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Thompson, Andrew John

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A thesis submitted by Andrew John Thompson BSc (London) in accordance with the requirements for the degree of Doctor of Philosophy in the University of Durham.

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ABSTRACT

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ANDREW J. THOMPSON

Three classes of legumin, encoded by the gene sub-families *legA, legJ* and *legS*, and a lectin encoded by a single gene, *lecA*, accumulate in the developing cotyledons of *Pisum sativum* L. Transcription rates for the genes encoding these proteins were measured in nuclei isolated from cotyledons at 12 and 16 days after flowering (DAF). The steady-state levels of the corresponding mRNA species were also measured, in absolute terms, throughout cotyledon development. It was found that the different legumin gene sub-families are not coordinately expressed and, in addition, members within the *legJ* sub-family show differential temporal expression. Also, it was demonstrated that the length of the poly(A) tail of the lectin mRNA is reduced during the period when the steady-state level of this mRNA is in decline.

When transcription rates and steady-state mRNA levels of the different gene families are compared, there is little correlation. This suggests a posttranscriptional regulation of the quantitative level of expression of these genes.

Expression of the legumin genes is known to be seed-specific, whereas expression of the lectin gene occurs in the root as well as the seed. When transcription rates were measured in leaf nuclei the levels of legumin and lectin transcripts detected approached background levels, indicating that these genes are either inactive or transcribed at very low levels in leaf; however, the rate of transcription of the chlorophyll a/b binding-protein gene was high. This suggests transcriptional control as the major factor in the organ-specificity of legumin and lectin expression.

The apparent posttranscriptional regulation of the quantitative level of expression of different seed-protein genes was investigated further by pulse-chase labelling the RNA of pea cotyledons grown in culture. Also, the possibility of using cell-free extracts to assay the cytoplasmic stability of specific polysomal mRNAs was investigated.

CONTENTS.

FIGURES.

TABLES

MEMORANDUM

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

Croy, R.R.D, Evans, I.M., Yarwood, J.N., Harris, N., Gatehouse, J.A., Shirsat, A.H., Kang, A., Ellis, J.R., Thompson, A., Boulter, D. (1988) "Expression of pea legumin sequences in pea *Nicotiana* and yeast." *Biochem. Physiol. Pflanzen.* **183**, 183-197.

Thompson, A.J., Evans, I.M., Croy, R.R.D., Boulter, D., Gatehouse, J.A. (1989) "Transcriptional and posttranscriptional regulation of seed storage protein gene expression in pea (*Pisum sativum* L.)." *Planta* **179** 279-287

Boulter, D., Croy,R.R.D., Evans, I.M., Gatehouse, J.A., Harris, N., Shirsat, A., Thompson, A. (1989) "The molecular biology of pea seed development with particular reference to the storage protein genes." In: Genetic Engineering of Crop Plants (49th Nottingham Easter School), eds. Grierson, D., Lycett, G. *In press.*

ABBREVIATIONS

A_{260}	absorbance at 260nm
ABA	abscisic acid
Act-D	actinomycin D
ATA	aurintricarboxylic acid
ATP	adenosine-5′-triphosphate
bp	base pair
BSA	bovine serum albumin
CCMV	cowpea chlorotic mottle virus
CDP	cytidine-5′-diphosphate
cDNA	copy DNA
CP	creatine phosphate
CPK	creatine phosphokinase
cpm	counts per minute
cRNA	copy RNA
CTP	cytidine-5′-triphosphate
cv.	cultivar
DAF	days after flowering
DAPI	4,6-diamidino-2-phenylindole
dATP	deoxyadenosine-5′-triphosphate
dCTP	deoxycytidine-5′-triphosphate
DDT	dithiothreitol
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine-5′-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPM	disintegrations per minute
dTTP	deoxythymidine-5′-triphosphate
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ELISA	enzyme linked immunosorbant assay
HPLC	high performance liquid chromatography
HPRI	human placental RNase inhibitor
IAA	isoamyl alcohol
kb	kilobase

kDa	kiloDalton
GTP	guanosine-5′-triphosphate
LSC	liquid scintillation counting
MES	2[N-mopholino]ethanesulfonic acid
mRNA	messenger RNA
MW	relative molecular weight
nt	nucleotide
NTP	nucleotide-5′-triphosphate
oligo(dT)	oligodeoxythymidylic acid
PABP	poly(A)-binding protein
PAGE	polyacrylamide gel electrophoresis
PIPES	piperazine-N,N′-bis[2-ethane-sulfonic acid]
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
pre-mRNA	precursor mRNA
pre-rRNA	precursor rRNA
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TCA	trichloroacetic acid
tRNA	transfer RNA
UDP	uridine-5′-diphosphate
UDPG	uridine-5 -diphospho-D-glucose
UMP	uridine-5′-monophosphate
UTP	uridine-5′-triphosphate
UV	ultraviolet

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1. INTRODUCTION

1.1. General Introduction.

Over the last few years a considerable mass of information has accumulated on the way in which gene expression is regulated in higher plants. The availability of routine techniques for the cloning and manipulation of genes, and for the transfer of these genes into plants, together with the recognition of the great agronomic potential of transgenic plants (Gasser and Fraley, 1989), has merited this burst of research activity.

Most of the research has been directed at genetic systems which are unique to plants, and are amenable to study by the techniques of molecular biology because of their high levels of gene expression. The systems studied include light regulated genes, particularly the highly expressed genes encoding the small sub-unit of ribulose-1,5-bisphosphate carboxylase and the chlorophyll a/b binding protein, seed protein genes, genes involved in plant-microbe interactions and genes responsive to adverse environmental conditions such as osmotic and anaerobic stress, and wounding (Kuhlemeier *et al.* 1987, Goldberg 1988). Today, the technology is available to clone any plant gene with a scorable mutant phenotype, in the absence of any biochemical information, by the methods of transposon tagging (Federoff *et al.* 1984, Martin *et al.* 1985, Schmidt *et al.* 1987), insertional mutagenesis using the T-DNA of *Agrobacterium tumefaciens* (Feldmann *et al.* 1989) or by chromosome walking (Meyerowitz *et al.* 1989). The application of these techniques to model plant species such as *Arabidopsis thaliana* (Finkelstein *et al.* 1988), and the identification and characterization of regulatory mutants (Meyerowitz *et al.* 1989), may provide a new phase in the understanding of plant gene regulation in the future.

Plant embryogenesis, the system under study here, is initiated by the fusion of sperm and egg nuclei in the embryo sac of the ovary. The resulting zygote undergoes a developmental program that generates the axis and cotyledons. The fusion of a second sperm nucleus with the diploid central nucleus of the embryo sac gives rise to the non-embryonic triploid endosperm. The axis contains

the shoot and root meristems that will determine the development of the mature sporophyte, whereas the cotyledons and endosperm are terminally differentiated organs whose function is to provide the germinating seedling with nutrients before the onset of photosynthesis (Raghavan 1976).

Plant embryos develop from a single cell, the fertilized egg cell, into a collection of different cell types, each performing a specialized function in an ordered spatial arrangement. All these cell-types contain the same genetic material, as emphasized by the capability of many cell types to dedifferentiate and regenerate into complete new plants (Steward 1967). The different cell types must therefore be generated through the differential expression of the same genetic information. The central question is what is it that regulates this differential expression?

At present very little is known about the factors that control the developmental program although embryogenesis in an algal species, *Fucus*, provides a convenient experimental system to study the early events of polarity and determination during development (Kropf *et al.* 1988). However, one particular set of genes that are under developmental control, and are highly expressed in the cotyledons or endosperm or both, have been studied in great detail. These are the seed storage protein genes.

Pernollet and Mossé (1983) described a series of conditions that must be satisfied before a protein can be considered a seed storage protein; such proteins must accumulate in a seed-specific manner in the cotyledon or endosperm tissues, they must be broken down during germination to provide an amino-acid and reduced-nitrogen source for the seedling plant and are found in relative abundance over other seed nitrogen compounds, they are localized in storage organelles of vacuolar origin called protein bodies, and they have a characteristic amino acid composition; in the case of legumes there is an abundance of nitrogen-rich residues and a dearth of sulphur amino acids (Higgins 1984). In addition to the storage proteins, seeds contain a battery of "house-keeping" proteins responsible for general cell metabolism.

The enormous interest in the seed storage protein genes stems from the great economic importance of the proteins they encode.

The seed storage proteins of cereal, legume and other crop plants, whether directly or indirectly, provide most of the protein component of the human diet (Payne 1983). Furthermore seed storage proteins are believed to be one of the best candidates for the successful application of genetic engineering for crop improvement (Croy and Gatehouse, 1985; Larkins, 1987; Higgins, 1984). Genetic engineering could be used to alter the storage proteins to improve nutritional quality or food processing properties. Foreign genes could be expressed in the seed to provide favourable phenotypes such as pest resistance, or novel, commercially valuable proteins could be produced in the seed as an alternative to production by microbial fermentation or mammalian cell culture. Indeed peptide hormones have recently been produced in transgenic *Brassica napus* seeds (Vandekerckhove *et al.* 1989). However, in order to realize the full potential for genetic engineering of seeds we must first have a thorough knowledge of the biochemistry and molecular biology of seed development.

Seed storage protein genes are also of purely scientific interest because they show differential expression in terms of tissue specificity, developmental stage and quantitative amount. They are, thus, a useful model system to study the way in which differential expression is regulated.

The large amount of research into the seed storage proteins and their genes has been the subject of many reviews (Boulter, 1981; Larkins, 1981; Higgins, 1984; Vol. 304B of Phil. Trans. R. Soc. Lond., pp275-407, 1984; Croy and Gatehouse, 1985; Gatehouse *et al.*, 1986; Goldberg *et al.*, 1989). Many genes and cDNAs of storage proteins have been cloned, sequenced and subsequently used as probes in the study of gene regulation at the transcriptional and mRNA levels. Functional analysis of *cis*-regulatory elements has been carried out for seed storage protein genes in a number of plant species (reviewed by Goldberg *et al.*, 1989). This is achieved by analyzing the expression, in transgenic plants, of homologous, or reporter gene constructs, whose 5′ or 3′ flanking sequences have been altered *in vitro*. *Trans*-acting factors have also been studied by investigating the *in vitro* interactions between DNA binding proteins from nuclear extracts and cloned DNA

fragments from the 5′ region of seed protein genes.

1.2. Regulation of eukaryotic gene expression.

1.2.1 The pathway of mRNA synthesis.

The activation of transcription of eukaryotic genes requires firstly the decondensation of the tightly packed chromatin structure followed by the interaction of regulatory proteins (*trans*-acting factors) with specific DNA sequences (*cis*-acting elements) (reviewed by Thangue and Rigby 1988, Hatzopoulos *et al.* 1988). These interactions regulate the rate of transcription of a given gene. Transcription of all genes encoding mRNAs is initiated by the binding of RNA polymerase II, followed by elongation of the nascent RNA chain and finally transcription termination.

Primary transcripts (nuclear pre-mRNAs) undergo the following extensive processing events in order to generate mature mRNAs.

(i) Capping.

The first nucleotide at the 5′-end of the message (corresponding to the transcription start) is capped by the addition of a guanosine residue, by a 5′-5′ triphosphate linkage, catalyzed by guanylyl transferase. The N7 position of this guanosine, and the 2′-O positions of the first and sometimes the second nucleotides, are then methylated by a methyltransferase to produce the final cap structure.

(ii) 3′ end processing.

In animals the 3′-end of the mRNA is generated by an endonucleolytic cleavage upstream from the transcription termination site, followed in most cases by the enzymatic addition of an average of 260 adenine nucleotide residues (polyadenylation). The site at which polyadenylation occurs is determined by the poly(A) signal, 10-30 bp 5′ to the cleavage site, with consensus sequence AATAAA (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981). There is also a conserved GT or T rich sequence immediately 3′ to the site (Gil and Proudfoot 1987). The plant poly(A) site, as in animals, is usually 27±9 bases downstream of the same AATAAA consensus poly(A) signal

(Joshi 1987). Ingelbrecht *et al.* (1989) have recently performed a functional analysis of the plant poly(A) site. Firstly, they demonstrated, using the reporter gene neomycin phosphoenolpyruvate transferase II (nptII) with and without the 3′ region from the octopine synthase gene, that a functional 3′ region is necessary for efficient expression in transgenic tobacco plants. Also a series of deletions in the 3′ region of the octopine synthase gene showed that both the AATAAA signal and a GT rich element, similar to those characterized in animal systems, are required for efficient expression in electroporated tobacco protoplasts. In addition, they showed that 3′ non-coding regions from several different plant genes linked to the same nptII reporter gene gave mRNA levels differing by up to 60-fold in transgenic tobacco; the 3′ region from a highly expressed ribulose bisphosphate carboxylase small sub-unit gene gave 60-fold higher expression than the 3′ region from chalcone synthase, a gene normally transiently expressed in response to UV illumination, and 15-fold higher expression than the 3′ region from the 2S seed storage protein gene from *Arabidopsis*. Such differences were speculated to be due to either differential efficiency of 3′ processing, or differential mRNA stability.

In animal systems any deviation from the consensus poly(A) signal has been shown to drastically reduce the efficiency of processing, and the variation ATTAAA, amongst others, occurs in 10% of known genes (Proudfoot and Whitelaw 1988). Thus, one way of regulating the mRNA level could be by use of poly(A) sites which are processed with different efficiency. In plants, there is greater variation in the signal sequence used; 54% of 46 putative poly(A) signals surveyed had a one base deviation from the consensus (Joshi 1987). It was shown that when human and animal genes were expressed in tobacco cells they showed aberrant processing (Hunt *et al.* 1987), so plant and animal signals, although similar, are not functionally interchangeable.

In many plant genes, including the seed storage protein genes, there are multiple poly(A) signals and often more than one signal is used (Dean *et al.* 1986), or the position of the poly(A) site from a single poly(A) signal may vary (Joshi 1987). The

differential use of poly(A) sites in some animal systems has been shown to allow more than one protein to be produced from a single gene (reviewed by Leff *et al.* 1986). The immunoglobulin μ constant region gene, by utilizing either a poly(A) site in the 3′ non-coding region or in an exon, generates two forms of mRNA by differential splicing patterns. One mRNA encodes a larger membrane bound protein and the other a smaller secreted protein. The two different mRNAs are produced in a cell-specific manner controlled by cell-specific poly(A) site selection (Proudfoot and Whitelaw 1988). The calcitonin gene demonstrates tissue-specific differential processing, which results in the predominance of calcitonin in thyroid tissue, whereas in brain tissues a calcitonin gene-related peptide (CGRP) is also produced. In this case, it is thought that two poly(A) sites are both used in thyroid but this tissue lacks the ability to splice the CGRP transcript, which consequently accumulates in the nucleus (Leff *et al.*, 1986).

An alternative theoretical role for differential selection of poly(A) sites could be the generation of different 3′ ends that alter the cytoplasmic stability of the resulting transcript (see discussion in section 4.2.3, and Vodkin *et al.*, 1983).

(iii) Splicing.

The vast majority of animal nuclear pre-mRNAs are composed of exons interrupted by intervening sequences (nuclear pre-mRNA introns) which may vary in size from 31 nucleotides (SV40 late transcript, Ghosh *et al.* 1978) to 105,000 nucleotides (dystrophin, Burghes *et al.* 1987). The few examples of intronless animal genes include some histone, heat shock and interferon genes. Plant genes tend to contain one or more introns, usually less than 600 nucleotides, although there are many examples of intronless plant genes including all the lectin genes characterized; pea lectin (Gatehouse *et al.* 1987), soybean lectin (Vodkin *et al.* 1983) and ricin (Halling *et al.* 1985), all maize zein storage protein genes characterized (Pederson *et al.* 1982), the 15 kDa seed protein gene from soybean (Fischer and Goldberg 1982), and some chlorophyll a/b binding protein genes (Cashmore 1984).

During the formation of mature mRNA, introns are excised and adjacent exons joined, by a series of cleavage and ligation reactions (splicing). In yeast and mammals the mechanism of splicing, within protein/RNA complexes known as spliceosomes, has been extensively studied using *in vitro* splicing extracts (reviewed by Sharp 1987). In contrast, very little information is available about the mechanism of splicing in plants; however, it has been demonstrated that a plant intron can be spliced in a HeLa nuclear splicing extract (Brown *et al.* 1986), although a human growth hormone transcript is not spliced in transgenic tobacco plants (Barta *et al.*, 1986). Plant nuclear pre-mRNA introns and their 3′ and 5′ splice site consensus sequences are more similar to those of animals than those of yeast. There are minor differences between plants and animals; the former have AU-rich sequences in the intron, more relaxed branch site criteria, and do not require a 3′-polypyrimidine tract (Brown, 1986; Wiebauer *et al.*, 1988; Goodall and Filipowicz, 1989).

An example of alternative splicing of a plant intron has recently been reported (Werneke *et al.*, 1989). This process results in the production of two polypeptides from one gene encoding rubisco activase.

(iv) mRNA editing.

It has been found, in some mammalian and protozoan systems, that individual bases in mRNA molecules can be substituted posttranscriptionally, to produce an uncoded mRNA nucleotide sequence (reviewed by Lamb and Dreyfuss 1989). This process was recently discovered to occur in wheat mitochondria by the conversion of cytidine residues to uridine in the mRNA encoding cytochrome c oxidase (Covello and Gray, 1989)

(v) Nucleocytoplasmic transport.

The nuclear pre-mRNAs (also called heteronuclear RNAs) exist in the nucleus as RNA:protein complexes called heteronuclear ribonuclear protein (hnRNP) particles. The proteins associated with the nuclear pre-mRNAs are different to those associated with the mature mRNA in the cytoplasm, where the mRNA is found in

cytoplasmic messenger ribonuclear protein (mRNP) particles (reviewed by Dreyfuss 1986). Thus, at some point in the transport of processed mRNA from the nucleus to the cytoplasm, a poorly understood process believed to take place through the nuclear pores, the nuclear proteins must be stripped from the mRNA and replaced by their cytoplasmic counterparts.

Once mRNP has reached the cytoplasm it can then be found in the non-polysomal fraction as small mRNP particles, or in the free or membrane bound polysomal fractions. The non-polysomal fraction may contain stored mRNAs such as those synthesized during late plant embryogenesis which are stored in the seed until germination (Payne 1976), or the maternal mRNAs stored in sea urchin oocytes until fertilization and subsequent development (Denny and Tyler 1964).

1.2.2. The pathway of mRNA degradation.

In comparison to the vast knowledge that has been accumulated on the mechanisms and control of mRNA biosynthesis, very little is known about the processes involved in mRNA degradation.

Although there are a number of well explored examples where regulation of gene expression has been observed at the level of differential mRNA degradation (see section **1.2.3.**), only recently, due to the advent of cell-free assays for mRNA degradation (section **1.3.2.**), has information become available on the mechanisms of mRNA degradation in the cytoplasm. Information concerning turnover of nuclear pre-mRNAs in the nucleus is virtually nonexistent, although it is known from studies on the complexity of nuclear and cytoplasmic mRNAs in higher eukaryotes that a large proportion of hnRNA is under-represented in, or absent from, the cytoplasm; in animals the complexity of hnRNA is 4-10 fold higher than the complexity of the mRNA and only 28% of tobacco leaf hnRNA is represented in polysomal mRNA (Goldberg *et al.* 1978), and Morton *et al.* (1983) estimated that only 50% of nuclear poly(A)$^+$ RNA from pea embryos at 9 DAF is present in the polysomal poly(A)$^+$ fraction. This is due, in part, to loss of complexity due to processing of hnRNA, but also points to an extensive turnover of hnRNA in the nucleus. In contrast, in lower

eukaryotes such as protists and fungi, no difference in the complexity of hnRNA and mRNA was observed (Goldberg *et al.*, 1978).

Cytoplasmic mRNA degradation is mediated by the interaction of nucleases and mRNP particles, and this process may be affected by the cellular compartmentalisation of these factors. The nucleases may recognize specific structural motifs within the mRNP particles either at the level of primary or secondary mRNA structure, or the tertiary structure within the protein:RNA complex.

It is believed that the unusual terminal structures of mRNAs, namely the cap and the poly(A) tail, serve to protect the mRNA from attack by exonucleases (Brawerman 1987). The use of chimeric gene constructs has enabled other structural motifs involved in the regulation of mRNA stability to be identified (reviewed by Brawerman 1989). For example the 3'-stem-loop of histone mRNA couples RNA degradation to the cycle of DNA replication (Levine *et al.* 1987) and may act as a target site for nuclease attack. Also the 3' stem-loop of the transferrin receptor mRNA promotes rapid degradation of this mRNA in the presence of iron (Müll-er and Kühn 1988). 3' AU rich sequences, including the AUUUA motif, are also implicated in regulation of stability, and seem to be required for the destabilization of some transiently expressed mRNAs such as *c-fos* (Wilson and Treisman 1988). The 5' region of *c-myc* and histone mRNAs have also been shown to be involved in the regulation of mRNA stability, possibly by interaction with other regions within the mRNA molecule (Brawerman 1987). Regulation of mRNA stability by the proteins encoded by the same mRNA (autoregulation) has been reported in two systems; Yen *et al.* (1988) have shown destabilization of β-tubulin mRNA by interaction of the free β-tubulin pool with the nascent N-terminus of β-tubulin as it is synthesized on polysomes, and Ross (1989) has claimed that addition of histones to polysomes *in vitro* destabilizes histone mRNA. Other indications that mRNA stability and translation are closely linked are provided by the effects of nonsense and frameshift mutations. Jofuku *et al.* (1989) suggested that a frameshift mutation in the soybean Kunitz inhibitor gene, that prevents accumulation of mRNA, operates by reducing mRNA stability. Also Daar and Maquat (1988) found that all *in vitro*

mutagenized triosephosphate isomerase alleles that had nonsense or frameshift mutations showed lower mRNA stability in mouse L-cells.

Recently Bernstein *et al.* (1989) proposed a general model to explain the differential rates of degradation of specific mRNAs based on experiments using cell-free systems derived from human erythroleukemia cell lines (see section **1.3.2.**). They discovered that the rate limiting step in the degradation of a specific mRNA was the rate at which the poly(A) tail is degraded. Once the poly(A) tail is shorter than approx. 25-32 residues the remaining mRNA molecule is rapidly degraded by a 3'-5' exonuclease activity associated with polysome preparations. They proposed that the half-life of a mRNA molecule is directly related to the half life of the poly(A) tail. Furthermore, they demonstrated that the binding of a component of mRNP, the 72 kDa poly(A)-binding protein (PABP), along the length of the poly(A) tail, prevented poly(A) tail degradation. Since from electron microscopy studies it has been estimated that a single PABP occupies approx. 25 nucleotides it was proposed that when the poly(A) tail is less than 25-32 nucleotides it can no longer bind a single PABP and is opened for attack by nucleases. In this model, differential stability of mRNA is achieved by a differential affinity of the poly(A) tail for PABP as mediated by either competing PABP sites elsewhere on the mRNA, such as the 3' AU-rich sequences, or by interaction of specific regulatory proteins. This model has recently been contradicted by the results of Sachs and Davies (1989). These authors used yeast mutants deficient in the PABP to show that PABP is *required* for poly(A) shortening and for the initiation of translation *in vivo*, and suggested that PABP protected poly(A) from non-specific nucleases in the *in vitro* extracts of Bernstein *et al.* (1989). Sachs and Davies (1989) also suggested, in agreement with Brawerman (1981), that mRNA degradation may be initiated by a specific cleavage resulting in poly(A) *removal* rather than a gradual poly(A) *shortening*. Furthermore, there is also evidence, reviewed by Brawerman (1981), that suggests that the size of the poly(A)-tail has no bearing on the stability of an mRNA molecule; this is based on the kinetics of mRNA decay which suggests that it is a random process that is not dependent on the

age of an mRNA molecule.

In another model proposed by Wilson and Treisman (1988) the poly(A) tail base-pairs with the AU-rich regions and the mismatched regions are then cleaved by polysome-associated nucleases. It is suggested that the extent of base-pairing determines the mRNA stability. The role, then, of the poly(A) in mRNA stability and translation is still controversial.

The only plant PABPs known to be reported are those isolated from germinating pea seeds (Sieliwanowicz 1987). These include a 60 kDa PABP which is not present in dry seeds but appears on germination. The function of this protein is unclear, although it is suggested that it may be involved in the translational activation of mRNAs stored in dry seed. There is little information concerning the mechanism of mRNA degradation in plants although it was recently shown that the addition of a poly(A) tail of 25 residues or greater stabilized *in vitro* synthesized β-glucuronidase transcripts in electroporated tobacco protoplasts (Gallie *et al.*, 1989).

1.2.3. The points of regulation in the gene expression pathway.

The rate of synthesis of a specific protein in a cell is generally proportional to the corresponding level of its mRNA, although there are also many examples of translational and posttranslational control (Gilbert, 1988; Hershey *et al.*, 1986). The cellular mRNA level is a balance between the rates of nuclear transcription, processing and export, and the rates of cytoplasmic degradation. Much attention has been aimed at determining at which steps the final level of mRNA is regulated. It is now generally accepted that the major rate-limiting step in mRNA accumulation is the initiation of transcription (Darnell, 1982; Hatzopoulas *et al.*, 1988), as mediated by *trans* and *cis* acting factors. In spite of this, over the last few years, it has become increasingly obvious that regulation of mRNA level by transcriptional termination (Proudfoot and Whitelaw, 1988) and posttranscriptional processes are also very important.

Posttranscriptional regulation in this context encompasses regulation at the levels of hnRNA processing and degradation in

the nucleus, nucleocytoplasmic transport and cytoplasmic degradation of mRNA. It does not include translational events and beyond although it must be recognized that mRNA degradation and translation may be closely linked (see section **1.2.2.**).

Posttranscriptional regulation of mRNA levels can be classified into two types: different mRNAs could reach different steady-state mRNA levels in the same cell although their genes are transcribed at similar rates, or differential accumulation of the same species of mRNA, either under different conditions, or in different cell/tissue types, could occur, even though its transcription rate remained constant.

Evidence for the former type has been obtained by Carniero and Schibler (1984) who reported that the steady-state mRNA levels of the constitutive or "house-keeping" genes of mouse L-cells correlate with mRNA half-lives rather than with transcription rates. Also Cabrera *et al.* (1984) concluded that in sea urchin embryos the turnover of cytoplasmic mRNA was a major variable in determining the level of expression of embryo genes. A similar type of regulation of mRNA levels has been observed in soybean cotyledons in which *in vitro* transcription rates of seed protein genes and non-seed protein genes in nuclei isolated from 70 DAF embryos are similar, but their mRNA levels vary up to 10,000-fold (Walling *et al.* 1986).

In the case of the latter type, an increase in mRNA half-life could accelerate the induction of mRNA levels in response to a particular stimulus (environmental, physiological or developmental), for example the induction of vitellogenin mRNA in *Xenopus* liver cells by the hormone estrogen is caused by a 20-fold increase in transcription rate but also a 30-fold increase in mRNA half-life (Brock and Shapiro, 1983a; 1983b). Alternatively, an increased turnover of mRNA would be an advantage if a rapid down-regulation of expression is required in response to a stimulus. In this case, a reduction in the transcription rate would more rapidly result in a decrease in cytoplasmic mRNA levels. Such a strategy has been suggested for the down regulation of oat phytochrome mRNA levels by light. In this case the *in vitro* transcription rate in isolated nuclei rapidly falls by three fold

in response to light treatment but the cytoplasmic mRNA level drops by 10-50 fold. Although no direct measurements of mRNA half-life were made, the implication is that light not only decreases the transcription rate but that it also increases the rate of mRNA degradation (Quail *et al.*, 1986). Using the same experimental approach, a similar observation was reported concerning the down-regulation of legumin mRNA in developing pea seeds in response to sulphur deprivation (Evans *et al.*, 1985; Beach *et al.*, 1985).

Other examples of regulation of mRNA degradation in animals, including regulation of the β-tubulin, histone, casein and prolactin genes amongst others, have been the subject of several recent reviews (Raghow, 1987; Shapiro *et al.*, 1987; Ross, 1989; Brawerman, 1989; Marzluff and Pandey, 1988).

There are relatively few reports of regulation of gene expression by posttranscriptional nuclear events although Berkvens *et al.* (1988) were able to conclude from measurements of "run-on" transcription, steady-state mRNA levels in both the nuclear and total RNA fractions, and cytoplasmic mRNA stability, that a 20-fold difference in adenosine deaminase activity between two different human cell lines was controlled at this level. Also, regulation of gene expression at the level of nucleocytoplasmic transport has been reported for the down regulation of 2B5 mRNA in the retanoic acid induced differentiation of human HL60 cells (Graham and Birnie, 1988). In this system, the first reported to show this type of regulation, although transcription rate is increasing, and the transcripts are polyadenylated and apparently normally processed, they accumulate in the nucleus and the cytoplasmic mRNA level declines. In the case of light-regulated plant genes, Sagar *et al.* (1988) measured nuclear and cytoplasmic mRNA levels in pea leaves under various light conditions, and found that each of 6 specific mRNAs exhibited a characteristic "nucleocytoplasmic partitioning coefficient". They concluded that posttranscriptional events regulated light-induced mRNA accumulation by either: (a) differential nucleocytoplasmic transport efficiency or, (b) differential degradation of mRNA in the nuclear or cytoplasmic compartments.

1.3. Methods for studying the regulation of gene expression.

In order to identify the regulatory steps in the accumulation of a specific mRNA we must have methods of measuring the rates of particular processes in the expression pathway. Comparisons of the rates of mRNA synthesis and degradation and measurements of steady-state levels of mRNAs in nuclear and cytoplasmic or total RNA allow conclusions to be drawn about the points of regulation. These methods are outlined below.

1.3.1. Transcriptional regulation.

The simplest approach to measuring the rate of synthesis and degradation of a specific mRNA is to measure the time course of changing mRNA levels. In this method some form of stimulus is used to cause a change in the steady-state level of the mRNA, and then the kinetics of the approach to the new steady-state level are determined. Under these circumstances the rate of mRNA accumulation, dC/dT (molecules mRNA per cell/min), is determined by the zero-order rate constant of synthesis, k_s (molecules mRNA/cell/min), and the first-order rate constant of degradation, k_d.$(min)^{-1}$ according to the expression:

$$\frac{dC}{dt} = k_s - k_d C_t \quad \text{(Kafatos, 1972; Guyette } et\ al.\text{, 1979)}$$

where C_t represents molecules mRNA per cell at time t. A plot of dC/dt against C_t provides values for k_s (y-intersect) and k_d (slope).

In order to measure k_s and k_d for mRNAs that are not changing but are at a constant steady-state level, the kinetics of radioactive labelling of mRNA must be determined; for example, Cabrera *et al.* (1984) labelled sea urchin embryos continuously with ^{3}H-guanosine and then measured the specific activity of the GTP precursor pool, S_t (DPM/mol nucleotide), and the incorporation of radioactivity into specific mRNAs, C_t^r (DPM specific mRNA/cell) throughout the labelling period. In this method k_s is dependent on S_t and it is possible to compute estimated values for k_d and k_s from the following expression:

$$\frac{dC^r}{dt} = k_s S_t - k_d C^r_t$$

An alternative method of measuring transcription rates is to label nascent transcripts *in vivo* by a pulse-labelling period sufficiently short that no significant processing or degradation of primary transcripts can occur. The labelled nuclear RNA is then measured by hybridization to specific DNA clones. This approach has proved successful in mammalian cell culture where a 5 min labelling period using ^{3}H-uridine was sufficient to allow measurement of labelled globulin nuclear RNA (Ganguly and Skoultchi, 1985); similarly a 30 min pulse-label allowed Guyette *et al.* (1979) to measure transcription rate of casein mRNA in mammary gland organ culture.

Labelling of nascent transcripts can also be achieved in isolated nuclei. In this method, first described by Cox (1976) and first applied to plant nuclei by Gallagher and Ellis (1982), nuclei are isolated and incubated under physiological conditions in the presence of ^{32}P-labelled nucleoside triphosphates. In this method, also known as "run-on" or "run-off" transcription, transcripts initiated *in vivo* are elongated in the isolated nuclei and become labelled. Hybridization of the transcripts to DNA clones then gives a measure of the density of preinitiated transcripts on a given gene. It is generally accepted from studies on animal nuclei that little or no initiation occurs *in vitro* (Weber *et al.*, 1977; Groudine *et al*, 1981) and that the transcription in isolated nuclei gives a good estimation of the relative transcription rates *in vivo* (reviewed by Darnell 1982). However, in at least one case, transcription in isolated nuclei gives different results to *in vivo* pulse-labelling (Ganguly and Skoultchi 1985). In this example a 20-fold increase in mouse globin *in vivo* transcription rate was measured as a 5-fold increase in isolated nuclei. Such differences were speculated to be due to loss of factors or changes in chromatin structure caused by the procedure for isolation of nuclei.

Pulse labelling and kinetic labelling techniques *in vivo* are the ideal method for measuring transcription rates but are not

always applicable due to lack of suitable cell or organ culture systems and are only suitable for studying highly transcribed genes so that sufficient label can be incorporated. Transcription in isolated nuclei is a more generally applicable technique and is more sensitive, although it is open to the criticism that it may not reflect the *in vivo* situation. In higher plants, there are no reports known to the author of transcription rates measured by pulse or kinetic labelling techniques, or from measurements of kinetics of approach to steady-state levels. However, many studies using transcription in isolated nuclei as the only method of measuring transcription rates have been reported (Gallagher and Ellis, 1982; Evans *et al.*, 1984; Walling *et al.*, 1986; Hagen and Guilfoyle, 1988; Schoffl *et al.*, 1987; Chappell and Chrispeels, 1986; Beach *et al.*, 1985; Mosinger *et al.*, 1987; Sørensen *et al.*, 1989). These workers make the assumption, which is correct in the majority of animal systems, that the behavior of isolated nuclei reflects the *in vivo* situation.

1.3.2. Posttranscriptional regulation.

It is technically difficult to measure the rate of posttranscriptional nuclear events and the little that is known about their role in regulating the rate of entry of mRNA into the cytoplasm has been deduced from comparisons of nuclear steady-state mRNA levels with transcription rates, cytoplasmic steady-state levels and cytoplasmic turnover (Berkvens *et al.*, 1988; Graham and Birnie, 1988).

The rate of degradation of mRNA in the cytoplasm is more amenable to study and various techniques have been used to measure mRNA half-lives in animal systems. The kinetics of approach to steady-state and kinetic labelling as described in section 1.3.1 can be used. Also pulse-labelling of cell or organ culture RNA with radioactive nucleosides for a short time (relative to the half-life of the mRNA under study), followed by a "chase" with cold nucleosides, has also been used successfully as exemplified by Brock and Shapiro (1983b). Another often used alternative is to uncouple cytoplasmic degradation and transcription by the application of transcription inhibitors such as actinomycin D,

followed by measurement of the decline in cytoplasmic mRNA.

More recently, *in vitro* mRNA degradation systems have been developed from animal cells in which the rank order of mRNA half-lives is the same as that measured *in vivo* (see also section 1.2.2). The original system developed by Ross and Kobs (1986) consisted of polysomes isolated from a human erythroleukemia cell line incubated in conditions previously described for *in vitro* translation reactions. The half-lives of the endogenous polysomal mRNAs could then be assayed by Northern blotting and S1-mapping. Sunitha and Slobin (1987) were able to demonstrate differential mRNA degradation in a system consisting of the 40-80S mRNP particles from Friend erythroleukemia cells whilst Pei and Calame (1988) used a crude cell extract from mouse plasmacytoma cells from which the nuclear, mitochondrial and lysosomal fractions had been removed by centrifugation. Also, as part of their investigations into the translation of TMV RNA, Sleat *et al.* (1988) measured the half-lives of ^{32}P-labelled *in vitro* synthesized mRNAs with different 5′ leader sequences in wheat germ translation extracts.

These *in vitro* systems have been used to determine the mechanism of degradation (Ross and Kobs, 1986; Brewer and Ross, 1988; Bernstein *et al.*, 1989), to identify an enzyme responsible for differential histone mRNA degradation (Ross *et al.*, 1987; Peltz *et al.*, 1987), and, by using chimeric DNA constructs in conjunction with *in vitro* transcription systems, to identify mRNA elements involved in differential degradation (Pei and Calame, 1988).

An alternative method that has been used in the investigation of mRNA stability in plant systems is the introduction of ^{32}P-labelled *in vitro* synthesized transcripts into protoplasts (Gallie *et al.*, 1989). Such a system could also be used to investigate the mechanism of degradation.

1.4 The structure and assembly of pea storage proteins and their genes.

Most of the work on the seed proteins of the Leguminosae has been done on four species: *Pisum sativum* (garden pea), *Vicia faba* (broad bean), *Glycine max* (soybean) and *Phaseolus vulgaris* (French

or common bean). In these species the major storage proteins are extracted in the globulin fraction, the fraction defined by Osbourne (1924) as that "soluble in dilute salt solutions at neutral pH". Legume globulin can be separated into the 7S vicilin and the 11S legumin fraction by density gradient centrifugation (Danielsson, 1949). In pea the globulins represent 60-70% of the total seed proteins. The proportions of the two fractions present varies between the different legume species and also between the different varieties of pea (Casey *et al.*, 1982, Schroeder, 1982).

1.4.1. The pea legumin fraction.

The proposed model for the structure of pea legumin (Croy *et al.* 1979), like that of broad bean (Wright and Boulter 1974) and soybean (Nielsen *et al.* 1989) is a hexameric protein of 360 to 410 kDa. It consists of six sub-unit pairs, each pair having an acidic (α) sub-unit (pI 4.8-6.2) of approximately 40 kDa linked by a single disulphide bond to an approximately 20 kDa basic (β) sub-unit (pI 6.2-8.0). These sub-units are homologous to the A and B polypeptides of soybean (Casey *et al.* 1981).

Each sub-unit pair is synthesized as a precursor, as demonstrated by *in vitro* translation of pea seed mRNAs (Croy *et al.*, 1982; Domoney and Casey, 1984), and subsequently proteolytically cleaved. Thus the α and β sub-units of each pair are specifically associated in the mature protein. Five classes of sub-unit pair have been identified by Matta *et al.* (1981), by 2-dimensional gel electrophoresis, and designated L1-L5. The sub-units show heterogeneity with respect to both charge and size. The heterogeneity is more pronounced for the α sub-units than for the β sub-units thus indicating greater evolutionary constraints on the structure of the β sub-units (Domoney *et al.* 1986a). The hexameric protein shows at least three forms on non-dissociating PAGE, each consisting of a non-random selection of several types of sub-unit pair (Matta *et al.* 1981).

The current understanding of the identity of the sub-unit pairs is as follows (see also Fig.1):

On the basis of relative protein abundance in mature seed the L4 class, running at 54 kDa in non-reducing gels, constitute the

"major" sub-unit pairs. They are synthesized as 60 kDa precursors (Croy *et al.* 1982) and are encoded by the *legA* gene family (Gatehouse *et al.* 1988).

The "minor" sub-unit pairs, L1-L3 and L5, were designated "big" and "small" respectively by Matta *et al.* (1981), because, in non-reducing PAGE, L1-L3 run as 55-58 kDa bands and L5 runs at 35 kDa. This classification is confusing because it is now known that the L2 "big minor" sub-unit pair is synthesized as a 63 kDa precursor (Domoney and Casey 1984) and is encoded by the *legJ* gene a closely homologous gene, or both (Gatehouse *et al.* 1988); the L5 "small" minor sub-unit pair, however, is synthesized as an 80 kDa precursor (Domoney and Casey 1984) and subsequently cleaved into three polypeptides - a basic sub-unit of 20.7 kDa and two acidic sub-units of 24.5 and 26.5 kDa (John Gatehouse, unpublished protein sequence data compared to cDNA pAD9.2 [Delauney 1984]). The 24.5 kDa acidic sub-unit is disulphide bonded to the basic sub-unit, forming the protein band running at 35 kDa observed on non-reducing PAGE; however, the 26.5 kDa acidic sub-unit is held by non-covalent interactions only and is released from the protein under these conditions and was previously thought to be a non-legumin contaminant (Matta *et al.* 1981).

cDNA clones encoding the L5 sub-unit pair have been isolated (pCD32; Domoney and Casey 1984, pAD9.2; Delauney 1984) and the corresponding gene has been designated *legS* (Croy *et al.* 1988), although no genomic clones have been characterized.

The protein sequence of L1 has recently been reported (March *et al.* 1988) and found to be distinct from *legA, J* or S type products. L1 is therefore probably a product from a forth class of legumin genes for which cDNA or genomic clones have not yet been isolated. The hypothetical gene(s) is designated *legX* (Croy *et al.* 1988).

No protein sequence is available for the L3 sub-unit pair, although it has been tentatively assigned as the product of the *legK* gene (Croy *et al.* 1988).

The legumin polypeptides are synthesized on membrane bound polyribosomes and are then assembled into an intermediate 7S trimeric form in the endoplasmic reticulum (Chrispeels *et al.*

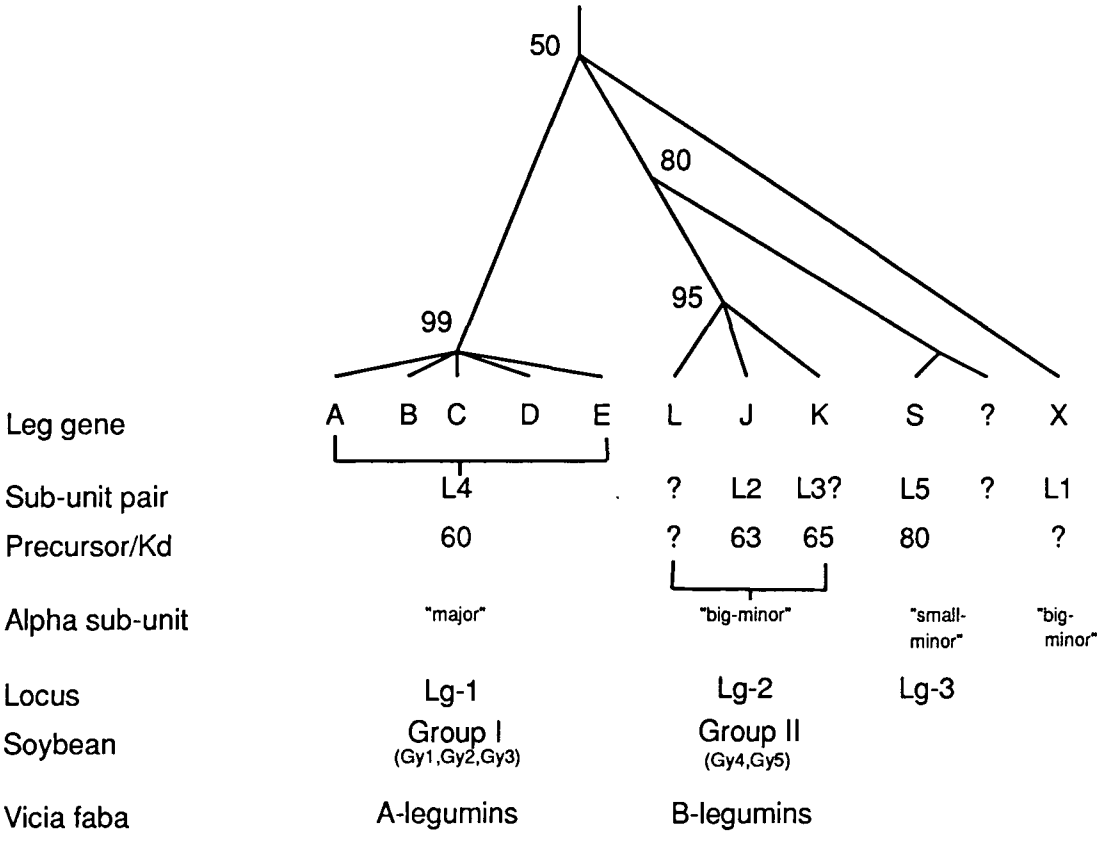


Figure 1: The legumin gene family.

Summary of the legumin gene family of *Pisum sativum* and its protein products. The numbers at branch points refer to approximate percentage nucleotide homology in the coding regions.The homologous gene families of Soybean and *Vicia faba* are also indicated. See text for details.

1982a). These trimers are then transported to the protein bodies where they are rapidly proteolytically processed and then slowly assembled into the final hexameric form (Chrispeels *et al.* 1982b). The legumin amino acid sequences predicted from gene and cDNA sequences reveal the presence of a leader sequence (Lycett *et al.* 1984) that is probably co-translationally removed.

1.4.2. The organization, inheritance and evolution of the legumin genes.

The genes encoding the 11S proteins of members of the Leguminosae show considerable evolutionary conservation. They can be divided into two main types that are conserved between pea, soybean and field bean as outlined below.

In pea, genetic variation in the banding pattern of legumin α sub-units on SDS-PAGE (Matta and Gatehouse 1982, Matta *et al.* 1981, Davies 1980) and restriction fragment length polymorphisms using three classes of legumin cDNA probes (Domoney *et al.* 1986b) has allowed the legumin genes to be mapped to three distinct loci, *Lg*-1, *Lg*-2 and *Lg*-3.

Lg-1 is the locus of the "major" legumin class, encoded by the *legA* gene sub-family, and maps 11, 7, or 17 map units from the *r* locus on chromosome 7 as reported by Domoney *et al.* (1986b), Davies (1980) or Matta and Gatehouse (1982), respectively. The *legA* sub-family is estimated to contain five tightly clustered genes four of which are in direct tandem repeat (Domoney and Casey 1985, Casey *et al.* 1986). Four of these genes, *legA* (Lycett *et al.* 1984), *legB* and *C* (Lycett *et al.* 1986, John Gatehouse, unpublished) and *leg D* (Bown *et al.* 1985) have been isolated from a genomic library and sequenced. These four genes show approximately 99% nucleotide sequence homology within their coding regions (Fig1). *legA* has been shown to be faithfully expressed in transgenic tobacco plants (Ellis *et al.* 1988), and polypeptide sequence data has shown that sub-units encoded by *legA* and *legB* or *legC* genes are present in the seed. The polypeptide sequence data cannot distinguish between *legB* or *legC* so is not known whether or not *legC* is expressed, however, the presence of a transposon like structure in its 5′ flanking region may affect its expression

(Shirsat 1988). *legD* was shown to be a pseudogene containing two in frame stop codons, other frame shift errors and minor deletions when compared to *legA*. No mRNA could be detected for this gene (Bown *et al.* 1985).

The *Lg*-2 locus maps 7 units from the *a* locus of chromosome 1 (Domoney *et al.* 1986b) and is estimated to contain three genes (Casey *et al.* 1986) belonging to the *legJ* sub-family encoding the "minor" legumin 63-65 kDa precursors (Fig1). Within this locus the *legK* and *legJ* genes lie in direct tandem repeat separated by 6 kb. The complete *legJ* gene and the 3′ region of the *legK* gene have been sequenced (Gatehouse *et al.* 1988), these genes are approximately 94% homologous to each other but are only 50% homologous to members of the *legA* sub-family.

The *Lg*-3 locus also maps close to the *a* locus and contains one or two genes (Domoney and Casey 1985) homologous to the cDNA clones pCD32 and pAD9.2. These genes, which have not yet been cloned, have been designated the *legS* sub-family (Croy *et al.* 1988). The *legS* cDNAs show 80% homology to the coding regions of members of the *legJ* sub-family and 50% homology to coding regions of the *legA* sub-family members (John Gatehouse, unpublished). The three classes of legumin cDNA-derived amino acid sequences show a high degree of variation in a polar region just to the N-terminal side of the α-β processing site. This region is known as the helical insert and is expected to be on the outside of the protein molecule. The helical insert is 56, 27 and 100 amino acids long in *legA*, *legJ* and *legS*, respectively (Domoney *et al.*, 1986a).

Glycinin (the legumin protein of *Glycine max* L. (soybean)), has five "major" α sub-units each of which can be assigned, from comparisons of DNA and protein sequences, as the product of one of each of the five glycinin genes designated Gy_1 to Gy_5 (Nielsen *et al.* 1989). From their sequences these five genes can be divided into two groups. Gy_1, Gy_2, and Gy_3 (Sims *et al.*, 1989; Thahn *et al.*, 1989; Cho and Nielsen, 1989) constitute group I (Nielsen *et al.* 1989), which is homologous to the pea *legA* sub-family and the A-legumins of field bean. Gy_4 and Gy_5 are classified as group II glycinins and they are homologous to the *legJ* sub-family of pea, and the B-legumins of field bean (Bäumlein *et al.*, 1986; Gatehouse

et al., 1988) (Fig1). The homologies within these two types of legumin gene sub-families suggests that the divergence of a group I and group II gene preceded speciation of *Pisum sativum, Glycine max* and *Vicia faba*.

With respect to intron/exon structure, all glycinin genes, both group I and group II, contain three introns. The pea group I legumin genes (*legA* subfamily) also contain three introns in similar positions. However, the group II legumin genes of pea (*legJ* and *legK*) and *Vicia faba* (LeB4) contain only two introns; **the first intron is absent.** The presence or absence of intron 1 agrees with the phyllogenetic relationships of these three species since pea and *Vicia* are more closely related and soybean is more distantly related.

Restriction fragment length polymorphisms and genetic segregation analysis have shown the glycinin genes to be arranged in four independently segregating genetic loci (Cho *et al.* 1989b). At one locus, Gy_1 and Gy_2 are arranged as a direct tandem repeat separated by 1.5 kb (Nielsen *et al.* 1989). The other three loci each contain one of the genes Gy_3, Gy_4 and Gy_5.

The fact that there are two loci for each of the group I and II soybean legumins, whereas there is only one locus for each of the two groups in pea (a true diploid), provides evidence that soybean is a stable tetraploid with a diploidized genome (Cho *et al.* 1989). The two 42 kb homoeologous domains containing the Gy_1/Gy_2 pair and the Gy_3 gene (ie. the two group I loci) also support the theory that these two loci have been generated from homoeologous chromosomes (Nielsen *et al.* 1989).

1.4.3. The pea vicilin fraction.

The pea vicilin fraction contains two separable components, the 140-170 kDa vicilin fraction and the 210-280 kDa convicilin fraction (Gatehouse *et al.*, 1981; Croy *et al.*, 1980a). On SDS-PAGE the 140-170 kDa fraction is shown to consist of 50, 33, 19, 16, 13.5 and 12.5 kDa sub-units. This heterogeneity is believed to arise from processing of preprovicilin precursors, and from glycosylation. *In vivo* pulse-labelling, *in vitro* translation of mRNA and polysomes, and hybrid release translation of vicilin cDNA

clones have all shown that vicilin is synthesized as precursors of 47 and 50 kDa (Croy *et al.* 1980b, Delauney 1984). Pulse-chase experiments have also demonstrated that the 33, 19, 13.5 and 12.5 sub-units are derived from these precursors. The 16 kDa sub-unit is a glycosylated version of the 12.5 kDa sub-unit, and the 50 kDa sub-unit is a glycosylated product from the 50 kDa precursor (Davey *et al.* 1981, Davey and Dudman 1979). As with legumin, the vicilin precursors are synthesized on membrane bound polysomes and contain N-terminal leader sequences. Leader sequences have been shown to be co-translationally removed by pulse-chase labelling experiments (Higgins and Spencer 1981, Chrispeels *et al.* 1982a). Further pulse-chasing shows that assembly of 7S trimers and glycosylation of specific polypeptides occurs in the endoplasmic reticulum before the 7S trimers are transported to the protein bodies (Chrispeels *et al.* 1982b). Proteolytic cleavage of the trimer sub-units then occurs relatively slowly (6-12 hours) in the protein bodies. The trimers retain their overall structure by non-covalent interactions only; no sulphur amino-acids are present so disulphide bonding is precluded.

A model for the idealized vicilin precursor polypeptide has been proposed (Lycett *et al.*, 1983; Gatehouse *et al.*, 1983) consisting of three regions called α, β, and γ running in that order from the N-terminus to the C-terminus. The consensus amino-acid sequence Gly-Lys-Glu-Asn is present immediately prior to the α-β and β-γ cleavage sites. These sites, as determined by hydrophobicity plots are considered to be in hydrophilic surface regions of the trimeric protein (Croy and Gatehouse 1985). It was suggested, on the basis of comparisons of cDNA and polypeptide sequences, that there were at least three types of precursor that were proteolytically processed to completion in the following ways; A, no cleavage, B, cleavage at the β-γ site only or C, cleavage at both α-β and β-γ sites (Lycett *et al.* 1983). There is now evidence that a single precursor, the product of a vicilin gene cloned from the *Vc-4* locus and transferred into tobacco, undergoes partial processing at each of the two sites (Higgins *et al* 1988).

The convicilin fraction consists of trimers or tetramers of an

approximately 70 kDa polypeptide which is synthesized as a precursor of approximately the same size (Croy *et al.* 1980a, Domoney and Casey 1983). The possibility that some protein species may contain both vicilin and convicilin sub-units has not been excluded. The vicilin and convicilin proteins are immunologically related and a comparison of vicilin cDNA and convicilin gene sequences reveals that they are 79% homologous and that convicilin differs from vicilin by an insertion of 121 amino-acids near the N-terminus of the protein (Bown *et al* 1988). The convicilin genes should, therefore, be regarded as a subfamily of the vicilin gene family.

1.4.4 The organization and inheritance of the vicilin genes.

Genetic mapping has shown that pea contains five vicilin loci, named *Vc-1 to Vc-5*, and a single convicilin locus, *Cvc* (Ellis *et al.* 1986). From Southern blotting of genomic DNA the *Cvc* locus is thought to contain two genes, in both pea cultivars "Feltham First" and "Dark Skinned Perfection", one of which, *cvcA*, from "Dark Skinned Perfection", has been cloned and sequenced (Bown *et al.* 1988).

The pea vicilin gene family forms the largest, and least well defined, of the pea seed storage protein gene families. Domoney and Casey (1985) have estimated that there are 5-7 and 4-6 vicilin genes encoding the 47 and 50 kDa precursors, respectively. Of the five vicilin loci, *Vc-1 to Vc-5*, only *Vc-1* to *Vc-3* have been mapped (Ellis *et al.* 1986); they all map close to the *r* locus on chromosome 7. Ellis *et al.* (1986) isolated cDNA and genomic clones representing four of the five loci. Two genes from locus *Vc-4*, of the class encoding a 50 kDa precursor, have been cloned and sequenced. The first, *vicB*, contains an insertion 12 amino-acids into exon 6, and is probably not expressed (Lavasseur 1988). The second gene is fully functional as demonstrated by its faithful expression in transgenic tobacco plants (Higgins *et al.* 1988). A *vicC* gene of a class encoding a 50 kDa precursor, but only approximately 85% homologous to *vicB*, and lying at locus *Vc-5*, has also been partially sequenced. This gene is unusual in that it has an in-frame stop codon after only 28 residues which may render it

non-functional, although it may be transcribed since a homologous cDNA has been isolated (John Gatehouse, unpublished). Locus *Vc-2* is thought to contain genes that encode the 47 kDa precursors. The *vicJ* gene from this locus has been sequenced but it may not be expressed because of an insertion, similar to that of *vicB*, near its 3′ end (D. Bown and J.A. Gatehouse, unpublished). The *Vc-4* locus has been estimated to contain a subfamily of at least 4 genes of greater than 95% homology (Higgins *et al.* 1988). As with the legumin gene families (Domoney *et al.* 1986b) the homology of genes within a locus exceeds the homology between loci (Ellis *et al.* 1986); the homology between cDNAs at the vicilin loci *Vc-2*, *Vc-4* and *Vc-5* is approximately 85%.

