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PISUM SATIVUM L.

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ABSTRACT

The temporal and spatial patterns of differential *leg*A expression during early *Pisum* embryogenesis have been examined. An *in situ* hybridisation protocol has been empirically refined to allow analysis of low level *leg*A expression, at the mRNA level, whilst ELISA and immunocytochemistry have been used to examine both protein levels and spatial distribution within the early seed.

Low levels of *leg*A mRNA were detected in very early globular embryos which were present at 4-5 d.a.f. High levels of *leg*A expression were detected in the transient endosperm tissue at both the mRNA and protein level. High levels of expression, at the mRNA level, were also detected within the suspensor, although a similar level of expression at the protein level was not detected. It is proposed that *leg*A expression within both the endosperm and suspensor is of nutritional significance for the development of the embryo.

Comparison of *leg*A mRNA and protein localisation studies, at later stages of embryo development, demonstrated a very strong correlation, both quantitatively and spatially, between mRNA and protein levels, indicating the importance of transcription level regulation in the control of differential *leg*A expression.

A cDNA complementary to a mRNA species present at elevated levels in *Pisum* root was used to examine embryonic root (radicle) ontogeny. The mRNA species was found to be absent from embryonic root, however its presence in primary roots of seedlings four days post imbibition has been demonstrated.

CONTENTS

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ABBREVIATIONS

Abbreviations were used according to the Biochemical Journal's *Instructions to Authors*, Biochem. J. 209: 1-27 (1983), with the following additions:-

ABTS	Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
d.a.f.	days after flowering
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
Etd Br	Ethidium bromide
GAR	Goat-anti-rabbit
hnRNA	heterogeneous nuclear RNA
HGT	high gelling temperature
Kb	kilobase
LMP	low melting point
Mr	relative molecular weight
mRNA	messenger ribonucleic acid
MOPS	3-[N-Morpholino]propanesulphonic acid
nRNA	nuclear RNA
O.D.	optical density
O.L.B.	Oligonucleotide labelling buffer
PBS	phosphate buffered saline
PBST	phosphate buffered saline plus 0.5% Tween

PBSTB	phosphate buffered saline plus 0.5% Tween; 0.1% BSA
PEG	polyethylene glycol
PVP	poly vinyl pyrrolidone
RAG	Rabbit-anti-goat
SDS	sodium dodecyl sulphate
Tris	[2-amino-2-(hydroxymethyl)propane-1,3-diol,(tris)]
tRNA	transfer ribonucleic acid
u.v.	ultra-violet
5'	5' terminal phosphate of DNA/RNA molecule
3'	3' terminal hydroxyl of DNA/RNA molecule

ABBREVIATIONS USED IN THIS THESIS

AM	apical meristem
C	corpus
CC	central cylinder
Chl	chloroplast
CI	columella initials
CP	cotyledon primordia
COT	cotyledon parenchyma
E	embryo
EB	embryo sac boundary wall
EP	epidermis
EN	endosperm
ES	extra-embryonic sheath
GM	ground meristem
INT	integument/ovular tissue
NO	nucleolus
P	protoderm
PA	parenchyma
R	radicle
S	suspensor
ST	starch
T	tunica
V	vascular elements

1: INTRODUCTION

1.1 GENERAL INTRODUCTION

The main aim of this research was to investigate angiosperm embryogenesis using a variety of morphological, cytological and molecular techniques. *Pisum* was chosen as a model system for three main reasons:- (i) the relatively large size of the embryo; (ii) the non-cellular endosperm allows comparatively easy access to the embryo; and (iii) the importance of *Pisum* as a food crop. Within the mature seed of *Pisum* the storage protein, legumin, is differentially expressed, being synthesised and deposited mainly within the cotyledon parenchyma cells. It was therefore proposed to use a probe, to *leg*A mRNA, as a "molecular marker" of cotyledon parenchyma cell development during early embryogenesis. Via this approach it was hoped to follow cell lineages in the developing embryo and thereby ascertain the role of cell fate and cell division in the process of differentiation and ontogeny during embryo development. However, a uniform low level, cytoplasmic distribution of *leg*A expression was detected throughout the early embryo. The *leg*A cDNA probe was therefore found to be of limited value as a "marker" of cell lineage patterns. Differential levels of *leg*A expression were, however, detected within the various components of the early seed. Therefore application of the *leg*A probe did provide information on early seed formation, the molecular events underlying storage protein gene regulation and the possible role of legumin in embryo nutrition.

The advent of DNA manipulation techniques, which allow analysis of gene structure, and molecular hybridisation techniques, that enable quantification of gene number and gene product, have resulted in analysis of the molecular events underlying developmental processes in eukaryotes. Despite being morphologically simple, (during the life cycle of a flowering plant only three vegetative organ systems, leaf, stem and root, and three reproductive organ systems, petal, stamen

and pistil, are formed), plants have molecular genetic processes equivalent in complexity to those found in animals. Plant genomes are as large and complex as those found in the Animal Kingdom (Goldberg, 1983a; 1986). DNA sequencing studies and functional analysis of plant genes in transformed plants indicate that they possess developmental control elements, splice junctions and promoters analogous to those found in the Animal Kingdom (Heidecker and Messing, 1986). In addition, both transcriptional and post-transcriptional processes have been shown to be involved in the regulation of plant gene expression (e.g. Chappell and Chrispeels, 1986). Plants therefore provide a morphologically simple biological system for the analysis of eukaryotic gene expression.

The majority of information concerning gene expression in higher plants has been obtained from a few intensively studied systems in which the genes encode abundant product, i.e. seed storage protein genes (Goldberg *et al.*, 1981a and b; 1983b; Gatehouse *et al.*, 1982), light regulated genes (Kuhlemeier *et al.*, 1987); gene responses to environmental pertubation, e.g. sulphur deficiency (Evans *et al.*, 1985; Beach *et al.*, 1985), heat shock (Schoffl *et al.*, 1986; Baumann *et al.*, 1987) and wounding (Thornburg *et al.*,1987). The advent of gene transfer technology, which allows such novel genetic traits as insect and herbicide resistance to be introduced into crop plants (e.g. Shah *et al.*, 1986; Vaeck *et al.*, 1987) and the possibility of improving the nutritional status of seed crops via genetic manipulation, has resulted in renewed interest in the physiological, biochemical and molecular processes in plants.

Seed storage proteins represent an extremely attractive experimental system for two primary reasons:-

(i) The genes encoding these proteins are expressed at high levels and only in the tissues of the seed at a single point in the life cycle of the plant (Goldberg *et al.*, 1981a; Gatehouse *et al.*, 1982). The high degree of spatial and

temporal control of these genes provides an excellent system for the study of differential gene regulation.

(ii) The nutritional importance of seed crops for both humans and livestock. Plants directly supply 90% of human calorie intake and 80% of protein intake, the remainder being derived from animal products which have ultimately derived their nutrition from plants (Mantell *et al.*, 1985). As the global human population continues to expand there is concern about the finite number of people that world agriculture can support (Chrispeels and Sadeva, 1977). Hence there is a great need for the development of high yielding crops and improved agricultural practices.

Most cereals are deficient in lysine and threonine whilst legumes are deficient in sulphur-rich amino acids (Higgins, 1984). The genes encoding a number of seed storage proteins have been cloned and characterised, e.g. legumin and vicilin (Croy *et al.*, 1982), phaseolin (Sun *et al.*, 1981), and hordein (Forde *et al.*, 1981). The possibility of altering the amino acid composition of storage proteins to produce seeds of greater nutritional value via site-directed mutagenesis of the encoding gene (Mantell *et al.*, 1985), or of introducing additional novel proteins into the seed via vector mediated gene transfer (Murai *et al.*, 1983; Ellis *et al.*, 1988), is an attractive prospect. Plants are amenable to such forms of manipulation since in many species entire fertile plants can be grown from single cells in culture (Steward, 1958). Interest has centered mainly on dicotyledons since in cereal crops protoplast regeneration is difficult and no natural vector system is available (Mantell *et al.*, 1985). Regulation of seed storage protein genes, however, is tightly controlled and it is essential that this control is maintained over the inserted genes and that they are expressed in the correct developmental context. In addition, correct packaging of the proteins within the cell may be perturbed as a result of alteration of their amino acid structure. Therefore a complete

knowledge of all the processes that occur during seed development is required before DNA recombinant technology can be utilised to produce crops of superior nutritional quality.

1.2 ANGIOSPERM EMBRYOGENESIS

Plants are characterised by a life-cycle that exhibits an alternation of a diploid spore producing generation (sporophyte) and a haploid gamete-producing generation (gametophyte). In vascular plants the sporophytic phase dominates and the gametophyte is dependant upon the sporophyte for nutrition and support (Esau, 1977). The most highly evolved class of plants, the Angiospermae, has evolved a specialised reproductive structure, the flower, whose function is the production of eggs and sperm. The egg is formed within the female gametophyte (embryo sac) which is enclosed within the integument and nucellus layers of the ovule whilst sperm are produced within the anther locule. Following fertilisation, the enclosed zygote enters a pathway of cell division and differentiation to produce an embryo. The outer integument of the embryo sac differentiates into the testa (seed coat) and the funiculus, connecting ovule to ovary, degenerates resulting in a nutritionally closed system. Finally, desiccation occurs, the ovule loses water to the surrounding environment and the testa sclerifies and dies. The resulting seed which consists of a dormant embryo encased in a protective coat is shed as an independent propagule (Dure, 1975).

1.2.1 Gamete Formation and Fertilisation

During embryo sac formation meiotic division of the megasporocyte (product of archesporial cell division) results in the formation of a linear tetrad of haploid megaspores. Generally only one persists to produce, via three mitotic divisions, the eight nuclei characteristic of the mature female gametophyte. The

egg apparatus, consisting of an egg nucleus flanked by two pear-shaped synergids arranged in a triangular configuration, is located at the micropylar end of the embryo sac. Three nuclei are located at the chalazal pole and termed the antipodals whilst two are centrally located within the embryo sac and termed the polar nuclei (Jensen, 1964; Esau, 1977; Kapil and Bhatnagar, 1981). The mature embryo sac derived from a single megaspore, as described, is termed the monosporic type. Not every angiosperm species follows this pattern with deviations depending upon the number of megaspores participating in embryo sac development (Raghavan, 1986).

Ultrastructural and histochemical analyses have revealed putative functions for these nuclei (Kapil and Bhatnagar, 1981). The synergids are thought to perform three main roles: the absorption and transport of nutrients into the embryo sac, the production and secretion of chemotrophic substances which attract the pollen tube and entry of the pollen tube and dissemination of male gametes (e.g. soybean, Dute *et al.*, 1989). The central cell, which is formed by fusion of the polar nuclei, is characterised by a high rate of metabolic activity and contains adequate food reserves for utilisation during fertilisation and initial growth of the endosperm (Schulz and Jensen, 1973). Analysis of the egg cell indicates that it exists in a quiescent metabolic state prior to fertilisation with adequate reserves of starch, lipid bodies and ribonucleoproteins. In both cotton (Jensen, 1965) and *Capsella* (Schulz and Jensen, 1968a) the egg is strongly polarised with a large vacuole towards the micropylar end and an aggregation of cytoplasmic elements towards the chalazal pole. This polarity is thought to be significant in determining the initial cleavage pattern of the developing zygote (Graham and Wareing, 1984).

Production of male gametes involves meiotic division of the microspore mother cell to produce a tetrad of four microspores that mature into pollen grains. During gametogenesis the nucleus of the pollen grain divides asymmetrically to

produce a large vegetative nucleus and small generative nucleus (Heslop-Harrison, 1971; Esau, 1977). After transfer of the pollen grain to the stigmatic surface, the generative and vegetative nuclei migrate into the emerging pollen tube. The generative nucleus divides to produce the two sperm involved in the event that is unique to angiosperms - that of double-fertilisation within the embryo sac (Raghavan, 1986). One of the male gametes fuses with the egg cell to produce the zygote. The second fuses with the previously fused polar nuclei to produce the triploid primary endosperm nucleus, repeated division of which produces the nutritive endosperm tissue.

1.2.2 Embryo Development

The ontogenic events which occur during embryogenesis establish the morphological pattern of the embryo and produce the meristematic tissues required for post-germination development (Goldberg *et al.*, 1989). Embryo ontogeny has been described in a number of species (Maheshwari, 1950; Davis, 1966). Although embryo ontogeny varies among the taxa, the following is a general description of embryogenesis in the Dicotyledoneae in terms of morphology, structure and cellular patterns.

The polarity established in the egg is maintained in the zygote, e.g. *Capsella*, (Schulz and Jensen, 1968a), soybean (Dute *et al.*, 1989). In cotton (Jensen, 1968) cytoplasmic organelles begin to flow around the egg after fertilisation and take up positions at the chalazal end, forming an investment around the nucleus, accentuating the polarity already present in the unfertilised egg. The first zygotic division is generally transverse and asymmetric (Wardlaw, 1955; Jensen, 1964), and results in the formation of a large basal cell and a small apical/terminal cell (though there are some exceptions, Sivaramakrishna, 1978). This first asymmetric division is thought to be as a result of zygote polarity and indicates the presence

of an underlying cytoplasmic programme that determines the position of the mitotic spindle and the orientation of the cell wall (Raghavan, 1986). In general, the division products of the small apical cell give rise to the embryo proper, whilst derivatives of the basal cell produce the suspensor, e.g. cotton (Jensen, 1964), *Capsella* (Schulz and Jensen, 1968a). However, there is considerable variation in the degree to which the basal cell contributes to the formation of the organogenetic part of the embryo and subtending suspensor. On the basis of these variations and the plane of division, five different types of embryo ontogeny have been described by Maheshwari (1950).

Soueges (1937) developed a complex set of laws to explain angiosperm embryogenesis based upon the origin of cells, the number of cells produced per generation and the deterministic fates of cells produced during the early division phase of embryogenesis. However, although the early division sequences of embryo development are extremely regular in some species, e.g. *Capsella* (Soueges, 1919), allowing designation of certain cells as giving rise to specific organs in mature seeds, in others, e.g. cotton (Jensen, 1964), early cleavage patterns are highly irregular.

Continued division of the terminal cell results in the formation of an eight-celled embryo, periclinal divisions of which produces eight external protodermal cells and eight internal cells (Raghavan, 1976; 1986). Cells of the embryonic protoderm continue to divide anticlinally to keep pace with the increasing volume of the embryo, to produce what is termed the globular embryo. A striking event in embryogenesis is the transition from the globular embryo, with radial symmetry, to heart-shaped embryo with bi-lateral symmetry and polarisation to produce the root/shoot axis (Goldberg *et al.*, 1989). Lateral expansion of the embryo results from periclinal divisions in the terminal lateral lobes, resulting in the formation of cotyledon primordia, which produce the so-called heart-shape embryo. Division

and differentiation of cells in the basal tier of the embryo produces the hypocotyl. During cotyledon initiation a mound of rapidly dividing cells becomes organised in the depression between the cotyledons; this mound gives rise to the future shoot apex. Differentiation of the shoot apex is via a concentration of mitoses in the apical portion of the embryo with subsequent differentiation into peripheral and central zones (Mahlberg, 1960). The embryo either remains in this form with continued division resulting in the enlargement of cotyledons only (e.g. pea, Marinos, 1970a) or an increase in the number of divisions in the axis results in the extension of the axis and the production of a torpedo-shaped embryo, e.g. cotton and *Capsella*. Ontogeny of the embryonic root (radicle), consisting of root meristem and root cap, varies depending upon the extent to which these tissues are formed from derivatives of the apical or basal cells. For example, in *Capsella* the embryonic root cap is derived from a single cell of the suspensor termed the hypophysis (Schulz and Jensen, 1968b).

The embryo therefore consists of two organ systems - the axis, comprising the root and shoot meristems, and the cotyledons, which are terminally differentiated organs that senesce during germination (Goldberg *et al.*, 1989). Analysis of DNA-RNA hybridisation results, using mRNA populations obtained at different times during soybean embryo development, indicated that approximately 20000 diverse genes were expressed at the cytoplasmic mRNA level (Goldberg, 1986). This mRNA complexity is equivalent to that found in mature plant organs (Kamalay and Goldberg, 1980) and sea urchin gastrula embryos (Galau *et al.*, 1974). The level of gene expression during soybean embryogenesis was found to remain relatively constant throughout development (Goldberg, 1986). This contrasts with animal embryo development where a reduction in the number of genes expressed, i.e. cytoplasmic mRNA complexity, occurs as embryogenesis proceeds (Galau *et al.*, 1974; Graham and Wareing, 1984; Davidson, 1986). Therefore, despite being

morphologically simple, angiosperm embryos exhibit a high degree of complexity at the molecular level.

1.2.3 Embryo Nutrition:- Structure and Function of Suspensor and Endosperm

In angiosperms there is no direct vascular connection between the vegetative plant and developing embryo, vascular elements of the ovarian tissue pass along the funiculus and end in the integument tissue of the ovule (Dure, 1975). In *Pisum* nutrient transfer from the vegetal plant to the growing embryo has been demonstrated (McKee *et al.*, 1955; Pate and Flinn, 1973); substrates being carried to the seed mainly in the phloem (Pate *et al.*, 1974). Hardham (1976) showed that beyond the funiculus the vasculature divided into three arms, two of which were phloem. Nutrients arriving in the seed coat must therefore be "unloaded" from the phloem cells and pass through the parenchymatous integument tissue prior to release into the embryo sac. Murray (1979, 1980) demonstrated that the cells of the integument can modify nutrients during this transfer since the acid phosphatase activity of the seed coat was shown to be responsible for the conversion of maternal phosphate esters into organic phosphate, prior to release into the embryo sac, for utilisation by the developing embryo.

Results of angiosperm embryo culture demonstrate that, in general, progressively younger embryos have more stringent nutrient requirements whilst older embryos can be grown successfully using solutions consisting only of inorganic salts (Raghavan, 1976; Steeves and Sussex, 1972; Raghavan, 1986). These results have been interpreted as demonstrating a progressive transition of the embryo from a heterotrophic mode of nutrition towards autotrophy (Steeves and Sussex, 1972). The energy required for the synthetic activities of the embryo before it becomes self-supporting is assumed to be supplied by the triploid nutritive endosperm (Raghavan, 1976; 1986). In general, fusion of male gametes

with both the egg and fused, diploid, polar nucleus are synchronous events. However, in the majority of angiosperms the triploid endosperm nucleus divides prior to the zygote (Jensen, 1964; Raghavan, 1976, 1986). In the few species where the triploid endosperm nucleus and zygote commence division concurrently the rate of mitosis in the endosperm exceeds that of the embryo (Raghavan, 1976), thereby ensuring the availability of reserve nutrient materials for initial growth of the embryo. The development of highly differentiated organelles in the endosperm cytoplasm immediately after nuclear division in *Capsella* (Schulz and Jensen, 1974) indicates its high metabolic activity. Three types of endosperm ontogenesis, based upon the degree of cellularisation, have been described (Maheshwari, 1950; Bhatnagar and Sawhney, 1981). Evidence for the nutritive function of the endosperm can be derived from examination of embryogenesis in those species in which the endosperm is cellular. For example, a progressive breakdown of the cellular endosperm occurs in the vicinity of the developing cotyledons of *Aesculus woerlutzensis* (List and Steward, 1965). The extent to which the embryo consumes the endosperm during development varies among the taxa, ranging from total absorption (exalbuminous seeds) to almost no absorption (albuminous seeds). In the case of the latter, accumulation of storage products; starch, proteins and lipids, occurs within the endosperm and it persists as a permanent organ of the mature seed, providing a source of energy during seed germination.

Endosperm haustoria in the Angiospermae attain a complex morphology, protruding into the nucellus or integument. Many haustoria establish contact with the vascular supply of the ovule (Bhatnagar and Sawhney, 1981; Raghavan, 1986). The intimate association of the haustoria with both endosperm and ovular tissue has resulted in the hypothesis that they are involved in the transfer of nutrients from the maternal tissue to the endosperm and hence to the developing embryo (Raghavan, 1986).

The endosperm of *Pisum* exists in two phases:- a coenocytic phase which is located at the periphery of the chalazal portion of the embryo sac, and a liquid endosperm phase that fills the remainder of the embryo sac vacuole. Initially, the liquid endosperm was thought to be derived from the breakdown of the transient nucleate endosperm (Hocking and Pate, 1977). However, Murray (1980) showed, via experiments analysing translocation of maternal nutrients across the seed coat, that the liquid accumulates as a secretion from the inner wall of the integument and that it represents an intermediate reservoir of nutrients for utilisation by the expanding embryo after utilisation of the coenocytic endosperm by the immature embryo. Analysis of ovular tissue in the vicinity of the embryo (*Pisum sativum*) revealed structural adaptations (large intercellular spaces between ovule cells, protrusions of embryo sac boundary wall into the endosperm), that were postulated to be involved in nutrient entry from the ovule tissue into the embryo sac (Marinos, 1970a).

During the very early phase of embryo development rapid endosperm multiplication occurs. This process (itself) may require quantities of cellular constituents and precursors resulting in the theory that it is improbable that the endosperm provides nutrition during the early cleavage phase of embryo development. It is postulated that during the early division phase the embryo utilises metabolites provided by the suspensor and degenerating synergids/antipodals (Raghavan, 1976; Bhatnagar and Sawhney, 1981). Wall ingrowths have been noted in cells of the soybean pro-embryo that are in contact with the degenerating synergid and postulated to be involved in nutrient transfer from the synergid to the developing embryo (Dute *et al.*, 1989).

The suspensor is the product of basal cell division. It is a short-lived organ that attains full development at the globular stage and is completely obliterated by the embryo during development (Raghavan, 1986). The suspensor varies greatly in

size and shape. Considerable morphological diversity is displayed in the Leguminosae within which four distinct forms of suspensor have been identified (Lernsten, 1983). In *Pisum* the suspensor consists of an elongated pair of multinucleate, saccate cells (Marinos, 1970b). Classically the suspensor has been assigned the function of maintaining the embryo in a fixed position within the embryo sac. However, evidence indicates that during the very early stages of embryogenesis the suspensor is the site of nutrient uptake from the endosperm. Electron microscopic studies (Schulz and Jensen, 1969 [*Capsella*]; Marinos, 1970 [*Pisum*]) have shown the suspensor to possess a morphology similar to that of transfer cells (Gunning and Pate, 1969). In *Capsella* the outer walls of the suspensor were shown to possess invaginations which increased in number and complexity as the embryo developed from the globular to the heart-shape stage. Plasmodesmata were also shown to connect the embryo and suspensor cells, resulting in the solute absorption theory of suspensor function (Schulz and Jensen, 1969). According to this theory metabolites from the endosperm, or surrounding ovule cells, are transferred to the embryo through the suspensor. Wall invaginations facilitate transfer by increasing the surface area of absorption, whilst plasmodesmata that cross the embryo-suspensor boundary provided a route for the transfer of nutrients to the embryo. Much evidence has since been obtained to support this theory. Wall invaginations have been documented in the suspensor cells of a wide variety of species (Raghavan, 1986). In *Phaseolus vulgaris* suspensor cell wall invaginations occur early in development, increasing in number and complexity as embryo development proceeds. A concomitant increase in organelle number also occurs; endoplasmic reticulum, dictyosomes and mitochondria being located in close proximity to wall invaginations, strongly indicating their role in nutrient transfer (Yeung and Clutter, 1979). Yeung (1980) demonstrated nutrient uptake via the suspensor in *Phaseolus coccineus*. The

administration of ^{14}C, either through the pods or isolated embryos (with attached suspensor), resulted in localisation of radioactivity in the suspensor and the suspensor end of the embryo. The uptake pattern changed at embryo mid-maturation when the cotyledons became the major nutrient uptake site. Cytochemical localisation of a number of enzymes in the suspensor cells of *Brassica campestris* showed an increase in enzymatic activity during embryo development, reaching its maximum when the embryo was at the heart-shape stage (Malik *et al.*, 1976). The high metabolic activity was postulated to be involved in the absorption and translocation of substances to the embryo.

The suspensor organs of a number of species possess haustoria (Maheshwari, 1950; Masand and Kapil, 1966) that penetrate the surrounding ovular tissue and are thought to be involved in the translocation of nutrients from the somatic cells of the ovule to the developing embryo (Raghavan, 1976). For example, in *Sedum ochroleucum* thin-walled tubular extensions arise from the basal cell of the suspensor which invade and actively digest cells of the nucellus and integument (Raghavan, 1976). Suspensor haustoria have been described in the Leguminosae (Maheshwari, 1950); however, their function in nutrient transport is uncertain (Raghavan, 1976).

Endoreduplication often occurs in the suspensor cells of a number of angiosperm species. However, results are inconclusive as to whether gene amplification occurs or not (Raghavan, 1986). Studies of RNA and protein metabolism of suspensor cells indicate high transcriptional and translational activities. Analysis of RNA/protein content and RNA/protein synthesis rates in *Phaseolus coccineus* suspensor cells (Walbot *et al.*, 1972) showed low levels of activity during the early stages of embryo development, increasing to a maximum at the late heart-shape stage, whilst the embryo proper exhibited very low levels of RNA/protein metabolism. The high template activity exhibited by suspensor cells,

in conjunction with endoreduplication of the genome, has resulted in the postulation that the suspensor may be responsible for the synthesis and translocation of some gene product required for early embryo growth (Raghavan, 1986).

1.3 BASIC CONCEPTS OF EMBRYOGENESIS

Experimental embryology has been invaluable in elucidating the control mechanisms responsible for early animal embryogenesis and morphogenesis (reviewed:- Graham and Wareing, 1984; Davidson, 1986; Gilbert, 1988). In comparison, little experimental work has been carried out on plant embryos for three main reasons:- (i) at early stages of development the embryo is embedded in maternal tissue and is relatively difficult to dissect; (ii) difficulties in obtaining large numbers of young embryos at similar developmental stages; (iii) relative difficulty in culturing zygotic embryos during the early stages of development. As a consequence apical meristems of mature plants have been used to investigate differentiation processes (Stebbins, 1965; Steeves and Sussex, 1972), whilst somatic embryos have been used to analyse the biochemical and molecular aspects of embryogenesis (Nomura and Komamine, 1986).

The following is a general account of the main processes postulated to be of importance in mediating cell differentiation and morphogenesis during embryo ontogeny. Due to the relative lack of literature detailing research on plant embryos there is, by necessity, a bias towards animal model systems.

1.3.1 Determination

Determination involves the progressive and successive limitation of the developmental potential of descendant cells (Graham and Wareing, 1984). This occurs via:-

(i) Cytoplasmic segregation of determinative molecules during embryonic cleavage. Cleavage planes separate qualitatively different regions of the zygote cytoplasm into different daughter cells. Embryos that develop in this manner are termined mosaic.

(ii) Interaction of cells during development. Cells develop according to their position within the embryo - termed regulative development.

However, these modes of development are not mutually exclusive, since in animal embryos both mechanisms have been shown to occur to some extent, e.g. the nematode, *Caenorhabditis elegans*. These embryos have been classically regarded as being highly mosaic, however, recent experiments involving ablation of individual cells has shown induction of one cell lineage by another (Kenyon, 1988).

1.3.1.1 Mosaic

Mosaic development is dependent upon the selective partitioning, via cell division, of substances synthesised and laid down in the egg. In the extreme, the developmental fate of the products of the first zygotic division are programmed in different directions as a consequence of heterogeneity within the egg (Graham and Wareing, 1984). For example, the tunicate *Styela partita*; immediately after fertilisation the cytoplasm of these eggs becomes segregated to form a series of coloured regions (Gilbert, 1988). Following the cell lineage of each blastomere it was found that each coloured plasm had a specific embryonic fate, i.e. a fate map could be drawn upon the zygote (Gilbert, 1988). Whittaker (1973) stained tunicate embryos for acetylcholinesterase, an enzyme specific to larval muscle tissue. At the two-celled stage both cells stained positively showing that they were capable of producing muscle cells, however, positive staining of only the two posterior blastomeres (known from cell lineage studies to produce muscle) occurred in the

eight-celled embryo. Therefore, selective partitioning of cell determinants had occurred.

Cytoplasm and nuclei transfer experiments using early tunicate blastomeres (Whittaker, 1982) showed that it was the cytoplasm and not the nucleus that determined the developmental fate of the cell, i.e. morphogenetic determinants exist in the cytoplasm that selectively activate/de-activate certain genes in the nuclei. Studies indicated that the morphogenetic determinants acted at both the transcription and translation level. Meedel and Whittaker (1984) demonstrated the absence of acetylcholinesterase activity in embryos grown in the presence of a transcription inhibitor, actinomycin D, suggesting that the gene encoding the enzyme had not been activated by the morphogenetic determinants. Expression of intestinal alkaline phosphatase, however, was found to be regulated at the translation level. It is postulated that the cytoplasmic determinants are attached to the cell cytoskeleton and that by this process they are localised within the cell (Gilbert, 1988).

As previously described (Section 1.2.2) an asymmetric first division of the zygote is extremely important in angiosperm embryogenesis, the cell derivatives of this unequal division giving rise to different organs:- the embryo and the suspensor.

1.3.1.2 Regulative

Driesch (reviewed Gilbert, 1988) showed that each of the cells produced by the first cleavage of the sea urchin zygote was capable, upon separation, of developing into a complete, normally organised larva. Changing the direction of the third embryo cleavage via gentle pressure and thereby altering the positions of the blastomeres within the embryo, still resulted in the development of normal larvae, showing that segregation of cytoplasmic/nuclear determinants was not

occurring during initial sea urchin embryogenesis since, otherwise, the resulting embryo would have been disorganised.

Horstadius (reviewed Gilbert, 1988) demonstrated, by experiments involving recombination of animal and vegetal portions of sea urchin embryos, the presence of two opposing gradients within the developing embryo; the vegetalising gradient, with its maximum activity at the vegetal pole, and the animalising gradient with its maximum at the animal pole. Therefore, a model for regulatory development can be based upon relative concentration gradients established within the oocyte and early embryo. Cells under the influence of particular ratios of gradient substances will differentiate to produce particular cell types (Gilbert, 1988). Changing the concentration ratios, via transplant experiments (reviewed by Tiedmann, 1976), results in cells altering their developmental fate and differentiating into cells characteristic of their new environment. The transplantation of prospective neural tissue from an early gastrula newt embryo to the region fated to become belly skin in a similar stage embryo, resulted in the transplanted cell differentiating into epidermal cells (Spemann, 1962). Therefore, cells at this early stage are not committed to a specific type of differentiation, they exhibit dependent development, cell fate being dependent upon location within the embryo. However, when the same experiment was performed at the late gastrula stage the transplant cells exhibited independent (autonomous) development. Prospective neural tissue differentiated into brain tissue irrespective of transplant location, i.e. as embryo development proceeds, cell fate becomes fixed, and cell differentiation occurs independently of embryonic location (Spemann, 1962).

Transplant experiments involving the dorsal blastopore lip of early newt embryos (Spemann and Mangold, 1924) showed that this embryonic region exhibited self-determination, it did not regulate according to its environment but invaginated (according to its normal developmental pattern). Host cells in the

region of the invagination differentiated into a range of tissues and cells that they would not normally have formed, resulting in the formation of a secondary embryo connected to the host. Therefore, the dorsal blastopore lip is an embryonic region that interacts with other cells causing them to differentiate in a particular direction, i.e. it induces them.

In a number of amphibian embryos ectoderm and endoderm have been shown to be autonomous. However, these tissue layers induce mesodermal tissue to produce mesodermal structures, e.g. muscle, kidney cells (Gilbert, 1988). Sargent *et al.* (1986) dissociated the cells of early blastula *Xenopus* embryos and grew the separated cells in culture, during the period when induction is thought to occur, in order to examine whether the separated cells could synthesise mRNAs characteristic of particular germ layers in the absence of inductive interactions. Analysis showed that mRNAs specific to ectoderm and endoderm differentiation were expressed at normal levels, indicating that the encoding genes were regulated autonomously by some internal cytoplasmic factors. However, mesoderm specific mRNA was not synthesised, indicating that expression of the gene required the inductive interaction of the cells of the other germ layers to activate it, since gene activation occurred when the dispersed cells were reaggregated. Those cells that induce others to differentiate must produce substances that pass into the cell and produce the induction response. The identity of inducer molecules is not yet known, though experimental work indicates that they are similar to mammalian growth factors. The formation of mesodermal tissue can be induced by exposure to low levels of fibroblast growth factor (Slack *et al.*, 1987). *Xenopus* cells treated with FGF have been shown (Kimelman and Kirschner, 1987) to transcribe the α -actin gene (a mesoderm specific mRNA), i.e. the gene is induced by FGF.

In the embryo of cotton, cell size during embryo development progressively decreases, until at the 100 cell stage average cell size is one-twentieth that of the

zygote (Pollock and Jensen, 1964). It has been postulated that the onset of histogenesis/organogenesis in angiosperm embryos requires compartmentalisation of the protoplasm via a rapid increase in cell number with a minimum increase in total embryo mass (Steeves and Sussex, 1972). Stebbins (1965) compared mitotic rhythm, nucleic acid synthesis and morphogenesis in higher plant meristems. Mitotic rhythm was shown to be important in the differentiation of reproductive meristems from vegetative meristems, cell patterns being determined by whether the rate of cell division exceeded or lagged behind the rate of cell expansion. In the case of the former a solid three-dimensional structure is produced, consisting of cells that become progressively smaller with each division, whilst in the case of the latter, cell size enlarges with successive division cycles. Transitions between elongation-determined and mitosis-determined cell patterns are thought to be important in the differentiation of meristems during angiosperm embryogenesis (Stebbins, 1965).

1.3.2 Morphogenesis

Development proceeds via the differentiation of cells and their subsequent morphogenesis to produce multicellular arrangements (organs/tissues) that are precisely located within the developing embryo (Graham and Wareing, 1984; Gilbert, 1988). Reaggregation experiments (reviewed by Gilbert, 1988) showed that when dissociated cells from a variety of amphibian germ layers were allowed to aggregate the cells became spatially segregated, each cell type positioned itself within the aggregate according to its original position within the embryo. For example, reassociation of ectoderm and mesoderm cells resulted in aggregates consisting of central mesoderm cells and peripheral ectoderm cells. Using rotary culture techniques Giudice (1962) obtained the reconstruction of sea urchin larvae from single cell suspensions of dissociated larvae cells. These experiments indicate

that animal embryonic cells possess the ability to determine and take up their correct position within the embryo and that they retain this morphogenetic information since, following dissociation, they are able to reform organs/tissues. Although in plants there is no movement and positioning of cells during morphogenesis, the cells are in contact and there is a requirement for control over the planes and number of divisions.

1.3.2.1 Cell recognition/interaction

The formation of tissues and organs is mediated by events occurring at the cell surfaces of adjacent cells (Gilbert, 1988; Graham and Wareing, 1984). Tissue-specific membrane components can be recognised by antisera and are therefore termed differentiation antigens. Monoclonal antibodies raised against specific cell types have been used to demonstrate the temporal and spatial arrangements of these differentiation antigens, e.g. temporal changes in the cell membrane of the *Drosophila* epithelial cell, as it differentiates into a retinal photoreceptor (Zipursky *et al.*, 1984). The local patterns of expression of cell surface molecules are postulated to provide a major link between the one dimensional genetic code and the three dimensional organism; by modulating the spatio-temporal expression of these molecules the genome can regulate the mechanical processes of morphogenesis (Gilbert, 1988). Cell membrane structure has been summarised (Singer and Nicholson, 1972) in the Fluid Mosaic model, in which some proteins traverse the membrane and hence have their active sites on the outer surface, capable of interaction with the extracellular environment. Three classes of cell membrane molecules (usually proteins) that are involved in specific interactions with adjacent cells have been identified (Edelman and Thiery, 1985):- (i) cell adhesion molecules (CAMs); (ii) substrate adhesion molecules; and (iii) junctional molecules.

(i) Using fluorescent antibodies, Edelman *et al.* (1983) demonstrated the spatio-temporal distribution of cell adhesion molecules (CAMs) during chick embryo development. A calcium dependent CAM has been shown to be expressed primarily on the mouse uterine wall (Nose and Takeichi, 1986), postulated to be involved in embryo implantation in the uterus. Differential CAM expression is proposed to be of importance in maintaining boundaries between differing cell types; cell segregation being as a result of the different cell types expressing different CAMs on their membrane surface (Gilbert, 1988). Investigations of cell recognition in plants have included examination of self-incompatible pollen:stigma interactions, organ transplants (grafting) and callus culture. In general, grafts between closely related species of the same family are compatible, whilst those between members of different families are incompatible (Graham and Wareing, 1984). Interactions between calli also depend upon how closely related are the genera; adjacent calli initiated from different families fail to grow together in culture. Therefore callus cells, which possess a cellulose cell wall, possess the ability to discriminate between self and non-self. Callus cells have been shown to display both species and organ specific antigens (Raff *et al.*, 1979), which may play a role in cell recognition and the acquirement, by cells, of correct positioning within the developing structure. Antisera have also been used to identify cell surface glycoproteins involved in self-recognition; using style exudates the products of self-incompatibility genes have been identified immunologically (Mau *et al.*, 1982).

(ii) Substrate adhesion molecules are involved in binding cells to their extracellular substrates and are particularly involved in cell migration.

(iii) In animals three main forms of cell junctional modifications occur:- tight junctions, that separate the extracellular compartments on either side of the cell;

desmosomes, which occur when cells are joined together to form an impermeable tissue; and gap junctions.

Gap junctions serve as communication channels between adjacent cells, allowing small molecules and ions to pass from one cell to another (Pitts, 1984). The junctional structure (plasmodesmata) joining plant cells is different to that found in animal systems, however, its permeability and function is similar (Pitts, 1984). Gap junctions have been shown to be important for the communication of developmental information between cells during amphibian and mammalian development. The injection of antibodies, to gap junction proteins, into one of the cells of an eight-celled *Xenopus* embryo results in morphologically abnormal tadpoles, the defect being specifically related to the developmental fate of the injected cell (Warner *et al.*, 1984). Therefore, the progeny of injected cells are unable to undergo their normal development as a consequence of their inability to "communicate". Lo and Gilula (1979) demonstrated the presence of gap junctions in the eight-celled stage mouse embryo. Dye injected at the pre-implantation blastocyst stage spread throughout the whole embryo, whilst later in development the cells of the inner and outer mass were coupled only to themselves and not to each other, i.e. compartmentalisation of coupling occurred. It was suggested that inside/outside positional information could be generated by intracellular gradients being established by coupled cells.

1.3.2.2 Secondary induction

Secondary induction occurs when one group of cells alters the behaviour of an adjacent set of cells causing them to differentiate. These proximate cell interactions result in co-ordinated organ development, e.g. a cascade of inductive interactions results in formation of the eye:- the notochord induces the neural tube to form an optic bulge which then induces the formation of a lens which

subsequently induces cornea formation (reviewed by Gilbert, 1988; Graham and Wareing, 1984). Proximate induction occurs only when the inducing tissue is in close proximity to a competent, responding tissue. Although the nature of inducer molecules is not known (see Section 1.3.1.2), three types of cell interaction have been postulated to be responsible for the induction processes:- cell:cell contact; cell:matrix contact; diffusion of soluble substances (Saxen *et al.*, 1976).

1.3.2.3 Acquirement of positional information

(i) Wolpert (1969) proposed a model whereby a gradient of morphogenetic substances is established across a developmental region. Cells interpret their gradient levels according to a code of threshold responses, realise their relative positions within the embryo and hence what structure they should differentiate into. Grafting experiments using the coelenterate *Hydra* have provided evidence for the presence of morphogenetic gradients and their role in differentiation. *Hydra* basically consists of a narrow tube with basal disc and apical hypostome (head region). MacWilliams (1983a and b) demonstrated the presence of a head activator and head inhibitor gradient, whose concentrations decreased from the apical region toward the base. Although regeneration experiments showed that any portion of *Hydra* can regenerate a new hypostome, lateral heads are prevented from forming by the inhibitory influence of the apical hypostome. Removal of the apical region results a in loss of the inhibitory gradient, resulting in the formation of a hypostome in the apical portion of tissue, where the concentration of head activator is highest. The injection of antibodies raised against gap junction proteins resulted in a loss of the head inhibitor gradient (Fraser *et al.*, 1987), indicating that the establishment of the morphogenetic gradients was via junctional communication between cells.

(ii) Analysis of regeneration in vertebrate and insect limbs resulted in the proposal (French *et al.*, 1976) of a polar co-ordinate model by which cells specify their position within the developing organ/tissue. This model proposes that cells assess their physical location via a system of polar co-ordinates, each cell possessing a circumferential value and a radial value, and it has been used successfully to predict patterns of limb regeneration in amphibian transplant experiments (Bryant *et al.*, 1981).

1.3.2.4 Surgical experiments on angiosperm embryos

Ball (1956) surgically divided the root apex of an excised *Ginkgo biloba* embryo into two halves. During subsequent growth in culture the two portions of the embryonic root regenerated to produce two root apices. This has subsequently been shown to occur in a number of plant species; the surgical splitting of embryonic root or shoot resulting in the formation of supernumerary organs (Raghavan, 1976). The results of these experiments indicate that only a small number of apex initials are required to regenerate the entire organ, resulting in the conclusion that "embryonic initials of root and shoot are not predetermined to produce specific tissues, but function according to the position they occupy" (Raghavan, 1976). Results obtained from the culture of embryo segments of several plant species (Raghavan, 1976) showed that only shoot segments were capable of developing into complete seedlings. Culturing of cotyledons and the region below the shoot resulted in callus formation, whilst embryonic root and hypocotyl produced lateral roots but no shoots. Interpretation of these results (Raghavan, 1976) suggests that the apical shoot region exhibits a high degree of autonomy, whilst the developmental pattern of the other embryo regions is established during embryogenesis, this set developmental fate being maintained when embryo segments are isolated.

1.3.3 Hormones as Mediators of Development

A major example of hormone action in animals is the process of metamorphosis during which developmental processes are reactivated, via specific hormones, resulting in the transition from the larval to adult form. Amphibian metamorphosis has been fully reviewed (Gilbert, 1988; Graham and Wareing, 1984) and is a result of the secretion of thyroid hormones. The various organs of the larval form respond differently to the hormonal stimulation, e.g. some tissues degenerate, whilst others develop and differentiate. The hormone response is intrinsic to the organ and independent of surrounding tissue, i.e. the cell response is pre-programmed. Different levels of hormone are required to produce different, specific responses, termed the threshold response. The timing of metamorphic events is regulated by the competency of different tissues to respond to increased hormone levels. The molecular events of metamorphosis in amphibians have been studied using RNA hybridisation techniques (Mathison and Miller, 1987). Three types of response to metamorphic hormone were demonstrated:- (i) increase in transcription rate; (ii) decrease in transcription rate; (iii) transcription rate remains unchanged. Although the possibility of hormone action occurring at later stages in the gene expression pathway was not excluded, it was concluded that, during metamorphosis in amphibians, hormone action occurred primarily at the level of transcription. In other animal systems hormone action has been demonstrated to occur via the stabilisation of mRNA molecules within the cytoplasm. The synthesis of casein by mammary gland tissue in lactating mammals is stimulated by the hormone prolactin. However, the hormone increases the transcription rate only two-fold. It exerts its stimulatory effect by stabilising the casein mRNA molecules resulting in a greater number of peptides being synthesised from each message (Guyette *et al.*, 1979).

Little conclusive evidence of the role of hormones in angiosperm embryogenesis has been obtained resulting in the conclusion, "no evidence yet exists that demonstrates or excludes the involvement of a specific hormone in the embryo morphogenesis of a wide range of plants" (Raghavan, 1976). Using cultured excised embryos from a variety of plant species Rietsema *et al.* (1953) demonstrated an inhibition of embryonic root initiation and growth at high auxin concentrations, whilst very low auxin levels were shown to be stimulatory. Subsequent work has shown that, in general, low auxin concentrations stimulate embryo growth, whilst high concentrations inhibit growth (Raghavan, 1976). A gradient of auxin sensitivity was demonstrated to exist in the organ systems of *Phaseolus vulgaris* embryos, the embryonic shoot having a higher auxin optimum than the root with intermediate regions between shoot and root exhibiting intermediate auxin optima with respect to inhibition or promotion of growth (Raghavan, 1976). The addition of gibberellins to embryo culture growth media appears to exert non-specific growth promoting effects, since gibberellic acid (GA) stimulation of both root and shoot primordia has been demonstrated in a variety of cultured zygotic embryos (Raghavan, 1976). However, in cotton embryos this response has been shown to be correlated to the physiological age of the embryo. In immature cotton embryos, GA resulted in enhanced cotyledon maturation and cell elongation whilst in older embryos accelerated cell division, cell elongation and axis growth were noted. Furthermore, the inhibitory effects of high auxin concentrations were reduced by GA addition (Dure and Jensen, 1957). A possible role for the interactions of hormone levels during embryogenesis has been obtained from manipulations of auxin/kinetin concentration ratios in callus growth media (Hall, 1984). Equal auxin and kinetin concentrations result in the development of an amorphous, undifferentiated tissue mass. However, a high auxin/kinetin ratio was found to induce the formation of roots, whilst low auxin/kinetin ratios

induced shoot formation. The cells of the callus culture could therefore be directed along one of two developmental pathways as a result of altered hormone ratios. Although no direct evidence exists for the role of hormones in the differentiation of angiosperm embryos it has been postulated that abscisic acid (ABA) is important during the later stages of embryo development. Eeuwens and Schwabe (1975) demonstrated a rise in ABA activity during late pea seed development, levels then declined during desiccation and were undetectable in the mature, desiccated seed. The germination capacity of mature excised seeds was shown to be as a result of decreasing ABA levels and not due to any restrictions imposed by the testa. Therefore, ABA has been hypothesised to be involved in maintaining an embryonic pathway and preventing germination during the later stages of embryo development (Dure, 1985). ABA has also been implicated in the modulation of storage protein genes in some plants. For example, in precociously germinating embryos of *Brassica napus*, synthesis of Cruciferin occurs at low rates, however, under conditions that suppress precocious germination, e.g. high osmoticum or the addition of ABA, the cultured embryos accumulate the storage protein to the same degree as embryos *in situ* (Crouch and Sussex, 1981). It is not certain whether ABA modifies gene expression or exerts its effect by forcing the embryo to continue along its normal developmental pathway (Raghavan, 1986). In contrast, recent work (Barratt *et al.*, 1989) resulted in the conclusion that ABA played no role in the regulation of either normal development or storage protein synthesis in embryos of *Pisum sativum.*

In angiosperms, both the suspensor and endosperm have been implicated as sites of hormone synthesis. Early development of the pea fruit is characterised by rapid elongation and enlargement of the pod (ovary) whilst the seeds increase very little in weight or size. Analysis of hormone concentrations (Eeuwens and Schwabe, 1975) demonstrated that pod elongation was correlated with high auxin

and gibberellin activity in the liquid endosperm of the seed. Fruits in which the seeds had been killed failed to elongate, whilst pod growth was re-instated via the application of an auxin/gibberellin mixture. It was therefore concluded that some, if not all, of the hormones required for pod growth were supplied by the developing seeds. Increase in seed growth rate was also found to be associated with the presence of high gibberellin and auxin concentrations in the endosperm (Eeuwens and Schwabe, 1975). Analysis of gibberellic acid (Alpi *et al.*, 1975) and cytokinin levels (Lorenzi *et al.*, 1978) during *Phaseolus coccineus* embryo development showed that, at the heart- shape developmental stage, high hormone levels were present in the suspensor though low levels were detected in the embryo proper. A complete reversal in the pattern of localisation was observed at the cotyledon stage of development. Heart-shaped embryos of *Phaseolus coccineus* failed to grow in culture when deprived of the suspensor, however, its stimulatory effect on early embryo growth could be replaced by gibberellic acid. In addition, the stimulatory effect was stage-specific, embryos at the cotyledon stage of development being successfully cultured in the absence of the suspensor (Yeung and Sussex, 1979).

