Gene expression in the precocious germination of late maturation *Phaseolus vulgaris* L. seeds.

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1-D	one dimensional
2-D	two dimensional
A_{260}	absorbance at 260nm
ABA	abscisic acid
AU	absorbance unit
AU*mm	absorbance unit times millimetres(a measure of peak area)
cpm	counts per minute
DAA	days after anthesis
DEPC	diethylpyrocarbonate
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
kb	kilobase
kD	kilodalton
LEA	late embryogenesis abundant(protein)
MOPS	3-[N-Morpholino]propanesulfonic acid
MW	molecular weight
PCR	polymerase chain reaction
pI	isoelectric point
ppm	parts per million
PPO	2,5-diphenyloxazole
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TBE	Tris-borate EDTA
TCA	trichloroacetic acid
TEMED	N,N,N′,N′,-tetramethylethylenediamine
TMV	tobacco mosaic virus

ABSTRACT

Ethylene induces precocious germination in late maturation embryos (32-40 days after anthesis) of *Phaseolus vulgaris* L. cv. Seminole, thus overriding the endogenous controls which normally maintain quiescence. The possibility that ethylene exerts its effects at the gene expression level was investigated by in vitro translation of RNA extracted from embryo axis tissue of seeds induced to germinate precociously by incubation with ethylene. ^{35}S-labelled products so produced were analyzed by electrophoresis, fluorography, and scanning densitometry. Results were compared with normally germinating seeds and with embryos incubated in the absence of ethylene. Ethylene was found to induce a qualitative and quantitative change in gene expression in late maturation embryos detectable within 6 hours of ethylene exposure. Two products (37-38kD and 27kD) were up-regulated within 24 hours in both ethylene-induced precocious germination and normal germination. Four products (71kD, 67-68kD, 65-66kD, and 41-42kD) which appeared in normal germination were evidently not required for ethylene-induced precocious germination. In contrast with the findings of Misra & Bewley (1985;1986) for maize(*Zea mays* L.) no products could be identified as being unique to the developmental phase, however two products (38-39kD and 28kD) were strongly present in development but disappeared shortly after germination. A product of 22-23kD was apparently unique to the ethylene-induced precocious germination treatment and may represent a gene regulated by ethylene. This product was not seen until 24 hours after ethylene introduction. An attempt was made using SDS-PAGE to identify the major storage proteins of *P.vulgaris* to use as markers of the developmental phase, however this was only partially successful. Suggestions are made as to approaches and methods for future research.

INTRODUCTION

In dicots from the time of fertilization, seed formation goes through three distinct developmental stages: histodifferentiation; maturation; and desiccation. During histodifferentiation the basic body plan of the root-shoot axis is laid down and the basic tissues from which the plant is derived are formed as follows. After fertilization the newly formed zygote undergoes division to form two cells; one of which will form the suspensor, and one of which will form the embryo proper. The epidermis is the first of the three basic tissues(ground, vascular, epidermis) to appear as the embryo undergoes rapid cell division to form the globular phase with its outer layer of cutin-producing epidermal cells. At the end of the embryo away from the suspensor, a period of localized cell division activity results in the formation of two primitive cotyledons and also results in the formation of a bilaterally symmetrical heart-shaped embryo. Between these two cotyledons an apical meristem forms which will give rise to the shoot system. At the opposite end of the embryo(i.e. the suspensor end) the root apical meristem is created and so the basic root-shoot axis is formed. The tissue destined to become the vascular tissue begins differentiation at the heart stage to create a cylinder of cells which traverses the length of the hypocotyl and joins a continuous provascular core located in the root end of the embryo. The remaining tissue in the embryo is termed ground tissue and these three tissue types will be the basic constituents for all further organogenesis throughout the plants life(Maheshwari, 1958).

Following the heart stage, a massive synthesis of storage reserves causes the cotyledons to swell greatly in size to become the dominating features of the embryo. These storage reserves are crucial to the plants future survival as they will be the sole source of nutrition in germination until the young seedling can assemble its photosynthetic machinery and become fully autotrophic.

Once reserve deposition is completed, the embryo and its surrounding tissues begin to lose water and desiccate to finally yield a mature dry seed. This is best

illustrated by looking at a generalized scheme of whole seed fresh and dry weights over the duration of seed development. During the histodifferentiation phase the young embryo rapidly increases in fresh weight while dry weight at this stage is comparatively negligible. Reserve deposition is marked by a plateauing of fresh weight while dry weight increases in a rapid linear fashion. This increasing proportion of dry weight as a percentage of total weight leads to a steady decrease in water content of the tissues as reserve materials displace the water within the plants cells. The desiccation phase is marked by a peaking of dry weight and a decline in fresh weight resulting in a seed which has a very low water content, is metabolically quiescent, and is ready for seed dispersal.

The ability of many seed plants to survive desiccation during seed dispersal has given them a major evolutionary advantage over plants which cannot survive desiccation, as it allows for greatly enhanced spatial and temporal distribution of their progeny. Several mechanisms have been hypothesized to provide desiccation tolerance in developing seeds. Carbohydrates such as sucrose and the oligosaccharides raffinose and stacchyose have been suggested to provide a stabilizing matrix for cell membranes by the hydrophillic binding of the hydroxyl groups of sucrose to the polar ends of the membranes phospholipids (Leopold & Vertucci, 1986). The oligosaccharides are proposed to interact with the sucrose molecules in such a way as to prevent the sucrose from crystallizing. Membrane stability may also be provided by an increased proportion of unsaturated fatty acids in membrane phospholipids(Dasgupta et al., 1982; Kuiper, 1985) and the presence of lipid soluble antioxidants such as tocopherols may limit free-radical mediated damage to the phospholipids(Senaratna et al., 1985a & b). Another putative mechanism of desiccation tolerance is the accumulation during late embryogenesis of a class of highly abundant proteins called LEA(Late Embryogenesis Abundant) proteins. These LEA proteins are strongly hydrophobic and are resistant to denaturation, both qualities which make this group of proteins ideal candidates for desiccation protectants. It has been suggested by Baker et al.(1988) that the many hydrophillic groups present in LEA proteins combined with the tendency of some of them to exist as

amorphous coils may enable them to act as "water-substitutes" to stabilize membranes and other cellular components. LEA proteins have been described from a evolutionary diverse variety of plants including tobacco(*Nicotiana tabacum*), soybean(*Glycine max*), and loblolly pine(*Pinus taeda*)(D.W.Hughes & G.A.Galau, unpublished data) perhaps indicating a fundamental role in desiccation tolerance, and they are known to be regulated at the gene level as a coordinately expressed gene set(Galau et al., 1987; Galau & Hughes, 1987).

A common observation relating to seed development is the ability of embryos isolated from the parent plant to germinate precociously when placed in culture conditions(reviewed in Norstog, 1979; Long et al., 1981) This has been reported for *P.vulgaris*(Long et al., 1981) and for a number of other angiosperms(Norstog, 1979). As they do not normally germinate precociously on the parent plant this has been interpreted by some as being evidence that they are prevented by the parent plant from doing so(Crouch, 1987), most probably through a factor which either maintains embryogeny, inhibits germination, or both.

The exact nature of this controlling factor is not clearly known and has been the subject of much interest. To date, two factors abscisic acid and low water potential have been widely involved in the inhibition of precocious germination. The plant growth regulator abscisic acid(ABA) has received much attention as a possible regulator of many different aspects of seed development. These vary from the accumulation of storage reserves in the embryos of bean(*Phaseolus vulgaris*)(Sussex & Dale, 1979), soybean(*Glycine max*)(Ackerson, 1984; Bray & Beachy, 1985; Eisenberg & Mascarenhas, 1985), rape(*Brassica napus*)(Crouch & Sussex, 1981; Finkelstein et al., 1985), rice(*Oryza sativa*)(Stinissen et al., 1984), and wheat(*Triticum aestivum*)(Triplett & Quatrano, 1982; Williamson et al., 1985), to the induction of enzyme activities such as malate synthase in late embryogenesis(Choinski & Trelease, 1978; Choinski et al., 1981)and the inhibition of other enzyme activities in late embryogenesis such as isocitrate lyase(Choinski et al., 1981). ABA has also been attributed with the ability to bring about the production of certain mRNA and protein species(LEA's) in late embryogenesis

of cotton and other species(Dure et al., 1980; Galau & Dure, 1981; Galau et al., 1986). The evidence for ABA being a preventer of precocious germination is varied but largely indirect: ABA levels are known to be high during the mid phases of seed development in many species and are inversely correlated with lag times for precocious germination in vitro(Hsu, 1979; Prevost and Le-Page Degivry, 1985a & b; Van Onckelen et al., 1980; Ackerson, 1984; Finklestein et al., 1985); precocious germination of developing seeds in culture can be inhibited by the application of exogenous ABA to the growth medium(Van Onckelen et al., 1980; Long et al., 1981; Quatrano, 1986); mutants of both *Arabidopsis* and maize which are either ABA insensitive or deficient, germinate precociously on the parent plant or show reduced dormancy times from wild type lines(Robertson, 1955; Karssen et al., 1983; Koornneef et al., 1984; Koornneef et al., 1989); and application of the ABA synthetic inhibitor fluridone to developing maize seeds results in reduced ABA levels and precocious germination but only within a restricted developmental window(Fong et al., 1983).

Low water potential has been noted to be as effective as abscisic acid in preventing precocious gemination(Norstog, 1979; Crouch & Sussex, 1981; Obendorf & Wettlaufer, 1984; Morris et al., 1985; Barratt, 1986). In *Brassica* this inhibitory effect of low water potential is effective over a wider range of developmental stages than ABA. Interestingly embryos which are prevented from germinating precociously by high osmoticum do not show an increase in endogenous ABA levels. In muskmelon, a fleshy fruited species, the water potential of the fruit tissue surrounding the seed has been shown by Welbaum et al.(1990) to be low enough to preclude precocious germination. By incubating seeds of several developmental ages in varying combinations of ABA and osmoticum they were able to show that in their system ABA and water potential have a linear additive interaction with regard to inhibiting germination. As ABA had no effect on solute potential or water content of the seeds they concluded that ABA and water potential appear to work via different mechanisms with ABA increasing the minimum turgor required for germination and water potential reducing turgor by reducing seed water content.

Developing seeds of *Phaseolus vulgaris* at the late maturation stage (Fig.1) may be induced to germinate precociously in vivo(on the parent plant) by the injection of water into the pod surrounding the seeds. This has been interpreted as being further evidence that in the later stages of seed development water relations may be more important than abscisic acid in preventing precocious germination(Fountain & Outred, 1990).

One approach to identifying factors which prevent precocious germination at late maturation is to characterize treatments which disrupt the normal control mechanisms. The incubation of late maturation *Phaseolus vulgaris* embryos in the presence of the plant growth regulator ethylene, has been found to promote precocious germination. This promoting effect takes place regardless of whether the embryo has been supplied with free water or not, so presumably water for axis elongation may be somehow made available from the cotyledons. By injecting ethylene into the pod it has been shown that ethylene may also cause precocious germination in vivo(Fountain & Outred, 1990).

The occurrence of ethylene-induced precocious germination in *P.vulgaris* combined with the conveniently large seed size make it an attractive model system for the study of the controls of seed development. This large seed size makes for easy dissection and also provides ample tissue mass for physiological, biochemical, and molecular studies. In addition it is easy to grow in glasshouse conditions and flowers and sets seed all year round, thereby providing the researcher with access to a number of developmental stages at any one time.

In other plant systems ethylene is known to induce changes in gene expression at the transcriptional and translational levels. Broadly speaking the genes concerned with these changes may be related to the processes of fruit ripening, plant senescence, or plant stress, including responses to flooding and oxygen deprivation, wounding, and pathogen attack(reviewed in Broglie & Broglie, 1991). To date no ethylene related changes in gene expression have been reported from plant embryos, although ethylene responsive chitinase mRNA's

have been reported from both tomato and bean seedlings(Broglie et al., 1986) and from the aleurone layer of barley grains(Swegle et al., 1989), ethylene-induced peroxidases have been described from cucumber(*Cucumis sativus*) seedling cotyledons(Abeles et al., 1988) and the ethylene-induced senescence-related protein DIN1 has been described from radish(*Raphanus sativus*) seedling cotyledons(Azumi & Watanabe, 1991).

Among the thirteen stress-related ethylene-induced genes listed on the EMBL database(as at November 1993), seven of these encode for an endochitinase-type product. The putative function of this enzyme is to digest away the cell walls of fungal and also some bacterial plant pathogens and thereby limit the spread of infection(Boller et al., 1983). These endochitinase genes were isolated from a variety of plant species including barley(*Hordeum vulgare*), *Arabidopsis*, tobacco(*Nicotiana tabacum*), and bean(*Phaseolus vulgaris*), and are expressed in a number of organs ranging from leaves(tobacco, *Arabidopsis*), roots(*Arabidopsis*), and flowering shoots(*Arabidopsis*) to the aleurone layer(barley).

Three other genes with a similar function to the chitinases are listed on the EMBL database as being ethylene-inducible; these are the β-1,3-glucanases which are thought to act on different components of fungal cell walls than the chitinases(Felix & Meins, 1987). One β-1,3-glucanase gene has been isolated from rice(*Oryza sativa*) and two from tobacco suggesting that β-1,3-glucanases may be a multigene family.

Of the remaining three genes no clear physiological function can yet be ascribed to any of them. The pattern of expression of two of them, tomato ethylene-inducible P1(p14) protein and tobacco pathogenesis-related protein 1b, suggests that they may be pathogenesis related(Eyal et al., 1992), while the remaining gene encodes for a potato heat-shock protein the exact function of which is unknown.

Five ethylene-induced genes listed on the EMBL database relate to plant

senescence. Abscission cellulase has been isolated from *P.vulgaris* where it is thought to degrade cell walls in the abscission zone located at the bases of leaves(Tucker et al., 1988). A peroxidase has been isolated from senescing cucumber(*Cucumis sativus*) cotyledons where its exact function remains uncertain(Morgens et al., 1990), and a glutathione-S-transferase gene has been described from senescing carnation(*Dianthus caryophyllus*) petal tissue where its precise function is once again unknown(Meyer et al., 1991). The remaining two senescence-related genes have yet to be connected with an enzymatic activity. The first of these encodes for SR12, a product isolated from senescing carnation petals(Ragothama et al., 1991). The second of these encodes for the previously mentioned DIN1 protein and was isolated from senescing radish cotyledons where it is thought to act in the early stages of senescence(Azumi & Watanabe, 1991).

Two ethylene-inducible genes have been isolated which are associated with the process of fruit ripening. A cytochrome P450-type product has been described from the mesocarp of ripening avocado(*Persea americana*) fruit where it is hypothesized to be involved in the development of flavour, possibly by acting as a trans-cinnamic acid 4-hydrolase(Bozak et al., 1990), and a proteinase inhibitor I, showing some similarity to potato chymotrypsin inhibitor I, has been isolated from ripening tomato fruit(Margossian et al., 1988).

The exact mechanism by which ethylene-induced precocious germination occurs in *P.vulgaris* is not clear and is the focus of experiments reported in this thesis. These experiments are based on the following hypothesis: "That late maturation *Phaseolus vulgaris* seed axes triggered by ethylene to germinate precociously, will show qualitative and quantitative changes in translatable mRNA from normally germinated seed axes".

METHODS

Plant Material

French Bean(*Phaseolus vulgaris* L. cv Seminole) plants were grown in pots in a glasshouse, by weekly plantings from seed, so that a wide variety of developmental stages were available at any one time. The growing medium consisted of 50% peat, 25% sand, and 25% pumice into which 500g dolomite, 200g 3-month Osmocote(Sierra Chemicals, Holland), and 150g superphosphate had been added per 30 litres of medium. The glasshouse temperature varied between 25°C-30°C during the day and 15°C at night during summer, to 20-25°C during the day and 12°C at night during winter, and a natural photoperiod was experienced by the plants.

Incubation conditions

Seeds from the late maturation stage (32-40 days after anthesis; Fig.1) were removed from the pod and the testa of each was carefully removed using a scalpel blade. These embryos were placed on a petri dish in gas-tight 600ml plastic containers(Fig.2), in which high humidity was maintained by a 15x250mm piece of Whatman 3MM chromatography paper, moistened with distilled water, which lined the container. An ethylene gas mixture(100ppm) was prepared by inflating a rubber balloon, in which the neck had been stoppered with a rubber gas port, with ethylene(New Zealand Industrial Gases), then removing 6ml with a syringe and injecting this volume into a 600ml container from which 6ml of air had been previously removed. 6ml of gas was then removed from this mixing container and injected immediately into the final 600ml incubation container, from which 6ml air had also been previously removed.

FIGURE 1

Stages of pod and seed development in *Phaseolus vulgaris*. At the late maturation stage (the stage primarily studied in this thesis) the pods are achlorophyllous but still discernably fleshy. The testa of the seed is a mottled red colour.

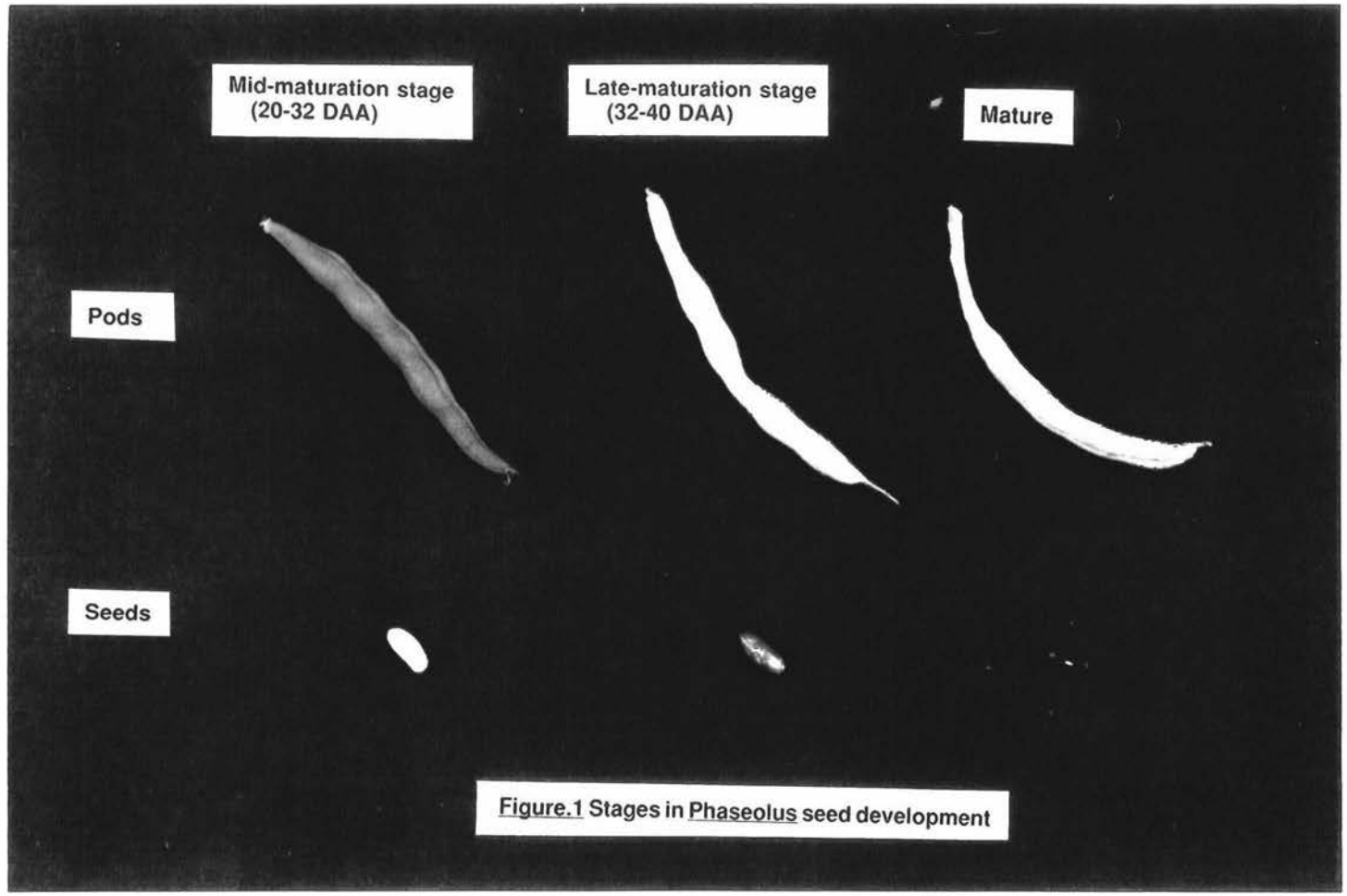
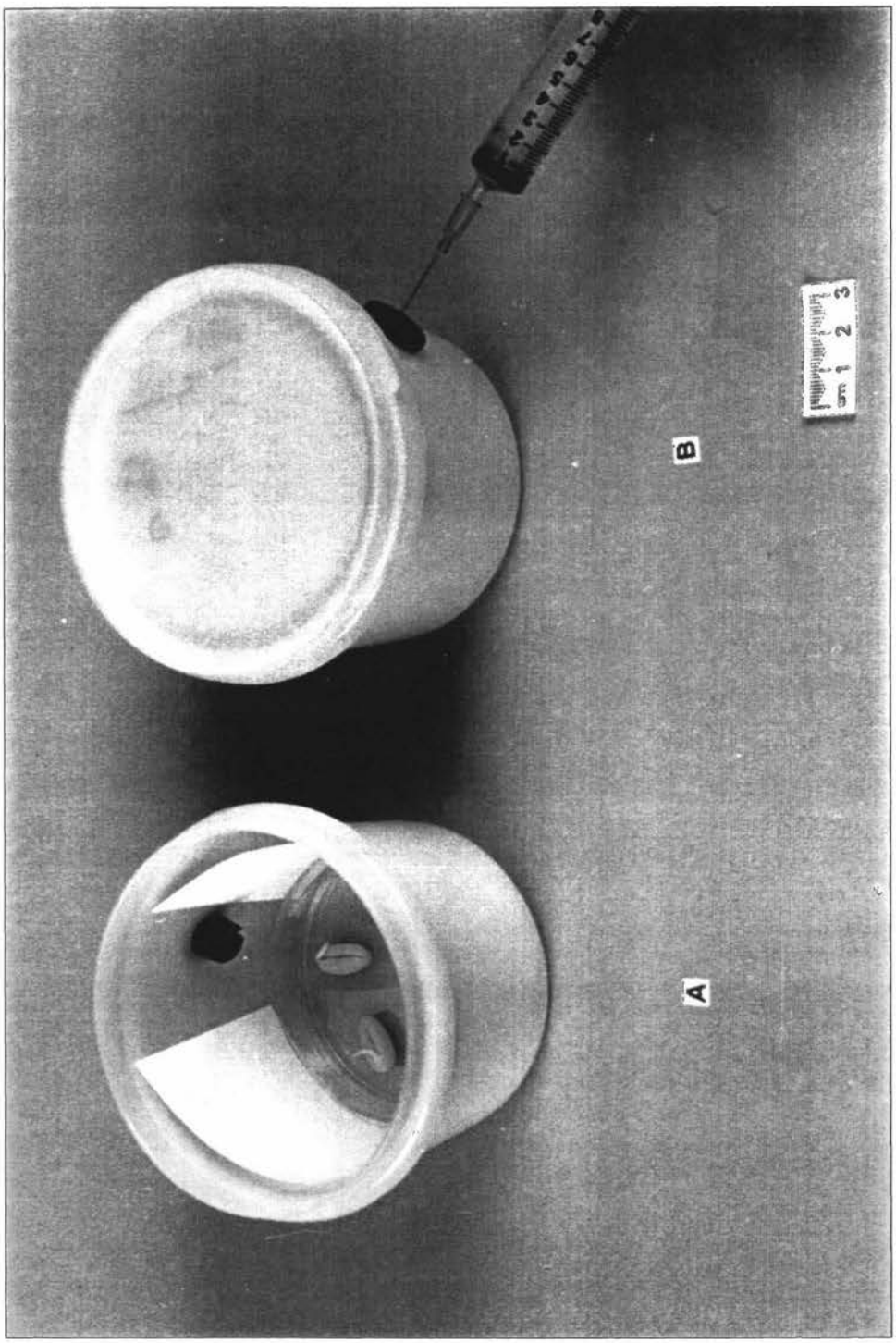


Figure.1 Stages in Phaseolus seed development

FIGURE 2

Gas-tight containers which were used for the incubation of *Phaseolus vulgaris* embryos. (A) Embryos were placed on a dry plastic petri dish and high humidity was maintained by a moist strip of paper which lined the container. (B) Ethylene was introduced into these containers by injecting through the gas ports in the container walls.


