Control of storage-protein synthesis during seed development in pea (*Pisum sativum* L.)

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The tissue-specific syntheses of seed storage proteins in the cotyledons of developing pea (*Pisum sativum* L.) seeds have been demonstrated by estimates of their qualitative and quantitative accumulation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and rocket immunoelectrophoresis respectively. Vicilin-fraction proteins initially accumulated faster than legumin, but whereas legumin was accumulated throughout development, different components of the vicilin fraction had their predominant periods of synthesis at different stages of development. The translation products *in vitro* of polysomes isolated from cotyledons at different stages of development reflected the synthesis *in vivo* of storage-protein polypeptides at corresponding times. The levels of storage-protein mRNA species during development were estimated by 'Northern' hybridization using cloned complementary-DNA probes. This technique showed that the levels of legumin and vicilin (47000- M_r precursors) mRNA species increased and decreased in agreement with estimated rates of synthesis of the respective polypeptides. The relative amounts of these messages, estimated by kinetic hybridization were also consistent. Legumin mRNA was present in leaf poly(A)⁺ RNA at less than one-thousandth of the level in cotyledon poly(A)⁺ (polyadenylated) RNA, demonstrating tissue-specific expression. Evidence is presented that storage-protein mRNA species are relatively long-lived, and it is suggested that storage-protein synthesis is regulated primarily at the transcriptional level.

Seed development in pea (*Pisum sativum* L.) has been divided into seven physiological stages (reviewed by Marinos, 1970). These stages cover fertilization to the presence of a developed (but undifferentiated) embryo, followed by differentiation of the embryo tissues to form axis and cotyledons. Cell division in the embryo is known to cease after differentiation (Cullis, 1976) and growth is subsequently by cell expansion. Little increase in size or weight occurs until after differentiation; thereafter, rapid accumulation of storage reserves occurs, (stages 5 and 6) and continues until the final stage of maturation and drying out of the seed.

Previously authors have been able to detect the cotyledon-specific proteins, primarily the storage proteins, legumin and the vicilin fraction, shortly

after differentiation, with the vicilin fraction appearing 1 day before legumin (Millerd *et al.*, 1975). More recent work (Domoney *et al.*, 1980) employing very sensitive immunological techniques has shown that storage proteins are present throughout embryo development, but only in very small quantities before differentiation. Storage proteins have not been detected in other tissues of the plant. Characterization of the pea storage proteins legumin, and the components of the vicilin fraction, vicilin and convicilin, has been extensively described (Matta *et al.*, 1981; Gatehouse *et al.*, 1981; Croy *et al.*, 1980a; Casey, 1979).

Both polysomes and poly(A)⁺ RNA isolated from developing pea seeds have been shown to synthesize storage-protein precursors when translated in cell-free synthesizing systems (Evans *et al.*, 1978; Croy *et al.*, 1980b,c; Spencer & Higgins, 1980; Higgins & Spencer, 1981). Characterization of the mRNA species themselves has also been reported (Croy *et al.*, 1982). As part of a continuing study, the present paper reports experiments

Abbreviations used: cDNA, complementary DNA; d.a.f., days after flowering; poly(A)⁺ RNA, polyadenylated RNA; poly(A)⁻ RNA, non-polyadenylated RNA; SDS, sodium dodecyl sulphate; SSC, 0.15 M-NaCl/15 mM-sodium citrate, pH 7.0.

designed to relate protein synthesis and mRNA production during pea seed development, and attempts to set out a model to serve as a basis for designing further experiments to investigate control of the expression of genes coding for cotyledon proteins.

Materials and methods

Plant materials

Seeds of *Pisum sativum* L., var. 'Feltham First' (Suttons Seeds Ltd., Reading, Berks., U.K.) were germinated and grown by water culture in a controlled-environment cabinet as previously described (Evans *et al.*, 1978). Plants were limited to the first two pods set per plant. Seed development as judged by fresh and dry weights, and protein synthesis and accumulation, was uniform to ± 1 day. All assays and results described in the present paper were obtained from a sample of at least 20 seeds at each developmental stage.

