pod RNA 4 - 6 DAF, prepared from four pea lines - L59, Feltham First, L58 and L1390 - probed with radio-labelled LP19 cDNA, isolated from a differential screen of a cDNA library constructed from 4 - 6 DAF L59 pods. The filters were exposed to sensitised film for 1 week at -80 °C.

(A)


L59
L58
L1390
FF
E.coli
rRNA

(B)


L1390
L58
FF
L59
kb
3.6
3.1
2.6
2.1
1.8
1.6
1.0

(Rochester *et al.*, 1986; Winter *et al.*, 1988). The minor 1.0 kb transcript has been observed in northern analysis of *Hsp70* expression in maize (Rochester *et al.*, 1986).

3.6.4 Genomic Analysis of LP19

The LP19 cDNA was hybridised to pea genomic DNA from L59, L58 and L1390 in a Southern blotting experiment (Figure 3.29) in order to establish the presence of the LP19 gene in the three experimental pea lines. Washing to high stringency (0.1 x SSC/0.1 % SDS, 65 °C) showed the LP19 probe hybridised to a single band in each of the two digests with different restriction enzymes, in all three pea lines, suggesting that a single gene probably encodes this polypeptide. All three experimental pea lines have a gene homologous to the LP19 transcript on the same restriction fragment.

3.6.5 Screening of the L59 Lignified Pod cDNA Library for a Full-length LP19 cDNA

Duplicate plaque lifts of 5 x 10^4 clones of the L59 lignified pod cDNA library were screened with ^{32}P labelled LP19 insert (as described 2.2.23.12) in an attempt to isolate a full-length LP19 cDNA. The primary screen yielded nine putative positive clones (Figure 3.30). The putative positive clones, designated LP19/1 - LP19/9, were cored from the primary stock plate and subjected to secondary screening. Strongly hybridised positive clones from the secondary screen were cored and plasmid rescued (as described 2.2.23.10). Plasmid DNA prepared from the putative LP19 homologues was EcoRI - XhoI cleaved and electrophoresed (Figures 3.31A and 3.32A). The EcoRI - XhoI cleaved putative LP19 homologues were Southern blotted onto nylon filters by alkaline transfer (as described 2.2.18.1). The Southern blotted filters were then hybridised (as described 2.2.18.2) with ^{32}P labelled LP19 probe. Positive hybridisation was observed for clones, LP19/1, LP19/2, LP19/3, LP19/4, LP19/6, LP19/7 and LP19/9 (Figures 3.31B and 3.32B).

Figure 3.29 Southern blot hybridisation of total pea leaf DNA from L59, L58 and L1390 with LP19 cDNA probe. DNA (20 µg) was digested with BamHI (L59 lane A; L58 lane C; L1390 lane E) or HindIII (L59 lane B; L58 lane D; L1390 lane F), electrophoresed on 0.7 % agarose gel, denatured and transferred to a nitrocellulose filter. The filter was hybridised with a radio-labelled LP19 cDNA, washed to a final stringency of 0.1 x SSC at 65 °C and exposed to sensitised film for 7 days at -80 °C.

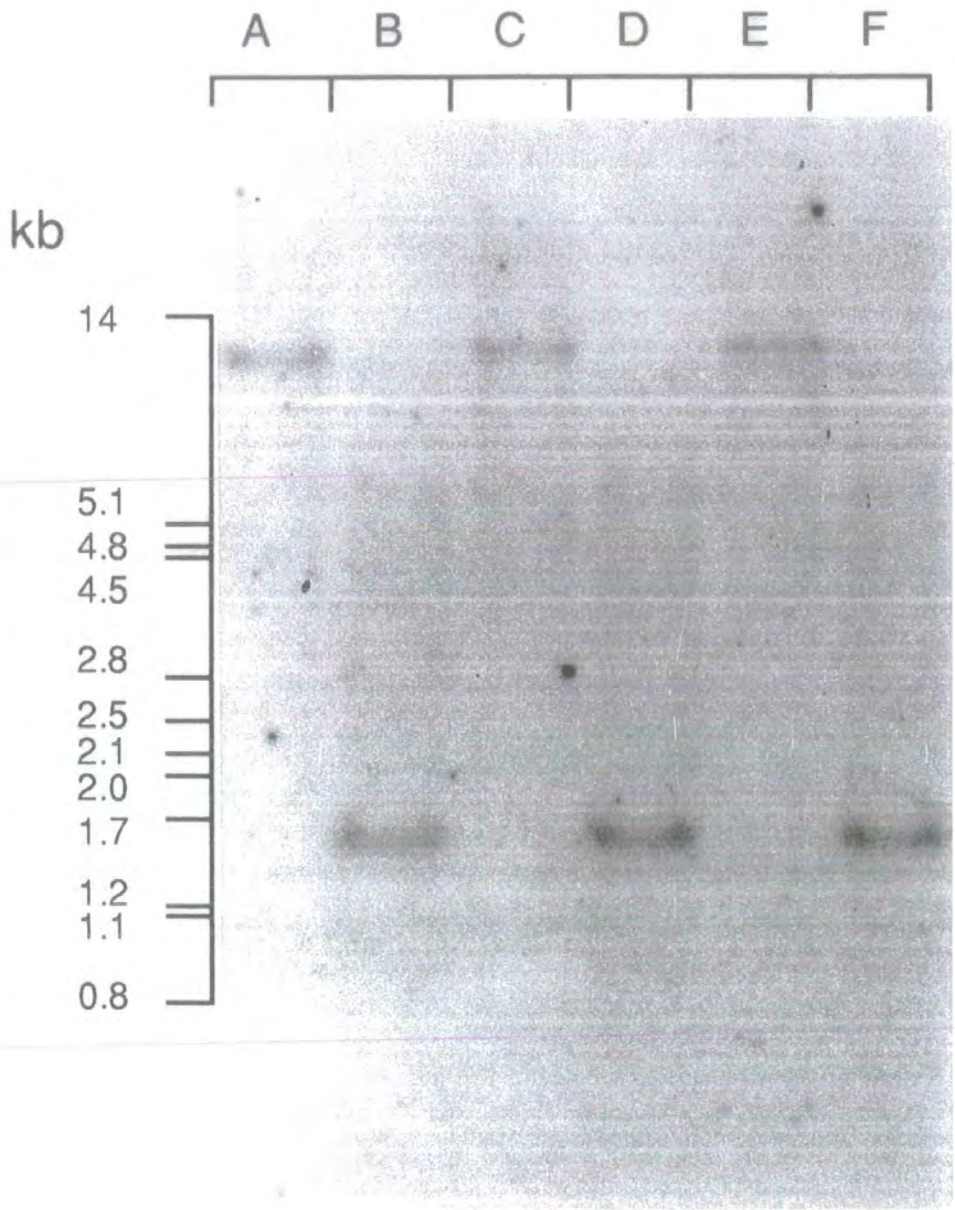

A
B
C
D
E
F
kb
14
5.1
4.8
4.5
2.8
2.5
2.1
2.0
1.7
1.2
1.1
0.8

Figure 3.30 Re-screen of L59 lignified pod cDNA library for full-length LP19 cDNA. Duplicate primary screen filters were hybridised with ^{32}P-labelled LP19 cDNA insert. The filters were washed to a final stringency of 0.1 x SSC at 65 °C and exposed to sensitised film for 7 days at -80 °C.