A
B

The embryos were incubated at 22°C and 100% relative humidity in darkness in the presence of 100ppm ethylene for 3,6,12, or 24 hours. As a control to this condition similar embryos were incubated at 22°C and 100% relative humidity in the absence of ethylene, for 0,3,6,12, or 24 hours. Mature *P.vulgaris* seeds were incubated, on their sides, on a cotton wool bed which enabled 50% immersion in distilled water, at 22°C and 100% relative humidity, for 0,6,12, or 24 hours. These conditions simulated a normal germination regime.

Fresh Weight and Radicle Length measurements

Twenty embryos per treatment(i.e. precocious germination, normal germination, and the control) were weighed at 0,3,6,12, and 24 hours and the mean was calculated for each timepoint. At these timepoints the length of each embryo's radicle(from the radicle tip to the outside point of attachment with the cotyledons) was measured using callipers. Care was taken during these measurements to ensure that the embryos did not dry out. In the case of the ethylene treatment, this was measured after the other treatments(to minimise risk of exposing the others to ethylene) and ethylene was reintroduced to the incubation containers after measurement.

RNA Isolation

For each treatment 10 axes (approximately 0.1g fresh weight; FW) were used immediately after embryo incubation for RNA extraction. RNA extraction was by a modification of Wadsworth et al.(1988)(see Appendix 1). All pipette tips, pasteur pipettes, and centrifuge tubes were autoclaved for 15 minutes before use. Where possible solutions were autoclaved before use. Phenol/chloroform/isoamyl alcohol was stored at 4°C in the dark, all other solutions were stored at room temperature.

For the axis samples, tissue was placed in a sterilized plastic bag with 500µl extraction buffer (4M guanidinium thiocyanate(Fluka, Switzerland), 25mM sodium citrate pH 7.0, 1.5%(w/v) sodium N-lauryl sarcosinate(N-methyl-N-(1-oxododecyl)glycine sodium salt), 100mM 2-mercaptoethanol),sealed, and crushed using a rolling-pin or similar. The resulting homogenate was transferred to a sterile 1.5ml eppendorf tube, containing an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) by cutting the corner of the plastic bag with a sterile scalpel blade and squeezing the contents out. The tube was mixed using a vortex mixer and then centrifuged in an Eppendorf 5414S benchtop microfuge for 10 minutes at 10000xG. The top phase was transferred to another tube containing an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and was mixed and centrifuged as before. The top phase was again transferred to another tube and the nucleic acids were precipitated with 2-2.5 volumes of ice-cold 95% ethanol for at least 30 minutes, and then centrifuged for 10 minutes at 10000xG. The supernatant was discarded and the pellet was washed with 1ml 70% ethanol and centrifuged for 10 minutes at 10000xG. The supernatant was discarded and the pellet dried under vacuum for 30 seconds.

The pellet was resuspended in 500µl sterile water and then 0.1 volume 3.0M sodium acetate (pH 6.0) and 0.8 volume isopropanol was added before incubating at -80°C for 30 minutes to precipitate nucleic acids. The tube was centrifuged for 20 minutes at 14000xG and 4°C, and the supernatant discarded. 1ml of 3M sodium acetate (pH 6.0) was added, the pellet was resuspended, and the tube was then centrifuged for 20 minutes at 14000xG and 4°C. After the supernatant was discarded, 1ml of 3M sodium acetate (pH 6.0) was added, the pellet resuspended, and the tube again spun for 20 minutes at 14000xG and 4°C. The supernatant was discarded, 1ml of 80% ethanol/20% 0.5M potassium acetate (pH 6.0) was added, the pellet resuspended, and the tube was then centrifuged for 20 minutes at 14000xG and 4°C. Again the supernatant was discarded, 1ml of 80% ethanol/20% 0.5M potassium acetate (pH 6.0) was added, the pellet resuspended, and the tube was then centrifuged for 20 minutes at 14000xG and

4°C. The supernatant was discarded, 1ml of 80% ethanol added, the pellet resuspended, and the tube was then centrifuged for 20 minutes at 14000xG and 4°C. The supernatant was carefully discarded, taking care not to disturb the pellet, which was then dried under vacuum for 30 seconds. The pellet was then resuspended in 50µl sterile water.

3µl of this RNA solution was added to 750µl sterile water in a 1ml quartz cuvette and then scanned in a Shimadzu UV-160A spectrophotometer. The ratio between the absorbance of the sample at 260nm and at 280nm was used as an estimate of the purity of the RNA sample(Sambrook et al.,1989).

Horizontal agarose gel electrophoresis

6µl RNA sample was loaded with 1µl loading buffer (30%(w/v) Ficoll(Type 400; Pharmacia), 0.25%(w/v) bromophenol blue, 0.25%(w/v) xylene cyanol FF, 1mM EDTA.Na_2, sterile filtered and stored in aliquots at -20°C) onto a horizontal gel consisting of 1% agarose, 0.045M Tris-borate, 0.001M EDTA, and 0.001% ethidium bromide. 1µl of λ DNA/HindIII double-stranded DNA markers(Bethesda Research Laboratories, USA) was loaded with 1µl loading buffer, to ensure that the gel ran truely. The gel was run in 1x TBE running buffer (0.045M Tris-borate, 0.001M EDTA) at 100 V constant current for approximately 45 minutes and photographed under ultraviolet light using Polaroid 667 Professional film.

In vitro translation

In vitro translation was performed according to Krawetz et al.(1983) using a Type 1, Reticulocyte Translation Kit(amino-acid depleted; Boehringer Mannheim, Germany). For each reaction 2μg RNA in 8.5μl sterile water was added to 2μl L-[^{35}S] methionine (15mCi/ml,>1000Ci/mmol; code no.SJ1515, Amersham, Australia), 2μl translation reaction mix, 1.5μl magnesium acetate(final concentration 1.5mM), and 1μl potassium acetate(final concentration 100mM), in a sterile 1.5ml eppendorf tube. This was mixed using a vortex mixer, and then centrifuged at 10000 rpm in a benchtop microfuge for a few seconds to return contents to the bottom of the tube. The rabbit reticulocyte lysate was removed from the freezer, quickly thawed, and 10μl added to the translation reaction tube which was then incubated at 30°C for 60 minutes to allow translation to occur. The reaction was stopped by the addition of 1μl RNaseA(10mg/ml, Sigma,USA; boiled for 10 minutes) and subsequent incubation at 30°C for 15 minutes. For each batch of translation reactions one reaction blank was prepared as above but with 8.5μl sterile water substituted for the RNA solution.

Incorporation of labelled methionine into TCA-precipitable protein was assayed as follows. 1μl of translated reaction was spotted onto approximately 0.5 cm^2 of Whatman 3MM chromatography paper, attached to disposable syringe needles for ease of handling, and allowed to air dry. This was done for each reaction. The papers were placed in ice-cold 10%(w/v) trichloroacetic acid(TCA), containing 0.5%(w/v) unlabelled methionine, for 10 minutes, before being transferred to 5%(w/v) TCA and boiled for 10 minutes. They were then transferred to ice-cold 5%(w/v) TCA for 10 minutes, and then rinsed in 100% ethanol for 10 minutes, before being allowed to air dry. An additional 1μl sample was taken from the blank reaction in order to estimate the total radioactivity present in the reaction tube. This was spotted onto paper and air dried as above but it did not undergo the TCA and ethanol washes. Each paper sample was placed in 10ml of BCS scintillant(code no.nbcs104, Amersham, USA) and then counted in an LKB 1219 Rackbeta liquid scintillation counter using the

^{14}C channel.

One Dimensional Gradient SDS-PAGE

Vertical gradient gels of 13.5cm x 11.5cm were poured in ethanol cleaned plates at 1.0mm thickness. Gradients were made using an LKB Multiphor II gradient former consisting of two chambers joined at the base by a valved channel. A length of peristaltic tubing lead from the mixing chamber through a peristaltic pump via a glass capillary tube to the base of the gel plates. A small mixing flea was placed in the mixing chamber and the gradient former sat in ice on a magnetic stirrer. The gel solutions were poured into the pots with the higher percentage solution (17% acrylamide= 6.3ml 50% acrylamide/1% bisacrylamide, 4.5ml 50% sucrose, 2.25ml distilled water, 1.35ml ammonium persulphate, 4.6ml Separating gel buffer [2M urea, 1.5M Tris-HCl pH 8.5, 0.4%(w/v) SDS], 10µl TEMED) being added to the reservoir chamber, and the lower percentage solution (15% acrylamide= 5.7ml 50% acrylamide/1% bisacrylamide, 4.5ml 50% sucrose, 2.85ml distilled water, 1.35ml 0.3% ammonium persulphate, 10µl TEMED, 4.6ml Separating gel buffer[2M urea, 1.5M Tris-HCl pH 8.5, 0.4%(w/v) SDS]) being added to the mixing chamber. Solution was removed from the mixing chamber by the action of the pump and delivered to the base of the gel plates. This was constantly replaced by solution from the reservoir chamber thus increasing the acrylamide percentage in the mixing chamber. This increasingly dense solution flowed along the base of the plates forcing the earlier added less dense solution upwards. Before the gel was poured 5-10ml distilled water was added to the plates to help form a smooth surface for the gel. All solutions were kept on ice until ready for mixing gradients. This separating gel was left to set overnight, then the water was removed, the gel comb put in place, and the stacking gel (1.2ml 50% acrylamide/1% bisacrylamide, 1.3ml distilled water, 2.5ml 0.3% ammonium persulphate, 5.0ml Stacking gel buffer [2.66M urea, 0.5M Tris-HCl pH 6.8, 0.4%(w/v) SDS], 5µl TEMED) was poured using a pasteur pipette.

Protein sample volume was adjusted to give 100000 cpm per lane. The sample was added to 20µl sample loading buffer (25%(w/v) sucrose, 5%(v/v) 2-mercaptoethanol, 2.5%(w/v) SDS, 50mM Tris, bromophenol blue till desired colour is reached), boiled for 4 minutes, and cooled before loading onto the gel. 2µl of Rainbow [^{14}C] methylated protein molecular weight markers (MW 2350-46000, Amersham) was loaded per standard lane.

Gels were run at 30mA (per gel) constant current, in running buffer(2.88%(w/v) glycine, 0.6%(w/v) Tris, 0.1%(w/v) SDS), until the dye front reached the base of the gel (3-4 hours).

Fluorography for protein gels

Gradient gels were fixed for 30 minutes-2 hours in 25% glacial acetic acid /40% ethanol. The fixative was discarded and the gels were then impregnated for 30 minutes with an enhancer consisting of 668ml glacial acetic acid, 280ml methanol, 50g 2-methylnapthalene, 5g 2,5-diphenyloxazole(PPO). Following this the gel was thoroughly rinsed with water to remove excess enhancer before being dried down at 68°C for approximately 90 minutes, using an Atto AE-3700 gel drier, on to Whatman 3MM paper. This dried gel was then placed in an X-Omatic Regular cassette(Kodak, USA) and used to expose X-Omat K x-ray film(Kodak, USA) for 5 days at -80°C. The image formed on this film was then scanned with an LKB Ultroscan XL densitometer with the following settings: smoothing:yes ; X-width:3 ; peak-width:3 ; type of beam:line ; base line:type 1. Care was taken to ensure that the scan length for each lane was identical as it was noted that this affected the baseline setting procedure. In each case the dye front was not scanned as this interfered both with base line setting and with data interpretation.

Protein extraction and analysis

Protein bodies were extracted by a modification of the method of Chrispeels et al.(1982). Axis tissue (30 axes/treatment) was finely chopped with a scalpel blade in 1ml homogenization buffer(12%(w/v) sucrose, 2mM $MgCl_2$) before being passed through a 200μm nylon mesh. The resulting liquid was carefully layered on top of 1ml 16%(w/v) sucrose/2mM $MgCl_2$ and centrifuged at 1000xG for 10 minutes in a Heraeus Sepatech Megafuge 1.0(3360 rotor), before the sample was separated into a supernatant fraction(protein body depleted) and a pellet fraction(protein body enriched). The pellet fraction was resuspended in 100μl 0.05%(w/v) SDS and boiled for 5 minutes to disrupt protein bodies and solubilise their contents.

Samples were assayed for total protein content using a modification of the method of Bradford(1976). 1ml of Bradford's reagent was added to 20μl sample and the absorbance at 595nm was measured after 2 minutes in a Pye Unicam SP8-400 UV/Vis spectrophotometer which had been zeroed with an appropriate blank. In the case of the pellet fractions this was 20μl 0.05% SDS + 1ml Bradford's reagent. In the case of the supernatant fractions this blank was 20μl 14%(w/v) sucrose/2mM $MgCl_2$ + 1ml Bradford's reagent. Total protein content was calculated by plotting the absorbance reading for each sample against a standard curve, constructed by determining the absorbance at 595nm of protein standards. The protein standards, made with Bovine Serum Albumin(fraction V,BDH, England), were between the range of 0-1000μg/ml, and were made up in 0.05% SDS in the case of the pellet fractions or water in the case of the supernatant fractions. Their absorbance at 595nm was determined by adding 100μl protein standard to 5ml Bradford's reagent and assaying 1ml of this in the spectrophotometer.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to Laemmli(1970) using a mini-PROTEAN II slab cell (Bio-Rad, California). Briefly, the separating gel (12% acrylamide, 1.07%

Bisacrylamide, 0.1% SDS, 0.375M Tris pH 8.8, 0.05% ammmonium persulphate, 0.05% TEMED) was cast to dimensions of 8.3cm x 5.5cm between ethanol cleaned plates spaced 0.75mm apart. Once the separating gel had set beneath a layer of water, the water was poured off, the comb inserted and the stacking gel (4% acrylamide, 0.357% bisacrylamide, 0.1% SDS, 0.125M Tris pH 6.8, 0.05% ammonium persulphate, 0.1% TEMED) was poured.

Protein sample volumes were adjusted to give 1.5μg protein per lane. The samples were diluted 1:4 with sample loading buffer(0.0625M Tris-Cl pH6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.125% Bromophenol blue) boiled for 5 minutes, then allowed to cool before being loaded onto the gel. 2μl SDS-PAGE standards(MW 14400-97400, Bio-Rad, California) was diluted with 2μl water and 16μl sample loading buffer then boiled for 5 minutes. 1μl of this mixture was loaded per standards lane.

Gels were run at 200V constant voltage, in running buffer(0.0124M Tris pH 8.3, 0.096M glycine, 4.8%(w/v) SDS), for approximately 35-40 minutes until the dye front reached the base of the gel. After this electrophoresis, gels were stained with Coomassie blue stain (40% (v/v) methanol, 10%(v/v) acetic acid, 0.1% Coomassie blue R-250) for at least 30 minutes, then destained for 60-90 minutes in destain solution (40%(v/v) methanol, 10%(v/v) acetic acid), before being photographed. The gels were then completely destained by incubating them in destain solution at 40°C for 90-120 minutes, before being silver stained. This silver staining was achieved using a Bio-Rad(California, USA) silver stain kit derived from the method of Merril et al.(1981). This kit was used in the following manner. In a chloride-free glass dish, gels were soaked for 15 minutes in 200ml fixative (10%(v/v) ethanol/5%(v/v) acetic acid); 15 minutes in another 200ml fixative; 5 minutes in 100ml oxidizer(Bio-Rad); 5 minutes in 200ml deionized water; 5 minutes in another 200ml deionized water; 20 minutes in 100ml silver reagent(Bio-Rad); and 1 minute in 200ml deionized water. In between each stage the gels were rinsed briefly with deionized water. 100ml developer(Bio-Rad) was then added and the dish containing the gel was placed

over a light box to monitor the development of the staining. If the developer turned a smokey grey colour before the gel was sufficiently stained the developer was discarded and fresh developer added. When the gels were sufficiently stained the developing reaction was stopped with the addition of 200ml 5%(v/v) acetic acid, and the gel was then photographed.

RESULTS

Fresh Weight Changes and Radicle Elongation

Embryos isolated from late maturation stage seeds by removing them from the enclosing testa, were incubated in conditions designed to prevent water loss from the whole embryo. Figure 3 shows typical responses of embryos incubated over 24 hours and shows a comparison with mature seeds incubated with access to water. For both the control treatment and the ethylene treatment, average fresh weight showed only a slight decrease over the 24 hour period. For the control treatment this decrease was from 0.667g(mean fresh weight of 20 embryos) to 0.649g, a decrease of 2.7%, while for the ethylene treatment this decrease was from 0.660g to 0.640g, a decrease of 3.0%. In contrast, the mature seeds incubated with access to water showed a noticeable linear increase in average fresh weight over the 24 hour period, from 0.499g to 0.867g, an increase of 73.7%.

Radicle length in the control treatment showed no detectable change throughout the incubation time(Fig.4). The ethylene treatment however, induced a dramatic increase in radicle length by the end of 24 hours, from 6.56mm to 14.82mm, an increase of 127%(Fig.4). This increase was not apparent at the 6 hour mark but had become detectable by 12 hours. Radicle length was not directly observable in the mature seeds over this time period owing to the presence of the testa. Radicle emergence in this treatment however was observed to occur between 24 and 48 hours.

RNA Isolation

RNA isolated from axes by the described method gave yields between 145ug/g FW for the 12hr control treatment, and 1444ug/g FW for the 0hr normal

FIGURE 3

Typical responses of *Phaseolus vulgaris* seeds and embryos to the experimental treatments.