1.4.5. Pea lectin.

Lectins have been defined as proteins or glycoproteins, of non-immune origin, that bind to sugars and agglutinate cells (Goldstein *et al.* 1980). They are widely distributed in nature but their most abundant source is the seeds of legumes. The function of the seed lectins is uncertain although they have been implicated in insect (Gatehouse *et al.*, 1984) and nematode (Jeyaprakash *et al.*, 1984; Marban-Mendoza *et al.*, 1987) resistance, and in the specificity of the *Rhizobium*-legume symbiosis by interaction with carbohydrates on the bacterial surface (Diaz *et al.*, 1986, Etzler 1986). They are also broken down upon germination and contribute to the nutrition of the seedling.

Pea seed lectin is localized in the protein bodies of seeds along with the major storage proteins (Van Driessche *et al.* 1981). The mature protein exists in three isolectin forms each consisting of a 47 kDa dimer made up of two sub-unit pairs. The latter are synthesized as preproprotein precursors of 28 kDa (275 amino-acids [a.a.]). A 30 a.a. N-terminal signal peptide is removed co-translationally in the endoplasmic reticulum to give a 25 kDa proprotein. This protein is transported to the protein bodies via the Golgi apparatus, where it is proteolytically cleaved to give a 17 kDa (187 a.a.) β-sub-unit common to all three isolectins and a 58 a.a. α-sub-unit (Higgins *et al.* 1983). The α-sub-unit is

processed further at its C-terminal end to give 54 and 52 a.a., 6 kDa α-sub-units. The single β-sub-unit and the 54 and 52 a.a. α-sub-units combine in the mature protein to produce the three isolectin forms observed (Rini *et al.*, 1987).

This protein is encoded by a single intronless gene which has been cloned, sequenced (Gatehouse *et al.* 1987), and expressed in transgenic tobacco plants under control of the constitutive CAMV promoter (Edwards 1988). It is highly expressed in developing cotyledons (Chandler *et al.*, 1984) and mRNA has also been detected in root, at a level 4000 times lower than that of cotyledon (Buffard *et al.* 1988).

1.5. Regulation of pea seed storage protein gene expression.

1.5.1. Accumulation of mRNA and protein.

After fertilization the pea embryo differentiates to form the cotyledons and axis. When differentiation is complete cell-division ceases and further growth occurs by cell-expansion accompanied by DNA endoreduplication (Marinos, 1970). During the cell-expansion phase there is a rapid accumulation of storage reserves, including the storage proteins, which continues until the final stages of development; maturation and desiccation (reviewed by Marinos, 1970).

The accumulation of the different storage proteins shows differential temporal expression in that the vicilin polypeptides are more abundant relative to the convicilin and legumin polypeptides at earlier points in the cell-expansion phase (Gatehouse *et al.*, 1982). Legumin has also been detected at very low levels at much earlier stages of development (2-3 mg fresh weight embryos), before the onset of its rapid accumulation (Domoney *et al.*, 1980). No storage proteins have been detected in non-seed tissues.

The mRNA levels for the seed storage proteins increase dramatically during cell-expansion and then fall again during the desiccation phase. As is the case for protein accumulation, the vicilin mRNAs accumulate before the legumin and convicilin mRNAs (Boulter *et al.*, 1987). The differential temporal expression of vicilin and *legA* mRNAs has been shown by Evans *et al.* (1984), by

measuring relative transcription rates in isolated nuclei throughout development, to be regulated mainly at the transcriptional level although posttranscriptional events may have also been involved. It was also shown that transcription of vicilin and *legA* genes does not occur in isolated leaf nuclei. Similar patterns of mRNA accumulation have been reported in other legumes such as soybean (Walling *et al.*, 1986) and *Phaseolus vulgaris* (Chappell and Chrispeels, 1986).

1.5.2. Storage protein gene *cis* and *trans* acting factors.

The finding that the regulation of these genes occurs, at least in part, at the transcriptional level stimulated the search for *cis*-acting DNA elements in their 5′ flanking regions. Many studies have now been reported (reviewed by Goldberg *et al.*, 1989) concerning the expression, in transgenic plants, of reporter or homologous genes under the control of the 5′ promoter regions of seed-storage protein genes. By producing systematic deletion mutations in these promoter regions and then observing their effect on expression, it is possible to map DNA elements responsible for regulating differential expression. Using this approach, for the pea *legA* gene transformed into tobacco, Shirsat *et al.* (1989) found that the promoter sequence between 97 and 549 bp upstream of the transcription start contained all the elements necessary for correct tissue-specificity and temporal regulation in tobacco. Additional enhancer sequences that increase expression lie between nucleotides -549 and -1203. The -97 to -549 region contains the 28 bp "legumin-box", a conserved element found in the 5′ region of legumin genes from many species (Gatehouse *et al.*, 1986; Bäumlein *et al.*, 1986). Also, a 200 bp upstream region of the soybean β-conglycinin gene has been shown, when placed either 5′ or 3′ to the CAT gene under control of the CaMV promoter, to act as a seed-specific enhancer in transgenic tobacco (Chen *et al.*, 1988).

In order to move towards an understanding of the mechanisms of regulation of storage protein genes attempts have been made to identify and characterize proteins (*trans* acting factors) that bind to the types of regulatory elements described above. Jofuku

et al. (1987) identified a 60 kDa DNA binding protein that interacts with the 5′ region of the soybean lectin gene. This protein is only found in seed tissue and its activity parallels the lectin gene transcription rate during embryogenesis. Also Maier *et al.* (1987) described the binding of a nuclear factor to a 22 nucleotide binding site in the 5′ flanking region of a maize zein gene. This binding site contains a sequence conserved in the promoter regions of all zein genes.

Once *cis*-regulatory elements have been identified and characterized, it should be possible to clone cDNAs encoding the corresponding DNA binding proteins by probing cDNA expression libraries with double stranded oligonucleotide probes derived from the sequences of the *cis*-elements. By using such a strategy it may be possible to determine some of the steps in the transduction pathway that lead to the transcriptional activation of seed-storage protein genes.

1.6. Objectives of this work.

The objective of this work was to establish the points of regulation in the differential expression of the seed storage protein genes during pea embryogenesis. Seed-specific and temporal differential expression as well as the quantitative differences in the expression of the different gene families and subfamilies were the subjects of investigation.

2. MATERIALS

2.1 Glassware and plasticware.

All plasticware used for DNA or RNA manipulations was siliconised with dimethyldichlorosilane solution (2% v/v in 1,1,1-trichloroethane) and autoclaved.

Siliconised glassware was autoclaved for use in DNA work, or heat sterilised overnight in a 180°C oven for RNA work.

Cryotubes and Cell-Well™ plates were obtained from Inter-Med, NUNC, Kamstrup DK-4000, Roskilde, Denmark.

2.2 Chemical and biological reagents.

Reagents, unless otherwise stated, were obtained from BDH Chemicals Ltd, Poole, Dorset, UK and were of "AnalaR" grade or the best available. The following reagents were obtained from alternative suppliers:

E.coli rRNA, *E.coli* tRNA, yeast tRNA, polyadenylic acid, herring sperm DNA, amino-acids, sucrose (grade 1), uridine, cytosine, UDPG, ampicillin and tetracycline; Sigma Chemical Co., Poole, Dorset.

Phostrogen; Phostrogen Ltd, Corwen, Clyde, UK.

Restriction endonucleases, T4-DNA ligase, T4-polynucleotide kinase; Northumberland Biologicals Ltd., Cramlington, Northumberland, UK.

RNaseH, X-gal, nucleoside triphosphates, Klenow fragment (sequencing grade); Boehringer Mannheim (BRL), GmbH, Mannheim, FRG.

High-Gelling-Temperature agarose; FMC Bioproducts, Rockland, ME, USA.

Percoll™, Sephadex-G50 and G25, Oligo(dT)$_{12-18}$, random hexadeoxynucleotides; Pharmacia Fine Chemicals, Uppsala, Sweden.

3MM paper; Whatmann Ltd, Maidstone Kent, UK.

Radiochemicals, Nick Translation Kit; Amersham International p.l.c., Amersham, Berks., UK.

MS salts; Flow Laboratories, Rickmansworth, Herts, UK.

PPO, POPOP; Koch-Light Ltd, Colnbrook, Berks, UK.

SP6 and T7 RNA polymerases and pGEM™-blue; Promega Biotec., Madison, WI, USA.

Ecoscint A; National Diagnostics, Manville, New Jersey, USA.

Optifluor O; Canberra Packard, Pangbourne, Berks, UK.

Fuji RX X-ray film; Fuji Photo Film Co. Ltd, Japan.

HiSpeed-X intensifying screens and film cassettes; Genetic Research Instrumentation Ltd, Dunmow, Essex, UK.

Yeast extract; Biolife S.l.r., Milan, Italy. Bacto-agar; Difco, Detroit, Michigan, USA. Bacto-tryptone; Oxoid Ltd, Basingstoke, Hants, UK.

3. METHODS.

3.1 Plant growth conditions.

In order to obtain pea cotyledons, *Pisum sativum* L. var. Feltham First seeds were germinated in a dark spray room for 6 days and then grown hydroponically in pots containing Phostrogen ($0.55g.dm^{-3}$) which was regularly replenished. The plants were grown under standardised conditions in a growth cabinet. The lighting conditions were as follows; fluorescent lighting from 06:00hrs to 18:00hrs with doubled intensity from 08:00hrs to 16:00hrs and incandescent lighting from 15:00hrs to 18:00hrs. The temperature was 25°C from 06:30hrs to 20:30hrs and 17°C during the remaining hours. The cabinet contained two humidifiers, providing approximately 70% relative humidity. Flowers were tagged on the day they opened and then cotyledons were harvested on different days after flowering (DAF). Pea leaf material was obtained from Feltham First plants, which were germinated as above and then grown in the growth cabinet in vermiculite soaked in Phostrogen ($0.55g.dm^{-3}$) for 9 days.

3.2 Manipulation of DNA.

Cleavage of DNA with restriction endonucleases, alcohol precipitation, phenol extraction, ligation of restriction fragments and transformation of *E. coli* with plasmid DNA were routine techniques performed as described by Maniatis *et al.* (1982).

3.3 Preparation of plasmid DNA from *E. coli*.

Small scale preparation of plasmid DNA was by the "miniprep" method as described by Maniatis *et al.* (1982). Preparation of plasmids used as DNA templates for the production of synthetic RNAs was by the method of Melton *et al.* (1984). Plasmids used in DNA dot blotting were more extensively purified by centrifugation through two successive CsCl gradients, as described by Godson and Vapnek (1973). The concentration of DNA solutions was determined by measuring A_{260} using a Pye Unicam SP8-150 UV/Vis spectrophotometer, assuming $1mg.ml^{-1}$ to give A_{260}= 20 (1cm path length).

3.4 Radioactive labelling of DNA

3.4.1 Labelling DNA for use as hybridisation probes.

Restriction fragments of DNA recovered from agarose gels by electroelution (section 3.6.2) were labelled with [α-^{32}P]dCTP by Nick Translation using a kit supplied by Amersham International plc, as described in the product handbook, or by random priming as described by Feinberg and Vogelstein (1983).

3.4.2 End labelling of plasmid DNA for calibration dots.

Plasmid DNA linearised by restriction was 5'-end-labelled with [γ-^{32}P]ATP (5000 Ci.mmol^{-1}) using T4 polynucleotide kinase as described by Maniatis *et al.* (1982).

3.4.3 Oligodeoxyribonucleotide probes.

Oligodeoxyribonucleotides were synthesised using an Applied Biosystems Model 381A DNA synthesiser and purified by reversed phase chromatography. The extinction coefficient of each oligodeoxyribonucleotide was calculated from the sum of the extinction coefficients of the constituent nucleotides and the concentration determined by measuring A_{260}. 10 pmol of each oligodeoxyribonucleotide was labelled using 100μCi of [γ-^{32}P]ATP at 5000 Ci.mmol^{-1} (20 pmol ATP). The labelling reaction contained the following components; 40mM Tris-Cl, pH 7.5, 10mM $MgCl_2$, 5mM DTT and 5 units of T4 polynucleotide kinase. The reaction was incubated at 37°C for 60 min.

3.4.4 Separation of labelled DNA from unincorporated nucleotides.

Unincorporated label was removed by gel permeation chromatography using Sephadex G-50, or Sephadex G-25 for oligodeoxyribonucleotides, in a disposable 10ml pipette with a buffer consisting of 0.3M NaCl, 0.1% SDS, 50mM Tris-Cl pH 7.5, 10mM EDTA.

3.5 Extraction of total RNA.

Plant material for total RNA extraction was dropped into liquid nitrogen immediately after harvesting and then stored at -80°C until required. Total RNA was extracted from pea tissues

using the "hot-SDS" method based on that of Hall *et al.* (1978).

Tissue was homogenised in an equal volume of boiling 0.2M sodium borate, pH 9.0, 1% SDS, 30mM EGTA using a Willems' Polytron for 2 x 10 seconds at full speed. Immediately prior to homogenisation, DTT was added to a final concentration of 5mM and a few drops of isoamylalcohol were added as an antifoaming agent. The homogenate was cooled to below 50°C and proteinase K added to $5\mu g.ml^{-1}$, before incubating at 37°C for 60 min. 1/13 volume of 2M KCl was then added and the mixture cooled on ice for 10 min. The potassium dodecyl sulphate precipitate thus formed was removed by centrifugation at 10000g for 10 min at 4°C. 1/5 volume of 12M LiCl was then added and the RNA allowed to precipitate overnight at 4°C. The precipitate was recovered by centrifugation at 10000g for 10 min at 4°C and washed twice in 2M LiCl. The pellet was dissolved in a suitable volume of DEPC-treated water and made up to 0.2M potassium acetate pH 5.5. At this stage, any undissolved material was removed from the solution by centrifugation. RNA was then precipitated overnight at -20°C by addition of 2.5 volumes of ethanol. The precipitate recovered by centrifugation at 10000g for 10 min at 4°C was dried *in vacuo* and redissolved in a suitable volume of 0.2M potassium acetate pH 5.5. This solution was extracted twice with phenol/chloroform/isoamylalcohol (24:24:1 v/v) and then ethanol precipitated as before. The pellet was washed in 70% ethanol, dried *in vacuo* and finally dissolved in water. The concentration was determined spectrophotometrically assuming $1mg.ml^{-1}$ to give A_{260}= 24. The yield of RNA was approximately 1mg per gram fresh weight of tissue.

RNA could be extracted from up to 0.5g fresh weight of cotyledon tissue by homogenisation in a 1.8ml Nunc cryotube using Polytron head PT7. All subsequent steps could then be carried out in 1.5ml polypropylene centrifuge tubes, using a Sarstedt MH 2-K refrigerated microfuge. For larger amounts of tissue all procedures were performed in 15ml or 30ml Corex tubes.

3.6 Electrophoresis of nucleic acids in agarose gels.

3.6.1 Electrophoresis of DNA.

DNA was size fractionated by electrophoresis through agarose gels cast in TEA gel buffer (40mM Tris-acetate pH 7.7, 2mM EDTA) as described by Maniatis *et al.* (1982). Inclusion of $0.5\mu g.ml^{-1}$ ethidium bromide in the gel and running buffer allowed visualisation of DNA under 300nm ultra-violet (UV) light. Gels were photographed under UV illuminatiom with a Polaroid MP-4 Land Camera through a Kodak 23A Wrattan filter, using Polaroid type 667 film.

3.6.2 Recovery of DNA fragments from agarose gels.

DNA fragments were recovered by electroelution into treated dialysis tubing containing TBE buffer (89 mM Tris-borate pH8.0, 89mM sodium borate, 2mM EDTA), as described by Maniatis *et al.* (1982). Eluted DNA was purified by three phenol extractions and two ethanol precipitations.

3.6.3 Formaldehyde-agarose gel electrophoresis of RNA.

RNA was electrophoresed through denaturing agarose gels containing 2.2M formaldehyde, and stained with ethidium bromide, as described by Miller (1987), except that all volumes were scaled to fit a 19 x 15cm gel former, or a minigel apparatus. The ribosomal bands of total RNA from pea and *E.coli*, together with standard DNA restriction fragments, were used as size markers.

3.7 Blotting of nucleic acids onto membranes.

3.7.1 Southern blotting for *in vitro* transcription assays.

Plasmid DNAs were digested with restriction endonucleases to remove cDNA and genomic DNA inserts from their vector sequences. 5µg of restricted DNA was run per track on a 0.75% agarose gel at 130 volts for 2.5 hours and then the DNA was transferred to nitrocellulose by Southern blotting (Southern, 1975). This was done as follows: the DNA was first denatured by incubating the gel in 0.5M NaOH, 1.5M NaCl for 2 x 15 min with shaking at room temperature and then neutralised by incubation in 0.5M Tris-HCl pH 7.5, 1.5M NaCl, 1mM EDTA for 2 x 15 min shaking at room temperature. The gel was then blotted onto nitrocellulose using 20

× SSC as transfer buffer, as described by Maniatis *et al.* (1982). After blotting, filters were allowed to air dry and then baked in a vacuum oven at 80°C for 90 min.

3.7.2 DNA dot blotting.

DNA dot blotting was based on the procedure of Marzluff and Huang (1982). Plasmid DNA was linearised by restriction using an enzyme with a single site in the vector only. The DNA was then phenol, phenol/chloroform and chloroform extracted and then ethanol precipitated with 2.5 volumes of ethanol at -20°C for 2 hours. The ethanol precipitate was washed with 70% ethanol, redissolved in water and the restriction confirmed by agarose gel electrophoresis of a 0.5μg sample. The remaining solution was adjusted to 0.2M NH_4OH, 0.2M NaCl at a final DNA concentration of 50μg/ml. The DNA was then denatured by boiling for 3 min followed by rapid cooling on ice. The DNA solution was dotted onto nitrocellulose (previously wetted in water and presoaked in 2 × SSC for 20 min) in 100μl aliquots (5μg) using a BRL 96 well Hybridot™ Manifold. The vacuum was adjusted so that each sample took 30 seconds to pass through the filter. Following application of the DNA, the bound DNA was washed by passing 50μl of 2 × SSC through each well. After washing the vacuum was increased 5 fold, the apparatus disassembled and the filter transferred to a sheet of 3MM paper and allowed to air dry. The filter was then baked in a vacuum oven for 90 min at 80°C, and then cut into strips ready for hybridisation.

3.7.3 Analysis of RNA by Northern blotting.

Formaldehyde agarose gels were run, with DNA and RNA standards in the tracks on one edge of the gel, and RNA samples to be blotted in the remaining tracks. The tracks containing standards were cut from the gel after electrophoresis and stained as described in section 3.6.3. The remaining tracks of the gel were blotted onto nylon membranes (Hybond-N, Amersham International p.l.c.) using 20 × SSC as transfer buffer and the blotting apparatus as described by Maniatis *et al.* (1982). After blotting, the nylon membranes were UV irradiated by wrapping in cling film and placing on a UV transilluminator for 2 min, and then baked *in*

vacuo at 80°C for 20 min. Blots were stored at room temperature in sealed polythene bags.

3.7.4 Immobilisation of RNA by dot-blotting onto nitrocellulose.

Pea total RNAs prepared by the method of Hall *et al.* (1978) were dot blotted by a procedure based on that of White and Bancroft (1982). RNA was denatured in 12% formaldehyde, 6 × SSC for 15 min at 60°C at an RNA concentration not exceeding 0.1μg/μl. This solution was then adjusted to 0.02μg/μl RNA, 15 × SSC and could be stored on ice for up to a few hours. 2μg aliquots of RNA were dotted onto nitrocellulose, previously wetted in water and equilibrated in 15 × SSC for 20 min, by loading 100μl samples into the wells of a BRL Hybridot™ manifold. After application, each well was washed with 100μl of 15 × SSC. A vacuum was applied to the manifold using a vacuum pump with a bleed valve and samples were aspirated at a rate of 3 min per 100μl. ^{3}H-cRNA standards were denatured as above at a concentration of 1 femtomole/μl and then serial dilutions were performed with 15 × SSC, 0.02μg/μl *E.coli* rRNA. 100μl aliquots were dotted onto nitrocellulose as above to provide 2:1 serial dilutions starting at 10 or 5 fmol per dot with 2μg carrier *E.coli* rRNA per dot.

3.8 Fluorography of Northern Blots.

Northern blots of cotyledon total RNA labelled with ^{3}H in culture were sprayed with "Amplify™" spray (Amersham International p.l.c.) according to the manufacturers instructions, and then autoradiographed.

3.9 Removal of poly(A) sequences from mRNA by digestion with RNaseH in the presence of oligo(dT)$_{12-18}$.

Poly(A) sequences were removed from mRNA essentially as described by Vournakis *et al.* (1975). 12μg of total RNA was incubated in 25mM $MgCl_2$, 20mM Tris-Cl pH 8.0, with 2μg oligo(dT)$_{12-18}$ at 25°C for 15 min in a volume of 40μl. 1.3 units of RNaseH were then added before incubating at 37°C for 20 min. The RNA was then recovered by two phenol/chloroform extractions and ethanol precipitation, washed in 70% ethanol and then analysed by formaldehyde gel electrophoresis and Northern blotting.

3.10 Hybridisation of nuclear "run-on" transcripts to DNA dot blots and Southern blots.

Blots were prehybridised for 4 hours at 41°C in the following buffer: 50% deionised formamide, 0.5M NaCl, 40mM PIPES-NaOH pH 6.5, 0.4% SDS, 1mM EDTA, 100μg/ml polyadenylic acid and 100μg/ml *E.coli* tRNA. Volumes used were: 1ml for dot blots and 10ml for Southern blots. The nuclear "run-on" transcripts were then added to a buffer identical to the prehybridisation buffer (750μl for dot blots, 2ml for Southern blots) and hybridised to the blots for 72 hours at 41°C. Blots were then washed as follows: 4 times 1.4 × SSC, 0.1% SDS at 60°C, 2 times 0.1 × SSC at 60°C, once in 0.3M NaCl, 10μg/ml RNase A at room temperature and once in 0.3M NaCl, 0.2% SDS at room temperature. Each wash was for 30 min. In each case the transcripts were saved and hybridised to a second prehybridised blot to estimate the extent of the first hybridisation. All hybridisations and washes were carried out in heat sealed polythene bags. Blots were autoradiographed and then dots or bands were cut out and dissolved in 5ml of the following fluid: 60% toluene, 40% methoxyethanol, 1.1g.dm^{-3} omnifluor and radioactivity measured by liquid scintillation counting.

3.11 Hybridisation of double stranded DNA probes to Northern blots and RNA dot blots.

Blots were prehybridised in 20ml of 50% deionised formamide, 0.5M NaCl, 1mM EDTA, 40mM PIPES-NaOH pH 6.5, 0.4% SDS, 100μg/ml denatured herring sperm DNA, 100μg/ml polyadenylic acid and 5 × Denhardts' solution. Prehybridisation was for 8-16 hours with blots sealed in polythene bags, inside a plastic box in a shaking water bath at 41°C. The DNA probe, [α-^{32}P]dCTP labelled by nick-translation or by random priming, was then denatured by boiling for 6 min followed by rapid cooling on ice. Denatured probe was then added directly to the prehybridisation buffer. The DNA probe typically was in a volume of 0.8 to 1.2 ml. The probe was hybridised to the blots for 40-48 hours and then washed as follows: 4 times 1.4 × SSC, 0.1% SDS then 2 times 0.1 × SSC. All washes were at 60°C for 30 min. Excess liquid was then removed from the blots and they were autoradiographed while still wet.

Nylon Northern blots were probed more than once by removing the previously hybridised probe by boiling for 20 min in 0.1% SDS. These blots were then checked by autoradiography, prehybridised and hybridised as before. Probes in the formamide buffer system could also be reused if stored at -20^{o}C and denatured again before use by heating to 65^{o}C for 10 min.

3.12 Analysis of autoradiographs of nucleic acid dot blots hybridised to ^{32}P-labelled probes.

Autoradiograghs were scanned using a LKB Ultroscan XL laser densitometer with parameters set to: X-width=2, Peak width=10, Y-step=10, with Smoothing. Calibration of autoradiographs of DNA dot blots from transcription assays was achieved by exposing the blots together with radioactive dots of known radioactivity. The latter dots were produced by dotting serial dilutions of linearised pBR322 DNA end-labelled with [γ-^{32}P]ATP onto nitrocellulose. Before dotting, the end labelled DNA was purified by gel permeation chromatography (Sephadex G-50), ethanol precipitated, redissolved in water and counted by LSC. That the radioactive DNA bound quantitatively to the nitrocellulose was shown by cutting out dots from a duplicate set of radioactive dots and counting them in 60% toluene, 40% 2-methoxyethanol, 0.55g.dm^{-3} omnifluor (nitrocellulose dissolving liquid scintillation fluid).

3.13 Transcription in isolated nuclei.

3.13.1 Isolation of nuclei from pea cotyledon and leaf tissue.

The method for isolation of nuclei was based on the method of Willmitzer and Wagner (1982). Pods were harvested into an ice bucket and cotyledons removed from their seed coat and separated from their axes. All manipulations were carried out on ice unless otherwise stated. All centrifugation steps were carried out at 4^{o}C using swing-out rotors and no brakes. Cotyledons were chopped in buffer A using a razor blade, filtered through 125μm nylon mesh (sterilised by boiling for 10 min) and washed in buffer A. Chopped cotyledons were incubated in buffer B at 25^{o}C for 4 hr then filtered through 125μm nylon mesh, washed in buffer C and resuspended in buffer C at 20ml.g^{-1}. Cotyledons were homogenised gently using a Quik-fit homogeniser (for less than 5g of material)

or, for larger amounts of material, a Cyclotrol 8 Osterizer (John Oster MFG Co., Milwaukee, Wisconsin, USA) using 3 x 5 second bursts on the "mix" setting. The homogenate was filtered through 125μm nylon mesh and the filtrate centrifuged at 2000g for 10 min. The pellet was resuspended in buffer C at 2g buffer C per g cotyledon. The resulting suspension was weighed and then 1.5 times this weight of buffer D was added. At this final Percoll concentration of 47.6%, nuclei and starch sediment and the majority of cell wall material remains buoyant. This suspension was filtered through 53μm nylon mesh and centrifuged at 1000g for 5 min. The supernatant was then thoroughly mixed and centrifuged again at 1000g, this was repeated 2-3 times and then all pellets were combined by resuspending in 5-10ml of buffer E. This suspension was centrifuged at 5000g for 15 min. The floating layer (containing nuclei) was removed and diluted with 5-10 volumes of buffer C, and centrifuged for 10 min at 1000g. The pellet was washed twice with buffer C and then resuspended in 0.5-1ml of transcription buffer I containing 50% glycerol. Nuclei were examined by fluorescence microscopy after dilution with buffer C and staining with 20μg/ml DAPI. Stained nuclei were counted by haemocytometer. The nuclei suspensions were stored at -80^{o}C for up to 6 months. A typical nuclei preparation used between 20 and 60 seeds. Mean values for mass per pair of cotyledons were 38mg (12 DAF) and 259mg (16 DAF). Mean yields of nuclei per pair of cotyledons were 4.7×10^{4} (12 DAF) and 3.4×10^{4} (16 DAF). Leaf nuclei were prepared in the same way as above. 9.42g pea leaves yielded 7.2×10^{7} nuclei.

3.13.2 Transcription reactions *in vitro* using isolated nuclei.

The method used was adapted from McKnight and Palmiter (1979). A known number of nuclei stored in 50% glycerol were diluted to 15% glycerol with glycerol free buffer I. They were centrifuged for 5 min at 1000g at 4^{o}C in a 15ml Corex tube using a swing-out rotor. The pellet was washed twice with buffer I containing 15% glycerol. The washed nuclei were resuspended in 50μl buffer I (15% glycerol), transferred to a 1.5ml microcentrifuge tube and made up to 100 μl with buffer I (15% glycerol). 20μl of buffer II was then added and the suspension incubated on ice for 15 min. An aliquot

of [α-^{32}P]UTP (410Ci/mmole) was lyophilised and redissolved in 20μl buffer V containing 140 units of human placental RNasin (HPRI). To this solution the suspension of nuclei was added and immediately incubated at 26^{o}C for 10 min (final reaction volume is 140 μl). The transcription was stopped by digestion with 10μg.ml^{-1} DNaseI for 5 min at 26^{o}C. The reaction was then deproteinised by digestion with 100μg.ml^{-1} proteinase K in 1 x SET (10mM Tris-Cl pH 7.5, 5mM EDTA, 1% SDS) at 45^{o}C for 30 min in the presence of 25μg of purified yeast tRNA. The mixture was then extracted with phenol/chloroform and the organic phase back extracted with 50μl 10mM Tris-Cl pH 7.5. The combined aqueous phases were extracted once with chloroform. The solution was then precipitated overnight at -20^{o}C by adding NaCl to 0.1M and 2.5 volumes of ethanol. After washing the ethanol precipitate with 40mM NaCl, 66% ethanol, the nucleic acid was dissolved in 100μl of DNaseI buffer and incubated for 1 hour at 26^{o}C with 10μg.ml^{-1} DNaseI and 140 units of RNasin (HPRI). The reaction was then adjusted to 0.2 x SET and digested again with 100μg.ml^{-1} proteinase K for 30 min at 45^{o}C, and again phenol/chloroform and chloroform extracted. The RNA was then separated from unincorporated [α-^{32}P]UTP by gel filtration using Sephadex G-50 and a column buffer consisting of 0.3M NaCl, 0.1% SDS, 100mM Tris-Cl pH 7.5 and 1mM EDTA. Fractions from the first peak were precipitated overnight at -20^{o}C by adding 2.5 volumes of ethanol. The precipitate was washed with 70% ethanol and redissolved in water. At this point aliquots were taken for formaldehyde gel analysis and liquid scintillation counting. The solution was then made to 0.1% SDS and stored, if necessary, for a few hours at -20 ^{o}C, before hybridisation.

3.13.3 Buffers used in the isolation of nuclei and for nuclear transcription.

Buffer A: 0.7M mannitol,
10mM MES,
5mM EDTA,
0.1% BSA,
0.2mM PMSF.

BufferB: Buffer A supplemented with 0.1mg/ml cellulase and 0.05mg/ml pectinase. In order to dissolve enzymes in buffer A the pH was raised to 11 with 2M NaOH for 3 min and then returned to 5.8 with acetic acid. PMSF was added after dissolving enzymes.

Buffer C: 0.25M sucrose (grade I, Sigma),
10mM NaCl,
5mM EDTA,
0.15mM spermine,
0.5mM spermidine,
20mM β-mercaptoethanol,
0.2mM PMSF,
0.6% Nonidet P-40,
0.1% BSA.

Buffer D: 30g 5 X buffer C (except Nonidet-P40 is kept at 0.6%) added to 116g Percoll. Adjust pH to 6.0 with 1N HCl.

Buffer E: 12g 5 x buffer C (except Nonidet-P40 is kept at 0.6%) added to 90g Percoll. Adjust pH to 6.0 with 1N HCl.

Buffer I: 20mM Tris-Cl pH 7.4,
5mM $MgCl_2$,
0.1mM EDTA,
±15% (w/v) glycerol,
0.5% BSA.

Buffer II: Buffer I supplemented with 240 mM $(NH_4)_2SO_4$

Buffer V: Buffer II supplemented with 2mM ATP, 2mM GTP, 2mM CTP.

3.14 Synthesis of cRNA transcripts trace labelled with ^{3}H or ^{32}P.

DNA clones were subcloned into the multiple cloning site of pGEM™-blue vector (Promega Biotec) which contains promoters for SP6 and T7 RNA polymerases flanking the multiple cloning site and allows selection of recombinants by inactivation of β-galactosidase activity (blue/white colonies). The resulting plasmids were prepared either by maxiprep or by a method developed by Melton *et al.* (1984), (section 3.3). The plasmids were linearised with a suitable restriction endonuclease and then extracted with 2 × phenol, 1 × phenol/chloroform, 3 × chloroform and 2 × ether. The DNA was then ethanol precipitated, washed in 70% ethanol and redissolved in sterile water. Complete restriction was then confirmed by agarose gel electrophoresis of 0.5μg samples. cRNA of specific activity 5×10^{5} cpm/μg (assuming 25% of RNA bases to be uridines) was then transcribed from the linearised DNA template by a method modified from Melton *et al.* (1984). This was done by mixing the following components in order at room temperature and then incubating at 37°C for 180 min:

1. sterile water to 100μl.
2. 20μl 5 × transcription buffer (200mM Tris-Cl pH 7.5, 30mM $MgCl_2$, 10mM spermidine, 50mM NaCl).
3. 10μl nucleotide mixture (5mM each of ATP, GTP, CTP and UTP).
4. 14.6μCi [5,6-^{3}H]UTP (40 Ci/mmol) or [α-^{32}P]UTP (410 Ci/mmol).
5. 10μl 100mM DTT.
6. 100 units HPRI.
7. 2μg linearised DNA template.
8. 10-20 units SP6 or T7 RNA polymerase.

The reaction was stopped and the DNA template removed by digestion with 10μg/ml DNase I for 15 min at 37°C. Then the solution was extracted with 2 × phenol/chloroform and 2 × chloroform and then unincorporated nucleotides removed by gel permeation chromatography followed by ethanol precipitation as in section 3.13.2. The ^{3}H-cRNA was finally dissolved in sterile water, counted in triplicate by LSC, and stored in a liquid nitrogen dewar. ^{32}P-cRNA was dissolved in sterile water, denatured in formaldehyde/formamide (see section 3.6.3) and stored at -80°C for up to 7 days before being analysed by formaldehyde gel electrophoresis. Gels were dried and autoradiographed. The

concentration of ^{3}H-cRNAs was calculated from the DPM/μl since the specific activity of the cRNA can be calculated from the final specific activity of the [5,6-^{3}H]UTP in the transcription reaction and the uridine content of each cRNA, as predicted by its corresponding DNA sequence.

3.15 Culture of pea cotyledons *in vitro* .

The method of cotyledon culture was based on that of Stafford and Davis (1979) with the modifications of Domoney *et al.* (1980). Pods of a known age (DAF) were harvested and immediately surface sterilised by submersion in a solution of calcium hypochlorite (1.2% w/v available chlorine), 0.1% Nonidet P-40 for 20 min. All subsequent manipulations of cotyledon tissue were carried out aseptically in a laminar flow hood. The pods were then rinsed thoroughly in sterile water to remove the sterilizing solution and cotyledons were isolated by removal of seed coat and embryonic axis. Where appropriate cotyledons were placed in 1.5ml centrifuge tubes each containing a drop of sterile culture medium and their fresh weight determined. Cotyledons were then transferred to a suitable culture vessel containing a specified volume of medium and incubated in a growth cabinet under the following conditions: 60 rpm shaker, 23°C, 16 hour photoperiod, 500-700 lux.

In pulse-chase experiments, RNA was labelled by including [5,6-^{3}H]uridine (>40 Ci/mmol) in the culture medium. During the chase 10mM unlabelled uridine was included in the medium.

The culture medium used consisted of Murashige and Skoog plant salt mixture supplied by Flow laboratories (Murashige and Skoog, 1962), 4-18% sucrose and the mixture of 15 amino acids as described by Stafford and Davis (1979). Abscisic acid (synthetic mixed isomers, Sigma) was added to the medium in some experiments to a concentration of 10^{-5}M. The pH of the medium was adjusted to 5.0 with 1N NaOH.

3.16 High performance liquid chromatography (HPLC) of nucleotides from pea cotyledons.

3.16.1 Extraction of nucleotides from cotyledon tissue.

Nucleotides were extracted based on the method of Standard *et al.* (1983). Approximately 0.5g samples of cotyledon tissue, which had been stored in a liquid nitrogen cryostat, were homogenised in 500µl of 8% trichloroacetic acid (laboratory grade reagent, BDH), 20% methanol (HPLC grade, BDH) using a Willems' Polytron at full speed for 20 seconds. The homogenate was left on ice for 15 min and then centrifuged at $0^{\circ}C$, 7500g for 10 min. The supernatant was extracted six times with 5ml of ice cold diethyl ether and then the aqueous phase was freeze-dried on an Edwards Modulyo freeze drier to a volume of 200µl. 50µl aliquots of this extract were then analysed by anion exchange HPLC.

3.16.2 HPLC analysis of nucleotide extracts.

Crude nucleotide mixtures were separated by anion exchange HPLC essentially as described by Perrett (1986). The equipment used was as follows: anion exchange column, Partisil 10SAX; chromatograph, Varian 5000 LC; detector, Varian UV50; chart recorder, Varian 9176 recorder; fraction collector, Gilson Model 201. The reagents used were: KCl - AnalaR, BDH; KH_2PO_4 - ARISTAR, BDH; methanol - HPLC grade, BDH. All buffers were made up in, and all glassware rinsed in, freshly deionised double-distilled water. All buffers were filtered through 0.45µm nitrocellulose before use. It was necessary to use ARISTAR grade phosphates and the purest water available to prevent a large baseline rise during chromatography (Perrett, 1986).

Nucleotides were eluted at 1ml/min on a multilinear gradient starting with 100% buffer B (0.04M KH_2PO_4 pH 2.8) for 10 min then changing to 68% buffer B, 32% buffer A (0.5M KH_2PO_4, 0.8M KCl pH 2.7) over the next 18 min and then to 55% buffer B, 45% buffer A over the next 20 min. Occasionally the column was regenerated by flushing with water followed by methanol and then again with water.

Elution of nucleotides was monitored by UV absorbance at 262nm (the peak absorbance for uridine) and 750µl fractions were collected for liquid scintillation counting. 50µl aliquots from

each fraction were counted in 5ml of Ecoscint A. Nucleotide peaks were identified by their co-chromatography with commercial preparations. UTP and CTP were quantified by cutting out peaks from paper chromatographs and weighing them; peak weight was then compared to a standard curve constructed by running known amounts of pure CTP and UTP on the HPLC and plotting peak weight against moles of nucleotide.

3.17 Hybridisation of total cotyledon RNA, labelled by *in vitro* culture, to DNA dot blots.

This hybridisation protocol is based on that of Gallagher and Ellis (1982). Linearised plasmid DNA was dot blotted onto nitrocellulose (5μg per dot) as described in section 3.7.2. Dots were cut out with a razor blade and placed in 2ml screw capped glass vials (S and H Scientific, Blyth, Northumberland, UK) together with 500μl of prehybridisation buffer (see section 3.10). Each vial contained only two filters; one carrying vector DNA only (pGEM-blue, Promega Biotech.), and one carrying the same vector DNA containing the subcloned cDNA insert either from pDUB6 or pCD40 (plasmids named pGEM4.4 and pGEM40, respectively). The DNA dot blots were prehybridised for 18 hrs at 42°C. The prehybridisation buffer was replaced by 80μl of an identical buffer, but also containing ^{3}H-labelled total RNA from cotyledons, and ^{32}P-labelled synthetic mRNA (see section 3.14) complementary to the cDNA insert in the DNA dot blots. The synthetic RNA was labelled to an activity of 2×10^{6} DPM/μg and served as an internal measure of hybridisation efficiency. The hybridisation buffer was overlaid with 500μl of paraffin oil which had been previously equilibrated with prehybridisation buffer. After hybridisation for 66 hr at 42°C in a shaking water bath the nitrocellulose filters were transferred to 10ml polypropylene tubes and washed as follows: once in chloroform for 5 min; 5 times 1.4 × SSC, 0.2% SDS, 20 min at 60°C; 2 times 0.1 × SSC, 20 min at 60°C; once in 2 × SSC, 10μg/ml RNaseA, 45 min at 37°C; once in 2 × SSC, 10μg/ml proteinase K, 45 min at 37°C; 2 times in 2 × SSC, 0.2% SDS, 20 min at 60°C. The volume of each wash was 5ml for each pair of filters.

After the last wash, in order to hydrolyse the RNA, each

filter was transferred to a 5ml disposable LSC vial which contained 250μl of 40mM NaOH and incubated at room temperature for one hour. Then 100μl of 0.1M acetic acid followed by 4ml of Ecoscint A were added. Vials were counted by conventional $^{3}H/^{32}P$ dual counting using a quench curve as described in the Hewlett-Packard 2000 CA Tri-Carb Liquid Scintillation analyser users' manual. It was necessary to hydrolyse the RNA before LSC to prevent under spill of the ^{32}P energy spectrum into the ^{3}H counting region.

3.18. Degradation of polyribosomal RNA in a cell free system.

3.18.1. Isolation of polyribosomes from pea cotyledons.

"Total" polyribosomes, ie. free polyribosomes and membrane-released polysomes, were isolated from freshly harvested pea cotyledons (14 DAF) as described by Evans *et al.* (1979). The procedure involved centrifugation of a post-mitochondrial pellet through a 70% sucrose cushion. The final polyribosome pellet was briefly rinsed in sterile water and then resuspended in 20mM Tris-Cl pH 7.6, 100mM KCl, 3mM Mg^{2+} acetate and then concentration was determined by absorbance at 260nm assuming 1mg/ml gives A_{260} = 10. Polysomes were stored in aliquots at -80°C.

3.18.2. Preparation of a cell free extract from pea cotyledons.

Cell-free extract was prepared from pea cotyledons as described by Beevers and Poulson (1972); alternatively, the method used to prepare a cell-free translation extract from dry pea primary axes by Peumans *et al.* (1980a) was employed, as described below:

1.45g of 14 DAF cotyledons were harvested and immediately homogenised using a hand-held "Jencons UniForm" glass-glass homogenizer in 6ml of ice cold 20mM Na-HEPES pH 7.8, 2mM Mg^{2+}acetate , 120mM KCl, 6mM β-mercaptoethanol. All the following procedures were carried out in a 4°C cold room. The homogenate was filtered through Miracloth and then a 1ml aliquot was centrifuged at 10000g at 4°C for 4 min in a Sarstedt MH 2-K refrigerated microfuge using a swing out rotor. The supernatant was still very turbid so the centrifugation step was repeated as above. 800μl of the second supernatant was run down a 10ml Sephadex G-25 column

using the homogenisation buffer given above. The green fraction was collected from the column by eye and stored as 20μl beads in a liquid nitrogen cryostat. 1μl of the extract in 1ml of water gave an A_{260} = 0.020.

3.18.3. RNA degradation assays.

Polyribosomes and cell-free extracts were incubated together under conditions known to sustain protein synthesis. Incubations were performed in a total volume of 30 to 60μl in 0.5ml microcentrifuge tubes. Each assay contained cell-free extract at a concentration of 0.0068 A_{260} units per μl and polyribosomes at 1.9μg/μl. Incubations were performed in 1 × SEA buffer (salts, energy and amino acids) which was made as a 5 × stock and stored at -80°C. 1 × SEA consists of: 0.05mM of each of 19 unlabelled amino acids (without leucine), supplied by Amersham International plc as a 1mM stock, 0.05 mM leucine, 2mM DTT, 1.2mM Tris-Cl pH 7.5, 20μm GTP, 1mM ATP, 75μm spermine, 5mM KCl. Where specified, 50μg/ml creatine phosphokinase (type I from rabbit muscle, Sigma), and 80mM creatine phosphate (Sigma) were added. EDTA, Mg^{2+}acetate and ribonuclease inhibitors were also added, where specified. Assays were set up on ice with the polyribosomes as the last component to be added. After mixing, an aliquot was removed as a time zero sample, and then the tubes were transferred to a water bath at 30°C. Aliquots were removed at time intervals and pipetted into tubes containing an equal volume of polyribosomal RNA extraction buffer (see section 3.18.4) and immediately frozen in liquid nitrogen.

3.18.4. Extraction of polyribosomal RNA.

This method is based on the method described by Mechler (1988). Tubes containing equal volumes of assay sample and extraction buffer (1% SDS, 200mM NaCl, 20mM Tris-Cl pH 7.5, 40mM EDTA) which had been stored in liquid nitrogen were allowed to warm to 0°C on ice, were boiled for 2 min and then returned to ice. Proteinase K was then added to 50μg/ml and the tubes were incubated at 37°C for 15 min. The mixture was then adjusted to 0.1M Tris-Cl pH 9.0, 0.5% SDS in a total volume of 100μl and then extracted three times with phenol/chloroform/IAA (24:24:1) which

had previously been equilibrated under 0.1M Tris-Cl pH 9.0. The RNA was then precipitated overnight by addition of 1/10 vol of 2M Na^+acetate pH 5.0 and 2.5 vol of ethanol. The recovered RNA was washed in 70% ethanol, dried and resuspended in denaturing buffer in preparation for formaldehyde agarose gel electrophoresis.

3.18.5. *In vitro* translation in the cell free extract.

Polyribosomes and cell-free extract were incubated together as described in section 3.18.3. except that SEA buffer contained no unlabelled leucine and each reaction was in a total volume of 15μl. 9μCi of [4,5-^{3}H]leucine (120 Ci/mmole) was added to give a final concentration of 50μM. The latter was freeze-dried before use. Incubations were for 1 hour.

The extent of radioactive incorporation was estimated by measuring total and TCA precipitable counts. Duplicate samples were pipetted onto separate G/FC filters and allowed to air-dry. Total counts were recorded by LSC of one filter in Optiscint O. The other filter was treated with TCA as follows: 10% TCA (1 hour, 0°C), 5% TCA (15 min, room temp.), 5% TCA (10 min, boiling), 5% TCA (2 times 15 min, room temp.). Ethanol was then passed through the filters using a Millipore filtration device. The filters were air dried and then counted in Optiscint O.

The labelled proteins were separated by denaturing SDS-polyacrylamide gel electrophoresis, using 17% polyacrylamide in the main gel, essentially as described by Laemmli (1970). The gels were fluorographed by treating the gel as follows: 1 hour in 10% TCA, 30% methanol, 2 times 30 min in DMSO, 3 hours in DMSO containing 22g PPO per 100ml, and 1 hour in 7% acetic acid; the gel was then dried before being exposed to X-ray film.

4. RESULTS AND DISCUSSION.

4.1 DNA clones used as probes in hybridisation assays.

In order to detect transcripts representing seed-protein gene families, in both nuclear transcription assays and RNA blotting experiments, the following DNA clones were used:

legA subfamily (major legumin polypeptides); this subfamily contains five genes; *legA,B,C,D* and *E* (see section 1.4.2 for details of their expression). All of these gene sequences hybridise strongly to plasmid pDUB6 (originally called pAD4.4; Delauney 1984) which contains a 1.1 kb cDNA derived from the 3′ coding and 3′ flanking region of the *legA* gene, starting 850 bp downstream of the transcription start.

legJ subfamily (big minor legumin); this subfamily consists of two or three genes of which *legJ* and *legK* have been characterised (section 1.4.2). pCD40 contains a 3′ *legK* cDNA of 0.91 kb which extends to the poly(A) signal, (Domoney *et al.*, 1986a). The 5′ end of this mRNA has not been mapped, but if the *legK* gene is similar to the *legJ* gene then pCD40 probably begins approximately 800 bp downstream of the transcription start. pJC5.2 is an EcoR1 genomic clone containing 1.42kb of the transcriptional unit of *legJ* (Gatehouse *et al.*, 1988). The clone begins 505 bp downstream from the transcription start, includes both introns (138 and 98 bp) and extends 544 bp downstream of the first poly(A) signal. When aligned, and allowing for deletions and introns, pCD40 and pJC5.2 show 94% homology and show extensive cross-hybridisation under the experimental conditions used (see section 4.2.1.1).

legS subfamily (small minor legumin); this subfamily has probably two genes (Domoney and Casey 1985) and can be detected using plasmid pAD9.2 whose insert is a near full length cDNA of 1.85kb (Delauney 1984) containing a start codon and poly(A) tail. Since the probes used for the other legumin subfamilies are all derived from 3′ regions of the genes it was decided to remove 420bp of 5′ region from the full length pAD9.2 cDNA when used in nuclear

transcription assays. This should make results more comparable between the three subfamilies because it is possible that incorporation into "run-on" nuclear transcripts is not constant along the length of the transcript. Nonlinear labelling, apparently as a result of premature transcription termination, has been previously observed in this type of assay (Mechti *et al.*, 1986). Moreover, it would be predicted that if transcripts are only derived from transcription initiated *in vivo* then a portion of the 5′ region of each transcript would always be unlabelled.

lecA (pea seed lectin); pea seed lectin is encoded by a single gene (section 1.4.5). Plasmid pLG4.10 contains a 0.85kb lectin cDNA beginning approximately 200 bp downstream of the transcription start site and extending to the poly(A) tail (Gatehouse 1986; Gatehouse *et al.*, 1987).

cvc subfamily (convicilins); this subfamily contains two genes (section 1.4.4). The probe sequence used was the insert from plasmid pDB9.08, a 2.9kb genomic clone containing the entire *cvcA* gene with its five introns. The size of the transcriptional unit of this gene (transcription start to poly(A) signal) is 2.45kb. The transcription start is about 390bp downstream of the 5′ end of the clone.

CAB gene family (chlorophyll a/b binding protein); this family has a relatively large gene copy number showing at least 8 bands on Southern blots (Coruzzi *et al.*, 1983). The probe sequence used is plasmid pFa/b31 which contains a 0.83kb cDNA insert (Bennett *et al.*, 1984).

4.2 Steady-state levels of mRNA species.

4.2.1 Quantification of steady-state mRNA levels.

Total RNAs were prepared from pea cotyledons at various stages of development. Under the growth conditions used the development period of pea seeds, from anthesis to cessation of dry weight gain, was 22 days (Evans *et al.*, 1979).

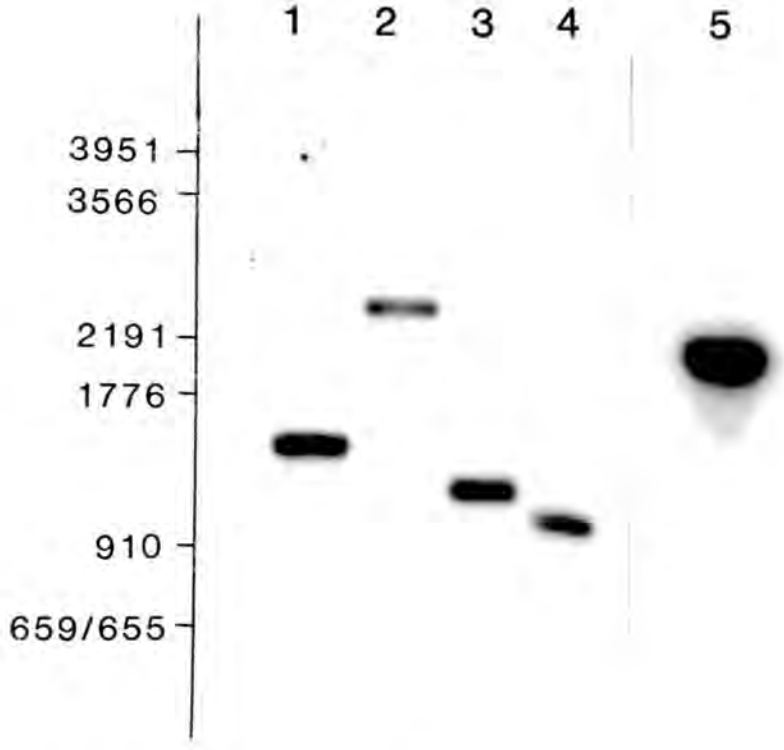


Figure 2. Analysis of ^{32}P-labelled synthetic mRNAs.

The inserts from plasmids pDUB6, pJC5.2, pCD40, pAD9.2, and pLG4.10 were excised from their vectors using restriction endonucleases BamHI, EcoRI, PstI, EcoRI and BamHI respectively. pCD40 contains a legK cDNA cloned into the PstI site of vector pAT153, however, the cDNA also contains two PstI sites and thus a partial digestion with PstI was required to isolate the insert. The inserts were subcloned into pGEM™-blue and the orientation of the inserts determined by restriction mapping. The new plasmids were renamed pGEM4.4, pGEM5.2B, pGEM40, pGEM9.2 and pGEM5B respectively.

Sense strand RNA was synthesized from these plasmids using SP6 or T7 RNA polymerases as described in section 3.14 after linearizing with a suitable restriction endonuclease. Track: 1, pGEM4.4, EcoRI, SP6; 2, pGEM5.2B, AvaI, T7; 3, pGEM40, HindIII, T7; 4, pGEM5B, SmaI, T7; 5, pGEM9.2, AccI, T7.

An aliquot from each sample of ^{32}P-labelled synthetic mRNA was run on an agarose formaldehyde gel which was then dried and autoradiographed. Size markers were provided by E.coli and Pea rRNAs and pBR322 AluI restriction fragments.

Figure 3. Autoradiograph of a single Northern blot probed with legumin and lectin cDNA probes.

Tracks labelled "DAF" contain 5μg of cotyledon total RNA from 12 to 28 DAF. These are the same RNA samples as used in Fig. 5, columns A and B. Tracks labelled "cRNA" contain 2 femtomoles of synthetic mRNAs (trace labelled with [5,6-^{3}H]UTP) transcribed, as in fig. 2., from the following plasmids; pGEM4.4, EcoRI, SP6 (*legA*); pGEM40, HindIII, T7 (*legJ*); pGEM5B, SmaI, T7 (*lec*); pGEM9.2, AccI, T7 (*legS*). The blot was probed sequentially with cDNA inserts isolated from the above plasmids and labelled by random oligopriming. The sizes of hybridizing bands are given in nucleotides (NT).

