Plant Physiol. (1983) 71, 47-54
0032-0889/83/71/0047/08/$00.50/0

Regulation of Legumin Levels in Developing Pea Seeds under Conditions of Sulfur Deficiency

RATES OF LEGUMIN SYNTHESIS AND LEVELS OF LEGUMIN mRNA

Received for publication May 5, 1982 and in revised form August 16, 1982

PETER M. CHANDLER, THOMAS J. V. HIGGINS, PETER J. RANDALL, AND DONALD SPENCER
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra City, A.C.T. 2601, Australia

ABSTRACT

It was shown previously that when peas (*Pisum sativum* L.) are grown with suboptimal sulfur supply the level of legumin (the more S-rich of the two major seed storage proteins) in the mature seed is selectively reduced (Randall, Thomson, Schroeder, 1979 Aust J Plant Physiol 6: 11-24). This paper reports a study of the cellular mechanisms involved in regulating legumin synthesis under these conditions. Pulse and pulse-chase labeling experiments were carried out with excised, immature cotyledons from normal and S-deficient plants. Legumin was isolated from cotyledon extracts by immunochromatography, and the proportion of legumin synthesis relative to total protein synthesis was determined. Results showed that reduced legumin accumulation could largely be accounted for by a greatly reduced level of legumin synthesis (80-88% reduction) rather than by a major increase in legumin breakdown.

Legumin mRNA levels were assayed by two methods. *In vitro* translation of polysomal RNA from cotyledons of normal and S-deficient plants indicated a reduction of 60 to 70% in synthesis of legumin-related products by preparations from S-deficient plants. A legumin cDNA clone was constructed, characterized, and used to measure the levels of legumin mRNA in polysomal and total RNA preparations from developing cotyledons. Legumin mRNA levels were reduced by 90% in preparations from S-deficient plants.

When restored to an adequate S supply, S-deficient plants (or pods taken from such plants) recovered normal levels of legumin synthesis (*in vivo* and *in vitro*) and of legumin mRNA. These results indicate that reduced legumin accumulation under conditions of S deficiency is primarily a consequence of reduced levels of legumin mRNA.

Legumin and vicilin are the two storage proteins accumulated in large quantities during the formation of pea (*Pisum sativum* L.) seeds. In the cultivar used in our experiments, legumin makes up approximately 15% of the total protein in the seed at maturity (25). Legumin has a higher content of cysteine and methionine than vicilin and could therefore be considered to be the more desirable protein from the point of view of animal nutrition. Its physicochemical properties are typical of one of the storage proteins found in a range of legume seeds including broad bean (*Vicia faba*), lupin (*Lupinus angustifolius*), and soybean (*Glycine max*) (8).

It was shown earlier (19) that when peas are grown with suboptimal sulfur (S) nutrition there is a marked, selective reduction in the level of accumulated legumin relative to the total protein level. Legumin was virtually undetectable under severe S deficiency. Similar changes were found in the more S-rich component of the storage proteins of lupin under S deficiency conditions (1).

This paper describes experiments that were designed to study the cellular mechanisms responsible for the reduction in the level of legumin in pea seeds developing under conditions of S deficiency. The following three mechanisms were tested: (a) a normal rate of legumin synthesis but an increased rate of degradation; (b) a normal level of legumin mRNA but reduced translation; and (c) reduced levels of legumin mRNA. The results indicate that a reduced level of legumin mRNA is the main factor responsible for the reduced accumulation of legumin.

MATERIALS AND METHODS

Plant Material. Peas (*Pisum sativum* L.) line PI/G 086, selected from cv Greenfeast, were grown in artificially lit cabinets at 20°C with a 16-h photoperiod as described (16) and pods of a known age, specified as DAF[1], were used. Plants were grown in sand with nutrient solution containing combined nitrogen (19). Sulfur was supplied as $MgSO_4$ at 1 mM in the control nutrient throughout growth and 0.05 mM in the S-deficient nutrient until first flower and omitted thereafter. The level of Mg^{2+} was kept constant by varying $MgCl_2$. Under these conditions, S-deficient plants showed characteristic symptoms and were smaller with fewer pods. Mean seed weight at maturity was little affected in pods from lower nodes but considerably reduced in later formed pods. For uniformity, only pods from the third to the sixth flowering node were used. Mature seeds from these pods had a mean weight of 280 mg in controls compared with 265 mg in S-deficient plants. S contents of these seeds differed considerably between the two treatments being, respectively, 0.20% and 0.05%. From these analyses, the degree of deficiency and the relative severity of the effects on aspects of seed composition can be gauged by reference to previous work (19).