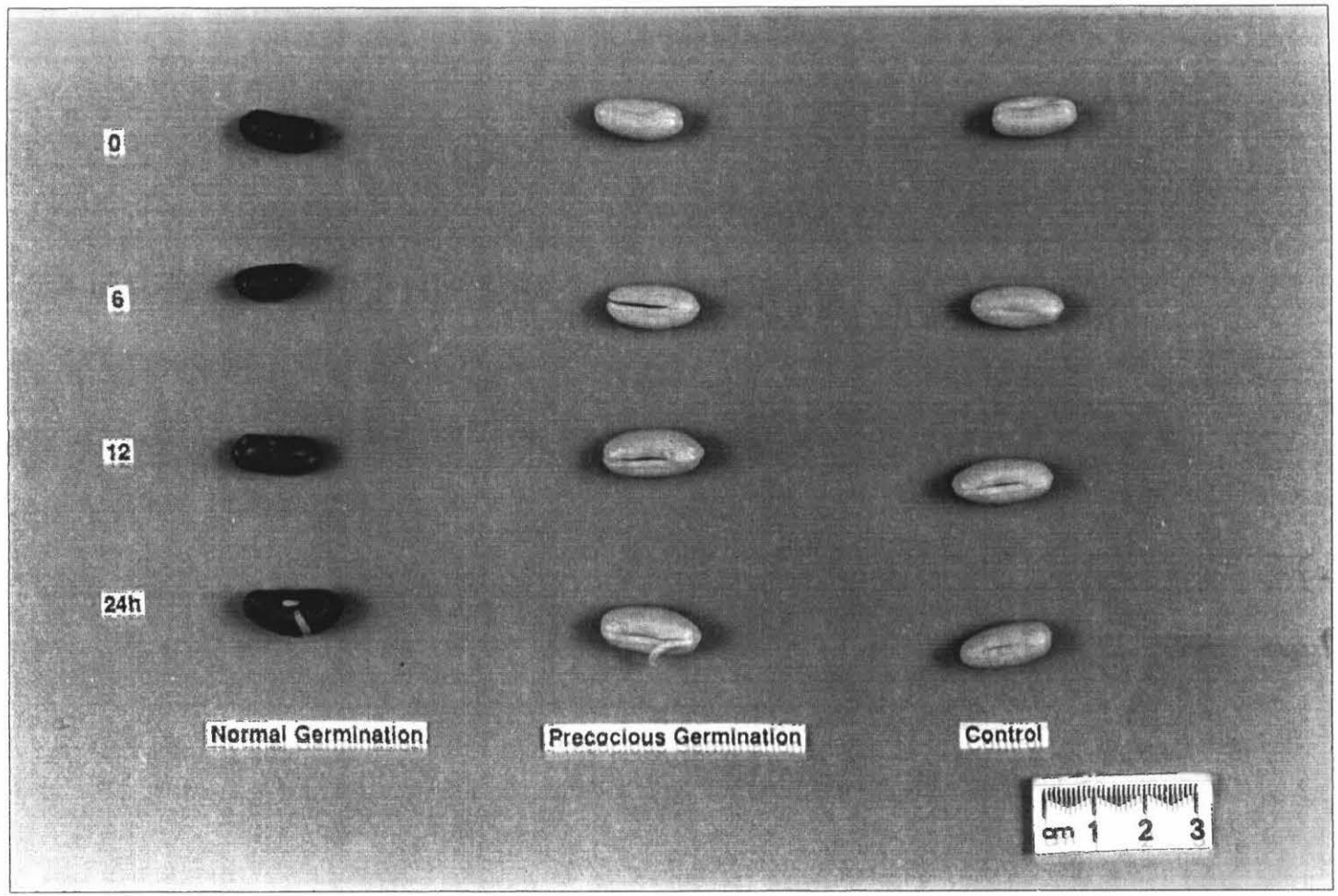
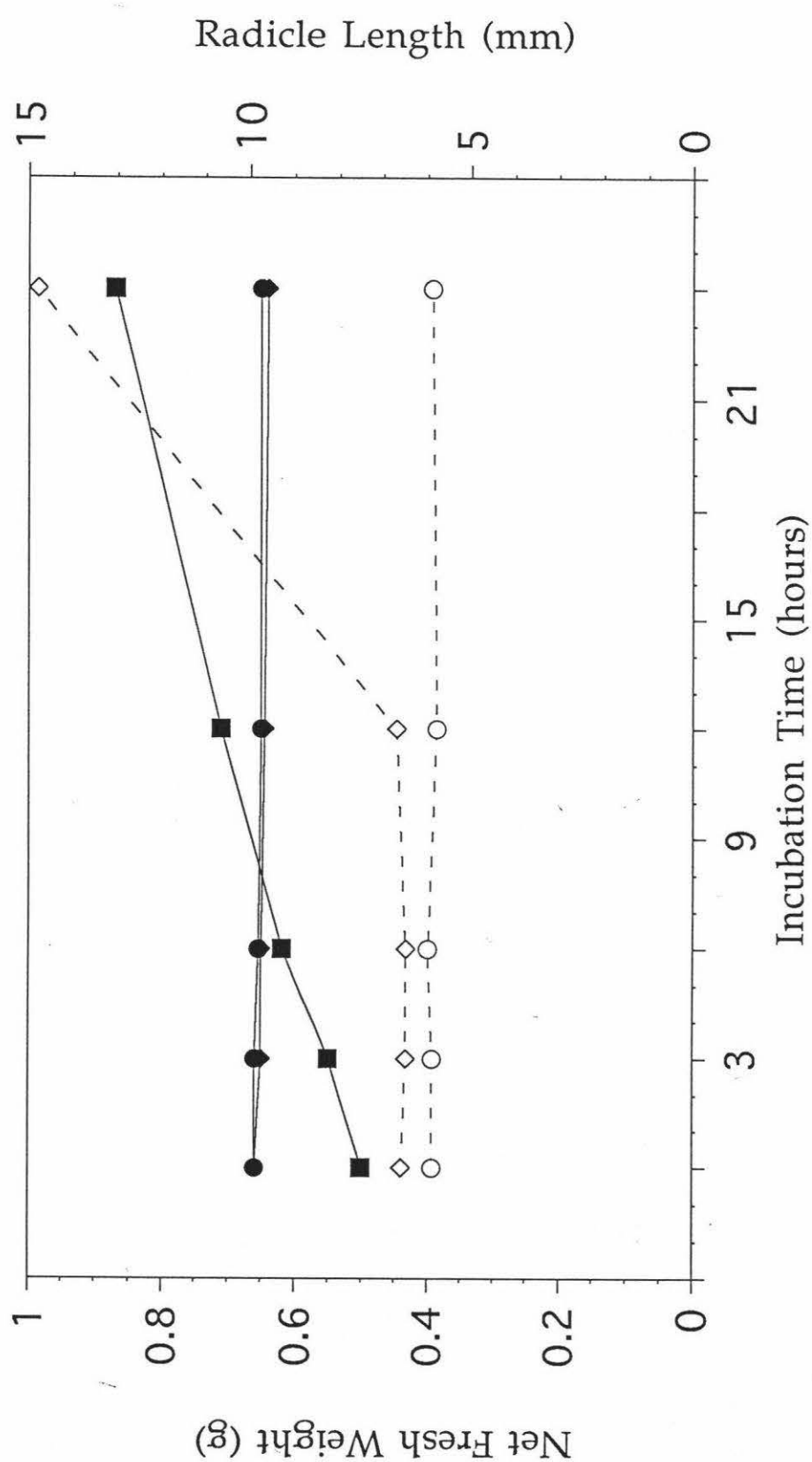

0
6
12
24h
Normal Germination
Precocious Germination
Control
cm 1 2 3

FIGURE 4

Fresh weight changes and radicle elongation of *Phaseolus vulgaris* embryos and seeds during incubation. Symbols are as follows: ●= Fresh weight of the control treatment; ♦= Fresh weight of the ethylene treatment; ■= Fresh weight of the normal germination treatment; ○= Radicle length of the control treatment; ◊=Radicle length of the ethylene treatment. Measurement of the radicle lengths for the normal germination treatment was not possible owing to the presence in this treatment of the testa.


Radicle Length (mm)
15
10
5
0
1
0.8
0.6
0.4
0.2
0
3
9
15
21
Incubation Time (hours)
Net Fresh Weight (g)

germination treatment. Table 1 shows details of the yields obtained for the RNA samples used for the in vitro translation experiments. This RNA was generally of sufficient purity to enable further manipulation. The ratio between the absorbance at 260nm and the absorbance at 280nm for the axes samples ranged between 1.55 for 0hr normal germination treatment and 2.46 for the 6hr control treatment. These ratios (a measure of RNA purity) are presented in Table 1.

RNA Integrity

Integrity of the axis RNA was assayed by electrophoresis on horizontal agarose gels. To ensure that the gels ran truely, λ DNA/HindIII fragments were run next to the RNA samples. Because these fragments are double-stranded and because the single-stranded RNA in these gels cannot be assumed to be completely denatured, the λ DNA/HindIII, while being of known size, cannot be used to accurately size the RNA molecules. RNA banding patterns however will be described in terms of the locus they are at with respect to the λ DNA/HindIII fragments. It is important to stress that these loci do not necessarily correspond with the actual size of the RNA molecules but are arbitrary names awarded to the bands to enable a comparison between samples to be made.

Two separate gel runs were made to assay RNA integrity. In gel A(Fig.5), in all samples tested a band at approximately the 17kb locus was present. In two of the samples tested (0hr germ. axes 10.1.92 and 12hr germ. axes 11.1.92) a band was present at the 2.5kb locus. One sample (24hr germ. axes 11.1.92) yielded a band at the 1.9kb locus which was not identifiable in any other sample. A band at the 1.6kb locus was present in all but two samples (24hr ethylene axes 31.12.91 and 24 germ. axes 11.1.92) although to varying degrees (compare 3hr ethylene axes 23.12.91 with 3hr ethylene axes 14.11.91). In one sample (24 germ. axes 11.1.92) a band at the 1.5kb locus was detectable which was not in any others. A band at the 1.4kb locus was present, with varying intensity, in 19 of

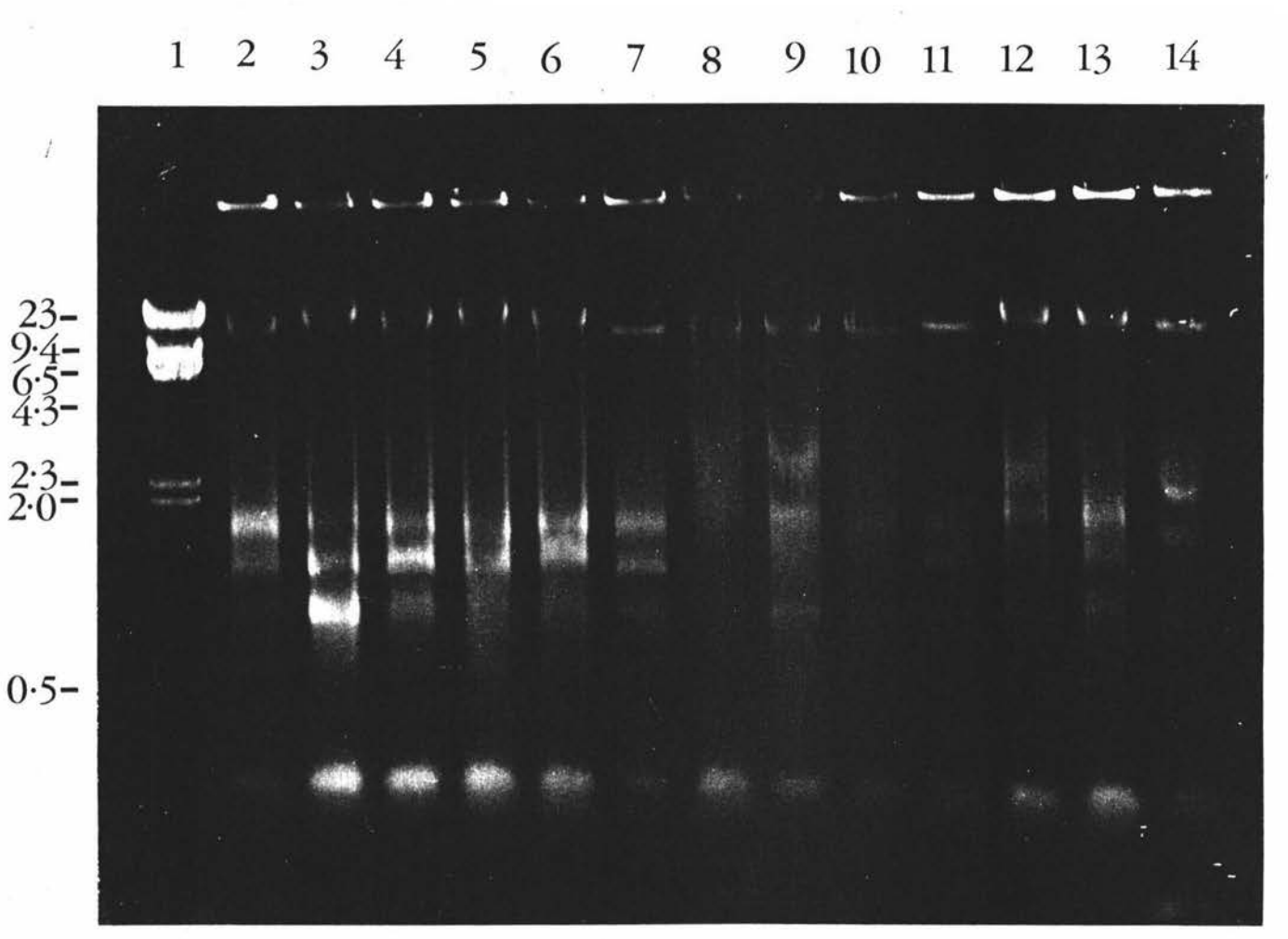
TABLE 1

Yields and purity of *Phaseolus vulgaris* RNA isolated using guanidinium and phenol/chloroform extraction procedures. The abbreviation "germ." refers to the normal germination treatment.

Sample	Yield (μg/g FW)	Purity (A_{260}/A_{280})
3hr ethylene axes 23.12.91	413	1.76
6hr ethylene axes 27.12.91	896	1.82
12hr ethylene axes 8.1.92	274	1.84
24hr ethylene axes 31.12.91	587	1.99
0hr control axes 9.1.92	357	1.69
3hr control axes 9.1.92	675	1.70
6hr control axes 29.11.91	297	2.46
12hr control axes 11.1.92	145	2.39
24hr control axes 31.12.91	1332	2.04
0hr germ. axes 10.1.92	1444	1.55
6hr germ. axes 11.1.92	272	1.62
12hr germ. axes 8.1.92	264	1.90
24hr germ. axes 8.1.92	810	1.68

FIGURE 5

Agarose gel electrophoresis of *Phaseolus vulgaris* RNA isolated using guanidinium and phenol/chloroform extraction procedures(Gel A). The lanes are as follows: 1. λ/Hind III markers; 2. 3hr ethylene axes 23.12.91; 3. 3hr ethylene axes 14.11.91; 4. 6hr ethylene axes 27.12.91; 5. 6hr ethylene axes 29.11.91; 6. 12hr ethylene axes 11.1.92; 7. 12hr ethylene axes 8.1.92; 8. 24hr ethylene axes 31.12.91; 9. 0hr normal germination axes 10.1.92; 10. 6hr normal germination axes 11.1.92; 11. 12hr normal germination axes 8.1.92; 12. 12hr normal germination axes 11.1.92; 13. 24hr normal germination axes; 14. 24hr normal germination axes 11.1.92. Samples showing the least degradation were used for subsequent in vitro translation analysis. Units of the markers are kilobases.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
23-
9·4-
6·5-
4·3-
2·3-
2·0-
0·5-

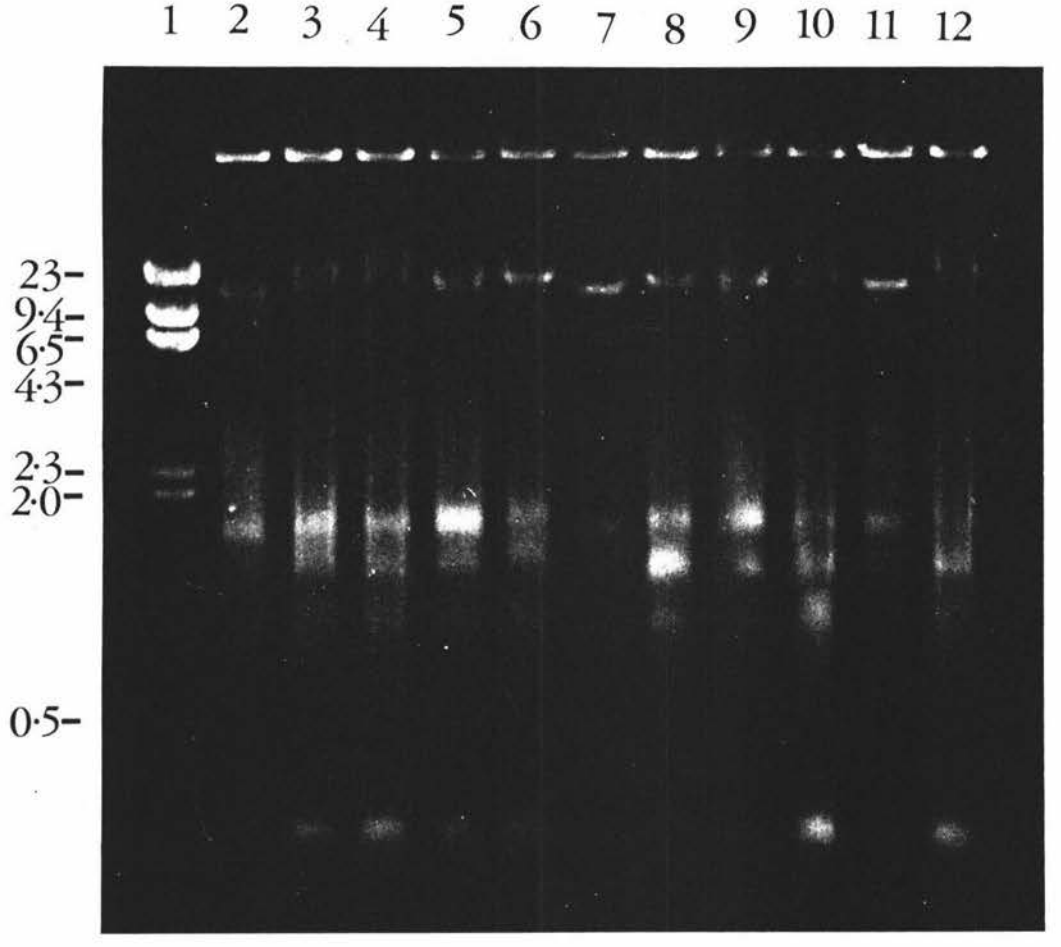
the 24 samples tested. In at least 21 of the samples a band at the 0.8kb locus was distinguishable. Without exception all of the samples showed a band at a locus of less than 0.5kb(0.27kb by extrapolation of the standard curve), again with varying intensity. The 24hr ethylene axes (31.12.91) sample showed only the 17kb locus and the <0.5kb locus bands, however this lack of some of the putative rRNA bands did not apparently indicate serious degradation of the mRNA in this sample as it was later translated in vitro at a rate comparable with other samples(Table 3; Fig.7). Alternatively degradation may have been confined to the aliquot of the sample that was used for electrophoresis. Interestingly the two samples which showed a band at the 2.5kb locus did not have recognizable bands at the 1.4kb locus. Also the sample (24hr germ. axes 11.1.92) which contained two apparently unique bands (at the 1.9kb locus and the 1.5kb locus), did not contain the otherwise common 1.6kb locus and 1.4kb locus bands, but did have the 17kb locus and <0.5kb locus bands present.

All of the samples run on gel B(Fig.6) had a band present between the loci of 30kb and 13kb, this band was crisp in some lanes but quite diffuse in others. A band at the 1.7kb locus was present to at least some degree in all samples, being most strongly represented in the 3hr.control axes(9.1.92) sample and least so in the 6hr.control axes(10.1.92) sample and the 24hr. control axes(31.12.91). Also present in all samples was a band at the 1.3kb locus, and once again the intensity of this band varied across the lanes, being strongly present in some samples(3hr.control axes 9.1.92, 6hr.control axes 29.11.91) and barely perceptible in others(0hr.control axes 9.1.92, 12 hr.control axes 11.1.92). Most samples showed a somewhat diffuse band at around the 0.9kb locus, however in a few(generally weak) samples this was barely detectable. A band at a locus of less than 0.5kb(0.25kb by extrapolation of the standard curve) occurred in all samples and as with most other bands its intensity varied between samples.

In general the samples on gel B(all control treatment samples) showed a greater consistency in their banding pattern than did samples on gel A(ethylene treatment and normal germination treatment samples) where the ethylene

FIGURE 6

Agarose gel electrophoresis of *Phaseolus vulgaris* RNA isolated using guanidinium and phenol/chloroform extraction procedures(Gel B). The lanes are as follows: 1. λ/Hind III markers; 2. 0hr control axes 9.1.92; 3. 0hr control axes 23.12.91; 4. 3hr control axes 27.12.91; 5. 3hr control axes 9.1.92; 6. 3hr control axes 23.12.91; 7. 6hr control axes 10.1.92; 8. 6hr control axes 29.11.91; 9. 6hr control axes 27.12.91; 10. 12hr control axes 8.1.92; 11. 12hr control axes 11.1.92; 12. 24hr control axes 31.12.91. Samples showing the least degradation were used for subsequent in vitro translation analysis. Units of the markers are kilobases.


1 2 3 4 5 6 7 8 9 10 11 12
23-
9·4-
6·5-
4·3-
2·3-
2·0-
0·5-

treatment samples showed a more consistent pattern than did the normal germination treatment samples. It was only in some of these normal germination treatment samples that a band at the 2.5kb locus was noted.

In vitro translation

Incorporation of ^{35}S-methionine into TCA-precipitable protein

RNA extracted from bean axes, when translated in vitro, gave stimulation over the blank of between 211% and 568%(Tables 2 and 3). Surprisingly the sample(24 hr ethylene axes) which showed an absence of several putative rRNA bands in the horizontal gel electrophoresis(Fig.5) gave the greatest activity in the rabbit reticulocyte in vitro translation system. No correlation was obvious between the presence and strength of putative rRNA bands on the horizontal agarose gel and the activity of the RNA in the in vitro translation system. The TMV RNA control reaction, supplied with the in vitro translation kit as a control, yielded a stimulation over the blank of 4675%.

The amount of radioactive label incorporated into TCA-precipitable protein varied from 0.3% for the blank of the first batch of reactions, 0.6% for the blank of the second batch of reactions, 0.6% for the least active (0hr germ. axes 10.1.92 and 3hr control axes 9.1.92) bean RNA samples from the first batch of reactions, 1.2% for the least active (24hr control axes 31.12.91) bean RNA sample in the second batch of reactions, 1.1% for the most active (24hr germ. axes 8.1.92) bean RNA sample from the first batch of reactions, 3.2% for the most active bean RNA sample (24hr ethylene axes 31.12.91) from the second batch of reactions, and 11.7% for the TMV RNA control reaction from the first batch of reactions. No TMV RNA control reaction was performed in the second batch of reactions owing to an insufficient amount of ^{35}S-methionine being available.

TABLE 2

Incorporation of ^{35}S-methionine into TCA-precipitable protein by in vitro translation of *Phaseolus vulgaris* RNA. The abbreviation "germ." refers to the normal germination treatment. Total radioactivity/µl as calculated from the blank was 2696905 cpm/µl and total radioactivity/reaction was 67422625 cpm.