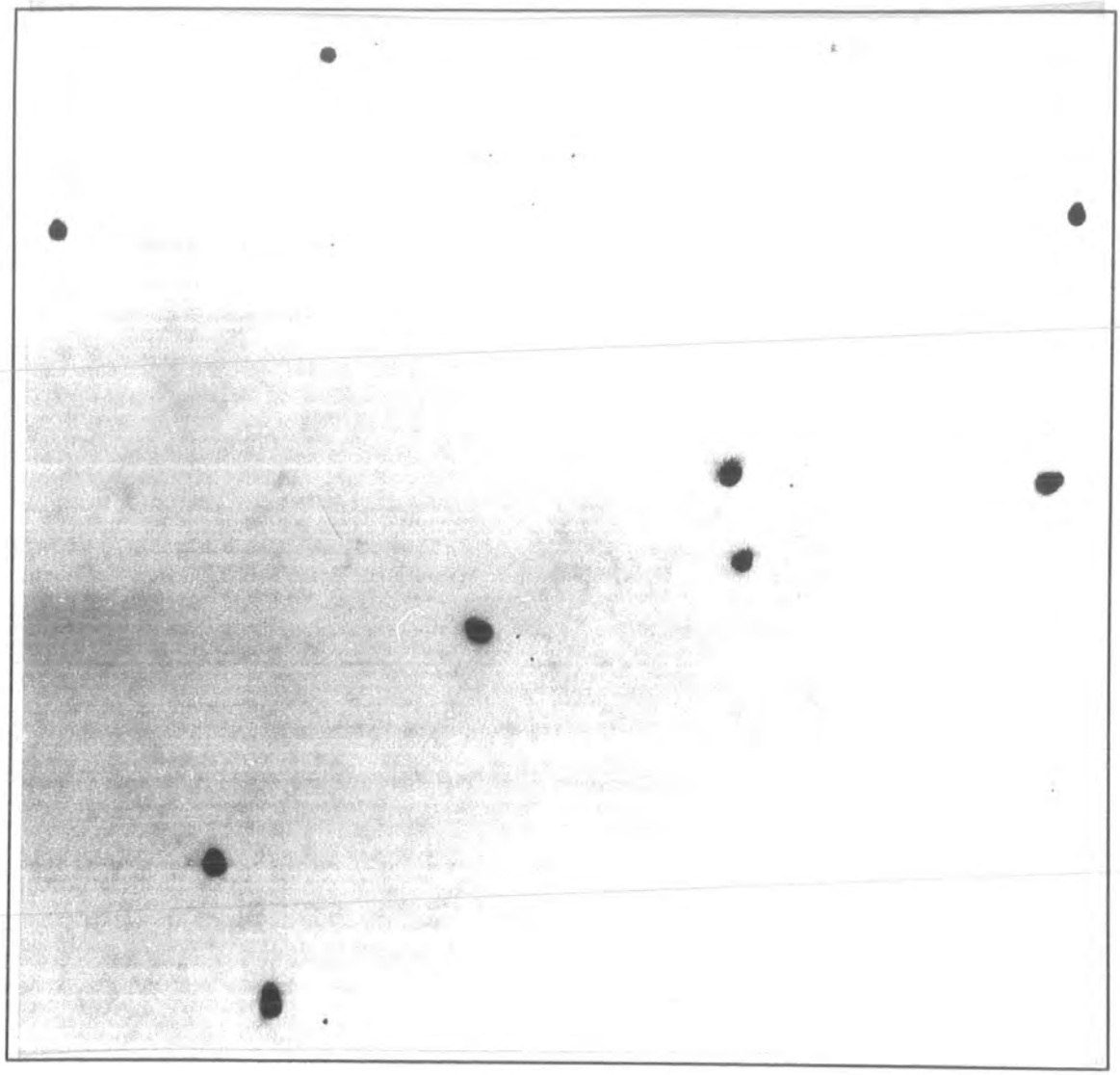
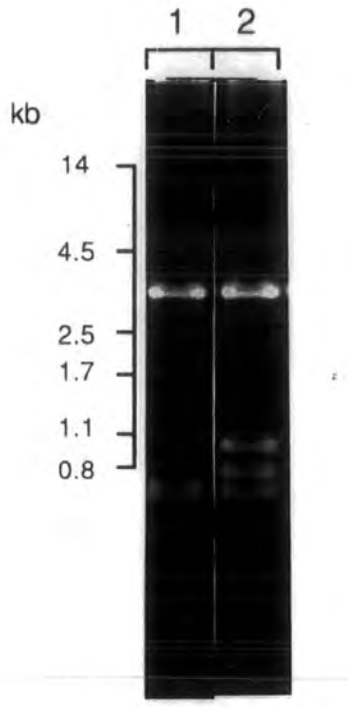


Figure 3.31 Southern blot hybridisation of EcoRI/XhoI cleaved pLP19 (lane 1) and the pLP19/3 homologue (lane 2) isolated from the re-screen of the L59 lignified pod cDNA library. (A) The cleavage products were electrophoresed on 0.7 % agarose gel, and transferrred to a nylon filter by alkali transfer. (B) The filter was hybridised with a radio-labelled LP19 cDNA. The filter was washed to a final stringency of 0.1 x SSC at 65 °C and exposed to sensitised film for 24 hours at -80 °C.

(A)



(B)

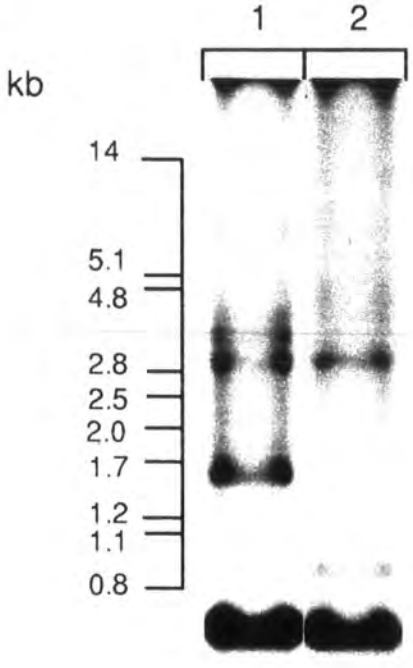
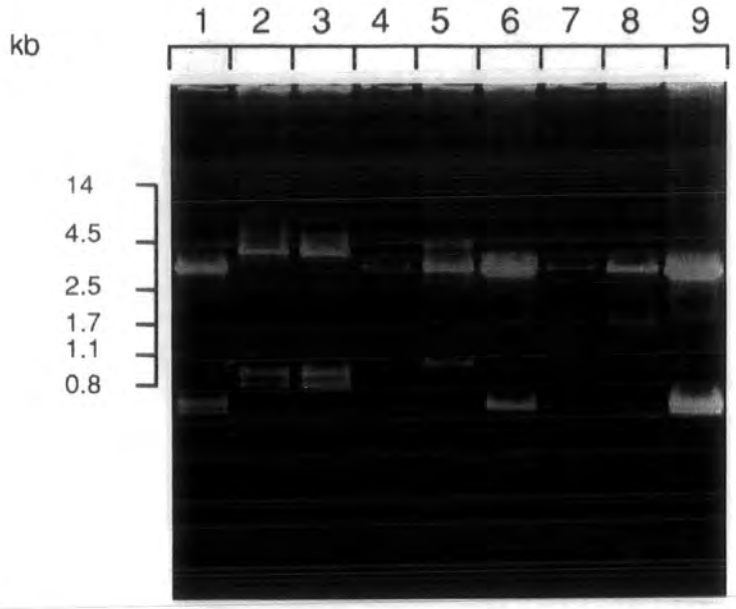
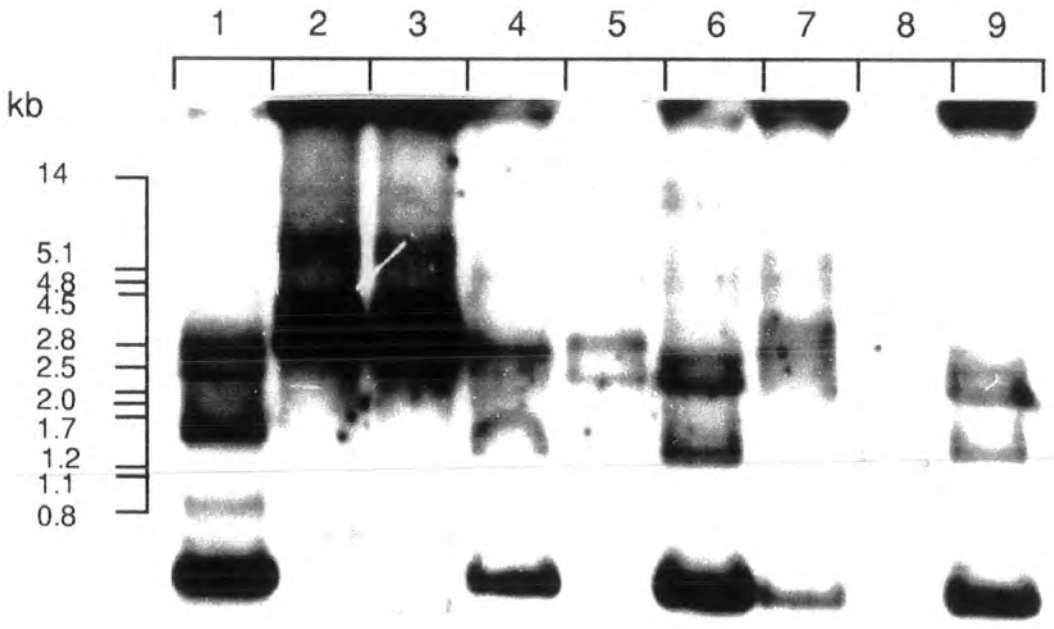


Figure 3.32 Southern blot hybridisation of EcoRI/XhoI cleaved pLP19 (lane 1) and pLP19 homologues (pLP19/1, lane 2; pLP19/2, lane 3; pLP19/4, lane 4; pLP19/5, lane 5; pLP19/6, lane 6; pLP19/7, lane 7; pLP19/8, lane 8; pLP19/9, lane 9) isolated from the re-screen of the L59 lignified pod cDNA library. (A) The cleavage products were electrophoresed on 0.7 % agarose gel, and transferrred to a nylon filter by alkali transfer. (B) The filter was hybridised with a radio-labelled LP19 cDNA. The filter was washed to a final stringency of 0.1 x SSC at 65 °C and exposed to sensitised film for 24 hours at -80 °C.