Chemicals

Chemicals used in the work described in the present paper were obtained from BDH Chemicals, Poole, Dorset, U.K., unless otherwise stated, and were of analytical grade or the best available. Trizma base, glyoxal, Acridine Orange, α -amanitin and Dextran-40 were purchased from Sigma Chemical Co., Poole, Dorset, U.K.; agarose, *Escherichia coli* RNA, globin mRNA and calf thymus DNA were from Miles Laboratories Ltd., Stoke Poges, Slough, Berks., U.K. All radiochemicals were supplied by Amersham International. Percoll and Ficoll were products of Pharmacia Ltd., Uppsala, Sweden. Restriction enzymes were obtained from BRL Ltd., Cambridge, U.K., oligo-(dT)-cellulose from Collaborative Research, Waltham, MA, U.S.A., and nitrocellulose (type BA 85) from Schleicher and Schuell, Dassel, Germany.

Protein analysis

Finely ground meals from freeze-dried cotyledons (5–40 mg of meal/ml of buffer, depending on developmental stage) were extracted overnight with constant agitation at 4°C in the appropriate buffer for protein analysis, and subsequently centrifuged at 9000 g for 5 min. A portion of the clear supernatant was subsequently taken for analysis. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970), using 12% (w/v) acrylamide in the separating gel. Rocket immunoelectrophoresis for protein quantification was carried out on the basis of the methods described by Weeke (1973). Tris/borate buffer (0.5 M-Tris/10 mM-EDTA adjusted to pH 8.6 with solid H_3BO_3) was used as extraction medium and electrophoresis buffer. Anti-

sera were raised in rabbits and purified as described previously (Croy *et al.*, 1980b). Their specificity was checked by double diffusion (Ouchterlony & Nilsson, 1978). Samples (5 or 10 μ l) of extracts were run in duplicate on horizontal 1%-agarose gel slabs (30 ml, containing 100–600 μ l of antisera, as appropriate); electrophoresis was at 100 V for 16 h. The resulting rockets were made visible by pressing, washing and staining with Kenacid Blue R [0.25% in methanol/acetic acid/water (50:7:43, by vol.)], and were compared with those produced by known amounts of purified legumin and vicilin (Gatehouse *et al.*, 1980; Croy *et al.*, 1980a) to quantify proteins in the extracts. To obtain reliable results when using this method, it was found necessary to run at least duplicate plates, and to compare results with estimates of protein contents made by other methods.

Isolation of RNA from developing cotyledons

Polysomes were isolated from cotyledons of developing seeds by using the method described by Evans *et al.* (1978). Amounts of polysomes were estimated by absorbance, assuming $A_{260}^{1\text{mg/ml}} = 10$. A minimum of 4 g of frozen cotyledons (8 d.a.f.) was used; at most developmental stages, 10 g of cotyledons were used. Poly(A)⁺ RNA and poly(A)[−] RNA were purified from polysomes by chromatography on oligo-(dT)-cellulose, and total polysomal RNA was purified from polysomes by an SDS/proteinase K digestion method followed by phenol/chloroform/3-methylbutan-1-ol extraction and precipitation with ethanol (Evans *et al.*, 1980). Total RNA was also purified from cotyledons by the SDS/proteinase K method described by Hall *et al.* (1978) and Girard (1967). RNA was assayed by absorbance, assuming $A_{260}^{1\text{mg/ml}} = 25$.

Translation of RNA

Polysomes, poly(A)⁺ RNA and RNA were translated in the rabbit reticulocyte and wheat-germ cell-free synthesizing systems with [³H]leucine as radioactive label, under the conditions described by Evans *et al.* (1978) and Croy *et al.* (1980b). The translation product were analysed by SDS/polyacrylamide-gel electrophoresis on 17% (w/v)-acrylamide gels and fluorography (Bonner & Laskey, 1974).

Hybridization of mRNA species with cloned cDNA species

The preparation, cloning and identification of cDNA species for the major storage-protein polypeptides of developing pea seeds have been previously described (Evans *et al.*, 1980; Croy *et al.*, 1982). Three cDNA clones were used in the present study: pRC 2.2.4 contained an insert of approx. 680 base-pairs and hybrid-selected mRNA coding for the

60 000- M_r legumin precursor polypeptide; pRC 2.2.1 contained an approx.-900-base-pair insert and hybrid-selected mRNA coding for the 50 000- M_r vicilin precursor polypeptide; pRC 2.2.10 contained an approx.300-base-pair insert and hybrid-selected mRNA coding for the vicilin 47 000- M_r precursor polypeptide (Croy *et al.*, 1980*b,c*, 1982; Gatehouse *et al.*, 1981).