Sample	No. of TCA-precipitable counts /µl	No. of TCA-precipitable counts /reaction	% Incorporation	% Stimulation over blank
TMV control	314733 cpm	7868325 cpm	11.7%	4676%
0hr germ.axes 10.1.92	15119 cpm	377975 cpm	0.6%	224%
6hr germ.axes 11.1.92	23812 cpm	595300 cpm	0.9%	353%
12hr germ.axes 8.1.92	20563 cpm	514075 cpm	0.8%	305%
24hr germ.axes 8.1.92	28835 cpm	720875 cpm	1.1%	428%
0hr control axes 9.1.92	18937 cpm	473425 cpm	0.7%	281%
3hr control axes 9.1.92	15412 cpm	385300 cpm	0.6%	229%
Blank	6731 cpm	168275 cpm	0.3%	-

TABLE 3

Incorporation of ^{35}S-methionine into TCA-precipitable protein by in vitro translation of *Phaseolus vulgaris* RNA. Total radioactivity/µl as calculated from the blank was 623941 cpm/µl and total radioactivity/reaction was 15598525 cpm.

Sample	No. of TCA-precipitable counts/µl	No. of TCA-precipitable counts /reaction	% Incorporation	% Stimulation over blank
3hr ethylene axes 23.12.91	8138 cpm	203450 cpm	1.3%	230%
6hr ethylene axes 27.12.91	11670 cpm	291750 cpm	1.9%	330%
12hr ethylene axes 8.1.92	10389 cpm	259725 cpm	1.7%	294%
24hr ethylene axes 31.12.91	20066 cpm	501650 cpm	3.2%	568%
6hr control axes 29.11.91	12497 cpm	312425 cpm	2.0%	353%
12hr control axes 11.1.92	15530 cpm	388250 cpm	2.5%	439%
24hr control axes 31.12.91	7454 cpm	188625 cpm	1.2%	211%
Blank	3532 cpm	88300 cpm	0.6%	–

Gradient SDS-polyacrylamide gel electrophoresis of in vitro translation products

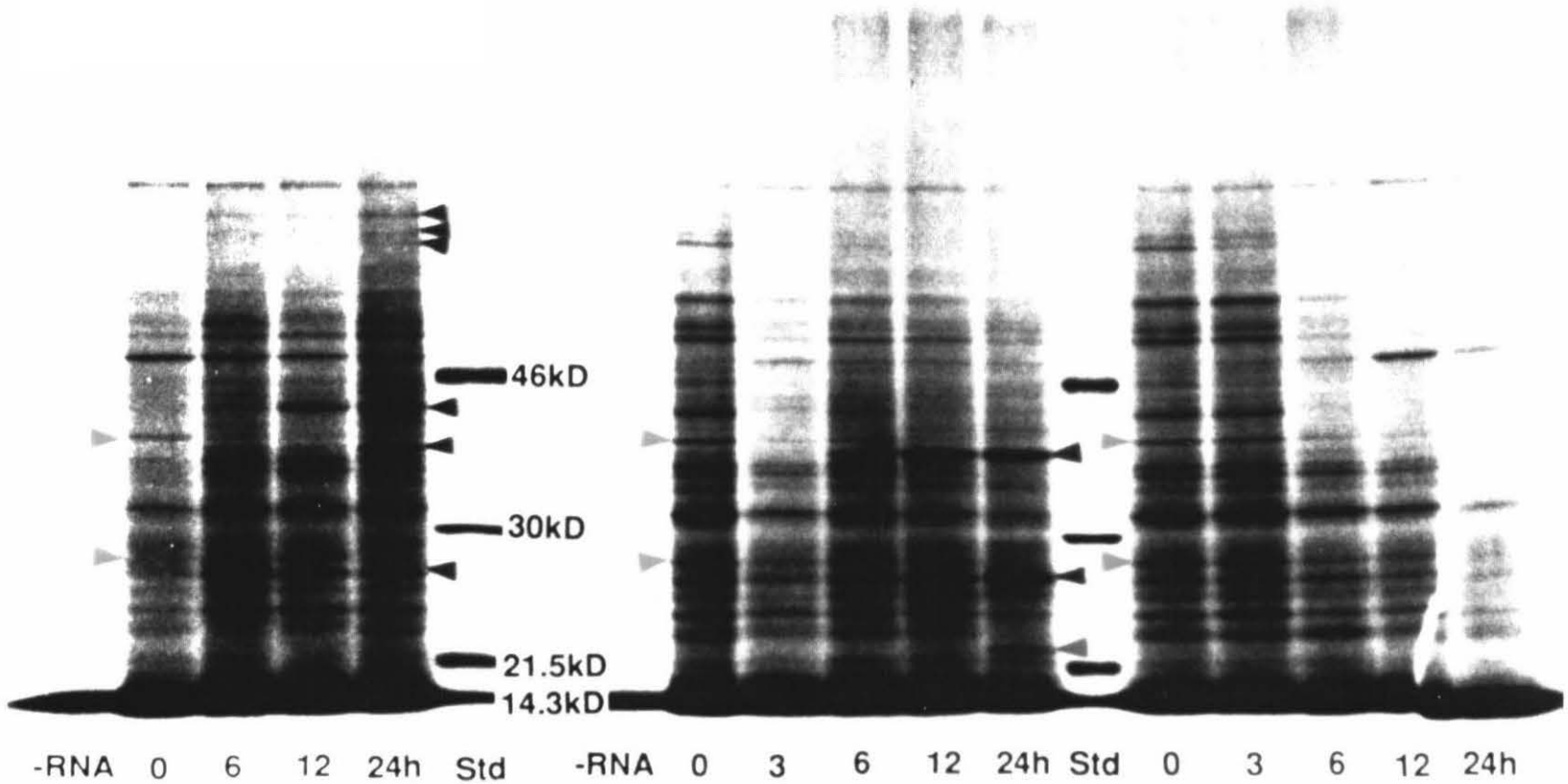
Electrophoresis and subsequent fluorography of TCA-precipitated proteins from the -RNA in vitro translation reaction (i.e. the blank) yielded two clear bands at 76-77kD and 47-48kD respectively, in addition to a heavy dye front(Fig.7). These bands were also present in all other translated samples although there were differences between lanes in the darkness of these bands. The 76-77kD product showed a fairly constant level of expression in each lane, while the 47-48kD product varied from being strongly present in the 12hr control lane and the 0hr normal germination lane, to being barely discernable in the 0hr control lane. While the dye front was very dark in the -RNA reaction, indicating the presence of endogenous products of 22kD or smaller, it was in all cases darker in reactions containing bean RNA.

Gene expression in development and germination

Late maturation embryo axes exhibited a pattern of gene expression which was similar, but not identical, to the pattern shown by normally germinating seed axes after 24hrs(Fig.7). Specifically, two products (71kD and 37-38kD), were present in germinating seed axes but not significantly in developing embryo axes. In normal germination the 37-38kD product had become apparent by 6hrs and was strongly present by 24hrs. Likewise the 71kD protein was detectable by 6hrs and was more strongly present by 24hrs. No products could be identified as being unique to the late maturation developmental state. One protein (28kD) which was strongly present in developing embryo axes was not present in mature dry axes but was synthesized during the earlier stages of normal germination before decreasing by the 24 hour mark. The 38-39kD product, which made up a major component of the translatable RNA in the mature dry seed, and was strongly expressed in the late maturation embryo, was a relatively minor component by 24hrs of germination.

FIGURE 7

Gradient SDS-polyacrylamide gel electrophoresis of in vitro translation products. 100000 cpm from each in vitro translation reaction was loaded per lane. After electrophoresis the gel was fixed, impregnated with scintillant, dried under vacuum, and then used to expose Kodak X-Omat K film. Blue arrows indicate products (71kD, 67-68kD, 65-66kD, and 41-42kD) which appeared in normal germination but not in ethylene-induced precocious germination. Yellow arrows indicate products (38-39kD and 28kD) which were present in development but disappeared in both ethylene-induced precocious germination and normal germination. Green arrows indicate products (37-38kD and 27kD) which were upregulated in both ethylene-induced precocious germination and normal germination. The red arrow indicates a product (22-23kD) which was apparently unique to the ethylene-induced precocious germination treatment.


46kD
30kD
21.5kD
14.3kD
-RNA 0 6 12 24h Std
-RNA 0 3 6 12 24h Std 0 3 6 12 24h
Normal Germination
Precocious Germination
Control

Densitometry was performed to attempt to quantitate the relative signal strength of bands detected. Three parameters can be obtained for each band: absorbance; area(absorbance unit times millimetres or AU*mm); and relative area. Although the 38-39kD product appears to the naked eye to decrease over time, the densitometry data in relation to this is somewhat ambiguous. For example, taking the variable absorbance(see Appendix 2), the 38-39kD protein showed an increase from 0.34 at 0hrs to 0.47 at 24hrs. In contrast the variables area (AU*mm) and relative area indicated an overall decrease. For area these figures were 1.02 at 0hrs, 1.12 at 6hrs, 0.68 at 12hrs, and 0.63 at 24hrs. For relative area these figures were 4.2% at 0hrs, 4.9% at 6hrs, 3.4% at 12hrs, and 2.6% at 24hrs. The products 41-42kD, 40-41kD, 37-38kD, 36-37kD, 35-36kD, 27kD, and 25kD were all up-regulated during normal germination.

Gene expression in ethylene-induced precocious germination

While embryos induced to precociously germinate showed radicle elongation, the pattern of gene expression in the axis was different from that in normal germination. Four products (71kD, 67-68kD, 65-66kD, and 41-42kD) which were up-regulated in normally germinating seed axes were absent in precocious germination(Fig.7).

An immediately noticeable feature of precocious germination was the dramatic increase in the expression level of the 37-38kD and 27kD products(Fig.7). So strong was this up-regulation that in the 24hr ethylene lane these two products accounted for 16.1% of the radioactive signal above the "dye front", as assessed using relative area. The 37-38kD protein which was barely detectable in late maturation increased over the 24hr period from 0.54AU to 1.00AU, from 0.89AU*mm to 1.63AU*mm, and from 3.2%(relative area) to 7.2% (relative area), representing increases of 85%, 83%, and 125% in these respective variables. The 27kD protein which was present in late maturation increased over the 24hr

period from 0.79AU to 1.05AU, from 1.74AU*mm to 2.02AU*mm, and from 6.2% (relative area) to 8.9% (relative area), representing increases of 33%, 16%, and 43% in the respective variables. These above gene products are all candidates for "ethylene-modulated" gene expression(see discussion).

Many products (67-68kD, 65-66kD, 56-57kD, 52-53kD, 51kD, 45-46kD, 41-42kD, 38-39kD, 36-37kD, 35-36kD, 31-32kD, 28kD, and 23-24kD) appeared down-regulated after exposure of the embryo to ethylene, however this may be artifactual(see discussion for explanation).

One protein (22-23kD), which was only barely detectable in other treatments, was strongly up-regulated after 24 hours incubation with ethylene(Fig.7). This product was faintly present at 0 hours but could not be reliably identified at 3,6, or 12 hours after the exposure of the embryos to ethylene. The induction of this product therefore takes place between 12 and 24 hours after exposure to ethylene. This gene product is the single candidate for an ethylene induced gene found in this study(see discussion).

Gene expression in detached non-germinating embryos (i.e. the control treatment)

The most striking feature of this treatment was an apparent decline in translatable RNA over 24hrs between the range of 22kD-77kD(Fig.7). Three bands(67-68kD, 65-66kD, and 41-42kD) had almost completely disappeared by 6hrs after the detachment of the embryo from the parent plant and the removal of the testa. The two products (37-38kD and 27kD) which were so strongly up-regulated in ethylene-induced precocious germination were not apparently up-regulated in this treatment. The 37-38kD product was at best barely present and declined over 24hrs from 0.56AU to 0.21AU, from 0.87 AU*mm to 0.26AU*mm, and from 2.9% (relative area) to 2.4% (relative area) representing decreases of 62.5%, 70.1%, and 17.2% in the respective variables. The 27kD product was

present in late maturation but declined over 24hrs from 0.84AU to 0.33AU, and from 1.58AU*mm to 0.80AU*mm, representing decreases of 60.7%, and 49.3% for the respective variables. The relative area variable however showed no clear trend. The 22-23kD product which was up-regulated after 24hrs exposure to ethylene was barely detectable in the late maturation embryo axis at 0hrs and was entirely absent after 24hrs incubation without ethylene.

In an exception to the above trend, the band at 47-48kD showed an apparent increase 12hrs after the start of incubation but had decreased at the 24hr mark.

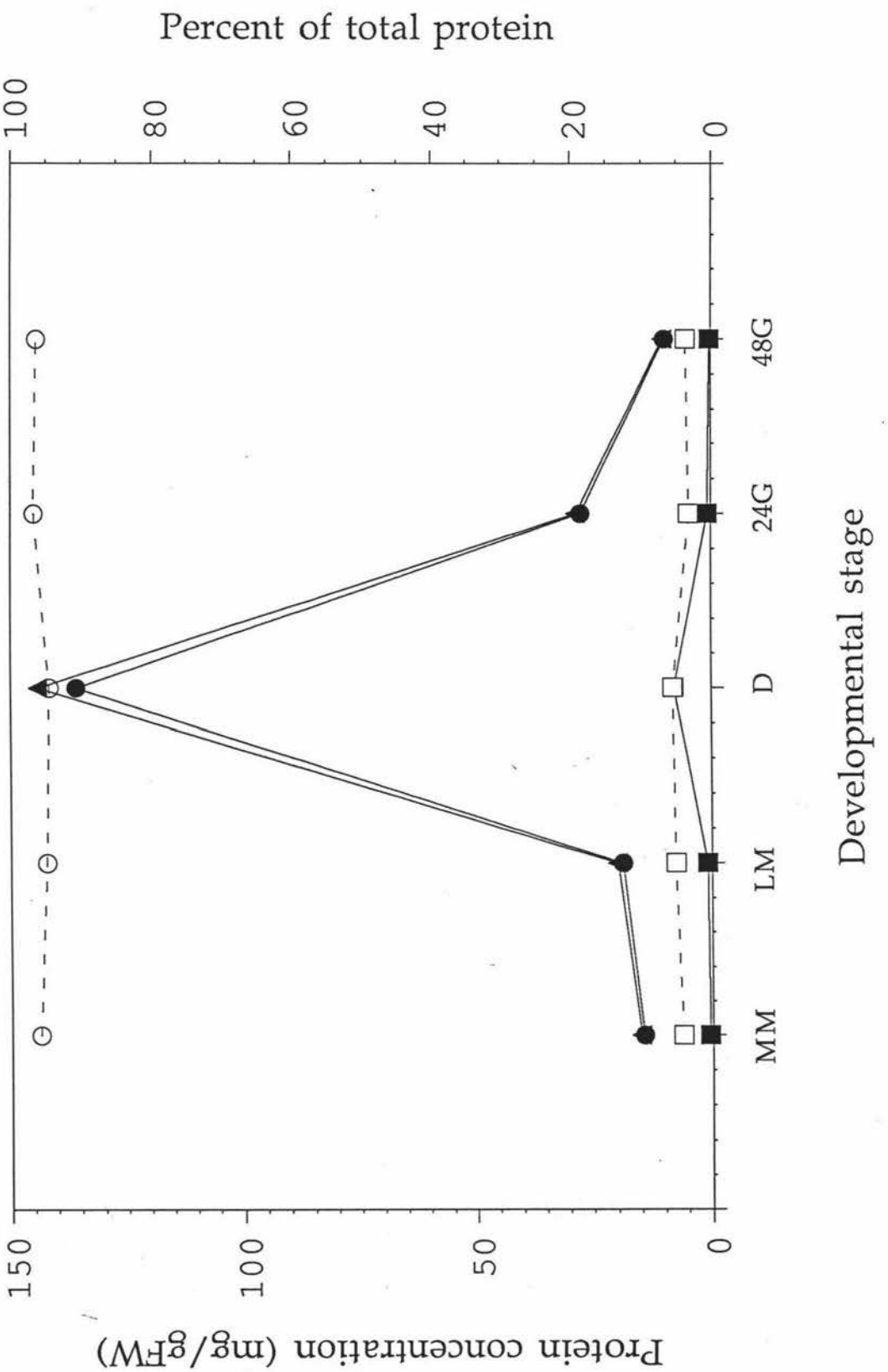
The dye front in the 24hr control lane was generally similar in appearance and size to the dye front in the 0hr control lane, but was noticeably thicker and darker than the dye front in the -RNA lane.

Protein Extraction, Fractionation, and Analysis

Analysis of total protein content by Bradford's assay revealed that axes from the mid-maturation stage(20-32 days after anthesis) contained a total of 15.1mg protein/g FW of tissue(taken as the mean of 30 axes) at the time of sampling(Fig.8). Of this, 14.5mg/gFW separated into the supernatant after centrifugation, representing 95.9% of the total protein, while 0.62mg/gFW separated into the pellet, representing 4.1% of the total protein. By late-maturation(32-40 DAA) total protein to fresh weight had risen to 19.9mg/gFW(mean of 30 axes), of which 18.9mg/gFW(94.9% of the total) was found in the supernatant and 1.02mg/gFW(5.1% of the total) was found in the pellet fraction. In mature dry seed axes the total protein expressed relative to fresh weight was 144.2mg/gFW. This high figure represents not so much a massive increase in protein but rather a dramatic loss of water (and therefore a loss in fresh weight) from the late maturation stage to a mature seed. The supernatant fraction contained 136.3mg/gFW(94.5% of the total) while 7.9 mg/gFW(5.5% of the total) partitioned towards the pellet. Germinating axes at

FIGURE 8

Protein content of putative protein-body enriched (i.e. pellet) and protein-body depleted (i.e. supernatant) fractions over development and germination. Fractions were prepared from 30 *Phaseolus vulgaris* axes by the method of Chrispeels et al.(1982). Symbols are as follows: ▲= Total protein concentration (i.e. unfractionated); ■= Protein concentration of protein-body enriched fractions; ●= Protein concentration of protein-body depleted fractions; □= Percent of total protein of protein-body enriched fractions; O= Percent of total protein of protein-body depleted fractions. Developmental stages are as follows: MM= Mid-maturation (20-32 days after anthesis); LM= Late-maturation (32-40 days after anthesis); D= Dry mature seeds; 24G= 24 hours of germination; 48G= 48 hours of germination.


Percent of total protein
100
80
60
40
20
0
150
100
50
0
Protein concentration (mg/gFW)
MM
LM
D
24G
48G
Developmental stage

24 hours after imbibition yielded 28.6mg/gFW of total protein. 27.7mg/gFW(96.8% of the total) of this was found in the supernatant and 0.90mg/gFW(3.2% of the total) was found in the pellet. At 48 hours after imbibition total protein expressed relative to fresh weight had dropped to 10.34mg/gFW, possibly due to an increase in the water content of the seeds axes. The supernatant fraction contained 9.97mg/gFW(96.4% of the total) while the pellet fraction contained 0.37mg/gFW(3.6% of the total).

Electrophoresis of presumptive protein body depleted(i.e. supernatant) and protein body enriched(i.e. pellet) samples showed that, while the fractions differed in general appearance, both fractions had a similar complexity of protein species in each developmental stage(Figs.9 & 10). At mid-maturation at least 25 bands could be identified in the supernatant fraction while the pellet contained at least 28. In late-maturation the supernatant contained at least 32 bands and the pellet at least 29. After 24 hours of germination the supernatant contained at least 36 bands and the pellet had at least 33 bands. After 48 hours of germination at least 42 bands could be found in the supernatant and at least 36 bands in the pellet. However, supernatant extracted from mature seed axes contained at least 44 bands while only 28 bands could be identified in the pellet fraction.

The supernatant of the mid-maturation extract (Fig.9) was notable largely for a reduced presence or entire absence of many bands which were present in later stages. Polypeptides of 26.5kD, 27.5kD, 28kD, 29kD, 30kD, 33kD, 34kD, 35kD, 36kD, 41kD, and 130kD were completely absent, while the bands of 17.5kD, 20kD, 25kD, 62kD, 68kD, and 81kD were at their weakest when compared with later phases in development and germination. However in contrast to this, the two bands of 49kD and 85kD were at their strongest at this stage.

The pellet fraction at mid-maturation (Fig.10) was the most notably different of all the pellet fractions. This noticeable difference was due to the absence of the 14kD and 17.5kD bands, and to the weak presence of the 20kD, 29kD, 42kD,and

FIGURE 9

SDS-polyacrylamide gel electrophoresis of protein-body depleted fractions prepared from development and germination stages of *Phaseolus vulgaris*. Fractions were prepared from 30 axes by the method of Chrispeels et al.(1982). Lanes are as follows: 1. SDS-PAGE standards (MW14400-97400; BioRad, California); 2. Mid-maturation (20-32 days after anthesis); 3. Late-maturation (32-40 days after anthesis); 4. Mature seed; 5. 24 hours of germination; 6. 48 hours of germination. Units of the markers are kilodaltons.