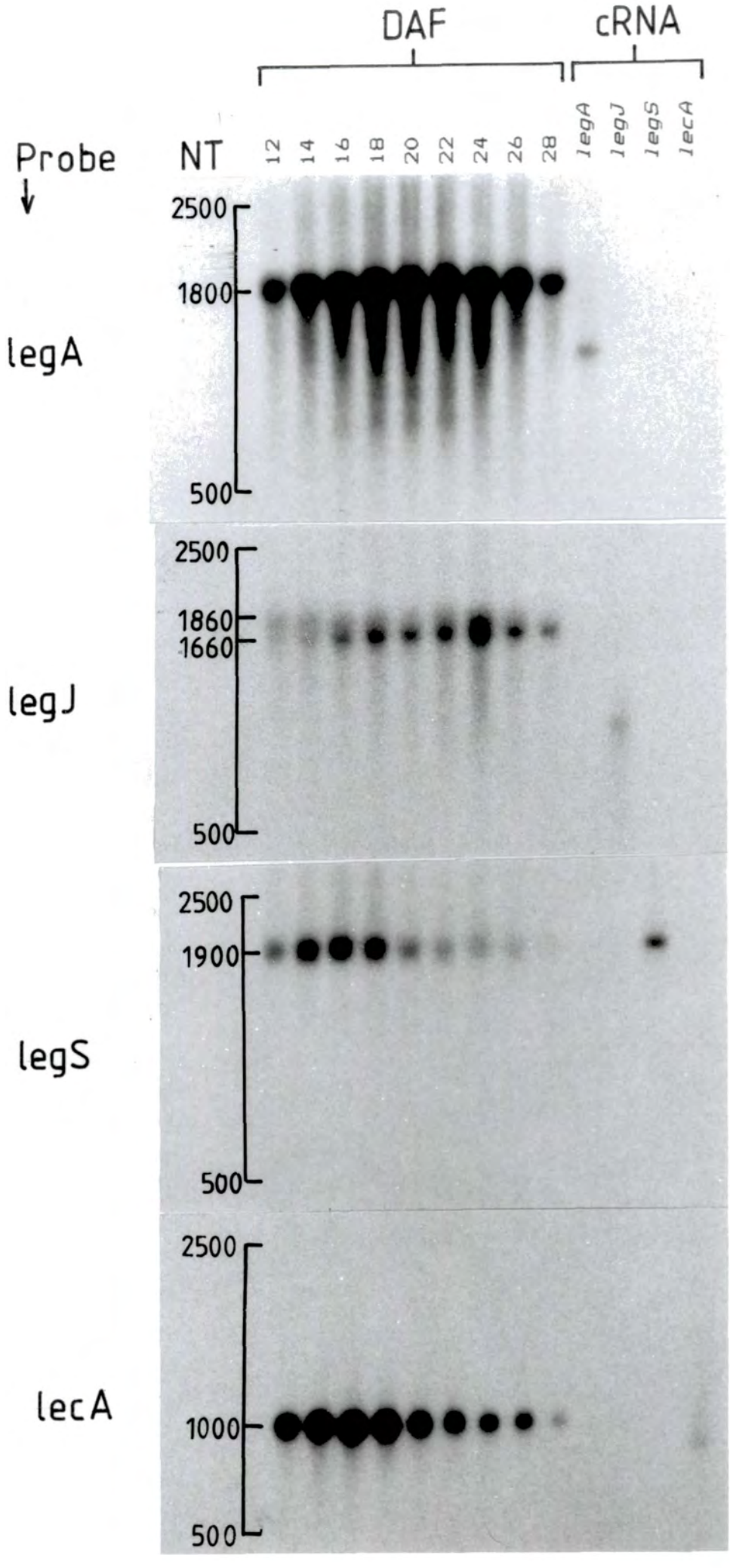
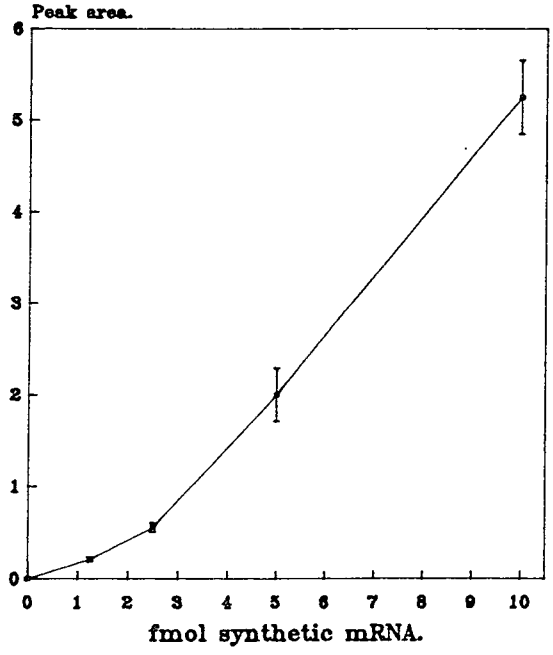

DAF
cRNA
Probe
NT
12
14
16
18
20
22
24
26
28
legA
legJ
legS
lecA
2500
1800
500
legA
2500
1860
1660
500
legJ
2500
1900
500
legS
2500
1000
500
lecA

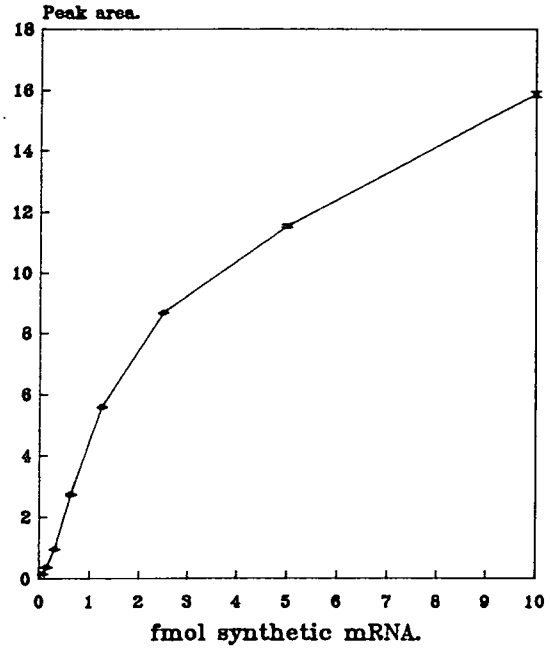
Figure 4. Example of standard curves used to quantify RNA dot blot assays.

A dot blot containing duplicate dots of cotyledon total RNAs and duplicate dots of a serial dilution of synthetic *legA* mRNA was probed with the insert from plasmid pDUB6 and autoradiographed for the different times indicated (Fig. 5 shows the 5 hour exposure of this blot). Autoradiographs were scanned using a laser densitometer and the integrated peak area of each dot was plotted against the molar amount of synthetic mRNA present.

20 min exposure.



5 hour exposure.



45 hour exposure.

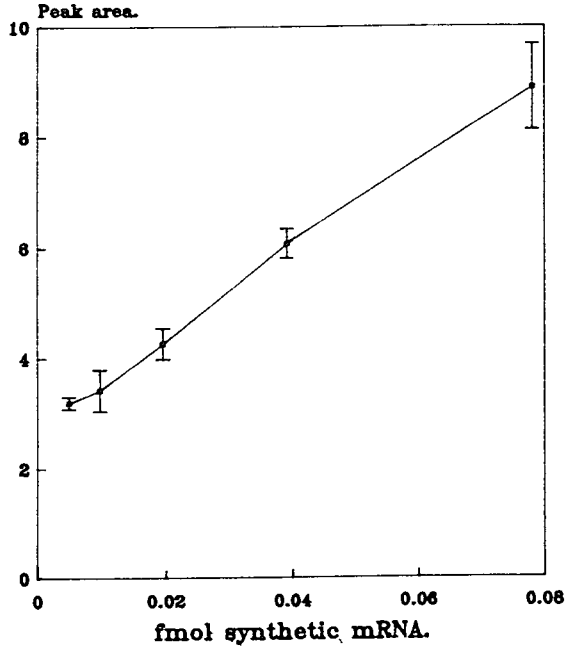


Figure 5. Autoradiograph of RNA dot blots used to quantify steady state mRNA levels.

Separate blots were probed with random primed inserts from the following plasmids: (i), pJC5.2; (ii), pCD40; (iii), pAD9.2; (iv), pDUB6. The X-ray film exposure times (at room temperature) were: blots (i) and (ii), 19 hours; blots (iii) and (iv), 5 hours.

Columns A-D show duplicate dots each containing 2μg total RNA extracted from cotyledons at various developmental stages (DAF). The numbers shown in the following tables under columns A-D indicate the developmental stage (DAF). Each RNA sample is also designated A or B depending on which of two batches of pea plants it originated.

Duplicate 2μg samples of total RNAs from pea leaf, root and stem, and *E.coli* rRNA were also included as indicated in the tables.

Columns E to T contain 2:1 serial dilutions of various synthetic mRNAs, in the amounts indicated in the table (given as fmol per dot), as follows:

(i) E and F, 1-12, *legJ*; G and H, 1-3, *legK*; G and H, 5-7, *legS*;
(ii) I and J, 1-12, *legK*; K and L, 1-3, *legJ*; K and L, 5-7, *legS*;
(iii) M and N, 1-12, *legS*; O and P, 1-3, *legJ*; O and P, 5-7, *legK*;
(iv) Q-T, *legA*.

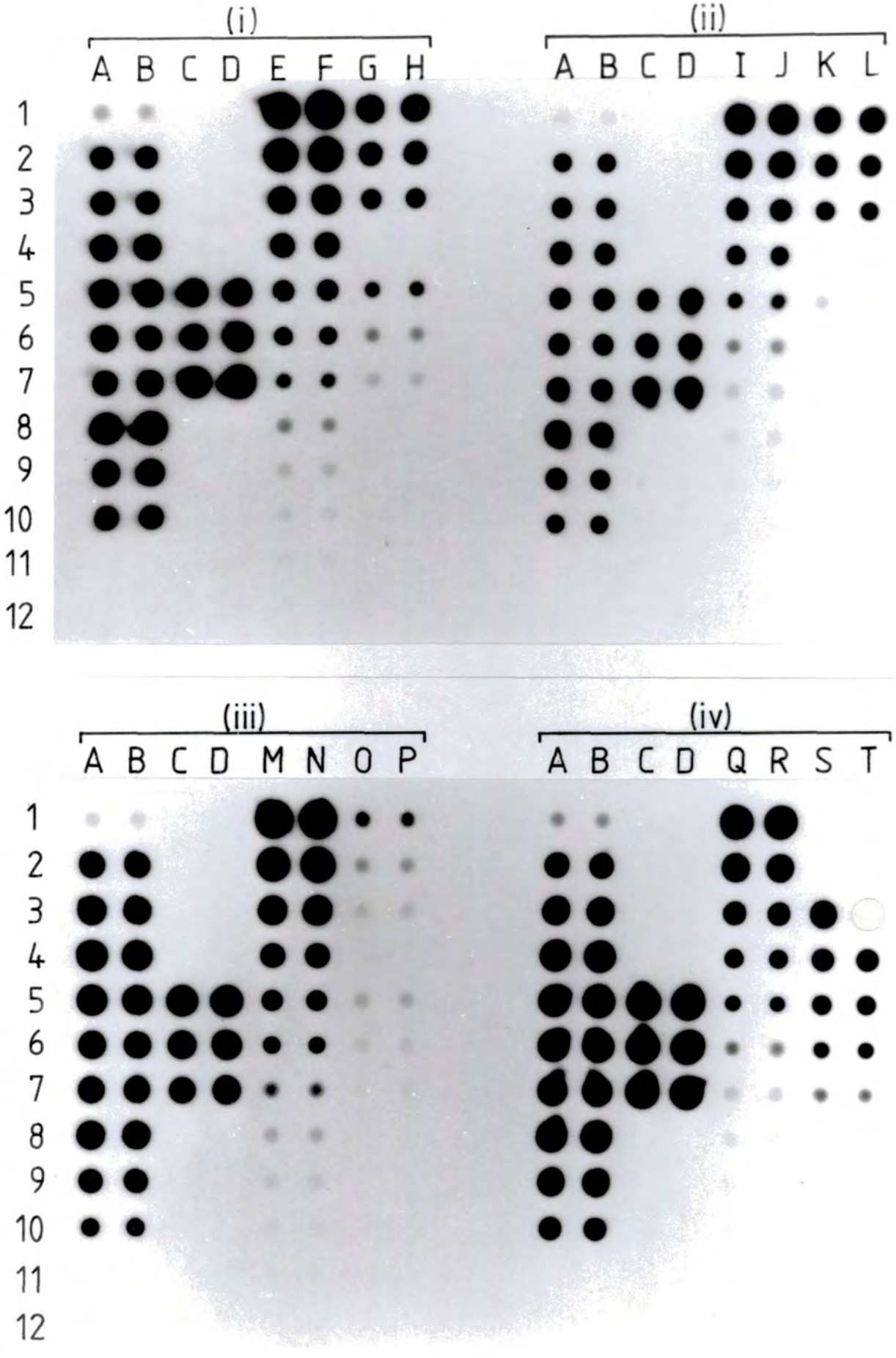

(i)
A B C D E F G H
1
2
3
4
5
6
7
8
9
10
11
12
(ii)
A B C D I J K L
(iii)
A B C D M N O P
(iv)
A B C D Q R S T

Figure 5 continued.

(i)-(iii)

	A	B	C	D	E/I/M	F/J/N	G/K/O	H/L/P
1	8-9A	8-9A			5	5	5	5
2	12A	12A			5/2	5/2	5/2	5/2
3	14A	14A			5/4	5/4	5/4	5/4
4	16A	16A	*E.coli*	*E.coli*	5/8	5/8		
5	18A	18A	18B	18B	5/16	5/16	5	5
6	20A	20A	20B	20B	5/32	5/32	5/2	5/2
7	22A	22A	22B	22B	5/64	5/64	5/4	5/4
8	24B	24B			5/128	5/128		
9	26B	26B			5/256	5/256		
10	28B	28B	Leaf	Leaf	5/512	5/512		
11	-	-	Root	Root	5/1024	5/1024		
12	-	-	Stem	Stem	5/2048	5/2048		

(iv)

	A	B	C	D	Q	R	S	T
1	8-9A	8-9A			10	10		
2	12A	12A			10/2	10/2		
3	14A	14A			10/4	10/4	4	4
4	16A	16A	*E.coli*	*E.coli*	10/8	10/8	4/2	4/2
5	18A	18A	18B	18B	10/16	10/16	4/4	4/4
6	20A	20A	20B	20B	10/32	10/32	4/8	4/8
7	22A	22A	22B	22B	10/64	10/64	4/16	4/16
8	24B	24B			10/128	10/128		
9	26B	26B			10/256	10/256		
10	28B	28B	Leaf	Leaf	10/512	10/512		
11	-	-	Root	Root	10/1024	10/1024		
12	-	-	Stem	Stem	10/2048	10/2048		

In order to quantify the absolute amounts of specific mRNAs per μg of total RNA, a constant amount of total RNA from each developmental stage and femtomolar amounts of synthetic mRNA standards (cRNAs), were immobilised in duplicate by dot blotting and hybridised to a mass excess of [α-^{32}P]dCTP-labelled legumin and lectin cDNA probes. The synthetic mRNAs were produced from the same DNA clones that were used as probes, using the *in vitro* transcription system first described by Melton *et al.* (1984) (see methods section 3.14). The exact molar concentration of the synthetic mRNAs was determined by trace labelling with [5,6-^{3}H]UTP of known specific activity during synthesis, followed by liquid scintillation counting. The integrity and identity of these cRNAs was confirmed by synthesis of [α-^{32}P]UTP-labelled cRNAs in otherwise identical reactions. These transcripts were then analysed by formaldehyde agarose gel electrophoresis followed by autoradiography of the dried gels (Fig. 2). The same [^{3}H]-labelled cRNA samples that were used as standards in the dot blot assays were also later analysed by Northern blotting as shown in Fig. 3 and were found to be greater than 90% intact, and of the correct size.

Autoradiographs of the RNA dot blots were quantified by densitometric analysis. Standard curves were constructed of femtomoles of cRNA against peak area (for example Fig. 4) and then the amount of specific mRNA per μg total RNA was determined from the linear region of this curve. As shown in Fig. 4, the shape of the standard curve and the linear region varies with the time of film exposure used. Thus, in order to quantify accurately the mRNA levels over the entire range, several exposures and corresponding standard curves of the same RNA dot blot were produced. Fig. 5 shows examples of RNA dot blots probed with 4 different legumin DNA clones. Data from these RNA dot-blots, and others probed with a lectin cDNA probe, were quantified and plotted in Fig. 6.

4.2.1.1 Cross hybridisation between *legJ*, *legK* and *legS* mRNA in RNA dot blot assays.

In each of the dot blot assays used for determining steady-state mRNA levels of *legJ*, *legK* and *legS* mRNAs in total

RNA, serial dilutions of synthetic mRNAs representing all three mRNAs were included (see Fig. 5). Thus the amount of cross-hybridisation of each DNA probe with the three types of mRNA, under the experimental conditions used, could be determined. This information is useful for interpretation of the data for the steady-state levels of these mRNAs.

The degree of cross-hybridisation between a probe sequence and a non-homologous synthetic mRNA was calculated as the ratio between hybridisation of the probe to the non-homologous synthetic mRNA and hybridisation to an equal amount of 100% homologous synthetic mRNA.

In practice this was done by measuring, from a standard curve of fmol homologous synthetic mRNA vs. peak area (for example see Fig. 4), the equivalent mass of a known mass of synthetic non-homologous mRNA. Thus, for example, the peak area generated by the *legJ* probe (pJC5.2) hybridising to 5 fmol of *legS* synthetic mRNA was read from the standard curve as equivalent to 0.09 fmol of *legJ* synthetic mRNA. The cross-hybridisation was, therefore, 0.09/5 = 1.7%. The data were then corrected according to the proportion of the probe that could theoretically hybridise to the non-homologous synthetic mRNA on the basis of the proportion of the probe sequence that could be aligned to it. Thus, extending the previous example, since only 1180 bp of the total 1906 bp of pJC5.2 aligns to the *legS* synthetic mRNA the true degree of cross-hybridisation becomes 1906/1180 x 1.7 = 2.7%. This adjustment was necessary to avoid under-estimating the degree of cross-hybridisation, because a labelled DNA clone used as a probe could hybridise completely to the synthetic mRNA derived from it, but regions of the probe may be unable to hybridise to non-homologous synthetic mRNAs derived from incomplete genomic or cDNA clones.

These data show that under the washing conditions employed, which were designed to be of high stringency (final wash 0.1 x SSC, 0.1% SDS at 60°C), there is a low, but insignificant, level of cross-hybridisation (<3.2%) between *legS* mRNA and both the *legJ* and *legK* mRNAs. Since the expression of *legS* mRNA is apparently of the same order of magnitude as the *legJ/K* mRNA this level of

cross-hybridisation does not prevent separate measurement of these two species by the dot blot procedure. However, the *legJ* and *legK* mRNAs, because of their very close nucleotide homology (94%) show approximately 50% cross-hybridisation to each other even under the high stringency washing employed. This prevents the expression of these two mRNAs from being studied independently by this method (see also section **4.2.3**).

The *legA* sub-family of mRNAs, which are approximately 99% homologous within this sub-family, are <60% homologous to other legumin mRNAs sub-families and show no detectable cross-hybridisation to them (data not shown).

Table 1. Nucleotide homology and Cross-hybridisation between *legJ*, *legK* **and** *legS* **mRNAs.**

CH = percentage cross-hybridisation, H = percentage nucleotide homology in the coding region. Nucleotide homology was determined by Dr. J.A. Gatehouse after alignment of the sequences using the NUCLAN program based on the algorithm of and Wilbur and Lipman (1983) with parameters K-tuple=3; window size=3; gap penalty=20. The two intron sequences were removed from the *legJ* genomic sequence (pJC5.2) before alignment.

	Synthetic mRNA					
Probe	*legS*		*legJ*		*legK*	
	CH	H	CH	H	CH	H
legS	--	--	3.2	72	1.4	75
legJ	2.7	72	--	--	49.2	94
legK	1.4	75	48.6	94	--	--

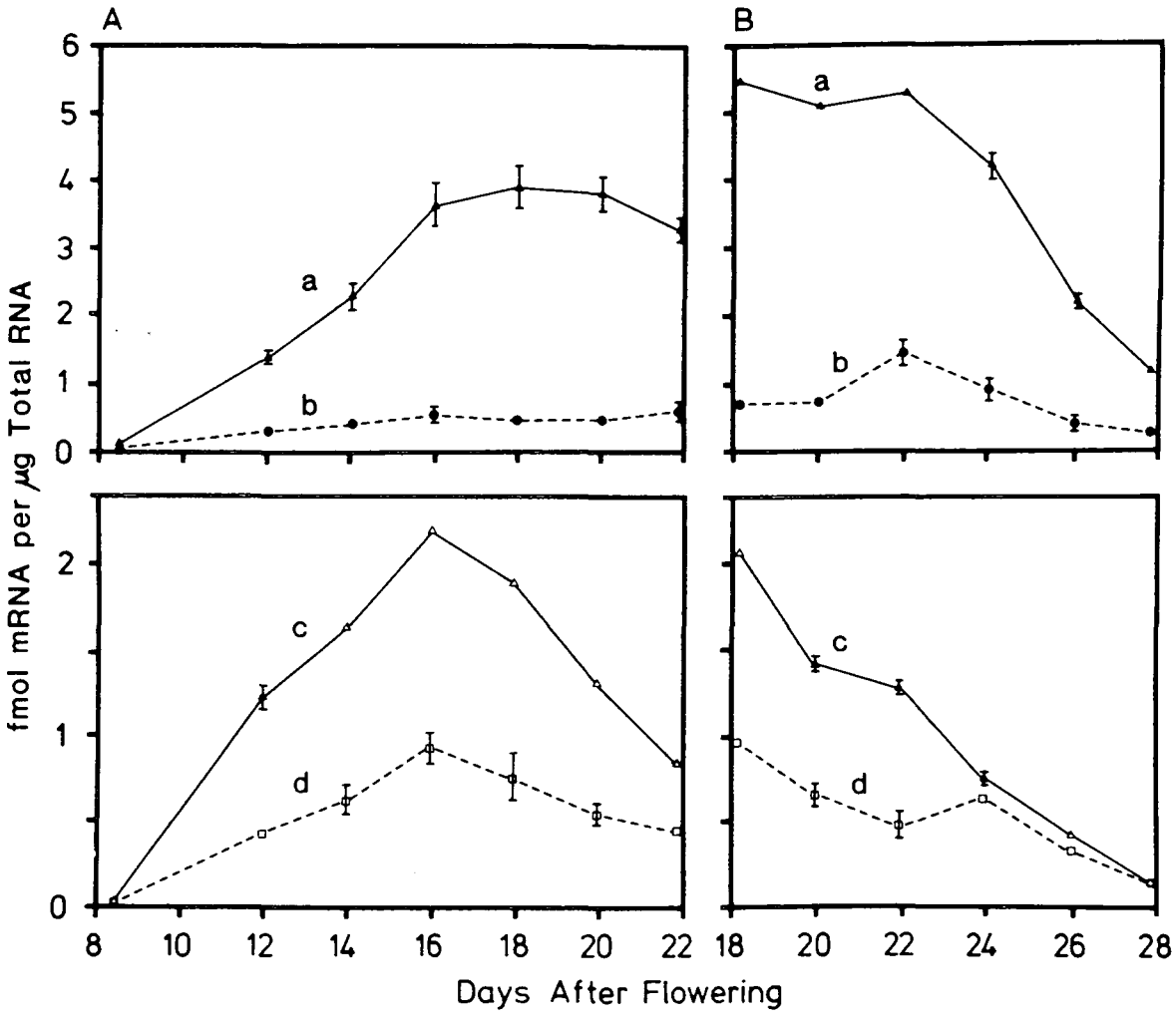


Figure 6. Steady state levels of legumin and lectin mRNAs throughout cotyledon development.

Autoradiographs of RNA dot blots probed with cDNA probes were quantified by laser densitometry using standard curves as exemplified by fig.4; each point represents the mean of duplicate dots. Error bars indicate the variation in duplicates. Error bars are absent when the variation is less than the size of the graph symbols used. The analysis was performed on cotyledons derived from two independent batches of plants (A and B). Cotyledons from batch A were from 8-9 to 22 DAF, whereas cotyledons from batch B were from 18 to 28 DAF.

Probes used were: a, pDUB6 (*legA*), (—▲—); b, pCD40 (*legJ*), (--●--); c, pLG4.10 (*lec*), (—△—); d, pAD9.2 (*legS*), (--□--);

4.2.2 The pattern of mRNA accumulation during embryogenesis.

The mRNAs assayed are present at low levels at the early cell expansion phase (8-9 DAF); they then increase at least 35-fold in each case to a peak level as cell-expansion proceeds and then decline as the seed begins to desiccate (Fig. 6, Table 2). The *legA* mRNA peaks at 18 DAF, whereas the *legS* and *lecA* mRNAs peak slightly earlier, at 16 DAF. The mRNA hybridising to the probe representing the *legJ* sub-family forms a doublet (Fig. 3) and peaks at 16 DAF before declining slightly and then reaching a second peak at 22 DAF. The expression of the *legJ* family is investigated further in section 4.2.3.

There are differences in the mRNA contents of the two batches of pea plants analysed (A and B, Fig. 6). Although the ratio of the three types of legumin mRNA does not vary significantly in the two batches, the absolute levels of these mRNAs at 18 DAF is 30-40% higher in batch B. However, lectin mRNA is only elevated by approximately 10% in batch B when compared to batch A, at 18 DAF. These differences may be due to some unknown variable in the plant growth conditions.

The quantitative levels of mRNA species, as a proportion by mass of total RNA, are comparable to those previously reported for *legA* and vicilin (0.2-0.7%; Boulter *et al.*, 1987); peak levels are 0.3% (*legA* at 18 DAF [batch A]), 0.15% (*lecA* at 16 DAF), and 0.05% (*legJ* and *legS* at 16 DAF). The Northern blot in Fig. 3 also shows, as discussed in section 4.2.1.1, that all the total RNAs used were in a largely nondegraded state and that cross-hybridisation between the three classes of legumin synthetic mRNAs was negligible.

These results show some similarities to those reported by Domoney and Casey (1987) for the developmental accumulation of the three classes of legumin mRNAs from *Pisum sativum* var. Birte. These authors reported a peak for *legA* (cDNA probe pCD43) at 24 DAF and also that *legS* and *legJ* (pCD32 and pCD40, respectively) peak earlier at 19 DAF. They did not observe a later peak for *legJ*. The relative levels of the three legumin mRNAs reported were very similar to our data at the stage where the *legA* mRNAs peak, but at earlier stages there are differences; at 8-9 DAF we show

that *legA* mRNA is at least 6 fold higher than *legS* and *legJ* (see Table 2) whereas at an equivalent stage (14 DAF) Domoney and Casey (1987) show that the three types of legumin mRNAs are present at approximately the same level. It can be speculated that such differences may be due to the use of a different pea genotype, or different growth conditions.

Table 2. Steady state levels of mRNAs in pea cotyledon total RNA. Total RNAs from different stages of cotyledon development (DAF) from two batches of plants (A and B) were analysed by dot blotting in duplicate [(i) and (ii)], followed by hybridisation to the inserts from the specified plasmids, as described in the methods section. Some of these data are plotted in Fig. 6.

DAF	fmol/μg total pea cotyledon RNA.									
	pJC5.2		pGEM40		pAD9.2		pAD4.4		pLG4.10	
	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
8-9A	0.015	0.014	0.02	0.02	0.012	0.014	0.12	0.12	0.026	0.026
12A	0.22	0.22	0.32	0.31	0.43	0.42	1.33	1.48	1.28	1.17
14A	0.11	0.24	0.41	0.42	0.70	0.56	2.10	2.46	1.64	1.63
16A	0.41	0.46	0.48	0.66	1.01	0.85	3.36	3.96	2.20	2.24
18A	0.54	0.56	0.52	0.45	0.90	0.63	3.61	4.27	1.92	1.91
20A	0.30	0.42	0.42	0.48	0.59	0.49	3.59	4.08	1.31	1.31
22A	0.35	0.26	0.51	0.70	0.47	0.42	3.12	3.45	0.84	0.84
18B	0.50	0.60	0.85	0.51	0.96	1.01	5.55	5.50	2.13	2.15
20B	0.68	0.35	0.82	0.69	0.60	0.70	5.11	5.12	1.40	1.45
22B	1.35	1.00	1.65	1.33	0.39	0.56	5.32	5.29	1.24	1.30
24B	1.17	0.89	0.80	1.06	0.65	0.61	4.08	4.40	0.72	0.78
26B	0.51	0.44	0.34	0.47	0.33	0.32	2.16	2.28	0.40	0.43
28B	0.26	0.26	0.23	0.25	0.13	0.15	1.02	1.15	0.14	0.14

4.2.3 Further analysis of the expression of the members of the *legJ* gene sub-family in cotyledon tissue by Northern blotting.

In the previous section (4.2.2) it was noted from the Northern blot probed with a *legK* cDNA (Fig. 3), known to cross-hybridise to three members of the *legJ* gene sub-family (Domoney and Casey 1985; section 1.4.2), that a broad region of hybridisation, divided into two ill-defined bands (as previously reported by Gatehouse *et al.* 1988) of size approximately 1660 and 1860 nucleotides, was

observed. It was also apparent from Fig. 3 that the upper and lower bands were of equal intensity at earlier stages of development (12-14 DAF) whereas at later stages (16-28 DAF) the lower band predominated. The size heterogeneity could be due to variation in size between the different members of the *legJ* gene sub-family, to multiple transcription starts of a single gene or, alternatively, to differential processing of a single species of primary transcript. A DNA restriction fragment derived from the 3′ non-coding region of the *legJ* gene, the region of least homology between the available *legJ* and *legK* sequences, has previously been shown to hybridise more strongly to the lower band and thus it appeared that this band represented the *legJ* mRNA (Gatehouse *et al.* 1988).

In order to try to improve the resolution of the observed broad doublet of hybridisation, the total RNA samples were hybridised to oligo(dT)$_{12-18}$ and then incubated with RNaseH in order to hydrolyse any resultant DNA:RNA hybrids. Such a treatment is designed to remove poly(A) tracts from the 3′ ends of mRNAs (Vournakis *et al.*, 1975). Since poly(A) tail length is variable, removal of the poly(A) tails should reduce mRNA size heterogeneity and may improve resolution of multiple, cross-hybridising mRNA species. Total RNAs with and without this treatment were analysed by Northern blotting and Fig. 7(a) and (b) shows one such blot probed with a *legK* cDNA. The first observation is that the treatment reduced the mean size of the hybridising region by 100-200 nucleotides, although no accurate change in size can be determined due to the small shift in mobility; this is consistent with successful removal of the poly(A) tails. It is also apparent that three, or possibly four, bands were resolved in the absence of poly(A) tails. The autoradiograph shown in Fig. 7(b) loses clarity on photography so the tracks containing treated RNA were analysed by laser densitometry (Fig. 8a). Three distinct bands were apparent, most easily seen at 20 DAF due to their approximately equal intensity, at mobilities of 70mm, 72mm and 74mm.

At 14 and 16 DAF the 70mm and 72mm bands were of approximately equal intensity, with the 74mm band forming a minor component;

Figure 7. Analysis of the expression of the *legJ* gene subfamily by Northern blotting.

Total cotyledon RNAs from the indicated days after flowering (DAF) were hybridized to oligo(dT)$_{12-18}$ as described in section 3.9 and then incubated with or without RNaseH (labelled as poly(A) tail - and + respectively). 1μg samples of RNA were then run on agarose formaldehyde gels and Northern blotted. Blots were hybridized to the following probes;

(a), cDNA insert from pGEM40 (*legK* cDNA), 5 hours exposure;

(b), cDNA insert from pGEM40, 16 hours exposure;

(c), *legJ* oligo1;

(d), *legJ* oligo2;

(e), *legK* oligo.

Size markers were provided by pea rRNA bands and by pBR322 AluI DNA restriction fragments. In addition to total RNAs, blots (c) and (e) also included, in the track labelled "cRNA", a mixture of 1 fmol of *legJ* synthetic mRNA (produced by T7 RNA polymerase from pGEM5.2B linearized with AvaI) and 1 fmol of *legK* synthetic mRNA (produced by T7 RNA polymerase from pGEM40 linearized with HindIII).

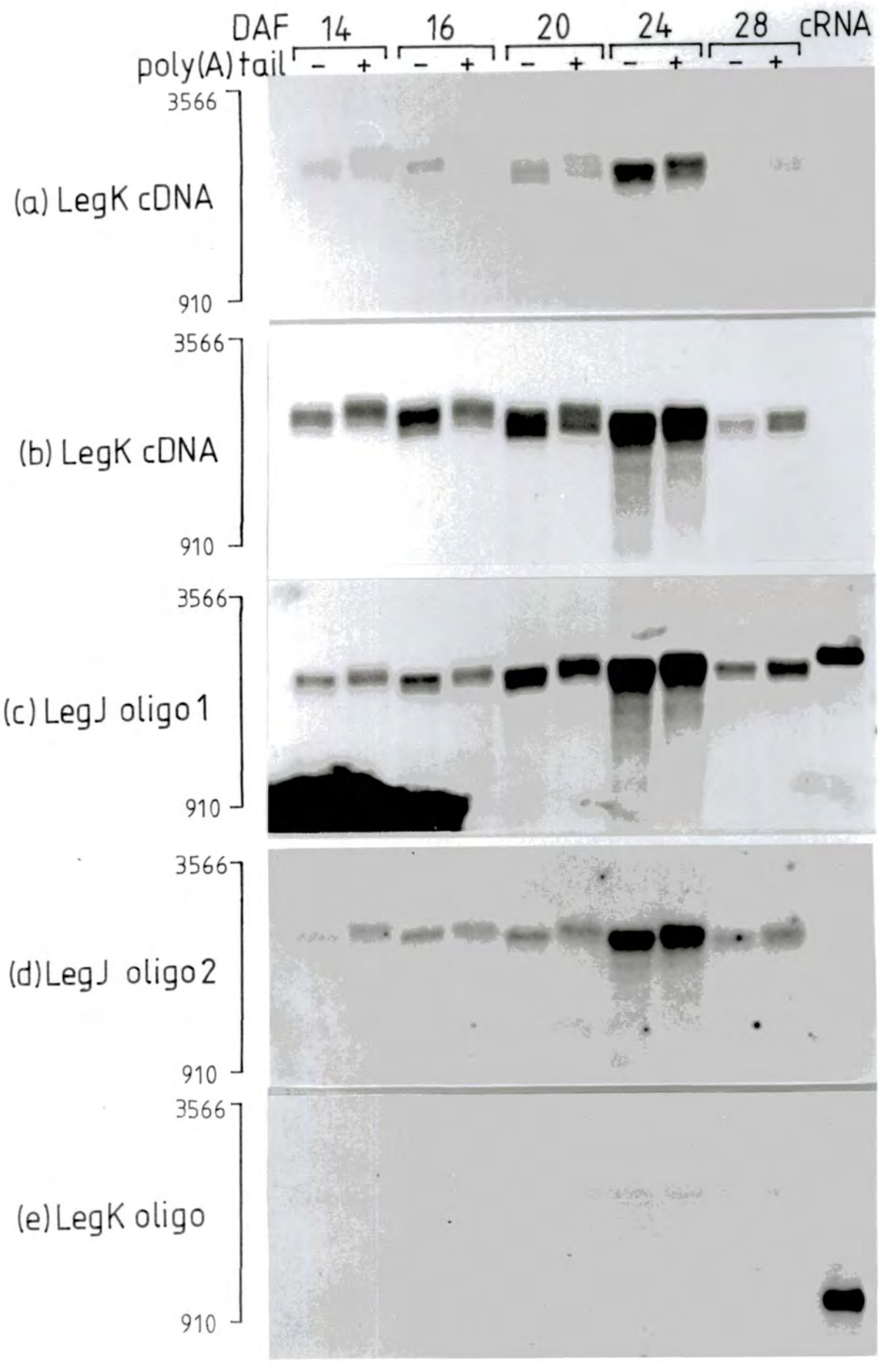

DAF 14 16 20 24 28 cRNA
poly(A) tail – + – + – + – + – +
3566
(a) LegK cDNA
910
3566
(b) LegK cDNA
910
3566
(c) LegJ oligo 1
910
3566
(d) LegJ oligo 2
910
3566
(e) LegK oligo
910

Figure 8. Densitometric analysis of autoradiographs shown in Figure 7. Individual tracks from the autoradiographs of the Northern blots represented in Figure 7 were scanned using a laser densitometer as described in section 3.12. Only the tracks containing RNA samples that had been treated with oligo(dT)$_{12-18}$ and RNaseH were scanned. The developmental stage (DAF) of the RNA analysed is indicated on the vertical axis. The dotted lines represent the baseline for each scan (the baseline is defined as the average of the lowest 16 points on the absorbance scale). The probes were as follows:

(a), cDNA insert from pGEM40 (*legK* cDNA);

(b), *legJ* oligo1;

(c), *legJ* oligo2.

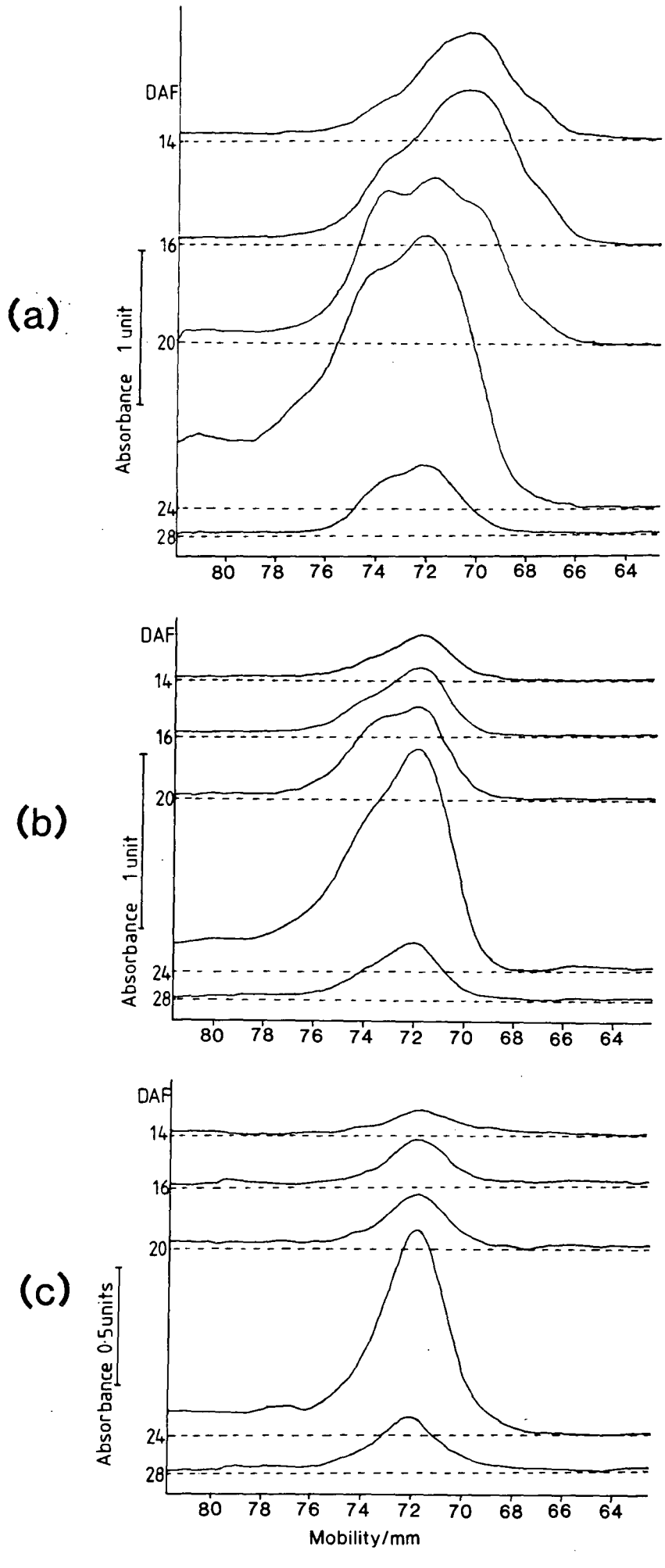

(a)
DAF
14
16
20
24
28
Absorbance 1 unit
80 78 76 74 72 70 68 66 64
(b)
DAF
14
16
20
24
28
Absorbance 1 unit
80 78 76 74 72 70 68 66 64
(c)
DAF
14
16
20
24
28
Absorbance 0·5units
80 78 76 74 72 70 68 66 64
Mobility/mm

Figure 9. Alignment of the 3′ coding and non-coding regions of *legJ* and *legK*; position of oligonucleotide probes.

The 3′ coding and non-coding regions of *legJ* and *legK* were aligned (taken from Gatehouse *et al.* 1988, with minor changes to the numbering scheme) and the positions of oligonucleotide probes used in section 4.2.3 are indicated as follows (sequences complementary to the oligos are underlined): *legK* oligo and *legJ* oligo1, 1516-1530; *legJ* oligo2, 1887-1910. Amino acid sequence and the position of consensus poly(A) signals are also given.

there also appeared to be an additional faint band at approximately 68.5mm, which was not observed at stages later than 16 DAF. At 20 DAF the 74mm band had increased in intensity to approximately the same as the 70mm and 72mm bands. At 24 DAF the 70mm band is no longer detected whereas the intensity of 72mm and 74mm bands have dramatically increased. At 28 DAF, again only the 72mm and 74mm bands are detected, albeit at a lower intensity.

In order to provide information about the identity of these different bands, oligonucleotides complementary to *legJ* and *legK* specific sequences were synthesised. Fig. 7(c) shows hybridisation to a 14-mer oligonucleotide, named *legJ* oligo1, of sequence 5'-ACACCGTGTTTCCT-3' (circumflexes indicate mismatches to the *legK* cDNA sequence) complementary to the *legJ* region 1517-1530 bp (see Fig. 9). This oligonucleotide hybridises to the 72mm and 74mm bands; no hybridisation to the 70mm and 68.5mm bands could be detected (Fig. 7(c), Fig. 8(b)). Synthetic mRNAs representing *legJ* and *legK* were both present as controls and the *legJ* oligo1 hybridises to the *legJ* synthetic mRNA only.

Because two bands hybridise to the *legJ* oligo1 it was possible that they were generated by differential processing of the same *legJ* primary transcript and it can be hypothesised that these two bands resulted from alternative use of the two poly(A) signals present (Gatehouse *et al.* 1988). To test this hypothesis an oligonucleotide was synthesised that was complementary to a region lying between the poly(A) signals of the *legJ* gene, and that contained mis-matches to the equivalent *legK* sequence. The 23-mer, 5'-TATAGGAAGTGAAATTTTTTACT-3' (*legJ* oligo2), was designed so that it would hybridise to *legJ* mRNA utilising poly(A) signal 2 but not that utilising poly(A) signal 1 (see Fig. 9). However, this 23-mer starts 16 bp downstream of the last bp of the poly(A) signal 1, thus, it may hybridise to mRNAs generated from both poly(A) signals if the site generated from signal 1 is greater than approximately 25 bp downstream of signal 1.

As shown in Fig. 7(d) and Fig. 8(c) this oligo hybridises to the 72mm band and not to the 74mm band. This suggests that the higher molecular weight band is the *legJ* mRNA with polyadenylation directed by poly(A) signal 2 and is consistent with the above

hypothesis. However, since Southern blotting has shown that the *legJ* gene sub-family has three members, one of which, *legL*, has yet to be isolated, the possibility can not be excluded that the 74mm band is a *legL* mRNA which, fortuitously, is identical in sequence in the region complementary to *legJ* oligo1, but different in the region complementary to *legJ* oligo2. This possibility may be considered unlikely.

In order to identify bands corresponding to *legK* mRNA an oligonucleotide of sequence 5'-ACAC^T^GC^G^TC^T^CCT-3 was synthesised (*legK* oligo) which would hybridise to *legK* but not *legJ* (see Fig. 9). Fig. 7(e) shows that, although this oligonucleotide hybridises to the positive control of synthetic *legK* mRNA (and not to the synthetic *legJ* mRNA), no hybridisation to the total RNA samples could be detected; the visible bands in the total RNA tracks are due to background hybridisation to 18S rRNA.

At this stage it must be pointed out that the various sequence information and RNA samples used in this study were derived from several different cultivars (cv.) of *Pisum sativum* L.: the *legK* cDNA, pCD40 (Domoney and Casey, 1984), used as a probe and as template for synthesis of *legK* synthetic mRNA, was isolated from cv. Birte, whereas a genomic clone containing the complete *legJ* gene and the 3' region of the *legK* gene, λJC5 (Gatehouse *et al.* 1988), was isolated from cv. Dark Skinned Perfection (DSP). The partial cDNA sequence of pCD40 and the sequence of the corresponding region of the *legK* gene of λJC5, after removal of introns, are identical in the region of the *legK* oligo, but are otherwise different by two base substitutions (Gatehouse *et al.* 1988); these base substitutions demonstrate the existence of legumin gene sequence variation between different cultivars. The total RNA samples used in this study were isolated from cv. Feltham First and, currently, there is no sequence data for the *legK* and *legJ* genes of this cultivar except that from a 5' fragment of a cDNA with sequence identical to the coding region of the DSP *legJ* coding region (Gatehouse 1986). This sequence does not cover the regions complementary to the oligonucleotides used in this study.

The lack of hybridisation of the *legK* oligo to Feltham First

total RNA, therefore, suggests two possible hypotheses; (1) in cv. Feltham First, *legK* is a pseudogene, or expressed at a level undetectable in this experiment, although the gene must be expressed in cv. Birte because a cDNA has been isolated; (2) the *legK* gene is expressed in cv. Feltham First but no hybridisation to *legK* oligo occurs. This could be because the gene sequence of *legK* differs by some form of mutation in the region of the *legK* oligo between the cvs. Birte/DSP and cv. Feltham First. Relevant to these results is a report that the *legK* gene is not expressed in cv. DSP; Domoney and Casey (1984) isolated poly(A)$^+$ RNA from cotyledons of pea cultivars Birte and DSP, selected mRNAs hybridising to plasmid pCD40 (*legK* cDNA), translated them *in vitro*, and then immunoprecipitated the legumin proteins. They found that with cv. Birte, pCD40 selected mRNAs that synthesised a 63000 MW polypeptide (*legJ* precursor, see section 1.4.2 and Fig. 1) and a 65000 MW polypeptide (the *legK* precursor); however, cv. DSP only selected mRNA that synthesised the 63000 MW polypeptide (in both cvs., incidentally, pCD40 also selected the cross-hybridising *legS* mRNA encoding the 80000 MW polypeptide). These results suggest that *legK* may be a pseudogene in cv. DSP and cv. Feltham First, but not in cv. Birte from which the *legK* cDNA was derived.

The 70mm and 68.5mm bands hybridising to the complete *legK* cDNA could be either *legK* and possibly *legL* mRNA assuming hypothesis (2) is correct or could represent only *legL* mRNA if hypothesis (1) is correct.

The tentative conclusions regarding the temporal regulation of the 4 observed bands can be summerised as follows: the 70mm and 68.5mm bands (*legK* and/or *legL*) peaked early, at approximately 16 DAF, whereas the 74mm and 72mm bands peaked much later at 22-24 DAF. In addition, it can be seen that the ratio of the bands 74mm and 72mm changed during development; at 14 and 16 DAF the 72mm band was of 2 to 3-fold higher intensity than the 74mm band whereas at 20 the intensity of the 74mm band had increased to approximately equal that of the 72mm band (see Fig. 7b). At 24 and 28 DAF the relative intensity of the 74mm band had declined again. This indicates, with the proviso that the identity of the 74mm and

72mm bands is not proved beyond doubt (see above), that the *legJ* poly(A) signal 2 is used more often than poly(A) signal 1 at the earlier and later stages of development, but at 20 DAF they are used with approximately equal frequency.

The occurrence of multiple poly(A) sites is a common feature of plant genes (Dean *et al.* 1986; Joshi, 1887), but the functional significance of this remains obscure. Poly(A) site variation also occurs in pea seed lectin; two independently isolated cDNA clones have poly(A) sites differing by 24bp, although there only appears to be a single simple poly(A) signal motif (Gatehouse *et al.*, 1987; Higgins *et al.*, 1983); also, Vodkin *et al.* (1983) mapped the poly(A) sites of soybean lectin mRNA and showed the presence of two sites. They showed that the use of site 2 rather than site 1 might prevent the formation of a 3'-terminal stem-loop structure, which they speculated might be of significance for the stability of the mRNA. A *legS* mRNA species of pea has also been shown, by the sequencing of two distinct cDNAs, to have more than one poly(A) site; pAD9.2 (J.A. Gatehouse, unpublished) is polyadenylated 13 bp downstream of the 3rd AATAAA motif, whereas pCD32 (Domoney *et al.* 1986a) is polyadenylated 106bp downstream of the pAD9.2 site. This site is 33bp downstream of an AATAAT motif.

In order to back-up the tentative conclusions from these experiments, it is necessary to show that the sequences of the *legJ* and *legK* genes from cv. Feltham First, in the regions complementary to the oligo probes used here, are identical to those of Birte/DSP. This information could be obtained from a genomic clone, recently isolated from cv. Feltham First (Sami Yaish, University of Durham; personal communication), containing *legK* and *legJ* sequences, either by direct sequencing, or by hybridisation of the DNA clones to *legJ* oligo 1 and the *legK* oligo. Alternatively, the experiments could be repeated using total RNA derived from Birte and/or DSP.

More powerful techniques for studying the expression of individual genes within a gene family with highly homologous members are provided by RNaseA/T1 and S1 nuclease protection assays, which utilise the greater resolution afforded by

polyacrylamide sequencing gels. Using this approach, the precise position of poly(A) sites for each mRNA species could be mapped, and their frequency of use estimated, by using genomic DNA probes from 3′ flanking regions which overlap these sites.

4.2.4 Steady-state levels of seed-protein mRNAs in non-seed tissues.

In order to determine whether any of the seed storage protein mRNAs were detectable in any tissue other than developing cotyledons, total RNAs from leaf, stem and root (kindly donated by L. Gatehouse and R. Swinhoe) were included in the dot blots shown in Figure 5. When these blots were exposed for long periods (Fig. 10(i)) a signal was obtained for these non-seed organs. However, the level of hybridisation was less than the hybridisation to the lowest dilution of synthetic mRNA used for the construction of the standard curves (each dot in the dilution series also contained 2μg *E.coli* rRNA as carrier). Also, extrapolation of the standard curve of peak area against fmol synthetic mRNA shows that this curve does not pass through zero (see Fig. 4); the intersect on the vertical axis indicates the level of background hybridisation to the carrier RNA. The dots of *E.coli* rRNA alone, which were included in these blots to indicate more directly the level of background hybridisation, were, unfortunately, obscured by the very high level of hybridisation to neighboring cotyledon RNA.

Thus, from these blots, it was unclear whether the signal was real, or due to background hybridisation. To overcome this problem serial dilutions of far lower concentrations of synthetic mRNAs were made using a variety of different sources of carrier RNA. These were dot blotted onto nitrocellulose together with sample RNAs and probed with very high specific activity (6×10^8 to 8×10^8 cpm/μg) synthetic antisense RNA probes complementary to *legK* and *legA* mRNA (Fig. 10(ii)). This shows that the background hybridisation was different according to the type of carrier RNA used. Very high background was observed with *E.coli* rRNA and with total RNA from leaves of *Nicotiana tabacum*, whereas the background hybridisation to the RNA genome from the plant virus CCMV (Cowpea Chlorotic Mottle Virus) was barely detectable in this assay; 3μg

of total RNA from *Nicotiana tabacum* gave a signal equivalent to between 0.006 and 0.015 fmol of synthetic mRNA (using 3μg of CCMV RNA as carrier). 0.01 fmol of legumin mRNA represents 6.1 picograms. When no carrier RNA was used the hybridisation signal was reduced approximately two to four-fold relative to the use of CCMV RNA as a carrier. This may be due to loss of the ng quantities of synthetic mRNAs by absorption to plasticware, or to lack of protection against degradation by trace amounts of contaminating nuclease activity in the absence of carrier.

From the dot blots shown in Fig. 10(ii) it is possible to place a maximum value on the expression of the cotyledon abundant mRNAs in light leaf (preparation (1) only), dark grown leaf and root; we can say that the levels of the *legJ* and *legA* classes of mRNA in these samples are less than 0.00025% of total RNA, ie. at least 2500-fold lower than the expression of *legA* type mRNA at its peak of expression in the cotyledon. However, it can also be seen from Fig. 10(ii) that a level of hybridisation significantly above background is obtained for the stem total RNA preparation (1) and for light leaf total RNA preparation (2). The Northern blot in Fig. 11 confirms that the above background signal in stem preparation (1) is due to presence of mRNAs of the correct size in the case of both *lecA* and *legS* probes. This surprising result was investigated further by assaying other preparations of stem and light leaf RNA (also gratefully received from L.Gatehouse and R. Swinhoe) (Figs. 10 (iii) and 11). RNA preparations from three other light leaf samples and one other stem sample showed no hybridisation above background. This would suggest that either the stem (1) and light leaf (2) preparations were contaminated with cotyledon mRNA, or that the expression of the seed-protein mRNAs is developmentally or environmentally regulated in these tissues, and that variation between samples was due to some unknown variable. The first explanation seems more likely and is consistent with the observation that the relative proportions of the different seed mRNAs in stem preparation (1) are identical to the proportions in the cotyledon. The presence of seed protein mRNAs in these samples was not investigated further.

In an attempt to avoid the problem of background hybridisation

Figure 10. Dot blots of RNA from non-seed tissues hybridized to probes complementary to seed-storage protein mRNAs.

(i): autoradiographs (a) and (b) show 45 hour exposures of the RNA dot blots shown in Fig. 5 (iii) and (iv) respectively, ie. probed with *legS* and *legA* random primed DNA clones respectively. Rows and columns are labelled as in Fig 5.

(ii): autoradiographs (c) and (d) show RNA dot blots hybridized to synthetic antisense mRNAs complementary to *legK* cDNA and *legA* cDNA clones respectively. Columns A-L contain 2:1 serial dilutions of synthetic sense mRNAs, complementary to the probes used, in the range 0.05 to 0.05/1024 fmol. Each of these dots also contained 3μg of the following carrier RNAs: A,B,I and J, CCMV RNA; C,D,G and H, *N.tabacum* leaf total RNA; E,F,K and L, no carrier. Columns Y and Z contain duplicate samples of various RNAs as follows (all samples contain 3μg of RNA and are total RNAs from *P. sativum* unless otherwise stated): 1, 14ng 14 DAF cotyledon; 2 and 3, blank; 4, light leaf (1); 5, root (1); 6, stem (1); 7, light leaf (2); 8, dark leaf; 9, *E.coli* rRNA; 10, CCMV RNA; 11, *N. tabacum* leaf.

(iii): autoradiographs (e) and (f) show RNA dot blots hybridized to synthetic antisense mRNAs complementary to *lecA* cDNA and *legS* cDNA clones respectively. Columns A-D, rows 1-11, contain 2:1 serial dilutions of sense synthetic mRNAs, complementary to the probes used, in the range 0.2 to 0.2/1024 fmol. 3μg of CCMV carrier RNA was used throughout. Columns A-D, row 12, contain 3μg of CCMV RNA only. Columns Y and Z contain duplicate samples of various RNA samples as follows (3μg of *P. sativum* total RNA unless otherwise specified): 1, 20ng 14 DAF cotyledon; 2, blank; 3, light leaf (1); 4, light leaf (3); 5, light leaf (4), 6, dark leaf; 7, root (1); 8, root (2); 9, stem (2); 10, stem (1); 11, *E.coli* rRNA; 12, *N. tabacum* leaf. Numbers in brackets indicate the identity of different RNA preparations from the same type of tissue as also used in Fig. 11.