(A)


1 2 3 4 5 6 7 8 9
kb
14
4.5
2.5
1.7
1.1
0.8

(B)


1 2 3 4 5 6 7 8 9
kb
14
5.1
4.8
4.5
2.8
2.5
2.0
1.7
1.2
1.1
0.8

3.6.6 Partial Sequencing of LP19 cDNA Homologues

DNA from the clones containing the largest inserts, as determined from the band pattern observed in gels containing the EcoRI - XhoI cleaved pLP19 homologues (see Figures 3.31A and 3.32A), was prepared for sequencing. LP19/1 and LP19/2 yielded similar EcoRI - XhoI cleavage products. It was suspected that one of the cloning sites was lost as the linearised plasmid fragment was slightly larger than pBluescript. Subsequent cleavage with either XhoI or EcoRI revealed that the XhoI cloning site appears to have been lost. Cleavage with EcoRI yielded two restriction enzyme fragments of approximately 600 bp and 800 bp. The linearised plasmid fragment was estimated to contain the remainder of the insert, approximately 300 bp. LP19/3 and LP19/7 gave similar EcoRI - XhoI cleavage products. The insert size was estimated to be approximately 1.7 kb. Therefore, pLP19/1, pLP19/2, pLP19/3 and pLP19/7 were sequenced with the reverse primer confirming the clones were homologous to the LP19 insert and sequenced with the forward primer to establish whether the inserts were full length cDNAs.

LP19/3 and LP19/7 were identical clones, 21 amino acids short of the methionine start residue and LP19/1 and LP19/2 were identical clones, missing only two nucleotides of the methionine start codon, by comparison with the homologous maize and petunia Hsp70 amino acid sequences.

Hence, a full-length clone was not isolated. However, an interesting observation was made with regard to the 3′ UTRs of the LP19 homologues. The LP19 transcripts contained heterogeneous 3′ UTRs with transcription termination and poly (A) addition occurring at different sites (Figure 3.33). Therefore, it was decided to sequence the other LP19 homologues, pLP19/4, pLP19/6 and pLP19/9, which gave positive hybridisation signals (see Figure 3.32B). These three clones appeared to encode identical transcripts with yet another termination and poly (A) addition site and identical truncated 5′ termini (Figure 3.33).

Figure 3.33 The nucleotide sequence of the 3′ untranslated regions of the LP19 cDNAs. The putative polyadenylation signal of LP19/1 and LP19/2 is underlined. Identical 3′ non-coding sequence is represented by the dashed line. The numbering indicates the position of nucleotides downstream of the termination codon, TAA/*.

```
                                                                +25
LP19/1 and LP19/2   TAA AGA AGC CAT ACC AGG GCT AGG GCC TAG GGG CAT GTC TGT TTT TAA

LP19                 *  ------------------------------------------------------------

LP19/4, LP19/6 and   *  ------------------------------------------------------------
LP19/9

LP19/3 and LP19/7    *  ------------------------------------------------------------


                        +50                                 +75
LP19/1 and LP19/2   GAC CTT GGT TGG GTC TAT GGA GTT ACT ATG AAA GTG TCT TAT AAC TTG

LP19                ------------------------------------------------------------

LP19/4, LP19/6 and  ------------------------------------------------------------
LP19/9

LP19/3 and LP19/7   ------------------------------------------------------------


                              +100                                  +125
LP19/1 and LP19/2   TGT ATG TTA TGT TAT AGA ATG TAG GTT TGG TAA AGT GGG TTT GTT GAG

LP19                ------------------------------------------------------------

LP19/4, LP19/6 and  ------------------------------------------------------------
LP19/9

LP19/3 and LP19/7   ------------------------------------------------------------

                         +150                                  +175
LP19/1 and LP19/2   TCT TTA TGT TTT TTA AGC TTT TTG TGA ACT ATG TAT TTT GTT TTT  TTA

LP19                ------------------------------------------------------------

LP19/4, LP19/6 and  ------------------------------------------------------------
LP19/9


LP19/3 and LP19/7   ------------------------------------------------------------


                              +200                                  +225
LP19/1 and LP19/2   TCA AGT ACT TAT GAA TAC TGT AAT TTC GAG AGA TGA AGA TGA ATG TAA

LP19                ------------------------------------------------------------

LP19/4, LP19/6 and  ------------------------------------------------------------
LP19/9

LP19/3 and LP19/7   ------------------------------------------------(A)n


                             -250                                  +275
LP19/1 and LP19/2   TTT AAT TTT CTT TAT CAA TAT ATC ACT TAA ATC CCT GTT

LP19                ---------------------------(A)n

LP19/4, LP19/6 and  -------------------(A)n
LP19/9


LP19/1 and LP19/2   AGA TAT GTA(A)n
```

LP19/1 and LP19/2 have the longest 3' UTR of 285 bp, followed by LP19 with a 3' UTR of 258 bp (Figure 3.33). LP19/4, LP19/6 and LP19/9 have a 3' UTR of 252 bp and LP19/3 and LP19/7 have the shortest 3' UTR of 226 bp (Figure 3.33).

4. DISCUSSION.

4.1 Investigation of the cDNA Clone, pPP406, Selected from a Differential Screen of Purple- and Green-podded Pea Lines

4.1.1 Isolation and Identification of the cDNA clone, pPP406

The cDNA clone, pPP406, was isolated from a differential screen of a purple pea pod cDNA library in an attempt to isolate clones associated with the purple-podded phenotype. The clone was initially selected on the basis of an apparently higher degree of hybridisation to PP (purple-pod) total cDNA probe compared to green-podded total cDNA probe. Subsequent northern analysis revealed strong and invariant expression of PP406 in total RNA from purple- and green-podded pea lines. The polypeptide predicted by the PP406 cDNA, designated Psa-rab, encoded a polypeptide with high homology to BRL-ras and Rab7 and lower overall homology with a number of ras-related proteins, particularly the Rab/Ypt subfamily. The Psa-rab predicted polypeptide has several domains conserved in small ras-related GTP-binding proteins including the consensus motifs for GTP-binding, GTP hydrolysis and GTPase activity and the YRG motif highly conserved in Rab family members. The identification of a novel plant *ras*-related cDNA was considered worthy of further investigation as very few plant *ras*-related genes had been investigated at the time.

The Rab proteins and their counterparts in yeast, Ypt proteins, are known to be involved in vesicular trafficking of membrane bound vesicles through the secretory pathway. Immunolocalisation experiments have shown that Rab and Ypt proteins are associated with endoplasmic reticulum, Golgi apparatus and early to late endosomes. The closest functional homologue to Psa-rab, the canine Rab7 gene product, is associated with late endosomes (Chavrier *et al.*, 1990), and may link early to late endosomes, late endosomes to lysosomes, or late endosomes to the trans Golgi network (Goda and Pfeffner, 1988). Few details of vesicle-mediated intra- and inter-cellular transport in plants has been investigated. However, a similar role for Psa-rab is possible

on the basis of sequence similarity with the Rab/Ypt sub-family of ras-related proteins.

CLUSTAL analysis further indicates that the Rab7, BRL-ras and Psa-rab amino acid sequences are closely related. The Psa-rab predicted polypeptide is less similar to the other plant ras-related amino acid sequences isolated, sharing more overall sequence homology with the mammalian Rab7 and BRL-ras amino acid sequences. This may reflect functional differences between the Psa-rab/Rab7/BRL-ras cluster, which all have identical effector-binding regions in the N-terminal portion of the protein, and the other Rab homologues analysed.

The C-terminal cysteine residues of the Psa-rab predicted polypeptide are similar to the C-terminal CXC motifs of BRL-ras, Rab3, Rab4 and Rab7 proteins (C = cysteine and X = any amino acid), and differ from those found in a number of the ras-related GTP-binding proteins so far identified in plants. The C-terminal motifs found in the plant GTP-binding proteins ara (Anai *et al.*, 1991), yptm (Palme *et al.*, 1992), rha1 (Anuntalabhochai *et al.*, 1991) and rgp1 (Sano *et al.*, 1991) have tandem Cys residues followed by two to three amino acids, similar to the CAAX (C = cysteine, A = any aliphatic amino acid and X = any amino acid) motif found in most ras proteins. It has been observed that Rab proteins with the CXC motif are substrates for the addition of geranyl-geranyl moieties (Farnsworth *et al.*, 1991; Khosravi-far *et al.*, 1991; Kinsella and Maltese, 1992) and that those with CC or CAAX motifs are modified by isoprenylation. Psa-rab has an acidic region, four consecutive Glu residues, upstream of the terminal Cys residues, as opposed to some members of the ras, rho and ral subfamilies, which have a basic sequence upstream of the Cys residues (Hall, 1990), thought to be an additional signal for palmitoylation. Neither basic nor acidic sequences upstream of the terminal Cys residues is common in the Rab/Ypt families previously characterised.