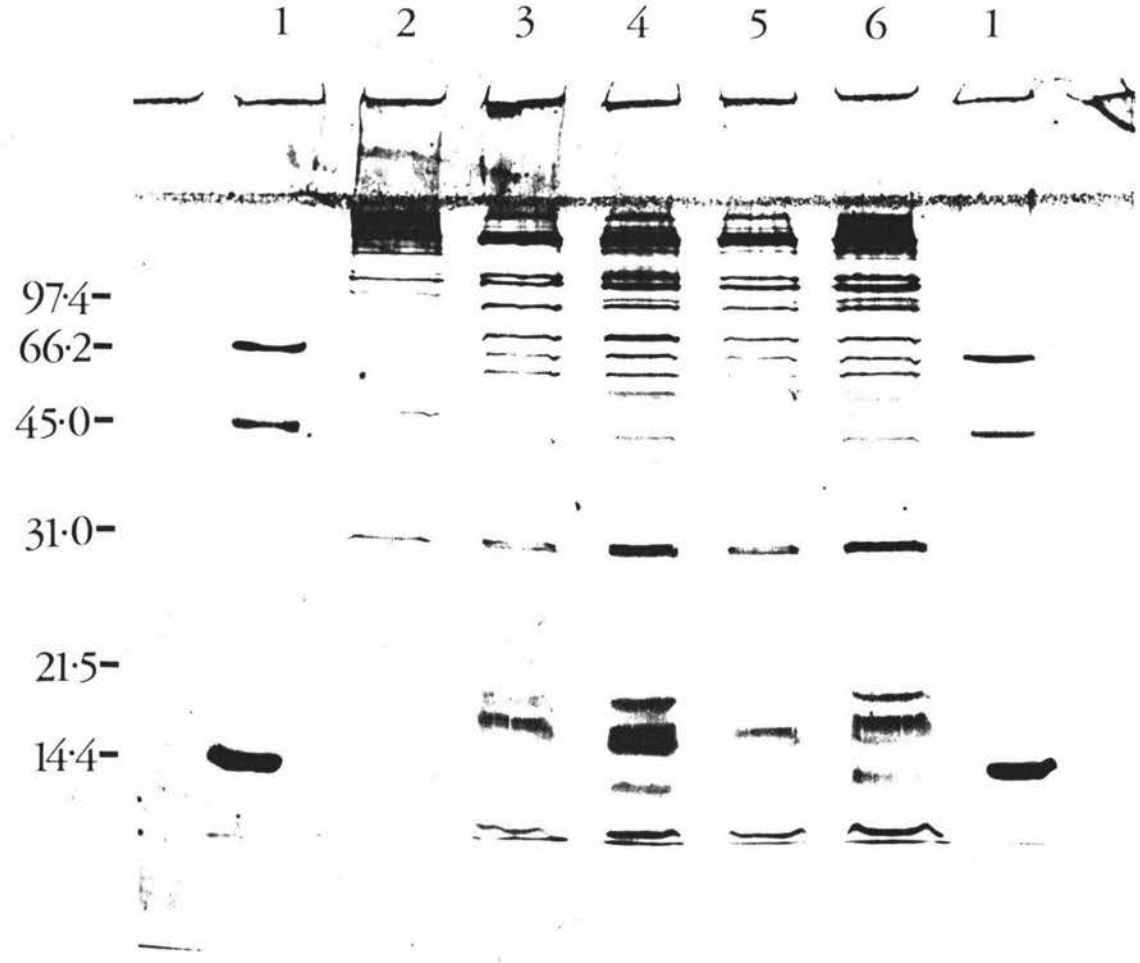
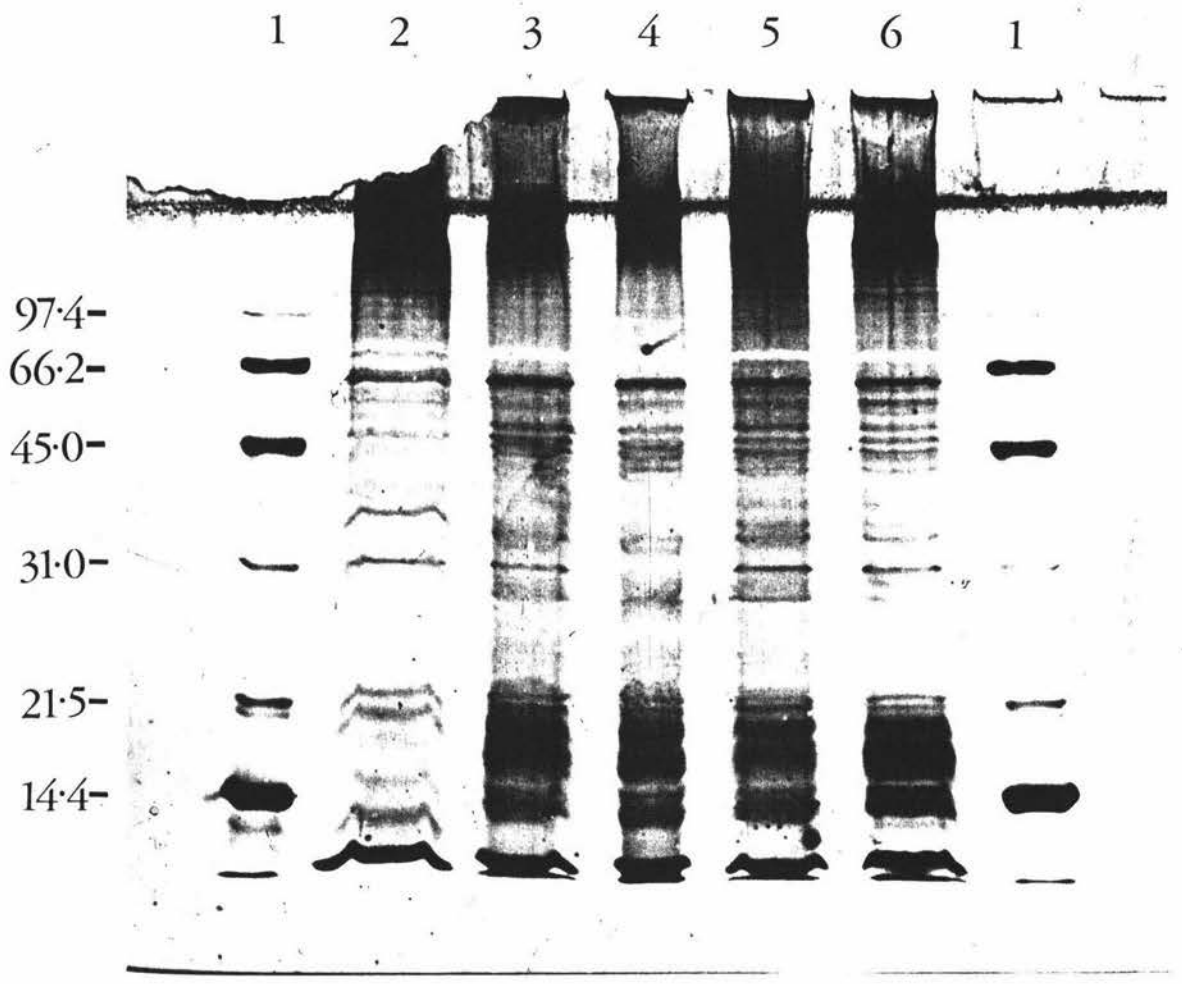

1
2
3
4
5
6
1
97·4-
66·2-
45·0-
31·0-
21·5-
14·4-

FIGURE 10

SDS-polyacrylamide gel electrophoresis of protein-body enriched fractions prepared from development and germination stages of *Phaseolus vulgaris*. Fractions were prepared from 30 axes by the method of Chrispeels et al.(1982). Lanes are as follows: 1. SDS-PAGE standards (MW14400-97400; BioRad, California); 2. Mid-maturation (20-32 days after anthesis); 3. Late-maturation (32-40 days after anthesis); 4. Mature seed; 5. 24 hours of germination; 6. 48 hours of germination. Units of the markers are kilodaltons.


1
2
3
4
5
6
1
97·4
66·2
45·0
31·0
21·5
14·4

45kD bands, all of which were more strongly present in subsequent stages. In addition to this, two bands 13kD and 72kD were at their strongest relative to the following stages, and a sharp band at 37kD was apparently unique to this sample. In common with all pellet samples a very strong band was present at 11kD and the area above 150kD was continuously stained.

Late-maturation was marked by the appearance in the supernatant fraction of bands at 13.5kD, 28kD, 35kD, 36kD, 38kD, 41kD, and 73kD, and by the increase in prominence of the bands at 17.5kD, 20kD, 62kD, 68kD, 81kD, and 98kD. The supernatant also showed a decline in the 49kD and 85kD bands and in the vicinity of 175kD. One band of 140kD disappeared from the supernatant altogether.

In the pellet fraction of late-maturation bands appeared at 14kD, 17.5kD, and 22.5kD while those at 20kD, 29kD, 33kD, 34kD, 51kD, and 62kD showed an increase. A decline in the level of the bands at 41kD, 72kD, 115kD, and 123kD was observed and bands at 13kD and 37kD were now both absent.

Supernatant from mature axes showed the appearance of bands at 23kD, 24kD, 26.5kD, 27.5kD, 29kD, 30kD, 33kD, 34kD, 39kD, and 95kD. The bands at 28kD, 31kD, 32kD, 38kD, 49kD, 58kD, 65kD, 68kD, 81kD, 113kD, 120kD, 150kD, and 175kD showed some increase and those at 13.5kD, 17.5kD, and 20kD increased further.

The pellet fraction obtained from mature axes was very similar in appearance to that obtained from late-maturation. The only observed differences were the almost complete absence of the 31kD band, the absence of the bands at 115kD and 123kD, and the possible darkening of the 43kD band, however an irregularity of the gel in the late-maturation lane partially obscures this region and makes judgement difficult.

In the 24 hour germination samples the supernatant was missing the 12kD,

24kD, 33kD, 39kD, and 95kD bands and a large number of bands(13.5kD, 17.5kD, 20kD, 23kD, 29kD, 30kD, 31kD, 32kD, 34kD, 38kD, 40kD, 41kD, 44kD, 49kD, 52kD, 58kD, 62kD, 65kD, 68kD, 81kD, 98kD, 113kD, 120kD, 150kD, and 200kD) were slightly diminished. No bands were noticeably more prominent than in the corresponding mature axes sample.

The pellet fraction at this stage showed an increase of the 31kD, 35kD, 38kD, and 72kD bands, a slight decline in the 17.5kD band and the reappearance of the bands at 115kD and 123kD.

At 48 hours of germination bands at 12kD, 24kD, 35kD, 73kD, 95kD, and 140kD had reappeared in the supernatant and a band at 88kD was apparent for the first time. Many bands(13.5kD, 17.5kD, 20kD, 25kD, 31kD, 32kD, 38kD, 40kD, 41kD, 44kD, 49kD, 55kD, 58kD, 62kD, 105kD, 113kD, 120kD, 175kD, and 200kD) were more strongly present at this stage than in the preceding 24 hour lane. One band of 65kD was absent .

In the pellet fraction of 48 hours germination, new bands were found at 12kD, 13kD, 30kD, and 55kD, and bands at 14kD, and 17.5kD were noticed to have increased. The 94kD band was weaker and the 22.5kD and 24kD bands were missing.

DISCUSSION

The techniques of in vitro translation and fluorography were used as an assay for gene expression. The appearance of labelled protein species(i.e.the assay) is dependant upon the presence of specific mRNA species. The mRNA population present at the time of RNA extraction is the resultant of a number of processes acting upon the pool, namely: transcription itself; post-transcriptional modification; transport processes through the nucleopore complexes of the nuclear membrane; and its availability for translation in terms of its half-life, a function of sequestering mechanisms. For the latter, ribonucleoprotein complexes have long been hypothesized as message storage mechanisms in seed tissues(Weeks & Marcus, 1971; Filimonov et al., 1977; Peumans et al., 1980) and whether these, if they exist, are immediately available to support translation in a cell free protein synthesizing is a matter of some question. The issue is further complicated by the extraction procedure used for RNA isolation in which phenol might be expected to dissociate such complexes and remove the masking protein component. Despite these complexities, in vitro translation using RNA extracted from tissues of interest has been used as a broad indicator of gene expression activity in many seed studies(Weeks & Marcus, 1971; Peumans et al., 1980).

Fluorographs of in vitro protein synthetic products from RNA taken from normally germinating *P.vulgaris* seeds, showed good translation activity at all times tested (0,3,6,12, and 24hrs). A wide range of molecular weight(MW) species was expected to be seen and this was found to be so between the ranges of 22kD-77kD. Translation products of molecular weights greater than 77kD were not seen and the reason for this is not clear. This was particularly surprising because the rabbit reticulocyte translation system was claimed to favour translation of higher molecular weight products(Krawetz et al., 1983). A Wheat Germ translation system(Promega; data not shown) used in preliminary experiments gave a similar range of products with bean RNA with little evidence of improvement in representation of higher molecular weight species. There was no accumulation of radioactivity in the wells of the gel, indicating

insolubilization or the entry into the gel of higher MW products was not a problem.

The heavy accumulation of radioactivity in the dye front is possibly due to translation products smaller than 22kD, which were not further resolved in this gradient gel, or to ^{35}S-methionine from the translation reaction. Thus all comparisons between the treatments are confined to products between the range of 22kD-77kD. The well known group of late embryogenesis abundant(LEA) proteins normally accumulate in the late maturation of seeds, and translation products would be expected to be present in at least the 0hrs precocious germination treatment, but from published estimates (Dure et al., 1989; Roberton & Chandler, 1992) of their molecular weights the majority of these would be unresolved in these gels.

As each lane contained equal amounts of radioactivity this may cause problems of interpretation, thus in a lane where one product heavily dominates (see Fig.7 normal germination 0hr), other translation products will be less evident. This becomes particularly significant when comparing different lanes. To explain, because each lane was loaded with the same amount of radioactivity, a major apparent increase in the expression level of one or two products, such as the 37-38kD and 27kD proteins observed for precocious germination (Fig.7), could be an accurate conclusion or it could be the result of a global decrease of the other products in the lane.

In summary, <u>in vitro</u> translation is a good screening technique for monitoring qualitative changes in gene expression of transcription products but it is difficult to get unequivocal quantitative measures of evidence for up- or down-regulation of single genes. These limitations will be borne in mind when conclusions are drawn regarding the effect of ethylene on gene expression as individual bands(representing translation products) are compared across lanes in the course of each experiment.

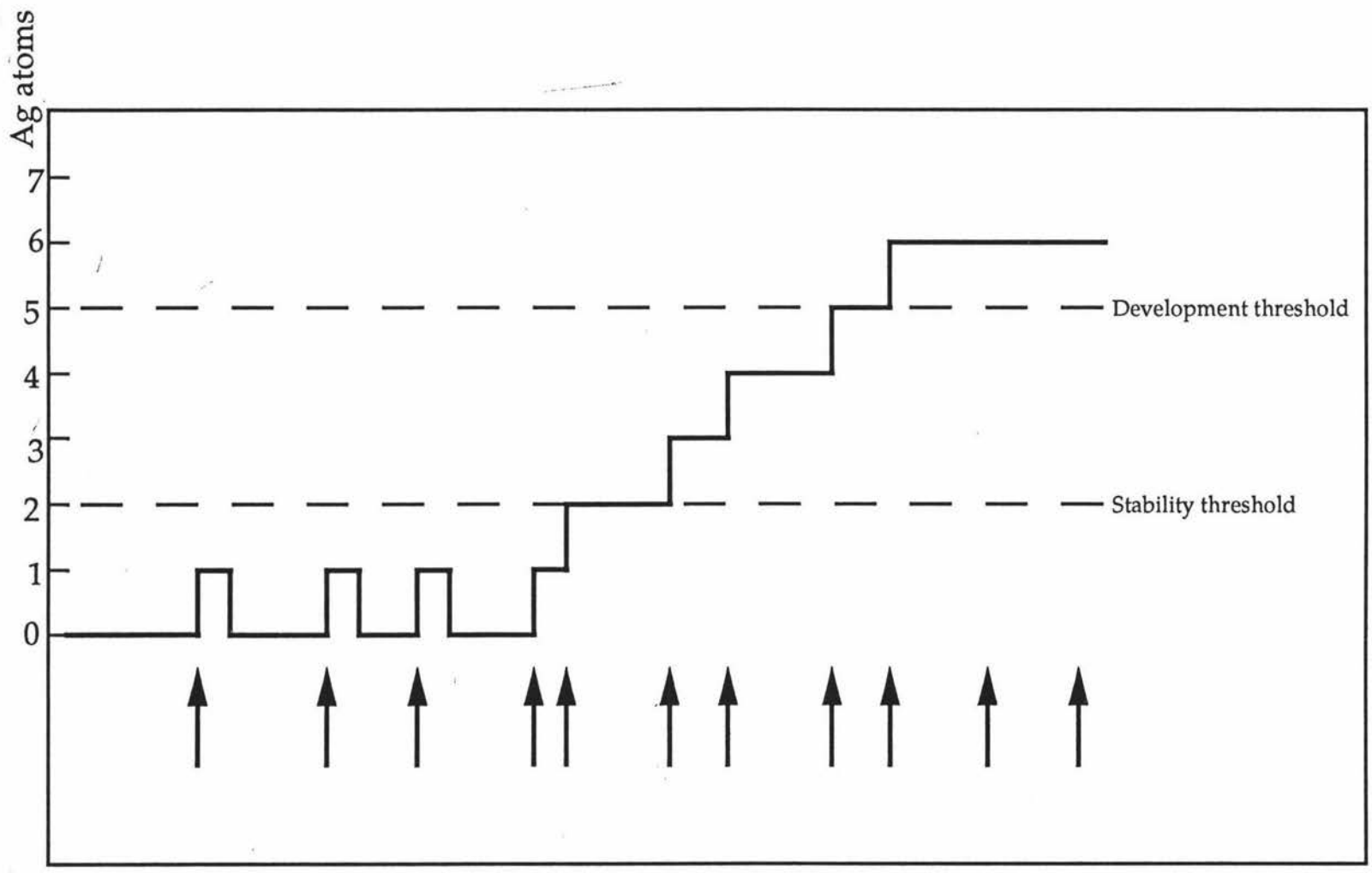
Densitometry was employed primarily as a backup to observation where subjectivity is likely to impede accurate assessment, however it's usefulness in providing evidence for up-regulation or down-regulation is limited by this potential loading artifact. Thus the apparently hard data obtained from the densitometer(see Appendix 2) of relative area of a circumscribed peak, or maximum absorbance of any band, is information primarily useful to that gel lane only. Comparisons can be drawn between gel lanes, however consideration must be given to differences in base line setting, peak integration, and scan length of the densitometer. Peak matching between scans of lanes can also be a problem and this was done by identifying some landmark bands(e.g. the endogenous translation products) and comparing the position of unknown bands relative to these.

Another problem with fluorography which must be kept in mind when conclusions are drawn from the results, is the non-linear exposure kinetics of X-ray film to photons (Fig.11). Beta-particles emitted from ^{35}S atoms in the methionine tracer are absorbed by the fluor(PPO) molecules in the dried gel, and energy gained from this absorption stimulates the release of photons. These photons are then absorbed by silver iodide crystals in the X-ray film emulsion. As the above graph shows, the first few photons cause no darkening of an individual crystal, then there is a linear response phase, before saturation occurs and the crystal has reached it's maximum darkness, and further photons cause no further darkening. This saturation was observed to occur when densitometry was performed on the dye front of the gradient gel. This densitometry yielded a characteristic flat topped peak, the height of which corresponded to the maximum absorbance of the film(data not presented).

The quality and integrity of the results obtained from <u>in vitro</u> translation experiments reported here depended upon the successful extraction of mRNA of high quality. Initially attempts were made to purify a poly(A)$^+$ fraction using the technique of oligo-dT-cellulose chromatography (see Appendix 1) however this gave only limited success due to the initial difficulty in extracting pure,

FIGURE 11

The response to energy capture by individual silver halide grains in film. A single silver atom is unstable. Once the stability threshold of 2 Ag atoms per grain is reached the reversible stage is by-passed allowing immediate progress to the development threshold of approximately 5 Ag atoms per grain. These catalyse development of the grain by the developer solution (after Laskey, 1984).



Exposure time (arrows represent energy capture)

undegraded, and resoluble total RNA from bean axis and cotyledon tissue (see Appendix 1). Specifically, initial extraction procedures had difficulty coping with the high protein and especially carbohydrate content of the bean seed tissue, and initially vacuum drying procedures were excessive so as to make the total RNA insoluble and therefore not amenable to further manipulation. Despite these problems, oligo-dT-cellulose chromatography gave evidence of separation of the total RNA sample into a poly(A)$^+$ fraction and an eluted fraction. Yields of poly(A)$^+$ RNA were low due to the low solubility as noted above, and as initial attempts to translate this gave no substantial incorporation over the blank (i.e. no result this approach was not pursued. Subsequently, the RNA extraction procedure was improved to such an extent as to make in vitro translation possible with a total RNA sample (see Appendix 1 where full details of the technique development are given).

Results reported in the body of the thesis are for axis RNA, as cotyledon RNA proved difficult to purify owing to high carbohydrate levels. These carbohydrates result in a high viscosity in the aqueous phase of the tube containing the phenol/chloroform/isoamyl alcohol and the extraction buffer, and make partitioning impossible. In addition, high protein content results in a very thick interface between layers. Two possible solutions to this problem were seen: decreasing the amount of tissue used; or increasing the volumes of extraction buffer and phenol/chloroform/isoamyl alcohol. Neither were feasible here because in the first case initial indications were that this tissue contained very little RNA, and in the second case suitable equipment was not immediately available. In response to this problem a decision was made to use only axis RNA in these experiments as this is where the growth response appears. The cotyledon RNA would have been of interest for in vitro translation studies as this is another possible site of ethylene perception (Sisler, 1979; Hall et al., 1990) and at the very least would be an interesting control.

Total RNA yield for axis tissue varied widely between the figures of 145ug/gFW and 1444ug/gFW (see Table 1) and no trend could be observed in relation to

any particular treatment. It can be assumed that the RNA yielded by this method does not reflect the RNA level present in the tissue. No correlation was apparent between the yield of RNA and the purity of that RNA. RNA purity estimated from the A_{260}/A_{280} ratio also varied widely for the samples, with values ranging between 1.55 and 2.46. Sambrook et al.(1989) indicate that pure RNA preparations give an A_{260}/A_{280} value of 2.0 and that a figure significantly less than this indicates protein or phenol contamination. Given this, it is hard to account for the A_{260}/A_{280} figures of 2.46 and 2.39 for the 6hr axis control sample and the 12hr axis control sample. Carbohydrates, when present, contributed to a peak centred on 180nm, and it is possible that the shoulder of such a peak may contribute to the absorbance at 260nm, so inflating the A_{260}/A_{280} figure. Although some samples yielded low A_{260}/A_{280} values (e.g. 0hr germination axes with 1.55, 6hr germination axis with 1.62, and 24hr germination axis with 1.68), these samples were later shown to translate adequately (see Tables 2 and 3) indicating that the actual level of possible protein or phenol contaminants was not high enough to cause inhibition of the translation reaction. In general, no correlation was apparent between the purity of the sample (as estimated by A_{260}/A_{280}) and the translational activity. RNA samples were also monitored for their absorbance at around 180nm(results not presented) because as mentioned above this is an indicator of possible carbohydrate contamination. These carbohydrates were found to be abundant just after the first vacuum drying stage in RNA extraction but the subsequent washing steps removed much of this contaminant from the axis samples. The washing steps were also designed to remove sodium ions which are known to interfere with the in vitro translation process(Krawetz et al., 1983).