Detection and quantitative comparison of single mRNA species in RNA preparations from developing seeds were carried out by the 'Northern Blot' technique. RNA species were denatured with glyoxal (ethanedial) (McMasters & Carmichael, 1977) and subjected to electrophoresis on 1.5% agarose gels in 10 mM-sodium phosphate buffer, pH 6.9. Gel slabs were run at 100 V for 5 h in a 'submarine' tank with continuous buffer recirculation by stirring. Gel tracks containing standard RNA species were stained with Acridine Orange; the RNA species on remaining tracks were transferred to nitrocellulose sheets and fixed at 80°C *in vacuo* for 2 h (Thomas, 1980). The nitrocellulose sheets were then pre-hybridized and hybridized at 65°C in $3 \times \text{SSC}$ with DNA probes as described by Southern (1979) (final wash $0.1 \times \text{SSC}$). DNA probes were prepared by ^{32}P -labelling of inserts excised from the above plasmids with endonuclease *Bam*H1 and purified by agarose-gel electrophoresis; labelling was carried out by nick-translation (Maniatis, 1975; Amersham International, 1980) to a specific radioactivity of $>10^7$ c.p.m./ μg of DNA. After hybridization, filters were dried and autoradiographed at -70°C with an intensifying screen (Fuji X-ray film; du Pont screen).

Comparison of the amounts of different mRNA species in a preparation of poly(A)⁺ RNA species from 19-d.a.f. pea cotyledons and leaves was carried out by a method analogous to that described by Le Meur *et al.* (1981). Discs of diazobenzyloxymethyl-paper (1.7 cm diameter) were prepared as described by Alwine *et al.* (1977) and increasing amounts (1 μg –10 μg) of poly(A)⁺ RNA were spotted on to the discs, with *Escherichia coli* RNA added to keep the total amount of RNA constant (20 μg). The discs were allowed to bind RNA overnight at 4°C in the dark and treated as described by Alwine *et al.* (1977). Nick-translated cDNA probes, as described above, were denatured by boiling for 6 min before being hybridized to the discs (1 μg of probe/filter in a total volume of 50 μl). The discs were allowed to hybridize for 18 h at 42°C and then washed as described by Le Meur *et al.* (1981). Dried discs were counted by Cerenkov radiation (efficiency 20%). Hybridization values obtained were normalized, taking the highest value as 100%, which represented 9 and 11% of the input DNA counts for pRC 2.2.4 and 2.2.10 respectively. A constant 1.5% background of non-specific hybridization was subtracted. Hybridization curves were computer-fitted.

Cotyledon culture

Cotyledons from developing pea seeds were transferred to culture medium (1 ml/cotyledon) prepared as described by Millerd *et al.* (1975) and incubated at 28°C in light for various periods of time. To estimate inhibition of protein synthesis, α -amanitin was added at concentrations of 5–100 $\mu\text{g}/\text{ml}$ to cotyledons, which were incubated for 2 days. The amounts of legumin and vicilin present were then compared with those in control cotyledons by rocket immunoelectrophoresis. The two cotyledons of a single seed provided material for each experiment and its control. To estimate the inhibition of transcription by α -amanitin, various amounts (concentrations 1–100 $\mu\text{g}/\text{ml}$) were added to cotyledons incubated in the presence of [^3H]-uridine (10 $\mu\text{Ci}/0.4$ pmol per cotyledon). After 2 days, total RNA was isolated from the cotyledons and fractionated into poly(A)⁻ and poly(A)⁺ RNA as previously described. The incorporation of [^3H]-uridine into each fraction was estimated. A similar procedure was used to perform a pulse-chase labelling experiment with [^3H]uridine; cotyledons were incubated for 4 h in the presence of the label (10 $\mu\text{Ci}/0.4$ pmol per cotyledon), then transferred to fresh culture solution containing non-radioactive uridine (1 mg/ml) and incubated for various times.

Results

Qualitative and quantitative changes in seed proteins during seed development

(i) *Seed development.* Under the growth conditions employed, differentiation of the embryo to form visible cotyledons and axis commenced 7 d.a.f., and seed development was essentially complete by 21 d.a.f., as shown in Fig. 1.