Culture of Detached Pods. Pods were cut from the plant at the base of the peduncle, quickly re-cut under water, and the peduncle immersed in nutrient solution. The nutrient with S contained (in mM): sucrose, 292; L-asparagine, 117; L-glutamine, 39; $CaCl_2$, 4; KH_2PO_4, 6; K_2SO_4, 2; $MgSO_4$, 4; and iron/EDTA and micronutrients as for whole plant culture. For treatments without S, sulfates were replaced by chlorides to give the same cation concentrations. NaOH and HCl were used to give a final solution pH of 5.0. Precautions to minimize microbial growth included treating peduncles with 0.5% NaOCl for 10 min before detachment from the plant, filter-sterilizing nutrient media before use, and immers-

[1] Abbreviations: DAF, days after flowering; MMH, methylmercuric hydroxide; PAGE, polyacrylamide gel electrophoresis; IgG, immunoglobulin G, AMV, avian myeloblastosis virus.

ing the culture vessels in a water bath at 2°C. Pods were supported by a polystyrene sheet and held in air at 20°C in an artificially lit growth cabinet as used for growing the plants. A 1- to 2-mm section was cut from the base of the peduncle every 48 h. In preliminary experiments, we found that during the first 24 h in culture under these conditions there was no increase in fresh weight or dry weight of seeds, but during the following 7 d the rate of increase of seed fresh weight, dry weight, and protein was comparable with that of equivalent pods developing on the plant. The normal progressive changes in protein synthetic patterns continued in detached pods cultured in this way. This was established in preliminary experiments in which pods from control plants, 17 DAF, were cultured for periods of up to 6 d. Similar *in vivo* pulse-labeling patterns were obtained from cotyledons harvested during this period from either the cultured pods or from equivalent pods left on the plant (Fig. 1). In both cases, there was a progressive decline in the synthesis of the major vicilin polypeptide (mol wt = 50,000) and a progressive increase in legumin synthesis (mol wt 60,000, 40,000, and 19,000).

Extraction of RNA. Polysomal RNA containing RNA from both free and membrane-bound polysomes was isolated from cotyledons as previously described (12). Total RNA was isolated by grinding frozen cotyledons in liquid N_2 in a mortar and pestle and thawing the powder in a 1:1 mixture of phenol-chloroform-isoamylalcohol (50:48:2) and 0.1 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% SDS. The mixture was centrifuged at 10,000*g* for 5 min, reextracted if necessary, and Na-acetate (pH 5.8) was added to the aqueous phase to a final concentration of 0.2 M and 2 volumes of ethanol. After storage overnight at −20°C, the precipitated nucleic acids were collected by centrifugation, washed in 70% ethanol, dried under vacuum, and dissolved in water. An equal volume of 4 M Li-acetate (pH 6.0) was added, the mixture kept on ice for 3 h, centrifuged at 10,000*g* for 10 min, and the precipitated high mol wt RNA dissolved in H_2O and reprecipitated from ethanol before storage in water. Although such RNA preparations lack low mol wt RNA species (principally 4S and 5S RNA), we shall refer to them as total RNA preparations to distinguish them from polysomal RNA preparations.