RNA which was assayed by electrophoresis generally showed similar patterns of banding with regard to the sizes of band present, however the intensity of these bands varied widely between some samples. The topmost of these bands(17kb locus) most probably represents genomic DNA which was not totally removed during RNA isolation. The bands which appear at the 1.6kb, 1.4kb, and 0.8kb loci on gel A and at the 1.7kb,1.3kb, and 0.9kb loci on gel B are

putative ribosomal RNA bands derived from the subunits of both cytoplasmic and organellar ribosomes. The band present at the <0.5kb locus(0.27kb locus by extrapolation of the standard curve) is the likely location of both small RNA species such as tRNA and degradation products of previously larger RNA species. Hence an increase in the strength of this band combined with a reduction in strength of bands "higher up" the gel indicates that some RNA degradation has taken place, most probably mediated by RNase enzymes contaminating the RNA sample. Using such indicators, degradation may be said to have taken place in the 24hr ethylene(31.12.91) sample. It is interesting to note, however, that in subsequent in vitro translation experiments this sample yielded the highest stimulation over the blank of any of the bean RNA samples translated. Other bands, which appeared at the 2.5kb, 1.9kb, and 1.5kb loci in some samples on gel A may represent genuinely different ribosomal RNA species but it is more likely that these bands are the product of aberrant gel running conditions. Such aberrant conditions may have been caused by high carbohydrate content of RNA samples or incomplete denaturation of RNA in these electrophoresis conditions.

In general, RNA extracted from bean seeds, as described earlier, was of good integrity. Samples which showed some degradation were not noticed to translate differently from samples which showed no degradation. Likewise no correlation was apparent between the integrity of RNA samples as determined by gel electrophoresis and the purity of the samples as estimated by the A_{260}/A_{280} ratio.

All RNA samples prepared by the improved extraction method showed at least a 200% stimulation over the blank. While this has been shown here to be quite sufficient to yield products for further analysis(i.e. electrophoresis and fluorography) it is a relatively low stimulation when compared with a pure and active mRNA such as the tobacco mosaic virus(TMV) control RNA(provided with the rabbit reticulocyte kit) which gave a stimulation of 4676%. Although the percent stimulation figures were similar for the two batches of reactions

performed, there was a difference in percentage of radioactive label incorporated. In the first batch of reactions, tubes containing bean RNA showed an average of 0.78% incorporation while in the second batch of reactions the average incorporation for reactions containing bean RNA was 1.97%. Whether this is due to the treatments involved(i.e. ethylene, normal germination) or to an irregularity in the preparation or performance of the reactions or in the TCA-precipitation of radiolabelled products, is not known. The absolute amounts of radioactivity incorporated into TCA-precipitable protein, on average, decline between the first batch and the second batch(see Tables 2 and 3). As these batches were performed seven days apart this may be explained as being caused partly by a loss in radioactivity of the ^{35}S isotope used, and is a pertinent reminder that once obtained this isotope must be used rapidly before it's activity is lost. A loss in the activity of the in vitro translation kit due to repeated freezing and thawing may have contributed to this decrease in absolute radioactivity incorporated. Another possible cause of this apparent decrease is that a pipetting error may have occurred when removing the 1ul aliquot for measurement. Translational activity of the RNA from the control treatment did not show a progressive decline over time, despite a progressive decline in the expression of many translation products between the ranges of 22kD-77kD, being observed in this treatment in the fluorograph (Fig.7). Because an equal amount of radioactivity was loaded in each lane, and this radioactivity seems to progressively decrease between the ranges of 22kD-77kD over time, it seems reasonable to surmise that this radioactivity becomes increasingly present in the dye front.

The rabbit reticulocyte in vitro translation system used in these experiments yielded at least two products which were considered to be endogenous(i.e. of rabbit origin, see Fig.7). The first of these, at 76-77kD showed a fairly constant level of expression in each lane. The second product (47-48kD) however varied widely in it's apparent level of expression. It appeared strongly at 0hrs of germination(i.e. in a mature dry seed) but seemed to decline over the next 24hrs of germination. In precocious germination this band was very weakly present

at 0hrs (i.e. a late maturation seed) but appeared more strongly between 3-12hrs before perhaps declining in the 24hr lane(although this apparent decline may be an artifact as explained before). In the control treatment the 47-48kD band was weakly present initially but may be interpreted as increasing to a maximum at 12hrs before declining in the 24hr lane. Because this wide variation would not be expected from a product endogenous to the in vitro translation kit, the best explanation is that a product of bean origin also contributes to the band at this location. The pattern of expression, particularly in relation to the enforced abscission induced in the control treatment, suggests the possibility of this product being an LEA protein, however if this is so then it is unlikely to be closely related to previously described LEAs the nearest of which in size are 30.3kD(Harada et al., 1989) and 66kD(Choi et al., 1987).

An issue which must be considered when drawing conclusions from in vitro translation studies of seeds is the possibility of stored mRNA(Weeks & Marcus, 1971; Filimonov et al., 1977; Mori et al., 1978; Peumans et al., 1980; Thompson & Lane, 1980; Laroche-Raynal et al., 1984). In this study the term "stored mRNA" refers to mRNA species which are stable for some period of time by whatever mechanism (rather than to the possibility of mRNA species be sequestered into organelles or analogous structures). If such highly stable mRNA species do exist then in vitro translation will not be an indication of real time transcription and time at which a hypothetical gene switches on or off will not be directly observable. The fact that dry seeds (i.e. 0hrs at normal germination) did yield translatable RNA argues that stored mRNA does exist in at least mature dry *P.vulgaris* seeds.

Gene expression in development and germination.

The pattern of gene expression observed in late maturation embryos using 1-D SDS-PAGE was similar but not identical to the pattern shown by normally germinating seed axes after 24hrs. The majority of products were present in

both late maturation and after 24hrs of germination, and it is possible that these products are required for the routine maintenance of metabolism in the axis. Surprisingly no products could be identified as being unique to the developmental state studied (i.e. late maturation). It might be expected that in the late maturation stage, mRNAs for seed storage proteins and for LEA proteins would be abundant and that these mRNAs would be strongly down-regulated (if present at all) in normal germination. This lack of developmental products contrasts with the findings of Misra & Bewley(1985; 1986). The difference between these two results may be due to the difference in analytical techniques employed. The 2-D gels produced by Misra & Bewley appear to yield a much greater variety of products than can be observed in the 1-D gels used here. As mentioned earlier the reported molecular weights for most LEA proteins would result in them being present in the dye front of these gradient gels, if they were present at all, and so they would be unobservable. In the case of storage protein mRNAs, although the storage proteins are known to be present in late maturation seed axes (Derbyshire et al., 1976) they do not dominate the protein profile the way they do in cotyledon tissue and it is possible that at the late maturation stage the synthesis of these products is largely over.

Despite the lack of evidence of products which were exclusive to the developmental stage, two products showed patterns of expression which may indicate that they are more important in development than in germination. The 28kD product was strongly present in developing axes, was not detectable in mature dry axes, but was synthesized during the earlier stages of germination, however by 24hrs of germination it's presence had decreased. While the 38-39kD product appeared to the naked eye to decrease in expression over germination, the densitometry data revealed a more confusing picture (see Appendix 2). Depending on which variable was considered, two contrasting interpretations could be drawn. Considering the variable "absorbance", this product appeared to increase over a 24hr period although somewhat erratically. For both the variables "area" and "relative area" a decrease in the expression of this product was indicated. This ambiguity points to the need for caution when

using densitometry to detect subtle changes in gene expression.

Two products (71kD and 37-38kD) were predominantly (i.e. not quite exclusively) present in germinating seed axes but not in late maturation axes. This pattern of some products being exclusive to germination is in line with Misra & Bewley's work, however the difference in analytical techniques used (i.e. 1-D vs. 2-D gels) means that direct comparisons between these two products and those described Misra & Bewley are not possible. It may be noted here that these two products later showed different behaviour with regard to ethylene-induced precocious germination. In this treatment the 37-38kD product was very strongly up-regulated while the 71kD polypeptide was undetectable. This indicates that the controlling mechanisms of the respective genes may not be identical.

Ethylene-induced precocious germination

By using the techniques of <u>in vitro</u> translation, SDS-PAGE and subsequent fluorography it has been shown that ethylene does induce a change in translatable mRNA in late maturation *P.vulgaris* embryos. Simple measurements of radical length in which the ethylene-exposed embryos showed an increase of 127%, while the control embryos exhibited no detectable change, show that this change in gene expression coincides with precocious germination.

Measurement of fresh weight of the embryos and seeds shows that for the embryos in these incubation conditions a slight loss in fresh weight occurs. The most likely explanation of the net loss in fresh weight is that fresh weight loss was due to losses from water loss and perhaps respiration losses. When embryos were removed from their containers for measurement every effort was made to ensure that water loss was minimised, however given the dry air conditions in a laboratory some water loss was still likely. Alternatively the net loss in fresh weight may be explained as being solely due to respiration losses.

If this was the case and respiration losses were large enough they may mask a possible uptake of water. Although the second explanation is less likely the experiments presented here cannot rule it out. In contrast to embryos the mature seeds had access to a source of free water and their fresh weight increased steadily over the incubation period due almost certainly to the imbibition of water.

Gene expression in ethylene-induced precocious germination.

The pattern of gene expression in precociously germinating embryonic axes is different from the pattern observed in normally germinating axes. There are, however, some factors which must be considered before a more detailed comparison between these two treatments can be made. Precociously germinating embryos were started from the late maturation stage, the stage which immediately precedes desiccation, and therefore these axes were not at minimal water content unlike mature dry axes. It may be assumed then that these axes were more readily available to show changes in gene expression than were normally germinating seed axes which were started from a dry state. In these normally germinating seed axes a lag phase in changes in gene expression might be expected corresponding with the time taken for the seed axis to fully imbibe. It has been shown that for imbibing seeds in vivo protein synthesis is detectable within 7hrs for lettuce(Fountain & Bewley, 1973), 12hrs for *Brassica napus*(Payne et al., 1976), and 12hrs for maize(Oishi & Bewley,1992).

The testa in bean seeds is an encapsulating layer of tissue which provides a stable developmental environment for the developing embryo. There is no vascular connection between the embryo and the testa and this permits it's easy removal at late maturation(Maheshwari, 1950; Norstog, 1979). Normally germinating seed axes were incubated with an intact testa (as it proved impossible to remove the testa from a dry seed without causing substantial damage to the seed) in contrast to both the precociously germinating treatment

and the control treatment in which the testa was removed before incubation. The presence of the testa in normally germinating seeds but not in the other treatments may complicate comparisons in several ways. Firstly as the testa forms a complete tissue layer around the embryo it might be expected that it would form a barrier to water in normal germination and a barrier to ethylene (if it had been present) in precocious germination. In the latter example it has been shown (Fountain & Outred, 1990) that the presence of the testa does delay the response of late maturation embryos to ethylene. Secondly, the testa cannot be ruled out as a potential source of plant growth regulators. Indeed, Fountain & Outred have suggested that the testa may be a producer of ethylene under some conditions. Thirdly, in the normal germination treatment it is possible that the testa may act as a mechanical constraint to radicle elongation, this constraint being absent on the other treatments, so allowing quicker radicle elongation.

Despite the above differences in timing and presence or absence of the testa, apparent changes in gene expression between precocious germination and normal germination can be noted.

Four products (71kD, 67-68kD, 65-66kD and 41-42kD) were up-regulated in normally germinating seed axes but were absent from precociously germinating axes. These products then, whatever their identity are apparently not required for radicle elongation as this occurred in their absence. Three of these products (67-68kD, 65-66kD and 41-42kD) were clearly present in later maturation however 3hrs after the exposure of embryos to ethylene these products had effectively disappeared. The fourth product (71kD) was at best only weakly present in late maturation but likewise disappeared after exposure to ethylene. It may be interpreted that this product in particular is of greater importance in germination than it is in late maturation. This observation raises the question of what is responsible for this apparent down-regulation. One possibility is that ethylene may have a direct effect in repressing the expression of these products. Alternatively this down-regulation may be a secondary effect of precocious germination and may take place with or without the presence of ethylene. It is

interesting to note that this phenomenon of products being upregulated in normal germination but not being required for precocious germination, appears to exist in other plant species. Oishi & Bewley(1992) used in vitro translation and 2-D electrophoresis to assay gene expression in normally germinating maize embryos and embryos induced to precociously germinate by incubating them in water. One product (approx. 45kD and 5.0pI) appeared strongly in fluorographs of normally germinating embryos but was absent from fluorographs of precociously germinating embryos. Of additional interest is the apparent occurrence of the phenomenon in a monocot such as maize and it is possible that this phenomenon may have similar controls despite the evolutionary differences between species, however this does not mean that the products themselves will necessarily be related. If this is so, then this phenomenon may represent a basic process in seed development. Elucidating such common changes in gene expression, both across species and within the same individual and determining the controls of these may prove more useful to understanding the controls of seed development, than studies which focus on the overall germinability (or other gross measurements) of seeds or embryos exposed to various culture treatments. While this similarity is of great interest once again it is worthwhile noting points which complicate such comparisons.

The difficulty of accurately identifying equivalent timepoints in a germination time-course which includes an imbibition phase(i.e. normal germination) and in one which does not(i.e. precocious germination) remains. A casual consideration of this difference between treatments could lead one to expect that a change in gene expression would be more rapid in the precocious germination treatment. However without a more complete description of changes in gene expression in these two treatments (i.e. more timepoints, particularly at later times) some doubt must remain as to the interpretation that in the maize system of Oishi & Bewley(1992) one product does appear in normal germination but not in precocious germination. Another complication is the difference between the tissue type used by Oishi & Bewley and that reported on here. Oishi & Bewley used whole maize embryos (including the axis and scutellum) while this study

focused on axis tissue alone.

Under the classification system developed by Galau et al.(1991) these four products may be classified as having at least a MAT component as they are present in late maturation but disappear upon the enforced abscission from the parent plant that they experience in the ethylene and control treatments. If their model of seed development holds true then these products might be regulated, at least in part, by an endogenous MAT factor (which is lost upon abscission) or perhaps be down-regulated by the PA factor which is proposed to accumulate after abscission. Because germination occurs in the ethylene treatment yet these four products are down-regulated we may speculate that ethylene may interact with the putative PA factor. Clearly these hypotheses await further testing. Due to the timepoints sampled in this study further classification under Galau's system is not possible .

Two products which are normally up-regulated in normal germination (37-38kD, 27kD) are dramatically up-regulated in precocious germination. As one of these products (27kD) is clearly present in late maturation and the other (37-38kD) is not, it is reasonable to suppose that while these products show a similar pattern of expression and may have similar controls with regard to precocious germination/ethylene, in precocious germination their controlling mechanisms (at least for late maturation) may differ. To further support this the 27kD product is clearly present in a dry seed (see Fig.7) while the 37-38kD product is not.

Why these products are up-regulated to such a degree is not clear, however it is possible that the 37-38kD product may be involved in reserve catabolism as it only appears in germinating axes and does so in large quantities. The 27kD product, however, does not fit with being such a degradative enzyme as it is present both in late maturation axes and in the dry seed. One possibility is that it may be a plant defence product. An ethylene-inducible chitinase has already been characterised in leaves of *P.vulgaris*(Boller et al., 1983), however this protein

exists in vivo as a polypeptide of 30kD. A β-1,3-glucanase activity has likewise been described in *P.vulgaris* leaves and has been attributed to two polypeptides of 28kD and 30kD respectively(Awade et al., 1989), however little is known of the regulation of these two proteins. It cannot be explained here why these products are so predominant in precocious germination but once again the possibility of direct regulation by ethylene remains.

A product of 22-23kD appeared up-regulated after 24hrs exposure to ethylene. This product was not significantly present in precocious germination before this time and was also not significantly present in any other treatment. Because this product is only present in the ethylene treatment it is possible then that this is an ethylene induced product. Interestingly an ethylene-inducible polypeptide of 22kD, identified as a thaumatin-like protein, has been reported from the abscission zones of *P.vulgaris* leaves(del Campillo & Lewis, 1992). It must be strongly borne in mind however that the experiments presented here cannot distinguish between a direct ethylene effect on gene expression and an effect bought about by precocious germination itself. To remedy this, comparisons must be made between the ethylene-induced precocious germination achieved through other means. This later treatment might be accomplished by incubating late maturation embryos with access to water. Such a comparison would be expected to provide clues as to the regulation of specific products, for example, if the 22-23kD product mentioned here was solely ethylene-inducible then it would be absent from a water-induced precocious germination time-course, conversely if it were a normal feature of precocious germination and ethylene did not affect it's expression levels then it would be present in both (i.e. ethylene and water) treatments, at similar levels.

Several ethylene-induced genes are now known from other plant systems. These genes generally relate to one of three broad categories: fruit ripening, senescence, or plant stress(Broglie & Broglie, 1991). If ethylene-inducible genes are present (in an inducible state) in late maturation *P.vulgaris* embryos it seems less likely that they are genes normally associated with fruit ripening or senescence. A

more likely prospect is that they may be products which in other systems have been linked with plant stress. These plant stresses include oxygen deficiency and flooding, wounding and pathogen attack, all of which have been associated with an increase in ethylene production, and all of which are possible stresses for an establishing seedling(Broglie & Broglie, 1991). Ethylene-inducible products which accumulate in response to some of those stresses include a chitinase from *P.vulgaris*(Boller et al., 1983), a β-1,3-glucanase from tobacco(*Nicotiana tabacum*)(Felix & Meins, 1987), hydroxyproline-rich glycoproteins from melon (*Cucumis melo*)(Roby et al., 1985) and carrot (*Daucas carota*)(Ecker & Davis, 1987), and a number of enzymes involved in phytoalexin biosynthesis in carrot(Ecker & Davis, 1987). None of these products however are known to have a molecular weight of 22-23kD. Another possible alternative is that ethylene-inducible genes may exist which are unique to this plant system (i.e. precocious germination or bean seeds).

It is worthwhile mentioning that this product does not appear until at least 12hrs after the exposure of the embryo to ethylene. In other plant systems mRNA synthesis in response to ethylene happens within half an hour for tomatoes, 2hrs for bean glucanase, and 3hrs for carnation petals(Abeles et al., 1992). This argues that if the appearance of this product is ethylene-induced then it is probably through an indirect means. Such a delay in gene response might be expected if the site of ethylene perception were distant from the place of ethylene response (i.e. the axis). Hall et al.(1990) have demonstrated that an ethylene binding protein exists in immature *P.vulgaris* cotyledons associated with endoplasmic reticulum and protein body membranes, and that this protein shows the expected characteristics of a putative receptor. Such speculation, however, will remain fanciful until the mechanism of ethylene signal transduction is further elucidated.

Gene expression in detached non-germinating embryos.

Detached non-germinating embryos generally showed an apparent decline in translatable RNA between the range 22-77kD (i.e. above the dye front). The band at 47-48kD, however, showed an apparent increase 12hrs after the start of incubation.

Because an equal amount of radioactivity was loaded onto each lane (assuming no loading error occurred) and because this signal is largely absent above the dye front then the only apparent place for this signal to be is in the dye front itself. The signal in the dye front could be due to ^{35}S-methionine from the translation reaction, endogenous products from the in vitro translation kit, or translation products of bean origin. There are several possible interpretations of this.

Firstly, if products of less than 22kD in size had been dramatically up-regulated in *P.vulgaris* then the expression of products outside of this range would appear to decrease correspondingly. In cotton abscission of the ovule from the parent plant strongly correlates with the appearance of LEA proteins and it has been proposed that this may in some way stimulate their synthesis(Galau et al., 1991). It is possible then that the enforced abscission experienced by the bean control treatment resulted in the synthesis of such LEA proteins. Of the 9 LEA proteins of known sequence, 7 of these are smaller than 22kD and so would appear in the dye front if present in these gels. Under this interpretation the 47-48kD band may consist of an endogenous product and a product that may be a component of the PA program. It must be pointed out, however, that such an ovule abscission is not known to occur in *P. vulgaris*.

Secondly, if a global decrease in gene expression occurred, then translation which occurred in the 24hr control reaction would be predominantly due to mRNA endogenous to the translation kit and the dye front would consist largely of products endogenous to the in vitro translation kit and radiolabelled

methionine. If this were so however, then the two known endogenous bands might be expected to darken considerably and the amount of TCA-precipitable radiolabelled protein might be expected to decline in a linear fashion over the 24hr period. Neither of these effects was observed.

Thirdly, if an increase in RNase activity occurred within the bean axes then as in the above interpretation translation which occurred in the 24hr control reaction would be predominantly due to mRNA endogenous to the translation kit and the dye front would consist largely of products endogenous to the in vitro translation kit and radiolabelled methionine. This explanation is unlikely as not only do the expected effects of endogenous band darkening and a steady decrease in TCA-precipitable protein not occur but also the RNA samples when run on a horizontal agarose gel did not show progressive degradation.

Whichever of the above interpretations is correct it is clear that a change in translatable RNA does occur in late maturation *P.vulgaris* embryos separated from the parent plant and incubated under the conditions described here. The cause of this change is not clear, however a number of possibilities suggest themselves. Removal from the parental environment may result in the loss in supply of a maternal factor as suggested by Galau et al.(1991). Whether this putative factor is ABA or another yet-to-be-identified compound, has yet to be determined, however it is important to remember that embryos in the pod at this stage still have an indirect vascular connection to the parental plant.