(ii) *Qualitative variation in proteins.* Analysis of total protein extracts of cotyledons of developing pea seeds by SDS/polyacrylamide-gel electrophoresis, shown in Fig. 2, was used to demonstrate the tissue-specific synthesis of a limited number of protein subunits during seed development. A band pattern without dominating cotyledon-specific proteins, i.e. storage-protein subunits, was obtained at early stages (7–9 d.a.f.), but by the end of seed development (21 d.a.f.), storage-protein subunits were the major components (more than 80% of the total stained material). The band patterns show some evidence of differential increased synthesis of different storage-protein polypeptides in that vicilin subunits are present in larger amounts earlier than others, whereas legumin and convicilin subunits accumulate in larger amounts relative to vicilin later in development (after 15 d.a.f.).

(iii) *Quantitative variation in proteins.* The accumulation of the storage proteins legumin and

Fig. 1. Cotyledon dry weight accumulation (■) and accumulation of the cotyledon storage proteins legumin (○) and the vicilin fraction (●) in developing pea seeds

the vicilin fraction [i.e. vicilin and convicilin, defined by Gatehouse *et al.* (1981)] was quantified by rocket immunoelectrophoresis. Material reacting with both anti-legumin and anti-vicilin antibodies was detected in whole seeds as early as 4 d.a.f. [in agreement with the results of Domoney *et al.* (1980)]. When cotyledons were assayed, the pattern of accumulation shown in Fig. 1 was obtained. Vicilin-fraction proteins were detected in appreciable amounts before legumin (as reported by Millerd *et al.*, 1975) and the rate of accumulation of legumin in this early period was always lower than that of vicilin. The rate of accumulation of both proteins was not constant, but increased with time so that the total accumulated protein followed an upward curve. This phase lasted 8–11 d.a.f. for vicilin and 9–13 d.a.f. for legumin. After this accelerating rate of protein synthesis the rate for legumin synthesis remained virtually constant (i.e. the amount of protein increased linearly) until the end of development (20–21 d.a.f.). The accumulation of vicilin followed a more complex pattern, with a period of roughly constant synthesis (11–14 d.a.f.) followed by decreased synthesis (14–16 d.a.f.) followed by increased synthesis (16–20 d.a.f.), resulting in a two-step accumulation curve. The results of SDS/polyacrylamide-gel electrophoresis suggest that a significant part of the second 'step' in the vicilin-accumulation curve is due to convicilin synthesis, since the ratio of convicilin (71000- M_r ,

polypeptides) to vicilin (50000- M_r polypeptides) changes significantly over the period 15–19 d.a.f. as judged by the relative intensities of the stained bands on gel.

Qualitative and quantitative changes in mRNA during seed development

(i) *Qualitative changes.* Changes in the relative amounts of mRNA species present during seed development were assessed by purifying polysomes from seeds of different ages and translating them *in vitro* in the reticulocyte cell-free synthesizing system. The resulting polypeptide band patterns after analysis by SDS/polyacrylamide-gel electrophoresis and fluorography are shown in Fig. 2(b). Similar results were obtained when poly(A)⁺ RNA purified from polysomes was translated. Bands due to legumin and vicilin-fraction polypeptide precursors were identified by immunoprecipitation as described previously (Croy *et al.*, 1980b,c; Gatehouse *et al.*, 1981). Vicilin polypeptide precursors (at 50000 and 47000 M_r) became significant components in the translation products *in vitro* before legumin polypeptide precursors (at 60000 M_r), (8–9 d.a.f. as against 10–11 d.a.f.), in agreement with the protein-accumulation data. Towards the end of development (17–19 d.a.f.) vicilin bands at 50000 and 47000 M_r became less prominent, whereas those of legumin at 60000 M_r and convicilin at 71000 M_r were unchanged, again in agreement with protein accumulation. The polypeptide band patterns of the translation products indicate that no new major mRNA species appear after 10–11 d.a.f.

(ii) *Quantitative changes in individual mRNA species.* A quantitative assessment of the changes in concentration of individual mRNA species during seed development was made by a 'Northern blot' technique. The hybridizations were carried out with probe in excess and the relative intensities of the subsequent bands on the autoradiographs were taken as a measure of the relative amounts of mRNA species present. A control experiment carried out with known amounts of globin mRNA and a globin cDNA probe was used to demonstrate the validity of this assumption. Since identical amounts of RNA were loaded in each track of the gel, the intensity of the hybridized band is a measure of the proportion of the total RNA (or polysomal RNA) contributed by the specific mRNA. The results of these experiments are shown in Fig. 3. The levels of all three mRNA species assayed increase during the earlier part of seed development (8–14 d.a.f.), but not equally. mRNA for the vicilin 47000- M_r precursor (probed with cDNA 2.2.10; see the Materials and methods section) is not detectable at 8 d.a.f. (present at less than 0.01% of total RNA), but increases continuously in proportion over 10–14 d.a.f. (approx. 5–10-fold). It is then present only in