Construction of cDNA Clones. Poly(A) RNA (25 μg) from polysomes of immature cotyledons (22 DAF) was used for cDNA synthesis in a reaction containing 80 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 10 mM DTT, 4 mM Na-pyrophosphate, 10 μg/ml oligo $(dT)_{12-18}$, 25 μg/ml poly(A) RNA, 500 μM each deoxynucleoside triphosphate, 25 μCi[α-^{32}P]dATP (carrier-free), and 250 units/ml AMV reverse transcriptase (18). Following a 1-h incubation at 41°C, NaOH was added to a final concentration of 0.1 M and RNA hydrolyzed at 60°C for 1 h. The sample was neutralized with Tris·HCl, extracted with buffer-saturated phenol, and cDNA precipitated by addition of 2 volumes of ethanol. Synthesis of second-strand DNA by AMV reverse transcriptase was carried out at 41°C for 5 h in conditions used for first-strand synthesis except for the omission of oligo dT, RNA, and Na-pyrophosphate. After extraction with phenol and precipitation with ethanol, the DNA was dissolved in 90 μl of H_2O, then 10 μl of 10 × S1 buffer (10 × S1 buffer = 3 M NaCl, 0.3 M Na-acetate, 30 mM $ZnSO_4$, pH 4.5), and 100 units S1 endonuclease (Miles Laboratories) were added and incubation allowed to proceed at 37°C for 30 min. The mixture was extracted with phenol, DNA precipitated with ethanol, and then fractionated on a Bio-Rad A150m column (280 x 7 mm) in 10 mM Tris (pH 7.5) and 1 mM EDTA. Fractions containing the leading half of the DNA peak (determined by radioactivity) were pooled, and the DNA precipitated with ethanol and dissolved in water. Conditions for dC-tailing were as previously described (20), and an estimated 15 dC residues were added per 3'OH terminus. Pst I-linearized pBR322 DNA was similarly tailed with dG residues. Samples were extracted with phenol, the DNAs precipitated with ethanol and then mixed in approximately equal molar proportions (double-stranded cDNA: pBR322) in 50 μl 0.2 M NaCl. The mixture was heated to 60°C, then cooled to room temperature over a 2-h period before addition of 200 μl of competent *Escherichia coli* K-12 RRI cells (2). Following 30 min incubation on ice and then 5 min at 42°C, 1 ml L broth (10 g Difco Bacto-Tryptone, 5 g Difco Yeast Extract, 10 g NaCl/l) was added, the cells were incubated 30 min at 37°C, and transformants selected on L agar containing tetracycline (20 μg/ml). Colonies containing plasmids with inserts were detected by subsequent failure to grow on L agar containing ampicillin (100 μg/ml).

Selection of Clones Containing Legumin mRNA Sequences. Colonies were screened by hybridization (10) using ^{32}P-labeled cDNA prepared from poly(A) RNA to reveal those inserts representing abundant mRNA sequences. Plasmids were isolated from colonies giving the strongest hybridization signal and tested for cross-hybridization of inserts. One of the major hybridization families detected had properties expected of a cloned legumin mRNA sequence (see "Results" for details).

Hybrid Release Translation. Full details of the procedure used are described elsewhere (4). An insert from pPS15-75 (a legumin cDNA plasmid) was subcloned into the Pst I site of phage fd103 replicative form DNA (11). The phage derivative containing the strand complementary to legumin mRNA was identified (fd103-75L) and 1 μg of this single-stranded DNA mixed with 0.5 μg poly(A) RNA from cotyledons 22 DAF in 25 μl of 0.2 M NaCl, 0.02 M Tris-HCl (pH 7.5), 2 mM EDTA, 0.1% SDS. Following hybridization at 65°C for 20 min, the mixture was fractionated on a Bio-Rad A150m column. Nucleic acids in the excluded peak (fd103-75L plus mRNA hybridized to the insert) were precipitated with ethanol and added to an *in vitro* translation system before and after treatment with 7 mM MMH (Alfa Chemicals, Ventron Corporation, Danvers, MA). Fractionation of the radioactive polypeptides by SDS-PAGE followed by fluorography revealed polypeptides whose mRNAs were hybridized to the insert in the phage DNA and released by MMH treatment.