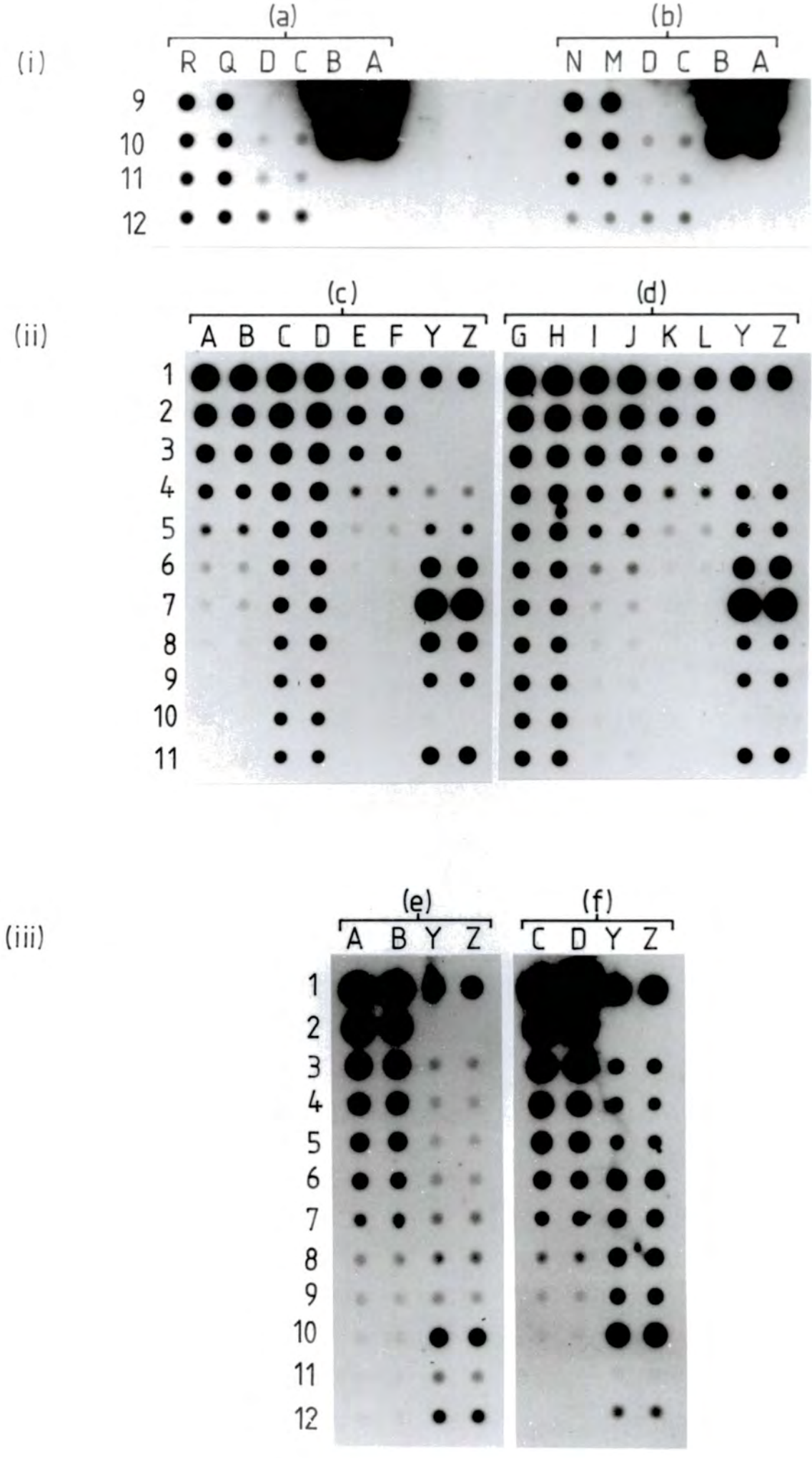
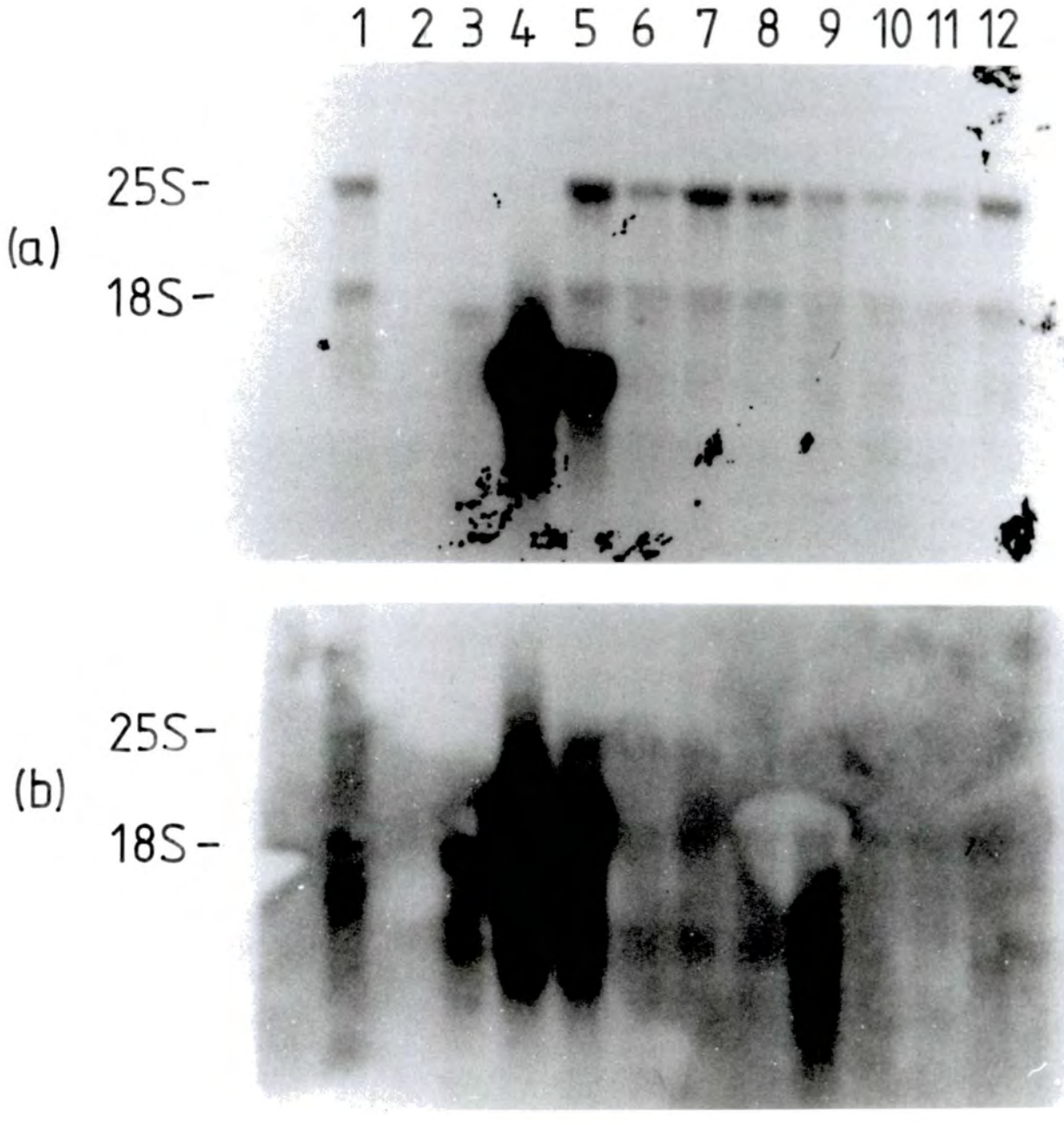

(i)
(a)
R Q D C B A
(b)
N M D C B A
9
10
11
12
(ii)
(c)
A B C D E F Y Z
(d)
G H I J K L Y Z
1
2
3
4
5
6
7
8
9
10
11
(iii)
(e)
A B Y Z
(f)
C D Y Z
1
2
3
4
5
6
7
8
9
10
11
12

Figure 11. Northern blot of total RNAs from non-seed tissues probed with antisense synthetic seed protein mRNAs.

Autoradiographs show a single Northern blot that has been probed first with lectin antisense mRNA (a). The blot was then treated with boiling 0.1% SDS to remove the probe and then reprobed with a *legK* antisense mRNA (b). Tracks contain the following RNA samples (each track contains 7μg of total RNA from *P. sativum* unless otherwise specified): 1, *N. tabacum* leaf; 2, CCMV; 3, *E.coli* rRNA; 4, 50ng 14 DAF cotyledon; 5, stem (1); 6, Stem (2); 7, root (1); 8, root (2); 9, dark leaf; 10, light leaf (1); 11, light leaf (3); 12, light leaf (4). Numbers in brackets indicate the identity of different RNA preparations from the same type of tissue as also used in Fig. 10.


1 2 3 4 5 6 7 8 9 10 11 12
(a)
25S-
18S-
(b)
25S-
18S-

(the limiting factor in sensitivity of the RNA dot blot assay) a Northern blot was produced. By using Northern blots it was hoped that the background hybridisation could be size fractionated from any true signal. The Northern blot shown in Fig.11(a) shows that no band for lectin mRNA of the expected size is detected in non-seed tissues. The limit of detection here is about 4 to 5-fold lower than the observed hybridisation to the rRNA bands. Thus, considering that the tracks of the Northern blot were loaded with 7μg of total RNA compared to the 3μg loaded in the dot-blot assay, this allows us to increase the lower limit of detection to approximately 2×10^4-fold lower than the peak lectin expression in the cotyledon. It would be, unfortunately, more difficult to detect very low levels of legumin mRNAs by Northern blotting of non-seed tissues because these mRNAs co-electrophorese with the 18S rRNA band, which hybridises non-specifically. The Northern blots show that the background hybridisation of the *lecA* RNA probe was localised to the 16S *E.coli* rRNA, and both the 25S and 18S rRNA bands of the total RNA from *Nicotiana tabacum* and *Pisum sativum*. The pattern of background hybridisation to the *legK* RNA probe was different; there was a more general signal covering the whole range of molecular weights in the samples.

It is not possible to say categorically that the genes are switched off in these non-seed tissues because of the limited sensitivity of the assay. Much greater sensitivity in detection of mRNAs could be achieved by the use of poly(A)$^+$ fractionated RNA or by using the polymerase chain reaction, which could theoretically detect a single molecule of mRNA per reaction vessel (stringent controls would be required to prevent cross-contamination of abundant mRNAs from tissues used in the same lab.).

Walling *et al.* (1984), when measuring the abundance in non-seed tissues of five classes of mRNAs known to be abundant in cotyledons of soybean, claim to have a lower limit of detection of $\simeq$ 50 molecules per cell. Using their assay (dot blotting of poly(A)$^+$ RNA) they were unable to detect any of the five classes of mRNA in 14 day postgermination cotyledons, leaf, stem or root. Since the most highly expressed mRNAs (β-conglycinin, and glycinin) represented 25000 molecules per cell (10% of mRNA) their

lower limit of detection was 500-fold lower than the peak expression detected. These authors also claim to have detected, in all plant mature organ systems, by unspecified "other procedures", ≃ 5 molecules per cell of Kunitz trypsin inhibitor, but could not detect β-conglycinin, glycinin and 15kDa protein mRNAs at a level of <0.1 molecules per cell. This latter detection level is 2.5 x 10^5-fold lower than peak cotyledon expression.

Soybean lectin mRNA could be detected in soybean roots, but not leaf or stem, by Northern blotting of 10μg quantities of poly(A)$^+$ RNA, at a level of ≃ 0.2 molecules per cell (Okamuro *et al.*, 1986). This is 20000-fold lower than the peak expression of the same lectin gene, L1, in soybean cotyledons. This parallels the detection of pea lectin mRNA, in the poly(A)$^+$ RNA fraction of pea roots taken from seedlings 21 days after sowing, at a level ≃4000-fold lower than that found in immature pea seed (Buffard *et al.*, 1987). This level of expression in pea roots should be within the limits of detection of the Northern blot shown in Fig. 11. Buffard *et al.* (1987) have shown that lectin mRNA was detectable in 4 day roots, reaches a peak in 10 day roots and had then diminished in 21 day roots. In addition, they found that prevention of nodulation by the use of nitrate or by not inoculating with *Rhizobium leguminosarum* did not reduce lectin expression in roots. The roots used in this experiment were 13 days old, the seeds having been germinated in a dark spray room for 4 days, grown in the growth cabinet described in section 3.1 for 4 days and then transferred to the dark for 5 days. They were grown in alcathene granules soaked in Phostrogen solution, a complete nutrient medium containing nitrate, and were not exposed to *R. leguminosarum*. The inability to detect lectin mRNA in these roots may be due to the differences in growth conditions, the only obvious difference being the 5 day dark treatment. Lectin expression in roots is thought to be involved in legume-*Rhizobium leguminosarum* interaction at the onset of nodulation (see section 1.4.5).

It is now known that leaves can act as a nitrogen sink and indeed a protein with many of the characteristics of the seed-storage proteins has been identified in all the vegetative

tissues of *Glycine max* (Wittenbach, 1983; Staswick, 1988). This protein is synthesised on the RER and accumulates in the vacuoles of paraveinal mesophyll cells in response to depodding. It may be broken down later and used as a nitrogen source during seed development. Thus, the expression of seed-storage proteins in non-seed tissue would not be totally surprising. However, the vegetative storage protein of *Glycine max* bears no obvious structural or sequence homology to *Glycine max* seed-storage proteins and is not expressed in the seed (Staswick, 1988).

In conclusion, these experiments have shown, in pea, in common with all other previous studies on the tissue specificity of seed-storage proteins, that no seed-storage protein mRNAs can be detected in non-seed tissues. However, some abundant seed proteins whose functions extend beyond nutrient storage (eg. soybean Kunitz trypsin inhibitor and seed lectins) have detectable mRNA levels in other tissues, albeit at a far lower level.

4.3 Transcription assays in isolated nuclei.

4.3.1 Quantification of transcription rates in isolated nuclei.

Nuclei isolated from developing pea cotyledons (12 and 16 DAF) and pea leaves were allowed to synthesise transcripts *in vitro*. Transcripts were labelled by synthesis in the presence of [α-^{32}P]UTP, and specific sequences in the transcripts were then detected by hybridisation to excess DNA immobilised on nitrocellulose by dot blotting (Fig 12). The amount of radioactivity hybridised to each dot was then quantified by calibrated autoradiographs or by liquid scintillation counting as described in sections 3.10 and 3.12.

The assay was characterised according to the following parameters:

4.3.1.1 The DNA immobilised in dots was in excess over the nuclear transcripts in solution.

Immobilised DNA was confirmed to be in excess by varying the amount of pDB9.08 DNA in each dot blot. Fig 12a, columns D,E and F, row 3, show that the amount of hybridisation of transcripts to pDB9.08 is independent of mass of DNA immobilised in the range 2.5 to 10μg.

4.3.1.2 The relationship between input radioactivity and the radioactivity hybridised.

A linear relationship between input of radioactive transcripts and cpm hybridised has been previously demonstrated in this type of assay (Evans *et al.*, 1984).

4.3.1.3 Specificity of hybridisation.

The specificity of hybridisation to the DNA dots was demonstrated by insignificant background hybridisation to dots of pBR322. Also, when plasmids were restricted to remove their recombinant inserts, electrophoresed through agarose gels and Southern blotted, nuclear transcripts hybridise to the inserts only and not to the vector sequences (Fig. 13).

Figure 12. Transcription assays in isolated nuclei.

Autoradiographs of DNA dot blots, hybridized to transcripts synthesized in nuclei isolated from (a), 12 and 16 DAF cotyledon and (b), leaf, are shown.

In (a), 3 X 10^8 nuclei from both 12 and 16 DAF cotyledons were incubated with 0.75 and 1.0 mCi [α-^{32}P]-UTP, respectively. The labelled transcripts in each case were divided into three equal aliquots (7.8 X 10^7 cpm and 1.7 X 10^8 cpm per aliquot for 12 and 16 DAF, respectively) and hybridized to separate dot blots (A-F). DNA dots are as follows:

Row 1: A-F, 5μg pDUB6.

Row 2: A,B,D and E, 5μg pLG4.10; C and F, no DNA.

Row 3: A and B, 5μg pDB9.08; C, 5μg pDB9.08 with HpaI/XbaI fragment removed; D,E and F - 2.5, 5 and 10μg, of pDB9.08 respectively.

Row 4: A and D, 5μg pAD9.2 with EcoR1 fragment removed; B and E, 5μg pCD40; C and F, 5μg pJC5.2.

Row 5: A,B,D and E, 5μg pFa/b31; C and F, no DNA.

Row 6: A-F, 5μg pBR322.

In (b), 10^7 nuclei from leaf tissue were incubated with 250 μCi [α-^{32}P]UTP and the transcripts (4.2 X 10^7 cpm) were hybridized to a single blot. All dots contained 5μg of DNA. Plasmids used were as follows:

legA, pDUB6;

legS, pAD9.2 with EcoR1 fragment removed;

legJ, pCD40;

lec, pLG4.10;

CAB, pFa/b31 (this dot was cut out before autoradiography to avoid obscuring other dots on prolonged exposure);

conv, pDB9.08.

Calibration dots of serially diluted, end-labelled pBR322 cover the ranges 20 to 860 cpm (12 DAF), 5 to 3600 cpm (16 DAF) and 3 to 22 cpm (leaf).

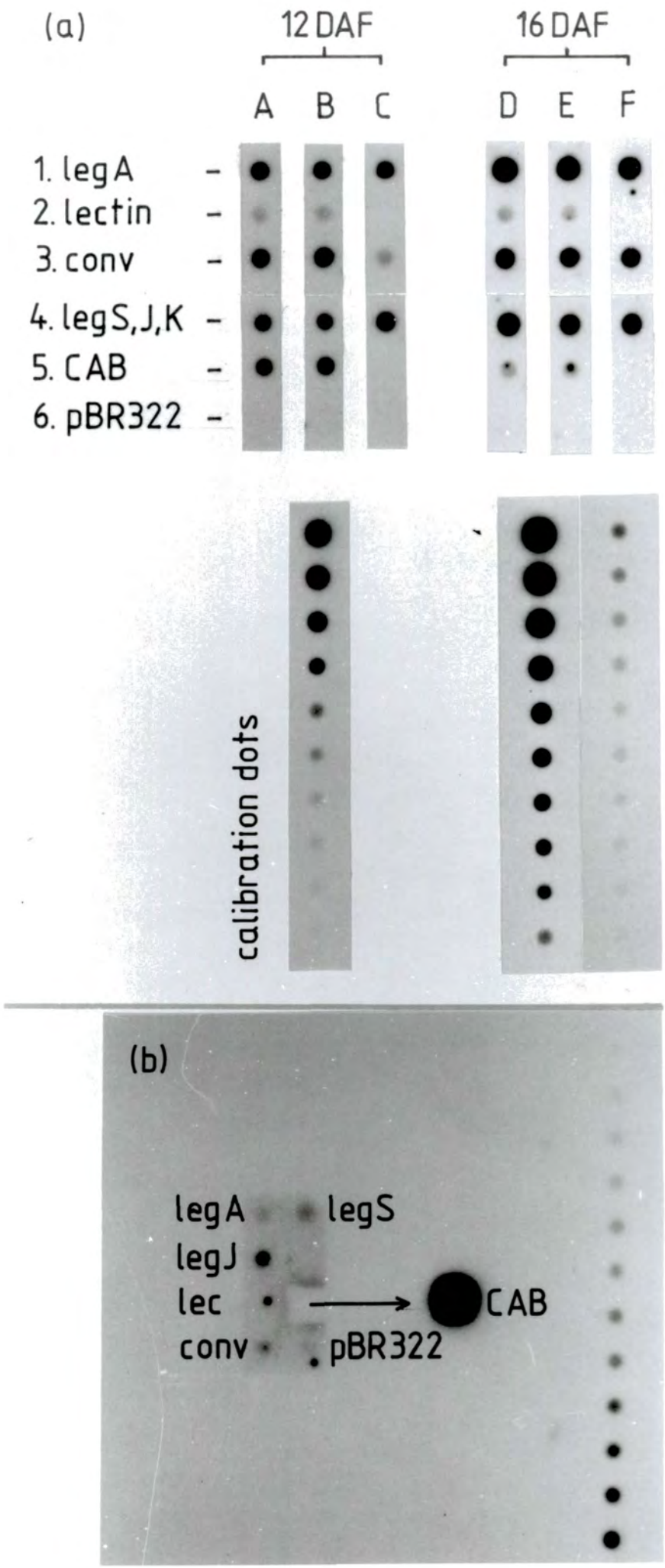

(a)
12 DAF
16 DAF
A
B
C
D
E
F
1. legA
2. lectin
3. conv
4. legS,J,K
5. CAB
6. pBR322
calibration dots
(b)
legA
legS
legJ
lec
conv
pBR322
CAB

Figure 13. Southern blot hybridized to ^{32}P-labelled transcripts synthesized in nuclei isolated from 16 DAF cotyledons.

Plasmids restricted to excise DNA inserts were run on 0.7% agarose gels and then Southern blotted. Tracks 1-4 and 5-8 were hybridized separately to identical aliquots of transcripts each containing 1.9×10^8 cpm. Transcripts were synthesized by incubation of 1mCi [α-^{32}P]UTP with 2.9×10^6 nuclei.

Track:

1, pDUB6 + BamHI;

2, pAD9.2 + EcoRI + BamHI;

3, pFa/b31 + BamHI;

4, pLG4.10 + EcoRI;

5, pDUB6 + BamHI;

6, pCD40 + PstI;

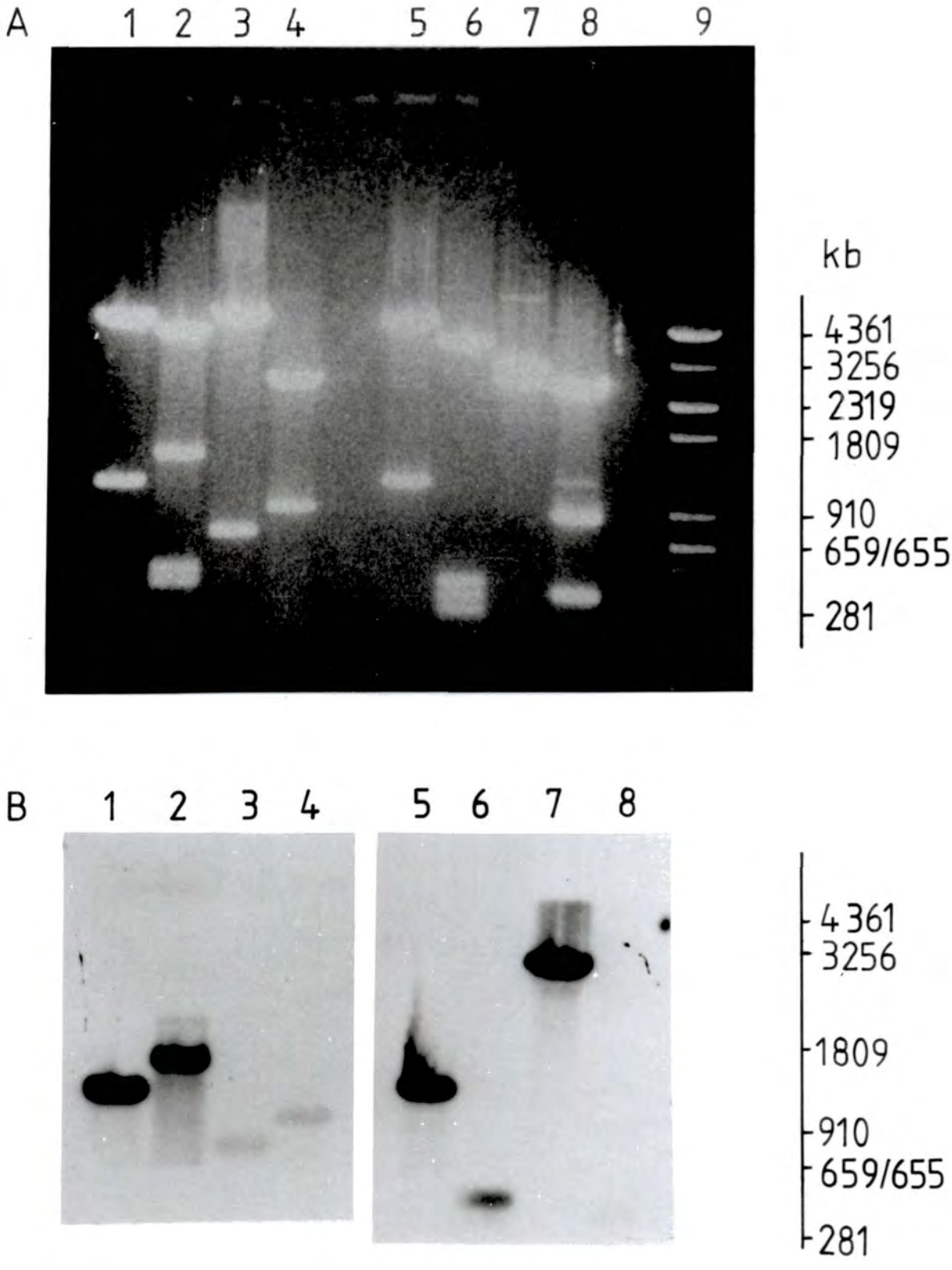
7, pDB9.08 + SphI + EcoRI;

8, pLG4.135 + EcoRI;

9, Markers: pBR322 separately digested with (a) EcoRI, (b) HincII, (c) BglI, and (d) HinfII and then mixed together.

pLG4.135 is a plasmid containing two clones from a pea seed cDNA library, the smaller insert is unknown and the larger insert is a pea albumin cDNA (Gatehouse 1986).

By restricting pAD9.2 with both EcoRI and BamHI a 420 bp 5′ fragment is released from the cDNA insert (see section 4.1 and 4.3.1.6). This fragment, together with a 375 bp fragment from the vector, were later removed from the blot before hybridisation by trimming with a razor blade.


A
1 2 3 4 5 6 7 8 9
kb
4361
3256
2319
1809
910
659/655
281
B
1 2 3 4 5 6 7 8
4361
3256
1809
910
659/655
281

4.3.1.4 Efficiency of hybridisation.

Under the conditions employed, the extent of hybridisation of the specific nuclear transcripts to immobilised probe sequences was estimated by the method of Marzluff and Huang (1984). In this method, transcripts are rehybridised to fresh blots and then, assuming that the percentage hybridisation was the same in first and second hybridisations, and allowing for radioactive decay, the proportion of transcripts remaining in solution after the first hybridisation was calculated. It was found that the second hybridisation signal was 10% of the first and thus the efficiency of hybridisation is approximately 90%.

The ideal method for measuring extent of hybridisation, as used by McKnight and Palmiter (1979), is to include synthetic mRNAs labelled with ^{3}H in the hybridisation solution and then, by dual liquid scintillation counting, to measure the proportion of input radioactivity that hybridised to the DNA dots. The method of Marzluff and Huang (1984) employed here suffers the disadvantage that the hybridisation efficiency of ^{32}P labelled nuclear transcripts may change over time due to the fragmentation caused by intramolecular radioactive decay (fission of the RNA chain caused by the transition from ^{32}P to ^{32}S) of RNA labelled to high specific activity (Mueller and Getz, 1987). The measurement of hybridisation efficiency allows specific nuclear transcription rates to be expressed in absolute terms as ppm of total transcripts. Since there is a linear relationship between cpm input and cpm hybridised, any error in the measurement of hybridisation efficiency will not influence the calculation of relative transcription rates although it will distort values obtained for absolute transcription rates.

4.3.1.5 Cross-hybridisation between transcripts within the three legumin gene subfamilies investigated.

DNA dot blots containing *legJ*, *legK* and *legS* sequences were hybridised to transcripts in separate, but otherwise identical, hybridisation assays. This was done because *legJ* and *legK* sequences are 94% homologous and *legJ/K* sequences are 75% homologous to *legS* sequences (see also section 4.2.1.1). If DNA

dots representing these sequences had been present in the same hybridisation reaction, cross-hybridisation may have occurred with nuclear transcripts during the low stringency hybridisation, followed by loss of these cross-hybrids during higher stringency washes, thus leading to an underestimate of the transcription rate of these gene sub-families. The final washes employed for these hybridisation assays were the same as those used in the assays of steady-state mRNA levels; this meant that the two sets of data were comparable with respect to the members of the legumin gene family that cross-hybridise to a given probe.

4.3.1.6 Non-linear labelling of nuclear transcripts.

To test the hypothesis that incorporation of label into nuclear transcripts is not linear along the length of a gene, the 3'-half of the *cvcA* gene was removed by restriction of pDB9.08 with Hpa1 and Xba1. If hybridisation of 12 DAF nuclear transcripts to duplicate dots of the complete 2.45kb gene sequence is given a relative transcription rate of 1.0 per kb, hybridisation to 1.1kb of 5' gene sequence gave a relative value of 0.67 per kb. This indicates a higher density of labelling towards the 3' end of this nuclear transcript. To reduce any effects of non-linear labelling on measurement of relative transcription rates, as far as possible, the probe sequences used in DNA dots covered the same region of the gene. For the 3 legumin sub-families, cDNA clones pDUB6, pCD40, and pAD9.2 restricted with EcoR1, begin 850, 800 and 440 bp downstream from the transcription start respectively and all extent to the poly(A) signals. The lectin clone is a 850 bp sequence starting in the coding region approximately 200 bp downstream of the transcription start and continuing to the poly(A) tail.

4.3.2 Results from transcription rate assays.

The measured transcription rates are shown in Table 3. The range of transcriptional activities of the genes tested in developing cotyledons is limited to approximately a 10-fold variation; the range is 0.9-4.1 $\times 10^{-6}$ of total transcripts at 12 DAF (ie. the earlier half of the cotyledon cell-expansion phase),

and 0.5-6.1 x 10^{-6} at 16 DAF (ie. the later half of the cotyledon cell-expansion phase). This similarity in transcriptional activities encompasses both the seed protein genes and the *CAB* light regulated genes.

If the transcription activities are expressed on a per gene basis, the similarity in expression of the seed protein genes becomes even more marked (Table 4.); the range over the legumin and lectin genes is less than 1.5-fold at 12 DAF and less than 3-fold at 16 DAF. Similarly, Sørensen *et al.* (1989) found that the "run-on" nuclear transcription rates of the B,C and D hordein genes in barley endosperm correlated well with their copy-number in the genome. However, the validity of expressing the transcription data on a per gene basis is uncertain because only two of the 4 *legA* sub-family genes (*legA* and *legB* and/or *legC*) are actually proven to be expressed (section 1.4.2), and it is not known whether 2 or 3 of the *legJ* genes are expressed (section 4.2.3). Also individual genes within a sub-family can show differential regulation, as in the case of soybean β-conglycinin (Harada *et al.*, 1989), pea *legJ* sub-family (section 4.2.3) and pea *rbcS* multigene family (Fluhr *et al.*, 1986). Also, since different patterns of cross-hybridisation are observed within different gene (sub-)families, the true transcription rate on a per gene basis may be distorted.

In contrast to the cotyledon, in leaves transcriptional activities range from undetectable (<0.02 x 10^{-6} of total transcripts), for the *lecA* gene and the *legA* gene sub-family, to 22.0 *X* 10^{-6}, for *CAB;* ie. at least 1000-fold variation. This demonstrates the organ-specific nature of the transcription of the seed protein genes, in contrast to the genes encoding the chlorophyll a/b binding protein, which are expressed in all green tissues. A low level of hybridisation of leaf nuclear transcripts to *legJ, legS* and cvcA DNA was detected; however, the level of detection was close to background hybridisation to pBR322 and it is not clear from this single assay whether the signal represents a basal low level of transcription or a variable background hybridisation. As discussed in section 4.2.4 no seed-storage protein mRNA could be detected in leaf, stem or root total RNA.

The *legA* subfamily genes gave transcripts equivalent to 3.5×10^{-6} of total transcripts for 12 DAF cotyledons and 6.1×10^{-6} for 16 DAF cotyledons. None of the other genes assayed showed this marked increase in transcription with cotyledon development; over the same interval the *legJ* and *legS* subfamily genes showed a constant proportion of total transcripts, whereas the *cvcA* gene family and *lecA* gene transcripts decreased by approximately 40%, and *CAB* decreased by approximately 80%.

Beach *et al.* (1985) measured transcription rates for *legA* in isolated nuclei from cotyledons of *Pisum sativum* L. cv. Greenfeast at 11, 13 and 19 DAF. In terms of seed development 19 DAF is approximately equivalent to 16 DAF for the peas (cv. Feltham First) used in this work. However, the transcription of *legA*, measured by Beach *et al.* (1985) at this stage, expressed as parts per million (ppm) of total nuclear transcripts, is approximately 35-fold higher than that measured here. Also Beach *et al.* (1985) measured transcription in nuclei isolated from leaf, and the ppm of transcripts hybridising to pFa/b31 is higher than our measurements by approximately 9 fold. Such differences could be explained by the use of different varieties of pea, grown under different conditions, or by the variation in experimental procedures for isolation of nuclei, *in vitro* transcription and hybridisation. However, it is the relative transcription rates of different gene families, as assayed under identical conditions, and not transcription rates as ppm, that are important in drawing the following conclusions from this data.

Table 3. Transcription in isolated nuclei.

Hybridisation of "run-on" nuclear transcripts to DNA dot blots was quantified and expressed as a proportion of total transcripts. Values were calculated by subtracting background hybridisation and then normalising according to the length of probe sequence, using pDUB6 (1.1 kb cDNA) as a reference. Data were then divided by the hybridisation efficiency (0.9). Error values represent variation of duplicate or triplicate hybridisations. In the case of the plasmid pAD9.2 the 5′ 430bp was removed from the cDNA insert by restriction with EcoR1 and then electroelution (see section 4.3.1.6). pDB9.08 was used as the complete plasmid (a) or with a 1.35bp 3′ fragment of the insert removed by restriction with Hpa1 and Xba1 followed by electroelution (b).

Gene family	Probe	Proportion of total transcripts $\times 10^{-6}$		
		12 DAF	16 DAF	Leaf
legA	pDUB6	3.5 ± 0.2	6.1 ± 0.1	ND
legS	pAD9.2	2.3	2.7	0.04
legJ	pJC5.2	3.2	3.0	-
legJ	pCD40	3.3	3.2	0.31
lecA	pLG4.10	0.9 ± 0.1	0.5 ± 0.05	ND
CAB	pFa/b31	4.1 ± 0.3	0.9 ± 0.06	22.0
cvcA	pDB9.08[a]	1.8 ± 0.2	1.2 ± 0.1	0.02
cvcA	pDB9.08[b]	1.2	-	-

4.3.3 A comparison of steady-state mRNA levels with transcription rates in isolated nuclei.

A comparison of data from nuclear "run-on" transcription rates and steady-state mRNA levels provides information about the control of gene expression with respect to organ-specificity, timing of expression during seed development and also the level of expression in the seed.

4.3.3.1 Organ specificity.

The transcription assays in leaf and cotyledon nuclei presented in this thesis, and previously (Evans *et al.*, 1984; Beach *et al.*, 1985) indicate that the seed-specific expression of legumin and vicilin genes is regulated principally at the transcriptional level. Transcription assays indicate a possible low level of transcription of the *legJ*, *legS* and *cvcA* gene families in leaf nuclei so it may be that for these genes seed-specific expression is a result of greatly enhanced transcription rather than an "on-off" switching mechanism. If transcription does occur in leaf then the inability to detect steady-state *legJ* or *legS* mRNA in leaf tissue, by an assay more sensitive than the transcription assays, would imply an additional posttranscriptional control to prevent accumulation of the transcripts.

Similarly Walling *et al.* (1986) found that the soybean seed storage protein genes for β-conglycinin, glycinin and the 15 kDa protein were transcribed in leaf nuclei at a level 50 to 100-fold below the level in embryo nuclei, whereas the steady-state levels in nuclear RNA were more than 10,000-fold lower in leaf nuclei than in embryo nuclei.

Pea lectin is expressed in both seed and roots but not in leaf (Buffard *et al.*, 1988). Nuclear "run-on" transcription assays described in this thesis indicate that the absence of expression in leaf is due to a lack of transcription.

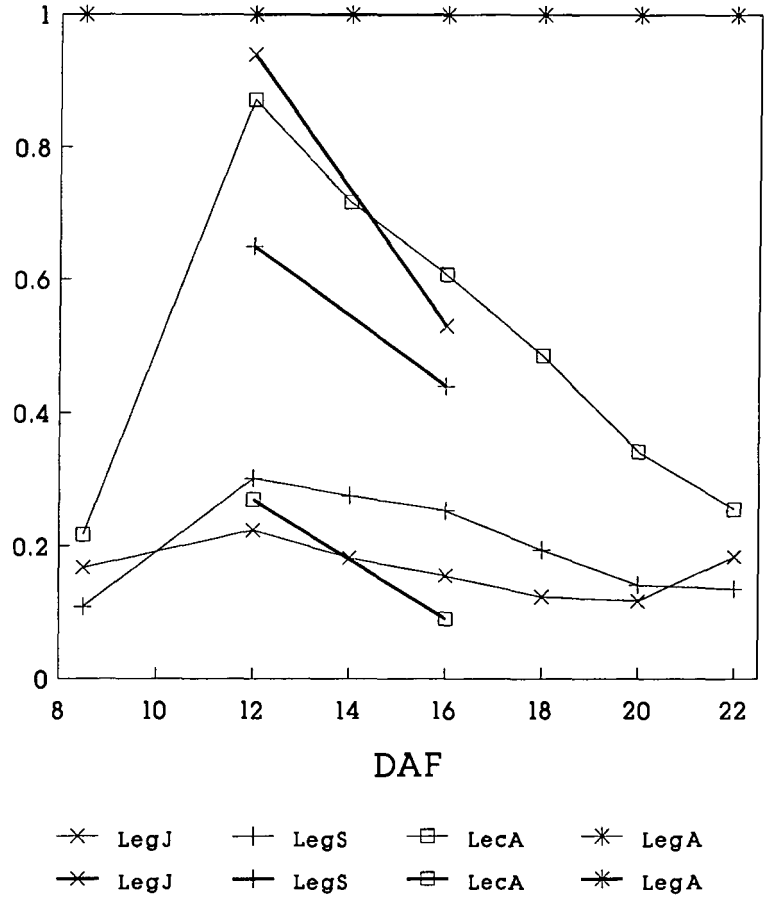


Figure 14. Comparison of nuclear transcription assays and steady-state mRNA levels in cotyledons at 12 and 16 DAF.
The vertical axis represents relative transcription rate or mRNA levels. All data is plotted relative to the *legA* family. Thick lines represent transcription rates and thin lines represent mRNA levels.

4.3.3.2 Timing of expression.

If we consider the timing of expression of the seed storage protein genes of pea, the transcription of these genes has previously been shown to be activated early in the mid-phase of cotyledon development, and then to diminish during seed desiccation. Furthermore the differences in timing of the accumulation of vicilin and legumin mRNA in pea can be accounted for, in part, by the timing of the increases in transcription rates and partly by some posttranscriptional effect (Evans *et al.*, 1984; Beach *et al.*, 1985). Also, Harada *et al.* (1989) have shown that the transcriptional activation of two classes of soybean β-conglycinin genes is co-ordinated, but that the corresponding mRNA levels accumulate and decay at different times during embryogenesis, thus indicating a role for posttranscriptional temporal regulation.

The differences in the timing of accumulation of the three classes of legumin mRNA and lectin mRNA shown in this thesis are in reasonable agreement with the corresponding changes in the relative transcription rates. In particular the transcription rate of the *legA* gene family increases relative to all other gene families investigated over the period from 12 to 16 DAF and this is reflected in a relative increase in *legA* mRNA over the same period (Fig. 14). Thus the differential timing of expression of the gene families studied appear to be mainly regulated at the transcriptional level, although posttranscriptional regulation may also occur.

4.3.3.3 Quantitative level of expression.

Table 4 summarises the data for steady-state mRNA levels and transcription rates relative to *legA* at both 12 and 16 DAF. These data are plotted in Fig. 14. It can be seen, at a given developmental stage, that there is no clear correlation between relative transcription rates and relative steady-state mRNA levels. The steady-state levels of *legJ* type mRNA relative to *legA* are 0.20 at 12 DAF, and 0.14 at 16 DAF. However, the corresponding transcription rates of *legJ* genes relative to *legA* genes are approximately 4-fold higher, ie. 0.93 at 12 DAF and 0.51 at 16

DAF. In the case of *legS*, the transcription rates are approximately two fold higher than would be predicted from the steady-state mRNA levels, whereas the *lecA* transcription rates appear to be approximately 3 and 6-fold lower than would be predicted from the steady-state mRNA levels at 12 and 16 DAF, respectively.

These discrepancies provide evidence for posttranscriptional control of the quantitative levels of mRNAs present at any one point in embryogenesis. The results also indicated that the three legumin gene families and lectin gene have relatively similar transcription rates on a per gene basis (see Table 4. and section 4.3.2), thus the final level of expression appears to be influenced more by gene copy number and posttranscriptional effects than by the transcription rates of individual genes.

Posttranscriptional control of the final level of legumin gene expression has previously been observed in response to an environmental change. Sulphur deficiency in pea plants results in a large depletion of legumin mRNA levels in pea cotyledons whereas the corresponding transcription of the legumin genes in isolated nuclei showed only a relatively small decrease. Vicilin expression remains unaffected by the sulphur deficiency (Evans *et al.* 1985; Beach et al., 1985). The physiological significance of this response is to direct the protein synthesis machinery towards synthesis of sulphur-free proteins such as vicilin and away from the synthesis of relatively sulphur-rich proteins, such as legumin.

The data presented here suggest an alternative type of posttranscriptional control of the level of gene expression during seed development. In this case, instead of a single mRNA species having an apparently different half-life under different environmental conditions, several different mRNA species have apparently different half-lives under identical conditions. This allows two genes to be transcribed at the same rate although their mRNAs accumulate to different levels.

A similar type of posttranscriptional control has been observed in soybean cotyledons in which "run-on" nuclear transcription rates of seed protein genes and non-seed protein

genes in 70 DAF embryos are similar, but their mRNA levels vary up to 10,000-fold (Walling *et al.*, 1986).

More recent studies of "run-on" transcription in soybean cotyledon nuclei have concentrated on the transcription of different members of the soybean legumin gene family - the glycinin genes (Nielsen *et al.*, 1989). In this report it was shown that the mRNAs representing the group I (*legA* type) and group II (*legJ* type) genes were co-ordinately regulated during development; furthermore, the developmental regulation of the mRNAs levels was exclusively controlled at the transcriptional level. However, in agreement with the results presented here, Nielsen *et al.* (1989) also stated that there was not an absolute correlation between relative gene transcription rate and mRNA prevalences. In particular, the mRNA level of the glycinin related gene, G^{*}, was low in comparison to group I and II glycinins, but its transcription rate was approximately the same. There were no apparent discrepancies suggesting posttranscriptional control of the group I and group II glycinin mRNA levels, which would be directly analogous to the results presented in this thesis; however, Nielsen *et al.* (1989) measured relative steady-state mRNA levels by hybridising RNA dot blots to different glycinin probes of the same specific activity. No other attempt was made to standardize their hybridisation signals, thus the accuracy of their relative mRNA level determinations is uncertain.

Similar conclusions concerning posttranscriptional regulation of seed-storage protein mRNA levels have also been presented for the hordeins present in barley endosperm (Sørensen *et al.*, 1989). In this case the mRNA levels of the B and C hordeins are up to 35-fold higher than the mRNA level of D hordein, but there is no great difference in nuclear "run-on" transcription rate.

It is suggested, then, that posttranscriptional regulation may be an important and widespread factor in determining the final level of expression of different seed-storage protein genes.

The posttranscriptional regulation could take the form of differential processing in the nucleus, or differential rate of nucleocytoplasmic transport, which might ultimately affect the rate of mRNA degradation in the nucleus, or, alternatively,

differential degradation in the cytoplasm. The physiological significance, if any, of this posttranscriptional control is unknown.

Similar regulatory processes are well known in animal systems: the steady-state mRNA levels of the constitutive or housekeeping genes of mouse L-cells correlate with mRNA half-lives rather than their transcription rates (Carneiro and Schibler 1984), and Cabrera *et al.* (1984) concluded that in sea urchin embryos cytoplasmic RNA turnover was a major variable in determining the level of expression of embryo genes.

Table 4. Comparison of steady-state mRNA levels with nuclear transcription rates in pea cotyledon at 12 and 16 days after flowering. Data are expressed relative to *legA*.

Gene family	Copy no.	Probe	Steady-state mRNA levels		Transcription rates per gene family		Transcription rates per gene	
		DAF	12	16	12	16	12	16
legA	4	pDUB6	1.00	1.0	1.0	1.0	1.0	1.0
legJ	3	pCD40	0.20	0.14	0.93	0.51	1.24	0.68
legS	2	pAD9.2	0.30	0.25	0.65	0.44	1.32	0.88
lecA	1	pLG4.10	0.87	0.60	0.27	0.09	1.08	0.36

4.4 An investigation of posttranscriptional regulation of mRNA levels using pea cotyledon culture systems.

The results presented in the previous sections suggest that the levels of accumulation of different classes of seed protein mRNA are, to a large extent, determined by posttranscriptional events. These conclusions, however, are based on discrepancies between two sets of data, i.e. "run-on" transcription rates and steady-state mRNA levels. In order to provide more direct evidence of these processes it is necessary to measure the differential turnover of specific transcripts in the cell. Methods for measuring mRNA half-lives were discussed in section 1.3.2.

Goldberg *et al.* (1981) suggested that the half-lives of soybean storage protein mRNAs, in the period immediately prior to seed desiccation when storage protein accumulation had ceased, was 4-6 days. This conclusion was based on the observations that the half-life of the decline in steady-state level was 4-6 days and that the transcription of the mRNAs had probably stopped. There is no known report of a direct measurement of the half-life of a storage protein mRNA. The only known report of a direct measurement of a specific nuclear-encoded higher plant mRNA is that of Cornelissen (1989). In this case, the half-lives of the mRNAs from bialophos resistance gene and hygromycin phosphotransferase resistance gene were measured in tobacco leaf protoplasts after treatment with transcription inhibitor actinomycin D; half-lives were found to be 119 and 62 minutes respectively.

The seed storage protein mRNAs, because of their very high abundance in developing cotyledons, are amenable to kinetic labelling techniques since it should be possible to incorporate sufficient radioactivity into them to allow quantitative measurements. Gatehouse *et al.* (1982) have previously shown that it was possible to pulse-chase label the RNA of pea cotyledons grown in the culture system developed by Millerd *et al.* (1975). They were able, using this technique, to estimate the half-life of the poly(A)$^+$ fraction; it was suggested that 75% of the mRNA decayed relatively quickly with a half-life of less than 10 hours, whereas the remainder seemed to decay more slowly with a half-life

of greater than 20 hours. Also Silflow and Key (1979) performed a uridine pulse-chase labelling procedure using exponentially growing soybean suspension-culture cells and measured the decay of label from polysomal poly(A)$^+$ RNA; they found that after a one hour pulse, 90% of the newly synthesised mRNA turned over with a half-life of approximately 0.6 hours, and the remaining 10% turned over with a half-life of approximately 30 hours; in the steady state situation they found that the mRNA with the longer half-life formed the majority of the polysomal poly(A)$^+$. The occurrence of two major half-life components of the poly(A)$^+$ mRNA is also common to mammalian and insect cell cultures (Silflow and Key, 1979). No attempt, in either of the above plant studies, was made to measure the half-life of specific mRNAs.

It was decided, using a similar system to Gatehouse *et al.* (1982), to investigate turnover of *legA* and *legJ* mRNA, whose differential accumulation appeared to be posttranscriptionally controlled.

4.4.1 Culture of immature pea cotyledons.

The culture medium of Millerd *et al.* (1975) consisted of 4% sucrose, 18 amino acids, trace elements, vitamins, and minerals. In this culture system excised immature pea cotyledons continue to grow and to synthesise DNA, RNA, chlorophyll and protein, including legumin and vicilin. However, in order for legumin synthesis to occur in this culture system it must have been initiated in the mature plant (this was not the case for vicilin synthesis which could be initiated in culture).

Stafford and Davies (1979) used a similar culture medium but found that increasing the sucrose concentration to 18% improved fresh weight increase and protein synthesis rate in culture, and reduced the occurrence of premature germination. Domoney *et al.* (1980) used this culture system and a sensitive ELISA assay to show that legumin synthesis was initiated when very young pea embryos were cultured for 7 days. No analysis of legumin mRNA levels in cultured pea embryos has been published.

The culture of developing pea pods (Millerd *et al.*, 1975; Barrett, 1986) has been used successfully in pulse labelling of

storage proteins (Chandler *et al.* 1983); however, it was considered that this technique, although it gives a better approximation to seed development *in vivo* than embryo culture, may not allow sufficient uptake of tritiated uridine and furthermore would not be amenable to statistically meaningful sampling throughout a pulse-chase labelling experiment. Thus, the culture conditions of Stafford and Davies (1979), with the modifications of Domoney *et al.* (1980), were chosen for performing an RNA pulse-chase labelling experiment.

4.4.2 A feasibility study to discover if sufficient label can be incorporated into cotyledon RNA in culture.

In order to see if sufficient radioactivity could be incorporated into total RNA to allow measurement of specific mRNAs, 3 intact 12 DAF pea cotyledons, and three cotyledons which had been cut into two equally sized pieces with a razor blade, were incubated for 4 hours in 1ml of Stafford and Davies (1979) 18% sucrose culture medium containing 0.5 mCi/ml [5,6-^{3}H]uridine, as described in section 3.15. The cotyledons were then incubated in "chase" medium containing 10mM uridine instead of [5,6-^{3}H]uridine for a further 2 hours. Total RNA was extracted separately from the intact and bisected cotyledons and the radioactive incorporation determined by LSC. The three intact cotyledons yielded 114μg of total RNA at 1.54×10^{5} cpm/μg whereas the three bisected cotyledons yielded 104μg of total RNA at 2.13×10^{5} cpm/μg. Assuming that 1% of the total RNA represented mRNA, and that a specific seed storage mRNA formed at least 5% of newly synthesised mRNA, this would mean that the total RNA from three cotyledons would provide at least ≃7500 cpm of this specific mRNA, which would be ample for measurement.

4.4.3 Pulse-chase labelling of pea cotyledon RNA in culture.

A full scale experiment was then set up to pulse-chase the RNA of cultured cotyledons in order that the decay of legumin mRNAs could be measured. Also, because the efficacy of the chase of radioactivity from the RNA precursor pool would greatly affect the observed half-life of a specific mRNA (Brock and Shapiro 1982b),

it was decided to measure the specific activity of this ribonucleoside triphosphate (NTP) pool during the chase period.

70 x 12 DAF cotyledons were excised from pod and testa, bisected using a razor blade, and immediately labelled for 3 hours 25 min in 5ml of the culture labelling medium described in section 4.4.2. They were then rinsed thoroughly in the chase medium and 2 samples of 7 half-cotyledons were taken (at time zero after pulse). The remaining cotyledons were placed in 8 separate 100ml conical flasks sealed with cotton wool bungs; each flask contained 14 half-cotyledons and 10ml chase medium. These were incubated for different times up to 52 hours before removing 2 samples of 7 half-cotyledons as before.

Bisected cotyledons were used in this experiment because; (i) it was shown (section 4.4.2) to improve incorporation of tritium, and (ii) it gave an increased sample size which is important to give statistically meaningful results because of the variation in the development of cotyledons taken from 12 DAF pods.

This experiment, then, produced two samples of 7 half-cotyledons from each of nine time points during the chase period. From one of each pair of samples total RNA was extracted. Nucleotides were extracted from the other samples in order to measure NTP specific activity.

An aliquot from each total RNA sample was used to measure concentration and radioactivity. A second aliquot was run on a Northern blot and then the remainder of each sample was hybridised to DNA dots containing *legA* or *legJ* clones.

4.4.3.1 The specific activity of the ribonucleoside triphosphate pool.

Nucleotides were extracted and then separated by anion exchange HPLC. Fig. 15 shows an example of an HPLC chromatograph and the radioactivity of eluted fractions. Peaks of radioactivity co-elute with both pyrimidine nucleotides CTP (peak 5) and UTP (peak 6). Tritium becomes associated with CTP because the biosynthetic pathway for CTP proceeds by the following reaction (Goodwin and Mercer 1983):

$$UTP + NH_3 + ATP \xrightarrow{Mg^{2+}} CTP + ADP + P_i$$

Peak 1 corresponds to the elution front and probably represents uridine. Peaks 2, 4 and the shoulder to the left of peak 4 probably represent UMP, UDP and CDP, respectively, although no attempt was made to prove identities of these peaks.

Peak 3 co-elutes with uridine-5'-diphosphate-D-glucose (UDPG), which, as shown in Fig. 15, is very abundant and highly labelled. UDPG is utilised in developing seeds in the first step of starch biosynthesis, a process which is very active in this stage of developing pea seeds. This step is catalysed by a sucrose synthase, which has been shown in developing seeds of maize and mung bean to have a very high affinity for UDP, but low affinity for ADP, in the following reaction (Goodwin and Mercer 1983):

$$\text{Sucrose} + \text{NDP} \longrightarrow \text{NDP-D-glucose} + \text{D-fructose}$$

Similarly, when Edwards and Rees (1986) investigated UDP-glucose metabolism in developing pea embryos by incubation with [^{14}C]sucrose, they showed a substantial labelling of UDP-glucose.

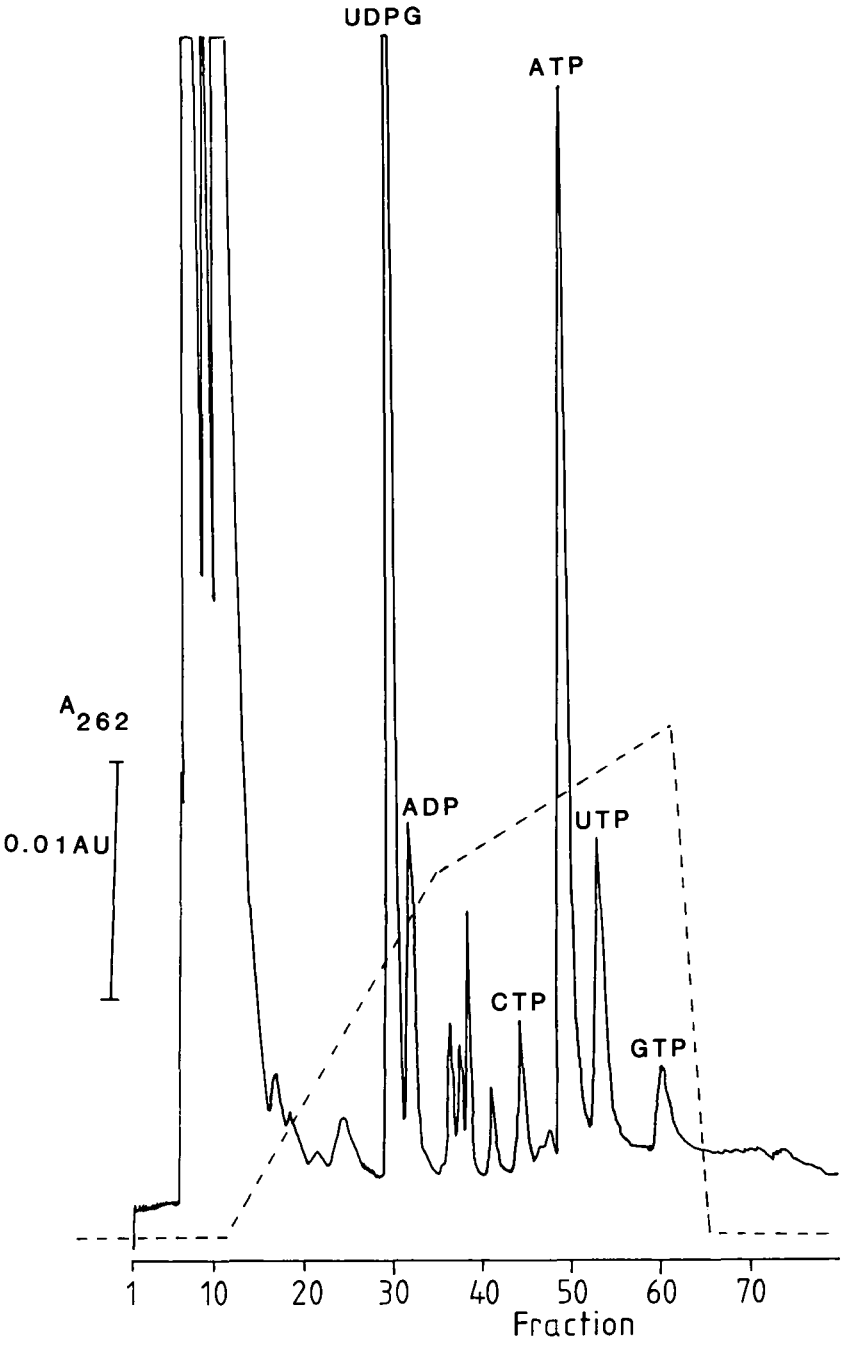
The specific activity of the UTP and CTP pool was calculated from the HPLC data and is plotted in Fig. 16(i). This plot shows that the specific activity declines with a half-life of approximately 10 hours and confirms that the chase of label did occur, although at a relatively slow rate. These data reflect the specific activity of the total cellular NTPs and the possibility must be considered that the specific activity is different in the nuclear compartment.