1.4 GENE EXPRESSION AND DEVELOPMENT

Developmental genetics is the study of how the inherited potential of the fertilised egg becomes progressively expressed during the life of an organism (Gilbert, 1988). Embryogenesis is a sequential process involving the divergence of cells, of a common lineage, into contrasting pathways of development, with specific regions of the embryo being destined to form a particular part of the mature organism. The progressive restriction of a cell's developmental potential is termed determination. The resulting product of this process is a differentiated cell that performs a specialised function within the organism (Graham and Wareing, 1984).

The process of determination is mediated via proteins, i.e. differentiated cells contain a characteristic complement of proteins that differs from those of other differentiated cell types. Since proteins are the product of gene activity the whole process of differentiation and morphogenesis is controlled at the level of gene expression.

The central hypothesis of developmental genetics is that cell differentiation occurs in the absence of any genetic alteration. Within the somatic cells of each organism is a complete, identical complement of genes; cell differentiation being the result of differential gene expression (Gilbert, 1988). Genomic equivalence has been demonstrated in a variety of animal systems, e.g. metaplasia (transformation of one differentiated cell type into another) in the salamander. Here the removal of the eye lens results in its regeneration from the pigmented cells of the iris (Dumont and Yamada, 1972). Another example is the transplantation of nuclei from differentiated frog skin cells to enucleated frog eggs which results in the formation of swimming tadpoles (Gurdon *et al.*, 1975). There is debate over the complete totipotency of fully differentiated animal cells. However, they are pluripotent since, for example, many inactivated genes in the frog skin cell can be re-activated to produce the cell types and tissues of a swimming tadpole (Gurdon *et al.*, 1975). It is only in plants that complete nuclear totipotency has been demonstrated, e.g. Steward (1958) obtained the regeneration of mature plants from differentiated carrot cells grown in culture.

1.4.1 Organisation of the Eukaryotic Genome

In eukaryotes the genome can be arbitrarily divided into three components on the basis of reassociation kinetics (Britten and Davidson, 1976; Flavell, 1980, 1982):-

(i) unique, single copy sequences;

(ii) moderately repetitive sequences;

(iii) highly repetitive sequences.

In most eukaryotes only a fraction of the DNA is unique in sequence, e.g. in *Pisum* only approximately 15% of the genome behaves as if present at one or few copies per cell (Murray *et al.*, 1978). Hybridisation experiments have shown that the majority of cellular mRNA is homologous to a small fraction of this single copy DNA (Goldberg *et al.*, 1978), i.e. most structural genes are present in the genome as few or single copies (exceptions being the histone and rRNA genes). The advent of gene cloning, i.e. the isolation and amplification of specific genes, and DNA sequencing techniques have allowed analysis of gene structure and the determination of sequences that control gene expression.

Eukaryotic genes exhibit a more complex organisation compared to those of prokaryotes in that each gene encompasses more DNA than is required to encode a polypeptide. This includes elements upstream and downstream of the coding region that are thought to be required for correct transcription and regulation. In addition, eukaryotic genes often contain internal, non-coding intervening sequences, termed introns, which are transcribed as pre-mRNA and removed by splicing reactions during mRNA formation. Intron splicing must be, by necessity, a highly accurate process since a one-nucleotide error would alter the reading frame of the message. Sequence comparisons of many splice junctions have shown that the intron begins with GT at its 5' end and finishes with AG at its 3' end; termed the GT/AG rule (Mantell *et al.*, 1985; Grierson and Covey, 1988; Gilbert, 1988).

Comparisons of the 5' extragenic regions of a large number of animal structural genes have revealed two conserved sequence elements.

(i) The TATA box, that resides in an A + T rich region (e.g. Gannon *et al.*, 1979), is located approximately 30 bp upstream from the site of initiation of RNA synthesis. *In vitro* transcription systems have shown the TATA box to be required for the specific initiation of RNA synthesis of certain genes, e.g. the rabbit globin gene (Grosveld *et al.*, 1981, 1982) and therefore it has been equated with the essential sequence of prokaryotic promoters.

(ii) The CAAT box (e.g. Benoist *et al.*, 1980; Hentschel and Birnstiel, 1981) is located approximately 80 bp upstream from the site of initiation of RNA synthesis and is thought to be involved in stimulating transcription, i.e. an enhancer sequence (Grierson and Covey, 1988).

The TATA and CAAT box have been found to be critical elements for the expression of numerous eukaryotic genes (Efstratiadis *et al.*, 1980). For example, Myers *et al.* (1986) found that site-directed mutagenesis of TATA, CAAT and enhancer elements further upstream of the β-globin gene, drastically reduced transcription.

The only notable conserved sequence in the 3' extragenic region is an AATAAA sequence postulated to serve as a signal for polyadenylation (Proudfoot and Brownlee, 1976)

Analysis of plant genes has shown that they contain conserved sequences analogous to those present in animal genes (Heidecker and Messing, 1986). For example, the leghaemoglobin gene possesses a TATAA and CCAAT sequence 30 bp and 90 bp upstream respectively, from the transcription initiation site; an AATAAA sequence upstream from the 3' end and introns that obey the GT-AG rule (Hyldig-Nielsen *et al.*, 1982). However, in comparison to animal genes, some plant genes exhibit only a limited homology to the CAAT-box, instead they possess a concensus sequence termed the AGGA-box (Messing *et al.*, 1983). In addition, many plant genes contain either no introns (e.g. lectin, zein) or a small number of

short introns, e.g. glycinin contains only one intron (Fischer and Goldberg, 1982), indicating that plant genes may be slightly simpler in structure than those of animals. However, where introns are present they follow the GT-AG rule (Lerner *et al.*, 1980), suggesting that similar RNA splicing mechanisms are involved. This has been verified by the splicing of plant nuclear pre-mRNAs using extracts of mammalian (HeLa) cells (Brown *et al.*, 1986)

1.4.2 Mechanisms of Differential Gene Expression

The pathway from gene to protein is very complex with many steps at which regulatory mechanisms can operate (Lewin, 1980). Compared with prokaryotes, eukaryotes have an extremely important extra area of gene expression control, that of the compartmentalisation of the cell into cytoplasm and nucleus, which allows post-transcriptional events to mediate regulation prior to translation whereas in prokaryotes transcription and translation are often coupled.

Transcription occurs both in the nucleolus (rRNA) and in the nucleoplasm. In common with animal cells, plant cells contain three types of RNA polymerase (Guilfoyle and Malcolm, 1980; Jendriask, 1980), distinguished on the basis of physical properties, localisation and function (Grierson and Covey, 1988; Mantell *et al.*, 1985). RNA polymerase II is responsible for transcribing genes that encode proteins, resulting in the production of a population of transcripts known as heterogeneous nuclear RNA (hn RNA), which includes the nuclear precursors of messenger RNA, termed pre-mRNA.

1.4.2.1 Transcriptional level

In animal systems the control of transcription has been shown to involve changes in chromatin structure, methylation of cytosine residues of DNA and the interaction of specific regulatory molecules with chromatin (Lewin, 1980).

(i) ***Cis* and *Trans*-acting regulatory factors**

Transcription level control is postulated to be mediated via the interaction of *trans*-acting factors with *cis*-regulators (Lewin, 1980). *Cis*-regulators are specific DNA sequences that exert their effect only upon adjacent genes and include promoter (TATA box) and enhancer (e.g. CAAT box) sequences. *Trans*-acting factors are soluble molecules (proteins and RNAs) made by one gene that interact with genes on the same or different chromosomes.

A number of DNA binding proteins (*trans*-acting factors) have been identified in both animal and plant systems. Two *trans*-regulatory proteins have been isolated from *Drosophila* nuclei, one of which has been demonstrated to bind to an upstream promotor element, whilst the other binds to the TATA box (Parker and Topol, 1984). Mammalian TATA box binding proteins have been isolated (Davison *et al.*, 1983) and in plants a nuclear protein factor, that binds to upstream sequence elements of the light responsive pea rubisco gene (Green *et al.*, 1988), has been reported. DNA-binding proteins involved in the heat-shock response of *Drosophila* have been isolated and shown to bind to both the TATA box and upstream enhancer element, collectively termed the heat-shock element (Wu 1984).

A number of *trans*-regulatory proteins isolated are specific to certain cell types and may be responsible for the tissue-specific expression of certain genes. Growth hormone factor 1 is a peptide specific to anterior pituitary cells. DNA foot-printing showed that the protein bound to the upstream promoter element of the human growth hormone gene and its addition to a non-pituitary nuclear extract resulted in growth hormone gene transcription (Bodner and Karin, 1987). In legumes, the protein lectin is synthesised at high levels in the embryo, where it constitutes a seed storage protein, and at low levels in roots (Okamuro *et al.*, 1986). A 60 kD nuclear protein has been isolated from soybean embryos that interacts

with the lectin gene 5' region. The DNA binding activity was shown to increase and decrease during embryo development in a manner that paralleled the transcriptional activity of the gene. The DNA binding protein was also detected at low levels in soybean root, whilst it was undetectable in other organ systems of the plant (Jofuku *et al.*, 1987).

An enhancer sequence has been identified in the octopine synthase gene (ocs) of *Agrobacterium tumefaciens*, its enhancer effectiveness was shown to decrease as distance from the site of transcription initiation was increased (Ellis *et al.*, 1987).

In addition to ubiquitous enhancer elements, e.g. CAAT box, both tissue-specific and temporal enhancer elements have been identified (Walker *et al.*, 1983; Krieg and Melton, 1987). Analysis of the numerous genes activated during the mid-blastula stage of *Xenopus* embryo development showed that they contained an homologous enhancer sequence. Fusion of the homologous 5' enhancer sequence to a *Xenopus* globulin gene, not normally transcribed until late in development, resulted, when replaced into a pre-blastula embryo, in its transcription during the mid-blastula transition i.e. the gene was temporally regulated by the fused upstream enhancer (Krieg and Melton, 1987).

(ii) **Template availability**

DNA within the nucleus is packaged into chromatin, the repeating unit of which is the nucleosome consisting of a specific aggregate of basic histone proteins with which the DNA is associated. Further condensation involves the coiling of the nucleosomes into a 30 nm solenoid structure (Nagl, 1982).

In any given cell type the majority of tissue-specific genes in the genome are not expressed (Gilbert, 1988), resulting in the hypothesis that chromatin normally exists in a repressed state; tissue-specific genes being activated as a

result of localised removal/inactivation of repressive factors (Weintraub, 1985). Gene repression is postulated to be as a result of the compaction of DNA into nucleosomes. In this tight configuration *trans*-acting regulatory factors are unable to bind to their corresponding promoter/enhancer sequences to initiate transcription (Schlissel and Brown, 1984).

The susceptibility of DNA to digestion by nucleases provides an indication of its chromatin configuration and its accessibility to nuclear proteins. Weintraub and Groudine (1976) showed that the chick ovalbumin gene from oviduct chromatin (where it is expressed) was susceptible to DNase 1 digestion, whilst the same gene in red blood cell chromatin (where it is not expressed) was not susceptible, i.e. was in a tightly bound chromatin configuration, protected from nuclease digestion by associated proteins. In addition, DNase 1 hypersensitive sites have been localised, most of which are found in developmentally regulated tissue-specific genes (Elgin, 1981). Studies using the glucocorticoid responsive enhancer element of the mouse mammary tumour virus showed that, prior to hormone addition, the enhancer sequence exhibited no DNase 1 sensitivity. However, following the addition of hormone, a discrete DNase 1 hypersensitive site developed in the enhancer region coinciding with the initiation of gene transcription (Zaret and Yamamoto, 1984). It was postulated that interaction between the hormone-receptor complex and the enhancer sequence altered chromatin configuration allowing transcription from the promoter. Analysis of DNase 1 sensitivity of legumin genes from cotyledon nuclei and leaf nuclei of *Pisum sativum* showed a greater nuclease sensitivity of the gene in cotyledon nuclei (where the gene is expressed) than in leaf nuclei (where gene is not transcribed) (Sawyer *et al.*, 1987). A similar correlation between transcriptional activity and DNase 1 susceptibility was shown in the phaseolin genes of *Phaseolus vulgaris* (Murray and Kennard, 1984). In neither case were hypersensitive sites detected.

(iii) **DNA methylation**

DNA methylation is the enzymatic conversion of the cytosine nucleotide into 5-methyl cytosine. Various studies have shown a correlation between gene activity and non-methylation in several developmental systems (Gilbert, 1988). Foetal liver cells that synthesise haemoglobin early in development contain unmethylated genes, whilst those in adult tissue, in which no synthesis occurs, are methylated (Van der Ploeg and Flavell, 1980). Organ-specific methylation patterns have been shown to occur in the chick ovalbumin gene which is unmethylated in oviduct cells, where it is expressed, but methylated in all other tissues where it is not (Mandel and Chambon, 1979; Groudine and Weintraub, 1981).

Methylation is an important mechanism of gene regulation, though it is not used in every system. For example, seed storage protein genes isolated from cotyledon tissue (where expressed) and leaf tissue (where not expressed) showed no significant differences in methylation pattern. Therefore, methylation has been demonstrated not to be involved in the differential expression of seed storage protein genes in both *Pisum* (Waterhouse, 1985) and soybean (Walling *et al.*, 1986).

(iv) **Transcription and differentiation**

The majority of examples in which gene expression has been shown to be controlled at the level of transcription have been obtained from genes whose products are as a result of differentiation, not the cause of it. Waddington (1940) proposed the existence of "switch genes" encoding products that would activate a wide range of genes characteristic of a particular cell type, i.e. would cause the cell to differentiate. Examples of such genes have been found, e.g. the lin-12 locus of *Caenorhabditis elegans.* Two neighbouring cells exist in the wild type embryo, one of which differentiates into a uterine anchor cell, the other into a uterine precursor cell. In recessive mutants of lin-12 the gene is not transcribed and both

cells differentiate into anchor cells, whilst in dominant lin-12 mutants the transcription level of the gene is abnormally high, resulting in both cells differentiating into uterine precursor cells (Greenwald *et al.*, 1983). The product of the lin-12 locus therefore controls the "switch" between two alternative developmental pathways. The Notch mutation in *Drosophila* is also an example of a gene whose product is responsible for the channelling of a bipotential cell along one of two developmental pathways. The ventral ectoderm cells of *Drosophila* can differentiate into either hypodermal or neural precursor cells. In the absence of Notch gene transcription the cells develop into neural precursors only, resulting in fatality of the embryo. The Notch gene has been cloned and demonstrated to be transcribed only during the early stages of embryogenesis (Kidd *et al.*, 1983), when the developmental fate of ectodermal cells is established.

1.4.2.2 Post transcriptional Control

Transcription, via RNA polymerase II, of structural genes results in the production of a population of transcripts, termed heterogeneous nuclear RNA (hnRNA). These transcripts are then processed, via a number of steps, to form the mature mRNA molecules that function in the cytoplasm.

(i) A "cap" structure, consisting of a 7-methyl guanosine residue in an inverted orientation, is added to the 5' end of the RNA transcript. Caps have been demonstrated in a number of plant species (Haugland and Cline, 1978) and may be involved in increasing the efficiency of translation, although uncapped mRNAs can also be translated (Mantell *et al.*, 1985).

(ii) The addition of a polyadenylic acid (poly-A) tail to the 3' end of transcript by the enzyme poly-A polymerase. Poly-A tails have been identified in many plant mRNAs, e.g. hordein (Matthews and Miflin, 1980), leghaemoglobin

(Verma *et al.*, 1974), legumin (Croy *et al.*, 1982). The length of the tail is variable, up to 200 residues (Mantell *et al.*, 1985).

(iii) The removal of introns. In addition to splice junctions (GT/AG) a further sequence has been identified that is necessary for correct cleavage and removal of introns, termed the branch site, located 20-50 bp upstream from the 3' splice site (Lewin, 1980).

Splicing reactions (reviewed by Gilbert, 1988) are directed by catalytic particles, composed of small nuclear RNAs and proteins (SnRNPs), which are capable of recognising splice/branch sequences and of cleavage and ligation reactions. Splicing occurs when a number of SnRNPs interact with one another to form a spliceosome on the pre-mRNA molecule. Splicing of RNA is a complex procedure and, although the mechanisms via which RNA splicing can regulate gene expression are not understood, there exist at least two theoretical points at which regulation could occur; at the binding of SnRNPs or formation of spliceosomes (Gilbert, 1988). In *Xenopus* heterogeneity within one class of SnRNP molecules has been demonstrated (Forbes *et al.*, 1984). The various forms of the molecule are detected at different stages during development, resulting in the hypothesis that the different forms of SnRNP bind to and splice different message precursors during development.

Primary hnRNA transcripts contain a 3' trailer sequence that extends beyond the point at which the gene message terminates. Within the trailer is a sequence essential for RNA cleavage and addition of the poly-A tail (Proudfoot and Brownlee, 1976). Regulation can occur via premature termination of transcription. The resulting transcript does not receive a poly-A tail and is rapidly degraded. This form of regulation has been shown to occur in the synthesis of proteins that are involved in regulating mammalian cell proliferation. Using HeLa cells it was shown that in non-growing cells gene transcription is

initiated, though not completed, whilst in growing cells the complete transcript is produced (Bentley and Groudine, 1987). A deletion in the 3' region of gene (where premature termination was shown to occur) results in the completion of gene transcription in cells that would prematurely terminate the transcript, resulting in the cells becoming malignant (Cesarman *et al.*, 1987).

Differential RNA processing has been found to control gene expression via the generation of multiple protein isoforms from single genes (Breitbart *et al.*, 1987). Different mRNAs can be formed by the splicing together of various exons of the mRNA precursor, e.g. the foetal and adult forms of myosin proteins (Nabeshima *et al.*, 1984). Brietbart and Nadal-Ginard (1987) showed that correct splicing together of exons to form a muscle-specific protein occurred only in muscle cells, whilst in non-muscle cells correct processing of the pre-mRNA did not occur. Therefore, the factor(s) required for correct processing of a cell-specific mRNA was shown to occur only in the cell type in which it is ultimately expressed at the protein level.

Very little is known about the mechanism of mRNA transport from the nucleus into the cytoplasm. It is assumed that exit is via the nuclear pores, interaction occurs with ribosomes that are situated at the cytoplasmic side of the nuclear envelope and the mRNA is pulled out of the nucleus during the process of translation (Gilbert, 1988). It is postulated that regulation could occur via the selection of only certain mRNA molecules for transport, however, no examples of such regulation have been reported.

1.4.2.3 Translational control

The initiation phase of protein synthesis involves the binding of the initiator tRNA (Met-tRNA) to the 40S ribosomal subunit, which is then competent to bind mRNA. The initiation complex scans along the mRNA to locate the

initiation codon (AUG) at which point the 60S ribosomal subunit joins the complex and translation proceeds (Hunt, 1983). The protein synthesis rate is directly proportional to both the initiation rate and elongation rate (rate at which ribosomes polymerise amino acids) (Hershey *et al.*, 1986). Control of translation could occur as a result of a decrease in elongation rate due to a shortage, within the cell, of a particular essential amino-acyl tRNA. However, no clear examples of this form of regulation have been demonstrated (Clark, 1983). In all cell types that have been evaluated, protein synthesis has been shown to be regulated by the initiation rate (Jagus *et al.*, 1982; Hunt, 1983).

An example of the role of translation control during differentiation is provided by the simple multicellular eukaryote, *Volvox carteri*, which consists of only two differentiated cell types. Cytodifferentiation occurs only upon exposure of the embryo to light and differences between the polypeptide spectrums of the two-cell types were shown to occur as a result of light exposure (Kirk and Kirk, 1983). The transition in the protein spectrum was unaffected by the addition of actinomycin D, i.e. was independent of transcription and *in vitro* transcription assays showed no significant differences in the mRNA populations of light and dark-grown cells (Kirk and Kirk, 1985; 1986). Therefore, it was concluded that changes in the protein spectrum were as a result of differences in the relative efficiency of translation of different mRNA species when exposed to differing environmental stimuli.

mRNAs are either associated with polysomes, in an active form, or bound in messenger ribonucleoprotein particles (mRNP) in an inactive form. Translational control can occur by changing the distribution of specific mRNAs between mRNP particles and polysomes without altering the actual efficiency of translation. This form of control is exemplified during amphibian oocyte development and early embryo development. Amphibian oogenesis is characterised by the synthesis and

accumulation of vast stores of mRNA that are stored in an inactive form and utilised by the embryo during early development, when much of the inactive maternal mRNA becomes mobilised and assembled into polysomes (Davidson, 1986; Gilbert, 1988). It is hypothesised that the oocyte mRNA is physically masked by proteins, in an inactive complex, and is therefore unable to bind to polysomes (Spirin, 1966). Within *Xenopus* oocytes a unique group of proteins have been isolated which have sedimentary co-efficients similar to non-translating ribonucleoprotein particles (Richter and Smith, 1983). In addition, a group of poly-A binding proteins have been demonstrated, via immunoprecipitation techniques, to be present in *Xenopus* oocytes which are postulated to bind to mRNA molecules and inhibit their translation (Richter *et al.*, 1986).

mRNA stability within the cytoplasm is also an important area for translational control of gene expression since, the greater the half-life of an mRNA species the greater the number of polypeptides that can be translated from it. The various mechanisms which determine mRNA stability have been reviewed (Brawerman, 1987). The ability to differentially degrade specific mRNAs can be critical for certain cell functions. The c-fos gene encodes a protein necessary for normal mammalian fibroblast cell division. Within the 3' region of its mRNA lies a large untranslated region rich in AU sequences. Deletion of this region (Meijlink *et al.*, 1985) results in an extended half-life of the molecule and cells divide continuously resulting in the formation of a tumour. The long half-life of mRNAs directing legumin protein synthesis in cotyledons of *Pisum sativum* has been shown to be important in the synthesis of protein during the later stages of embryo development (Gatehouse *et al.*, 1982).

1.4.2.4 Post translational control

Many newly synthesised polypeptides are inactive until post-translationally modified, by the removal of amino acids or the covalent attachment of smaller molecules (Gilbert, 1988). Many proteins that pass through the endoplasmic reticulum or Golgi apparatus are glycosylated by the addition of oligosaccharide side-chains containing N-acetylglucosamine and mannose (Chrispeels, 1985), e.g. the vicilin storage protein of *Pisum.* In addition to activation, via post-translational processes, proteins can also be deactivated. Rogers *et al.* (1986) demonstrated a correlation between the amino acid composition of protein terminals and their half-lives within the cell. From these results Bachmair *et al.* (1986) postulated that specific factor(s) bind to cellular proteins targeting them for degradation, the probability of this factor binding being dependent upon the terminal amino acid composition of the protein. Blobel *et al.* (1979) suggested a mechanism via which peptides are "targeted" to their correct position within the cell. According to the model a signal, at or near the N-terminus of the polypeptide, binds to a signal recognition particle (S.R.P.) which then binds to a signal recognition particle receptor (S.R.P.R.) within the target membrane. Reconstruction of this process *in vitro* (Walter *et al.*, 1984) showed that recognition of the signal peptide by a signal recognition particle arrests translation. Only when the signal peptide/S.R.P. complex binds to the correct S.R.P.R. in the target membrane is peptide elongation allowed to continue (Walter *et al.*, 1986). It is postulated that translation arrest is of great importance in the synthesis of certain proteins that would be detrimental to the cell if not correctly compartmentalised/secreted.

1.4.3 Differential Gene Expression and Differentiation

Kamalay and Goldberg (1980) demonstrated that qualitative changes in gene expression were required both to establish and maintain distinct differentiated

states. Using DNA/RNA hybridisation studies they compared the mRNA sequence sets of the organ systems (root, leaf, stem, ovary, anther, petal) of the tobacco plant, *Nicotiana plumbaginifolia.* Each organ system contained approximately 25000 diverse mRNAs. However, the mRNA composition of each organ system varied significantly. Each organ system contained a sub-set of mRNAs not detectable at the cytoplasmic level of other organ systems, e.g. leaf cells contained approximately 7000 unique mRNA species. The only exception was found to be leaf and petal which possessed similar mRNA spectrums, due to the fact that they are indistinguishable morphologically. Approximately one-third of the mRNA composition was common to all organ systems and presumed to be "housekeeping" mRNAs responsible for the synthesis of proteins required by all cells.

There are two model systems postulated to account for these differential mRNA profiles:-

(i) Transcriptional model:- Genes are uniquely expressed in a given organ system, whilst being repressed in others.

(ii) Post-transcriptional model:- All genes are constitutively expressed at the nuclear RNA level; post-transcriptional selection events operate to give rise to each organ-specific mRNA population.

Analyses of gene expression, in a number of animal and plant systems, indicate that both mechanisms operate. Analysis of nuclear RNA sets of tobacco organ systems (Kamalay and Goldberg, 1984) demonstrated that certain organ-specific mRNAs were detectable only in the nuclear RNA of that system, i.e. gene regulation was occurring at the level of transcription. However, post-transcriptional events were also shown to be involved in the establishment of organ specific mRNA populations since DNA/RNA hybridisation studies showed that approximately 20% of single copy DNA was present in nuclear RNA population,

whilst only 5% was represented in the cytoplasmic population (Kamalay and Goldberg, 1984).

Goldberg *et al.* (1981a), using nucleic acid hybridisation techniques estimated to be sensitive enough to detect 1 mRNA molecule/10 cells, analysed the expression of soybean embryo-specific super-abundant mRNA species (seed storage protein mRNAs) and demonstrated that certain embryo-specific mRNA species were not detected at the cytoplasmic level in either leaf, root or stem tissue. *In vitro* "run off" experiments using leaf nuclei demonstrated that leaf steady state nuclear RNA contained no embryo-specific mRNA molecules (Goldberg, 1983b). Control of soybean embryo-specific gene expression was therefore shown to be at the level of transcription resulting in the conclusion that, with the exception of a short period during embryogenesis, the genes encoding embryo-specific super-abundant mRNA species are effectively "off" during most of the soybean life-cycle (Goldberg *et al.*, 1981a; Goldberg, 1983a and b). Gallagher and Ellis (1982) demonstrated that the light-stimulated increase in mRNA levels encoding chloroplast polypeptides was mediated via an increase in transcription and not as a result of a decrease in mRNA degradation rates. Regulation of certain tuber-specific RNA species has been demonstrated to be at the transcription level, since they were absent from the nuclei of non-expressing cells, whilst other tuber-specific mRNAs were controlled at the post-transcription level since they were detected in nuclei from organs in which no accumulation of steady state mRNA was observed (Rosahl *et al.*, 1986).

Comparisons of nuclear RNA and cytoplasmic poly $(A)^+$ mRNA populations from a variety of animal organisms (Lewin, 1980) showed that only a portion of the nuclear RNA population was represented in the cytoplasmic mRNA, indicating an important role for post-transcriptional level regulation. For example, in sea urchin embryos only 10-20% of the nuclear RNA complexity is present in the

cytoplasmic mRNA populations (Galau *et al.*, 1976). During sea urchin development mRNA sequence set complexity decreases (Galau *et al.*, 1976). However, using blastula specific cDNA, Wold *et al.* (1978) demonstrated that all blastula-specific message sequences were present in the hnRNA of all adult tissues investigated, whilst only 12-13% was represented in their cytoplasmic mRNA populations. Both transcriptional and post-transcriptional controls have been shown to be responsible for gene regulation in *Phaseolus vulgaris*. The absence of phytohaemagglutinin (PHA) in a PHA deficient cultivar was shown to be primarily as a result of a reduced transcription rate, however, low transcript levels were detected in nuclei, whilst mRNA did not accumulate. It was postulated that post-transcriptional regulation occurred via instability of the low level of mRNA transcribed (Chappell and Chrispeels, 1986).

1.4.3.1 Abundance levels of mRNA and differentiation

mRNA populations can be arbitrarily divided into three classes:-

(i) Complex class mRNA - present at 1 to several copies per cell.

(ii) Moderately prevalent - present at a few hundred copies per cell.

(iii) Superprevalent - more than 10^4 copies per cell.

Superprevalent mRNAs occur in highly differentiated cell types and, although of crucial importance for certain cell types, they represent only a fraction of all the diverse gene products required by a cell. In addition, superprevalent mRNAs are not found in all cell types. Both moderately prevalent (Brandhorst, 1976) and complex class mRNAs (Galau *et al.*, 1976) are regulated. For example, in sea urchin less than 20% of the embryo complex class mRNA sequence set is ubiquitous (Galau *et al.*, 1976). Davidson and Britten (1979) proposed separate control mechanisms for genes encoding superprevalent mRNAs and moderately prevalent/complex class mRNAs. They concluded that the only clear evidence for

transcriptional level control of structural genes pertained to those genes encoding the superprevalent mRNA class, whilst structural genes encoding the complex/moderately prevalent class mRNAs are transcribed continuously, at relatively constant rates, with regulation occuring at the post-transcription level.

1.5 DIFFERENTIAL REGULATION OF SEED STORAGE PROTEIN GENES

1.5.1 Seed Storage Proteins

The proteins present in seeds can be subdivided into two types:-

(i) Metabolic proteins - both enzymatic and structural, which are involved in the general metabolic activities of the cell, including the synthesis of the second type of protein;

(ii) Storage proteins - seed storage proteins are generally defined as being any protein, accumulated in significant quantities in the developing seed, which is rapidly hydrolysed during germination to provide a source of reduced nitrogen for the early stages of seedling growth (Higgins, 1984). In addition, starch, oil, or both, may be deposited during seed formation to provide a source of carbon for subsequent germination.

Since reserve proteins exhibit no apparent catalytic properties they are classified on the basis of their physico-chemical properties. The first systematic analysis of seed proteins was carried out by Osborne (1924), who classified them on the basis of their solubility. Seed storage proteins are generally divided into four classes: albumins, globulins, prolamins and glutelins, which are soluble in water, salt, aqueous alcohol and acid/alkali solutions, respectively. In general, the major seed storage proteins of legumes and other dicotyledon species are globulins, whilst those of monocotyledon species (a major exception being oats) are prolamins and glutelins (Higgins, 1984).

In mature seeds the storage proteins are found predominantly in small organelles called protein bodies, which are surrounded by a single membrane of tonoplast or endoplasmic reticulum origin (Pernollet, 1978). Two main functions are attributed to the protein storage organelles:- the protection of the proteins from enzymatic digestion and the maintenance of specific environmental conditions required for the correct packaging and storage of the protein (Muntz, 1982).

Consistent with their role as a reserve of nitrogen for the germinating seedling, storage proteins tend to be rich in asparagine, glutamine and arginine or proline (Higgins, 1984). This bias in amino acid composition means that certain amino acids are under-represented. Generally legumes are deficient in cysteine, methionine and tryptophan (Danielsson, 1949), whilst in cereals lysine, threonine and tryptophan are present at reduced levels (Higgins, 1984). On average, the protein content of cereal grains is 10-15% of dry matter and in legume seeds 20-25% (Derbyshire *et al.*, 1976). In contrast, in a typical vegetative organ, e.g. leaf, only 3-5% of dry matter is protein. Hence seeds are an extremely important source of nutrition for both humans and animals.

1.5.2 Storage Proteins of the Leguminosae

The two main classes of the globulin storage proteins of the Leguminosae have been identified on the basis of their solubility (Osborne, 1924) and sedimentary coefficient (Danielsson, 1949). Danielsson (1949) showed that the globulin storage protein of the Leguminosae was characterised by the presence of two peaks when ultracentrifuged:- the 11S legumin and the 7S vicilin.

Examination of the seed storage proteins of 34 species of legumes (Danielsson, 1949) showed that, with few exceptions, all contained two classes of globulin of 11S and 7S, although they differed slightly in amino acid composition

and chemical properties (Derbyshire *et al.*, 1976). This indicates a wide distribution of these proteins throughout the genera. In addition, legumin and vicilin type proteins have been shown to occur in some non-legume species (Derbyshire *et al.*, 1976). An explanation for the homology of the 11S and 7S storage proteins within a wide variety of plant groups, has been suggested by Spencer (1984). The structure of a seed storage protein is required to fulfil certain requirements, for example, they must be able to withstand the osmotic changes that occur on desiccation and imbibition. It is postulated that once a protein, that is an efficient store of nitrogen, has evolved to meet all the criteria required of a seed storage protein, there may be evolutionary constraints against a change in structure.

1.5.2.1 Storage proteins of *Pisum*

Legumin is a hexameric protein (M_r 360-400000) comprised of six M_r 60000 subunits. Each subunit consists of a relatively acidic polypeptide (rich in glutamine/glutamic acid) of M_r 40000 disulphide linked to a M_r 20000 relatively basic polypeptide (rich in alanine, valine and leucine) (Derbyshire *et al.*, 1976). Legumin is synthesised as a M_r 60000 precursor polypeptide containing one copy each of the acidic and basic subunits covalently linked together (Croy *et al.*,1980a) and undergoes extensive co- and post- translational modification, including removal of the leader sequence and proteolytic cleavage (Boulter, 1983).

Vicilin (M_r 150000) consists of M_r 50000 subunits and non-equimolar amounts of a range of lower molecular weight subunits (Gatehouse *et al.*, 1981). Vicilin is also extensively co- and post-translationally modified including removal of the leader sequence, proteolytic cleavage and is glycosylated.

A third vicilin-type storage protein, convicilin, is present in *Pisum* (Croy *et al.*, 1980c). It consists of M_r 71000 subunits and apart from removal of the leader sequence, does not undergo any co-/post-translational modifications.

The subunits of legumin and vicilin exhibit considerable charge and size heterogeneity (Croy *et al.*, 1980a and b). This is partly as a result of extensive co- and post-translational modification and partly genetic in origin (Boulter, 1983), since both legumin and vicilin are encoded by small multigene families (Boulter *et al.*, 1987).

Hybridisation studies have shown the presence of ten legumin genes that can be divided into three sub-families (Boulter *et al.*, 1987).

(i) The five gene sub-family consisting of genes A-E (of which D is a pseudogene) that encode the major legumin subunit pairs which constitute approximately 80% of the legumin product.

(ii) Genes J-L which encode the minor legumins.

(iii) A possible third sub-family consisting of two genes M and X; the latter having not yet been isolated, the sub-family being postulated on the basis of amino acid sequences and size data of encoded proteins.

Knowledge of the vicilin multigene family is less advanced compared to legumin. Southern blotting indicates the presence of approximately fifteen genes in the pea genome which, based upon hybridisation studies, are divisible into 3 subgroups (Boulter *et al.*, 1987).

1.5.3 Late Legume Embryogenesis and Storage Protein Deposition

Seed growth characteristics, e.g. fresh weight, cotyledon length, generally show a characteristic sigmoidal growth curve until the onset of dormancy (Muntz, 1982). Storage protein synthesis and deposition occurs mainly on cessation of cell division, after expansion of the cotyledon parenchyma cells (Bain and Mercer,

1966). However, small amounts of protein, legumin (Domoney *et al.*, 1980), vicilin (Corke *et al.*, 1987), have been detected at embryonic stages prior to this. Bain and Mercer (1966) divided *Pisum* embryogenesis into four phases: (i) cell formation; (ii) cell expansion; (iii) synthesis of storage reserves; (iv) maturation and dormancy. Morphologically, phase II of development is characterised by an increase in cotyledon size due to rapid expansion of the parenchyma cells. At this stage the cells contain one or two large vacuoles with small, discrete protein deposits on the inner membrane. As maturation continues the large vacuole is progressively replaced by an increasing number of small vacuoles, resulting in an increase in the vacuolar surface/volume ratio. The amount of protein staining material within the vacuoles increases until their profiles stain uniformly, at which point they are termed protein bodies (Craig *et al.*, 1979a). Serial sections of developing cotyledons suggest that the large vacuole of early development becomes convoluted and fragments to give rise to the protein bodies present at maturity (Craig *et al.*, 1980a). Both legumin and vicilin are sequestered into the same protein body (Graham and Gunning, 1970) and this pattern of sequestration occurs throughout development (Craig *et al.*, 1980b). Synthesis of reserve protein precursors occurs on the rough endoplasmic reticulum, followed by transient sequestration within this organelle (Chrispeels *et al.*, 1982). Protein is deposited within the protein bodies via movement through the Golgi apparatus (Craig, 1988) where vicilin undergoes glycosylation (Craig, 1988). Subsequent transport to protein bodies is via Golgi-derived vesicles (Craig, 1988). However, within the Golgi apparatus differential sorting of storage proteins is reported to occur. Vesicles associated with the *cis*-face accumulate predominantly legumin, whilst vesicles derived from the *trans*-face accumulate vicilin (Harris *et al.*, 1989). The importance of the Golgi apparatus in protein transport is demonstrated by the disruption of Golgi function, by monensin (Craig and Goodchild, 1984), which

results in protein transport to the plasmalemma/cell wall compartment. However, disruption of glycosylation, via tunicamycin, demonstrated that glycosylation was not necessary for the assembly of vicilin into oligomers (Badennoch-Jones *et al.*, 1981). Cotyledon cells become highly polyploid during embryogenesis. Maximum ploidy levels in *Pisum sativum* (32-64C) are reached after cell divison and concomitant with the onset of maximum storage synthesis (Scharpe and V. Parijs, 1973). Reassociation kinetics of DNA from diploid and polyploid cells of *Vicia faba* showed that the increase in DNA was as a result of endoreduplication of the entire genome (Millerd and Whitfield, 1973) and not as a result of amplification of the storage protein genes. Analysis of genomic DNA isolated from *Pisum sativum* cotyledon and leaf showed no selective amplification or alteration of the legumin gene (Croy *et al.*, 1982). Therefore, selective gene amplification is not responsible for the synthesis of large quantities of the seed storage proteins that occurs during the late stage of legume embryogenesis. Polyploidy is postulated to provide a nucleotide source for the germinating seedling (Bain and Mercer, 1966).

1.5.4 The Control of Storage Protein Gene Expression

By the end of *Pisum* seed development approximately 80% of the total soluble protein consists of storage protein subunits (Gatehouse *et al.*, 1982) which result from the expression of approximately twenty genes organised into small multigene families. The high expression of the seed storage proteins, legumin (11S) and vicilin (7S), for a short period during the life cycle of *Pisum* provides an excellent system for the study of gene expression. Legumin and vicilin storage protein gene transcripts are either absent or present at undetectable levels in leaf (Evans *et al.*, 1984), i.e. they are seed-specific, tissue specificity being achieved primarily at the level of transcription. Both spatial and temporal differential synthesis and accumulation of storage proteins occurs within the seed.

Within the cotyledons of *Pisum sativum* cells do not accumulate storage proteins to the same level, both the epidermal and vascular elements have been shown, by immunocytochemistry, not to accumulate protein (Harris *et al.*, 1989). In addition, the pattern of protein deposition within the organs of the embryo varies. In soybean the major seed storage proteins have been shown to be expressed at lower levels in the embryo axis, compared to the cotyledons (Meinke *et al.*, 1981; Goldberg *et al.*, 1981b). The genetic basis of this spatial differential expression is not known, but may possibly be influenced by the differing cytological environments experienced by the different tissues/organs within the ovule, i.e. the differential spatial expression is determined by the overall embryonic developmental programme.

The seed storage proteins of *Pisum* exhibit an accumulation pattern which is sigmoidal (Gatehouse *et al.*, 1982), indicating an increasing rate of synthesis of gene product during the earlier stages of development which reaches a maximum and then declines during the later stages of development. The different storage proteins exhibit differing times of onset of rapid synthesis, maximal synthesis and synthetic decline. Vicilin subunits are present in larger amounts earlier in development, whereas legumin and convicilin accumulate in greater amounts, relative to vicilin, later in development (Gatehouse *et al.*, 1982). Using Northern blot analysis it has been shown (Gatehouse *et al.*, 1982) that for legumin and vicilin (M_r 47000 precursor) mRNA levels are in reasonable agreement with the levels of protein detected, indicating an important role for transcriptional control in the differential regulation of the storage protein genes.

Evidence to support the role of transcriptional control can be derived from analysis of protein and mRNA profiles of plants (*Pisum sativum*) grown under conditions of sulphur deficiency. Under such conditions there is a differential effect such that synthesis of the sulphur-rich protein, legumin, is decreased, whilst

accumulation of the other proteins is either unaffected or increased (Randall *et al.*, 1979). The reduced legumin content of seeds was shown to be largely as a result of a decrease in legumin mRNA production (Chandler *et al.*, 1983). However, nuclei transcription assays (Evans *et al.*, 1985) showed that, although transcription was the major factor in determining the gene expression response to sulphur deficiency, transcription rates were not reduced to a level low enough to fully account for the reduction in mRNA. Therefore, other factors such as mRNA stability are involved in modulating transcriptional control, a result verified by the work of Beach *et al.* (1985).

The increasing mRNA levels which occur during seed development may be as a result of increasing transcription rates, decreasing degradation rates, or both. Millerd and Spencer (1974) demonstrated a continuous decrease in the amount of RNA per cell and per cotyledon after cessation of cell division in *Pisum.* It was therefore postulated that production of storage protein mRNA species is restricted to the early phase of cotyledon development and that once maximal synthesis is achieved subsequent mRNA levels are determined by their relative rates of stability (Muntz, 1982). The results of mRNA inhibition, in *Pisum sativum* cotyledons, by α -amanitin substantiate the aforementioned theory since protein production remained constant over a two-day period despite the absence of mRNA synthesis (Gatehouse *et al.*, 1982), indicating that the storage protein mRNAs have relatively long half-lives.

Different pea varieties/species exhibit quantitative variability in the levels of legumin, vicilin and convicilin present within the seed. Analysis of gene copy number in the variant genotypes (Domoney and Casey, 1985) showed that there was no difference in the number of genes encoding the major storage proteins, even though protein compositions varied. Therefore, in *Pisum*, protein variations in

different genotypes are solely as a result of processes occurring at the transcriptional/post-transcriptional/ translational level.

Run-off transcription assays (Evans *et al.*, 1984) indicated that in 9 d.a.f. embryos of *Pisum sativum* the transcription rates of vicilin genes (M_r 47000; 50000 precursors) and legumin genes were similar. However, the cytoplasmic mRNA levels of the two gene sets varied indicating that during the early stages of embryo development, at least, cytoplasmic mRNA levels are regulated by post-transcriptional selective processing of heterogeneous nuclear RNA. This mechanism is postulated to be of importance in the control of gene expression in both plants (Kamalay and Goldberg, 1984) and animals (Bathurst *et al.*, 1980).

1.5.4.1 DNA sequence basis for the control of differential storage protein gene expression

It is postulated that genes which are expressed in a tissue-specific manner would possess a homologous DNA sequence, within their 5' flanking region, that is responsible for their tissue-specific expression (Goldberg, 1983a; Gatehouse *et al.*, 1986). Foreign seed-specific genes are expressed in a typical seed-specific manner when transformed into different genera (Okamuro *et al.*, 1986; Barker *et al.*, 1988; Ellis *et al.*, 1988), indicating that DNA sequences and regulatory factors are highly conserved in different species. However, comparison of the 5' flanking region of seed protein genes from different families has shown little homology, apart from the TATA box region (Heidecker and Messing, 1986).

Although no universal seed-specific sequence has been identified, putative control elements specific to gene families have been identified. Analysis of the 5' flanking region of legumin genes from four pea species, *Glycine max* and *Vicia faba* has revealed a conserved region of 28 bases approximately -100 relative to the transcription start, of which the first 14 bases are invariant (Baumlein *et al.*, 1986).

This element is absent from equivalent positions in all of the non-legumin and fungal genes examined. The region has been termed the "legumin box" and is postulated to have a function in the regulation of legumin gene expression. Examination of expression of deletion mutants of the *leg*A seed storage protein gene in transformed tobacco plants (Shirsat *et al.*, 1989) demonstrated the requirement of the legumin box and a further upstream enhancer element for correct gene expression. Analysis of the vicilin gene family has revealed the presence of a conserved region of 42 bp approximately -130 bases relative to transcription initiation. Within the 5' region of the conserved sequence 13 bases are invariant and include a sequence also found in the "legumin box" and identified as being homologous to the adenovirus enhancer sequence (Lycett *et al.*, 1984).