Sequencing of DNA. A Sau 3a fragment of the insert in pPS15-75 containing the appropriate sequence was 3'-end labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I, or 5'-end labeled with [γ-^{32}P] ATP and polynucleotide kinase (15). This fragment was digested with Hha 1, and a 200 base pair fragment labeled at a single 3' or 5'end sequenced by the procedure of Maxam and Gilbert (15).

Radioactive Labeling of DNA. One μg of plasmid or insert was incubated at 15°C for 90 min in a 25-μl reaction containing 25 mM Na-phosphate (pH 7.8), 5 mM $MgCl_2$, 20 ng/ml DNase 1, 10 μM each of dCTP, dGTP, and dTTP, 0.5 μM dATP, 50 μCi [α-^{32}P]dATP, and 1 unit DNA polymerase I (Klenow fragment). NaOH was added to 0.3 N before heating at 70 to 80°C for 5 min, neutralization, and chromatography on Sephadex G-50 (Pharmacia) in 10 mM Tris, 1 mM EDTA (pH 7.5). DNA in the excluded peak was precipitated in the presence of calf thymus DNA carrier with ethanol and dissolved in approximately 5 ml hybridization solution. DNA was denatured by heating at 80°C for 5 min before addition to filters.

RNA Transfers and Hybridization. Equal masses (20 μg) of total or polysomal RNA were electrophoresed in horizontal, 1.5% agarose gels (220 x 150 x 3 mm) containing 10 mM MMH. Gel buffer and precautions taken with MMH were as previously described (5). Electrophoresis was carried out at 7 v/cm, 50 mamp for 4 h.

Gels were prepared for RNA transfer by soaking in 200 ml 0.2 M NaOH containing ethidium bromide (2 μg/ml) and 2-mercaptoethanol (5 mM) for 30 min with gentle shaking. Then followed three successive 5-min washes in 200 ml 0.2 M K-phosphate (pH 6.5) containing 7 mM Na-iodoacetate, and two 5-min washes in 0.2 M Na-acetate (pH 4). The gel was photographed under UV light and placed on a stack of 10 sheets of Whatman No. 1 paper