Fig. 2. (a) SDS/polyacrylamide-gel electrophoretic analysis of total protein extracts of cotyledons of developing pea seeds and (b) SDS/polyacrylamide-gel electrophoretic analysis followed by fluorography of translation products *in vitro* of polysomes isolated from cotyledons of developing peas in the rabbit reticulocyte cell-free synthesis system

(a) Bands representing the major storage-protein polypeptides are marked Lg (legumin; α = acidic, β = basic subunits, Vc (vicilin) and Cvc (convicilin). Amounts of sample loaded are equivalent to 400 μ g of cotyledon tissue (7 d.a.f.), 300 μ g (9 d.a.f.), 200 μ g (10 d.a.f.), 150 μ g (11, 12 d.a.f.), 100 μ g (13, 14 d.a.f.), 75 μ g (15, 17 d.a.f.), 50 μ g [19, 21, 33 (M = mature) d.a.f.]. (b) Bands representing the major storage-protein polypeptide precursors are marked Lg (legumin), Vc (vicilin) and Cvc (convicilin). Approx. 10^5 c.p.m. of incorporated radioactivity ($[^3\text{H}]$ leucine label) were loaded in each gel track. Each translation reaction contained 100 μ g of polysomes and 10 μ Ci of $[^3\text{H}]$ leucine; the proportion of radioactivity incorporated into polypeptide material varied from 9.7 (8 d.a.f.), to 11.6 (13 d.a.f.) %. Under the conditions used the incorporation was limited by the amount of polysomes. M_r std. denotes M_r standard proteins run in this track.

small proportions (less than at 10 d.a.f.) at 16 d.a.f. and for the rest of development. mRNA for the vicilin 50000- M_r precursor (probed with cDNA 2.2.1) is also first detectable at 10 d.a.f., but remains a small proportion of total seed RNA species until 14 d.a.f., when it becomes a very prominent component. It then decreases to a relatively constant proportion over the period 16–18 d.a.f., and then decreases considerably, so that at 22 d.a.f. it is only present in small amounts. mRNA for the legumin 60000- M_r precursor (probed with cDNA 2.2.4) is hardly detectable at 12 d.a.f., and not at all earlier, in agreement with the later onset of rapid

synthesis of this protein. A rapid increase in this mRNA then takes place to 14 d.a.f., after which the overall proportion of this mRNA remains roughly constant to 19 d.a.f., i.e. over the period of legumin mRNA synthesis at a roughly constant rate. Similar results for total seed RNA and polysomal RNA preparations were observed for all three mRNA species, except that for legumin mRNA the proportion in polysomal RNA decreased after 19 d.a.f. but increased slightly in total RNA; this indicates that legumin mRNA is under-represented in the polysomal preparations from these late development stages. Significant degradation of mRNA

Fig. 3. 'Northern' blots of total and polysomal RNA species isolated from developing pea cotyledons

A 10 μ g portion of RNA was loaded in each slot on the original agarose gel, which has its origin at 'O'. Owing to variations in probe specific radioactivities, band intensities cannot be compared between plots, but are comparable on the same blot. The plate in Fig. 3(a) was probed with clone pRC 2.2.10 (vicilin 47000- M_r precursor), Fig. 3(b) with pRC 2.2.1 (vicilin 50000- M_r precursor), and Fig. 3(c) with pRC 2.2.4 (legumin 60000- M_r precursor). The messages selected by these probes have previously been sized at 17S (vicilin 50000- and 47000- M_r precursors) and 19S (legumin 60000- M_r precursors) (Croy *et al.*, 1982).

species was observed in some total RNA preparations, particularly at 16 d.a.f., and was considered to be an artefact of preparation.