1.6 RESEARCH AIMS

The general aim of this research is to examine, by cytological and molecular techniques, early seed formation and embryogenesis in *Pisum.* The events underlying embryogenesis in animals are well documented, however little is known about embryogenesis in higher plants. The following are therefore the main aims of this research:-

(i) to establish the basic structural and cytological events of early embryogenesis in *Pisum sativum* L.

(ii) to establish whether *leg*A is constitutively expressed or "switched on" during development. Previous workers (Gatehouse *et al.*, 1982; Boulter *et al.*, 1987) have failed to detect *leg*A mRNA or protein prior to 9 d.a.f. *In situ* hybridisation, using a probe to *leg*A mRNA, was utilised to detect *leg*A expression during the early stages of embryogenesis.

(iii) to determine the earliest embryonic stage at which LegA could be detected.

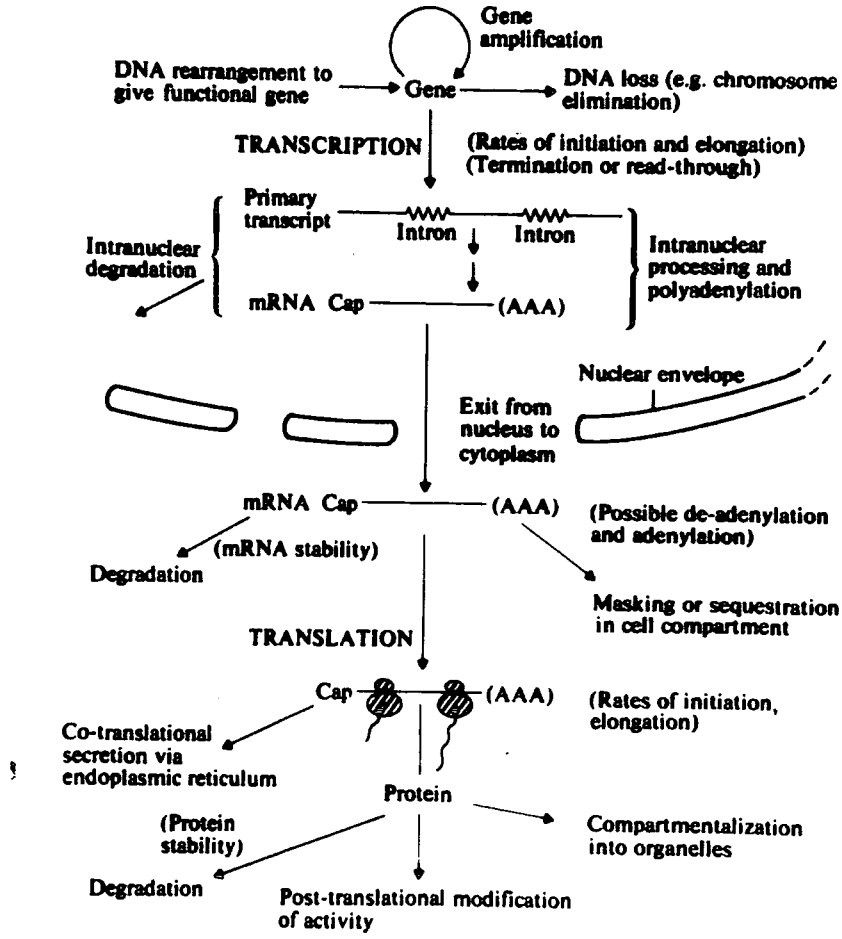
(iv) to determine the spatial distribution of legumin in early ovules via immunocytochemistry.

(v) to examine *leg*A expression in the various components of the seed. The majority of previous investigations of storage protein gene regulation have utilised homogenised whole seed extracts. However, the immature seed consists of four distinct components:- the embryo proper, suspensor, endosperm and integument whilst the mature seed is composed of embryo and integument.

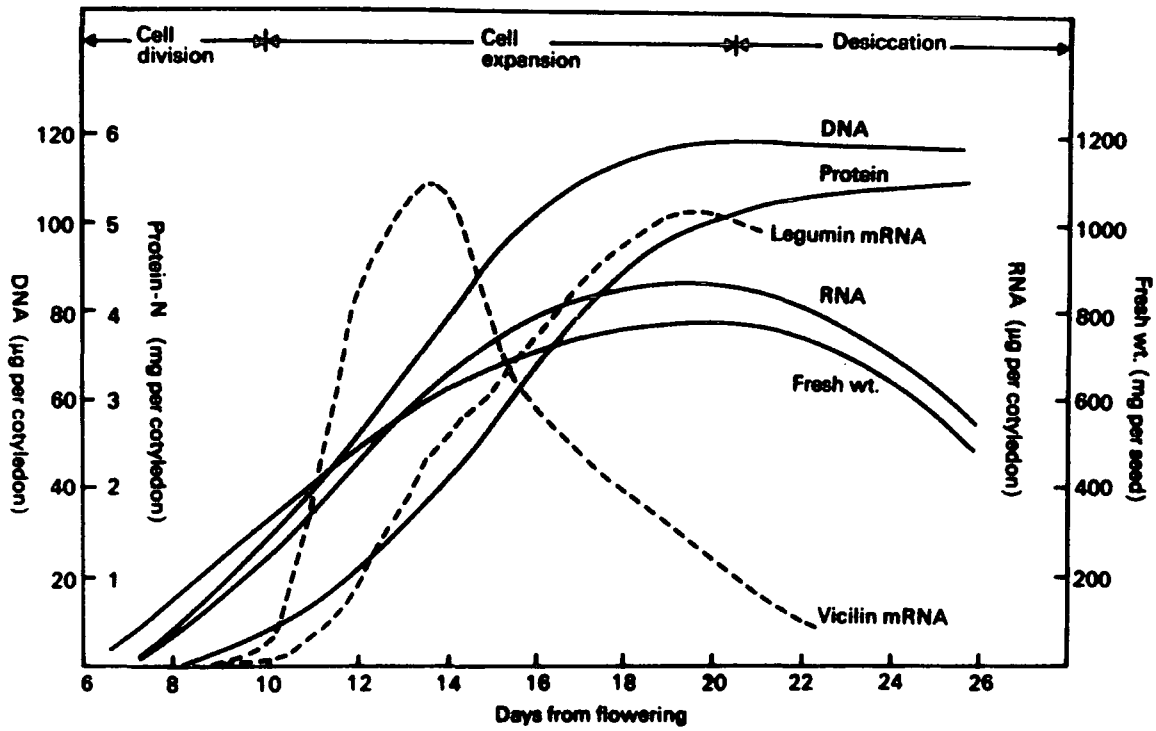
(vi) to determine the molecular events underlying *leg*A expression by correlated *in situ* hybridisation and immunocytochemistry.

(vii) to examine embryonic root ontogeny during development.

FIG 1



Possible points of regulation in the gene-to-protein pathway of class II nuclear genes. Taken from Graham and Wareing (1984).



Changes in the amount of DNA, RNA, protein, and vicilin and legumin mRNAs during development of pea seed cotyledons. Modified from Spencer and Higgins (1982), and Gatehouse *et al.* (1986).

2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Biological Materials

Seeds of *Pisum sativum* c.v. "Feltham First" were obtained from Sutton Seeds Ltd., Reading, Berks., U.K.

Anti-legumin polyclonal antisera were raised by the injection of purified legumin into New Zealand white rabbits and purified using immunoaffinity chromatography (Croy *et al.*, 1980a). The polyclonal antisera used during this research were provided by Drs. J.A. Gatehouse and R.R.D. Croy, Department of Biological Sciences, University of Durham. The monoclonal anti-legumin antibody was provided by Dr. A. Kang, Department of Biological Sciences, University of Durham.

The pAD 4.4 clone consists of a 1120 bp cDNA, complementary to 90% of the acidic and basic subunit pair coding region of the *leg*A mRNA, cloned into the Bam H1 site of pBR 322 (Delauney, 1984). The clone pRC 2.2.4 (Croy *et al.*, 1982) consists of a 638 bp cDNA complementary to the 3' end of the *leg*A mRNA, including the polyadenylation signal, cloned into the Bam H1 site of pBR 322. Both pAD 4.4 and pRC 2.2.4 were kindly provided by Dr. R.R.D. Croy, Department of Biological Sciences, University of Durham.

p^{PR} 179 was produced and provided by Dr. I. M. Evans and Mr. L. Gatehouse, Department of Biological Sciences, University of Durham. A cDNA library, prepared from poly $(A)^+$ mRNA from *Pisum sativum* root, was screened for mRNA species present at elevated levels in root tissue relative to other organ systems of the plant (Evans *et al.*, 1988). The clone p^{PR} 179 consists of a 470 bp cDNA complementary to a mRNA of approximately 640 bp which was found, by Northern blot analysis, to be more abundant in root tissue compared to other organ systems of the plant.

2.1.2 Chemical and Non-Biological Materials

Unless otherwise stated chemicals were obtained from either B.D.H., Poole, Dorset, U.K. or Sigma Chemicals, Poole, Dorset, U.K., and were of the purest grade available.

Spurr resin and fixatives (Taab Laboratories, Reading, Berks., U.K.); L.R. White resin and osmium tetroxide (Agar Aids, Stanstead, Essex, U.K.); streptavidin gold and silver enhancement reagents [Intense II] (Janssen Life Sciences, Wantage, Oxon., U.K.); Photoprobe biotin and Vectastain ABC-AP Kit (Vector Laboratories Inc., Bretton, Peterborough, U.K.); gold conjugated secondary antibodies (Bioclin Services Ltd., Cardiff, U.K.); K2 nuclear emulsion and photographic reagents (Ilford Ltd., Mobberley, Knutsford, Cheshire, U.K.); Decon 9 cleaning agent (Decon Laboratories Ltd., Hove, U.K.); Histomount (B.S. and S. [Scotland Ltd.], Edinburgh, U.K.); Soluene 350 (Canberra-Packard Ltd., Pangbourne, Berks., U.K.); radiochemicals and Nick-translation kit (Amersham International p.l.c., Amersham, Bucks., U.K.); restriction enzymes, agarose, L.M.P. agarose (B.R.L. Ltd., Cambridge, U.K.); H.G.T. agarose (I.C.N. Biomedicals Ltd., High Wycombe, Bucks., U.K.); Sephadex G-50, random hexameric nucleotides (P.L. Biochemicals Inc., Pharmacia [G.B.] Ltd., Milton Keynes, Bucks., U.K.); Nitrocellulose (Schleicher & Schuell, Dassel, Germany); dialysis tubing (Medicell International Ltd., London, U.K.); Murashige and Skoog culture medium (Flow Laboratories, Rickmansworth, Herts., U.K.); Fuji RX-100 x-ray film (Fuji Ltd., Swindon, Wilts., U.K.); Nunc maxisorp microtitre plates (Gibco BRL Life Technologies, Paisley, Scotland, U.K.).

2.2 METHODS

2.2.1 Growth Conditions of Plant Material

Pea seeds (*Pisum sativum* c.v. "Feltham First") were surface sterilised with 5% calcium hypochlorite for 15 min., prior to germination in the dark at 25°C.

After approximately three days the developing seedlings were transferred to individual containers and grown hydroponically in a growth cabinet under a lighting regime of twelve hours light, twelve hours dark, and a temperature regime of 25°C (16 h.-day); 18°C (8 h.-night).

2.2.2 Harvesting of Material

Flowers were tagged when the corolla was fully open (day 0) and pods subsequently harvested at various days after flowering (d.a.f.). In order to avoid constriction effects at the pod extremities (Linck, 1961) only the central three seeds from each pod were excised.

2.2.3 Formvar Coating of EM Grids

Clean glass microscope slides were dipped into a solution of 0.3% formvar in chloroform and allowed to dry for approximately 3 min. The slide was scored using a razor blade and the formvar coating floated onto the surface of water. Grids were placed onto the formvar, lifted off and allowed to dry thoroughly.

2.2.4 Processing of Material for EM/LM Examination of Morphology

Seeds of each developmental stage were fixed in 2.5% glutaraldehyde; 1.5% formaldehyde in 0.05 M sodium cacodylate buffer, pH 7.0. Prior to fixation the ovular tissue was "nicked", without damage to the embryo or suspensor, in order to allow greater penetration of the fixative and resin. Specimens that were to be embedded in epoxy resin for EM examination were post-fixed in 1% aqueous osmium tetroxide. Fixation times were varied, according to the size of the ovule, ranging from 2-7 h. Samples were then dehydrated, via an ascending graded alcohol series, prior to infiltration and embedding in either Spurr epoxy resin,

which was polymerised at 70°C, or L.R. White acrylic resin, which was polymerised anaerobically at 60°C.

For observation under the light microscope, L.R. White resin embedded material was sectioned at one micron thickness, on glass knives, using a Sorval M.T.2-B ultra-microtome (Du Pont Instruments). Sections were placed on glass slides and stained with 0.1% toluidine blue in 1% borax for approximately 30 sec. Sections were photographed using Kodak Tech Pan monochrome film, 100 A.S.A. on a Leitz Ortholux or Nikon Diaphot TMD microscope.

Ultra-thin sections of osmicated material, embedded in Spurr resin, were cut to a thickness of 900-1500Angstroms (gold interference colour) using a Sorval M.T.2-B ultra- microtome. Sections were collected on formvar-coated grids, stained with saturated aqueous uranyl acetate for 15 min., washed in distilled water and post-stained with lead citrate for a further 15 min. (Reynolds, 1963). Ultra-thin sections were viewed using a Phillips 400 transmission electron microscope, at an accelerating voltage of 80 kV, and recorded on Kodak electron image film 4489.

2.2.5 Preparation of Material for Immunological/Cytological Procedures

Fixation was carried out using 4% paraformaldehyde in P-S buffer (0.05 M phosphate, pH 7.4; 0.05 M sucrose) [for *in situ* hybridisation] or 3% paraformaldehyde; 1.25% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4 [for immunocytochemistry]. Fixation times were varied according to tissue type and thickness. Material was dehydrated and either directly infiltrated and embedded in polyethylene glycol (PEG) molecular weight 1500 or, following dehydration, was equilibrated with xylene prior to infiltration with wax. Ten micron thick sections of PEG or wax embedded material were cut, using metal knives, on a Leitz microtome and dried onto clean, subbed slides.

Alternatively, cryosections were prepared. Excised material was frozen immediately, via immersion in liquid nitrogen, and subsequently stored at -80°C until required. Sections were cut out, at either 12 or 24 micron thickness, on metal knives, using a cryostat (Bright Instrument Co. Ltd.) and transferred onto subbed slides previously maintained at ambient room temperature. Sections were fixed using 4% paraformaldehyde in P-S buffer for 30 min., washed in P-S buffer and stored at -20°C until required.

2.2.5.1 Slide preparation

Slides used during this work were routinely "subbed" - a protocol which subsequently results in an increased adhesion between section and slide. This is of great importance when protocols require sections to be incubated in solutions for prolonged periods, at high temperatures, or require numerous changes of solutions. Glass microscope slides were cleaned by soaking for at least 2 h. in an aqueous solution of 4% v/v Decon 9 cleaning agent, rinsed thoroughly in running tap water, then distilled water and allowed to dry. Cleaned slides were then dipped in subbing solution [1% w/v gelatin, 0.1% w/v chrome alum (Gall and Pardue, 1969)], and allowed to air-dry in a vertical position prior to storage in a dust-free container.

2.2.5.2 Rehydration of wax and PEG embedded material

PEG embedded material was rehydrated by immersion in water, with slight agitation, for approximately 1 min. Wax embedded material was firstly incubated in xylene, with agitation for approximately 30 min., followed by passage through a descending graded alcohol series and finally equilibrated with water.

2.2.6 Histochemical Staining of Tissue Sections for RNA

(i) Methyl green/pyronin

Sections were rehydrated and washed in 0.2 M sodium phosphate pH 6.0, then washed in water for 5 min. followed by a wash in Walpoles buffer (0.12 M sodium acetate, 0.8 N acetic acid pH 4.8). Sections were incubated with methyl green/pyronin (0.36% w/v methyl-green; 0.16% w/v pyronin Y; 28% v/v glycerol in Walpoles buffer; the solution was extracted overnight with chloroform to remove the methyl violet contaminant) for 25 min., rinsed in distilled water, blotted dry, dehydrated through a graded alcohol series and mounted in Histomount. Control sections were incubated at 37°C for 1 h. with Ribonuclease A at a concentration of 0.5 mg/ml in 0.2 M phosphate buffer pH 6.0, prior to staining with methyl green/pyronin.

(ii) Acridine Orange

Following rehydration, sections were incubated for 30 min., at room temperature, in the dark, in a solution of 0.5 mg/ml acridine orange in 0.2 M glycine-HCl, pH 2.0. Sections were washed in 0.2 M glycine, mounted in the same solution and examined using a fluorescence microscope with a blue excitation filter (400 nm) and 530 nm suppression filter.

2.2.7 Immunolabelling at LM Level using Gold and Silver Enhancement

Sections were rehydrated, incubated with Lugol's iodine (1% w/v iodine in 2% aqueous potassium iodide) for 5 min. and washed briefly in tap water. 2.5% sodium thiosulphate was added until the sections turned colourless and then rinsed in running water for 10 min.. Sections were blocked using 1% w/v BSA in PBS (10 mM phosphate, pH 7.4; 140 mM NaCl) for 10 min., followed by a 2 x 10 min. PBST (10 mM phosphate, pH 7.4; 140 mM NaCl; 0.5% Tween 20) wash.

Incubation with the primary antibody was for 1 h. at room temperature whilst control sections were incubated with pre-immune serum. After washing with PBST (3 x 15 min.) sections were incubated with the secondary antibody linked to colloidal gold (10 nm), diluted $^1/_{20}$ with PBST, for 30-40 min. Sections were washed in PBST (3 x 15 min.), then in distilled water, followed by silver enhancement using Janssen Intense II for 5-10 min.. Following a rinse in distilled water, sections were dehydrated to 70% ethanol, stained with either light green or safranin for 30 sec., dehydrated to 100% ethanol and mounted in Histomount.

2.2.8 Immunolabelling at EM Level

Ultrathin sections of Spurr-embedded material were collected on formvar coated nickel or gold grids. The labelling procedure was performed by floating the grids, section side down, on drops of solution placed on Nescofilm in a moist chamber. Sections were "etched", to expose antigenic sites, by incubation in saturated aqueous sodium periodate for 30 min. (optional). Following three water washes, sections were blocked using 1% w/v BSA in PBS for 10 min. Following 3 x 1 min. PBST washes, sections were incubated in primary antibody, diluted in PBST, for 1 h. Sections were then washed 10 x 1 min. in PBST followed by incubation with the secondary antibody, linked to gold (20 nm), diluted $^1/_{20}$ with PBST for 1 h. Following a further 10 x 1 min. PBST washes, sections were rinsed in a stream of distilled water, stained with aqueous uranyl acetate for 15 min., rinsed in distilled water, dried and viewed in the EM.

2.2.8.1 Optimisation of antibody dilution for immunolocalisation

The optimum concentration of each antibody batch used during this research was determined by performing an immunolocalisation using the antibody at various dilutions, i.e. $^1/_{10}$; $^1/_{100}$; $^1/_{1000}$, with a tissue known to contain the

antigen under investigation at relatively high levels. Subsequent analysis of results allowed determination of the optimal antibody concentration.

2.2.9 Enzyme Linked Immunosorbent Assay (ELISA)

Embryos and corresponding ovules, ranging in age from 2 days prior to anthesis to 11 d.a.f., were homogenised in 250 µl extraction buffer (0.3 M NaCl; 0.2 M Tris-HCl, pH 9.5) and centrifuged at 13000 x*g* for 10 min. to pellet cell debris.

Total protein content of samples was determined using the dye-binding method of Bradford (1976), with a range of BSA concentrations used as standards. The resultant colour reaction was measured by spectrophotometric absorbance at 818 nm.

1 µg of total protein from each extract, and a range of legumin protein standards, were added to the wells of a microtitre plate (Nunc maxisorp) and the volumes increased to 200 µl with extraction buffer. Plates were incubated at -4°C for 16-20 h. and then washed extensively by flooding with PBST (10 mM phosphate pH 7.2; 2.7 mM KCl; 140 mM NaCl; 0.5% Tween 20). All further incubations were carried out at room temperature. 300 µl aliquots of aqueous 1% w/v BSA in PBS were added to each well and incubated for 1 h. Plates were washed as described above. 200 µl aliquots of primary antibody (mouse monoclonal anti-legumin diluted $^1/_{10}$ with PBSTB (PBST plus 0.1% w/v BSA) were added to each well and incubated for 2 h.. Plates were washed as described and incubated for 2 h. with the enzyme conjugate (sheep anti-mouse peroxidase conjugate diluted $^1/_{1000}$ with PBSTB). Plates were then washed as before and 200 µl aliquots of enzyme substrate (1.09 mM ABTS in 0.12 M citric acid; 0.1% H_2O_2, pH 4.0) were added to each well. The reaction was allowed to proceed for 30 min. at room temperature. The quantity of hydrolysed substrate in each well was measured by spectrophotometric absorbance at 414 nm using a Titertek Multiscan MCC (Flow

Laboratories Ltd., U.K.). The legumin content of each extract was determined by comparison of the absorbance values obtained with those of the standards.

2.2.10 Treatment of Glassware and Solutions

All plasticware, glassware and solutions used for nucleic acid work were sterilised by autoclaving before use. For use in RNA manipulations all glassware was siliconised and dry heat sterilised by baking overnight at 170°C. Aqueous solutions were made up in redistilled, deionised water and treated with 0.1% diethylpyrocarbonate at 37°C, overnight, prior to autoclaving.

2.2.11 Restriction Analysis

cDNA inserts were removed from plasmid vectors via restriction endonuclease digestion using the appropriate restriction enzyme and buffer (Maniatis *et al.*, 1982). Restriction reactions were incubated for 2-3 h. at 37°C, and terminated by heat inactivation of the enzyme (70°C; 10 min.).

2.2.12 DNA Fractionation via Agarose Gel Electrophoresis

DNA digests were run on 0.8% agarose gels in order to separate and identify the DNA fragments produced as a result of restriction. The prepared gel (0.8% w/v agarose; 1 µg/ml EtdBr; 40 mM Tris-acetate pH 7.7; 2 mM EDTA) was poured into a perspex mould placed on a clean glass plate with vacuum grease used to produce a seal between mould and glass. Perspex combs were used to produce wells within the gel and it was allowed to cool on an even surface. Once set the gel was transferred to an electrophoretic tank with sufficient running buffer (40 mM Tris-acetate pH 7.7; 2 mM EDTA; 1 µg/ml EtdBr) to cover it. The DNA digest, previously mixed with tracking dye* at a ratio of 5:1, was loaded onto the gel and run overnight at 30 V. The digest of a previously characterised plasmid e.g. pBR

322, the restriction of which with a known enzyme produces fragments of a known size, was run alongside digests in order that the fragments produced could be sized by comparison. Localisation of DNA was determined by direct examination under u.v. light at a wavelength of 245 nm (the incorporation within both gel and buffer of the fluorescent, intercalating dye, ethidium bromide, allows DNA visualisation under such conditions) and recorded by photographing with Polaroid film type 667 (3,000 A.S.A.) through a red-orange filter (Kodak 23a Wratten).

*Tracking dye:- 10 mM Tris-HCl pH 8.0; 10 mM EDTA; 31.25% v/v glycerol; 0.2% w/v agarose; 0.1% w/v bromophenol blue - the mixture was autoclaved, allowed to solidify and extruded through a syringe needle.

2.2.13 Recovery of DNA from Agarose: Gel Electro-elution into Dialysis Bags

The method used was that of McDonnell *et al.* (1977). The DNA band required was cut out of the gel, using a sterile razor blade and placed in a previously treated* 200 mm length of dialysis tubing which was clipped at one end and filled with approximately 400 µl 1 x TBE buffer (89 mM Tris-borate pH 8.0; 2 mM EDTA; 89 mM sodium borate). The remaining open end of tubing was clipped and placed in a mini gel apparatus containing 30 mls of 1 x TBE buffer and run at 50-60 V for 1-2 h.. During this process the DNA is electro-eluted out of the gel and onto the inner wall of the dialysis bag. The current across the tubing was momentarily reversed, resulting in movement of DNA from the dialysis tubing into the buffer which was then pipetted off. Following phenol extraction and ethanol precipitation the resultant DNA pellet was dissolved in the required volume of distilled water for subsequent labelling reactions.

*Dialysis tubing was boiled for 10 min. in a large volume of 2% sodium bicarbonate; 1 mM EDTA, rinsed in distilled water, boiled for a further 10 min. in distilled water, allowed to cool and stored submerged at 4°C prior to use.

2.2.14 Phenol Extraction and Ethanol Precipitation of DNA

Nucleic acids in solution were deproteinised by the addition of phenol at a ratio of 1:1 v/v, vortexed and centrifuged for 10 min. The upper aqueous phase was retained and vortexed with an equal volume of phenol/chloroform 1:1 v/v and centrifuged, the lower phase being discarded. A further extraction was performed with chloroform at a ratio of 1:1 v/v. The upper phase was retained to which was added 3 M sodium acetate pH 5.2 to give a final concentration of 0.3 M and 2.5 vol. ethanol. The sample was maintained at -20°C overnight in order to precipitate the DNA which was subsequently pelleted by centrifugation (13000 x*g*) for 10 min. at room temperature. The resultant DNA pellet was washed in 70% ethanol, spun down, dried in a stream of nitrogen and resuspended in the required volume of distilled, sterile water.

2.2.15 Quantification of Nucleic Acid Concentration by u.v. Spectrophotometry

Absorbance readings of solutions were taken at 260 and 280 nm on an Pye Unicam SP8-150 uv/vis spectrophotometer using double-distilled, deionised water as a reference standard. Estimation of nucleic acid concentration was based on the assumption that a 1 mg ml $^{-1}$ solution of pure double-stranded nucleic acid has an A_{260} value of 24.000 (Maniatis *et al.*, 1982). Purity of preparations was ascertained by calculation of the 260:280 nm absorbance readings: the ratio being 1.8 for pure DNA and 2.0 for pure RNA

2.2.16 Labelling of Nucleic Acids: Probe Production

2.2.16.1 Biotinylation with "Photoprobe"

Equal volumes of nucleic acid (optimum labelling results were achieved using nucleic acid at a concentration of 1 µg/µl) and "Photoprobe" biotin stock solution were mixed and irradiated in an ice bath 10-15 cm below a mercury

tungsten 500 Watt bulb for 15 min. After labelling, the probe nucleic acid was recovered by increasing the volume of the sample to 100 µl by the addition of 0.1 M Tris-HCL pH 9, followed by the addition of 100 µl of 2-butanol. The sample was vortexed, centrifuged and the resultant upper phase discarded. The extraction was repeated with a further 100 µl of 2-butanol; the lower phase being retained to which was added 10 µg of bakers yeast tRNA. The nucleic acid was precipitated at -20°C with the addition of 0.75 µl 4 M NaCl and 100 µl of ethanol, collected by centrifugation, dried and dissolved in sterile water.

2.2.16.1.1 Detection of biotinylated nucleic acids on nitrocellulose using the Vectastain ABC-AP kit

Quantification of biotin labelling reactions was performed using a modified dot blot assay system. 5 µl aliquots of labelled nucleic acid and a range of biotin standards were dotted onto nitrocellulose previously equilibriated with TTBS (0.1M Tris-HCl, pH 7.5; 0.15M NaCl; 0.1% Tween 20) and baked for 2 h. at 80°C under vacuum. Filters were washed 3 x 5 min. in TTBS prior to incubation with the Vectastain ABC reagent, a preformed avidin:biotinylated horseradish peroxidase complex (Hsu *et al.*, 1981 a and b), for 30 min. Following 3 x 10 min. washes in TTBS filters were incubated, in the dark, for 15-30 min. in freshly prepared alkaline phosphatase substrate solution. After colour development, filters were washed 3 x 10 min. in TTBS, air-dried and stored protected from light.

2.2.16.2 Nick-translation

The protocol used was originally described by Rigby *et al.* (1977). Purified DNA was labelled with either [^{32}P] dCTP or [^{35}S] dATP using Amersham's Nick-translation kit (Code N.5000). A typical reaction mix yielding a specific activity, on average, of 1 x 10^8 c.p.m./µg was as follows:- 0.5-1 µg DNA; 20 µl nucleotide solution; 100-200 µCi radiolabelled nucleotide; 10 µl enzyme solution (DNase 1

plus DNA polymerase 1); sterile distilled water to produce a final volume of 100 µl. The reaction mixture was incubated at 15°C for 2 h.

2.2.16.3 Random priming

Restricted DNA was run on a 0.6% w/v low melting point agarose gel using a running buffer of 40 mM Tris HCl; 5 mM sodium acetate; 1 µg/ml EtdBr; 1 mM EDTA pH 8.0. The required fragments were removed from the gel using a sterile scalpel and placed in a preweighed 1.5 ml eppendorf tube. Sterile, distilled water was added at the ratio 3 ml per gram of gel and boiled for 7 min. The resultant DNA in molten agarose was either used directly or stored at -20°C.

Labelling Reaction

DNA in molten agarose, previously denatured by boiling and cooling on ice, was added to the following reaction mixture and incubated overnight at room temperature: 10 µl O.L.B. solution*; 2 µl BSA (10 mg/ml); 27 µl DNA in molten agarose; 50-100 µCi [^{32}P] dCTP; 2 µl Klenow enzyme; sterile distilled water to give final volume of 50 µl. This reaction protocol routinely yielded "probes" with specific activities of 2 x 10^9 c.p.m./µg.

*O.L.B. Solution was prepared as follows:-
Solution A:- 1.25 M Tris-HCl; 0.125 M $MgCl_2$ pH 8.0, 1.8% v/v B-mercapto-ethanol, 0.005 M dATP; dGTP, dTTP.
Solution B:- 2 M Hepes pH 6.6.
Solution C:- Hexadeoxyribonucleotides in T.E. buffer at 90 O.D. units/ml.
Solutions A, B and C were mixed in the v/v ratio of 1:2.5:1.5.

2.2.17 Gel Filtration

Labelled DNA was separated from unincorporated radionucleotides by gel filtration through a column of Sephadex G50 (fine grade), equilibrated and eluted with column buffer (50 mM NaCl; 10 mM EDTA; 50 mM Tris-HCl pH 7.5; 0.1% SDS). 400 µl aliquots of eluate were collected, from which 1 µl aliquots were

removed and dispersed in 5 ml of scintillation fluid for measurement using a Packard tri-carb liquid scintillation analyser. Fractions containing the excluded labelled DNA peak were pooled and either used directly as a probe, following denaturation, or, alternatively, following phenol extraction and ethanol precipitation the labelled DNA, dissolved in the required volume of water, was used for subsequent *in situ* hybridisation protocols.

2.2.18 Analysis of Probe Size Range

Both Nick-translation and random priming protocols result in the production of probes consisting of a wide range of DNA fragment sizes. A small sample of radiolabelled DNA probe was run on 0.8% w/v agarose mini gels, along with size marker DNA. After photographic recording of marker DNA under u.v. transillumination the wet gel was exposed to autoradiographic film at room temperature in a sealed, light proof container. Comparison of the resultant autoradiograph with the original marker DNA allowed determination of DNA probe fragment size range.

2.2.19 RNA Extraction in Guanidine Hydrochloride

The protocol described was adapted from Logemann *et al.* (1987). Freshly excised tissue was frozen immediately by immersion in liquid nitrogen and then ground to a powder, under liquid nitrogen, using a sterile pestle and mortar. Guanidine hydrochloride extraction buffer (8 M guanidine hydrochloride; 20 mM EDTA; 50 mM B-mercaptoethanol) was added at a ratio of 2 ml per mg of tissue and homogenisation continued. The homogenate was transferred to sterile corex tubes and centrifuged (10000 x*g*) at 4°C for 10 min. to pellet cell debris. The supernatant was transferred to a fresh tube and extracted once with phenol/chloroform. The phases were separated by centrifugation (10000 x*g*) at

room temperature for 20 min. The upper aqueous phase was transferred to a fresh tube to which was added 0.2 vol. of 1 M acetic acid and 0.7 vol. of ethanol and left overnight at -20°C in order for precipitation to occur. The precipitate was recovered by centrifugation at 10000 x*g* for 10 min. and the resultant pellet washed twice, at room temperature, with 3 M sodium acetate (pH 5.2). Following a further wash in 70% ethanol, the pellet was dried under nitrogen, dissolved in sterile, distilled water and stored at -80°C.

2.2.20 Gel Electrophoresis of RNA Samples

2.2.20.1 RNA denaturation via glyoxylation

The protocol used was essentially that of McMasters and Carmichael (1977). Samples of RNA (10 µg) were glyoxylated by incubation, in a solution containing 50% redistilled DMSO; 10 mM sodium phosphate (pH 7.0) and 2.1% w/v deionised glyoxal, for 1 h. at 50°C. Samples were mixed with 0.5 vol glyoxal loading buffer (10 mM sodium phosphate pH 6.8; 31% v/v glycerol; 0.2% w/v agarose; 0.1% w/v bromophenol blue), loaded onto 1.5% high gelling temperature gels (1.5% w/v H.G.T. agarose in 10 mM sodium phosphate pH 6.8) in an electrophoresis tank placed on two slow magnetic stirrers, with a peristaltic pump used to circulate the running buffer (10 mM sodium phosphate, pH 6.8). Electrophoresis was carried out for 6 h. at 100 V with constant recirculation of buffer.

Gels were either stained in acridine orange (30 mg/l) in 10 mM sodium phosphate pH 6.8, and destained in that buffer overnight prior to examination by u.v. transillumination, or blotted onto nitrocellulose filters by capillary action using 20 x SSC (0.3 M sodium citrate, pH 7.0; 3 M sodium chloride) as the transfer buffer. Capillary blotting of nucleic acids was performed by placing the gel on a blotting apparatus, consisting of a glass plate held above a reservoir containing transfer buffer with Whatman 3MM paper used as a "wick", allowing movement of

buffer from the reservoir up through the gel. A nitrocellulose filter, previously wetted and equilibrated with transfer buffer, was laid over the gel. Absorbant towels were placed on top and weighted, as described by Maniatis *et al.* (1982). Transfer was allowed to proceed overnight at 4°C.

2.2.20.2 RNA denaturation via formaldehyde

The procedure was that of Fourney *et al.* (1988). RNA samples (10 µg) were, after concentration via lyophilisation, incubated for 15 min. at 65°C in 25 ul electrophoresis sample buffer (53% v/v deionised formamide; 17% v/v formaldehyde; 7% v/v glycerol; 10.5% w/v MOPS pH 7.0; 5.6% w/v bromophenol blue). Following the addition of 1 µg Etd Br samples were mixed thoroughly and loaded onto 0.66 M formaldehyde gels using 1 x MOPS solution (0.02 M MOPS; 5 mM sodium acetate; 1 mM EDTA, pH 7.0) as the electrophoresis buffer.

Gels were blotted as described, however sponges were used to enhance capillary action and 10 x SSC was used as the transfer buffer. The addition of Etd Br directly to the RNA sample allowed direct visualisation of RNA bands in the gel and monitoring of transfer to membrane.

2.2.21 Northern Blot Hybridisation

Filters were baked at 80°C, under vacuum, for approximately 2 h.. Filters were prehybridised at 42°C for 16 h., in sealed plastic bags, in a solution containing 50% v/v deionised formamide; 6 x SSC; 5 x Denhart's reagent (0.1% w/v ficoll, 0.1% w/v P.V.P., 0.1% w/v BSA); 0.1% w/v SDS; 100 mg/ml denatured herring sperm DNA. Hybridisation with a radiolabelled cDNA probe was performed by the addition of the probe directly to the prehybridisation mixture, after denaturation of probe by heating to 80°C for 10 min. and rapid cooling on ice. Hybridisation was performed for 48 h. at 42°C. Filters were washed 3 x 15

min. with 2 x SSC; 0.1% w/v SDS, at room temperature, to remove formamide and subsequently washed to varying degrees of stringency depending upon the specificity of homology between cDNA and target RNA required. Filters were air-dried and exposed to pre-flashed X-ray film against an intensifying screen at -80°C.

2.2.22 RNA Dot Blots

RNA (at a concentration not exceeding 1 µg/ml) was denatured in a solution of 12% v/v formaldehyde; 6 x SSC for 15 min. at 60°C; diluted to 0.2 ug/ml with 15 X SSC and stored on ice. Nitrocellulose filters were equilibrated firstly with water for 5 min., followed by a 20 min. equilibration period with 15 x SSC. 100 µl aliquots of a range of RNA concentrations were transferred, under vacuum, onto nitrocellulose using a hybridot manifold. Each well was washed with 15 x SSC and aspirated for 5 min. Filters were baked at 80°C, under vacuum, and stored between sheets of 3 MM paper until required.

Nitrocellulose filters were prehybridised for 8-16 h. at 42°C, in the following solution:- 50% v/v deionised formamide; 6 x SSC; 5 x Denhart's reagent; 10% w/v dextran sulphate; 100 mg/ml denatured herring sperm DNA. Double-stranded DNA probes, denatured by heating at 80°C for 10 min. followed by rapid cooling on ice, were added directly to the prehybridisation mixture. Hybridisation was continued for 40-48 h. at 42°C. Filters were washed 4 x 30 min. with 2 x SSC; 0.1% w/v SDS followed by 2 x 60 min. washes with 0.1 x SSC; 0.1% w/v SDS at 60°C. Filters were blotted dry and exposed to X-ray film against an intensifying screen, at -80°C.

2.2.23 Tissue Printing onto Nitrocellulose

The protocol utilised was adapted from that of Cassab and Varner (1989). Freshly excised material was rinsed quickly in distilled water and placed on nitrocellulose previously equilibrated with 5 x SSC; 0.1% w/v SDS for 15 min.. The material was then carefully squashed onto the nitrocellulose using a sterile spatula tip, allowing transfer of proteins and nucleic acids present within the tissue onto the nitrocellulose. The resulting tissue blot was baked at 80°C, under vacuum, for 2 h.

Filters were incubated, for 2 h. at 37°C, in buffer (0.01M Tris-HCl, pH 7.8; 0.05M EDTA; 0.5% w/v SDS) containing 0.5 ug/ml proteinase K. Following a buffer rinse RNA detection was performed via the Northern blot hybridisation protocol.

Detection of protein transferred to nitrocellulose

Filters were incubated for 5 min. in a solution containing 0.1% Napthol Blue black; 45% methanol; 10% acetic acid, followed by incubation in 40% methanol, 10% acetic acid for 15 min. with constant shaking. Results were photographed immediately.

2.2.24 *In situ* Hybridisation of Double-Stranded cDNA Probe to RNA in Tissue Sections

Sections were rehydrated, re-fixed in 4% paraformaldehyde in P-S buffer for 15 min., and "blocked" with 1% w/v BSA in PBS (0.05 M phosphate pH 7.5; 0.7 M NaCl) for 3 x 5 min. Sections were acetylated via equilibration in 0.1 M triethanolamine (pH 8.0) for 10 min. at room temperature, followed by equilibration, for 10 min. in a solution of 0.25% v/v acetic anhydride in 0.1 M triethanolamine (Cox and Goldberg, 1988). Sections were then washed 2 x 15 min. in hybridisation buffer (50% v/v deionised formamide; 4 x SSC).

Pre-hybridisation (2 h.) and hybridisation (overnight) were carried out at 37°C, in moist chambers, with 20 µl per section of the following solution made up to final volume with distilled water:- 50% v/v deionised formamide; 30% v/v 6x SSC; 10% w/v dextran sulphate; 1 x Denhardt's reagent; 5 ng heat-denatured probe DNA per section; and a 600 x mass excess of sonicated, sheared salmon sperm DNA. During pre-hybridisation probe DNA was not included in the incubation medium. Hydrogen peroxide treated, siliconised cover slips were used to spread the hybridisation mixture evenly over the section and to minimise evaporation during hybridisation.

After hybridisation sections were washed in 2 x SSC at room temperature to remove unbound/loosely bound probe and hybridisation solution and subsequently washed at increasing stringencies as follows:- 1 x SSC at 37°C (2 x 1 h.); 0.5 x SSC at 37°C (2 x 1 h.); 0.1 x SSC at 37°C (2 x 30 min.).

2.2.24.1 *In situ* hybridisation control experiments

(i) Sections were incubated, at 37°C for 1 h., with ribonuclease A (0.5 µg/ml in phosphate buffer pH 6.0) prior to commencement of the *in situ* hybridisation protocol, in order to determine the extent of any non-nucleotide interactions.

(ii) Sections were hybridised with labelled, non-probe, DNA i.e. pBR 322; labelled to the same specific activity or degree of biotin incorporation (as verified by dot blots, Section 2.2.16.1.1) as the corresponding probe DNA.

(iii) Sections of material known not to contain the target sequence, e.g. leaf were processed in the same manner as experimental material in order to evaluate the degree of non-specific labelling of either tissue or slide.

(iv) A positive control was included in order to evaluate the sensitivity of the protocol; i.e. a section known to contain the target sequence in relative abundance; 16 d.a.f. cotyledon.

2.2.25 Hybrid Visualisation

2.2.25.1 Biotinylated probes

Various amplification procedures based upon the affinity between avidin, a basic glycoprotein of approximately 68000 daltons, and biotin, a low molecular weight vitamin (reviewed Bonnard *et al.*, 1984) have been used during the course of this work and their relative efficiencies evaluated.

Sections were "blocked" with 1% w/v BSA in PBS for 15 min., prior to commencement of detection protocols. All incubations were performed at room temperature and washes (3 x 20 min.) were with 0.1% BSA in PBS. Antibody dilutions were with PBS.

2.2.25.1.1 Via colloidal gold and silver enhancement

(i) **Avidin: biotin reactions**

Following primary incubation for 1 h. with streptavidin conjugated to colloidal gold (5 nm) diluted $^{1}/_{500}$, sections were washed and incubated with goat-anti-rabbit biotin at a concentration of 5 µg/ml for 30 min. Sections were washed and incubated with rabbit-anti-goat gold (5 nm) diluted $^{1}/_{100}$ for 1 h. and washed as described.

(ii) **Antibody reaction**

Primary incubation for 1 h. with goat-anti-biotin (diluted $^{1}/_{200}$) was followed by incubation with either:-

(a) rabbit-anti-goat IgG (diluted $^{1}/_{100}$) for 1 h. following washing sections were incubated with goat-anti-rabbit gold (diluted $^{1}/_{100}$) for 1 h.

(b) rabbit-anti-goat gold (5 nm) diluted $^{1}/_{100}$ for 1 h.

In all cases, following the amplification procedure, sections were rinsed in distilled water and silver enhanced (Holgate *et al.*, 1983; Danscher and Norgaard, 1983) with Intense II for 10 min., blot dried, counter-stained and mounted.

2.2.25.1.2 Via alkaline phosphatase

The alkaline phosphatase detection protocol was adapted from Singer *et al.* (1986). Sections were incubated for 1 h. with goat-anti-biotin diluted $^{1}/_{200}$, washed and incubated with rabbit-anti-goat biotin diluted $^{1}/_{100}$ for 1 h. Biotin was detected using the Vectastain ABC-AP system as described in Section 2.3.9.1.1, after which sections were counter-stained and mounted.

2.2.25.1.3 Via fluorescence

The detection of hybrids, in plant material, via fluorescent markers was originally described by Harris and Croy (1986). Sections were incubated with streptavidin rhodamine at a concentration of 5 µg/ml for 2 h. at 37°C. Excess streptavidin rhodamine was removed via PBS washes and sections were mounted in a water soluble mountant and viewed immediately using a fluorescent microscope with an excitation wavelength of 490 nm and a 515 nm suppression filter.

2.2.25.2 Radiolabelled probes - via autoradiography

Sections were rinsed in deionised water and allowed to air dry thoroughly at room temperature. Under dark-room conditions (Kodak O.C. filter) 12.5 ml of K2 autoradiographic emulsion was melted at 45°C and to this were added 11.75 ml distilled water and 0.24 ml glycerol. The emulsion was carefully stirred and allowed to stand until free of air bubbles. Sections were dipped into the emulsion and dried completely prior to storage at 4°C in a light proof container. Development times ranged from 48 h. to 4 weeks, depending upon the

radionucleotide used and the specific activity of the probe. Sections were developed, under dark room conditions, by placing in Kodak D19 developer for 4 min.. Following a rinse in distilled water sections were fixed in 30% aqueous sodium thiosulphate solution for 3 min. After washing in running tap water for 10 min. sections were dehydrated, stained and mounted.

2.2.26 Embedding of *In Situ* PEG Sections for EM Visualisation

After viewing and recording of *in situ* hybridisation results at LM level, slides were placed in Histosol for approx. 1 h. to enable removal of the coverslip. Sections were dehydrated via a graded alcohol series. A drop of Spurr resin:alcohol mixture at a ratio of 25:75 (v/v) was placed on the section and left overnight followed by a further overnight infiltration with pure Spurr resin. A Beem capsule (Agar Aids), filled with Spurr resin was carefully inverted over the section and polymerised at 70°C. The Beem capsule with attached slide was immersed in liquid nitrogen, rapid freezing resulted in separation of capsule and slide, with the original PEG section embedded in the polymerised resin and ready for ultrathin sectioning.

2.2.27 Pulse-Labelling of Cotyledons:- Fixation Optimisation

Cotyledon culture techniques were adapted from Millerd *et al.* (1975) and Stafford and Davies (1979). Pea pods ranging in age from 13 d.a.f. to 16 d.a.f. were surface sterilised by immersion in 5% calcium hypochlorite for 30 min. All subsequent procedures were performed aseptically in a laminar flow cabinet. After removal of the integuments, individual cotyledons were transferred to filter sterilised culture medium (5% sucrose, 1 x Murashige and Skoog basal plant salt mixture [4.71 g/l], pH 5.15) containing 100 μCi [^{3}H] uridine. Cotyledons were

cultured for 4-8 h. at 37°C with constant shaking, rinsed 3 x 5 min. in culture medium and weighed individually prior to further processing:-

(i) Placed immediately in 15 ml tissue solubiliser, Soluene 350, for 48 h. at 37°C.