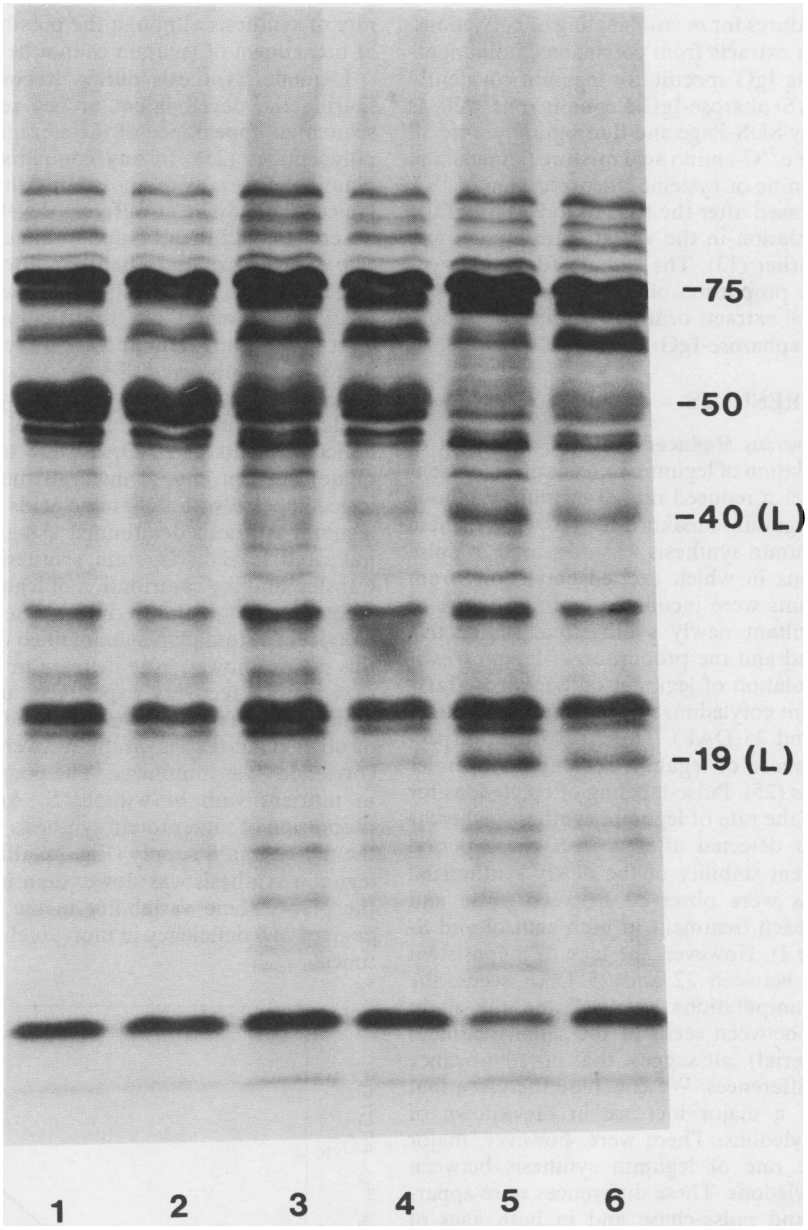

FIG. 1. A comparison of pulse-labeling patterns of proteins in cotyledons from detached pods in culture and from intact plants. At 17 DAF, pods were detached from plants and cultured for 6 d as described in "Materials and Methods". At 18, 20, and 23 DAF, cotyledons from detached pods and from pods of the same age on intact plants were pulse-labeled for 2 h with ^{14}C-amino acids, and the radioactive proteins fractionated by SDS-PAGE and detected by fluorography. Tracks 1, 3, and 5 show extracts of cotyledons from detached pods at 18, 20, and 23 DAF, respectively. Tracks 2, 4, and 6 show the corresponding extracts from cotyledons from intact plants. Each track was loaded with 200 μg of protein. The numbers indicate the mol wt $\times 10^{-3}$ of the major legumin (L) and vicilin polypeptides.

soaked in 0.2 M Na-acetate (pH 4).

Aminothiophenol paper (21) was activated in 200 ml of 1.2 M HCl containing 7.5 ml freshly prepared 1% (w/v) $NaNO_2$ for 10 to 15 min at room temperature, washed briefly in H_2O and cold 0.2 M Na-acetate (pH 4) before being placed on top of the gel and overlaid with two sheets of Whatman No. 1 paper and a small pile of absorbent paper. After transfer overnight, the filter was washed for 30 min in 10 mM Tris-HCl (pH 8) containing 1% (w/v) glycine.

Filters were prehybridized at 42°C in 50% formamide, 3 × SSC (SSC = 0.15 M NaCl, 15 mM trisodium citrate), 10 × Denhardt's solution (in w/v, 0.2% BSA, 0.2% PVP, 0.2% Ficoll 400; Pharmacia), 0.1% SDS, 25 μg/ml denatured calf thymus DNA, 10 μg/ml poly(A), 1% glycine for 6 to 18 h, and hybridized under the same conditions with approximately 5×10^6 cpm ^{32}P-labeled DNA for approximately 24 h. After hybridization, filters were washed in 2 × SSC containing 0.1% SDS at 70°C for 1 h, then 0.1 × SSC, 0.1% SDS at 50°C for 20 min before autoradiography with an intensifying screen.

Densitometry. Relative legumin mRNA levels in RNA preparations from different tissues were estimated by densitometry of the x-ray film and comparison of peak areas. Corrections for differences between lanes in loading of the gel or transfer to the filter were made by densitometry of x-ray film of the same filter hybridized with ^{32}P-labeled cDNA prepared from purified 25S rRNA by random priming. Relative legumin mRNA levels are therefore normalized to 25S rRNA levels. Under these conditions of RNA transfer and hybridization, there is a linear relationship between intensity of a mRNA band and amount of RNA loaded on the gel between 1 and 40 μg RNA/lane (lane width = 11 mm).