(iii) *Accurate relative levels of mRNA species.* The

accurate relative levels of legumin and vicilin mRNA species in 19-d.a.f. cotyledons, and legumin mRNA in cotyledons and leaves, were assessed by using a kinetic method based on hybridization of nick-

Fig. 4. Hybridization of labelled cDNA species to various amounts of immobilized poly(A)⁺ RNA species ●, cDNA 2.2.4 (legumin) hybridized to poly(A)⁺ RNA from 19-d.a.f. cotyledons; ○, cDNA 2.2.10 (vicilin 47000-*M_r* precursor) hybridized to poly(A)⁺ RNA from 19-d.a.f. cotyledons; ▲, cDNA 2.2.4 (legumin) hybridized to poly(A)⁺ RNA from leaves.

translated specific cDNA probes to different amounts of immobilized polysomal poly(A)⁺ RNA (Fig. 4). As shown in Fig. 4, the concentration of legumin mRNA in 19-d.a.f. poly(A)⁺ RNA was approximately three times that of vicilin (47000-*M_r* precursor) mRNA, in reasonable agreement with the relative amounts of these polypeptides synthesized *in vitro* by polysomes isolated from 19-d.a.f. cotyledon (Fig. 2b). The concentration of legumin mRNA in 19-d.a.f. cotyledon poly(A)⁺ RNA was at least one thousand times that in leaf poly(A)⁺ RNA, showing the tissue-specific accumulation of legumin mRNA.

(iv) *Estimation of mRNA half-lives.* mRNA half-lives were estimated by two methods. First, cotyledons were incubated in culture medium under conditions where continued synthesis of storage proteins had been demonstrated, both without and with the addition of α -amanitin at a concentration (5 μ g/ml) that had been shown to inhibit the incorporation of [³H]uridine into poly(A)⁺ RNA by over 95%. In the presence of α -amanitin, storage-protein synthesis was shown to continue over a 2-day period (in the range 9–13 d.a.f.) at a virtually unchanged rate by measurements of amounts of proteins present by rocket immunoelectrophoresis, indicating that the mRNA for the storage proteins was relatively stable over this period, since, if mRNA

had been unstable, synthesis would have ceased very rapidly.

Secondly, a pulse-chase experiment, assessing the incorporation of [³H]uridine into poly(A)⁺ RNA in cotyledons in culture was carried out. After a 4 h pulse with [³H]uridine, the radioactivity in poly(A)⁺ RNA was measured after various intervals of chase with non-radioactive uridine (Fig. 5). This experiment showed that, at most, 75% of the radioactivity incorporated into poly(A)⁺ RNA turned over relatively quickly [half-life (*t₁*) < 10 h]; a further part (20–30%) of the incorporated radioactivity had a much slower rate of turnover (*t₁* > 20 h). This part of the poly(A)⁺ RNA with slow turnover was considered to be equivalent to the long-lived mRNA deduced from the earlier experiment. Radioactivity was incorporated relatively slowly into poly(A)⁻ RNA, and did not appear to turn over with a *t₁* of less than 50 h.

Discussion

The present results provide a demonstration of the control of gene expression in the cotyledons of pea seeds during their development, comparable with the studies of Goldberg *et al.* (1981) and Meinke *et al.* (1981) on the expression of soya-bean (*Glycine max*) seed-protein genes.

Fig. 5. Pulse-chase experiment with $[^3\text{H}]$ uridine on 11-d.a.f. cotyledons being cultured *in vitro*

The radioactivity incorporated after a 4 h pulse ($10\mu\text{Ci}$ of $[^3\text{H}]$ uridine/cotyledon, 0.4 nM) and various chase times (with fresh culture medium containing 4 mM -uridine) per cotyledon is shown: ●, radioactivity in poly(A)⁺ RNA; ○, radioactivity in poly(A)⁻ RNA.

The accumulation of legumin follows a roughly linear increase over the latter part of seed development (13–19 d.a.f.) and thus the amount of legumin mRNA would be expected to be roughly constant over this interval. This is found to be the case if polysomal RNA at 14, 16 or 18 d.a.f. is considered (Fig. 3c). The increasing rate of legumin accumulation over the period 10–13 d.a.f. is also in agreement with the increase in the amount of legumin mRNA 10–14 d.a.f. in total and polysomal RNA (Fig. 3c). At the end of seed development, legumin synthesis ceases, and legumin mRNA declines as a component of polysomal RNA. At this time legumin mRNA seems to survive as a component of the total RNA, suggesting that the cessation of legumin synthesis is not due to non-availability of mRNA, but is a reflection of the decrease in the amount of polysomes (results not shown).