4.1.2 Genomic Analysis of Psa-*rab*

Hybridisation to pea genomic DNA under high stringency conditions indicates that there are possibly one to two copies of the Psa-*rab* genes in pea. This may indicate a small multigene family, or the presence of

an intron in the Psa-*rab* gene. Introns have been observed in other plant *rab* gene family members (Palme *et al.* 1993; Matsui *et al.*, 1989). Hybridisation at low stringency revealed a high degree of non-specific hybridisation of the Psa-*rab* sequence (data not shown). The high degree of non-specific hybridisation at low to moderate stringency of the small GTP-binding proteins has been observed by other investigators. Nagano and coworkers isolated 100 cDNAs from a pea leaf cDNA library using plant *Rab* homologues as probes. Half of the isolated clones were related to GTP-binding proteins and the other half were unrelated to GTP-binding proteins (Yukio Nagano, personal communication). Under low-stringency washing conditions the *rgp1* cDNA, a plant *Ypt3* homologue, was shown to cross-hybridise with DNA fragments from different plant species and even humans (Kamada *et al.*, 1992). Hence, pre-adsorbing Psa-*rab* probes to genomic blot filters prior to use in final hybridisation for genomic analysis at low stringency may have yielded better results (see Yoshida *et al.*, 1993).

4.1.3 Expression of Psa-*rab*

The strong and invariant expression of Psa-*rab* in PP, GP and FF pods observed from northern analysis did not provide evidence of a correlation between Psa-*rab* expression and the purple-podded phenotype. It was proposed that Psa-*rab* expression may be associated with the differentiated endocarp phenotype, as it was observed that all of these lines have a differentiated endocarp and intense intracellular transport activity is associated with the formation of lignified sclerenchyma in the differentiated pod endocarp phenotype (see 1.6). Alternatively, cell division in the middle layer of the differentiated endocarp phenotype may require the expression of Rab proteins, as plant cells divide by fusion of Golgi derived vesicles in one plane across the dividing cell wall that separates the daughter cells (Gunning, 1982). Small GTP-binding proteins have been associated with cytodifferentiation (Saxe and Kimmel, 1990), which is a prerequisite of the differentiation and formation of lignified sclerenchyma in the pod endocarp. However, the expression of Psa-*rab* in pods of L59, L58 and L1390, which have distinct morphological differences in endocarp formation (described 3.2.2) did not reveal any significant differences in

expression which could be correlated with the differentiated endocarp phenotype.

Considerable progress has been made in the identification and characterisation of plant ras-related genes during the past 2 years (Anai *et al*, 1991; Palme *et al*, 1992; Nagano *et al*, 1993; Cheon *et al.*, 1993; Bednarek *et al.*, 1994). Psa-rab homologues, sRab7p and vRab7p (71 % similarity with Psa-rab), were isolated from soybean and *Vigna aconitifolia*, respectively (Cheon *et al.*, 1993). Induction of *vrab7* was observed during nodulation involving the endocytosis of *Rhizobium* by fusion of newly synthesised vesicles. Antisense *vrab7* nodules were smaller in size and showed lower nitrogenase activity (Cheon *et al.*, 1993). Electron microscopy of nodules expressing antisense *vrab7* revealed an accumulation of many small unfused vesicles and some large multivesicular bodies.

The observation of Psa-*rab* expression in a number of phenotypically different pea pods (purple-podded, green-podded, with and without a differentiated or lignified endocarp) seems to indicate that Psa-rab has an essential function in developing pea pods. The pod total RNA for northern analysis was extracted from pods during the rapid expansion phase of growth when there is presumably an active production of secretory vesicles directed to the provision of new components for the rapid expansion of the plasma membrane and cell wall. Pea pod mesocarp cells have been observed to increase in size from 24 μm at day 0 to 80 μm at day +4 after fertilisation, and pod wall thickness to increase from 323 $\pm$ 24 μm at day 0 to 700 $\pm$ 83 μm at day 4 (Vercher *et al.*, 1984). The epidermal cells undergo elongation as the pod develops. It may be that Psa-rab is required by developing pods for the vesicle production necessary during rapid pod growth. Expression of a number of plant *rab* homologues has been observed during pollen development (Palme *et al.*, 1993), when rapid cell expansion is observed in the extending tip of germinating pollen tubes with a subsequent high vesicle production rate (Steer and Steer, 1989). Morre and van der Woude (1974) observed vesicle production rates of 1000 to 5000 vesicles per minute in pollen tubes of *Lilium* and *Tradescantia*.

The observation that the Psa-*rab* plant homologue, *vrab7*, is expressed during vesicular membrane proliferation, associated with *Rhizobium* infection and nodule formation, is consistent with a role for the plant vrab7 homologues in membrane formation in rapidly expanding tissue. The nodules expressing antisense *vrab7* produce only small nodules with abnormal vesicle formation. Expression of Psa-*rab* has also been observed to occur in pea leaf epidermal cells (Martin Cannell, personal communication). Perhaps expression of Psa-*rab*, associated with membrane proliferation, has a functional role in plant tissues which are undergoing cell expansion and elongation.

4.1.4 Complementation of the *YPT7* Null Mutant with Psa-*rab*

Functional classification of the effector-binding region of ras-related proteins has been extended to predict grouping of functional homologues (Haubruck *et al.*, 1989). Hence, phylogenetic comparisons may reflect function (Palme *et al*, 1993) (see CLUSTAL analysis, Figure 3.). This has led to the selection of plant and mammalian Rab homologues which successfully complement yeast strains with non-functional Ypt proteins (Cheon *et al.*, 1993; Palme *et al.*, 1993; Bednarek *et al.*, 1994). Thus, it was considered that the Psa-*rab* cDNA may be able to functionally complement the fragmented vacuole phenotype of the *Ypt7* null mutant, Y7L1.

Difficulties were experienced in inducing Y7L1 transformed with Psa-rab expression vector, pYP52 (see 3.1.5), which may be due to a reduction in vigour resulting in part from high expression levels from the GAL1 promoter. *Ypt1* mutant yeast strains complemented with the maize *Ypt1* homologues exhibited slow growth on minimal selective media with galactose (Palme *et al.*, 1993). Slow growth of the Y7L1 strain on glucose minus media was also observed by Dr. Dietrich Scheglmann (personal communication).

4.2 Investigation of the Genetic Basis for Lignification of the Pea Pod Endocarp

4.2.1 Lignification and Pod Endocarp Development in the Experimental Pea Lines

Histochemical staining with phloroglucinol established the onset of lignification in the endocarp of L59 and FF at around 6 DAF giving positive confirmation of a lignified endocarp phenotype and the corresponding, PV genotype. Lignified sclerenchyma were absent in the endocarp of L58 and L1390 at 6 DAF. The patches of lignin in the endocarp indicated by phloroglucinol staining of 12 DAF pods of L58 confirmed the partially lignified endocarp phenotype and Pv genotype. Pods of L1390 at 12 DAF did not lignify confirming the unlignified endocarp phenotype and pv genotype. The pattern of lignin formation in the pod endocarp was further characterised by examination of 1 μm resin embedded pod sections stained with toluidine blue. Distinct differences in endocarp morphology and development were observed. The differentiated endocarp with the characteristic meristematic layer which gives rise to the pre-sclerenchyma layer was absent in L1390 pods. The differentiated endocarp is present in L58. However, only a few of the pre-sclerenchyma cells undergo differentiation to form lignified sclerenchyma. The cells in the L58 endocarp which do differentiate to form sclerenchyma do so at a later stage of development than in L59.

4.2.2 Plant Growth Regulators and Pod Development

It is known that pod development is regulated by various plant hormones (Carbonell and Garcia-Martinez, 1980; Vercher *et al.*, 1987). Hence, it was considered that the abnormal development observed in the endocarp of L1390 and L58 pods may be due to some abnormality in the hormonal signalling required for normal pod development. The induction of pod development is thought to require correct temporal and spatial transduction of extracellular signals from the pollen and, or the fertilised ovule (Garcia-Martinez and Carbonell, 1980; Gillaspy *et al.*, 1993). Hence, plant growth regulators, GA3, 6-BAP and MVA known to be involved in fruit set and development were applied

directly to emasculated ovaries of L59, L58 and L1390 at day 0. GA3 applied to unpollinated ovaries has been observed to provide a major stimulus to enlargement of the mesocarp cells and differentiation of the endocarp (Vercher *et al.*, 1984). Cytokinins are well characterised as cell division factors (McGaw, 1988) and are also associated with early phases of differentiation to form lignifying xylem tissue (Aloni, 1982). Therefore, it was hypothesised that cytokinin may play a role in the cell division of the meristematic layer of the normal pod endocarp phenotype and, or subsequent differentiation to form lignifying sclerenchyma. MVA is the precursor to several compounds involved in fruit set and development (Bach, 1987; Gillaspy *et al.*, 1993). Application of MVA to unpollinated tomato produced parthenocarpic development. It was considered that exogenous application of MVA to the unpollinated pea ovaries may increase the precursor pools required for normal pod development. It was hoped that some correlation between pod endocarp phenotype and genotype, with regard to the hormonal signals received at day 0 would be identified.