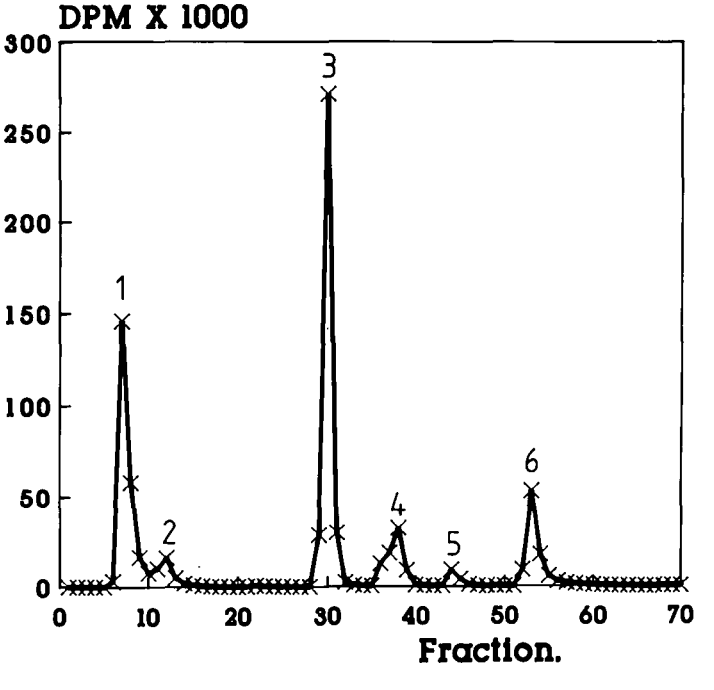
The fluorograph of total tritium labelled RNA, shown in Fig. 17(i), is able to detect very abundant classes of RNA. This shows further evidence for the efficacy of the chase because tritium is chased from a high molecular weight rRNA precursor (band A) into the final species, which are known to be generated by posttranscriptional processing, of size 25S, 18S, and 5.8S (bands C,E and F, respectively). In wheat, the most abundant pre-rRNA observed in S1 nuclease mapping experiments is a 6900 nucleotide species, which probably represents a relatively stable pre-rRNA intermediate which has already been processed at its 5' end by removal of 500 nucleotides of intergenic sequence (Vincentz and Flavell, 1989). In this experiment, then, it might be expected

Figure 15. Example of an anion-exchange HPLC chromatogram used to measure the specific activity of ribonucleotide triphosphates (NTP) during pulse-chase labelling experiments.

Top: nucleotides were extracted from cotyledon tissue pulse-chase labelled in culture with [5,6-^{3}H]uridine, at time zero (see text), and then separated by anion exchange HPLC as described in the methods section 3.16. The dotted line represents the proportion of buffer A in the multilinear elution gradient whereas the solid line shows the absorbance at 262nm. The labelled peaks were identified by co-chromatography with pure commercial standards.

Bottom: fractions from the HPLC column were collected and aliquots analysed by liquid scintillation counting with a quench curve. The total DPM per fraction was plotted against fraction number.


UDPG
ATP
A 262
0.01AU
ADP
UTP
CTP
GTP
1
10
20
30
40
50
60
70
Fraction


DPM X 1000
300
250
200
150
100
50
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
Fraction.

that an additional larger, unprocessed pre-rRNA transcript might be detectably labelled immediately after the pulse, however, no such species could be detected even with prolonged exposures.

Like the decline in specific activity of the total cellular NTP pool, the decay of the pre-rRNA precursor in this experiment occurs with a half-life of approximately 10 hours. The agreement of these two sets of data is consistent with the hypothesis that there is no difference in the specific activity of the nuclear and cytoplasmic pools of NTPs. Furthermore kinetic labelling experiments in *Euglena gracilis* and tomato cell suspension culture have shown that UTP specific activity was identical in the nucleolus and the nuclear matrix (Karnahl and Wasternack 1989).

In Fig. 17(i) band B declines in abundance at a rate similar to the pre-rRNA band and probably represents an intermediate in the processing of this precursor. At D there are two bands, an upper and a lower band; their identities are uncertain. They might represent further intermediates in the pre-rRNA processing pathway, organellar rRNAs, or very abundant classes of mRNA. The lower band at D apparently declines during the chase whereas the upper band appears to increase in intensity.

4.4.3.2 Incorporation of tritium into total RNA.

The yield of RNA isolated from each sample of 7 half-cotyledons ranged from 270 to 350 μg. Incorporation of tritium into each sample is plotted against time after pulse in Fig. 16(i). Incorporation was most rapid during the first 3 hours of the chase period (when the specific activity of the NTP pool was still relatively high), the rate then declined between 3 and 13 hours, after which there was no further significant increase in incorporation. The incorporated tritium, as illustrated by Fig. 17(i), is present predominantly in the rRNA, and is stable up to 52 hours. This is in agreement with the long half-life of the poly(A)$^-$ fraction demonstrated by Gatehouse *et al.* (1982). Also, the half-life of *Euglena gracilis* cytoplasmic rRNA and tRNA was measured, by the kinetics of approach to steady-state labelling in culture, as 45 hours (Karnahl and Wasternack 1989).

4.4.3.3 Incorporation of tritium into *legA* and *legJ* mRNA.

The remaining total RNA samples were each divided into two aliquots. One set of aliquots was hybridised, in glass vials, to nitrocellulose filters containing vector DNA alone and vector with a *legA* cDNA insert. The second set of aliquots was hybridised in an identical way except that the vector contained a *legJ* cDNA insert rather than a *legA* cDNA insert. It was found that hybridisations performed in small polythene bags, similar to the assays performed for nuclear transcriptions, gave unacceptably high variation in hybridisation efficiency and background, possibly because of the high concentrations of labelled total RNAs involved (data not shown). More consistent results could be obtained by performing hybridisations in small volumes in glass vials, and by not including more than two DNA dots per vial. Under these conditions it was still necessary to monitor hybridisation efficiency in each vial by inclusion of ^{32}P-labelled synthetic mRNAs (see section 3.17).

Results were quantified by dual label $^{32}P/^{3}H$ LSC and are presented in Table 5 and plotted in Fig. 16(ii). The mRNA hybridising to the *legA* clone was sufficient to measure the half-life of incorporation of tritium into this mRNA, under the culture conditions used. The incorporation continues to increase 6 hours into the chase period, whilst the specific activity of the NTP pool is still relatively high; it then declines with a half-life of approximately 10 hours. This rate of decline is very similar to the rate of decline of the specific activity of the NTP pool and thus the true half-life for this mRNA is probably considerably less than 10 hours (assuming transcription of the *legA* gene family continues), although no accurate value can be estimated from these data. From this type of experiment it is only possible to give an accurate estimate of mRNA half-life if it is considerably longer than the half-life of the NTP specific activity.

The hybridisation of mRNA to the *legJ* clone is not sufficiently above a highly variable background to draw any conclusions directly from these data (see Table 5). However, it may be viewed as significant that, during the 3.42 hour

Figure 16.

(i) Plot of specific activity of NTP, and radioactivity incorporated into total RNA during the pulse-chase labelling experiment.

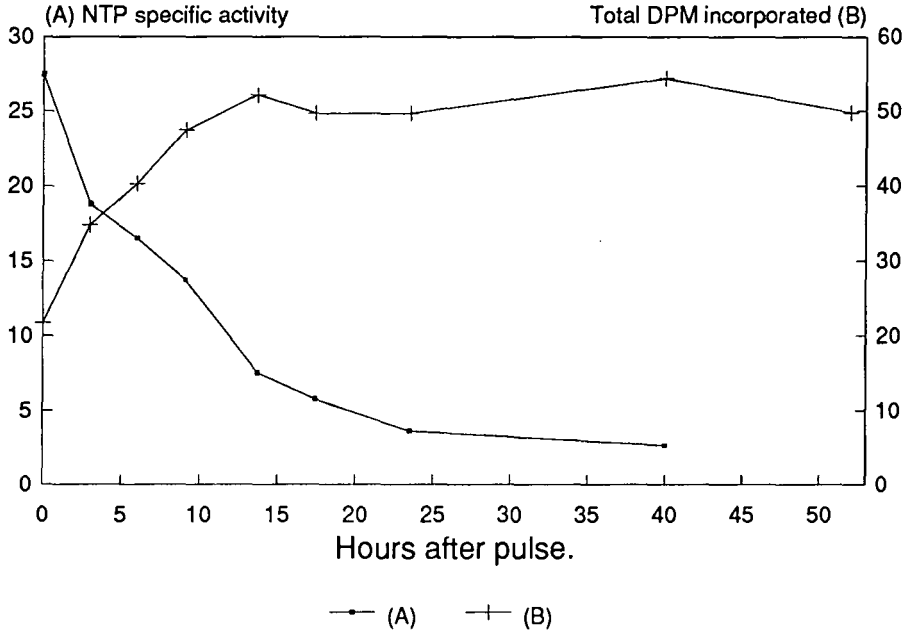
NTP specific activity was calculated from data such as illustrated in Fig. 15. This was done by dividing, for a given chromatogram, the molar amount of UTP by the associated DPM. This was repeated for CTP and the values added. This value was then divided by 4 in order to give the specific activity of newly synthesised RNA at each time point (assuming equal representation of the four NMPs in the RNA). These data are plotted on Y-axis (A) as DPM per nmol NTP $\times 10^{-4}$.

Total RNA was extracted from cotyledons by the "Hot-SDS" method and aliquots were counted by LSC. The radioactivity is plotted on Y-axis (B) as total DPM incorporated per sample (3.5 cotyledons) $\times 10^{6}$.

(ii) Radioactivity incorporated into *legA* and *legJ* mRNA during the pulse-chase period.

See Table 5 for details.

(i)


(A) NTP specific activity
Total DPM incorporated (B)
30
25
20
15
10
5
0
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50
Hours after pulse.
(A)
(B)

(ii)

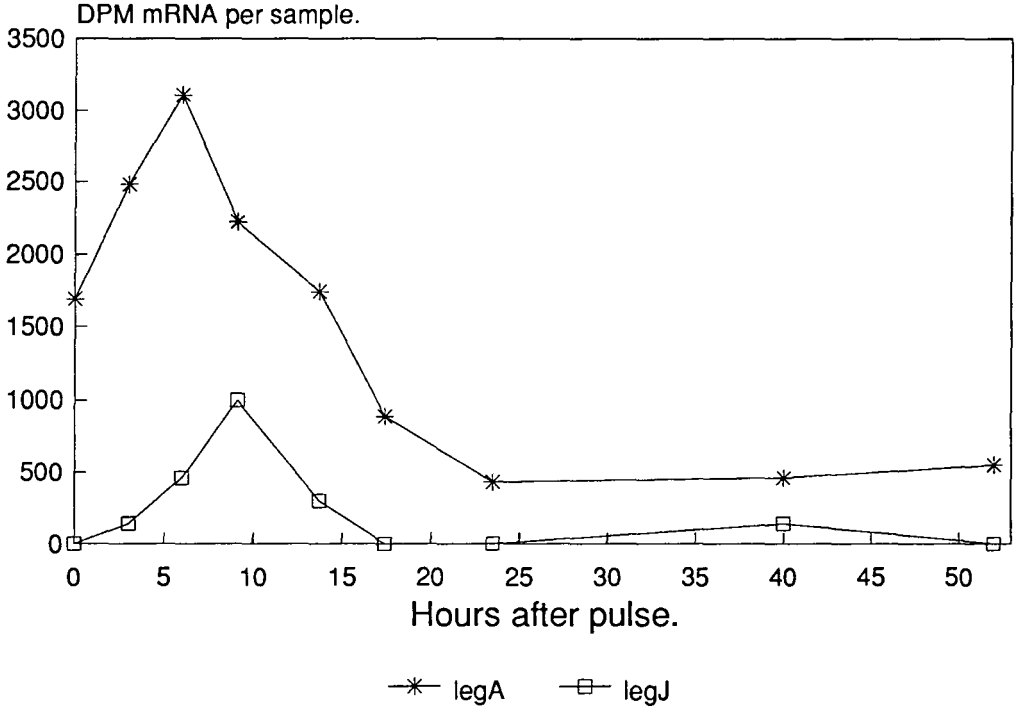

DPM mRNA per sample.
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50
Hours after pulse.
legA
legJ

Figure 17. Northern blot of tritium labelled total RNA from the pulse-chase RNA labelling experiment.

$1/60^{th}$ of each total RNA preparation, extracted from the cotyledon samples taken during the pulse-chase experiment, was run on a formaldehyde agarose gel and blotted onto Hybond-N nylon membrane. Tracks 1-8 contain samples from the time points 0, 3, 6, 9, 13, 17, 23, and 39 hours, respectively.

The blot was probed first with a *legJ* antisense, ^{32}P-labelled, synthetic mRNA probe and autoradiographed, (ii)B.

After removing the probe with boiling 0.1% SDS, the blot was reprobed with a *legA* antisense probe and again autoradiographed, (ii)A.

Then, the *legA* probe was removed as before and residual ^{32}P was allowed to decay until it was no longer detectable using a Geiger-Müller tube (≃ 6 half-lives). The blot was then sprayed with "Amplify™" and fluorographed to reveal the abundant ^{3}H-labelled RNA species, A-F, (i).

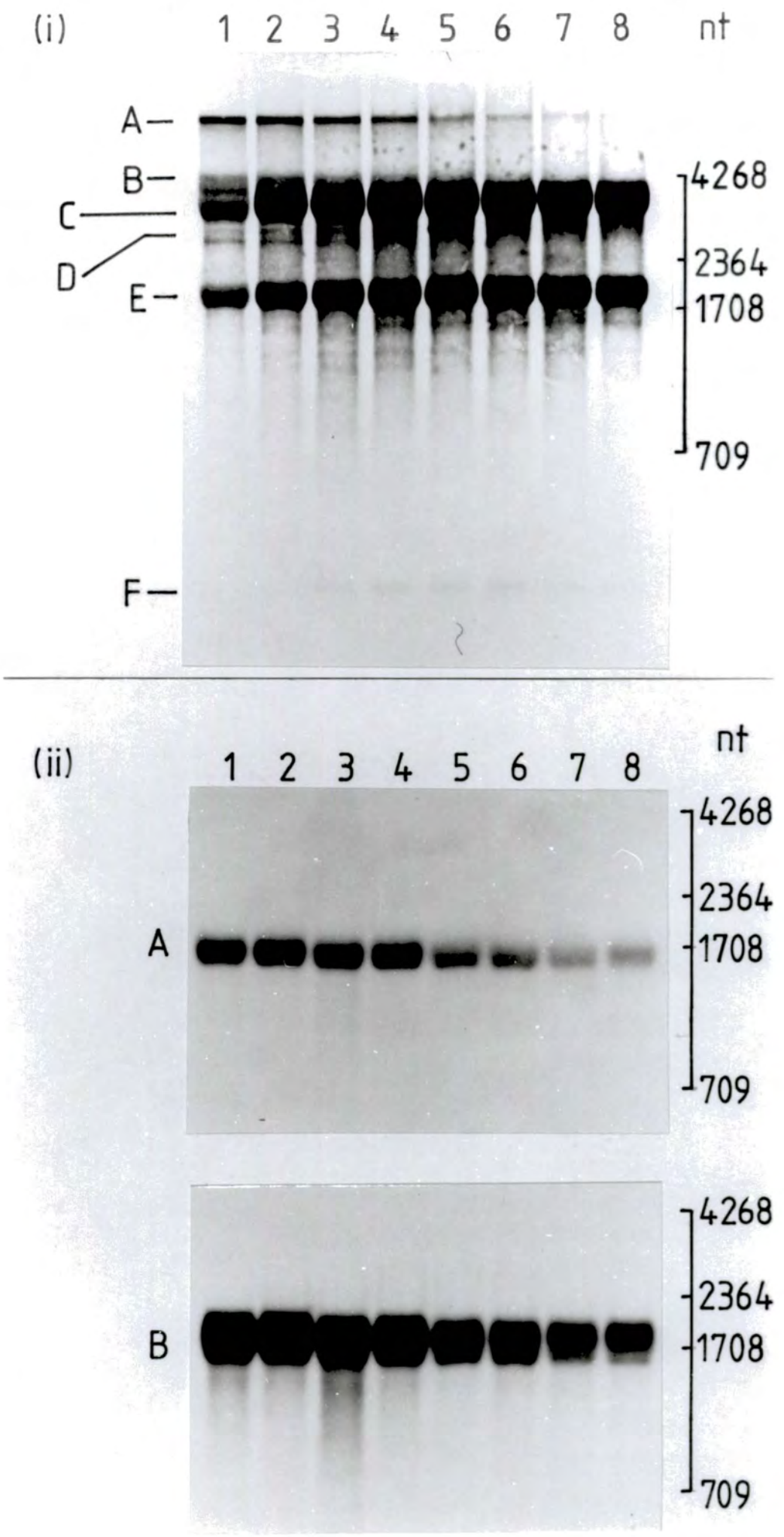

(i)
1 2 3 4 5 6 7 8
nt
A
B
C
D
E
F
4268
2364
1708
709
(ii)
1 2 3 4 5 6 7 8
nt
A
B
4268
2364
1708
709
4268
2364
1708
709

Table 5. Hybridization of tritium labelled total RNA to dot blots of *legA* and *legJ* cDNA clones.

Total RNA samples from each time point during the pulse-chase period were hybridised to DNA dot blots as described in section 3.17. The radioactivity hybridised to each dot was adjusted by subtracting background hybridisation and dividing by hybridisation efficiency. The latter was determined, in each separate hybridisation vial, by inclusion of 550 DPM (equivalent to 275 pg) of sense strand synthetic mRNA (homologous to either *legA* or *legJ* cDNA clones) labelled to 2×10^6 DPM/μg.

legA (pGEM4.4 clone).

Chase time/ hours	DPM		*z*	*(x-y)/z*
	x pGEM4.4	*y* pGEM-blue	Hybridization efficiency	
0	656	267	0.23	1690
3	828	208	0.25	2480
6	1329	178	0.37	3110
9	788	121	0.30	2220
13	1038	133	0.52	1740
17	772	389	0.43	890
23	498	349	0.35	430
39	353	251	0.22	460
52	341	112	0.42	550

legJ (pGEM40 clone)

Chase time/ hours	DPM		*z*	*(x-y)/z*
	x pGEM40	*y* pGEM-blue	Hybridization efficiency	
0	70	82	0.26	-
3	129	90	0.29	140
6	251	119	0.29	460
9	332	92	0.24	1000
13	226	124	0.34	300
17	204	256	0.13	-
23	111	151	0.16	-
39	249	209	0.28	140
52	163	897	0.42	-

pulse-labelling, considerably less *legJ* mRNA accumulated than did *legA* mRNA. Thus, since at 12 DAF the "run-on" transcription rate of *legA* and *legJ* gene sub-families is approximately equal, the relatively low accumulation of *legJ* transcripts during this 3.42 hour pulse suggests that the *legJ* transcripts are rapidly degraded, with a half-life less than the duration of the pulse and less than that of *legA* mRNA.

4.4.3.4 Steady-state levels of legumin mRNAs during the pulse-chase experiment.

Since for the culture conditions used it was reported that legumin protein synthesis continued, and indeed could be initiated in culture (Domoney *et al.* 1980), it was assumed that legumin mRNA accumulation would also continue as *in vivo*. The cotyledons used in this experiment were harvested at 12 DAF, approximately three days after the onset of the rapid accumulation of legumin mRNAs.

In order to check this assumption, aliquots of the tritium labelled total RNAs were Northern-blotted and probed with ^{32}P-labelled *legA* and *legJ* probes. The resulting autoradiographs are shown in Fig. 17(ii). This shows that during the chase period (up to 39 hours) the steady-state levels of both classes of mRNA decline steadily to approximately 20% of their initial level.

Relevant to this are the recent observations of Wang *et al.* (1988) who cultured *Glycine max* embryos *in vitro* and measured lipoxygenase mRNA levels during the first 40 hours of culture; they found that these mRNA levels had dropped to 5-14% of their original values during the first 4 hours. The mRNA levels then increased during the following 36 hours of culture. They also found that the level of translatable mRNAs for other unspecified proteins declined transiently in the first few hours of culture. They concluded that these changes, when cotyledons are first placed into culture, are an adaptive phenomenon. Furthermore, they recommended that care needs to be taken in the use of newly cultured cotyledons for biochemical or physiological studies, and that a period of 1 day acclimation should be allowed for resumption of regular protein and RNA synthesis. In addition, Crouch and Sussex (1981) investigated the effect of various

culture conditions on the synthesis of legumin-like 12S storage protein (cruciferin) in the cultured embryos of *Brassica napus*. They found that in embryos grown in 12% sucrose medium there was a 2 day lag period in the increase in 12S storage protein synthesis compared to that observed when the embryos were grown in medium containing 2% sucrose and 10^{-6}M abscisic acid. Later, Finkelstein and Crouch (1986) studied the effect of osmoticum and ABA on the cruciferin mRNA level in cultured embryos; in a basal medium containing 1% sucrose with the addition of 12.5%, 8.7% or no sorbitol, they showed that after 3 days in culture the changes in mRNA level showed a positive dose-response to osmoticum. Using 12.5% sorbitol, at early developmental stages, mRNA accumulated to a higher level than it did in the equivalent period *in vivo*. However, at later stages mRNA level *in vitro* declined to a lower level than it did *in vivo*. The time course of these changes in mRNA level over the three day incubation was not reported, but the kinetics of reinduction of mRNA levels after incubation on basal medium were investigated; when embryos (27 DAF) were cultured on basal medium (low osmoticum) for ten hours there is a drop in mRNA level (at least 5-fold) with a half-life of about 5 hours. Then, when embryos are transferred to basal medium containing 10.9% sorbitol, or 10μM ABA, there is a rapid increase in mRNA level (this increase is more rapid for sorbitol than for ABA).

Thus it is clear that mRNA and protein synthesis in embryos, not surprisingly, can be transiently perturbed by transfer to culture, and this can be affected drastically by the osmoticum and ABA content of the medium.

As demonstrated in section 4.4.4 bisecting the cotyledons was shown not to prevent the accumulation of legumin mRNA during a 4 day culture period, albeit in a different media to that used in this experiment (5% sucrose, 10^{-5}M ABA), although it may have contributed to any transient decrease in mRNA level.

Fig. 17(ii) shows that the decline in mRNA levels of *legA* and *legJ* follow apparently identical kinetics under the culture conditions employed. This suggests, assuming that the down regulation of the transcription of these two gene sub-families is co-ordinate, that the stability of these two mRNAs in the

cytoplasm (where the bulk of detectable mRNA is found) is the same.

4.4.3.5 Conclusions from pulse-chase experiment.

The results from this experiment were unsatisfactory for the following reasons: firstly, it was shown that the cotyledon culture conditions used did not allow continued accumulation of legumin mRNAs and, thus, any results obtained may not be relevant to the developing seed on the mature plant; secondly, the measured half-life of the tritium-labelled *legA* mRNA was too short relative to the half-life of the specific activity of the NTP pool to give an accurate estimate of the true half-life; we can only say that it is less than 10 hours; however, since legumin steady-state mRNA levels were declining during the chase period it is possible that the corresponding transcription rates were also suppressed and so further incorporation of tritium into these mRNAs would not occur anyway (thus making the chase of label from the mRNAs more representative of the true half-life); finally, the incorporation of label into *legJ* mRNA, in contrast to *legA* mRNA, was too low to be measured accurately.

This last observation, considering that "run-on" transcription of these two genes sub-families is similar, and that the kinetics of their down regulation in the cytoplasm when placed into culture is similar, would be consistent with the hypothesis that *legJ* transcripts are degraded faster than *legA* transcripts in the nucleus. However, the abnormal behaviour of mRNA levels in culture brings the validity of these results to the *in vivo* situation into question. Also, the main objective of this experiment, to measure directly a differential half-life between *legA* and *legJ* mRNA, has not been realised because the conclusions drawn are again based on comparisons with "run-on" transcription data. If this differential turnover was indeed due to rapid nuclear events, then the pulse-chase experimental approach would be unable to detect it in this case, because of the short time of the pulse-label required.

It would be more efficient for a plant to accumulate a storage protein mRNA if the mRNA had a relatively long half-life, unless very rapid modulation of mRNA levels in response to environmental

stimuli was required. Indeed, in several mammalian developmental systems, cell specialisation is associated with unusual stability of the mRNA coding for the major protein synthesised in that cell type (Kafotos 1972;, Brock and Shapiro 1983b). It is possible, then, that the observed half-life of *legA* mRNA is lower than it is in the normal developing seed because a reduced half-life is part of the mechanism of down regulation of this mRNA in response to the stress of the culture conditions. Also, the relatively short half-life of cruciferin steady-state mRNA levels (approximately 5 hours) in low osmoticum culture medium (Finkelstein and Crouch, 1986) may not reflect the true half-life during normal cotyledon development because the low osmoticum favours germination and it would be desirable to rapidly stop storage protein synthesis during mobilisation of seed reserves.

The solutions to the problems outlined above seemed to be in the development of a cotyledon culture system in which cotyledon development more accurately reflected the *in vivo* situation and, in particular, it was necessary to apply the pulse label at a time when legumin mRNAs were rapidly accumulating. Under these conditions it is possible that incorporation into specific mRNAs during the pulse would be higher and that half-lives would be sufficiently long relative to that of the NTP pool specific activity to allow more accurate measurement.

4.4.4 An investigation of the effect of culture conditions on the accumulation of legumin mRNAs.

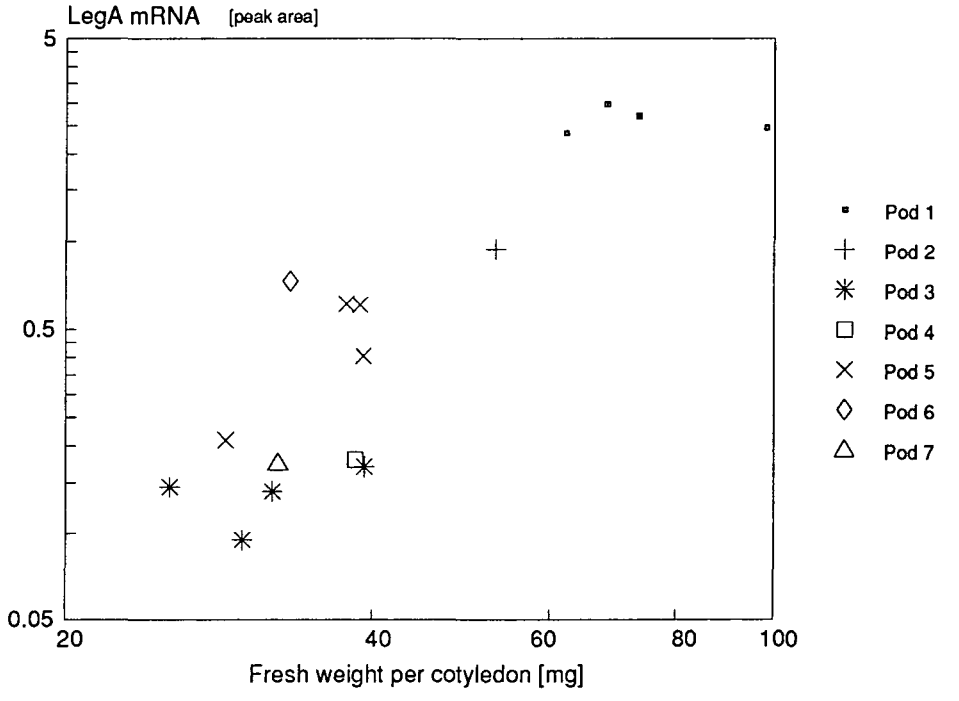
In order to find culture conditions that would promote continued legumin mRNA accumulation the effects of different culture conditions on mRNA levels were determined by dot blotting.

These experiments were performed by using one cotyledon from a single seed as an uncultured control, while the other cotyledon was cultured in defined conditions. 12 DAF cotyledons were used throughout. Legumin mRNA levels in individual cotyledons were found to correlate well with their initial fresh weight, as shown in the log-log scatter plot of initial fresh weight vs. *legA* mRNA level (Fig. 18). Also, the scatter plot of initial fresh weight of one cotyledon vs. the fresh weight of the other cotyledon from the

Figure 18.

Top: Relationship between *legA* mRNA and cotyledon fresh weight.
Total RNA was prepared from individual cotyledons excised from seeds from 7 different 12 DAF pods. *LegA* mRNA level was determined for each total RNA sample by hybridising an antisense synthetic mRNA to RNA dot blots. Data were quantified by scanning with a laser densitometer and are standardised according to the total RNA content of each sample. The peak area of each scan was plotted against cotyledon fresh weight on a log-log scale.

Bottom: Correlation between fresh weights of individual cotyledons from the same seed.
Using the same pods as above, fresh weight from one cotyledon of a given seed was plotted against the fresh weight of the other cotyledon from the same seed.


LegA mRNA [peak area]
5
0.5
0.05
20
40
60
80
100
Fresh weight per cotyledon [mg]
Pod 1
Pod 2
Pod 3
Pod 4
Pod 5
Pod 6
Pod 7

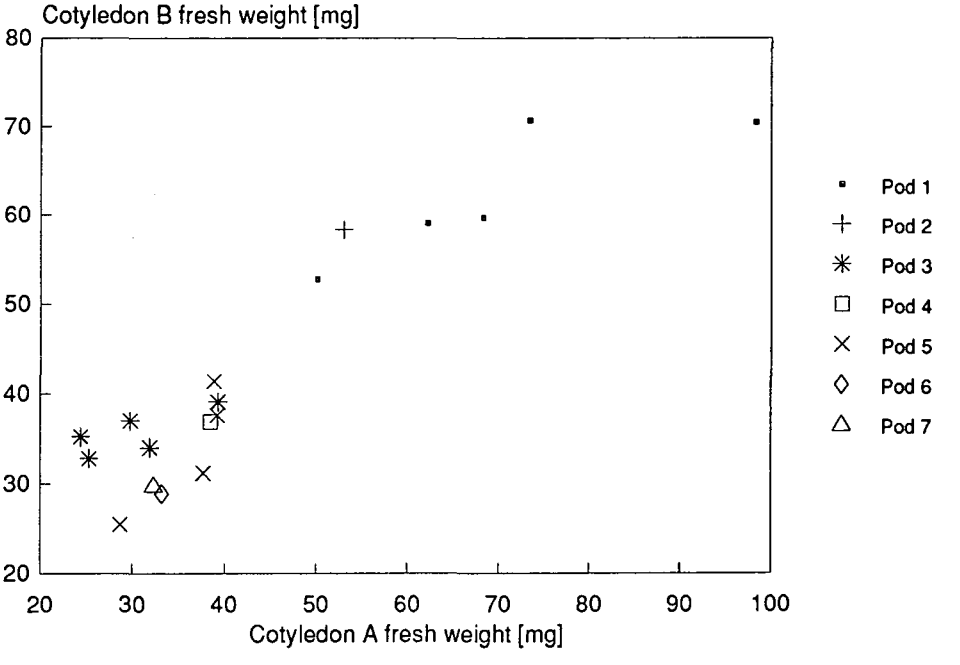

Cotyledon B fresh weight [mg]
80
70
60
50
40
30
20
20
30
40
50
60
70
80
90
100
Cotyledon A fresh weight [mg]
Pod 1
Pod 2
Pod 3
Pod 4
Pod 5
Pod 6
Pod 7

Figure 19. *legA* **mRNA levels in cotyledons cultured under various conditions.**

(a) Effect of sucrose, ABA, and axis.

3 seeds were taken from pod 5 (see Fig. 18) and total RNA was extracted from one cotyledon from each seed to determine the initial mRNA level, Ai. The other cotyledons from each of these seeds (sibling cotyledons) were cultured in the medium of Stafford and Davies (1979) containing 5% sucrose and 10^{-5}M ABA and the final level of mRNA was determined after 4 days in culture, Af.

This procedure was repeated for a medium containing 18% sucrose and no ABA using one seed from each of pods 5, 6 and 7; Bi represents initial mRNA level and Bf the final mRNA level.

Two complete embryos including axes were excised from pod 5 and one also from pod 6. These were cultured in 18% sucrose medium for 4 days and then their final mRNA level, Cf, determined. The initial mRNA level for these embryos was assumed to be similar to Ai and Bi, since seeds arose from the same pods.

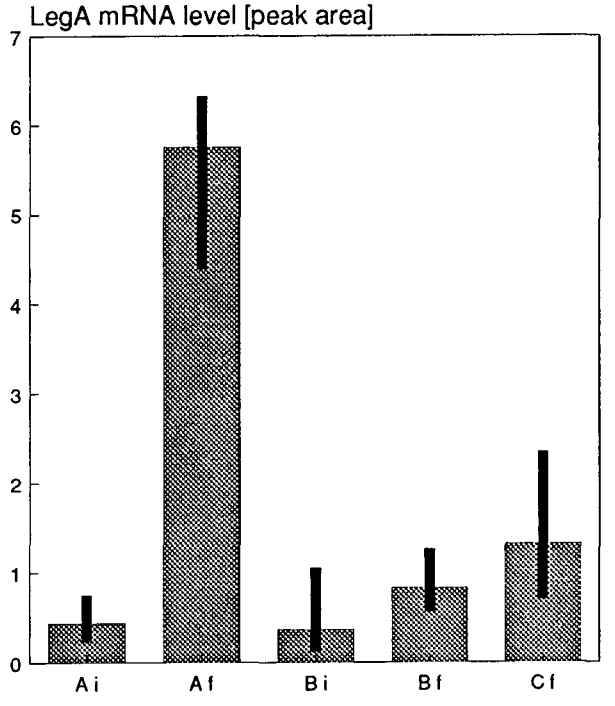
(b) Effect of sucrose concentration.

Duplicate cotyledons from the seeds of pods 3 and 4 were incubated for 4 days in Stafford and Davies medium containing 5, 10, or 18% sucrose and the final (f) level of mRNA was determined. For each cultured cotyledon its sibling was taken and used as a measure of the initial mRNA level (i).

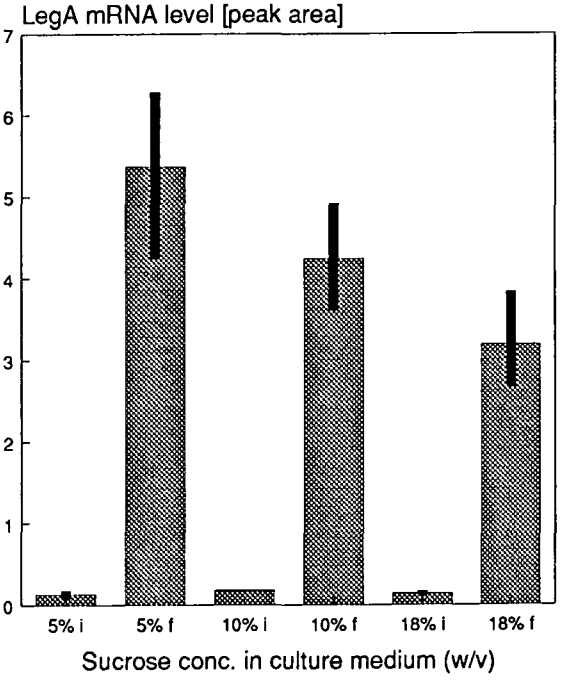
(c) The effect of wounding on mRNA levels in culture.

Two seeds from a single 12 DAF pod (not shown on Fig. 18) were used. One cotyledon from each seed was used to determine initial mRNA level (Ai and Bi). Sibling cotyledons were either left intact (Bf) or cut into four approximately equal pieces using a razor blade (Af) and then cultured for 4 days in Stafford and Davies medium containing 5% sucrose, 10^{-5}M ABA.

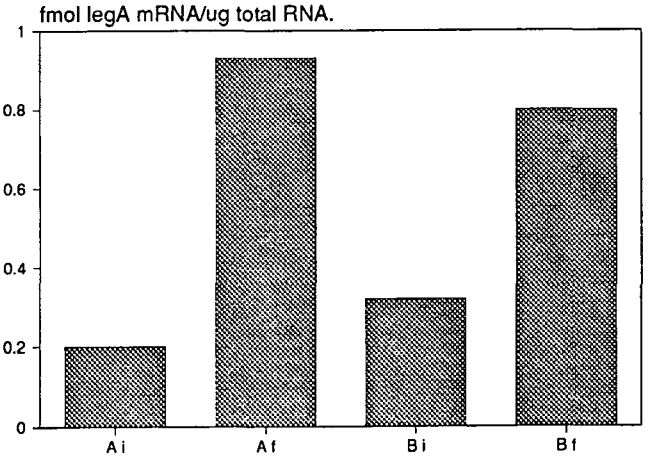
For (a) and (b) mRNA levels were quantified as in Fig. 18; histograms represent the mean of each sample, and the error bars the extremes of variation within each triplicate or duplicate experiment. For (c) mRNA levels were determined in absolute terms using a standard curve of *legA* mRNA against densitometric peak area.


LegA mRNA level [peak area]
7
6
5
4
3
2
1
0
Ai
Af
Bi
Bf
Cf

(a)


LegA mRNA level [peak area]
7
6
5
4
3
2
1
0
5% i
5% f
10% i
10% f
18% i
18% f
Sucrose conc. in culture medium (w/v)

(b)

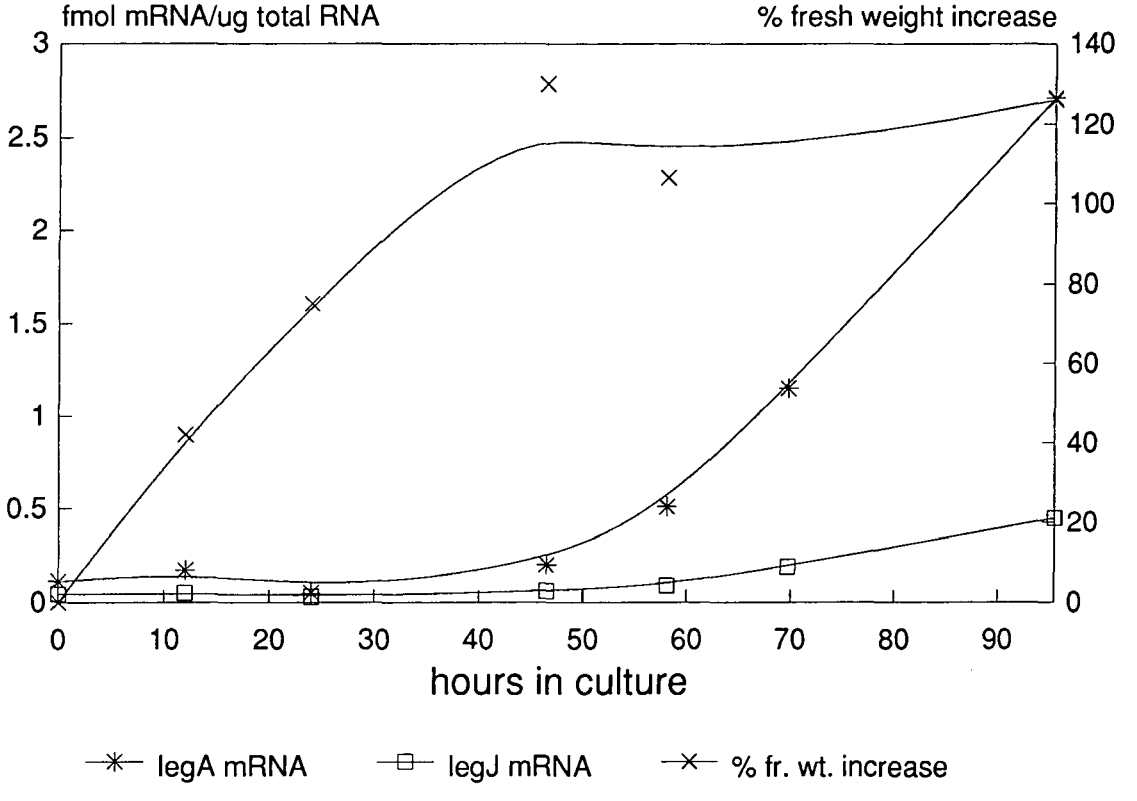

fmol legA mRNA/ug total RNA.
1
0.8
0.6
0.4
0.2
0
Ai
Af
Bi
Bf

(c)

Figure 20. Time course of *legA* and *legJ* mRNA accumulation in 12 DAF cotyledons cultured in a medium containing 5% sucrose and 10^{-5}M ABA.

8 cotyledons from a single pod were cultured individually in the wells of a Cell-Well plate for varying times. Total RNA was then extracted from each cotyledon and 5μg aliquots dotted on to nitrocellulose. Also on the same blots were included dilution series of 14 DAF total RNA as used in Fig. 5, which had a known content of the three different classes of legumin mRNA and was thus suitable to use to calibrate the RNA dot blot. Separate blots were probed with ^{32}P-labelled antisense synthetic RNA probes complementary to *legA* and *legJ* mRNA. Absolute mRNA levels in each sample were determined from a suitable standard curve.

Percentage fresh weight increases were calculated as 100 × (final weight-initial weight)/initial weight.


fmol mRNA/ug total RNA
% fresh weight increase
3
2.5
2
1.5
1
0.5
0
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
hours in culture
legA mRNA
legJ mRNA
% fr. wt. increase

same seed (the sibling cotyledon), as shown in Fig. 18, shows a good correlation and indicates the validity of using paired cotyledons as control and treated samples, as in the work of Spencer *et al.* (1980). For comparisons of different treatments, or for duplicate or triplicate assays, the seeds from a single pod were used, where possible. This was necessary, because it was found that the variation of cotyledon fresh weight, and thus the legumin mRNA level, was considerable between different 12 DAF pods (Fig. 18), but was low within the seeds of a single pod. This is a consequence of the very rapid increase in cotyledon fresh weight over a 24 hour period at this stage of development.

Fig. 19(b) shows the *legA* mRNA level before and after 4 days in culture in 5, 10 and 18% sucrose medium. This shows that after 4 days the level had increased at all three sucrose concentrations, although the trend was for higher increases at lower sucrose concentrations. These data are only semi-quantitative because they are expressed as the peak area of a densitometric scan; no standard of curve peak area against mRNA level was used.

In a second experiment, excised cotyledons were cultured in (A) 5% sucrose medium, with the addition of 10^{-5}M abscisic acid, (B) in 18% sucrose medium, and (C) complete embryos, with intact axes, were cultured in 18% sucrose medium. The mRNA levels before and after 4 days of culture are shown in Fig. 19(a). This shows that the 5% sucrose/ABA medium produced a far more dramatic increase in *legA* mRNA level than did 18% sucrose medium with or without intact axes. The increase obtained with 5% sucrose/ABA medium shown in Fig. 19(a) is comparable to that obtained with 5% sucrose medium alone in Fig. 19(b), thus the presence of ABA does not appear to effect mRNA levels. It was not clear why the increase in mRNA level in the 18% sucrose medium was significantly lower in 19(a) than in 19(b).

From these results it is clear that the 5% sucrose/ABA medium was capable of producing an increase in *legA* mRNA after 4 days in culture similar to that observed *in vivo*. Using this medium the effect of wounding the cotyledon tissue by bisecting with a razor blade on mRNA accumulation after 4 days was assayed. Fig. 19(c)

shows that the level of *legA* mRNA increased 4.7-fold in the bisected tissue, and by 2.5-fold in the whole cotyledons, thus it would appear that the wounding did not prevent *legA* mRNA accumulation. The difference in increase between these two single samples is within the variation shown by individual cotyledons.

The time course of *legA* and *legJ* mRNA accumulation, and fresh weight changes in culture were then investigated in the 5% sucrose/ABA medium as shown in Fig. 20. This shows that when 12 DAF cotyledons are placed in culture there is a lag period of approximately 45 hours before the legumin mRNAs begin to accumulate. During this period the percentage increase in fresh weight increased steadily. After 45 hours there is no further significant increase in fresh weight and legumin mRNA levels then begin to rapidly accumulate; they are continuing to do so at 100 hours. The ratio of *legA* to *legJ* mRNA in this experiment is 6.3 at 100 hours, this is within the range observed at a similar stage *in vivo* (5.0 at 12 DAF and 7.1 at 16 DAF). Thus, the factors which determine the differential accumulation of these two classes of mRNA are preserved in this culture system.

4.4.5 Repeat of RNA pulse-chase labelling experiment using modified culture conditions.

From the information obtained about the behavior of legumin mRNA levels in 12 DAF cotyledons cultured in the 5% sucrose/ABA medium, it was decided to delay the onset of the pulse-chase RNA labelling procedure until after the cotyledons had been in culture for 3 days. Using this system it was hoped that RNA could be labelled during the rapid accumulation of legumin mRNA, i.e. after the initial lag observed in culture.

The results of such an experiment are shown in Fig. 21(a). The plot of *legA* mRNA level during the culturing period shows that there is a dramatic rise (16-fold) between the harvesting of the 12 DAF cotyledons and the next measurement, 74 hours and 45 min later (immediately after the pulse labelling). However, during the next 6 hours there appears to be a drop in mRNA level, before a further increase beginning 16 hours after the end of the pulse. This brief drop may be analogous to that observed by Wang *et al.*

Figure 21.

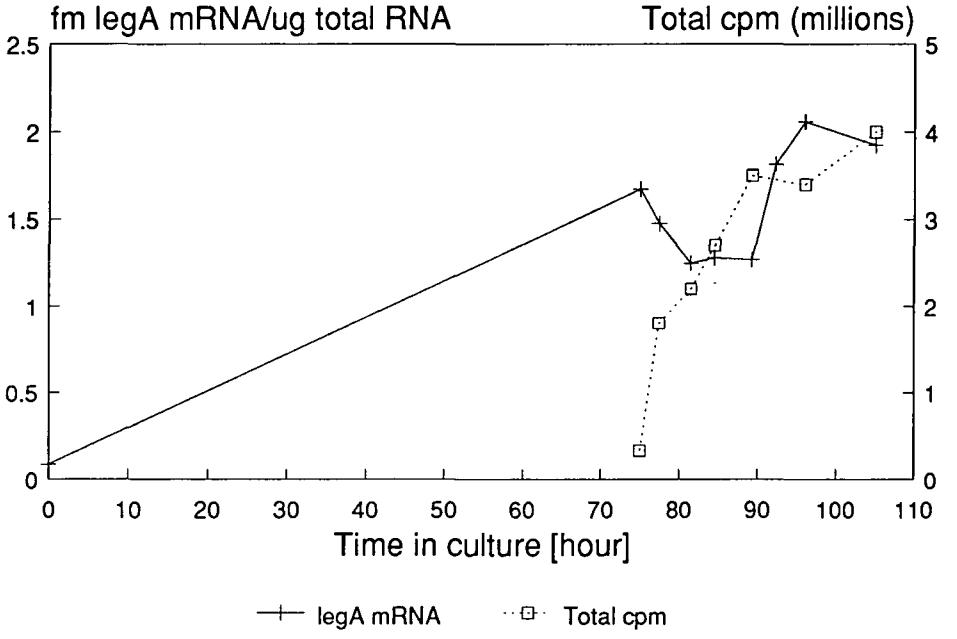
(a) Pulse-chase RNA labelling of 12 DAF cotyledons after 3 days in culture.

Approximately 70 cotyledons were isolated from 12 DAF pods, each was bisected with a razor blade. A sample of 11 half-cotyledons was taken at this point. Equal numbers were then placed in each of two flat bottomed glass pots, covered with Petri dish lids, with 35ml of Stafford and Davies (1979) medium containing 5% sucrose and 10^{-5}M ABA. These were cultured for 72 hours. At the end of this period the two sets of cotyledons were pooled, rinsed in fresh medium, and then placed in 3ml of labelling medium (see Table 5) for 2 hours 45 min. A sample of 11 half-cotyledons was taken at this point and then the remainder were divided into 9 samples of 11 half-cotyledons and each cultured in 10ml of chase medium in small glass pots for the times indicated. Total RNA was then extracted from each sample and radioactive incorporation determined by LSC. *LegA* mRNA levels were then assayed by dot blotting as Fig. 19(c).

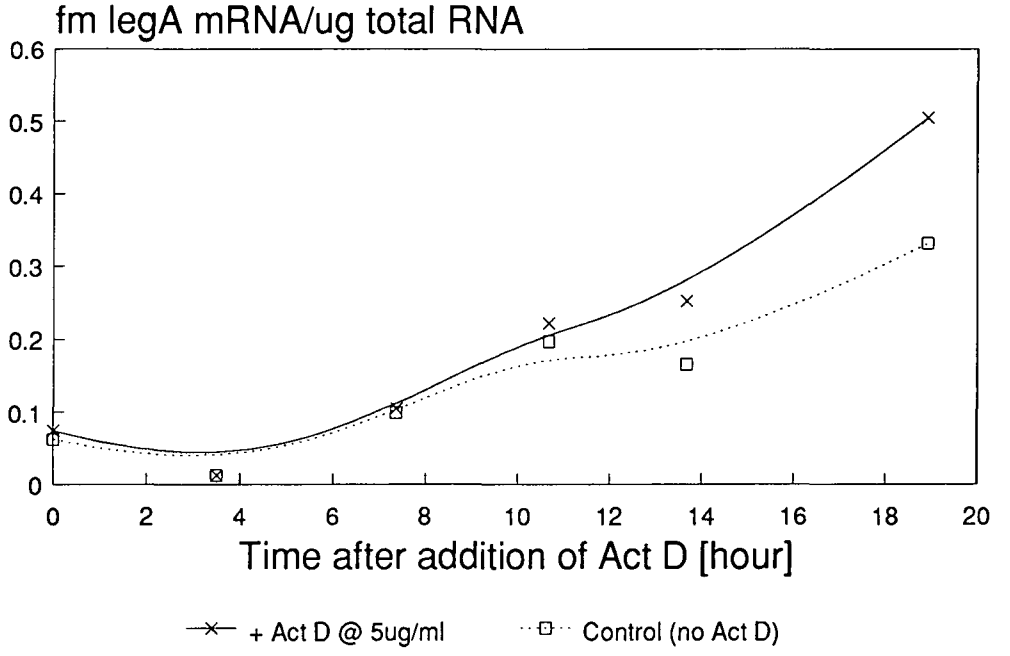
(b) Effect of actinomycin D.

12 cotyledons taken from the six seeds of a single 12 DAF pod were cultured individually in 2.5mls of Stafford and Davies (1979) medium containing 5% sucrose and 10^{-5}M ABA, in the wells of a Cell-Well plate. The cotyledons were cultured for 31 hours and then the medium was changed to fresh medium either containing $5\mu g.ml^{-1}$ actinomycin D (Act D), for 6 of the cotyledons, or no actinomycin D for the other 6. One cotyledon was then removed, from each set of 6 at random, at time points over the next 19 hours and total RNA extracted. $2\mu g$ aliquots of total RNA from each sample were then assayed for *legA* mRNA content by dot blotting as in Fig. 19(c).

(a)


fm legA mRNA/ug total RNA
Total cpm (millions)
2.5
2
1.5
1
0.5
0
5
4
3
2
1
0
0 10 20 30 40 50 60 70 80 90 100 110
Time in culture [hour]
legA mRNA
Total cpm

(b)


fm legA mRNA/ug total RNA
0.6
0.5
0.4
0.3
0.2
0.1
0
0 2 4 6 8 10 12 14 16 18 20
Time after addition of Act D [hour]
+ Act D @ 5ug/ml
Control (no Act D)

(1988) for lipoxygenase mRNA when soybean embryos are first placed into culture (see section 4.4.3.4), and could be due to the change in culture conditions during the pulse-labelling period (fresh medium, presence of uridine, smaller volume). Alternatively, since the kinetics of the mRNA increase before the application of the pulse were not measured, the mRNA level may have already been decreasing at 72 hours, although this would contradict the results shown in Fig. 20.

The radioactivity incorporated into the total RNA was very low in comparison to that when cotyledons were labelled immediately after harvest (Fig. 16i); the peak of total incorporation was 10-fold lower. This may be related to the kinetics of fresh weight increase during the culturing period (see Fig. 20), where there appears to be no fresh weight increase after the first 50 hours in culture even though *legA* mRNA level begins to rapidly increase. There may be a generally reduced uptake of nutrients from the culture medium, after the three days in culture when the pulse was applied, including reduced uridine uptake, thus preventing high activity labelling of the RNA.

The 10-fold reduction in incorporation precludes any attempt to measure radioactivity incorporated into specific legumin mRNAs.

4.4.6 Effect of application of actinomycin D.

Actinomycin D (ActD), an inhibitor of transcription, has frequently been used in studies of RNA turnover, including in tobacco protoplasts (Cornelissen, 1989). It was hoped that application of ActD to a culture of 12 DAF cotyledons at a time when legumin mRNAs were accumulating would inhibit transcription and the resulting decline of steady-state levels of mRNAs would give an indication of their half-lives. The results of such an experiment, plotted in Fig. 21(b), show that *legA* mRNA levels continue to increase in the presence and absence of ActD thus indicating that 5μg/ml ActD was unable to inhibit transcription in this system. This may be due to a low uptake of ActD by the cotyledons after they have been in culture for 31 hours (the time of application of ActD), similar to the way that the reduced incorporation of tritium into total RNA could have been due to

reduced uptake of [^{3}H]-uridine (section 4.4.5).

This problem might be overcome by using higher concentrations of ActD, but no further experiments were performed.

4.4.7 Final conclusions from cotyledon culture experiments.

Using this culture system, there are two seemingly insurmountable problems. Firstly, when 12 DAF cotyledons are harvested and immediately pulse labelled in culture, they readily incorporate tritiated uridine into total RNA in amounts sufficient to measure incorporation into specific mRNAs. However, *in vitro* there is a 2-3 day lag period before legumin mRNA resumes the accumulation observed *in vivo*, thus the immediate pulse-label occurs at a time when mRNAs are not accumulating, and thus any measured half-life may be artifactual. If, however, we try to avoid this problem by applying the pulse later in culture when legumin mRNAs *are* accumulating, then incorporation of tritium into total RNA is so low that it would be very difficult to measure radioactivity of specific mRNAs.

Secondly, the pulse labelling procedure, even when applied at a time when legumin mRNA levels were shown to be increasing, appeared to produce a transient drop in mRNA level and thus the attempts to pulse-label mRNAs under conditions in which they are accumulating may not be possible.

More extensive characterisation and manipulation of cotyledon culture conditions are required before they can be used further in studies on the control of gene expression.

4.4.8 Discussion of the behaviour of pea embryos in culture.

A series of papers have recently been published concerning the use of embryo culture to investigate seed development in pea. In this section the results presented in these papers will be discussed, where relevant to the culture experiments presented in the previous sections.

4.4.8.1 The effect of ABA and osmoticum on precocious germination, and storage proteins accumulation.

Cook *et al.* (1988) and Barrett *et al.* (1989) investigated precocious germination of pea embryos *in vitro* under various different conditions; Cook *et al.* (1988) found that precocious germination was stimulated by media of low osmoticum, but that no precocious germination could occur in the medium of Stafford and Davies (1979), as used here, even in sucrose concentrations as low as 2%, because of the high concentrations of amino-acids included in the medium. It is important to use a medium that does not permit precocious germination in studies on seed development because storage protein synthesis might be expected to cease during *in vitro* germination.

ABA was not implicated in the inhibition of precocious germination because embryos from later stages of development, known to contain the peak levels of endogenous ABA (Wang *et al.*, 1987), are still able to precociously germinate *in vitro* (Cook *et al.*, 1988). Wang *et al.* (1987) showed that the increases in ABA levels in testa and embryo *followed* closely the periods of rapid growth of these tissues and did not *cause* them. Barrett *et al.* (1989) further showed that ABA was not required for inhibition of precocious germination, accumulation of storage proteins or desiccation tolerance. This was achieved by culturing 3 day pea pods *in vitro* for 21 days in the presence of fluridone, an inhibitor of biosynthesis of one of the precursors to ABA. The fluridone prevented any detectable ABA from accumulating in seed tissues, but did not interfere with the normal seed development. When culturing embryos in a medium equivalent to an osmotic pressure of 2% sucrose, Barratt *et al.* (1989) found that ABA concentrations as high as 0.1M were not able to inhibit precocious germination, however, ABA was able to reduce precocious germination in embryos cultured in 5% sucrose medium. They concluded that it was the water relations of the seed, as first suggested by Walbot (1978), and not ABA, that were important in seed development.