(ii) Fixed for 12 h. in a variety of fixatives routinely used for *in situ* hybridisation protocols, prior to subsequent solubilisation as described.

(iii) Fixed, dehydrated and embedded in P.E.G., prior to solubilisation.

Once solubilised, the samples were vortexed and 5 µl aliquots were suspended in 5 ml scintillation fluid. Radioactive incorporation was measured using a liquid scintillation counter.

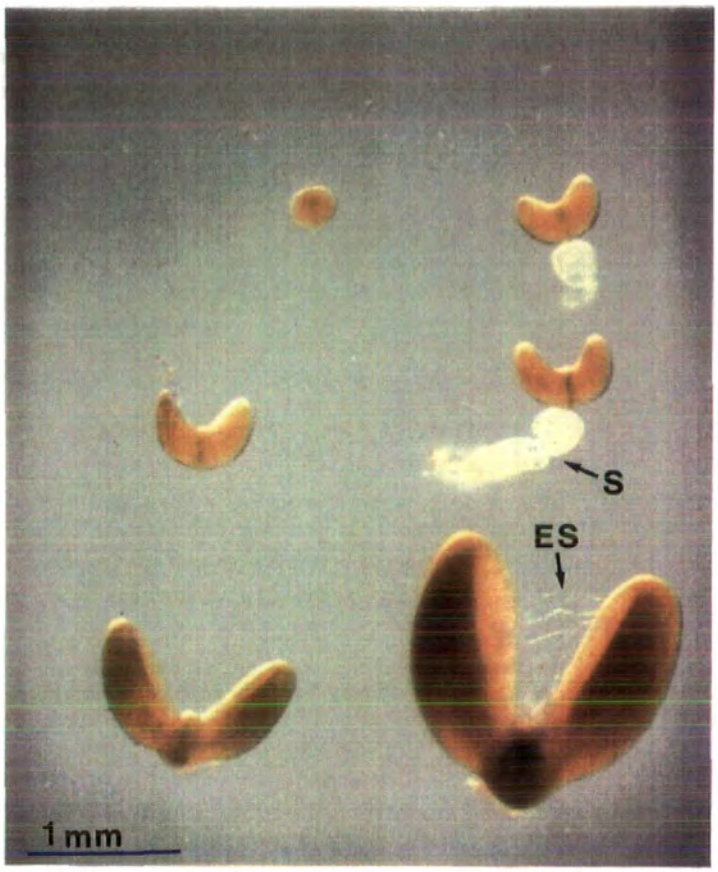
3: RESULTS AND DISCUSSION

3.1 MORPHOLOGY AND CYTOLOGY OF EARLY EMBRYOGENESIS IN *PISUM*

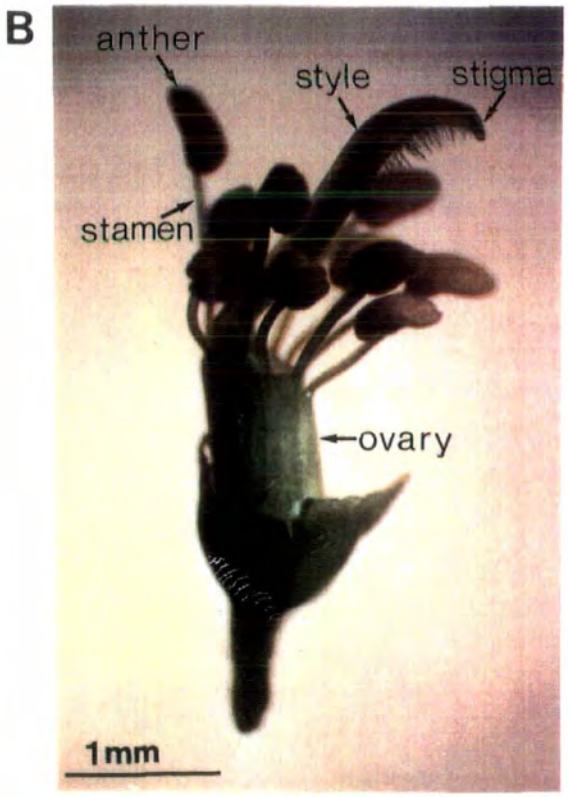
Much information has recently been obtained on the molecular aspects of late *Pisum* embryogenesis (Gatehouse *et al.*, 1982; Gatehouse *et al.*, 1986; Boulter *et al.*, 1987), however, this knowledge generally pertains to the seed storage proteins; most of which has been obtained independently of cellular/cytological procedures. Although it has been accepted that the process of seed storage protein gene expression is under the ultimate control of an overall developmental programme (Gatehouse *et al.*, 1986), little work has been carried out to determine the morphological events of pea embryogenesis and still less is known about the process of differentiation. Following the initial work of Cooper (1938) [*P. sativum* cv. "Little Marvel" and "Asgrow Pride"] and Reeve (1948) [*P. sativum* cv. "Alaska"] only one further study of histogenesis during *Pisum* embryo development has been reported (King and Heyes, 1986) [*P. sativum* cv. "Alaska"]. Advancement in the area of angiosperm embryogenesis is hindered by the lack of co-ordination, between researchers, with regard to genotype and growth conditions used. This produces difficulties in comparing results, a problem noted also by King and Heyes (1986), who were unable to determine whether they were working with the same developmental stage as Reeve (1948).

Therefore, an embryonic time-scale and developmental index was established for the cultivar "Feltham First" (Fig. 2), based upon three criteria:- (i) gross morphological appearance (Fig. 4A); (ii) age of ovule; (iii) weight of ovule/embryo (Figs. 5 and 6).

FIG 4



Isolated early embryos of *Pisum*.



Structure of anther and pistil prior to fertilisation.

FIG 5

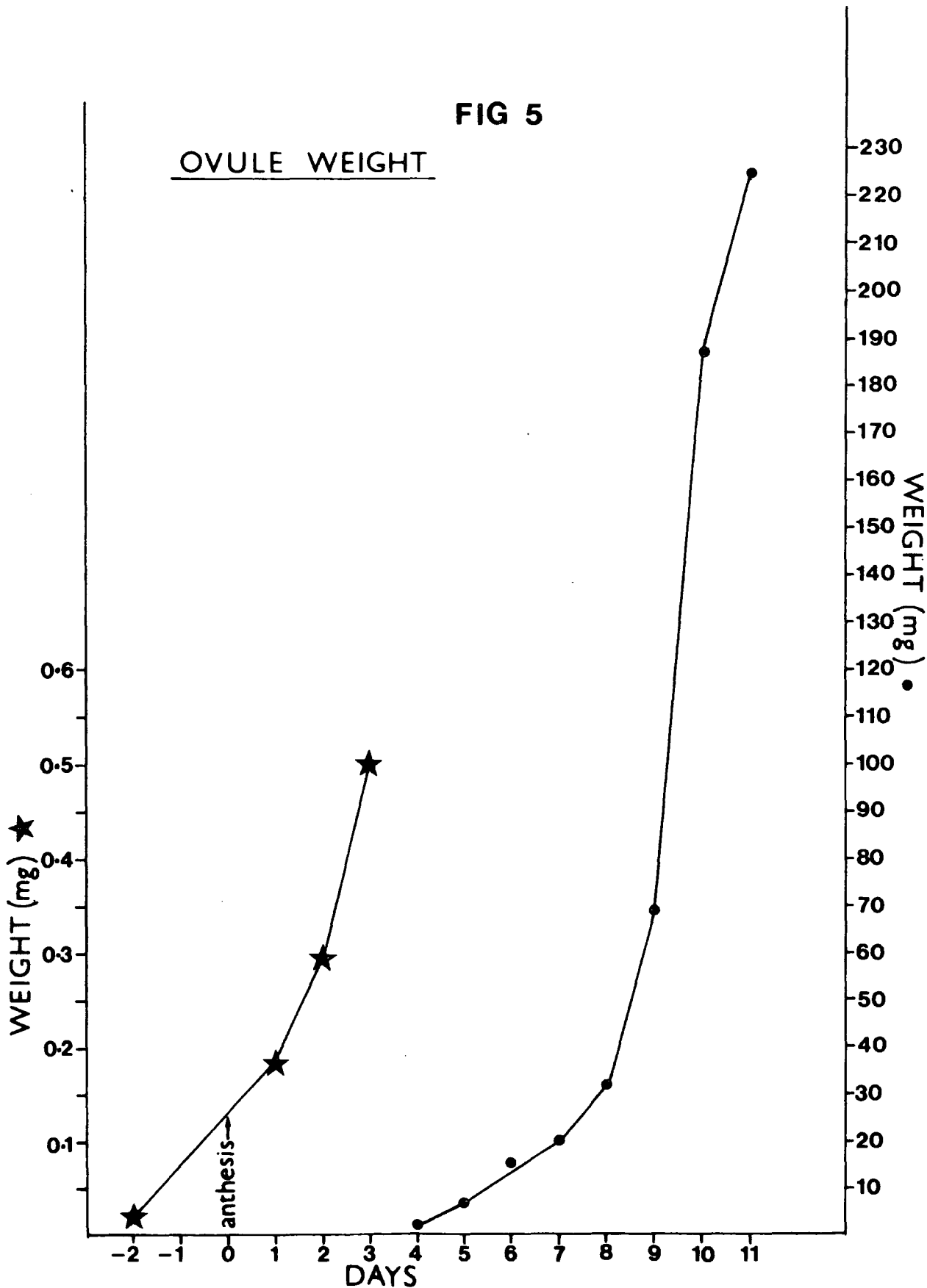

OVULE WEIGHT
WEIGHT (mg) ★
WEIGHT (mg) ●
0·6
0·5
0·4
0·3
0·2
0·1
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
anthesis
−2 −1 0 1 2 3 4 5 6 7 8 9 10 11
DAYS

FIG 6

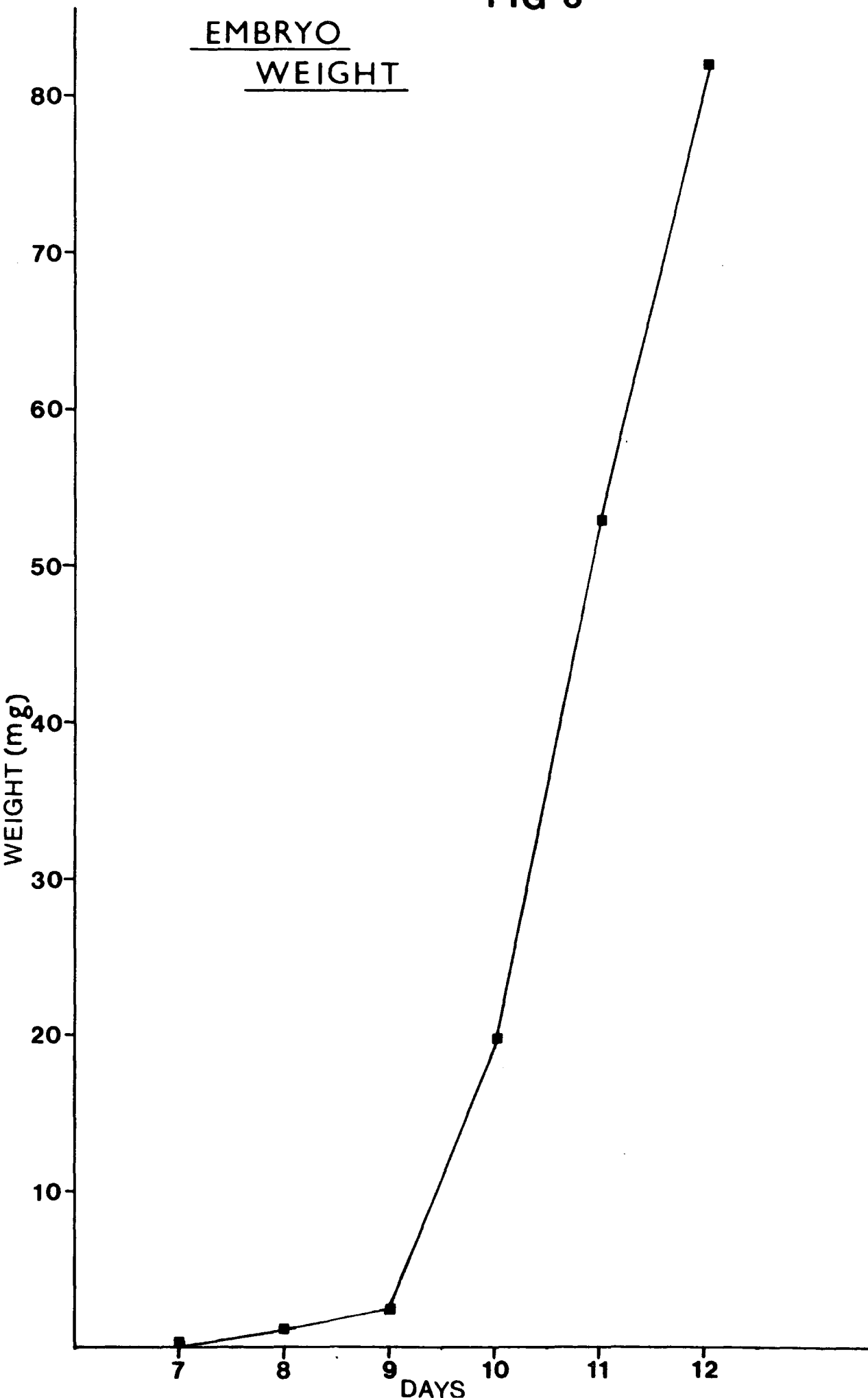


Figure 2

Morphology	Age (d.a.f.)	Mean weight (mg) embryo	Mean weight (mg) ovule	Designation
Globular	4-5	*	2-7	Stage (1)
Flattened globose-initial development of tunica-corpus	5	*	7	Stage (2)
Early heart-shape - initiation of cotyledon primordia	6	*	15	Stage (3)
Middle heart-shape - elongation of cotyledons; continued axis development	7-8	0.1-1.2	15-30	Stage (4)
Late heart-shape - cotyledons and apex fully formed. Prior to cotyledon expansion	8-9	1.12-2.3	30-70	Stage (5)

* = not determined.

In *Pisum sativum* cv. "Feltham First", under the growth conditions used, the embryo was in the globular form until approximately 4 to 5 d.a.f., after which histogenesis proceeded rapidly. Embryos underwent stages (2) and (3) of development very quickly and were hence difficult to harvest. It was therefore necessary to tag flowers at 3 h. intervals in order to allow harvesting of specific stages. A similar pattern of development was noted in the cultivar "Alaska" by King and Heyes (1986), who proposed that the embryonic sequence in *Pisum* was characterised by an initial period of slow mitotic activity followed by rapid development, accompanied by differentiation, with the embryo then undergoing a phase of slower growth and further differentiation. Morphogenesis was completed by 9 d.a.f., the embryo at this stage consisting of two cotyledons and central axis (Fig. 4A). Continued growth of the embryo is mainly via cell expansion, resulting

in lateral expansion of the cotyledons. However, cellular differentiation continues at the axis apices resulting in the formation of the radicle and the epicotyl (first leaf primordium).

Figure 4A shows dissected embryos at varying stages of development and demonstrates clearly the morphological transition that occurs during early embryogenesis. The extra-embryonic sheath, postulated to be responsible for the attachment of the embryo to the embryo sac wall (Marinos (1970a), is evident at the later stages of development. At early stages the attached suspensor, consisting of two large, saccate cells, can be dissected out of the ovule with the embryo. The suspensor, in the cultivar "Feltham First", is short-lived and persists only until stage (4) of development. Within the axis of the developing embryos a prominent dark green "core" is evident. A cross-section through a stage (4) embryo (Fig. 11A) shows that cells in this region are highly cytoplasmic, compared to the cells of the developing cotyledon. The axis, consisting of cells of a high cytoplasmic density, therefore appears darker when viewed under the binocular microscope with transmitted light.

Figures 7, 8 and 9 show serial sections through the embryo of *Pisum* at various stages of development.

At the globular stage (Fig. 7) there is no clear evidence of gross differentiation. However, the cells are not as embryonic, i.e. undifferentiated in terms of morphology, as King and Heyes (1986) reported. In terms of the absence of any putative root or shoot the globular embryo is undifferentiated. However, the cells at this stage are not as uniform as those previously reported (Reeve, 1948; King and Heyes, 1986), since they are of widely differing sizes, though the majority are roughly iso-diametric in shape. The nuclei of the cells are large, prominent and generally centrally located. King and Heyes (1986) reported the presence within most nuclei of two nucleoli, however this pattern is not evident in

the cultivar "Feltham First". A major difference between the embryo at this stage and those at a more advanced stage of embryogenesis is the almost complete lack of cellular vacuolation since the presence of only a few small vacuoles can be visualised. In their examination of this stage of development King and Heyes (1986) noted that the vacuoles were small and arranged around the periphery of the nucleus. However, no such perinuclear arrangement is visible in this cultivar. Initiation of protoderm development has occurred at the globular stage; surface cells being generally of a similar size and shape, and dividing periclinally. This observation differs from that of Reeve (1948) who stated "At this stage (globular) a continuous protoderm is not present over the embryo and many of the surface cells remain large and undifferentiated". The degree to which a cell is differentiated is open to personal interpretation, however Reeve (1948) continued to state, "In *Pisum* it is clearly evident that the surface cells remain undifferentiated until periclinal divisions initiate the epicotyl apex". The results presented in this thesis contradict that statement since a protoderm is present at stage (2) (Fig. 10A), prior to any epicotyl apex initiation. Reeves (1948) reported morphological differentiation at the poles of the embryo where he noted that the cells were "large, somewhat vacuolate and with deeply staining nuclei". No such evidence of differentiation at the poles is evident at LM level in the example presented here (Fig. 7). Both Cooper (1938) and Reeve (1948) showed that, prior to histogenesis, the embryo was spherical or globular, whilst King and Heyes (1986) reported the embryo, at this stage, to possess a flattened, globose shape. The embryo in the cultivar "Feltham First" is globular prior to histogenesis and it is apparent that embryo development differs slightly in the various cultivars.

Stage (2) of embryo development (Fig. 8) exhibits a far greater degree of differentiation than was visible at the globular stage. However, cellular organisation into discrete, recognisable structures is not yet evident. At this stage

initial development of the tunica-corpus structure is visible. The apical surface cells do not yet exhibit the rectangular shape characteristic of the tunica, nor are they in a double layer. However, they possess a high cytoplasmic density; this region of the embryo being characterised by a low level of vacuolation. The embryo possesses a slight bulge at the apex, an indication of corpus development. Beneath the surface layer of apex cells (tunica initials) a V-shaped zone of small, non-vacuolated cells is present (Fig. 10A). These cells are either the initials of the corpus or they may be fully differentiated corpus cells. The fact that the corpus region, at this stage, is more highly developed compared to the tunica appears to indicate that in this cultivar, or in the species of *Pisum* as a whole, the formation of the corpus region occurs prior to cellular organisation into the overlying tunica. Ground meristem development has occurred by this stage of development. Cytologically ground meristem cells are characterised by their large size compared to cells from other embryonic regions, i.e. tunica, corpus, columella. The small zone of differentiated cells centrally located within the embryo, are the initials of the stele promeristem from which procambium develops (Fig. 10A). The rectangular cells of the protoderm cover the entire embryo except at the radicle pole region (Fig. 10A). Columella initials are not clearly visible at this stage and the cells at the putative radicle pole appear to be at a low level of mitotic activity, i.e. are large and vacuolate. This indicates that shoot apex formation is precocious, in comparison to radicle pole development, in this cultivar. The difference in shape between the embryo at Stage (2) of development and the globular embryo is as a result of two main changes in the pattern of cell division within the embryo. Firstly, cells in the apex region are in a state of rapid division, i.e. highly meristematic, resulting in the apical bulge. Secondly, cells of the ground meristem are dividing to produce an increase in girth of the embryo in preparation for cotyledon initiation. The most salient feature of the embryo at this stage (Fig.

10A) is the decreasing gradation in the degree of vacuolation from the radicle pole to the shoot apex. This gradation in vacuolation is coupled with a corresponding gradation in cell size. Esau (1977) correlated cell vacuolation with cell maturity, this, in connection with the greater cell size present at the root pole, indicates that these cells are dividing far less rapidly than those in the apical region and provides further evidence for the retarded formation of the root apex.

By the time embryogenesis has advanced to Stage (3) (Fig. 9) cellular differentiation has occurred to such an extent that tissue systems and organs can be identified. The shoot apex has developed in the form of a tunica-corpus structure and lateral outgrowths, the initial development of the cotyledons, are present (Fig. 10B). The tunica-corpus structure is now similar to that described by King and Heyes (1986); a corpus region organised into a cup-shaped structure with an overlying two-layered tunica, the cells of which divide periclinally. Cells of the tunica-corpus region are smaller and less vacuolate than cells of the ground meristem with prominent nuclei often centrally located (Figs. 9D and 10B). Differentiation at the radicle pole has occurred, tiers of 2-3 cells of similar shape in cross-section being visible (Fig. 9G). These are the columella initials, division of which will eventually give rise to the root cap. At this stage a continuous epidermis, consisting of a single layer of uniform, rectangular cells, is present (Fig. 9B). The depression that can be seen at the radicle pole region (Fig. 9F) is proposed, by Reeve (1948), to be as a result of rapid growth of the cotyledons and a delayed elongation of the axis to compensate for such growth.

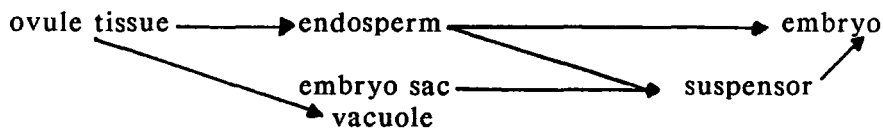
The embryo at stage (4) is highly differentiated with highly developed cotyledons and epicotyl axis (Fig. 11A). Cells of the abaxial layer of the cotyledon possess highly cytoplasmic contents with little vacuolation, whereas adaxial cells appear highly vacuolate. The non-vacuolated state of the abaxial layer possibly indicates a nutrient uptake role. A cross-section through the apical

shoot (Fig. 11A) reveals that the central "core" cells possess prominent nuclei and are cytoplasmically dense, whilst the surrounding cells are highly vacuolate. The shoot apex therefore exhibits regional zonation in which the central zone is the least differentiated, i.e. most highly meristematic. Due to the functional nature of this zonation it has been defined as the primordial meristem proper (Reeve, 1948).

Examination of ovular tissue reveals regional differences, described by Marinos (1970a), and postulated to be of nutritional significance. In the vicinity of the embryo (Fig. 11B) and suspensor (Fig. 11C) the ovular tissue adjacent to the embryo sac wall consists of loosely packed, round, cells with large intercellular spaces, whilst in all other regions the ovular cells are polyhedral and tightly packed. However, ovular tisue in the vicinity of the suspensor exhibits a greater degree of "cellular organisation" than that in the vicinity of the embryo (compare Figs. 11B and 11C). Results of cytological analysis of ovular cells in regions close to the embryo/suspensor, in conjunction with their loose packaging, resulted in the proposition that nutrient transfer from ovular cells into intercellular spaces and then into the embryo sac occurs (Marinos, 1970a). Examination of the integument of *Vicia faba* seeds (Offler *et al.*, 1989) demonstrated that the inner integument was composed of three cell layers; the vascular, the thin-walled parenchyma and the inner layer of thin-walled parenchyma/transfer cells. A similar pattern of cellular zonation is demonstrated in the *Pisum* integument (Figs. 11B and 11C), the parenchyma layers being designated PA1; PA2, respectively. Offler *et al.* (1989) noted that the layer of parenchyma cells adjacent to the embryo sac boundary wall possessed uniform secondary thickening and they postulated that, during the mid-maturation phase of embryo development, these cells were the cellular site of photosynthate exchange to the embryo apoplast, however, large intercellular spaces were not present.

The triploid endosperm is present at all the stages of development examined, and consists of a thin layer of multinucleate cytoplasm adjacent to the embryo sac boundary wall, mainly in the chalazal portion of the ovule (Fig. 8A). Examination of Stages (1) and (2) of development (Figs. 7, 8and 10A) shows that the endosperm cytoplasm projects from the embryo sac boundary wall and surrounds the embryo. Analysis of the endospermic cytoplasm of *Pisum sativum*, cv. "Greenfeast" (Marinos, 1970a) showed that it possessed motility, continually producing and retracting pseudopodia. The endosperm cytoplasm is in direct contact with both the embryo sac boundary wall and embryo, indicating its role as a possible site of nutrient transfer between the two. In the earlier stages of development (Figs. 8C and 8D) the endosperm can also be seen to be in contact with the suspensor which is demonstrated, at all the stages where it is present, to be in direct contact with the embryo. A salient feature of the embryo at Stage (2) of development (Fig. 10A) is the lateral gradation of vacuolation, cells in the portion of the embryo closest to the embryo sac boundary wall being less vacuolated, compared to those that are more distal. It is possible that those cells exhibiting a low degree of vacuolation are involved in nutrient uptake, either directly from the embryo sac vacuole or indirectly via the endosperm cytoplasm.

The proposed pathways of nutrient entry into the developing embryo of *Pisum sativum*, based upon morphological observations is therefore as follows:-



Figures 5 and 6 show the mean fresh weight of ovules and embryos at various stages of development. Initially weight increase is exponential indicating

Toluidine blue stained 1 um serial sections of globular (Stage (1) embryo. Measurements underneath each photograph represent the distance of each section through the embryo (um).

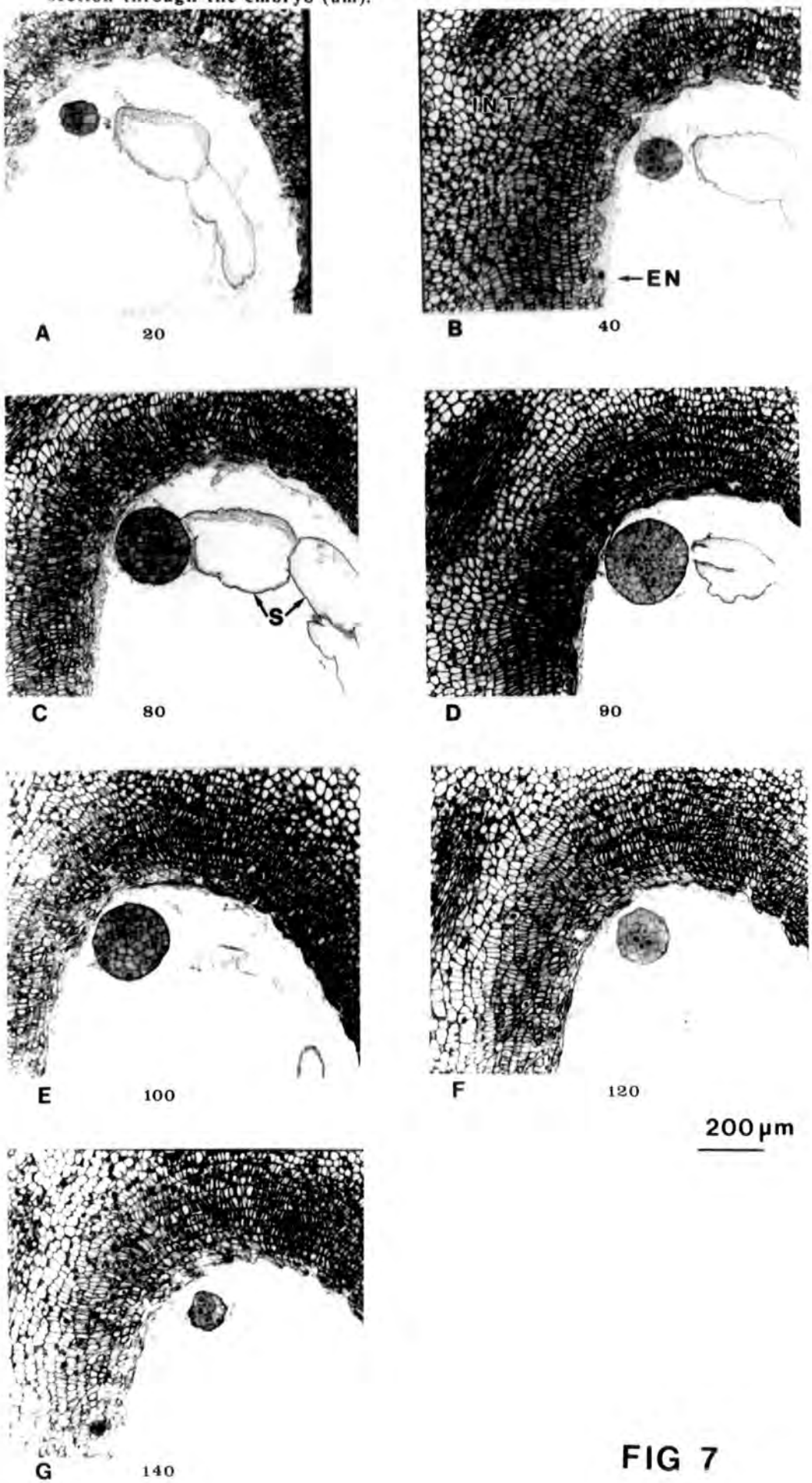
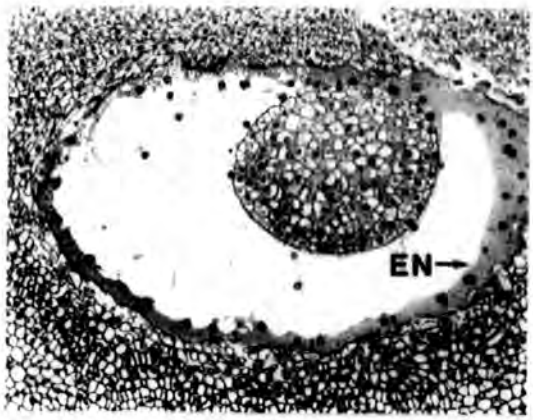


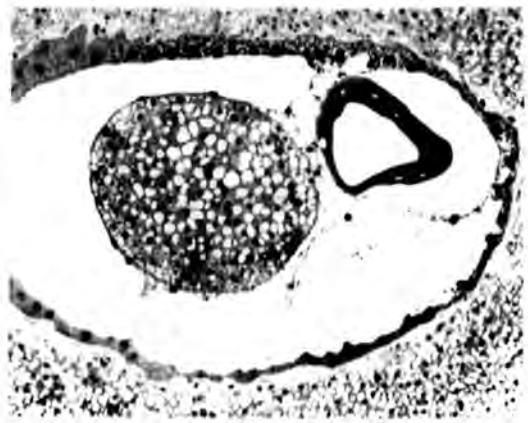
FIG 7

FIG 8

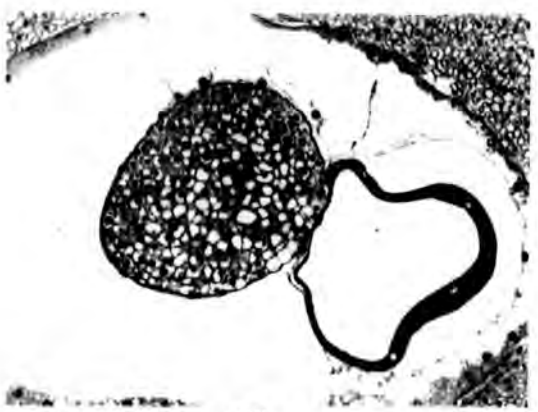
Toluidine blue stained 1 um serial sections of Stage (2) embryo. Measurements underneath each photograph represent the distance of each section through the embryo (um).



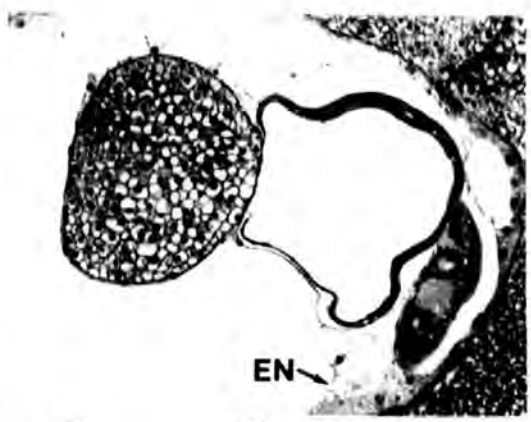
A 30



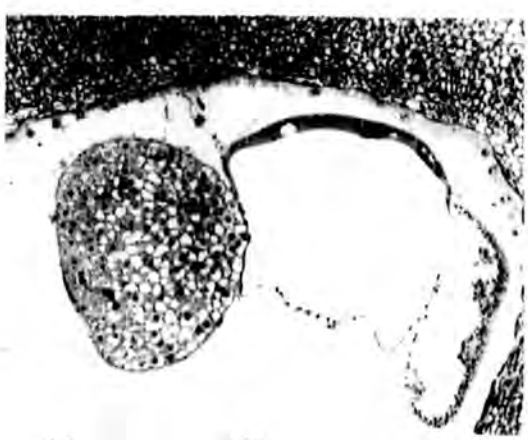
B 50



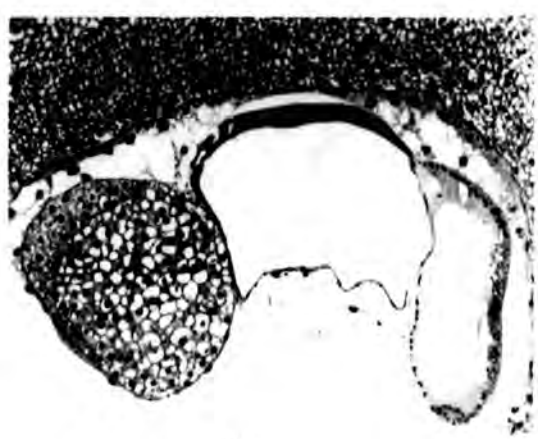
C 60



D 70



E 80



F 90

200 µm

FIG 9

Toluidine blue stained 1 um serial sections of Stage (3) embryo. Measurements underneath each photograph represent the distance of each section through the embryo (um).

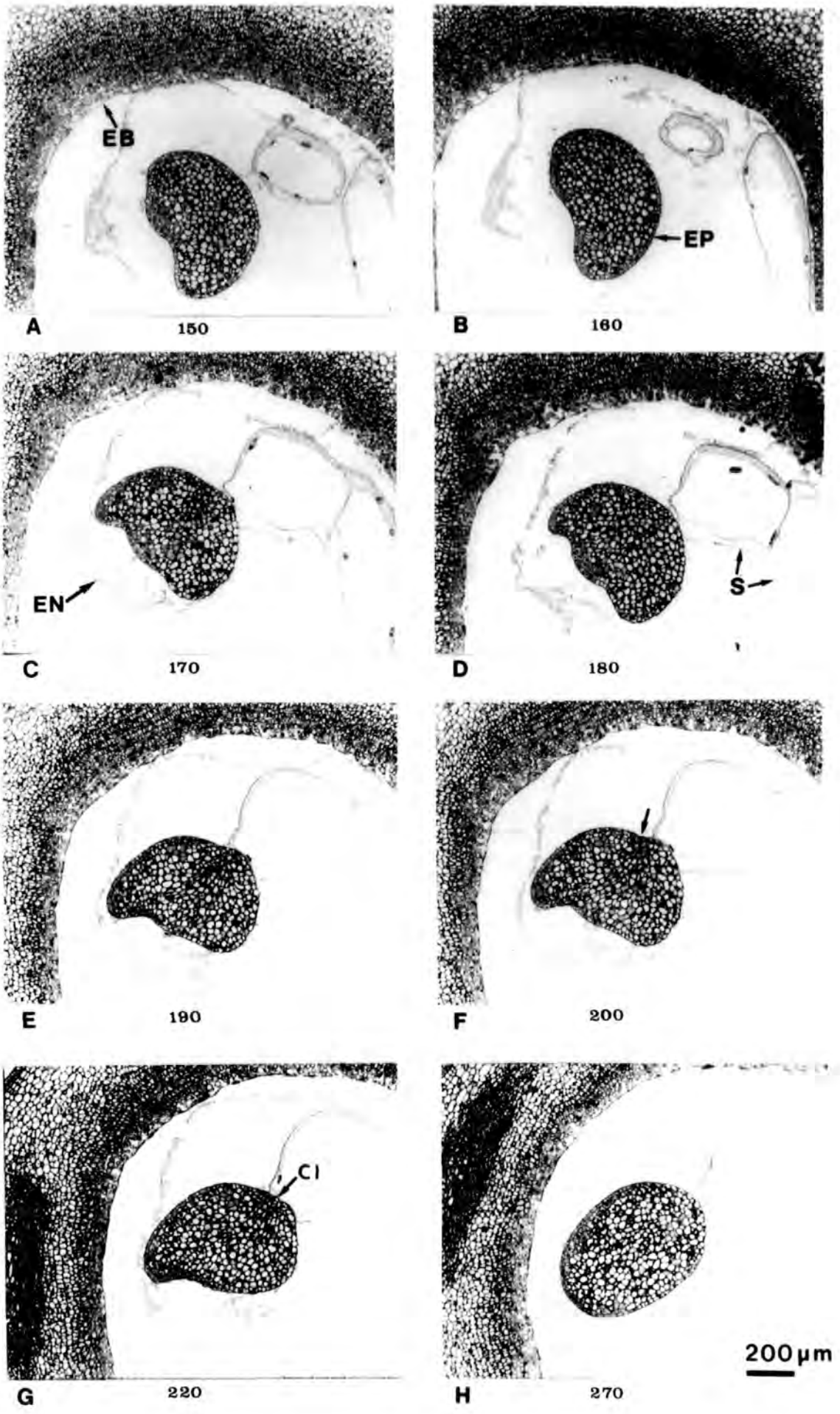
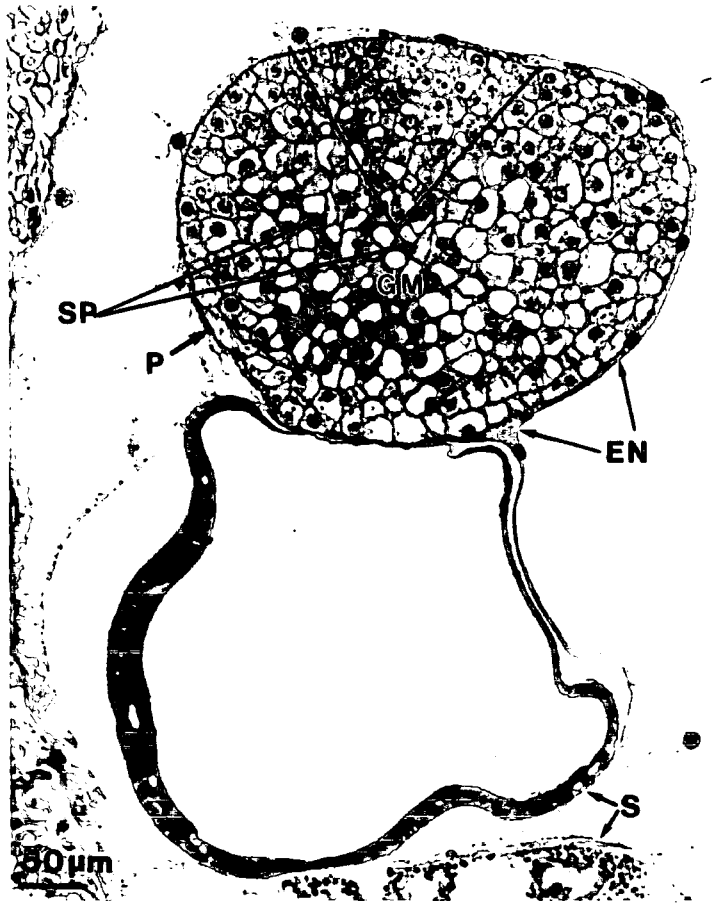
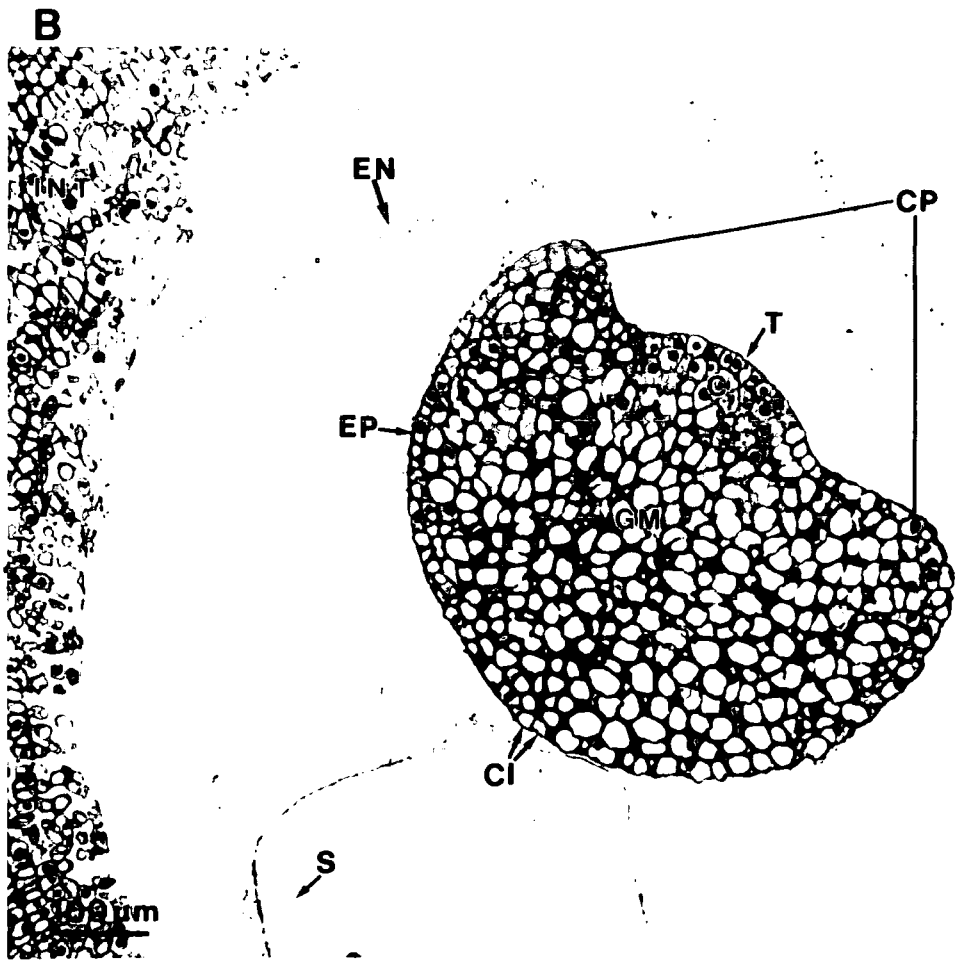


FIG 10

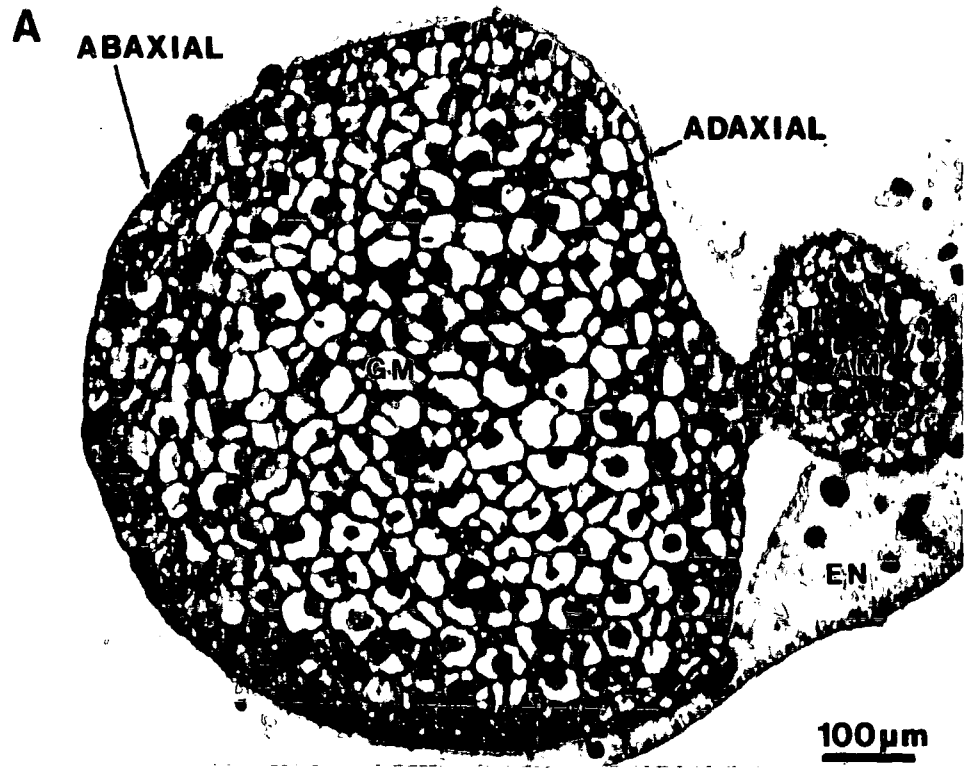


Transverse section through Stage (2) embryo; stained with toluidine blue.