A number of interesting observations were made with regard to development of the emasculated pods receiving plant growth regulator treatments. Endocarp phenotype remained true to the corresponding genotype, regardless of the plant growth regulator treatment applied or in the absence of plant growth regulator treatment, as in the tween treated controls (see Plates 3.5, 3.6 and 3.7). However, the growth of each pod in response to a particular plant growth regulator appeared to differ depending to which experimental line the pod belonged. For example the response of emasculated pods to GA3 varied. While GA3 applied to emasculated L59 pods enhanced the rapid growth and lignification of the endocarp associated with normal pods of L59, the GA3 treated pods of L58 and L1390 appear to exhibit a reduced response to GA3 treatment (see Table 3.2). Consequently the GA3 treated pods of L58 and L1390 are smaller than the respective normal fertilised pods, while GA3 treated pods of L59 are longer than the corresponding normal fertilised pods. However, GA3 does appear to stimulate endocarp development in L58 pods (Plate 3.6F) in comparison to the endocarp development of L58 normal fertilised pods (Plate 3.6A).

The cytokinin-treated pods from each line also varied in their individual response. L59 pods appeared to have a positive growth response to application of 6-BAP, as these pods were larger than the Tween-treated controls. The cytokinin 6-BAP appears to considerably stimulate lignification in the endocarp of L59, as although the pods are small in comparison to normal fertilised pods of L59, they exhibit a considerable degree of lignification (Plate 3.5D). A slight stimulatory effect with 6-BAP treatment was also observed for L1390, but the 6-BAP treated L58 ovaries were smaller that the Tween-treated controls. This inhibitory effect in L58 appears to be overcome by GA3/6-BAP mixed applications, producing pods of approximately the same size as the normal fertilised L58 pods. The effect of the mixed GA3/6-BAP application enhanced pod growth in L1390 compared to the corresponding single applications. In contrast, the GA3/6-BAP mixed application applied to L59 produced smaller pods than the single GA3 application. Hence, it appears that the reduced response of unpollinated ovaries to single applications of GA3 or 6-BAP in L58 and L1390 is associated with the ratio of GA3 and 6-BAP. Thus, addition of a mixed application of GA3/6-BAP produces an enhanced growth response.

Absisic acid has been shown to inhibit pod development. This effect may be overcome by GA3 application (Garcia-Martinez and Carbonell, 1980). If the experimental pods have different levels of absicic acid, different amounts of GA3 would be necessary to overcome inhibition. Hence, it is possible that sub-optimal amounts of GA3 were applied to L58 and L1390 pods preventing stimulation to develop similar to their respective normal fertilised pods. However, this explanation would appear to contradict the observation that the Tween-treated emasculated pod controls develop to a greater extent in L58 and L1390. Conversely, the L1390 and L58 pods may have lower levels of absicic acid and, or higher levels of endogenous GA3, or the GA12 and GA20 precursors. Hence, the application of GA3 may lead to supra-optimal levels resulting in a feedback mechanism, causing down regulation of reception and , or, processing of GA3, resulting in a reduced growth response. The GA3 stimulus in L59 produces a positive growth advantage, possibly by enhancing endogenous auxin-like compounds.

GA3 application has been observed to produce such an effect in other fruits (Sastry and Muir, 1963).

The response to MVA was inconclusive. The single application of MVA appears to slightly enhance pod growth in L59, and produce some growth inhibition in L58 and L1390. When mixed application of MVA/GA3 are applied, the pod growth in L58 and L1390 is similar to single applications of GA3. This suggests that MVA has little or no effect on pod growth or the putative inhibitory effect is overcome by GA3. The mixed application of MVA/GA3 to L59 pods produces pods slightly smaller than single applications of GA3 indicating that exogenous application of MVA may have an inhibitory effect on pod development. The possibility of MVA inhibiting pod growth when applied singly, or in mixed applications, could perhaps be due to some feedback inhibition causing a down regulation of HMG-CoA reductase and reduction of some limiting compounds involved in pod development. Although MVA application has been observed to induce normal development in tomato, it may be that the application method used in this instance resulted in poor uptake by the pea ovary. The concentration of MVA received by the treated ovaries may have been sub-optimal as a result of poor uptake by the ovaries, too low a concentration or degradation of the MVA.

It was observed that Tween-treated, emasculated, control pods of L58 and L1390 were larger that L59 Tween-treated emasculated control pods. Fruit set of the Tween-treated controls would appear to indicate that endogenous signals from the ovary or signals from the surrounding sporophytic tissues are sufficient to induce parthenocarpic fruit set in the experimental pea lines. Subsequently, inductive signals appear to be greatest in L1390, followed by L58, or conversely, levels of inhibitory signals are reduced. A source of inhibition of unpollinated pea ovaries has been located in developing leaves (Garcia-Martinez and Carbonell, 1980). Haan (1930) observed parthenocarpic development of emasculated ovaries of some pea varieties.

4.2.3 Differential Screening of the L59 Lignified Pod cDNA Library

It was hoped that differential screening strategies would yield cDNAs associated with the lignified pod phenotype. Hence, a L59 lignified pod cDNA library was prepared from L59 4 - 6 DAF pod total RNA, as it was observed that L59 pods undergo differentiation and formation of lignified sclerenchyma during this stage of growth (see 3.2.1 and 3.2.2).

The *in vivo* excision of trial inserts indicated the presence of a range of differently sized inserts, 0.5 kb - 2 kb. To test whether full-length cDNAs typical of developing pods were present, a test screen of the L59 lignified pod cDNA library was performed using Psa-*rab* as a probe. Three identical full-length clones derived from the Psa-*rab* gene were isolated and designated LP4, LP12 and LP13. The clones were slightly longer than Psa-*rab*, having additional 5′ non-coding sequence and 3′ UTR. Hence, the L59 lignified pod cDNA library was considered to be suitable for performing a differential screen using pod total cDNA probes from 4 - 6 DAF pods of L59 and L1390. Clones were selected for further characterisation on the basis of consistent hybridisation to the L59 (phenotype, lignified endocarp; genotype, PV) pod total cDNA probe and not to the L1390 (phenotype, unlignified endocarp; genotype, pv) pod total cDNA probe.

The method used to identify cDNA clones representing differentially expressed RNAs was based on that of Olszewski *et al.* (1989). This method relies on labelling cDNAs prepared from the total pod RNAs of the lignified pods of L59 with ^{35}S. Total pod RNAs from the unlignified pods of L1390 are labelled with ^{32}P. The ^{35}S and ^{32}P decay, producing β^- particles of 0.1674 and 1.710 MeV respectively (Weast and Astle, 1980). The energy difference provides a means of detecting β^- emissions from the ^{35}S particles originating from clones which hybridise to the lignified pod total cDNA probe and not to the L1390 total cDNA probe, by using an attenuator (3 MM paper in this case, see Figure 2.3). (The ability of the 3 MM paper to block ^{35}S β^- emissions was ascertained by trial radiographs of [^{35}S] dCTP and [32P] dCTP spotted on NC filters.) In the absence of the attenuator both isotopes are detected.

However, problems were encountered in detecting hybridised clones representing the differentially expressed RNAs using this differential screening protocol. The longer exposure necessary for detection of the ^{35}S hybridised clones on radiographs led to over exposure of ^{32}P hybridised clones. Also, the presence of the attenuator led to the film recording ^{32}P alone being slightly out of focus, even when the intensifying screens were blocked. Therefore, only colonies giving a clear indication of differential expression were chosen. Subsequently, low numbers of true positive clones were selected from the differential screen. Perhaps a greater number of clones hybridising to RNAs that are more abundant in the lignified pods of L59, compared to unlignified pods of L1390, would have been isolated if the density of plaques per hybridised primary screen filter was reduced.