These results are in contrast to work published for other

plant species where there have been reports that ABA regulates storage protein accumulation (Bray and Beachy, 1985; Crouch and Sussex, 1981), and mutants of *Zea mays* (Neill *et al.*, 1987) and *Arabidopsis thaliana* (Karssen *et al.* 1983) deficient in ABA show precocious germination during seed development *in vivo*. More recently ABA has been implicated in the water stress transduction pathway during the desiccation stage of seed development: proteins and mRNAs that are abundant late in embryogenesis can be induced in other parts of rape, cotton, barley, rice and wheat plants (including young embryos) in response to water-stress or application of ABA (Dure *et al.*, 1989); however, desiccation tolerance was not affected in pea seeds that developed in an absence of detectable ABA (Barrett *et al.*, 1989).

In the results presented in Fig. 20, ABA was included in the medium at a concentration of 10^{-5}M, but there is no evidence that ABA was necessary for the accumulation of the legumin mRNA.

Stafford and Davies (1979) reported, using pea cultivar JI430, that 18% sucrose was the optimum concentration for fresh and dry weight increases in culture, although in a later study Wang *et al.* (1987) found that two pea lines, near-isogenic except for the *rugosus* (r) locus (produced from a cross between JI430 *rr* and JI145 *RR* followed by six back crossings to JI430 before multiple rounds of selfing and progeny selection), both showed optimal relative fresh weight increase in an identical medium containing 10% sucrose. However, the *RR* genotype attained higher fresh weight increases than the *rr* genotype under all sucrose concentrations, thus demonstrating the variable response to culture conditions of different genotypes. The cultivar used in this thesis is Feltham First, which has the round (*RR*) phenotype as judged by starch grain morphology (J.A. Gatehouse, personal communication). As discussed by Ambrose *et al.* (1987) (also see next section), the selection of a culture medium favouring optimum fresh weight increase also selected for conditions which favoured growth by cell-expansion rather than cell-division.

4.4.8.2 Cell populations during development and their their relationship to storage protein accumulation.

Ambrose *et al.* (1987) performed an investigation of the cell size distributions during embryo development both *in vitro* and *in vivo*. They concluded that *in vivo* embryos up to 1mg contained a single cell population of size similar to that of meristematic cells. During growth from 3mg to 100mg there was a progression towards a bimodal cell population consisting of the meristematic sized cells and a population of larger cells. At later stages of cotyledon development there is also a gradient of cell sizes in the storage parenchyma, with larger cells found towards the center. Corke *et al.* (1988) were able to show - using a triple staining technique for DNA, tubulin, and storage protein - that storage protein is only deposited in non-dividing, endoreduplicated cells with DNA contents above 5C, and that these cells were non-uniformly distributed throughout the cotyledon tissue.

Ambrose *et al.* (1987) raised the question of whether the accumulation of storage protein is due to an increased rate of synthesis in all storage cells, or due to an increase in the number or proportion of cells synthesising the storage protein. Harris *et al.* (1989) reported that high levels of legumin and *legA* mRNA only accumulate in the large, highly vacuolated storage parenchyma cells of cotyledons, but not in cotyledonary vascular and epidermal tissues. The onset of mRNA accumulation in pea cotyledons seems to be synchronised throughout the developing storage parenchyma, unlike the soybean seed protein mRNAs (β-conglycinin and Kunitz trypsin inhibitor) which were reported to accumulate in a wave from the outer to the inner storage parenchyma cells as development proceeds, in seed and somatic soybean embryos, and in transgenic tobacco embryos (Goldberg 1989). This latter work suggests control either by some diffusible factor or by the developmental age of individual parenchyma cells (larger cells are present towards the center of the cotyledon).

The cell population of embryos grown *in vitro* was markedly different to that *in vivo;* embryos from 0.1 to 15 mg fresh weight

were grown *in vitro* for 7 days in 10% sucrose medium and then cell populations analysed. *In vitro* grown embryos, of a similar weight to *in vivo* grown embryos, showed a larger mean and maximum cell area for the population of larger cells, and had a lower proportion of smaller cells; overall, cell numbers per embryo were lower by more than 2-fold (Ambrose *et al.*, 1987). This shift to an increased population of larger cells could be regarded as a premature aging of the tissue, although it is not clear how this could relate to the lag in mRNA accumulation in culture shown in Fig. 20.

In all of these studies of pea embryo culture, embryos were cultured for 7 days and no time course studies were performed. Thus, any transient effects on storage protein accumulation such as the lag observed in Fig. 20 would not be detected.

4.5 Investigation of differential mRNA stability using *in vitro* **systems.**

In vitro systems for studying the regulation of mRNA degradation in the cytoplasm would be extremely useful for mapping the regions of mRNA molecules that control their stability. This could be done by creating various chimeric and/or mutant mRNA constructs by transcription from recombinant DNA plasmids *in vitro*, followed by assaying the degradation of these synthetic mRNAs in cell-free systems. The systems could also be used as an assay for the purification of specific regulatory ribonucleases and other *trans*-acting factors.

Such systems have recently been developed in several laboratories from extracts of mammalian cell cultures as described in section 1.3.2. No such system is available in plants and thus it was decided to investigate the possibility of developing a system from pea cotyledon tissue.

Since the original cell-free extract successfully used by Ross and Kobs (1986) was based on the degradation of polysomal mRNA in a cell-free translation system, it was decided to begin by assaying mRNA degradation in the cell-free translation extract that had previously been developed from pea cotyledon tissue (Beevers and Poulson, 1972). This system was chosen so that the seed-storage protein mRNAs could be studied in a homologous system.

The cell-free extract of Beevers and Poulson (1972) consisted of a postmicrosomal supernatant from a pea cotyledon homogenate, and had been shown by these authors and by Morton *et al.* (1983) to promote polysome-driven translation with 8-16% incorporation of radioactive amino-acids. From studies using the inhibitor of translation initiation, aurintricarboxylic acid (ATA), it was shown that up to 44% of peptide synthesis in this extract was due to reinitiation (Morton, 1982).

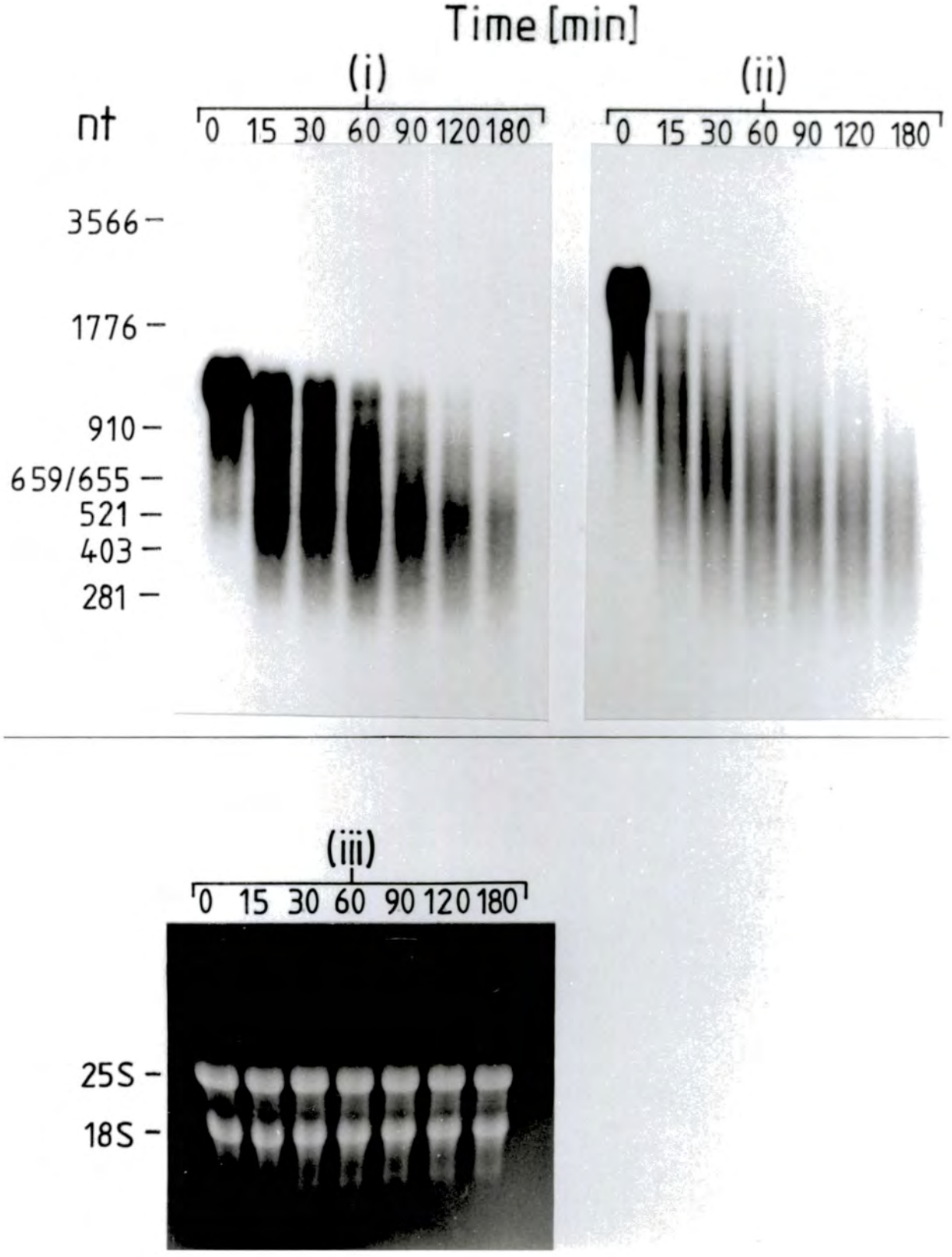
Figure 22. Degradation of *legJ* and *CAB* polysomal mRNAs in a pea cotyledon cell-free extract.

14 DAF pea cotyledon polysomes were incubated in a reaction mixture containing Beevers and Poulson (1972) cotyledon cell-free extract, as described in section 3.18.3; creatine phosphokinase and phosphocreatine were included in the reaction. Samples were taken at the times indicated, polysomal RNA was extracted and aliquots of RNA, equivalent to that extracted from 2-5μg of polysomes, were run in duplicate on a formaldehyde gel and Northern blotted. Size markers were provided by *E.coli* rRNA and a pBR322 AluI restriction digest. The two duplicate halves of the Northern blot were probed as follows:

(i) pFa/b31 insert (*CAB* cDNA);

(ii) pGEM40 insert (*legJ* cDNA).

(iii) 2μg aliquots of polysomal RNA from each time point were run on a separate mini-formaldehyde gel which was then stained with Ethidium bromide.


Time [min]
(i)
(ii)
nt
0 15 30 60 90 120 180
0 15 30 60 90 120 180
3566
1776
910
659/655
521
403
281
(iii)
0 15 30 60 90 120 180
25S
18S

4.5.1 Polysomal mRNA degradation in the pea cotyledon cell-free extract of Beevers and Poulson (1972).

A cell-free extract was prepared from 14 DAF cotyledons by the method of Beevers and Poulson (1972) and 14 DAF cotyledon polysomes were incubated in this extract for 180 min. Samples were taken at various times, the RNA was extracted, Northern-blotted and then probed with clones representing *legA*, *legJ* and *CAB*. Autoradiographs of blots probed with *CAB* and *legJ* clones are shown in Fig. 22. Half-lives of *legA* and *legJ* mRNA were indistinguishable in this experiment and only the *legJ* blot is shown.

It can be clearly seen that the *CAB* mRNA has a greater half-life than the *legJ* mRNA under these conditions; less than 1-2% of the legumin mRNA remained intact after 15 min, whereas approximately 30% of the *CAB* mRNA remained intact at this time point. As seen in Fig. 22(iii) rRNA is completely stable during the 3 hour incubation.

As the intensity of the intact *CAB* mRNA band decreased during the incubation it is clear that the size distribution of the mRNA hybridising to this probe also changed: at time zero a broad band hybridised, but as the incubation proceeded the band became less disperse, and tended towards becoming a narrow band at the lower limit of the size range observed at time zero. This is consistent with a 3' and/or 5'-exonuclease activity being at least partially responsible for the mechanism of the *CAB* mRNA degradation during the incubation, and is also consistent with the mechanism of mRNA degradation proposed by Bernstein *et al.* (1989) (described in section 1.2.2) in which poly(A)-tail shortening is the first step in degradation. However, because the *CAB* mRNA is transcribed from a large multigene family, the possibility can not be eliminated that the reduction in size heterogeneity during the incubation is due to differential degradation of several cross-hybridising mRNAs of different sizes.

A lower molecular weight smear hybridising to the *CAB* cDNA is also generated as the incubation proceeds; the smear does not appear to be random since there are two relatively high intensity regions centering on approximately 800 and 550 nucleotides. This

suggests the possibility of the production of intermediates in the degradation pathway that are relatively stable. The degradation of the legumin mRNAs is too rapid to make any comments on its mechanism.

4.5.2 Polysome driven translation, and mRNA degradation, in the cotyledon cell-free extracts.

In order to test whether or not the cell-free translation system of Beevers and Poulson (1972) was active in translation, an identical incubation to that of section 4.5.1 was set up, but replacing non-radioactive leucine with [^{3}H]-leucine. The polysome driven translation in this extract was not able to give more than 2.5% incorporation of radioactivity into TCA precipitable counts. This suggested that the high rate of degradation of polysomal mRNA promoted by this extract may have been responsible for the low level of incorporation.

Another method for producing a cell-free translation extract from pea cotyledons was then tried, based on the method that Peumans *et al.* (1980a) used for the preparation of a cell-free extract from dry pea primary axes. In their method, a 10,000xg supernatant from a pea axes homogenate was purified by passage through Sephadex G-25 and was found to be suitable for translation of purified poly(A)$^{+}$ mRNA, viral RNAs and polysomes (Peumans *et al.*, 1980a, 1980b; Morton, 1982). In contrast to the 2.5% incorporation obtained from the Beevers and Poulson (1972) extract, the cotyledon extract prepared (using the same batch of cotyledons) by the method of Peumans *et al.* (1980a) was able to incorporate 25-56% of [^{3}H]-leucine into the TCA precipitable fraction in a polysome driven translation. This degree of incorporation is similar to that reported for the axes system (Peumans *et al.*, 1980a).

Fig. 23 shows the time course of incorporation of radioactivity into the TCA precipitable fraction, with and without the addition of cycloheximide. Fig. 24a shows the protein synthesised during the time course in the absence of cycloheximide analysed by SDS-PAGE and fluorographed. From the same incubation tubes, samples were also taken for isolation of RNA, which was

Figure 23. Time course of amino-acid incorporation during the RNA degradation assay shown in Fig. 24, in the presence and absence of cycloheximide.

From the incubations shown in Fig. 24, as a measure of percentage incorporation of amino-acids into protein, samples were taken for determination of total and TCA-precipitable counts. Percentage incorporation is plotted against incubation time.

CH=cycloheximide.

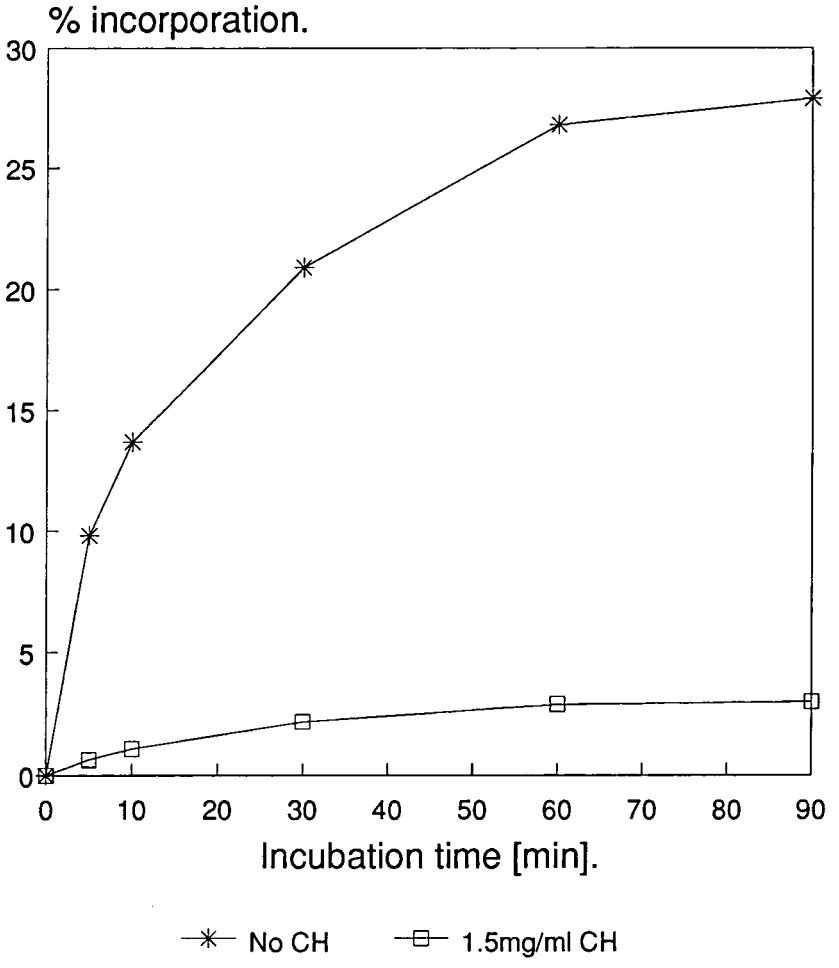

% incorporation.
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
Incubation time [min].
No CH
1.5mg/ml CH

Figure 24. Degradation of *vicJ* polysomal mRNAs and protein synthesis in a pea cotyledon cell-free extract.

14 DAF polysomes were incubated in a cotyledon extract prepared by the method of Peumans *et al.* (1980a) in the presence of [^{3}H]-leucine, and with or without cycloheximide, as described in section 3.18.5. During the incubations samples were taken from the same tube for RNA and protein analysis at the times indicated.

(a) Fluorograph of protein samples (24 hour exposure at -80°C).

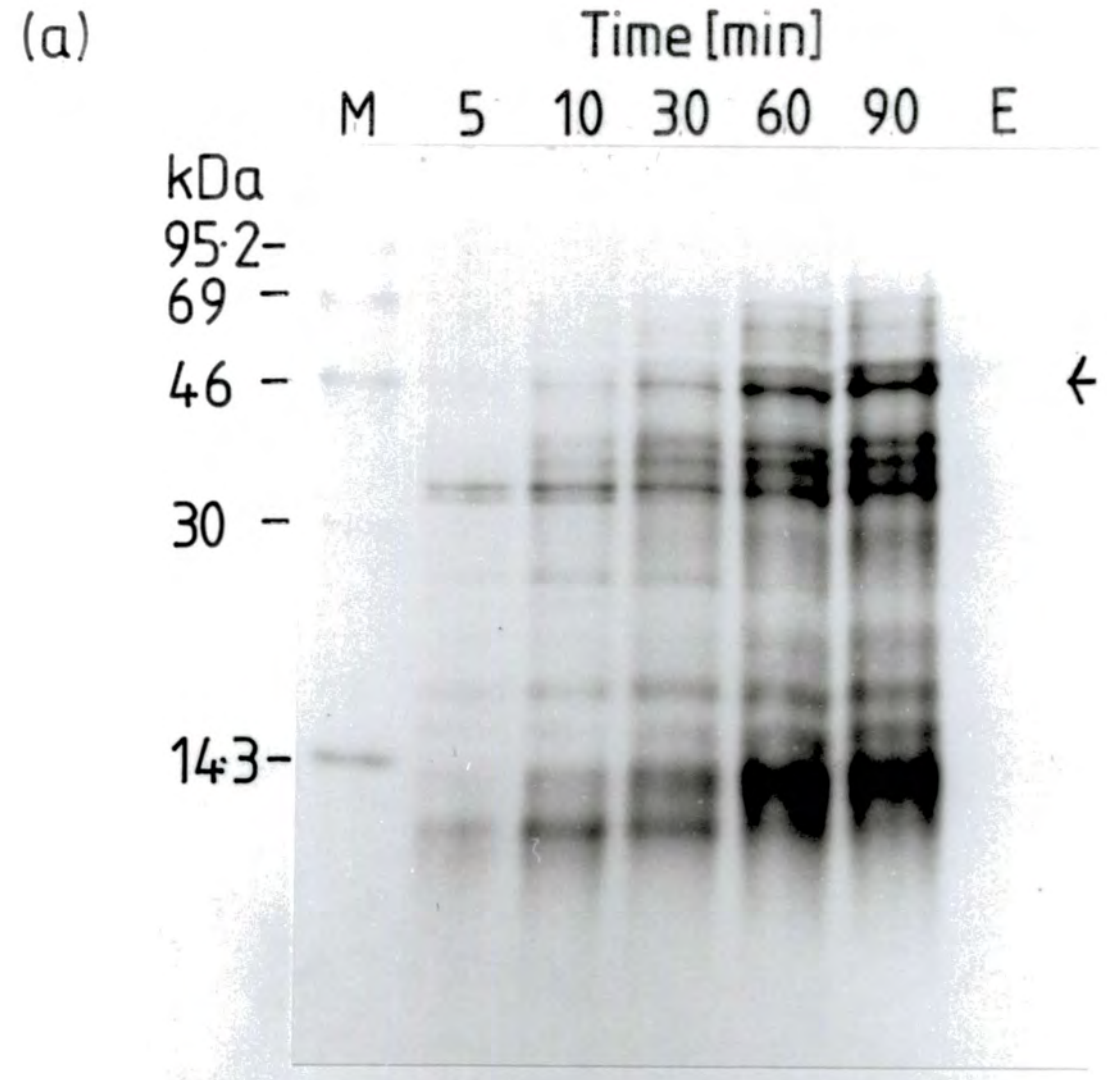
Size markers (M) are commercial [^{14}C]-labelled proteins from Amersham International, plc. Track E shows the endogenous protein synthesising activity of the extract in the absence of polysomes, after a 90 min incubation. The arrow shows the position of the 47,000 MW vicilin precursor polypeptide (encoded by *vicJ*).

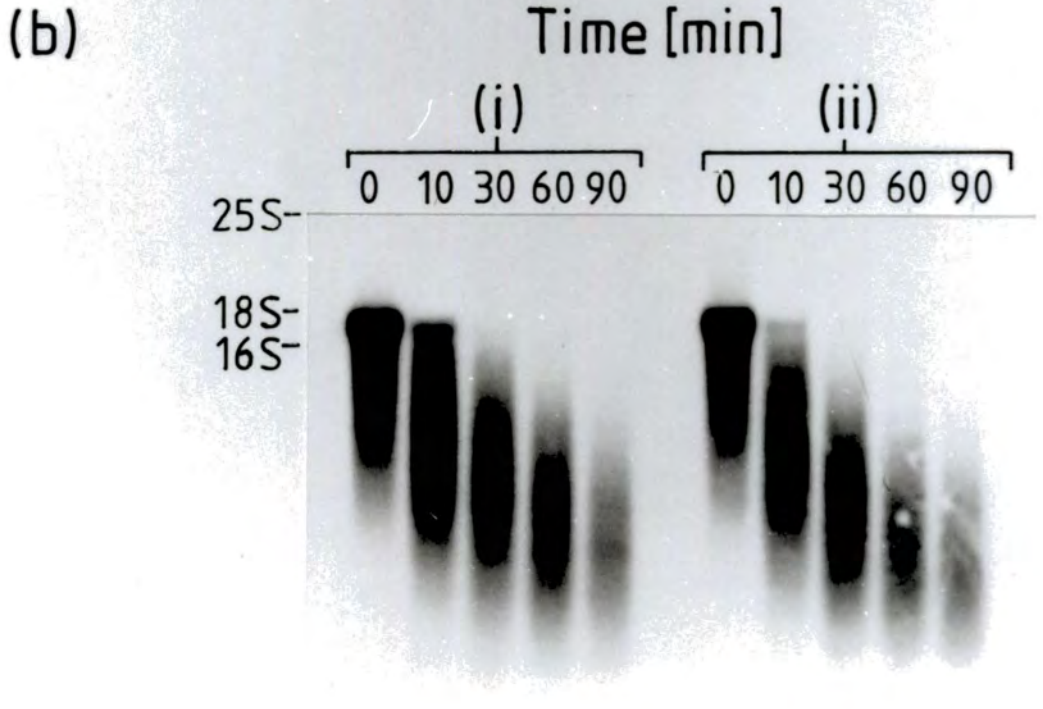
(b) RNA samples analysed by Northern blotting and probed with the insert from pAD3.4 (*vicJ* cDNA probe; Delauney, 1984).

Markers were provided by *E.coli* and pea rRNA.

(i) shows the samples taken from the same tube from which protein samples in (a) were taken.

(ii) shows samples taken from an identical reaction except that cycloheximide was included at 1.5mg/ml.


(a)
Time [min]
M
5
10
30
60
90
E
kDa
95·2-
69 -
46 -
30 -
14·3-


(b)
Time [min]
(i)
(ii)
0 10 30 60 90
0 10 30 60 90
25S-
18S-
16S-

then Northern-blotted and probed with pAD3.4, a cDNA probe representing the 47,000 MW vicilin precursor (*vicJ*), as shown in Fig. 24b.

A comparison of the translation and degradation of the *vicJ* mRNA gives a surprising result: the 47,000 MW vicilin precursor polypeptide (the band arrowed in Fig. 24b as identified by comparison to hybrid release translations performed in this laboratory [Delauney 1984]) appears to accumulate until the 60 min time point; there is an approximately 2-fold increase in the intensity of this band between 30 and 60 min and then there is no further increase. However, the *vicJ* mRNA is rapidly degraded such that the intensity of the band hybridising to the intact mRNA at 30 min is less than 1% that at time zero, and is undetectable after 60 min. At later stages, however, a high degree of hybridisation to a low MW smear occurs, indicating that a large proportion of the mRNA remains as smaller fragments.

The question arises: how does translation continue when the vast majority of mRNA has been degraded? Such a result could be explained if the vicilin mRNA was in vast excess over the amount required for maximum vicilin synthesis in this system; or, if 3'-fragments of mRNA molecules, still bound to translating ribosomes, were able to direct the completion of nascent polypeptide chains whose translation had been initiated on intact mRNA molecules.

The time course of accumulation of different protein bands differs wildly: some proteins such as vicilin accumulate relatively steadily throughout the incubation, whereas other bands, such as the upper band of the doublet at approximately 35,000 MW which is prominent at 5 min, are abundant after only 5 min and then do not increase substantially over the next 85 min. There are also bands which decay after becoming labelled during the first 5 min. Such differences could be related to differential mRNA turnover, or, alternatively, to differential translation initiation or elongation, or protein turnover.

This translation system, when compared to the Beevers and Poulson (1972) extract, caused only a marginally lower rate of mRNA degradation of *legA* mRNA (data not shown).

4.5.3 Effect of incubation conditions on degradation rates of mRNAs *in vitro*.

14 DAF polysomes were then incubated with or without the Peumans *et al.* (1980a) cotyledon extract under various conditions and the degradation of mRNAs were assayed over a time course by Northern blotting. Blots were probed with 6 different cDNA probes whose mRNAs were present in 14 DAF cotyledon polysomes; examples of autoradiographs are shown in Fig. 25.I and II. Autoradiographs were quantified by laser densitometry and plotted in Fig. 26.

Fig. 26(i) shows a densitometric analysis comparing *CAB* mRNA degradation, under various different conditions. The effects of these conditions on the degradation of *CAB* mRNA and other mRNAs are described below. The blots were also probed with 5 other probes (Fig. 26(1-6)) and the rank ordering of the effects of the different conditions on the half-lives of each individual class of mRNA was the same as that described for *CAB* mRNA.

The standard reaction (1) contained polysomes and cell-free extract; in this reaction *CAB* mRNA was degraded with a half-life of approximately 12 min [Fig. 25.I(a)(1) and 26(i)(1)]. The mRNA present in deproteinised cotyledon total RNA was shown to be completely stable during incubation in the 1xSEA solution alone, thus showing that no contaminating exogenous RNase activity was present in the assay (Fig. 25I.7).

4.5.3.1 Effect of cycloheximide.

Cycloheximide, an inhibitor of translation elongation, but not initiation, was added to see if this affected mRNA stability. This type of inhibitor, in a translation system with active initiation, should cause "ribosome loading", i.e. an increase in polysome size because ribosomes can attach to a mRNA but cannot be released. A relatively high concentration of cycloheximide (1.5mg/ml) was required to produce a 90% inhibition of translation (Fig. 23). One effect of ribosome loading that might be expected is the protection of the mRNA from soluble endonucleases, however mRNA degradation showed a slight increase rather than a decrease in the presence of cycloheximide; this increase, if it is significant

Figure 25. Northern blots showing degradation of several different species of polysomal mRNAs under various conditions *in vitro*.

I. RNA samples from mRNA degradation assays under conditions 1-7 were Northern-blotted and probed with the following probes:
Column (a) was probed with pFa/b31 DNA insert (*CAB* cDNA). Column (b) was probed with pDUB6 DNA insert (*legA* cDNA).
Incubation conditions used were as follows:

1. Standard reaction containing 14 DAF polysomes, 14 DAF cotyledon extract prepared as in Peumans *et al.* (1980a) , and 1×SEA, as described in section 3.18.3;
2. as 1., but with addition of creatine phosphokinase and creatine phosphate (CPK/CP), (see section 3.18.3 for details);
3. as 1., but with HPRI added to 2units/μl;
4. as 1., but with Na_2EDTA.NaOH pH 7.5 added to 6.7mM;
5. as 1., but with the cotyledon extract replaced by water;
6. as 5., but with the addition of 5mM Mg^{2+};
7. as 5., but polysomes were replaced with deproteinised 14 DAF total RNA dissolved in polysome resuspension buffer.

II. RNA degradation assays were performed under conditions 5 and 6 (see above) and then probed with the following cDNA inserts:
(c) pLG4.10 (*lecA*);
(d) pAD3.4 (*vicJ*);
(e) pGEM40 (*legJ*).

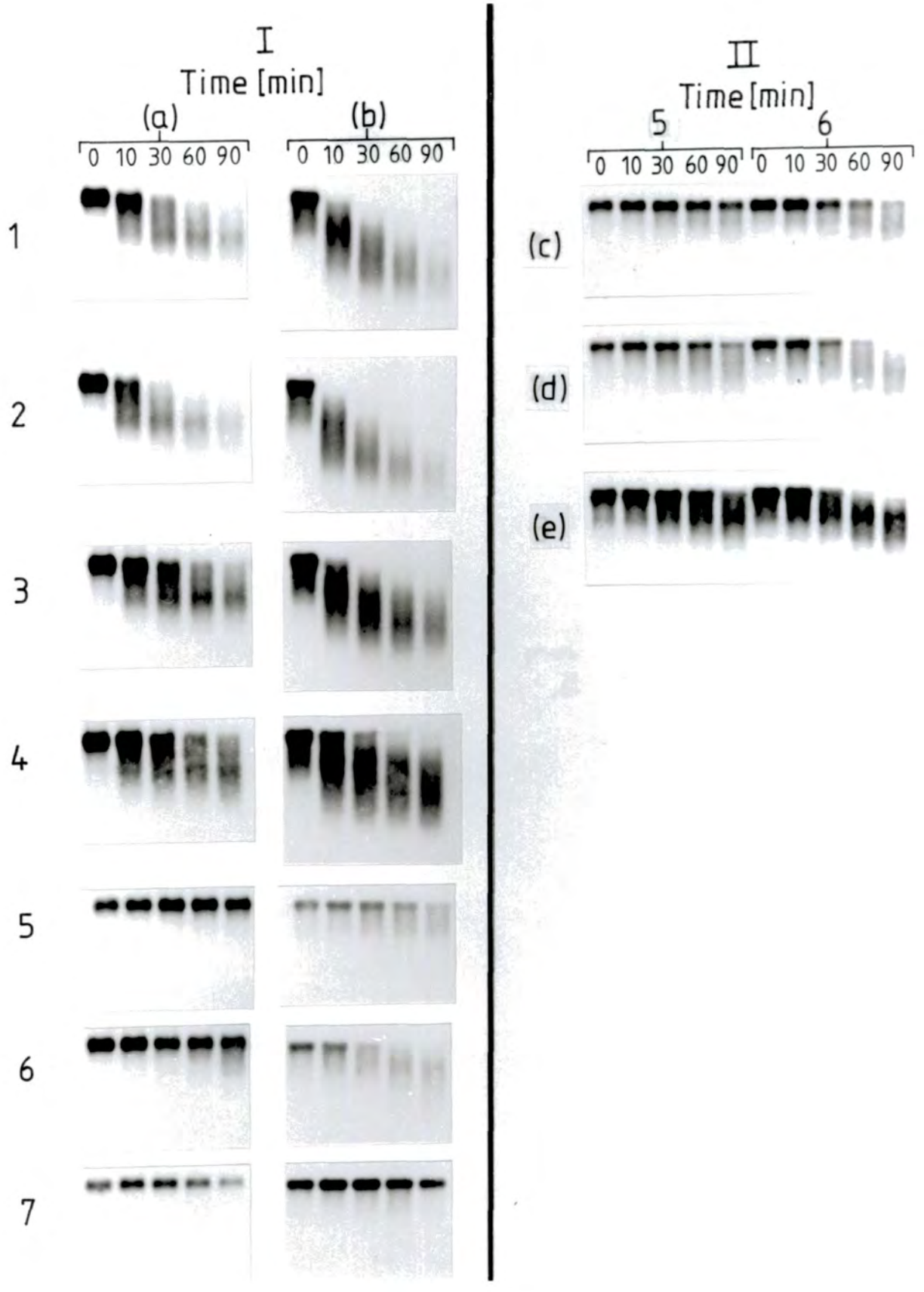

I
Time [min]
(a)
(b)
0 10 30 60 90
0 10 30 60 90
1
2
3
4
5
6
7
II
Time [min]
5
6
0 10 30 60 90
0 10 30 60 90
(c)
(d)
(e)

Figure 26. Degradation of 6 mRNA species *in vitro* under various conditions.

Data shown in Fig. 25, and other data produced by reprobing the same blots with other probes, were analysed by laser densitometer. Autoradiographs were scanned perpendicular to the direction of electrophoresis, across the width of the intact bands, and the percentage of maximum scan peak area (representing intact mRNA), was plotted against incubation time.

(i) the effect of various conditions on the decay of a single species of mRNA (*CAB*).

The different conditions are as follows (described in more detail in Fig. 27):

(1) polysomes + extract;

(2) polysomes + extract + CPK/CP;

(3) polysomes + extract + HPRI;

(4) polysomes + extract + EDTA;

(5) polysomes only;

(6) polysomes + Mg^{2+}.

(1),(3),(4),(6): a comparison of the decay of different mRNAs under each of four identical conditions.

Blots were probed with six different probes as follows:

(a) pFa/b31 (*CAB*);

(b) pDUB6 (*legA*);

(c) pLG4.10(*lecA*);

(d) pAD3.4 (*vicJ*);

(e) pGEM40 (*legJ*);

(f) pSAc3 (soybean actin).

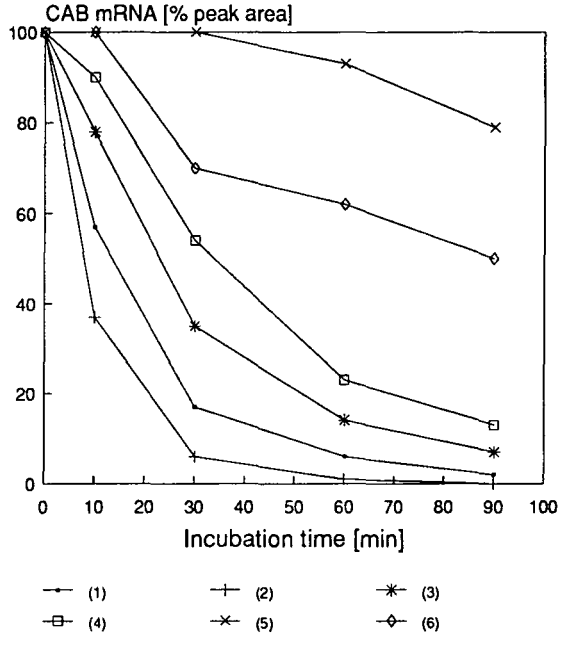
The conditions used (numbered as previously in Fig.25.I and 26(i)) are as follows:

Plot (1); polysomes + extract;

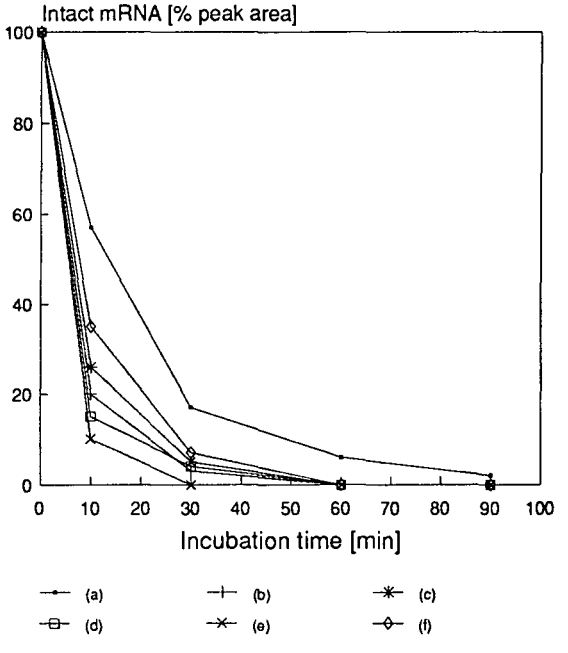
Plot (3); polysomes + extract + HPRI;

Plot (4); polysomes + extract + EDTA;

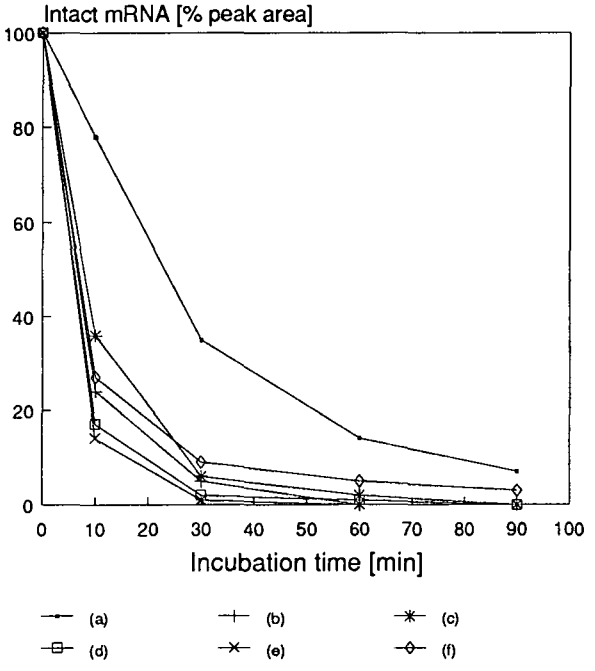
Plot (6); polysomes + Mg^{2+}.


CAB mRNA [% peak area]
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Incubation time [min]
(1)
(2)
(3)
(4)
(5)
(6)

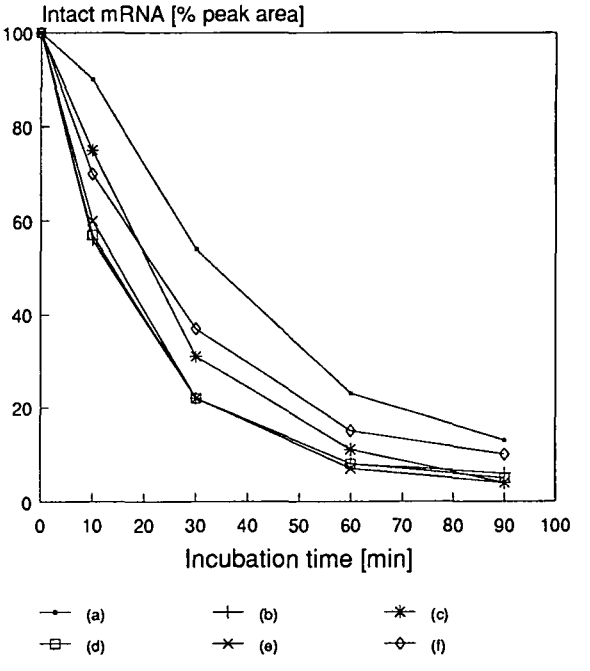
(i)


Intact mRNA [% peak area]
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Incubation time [min]
(a)
(b)
(c)
(d)
(e)
(f)

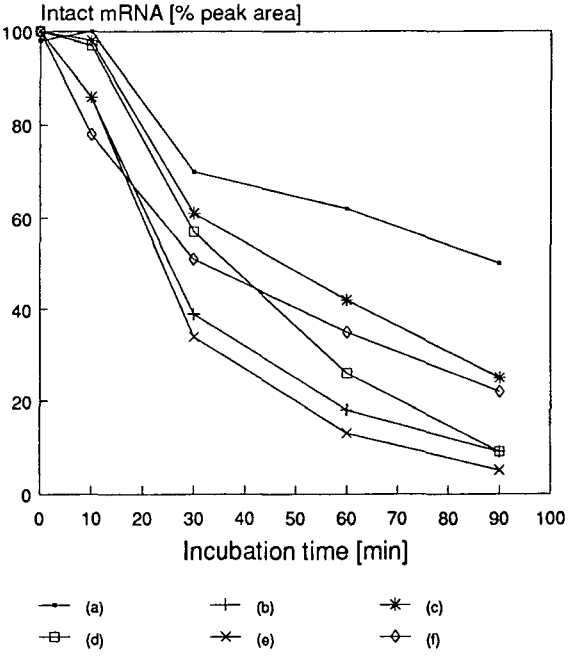
(1)


Intact mRNA [% peak area]
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Incubation time [min]
(a)
(b)
(c)
(d)
(e)
(f)

(3)


Intact mRNA [% peak area]
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Incubation time [min]
(a)
(b)
(c)
(d)
(e)
(f)

(4)


Intact mRNA [% peak area]
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Incubation time [min]
(a)
(b)
(c)
(d)
(e)
(f)

(6)

considering the variation in the assay (which is not clear), may be due to the action of a ribosome associated nuclease. The result also suggests that active elongation of the polypeptide chain is not required for degradation.

4.5.3.2 Effect of creatine phosphokinase and creatine phosphate.

Creatine phosphokinase (CPK) and creatine phosphate (CP), a system used for regeneration of ATP from ADP, as used in many *in vitro* translation systems including those used by Peumans *et al.* (1980a) and in the degradation assays used by Ross and Kobs (1986), was originally included in the standard degradation assay; however, it was previously reported by Peumans *et al.* (1980c) that commercial preparations of creatine phosphokinase were contaminated with ribonuclease activity, and its use in the pea axes system introduced more ribonuclease activity than was present in the extract itself. Addition of CPK and CP to the incubations in the experiments shown here had no overall effect on incorporation of [^{3}H]-leucine (data not shown), but considerably reduced the half-life of *CAB* mRNA from 12 to about 8 min [Fig. 26(i)(2), Fig. 25.I(a)(2)]. However, when total RNA was incubated with CPK/CP in an identical reaction, but in the absence of polysomes and extract, no degradation occurred (data not shown). This indicates that the CPK was not contaminated with nuclease, and thus it is possible that the CPK/CP-stimulated degradation of mRNA was related to its function in the energy generating system.

In contrast, Ross *et al.* (1987) found that ATP, GTP and CP/CPK were unnecessary for the *in vitro* exonucleolytic degradation of histone mRNA, but that these factors were required to maintain the characteristic relative stability of β-globin mRNA. They also reported that when synthetic ^{32}P-mRNA alone was incubated in the presence of creatine phosphokinase that no degradation occurred, thus eliminating the possibility that the enzyme was contaminated with RNase activity. They offered the explanation that ATP influenced the activity of a polysome associated poly(A)-polymerase/exonuclease activity; ATP would drive the equilibrium towards the polymerase activity and away from the exonuclease activity and therefore stabilise the β-globin mRNA.

4.5.3.3 The effect of human placental RNase inhibitor.

The addition of human placental RNase inhibitor (HPRI), to a final concentration of 2 units/μl had a stabilising effect on the *CAB* mRNAs, approximately doubling the half-life to 25 min (Fig. 26(i)(3)). It is not known whether the inhibitor activity was in excess over the target ribonuclease activity since no attempt was made to repeat assays using higher or lower concentrations of inhibitor; it may be that using increased levels of HPRI would further reduce any non-specific RNaseA activity.

HPRI is an specific inhibitor of neutral pancreatic-type enzymes (RNaseA-type), which occur at low levels in a variety of animal tissues in addition to being highly expressed in the pancreas; it does not inhibit lysosomal acid endoribonuclease of animal tissues (Blackburn and Moore, 1982). Since the RNaseA type enzyme does not fit into any of the 4 classes of plant nuclease described by Wilson (1982, see below) it is not certain if this enzyme occurs in plant tissues; however, the inhibition of mRNA degradation in the cotyledon extract caused by HPRI would argue for a plant enzyme of this type since HPRI is unlikely to inhibit other classes of plant RNase.

Vanadyl ribonucleoside complexes, which should inhibit the majority of cyclising phosphotransferase RNAses (Berger and Birkenmeier, 1979) including plant RNase I and II (section 4.5.5.2) and do not inhibit *in vitro* translation, might prove more useful than HPRI as an inhibitor of non-specific RNase activity in this system.

Pei and Calame (1988) found that HPRI had little or no effect on mRNA degradation in their mouse plasmacytoma cell-free extract; however, Ross *et al.* (1987) found that the addition of HPRI, or mouse liver pH5 factors (a protein fraction containing factors required for translation elongation and a ribonuclease inhibitor activity) was required in their mRNA degradation assays to prevent non-specific degradation of mRNA and rRNA, and to allow differential degradation of different mRNAs to become apparent. In contrast, in the system used here, 18S and 25S rRNAs are completely stable under all conditions used, as shown by ethidium

bromide staining of formaldehyde gels [eg Fig. 22(iii)]. However, HPRI in this system does reduce mRNA degradation, although there is no significant effect on the rank order of degradation of different species of mRNA.

4.5.3.4 The effect of EDTA.

The addition of 6.7mM EDTA to the standard reaction dramatically increased the half-life to 35 min. (Fig. 26(i)(4)). The divalent cation concentration in the reaction, containing both extract and polysomes, is 0.5mM, not including any contribution from the cotyledon tissue from which the extract was made, thus 6.7mM EDTA is likely to be in excess over divalent cation.

Since it is known that Mg^{2+} is required to stabilise polysomes, the chelation of divalent cations by the EDTA would have the effect of releasing the ribosomes from the mRNA. Mg^{2+}, and other divalent cations are also important in maintaining higher order structures in RNA molecules and are necessary cofactors for a variety of nucleases. Thus, the effect of EDTA could have been due to (i), release of ribosomes and associated ribonucleases from mRNA; (ii), inhibition of a divalent-cation-dependent nuclease, or (iii), changes in mRNA secondary structure resulting in reduced susceptibility of the mRNA to enzymatic degradation.

4.5.3.5 Degradation of polysomal mRNA in the absence of cell-free extract.

If the cell-free extract is omitted from the incubation and replaced by water so that polysomes are incubated in the 1xSEA mixture only, then the *CAB* mRNA is greatly stabilised such that at least 80% of the mRNA remains intact after a 90 min incubation (Fig. 26(i)(5)).

4.5.3.6 Degradation of polysomal mRNA in the absence of cell-free extract with the addition of 5mM Mg^{2+}.

The degradation of mRNAs in polysomes alone can be stimulated by the addition 5mM Mg^{2+} (Fig. 26(i)(6)); the possible explanations for this are simply the reverse of the explanations

for the stabilising effect of EDTA as given in section 4.5.3.4. The Mg^{2+} concentration before the addition of 5mM Mg^{2+} in this reaction was 0.15mM due to the presence of 3mM Mg^{2+} in the polysome resuspension buffer. Ross *et al.* (1987) observed that histone mRNA degradation (either endogenous polysomal mRNA or synthetic mRNA) was relatively slow in the Mg^{2+} concentration range 0.2 to 0.5mM but that degradation was maximal with a broad optimum of 5-20mM. Also in the mRNA degradation assay of Pei and Calame (1988), which contained from 0.5 to 1mM Mg^{2+} the degradation of F-*myc* and T-*myc* mRNA could be conveniently measured over a 50 min assay, but if 5mM Mg^{2+} was added degradation became so rapid that it could not be measured. It should also be noted that ribosomes begin to precipitate at 10mM Mg^{2+} (Palmiter, 1974).

Also a report by Larkins and Davies (1973) showed that during the incubation at 4°C of a post-mitochondrial supernatant from pea leaves, polysome degradation occurred if the Ca^{2+} concentration of their buffer was raised from 0 to 3mM. This degradation could be prevented by addition of EGTA and no divalent cation tested other than Ca^{2+} caused this effect. They concluded that degradation was likely to be due to the action of an endogenous Ca^{2+}-dependent, heat-labile endonuclease.

4.5.4 Differential degradation of different species of mRNA *in vitro*.

Fig. 26(1,3,4 and 6) shows plots of mRNA degradation of 6 different classes of mRNA under each of 4 different incubation conditions. Under each different condition, the 6 different classes of mRNA fall broadly, although the differences are not clear where mRNAs are degraded very rapidly [Fig. 26(1 and 3)], into the same rank order of stability:

$$CAB > \text{actin} \simeq lecA \geq vicJ \simeq legA \simeq legJ$$

The only possible exception to this ranking is the enhanced relative stability of *vicJ* mRNA when polysomes are incubated with 5mM Mg^{2+} in the absence of cell-free extract [Figs. 26(6); 25.II.6]. Under these conditions the *vicJ* mRNA is significantly more stable than the *legA* or *legJ* type mRNA. The reason for this is unknown.

The actin clone used, pSAc3, is a soybean actin cDNA (Shah *et al.* 1980; Hightower and Meagher 1985) and was included to represent a "housekeeping" mRNA as this protein is present in all cells as a component of the cytoskeleton.

This ranking of stability seems to have some degree of correlation with mRNA sizes;

Class of RNA	Size of mRNA [kb]
CAB	1.1
lecA	1.1
actin	1.7
vicJ	1.7
legA	1.8
legJ	1.75-1.85

A positive correlation between mRNA size and degradation rate would be expected if mRNA degradation occurred only by a non-specific random endonucleolytic attack. Under these conditions larger mRNA molecules form a larger target for the endonuclease and, thus, the rate of disappearance of intact mRNA would be expected to be higher. The results shown here cannot entirely be explained in these terms since *lecA* and *CAB* mRNA are practically identical in size whereas, under all conditions assayed, the difference in half-life is approximately 2-fold. Thus, there appears to be some specific factor causing the differential stability of these two classes of mRNA. Such a factor could be the differential susceptibility of mRNAs to non-specific nucleases due to mRNA secondary structure or mRNA-protein interactions within the polysomes, or could be due to attack of specific classes of mRNA by specific nucleases. The significance of this higher stability of *CAB* mRNA *in vitro* to the situation in the intact cell is uncertain. The *CAB* gene family has been extensively studied, and is highly expressed and light regulated in all photosynthetic tissues (Kuhlemeier *et al.* 1987).

The rank ordering of stability is maintained in the absence of cell-free extract, except for the increase in relative stability of *vicJ* mRNA, suggesting that the factors responsible are associated with the polysomes; however, the fact that the ranking

is also maintained when ribosomes are released by EDTA, contradicts this. The global destabilising effect of the extract is likely to be due to the presence of RNase I released from the lysosomal compartment during homogenisation (see sections 4.5.5.1 and 4.5.5.2), although stimulation of ribosomal bound enzymes by some ionic or other factor in the extract is possible; for example the addition of cell-free extract results in an increase in Mg^{2+} concentration from 0.15mM to 0.5mM. RNaseI, although it would have to operate at pH 7.6, outside its pH optimum of 5.0-6.0, might non-specifically degrade mRNAs having possibly a greater effect on longer mRNAs as discussed above.

4.5.5 Discussion of *in vitro* degradation assays.

4.5.5.1 The disadvantages of using plant tissues.

The mRNA degradation assays developed by Ross and Kobs (1986) and Pei and Calame (1988) were both derived from mammalian cell cultures. The isolation of polysomes and/or cell-free extracts from these cell-cultures involves gentle cell lysis, followed by removal of the nuclear and lysosomal fractions by centrifugation. Pei and Calame (1988) stated that RNase, released from broken lysosomes, was the primary source of non-specific RNase, and were able to estimate that their procedure removed 85-90% of the lysosomal enzymes due to successful sedimentation of intact lysosomes. They were also able to show that the RNase activity that caused mRNA degradation in their system was not due to a cyclising phosphotransferase RNase of the pancreatic, or any other, type.

In the system of Ross *et al* (1987), HPRI or pH5-factors were needed to inhibit non-specific RNAses when incubating polysome preparations with or without cell-free extract.

In plant cells the acid hydrolases, including RNAses which are analogous to those found in animal cell lysosomes, are present in the vacuole (Matile 1978); indeed it has been estimated that 80% of the total plant cell RNase activity is localised in the vacuole. During homogenisation of plant tissues it is not possible to avoid disruption of the vacuolar and nuclear compartments. This problem may be aggravated in the tissue used here because

cotyledon storage parenchyma cells in the mid-cotyledon expansion phase contain large endoreduplicated nuclei and a complex vacuole (Craig 1988); at this stage, during the rapid deposition of storage protein into the vacuole, the vacuole is present as one or two very complex discrete vacuoles that in serial sections show multiple interconnected profiles, then, later in the cotyledon expansion phase, these structures break up into many small discrete protein bodies.

Thus, nuclear and vacuolar RNAses will be present in the cell-free extract and may also become artifactually bound to ribosomes, or microsomal membranes; it is therefore necessary to rigorously demonstrate the specific nature, and significance to the intact cell, of any differential degradation observed in polysome incubations.

4.5.5.2 Plant nucleases.

Considering the large amount of work that has been done on the regulation of mRNA levels in plants, investigations of plant nucleases have been largely neglected over the last few years; however, over 100 papers published before 1982 have been reviewed (Wilson, 1982; Farkas, 1982).

Wilson (1975, 1982) was able, from many fragmentary reports, to group the plant nucleases into 4 classes:

(1) RNase I, the only plant RNase to be purified to near homogeneity and well characterised (Abel and Glund 1987). It is localised in the vacuole, and probably forms the majority of the soluble RNase found in plant tissue homogenates. It is a soluble endoribonuclease with an acid pH optimum of 5.0-6.0. It is a phosphotransferase and, characteristically for this type of enzyme, is not dependent on divalent cations and generates nucleoside 2'-3'-cyclic monophosphate products.

(2) RNaseII, microsome associated acid endoribonucleases (pH optimum 6.0-7.0) with low sensitivity to EDTA. Like RNase I this is a phosphotransferase.

(3) Nuclease I, particle or membrane bound acid endonucleases (pH optimum 5.0-6.5) that can attack DNA and RNA, can have high or low sensitivity to EDTA, and produce nucleoside 5'-monophosphates.

Mung bean nuclease falls into this class, and Blank and McKeon (1989) have recently reported physiological roles for this type of enzyme in wheat leaves.

(4) Soluble exonucleases (phosphodiesterase I), that are dependent on divalent cations, produce nucleoside 5'-monophosphates from a DNA or RNA substrate, and have a pH optimum of 7.0-9.0.

It was pointed out that some RNase activity was usually associated with the ribosomal fraction (Farkas, 1982; Rijven 1978), but that it had not been convincingly demonstrated that these activities were unique to ribosomes and their significance was questioned. Indeed, it was found that the subcellular localisation of some RNase activities was heavily influenced by the composition of the extraction medium used and that artifactual association of RNase with ribosomes was difficult to disprove (Farkas, 1982; Dyer and Payne, 1974). In a study by Rijven (1978), using ribosomal pellets isolated from the germinating cotyledons of Fenugreek and Soybean, it was found that a soluble endonuclease, probably identical to RNase I, could be washed from the ribosomal pellet in low salt buffer. This enzyme was probably loosely bound to the ribosomes, or not bound at all. Another RNase activity could be washed from the ribosomes in a buffer containing 0.5M KCl. This enzyme was similar to the RNase II class of enzyme, but had a unique specificity with respect to homopolymer digestion and is the best candidate for a genuine ribosome specific ribonuclease enzyme.