In the incubation conditions used, carbon dioxide emitted due to respiration by the bean embryos would accumulate in the incubation chamber and as no carbon dioxide-scrubbing system was employed the carbon dioxide concentration would be expected to rise over the incubation period. It may be hypothesized then that carbon dioxide had some effect on gene expression and that the ethylene supplied in the other treatment was antagonistic to this. Previous studies though, have indicated that at least for mature seeds, ethylene and carbon dioxide have a synergistic effect in promoting seed germination and

that carbon dioxide may enhance ethylene production (reviewed in Esashi, 1991). A further possibility is that the process of testa removal caused some kind of mechanical damage to the embryos and that the change in translatable RNA is as a result of this, however every care was taken in testa removal and no damage was visible on embryos used for this experiment. Given the problems with the two latter explanations, the interpretation that the change in translatable RNA for this treatment was due to the lack of an hypothesised maternal factor seems the most plausible here.

Protein extraction, fractionation, and analysis.

A timecourse picture of extant proteins in developing, mature, and germinating *P.vulgaris* seed axes was produced using one-dimensional SDS-PAGE as an analytical technique. It was initially hoped that these SDS-PAGE gels would assist in identification of storage proteins on the in vitro translation fluorographs as these proteins might be used as an indicator of the putative developmental or germination programs. Also it was hoped that in the case of products identified through the in vitro translation experiment as being of interest, a broad indication of their expression throughout development and germination could be obtained. Early experiments, however, revealed that owing to the large size diversity and similar expression levels of products in the axes, bands consistent with the storage protein subunits were not readily identifiable. Further complicating such an identification were the differing values recorded by Hall et al.(1978) and Bollini & Chrispeels(1978) for the subunits of vicilin, the major storage protein in bean(the other being phytohemagglutinin). Extraction and electrophoresis of storage proteins from mature bean seed cotyledons (Figs.9 & 10) was performed in order to determine their likely locus on the axis SDS-PAGE minigels, however even this showed that accurate identification was not possible owing to the inherent margin of error in electrophoresis due to such factors as how evenly the lanes ran and how the standard curve was constructed. Therefore a decision was made to separate the initial protein

extract from the bean axes into a protein-body enriched fraction and a protein-body depleted fraction to enable the storage protein subunit bands to be identified by a simple comparison of electrophoretic patterns of the two classes of fractions. The method of Chrispeels et al.(1982) was chosen due to the availability of suitable equipment and to its ease of performance. There is reason to believe, however, that this fractionation was not entirely successful. Firstly, the assay of total protein content in each fraction revealed that in all of the samples at least 94% of the total protein in the crude extract partitioned towards the supernatant(the supposed protein-body depleted fraction) and that the maximum value for the pellet fraction(the supposed protein body enriched fraction) was only 5.5% of the total protein extracted (this was recorded from the mature dry seed axis). Secondly, the electrophoretic patterns that these gels produced were not consistent with the expected pattern of fractionation. To elaborate it might be expected that bands at approximately 52kD, 49kD, 46kD, 36kD and 34kD (representing the 3 subunits of vicilin and the 2 subunits of phytohemagglutinin respectively) would be significantly darker in the protein body enriched fractions(i.e. the pellet) than in the protein body depleted fractions(i.e. the supernatant). However, this pattern was not observable in these gels. Indeed, in mature dry bean axes a very dark band of around 31-32kD appeared in the supernatant fraction, whether this band represents the phytohemagglutinin subunits though is uncertain. Similarly, while bands at around 52kD, 49kD, and 46kD were present in the pellet fraction they were by no means dominant and were also as strongly present in the supernatant fraction. It is possible that *P.vulgaris* protein bodies are sufficiently different from *Pisum sativum* protein bodies(as used by Chrispeels et al., 1982) and that either the majority of the protein bodies lysed or would not pellet under these conditions, however no differences can be determined between the method used by Chrispeels et al.(1982) and the method used here.

While products of interest could be identified from the fluorographs of in vitro translation studies, these same products could not be reliably identified on SDS-PAGE gels of simple extracts. Factors which hindered such an identification

included the possible post-translational processing of proteins extracted directly from bean tissue, the large variety of protein species present in the extracts and the margin of error inherent in the electrophoresis system used.

In view of these difficulties it may be concluded that the mini SDS-PAGE system used here, while useful as a quick and easy indicator of protein species present, is not suitable for the accurate identification of individual protein species. Rather it would be more appropriate that these proteins are studied with other techniques such as SDS-PAGE electrophoresis on a larger gel with greater resolving power, two-dimensional electrophoresis combining SDS-PAGE and isoelectric focusing, or in the case of the storage proteins (or LEAs) as antibodies to their subunits may be available Western blotting might be a possibility.

Conclusions

While this study has determined that ethylene-induced precocious germination does occur with a concomitant change in translatable RNA from the control treatment and from normal germination, the results presented here highlight the need for continuing work in this area.

Firstly,as mentioned earlier in the discussion, the up- or down-regulation of certain products in the ethylene-induced precocious germination treatment that then showed a different pattern of behaviour in normal germination, raises the question of whether these effects are due to precocious germination itself or to the direct effect of ethylene on gene expression. This may be answered, by inducing late maturation bean embryos to germinate precociously using a method other than ethylene and then assaying gene expression using <u>in vitro</u> translation. Most simply, this might be accomplished by incubating the embryos with access to an external source of water(as in Fountain & Outred,1990). A comparison between these two treatments would then provide information as to the ethylene inducibility of these products.

Secondly, although previous work has been done on the pattern of translatable RNA in development and germination of *P.vulgaris* (Misra & Bewley, 1985) because of factors discussed earlier (i.e. the type of gel system used, the timepoints chosen, etc.) direct comparison with the treatments performed here is not possible. Therefore, further characterization of earlier stages in development, and later stages in germination using the one-dimensional SDS-PAGE system used here would be of great interest. Data gathered from these extra timepoints might be expected to allow further provisional classification of some of these products under the system proposed by Galau et al.(1991). The timepoints so far analyzed allow analysis with regard to the GRM program but only limited information on the MAT program and others. Also it would be of great interest to see if the 22-23kD product did in fact appear later in normal germination or if it is unique to the ethylene treatment alone.

These above two lines of enquiry should enable further identification of products which show novel patterns of expression in seed development and germination. Central to this problem is the question of what regulates the expression of such products. To determine this it is desirable to be able to follow the expression of the target mRNA and ultimately to isolate the genes concerned. The strategy for achieving this is as follows. The protein of interest is purified to homogeneity using one or two-dimensional electrophoresis (and perhaps by other techniques if necessary) bearing in mind that not only does one have to separate this product from other translation products but also from proteins that were present but not labelled in the <u>in vitro</u> translation kit as well. Once a pure protein fraction is isolated, the amino acid sequence of at least part of the polypeptide can be obtained through the technique of protein sequencing(Wilkinson, 1986). This sequence can then be used to generate an oligonucleotide DNA probe. This oligonucleotide can in turn be used as a primer for the polymerase chain reaction(PCR) in the presence of cDNA synthesized from mRNA isolated from the developmental stage at which the target protein is most strongly expressed(Gurr & McPherson, 1991). The result of this is a probe highly specific to both the mRNA and the gene for the target

protein.

This probe can be used in a variety of ways to determine the tissue specificity and exact patterns of expression of the target gene. Dot blotting is a good first technique for screening tissues for the presence of the target mRNA, Northern blotting yields similar information but also provides an indication of the transcripts size, while the RNase protection assay can give us detailed information about the kinetics of transcript accumulation(Old & Primrose, 1989). Spatial distribution of transcripts can be ascertained using either tissue printing or in situ hybridization. Finally the probe can be used to screen a genomic library for the presence of transformants containing a portion of the target gene.

An alternative method for obtaining probes to genes which are developmentally regulated is to use the technique of differential screening. Briefly, (see Old & Primrose for a fuller description) this consists of preparing a cDNA library from the developmental stage or treatment for which novel probes are required. This library is then screened first with radiolabelled cDNA prepared from the total mRNA fraction from the developmental stages in question, then it is screened with radiolabelled cDNAs prepared from the total mRNA fraction from a different developmental stage. Clones which hybridize only to the probes prepared from the developmental stage in question contain cDNA which codes for transcripts found only in that developmental stage.

The possibility that the enforced abscission experienced by the control treatment induced the synthesis of LEA proteins may be investigated using the techniques of: i)Western blotting with an antiserum prepared against maize dehydrin protein(Close et al., 1989) or ii)Dot blotting, Northern blotting or RNase protection assay using the previously cloned LEA cDNAs(Galau et al., 1986) as probes. If this proves to be so it may well be worthwhile investigating the process of ovule abscission in normal seed development, particularly in view of the importance placed on this event by Galau et al.(1991) in the maturation of cotton seeds.

Finally, a practical method of extracting RNA from the cotyledons of *P.vulgaris* would enable the importance of these organs to the control of seed development to be investigated. As cotyledons are the main storage site of reserve materials(and these are especially abundant at late maturation) a method which quickly and efficiently separates these materials (and in particular carbohydrates) from mRNA would be highly desirable.

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APPENDIX 1: TECHNIQUE DEVELOPMENT

RNA Extraction

It was initially hoped that a picture of protein synthesis at precocious germination in both axes (where the growth response occurs) and cotyledons (where an ethylene binding site has been described; Sisler, 1979; Hall et al., 1990) could be obtained, however nucleic acid extraction from seed tissue, particularly from cotyledons which in *Phaseolus vulgaris* represents the major storage tissue, presents quite a technical challenge. A particular problem was the presence in cotyledon tissue of large amounts of carbohydrates which, due to their chemical similarity to the sugar-phosphate backbone of nucleic acids, tended to copurify with them.

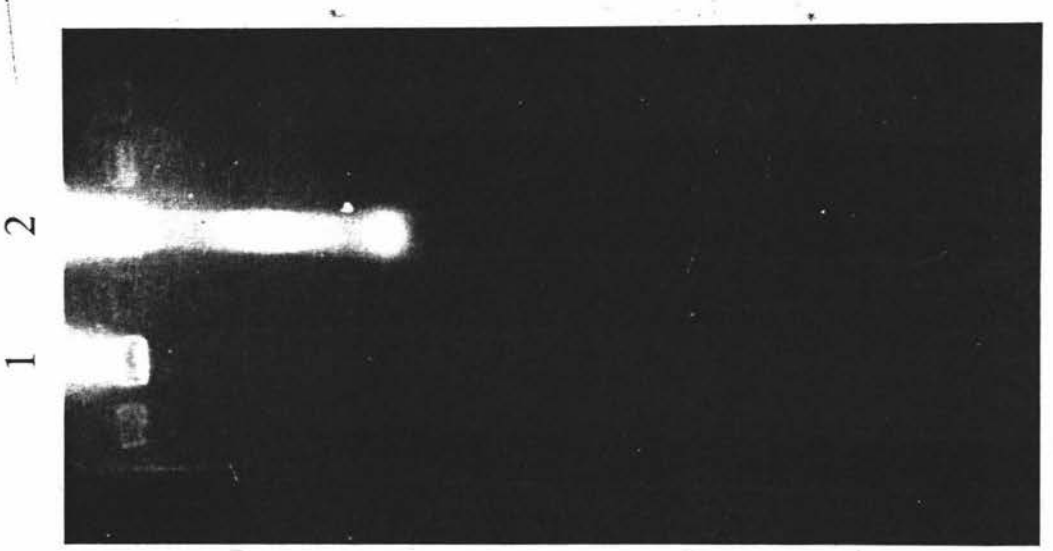
The first RNA extraction protocol used was similar to that described in the methods section but only one phenol/chloroform step was performed and the extraction procedure was stopped at the first drying step (i.e. none of the sodium acetate/ potassium acetate washes were performed). RNA extracted by this method from 0.1g FW late maturation axis tissue and 0.1g FW late maturation cotyledon tissue was analyzed by electrophoresis through a 1.2% agarose/0.66M formaldehyde gel(method given below). This revealed (Fig.12; Gel A) a clearly discernable smear of RNA in the axes lane, but an absence of RNA in the cotyledon sample. (Note: assessment of these samples by monitoring their absorbance at 260nm was complicated by the presence of a large amount of material which had a peak absorbance at 180-200nm and so obscured the nucleic acid peak. This extraneous material was assumed to be carbohydrates.)

It was suspected that proteins and other matter from the cotyledon tissue had not been fully removed by the RNA extraction protocol and may have resulted in the formation of an insoluble complex with the RNA. In an attempt to rectify

FIGURE 12

Agarose gel electrophoresis of RNA extracted from axes and cotyledons of *Phaseolus vulgaris*. (A) RNA extracted with a single phenol/chloroform step from: 1. 0.1g cotyledon tissue, and 2. 0.1g axis tissue. (B) RNA extracted with two phenol/chloroform steps from: 1. 0.1g cotyledon tissue, and 2. 0.1g axis tissue.

A



B

this a second phenol/chloroform extraction step was included in the protocol. RNA so extracted from 0.1g FW late maturation axis tissue and 0.1g FW late maturation cotyledon tissue was analyzed by electrophoresis. The axis sample showed the presence of RNA, however RNA was clearly lacking from the cotyledon sample(Fig.12; Gel B).

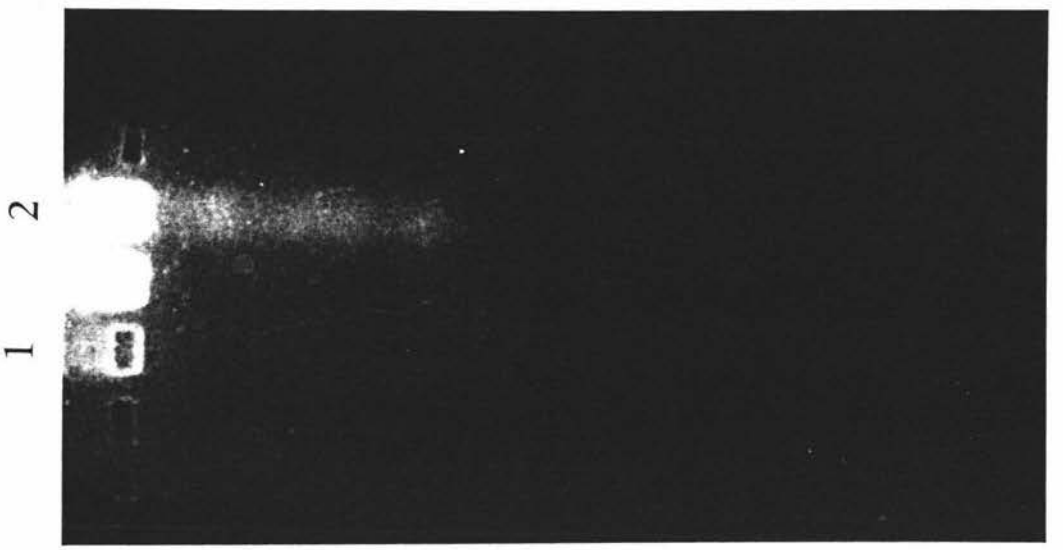
An alternative hypothesis for the absence of cotyledon RNA was that it was present in the tissue in very small amounts as a proportion of fresh weight. Given the primary role of the cotyledons as a site for the synthesis and storage this seemed plausible. To this end the amount of cotyledon tissue used for RNA extraction was doubled. Figure 13(Gel A) shows RNA extracted from 0.1g FW axis tissue and 0.2g FW cotyledon tissue analyzed by gel electrophoresis. As cotyledon RNA was still not apparent on the gel, the next extraction attempt utilised 0.1g FW of tissue for the axis sample and 1.0g FW tissue for the cotyledon sample. Figure 13(Gel B) shows that both the axis and cotyledon samples contain RNA. RNA so extracted was still difficult to manipulate (i.e. slow to dissolve, etc.) and so the sodium acetate/ potassium acetate steps were added to yield RNA capable of stimulating an in vitro translation reaction. Unfortunately cotyledon RNA so treated was apparently lost during these wash procedures (owing perhaps to an initial small yield) and so was not available for further experiments.

Oligo-dT-chromatography

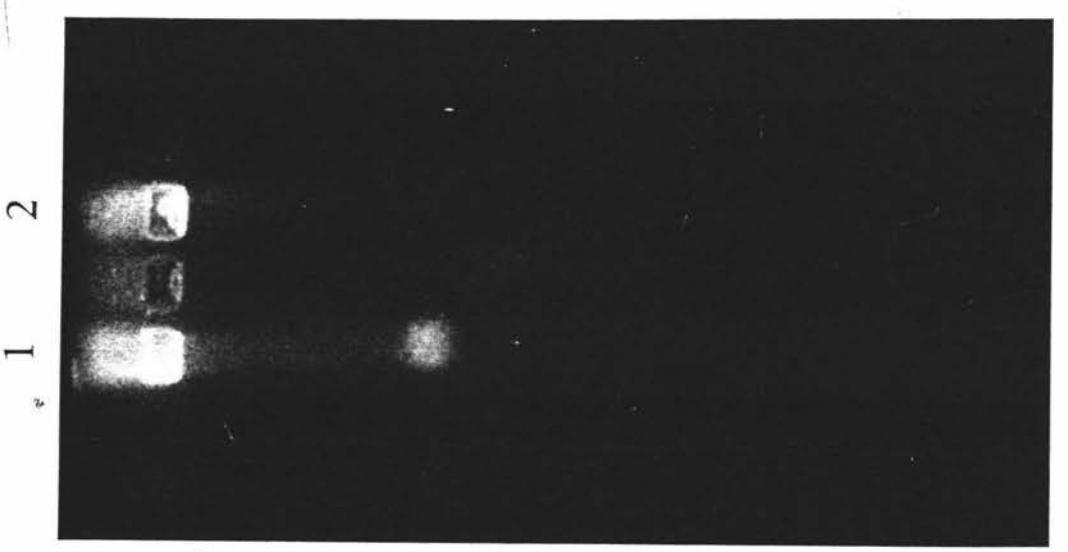
RNA extracted from axes and cotyledons via the above method (minus the sodium acetate/ potassium acetate wash steps) was subject to oligo-dT-chromatography (method described below) in an attempt to yield a reasonably pure poly(A)$^+$ fraction in the belief that this would result in a superior in vitro translation performance. However owing to previous extraction difficulties (including the overdrying of the RNA pellet stage, rendering most RNA extracted with this flawed method insoluble), the poly(A)$^+$ fraction yielded did

FIGURE 13

Agarose gel electrophoresis of RNA extracted from axes and cotyledons of *Phaseolus vulgaris*. (A) RNA extracted two phenol/chloroform steps from: 1. 0.2g cotyledon tissue, and 2. 0.1g axis tissue. (B) RNA extracted with two phenol/chloroform steps from: 1. 1.0g cotyledon tissue, and 2. 0.1g axis tissue.



A



B

not stimulate in vitro translation despite the apparent good separation obtained on most column runs (see Fig.14 for a typical column run).

RNA electrophoresis method

2µl RNA was added to 4µl formamide, 2µl 37%formaldehyde pH>4, 1µl 10x MOPS running buffer(0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA, made by dissolving MOPS in DEPC-treated water, adjusting pH to 7 with either sodium hydroxide or acetic acid, adding DEPC-treated sodium acetate stock and DEPC-treated EDTA stock, pH 8, and then adjusting final volume with DEPC-treated water before being filtered), and 1µl ethidium bromide (10mg/ml) then heated at 55°C for 15 minutes. After cooling on ice, it was loaded onto a 1.2% agarose gel containing formaldehyde and 1x MOPS running buffer and run at 80 V constant current for approximately 30 minutes. The gel was visualized under UV light and photographed using Polaroid film.

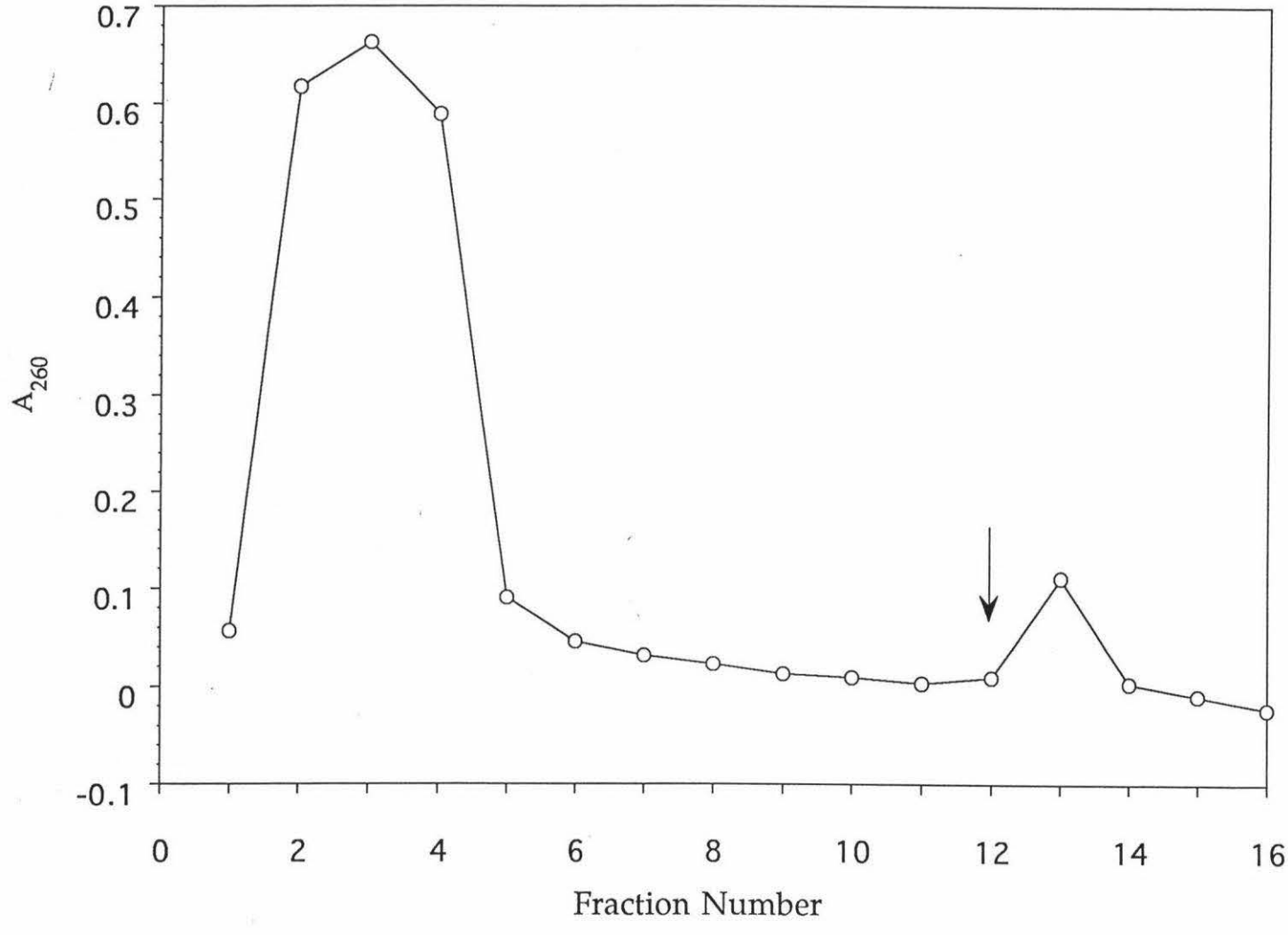
Oligo-dT-chromatography method

Oligo-dT-cellulose columns were prepared by placing 50mg of oligo-dT-cellulose(Pharmacia, U.S.A.) on a bed of glass wool in a pasteur pipette. Both the pasteur pipette and the glass wool had been previously immersed in 1% Siliclad(Clay Adams, U.S.A.) for 5-10 seconds and then baked at 180°C for at least 6 hours. The oligo-dT-cellulose was packed into place by washing with 1-2ml 0.05% DEPC.