The heterogeneity of the vicilin fraction of pea seeds does not allow such a clear comparison between mRNA levels and protein synthesis to be drawn. If the vicilin 47000- M_r precursor is considered, it may be deduced (Figs. 2a and 2b) that the synthesis of this polypeptide increases 8–10 d.a.f. and declines markedly after 15 d.a.f. This polypeptide is known to be a precursor ($t_{1/2}$ approx. 6h) of vicilin polypeptides of lower M_r (Gatehouse *et al.*,

1981; Higgins & Spencer, 1981) and thus disappears from accumulated vicilin when its rate of synthesis decreases; at the same time it is decreased as a component of polysomal translation products. The levels of its mRNA (Fig. 3a) thus reflect this pattern of synthesis. The results concerning the vicilin 50000- M_r precursor are not as readily interpreted. The relatively low levels of mRNA for this polypeptide until 14 d.a.f. (Fig. 3b) are not in agreement with the onset of vicilin synthesis and its rapid accumulation from 11 d.a.f. (Figs. 1 and 2). Vicilin is known to contain a number of molecular forms (Gatehouse *et al.*, 1981), and at least ten separable 50000- M_r polypeptides have been observed in accumulated vicilin on two-dimensional gel analysis (J. A. Gatehouse, unpublished work). In addition, multiple 50000- M_r vicilin polypeptides are present in translation products *in vitro* of pea seed mRNA (J. A. Gatehouse, unpublished work). It is thus possible that the clone pRC 2.2.1 is selectively detecting vicilin 50000- M_r precursors predominantly synthesized during the latter part of development, and is not hybridizing to the vicilin precursor(s) synthesized during the earlier part of development. The relative amounts of different molecular forms of vicilin are known to change during seed development (Millerd *et al.*, 1978). The washing conditions used for blots are sufficiently stringent that incomplete homology could prevent hybridization; this is illustrated by the failure of the vicilin 50000- M_r and 47000- M_r precursor clones to cross-hybridize, despite extensive sequence homology (G. W. Lycett, unpublished work). Clarification of the results obtained with this cDNA will thus necessitate precise characterization of the vicilin polypeptide it specifies. Although results obtained with the legumin and vicilin 47000- M_r precursors are in reasonable agreement with the assumption that protein synthesis depends only on mRNA amounts, and the relative levels of legumin and vicilin (47000- M_r) messages measured in poly(A)⁺ RNA from 19 d.a.f. cotyledons are also in reasonable agreement with this simple model, this seems unlikely to be the only controlling factor in seed-protein synthesis.

At any time in seed development t , for any particular mRNA species, we may write:

$$\text{Amount of mRNA} = \int_0^t S \, dt - \int_0^t D \, dt$$

where S is the rate of message production at time t , and D is the rate of message breakdown at time t . The increasing amounts of seed protein mRNA species observed during cotyledon development may thus be the result of increased synthesis, decreased degradation, or both. Although the present results do not allow firm conclusions to be drawn, it can be suggested [in agreement with the conclusions of Goldberg *et al.* (1981)] that transcription of cotyledon protein genes is restricted to differentiated

cotyledon cells, and that the increase in these mRNA species is a result of increased synthesis. Since transcription decreases as cotyledon development continues (Millerd *et al.*, 1975) and mRNA production (as measured by α -amanitin inhibition) is also decreased after 12 d.a.f. (J. A. Gatehouse, unpublished work), it may be assumed that production of storage-protein mRNA species is restricted to this initial part of cotyledon development (in agreement with the measured increases in mRNA species; Fig. 3) and that the subsequent mRNA levels are determined by their relative rates of degradation. Since mRNA levels do not decline rapidly, half-lives for these mRNAs must be relatively long (>20h) as suggested here (Fig. 5), and as measured more rigorously for soya-bean mRNA species by Goldberg *et al.* (1981).

At present we have not determined whether cotyledon-specific protein genes are transcribed in other tissues of the plant. The immunological detection of legumin and vicilin in very young (4 d.a.f.) seeds suggests the possibility of expression of these genes in the undifferentiated embryo, or in the endosperm tissue present at this stage of development. In leaf tissue the results in Fig. 5 show that if these genes are being transcribed, degradation of the resulting transcript must occur very rapidly, possibly as nuclear post-transcriptional processing, as no legumin mRNA could be detected [limit of detection less than 0.01% of poly(A)⁺ RNA]. It must therefore be concluded that, although transcriptional control is the most likely method for regulating the expression of cotyledon-specific protein genes, it is possible that other forms of regulation also play significant roles.

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