The purified exonuclease used in the studies of Ross *et al.* (1987) and Peltz *et al.* (1987), which exhibited differential degradation of histone and β-globin mRNA *in vitro*, was also released from polysomes by a high-salt wash, as was the RNase activity associated with the 40-80S mRNPs in the system of Sunitha and Slobin (1987).

4.5.5.3 Future prospects of *in vitro* degradation assays from plant systems.

The pea cotyledon mRNA degradation assays, from which these preliminary results have been obtained, are obviously in need of much further characterisation. The use of cell-free extracts may

prove to be hampered by the presence of vacuolar RNAses; however, since in the animal systems discussed above differential mRNA degradation occurs in polysomes in the absence of soluble extract, and the high stability of *CAB* mRNA is also maintained in the cotyledon polysomes, it may be possible to use plant polysomes to isolate factors responsible for differential degradation. This will only be possible if polysomes can be isolated under ionic conditions that prevent artifactual binding of RNases which normally occupy other compartments in the intact cell, but do not cause dissociation of the native enzymes. Isolation of polysomes after lysis of protoplasts to allow removal of intact vacuolar and nuclear compartments by centrifugation may be useful in this respect.

4.6 The shortening of mRNA poly(A)-tails.

The progressive shortening of mRNA poly(A)-tails in animal systems is a general phenomenon which has been known for many years, however, the significance of this phenomenon, and of the role of the poly(A)-tail in general, which may be related to mRNA stability, translation efficiency, nuclear processing of primary transcripts, or nucleocytoplasmic transport, is still disputed today (Brawerman, 1981; Carter *et al.*, 1989).

Sheiness and Darnell (1973) first showed, by pulse-chase labelling of RNA in HeLa cells, followed by release of poly(A) sequences by digestion with pancreatic ribonuclease and RNAse T1, that the size of poly(A) sequences became more heterogeneous during the chase, due to their progressive shortening. Since then many examples of shortening of poly(A)-tails of specific mammalian mRNAs have been reported (eg. Mercer and Wake, 1985; Zingg *et al.*, 1988; Carter and Murphy, 1989; Carter *et al.*, 1989); these studies concerned inducible mRNAs whose levels declined after the stimulus was removed: when the mRNAs are first induced they have long, relatively homogeneous poly(A)-tails, but after the induction of transcription has ceased, and the mRNAs age in the cytoplasm, their poly(A)-tails shorten, giving a heterogeneous size population. Kleene (1989) recently reported evidence that poly(A) shortening was related to translation, because during mouse spermiogenesis several mRNAs, when they are stored in mRNPs, have long homogeneous poly(A)-tails of approximately 150 nucleotides, but when they are translationally activated and become associated with polysomes the poly(A)-tails become shorter and more heterogeneous in length.

It has been suggested that the phenomenon of poly(A)-shortening is related to the mechanism of mRNA degradation according to the model proposed by Berstein *et al.* (1989); this model and evidence against it are discussed in section 1.2.2. The role of poly(A)-shortening in mRNA stability is still controversial, although it is accepted that a short poly(A)-tail of at least approximately 30 nucleotides is required to stabilize most mRNAs, since most mRNAs do not accumulate in the cytoplasm in a deadenylated form.

Poly(A)-tails are initially added to the 3'-end of primary transcripts in the nucleus by a poly(A)-polymerase; in mammalian systems the average poly(A)-tail length added in the nucleus has been estimated to be 260 nucleotides and is relatively homogeneous. After entry into the cytoplasm, the poly(A)-tails shorten rapidly during the first 2-3 hours and during this time the spread in poly(A)-tail lengths remains narrow. The tail length distribution after this rapid shortening may be unique to each type of mRNA (Sachs and Davies 1989). Subsequent to this rapid shortening, there is a slower shortening process which results in increased heterogeneity in tail lengths (Brawerman, 1981).

Cytoplasmic activity of poly(A)-polymerase is also known to occur: Rosenthal *et al.* (1983), for example, showed that 4 classes of mRNA with short poly(A)-tails that are present, but not translated, in *Spisula* oocytes show an increase in poly(A)-tail length and recruitment to actively translating polysomes when the oocytes are fertilized, presumably as a result of a cytoplasmic polyadenylation. Diez and Brawerman (1974) concluded from kinetic labelling studies in cultured mammalian cells that the rate of cytoplasmic poly(A)-tail extension was of the same order of magnitude as the rate of entry of poly(A) from the nucleus; they suggested that the length of poly(A)-tails in the cytoplasm was determined by a balance between cytoplasmic polyadenylation and 3'-exonuclease activities.

A poly(A)-polymerase activity, that is translated *de novo* during germination of wheat embryos, has recently been reported and it may be involved in the translational activation of stored mRNAs upon germination (Lakhani *et al.*, 1989) although data conflicting with this hypothesis was reviewed by Payne (1976).

Since there were no known studies of changes in poly(A)-tail length for mRNAs of higher plants, it was decided to investigate the poly(A)-tail length of a seed protein mRNA during late embryogenesis when the mRNA was in decline and might, therefore, be expected to consist of an aging population of mRNA molecules, a situation analogous to the period following the transient induction of the mammalian mRNAs described above.

4.6.1 Changes in the length of lectin mRNA poly(A)-tails during pea embryogenesis.

A blot similar to that used in Fig. 7, containing pea cotyledon total RNA treated with and without RNaseH/oligo(dT) to remove poly(A) sequences, was probed for lectin mRNA (Fig. 27). This mRNA species was chosen because it was sufficiently short (approximately 1100 nucleotides) so that the the removal of the poly(A)-tail would produce a measurable shift in mobility on agarose gel electrophoresis. There are no data on the transcription rates of the pea lectin gene beyond 16 DAF, although there already appears to be a decline in the "run-on" transcription rate from 12 to 16 DAF (see Table 3). In soybean, however, lectin "run-on" transcription rates do decline in late soybean embryogenesis; at 95 DAF, the latest stage measured, the rate had declined to 15% of the maximum (Walling *et al.*, 1984).

The autoradiograph shown in Fig. 27, and lower exposures of the same blot, were analysed by laser densitometry (Fig. 28). This shows that the change in mobility caused by RNaseH/oligo(dT) treatment at 14 DAF, the stage 2 days before the peak in the level of lectin mRNA, was approximately 2.5mm (equivalent to 80-100 nucleotides). But gradually, from 16 to 28 DAF, the difference was reduced to approximately 0.5 mm (15-30 nucleotides). This provides evidence that the poly(A)-tail of the lectin mRNA becomes shorter during the period when the steady-state level of this mRNA is in decline.

Although the RNaseH/oligo(dT) treatment causes a shift in the mobility of each band it does not appear to have any significant effect on peak width for a given developmental stage (Fig. 28). This suggests that the lengths of the poly(A)-tails in the population of lectin mRNA molecules, at a given developmental stage, are relatively homogeneous.

Changes in the polyadenylation of other, larger mRNAs, such as legumin mRNAs, could be studied by the same technique provided they can be first shortened by a specific endonuclease treatment. This is necessary to allow accurate measurement of the small changes in mobility generated by removal of a poly(A)-tail, which was measured to have an average length of 100-140 nucleotides in

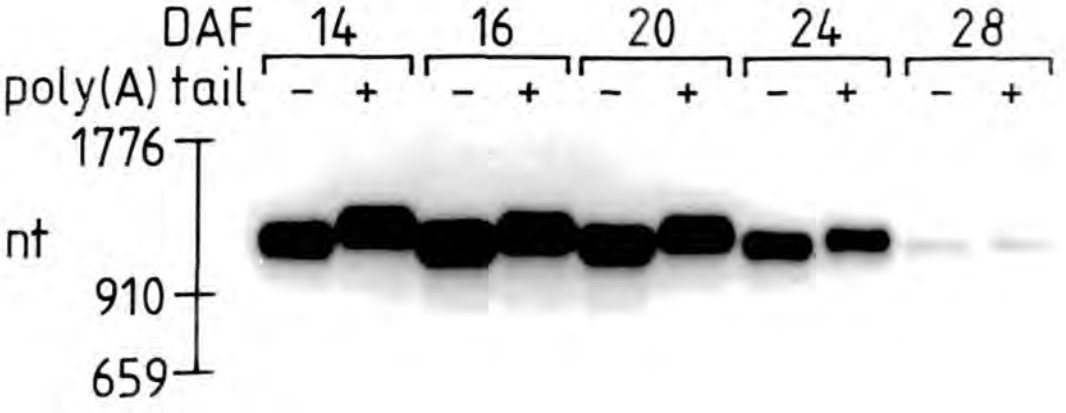
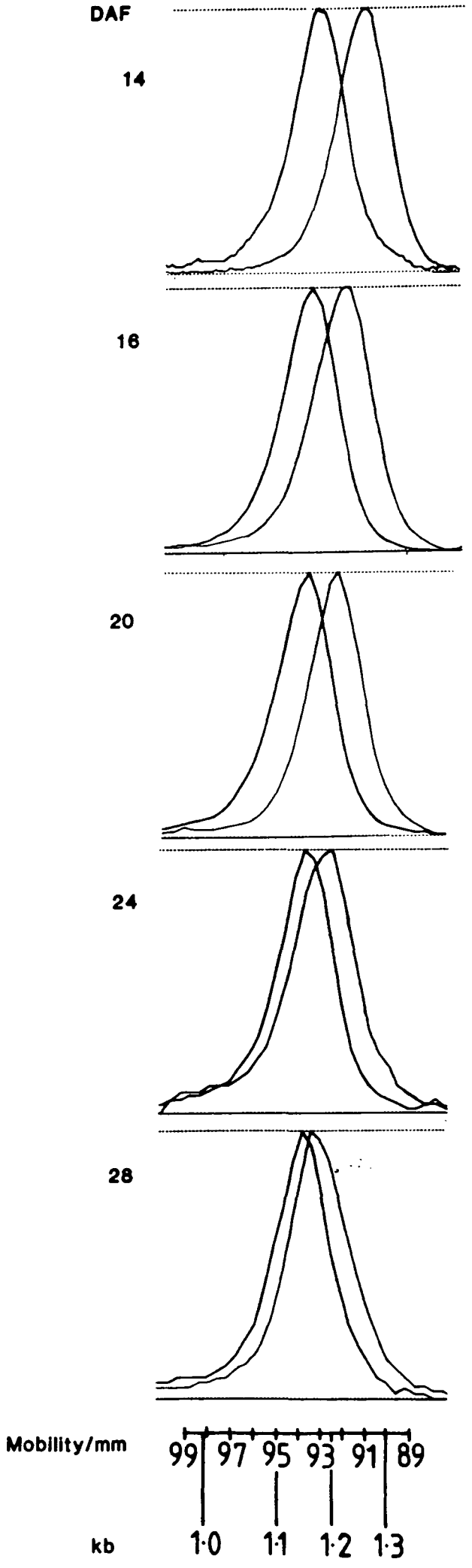


Figure 27. Poly(A)-tail length of lectin mRNA during the pea embryogenesis, I.

Total RNA samples from 14 to 28 DAF cotyledons were treated with oligo(dT)$_{12-18}$ and RNaseH (poly(A) tail -), or left intact (poly(A) tail +), then run on a formaldehyde agarose gel and Northern-blotted. The blot was probed with a lectin cDNA probe (insert from plasmid pLG4.10). Size markers are derived from a pBR322 AluI digest and 16S *E. coli* rRNA.

Figure 28. Poly(A) tail length of lectin mRNA during the pea embryogenesis, II.

The tracks from the autoradiograph shown in Fig. 27, showing 28 DAF total RNA, and the tracks from a lower exposure of the same blot, showing 14-24 DAF total RNA, were scanned with a laser densitometer. The scans for with and without poly(A)-tail for each developmental stage were superimposed.


DAF
14
16
20
24
28
Mobility/mm
99
97
95
93
91
89
kb
1·0
1·1
1·2
1·3

soybean suspension cultures (Silflow and Key 1979). The smaller the defined 3′ mRNA fragment to which the poly(A)-tail is attached the easier it is to detect mobility changes when the tail is removed. This needs to be offset against the need for a fragment large enough to provide a target for specific hybridisation probes. A specific endonucleolytic cleavage could be provided, as first suggested by Mercer and Wake (1985), by hybridization to an oligodeoxyribonucleotide, say 200-400 nucleotides upstream of the poly(A)-signal, followed by RNaseH treatment; this method has recently been applied by Brewer and Ross (1988). Alternatively, suitable fragments may be generated by catalytic RNA molecules which cleave at CUCU motifs and are now commercially available (United States Biochemical Corporation, Cleveland, Ohio).

The shortening of the lectin poly(A)-tail could be explained by three possible hypotheses: (i) transcription declined in later development, the mRNA turned over relatively slowly and, thus, the population of lectin mRNA molecules began to age. The poly(A)-tail while still attached to intact mRNA was then progressively degraded in the cytoplasm by an 3′-exonuclease activity, possibly according to the model of Bernstein *et al.* (1989) (section 1.2.2), and thus became shorter in older molecules; (ii) transcription continued throughout development, albeit declining in rate, and the mRNA turned over relatively rapidly by a process initiated by an endonucloelytic cleavage; under these conditions a homogeneous but diminishing poly(A)-tail size could be observed if the poly(A)-tail entering the cytoplasm from the nucleus became shorter as development proceeded; (iii) the length of poly(A)-tail was controlled in a precise manner by the balance of cytoplasmic poly(A)-polymerase and 3′-exonuclease, regardless of the age of the mRNA. It is not possible from these results to distinguish between the above hypotheses although hypothesis (i) seems more likely.

The reduction in the poly(A)-tail size of lectin mRNA during late development may be a consequence of the mechanism for mRNA degradation or translation, or may itself be a controlling factor in these processes.

4.7 Concluding Discussion.

The central problem concerning the control of gene expression is to discover what factors ultimately determine the amount of a gene product that accumulates in a particular cell or tissue type.

In the case of legumin, the 11S globulin of pea seeds, there are many different protein sub-units, each encoded by a different gene or gene sub-family, that are each expressed at a characteristic level and can be defined as major and minor sub-units. In this study, the question becomes: why are some legumin sub-units more abundant than others in the mature seed?

Although there are no good quantitative data for protein sub-unit abundance, it can be seen from semi-quantitative SDS-PAGE and 2-D gel analysis of total soluble proteins from pea seed (eg. Gatehouse *et al.*, 1982; Matta *et al.* 1981), that the level of accumulation of a given legumin sub-unit is well correlated with the corresponding mRNA steady-state level that has been quantified throughout development. Specifically, the minor sub-units are encoded by *legS* and *legJ* mRNAs, which are of low abundance in comparison to the *legA* mRNA, which encodes the major sub-units.

In order to determine whether the level of mRNA is controlled by gene transcription rate or by some other posttranscriptional process, a comparison of "run-on" transcription rates and steady-state mRNA levels was made for the *legA*, *legJ* and *legS* gene sub-families and the single lectin gene. It was concluded that the steady-state level of mRNA was determined, to a large extent, by posttranscriptional processes, and that transcription rates on a per gene basis were probably relatively constant. Transcription appeared to be the important factor in the tissue-specificity of gene expression and in the developmental regulation of expression rather than the actual level of expression at any particular time.

The observation of posttranscriptional regulation of gene expression has been observed in many plant systems by workers employing a similar experimental approach to that described above, but, as yet, there is little or no insight into the mechanism of

this "posttranscriptional" control.

In order to investigate this further, attempts were made to measure the turnover of specific seed-protein mRNAs in cultured pea cotyledons by pulse-chase labelling of RNA, and by the use of the transcription inhibitor actinomycin D. No conclusive evidence could be obtained from these studies because of the aberrant behaviour of legumin mRNAs during *in vitro* culture, and because of the reluctance of the tissue to respond to actinomycin D and to incorporate ^{3}H-uridine into RNA. However, during a 3.4 hour pulse-labelling of developing cotyledons *in vitro*, *legA* mRNA was labelled highly enough to allow measurement, whereas *legJ* mRNA was not. This, together with the approximately equal "run-on" transcription rates for these two gene sub-families, is suggestive of a selective, rapid turnover of *legJ* transcripts, possibly in the nucleus. Pea embryogenesis is probably not the most suitable system for further study on posttranscriptional nuclear events due to the difficulty in obtaining large amounts of purified nuclei.

A method of studying differential mRNA turnover in the cytoplasm by the use of cell-free extracts was investigated. Such a system would be very useful for studying the relationship between the structure of mRNA molecules and the mechanism and rate of their degradation. When polyribosomes isolated from pea cotyledons were incubated with or without a cell-free translation extract, no difference could be detected between the degradation of the endogenous *legA* and *legJ* mRNAs. This, however, does not exclude the possibility of differential cytoplasmic degradation *in vivo*, because of the possibility of artifactual loss of nuclease activity, or association with polyribosomes of nucleases that are normally present in other cellular compartments. The validity of such a system could more easily be assessed if relative mRNA degradation rates could also be measured *in vivo* or in cotyledon culture.

Most of the successful studies of posttranscriptional gene regulation reported in animal systems have utilized differentiated cell lines grown in culture. Such systems, which unfortunately are not available in plants because of the inability to culture cells in a differentiated state, are more suited to RNA labelling and

transcription inhibitor studies than are systems based on organ culture.

One approach to the problem of uncoupling the transcription rate from posttranscriptional events could be the use of transgenic plants. Constructs could be made that place defined transcription units under the control of well characterised inducible promoters (eg. heat shock or light induced promoters). The inducible promoters could be transiently switched on and then off, by the appropriate environmental stimuli, to produce a defined pulse of transcripts whose fate could then be followed by measuring the amount of transcripts in various cellular fractions.

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Transcriptional and posttranscriptional regulation of seed storage-protein gene expression in pea (*Pisum sativum* L.)

Andrew J. Thompson, I. Marta Evans, Donald Boulter, Ronald R.D. Croy, and John A. Gatehouse
Department of Biological Sciences, University of Durham, Durham DH1 3LE, UK

Abstract. At least three classes of legumin, encoded by the gene families *legA*, *legJ* and *legS*, and a lectin, encoded by a single gene, accumulate in the developing cotyledons of *Pisum sativum* L. Transcription rates for the genes encoding these proteins were measured in nuclei isolated from cotyledons at 12 and 16 days after flowering (DAF). The steady-state levels of the corresponding mRNA species were also measured in absolute terms throughout cotyledon development, from 8–9 to 28 DAF. When transcription rates and steady-state mRNA levels of the different gene families are compared, there is little correlation. This indicates a posttranscriptional regulation of the level of expression of these storage proteins in the developing cotyledons. Expression of the legumin genes is known to be seed-specific, whereas expression of the lectin gene is found in both seed and root. When transcription rates were measured in leaf nuclei the levels of legumin and lectin transcripts detected approached background levels, indicating that these genes are either inactive or transcribed at very low levels in leaves; however, the rate of transcription of the chlorophyll a/b-binding protein gene was high. This points to transcriptional control as the major factor in the organ-specificity of legumin and lectin expression.

Key words: mRNA levels – Legumin – Lectin – *Pisum* (storage-protein genes) – Posttranscriptional regulation – Seed storage protein – Transcription in isolated nuclei

Introduction

Storage proteins accumulate in cotyledons to a high level during pea seed development. These proteins comprise two major species, the legumins and vicilins. The different families of genes encoding these proteins show differential expression with respect to organ specificity, timing of expression within the developing seed and level of expression (Gatehouse et al. 1986). The control of differential expression could be at the level of transcription, transcript stability and processing in the nucleus, transport of mRNA to the cytoplasm, stability of mRNA in the cytoplasm or at the levels of translation and protein turnover.

The components of the legumin fraction of the seed storage proteins of pea have been classified into major and minor legumins on the basis of protein abundance (Casey 1979). The *legA* gene family (Croy et al. 1982; Domoney and Casey 1985) encodes the major legumins whilst the *legJ* (Gatehouse et al. 1988) and *legS* (Croy et al. 1988) gene families encode the 'big' and 'small' minor legumins' respectively (Matta et al. 1981). In contrast, a single gene encodes the pea lectin which accumulates in the pea cotyledon (Gatehouse et al. 1987) although it is also expressed in the roots (Buffard et al. 1988). In order to determine whether differential expression of these genes is regulated at the transcriptional or posttranscriptional level, steady-state mRNA levels in developing pea cotyledons have been compared with the corresponding gene transcription rates. The ideal method of measuring the instantaneous transcription rate of a given gene is to pulse-label RNA in vivo for a period sufficiently short such that nascent unprocessed transcripts can be measured. This type of experiment is not presently possible in the pea cotyledon because of the inability to incorporate enough label into the RNA in a short in-vivo pulse. The only alternative method available for measuring transcription rates is the incorporation of labelled uridine 5'-triphosphate (UTP) into transcripts synthesised in vitro in isolated nuclei (nuclear 'run-on'

Abbreviations: bp = base pairs; cDNA = copy DNA; DAF = days after flowering; kb = kilobase; UTP = uridine 5'-triphosphate

transcription). While this approach is not ideal because it has never been demonstrated that nuclei isolated from plant tissues behave in the same way as in vivo, it has been demonstrated in mouse liver that the relative transcription rates of different genes are the same whether measured by in-vivo pulse labelling or by transcription in isolated nuclei (Derman et al. 1980). Other evidence supporting the validity of nuclear 'run-on' transcription has been reviewed (Darnell 1982). Thus, although it has not yet been established that transcription in isolated plant nuclei represents the situation in vivo, many studies on the regulation of plant gene expression have used nuclear 'run-on' transcription assays as the only method of determining transcription rates (Gallagher and Ellis 1982; Evans et al. 1984; Beach et al. 1985; Hagen and Guilfoyle 1985; Walling et al. 1986; Schoffl et al. 1987; Mosinger et al. 1987).

In this paper, differences between steady-state mRNA levels and nuclear 'run-on' transcription rates from the legumin and lectin genes in pea cotyledons will be described.

Material and methods

Plant material. Seeds of *Pisum sativum* L., cv. Feltham First were obtained from Sutton Seeds, Reading, Berks, UK. After 6 d germination in a dark spray room, plants were transferred to a growth cabinet and grown hydroponically in Phostrogen (0.55 g/l) under controlled conditions as described previously (Evans et al. 1979). Pea leaves used for isolation of nuclei were obtained from plants 9 d after transfer to the growth cabinet.

Materials. Nitrocellulose (BA85 0.45 µm) was from Schleicher and Schuell, Dassel, FRG. Deoxyribonuclease (DNase) I was from Worthington Biochemicals, Bury St. Edmunds, Suffolk, UK. Radiochemicals and Hybond-N nylon membranes were from Amersham International, Amersham, Bucks, UK. SP6 and T7 RNA polymerases were from Promega Biotech, P and S Biochemicals, Liverpool, UK. Placental ribonuclease (RNase) inhibitor and ribonucleotides were from Boehringer, Lewes, East Sussex, UK. Restriction enzymes were from Northumberland Biologicals, Cramlington, Northumberland, UK. Phostrogen was from Phostrogen, Corwen, Clwyd, UK. All other chemicals were of AnalaR grade from BDH Chemicals, Poole, Dorset, UK.

Isolation of total RNA. Pods were removed from plants at different stages of development and cotyledons isolated aseptically. After freezing in liquid nitrogen cotyledons were stored at −80° C. Total RNA was extracted, from a minimum of twenty cotyledons per preparation, by the method of Hall et al. (1978), and its concentration determined by absorbance at 260 nm assuming 1 mg/ml gives $A_{260} = 24$. RNA was stored in liquid nitrogen.

RNA blotting. RNA was subjected to electrophoresis on formaldehyde agarose gels and Northern-blotted onto nylon membranes, as described by Maniatis et al. 1982. Size markers were provided by restriction fragments of lambda DNA and by ribosomal RNAs from pea and *Escherichia coli*. These were visualized by ultraviolet light after staining with ethidium bromide. RNA was dot-blotted onto nitrocellulose by the method of White and Bancroft (1982) using a BRL Hybridot manifold (Bethesda Research Laboratories Inc., Cambridge, UK). Northern and dot blots were hybridized with DNA probes labelled with α[^{32}P]deoxycytosine 5'-triphosphate (α-[^{32}P]dCTP) by the method of random oligo-priming as described by Feinburg and Vogelstein (1983). The blots were prehybridized for 4 h at 42° C in the following buffer: 50% formamide, 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 40 mM Na-1,4-piperazinediethanesulfonic acid (Pipes) buffer pH 6.5, 0.4% sodium dodecyl sulfate (SDS), 100 µg/ml denatured herring-sperm DNA, 100 µg/ml polyadenylic acid and 5 × Denhardt's solution (0.1% Ficoll, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone). The denatured DNA probe was then added and the blots incubated at 42° C for a further 48 h. Blots were washed four times in 1.4 × SSC, 0.2% SDS and twice in 0.1 × SSC, 0.2% SDS, each for 20 min at 60° C (1 × SSC is 0.15 M NaCl, 0.015 M sodium-citrate buffer pH 7.2). Prior to reprobing Northern blots the previous probes were removed from the Hybond-N membranes by boiling in 0.1% SDS for 2 × 10 min. Autoradiographs of RNA dot blots were quantified by scanning with an LKB (Bromma, Sweden) Ultrascan XL laser densitometer. Standard curves were produced by scanning dots of serial dilutions of synthetic mRNAs.

Synthetic mRNA standards. Synthetic mRNAs were produced by sub-cloning copy(c)DNA sequences into pGEM-blue plasmid (Promega Biotech.) which contains promoters for SP6 and T7 RNA polymerases flanking a multiple cloning site. Synthetic mRNAs were transcribed from these plasmid templates in vitro by the method of Melton et al. (1984). The concentration of the synthetic mRNAs was determined by including trace amounts of [5,6-^{3}H]UTP in the in-vitro synthesis reaction to give a final specific activity of $5 \cdot 10^5$ cpm/µg RNA, assuming 25% of incorporated nucleotides to be uridine 5'-monophosphate (UMP). The specific activity was then recalculated from the known UMP content based on the cDNA nucleotide sequences. After synthesis the mRNAs were purified as follows: template DNA was removed by adding DNAse I to 10 µg/ml and incubating at 37° C for 15 min. The solution was extracted twice with phenol:chloroform:*iso*amylalcohol (24:24:1, by vol) and then unincorporated nucleotides were removed by Sephadex G-50 chromatography in 0.3 M NaCl, 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl pH 7.5, 1 mM EDTA, 0.1% SDS. The synthesised mRNA was recovered by ethanol precipitation. The precipitate was washed in 70% ethanol, dissolved in sterile water and its radioactivity determined by liquid scintillation counting before being stored in liquid nitrogen.

Isolation of nuclei. Nuclei were isolated from freshly harvested cotyledon and leaf tissue by a method described previously (Evans et al. 1984). Nuclei could be stored for several months at −80° C in buffer I (see below) containing 50% glycerol.

Transcription in isolated nuclei. The method used was based on that of Evans et al. (1984). Nuclei were washed three times in buffer I (20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5% BSA, 15% glycerol) by centrifugation for 5 min at 1000·*g* at 4° C using a swing-out rotor. The nuclei were resuspended in 100 µl buffer I, then 20 µl of buffer II (buffer I supplemented with 240 mM ammonium sulphate) was added and the mixture incubated on ice for 15 min. An aliquot of α-[^{32}P]UTP (15.2 TBq/mmol) was lyophilized and redissolved in 20 µl buffer V (buffer II supplemented with 2 mM ATP,

2 mM guanosine 5'-triphosphate, 2 mM CTP and 7 units/μl of human placental ribonuclease inhibitor (RNAsin). The suspension of nuclei was immediately transferred to this solution and the mixture incubated for 10 min at 26° C. The reaction was stopped by addition of DNase I to 10 μg/ml followed by incubation at 26° C for 15 min. RNA was isolated from transcribed nuclei as described by McKnight and Palmiter (1979).

Dot blotting of DNA. Immobilisation of DNA in dot blots using a BRL Hybridot manifold was as described by Marzluff and Huang (1984).

Hybridisation of nuclear transcripts to DNA dot blots. The method used was based on that of Gallaghar and Ellis (1982). Each dot blot was prehybridized in 1 ml of 50% formamide, 0.5 M NaCl, 40 mM Na-Pipes buffer pH 6.5, 0.4% SDS, 1 mM EDTA, 100 μg/ml polyadenylic acid, and 100 μg/ml *E. coli* transfer RNA for 4 h at 41° C. Aliquots of nuclear transcripts were made up to 750 μl in an identical buffer and hybridized to the dot blots for 72 h at 41° C. Blots were then washed as follows: four times in 1.4 × SSC, 0.1% SDS at 60° C, two times in 0.1 × SSC at 60° C, once in 0.3 M NaCl, 10 μg/ml RNase A at 37° C and once in 0.3 M NaCl at 37° C. Each wash was for 30 min. Blots were then autoradiographed with intensifying screens at −80° C together with dots of known radioactivity. The latter were produced by 5'-end-labelling (Maniatis et al. 1982) of linearized pBR322 with γ-[^{32}P]ATP and dotting onto nitrocellulose. Autoradiographs were quantified by scanning with a laser densitometer or dots were counted directly by liquid scintillation counting in 60% toluene, 40% 2-methoxyethanol, 0.55 g/l Omnifluor (98%, w/w, 2,5-diphenyloxazole; 2%, w/w, *p*-bis(*o*-methylstyryl)-benzene).

Results

DNA clones used as probes in hybridisation assays. In order to detect nuclear transcripts and mRNA species representing the major gene families of the seed proteins the following DNA clones were used:

LegA subfamily ('major' legumin polypeptides); this subfamily contains four active genes *legA*, *B*, *C* and *E* (Lycett et al. 1984) and one pseudogene, *legD* (Bown et al. 1985); all of these sequences hybridize strongly to plasmid pDUB6 (Delauney 1984) which contains a 1.1-kilobase (kb) cDNA derived from the 3' coding and 3' flanking region of the *legA* gene.

LegJ subfamily ('big minor' legumin); this subfamily consists of two or three genes (Domoney and Casey 1985) of which *legJ* and *legK* have been characterized (Gatehouse et al. 1988). pCD40 contains a *legK* cDNA of 0.91 kb from the coding and 3' flanking region (Domoney et al. 1986). pJC5.2 is an EcoR1 genomic clone containing 1.42 kb of the transcriptional unit of *legJ* (Gatehouse et al. 1988). This covers the 3' end of the gene and includes two introns of approximately 100 base pairs (bp) each. Allowing for deletions and introns these two clones show 94% homology and should detect the same transcripts.

LegS subfamily ('small minor' legumin); this subfamily has probably two genes (Domoney and Casey 1985) and can be detected using plasmid pAD9.2 which contains a near full-length cDNA of 1.85 kb. Since the probes used for the other legumin subfamilies are all derived from 3' regions of the genes it was decided to remove 420 bp of 5' region from the full-length pAD9.2 cDNA when used in nuclear transcription assays. This should make results more comparable between the three subfamilies because it is possible that incorporation into 'run-on' nuclear transcripts is not constant along the length of the transcript. Nonlinear labelling has been previously observed in this type of assay (Mechti et al. 1986) and it would also be predicted that if transcripts are only derived from transcription initiated in vivo then a portion of the 5' region of each transcript would always be unlabelled.

Lec (pea seed lectin); pea seed lectin is encoded by a single gene (Gatehouse et al. 1987); plasmid pLG4.10 contains a 0.85 kb lectin cDNA covering coding and 3' flanking regions (Gatehouse 1986).

Cvc subfamily (convicilins); this subfamily contains two genes (Bown et al. 1988). The probe sequence used was pDB9.08 which contains a 2.9-kb genomic clone with the complete *CvcA* gene with a transcriptional unit of 2.45 kb.

CAB gene family (chlorophyll a/b-binding protein); this family has a relatively large gene copy number showing at least eight bands on Southern blots (Coruzzi et al. 1983). The probe sequence used is plasmid pFa, b31 which contains a 0.83-kb cDNA insert (Bennett et al. 1984).

Steady-state levels of mRNA species. Total RNA was prepared from pea cotyledons at various stages of development. Under the growth conditions used the development period of pea seeds was 22 d from anthesis to cessation of dry-weight gain (Evans et al. 1979). Total RNAs together with femtomolar amounts of synthetic mRNA standards were immobilized by dot and Northern blotting and hybridised to a mass excess of ^{32}P-labelled

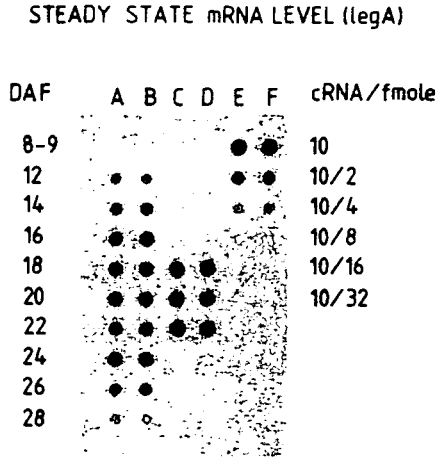

Fig. 1. Autoradiograph of an RNA dot blot used to quantify steady-state mRNA levels. Columns *A–D* show duplicate dots each containing 2 μg total RNA extracted from pea cotyledons at developmental stages between 8–9 DAF and 28 DAF. RNA used in columns *A* and *B* from 8–9 to 22 DAF originated from one batch of plants and RNA used in columns *A* and *B* from 24 to 28 DAF and in columns *C* and *D* from 18 to 22 DAF originated from a second batch of plants. Columns *E* and *F* contain known amounts of synthetic *legA* mRNA serialy diluted and dotted in duplicate. The amounts of synthetic mRNA shown are in the range 10 fmol to 10 32 fmol. Each dot of synthetic mRNA also contains 2 μg *E. coli* rRNA as carrier. The blot was probed with the cDNA insert from pDUB6 (0.5 μg) labelled by random oligopriming

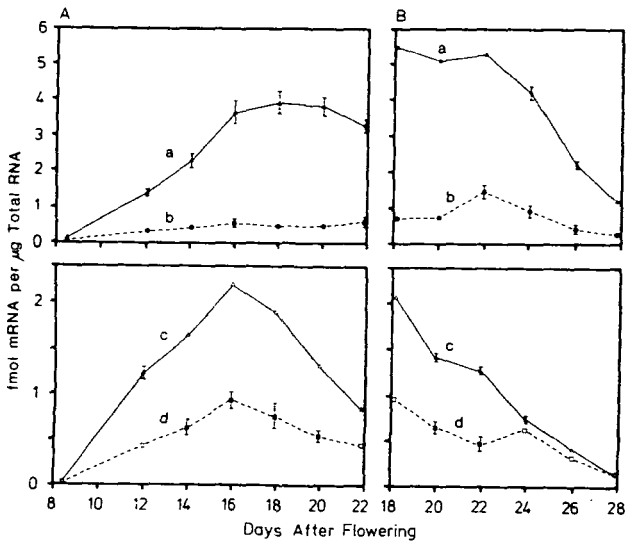

Fig. 2A, B. Steady-state levels of legumin and lectin mRNAs throughout development of pea cotyledons. Autoradiographs of RNA dot blots probed with cDNA probes were quantified by laser densitometry; each point represents the mean of duplicate dots. Error bars indicate the variation in duplicates. Error bars are absent when the variation is less than the size of the graph symbols used. The analysis was performed on cotyledons derived from two independent batches of plants (**A** and **B**). Cotyledons from batch **A** were from 8–9 to 22 DAF, whereas cotyledons from batch **B** were from 18 to 28 DAF. Probes used were: pDUB6 (*legA*) (▲—▲, *a*), pAD9.2 (*legS*) (□---□, *d*), pCD40 (*legJ*) ●---●, *b*), pLG4.10 (*lec*) (△—△, *c*)

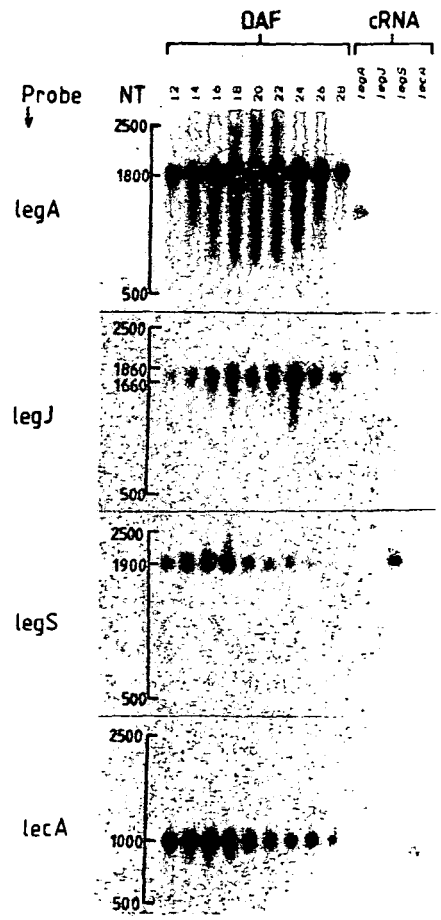

Fig. 3. Autoradiograph of a single Northern blot probed with legumin and lectin cDNA probes. Tracks labelled 'DAF' contain 5 μg of cotyledon total RNA from 12 to 28 DAF. These are the same RNA samples as used in Fig. 1, columns A and B. Tracks labelled 'cRNA' contain 2 fmol of synthetic mRNAs transcribed from the pGEM-blue vector containing cDNA inserts from pDUB6 (*legA*), pCD40 (*legJ*), pAD9.2 (*legS*) and pLG4.10 (*lec*). The blot was probed sequentially with cDNA inserts isolated from the above plasmids and labelled by random oligopriming. The sizes of hybridizing bands are given in nucleotides (*NT*)

legumin and lectin cDNA probes. RNA dot blots were used for quantification of mRNA levels. Figure 1 shows an example of an RNA dot blot probed with a *legA* probe. Data from quantified RNA dots are plotted in Fig. 2. This shows that the mRNAs assayed are present at low levels at the early cell-expansion phase (8–9 days after flowering, DAF), they then increase at least 35-fold in each case to a peak level as cell expansion proceeds and then decline as the seed begins to desiccate. The *legA* mRNA peaks at 18 DAF, whereas the *legS* and *lec* mRNAs peak slightly earlier at 16 DAF. *LegJ* mRNA peaks at 16 DAF then declines slightly before reaching a second peak at 22 DAF. This diphasic accummulation of *legJ*-type mRNA may be the result of differential expression of genes within the *legJ* gene subfamily. As reported previously (Gatehouse et al. 1988), a Northern blot (Fig. 3) shows two bands hybridizing to the *legJ* probe; the upper and lower bands appear to be of equal intensity up to 16 DAF then the lower band increases in intensity at later stages of development. The relationship of these two components to the members of the *legJ* gene subfamily is uncertain.

There are differences in the mRNA contents of the two batches of pea plants analysed (A and B, Fig. 2). Although the ratio of the three types of legumin mRNA does not vary significantly in the two batches, the absolute levels of these mRNAs at 18 DAF is 30–40% higher in batch B. However, lectin mRNA is only elevated by approx. 10% in batch B when compared with batch A at 18 DAF. These differences may be the consequence of some unknown variable in the plant growth conditions. The quantitative levels of mRNA species, as a proportion by mass of total RNA are comparable to those previously reported

for *legA* and vicilin (0.2–0.7%; Boulter et al. 1987); peak levels are 0.3% (*legA* at 18 DAF (batch A)), 0.15% (*lec* at 16 DAF), and 0.05% (*legJ* and *legS* at 16 DAF). The Northern blot in Fig. 3 shows that all the RNAs used were in a largely intact state and that cross-hybridisation between the three classes of legumin synthetic mRNAs was negligible.

These results show some similarities to those reported by Domoney and Casey (1987) for the developmental accumulation of mRNAs of the three classes of legumin from *Pisum sativum* cv. Birte. They report a peak for *legA* (homologous to pCD43) at 24 DAF and also that *legS* and *legJ* (pCD32 and pCD40 respectively) peak earlier at 19 DAF. They did not observe a later peak for *legJ*. The relative levels of the three legumin mRNAs reported were very similar to our data at the stage where the *legA* mRNAs peak but at earlier stages there are differences; at 8–9 DAF we show that *legA* mRNA is at least six-fold higher than *legS* and *legJ* whereas at an equivalent stage (14 DAF) Domoney and Casey (1987) show that the three types of legumin mRNAs are present at approximately the same level. Such differences may result from the use of a different pea genotype, or different growth conditions.

Transcription assays in isolated nuclei. Nuclei isolated from developing pea cotyledons (12 and 16 DAF) and pea leaves were allowed to synthesize transcripts in vitro. Transcripts were labelled by synthesis in the presence of α-[^{32}P]UTP, and specific sequences in the transcripts were then detected by hybridisation to excess DNA immobilized on dot blots (Fig. 4). Immobilized DNA was confirmed to be in excess by varying the amount of DNA in each dot blot. Figure 4a (columns D, E and F, row 3) shows that the amount of hybridisation of transcripts to pDB9.08 is independent of mass of DNA immobilized in the range 2.5 to 10 µg. A linear relationship between input of radioactive transcripts and counts per minute hybridized has been previously demonstrated in this type of assay (Evans et al. 1984). The specificity of hybridisation to the DNA dots was demonstrated by insignificant hybridisation to pBR322 and by specific hybridisation of transcripts to plasmid inserts which had been excised from their vectors, separated on agarose gels and Southern-blotted (data not shown). Under the conditions employed, the extent of hybridisation of the specific nuclear transcripts to immobilized probe sequences was approx. 90% as estimated by rehybridisation of transcripts to fresh blots.

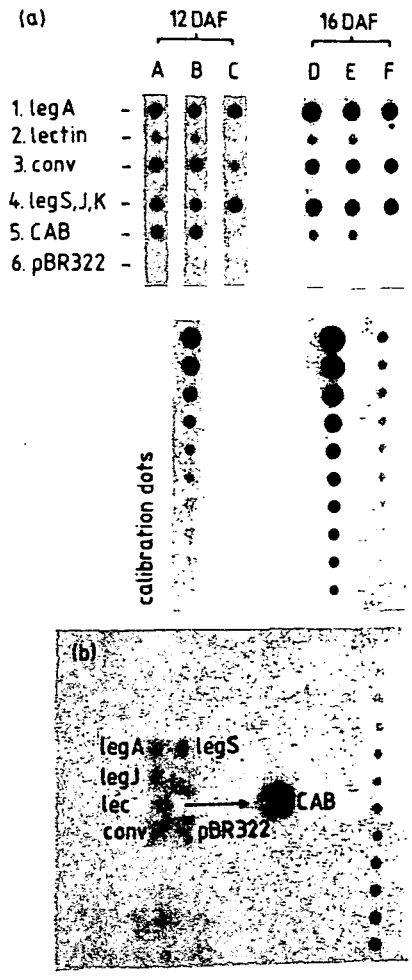

Fig. 4a, b. Autoradiographs of DNA dot blots hybridized to transcripts synthesised in nuclei isolated from **a** 12- and 16-DAF cotyledons and **b** leaves of pea. In **a** $3 \cdot 10^8$ nuclei from both 12- and 16-DAF cotyledons were incubated with 27.8 MBq and 37 MBq α-[^{32}P]UTP, respectively. The labelled transcripts in each case were divided into three equal aliquots ($7.8 \cdot 10^7$ cpm and $1.7 \cdot 10^8$ cpm per aliquot for 12 and 16 DAF, respectively) and hybridized to separate dot blots (*A–F*). DNA dots are as follows. *Row 1: A–F*, 5 µg pDUB6. *Row 2: A, B, D, E*, 5 µg pLG4.10; *C, F*, no DNA. *Row 3: A, B*, 5 µg pDB9.08; *C*, 5 µg pDB9.08 with HpaI/XbaI fragment removed; *D, E, F*, 2.5, 5 and 10 µg of pDB9.08 respectively. *Row 4: A, D*, 5 µg pAD9.2 with EcoR1 fragment removed; *B, E*, 5 µg pCD40; *C, F*, 5 µg pJC5.2. *Row 5: A, B, D, E*, 5 µg pFa b31; *C, F*, no DNA. *Row 6: A–F*, 5 µg pBR322. In **b**, 10^7 nuclei from leaf tissue were incubated with 9.3 MBq α-[^{32}P]UTP and the transcripts ($4.2 \cdot 10^7$ cpm) were hybridized to a single blot. All dots contained 5 µg of DNA. Plasmids used were as follows: *legA*, pDUB6; *legS*, pAD9.2 with EcoR1 fragment removed; *legJ*, pCD40; *lec*, pLG4.10; *CAB*, pFa b31 (this dot was cut out before autoradiography to avoid obscuring other dots on prolonged exposure); *conv*, pDB9.08. Calibration dots of serially diluted, end-labelled pBR322 cover the ranges 20 to 860 cpm (12 DAF), 5 to 3600 cpm (16 DAF) and 3 to 22 cpm (leaf)

Table 1. Transcription in nuclei isolated from pea cotyledons and leaves. Hybridisation of 'run-on' nuclear transcripts to DNA dot blots was quantified and expressed as a proportion of total transcripts. Values were calculated by subtracting background hybridization and then normalizing according to the length of probe sequence using pDUB6 (1.1-kb cDNA) as a reference. Data were then divided by the hybridisation efficiency (0.9). Error values represent variation of duplicate or triplicate hybridisations. In the case of the plasmid pAD9.2 the 5′ 430 bp was removed from the cDNA insert by restriction with EcoR1 and then electroelution (see *Results* section).

Gene family	Probe	Proportion of total transcripts $\times 10^{-6}$		
		12 DAF	16 DAF	Leaf
legA	pDUB6	3.5 ± 0.2	6.1 ± 0.1	ND
legS	pAD9.2	2.3	2.7	0.04
legJ	pJC5.2	3.2	3.0	–
legJ	pCD40	3.3	3.2	0.31
lec	pLG4.10	0.9 ± 0.1	0.5 ± 0.05	ND
CAB	pFa,b31	4.1 ± 0.3	0.9 ± 0.06	22.0
cvcA	pDB9.08[a]	1.8 ± 0.2	1.2 ± 0.1	0.02
cvcA	pDB9.08[b]	1.2		

[a] pDB9.08 was used as the complete plasmid
[b] pDB9.08 was used with a 1.35-bp 3′ fragment of the insert removed by Hpa1 Xba1 restriction and electroelution
ND = not detected

DNA dot blots containing *legJ*, *legK* and *legS* sequences were hybridized to transcripts in separate but otherwise identical hybridisation assays. This was done because *legJ* and *legK* sequences are 94% homologous and *legJ/K* sequences are 75% homologous to *legS* sequences. If DNA dots representing these sequences had been present in the same hybridisation reaction, cross-hybridisation may have occurred with nuclear transcripts during the low-stringency hybridisation, followed by loss of these cross-hybrids during higher-stringency washes.

To test the hypothesis that incorporation of label into nuclear transcripts is not linear along the length of a gene, the 3′ half of the *cvcA* gene was removed by restriction of pDB9.08 with Hpa1 and Xba1. If hybridisation of 12-DAF nuclear transcripts to duplicate dots of the complete 2.45-kb gene sequence is given a relative transcription rate of 1.0 per kb, hybridisation to 1.1 kb of 5′ gene sequence gave a relative value of 0.67 per kb. This indicates a higher density of labelling towards the 3′ end of this nuclear transcript.

Results of hybridisation to DNA dot blots were quantified as described in *Material and methods* and are shown in Table 1. The range of transcriptional activities of the genes tested in developing cotyledons is limited to approx. a 10-fold variation; the range is $0.9 \cdot 10^{-6} - 4.1 \cdot 10^{-6}$ of total transcripts at 12 DAF (i.e. the earlier half of the cotyledonary cell-expansion phase), and $0.5 \cdot 10^{-6} - 6.1 \cdot 10^{-6}$ at 16 DAF (i.e. the later half of the cotyledonary cell-expansion phase). This similarity in transcriptional activities encompasses both the seed-protein genes and the *CAB* light-regulated genes. If the transcriptional activities are expressed on a per-gene basis, the similarity in expression of the seed-protein genes becomes even more marked (Table 2); the range over the legumin and lectin genes is less than 1.5-fold at 12 DAF and less than threefold at 16 DAF. By contrast, in leaves, transcriptional activities range from undetectable ($< 0.02 \cdot 10^{-6}$ of total transcripts), for the *lec* gene and the *legA* gene subfamily, to $22.0 \cdot 10^{-6}$, for *CAB*, i.e. at least a 1000-fold variation. This demonstrates the organ-specific nature of expression of the seed-protein genes, in contrast to the gene encoding the chlorophyll a/b-binding protein, which is expressed in all green tissues. A low level of hybridisation of leaf nuclear transcripts to *legJ*, *legS* and *cvcA* DNA was detected; however, the level of detection was close to background hybridisation to pBR322 and it is not clear from this single assay whether the signal represents a basal low level of transcription or a variable background hybridisation.

The *legA* subfamily genes gave transcripts equivalent to $3.5 \cdot 10^{-6}$ of total transcripts for 12-DAF cotyledons and 6.1×10^{-6} for 16-DAF cotyledons. None of the other genes assayed showed this marked increase in transcription with cotyledon development; over the same interval the *legJ* and *legS* subfamily genes showed a constant proportion of total transcripts, whereas the *cvc* and *lec* genes decreased by approx. 40%, and *CAB* decreased by approx. 80%.

A comparison of steady-state mRNA levels with transcription rates in isolated nuclei. Table 2 summarizes the data for steady-state mRNA levels and transcription rates relative to *legA* at both 12 and 16 DAF. It can be seen that there is no clear correlation between relative transcription rates and relative steady-state mRNA levels. The steady-state levels of *legJ*-type mRNA relative to *legA* are 0.20 at 12 DAF and 0.14 at 16 DAF. However, the corresponding transcription rates of *legJ* genes relative to *legA* genes are approx. four-fold higher, i.e. 0.93 at 12 DAF and 0.51 at 16 DAF. In the case of *legS*, the transcription rates are approx. twofold higher than would be predicted from the steady-state mRNA levels, whereas the lectin transcription rates appear to be approx. three- and sixfold lower than would be predicted from the

Table 2. Comparison of steady-state mRNA levels with nuclear transcription rates in pea cotyledons at 12 and 16 DAF. Data are expressed relative to *legA*

Gene family	Copy no.	Probe	Steady-state mRNA levels		Transcription rates per gene family		Transcription rates per gene	
			12 DAF	16 DAF	12 DAF	16 DAF	12 DAF	16 DAF
legA	4	pDUB6	1.0	1.0	1.0	1.0	0.25	0.25
legJ	3	pCD40	0.20	0.14	0.93	0.51	0.31	0.17
legS	2	pAD9.2	0.30	0.25	0.65	0.44	0.33	0.22
lec	1	pLG4.10	0.87	0.60	0.27	0.09	0.27	0.09

steady-state mRNA levels at 12 and 16 DAF, respectively. These discrepancies provide evidence for posttranscriptional control in the form of differential stabilities or selective degradation of specific mRNAs.

Discussion

The data from nuclear 'run-on' transcription rates and steady-state mRNA levels provide information about the control of gene expression with respect to organ-specificity, timing of expression during seed development and also the level of expression in the seed.

The transcription assays in leaf and cotyledon nuclei presented in this paper, and previously (Evans et al. 1984; Beach et al. 1985) indicate that the seed-specific expression of legumin and vicilin genes is regulated principally at the transcriptional level. Transcription assays indicate a possible low level of transcription of the *legJ*, *legS* and *cvcA* gene families in leaf nuclei so it may be that for these genes seed-specific expression is a result of greatly enhanced transcription rather than an on-off switching mechanism. If transcription does occur in leaves then our inability to detect steady-state *legJ* or *legS* mRNA in leaf tissue, (data not shown) would imply an additional posttranscriptional control to prevent accumulation of the transcripts.

Similarly Walling et al. (1986) found that the soybean seed storage-protein genes for β-conglycinin, glycinin and the 15-kDa protein were transcribed in leaf nuclei at a level 50 to 100-fold below the level in embryo nuclei, whereas the steady-state levels in nuclear RNA were more than 10000-fold lower in leaf nuclei than in embryo nuclei.

Pea lectin is expressed in both seed and roots but not in leaves (Buffard et al. 1988). Nuclear 'run-on' transcription assays described in this paper indicate that the absence of expression in leaf tissue is due to a lack of transcription.

Beach et al. (1985) measured transcription rates for *legA* in isolated nuclei from cotyledons of *Pisum sativum* L. cv. Greenfeast at 11, 13 and 19 DAF. In terms of seed development, 19 DAF is approximately equivalent to 16 DAF for the peas (cv. Feltham First) used in this work; however, the transcription of *legA* measured by Beach et al. (1985) at this stage, expressed as parts per million (ppm) of total nuclear transcripts, is approx. 35-fold higher than that measured here. Also Beach et al. (1985) measured transcription in nuclei isolated from leaves, and the ppm of transcripts hybridizing to pFa2b31 is higher than our measurements by approx. 9-fold. Such differences could be explained by the use of different varieties of pea, grown under different conditions or by the variation in experimental procedures for isolation of nuclei, in vitro transcription and hybridization. However, it is the relative transcription rates of different gene families, as assayed under identical conditions, and not transcription rates as ppm, that are important in drawing the following conclusions from our data.

If we consider the timing of expression of the seed storage-protein genes of pea, the transcription of these genes has previously been shown to be activated early in the mid-phase of cotyledon development, and then to diminish during seed desiccation. Furthermore, the differences in timing of the accumulation of vicilin and legumin mRNA in pea can be accounted for, in part, by the timing of the increases in transcription rates and partly by some posttranscriptional effect (Evans et al. 1984; Beach et al. 1985).

The differences in the timing of accumulation of the three classes of legumin mRNA and lectin mRNA shown in this paper are in reasonable agreement with the corresponding changes in the relative transcription rates. In particular, the transcription rate of the *legA* gene family increases relative to all other gene families investigated over the period from 12 to 16 DAF and this is reflected

in a more rapid accumulation of *legA* mRNA over the same period. Thus the differential timing of expression of the gene families studied appears to be mainly regulated at the transcriptional level, although posttranscriptional regulation may also occur.

Posttranscriptional control of the final level of legumin gene expression has been observed in response to an environmental change. Sulphur deficiency in pea plants results in a large depletion of legumin mRNA levels in pea cotyledons whereas the corresponding transcription of the legumin genes in isolated nuclei showed only a relatively small decrease. Vicilin expression remains unaffected by the sulphur deficiency (Evans et al. 1985; Beach et al. 1985). The physiological significance of this response is to direct the protein-synthesis machinery towards synthesis of sulphur-free proteins such as vicilin and away from the synthesis of relatively sulphur-rich proteins, such as legumin. The data presented in this paper indicate an alternative type of posttranscriptional control of the level of gene expression during seed development in which, instead of a single mRNA species having an apparently different half-life under different environmental conditions, several different mRNA species have apparently different half-lives under identical conditions. This allows two genes to be transcribed at the same rate although their mRNAs accumulate to different levels. The present results show that the three legumin gene families and lectin gene have relatively similar transcription rates on a per-gene basis (see Table 2), whereas their steady-state mRNA levels differ considerably. The latter is partly caused by variation in gene copy numbers for each gene family, but is mainly the result of some posttranscriptional effect, which may be a differential turnover of a specific mRNA in either the nucleus or in the cytoplasm. The physiological significance, if any, of this posttranscriptional control is unknown. A similar type of posttranscriptional control has been observed in soybean cotyledons in which transcription rates of seed protein genes and non-seed protein genes in 70-DAF embryos are similar, but their mRNA levels vary up to 10000-fold (Walling et al. 1986). It has also been reported that the steady-state mRNA levels of the constitutive or 'housekeeping' genes of mouse L-cells correlate with mRNA half-lives rather than their transcription rates (Carneiro and Schibler 1984), and Cabrera et al. (1984) concluded that in sea urchin embryos the turnover of cytoplasmic RNA was a major variable in determining the level of expression of embryo genes.