4.2.4 Isolation and Identification of the cDNA Clone, pLP18

The cDNA clone pLP18, isolated from the differential screen of the L59 lignified pod cDNA library, was shown to encode a predicted polypeptide, PBP, with significant homology to a number of blue copper proteins. PBP has the conserved copper binding residues (Vangaard, 1972; Guss *et al.*, 1988) and two conserved Cys residues, which have been shown in Sc (Engeseth *et al.*, 1984) and CPB (Guss *et al.*, 1988) to form a disulphide bridge. Crystallographic studies have confirmed that the geometry of the copper binding site is that of a distorted tetrahedron as a result of the extended Cu^{2+}-methionine bond distance (Fields *et al.*, 1991). The other copper ligands are two histidines and a cysteine. These Cu binding residues are conserved in a number of type I blue copper proteins such as azurin, plastocyanin and rusticyanin. Comparison with the Cu binding sites of CBP, Sc and CPC suggest the following Cu binding sites in LP18: His67, Cys111, His116 and Met121. CuII and CuI occupy almost identical positions in blue copper proteins, thus facilitating electron transfer with minimal geometric change and a reduction in structural changes in the protein. By analogy PBP likely binds Cu^{2+} and functions as an electron carrier. The PBP predicted polypeptide has a hydrophobic N-terminal leader sequence. The genes of various blue copper proteins appear to encode a polypeptide with a leader sequence, which serves as a signal for protein translocation over the cytoplasmic membrane into the

periplasmic space, with subsequent cleavage of the signal peptide (Canters and Gilardi, 1993).

The region rich in threonine and serine in PBP (residues 127- 161) corresponds to the region rich in serine, hydroxy-proline and threonine residues in BCB and serine and hydroxy-proline rich in CPC. The threonine and serine rich region is followed by a hydrophobic C-terminal. Van Gysel *et al.* (1993) proposed that the hydrophobic C-terminal domain is enzymatically cleaved in analogy to homologous regions in the variant surface protein of *Trypanosoma* and the cell adhesion proteins of *Dictyostelium* (Ferguson *et al.*, 1985; Low *et al.*, 1986; Noegel *et al.*, 1986). By analogy with BCB and the truncated stellacyanin and CBP amino acid sequences isolated from plant tissues, this site may be cleaved in PBP to produce a mature, membrane-bound protein.

4.2.4.1 Genomic Analysis of LP18

There appears to be one gene encoding PBP per haploid pea genome. The small family of type I blue copper proteins identified in plants show highly conserved amino acid sequence homology in the copper binding region, with the lower overall homology perhaps indicating different biological functions. Hence, there may be other type I copper genes in pea, but sequence divergence is likely to be such that cross-hybridisation with *PBP* would only occur under very low stringency washing conditions.

4.2.4.2 Correlation of LP18 mRNA Expression with Lignin Biosynthesis

Semi-quantitative RT-PCR for LP18 mRNA provided a more sensitive analysis of gene expression than northern analysis of total RNA from 4 - 6 DAF pods confirming differential expression of LP18 mRNA and the relative expression pattern of LP18 mRNA in each experimental pea line at four, five and six days after flowering. The incorporation of synthetic RNA, rRLP18, in each reaction, gave a precise internal control for both reverse transcription and PCR amplification, using the same primers P1 and P2 and producing a product differing from the target RNA in size. This method of RT-PCR allowed the calculation of a

quantitative index for the relative expression of LP18 mRNA in each pod line at four, five and six days after flowering relative to the 0.025 ng of internal standard incorporated in the initial reaction.

Several ways of counting the hybridised bands were considered e.g. staining DNA with ethidium bromide in gels (not sensitive enough for small amounts of DNA, and inaccuracies due to ethidium bromide complexed to the lambda fragment in the internal standard bands); Cerenkov counting of dry filters using ^{3}H channel of a scintillation counter (assay of ^{32}P is approximately 25 % counting efficiency, deemed unacceptable for small amounts of DNA); counting dry filters in scintillation fluid, using ^{32}P channel of a scintillation counter (efficiency almost 100 %) (Wallace, 1987). Hence, counting of dry filters in scintillation fluid using the ^{32}P channel was selected as the most accurate estimation of the radioactivity in the hybridised bands and the most suitable for samples containing low counts.

Small amounts of genomic DNA contamination were encountered using the guanidium thiocyanate method for preparation of total RNA, hence, the DNase treatment. However, this method was selected to produce high yields from small amounts of tissue allowing rapid processing of numerous samples. The size band at 650 bp observed in the calibration RT-PCR reactions was presumed to be amplification of residual genomic DNA containing the gene corresponding to LP18 plus intron. The band is not observed in PCR with RLP18 only. A similar result is obtained by Chang *et al.* (1993), presumably again caused by contamination by an intron containing gene (they also used a guanidium thiocyanate RNA extraction method). The effect on data calculations was not considered by Chang *et al.* (1993) and was also not considered here, since the purpose of the calibration, with a series amount of internal standard, was to ascertain an amount of internal standard which does not produce excessive competition for primers etc. Conditions in subsequent RT-PCR reduced amplification of the putative intron containing LP18 gene sequence to a minimum unlikely to alter quantitative estimations. It is possible that a "plateau effect" resulting from attenuation of product accumulation may be occurring at the highest concentration of internal standard. A consequence of reaching plateau is that an initially low

concentration of, in this case, a putative contaminating genomic DNA can continue to amplify preferentially (Innis and Gelfand, 1992).

The calibration reactions performed with a series amount of internal standard RNA with a fixed amount of pod RNA (L59 in this case, chosen as northern hybridisation indicated this sample was likely to contain target) determines an amount of internal standard, which may be included in the RT-PCR reaction as a control to test reaction efficiency, and eliminate sample-to-sample and tube-to-tube variations. The optimal amount is ascertained to prevent excessive competition with target amplification. Subsequently 0.025 ng was selected, as this amount was close to the linear region of the plot and detectable on blots. A smaller amount of internal standard may have produced more accurate estimation of target RNA. However, the use of very low amounts of internal standard may cause greater fluctuations by amplifying pipetting errors. Therefore, 0.025 ng was chosen as being a reasonable amount close to the linear region.

RT-PCR correlates LP18 expression with lignin biosynthesis. Expression of LP18 mRNA increases in L59 and FF as lignification in the endocarp is imminent (4 - 6 DAF). This expression pattern is observed in L58, but LP18 mRNA levels are reduced by comparison with L59 and FF. Expression of LP18 mRNA is lowest in L1390 and remains stable through the same stages of pod development (4 - 6 DAF), perhaps reflecting basal levels of LP18 mRNA necessary for lignification in developing vascular tissue.

It was proposed that if LP18 mRNA was necessary for lignin formation, it would be expressed throughout the plant. RT-PCR results revealed this to be the case in all of the experimental lines, with each line showing comparable levels of expression in the respective root, stem and leaf tissues. Highest expression levels were observed in the stem for all of the experimental lines consistent with a role for the PBP encoded by LP18 in lignification of vascular and support tissues of the growing stem.

4.2.5 Isolation and Identification of the cDNA Clone, pLP19

The cDNA clone pLP19 isolated from a differential screen of the L59 lignified pod cDNA library, was shown to encode the C-terminal fragment of an Hsp70 homologue. High homology was observed with a number of Hsp70 proteins from a diverse range of species. However, highest homology was with the plant Hsp70s from soybean (89.7 %) (Roberts and Key, 1991), tomato (83.2 %) (Lin *et al.*, 1991a), petunia (81.3 %) (Winter *et al.*, 1988), maize (79.9 %) (Rochester *et al.*, 1986) and carrot (79.2%) (Lin *et al.*, 1991b). The C-terminal of Hsp70s are known to be more divergent than the amino terminal, which would further suggest that the high homology observed indicates that the C-terminal fragment predicted by LP19 is the counterpart in pea of the plant Hsp70s listed above. The LP19 predicted amino acid sequence contains a motif, GPKIEEVD (residues 264 - 271), similar to the eukaryotic Hsp70 cytosolic consensus sequence, GPTIEEVD (Gupta *et al.*, 1994), suggesting that LP19 encodes the C-terminal of a cytosolic Hsp70. The other identifying feature of cytoplasmic Hsp70s is the lack of a leader sequence (Wierling, 1991). However, the absence of a leader sequence could not be verified as re-screening the L59 lignified pod cDNA library failed to yield a full-length LP19 cDNA.

4.2.5.1 Differential Expression of LP19 mRNA in Pods of the Experimental Pea Lines

The expression pattern of LP19 mRNA in 4 - 6 DAF pods indicates some correlation with the lignified endocarp phenotype. Expression is highest in pods of L59 and FF, which both have a differentiated endocarp with lignification commencing at around 6 DAF. Expression levels of LP19 mRNA are lower in L58, which has a differentiated endocarp, but differentiation of the pre-sclerenchyma layer is delayed and reduced, with small numbers of lignified cells only present in pods at a later stage of development. LP19 mRNA expression is absent in L1390 4 - 6 DAF pods, which do not have a differentiated lignified endocarp. This pattern of expression is also observed in northern dot blot analysis of 4 - 6 DAF pod total RNA from L59, FF, L58 and L1390. Genomic analysis revealed hybridisation to a single band in the experimental pea lines, L59, L58 and L1390, indicating that the LP19

gene is present in L1390 and the LP19 nucleic acid sequence from L59 is specifically hybridised to the corresponding sequence in L1390. This would suggest that the lack of hybridisation of the LP19 cDNA to L1390 4 - 6 DAF pod total RNA is due to an absence of LP19 expression or expression at levels too low to be detected.