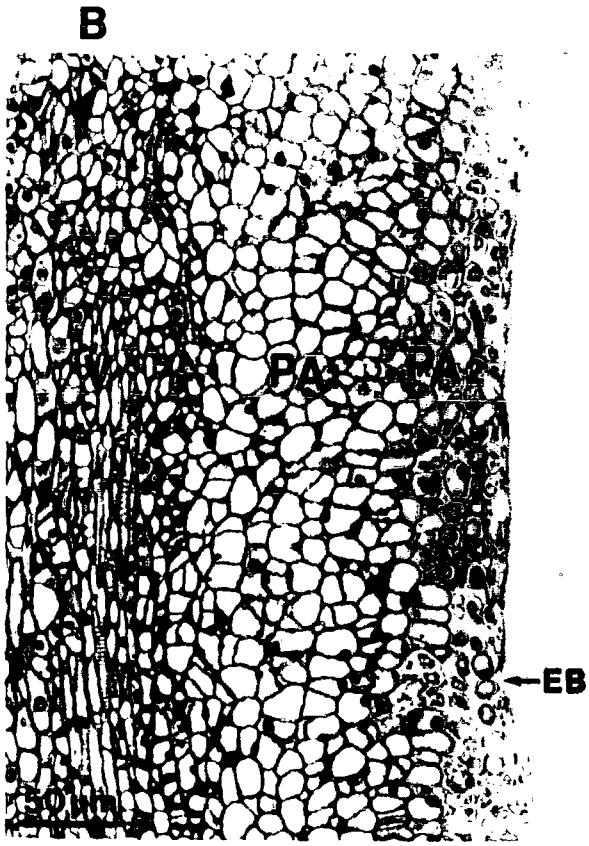


Transverse section through Stage (3) embryo; stained with toluidine blue.

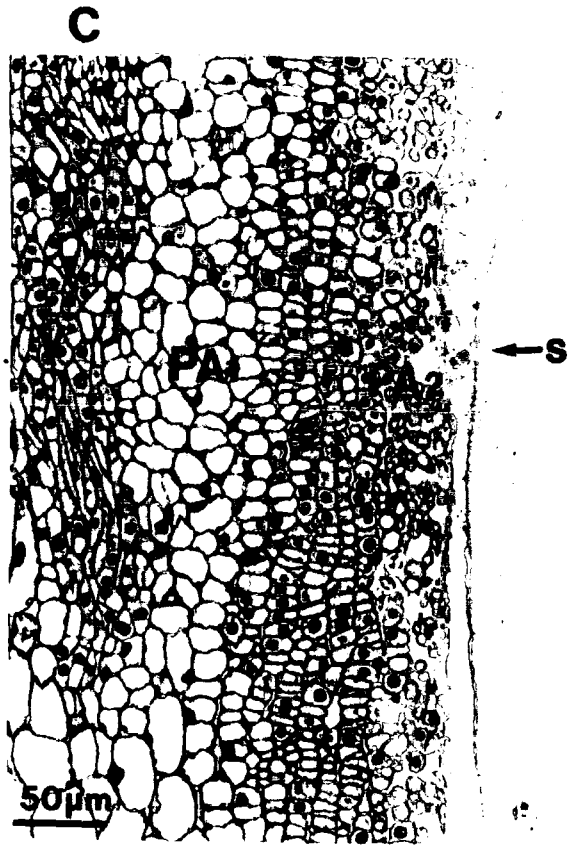
FIG 11



1 um cross-section of Stage (4) embryo; stained with toluidine blue.



Structure of ovular tissue adjacent to embryo at Stage (3) of development.



Structure of ovular tissue adjacent to suspensor at Stage (3) of development.

that growth is mainly due to cell division, whilst later in development weight increase is linear, indicating that both cell division and cell expansion are contributing to growth (Hedley and Smith, 1985). Comparison of ovule and embryo weight data (Fig. 3) shows that initial growth of the seed is due mainly to cell division and growth of the sporophytic ovular tissue, however, as development continues the embryo becomes an increasingly important component of the seed. Subsequent seed growth is due, primarily, to embryo weight increase.

Figure 3

D.a.f.	Embryo as % of total ovule (seed) weight
7	0.5
8	3.3
9	3.6
10	10.63
11	23.6
12	44.09

3.2 OPTIMISATION OF METHODS FOR mRNA AND PROTEIN LOCALISATION

3.2.1 *In situ* Hybridisation Histochemistry

In situ hybridisation histochemistry allows the detection of specific nucleic acid sequences within individual cells. The technique is more sensitive than conventional nucleic acid hybridisation/detection protocols which involve homogenisation of tissue and extraction of nucleic acids, resulting in a dilution effect for any one gene or gene product (Bresser and Evinger-Hodges, 1987). In addition, methods that involve tissue homogenisation sacrifice cellular integrity

and spatial resolution. *In situ* hybridisation is particularly useful for studying the developmental pattern of expression of specific genes in heterogeneous tissues (e.g. Gee and Roberts, 1983) and, when used in conjunction with protein immunolocalisation, can be used to identify the cellular site of peptide biosynthesis.

The variety of *in situ* hybridisation protocols and detection methods utilised (Bresser and Evinger-Hodges, 1987; Warford, 1988) indicates the absence of any single, standard *in situ* hybridisation protocol and the necessity to optimise methodology for each tissue type.

The *in situ* hybridisation protocol consists of many steps, each of which require optimisation:-

Fixation

Attempts to use the *in situ* hybridisation protocol on unfixed material resulted in a loss of target sequences and disruption of morphology (McCabe *et al.*, 1986). Fixation of tissue is therefore essential. The majority of *in situ* hybridisation protocols published involve the use of animal tissues and standard animal tissue fixatives, e.g. Carnoys, 1% glutaraldehyde (Angerer and Angerer, 1981). The few comparative studies on fixation protocols that have been undertaken (Singer *et al.*, 1986; McAllister and Rock, 1985; Bresser and Evinger-Hodges, 1987) provide little information for the plant scientist since they often utilise fixatives employed with animal tissues and have arrived at different conclusions with regard to optimal fixation.

Bresser and Evinger-Hodges (1987), after optimising fixation protocols for bone marrow and blood cells, concluded that "optimal fixatives need to be empirically determined for each tissue type assayed". Fixation optimisation has not been reported for plant tissue, hence it was necessary to undertake a comparative study of fixatives with regard to the tissue under investigation during

this research. Cotyledons, known to be at a high level of legumin mRNA synthesis (Boulter *et al.*, 1987), were cultured in a solution containing tritiated thymidine. Following subsequent processing the cotyledons were solubilised and the degree of RNA retention determined by scintillation counting. The experiment was performed in triplicate and the results, presented in Figure 12, are the mean values obtained. Retention of morphology was ascertained by methyl green/pyronin staining of sections cut from blocks of tissue which had been fixed using a range of different fixatives. Sections of tissue were stained, viewed and scored, by eye, ranging from plus one (poor morphology) to plus five (good morphology).

The results show that fixation by ethanol provides a relatively high degree of RNA retention, however, there is a loss of RNA of 17.9%, during the process of infiltration and embedding. In the case of the other precipitating fixatives, i.e. methanol and acetone, percentage retention increases when the tissue is fully processed as compared to fixation alone. It is possible that the increase in retention is as a result of the secondary fixation that the tissue undergoes as a result of dehydration through an ascending alcohol gradient. Ethanol has been shown to be a good primary fixative and it is proposed that the degree of RNA retention achieved, via secondary fixation in ethanol, is greater than the subsequent loss that probably occurs during the process of infiltration and embedding. This result, indicating improved RNA retention as a result of secondary fixation in ethanol, has been reported also by Bresser and Evinger-Hodges (1987). The cross-linking fixatives, in general, do not respond to a secondary fixation; RNA retention being reduced as a result of complete processing. However, tissue fixed in paraformaldehyde shows a considerable increase in RNA retention after secondary ethanol fixation. Paraformaldehyde is reported to cross-link proteins to a lesser extent than other glutaraldehyde-based

RNA RETENTION BY FIXED AND EMBEDDED ^{3}H LABELLED COTYLEDONS

Fixative		Counts per Gram of Tissue: Fixation only	% Retention	Fixed, Dehydrated & Embedded	% Retention	Morphology
CONTROL		5.2×10^6	100	-	-	-
70% Etoh: 30% acetic acid	R.T.	2.6×10^6	50	1.6×10^6	30.76	++
100% Etoh	R.T.	1.9×10^6	36.5	9.7×10^5	18.6	+
100% Methanol	-20°C	1.5×10^6	28.8	1.8×10^6	34.6	+
3% paraformaldehyde; 1.25% glutaraldehyde in 0.05 M sodium phosphate buffer	R.T.	9.3×10^5	17.88	7.8×10^5	15	++++
4% glutaraldehyde in PBS	R.T.	8.1×10^5	15.57	7.1×10^5	13.65	+++
4% paraformaldehyde in PS buffer	R.T.	6.7×10^5	12.88	1.6×10^6	30.76	++++
1% glutaraldehyde in PBS	R.T.	6.1×10^5	11.73	4×10^5	7.69	+++
2.5% glutaraldehyde; 1.5% paraformaldehyde in 0.05 M sodium cacodylate buffer	R.T.	2.9×10^5	5.57	5.16×10^5	9.92	+++
50% methanol:50% acetone	-20°C	8.5×10^4	1.63	1.2×10^6	23.07	++
100% acetone	-20°C	2.4×10^4	0.46	1.7×10^6	32.6	+

FIG 12

cross-linking fixatives (Singer *et al.*, 1986) and may therefore be amenable to a secondary precipitating fixation.

The precipitating fixatives resulted in poor retention of morphology, whilst cross-linking fixatives provided excellent morphological retention.

Paraformaldehyde, which provides a good degree of RNA and morphology retention was therefore chosen as the optimal fixative in this study. However, it should be noted that the success of *in situ* hybridisation depends not only upon the nucleic acid being retained but also upon its availability, within the section, for hybridisation. This experiment has indicated the extent of RNA retention within the tissue but it is possible that despite its retention it is inaccessible to the probe.

Embedding

16 d.a.f. cotyledons were fixed, using a variety of fixatives, and infiltrated and embedded either in PEG mol. wt. 1500, or paraffin wax. Sections were cut and stained for RNA with both methyl green/pyronin and acridine orange. Comparison of results showed that RNA detection by the histological stains, which provides an indication of RNA retention, was consistently greater in wax-embedded material, compared to PEG-embedded. However, retention of morphology was greater in the PEG-embedded material. Despite the slightly lower RNA retention, PEG was chosen as the embedding medium due to the excellent retention of morphology achieved.

Mounting of Sections

Some slides and cover-slips were washed in Decon 9 cleaning agent (Chapter 2.2.5.1) and incubated in a 0.5% v/v hydrogen peroxide solution in order to destroy any RNase contamination. Results of *in situ* hybridisations performed using both treated and non-treated slide/cover-slips showed no difference in signal level,

indicating little or no loss of retained RNA via RNase contamination of glassware. However, siliconisation of cover-slips was found to be essential. Un-siliconised coverslips were difficult to remove following hybridisation resulting in tissue damage. Various adhesive slide coatings have been utilised in *in situ* hybridisation protocols including denatured egg white, chrome alum gelatine and poly-L-lysine (Warford, 1988). During the course of this work chrome alum gelatine was found to be a sufficient adhesive when used in conjunction with strong heat (60°C). Sections that had been "heated down" onto the slide, and those that had not were stained, for RNA retention, using methyl green/pyronin. No differences in staining intensities were observed and it was concluded that neither RNA retention nor morphology were affected detrimentally by the "heating down" of sections.

Tissue Pre-treatments

One of the main factors limiting the efficiency of *in situ* hybridisation is the rate of diffusion of the probe through the section. A number of protocols therefore include the pre-treatment of tissue/cell samples in order to render target sequences more accessible to the probe. Pre-treatments include the use of proteinases (Angerer and Angerer, 1981), acid (Brahic and Haase, 1978) and heat denaturation (Brahic and Haase, 1978), which are thought to permeabilise the fixed, cellular matrix of the section, thereby facilitating probe entry and diffusion (Singer *et al.*, 1986).

A variety of tissue pre-treatments were assayed with respect to their effect upon hybridisation sensitivity. A series of *in situ* hybridisations were performed in which the protocol was kept constant with the exception of the tissue pre-treatment. Each pre-treatment was therefore assessed independently of any other variable. Pre-treatments were as follows: (i) proteinase K (1 µg/ml in 0.1 M Tris-HCl, 50 mM EDTA pH 8.0) for 10 min. at 37°C; (ii) 0.2 N HCl for 30 min. at room

temperature; (iii) 0.1% v/v Triton 20 for 15 min. at room temperature. Following pre-treatment, sections were post-fixed with 4% paraformaldehyde for 15 min. Treatment with proteinase K required careful control since over-digestion resulted in tissue damage and detachment of section from slide. A range of proteinase K concentrations were assayed with 1 ug/ml resulting in least tissue damage.

None of the pre-treatments were found to increase signal sensitivity and in general were detrimental to morphology. Pre-treatment with proteinase K without subsequent post fixation resulted in a loss of hybridisation signal, probably due to a loss of mRNA from the section. A similar loss of RNA, following protease treatment, has been reported also by Blum *et al.* (1983). It was therefore concluded that tissue pre-treatments were inappropriate in our protocol. Proteolytic treatment of glutaraldehyde fixed material is reported to increase sensitivity of hybridisation (Angerer and Angerer, 1981). However, Singer *et al.* (1986) using paraformaldehyde fixed material detected RNA loss following proteinase K digestion. It is probable, therefore, that the requirement of tissue pre-treatment is dependent upon the initial fixation protocol.

Other pre-treatments, involving the use of inhibitors of non-specific binding of nucleic acids, have also been utilised to decrease background "noise" and thereby increase hybridisation sensitivity. In this study the following inhibitors were found to be necessary:- acetic anhydride treatment, 1% BSA block, Denhardt's reagent, non-specific DNA (salmon sperm DNA, or calf thymus DNA). Blocking with 1% BSA was found to provide optimal signal:noise ratios. The use of 1% glycine reduced signal levels whilst a 0.1% solution of non-fat dried milk abolished the signal completely. This result contradicts those obtained by Duhamel and Johnson (1985), who found a 5% w/v solution of non-fat dried milk to be most effective at reducing non-specific binding, whilst a 5% w/v BSA solution was found to have only a moderate blocking effect. Acetylation was originally

introduced by Hayashi *et al.* (1978) to reduce background levels when using ^{125}I labelled probes and has subsequently been reported to improve signal:noise ratios when using ^{32}P-labelled probes (Lawrence and Singer, 1985) and tritiated probes (Angerer and Angerer, 1981). The treatment is proposed to reduce background levels via acetylation of positively charged groups, resulting in a decrease in non-specific electrostatic binding of nucleic acids (Angerer and Angerer, 1981). In contrast to the comparative study undertaken by Bresser and Evinger-Hodges (1987), acetylation of pea tissue sections was found to increase signal:noise ratios when using radio-labelled probes and was therefore included in our *in situ* hybridisation protocol. Contrary to the results of Bresser and Evinger-Hodges (1989), pre-hybridisation was also found to be essential in reducing background levels. In addition it is probable that the pre-hybridisation step, involving section incubation in 50% formamide at 37°C, results in permeabilisation of the tissue thereby increasing probe penetration. However, it is extremely important that the non-specific DNA is fully sheared and completely dissolved. In order to ensure homogeneity of the solution the non-specific DNA was sonicated directly; failure to do so resulted in localised high background levels.

Probe size and Concentration

An important factor in the *in situ* hybridisation protocol is probe sequence length since this, combined with tissue permeability, determines the rate of tissue penetration and hence hybridisation with target sequences. Probes produced by nick-translation and random priming are reported to be of optimal sequence length (Warford, 1988). Analysis of probe size range (Chapter 2.2.18) showed that nick-translation labelling of the 1.12 Kb *leg*A cDNA produced probes consisting of fragments ranging in size from 1 Kb to 50 bp, whilst random priming produced fragments ranging in size from 1 Kb to 400 bp. Various studies have reported a

positive correlation between increased hybridisation signal and reduced probe length (Brahic and Haase, 1978; Angerer and Angerer, 1981; Gee and Roberts, 1983). Probes of 30-50 bp are reported as being optimal though increased hybridisation temperature has been reported to increase signal levels when using larger probes, possibly due to enhanced diffusion of probes at higher temperatures (Gee and Roberts, 1983). The range of fragment sizes produced by nick-translation and random priming of the pAD 4.4 cDNA insert therefore provide appropriate probes for *in situ* hybridisation. Compared to random priming, nick-translation produces probes of a greater size range, however the process of random priming allows the labelling of DNA to a much higher specific activity (~2 x 10^9 c.p.m./μg) compared to nick translation (2 x 10^8 c.p.m./μg). Probes produced by either protocol are applicable to *in situ* hybridisation. Samples of photobiotinylated and non-photobiotinylated DNA were size-fractionated via agarose gel electrophoresis. As expected, the process of photobiotinylation was shown not to alter probe sequence length. Since the photobiotinylated *leg*A cDNA used during this study (isolated from the clone pAD 4.4 by restriction endonuclease digestion) was 1.12 Kb in length it was possible that probe size might be a factor limiting the sensitivity of the protocol. To test this the 638 bp cDNA insert of pRC 2.2.4. (Croy *et al.*, 1982) which is complementary to the 3' region of the pAD 4.4 insert cDNA was reduced in size, via double restriction with Bam HI and Pst 1, into fragments of 400 and 200 bp in length, and photobiotinylated. *In situ* hybridisations were performed using either the 1.2 Kb cDNA or the 200/400 bp cDNA probe mixture. However, no appreciable difference in signal intensity was observed. This result is consistent with those of Singer *et al.* (1986), who found that paraformaldehyde fixed material tolerated a wide range of probe size, ranging from 50 bp to 1.5 Kb. They suggested that this was due to the low level of cross-linking that occurs with

paraformaldehyde fixation resulting in the tissue being relatively permeable to probe penetration.

A ten-fold increase in hybridisation signal was reported when both insert and vector DNA was used in the hybridisation mix, as opposed to insert cDNA alone (Singer *et al.*, 1986). It was proposed (Singer *et al.*, 1986) that since DNase nicking, in the nick-translation process, is random many of the insert cDNAs would be linked to vector sequences, termed junction pieces. Following hybridisation to target sequences contiguous vector sequences would be available for hybridisation with other vector sequences, resulting in a network effect, amplifying the original signal. This hypothesis was tested via hybridisation of sections with nick-translated ^{32}P labelled pAD 4.4 cDNA only (following removal from vector) or with ^{32}P labelled vector (pBR 322) plus insert. The results were in contrast to those previously obtained by Singer *et al.* (1986) since hybridisation with both vector and insert resulted in extremely high levels of background, considerably reducing signal to noise levels. Therefore, in the course of this work, cDNA inserts were always removed from the vector prior to labelling and hybridisation.

Probe concentration affects several parameters of the *in situ* hybridisation reaction. High probe concentrations are used to increase diffusion, reduce hybridisation time and saturate all the available sequences, however, high concentrations generally result in increased background levels (Singer *et al.*, 1986). In general an increase in probe concentration results in a decrease in hybridisation time, however it has been reported that the rate of *in situ* hybridisation is one-tenth that of liquid hybridisation performed under the same conditions (Brahic and Haase, 1978). Hybridisation, overnight, with a low probe concentration was found to be optimal since increased background levels were detected when probe concentration was increased above 0.25 µg/ml, even when hybridisation time was

reduced. Since time was not a limiting factor in this study a convenient overnight incubation with probe at a concentration of 0.25 µg/ml was used.

Hybrid Detection

i) **Isotopic**

Target nucleic acid sequences, hybridised to radiolabelled probes, are detected via microautoradiography. Sections are coated with liquid emulsion and the silver grains produced, by interaction of B particles with the emulsion, are developed chemically. Three B emitting isotopes (^{32}P, ^{35}S, ^{3}H) have been utilised in *in situ* hybridisation protocols. ^{32}P has the highest energy and therefore provides the greatest sensitivity, however, the spread of emitted particles results in low resolution. ^{35}S is a lower energy emitter and therefore provides greater resolution. ^{3}H is a low energy emitter and provides excellent resolution, however ^{3}H B particles have an average path-length of 0.5 µm and therefore only hybrids in the upper portion of the section can be detected (McFadden, 1989). During this research an *in situ* hybridisation protocol was utilised in order to detect transcript levels that are too low to be detected by conventional filter hybridisation protocols. To increase the sensitivity of our protocol sections were of optimal thickness, in order to increase the number of target sequences available for hybridisation. Therefore, tritiated probes, which allow detection of only those hybrids in the upper portion of the section were of no use in this study. The ^{32}P and ^{35}S labelled probes provided good results in terms of signal:noise ratios and resolution. ^{35}S labelled probes provided optimal results, in terms of resolution (Fig. 19), however resolution using ^{32}P appeared not to be as greatly reduced as previously reported (Warford, 1988; McFadden, 1989). It is possible that the usefulness of ^{32}P for *in situ* hybridisation protocols is dependent upon the nuclear emulsion used. In

conjunction with Ilford K2 emulsion ^{32}P was found to provide acceptable resolution in this research.

ii) **Non-Isotopic**

Biotin, attached at the C-5 position in uridine via an allylanine spacer arm which renders it accessible to detection reagents, is the most common hapten utilised in *in situ* hybridisation protocols (e.g. Singer and Ward, 1982). The biotin moeity can be detected via either fluorescence, enzyme conjugates or colloidal gold and silver intensification. The biotin system, though providing much greater resolution compared to radiolabelled probes, is reported to be less sensitive (McFadden, 1989; Warford, 1988). However, studies have shown that multi-step detection methods can result in significant amplification of the signal (e.g. Groudy *et al.*, 1987).

A variety of amplification procedures were assayed using a filter detection system. A range of concentrations of biotinylated pAD 4.4 cDNA were dotted onto nitrocellulose. Following amplification the resultant biotin moeity was visualised by either enzymatic reaction (alkaline phosphatase, Chapter 2.2.16.1.1) or via colloidal gold and silver intensification (Chapter 2.2.25.1). Controls consisted of biotinylated DNA "dot blots" detected directly as described (Chapter 2.2.16.1.1).

Results (Fig. 13) demonstrated that optimal amplification was achieved using goat-anti-biotin: rabbit-anti-goat IgG, conjugated to either colloidal gold or biotin, with subsequent detection by either alkaline phosphatase or silver enhancement. The alkaline phosphatase detection protocol produces a colour reaction product and when used to detect low hybrid levels in tissue sections resulted in only a "grainy" coloration of the cytoplasm, which was unacceptable both photographically and quantitatively. Therefore amplification via goat-anti-

FIG 13 Detection of biotinylated DNA transferred to nitrocellulose. Figures beneath filters represent the amount of bound DNA (pg).

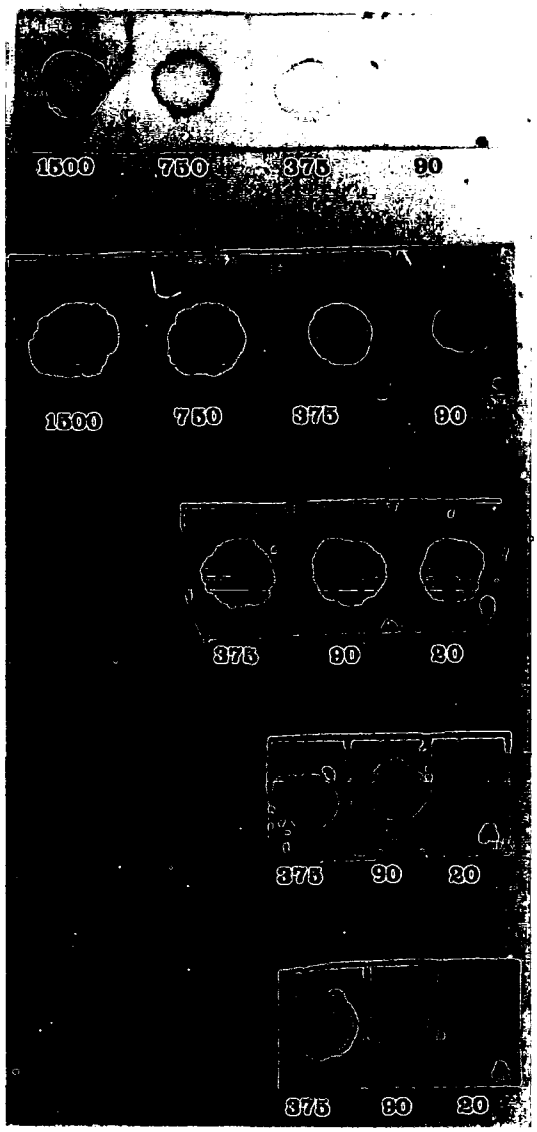
Biotin detection via ABC-AP Kit - no amplification.

Amplification via goat-anti-biotin; rabbit anti-goat biotin. Biotin detection via ABC-AP kit.

Amplification via goat-anti-biotin; rabbit-anti-goat gold, silver intensification.

Amplification via streptavidin gold; goat-anti-rabbit biotin rabbit-anti-goat gold silver enhancement.

Amplification via goat-anti-biotin, rabbit-anti-goat biotin. Biotin detection via ABC-AP kit.



DETECTION PROCEDURE	Detection limit (pg biotinylated DNA)	Amplification
Direct detection via alkaline phosphatase	<375	-
Goat anti biotin; R.A.G. biotin. Alkaline phosphatase	<20	>18.7
Goat anti biotin; R.A.G. gold. Silver enhancement	<<20	>>18.7
Streptavidin gold; Goat anti biotin; R.A.G. gold, silver enhancement	~20	~18.7

biotin: rabbit-anti-goat gold and visualisation via silver enhancement was the detection system chosen.

3.2.2 Immunological Techniques

A major aim of this research was to ascertain the earliest stage of *Pisum* embryo development at which legumin protein could be detected. Previous work by Domoney *et al* (1980) demonstrated that, with the use of very sensitive detection systems, legumin could be detected in developmental stages previously concluded to be quiescent, with respect to legumin storage protein synthesis. The ELISA protocol used in this study was a modified antibody capture technique (Harlowe and Lane, 1988). Two factors that affect the sensitivity of antibody capture techniques are; (i) the amount of antigen bound; and (ii) the avidity of the antibody for the antigen. The wells of a microtitre plate can only bind, via electrostatic forces, a finite amount of protein. Since a crude protein extract was being assayed the extraneous proteins would be in considerable excess and would compete with the antigen for the binding sites of the plate. Hence it was therefore necessary to optimise the sensitivity of the assay by determining the maximum amount of total protein (plus antigen) that could be bound to a well without competitive interactions resulting in a reduction of antigen binding. Increasing amounts of storage tissue protein, at a developmental stage (16 d.a.f.) known to contain relatively large amounts of legumin protein, were added to the wells of a microtitre plate and assayed as described (Chapter 2.2.9). The collated results, expressed in a graph of total protein against absorbance (not presented), showed that the maximum amount of total protein that could be bound to a well without competitive interactions resulting in a reduction of sensitivity was 1.1 µg, when using the monoclonal antibody and 0.3 µg when using the affinity purified polyclonal antibody. The result obtained with the polyclonal antibody correlated

with that obtained, from similar experiments, by Edwards (1988). The difference in values obtained for the two types of antibody is due to the fact that the polyclonal serum consists of multiple antibodies directed against different epitopes on the antigen, whilst the monoclonal will react only with a single epitope of LegA. The avidity of the antibody-antigen reaction, i.e. its stability, is intrinsic to the antibody. However, avidity can be increased by increasing the concentration of bound antigen (Harlowe and Lane, 1988). Optimisation of antigen binding, as previously described, therefore increases the detection limit of the protocol. Pea leaf and *Nicotiana* seed protein extracts were also assayed as controls and the low level of reaction that occurred with the monoclonal antibody was substracted from experimental data.

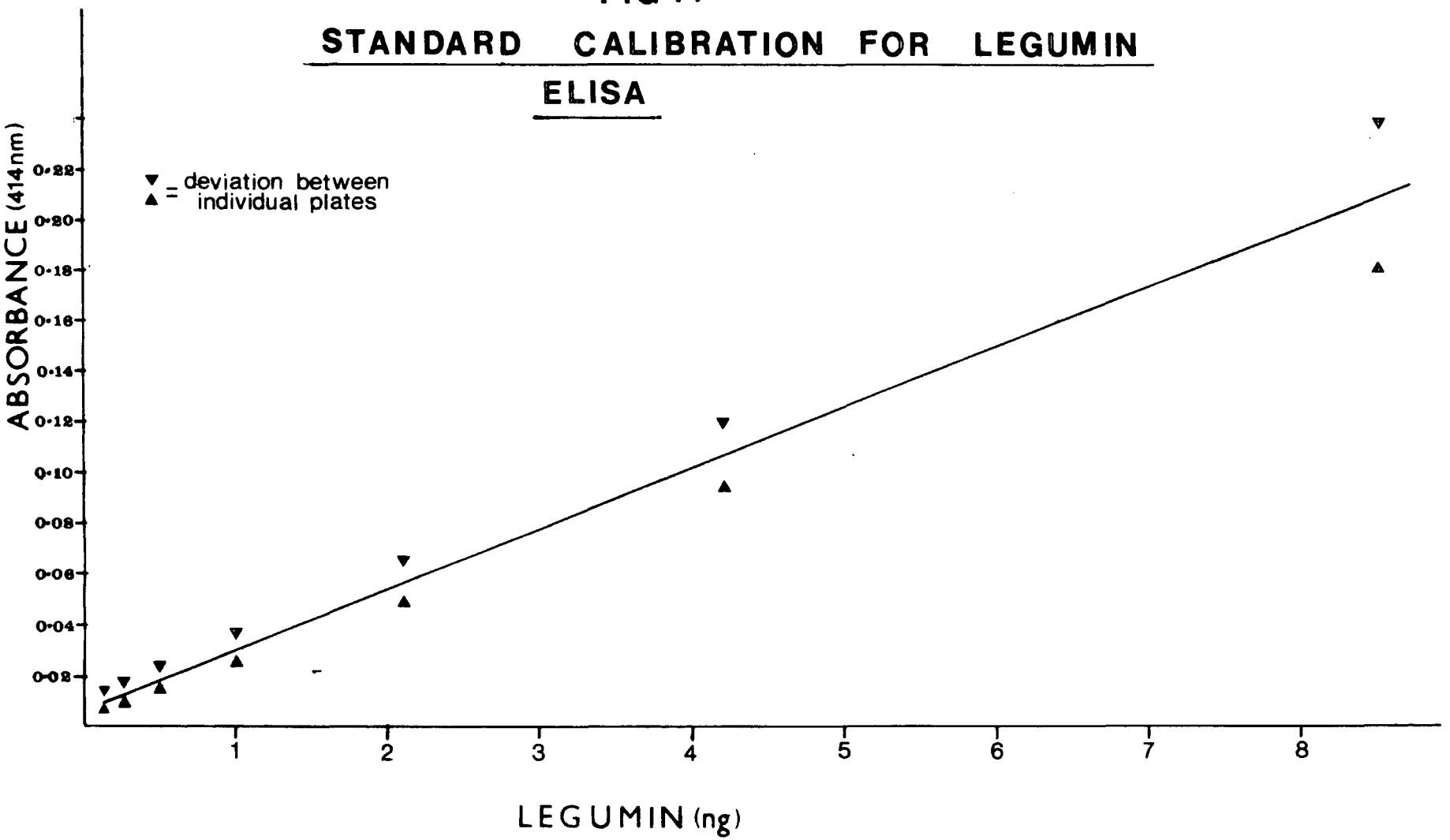
The sensitivity of the monoclonal anti-legumin antibody is demonstrated in Figure 14. The antibody consistently gave a linear resonse in the 1-10 ng antigen range and was therefore ten-fold more sensitive than that utilised by Domoney *et al.* (1980). However individual microtitre plates vary with respect to the amount of protein that can be bound. Therefore each sample set was assayed, in triplicate, and with three replicates.

A further factor affecting the sensitivity of the assay is that the antibody must be in excess. The wells of a microtitre plate were coated with purified legumin and assayed using the monoclonal antibody at varying dilutions; a $^1/_{10}$ dilution was found to be optimal.

As with all experimental protocols it is desirable to validate the results obtained via the use of a secondary technique. Immunohistochemistry was therefore used to localise the antigen in tissue sections, however, the different protocols require different optima with regard to conditions. Tissue used for immunolocalisation was fixed, embedded in PEG and sectioned. This protocol retains morphology, however, it is very harsh in terms of retention of epitope

FIG 14

STANDARD CALIBRATION FOR LEGUMIN ELISA



structure (Harlow and Lane, 1988). A monoclonal antibody only recognises a single epitope on the antigen surface, if the epitope is destroyed or altered during tissue processing then the antibody will not react. A polyclonal antibody that reacts with a broad range of epitopes is therefore the antibody of choice when localising an antigen in fixed samples in which some of the epitopes may have been altered by fixation. In addition, several antibodies may bind to a single antigen molecule, amplifying the signal and increasing resolution of results (Harlowe and Lane, 1988).

During the course of this work both the monoclonal and polyclonal anti-legumin antibodies were tested in the immunolocalisation protocol. As predicted superior results were obtained using the polyclonal at $^1/_{100}$ dilution. Since polyclonal antibodies react with a range of epitopes there is the increased possibility of cross-reaction occurring, i.e. recognising an epitope on an unrelated molecule. However, the polyclonal anti-serum used was affinity purified (Croy *et al.*, 1980) and sections were "blocked" with 1% BSA in order to reduce non-specific interactions.

3.3 DISTRIBUTION OF *leg*A mRNA AND PROTEIN IN DEVELOPING OVULES OF *PISUM*

3.3.1 Detection of mRNA by *in situ* Hybridisation Histochemistry

The distribution of *leg*A mRNA in a ten micron, PEG embedded section of stage (2) ovule is demonstrated in Figure 15A. The section was hybridised to ^{35}S labelled pAD 4.4 cDNA and hybrids were detected via autoradiography. A very high level of labelling is associated with the endosperm cytoplasm indicating a high level of *leg*A expression in this tissue, whilst very low levels of label are associated with the embryo and integument.

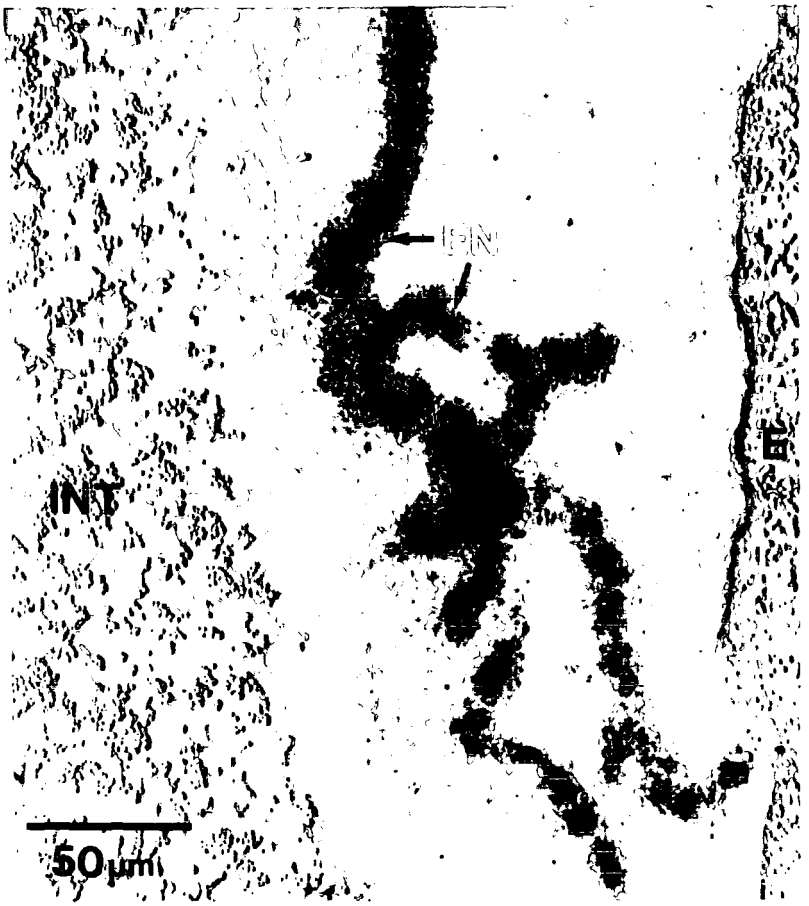
The result of an *in situ* hybridisation using a fixed, PEG embedded, ten micron section of stage (2) embryo is shown in Figure 16. Sections were hybridised with photobiotinylated pAD 4.4 cDNA. Hybrids were detected using goat-anti-biotin, rabbit-anti-goat gold and silver enhancement. Hybrids are detected dispersed throughout the cytoplasm and are present over the nuclei. Since the sections used were ten microns in thickness hybrids present over the nuclei may represent the detection of mRNA within a thin layer of cytoplasm above the nuclei. However, as shown in Figure 15B the probe is able to hybridise to *leg*A transcripts in the nucleus. The results of an LM level *in situ* hybridisation of stage (2) embryo (Fig. 16) taken to EM level as previously described (Chapter 2.2.26) is shown in Figure 15B. The micrograph shows an interphase nucleus, the central nucleolus and surrounding chloroplasts are unlabelled, whilst hybrids are localised both within the nucleoplasm and on the cytoplasmic side of the nuclear membrane. Within the nucleoplasm the darkly staining heterochromatin, known to consist of DNA in a condensed inactive form (Gilbert, 1988), is unlabelled, whilst hybrids are detected in those regions which are lightly stained and presumed to be euchromatin, i.e. DNA in an uncondensed, active form.

In situ hybridisation results (Figs. 15B and 16) therefore demonstrate that *leg*A expression occurs, at low levels, during the very early stages of *Pisum* embryo development (at the late globular stage). The detection, via EM analysis, of *leg*A transcripts in the nuclei of stage (2) embryos, their detection on the cytoplasmic side of the nuclear membrane (Fig. 15B) and their subsequent localisation in the cytosol (Fig. 16) demonstrates that low level *leg*A expression occurs in the nuclei of early embryos and that these transcripts are correctly processed, and transported from the nucleus.

There are two model systems postulated to control differential gene expression (Gatehouse *et al.*, 1986; Boulter *et al.*, 1987):-

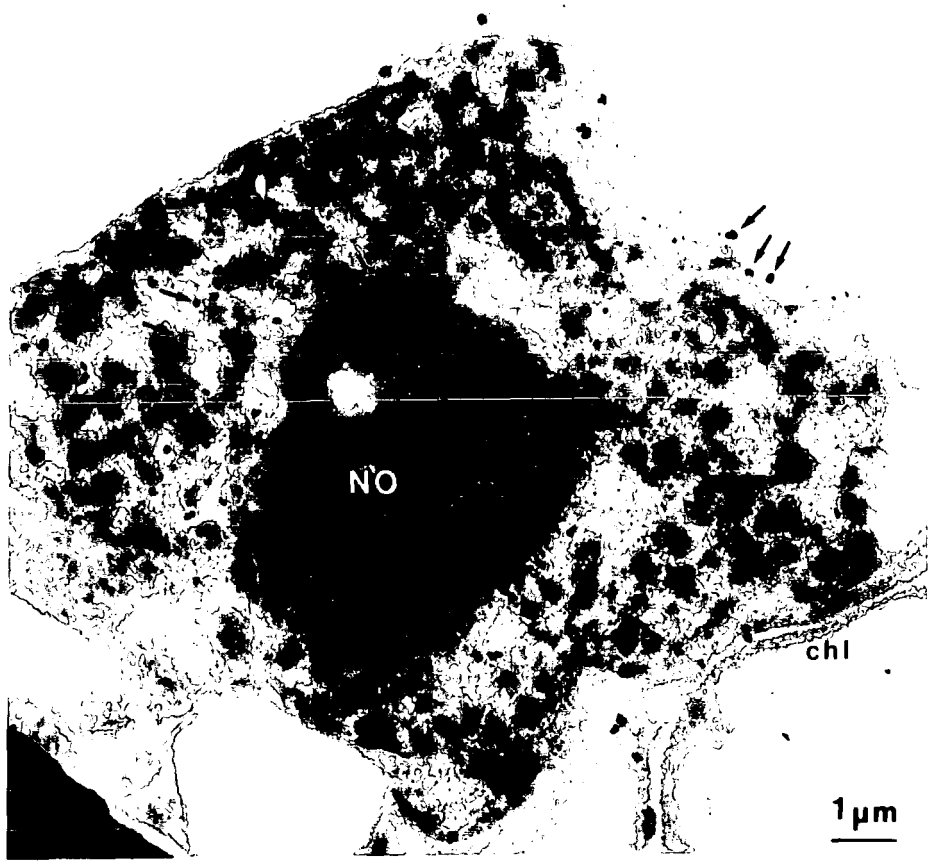
FIG 15

A



Distribution of *leg*A mRNA in Stage (2) ovule. The section was hybridised with the ^{35}S labelled probe to *leg*A and hybrids were detected via autoradiography.

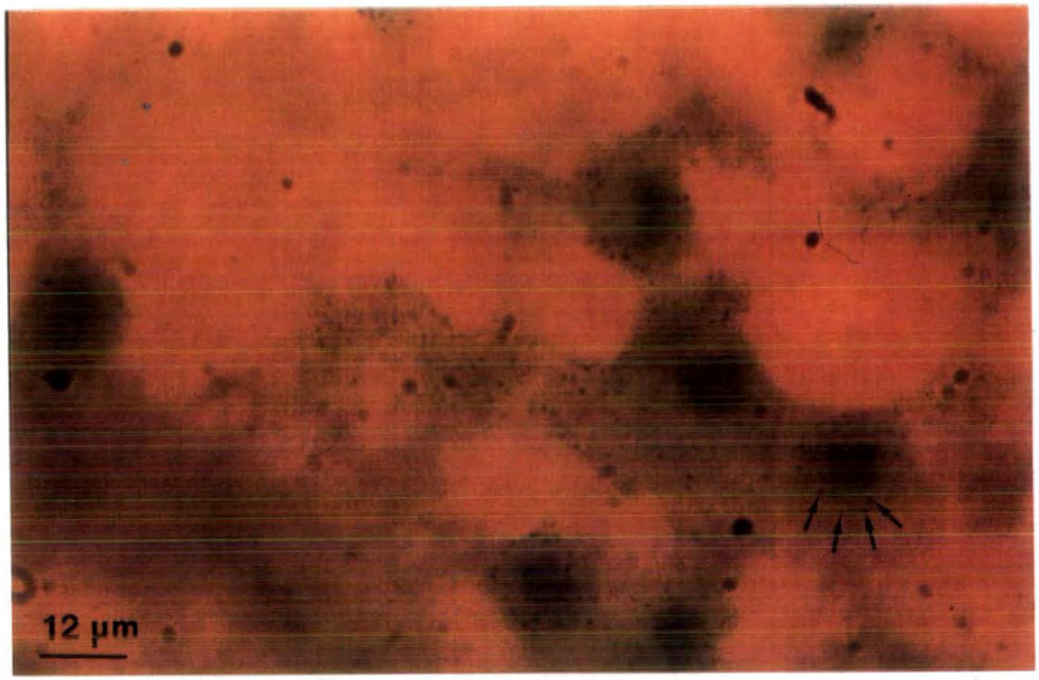
B

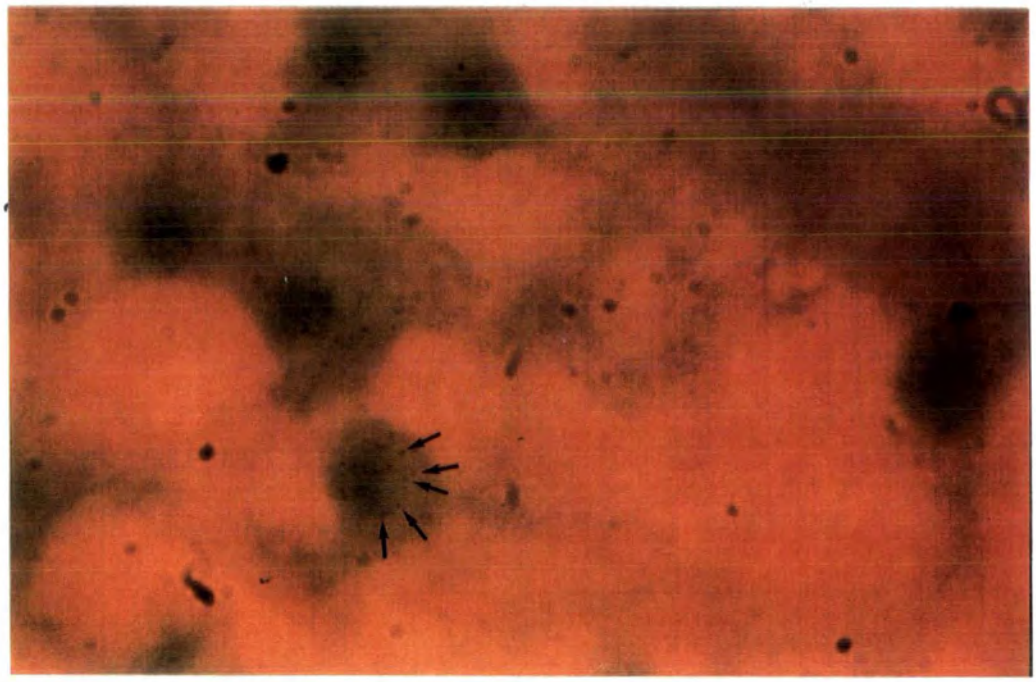


Localisation of *leg*A mRNA in nucleus of Stage (2) ovule. A section was hybridised with biotinylated probe to *leg*A, hybrids were visualised by gold and silver enhancement and subsequently re-embedded and sectioned for E.M. examination.

FIG 16

Detection of *leg*A mRNA in Stage (2) embryo. Sections were hybridised with the photobiotinylated probe to *leg*A and hybrids were detected via goat–anti–biotin; RAG gold and silver enhancement.





(i) via a "switch" mechanism:- the gene is transcriptionally inactive until developmentally switched on. Genes are either transcriptionally active, i.e. on, or transcriptionally inactive, i.e. off.