All examples of posttranscriptional regulation of plant gene expression so far described, have been determined by comparison of steady-state mRNA levels and transcription rates in isolated nuclei. Development of in-vivo labelling techniques in plant systems is necessary to substantiate these data. Understanding the mechanism of posttranscriptional control of the level of gene expression in plants is important in order to express foreign genes to a high level in transgenic plants. The mechanism can only be investigated by the direct measurement of RNA half-lives by kinetic labelling experiments and by the use of cell-free systems (Shapiro et al. 1987).

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Expression of Pea Legumin Sequences in Pea, *Nicotiana* and Yeast[1])

Ronald R. D. Croy, I. Marta Evans, Jenny N. Yarwood, Nick Harris, John A. Gatehouse, Anil H. Shirsat, Angray Kang, J. R. Ellis, Andrew Thompson and Donald Boulter

Plant Molecular Biology Group, Department of Botany, University of Durham, Durham, U.K.

Key Term Index: pea legumin, gene expression, transgenic plants, legumin gene family; yeast, *Nicotiana plumbaginifolia*

Summary and Introduction

The pea legumin seed storage proteins represent one of the most highly characterised group of proteins and genes in plants. The gene family consists of at least two classes of legumin genes which encode distinct but related polypeptides. In the homologous host (*Pisum sativum* L.) these genes are expressed to different degrees as judged by different levels of the proteins accumulated, steady-state messenger RNAs and nuclear transcription rates. Such experiments indicate that the control of gene expression is primarily at the transcriptional level, probably within the non-coding flanking gene sequences; however, post-transcriptional controls operate to modify expression at the translational level.

A major, active, pea legumin gene has been transferreed intact to *Nicotiana* and is expressed in the transgenic plants. The expression of this construct has been shown to retain its organ specificity, and pea legumin protein is accumulated in the *Nicotiana* seeds. An assessment of the levels and the sites of deposition of the legumin protein synthesised indicate that not only are the gene control sequences present and functioning in the heterologous host but the amino acid sequences which determine targeting and processing of the newly synthesised protein are also recognised in *Nicotiana*. Similarly such targeting sequences when synthesized in yeast also direct the legumin protein to membrane compartments.

Methods

1. *DNA Sequencing:* Gene and cDNA fragments for sequencing were subcloned in M13 mp18 or 19 and were sequenced by the dideoxy chain termination method using [α-^{35}S] dATP (Bown et al. 1985).

2. *Cloned DNA:* The following cloned and sequenced DNAs were used for production of gene constructs or as probes for pea seed expressed sequences: — pDUB3 — Leg A cDNA; pDUB6 — Leg A cDNA; pDUB7 — vicilin 47k cDNA; pDUB2 — vicilin 50 k cDNA; pCD40 — leg K cDNA; pJC5.2 — leg J gene; pDUB41 — lectin cDNA; pFa/b 31 — light harvesting protein cDNA; pDUB83 — a convicilin gene, a sub-clone from pJC4.100. pDUB24 — Leg A gene (Lycett et al. 1983, 1984a, 1984b; Domoney and Casey 1984; Domoney et al. 1986; Ellis et al. 1986). DNAs were labelled with [^{32}P]-dCTP by nick translation or by random primer extension. pCD40, pCJ5.2 and pCJ4.100 were kindly supplied by Dr. Claire Domoney and Dr. Noel Ellis (John Innes Research Institute, Norwich); pFa/b 31 was a gift from Dr. Steve Smith (Warwick University).

Abbreviations: NPT, neomycin phosphotransferase; NOS, nopaline synthase; PKG, phosphoglycerate kinase; ER, endoplasmic reticulum; d.a.f., days after flowering

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3. *Genomic DNA, RNA Blots and Transcription Assays:* Genomic DNA was isolated from plants and Southern blots prepared as described by ELLIS et al. (1988).

Total RNA was isolated from the cotyledons of developing pea seeds by the method of CHIRGWIN et al. (1979). Relative steady state levels of individual classes of seed mRNAs, were estimated by spotting known amounts of total RNA from selected stages of development onto nitrocellulose filters. The dot-blots were then hybridised with DNA probes specific for members of the legumin and vicilin gene families. Absolute levels of mRNAs were estimated by calibration of dot-blots with known amounts of SP6 transcripts from the appropriate cDNA sequences inserted in SP6 plasmids (MELTON et al. 1984).

To estimate the *in vivo* rates of transcription from different gene sets in developing cotyledons, nuclei were prepared from cotyledons at the appropriate stage of development and used for *in vitro* transcription run-off assays (EVANS et al. 1984). 32P-labelled transcripts were hybridised to dot blots or Southern blots containing an excess of cDNA or gene sequences encoding seed expressed proteins.

Hybridisation of radioactive probes was quantified by liquid scintillation counting of dots bands cut from the filters or by laser densitometric scanning of the autoradiographs.

4. *Nicotiana Transformation:* A 6.9 kbp DNA construct consisting of: — a 3.4 kbp pea genomic fragment containing Leg A, a major active legumin gene; a constitutively expressed nopaline synthase gene (NOS) and a chimaeric gene: neomycin phosphotransferase linked to the constitutive plant NOS promoter (NOS-NPT) encoding kanamycin resistance, was transferred to *Nicotiana plumbaginifolia* using the BIN19 binary Ti system (BEVAN 1984). Leaf discs were cut from young expanded leaves and infected with *Agrobacterium tumefaciens* (LBA4404) containing the construct. Clonal shoots which developed in the presence of 200 $\mu g/ml^{-1}$ kanamycin after about 4 to 6 weeks were excised and rooted and grown to full maturity. Leaf and seed materials were harvested at various stages of development for assessment of Leg A function. Full experimental details of this work have been published elsewhere (ELLIS et al. 1988).

5. *Constructs for Yeast Expression:* A full length, Leg A type, legumin cDNA was constructed from two overlapping cDNAs and a legumin gene and cloned in PUC18 (Fig. 10). The construct was then sub-cloned, in the correct reading frame, into the yeast expression vector pMA91 (MELLOR et al. 1983) containing the yeast phosphoglycerate kinase gene promoter upstream from the insertion site. This plasmid was then transferred to a yeast MC16 host by the lithium acetate transformation method. Extracts from clones containing the pea legumin construct were subsequently tested on Western blots for the synthesis of legumin protein.

6. *Antibodies, Western Blots and ELISA Assays:* Antibodies against purified pea legumin and vicilin were raised in New Zealand White rabbits and in mice by standard protocols. It was apparent that components in the rabbit anti-pea legumin antisera cross-reacted with material in *Nicotiana* seed extracts and tissue sections, possibly the *Nicotiana* 11S legumin-type proteins. Pretreatment of the antisera with untransformed *Nicotiana* seed extracts, effectively removed the cross reacting materials while still maintaining specific pea legumin recognition.

Western blotting was performed according to the method of TOWBIN et al. (1979) using a Sartorius semi-dry blotter system. Filters were blocked with 50 $mgml^{-1}$ low fat dried milk before reacting with anti-legumin antibodies (0.5 μgml^{-1}. Immunoreacting bands were visualized with goat anti-rabbits IgG conjugated with horseradish peroxidase using diaminobenzidine tetrahydrochloride substrate.

ELISA assays were performed in microtitre plates using legumin standards (0 to 1280 $ngml^{-1}$) and 50 μl aliquote of single or half *Nicotiana* seed extracts. Samples were incubated in the plates overnight at 4 °C followed by reaction for 2 h with anti-pea legumin IgG diluted 1 : 100. Bound anti-legumin IgG was estimated by binding goat anti-rabbit IgG conjugated with peroxidase, followed by reaction with ABTS [azinobis — (3-ethyl benzthiazoline sulphonic acid)]. Absorbances were measured on a plate reader and standard curves used to estimate pea legumin contents. Pea legumin contents of single seeds were corrected for protein levels after estimation by the BRADFORD dye binding assay (BRADFORD 1976).

7. *Immunocytochemistry:* Developing pea seeds were harvested at 13 d.a.f., fixed in glutaraldehyde/paralformaldehyde followed by osmium tetroxide, embedded in Spurr resin. Thin sections were

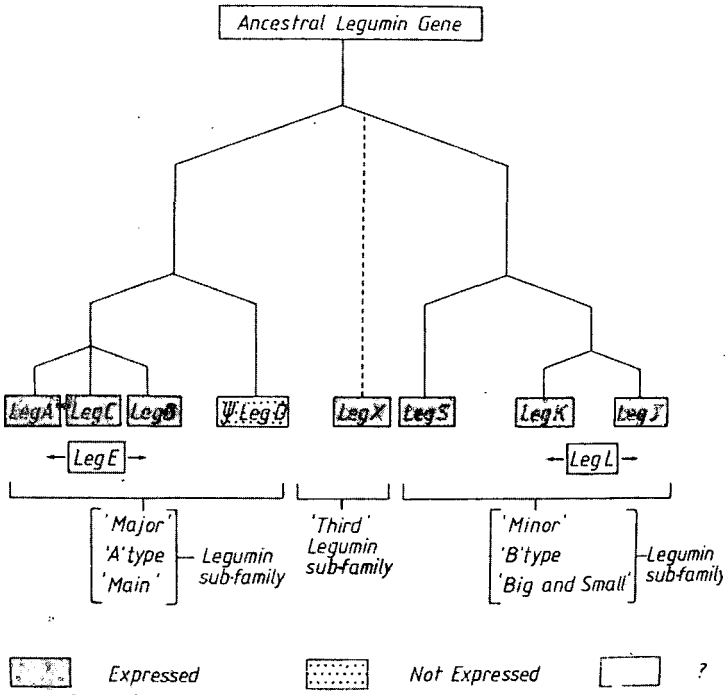


Fig. 1. *Tentative evolutionary tree of the Legumin Gene Family illustrating possible relationships between subfamilies.* Arrowed boxes indicates uncertainty in the positioning of uncharacterised genes.

mounted on gold grids and 'etched' with sodium periodate before incubating them with rabbit anti vicilin, and mouse anti-legumin polyclonal antibodies. The washed sections were subsequently incubated with goat anti-rabbit IgG and goat anti-mouse IgG antibody-gold conjugates. Colloidal gold labels of different sizes were used to distinguish legumin and vicilin. Sections were stained with uranyl acetate and lead citrate before being examined.

Control and transformed *Nicotiana* seeds were fixed as for pea seeds and embedded in LR White resin. Sections were incubated with pre-treated anti-legumin antibodies (see section 6) followed by goat anti-rabbit IgG antibody-gold.

Yeast cells expressing pea legumin were fixed in paraformaldehyde, infiltrated with sucrose as a cryoprotectant and then frozen in a Freon slush prior to cryosectioning. Sections were incubated with rabbit anti-legumin antibodies followed by goat anti-rabbit IgG antibody-gold as before.

Results and Discussion

1. *The Legumin Gene/Protein Family*

Complete and partial sequences of several isolated legumin genes, cDNAs and polypeptides have been obtained. This has enabled us to classify the genes into an evolutionary tree and to infer relationships between the different gene sequences (Fig. 1). In *Pisum* we have at least two classes or subfamilies of genes with the possibility of a third, as yet, uncharacterised class. Sequence comparisons between pea legumin genes and with legumin genes in other species (*Vicia faba* and *Glycine max*) suggest that the division of legumin genes into "Major", or Type A, and "Minor", or Type B, subfami-

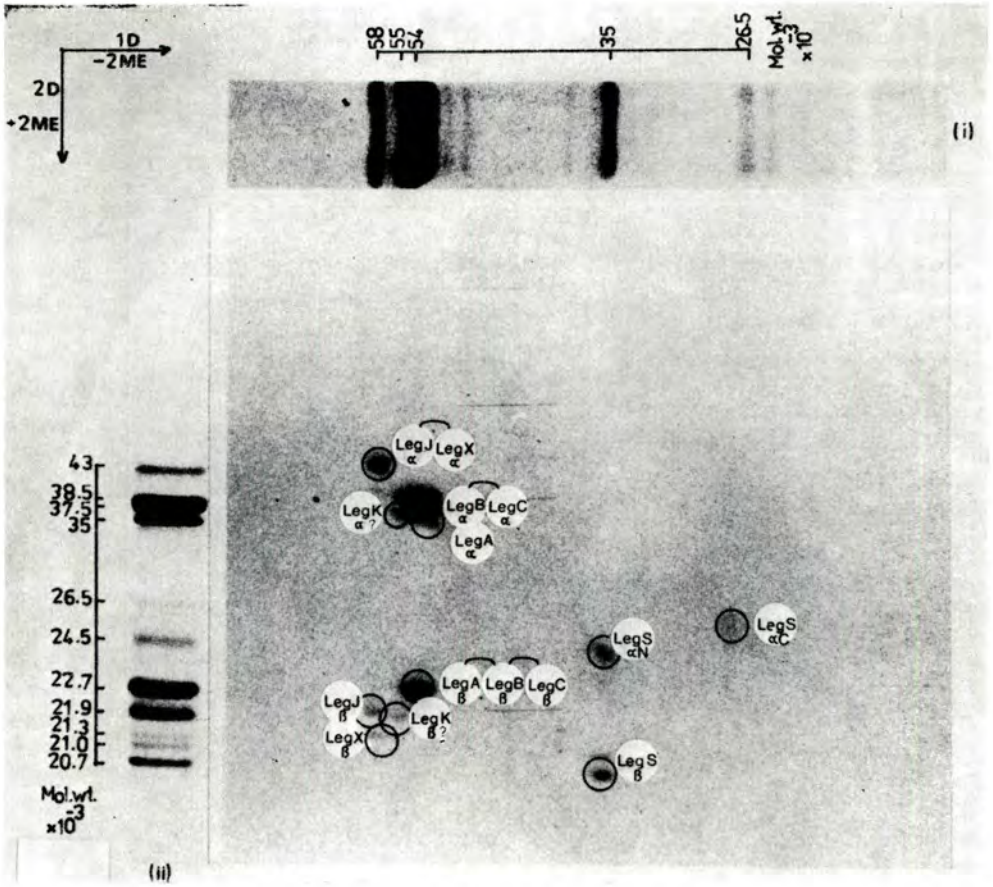
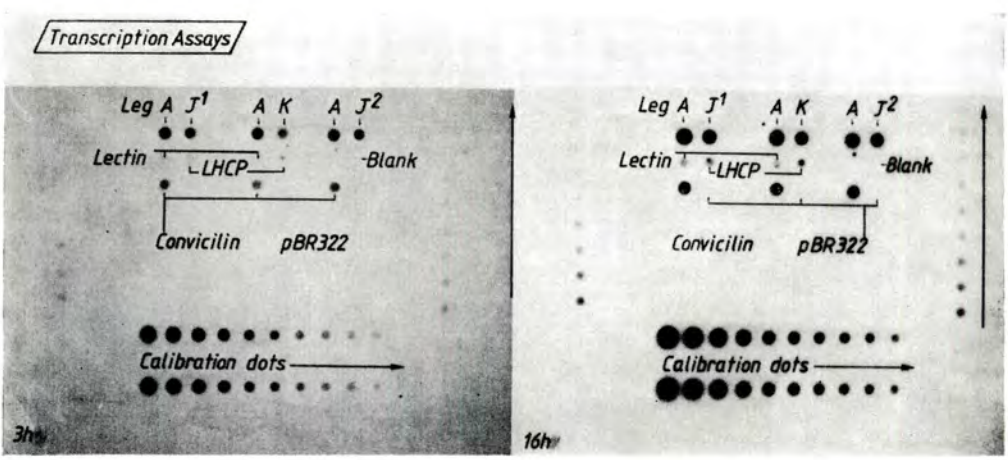


Fig. 2. *Two dimensional SDS gel separation of legumin proteins showing tentative assignment of major and minor polypeptides to specific genes.* i) First dimension gel (—2ME) non-reducing conditions, followed by ii) reducing conditions (+2ME) in the second dimension (Adapted from MATTA et al. 1981).



lies is a comparatively ancient event, having occurred prior to some speciation in the legumes. However, separation of genes within subfamilies is indicated to be a much more recent event. The very high level of homology between the genes in the Leg A subfamily, which extends for several hundred bases into the 5′ and 3′ flanking regions, suggests a very recent divergence. However, gene copy number estimates for the Leg A subfamily do not appear to differ significantly between modern and primitive pea lines. This suggests that selection pressure caused by cultivation for desirable characteristics (and possibly linked to higher legumin levels), has not resulted in gene duplication.

Mechanisms of evolutionary divergence in the legumin gene family seem to have relied at least as much on insertions and deletions as on base substitution, and there is little evidence for an excess of "silent" changes in the coding sequences. This reflects the relatively low level of constraint on the sequences of these proteins, although certain regions (e.g. around the inter-chain disulphide bridge) are relatively invariant and can therefore be identified as playing a vital role in maintaining the proteins structure or conformation.

The accumulated data from gene, cDNA and protein sequences as well as from the alpha and beta subunit properties, has allowed us to tentatively assign to specific genes, the major (A-type) and minor (B-type) legumin polypeptides accumulated in mature seeds as shown in Fig. 2, separated in 2D SDS gels.

2. *Expression of Legumin Genes in Pisum*

Quantitative assays of steady state levels of mRNA species during pea seed development (Gatehouse et al. 1986) has shown that the primary regulation of legumin and vicilin genes is at the transcriptional level. However the patterns of vicilin and legumin mRNA accumulation are temporally different. Vicilin mRNAs for example peak at around 14 days after flowering (d.a.f.) on our developmental time-scale. In contrast Leg A and convicilin mRNAs peak at 18 to 20 d.a.f. (Gatehouse et al. 1986; Boulter et al. 1987).

Recent work on the relative levels of different classes of legumin mRNAs indicates that at mid-development (16 d.a.f.) Leg A mRNA levels are 7-fold higher than Leg J. This is similar to the findings of Domoney and Casey (1987). However as shown in Fig. 3 the nuclear transcription rates of Leg A genes is only two fold higher than that of the Leg J genes. Thus steady state levels of different legumin mRNAs do not reflect the in vitro transcription rates suggesting a post-transcriptional control, possibly involving lowered transcript stability or export from the nucleus. Further studies are continuing to confirm this regulatory mechanism with the other genes shown in Fig. 3 and at several other developmental stages.

Immunolocation studies on pea cotyledons at the electron microscope level, using gold-labelled antibodies have clearly shown that the synthesis of both legumin and vi-

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Fig. 3. *'In vivo' transcription run-off assays from cotyledon nuclei at 16 d.a.f., showing the relative rates of transcription of a number of seed expressed genes including Leg A and Leg J (1 + 2 = different probes), lectin, convicilin, and light harvesting chlorophyll-binding protein (LHCP).* Exposures after 3 and 16 h are shown.

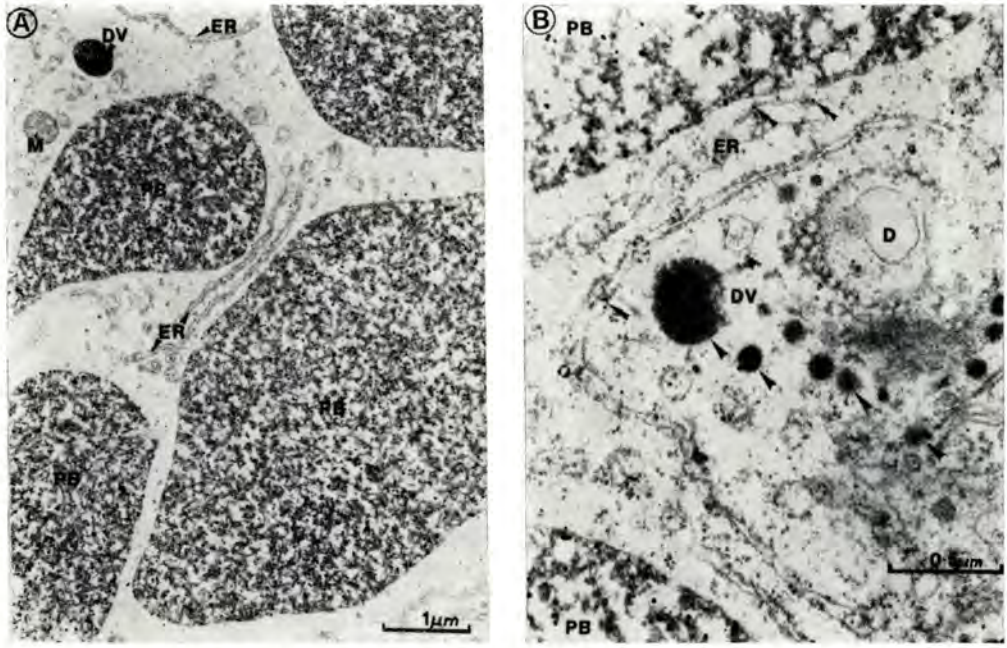

Fig. 4. *Immunolocation of sites of synthesis and deposition of legumin and vicilin in developing pea cotyledons at 13 d.a.f.* The larger gold particles indicate the vicilin sites and the smaller, the legumin sites. Protein bodies and vesicles containing both proteins are clearly visible.

cilin occurs at the endoplasmic reticulum. Transport through the ER lumen is followed by sequestration of both proteins into dictyosome vesicles and deposition of both legumin and vicilin within the same vacuolar protein bodies (Fig. 4 A and B).

3. *Expression of a Pea Legumin Gene in Nicotiana*

Evidence from cDNA and protein sequences and from S1 mapping and *in vitro* transcription experiments indicates that the Leg A is an intact, active gene which encodes one of the major legumin proteins accumulated in mature pea seeds (Lycett et al. 1984a, 1984b; Evans et al. 1985). A 3.4 kbp Bam fragment containing this gene has been used for *in vivo* functional assays in *Nicotiana*. The gene (Fig. 5A) comprises: 1,239 bp of 5′ sequence within which various putative modulating sequences have been identified; the transcription start; the coding region; and a 3′ non-coding region of 633 bp containing three polyadenylation signals. The coding region of 1,488 bp encodes the translation start, a leader sequence of 21 amino acids, 311 amino acids of alpha (acidic) polypeptide containing three major hydrophilic repeats (Lycett et al. 1984a), 185 amino acids of beta (basic) polypeptide and the translation stop codon. The coding region is interrupted by three introns (Fig. 5A).

A construct (Fig. 5B) containing this legumin gene plus a complete nopaline synthase (NOS) reporter gene, have been inserted into the disarmed binary Ti vector, pBIN19, linked to the chimaeric NOS promoter — neomycin phosphotransferase (NPT) gene (Fig. 5B). This construct has been designated BIN19-DUB53. The three genes in the T-DNA were conveniently flanked by a number of diagnostic restriction sites which

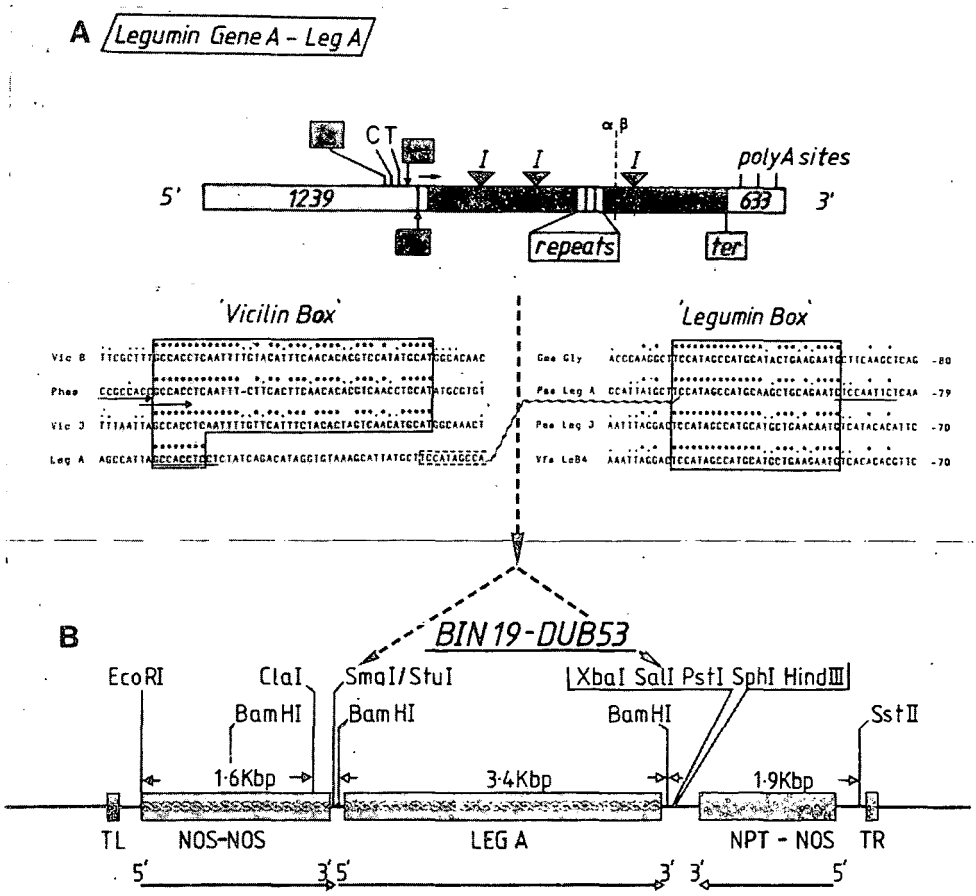

Fig. 5. *Detailed structure of the Leg A gene used for 'in vivo' functional assays:* ts = putative tissue specific sequences (enhancers) such as consensus vicilin and legumin boxes; C = CAAT box; T = TATA box; tcs = transcription start; tls = translation start; I = introns; ter = termination codon. Boxed regions indicate consensus sequences found in similar 5′ positions upstream from the transcription start in several seed specific genes. Homology in all sequences is indicated by a asterisk. Phas = Phaseolin (SLIGHTOM et al. 1983); Vic B and J = *Pisum* vicilin genes (GATEHOUSE et al. 1986); Gma Gly = *Gylcine max* glycinin gene (GATEHOUSE et al. 1986); Psa Leg A and J = *Pisum* legumin genes (LYCETT et al. 1984b; GATEHOUSE et al. 1986); Vfa LeB4 = *Vicia faba* legumin gene (BÄUMLEIN et al. 1986).

Fig. 5B. *Gene construct in the BIN19-DUB53 T-DNA, transferred to Nicotiana plumbaginifolia.* Long arrows indicate direction of transcription. Diagnostic restriction sites and fragment sizes are indicated. NOS-NOS = Nopaline synthase gene, NOS-NPT = NOS promoter and neomycin phosphotransferase gene.

could subsequently be used to assess the integrity of the genes in the transgenic plants (Fig. 5B).

BIN19-DUB53 was transferred to *Nicotiana plumbaginifolia* by leaf disc infection and transgenic plants grown to full maturity. The plants were analysed for the intact genes by Southern blotting, for NOS by opine assays, and for seed specific transcription

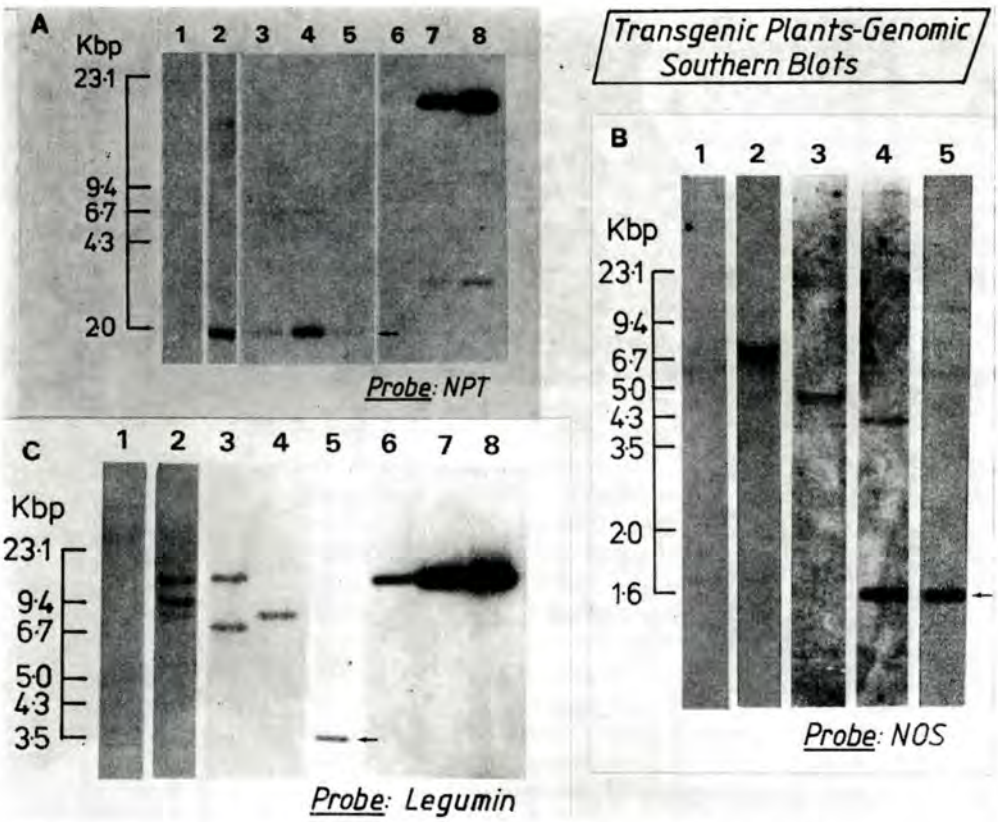

Fig. 6. *Southern blots of genomic DNA of four transgenic Nicotiana plants, restricted to excise secomponent genes in the original construct* (Fig. 5 B) A) *NPT gene probe, Bam HI plus SstII restrictions* B) *NOS gene probe, Eco RI plus Cla I restrictions.* C) *Legumin gene probe, Bam HI restrictions.* Tracks contain digests of DNA (10 μg) from 1) control (untransformed) plant; transformed plants 2) T-45; 3) T-10; 4) T-9; 4) T-20. Tracks 6), 7) and 8) represent 1,5 and 10 gene copies per haploid genome.

and translation by Northern blots and ELISA assays. Of 50 kanamycin selected plants legumin was detected in 22 seed samples while nopaline was found in only 10 leaf samples. There was no correlation between nopaline and legumin expressing lines.

Fig .6 shows Southern blots of genomic DNA from four selected phenotypes, restricted to excise the individual genes and probed as indicated. Blot A) shows clearly that the NPT gene which functions during the kanamycin selection, is present in all lines in its intact form. Blot B), probing for the NOS gene, shows that the gene fragment is intact in only one line, all the others are rearranged, explaining why some transformed plants were nopaline negative. Blot C) probed for the legumin gene, similarly shows only one line with an intact gene fragment which also showed legumin expression. Thus it is clear that gene rearrangements have occurred causing gene inactivation, though inactivation does not always accompany such recombination.

Western blots have been used to assess the integrity of the expressed pea legumin. Blots of untransformed *Nicotiana* seed proteins using pretreated antibodies showed no immuno-reacting bands (Fig. 7). Under non-reducing conditions an antigenic band was

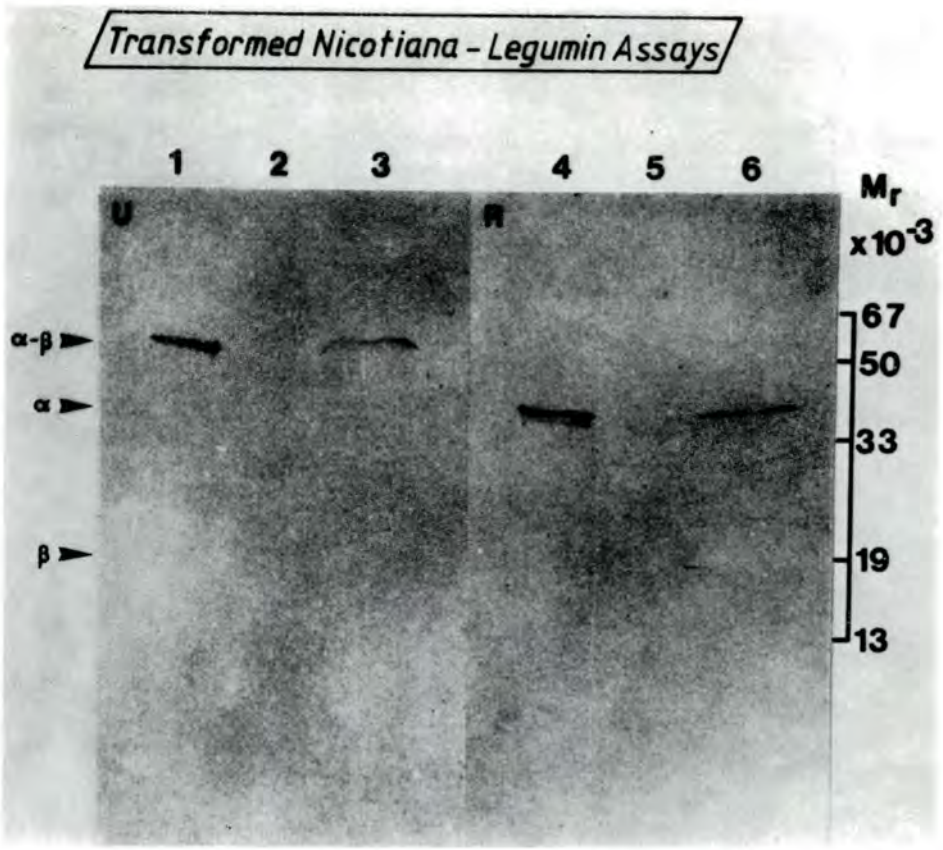

Fig. 7. *Western blot of transformed Nicotiana seeds.* Tracks 1 and 4 — authentic pea legumin, 2 and 5 — control seed extracts, 3 and 6 — transformed seed extract. U = unreduced samples, R = reduced samples.

detected in the transformed seeds which comigrated with authentic legumin (Fig. 7). Under reducing conditions this band was replaced by a smaller one which comigrated with the alpha polypeptide of authentic pea legumin. Failure to detect the beta (basic) polypeptides in either the standard or *Nicotiana* samples is a reflection of the small amounts of protein present, the poor transfer of basic polypeptides and the low antigenicity of these subunits. However the reduction in size of the single antigenic band provides indirect evidence for its presence disulphide linked to the alpha polypeptide. No antigenic protein was found in any other tissues of the transformed plants.

Immunocytochemical location of the pea protein in transgenic *Nicotiana* seeds was complicated by the presence of 11S legumin-type proteins. The use of pretreated antibodies (section 6) enabled the specific localisation of pea legumin. As shown in Fig. 8, pea legumin was deposited in vacuolar protein bodies. Nicotiana seeds contain several clearly distinguishable tissue types (Fig. 8 insert) and pea legumin deposition occurred not only in the embryo, as in pea but also in the endosperm tissue, which is not a storage tissue in mature pea seeds. It was also apparent that the accumulation of the pea protein in the endosperm protein bodies was greater than any of the embryonic protein bodies.

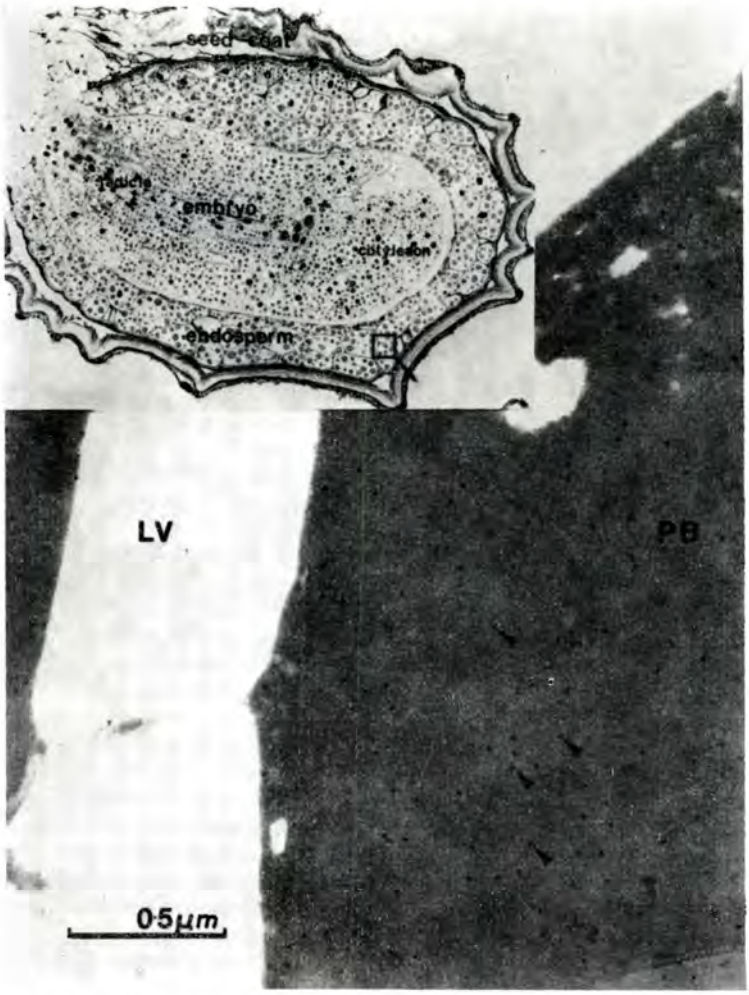

Fig. 8. *Immunolocation of pea legumin deposition in transgenic Nicotiana seeds.* Insert shows a T/S of a *Nicotiana* seed. The box indicates the approximate position of the electron micrograph which shows a close-up of an endosperm protein body labelled with gold particles (arrowed) at the site of pea legumin deposition. PB = protein body, LV = lipid vesicle.

Sensitive ELISA assays have been used to accurately estimate the levels of pea legumin in individual *Nicotiana* seeds from one transformed line (T45), using statistically meaningful methods. Positive values for legumin detection in individual seeds were determined statistically as those values greater than the mean value of the determination for control seeds plus two standard deviations. Our findings for the S1 and S2 seeds are shown as scatter plots in Fig. 9 (A and B). The 81 seeds showed a Leg A segregation ratio of 3 : 1 (74% legumin positive seeds) as would be expected for a single gene locus. The level of expression in these transformed seeds was greater than 0.1% of the total seed protein and up to 0.5% in some cases. While the levels of expression in S2 seeds were similar, greater than 90% of the seeds had legumin levels higher than the controls suggesting this line to be homozygous for the Leg A gene. Levels of foreign proteins in

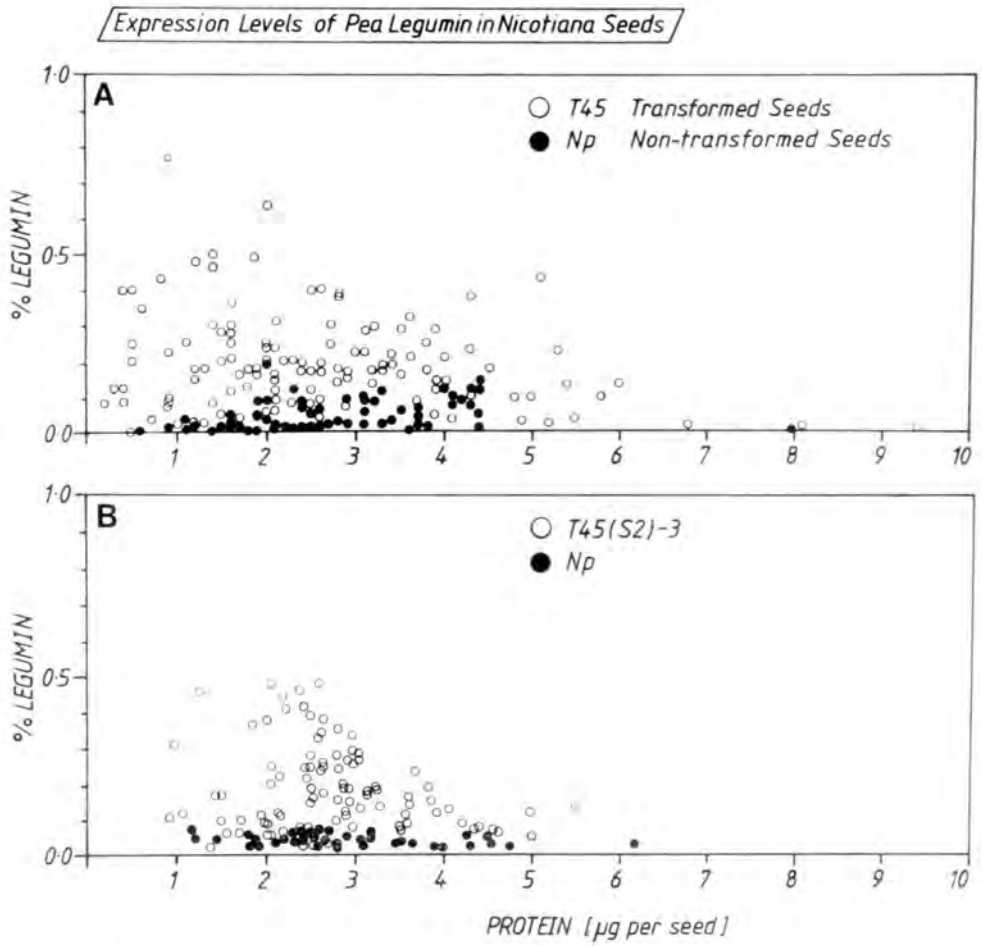

Fig. 9. *Scatter plots of pea legumin contents of single transformed and untransformed Nicotiana seeds.* T45 transformed line 45 and Np control, untransformed, *Nicotiana plumbaginifolia*. Fig. A shows results for S1 seeds and B results for the S2 (selfed) seeds.

Nicotiana seeds expressed under the control of the legumin gene promoter, may therefore be expected to be of the order of 10—40 ng per seed.

4. *Expression of Pea Legumin Polypeptides in Yeast (Saccharomyces cerevisiae)*

A full-length legumin cDNA molecule has been engineered which encodes the whole of a Leg A type legumin polypeptide including the authentic leader sequence (Fig. 10). The final construct consisted of the whole of the coding region encoding; the legumin leader sequence of 21 amino acids; the contiguous alpha and beta polypeptides; alpha to beta proteolytic processing site; four cysteines, two forming a disulphide bridge between the alpha and beta polypeptides; and finally a 3′ non-coding region of 139 base pairs containing multiple polyadenylation signals.

This construct has been placed in the correct reading frame downstream from the yeast phosphoglycerate kinase (PGK) promoter in the expression vector pMA91 (Mel-

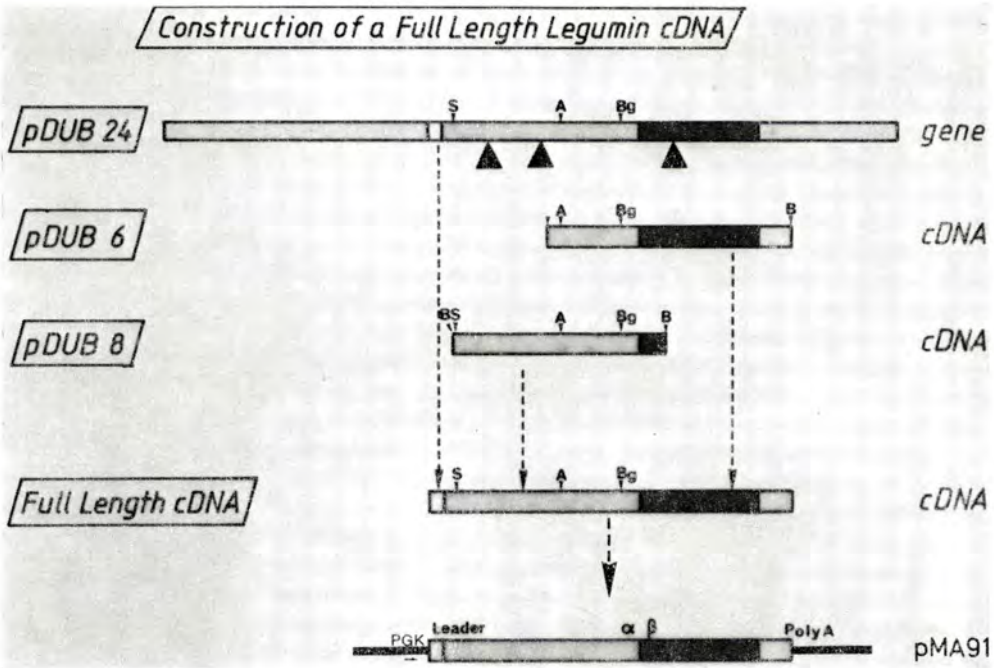

Fig. 10. *Construction of a full length Leg A type cDNA*. Figure shows the strategy and key restriction sites for the construction of the cDNA.

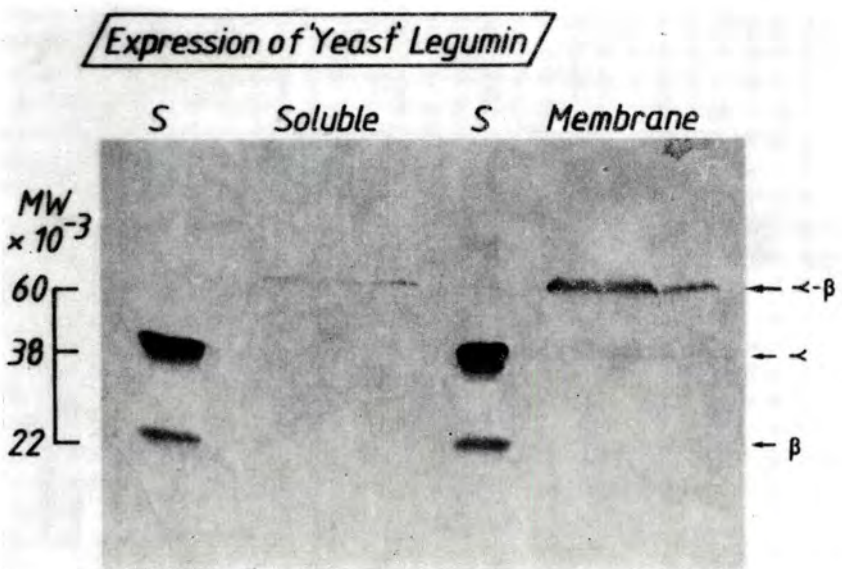

Fig. 11. *Western blot analysis of proteins expressed in yeast, transformed with the full length legumin cDNA*. S = standard pea legumin Soluble = proteins released into non-denaturing solution, from yeast cells, by sonication with glass beads. Membrane = proteins associated with the membrane fraction after sonication treatment. Samples were run on SDS gels after reduction.

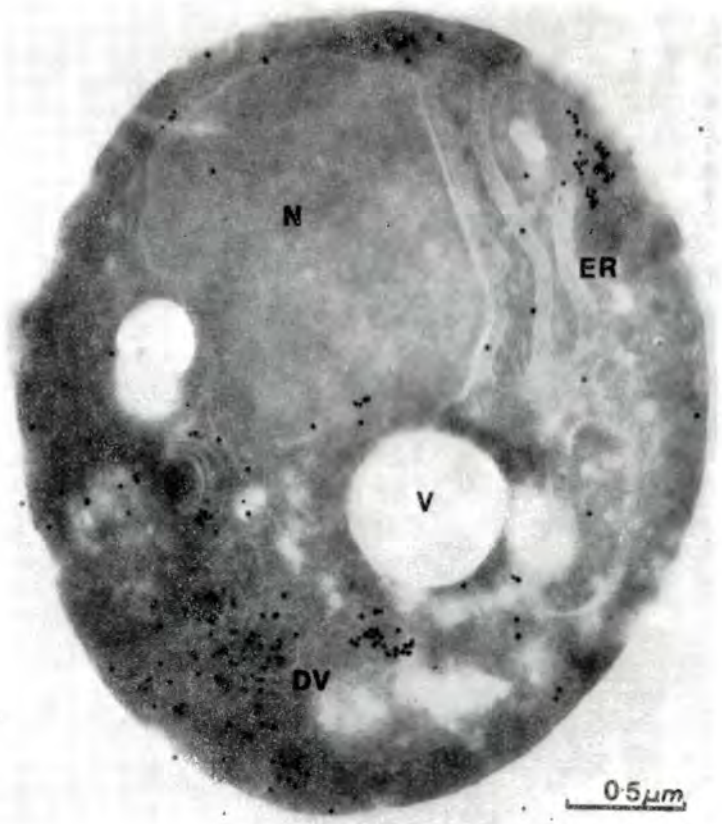

Fig. 12. *Immunolocation of yeast synthesized legumin.* Electron micrograph of a yeast cell synthesizing pea legumin polypeptides. Colloidal gold particles indicate the location of the polypeptides. Pea legumin is found on endoplasmic reticulum (ER) membranes but appears to be mainly sequestered into the dictyosome vesicles (DV). No legumin is located in the yeast vacuole (V). N = Nucleus.

LOR et al. 1983). The encoded legumin polypeptide was synthesised in yeast in high yields as judged by quantitative western blots reacted with anti-legumin antibodies (Fig. 11). The yeast legumin appears to be synthesized as a single, unreduceable polypeptide of Mr 60,000, unlike native pea legumin which is proteolytically processed to disulphide linked alpha (Mr 38,000) and beta (Mr 22,000) polypeptides, which are separable on SDS gels after reduction (Fig. 11). Thus it is apparent that the alpha-beta processing site is not recognised in yeast or that the Mr 60,000 precursor polypeptide is not accessible to the processing system.

Attempts to solubilize the yeast legumin in non-denaturing buffers by lysing the cells by sonication or glass beads, has largely failed and most of the legumin remains in the insoluble membrane fraction (Fig. 11).

Immunocytochemistry using specific anti-pea legumin antibodies has been used to locate the sites of synthesis and deposition of the legumin in the yeast cells. As shown in Fig. 12 the gold labelling patterns indicate an initial mode of synthesis closely resembling that in pea. Synthesis occurs at the endoplasmic reticulum and the legumin protein

is sequestered within vesicles as sociated with the Golgi apparatus. However there appears to be no further transport to other compartments. It seems likely therefore that the leader sequence encoded in the legumin cDNA allows the legumin polypeptides to enter the yeast membrane system but subsequently are not sufficiently well recognised for the protein to be released and transported to other cell compartments such as the vacuole. Failure of the protein to fold correctly and insolubilisation within these membrane compartments may also be involved.

Conclusions

The accumulated gene and protein sequence data has enabled us to construct a tentative evolutionary tree for the legumin gene family and to predict which members are active, missing or inactive.

While primary regulation of the expression of storage protein genes is at the transcriptional level it is clear that post-transcriptional processes operate to further modify expression at the level of translation.

A Leg A gene transferred to *Nicotiana plumbaginifolia* contains all the control sequences which determine seed specific expression and these are recognised by the endogenous *Nicotiana* control system. Furthermore the amino acid sequences which determine sites of legumin synthesis transport, proteolytic processing and deposition in pea, are also recognised by the *Nicotiana* processing enzymes and transporting system such that the protein is synthesized at the ER, is transported to vacuolar protein bodies and is also proteolytically processed to separate alpha and beta polypeptides in a manner entirely analogous to pea.

In yeast, however, it is apparent that the legumin protein sequences are only partially recognised or are inaccessible to the yeast processing systems.

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Author's address: Dr. Ronald R. D. Croy, Plant Molecular Biology Group, Department of Botany, University of Durham, South Road, Durham, DH1 3LE, U.K.

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Buchbesprechung

STAEHELIN, L. A., and ARNTZEN, C. J. (Eds.): Photosynthesis III. Photosynthetic Membranes and Light Harvesting Systems. Encyclopedia of Plant Physiology. New Series, Vol. 19. XXVIII, 902 Seiten, 163 Abbildungen. Springer-Verlag, Berlin-Heidelberg-New York-Tokyo 1986. Preis: DM 498.

In Struktur, Zusammensetzung und Funktion gehören die Thylakoide der photosynthetischen Grana- und Stromalamellen zu den bestuntersuchten Biomembranen. Diesen Eindruck gewinnt man unbedingt bei der Lektüre des vorliegenden Bandes 19 („Photosynthesis III") in der Reihe der großen Neuen Serie des Handbuches für Pflanzenphysiologie. Nach den beiden Photosynthesebänden 5 und 6 der Serie, deren Erscheinen 10 bzw. 8 Jahre zurückliegen, rechtfertigen die enormen Erkenntnisfortschritte in der Perzeption und Wandlung der Lichtenergie und im Aufbau der molekularen Dimensionen der Photosynthesemembran diesen speziellen Band vollauf. Die Herausgeber L. A. STAEHELIN und C. J. ARNTZEN haben sich die größte Mühe gegeben, nicht nur ein den modernsten Stand des Wissens repräsentierendes Nachschlagewerk zusammenzustellen, sondern auch versucht, neue Möglichkeiten zur Bändigung der Wissensflut auszuprobieren: Größere einführende Übersichten werden durch spezielle „Minireviews" über ein bestimmtes Thema ergänzt. Dafür ist eine beträchtliche Zahl an Mitarbeitern notwendig, und so steht dieser Band mit 49 Beiträgen auch mit Abstand an der Spitze der Autorenzahl (67) in den Enzyklopädie-Bänden. Ohne sich ein abschließendes Urteil anmaßen zu wollen, möchte der Rezensent ein Überwiegen der Vorteile konstatieren, die in der Prägnanz und in der Tiefe der Kurzbeiträge liegen — allerdings auch eine glückliche und feste Hand der Herausgeber voraussetzen. Doch auch die „Übersichten" sind exzellent geschrieben, up-to-date und in der Regel großartig illustriert (z. B. STAEHELINS Beitrag zur supramolekularen Struktur der Photosynthese-Membranen). Gleichwertig dem strukturellen Aspekt sind die Beiträge von THORNBER (Biochemie und Struktur der Pigment-Protein-Komplexe) und von ORT (Struktur und Mechanismen der Energietransduktion) an die Seite zu stellen. Jeweils mehrere Autoren beteiligen sich an den Großkapiteln über Vergleichende Biochemie der Lichtsammelsysteme, über den Prozeß des Lichteinfangens (trapping), über die Physik des Ladungstrennungsprozesses, über die Struktur und Proteinzusammensetzung der beiden Photosysteme, über den Energietransfer (Cytochrome, Plastocyanin, CF_1-CF_0), über die Topographie und das Assembly der Photosynthese-Membranen (vergleichende Betrachtungen bei unterschiedlichen Organismen, inklusive Chloroplastenmembranen in Mutanten; Prolamellarkörper; molekulare Organisation sowie Biosynthese der Membranlipide). — Es ist aus mehreren Gründen unmöglich, an dieser Stelle Einzelbeiträge wertend herauszustellen oder herabzumindern. Die durchweg anerkannten Spezialisten garantieren für gute Qualität und neueste Informationen (Literaturerfassung bis einschließlich 1984) über das zunehmend bessere molekulare Verständnis der Photosynthese am Anfang einer gentechnisch bestimmten Forschungsepoche. In seiner sachlichen Geschlossenheit und der schon bekannten vorzüglichen Ausstattung durch den Verlag repräsentiert dieser Band den prächtigen Abschluß eines glänzend gelungenen wissenschaftsreferierenden und Forschungstrends aufzeigenden sowie verlegerischen Großunternehmens. Allen dafür Verantwortlichen gebührt bleibender Dank.

B. PARTHIER, Halle (Saale)