Poly(A)$^+$RNA isolation was by the method of Cox & Goldberg(1988). Before the RNA sample was loaded onto the column, the column was washed with 20ml of 0.05% DEPC, 20ml of 0.1M sodium hydroxide (made with DEPC-treated and autoclaved water), elution buffer (10mM Tris-Cl[pH 7.6], 1mM EDTA, 0.1% sodium dodecyl sulphate, made with DEPC-treated water and autoclaved), and

FIGURE 14

Isolation from *Phaseolus vulgaris* total RNA of a poly(A)$^+$ fraction by the technique of oligo-dT-cellulose chromatography. 1ml fractions were collected from the column and their absorbance at 260nm determined. The spectrophotometer had previously been zeroed using elution buffer only. The arrow marks the point at which elution buffer was added to the column in place of binding buffer.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
A_{260}
0
2
4
6
8
10
12
14
16
Fraction Number

20ml of binding buffer (0.5M sodium chloride, 10mM Tris-Cl[pH 7.6], 1mM EDTA, 0.1% SDS, made with DEPC-treated and autoclaved water).

RNA was resuspended in 1ml elution buffer, heated at 68°C for 3 minutes, then cooled to room temperature on ice. Sodium chloride was added to a final concentration of 0.5M before the sample was loaded onto the column. The column was run at 1ml/minute and effluent was collected in 1ml volumes in sterile 1.5ml eppendorf tubes. When the sample had run into the column, binding buffer was slowly added until the effluent contained no RNA as determined by spectrophotometry at 260nm. Poly(A)$^+$RNA was retrieved from the column by the addition of elution buffer until the absorbance at 260 nm was zero.

APPENDIX 2: DENSITOMETRY DATA

The following tables contain the raw densitometry data obtained from scanning the fluorograph presented in Figure 7. Each table refers to one translation product and how it's various parameters (as assessed by densitometry) changed over the three experimental treatments and over the appropriate timepoints for each treatment. Tables are divided into three blocks in which the three densitometry variables are presented for each treatment. The abbreviations used in the tables may be interpreted as follows: trt.= treatment; germ.= the normal germination treatment; eth.= the ethylene-induced precocious germination treatment; cont.= the control treatment; AU= absorbance units; AU*mm= absorbance units times millimetres (i.e. a measure of the peak area); Rel.area%= the relative area of a peak expressed as a percentage of the total signal detected in that lane; and NA= not applicable.

22kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ.						
AU	0.12	0.40	NA	0.55	0.45	0.56
AU*mm	0.23	0.66	NA	0.95	0.82	0.83
Rel. area%	4.9	4.8	NA	4.3	4.6	3.5
eth.						
AU	0.08	0.69	0.50	0.65	0.70	0.69
AU*mm	-	1.84	1.06	1.31	1.12	0.93
Rel. area%	-	5.8	5.1	4.1	3.7	3.3

cont.						
AU	NA	0.67	0.68	0.65	0.46	0.37
AU*mm	NA	1.05	1.90	1.56	0.49	0.88
Rel. area%	NA	3.1	5.8	7.2	2.3	6.6

22-23kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.09	0.28	NA	0.47	0.36	0.46
AU*mm	-	0.25	NA	0.59	0.33	0.34
Rel. area%	-	1.8	NA	2.6	1.8	1.4
eth.						
AU	0.08	0.59	0.52	0.51	0.63	0.74
AU*mm	-	0.44	0.75	0.56	0.69	1.21
Rel. area%	-	1.4	3.6	1.8	2.3	4.3
cont						
AU	NA	0.55	0.57	0.50	0.43	0.34
AU*mm	NA	0.53	0.54	0.54	0.48	0.53
Rel. area%	NA	1.6	1.6	2.5	2.2	4.0

23-24kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	-	0.45	NA	0.57	0.51	0.54
AU*mm	-	0.67	NA	1.21	1.28	1.08
Rel. area%	-	4.1	NA	5.3	6.3	4.4
eth.						
AU	-	0.75	0.49	0.65	0.66	0.56
AU*mm	-	1.89	1.52	1.35	1.06	0.59
Rel. area%	-	6.8	9.9	5.3	4.3	2.6
cont						
AU	NA	0.77	0.81	0.74	0.53	0.38
AU*mm	NA	1.99	2.22	2.08	1.28	1.31
Rel. area%	NA	6.7	7.5	11.7	7.6	12.0

25kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.15	0.40	NA	0.76	0.60	0.63
AU*mm	0.29	1.01	NA	1.74	1.21	1.31
Rel. area%	5.4	6.2	NA	13.7	6.0	5.5

eth.						
AU	-	0.81	0.59	0.68	0.76	0.70
AU*mm	-	1.45	1.11	1.22	1.59	2.05
Rel. area%	-	5.2	7.2	4.8	6.4	9.0
cont						
AU	NA	0.83	0.92	0.61	0.52	0.36
AU*mm	NA	1.34	1.71	1.16	1.07	0.69
Rel. area%	NA	4.5	5.8	6.6	6.4	6.3

26kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	-	0.43	NA	0.58	0.48	0.54
AU*mm	-	0.55	NA	1.13	0.74	1.06
Rel. area%	-	3.3	NA	5.1	3.7	4.4
eth.						
AU	0.05	0.69	0.48	0.63	0.73	0.73
AU*mm	0.04	1.30	1.04	1.25	1.39	1.43
Rel. area%	2.2	4.7	6.8	4.9	5.6	6.3
cont						

AU	NA	0.73	0.82	0.56	0.53	0.30
AU*mm	NA	1.55	1.72	0.82	1.07	0.63
Rel. area%	NA	5.2	5.8	4.7	6.4	5.8

27kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.12	0.46	NA	0.82	0.71	0.66
AU*mm	0.35	0.83	NA	1.83	1.36	1.13
Rel. area%	6.6	5.1	NA	8.4	6.7	4.7
eth.						
AU	0.03	0.79	0.59	0.79	0.91	1.05
AU*mm	0.10	1.74	1.00	1.61	2.03	2.02
Rel. area%	5.9	6.2	6.5	6.3	8.2	8.9
cont						
AU	NA	0.84	0.87	0.67	0.57	0.33
AU*mm	NA	1.58	1.63	1.57	1.16	0.80
Rel. area%	NA	5.3	5.5	8.9	6.9	7.3

28kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.

germ						
AU	0.14	0.47	NA	0.67	0.64	0.56
AU*mm	0.25	0.72	NA	0.88	1.43	1.01
Rel. area%	4.7	4.4	NA	3.8	7.0	4.2
eth.						
AU	-	0.83	0.54	0.79	0.74	0.65
AU*mm	-	1.26	0.89	1.36	1.13	1.18
Rel. area%	-	4.5	5.8	5.3	4.6	5.2
cont						
AU	NA	0.85	0.86	0.59	0.57	0.30
AU*mm	NA	1.46	1.85	0.93	1.03	0.65
Rel. area%	NA	4.9	6.3	5.2	6.2	6.0

30kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.12	0.47	NA	0.51	0.53	0.57
AU*mm	0.27	0.54	NA	0.66	0.99	1.25
Rel. area%	5.1	3.3	NA	2.9	4.9	5.2
eth.						

AU	0.04	0.62	0.51	0.65	0.62	0.62
AU*mm	0.06	0.85	1.16	1.61	1.60	1.37
Rel. area%	3.3	3.0	7.6	6.3	6.5	6.0
cont						
AU	NA	0.67	0.71	0.87	0.43	0.29
AU*mm	NA	1.65	1.31	1.28	1.05	0.75
Rel. area%	NA	5.5	4.4	7.2	6.2	6.9

31-32kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	-	0.46	NA	0.82	0.65	0.74
AU*mm	-	1.16	NA	1.32	0.85	0.78
Rel. area%	-	7.1	NA	5.8	4.2	3.3
eth.						
AU	-	1.08	0.63	0.87	0.75	0.64
AU*mm	-	3.20	1.32	2.62	2.44	2.26
Rel. area%	-	11.5	8.6	10.2	9.9	12.0
cont						
AU	NA	1.08	1.04	0.83	0.72	0.38

AU*mm	NA	3.38	3.26	2.07	1.89	1.13
Rel. area%	NA	11.4	11.1	11.7	11.8	10.3

34kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.11	0.33	NA	0.55	0.48	0.55
AU*mm	0.10	0.28	NA	0.74	0.63	0.42
Rel. area%	1.8	1.8	NA	3.2	3.1	1.8
eth.						
AU	0.03	0.67	0.36	0.64	0.61	0.55
AU*mm	0.02	1.10	0.59	1.05	1.13	1.22
Rel. area%	1.3	4.0	3.8	4.1	4.6	5.4
cont						
AU	NA	0.66	0.72	0.46	0.42	0.22
AU*mm	NA	0.97	1.23	0.60	0.70	0.42
Rel. area%	NA	3.3	4.2	3.4	4.2	3.8

35-36kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						

AU	0.09	0.33	NA	0.60	0.64	0.61
AU*mm	0.10	0.62	NA	0.98	0.82	0.60
Rel. area%	1.9	3.9	NA	4.3	4.1	2.5
eth.						
AU	0.02	0.70	0.48	0.78	0.66	0.52
AU*mm	0.01	0.92	0.64	1.17	0.87	0.45
Rel. area%	0.8	3.3	4.2	4.6	3.5	2.0
cont						
AU	NA	0.72	0.87	0.60	0.50	0.21
AU*mm	NA	0.89	1.13	1.14	0.67	0.26
Rel. area%	NA	3.0	3.8	6.4	4.0	2.4

36-37kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.09	0.35	NA	0.68	0.63	0.65
AU*mm	0.11	0.44	NA	0.84	0.85	0.98
Rel. area%	2.0	2.7	NA	3.7	4.2	4.1
eth.						
AU	-	0.77	0.42	0.81	0.62	0.51

AU*mm	-	0.84	0.58	0.89	0.64	0.55
Rel. area%	-	3.0	3.8	3.5	2.6	2.4
cont						
AU	NA	0.78	0.75	0.51	0.41	0.22
AU*mm	NA	0.99	0.82	0.50	0.46	0.30
Rel. area%	NA	3.3	2.8	2.8	2.8	2.8

37-38kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.10	0.29	NA	0.49	0.38	0.67
AU*mm	0.15	0.32	NA	0.54	0.52	0.98
Rel. area%	2.8	2.0	NA	2.3	2.6	4.1
eth.						
AU	0.01	0.54	0.26	0.73	0.90	1.00
AU*mm	0.05	0.89	0.23	1.19	1.43	1.63
Rel. area%	3.2	3.2	1.5	4.6	5.8	7.2
cont						
AU	NA	0.56	0.55	0.37	0.26	0.21
AU*mm	NA	0.87	0.79	0.52	0.37	0.26

Rel. area%	NA	2.9	2.7	3.0	2.2	2.4

38-39kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.11	0.34	NA	0.41	0.35	0.47
AU*mm	0.36	0.67	NA	1.12	0.68	0.63
Rel. area%	6.8	4.2	NA	4.9	3.4	2.6
eth.						
AU	-	0.63	0.29	0.56	0.43	0.39
AU*mm	-	0.85	0.57	0.76	0.71	0.35
Rel. area%	-	3.0	3.7	3.0	2.9	1.6
cont						
AU	NA	0.62	0.66	0.35	0.27	0.19
AU*mm	NA	0.98	1.02	0.78	0.40	0.39
Rel. area%	NA	3.3	3.5	4.4	2.4	3.5

40-41kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	-	0.25	NA	0.38	0.34	0.50

AU*mm	-	0.43	NA	0.42	0.48	0.79
Rel. area%	-	2.7	NA	1.8	2.4	3.3
eth.						
AU	0.02	0.43	0.24	0.50	0.44	0.44
AU*mm	0.04	0.48	0.51	0.63	0.52	0.85
Rel. area%	2.3	1.7	3.3	2.5	2.1	3.8
cont						
AU	NA	0.44	0.49	0.26	0.21	0.17
AU*mm	NA	0.67	0.50	0.36	0.26	0.22
Rel. area%	NA	2.3	1.7	2.1	1.5	2.0

41-42kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.09	0.26	NA	0.47	0.56	0.61
AU*mm	0.22	0.63	NA	0.73	1.00	1.07
Rel. area%	4.2	3.9	NA	3.2	5.0	4.5
eth.						
AU	0.01	0.70	0.26	0.53	0.45	0.36
AU*mm	0.01	1.72	0.32	1.56	0.86	0.80

Rel. area%	0.8	6.2	2.1	6.1	3.5	3.5
cont						
AU	NA	0.70	0.72	0.28	0.20	0.15
AU*mm	NA	1.36	1.68	0.42	0.28	0.29
Rel. area%	NA	4.6	5.7	2.4	1.7	2.7

43-44kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.10	0.29	NA	0.41	0.33	0.49
AU*mm	0.19	0.30	NA	0.50	0.33	0.82
Rel. area%	3.6	1.9	NA	2.2	1.6	3.4
eth.						
AU	0.02	0.49	0.23	0.51	0.34	0.30
AU*mm	0.01	0.59	0.34	0.61	0.29	0.21
Rel. area%	0.7	2.1	2.2	2.4	1.1	0.9
cont						
AU	NA	0.49	0.48	0.25	0.20	0.13
AU*mm	NA	0.66	0.58	0.27	0.31	0.18

Rel. area%	NA	2.2	2.0	1.5	1.9	1.7

45-46kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.10	0.25	NA	0.38	0.28	0.51
AU*mm	0.19	0.60	NA	0.58	0.33	0.46
Rel. area%	3.6	3.7	NA	2.5	1.8	1.9
eth.						
AU	0.03	0.50	0.21	0.42	0.36	0.33
AU*mm	0.04	0.79	0.37	0.71	0.40	0.66
Rel. area%	2.1	2.8	2.4	2.8	1.6	2.9
cont						
AU	NA	0.51	0.46	0.23	0.20	0.14
AU*mm	NA	0.92	0.86	0.40	0.21	0.24
Rel. area%	NA	3.1	2.9	2.3	1.2	2.2

47kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.08	0.25	NA	0.27	0.18	0.43
AU*mm	0.07	0.25	NA	0.34	0.17	0.48

Rel. area%	1.2	1.5	NA	1.5	1.0	2.0
eth.						
AU	0.03	0.43	0.20	0.38	0.33	0.27
AU*mm	0.03	0.73	0.22	0.50	0.44	0.32
Rel. area%	1.9	2.6	1.5	2.0	1.7	1.4
cont						
AU	NA	0.45	0.47	0.22	0.23	0.15
AU*mm	NA	0.62	0.49	0.21	0.37	0.11
Rel. area%	NA	2.1	1.6	1.2	2.2	1.0

47-48kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.15	0.46	NA	0.47	0.43	0.48
AU*mm	0.22	0.73	NA	0.69	0.77	0.56
Rel. area%	4.1	4.5	NA	3.0	3.8	2.4
eth.						
AU	0.12	0.37	0.30	0.42	0.39	0.30
AU*mm	0.15	0.41	0.40	0.67	0.62	0.59
Rel. area%	8.8	1.5	2.6	2.6	2.4	2.6
cont						

AU	NA	0.43	0.45	0.29	0.57	0.25
AU*mm	NA	0.48	0.61	0.57	0.33	0.44
Rel. area%	NA	1.6	2.1	3.2	4.0	4.0

51kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	-	0.26	NA	0.34	0.33	0.57
AU*mm	-	0.32	NA	0.44	0.48	0.89
Rel. area%	-	2.0	NA	1.9	2.4	3.7
eth.						
AU	0.04	0.60	0.21	0.47	0.39	0.26
AU*mm	0.08	0.86	0.32	0.79	0.70	0.35
Rel. area%	5.0	3.1	2.1	3.1	2.8	1.5
cont						
AU	NA	0.60	0.59	0.23	0.17	0.12
AU*mm	NA	1.19	1.01	0.29	0.35	0.19
Rel. area%	NA	4.0	3.4	1.7	2.1	1.8

52-53kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.

germ						
AU	0.09	0.23	NA	0.40	0.27	0.46
AU*mm	0.35	0.41	NA	0.72	0.87	0.63
Rel. area%	6.5	2.5	NA	3.1	4.3	2.6
eth.						
AU	0.03	0.52	0.18	0.35	0.30	0.26
AU*mm	0.06	1.15	0.43	0.73	0.59	0.49
Rel. area%	3.3	4.1	2.8	2.9	2.4	2.2
cont						
AU	NA	0.50	0.51	0.18	0.17	0.12
AU*mm	NA	0.83	1.09	0.48	0.36	0.28
Rel. area%	NA	2.8	3.7	2.7	2.2	2.5

56-57kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.06	0.19	NA	0.19	0.15	0.29
AU*mm	0.16	0.32	NA	0.46	0.28	0.51
Rel. area%	3.0	2.0	NA	2.0	1.3	2.1
eth.						

AU	0.04	0.45	0.18	0.32	0.21	0.15
AU*mm	0.05	0.67	0.28	0.45	0.37	0.14
Rel. area%	3.1	2.4	1.8	1.7	1.5	0.6
cont						
AU	NA	0.55	0.58	0.20	0.12	0.13
AU*mm	NA	1.01	0.85	0.31	0.25	0.28
Rel. area%	NA	3.4	2.9	1.8	1.5	2.6

62kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.07	0.12	NA	0.11	0.09	0.16
AU*mm	0.06	0.34	NA	0.13	0.10	0.59
Rel. area%	1.2	2.1	NA	0.6	0.5	2.5
eth.						
AU	0.04	0.18	0.08	0.16	0.11	0.10
AU*mm	0.03	0.41	0.26	0.52	0.33	0.36
Rel. area%	2.0	1.5	1.7	2.0	1.3	1.6
cont						
AU	NA	0.27	0.24	0.09	0.09	0.08

AU*mm	NA	0.47	0.78	0.32	0.27	0.19
Rel. area%	NA	1.5	2.7	1.9	1.6	1.8

65-66kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.03	0.12	NA	0.07	0.09	0.11
AU*mm	0.03	0.07	NA	0.08	0.09	0.15
Rel. area%	0.5	0.4	NA	0.3	0.5	0.6
eth.						
AU	0.02	0.26	0.06	0.14	0.07	0.07
AU*mm	0.01	0.40	0.05	0.15	0.16	0.07
Rel. area%	0.7	1.4	0.3	0.6	0.6	0.3
cont						
AU	NA	0.34	0.22	0.06	0.07	0.08
AU*mm	NA	0.37	0.28	0.04	0.12	0.10
Rel. area%	NA	1.3	0.9	0.2	0.7	0.9

67-68kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						

AU	0.07	0.11	NA	0.08	0.07	0.12
AU*mm	0.05	0.13	NA	0.15	0.11	0.15
Rel. area%	1.0	0.8	NA	0.7	0.5	0.6
eth.						
AU	0.02	0.16	0.05	0.09	0.07	0.08
AU*mm	0.02	0.21	0.03	0.09	0.08	0.07
Rel. area%	1.2	0.7	0.2	0.3	0.3	0.3
cont						
AU	NA	0.24	0.23	0.07	0.05	0.04
AU*mm	NA	0.24	0.45	0.10	0.13	0.09
Rel. area%	NA	0.8	1.5	0.6	0.8	0.8

71kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.05	0.10	NA	0.08	0.08	0.15
AU*mm	0.03	0.10	NA	0.08	0.24	0.21
Rel. area%	0.6	0.6	NA	0.4	1.2	0.9
eth.						
AU	0.01	0.09	-	0.06	-	0.03

AU*mm	0.01	0.12	-	0.07	-	0.02
Rel. area%	0.8	0.4	-	0.3	-	0.1
cont						
AU	NA	0.15	0.13	0.03	0.07	0.06
AU*mm	NA	0.44	0.35	0.05	0.09	0.07
Rel. area%	NA	1.5	1.2	0.3	0.6	0.6

76-77kD

trt.	-RNA	0hrs.	3hrs.	6hrs.	12hrs.	24hrs.
germ						
AU	0.09	0.14	NA	0.15	0.16	0.10
AU*mm	0.10	0.15	NA	0.21	0.25	0.15
Rel. area%	1.9	0.9	NA	0.9	1.2	0.6
eth.						
AU	0.08	0.05	0.10	0.09	0.11	0.07
AU*mm	0.10	0.13	0.11	0.17	0.27	0.09
Rel. area%	5.8	0.5	0.7	0.7	1.1	0.4
cont						
AU	NA	0.13	0.15	0.07	0.14	0.14
AU*mm	NA	0.22	0.28	0.10	0.18	0.27

Rel. area%	NA	0.7	0.9	0.6	1.1	2.5