A number of transcript sizes were detected in the northern blot of 4 - 6 DAF pod total RNA. *Hsp70* transcripts of varying size have been observed by other researchers (Rochester *et al.*, 1986; Winter *et al.*, 1988). Large transcripts of 2.6 - 2.8 kb are presumed to be unprocessed mRNAs of the corresponding intron-containing *Hsp70* gene and smaller transcripts of 2.1 - 2.2 kb are presumed to be the processed transcript of the corresponding Hsp70 amino acid sequence (Rochester *et al*, 1986; Winter *et al.*, 1988). The putative unprocessed transcript is the major transcript apparent in 4 - 6 DAF pods of L59 and FF and is the only detectable transcript in L58. The processed transcript is detectable in L59, indicating a post-transcriptional control mechanism for LP19 expression. The 1.1 kb transcript may be a LP19 mRNA breakdown product, which hybridises to the 3' end of the LP19 mRNA. A similar hybridised transcript of 1 kb has been observed in northern analysis of maize *hsp70* (Rochester *et al.*, 1986).

Hsp70s have been proposed to play a role in differentiation and development (Bienz, 1984; Singh and Yu, 1984; Kurtz *et al.*, 1986; Lindquist, 1986). It is proposed that the lack of processed transcripts in L58 may be a result of the delay in commencement of differentiation and lignification of the discrete regions of pre-sclerenchyma in L58. Hence, LP19 mRNA expression may indicate the differentiation event occurring in the pre-sclerenchyma of the pod endocarp. Subsequently, there is no detectable LP19 mRNA expression in L1390 4 - 6 DAF pods, as the pre-sclerenchyma layer is absent. Expression of tomato *Hsc70* has been detected in secretory tissue and organs with rapidly dividing and differentiating cells (Duck *et al.*, 1989). It has been suggested that some conserved developmental function requires the aid of *Hsc/Hsp70* in ovaries (Winter and Sinibaldi, 1991).

Some of the *Hsp70* genes are classified as cognate (*Hsc*) genes, which are expressed at normal temperature and contain an intron. The LP19

Hsp70 is expressed at normal growth temperatures, but the presence of an intron can only be inferred by the presence of a putative unprocessed transcript in northern blots, similar to other plant *Hsp70s* (Rochester *et al.*, 1986; Winter *et al.*, 1988). However, this definition is further confused by the reports of heat-induced expression of maize*Hsp70s* which contain introns (Rochester *et al.*, 1986).

4.2.5.2 Heterogeneous 3′ UTRs of the LP19 mRNA Transcripts

The 3′ termini encoded by the LP19 transcripts isolated from the re-screen of the L59 lignified pod cDNA library (see 3.6.5) are identical up to the differing poly (A) addition sites, suggesting that they are all transcribed from one gene. This is further supported by genomic blot analysis, which indicates that the LP19 gene was present as a single copy gene and that hybridisation was specific at high stringency. Multiple polyadenylation sites have been observed in other eukaryotic genes (Setzer *et al.*, 1982; Parnes *et al.*, 1983; Yassemi *et al.*, 1983; Dean *et al.*, 1986; Hernandez-Lucas *et al.*, 1986).

Only the LP19/1 and LP19/2 transcripts have a polyadenylation signal, while the other heterogeneous LP19 3′ UTRs do not. The absence of polyadenylation signals in several of the heterogeneous 3′ UTRs of the mouse dihydrofolate reductase gene has also been observed (Setzer *et al.*, 1982). Dean *et al.* (1986) proposed that binding of proteins to different terminator sequences in the 3′ untranscribed region could alter the selection of the 3′ cleavage site.

Heterogeneous 3′ UTRs have been implicated in mRNA translation efficiency or stability (Gallie *et al.*, 1993). It may be that the heterogeneous 3′ UTRs exhibited by LP19 transcripts may play a role in mRNA stability. Ingelbrecht *et al.* (1989) observed a 60-fold difference in mRNA levels using different 3′ UTRs on reporter genes. Differentiating cells have a varying mRNA population depending on the stage of differentiation. Subsequently, rapid changes in the differentiated state of a cell will necessitate rapid changes in RNA transcription. Developmental regulation of 3′ end cleavage and poly (A) site addition has also been observed in the case of vimentin (Yassemi *et al.*, 1983) and pro α2(I) (Aho *et al.*, 1983). Hence, it may be

expected that if the LP19 *Hsp70* is involved in the differentiation event associated with pre-sclerenchyma in the pod endocarp, as suggested above, it would require tight transcriptional or translational control. This may be facilitated by altering the stability of the LP19 mRNA transcripts as cell differentiation and development progresses.

5. CONCLUDING DISCUSSION.

The clone pPP406 encoded a polypeptide, designated Psa-*rab*, belonging to the Rab sub-family of small GTP-binding proteins, which are involved in intracellular vesicular transport (Zahraoui *et al.*, 1989; Plutner *et al.*, 1990; Horazdovsky *et al.*, 1994). Northern analysis revealed invariant expression of Psa-*rab* in the different pod phenotypes investigated in this study, indicating an essential role for Psa-rab in developing pods. The observation that a number of plant *Rab* homologues are expressed in rapidly expanding tissues (Cheon *et al.*, 1993; Palme *et al.*, 1993) is consistent with the observation of Psa-*rab* expression in developing pods. Psa-rab may be associated with the active production of secretory vesicles directed to the provision of new components for the rapid expansion of the plasma membrane and cell wall during the rapid expansion phase of pod growth. One of the Rab family proteins is highly expressed in tomato fruit during the rapid expansion phase of growth (Gillaspy *et al.*, 1993).

A successful conclusion to the yeast complementation experiments subsequent to submission of this thesis would confirm the assumed functional role of Psa-rab. Transgenic pea lines expressing sense and antisense Psa-*rab* would be useful to enable further analysis of the functional role of Psa-rab. This approach has been successful with *rgp1* from rice (Kamada *et al.*, 1992) and *vrab1* and *vrab7* from *Vigna* (Cheon *et al.*, 1993).

Two differentially expressed clones, pLP18 and pLP19, encoding a putative blue type I copper protein, designated PBP, and the C-terminal of an Hsp70 homologue respectively, were isolated from a pea pod cDNA library representing poly $(A)^+$ RNA purified from L59 pea pods at 4 - 6 DAF (with embryos and main veins excised). The clones, pLP18 and pLP19, were isolated on the basis of apparent differential expression on screening the L59 pod cDNA library with total cDNA probes prepared from 4 - 6 DAF pod mRNAs from L59 (lignified endocarp) and L1390 (unlignified endocarp).

The predicted polypeptide encoded by LP18, PBP, has significant homology to a number of blue type I copper proteins. In analogy to these blue copper proteins, PBP probably binds copper and functions as an electron carrier. A putative signal peptide, residues 1 - 24, indicates that PBP may be translocated over the cytoplasmic membrane in analogy to the function of signal peptides of other blue copper proteins (Canters and Gilardi, 1993). The serine/threonine rich domain and the short hydrophobic C-terminal may play a role in membrane anchorage and/or targeting, as suggested for BCB (Van Gysel *et al.*, 1993). The higher expression levels and the pattern of expression in the experimental pods of L59 and FF compared to L58 and L1390 is consistent with a role in development of the lignified endocarp phenotype. In addition, the high expression of LP18 mRNA in stems from the pea lines L59, L58 and L1390 is also consistent with a role in lignification. It is suggested that this protein is involved in oxidative polymerisation reactions characteristic of lignification in plant cells (Dean and Eriksson, 1992). The cross-linking enzymes giving rise to lignin in plant cell walls are oxidases, including copper oxidases (Williams, 1988; Ohkawa *et al.*, 1989; McDougall, 1991; O'Malley *et al.*, 1993; Sato *et al.*, 1993; Liu *et al.*, 1994). The blue copper protein, PBP, may work in series with such oxidases to transfer electrons generated by the free radical polymerisation reactions of lignin monomers to each other or to cell wall components.