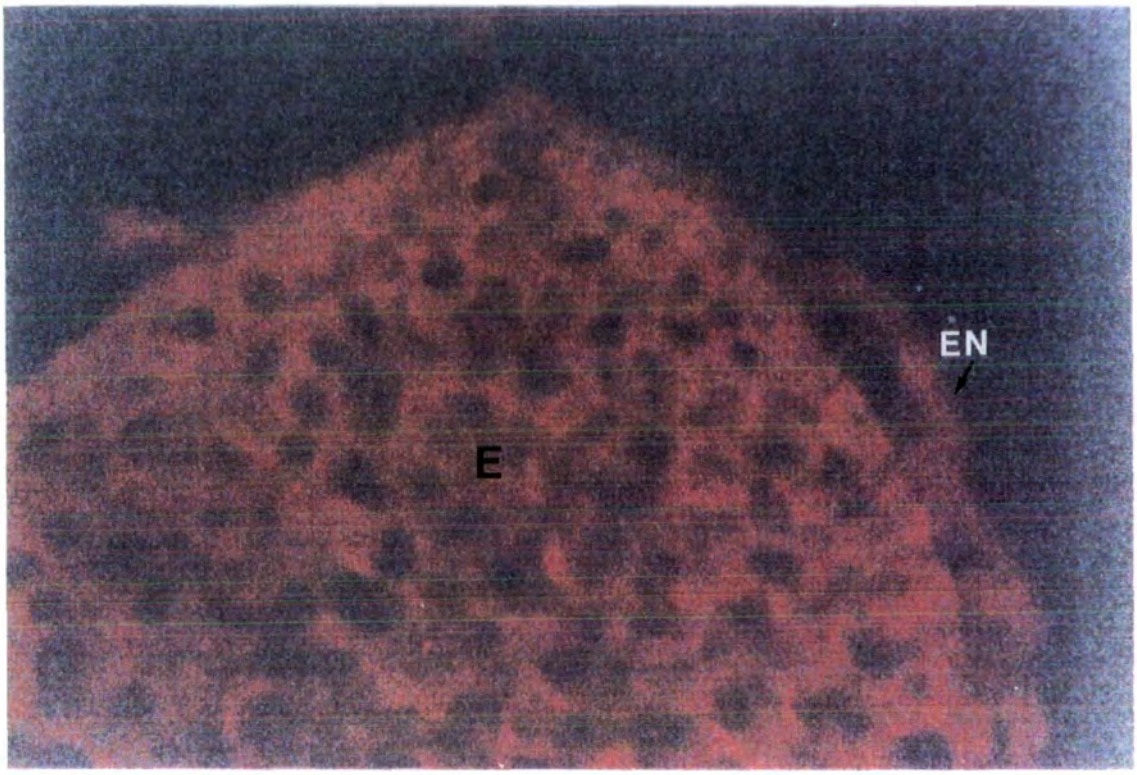
(ii) via a regulatory mechanism:- genes are constitutively transcribed, at low levels. Control of gene expression is achieved by regulating the level of transcription.

Previous researchers have failed to detect *leg*A mRNA in embryos prior to 9 d.a.f. (Gatehouse *et al.*, 1982, 1986; Boulter *et al.*, 1987) indicating that the gene is transcriptionally inactive during the early stages of embryo development. The results presented in this thesis indicate that control of *leg*A expression is via a regulatory mechanism. The gene is transcribed constitutively, the high levels of *leg*A mRNA detected at later stages of development are as a result of an increased transcription rate and not as a result of "switch on" of the gene at a certain point in embryo development. The failure, by previous researchers, to detect *leg*A mRNA in embryos at earlier stages of development was therefore probably due to the sensitivity limit of the detection methods utilised.

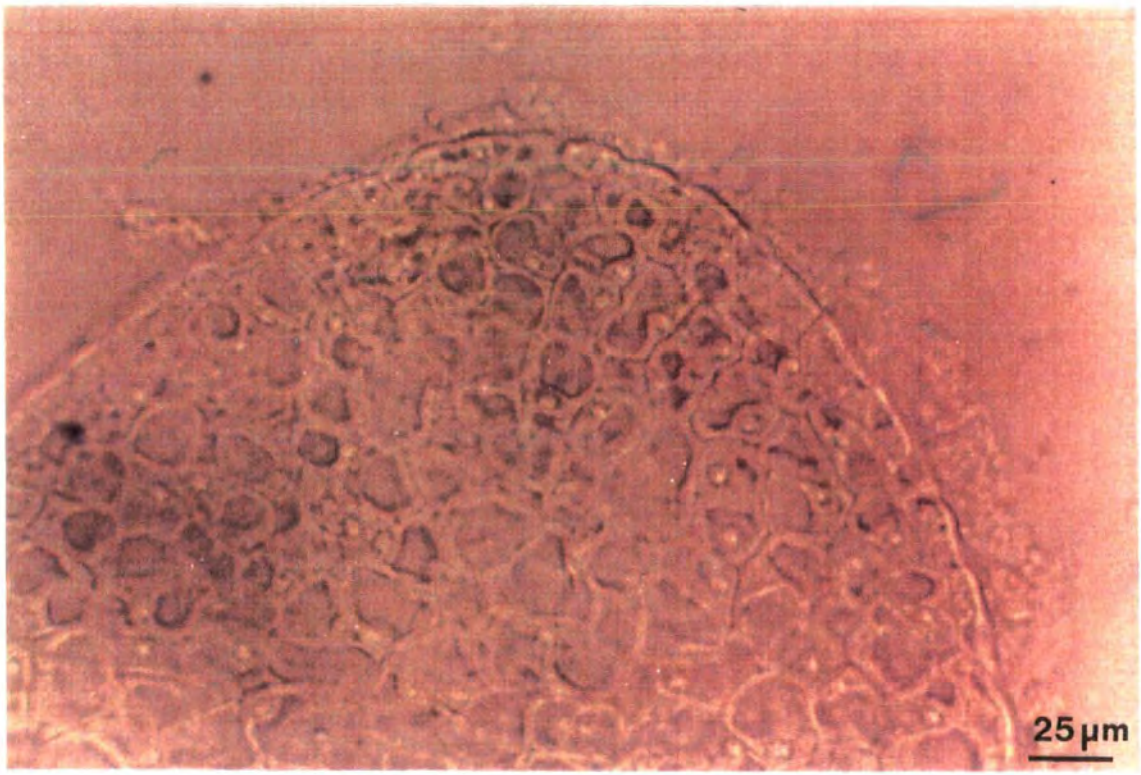
The spatial distribution of *leg*A mRNA in a ten micron PEG embedded section of a stage (3) ovule is demonstrated in Figures 17 and 18. The section was hybridised with photobiotinylated pAD 4.4 cDNA and hybrids were localised using streptavidin rhodamine. Uniform cytoplasmic distribution of labelling within the cotyledon tip of the embryo is evident (Fig. 17). The basal portion of the embryo with attached suspensor is demonstrated in Figure 18. The suspensor exhibits a very high level of labelling compared to the embryo. The endosperm cytoplasm, adjacent to the embryo sac wall, also exhibits a high level of labelling, whilst within the ovular tissue a low level of labelling is detected. Thus, within the ovule at stage (3) of development differential *leg*A mRNA levels can be detected in the various components of the seed.

FIG 17

Detection of *leg*A mRNA in Stage (3) embryo via hybridisation with the photobiotinylated probe to *leg*A and hybrid visualisation via streptavidin rhodamine.



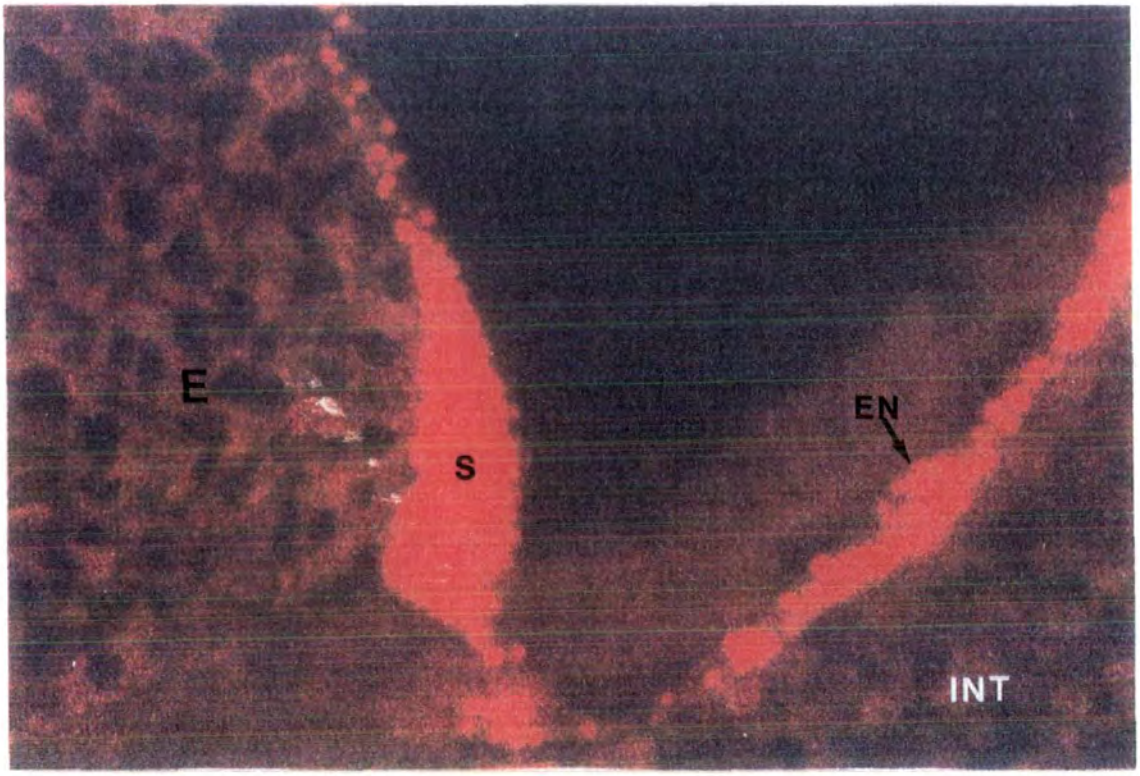
Viewed by epifluorescence optics using a 490 nm excitation wavelength and 515 nm suppression filter.



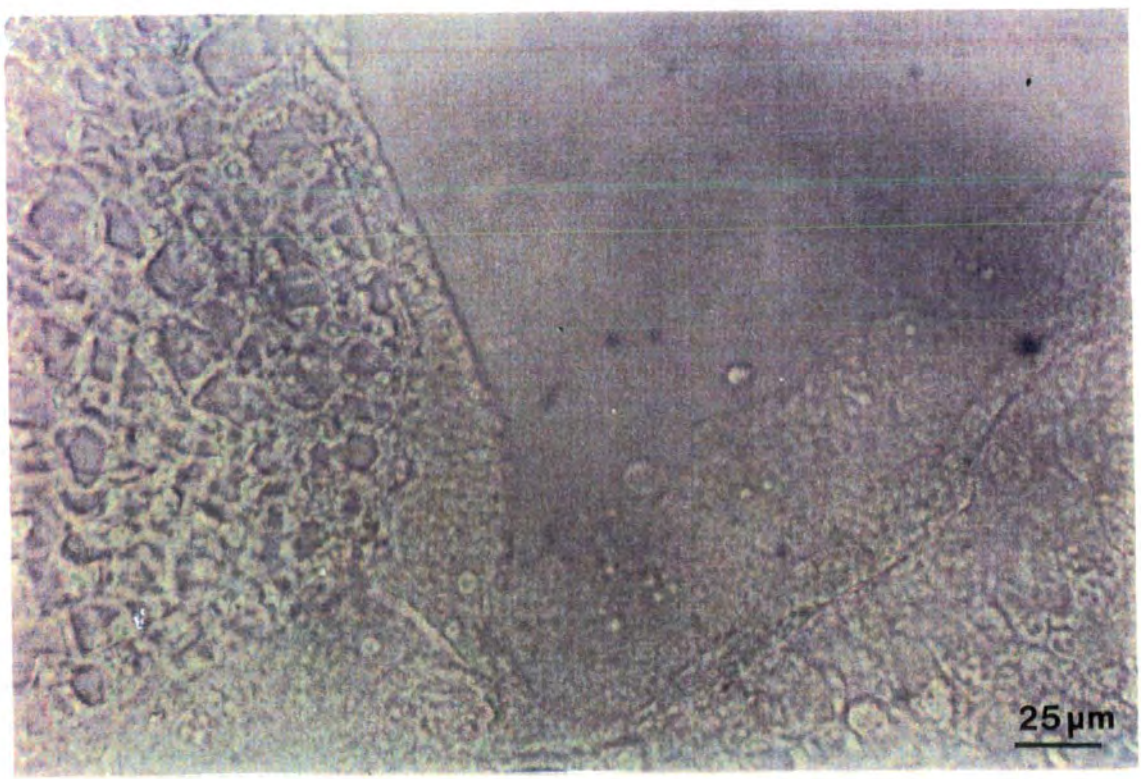
Viewed by Nomarski DIC

FIG 18

Spatial distribution of *leg*A mRNA in Stage (3) ovule.
Sections were hybridised with the photobiotinylated probe to *leg*A and hybrids were detected via streptavidin rhodamine.



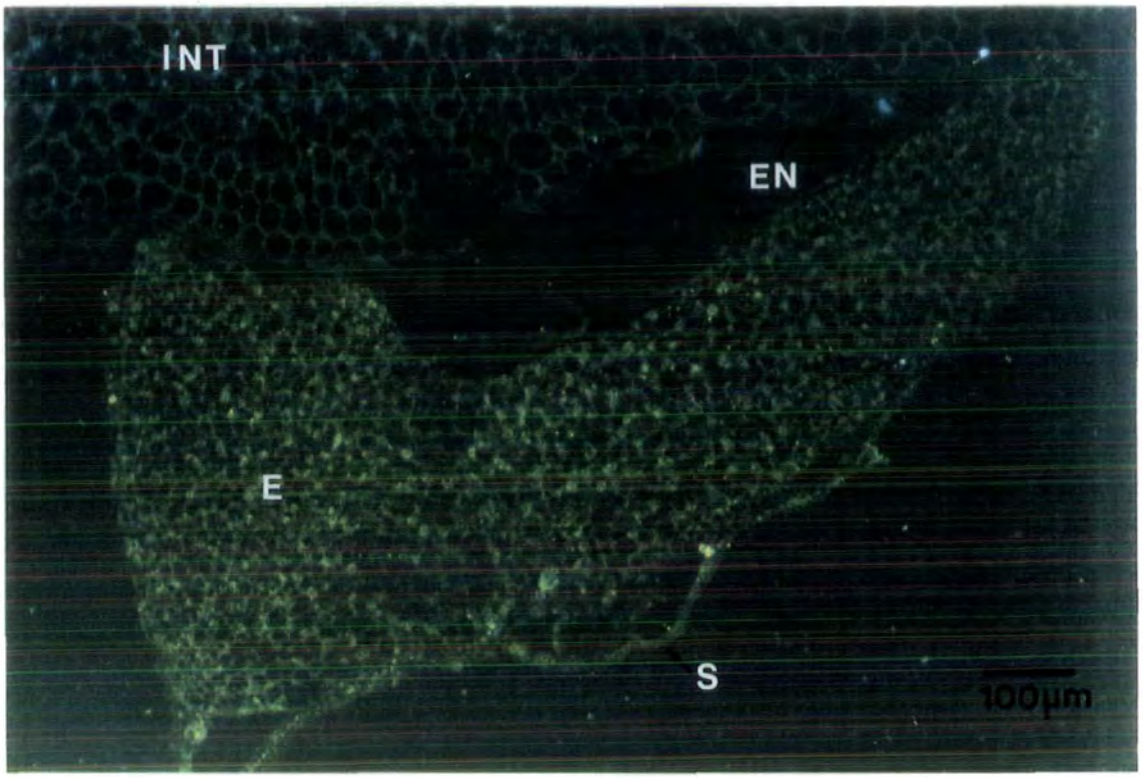
Viewed by epifluorescence optics using a 490 nm excitation wavelength and 515 nm suppression filter.



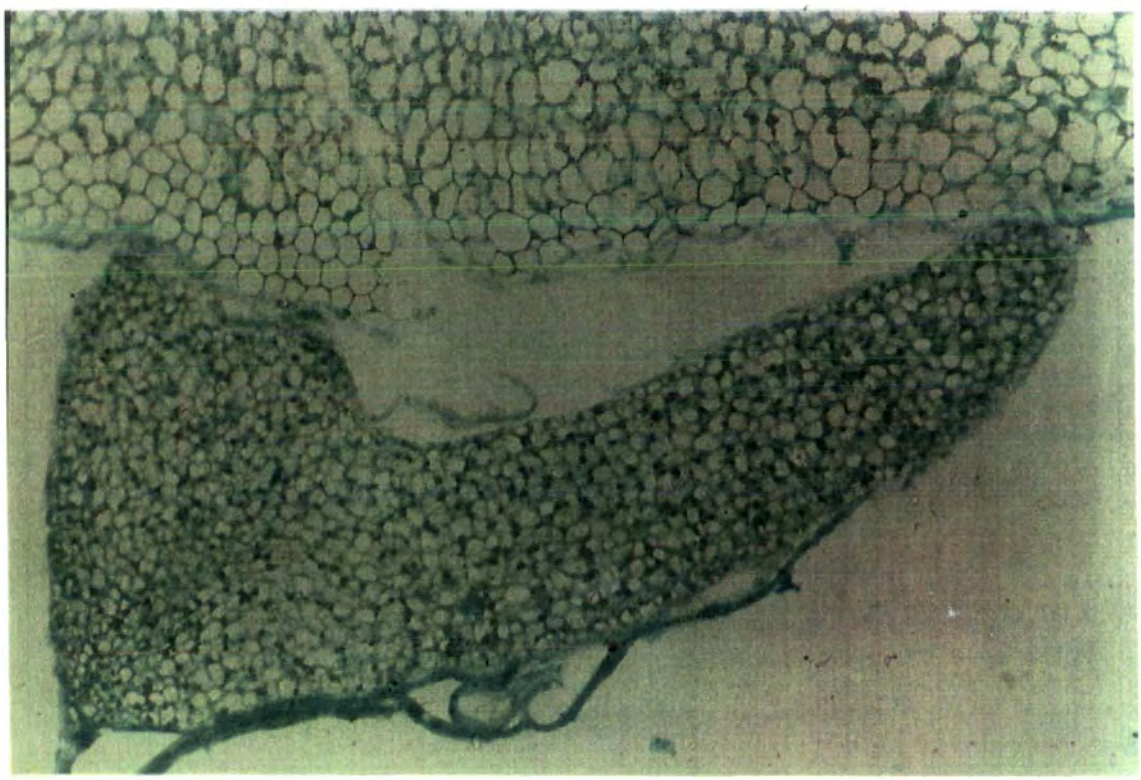
Viewed by Nomarski DIC

FIG 19

Distribution of *leg*A mRNA in ovule at Stage (4) of development. Sections were hybridised with the ^{35}S labelled probe to *leg*A; hybrids were detected via autoradiography.



Viewed using Nikon IGS optics.



Bright field.

Figure 19 shows the result of an *in situ* hybridisation using a ten micron PEG embedded section of stage (4) ovule hybridised to ^{35}S labelled pAD 4.4 cDNA. Hybrids were detected via autoradiography and viewed using Nikon IGS optics. Uniform cytoplasmic distribution of labelling is detected both within the developing cotyledon and central axis of the embryo, however, a lower level of labelling occurs in the very basal portion of the cotyledon. The attached suspensor, which has collapsed and commenced degeneration by this stage of development, displays a level of labelling similar to that of the embryo whilst, in comparison, a lower level of labelling is detected within both the degenerating endosperm cytoplasm and ovular tissue.

The use of *in situ* hybridisation has shown that, during the early stages of *Pisum* seed development, *leg*A expression is not embryo-specific since expression has been shown to occur in the suspensor, endosperm and integument. At early stages of development (Figs. 15A and 18) expression levels detected in the endosperm and suspensor are greater than those detected in the embryo proper. However, as development continues this expression pattern is reversed and by stage (4) of development (Fig. 19) the level of expression detected in the embryo is greater than that detected in the residual endosperm, whilst being of a similar level to that detected in the degenerating suspensor. Expression in the maternal ovular tissue was found to be at a constantly low level throughout development. Therefore, during initial development both temporal and spatial differential expression of the *leg* A gene has been detected within the various components of the seed.

3.3.2 Detection of Legumin Protein by ELISA and Immunocytochemistry

Since differential *leg*A expression had been detected, within the developing seed, at the mRNA level the next stage of research involved ascertaining whether

the gene was expressed at the protein level also. Using immunoelectrophoresis techniques legumin protein has been detected only in embryos greater than 9 d.a.f. However protein, reacting with both anti-legumin and anti-vicilin antibodies, was detected (though not quantified) in 4 d.a.f. ovules (Gatehouse *et al.*, 1982) and postulated to be as a result of legumin synthesis by the endosperm (Gatehouse *et al.*, 1986). Using an ELISA protocol Domoney *et al.* (1980) were able to detect legumin in *Pisum* embryos of 3 mg fresh weight in the cultivar JI 1068 and 2 mg in the cultivar JI 126, indicating low level legumin gene expression, at the protein level, in young embryos. However, these workers removed the testa from the seed, adsorbed the liquid endosperm onto filter paper and assayed only the embryo, although it is possible that there may have been contamination by the coenocytic endosperm.

Intact ovules and corresponding embryos (when large enough to be dissected and accumulated in sufficient quantities) were assayed for LegA content using a sensitive ELISA protocol. The results obtained are presented in Figure 20. LegA can be detected in embryos as young as 6 d.a.f. when the embryo is at the early heart-shape, i.e. prior to completion of morphogenesis. However, it is possible that legumin would be detected in embryos prior to 6 d.a.f. if sufficient numbers could be harvested and assayed. LegA levels in the embryo increase exponentially from 6-10 d.a.f., indicating an increasing rate of protein synthesis during this period of development. Subsequent increase is linear, indicating a constant rate of synthesis (Gatehouse *et al.*, 1982). Domoney *et al.* (1980) were unable to detect legumin in embryos with a fresh weight of less than 2 mg in the cultivar JI 126, however, they concluded that the ability to detect the onset of protein synthesis was directly related to the sensitivity of the detection technique. The detection of LegA in embryos with a fresh weight of less than 1.7 mg therefore reflects the use of a monoclonal antibody ten-fold more sensitive than that used by the aforementioned

workers. Previous research has correlated the onset of storage protein synthesis with both the cessation of cell division (Bain and Mercer, 1966; Dure, 1975) and endoreduplication to DNA levels of 32C and above (Scharpe and Van Parijs, 1973). From their results Domoney et al. (1980) were unable to conclude whether protein levels detected in early embryos were due to the initiation of legumin gene expression at the onset of embryo formation or the synthesis of protein in a small proportion of embryo cells that had ceased to divide. Corke *et al.* (1987) demonstrated vicilin accumulation in *Pisum sativum* JI 181 embryos of 1 mg fresh weight. Using fluorescence triple staining they were able to detect vicilin in embryo cells with a DNA content of 5C and above, i.e. much earlier than previously reported (Le Gal *et al.*, 1984), however, vicilin was never detected in mitotic cells. Ambrose *et al.* (1987) quantified changes in the cell populations of *Pisum sativum* cotyledons during development. Embryos of 0.3-3 mg weight consisted of a single population of similar sized cells (mean area of 100 μm^2), however, a small number of large cells (maximum area of 1060 μm^2) were also present at this stage of early development. The detection, within early embryos, of a small number of large cells was proposed (Ambrose *et al.*, 1987) to support the suggestion by Domoney *et al.* (1980) that small amounts of protein occurring in early embryos was possibly due to cessation of division of a small number of embryo cells. It is probable, therefore, that the detection of LegA in early embryos is due to cessation of division of a small number of cells. However, the results of Domoney *et al.* (1980) and Corke *et al.* (1987), in conjunction with the results presented in Figure 20, indicate that the relationship between the onset of storage protein synthesis and cessation of cell division and endoreduplication is not as clear as previously reported (op. cit.).

No previous work has analysed legumin levels in both ovules and corresponding embryos. The quantitative results of ELISA (Fig. 20) show that up

until 10 d.a.f. LegA levels in the intact ovule are greater than those in the excised corresponding age embryo, indicating that tissue(s), other than the embryo, are synthesising LegA. During early development LegA levels in intact ovules increase and peak at 7 d.a.f. After 7 d.a.f. LegA levels in the ovule fall, followed by an increase at 9 d.a.f. when the embryo becomes the major LegA synthesising component of the seed. An unexpected result obtained from this assay was the detection of legumin within pre-fertilised ovules. The detection, within very early embryos (Figs. 15B, 16 and 17) of low level *leg*A expression at the mRNA level resulted in the hypothesis that the *leg*A gene may be "switched on" by the physiological process of fertilisation. However, the detection of LegA in pre-fertilised ovules invalidates this.

Immunocytochemistry was used to localise legumin in tissue sections of young ovules (Fig. 21). Ten micron, immuno-fixed, PEG embedded sections of a 6 d.a.f. (stage (3)) ovule were incubated with affinity purified anti-legumin antisera. Bound polyclonal antibody was detected using GAR gold and silver enhancement and viewed using Nikon IGS optics. The endosperm cytoplasm exhibits a very high level of labelling (Figs. 21A and 21C) whilst lower level labelling of suspensor (Fig. 21C), embryo and integument occurs (Fig. 21A). Within the embryo label is associated only with the cytoplasm; vacuoles and nuclei being unlabelled. Within the maternal ovular tissue a lower level of labelling, compared to the embryo, can be detected, again vacuoles are unlabelled. Control sections, incubated with pre-immune serum demonstrated a very low level of non-specific background labelling (Figs. 21B and 21D). With regard to the embryo, endosperm and integument, protein levels correlate with the spatial differential *leg*A mRNA distribution localised via *in situ* hybridisation, i.e.high levels in the endosperm cytoplasm, low levels in the embryo and lower levels in the integument. The correlation between protein and mRNA levels indicates that translation rates of

FIG 20

TOTAL LEGA CONTENT

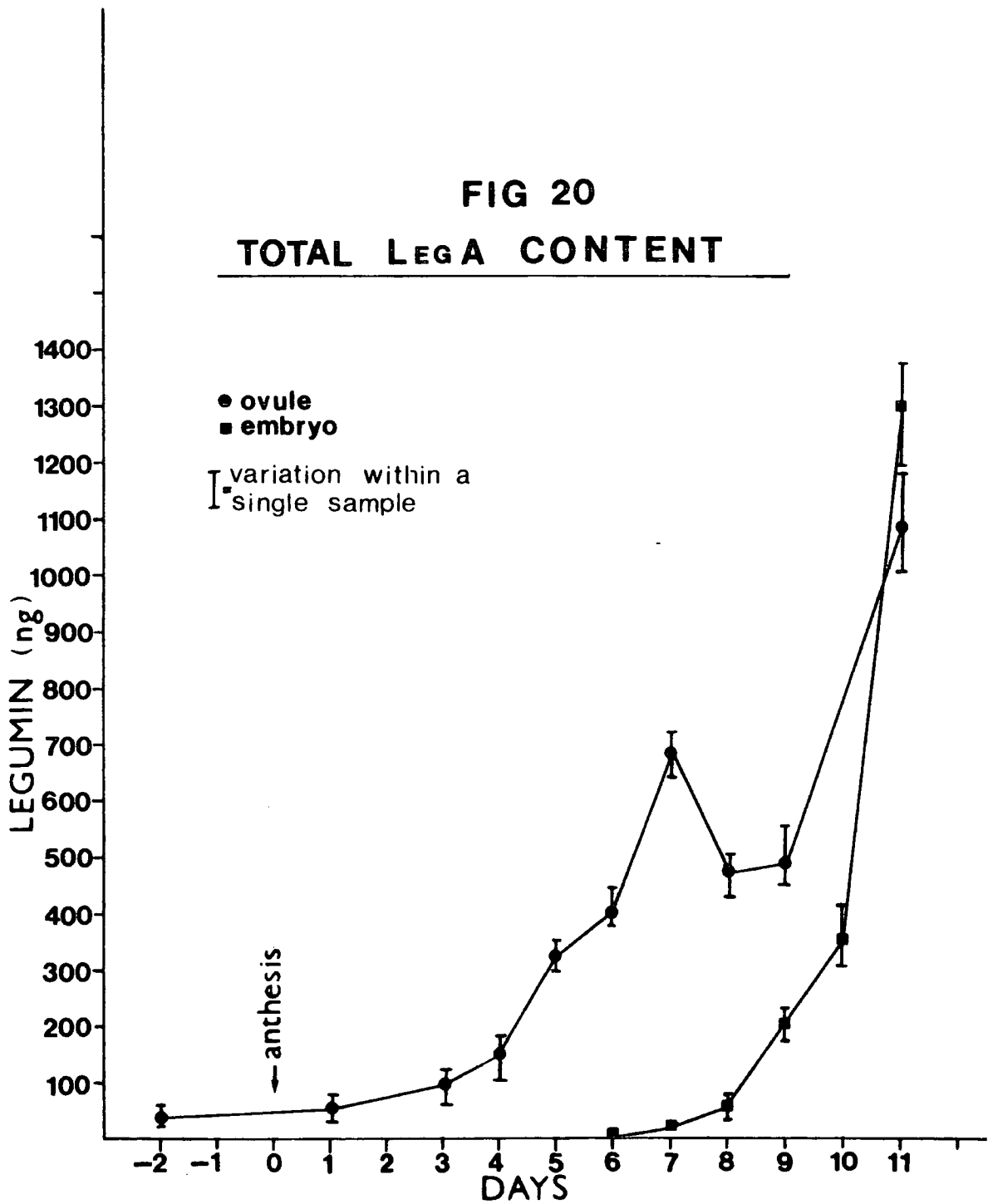
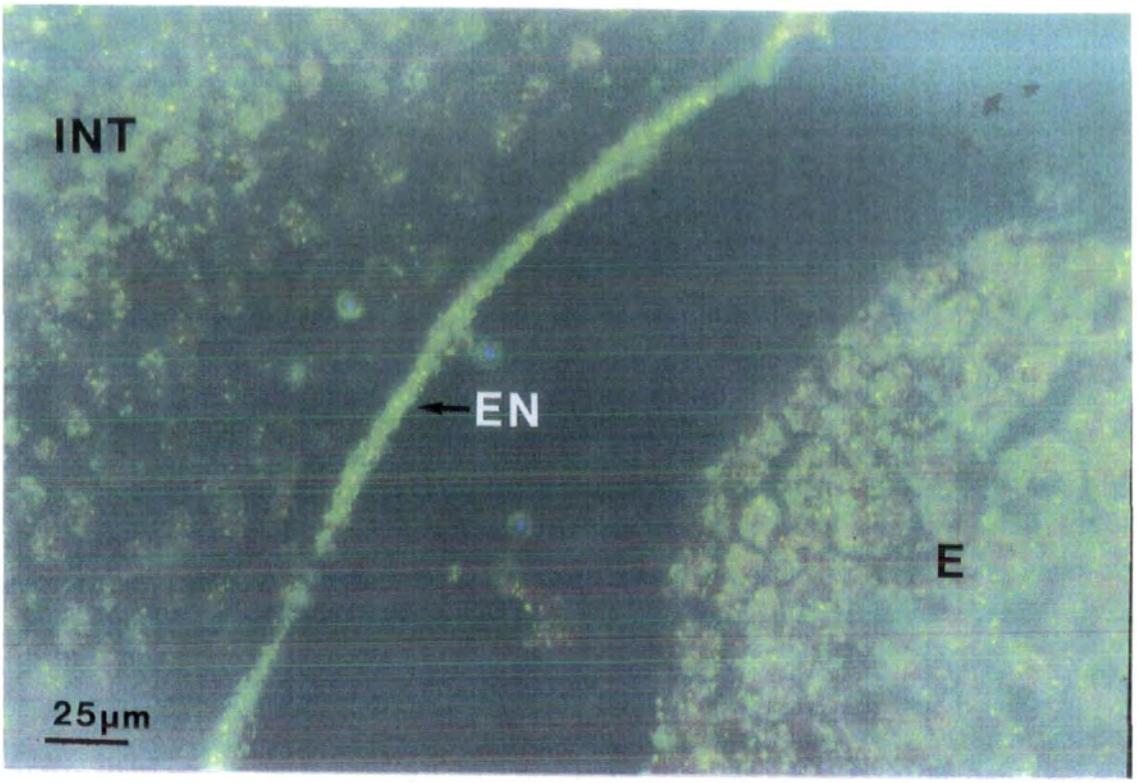


FIG 21

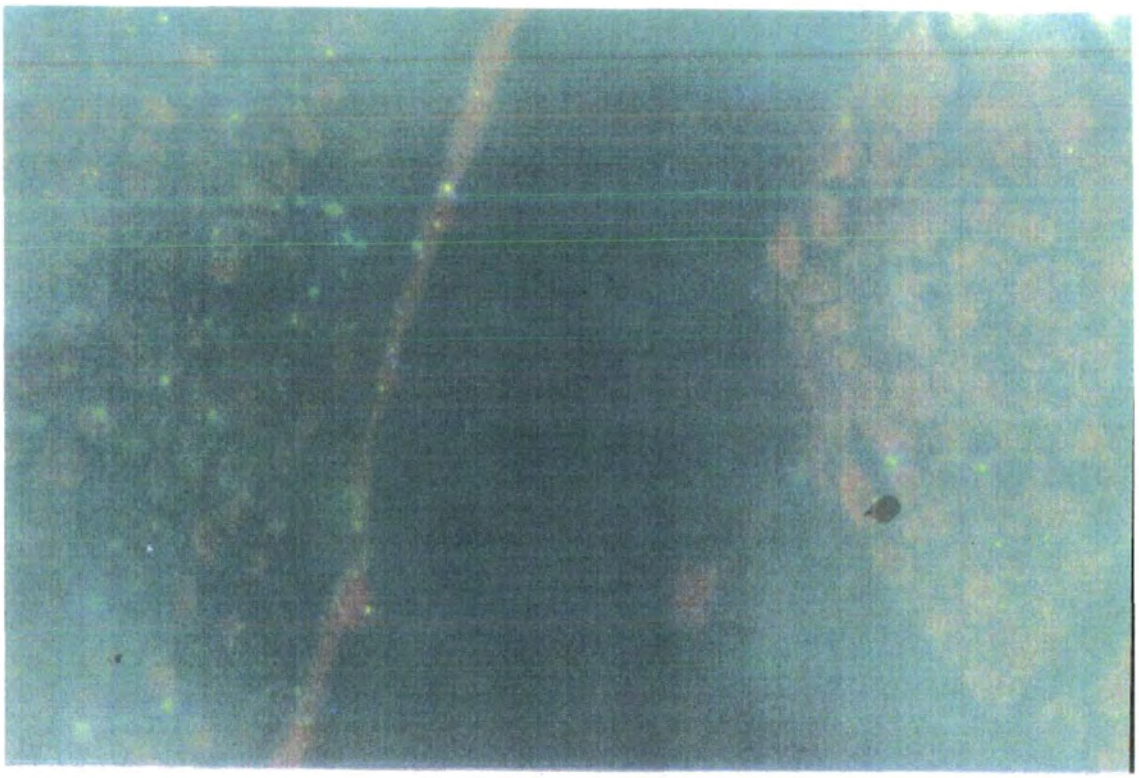
Distribution of legumin protein in ovule at Stage (3) of development.

A



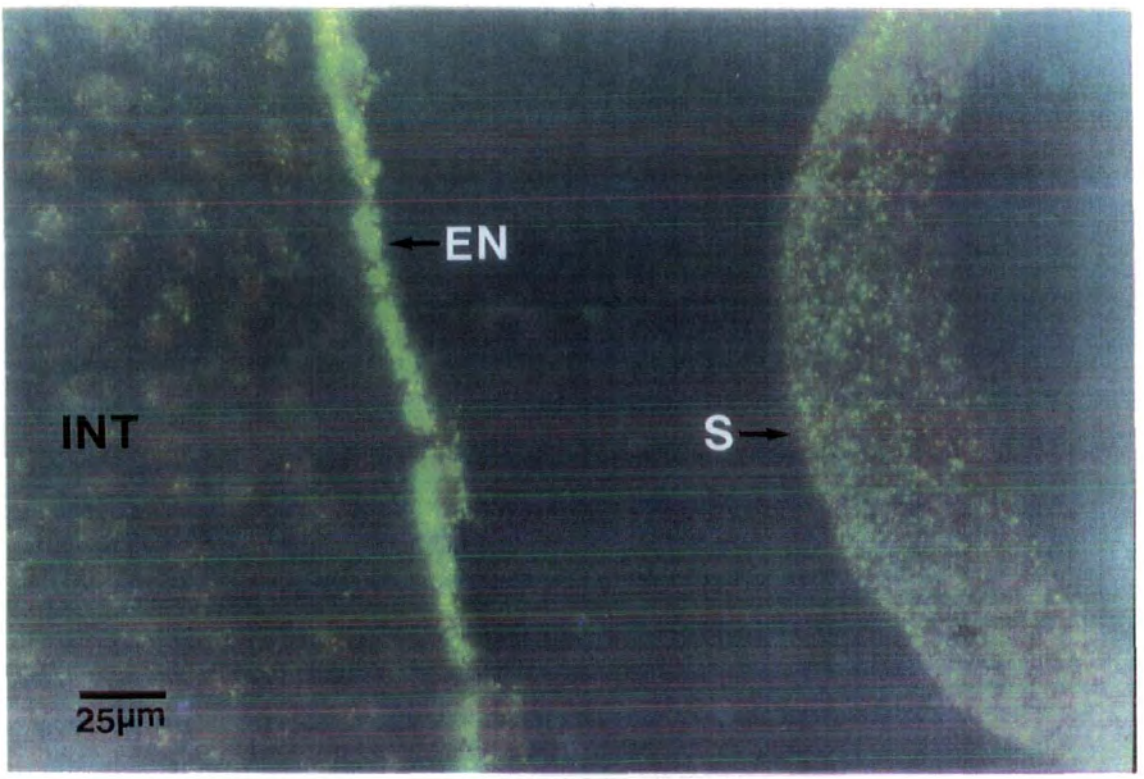
Experimental. Viewed using Nikon IGS optics.

B



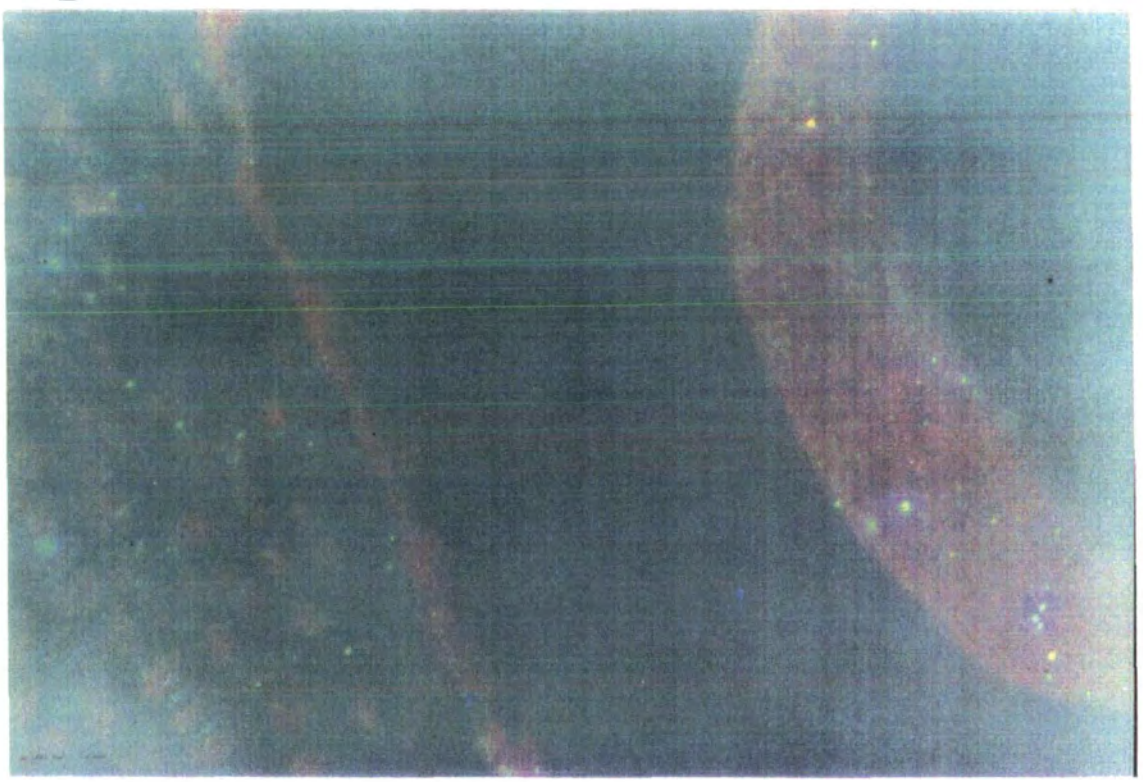
Control. Viewed using Nikon IGS optics.

C



Experimental. Viewed using Nikon IGS optics.

D



Control. Viewed using Nikon IGS optics.

*leg*A mRNA in the three seed components may be similar. An anomaly to this pattern concerns the suspensor which has been shown (Fig. 18) to contain a high level of *leg*A mRNA but a low level of protein (Fig. 21C). Two theories might explain this discrepancy:- (i) translation of *leg*A mRNA may occur at low levels resulting, therefore, in little protein accumulation; (ii) translation may occur at a similar rate as in the other seed components, however protein turnover, i.e. hydrolysis, may occur at relatively high levels resulting in only a low level of accumulation. As discussed (Chapter 1.5.1) legumin has evolved to provide a concentrated store of nitrogen for the germinating seedling, it is not inconceivable therefore that the same protein is utilised, during early seed development, to provide nutrition for the developing embryo. It is hypothesised that transient expression in the endosperm and suspensor is of nutritional significance. The suspensor is attributed a nutrient-transport role (Chapter 1.2.3). Legumin may be synthesised in the suspensor but then rapidly hydrolysed and the products of hydrolysis transported to the embryo proper via the embryo:suspensor plasmodesmata link. Both mature legumin and its constituent subunits (Chapter 1.5.2.1) are of a greater molecular weight than the size limit of the plasmodesmata [approx. 700-800 daltons (Tucker, 1982)]. If protein transfer occurs then hydrolysis must precede transport. Conversely the endosperm is regarded as providing a secondary source of nutrition for the developing embryo (Chapter 1.2.3) and hence it is possible that LegA accumulates in this tissue prior to mobilisation at a slightly later stage of embryogenesis.

From the results of immunocytochemistry it is possible to consider the quantitative data obtained from ELISA experiments (Fig. 20) in the following terms:- the increase in LegA levels in intact ovules from 2-6 d.a.f. may be a result of a steady increase in *leg*A gene expression, mainly by the endosperm, with a low level integument contribution. The protein status of the suspensor is unclear. The

detection of high legumin levels in the endosperm, by immunocytochemistry, indicates that the peak in protein levels at 7 d.a.f. is mainly as a result of transient high expression in the endosperm, though at this stage there is a small embryo contribution. The rapid decrease in legum protein levels in the ovule between 7 d.a.f. and 9 d.a.f. indicates that rapid hydrolysis of legumin, for utilisation by the developing embryo, may be occurring.

The localisation of legumin in the transient endosperm of *Pisum* correlates with the results of immunolocalisation of *Pisum* legumin in seeds of *Nicotiana plumbaginifolia* plants transformed with *leg*A under its own promoter (Ellis *et al.*, 1988). At the torpedo stage of *Nicotiana* development *Pisum* legumin was localised in the endosperm though not in the embryo proper. In *Nicotiana* the endosperm persists throughout development in a cellularised form. Immunolocalisation, at later stages of development, demonstrated the presence of *Pisum* legumin in both the embryo proper and the cellular endosperm (Abbott, 1988; Kang *et al.*, 1988). These results, in conjunction with legumin localisation in the transient endosperm of *Pisum* (Fig. 21A and 21C), indicate that the upstream *cis* region of *leg*A encodes information directing expression in the endosperm. In *Pisum*, where the endosperm cytoplasm exists only during the early phase of seed development, *leg*A expression is transient, however, when transformed into *Nicotiana* the upstream element continues to direct gene expression in the persisting endosperm. These results, demonstrating *leg*A expression at the protein level in both the transient endosperm of *Pisum* and the persistent endosperm of transformed *Nicotiana* seeds, contrast with the results of Barker *et al.* (1988). These workers examined, by *in situ* hybridisation, the temporal and spatial expression of the soybean storage protein gene, B-conglycinin, in seeds of transformed *Nicotiana*. The storage protein gene was expressed, at the mRNA level, only within specific cell types of the embryo and was not detected within the endosperm. They concluded that β-conglycinin

mRNA was either absent from the persistent endosperm of *Nicotiana* or was present at levels below the detection limit of the *in situ* hybridisation protocol. The spatial differential expression of the storage protein gene of *Phaseolus vulgaris*, B-phaseolin, in seeds of transformed tobacco is unclear. Both Sengupta-Golan *et al.* (1985) and Greenwood and Chrispeels (1985) reported the presence of low levels of phaseolin protein in transformed tobacco endosperm. However, Sengupta-Golan *et al.* (1985) concluded that the detection of phaseolin within the endosperm, by immunoblot techniques, was as a result of contamination of the endosperm by embryo proteins during dissection of the seeds. Greenwood and Chrispeels (1985) tentatively concluded that expression of the phaseolin gene in transformed tobacco was not restricted to embryonic tissue, though they pointed out that it was not known whether the phaseolin gene was expressed in the transient endosperm of *Phaseolus vulgaris.* Both conglycinin and phaseolin are 7S seed storage proteins, however, legumin is the 11S storage protein of *Pisum.* It is possible that the two main globulin storage proteins of the Leguminosae (11S and 7S) are under separate, distinct, control mechanisms.

It is proposed therefore, that upstream elements of *leg*A encode information directing temporal and spatial expression both within the embryo proper, within the endosperm and, from the results presented here, possibly within the suspensor and integument also. Goldberg *et al.* (1989) examined the spatial differential pattern of seed storage protein gene expression in a variety of legumes, e.g. soybean, pea, common bean. They concluded that, although qualitative and quantitative differences exist in the expression patterns of individual seed protein genes, the general pattern of expression was similar and that therefore "seed protein genes are regulated, in part, by common factors". However, the embryo specific expression pattern of the β-conglycinin subunits of soybean in transformed tobacco seeds (Barker *et al.*, 1989) contrasts with the expression pattern

of legumin in *Pisum*, reported here, and of the storage protein phaseolin (Greenwood and Chrispeels, 1985). Although the existence of a common mechanism underlying differential storage protein gene expression in the Leguminosae cannot be excluded, the results presented indicate that control mechanisms specific to each species must also operate.

Legumin gene expression at low levels, at both the mRNA and protein level, has been detected in the integument (Figs. 18 and 21A), however the nutritive role of the integument is unclear. Analysis of ovular tissue (Marinos, 1970a; this thesis, Section 3.1) revealed a structural organisation postulated by Marinos (1970a) to be involved in nutrient transport from the ovular tissue into the embryo sac vacuole; a proposal based solely upon morphological observations. Marinos (1970a) and Bakhtiar (1983) reported the absence of plasmodesmata in the embryo sac boundary wall and hence any nutrient movement must be a via trans-membrane uptake from the integument apoplast to the endosperm symplast. The endosperm in *Pisum* exists in two phases; the peripheral layer of coenocytic cytoplasm and a liquid portion that fills the remainder of the embryo sac. Extracts of liquid endosperm, obtained by carefully drawing the solution out of the embryo sac of 6 d.a.f. ovules using a fine hypodermic needle, were assayed for LegA via ELISA. Results showed that each microlitre of liquid endosperm contained 18 ng of LegA. Both untreated liquid endosperm and liquid endosperm total RNA were assayed for *leg*A mRNA using a dot blot assay. The results (not presented) showed the absence of *leg*A mRNA from the liquid phase. LegA detected in the liquid endosperm could therefore be derived from either; (i) transport from the ovular tissue into the embryo sac vacuole; or (ii) "leakage" from the endosperm cytoplasm since ultrastructural examination has revealed the complete absence of cell walls or cell wall formation in the endosperm cytoplasm of 6 d.a.f. ovules (Bakhtiar, 1983). It

is not possible, from present results, to determine the source of liquid endosperm LegA.

The ELISA protocol demonstrated the presence of LegA in pre-fertilised ovules. *Pisum* is self-fertilising. Fertilisation occurs early and by the time the flower is fully open the zygote is either in the process of division or a two-celled embryo is present (Cooper, 1938). Ovules were therefore dissected from ovaries two days prior to flower opening and prior to anther dehiscence (Fig. 4B). Immunocytochemistry was performed using ultrathin sections of immuno-fixed, L.R. White resin-embedded pre-fertilised ovules. However, no significant level of labelling, above background, was detected in any component of the ovule using either the monoclonal or polyclonal anti-legumin antibody. It is possible that the legumin content of the pre-fertilised ovule is below the detection limit of the immunocytological procedure.

3.4 CORRELATED *IN SITU* HYBRIDISATION AND IMMUNOCYTOCHEMISTRY

Correlated *in situ* hybridisation and immunocytochemistry was used to examine the molecular mechanisms underlying differential *leg*A expression. The histochemical localisation of *leg*A mRNA in a 20 d.a.f. pea embryo, known to synthesise large amounts of legumin message and protein (Gatehouse *et al.*, 1982; Boulter *et al.*, 1987) is demonstrated in Figure 22. Sections were hybridised with biotinylated pAD 4.4 cDNA and hybrids were detected via goat-anti biotin: RAG gold and silver enhancement.

A very high level of labelling occurs in the cotyledon storage parenchyma cells, whilst labelling is absent from the cotyledonary vascular tissue and epidermal cells. Within the radicle a lower level of labelling is detected. The cortical parenchyma cells of the radicle are labelled, though to a lesser level than cotyledon

parenchyma cells, whilst meristematic cells at the radicle tip and files of cells, produced by the tip, are unlabelled, i.e. exhibit little or no expression of the *leg*A gene.

Immunolocalisation of legumin in cotyledons of 20 d.a.f. embryo (Figs. 23A and 23B) demonstrates that within cotyledon parenchyma cells a high level of labelling occurs, whilst cells of the vascular bundle and epidermis are unlabelled. Legumin distribution in the radicle (Fig. 24) correlates with the spatial distribution of *leg*A mRNA.

These results demonstrate, firstly, the high degree of spatial differential expression of the *leg*A gene within the embryo of *Pisum* during the mid-maturation stage of development. Differential expression occurs at the organ level; labelling levels for both mRNA and protein being much lower in the radicle compared to the cotyledon parenchyma cells. A similar pattern of differential expression, at the organ level, has been reported for the storage proteins β-conglycinin (Meinke, 1981) and lectin (Walling *et al.*, 1986) in soybean. There is also a high degree of differential expression at the cellular level, since within the cotyledons certain cell types (vascular elements and epidermal) do not accumulate protein. Similarly within the radicle a gradation in the level of expression at the mRNA and protein level is evident from the base to the tip. The high degree of differential expression within specific embryonic cell types implies the possible involvement of cellular factors, e.g. tissue/cell specific DNA binding proteins, in the regulation of *leg*A expression, as has been discussed previously (Chapter 1.3; 1.4.2.1).

Secondly, the co-localisation of *leg*A mRNA and protein within certain cell types demonstrates that cell types that are the site of mRNA synthesis are also the site of peptide synthesis and protein deposition, i.e. little or no intercellular transport of *leg*A message or protein occurs.

Histochemical localisation of *leg*A mRNA in the cotyledon and radicle of 20 d.a.f. embryo. The section was hybridised with the photobiotinylated probe to *leg*A and hybrids were deteced via goat-anti-biotin, RAG gold and silver enhancement.

FIG 22

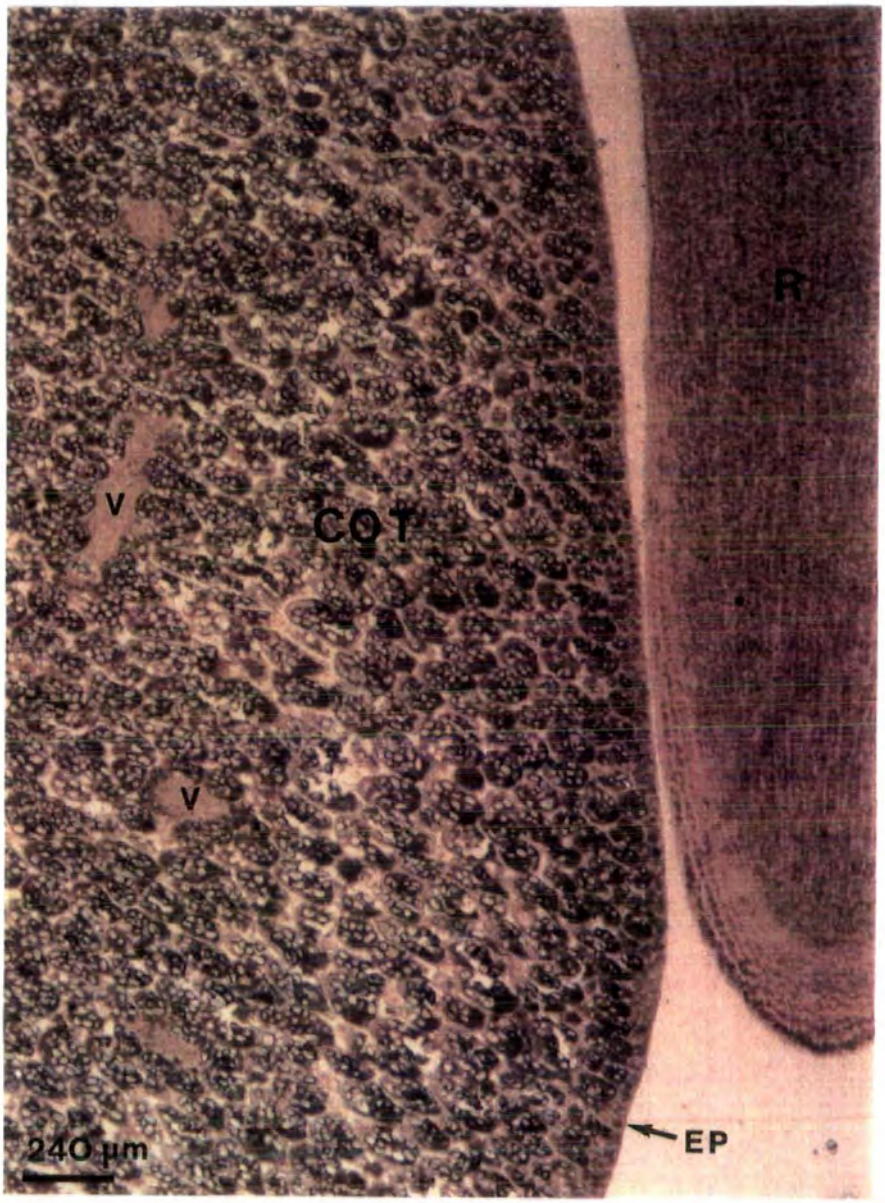
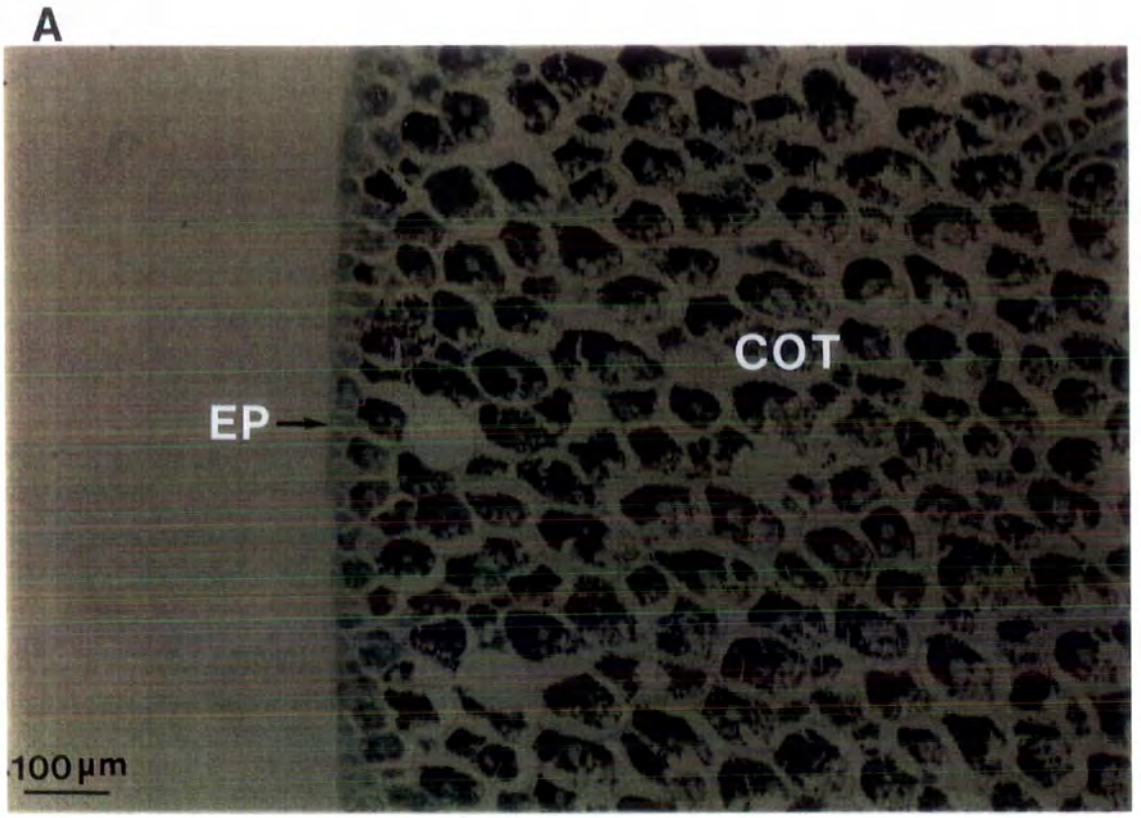


FIG 23

Immunocytochemical localisation of legumin in the cotyledon of 20 d.a.f. embryo.



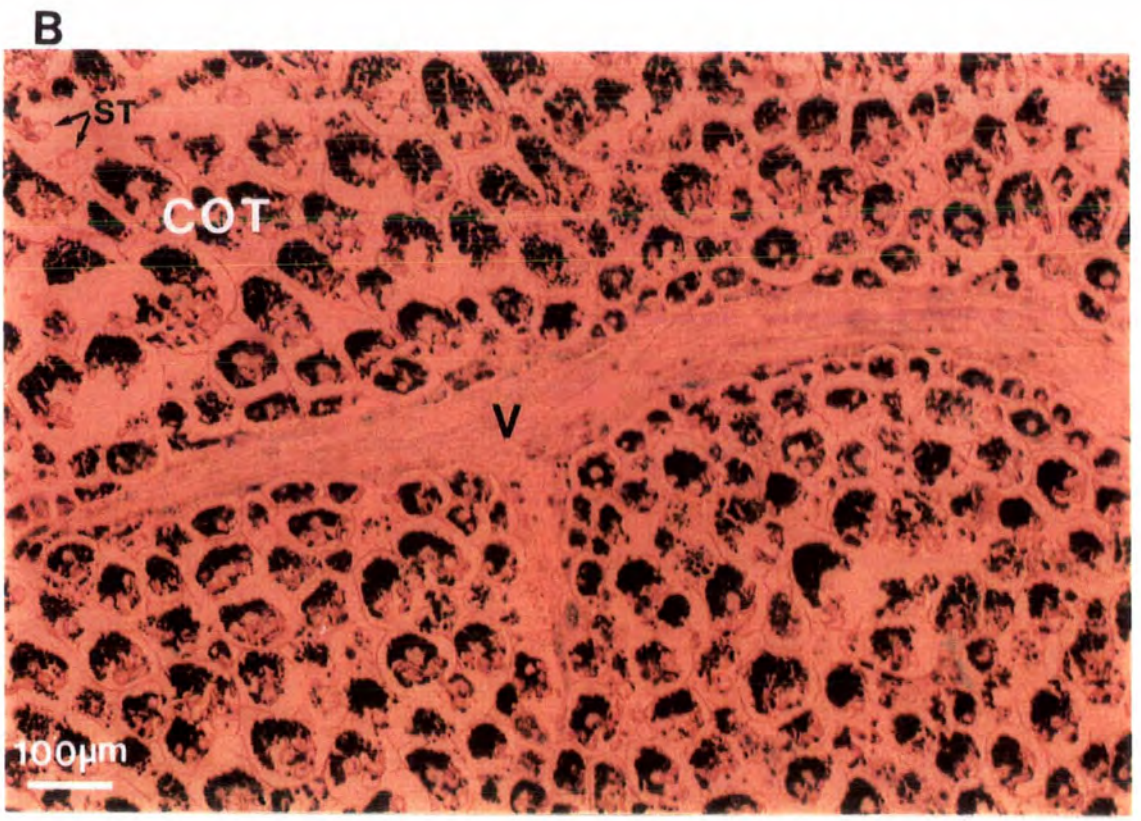
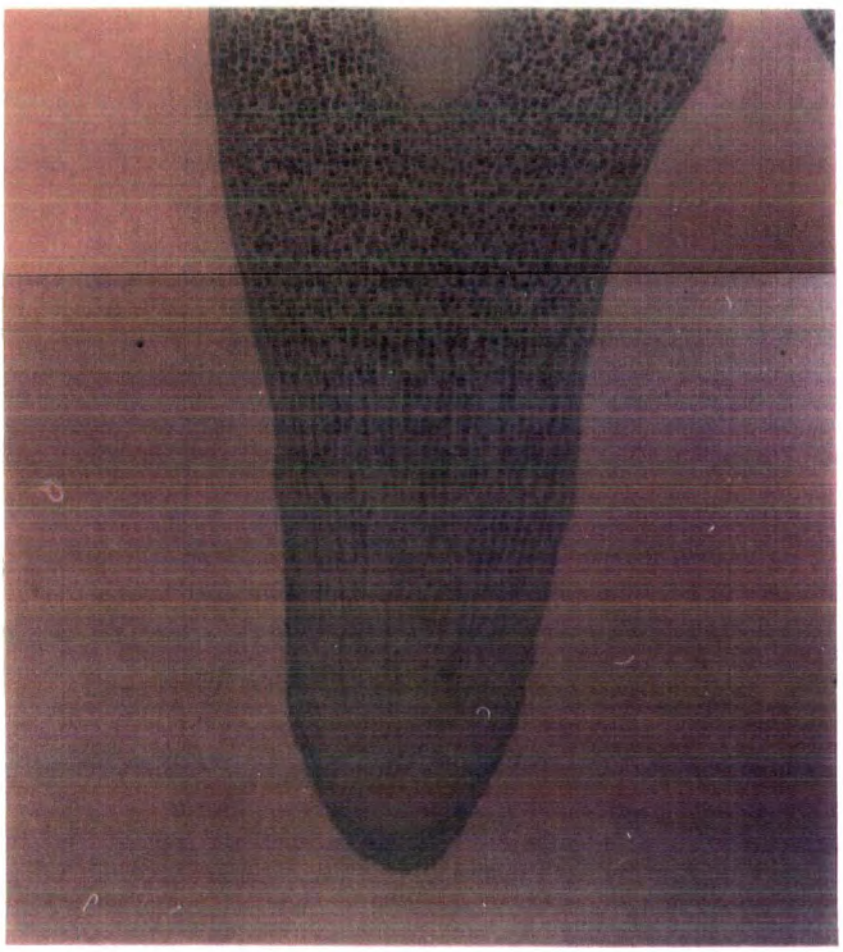


FIG 24

Immunocytochemical localisation of legumin in the radicle of a 20 d.a.f. embryo.



Thirdly, the strong correlation, both in terms of spatial distribution and relative abundance, between mRNA and protein levels within the embryo, indicates that transcription level regulation is an important factor in the control of differential *leg*A expression, although the role of other control processes, e.g. post-transcriptional, translational cannot be excluded. For example, Walling *et al.* (1986) demonstrated, using *in vitro* nuclear RNA synthesis and filter hybridisation protocols, that the seed protein mRNAs of soybean were less abundant in the axis compared to the cotyledons, however transcription rates in the axis were not reduced proportionally. They concluded that post-transcriptional processes were important in the establishment of differential seed protein mRNA levels.

3.5 ONTOGENY OF EMBRYONIC AND MATURE ROOT

The ontogeny of the embryonic root (radicle) was examined by the application of a probe (p^{PR} 179) complementary to a mRNA species known to be present at elevated levels in *Pisum* root (Evans *et al.*, 1988). However, *in situ* hybridisation results revealed that the p^{PR} 179 cDNA fragment failed to hybridise with radicle tissue, even when washing stringencies were reduced (to 1 x SSC at 37°C). Therefore the root probe was also of no use as a "marker" of radicle formation.

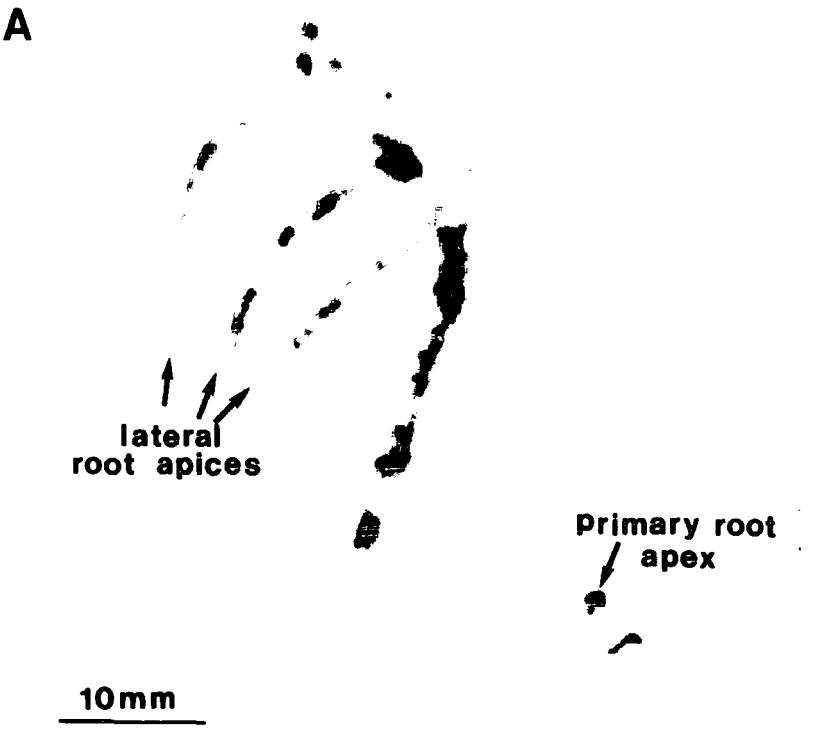
Tissue blot analysis (Chapter 2.2.23) was used to demonstrate the abundance and spatial distribution of the mRNA species complementary to the p^{PR} 179 cDNA, in the mature root system of *Pisum* (Fig. 25A). The blot was hybridised to excess ^{32}P labelled pPR 179 cDNA. The final wash was with 1 x SSC; 0.1% w/v SDS at 60°C, i.e. x 86% homology.

Figure 26C demonstrates the morphology of the pea root system. Along the longitudinal axis of the root successive overlapping zones can be distinguished, as described previously (Esau, 1977). The root cap is followed by the apical meristem,

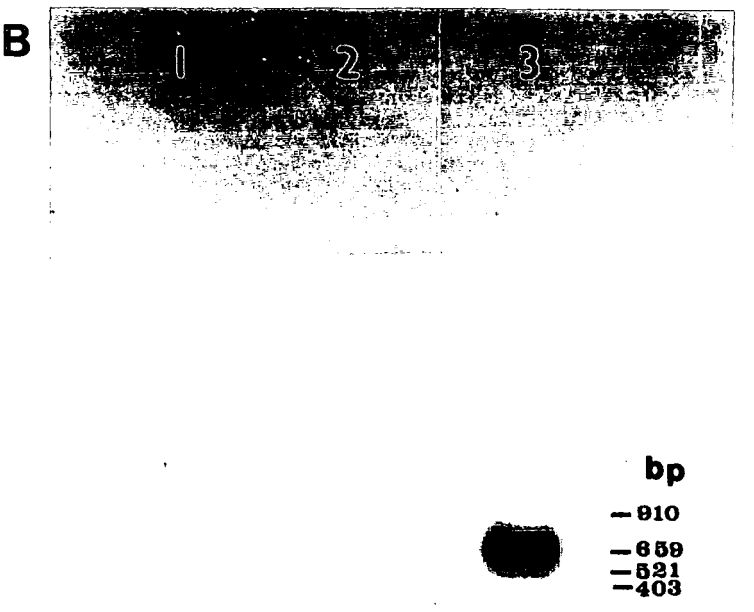
a zone of high mitotic activity where the majority of root cells are produced (Feldman, 1984). This is succeeded by a zone of elongation and finally a zone of differentiation in which vascular elements are present (Torrey, 1955). Comparison of morphological observations (Fig. 26C) with the tissue blot (Fig. 25A) shows that high hybridisation signals are associated mainly with the outer cortical parenchyma regions of the root system whilst hybridisation signal is absent from the root apices (the exception being the primary root apex) and from the central cylinder of the root axis. From these results it can be concluded that the mRNA complementary to the p^{PR} 179 cDNA is expressed at high levels in the zone of differentiation, whilst being absent from the mitotic cells of the root apex, and vascular elements of the central cylinder. It is not possible to ascertain whether the message is expressed in the zone of elongation, since the spatial resolution achieved using this technique is limited, however it is present, at high levels, in the zone of differentiation. The mRNA complementary to the p^{PR} 179 cDNA may either be involved in the process of cellular differentiation or may be a product of the differentiated state. The embryonic root exhibits limited histogenesis, consisting of files of equally-sized cells that radiate from the tip of the apex (Fig. 26A). The absence of a mRNA species, associated with differentiated root cell types, from the undifferentiated cells of the radicle is therefore not surprising.

Northern blot analysis was performed, as shown in Figure 25B. Ten micrograms of total RNA isolated from, (i) mature root (2 week post germination); (ii) germinating root (4 days post seed imbibition); and (iii) radicle (16 d.a.f. embryo) were electrophoresed using a formaldehyde agarose gel, blotted onto nitrocellulose and hybridised to excess ^{32}P labelled p^{PR} 179 cDNA (specific activity 1.5 x 10^9 cpm/μg). The filter was washed to approximately 100% homology with 0.1 x SSC; 0.1% w/v S.D.S. at 65°C (Evans *et al.*, 1988). The results show that the "root mRNA" is absent from the embryonic root (as demonstrated previously by *in*

FIG 25



Macroscopic localisation of pPR 179 in *Pisum* root.

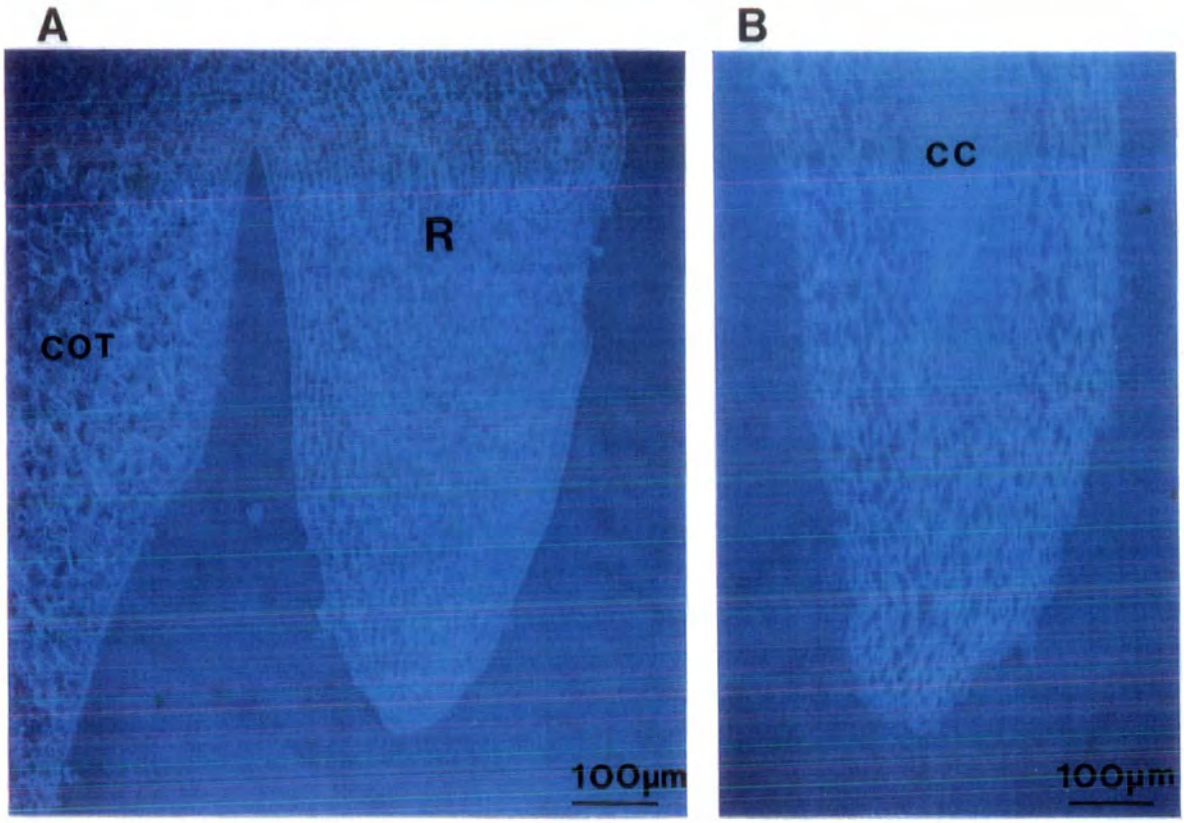


Northern blot analysis of total RNA (10 ug/track) isolated from (1) radicle (16 d.a.f.); (2) root (4 days post seed imbibition; (3) mature root (2 weeks post germination). The filter was hybridised with an excess of ^{32}P labelled pPR 179 probe and subsequently washed to approximately 100% homology. M_r size marker = pBR 322 digested with Alu 1.

situ hybridisation), whilst low levels are detected in primary roots produced by germinating seeds four days post imbibition. The relative abundance of the mRNA in the differing tissue types was quantified by scanning the autoradiograph with an L.K.B. Ultroscan XL laser densitometer. Analysis showed that the root enhanced mRNA species was 7.2 times more abundant in root tissue compared to germinating root. Since the hybridisation signal of the root tissue had saturated the x-ray film, this value is an under-estimate.

It is possible that the gene encoding the root enhanced mRNA is "switched on" by the physiological process of imbibition and germination. Alternatively the mRNA may be accumulated in the quiescent radicle during seed maturation and desiccation. Other workers have demonstrated the presence of mRNA species associated mainly with the desiccation period of seed formation, i.e. lea (late embryogenesis abundant) mRNAs (Goldberg *et al.*, 1989). Harada *et al* (1988) examined the spatial distribution of post-germination abundant mRNA species in *Brassica napus* seedlings. They demonstrated that most post-germination abundant mRNA species were present in the dry seed and it was concluded that they accumulated during seed maturation. However analysis of root ontogeny during the germination of *Zea mays* (Deltour *et al.*, 1989) resulted in the conclusion that root zonation, as previously described, was as a result of sequential differentiation and not as a result of differentiation of predetermined regions of the radicle. The structure of the embryonic and mature root is such that the radicle must undergo strong ontogenetic structural changes to produce the histological patterns characteristic of the mature root (compare Fig. 26A with Fig. 26C). The root in *Pisum*, four days post imbibition (Fig. 26B), consists of differentiated cell types typical of the mature root. Some of these differentiated cell types have been shown to be expressors of the root enhanced mRNA (Fig. 25A) and it is most

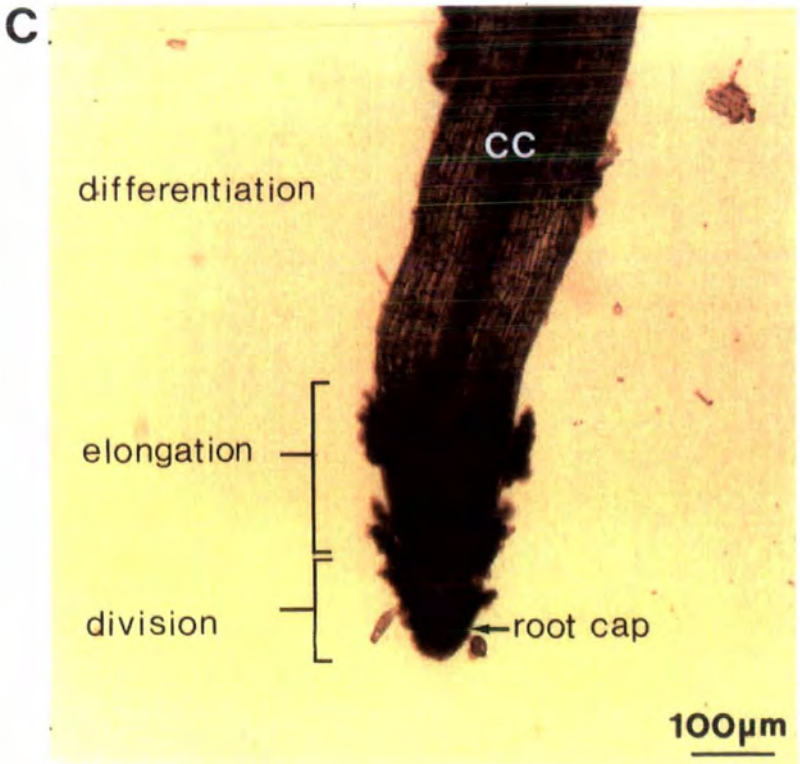
FIG 26



Structure of the radicle of 16 d.a.f. embryo

Structure of the primary root four days post imbibition

Sections were stained with 1% aqueous calcofluor and viewed by epifluorescence optics using a 380 nm excitation wavelength, 420 nm suppression filter and 400 nm dichroic mirror.



Zonation along differentiated lateral root of *Pisum*.

possible that the gene encoding the root mRNA species is "switched on" by the process of germination and subsequent ontogeny of the mature root.

3.6 PUTATIVE CONTROL MECHANISMS UNDERLYING EMBRYOGENESIS IN *PISUM*

The two basic modes of embryogenesis in animal systems have been described in Chapter 1.3.1. According to the mosaic pattern of development cell fate is determined during the very early cleavage stages of embryogenesis, whilst regulative development involves the progressive restriction of a cell's developmental potential as a result of interaction with the external environment. If embryogenesis in *Pisum* was of the mosaic type then *leg*A expression, during the very early stages of embryogenesis, would be expected to be restricted only to specific cell types within the embryo, i.e. the precursors of the cotyledon parenchyma cells, indicating that cell fate had been determined during the early stages of embryogenesis. The results of *in situ* hybridisation demonstrated that until stage (4) of development *leg*A expression was detected in all embryo cells, at a similar level (Figs. 17 and 18). Differential expression of *leg*A was, however, detected in mid-maturation stage embryos (Fig. 22). These results indicate that, during early development, cells within the embryo possess equal potentials with respect to *leg*A expression. This potential appears to be progressively restricted during development, resulting in the differential expression levels detected later in embryogenesis. Therefore, with respect to *leg*A expression, *Pisum* embryogenesis is not highly mosaic. The detection of differential expression only during the later stages of embryogenesis indicates that factors such as cell division, cell position within the embryo etc. (as discussed in Chapter 1.3.2), are important in the control of differential *leg*A expression, i.e. embryogenesis in *Pisum* appears to be regulative.

These conclusions are, however, derived from static images of embryo development and from the developmental expression pattern of a single gene. Only when culture protocols are improved, such that very young embryos can be successfully and routinely cultured *in vitro* will be the processes underlying angiosperm embryogenesis be elucidated via direct experimentation.

3.7 GENERAL CONCLUSIONS

This investigation of the spatial and temporal patterns of expression of the *Pisum* storage protein gene, *leg*A, during seed development has resulted in a number of original and interesting observations.

The identification, by sensitive ELISA protocol, of low levels of LegA accumulation in the pre-fertilised ovule was an unexpected result since previous work has indicated that the expression of the legumin storage protein gene was seed specific. Preliminary immunocytological studies failed to reveal the localisation of legumin within pre-fertilised ovules, however this would be of interest in any subsequent research. The presence of the storage protein in pre-fertilised ovules indicates that the encoding gene is not specifically "switched on", during embryogenesis, by the physiological process of fertilisation.

During early seed development both temporal and spatial differential *leg*A expression has been detected in the embryonic and non-embryonic tissues of the seed. Examination of stages (1)-(3) of ovule development, by *in situ* hybridisation, revealed a high level of *leg*A expression in both the endosperm and suspensor whilst the embryo during this stage of development exhibited only a low level of expression. Legumin protein was localised within the endosperm of 6 d.a.f. ovules, however only a very low level of labelling was associated with the suspensor. Immunolocalisation studies correlated with the quantitative data obtained from the ELISA protocol; the results of which demonstrated a peak in LegA levels in intact

ovules at 7 d.a.f., which is presumed to be primarily as a result of legumin synthesis within the endosperm. The correlation, within the endosperm, of mRNA and protein levels indicates that control of *leg*A expression in this tissue is via transcription level regulation. The detection within the suspensor, however, of only low protein levels indicates that post-transcriptional processes are involved in regulating *leg*A expression in this organ. The expression of *leg*A in both the endosperm and suspensor is proposed to be of nutritional significance for the developing embryo.

During stages (1)-(3) of development a uniform cytoplasmic level of label was detected throughout the developing embryos, indicating that control of *leg*A expression is via an "up/down" transcription regulation mechanism rather than an "on/off" switch. At stage (4) of development *leg*A expression levels in the degenerating endosperm and suspensor were lower than in the embryo proper, which at this stage of development demonstrated a low level of differential expression. The detection of differential levels of *leg*A expression only during the later stages of development implies that embryogenesis in *Pisum* follows a regulative mode of development.

During initial seed development *leg*A expression is therefore greatest in the non-embryonic tissues, however, as development continues this pattern of expression is reversed and the embryo becomes the major expressor of the seed storage protein gene. *Leg*A expression has been demonstrated, via use of combined cytological and molecular techniques to be neither embryo-specific nor seed-specific. Correlated localisation of both seed storage protein gene mRNA and protein during the mid-maturation phase of embryogenesis revealed a high degree of differential expression, compared to younger embryos, since in some cell types, e.g. vascular and epidermal, there was a complete absence, or a very low level of expression. The high degree of correlation between mRNA and protein levels

indicates the importance of transcription level regulation in the control of differential *leg*A expression.

In conclusion, a complex pattern of both spatial and temporal differential *leg*A expression has been detected within early ovules by the use of combined molecular and cytological techniques. The results have been discussed with regard to the control of gene expression, the use of legumin protein as a source of nutrition for the developing embryo and the possible mechanisms underlying embryogenesis in *Pisum*.

4: BIBLIOGRAPHY

Abbott, S.J. (1988). *The Immunocytochemical Localization of Transgenically-Expressed Proteins in Plants.* M.Sc. Thesis, University of Durham.

Alpi, A., Tognoni, F. and D'Amato, F. (1975). Growth regulator levels in embryo and suspensor of *Phaseolus coccineus* at two stages of development. *Planta* 127, 153-162.

Ambrose, M.J., Wang, T.L., Cook, S.K. and Hedley, C.L. (1987). An analysis of seed development in *Pisum sativum.* IV. Cotyledon cell populations *in vivo* and *in vitro. J. Ex. Bot.* 38, 1909-1920.

Angerer, L.M. and Angerer, R.D. (1981). Detection of poly A and RNA in sea urchin eggs and embryos by quantitative *in situ* hybridization. *Nucl. Acids Res.* 9, 2819-2840.

Bachmair, A., Finley, D. and Varshausky, A. (1986). *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* 234, 179-186.

Badennoch-Jones, J., Spencer, D., Higgins, T.J.V. and Millerd, A. (1981). The role of glycosylation in storage-protein synthesis in developing pea seeds. *Planta* 153, 201-209.

Bain, J.M. and Mercer, F.V. (1966). Subcellular organization of the developing cotyledons of *Pisum sativum* L. *Aust. J. Biol. Sci.* 19, 49-67.

Bakhtiar, E. (1983). *Structural Studies of Protein Deposition in Developing Legume Seeds.* Ph.D. Thesis, University of Durham.

Ball, E. (1956). Growth of the embryo of *Ginkgo biloba* under experimental conditions. II. Effects of a longitudinal split in the tip of the hypocotyl. *Amer. Jour. Bot.* 43, 802-810.

Barker, R.D.J., Derbyshire, E., Yarwood, A. and Boulter, D. (1976). Purification and characterization of the major storage proteins of *Phaseolus vulgaris* seeds and their intracellular and cotyledonary distribution. *Phytochemistry* 15, 751-757.

Barker, S.J., Harada, J.J. and Goldberg, R.B. (1988). Cellular localisation of soybean storage protein mRNA in transformed tobacco seeds. *Proc. Natl. Acad. Sci. USA* 85, 458-462.

Barratt, D.H.P., Whitford, P.N., Cook, S.K., Butcher, G. and Wang, T.E. (1989). An analysis of seed development in *Pisum sativum.* VIII. Does abscisic acid prevent precocious germination and control storage protein synthesis? *J. Exp. Bot.* 40, 1009-1014.

Bathurst, T.C., Craig, R.K., Herries, D.G. and Campbell, P.N. (1980). Differential distribution of poly(A) containing RNA sequences between the nucleus and post-nuclear supernatant of the lactating guinea-pig mammary gland. *Eur. J. Biochem.* 109, 183-191.

Baumann, G., Raschke, E., Beven, M. and Schoffl, F. (1987). Functional analysis of sequences required for transcriptional activities of a soybean heat shock gene in transgenic tobacco plants. *EMBO J.* **6**, 1161-1166.

Baumlein, H., Wobus, U., Pustell, J. and Kafatos, F.C. (1986). The legumin gene family: Structure of a type gene of *Vicia faba* and a possible legumin gene specific regulatory element. *Nucl. Acids Res.* **14**, 2707-2720.

Beach, L.R., Spencer, D., Randall, P.J. and Higgins, T.J.V. (1985). Transcriptional and post-transcriptional regulation of storage protein gene expression in sulphur deficient peas. *Nucl. Acids Res.* **13**, 999-1013.

Benoist, C., O'Hare, K., Breathnach, R. and Chambon, P. (1980). The ovalbumin gene-sequence of putative control regions. *Nucleic Acids Res.* **8**, 127-145.

Bentley, D.L. and Groudine, M. (1987). A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. *Nature* **321**, 702-706.

Bhatnagar, S.P. and Sawhney, V. (1981). Endosperm - Its morphology, ultrastructure and histochemistry. *Intern. Rev. of Cytol.* **73**, 55-102.

Blobel, G., Walter, P., Chang, G.N., Goldman, B.M., Erickson, A.H. and Lingappa, V.R. (1979). Translocation of proteins across membranes: the signal hypothesis and beyond. *Symp. Soc. Exp. Biol.* **33**, 9-36.

Blum, H.E., Stowringer, L. and Figus, A. (1983). Detection of hepatitis B virus DNA in hepatocytes, bile duct epithelium and vascular elements by *in situ* hybridisation. *Proc. Nat. Acad. Sci. USA* **80**, 6685-6688.

Bodner, M. and Karin, M. (1987). A pituitary-specific trans-acting factor can stimulate transcription from the growth hormone promotor in extracts of non-expressing cells. *Cell* **50**, 267-275.

Bonnard, C., Papermaster, D.S. and Kraehenbuhl, J.P. (1984). The streptavidin-biotin bridge technique: application in light and electron microscope immmunocytochemistry. In: *Immunolabelling for Electron Microscopy*, ed. J.M. Polak and I.M. Varndell. Elsevier, Amsterdam, pp. 95-111.

Boulter, D. (1983). Protein composition of grains of the Leguminosae. *Qual. Plant. Plant Foods Hum. Nutr.* **32**, 247-252.

Boulter, D., Evans, I.M., Ellis, J.R., Shirsat, A., Gatehouse, J.A. and Croy, R.D. (1987). Differential gene expression in the development of *Pisum sativum*. *Plt. Physiol. Biochem.* **25**, 283-289.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72**, 248-254.

Brahic, M. and Haase, T. (1978). Detection of viral sequences of low reiteration frequency by *in situ* hybridisation. *Proc. Nat. Acad. Sci. USA* **75**, 6125-6129.

Brandhorst, B.P. (1976). Two-dimensional gel patterns of protein synthesis before and after fertilization of sea urchin eggs. *Dev. Biol.* **52**, 310-317.

Brawerman, G. (1987). Determinants of messenger RNA stability. *Cell* **48**, 5-6.

Breitbart, R.E., Andreadis, A. and Nadal-Ginard, B. (1987). Alternative splicing: A ubiquitous mechanism for the generation of multiple protein isoforms from single genes. *Ann. Rev. Biochem.* **56**, 467-495.

Breitbart, R.E. and Nadal-Ginard, B. (1987). Developmentally induced muscle specific factors control the differential splicing of alternative and constitutive troponin T exons. *Cell* **49**, 793-803.

Bresser, J. and Evinger-Hodges, M.J. (1987). Comparison and optimization of *in situ* hybridization procedures yielding rapid, sensitive mRNA detections. *Gene Anal. Tehn.* **4**, 89-104.

Britten, R.J. and Davidson, E.H. (1976). Studies on nucleic acid reassociation kinetics: Empirical equations describing DNA reassociation. *Proc. Nat. Acad. Sci. U.S.A.* **73**, 415-419.

Brown, J.W.S., Feix, G. and Frendewey, D. (1986). Accurate *in vitro* splicing of two pre-mRNA plant introns in a HeLa cell nuclear extract. *EMBO J.* **5**, 2749-2758.

Bryant, S.V., French, V. and Bryant, P.J. (1981). Distal regeneration and symmetry. *Science* **212**, 993-1002.

Cassab, G.I. and Varner, J.E. (1989). Tissue printing on nitrocellulose paper: A new method for immunolocalization of proteins, localization of enzyme activities and anatomical analysis. *Cell Biol. Int. Reports* **13**, 147-152.

Cesarman, E., Dalla-Favera, R., Bentley, D. and Groudine, M. (1987). Mutations in the first exon are associated with altered transcription in c-myc in Burkitt lymphoma. *Science* **238**, 1272-1275.

Chandler, P.M., Higgins, T.J.V., Randall, P.J. and Spencer, D. (1983). Regulation of legumin levels in developing pea seeds under conditions of sulphur deficiency. *Plant Physiol.* **71**, 47-54.

Chappell, J. and Chrispeels, M.J. (1986). Transcriptional and post-transcriptional control of phaseolin and phytohemagglutinin gene expression in developing cotyledons of *Phaseolus vulgaris*. *Plt. Physiol.* **81**, 50-54.

Chrispeels, M.J. (1985). The role of the golgi apparatus in the transport and post-translational modification of the vacuolar (protein body) proteins. *Oxford Surv. Plant Mol. Cell. Biol.* **2**, 43-68.

Chrispeels, M.J. and Sadeva, D. (1977). *Plants, Food and People.* W.H. Freeman & Co., San Francisco.

Chrispeels, M.J., Higgins, T.J.V., Craig, S. and Spencer, D. (1982). Role of the endoplasmic reticulum in the synthesis of reserve proteins and the kinetics of their transport to protein bodies in developing pea cotyledons. *J. Cell Biol.* **93**, 5-14.