The C-terminal fragment of an Hsp70, encoded by the LP19 cDNA may also be correlated with the lignified endocarp phenotype. Expression is greatest in the L59 and FF pods (phenotype, lignified endocarp; genotype, PV), with reduced expression in L58 (phenotype, partially lignified endocarp; genotype, Pv), and no detectable expression in L1390 (phenotype, unlignified endocarp; genotype, pv). The transcriptional regulation of the *Hsp70* gene family is known to be very complex and they are implicated in a number of functional roles in cells (Lindquist, 1986). Hsp70 family members recognise and stabilise partially folded intermediates during polypeptide folding, assembly and disassembly (Beckmann *et al.*, 1990; Nelson *et al.*, 1992) and are involved in receptor recycling (Hutchison *et al.*, 1992; Pratt, 1993). They may be stress inducible (Fernandes *et al.*, 1994), while others may be expressed constitutively (Benaroudj *et al.*, 1994), or be induced at specific stages of

development (Hatayama *et al.*, 1993; Winter and Sinibaldi, 1991). The complexity of the *Hsp70* gene family is further indicated here, with the putative post-transcriptional processing of mRNA transcripts observed in northern analysis (see Figure 3.28) and the isolation of LP19 mRNA transcripts with heterogeneous 3′ UTRs (see Figure 3.33). The expression pattern of the LP19 *Hsp70* transcripts and the evidence for the involvement of Hsp70s in differentiation and development are consistent with the LP19 *Hsp70* homologue playing a role in the differentiating events in the pod endocarp leading to the formation of lignified sclerenchyma. LP19 expression may be regulated in the differentiating cells by some mechanism involving the generation of heterogeneous 3′ UTRs and subsequent RNA processing. The 3′ UTR of *Hsp83* in *Leishmania* is involved in temperature-dependent, regulated decay (Aly *et al.*, 1994). The *Leishmania* parasite undergoes stage differentiation triggered by a change in temperature, which results in an increase in steady state levels of *Hsp83* transcripts by a differential decay mechanism. The 3′ UTRs of several other genes are implicated in gene regulation and RNA processing (Birnsteil *et al.*, 1985; Petersen and Lindquist, 1988; An *et al.*, 1989; Ingelbrecht *et al.*, 1989). Further clues as to the mechanisms controlling LP19 expression may be gained by sequencing the LP19 gene. The identification of a promoter(s) and terminator(s), and the presence, or absence of an intron, would be useful to further characterise this particular pea *Hsp70*.

Conclusive proof of the involvement of the blue copper protein, PBP, and the LP19 Hsp70 homologue in development of the lignified endocarp phenotype awaits localisation in the pods of the experimental pea lines.

The PGR treatments failed to produce any radical change in the cell fate pathway of the cells of the inner pod wall in any of the pod phenotypes (see Plates 3.5, 3.6 and 3.7). The PGRs would appear to provide necessary stimuli for pod growth to a varying degree. However, they do not appear to control the formation of the differentiated lignified endocarp, although they can enhance, or inhibit its development where present (see Plates 3.5 and 3.6). This is particularly notable with regard to lignification in the endocarp of 6-BAP treated pods of L59,

which are small, but have a relatively advanced degree of lignification compared to the normal fertilised pods of L59.

The observation that tween treated control pods developed parthenocarpically would appear to indicate that endogenous stimuli and, or, stimuli from surrounding tissue also plays a significant role in pod development. This is also the case in developing tomato fruit (Gillaspy *et al*, 1993). Furthermore, levels of such stimuli would appear to vary in the different experimental pea lines. The experimental pea lines are closely related genetically. Hence, the differences in sensitivity to PGRs and the degree of parthenocarpic development may be linked to the pod genotypes.

There is strong evidence that each cell determines its position relative to others during development and differentiates accordingly, implying cell-cell communication (Verbeke, 1992). The gene programme for each cell can then be set or re-set in response to extracellular stimuli from surrounding tissues or adjacent cells (Greenwald and Rubin, 1992). Those cells receiving and transducing specific signals from surrounding cells and tissues respond by differentiating. Those not receiving or failing to transduce such signals follow the default pathway. Positional signalling occurs within groups of cells (Poethig, 1987; Dave and Freeling, 1991). Stochastic elements create the initial difference and feedback from surrounding cells confirm this initial difference by altering signalling or receiving potential. The cell recognition mechanisms are thought to involve the binding of signal molecules with membrane bound receptors (Verbeke, 1992). Hence, it may be hypothesised that the fate of the cells destined to form the pod endocarp are responding to positional effects and the subsequent perception of extracellular signals from surrounding cells and tissues. The completion of normal endocarp development then depends on successful transduction of these signals. If this is the case it would appear that this chain of events is disrupted in L58 and L1390.

The tissues that contribute to the growing fruit differentiate from cells in the carpel (Blixt, 1974). Hence, it is suggested that stochastic elements at the very early stages of ovary formation in the carpel of L1390 would appear to have failed to produce the initial differences in

the cells destined to form the inner pod wall. The gene programme of the cells of the inner pod wall then fail to set and subsequently follow a default pathway in pods of L1390. Conversely, the cells in the carpel destined to form the inner pod wall in L58 do respond to stochastic elements, creating the differences which lead to setting the gene programme for endocarp differentiation. Hence, it would appear that one functional P or V allele is sufficient for this early stage of endocarp formation. It is at a later stage of development that some of the cells of the pre-sclerenchyma layer of L58 pods fail to complete the gene programme for differentiation to form lignified sclerenchyma. It is suggested that the cell lineage resulting from cell division in the endocarp predicts those cells of the endocarp which differentiate. Cells in the carpel destined to form the endocarp then ultimately acquire a unique genetic programme depending on their position in the endocarp, analogous to the patterning processes described for other plant organs and tissues (Wareing and Philips, 1970; Poethig, 1987; Steeves and Sussex, 1989; Doerner, 1993; Dolan *et al.*, 1994). Different patterns of lignification are observed in the partially lignified endocarp of pV and Pv pods. The pV pods have a strip of lignified sclerenchyma close to the main veins, whereas the Pv pods form patches of sclerenchyma (Lamprecht, 1953; Bowling and Crowden, 1973). The P and V alleles may therefore direct differentiation specific events of different regions of the endocarp depending on the cell lineage.

However, the intricate cascade of signal pathways directing cell differentiation to form the differentiated endocarp phenotype are likely to remain elusive until the mechanisms controlling expression of the genes that are uniquely and highly expressed in the carpels and growing pod are characterised. Differential display techniques using mRNA isolated from the carpel of L59 and L1390 may enable the isolation of genes expressed at the early stages of pod development. Morphological analysis, tracing the origin of the endocarp cells, the pattern of cell division, cell expansion and cellular differentiation, may provide a more meaningful analysis of subsequent genetic analysis.

The "symptoms" exhibited by pods of pea lines carrying the p and v mutant alleles have been observed to be complex. However, differential screening as an investigative approach has provided some

insight as to the biochemical complexity of these effects, such as the differential expression of mRNAs encoding the putative blue copper protein and a member of the Hsp70 family of proteins. Investigation of such complexities is ultimately necessary for a complete and coherent description of the differentiation and development of the lignified endocarp phenotype. In addition, knowledge obtained may be of use in studying other tissues undergoing differentiation and , or lignification.

Differential display techniques have been used more recently to isolate differentially expressed mRNAs. However, the availability of commercially available cDNA library construction kits and the standard protocols developed for differential screening still make differential screening strategies a viable approach to isolating differentially expressed mRNAs. In addition, refinements such as subtractive hybridisation to eliminate or reduce the population of mRNAs encoding housekeeping genes and PCR techniques allowing construction of cDNA libraries from small amounts of tissue (making construction of a cDNA library solely from the pod endocarp possible) could lead to greater success in isolating differentially expressed mRNAs associated with the lignified endocarp phenotype.

The alternative approach, positional cloning, to identify the mutant genes at the P and V loci and determination of their function, presents an enormous task, perhaps exemplified by the efforts to identify mutant genes causing genetic diseases in humans. However, restriction-fragment-length-polymorphism (RFLP) techniques have proved of some use in the diagnosis of monogenic diseases in humans (see Davies and Tilghman, 1991). The construction of RFLP maps for *Pisum* and the identification of marker genes is presently being undertaken (Dr. John McCallum, personal communication) and may provide a useful resource for location of mutant genes of pea in the future. RAPD (random amplified polymorphic DNA) analysis has also been investigated as a means of identifying polymorphisms amplified by arbitrary primers, which can be used as genetic markers (Williams *et al.*, 1990). RFLP and RAPD analysis strategies may be employed using the experimental lines, L59, L58 and L1390, which are all closely related genetically, together with heterozygotes for the P and

V alleles obtained by crossing the experimental lines to confirm mutations in the gene fragments.

Ultimately, the identification and cloning of mutant genes and the study of their function requires an integrated approach combining molecular biology, biochemistry, genetics, together with an understanding of the anatomy and physiology of the observed phenotype(s) attributed to the mutant gene(s) under investigation.

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