Clark, B. (1983). The elongation step of protein biosynthesis. In: *DNA Makes RNA Makes Protein*, ed. T. Hunt, S. Prentis, and J. Tooze. Elsevier biomedical Press, pp. 213-219.

Cooper, D.C. (1938). Embryology of *Pisum sativum. Bot. Gaz.* **100**, 123-132.

Corke, F.M.K., Hedley, C.L., Shaw, P.J. and Wang, T.L. (1987). An analysis of seed development in *Pisum sativum.* V. Fluorescence triple staining for investigating cotyledon cell development. *Protoplasma* **140**, 164-172.

Cox, K.H., Deleon, D.V., Angerer,L.M. and Angerer, R.D. (1984). Detection of mRNA's in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Dev. biol.* **101**, 485-502.

Cox, K.H. and Goldberg, R.B. (1988). Analysis of plant gene expression. In: *Plant Molecular Biology. A Practical Approach*, ed. C.H. Shaw. IRL Press Ltd.

Craig, S., Goodchild, D.J. and Hardham, R.A. (1979). Structural aspects of protein accumulation in developing pea cotyledons. I. Qualitative and quantitative changes in parenchyma cell vacuoles. *Aust. J. Plant Physiol.* **6**, 81-89.

Craig, S., Goodchild, D.J. and Miller, C. (1980a). Structural aspects of protein accumulation in developing pea cotyledons. II. Three-dimensional reconstructions of vacuoles and protein bodies from serial sections. *Aust. J. Plant Physiol.* **7**, 239-237.

Craig, S., Millerd, A. and Goodchild, D.J. (1980b). Structural aspects of protein accumulation in developing pea cotyledons. III. Immunocytochemical localization of legumin and vicilin using antibodies shown to be specific by the enzyme-linked immunosorbent assay (ELISA). *Aust. J. Plant Physiol.* **7**, 339-351.

Craig, S. and Goodchild, D.J. (1984). Golgi-mediated vicilin accumulation in pea cotyledon cells is redirected by monensin and nigericin. *Protoplasma* **122**, 91-97.

Crouch, M.L. and Sussex, I.M. (1981). Development and storage protein synthesis in *Brassica npaus* L. embryos *in vivo* and *in vitro. Planta* **153**, 64-74.

Croy, R.R.D., Gatehouse, J.A., Evans, I.M. and Boulter, D. (1980a). Characterization of the storage protein subunits synthesized *in vitro* by polyribosomes and RNA from developing pea (*Pisum sativum* L.). I. Legumin. *Planta* **148**, 49-56.

Croy, R.R.D., Gatehouse, J.A., Evans, I.M. and Boulter, D. (1980b). Characterization of the storage protein subunits synthesized *in vitro* by polyribosomes and RNA from developing pea (*Pisum sativum* L.). II. Vicilin. *Planta* **148**, 57-63.

Croy, R.R.D., Gatehouse, J.A., Tyler, M. and Boulter, D. (1980c). The purification and characterization of a third storage protein (convicilin) from the seeds of pea (*Pisum sativum* L.). *Biochem. J.* **191**, 509-516.

Croy, R.R.D., Lycett, G.W., Gatehouse, J.A., Yarwood, J.N. and Boulter, D. (1982). Cloning and analysis of cDNAs encoding plant storage protein precursors. *Nature* **295**, 76-79.

Danielsson, C.E. (1949). Seed globulins of the Gramineae and Leguminosae. *Biochem. J.* **44**, 387-400.

Danscher, G. and Norgaard, J.O.R. (1983). Light microscope visualization of colloidal gold on resin embedded tissue. *J. Histochem. Cytochem.* **31**, 1394-1398.

Davidson, E.H. (1986). *Gene activity in early development*, 3rd edition. Academic Press London.

Davidson, E.H. and Britten, R.J. (1979). Regulation of gene expression: possible role of repetitive sequences. *Science* **204**, 1052-1059.

Davis, G.L. (1966). *Systematic Embryology of the Angiosperms.* John Wiley and Sons, New York.

Davison, B.L., Edgly, J.M., Mulvihill, E.R. and Chambon, P. (1983). Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promotor sequences. *Nature* **301**, 680-686.

Delauney, A.J. (1984). *Cloning and Characterization of cDNAs Encoding the Major Pea Storage Protein Genes, and Expression of Vicilin in E. coli.* Ph.D. Thesis, Dept. of Botany, University of Durham.

Deltour, R., Antoine, N. and Bronchart, R. (1989). Root ontogenesis during early germination of *Zea mays*. *Ann. Bot.* **64**, 107-116.

Derbyshire, E., Wright, D.J. and Boulter, D. (1976). Legumin and vicilin, storage proteins of legume seeds. *Phytochem.* **15**, 3-24.

Domoney, C. and Casey, R. (1985). Measurement of gene number for seed storage proteins in *Pisum*. *Nucl. Acids Res.* **13**, 687-699.

Domoney, C., Davies, D.R. and Casey, R. (1980). The initiation of legumin synthesis in immature embryos of *Pisum sativum* L. grown *in vivo* and *in vitro*. *Planta* **149**, 454-460.

Duhamel, R.C. and Johnson, D.A. (1985). Use of non-fat dry milk to block non-specific nuclear and membrane staining by avidin conjugates. *J. Histochem. Cytochem.* **33**, 711-714.

Dumont, J.N. and Yamada, T. (1972). Dedifferentiation of iris epithelial cells. *Dev. Biol.* **29**, 385-401.

Dure, L.S. (1975). Seed formation. *Ann. Rev. Plant Physiol.* **26**, 259-278.

Dure, L.S. (1985). Embryogenesis and gene expression during seed formation. *Oxford Surv. Plant Mol. Cell. Biol.* **2**, 179-197.

Dure, L.S. and Jensen, W.A. (1957). The influence of giberellic acid and indoleacetic acid on cotton embryos cultured *in vitro*. *Bot. Gaz.* **118**, 254-261.

Dute, R.R., Peterson, C.M. and Rushing, A.E. (1989). Ultrastructural changes of the egg apparatus associated with fertilization and proembryo development of soybean, *Glycine max* (Fabaceae). *Ann. Bot.* **64**, 123-135.

Edelman, G.M., Gallin, W.J., Delouvee, A., Cunningham, B.A. and Thiery, J.P. (1983). Early epochal maps of two different cell adhesion molecules. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4384-4388.

Edelman, G.M. and Thiery, J.P. (1985). *The Cell in Contact: Adhesions and junctions as Morphogenic Determinants.* Wiley, New York.

Edwards, G.A. (1988). *Plant Transformation Using an Agrobacterium tumefaciens Ti-Plasmid Vector System.* Ph.D. Thesis, University of Durham.

Eeuwens, C.J. and Schwabe, W.W. (1975). Seed and pod wall development in *Pisum sativum* L. in relation to extracted and applied hormones. *J. Ex. Bot.* **26**, 1-14.

Efstratiadis, A., Posakony, J.W., Maniatis, T., Lawn, R.M., O'Connell, C., Spritz, R.A., De Riel, J.K., Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C. and Proudfoot, N.J. (1980). The structure and evolution of the human B-globin gene family. *Cell* **21**, 653-668.

Elgin, S. (1981). DNase 1 hypersensitive sites of chromatin. *Cell* **27**, 413-415.

Ellis, J.G., Llewellyn, D.J., Walker, J.C., Dennis, E.S. and Peacock, W.J. (1987). The ocs element: a 16 base pair palindrome essential for activity of the octopine synthase enhancer. *EMBO J.* **6**, 3203-3208.

Ellis, J.R., Shirsat, A.H., Hepher, A., Yarwood, J.N., Gatehouse, J.A., Croy, R.R.D. and Boulter, D. (1988). Tissue-specific expression of a pea legumin gene in seeds of *Nicotiana plumbaginifolia*. *Plant Mole. Biol.* **10**, 203-214.

Esau, K. (1977). *Anatomy of Seed Plants*, 2nd edition. John Wiley and Sons Inc., U.S.A.

Evans, I.M., Gatehouse, J.A. and Boulter, D. (1985). Regulation of storage protein synthesis in pea (*Pisum sativum* L.) cotyledons under conditions of sulphur deficiency. *J. Biochem.* **232**, 261-265.

Evans, I.M., Gatehouse, J.A., Croy, R.R.D. and Boulter, D. (1984). Regulation of the transcription of storage-protein mRNA in nuclei isolated from developing pea (*Pisum sativum* L.) cotyledons. *Planta* **160**, 559-568.

Evans, I.M., Swinhoe, R., Gatehouse, L.N., Gatehouse, J.A. and Boulter, D. (1988). Distribution of root mRNA species in other vegetative organs of pea (*Pisum sativum* L.). *Mol. Gen. Genet.* **214**, 153-157.

Feldman, L.J. (1984). The development and dynamics of the root apical meristem. *Am. J. Bot.* 71, 1308-1314.

Fischer, R.L. and Goldberg, R.B. (1982). Structure and flanking regions of soybean seed protein genes. *Cell* 29, 651-660.

Flavell, R.B. (1980). The molecular characterization and organization of plant chromosomal DNA sequences. *Ann. Rev. Plant Physiol.* 31, 569-596.

Flavell, R.B. (1982). Chromosomal DNA sequences and their organization. In: *Nucleic Acids and Proteins in Plants. II. Structure, Biochemistry and Physiology of Nucleic Acids*, ed. B. Parthier and D. Boulter, pp. 46-74. Springer-Verlag, Berlin, Heidelberg and New York.

Forbes, D.J., Kirschner, M.W., Caput, D., Dahlberg, J.E. and Lund, E. (1984). Differential expression of multiple U1 small nuclear RNAs in oocytes and embryos of *Xenopus laevis*. *Cell* 38, 681-689.

Forde, B.G., Kreis, M., Bahramian, B., Mathews, J.A., Miflin, B.J., Thompson, R.D., Bartels, D.B. and Flavell, R.B. (1981). Molecular cloning and analysis of cDNA sequences derived from poly A^+ RNA from barley endosperm: identification of B-hordein clones. *Nucl. Acids Res.* 9, 6689-6707.

Fourney, R.M., Miyakoshi, J., Day, R.S. and Paterson, M.C. (1988). Northern blotting: efficient RNA staining and transfer. *Focus* 10, 5-7.

Fraser, S.E., Green, C.R., Bode, H.R. and Gilula, N.B. (1987). Selective disruption of gap junctional communication interferes with a patterning process in *Hydra*. *Science* 237, 49-55.

French, V., Bryant, P.J. and Bryant, S.V. (1976). Pattern regulation in epimorphic fields. *Science* 193, 969-981.

Galau, G.A., Britten, R.J. and Davidson, E.H. (1974). A measurement of sequence complexity of polysomal RNA in sea urchin embryos. *Cell* 2, 9-21.

Galau, G.A., Klein, W.H., Davis, M.M., Wold, B.J., Britten, R.J. and Davidson, E.H. (1976). Structural gene sets active in embryos and adult tissues of the sea urchin. *Cell* 7, 487-505.

Gall, J.G. and Pardue, M.L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. U.S.A.* 63, 378-383.

Gallagher, T.F. and Ellis, R.J. (1982). Light stimulated transcription of genes for two chloroplast polypeptides in isolated pea leaf nuclei. *EMBO J.* 1, 1493-1498.

Gannon, F., O'Hare, K., Perrin, F., Le Pennec, J.P., Benoist, C., Cochet, M., Breathnach, R., Royal, A., Garapin, A., Carni, B. and Chambon, P. (1979). Organization and sequences at the 5' end of a cloned complete ovalbumin gene. *Nature* 278, 428-434.

Gatehouse, J.A., Croy, R.R.D., Morton, H., Tyler, M. and Boulter, D. (1981). Characterisation and sub-unit structures of the vicilin storage proteins of pea (*Pisum sativum* L.). *Eur. J. Biochem.* **118**, 627-633.

Gatehouse, J.A., Evans, I.M., Bown, D., Croy, R.R.D. and Boulter, D. (1982). Control of storage protein synthesis during seed development in pea (*Pisum sativum* L.). *Biochem. J.* **208**, 119-127.

Gatehouse, J.A., Evans, I.M., Croy, R.R.D. and Boulter, D. (1986). Differential expression of genes during legume seed development. *Phil. Trans. R. Soc. London B.* **314**, 367-384.

Gee, C.E. and Roberts, J.L. (1983). *In situ* hybridization histochemistry: A technique for the study of gene expression in single cells. *DNA* **2**, 157-163.

Gilbert, S.F. (1988). *Developmental Biology*, 2nd edition. Sinaver Associates, Massachusetts.

Giudice, G. (1962). Restitution of whole larvae from disaggregated cells of sea urchin embryos. *Dev. Biol.* **5**, 402-411.

Goldberg, R.B. (1983a). Structure and function of plant genes. In: *Eukaryotic Genes:- their Structure, Activity and Regulation*, ed. N. Maclean, S.P. Gregory and R.A. Flavell. Butterworth & Co. Ltd.

Goldberg, R.B. (1983b). Organization and expression of soybean protein genes. In: *Genetic Engineering.* Rowman and Allanheld.

Goldberg, R.B. (1986). Regulation of plant gene expression. *Phil. Trans. R. Soc. London. Ser. B.* **314**, 343-353.

Goldberg, R.B., Barker, S.J. and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. *Cell* **56**, 149-160.

Goldberg, R.B., Hoschek, G., Ditta, S. and Briedenbach, R.W. (1981a). Developmental regulation of cloned superabundant embryo mRNA's in soybean. *Dev. Biol.* **83**, 218-231.

Goldberg, R.B., Hoschek, G., Kamalay, J.C. and Timberlake, W.E. (1978). Sequence complexity of nuclear and polysomal RNA in leaves of the tobacco plant. *Cell* **14**, 123-131.

Goldberg, R.B., Hoschek, T., Tam, S.H. and Ditta, G.S. (1981b). Abundance, diversity and regulation of mRNA sequence sets in soybean embryogenesis. *Dev. Biol.* **83**, 201-217.

Graham, C.F. and Wareing, P.F. (1984). *Developmental Control in Animals and Plants*, 2nd edition. Blackwell Scientific Publications.

Graham, T.A. and Gunning, B.E.S. (1970). Localization of legumin and vicilin in bean cotyledon cells using fluorescent antibodies. *Nature* **228**, 81-82.

Green, P.J., Yong, M.H., Cuozzo, M., Murakami, Y.K., Silverstein, P. and Chua, N.H. (1988). Binding site requirements for pea nuclear protein factor GT-1 correlate with sequences required for light dependant transcriptional activation of the rbcS-3A gene. *EMBO* 7, 4035-4044.

Greenwald, I.S., Sternberg, P.W. and Horvitz, H.R. (1983). The lin-12 locus specifies cell fates in *Caenorhabditis elegans*. *Cell* 34, 435-444.

Greenwood, J.S. and Chrispeels, M.J. (1985). Correct targeting of the bean storage protein Phaseolin in the seeds of transformed tobacco. *Plant Physiol.* 79, 65-71.

Grierson, D. and Covey, S.N. (1988). *Plant Molecular Biology*, 2nd edition. Blackie and Son Ltd.

Grosveld, G.C., Shewmaker, C.KI., Jat, P. and Flavell, R.A. (1981). Localization of DNA sequences necessary for transcription of the rabbit B-globin gene *in vitro*. *Cell* 25, 215-226.

Grosveld, G.C., de Boer, E., Shewmaker, C.K. and Flavell, R.A. (1982). DNA sequences necessary for transcription of the rabbit B-globin gene *in vivo*. *Nature* 295, 120-126.

Groudine, M. and Weintraub, H. (1981). Activation of globin genes during chick development. *Cell* 24, 393-401.

Guilfoyle, T.J. and Malcolm, S. (1980). The amounts, subunit structure and template engaged activities of RNA polymerases from germinating soybean axis. *Dev. Biol.* 78, 113-125.

Gunning, B.E.S. and Pate, J.S. (1969). Transfer cells. *Protoplasma* 68, 107-133.

Gurdon, J.B., Laskey, R.A. and Reeves, O.R. (1975). The developmental capacity of nuclei transplanted from keratinized cells of adult frogs. *J. Embryol. Exp. Morphol.* 34, 93-112.

Guyette, W.A., Matusik, R.J. and Rosen, J.M. (1979). Prolactin mediated transcriptional and post-transcriptional control of casein gene expression. *Cell* 17, 1013-1023.

Hall, M.A. (1984). Hormones and plant development: cellular and molecular aspects. In: *Developmental Control in Animals and plants*, ed. C.F. Graham and P.F. Wareing, pp. 313-330.

Harada, J.J., Dietrich, R.A., Comai, L. and Baden, C.S. (1988). Regulation of gene expression during seed germination and postgerminative development. In: *Temporal and Spatial Regulation of Plant Genes*, ed. D.P.S. Verma and R.B. Goldberg, pp. 28-39. Springer Verlag, N. York.

Hardham, A.R. (1976). Structural aspects of the pathways of nutrient flow to the developing embryo and cotyledons of *Pisum sativum*. *Aust. J. Bot.* 24, 711-721.

Harlow, E. and Lane, D. (1988). *Antibodies. A Laboratory Manual.* Cold Spring Harbor Laboratory.

Harris, N. and Croy, R.R.D. (1986). Localization of mRNA for pea legumin: *In situ* hybridization using a biotinylated cDNA probe. *Protoplasma* 130, 57-67.

Harris, N., Grindley, H. and Mulcrone, J. (1989). Correlated *in situ* hybridization and immunochemical studies of legumin storage protein deposition in pea (*Pisum sativum* L.). *Cell Biol. Int. Reports* 13, 23-35.

Haugland, R.A. and Cline, M.G. (1978). Capping structures at the 5' terminus of polyadenylated RNA in *Avena* coleoptiles. *Plant Physiol.* 62, 838-840.

Hayashi, S., Gillam, I.C., Delaney, A.D. and Tener, G.M. (1978). Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs from hybridization with ^{125}I-labelled RNA. *J. Histochem. Cytochem.* 26, 677-679.

Hedley, C.L. and Smith, C.M. (1985). Genetic variation for pea seed development. In: *The Pea Crop - A Basis for Improvement*, eds. P.D. Hebblethwaite, M.C. Heath and T.C.K. Dawkins. Butterworths.

Heidecker, G. and Messing, J. (1986). Structural analysis of plant genes. *Ann. Rev. Plant Physiol.* 37, 439-466.

Hentschel, C.C. and Birnstiel, M.C. (1981). The organization and expression of histone gene families. *Cell* 25, 301-313.

Hershey, J.W.B., Duncan, R. and Mathews, M.B. (1986). Mechanisms of translational control. In: *Translational Control. Current Comm. in Molecular Biology*, ed. M.B. Mathews. Cold Spring Harbor.

Heslop-Harrison, J. (1971). The cytoplasm and its organelles during meiosis. In: *Pollen: Development and Physiology*, ed. Heslop-Harrison, Butterworths, London.

Higgins, T.J.V. (1984). Synthesis and regulation of major proteins in seeds. *Ann. Rev. Plant Physiol.* 35, 191-221.

Hocking, P.J. and Pate, J.S. (1977). Mobilization of minerals to developing seeds of legumes. *Ann. Bot.* 41, 1259-1278.

Holgate, C., Jackson, P., Cowen, P.N. and Bird, C.C. (1983). Immunogold-silver staining: a new method of immunostaining with enhanced sensitivity. *J. Histochem. Cytochem.* 31, 938-944.

Hsu, S.M., Raine, L. and Fanger, H. (1981a). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. cytochem.* 29, 577-580.

Hsu, S.M., Raine, L. and Fanger, H. (1981b). The use of anti-avidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase techniques. *Am. J. Clin. Pathol.* 75, 816-821.

Hunt, T. (1983). The initiation of protein synthesis. In: *DNA Makes RNA Make Protein*, ed. T. Hunt, S. Prentis and J. Tooze. Elsevier Biomedical Press, pp. 205-212.

Hyldig-Nielsen, J.J., Jensen, E.O., Wiborg, O., Garret, J., Horgensen, P. and Marcker, K.A. (1982). The primary structures of two leghemoglobin genes. *Nucleic Acid Res.* 10, 689-701.

Jagus, R., Anderson, W.F. and Safer, B. (1982). The regulation of initiation of mammalian protein synthesis. *Progr. Nucl. Acids. Res. Mol. Biol.* 25, p. 127.

Jendriask, J. (1980). Purification, structures and functions of the nuclear RNA polymerases from higher plants. In: *Genome Organization and Expression in Plants*, ed. C.J. Leaver, pp. 77-92. Plenum, New York.

Jensen, W.A. (1965). The ultrastructure and composition of the egg and central cell of cotton. *Am. J. Bot.* 52, 781-797.

Jensen, W.A. (1968). Cotton embryogenesis: The zygote. *Planta* 79, 346-366.

Jensen, W.A. (1964). *Cell Development During Plant Embryogenesis.* Brookhaven Symposia in Biology No. 16, pp. 179-202.

Jofuku,K.D., Okamuro, J.K. and Goldberg, R.B. (1987). Interaction of an embryo DNA binding protein with a soybean lectin gene upstream region. *Nature* 328, 734-737.

Kamalay, J.C. and Goldberg, R.B. (1980). Regulation of structural gene expression in tobacco. *Cell* 19, 935-946.

Kamalay, J.C. and Goldberg, R.B. (1984). Organ specific nuclear RNAs in tobacco. *Proc. Natl. Acad. Sci. USA* 81, 2801-2805.

Kang, A.S., Abbott, S.J. and Harris, N. (1988). The assay of gene expression by immunodetection. In: *Manipulating Secondary Metabolism in Culture*, ed. R.J. Robins and M.J.C. Rhodes, pp. 273-283. Cambridge Univ. Press.

Kapil, R.N. and Bhatnagar, A.K. (1981). Ultrastructure and biology of female gametophyte in flowering plants. *Internat. Rev. Cytology* 70, 291-341.

Kenyon, C. (1988). The nematode *Caenorhabditis elegans. Science* 240, 1448-1453.

Kidd, S., Locket, T.J. and Young, M.W. (1983). The Notch locus of *Drosophila melanogaster. Cell* 34, 421-433.

Kimelman, D. and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-B and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. *Cell* 51, 869-877.

King, G.A. and Heyes, J.K. (1986). Morphology and cytology of pea embryos during histogenesis. *Ann. Bot.* **58**, 633-640.

Kirk, D.L. and Kirk, M.M. (1983). Protein synthetic patterns during the asexual life cycle of *Volvox carteri*. *Dev. Biol.* **96**, 493-499 .

Kirk, D.L. and Kirk, M.M. (1986). Translational regulation of protein synthesis and the onset of cytodifferentiation in *Volvox*. In: *Translational Control*, ed. M.B. Mathews, Cold Spring Harbor, pp. 185-192.

Kirk, M.M. and Kirk, D.L. (1985). Translational regulation of protein synthesis, in response to light, at a critical stage of *Volvox* development. *Cell* **41**, 419-424.

Krieg, P.A. and Melton, D.A. (1987). An enhancer responsible for activating transcription at the mid-blastula transition in *Xenopus* development. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2331-2335.

Kuhlemeier, C., Green, P.J. and Chua, N.H. (1987). Regulation of gene expression in higher plants. *Ann. Rev. Plt. Physiol.* **38**, 221-237.

Lawrence, J.B. and Singer, R.H.(1985). Quantitative analysis of *in situ* hybridisation method for the detection of actin gene expression. *Nucl. Acids Res.* **13**, 1777-1779.

Lawrence, J.B. and Singer, R.H. (1986). Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell* **45**, 407-415.

Lee, K.A.W., Guertin, D. and Sonenberg, N. (1983). mRNA structure as a determinant in cap recognition and initiation complex formation. *J.Biol. Chem.* **258**, p.707.

Le Gal, M.F., Hallet, J.N. and Rey, L. (1984). DNA and protein content relationship in the cells of mature cotyledons of *Pisum sativum*. *Protoplasma* **120**, 113-122.

Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.N. and Steitz, J.A. (1980). Are snRNPs involved in splicing? *Nature* **283**, 220-224.

Lernsten, N.R. (1983). Suspensors in Leguminosae. *Bot. Rev.* **49**, 233-257.

Lewin, B. (1980). *Gene Expression*, 2nd edition. Wiley Interscience, London.

Linck, A.J. (1961). The morphological development of the fruit of *Pisum sativum* var. Alaska. *Phytomorphol.* **11**, 79-84.

List, A. Jr. and Steward, F.C. (1965). The nucellus, embryo sac, endosperm and embryo of *Aesculus* and their interdependence during growth. *Ann. Bot.* **29**, 1-15.

Lo, C.W. and Gilula, N.B. (1979). Gap junctional communication in the pre-implantation mouse embryo. *Cell* **18**, 399-410.

Logemann, J., Schell, J. and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16-20.

Lorenzi, R., Bennici, A., Cionini, P.G., Alpi, A. and D'Amato, F. (1978). Embryo-suspensor relations in *Phaseolus coccineus*: cytokinins during seed development. *Planta* 143, 59-62.

Lycett, G.W., Croy, R.R.D., Shirsat, A.H. and Boulter, D. (1984). The complete nucleotide sequence of a legumin gene from pea (*Pisum sativum* L.). *Nucl. Acids Res.* 12, 4493-4506.

MacWilliams, H.K. (1983a). *Hydra* transplant phenomenon and the mechanism of hydra head regeneration. I. Properties of head inhibition. *Dev. Biol.* 96, 217-238.

MacWilliams, H.K. (1983b). *Hydra* transplantation phenomena and the mechanism of hydra head regeneration. II. Properties of head activation. *Dev. Biol.* 96, 239-272.

Maheshwari, P. (1950). *An Introduction to the Embryology of Angiosperms.* McGraw-Hill Brook Co., New York.

Mahlberg, P.G. (1960). Embryogeny and histogenesis in *Nerium oleander* L. I. Organization of primary meristematic tissues. *Phytomorphol.* 10, 118-131.

Malik, C.P., Singh, M. and Thapar, N. (1976). Physiology of sexual reproduction. IV. Histochemical characteristics of suspensor of *Brassica campestris*. *Phytomorphol.* 26, 384-389.

Mandel, J.L. and Chambon, P. (1979). DNA methylation differences: Organ specific variations in methylation pattern within and around ovalbumin and other chick genes. *Nucleic Acid Res.* 7, 2081-2103.

Maniatis, T., Fritsch, E.F. and Sambrook, S. (1982). *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, U.S.A.

Mantell, S.H., Mathews, J.A. and McKee, R.A. (1985). *Principles of Plant Biotechnology.* Blackwell Scientific Publications.

Marinos, N.G. (1970a). Embryogenesis of the pea (*P. sativum*). I. The cytological environment of the developing embryo. *Protoplasma* 70, 261-279.

Marinos, N.G. (1970b). Embryogenesis of the pea (*P. sativum*). II. An unusual type of plastid in the suspensor cells. *Protoplasma* 71, 227-233.

Masand, P. and Kapil, R.N. (1966). Nutrition of the embryo sac and embryo - a morphological approach. *Phytomorphol.* 16, 158-175.

Mathison, P.M. and Miller, L. (1987). Thyroid hormone induction of keratin genes: a two step activation of gene expression during development. *Genes Dev.* 1, 1107-1117.

Matthews, J.A. and Miflin, B.J. (1980). *In vitro* synthesis of barley storage proteins. *Planta* 149, 262-268.

Mau, S.L., Raff, J. and Clarke, A.E. (1982). Isolation and partial characterisation of components of *Prunus avium* L. styles including an antigenic glycoprotein associated with a self-incompatibility genotype. *Planta* 156, 505-516.

McAllister, H.A. and Rock, D.L. (1985). Comparative usefulness of tissue fixatives for *in situ* viral nucleic acid hybridization. *J. Histochem. Cytochem.* 33, 1026-1032.

McCabe, J.T., Morrell, J.I. and Ivell, R. (1986). *In situ* hybridisation technique to localise rRNA and mRNA in mammalian neurons. *J. Histochem. Cytochem.* 34, 45-50.

McDonnell, M.W., Simon, M.N. and Studier, F.W. (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* 110, 119-122.

McKee, H.S., Robertson, R.N. and Lee, J.B. (1955). Physiology of pea fruits. I. The developing fruit. *Aust. J. Biol. Sci.* 8, 137-163.

McMasters, G.K. and Carmichael, G.G. (1977). Analysis of single and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. U.S.A.* 74, 4835-4838.

Meedel, T.H. and Whittaker, J.R. (1984). Lineage segregation and developmental autonomy in expression of functional muscle acetylcholinesterase in the ascidian embryo. *Dev. Biol.* 105, 479-487.

Meijlink, F., Curran, T., Miller, A.D. and Verma, I.M. (1985). Removal of a 67 base pair sequence in the non-coding region of proto-oncogene fos converts it to a transforming gene. *Proc. Natl. Acad. Sci. U.S.A.* 82, 4987-4991.

Meinke, D.W., Chen, J. and Beachy, R.N. (1981). Expression of storage protein genes during soybean seed development. *Planta* 153, 130-139.

Messing, J., Geraghty, D., Heidecker, G., Hu, N.T., Kridl, J. and Rubenstein, I. (1983). Plant gene structure. In: *Genetic Engineering of Plants - An Agricultural Perspective*, ed. Kosuge, Meredith and Hollander. Plenum Press.

Millerd, A. (1975). Biochemistry of legume seed proteins. *Ann. Rev. Plant Physiol.* 26, 53-72.

Millerd, A. and Spencer, D. (1974). Changes in RNA synthesizing activity and template activity in nuclei from cotyledons of developing pea seeds. *Aus. J. Plant Physiol.* 1, 331-341.

Millerd, A., Spencer, D., Dudman, W.F. and Stiller, M. (1975). Growth of immature pea cotyledons in culture. *Aust. J. Plant Physiol.* 2, 51-59.

Millerd, A. and Whitfeld, P.R. (1973). Deoxyribonucleic acid and ribonucleic acid synthesis during the cell expansion phase of cotyledon development in *Vicia faba*. *Plant Physiol.* 51, 1005-1010.

Muntz, K. (1982). Seed development. In: *Nucleic Acids and Proteins in Plants*, eds. D. Boulter and B. Parthier. I. Encyclopaedia of Plant Physiology, New Series, vol. **14A**. Springer-Verlag.

Murai, N., Sutton, D.W., Murray, M.G., Slightom, J.L., Merlo, D.J. Reichert, N.A., Sengupta Gopalan, C., Stock, C.A., Barker, R.F., Kemp, J.D. and Hall, T.C. (1983). Phaseolin gene from bean is expressed after transfer to sunflower via tumor inducing plasmid vectors. *Science* **222**, 476-482.

Murray, D.R. (1979). Nutritive role of the seedcoats during embryo development in *Pisum sativum* L. *Plt. Physiol.* **64**, 763-769.

Murray, D.R. (1980). Functional significance of acid phosphatase distribution during embryo development in *Pisum sativum* L. *Ann. Bot.* **45**, 273-281.

Murray, M.G., Cuellar, R.E. and Thompson, W.F. (1978). DNA sequence organisation in the pea genome. *Biochemistry* **17**, 5781-5790.

Murray, M.G. and Kennard, W.C. (1984). Altered chromatin conformation of the higher plant gene Phaseolin. *Biochemistry* **23**, 4225-4232.

Myers, R.M., Tilly, K. and Maniatis, T. (1986). Fine structure genetic analysis of a B-globin promotor. *Science* **232**, 613-618.

Nabeshima, Y., Fuji-Kuriyama, Y., Muramatsu, M. and Ogata, K. (1984). Alternative transcription and two modes of splicing result in two myosin light chains from one gene. *Nature* **308**, 333-338.

Nagl, W. (1982). Nuclear chromatin. In: *Encyclopaedia of Plant Physiology*, eds. D. Boulter and B. Parthier, vol. **14B**, pp. 1-45. Springer Verlag, Berlin.

Nomura, K. and Komamine, A. (1986). Molecular mechanisms of somatic embryogenesis. *Oxford Surveys of Plant Molecular and Cell Biology* **3**, 456-466.

Nose, A. and Takeichi, M. (1986). A novel cadherin adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J. Cell Biol.* **103**, 2649-2658.

Offler, C.E., Herlich, S.M. and Patrick, J.W. (1989). Pathway of photosynthate transfer iin the developing seed of *Vicia faba* L. transfer in relation to seed anatomy. *J. Ex. Bot.* **40**, 769-780.

Okamuro, J.K., Jofuku, K.D. and Goldberg, R.B. (1986). Soybean seed lectin gene and flanking non-seed protein genes are developmentally regulated in transformed tobacco plants. *Proc. Nat. Acad. Sci. USA* **83**, 8240-8244.

Osborne, T.B. (1924). *The Vegetable Proteins*, 2nd edition. Longmans Green and Co., London.

Parker, C.S. and Topol, J. (1984). A *Drosophila* RNA polymerase II transcription factor binds to the regulatory site of an hsp 70 gene. *Cell* **37**, p. 273.

Pate, J.S. and Flinn, A.M. (1973). Carbon and nitrogen transfer from vegetative organs to ripening seeds of field pea (*Pisum arvense* L.). *J. Ex. Bot.* 24, 1090-1099.

Pate, J.S., Sharkey, P.L. and Lewis, O.A.M. (1974). Phloem bleeding from legume fruits - technique for study of fruit nutrition. *Planta* 120, 229-243.

Pernollet, J.C. (1978). Protein bodies of seeds: Ultrastructure, biochemistry, biosynthesis and degradation. *Phytochem.* 17, 1473-1480.

Pitts, J.D. (1984). Junctional communication between cells. In:- *Developmental Control in Animals and Plants*, eds. C.F. Graham and P.F. Wareing, 2nd edition. Blackwell Scientific Publications.

Pollock, E.G. and Jensen, W.A. (1964). Cell development during early embryogenesis in *Capsella* and *Gossypium*. *Am. J. Bot.* 51, 915-921.

Proudfoot, N.J. and Brownlee, G.G. (1976). 3' non-coding region sequences in eukaryotic messenger RNA. *Nature* 263, 211-214.

Quatrano, R.S. (1986). Regulation of gene expression by abscissic acid during angiosperm embryo development. *Oxford Surv. Plant Mol. Cell Biol.* 3, 467-476.

Raff, J.R., Hutchinson, J.F., Knox, R.B. and Clark, A.E. (1979). Cell recognition: antigenic determinants of plant organs and their cultured callus cells. *Differentiation* 12, 240-242.

Raghavan, V. (1976). *Experimental Embryogenesis in Vascular Plants.* Academic Press.

Raghavan, V. (1986). *Embryogenesis in Angiosperms.* Cambridge University Press.

Randall, P.J., Thomson, J.A. and Schroeder, H.E. (1979). Cotyledonary storage proteins in *Pisum sativum*. IV. Effects of sulphur, phosphorous, potassium and magnesium deficiencies. *Aust. J. Plant Physiol.* 6, 11-24.

Reeve, R.M. (1948). Late embryogeny and histogenesis in *Pisum*. *Am. J. Botany* 35, 591-601.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17, 208-212.

Richter, J.D., Lorenz, L.J., Crawford, D.R., Audet, R.G. and Swiderski, R.E. (1986). The control of translation by RNA binding proteins in *Xenopus laevis* oocytes. In: *Translational Control*, ed. M.B. Mathews, Cold Spring Habor, pp. 144-149.

Richter, J.D. and Smith, L.D. (1983). Developmentally regulated RNA binding proteins during oogenesis in *Xenopus laevis*. *J. Biol. Chem.* 258, 4864-4869.

Rietsema, J., Satina, S. and Blakeslee, A.F. (1953). The effect of indole-3-acetic acid on *Datura* embryos. *Proc. Natl. Acad. Sci. U.S.A.* 39, 924-933.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase 1. *J. Mol. Biol.* **113**, 237-251.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364-368.

Rosahl, S., Eckes, P., Schell, J. and Willmitzer, L. (1986). Organ-specific gene expression in potato: isolation and characterisation of tuber-specific cDNA sequences. *Mol. Gen. Genet.* **202**, 368-373.

Sargent, T.D., Jamrich, M. and Dawid, B. (1986). Cell interactions and the control of gene activity during early development of *Xenopus laevis*. *Dev. Biol.* **114**, 238-246.

Sawyer, R.M., Boulter, D. and Gatehouse, J.A. (1987). Nuclease sensitivity of storage protein genes in isolated nuclei of pea seeds. *Planta* **171**, 254-258.

Saxen, L., Lehtonen, E., Karkinen-Jaaskelainen, M., Hordling, S. and Wartiovaara, J. (1976). Are morphogenetic tissue interactions mediated by transmissable signal substances or through cell contacts? *Nature* **259**, 662-663.

Scharpe, A. and Van Parijs, R. (1973). The formation of polyploid cells in ripening cotyledons of *Pisum sativum* L. in relation to ribosome and protein synthesis. *J. Exp. Bot.* **24**, 216-222.

Schlissel, M.S. and Brown, D.D. (1984). The transcriptional regulation of *Xenopus* 5S RNA genes in chromatin. The roles of active stable transcription complex and histone H1. *Cell* **37**, 903-913.

Schoffl, F., Baumann, G., Raschke, E. and Bevan, M. (1986). The expression of heat-shock genes in higher plants. *Phil. Trans. R. Soc. London B.* **314**, 435-468.

Schulz, R. and Jensen, W. (1968a). *Capsella* embryogenesis: the egg, zygote and young embryo. *Am. J. Bot.* **55**, 807-819.

Schulz, R. and Jensen, W. (1968b). *Capsella* embryogenesis: the early embryo. *J. Ultrastructure Res.* **22**, 376-392.

Schulz, P. and Jensen, W.A. (1969). *Capsella* embryogenesis: the suspensor and basal cell. *Protoplasma* **67**, 139-163.

Schulz, P. and Jensen, W.A. (1973). *Capsella* embryogenesis: The central cell. *J. Cell Sci.* **12**, 741-763.

Schulz, P. and Jensen, W.A. (1974). *Capsella* embryogenesis: the development of the free nuclear endosperm. *Protoplasma* **80**, 183-205.

Sengupta-Gopalan, C., Reichert, N.A., Barker, R.F., Hall, T.C. and Kemp, J.D. (1985). Developmentally regulated expression of the bean B-phaseolin gene in tobacco seed. *Proc. Natl. Acad. Sci. USA* **82**, 3320-3324.

Shah, D.M. Horsch, R.B., Klee, H.J., Kishore, G.M., Winter, J.A., Tumer, N.E., Hironaka, C.M., Sanders, P.R., Gasser, C.S., Aykent, S., Siegel, N.R., Rogers, S.G. and Fraley, R.T. (1986). Engineering herbicide resistance in transgenic plants. *Science* **233**, 478-481.

Shirsat, A., Wilford, N., Croy, R.R.D. and Boulter, D. (1989). Sequences responsible for the tissue-specific promotor activity of a pea legumin gene in tobacco. *Mol. Gen. Genet.* **215**, 326-331.

Singer, R.H., Lawrence, J.B. and Villnave, C. (1986). Optimization of *in situ* hybridization using isotopic and non-isotopic detection methods. *Biotechniques* **4**, 230-250.

Singer, S.J. and Nicholson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. *Science* **175**, 720-731.

Sivaramakrishna, D. (1978). Size relationships of apical cell and basal cell in two celled embryos in angiosperms. *Can. J. Bot.* **56**, 1434-1438.

Slack, J.M.W., Darlington, B.G., Heath, J.K. and Goodsave, S.F. (1987). Mesoderm induction in early *Xenopus* embryos by heparin binding growth factor. *Nature* **326**, 197-200.

Soueges, R. (1919). Les premieres divisions de l'oeuf et les differenciation du suspenseur chez le *Capsella bursa pastoralis* Moench. *Ann. Sci. Naturl. Bot.* **1**, 1-28.

Soueges, R. (1937). *Les lois du developpement.* Hermann, Paris.

Spemann, H. (1962). *Embryonic development and induction.* Hafner Publishing Co., New York.

Spemann, H. and Mangold, H. (1924). Induction of embryonic primordia by implantation of organizers from a different species. In: *Foundations of Experimental Embryology*, eds. B.H. Willier and J.M. Oppenheimer, pp. 144-184. Hafner, N. York.

Spencer, D. (1984). The physiological role of storage proteins in seeds. *Phil. Trans. R. Soc.* **304**, 275-285.

Spirin, A.S. (1966). On "masked" forms of messenger RNA in early embryogenesis and in other differentiating systems. *Curr. Top. Dev. Biol.* **1**, 1-38.

Stafford, A. and Davies, D.R. (1979). Culture of immature pea embryos. *Ann. Bot.* **44**, 315-321.

Stebbins, G. L. (1965). Some relationships between mitotic rhythm, nucleic acid synthesis and morphogenesis in higher plants. *Brookhaven Symposia in Biology* No. **18**, pp. 204-221.

Steeves, T.A. and Sussex, I.M. (1972). *Patterns in Plant Development.* Prentice-Hall Inc., New Jersey.

Steward, F.C. (1958). Growth and organized development of cultured cells. III. Interpretation of growth from free cell to carrot plant. *Am. J. Bot.* **45**, 709-713.

Sun, S.M., Slightom, J. and Hall, T.C. (1981). Intervening sequences in a plant gene - comparison of the partial cDNA and genomic DNA of French bean phaseolin. *Nature* **289**, 37-41.

Thornburg, R.W., Gynheung, A., Cleveland, T.E., Johnson, R. and Ryan, C.A. (1987). Wound inducible expression of a potato inhibitor II chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 744-748.

Tiedmann, H. (1976). Pattern formation in early developmental stages of amphibian embryos. *J. Embryol. exp. Morph.* **35**, 437-444.

Torrey, J.G. (1955). On the determination of vascular patterns during tissue differentiation in excised pea roots. *Am. J. Bot.* **42**, 183-198.

Tucker, E.B. (1982). Translocation in the staminal hair of *Setcreasa purpurea.* I. A study of cell ultrastructure and cell to cell passage of molecular probes. *Protoplasma* **113**, 193-202.

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., de Beauckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. and Leemans, J. (1987). Transgenic plants protected from insect attack. *Nature* **328**, 33-37.

Van de Ploeg, L.H.T. and Flavell, R.D. (1980). DNA methylation in the human globin locus in erythroid and non-erythroid cells. *Cell* **19**, 947-958.

Verma, D.P.S., Nash, D.T. and Schulman, H. (1974). Isolation and *in vitro* translation of soybean leghemoglobin. *Nature* **251**, 74-77.

Waddington, C.H. (1940). *Organisers and Genes.* Cambridge. Univ. Press.

Walbot, V., Brady, T., Clutter, M. and Sussex, I. (1972). Macromolecular synthesis during plant embryogeny: rates of RNA synthesis in *Phaseolus coccineus* embryos and suspensors. *Dev. Biol.* **29**, 104-111.

Walker, M.D., Edlund, T., Boulet, A.M. and Rutter, W.J. (1983). Cell specific expression controlled by the 5' flanking region of the insulin and chymotrypsin genes. *Nature* **306**, 557-561.

Walling, L., Drews, G.N. and Golding, R.B. (1986). Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. *Proc. Natl. Acad. Sci. USA* **83**, 2123-2127.

Walter, P., Gilmore, R. and Blobel, G. (1984). Protein translocation across the endoplasmic reticulum. *Cell* **38**, p. 5.

Walter, P., Siegel, V., Hansen, W. and Garcia, P.D. (1986). Elongation control by the signal recognition particle. In: *Translational Control*, ed. M.B. Mathews, Cold Spring Harbor, pp. 158-161.

Wardlaw, C.W. (1955). *Embryogenesis in Plants.* Methuen & Co. Ltd., London.

Warford, A. (1988). *In situ* hybridisation; a new tool in pathology. *Med. Lab. Sci.* **45**, 381-394.

Warner, A.E., Guthrie, S.C. and Gilula, N.B. (1984). Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* **311**, 127-131.

Waterhouse, R.N. (1985). *The Control of Expression of Storage Protein Genes in Pisum sativum* L. Ph.D. Thesis, Department of Botany, University of Durham.

Weintraub, H. (1985). Assembly and propagation of repressed and derepressed chromosomal states. *Cell* **42**, 705-711.

Weintraub, H. and Groudine, M. (1976). Chromosomal subunits in active genes have an altered configuration. *Science* **193**, 848-856.

Whittaker, J.R. (1973). Segregation during ascidian embryogenesis of egg cytoplasmic information for tissue-specific enzyme development. *Proc. Nat. Acad. Sci. USA* **70**, 2096-2100.

Whittaker, J.R. (1982). Muscle cell lineage can change the developmental expression in epidermal lineage cells of ascidian embryos. *Dev. biol.* **93**, 463-470.

Wold, B.J., Klein, W.H., Hough-Evans, B.R., Britten, R.J. and Davidson, E.H. (1978). Sea urchin embryo mRNA sequences expressed in nuclear RNA of adult tissues. *Cell* **14**, 941-950.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular formation. *J. Theoret. Biol.* **25**, 1-47.

Wu, C. (1984). Activating protein factor binds *in vitro* to upstream control sequences in heat shock chromatin. *Nature* **311**, 81-84.

Yeung, E.C. (1980). Embryogeny of *Phaseolus*: the role of the suspensor. *Z. pflanzenphysiol.* **96**, 17-28.

Yeung, E.C. and Clutter, M.E. (1979). Embryogeny of *Phaseolus coccineus*: the ultrastructure and development of the suspensor. *Can. J. Bot.* **57**, 120-136.

Yeung, E.C. and Sussex, I.M. (1979). Embryogeny of *Phaseolus coccineus:* the suspensor and the growth of the embryo-proper *in vitro*. *Z. pflanzenphysiologie* **91**, 423-433.

Zaret, K.S. and Yamamoto, K.R. (1984). Reversible and persistent changes in chromatin structure accompanying activation of a glucocorticoid-dependant enhancer element. *Cell* **38**, 29-38.

Zipursky, S.L., Ven Katesh, T.R., Teplow, D.B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.