Other Procedures. Procedures for *in vivo* labeling of cotyledons, preparation of total protein extracts from cotyledons, immunoaffinity chromatography using IgG specific for legumin covalently coupled to Sepharose-4B (Sepharose-IgG-Legumin), as well as polypeptide fractionation by SDS-Page and fluorography were all described elsewhere (24). The ^{14}C-amino acid mixture (Amersham, U.K.) contained no methionine or cysteine. Incorporation of ^{14}C-amino acids into protein ceased after the first 10 to 20 min of the chase period. *In vitro* translation in the wheat germ system was carried out as described earlier (13). The extent of legumin synthesis was expressed as the proportion of labeled protein (TCA-insoluble counts) in the total extracts or in the *in vitro* translation products which bound to Sepharose-IgG-legumin.

RESULTS

Increased Degradation *versus* Reduced Rate of Synthesis of Legumin. Reduced accumulation of legumin in seeds of S-deficient plants could be due to either a reduced rate of legumin synthesis or an increased rate of legumin breakdown. To resolve these alternatives, the rate of legumin synthesis was measured in pulse and pulse-chase experiments in which excised cotyledons from control and S-deficient plants were incubated with a mixture of ^{14}C-amino acids. The resultant newly synthesized, radioactive proteins were then extracted and the proportion of legumin estimated after quantitative isolation of legumin on Sepharose-IgG-legumin. For this experiment cotyledons were taken at two stages of seed development (22 and 25 DAF). These represent, respectively, the early and mid-phase of legumin accumulation under our experimental conditions (25). Pulse-labeling of cotyledons for 10 min gave an estimate of the rate of legumin synthesis, whereas the proportion of legumin detected after a 20-h chase period should reflect the subsequent stability of the newly synthesized legumin. Small differences were observed between pulse and pulse-chase values within each treatment in both control and S-deficient cotyledons (Table I). However, the lack of a consistent trend in these differences between 22 and 25 DAF seeds, the complex nature of the manipulations involved and the slight variability known to exist between seeds of the same treatment (especially S-deficient material), all suggest that no significance can be attached to these differences. We conclude therefore that there was no evidence of a major increase in breakdown of legumin in S-deficient cotyledons. There were, however, major differences in the relative rate of legumin synthesis between control and S-deficient cotyledons. These differences were apparent after both the pulse and pulse-chase and in both ages of cotyledon. This relative rate was reduced by 88% at 22 DAF and by 80% at 25 DAF compared to that in control plants (Table I). These results indicate that the reduced accumulation of legumin in mature seeds of S-deficient plants is largely due to a decreased rate of synthesis although the possibility of small changes in rate of breakdown of legumin cannot be excluded.

Table I. *Legumin Synthesis, as a Percentage of Total Protein Synthesis, in Cotyledons from Control and Sulfur-Deficient Plants at Two Stages of Seed Development*

Cotyledons were pulse-labeled for 10 min with ^{14}C-amino acids with or without a subsequent 20-h chase period.

Development Stage	Cotyledon Source	Labeling Time	[^{14}C]Legumin (% of Total ^{14}C-Protein)
22 DAF	Control	10 min	8.3
		20 h	10.5
	S-deficient	10 min	1.0
		20 h	1.6
25 DAF	Control	10 min	14.8
		20 h	12.8
	S-deficient	10 min	2.9
		20 h	1.9

Legumin Synthesis during Recovery from Sulfur Deficiency. During the development of pea seeds, there is a well-defined sequential appearance of the storage proteins and corresponding polypeptides (25). In any comparison of healthy and deficient plants, there exists the possibility that seeds of the same chronological age may be at different developmental stages. Differences observed could then be due to the effect of the deficiency on the rate of development rather than directly on the particular parameter measured. In recovery experiments carried out over 1 or 2 d, possible differences due to developmental age are minimized at least in the short term and the direct effects of the deficiency are more readily observed. Recovery experiments were therefore undertaken to test whether legumin synthesis in S-deficient plants could be restored by application of S during seed formation. S-deficient plants at 20 DAF were transferred to control (S-adequate) nutrient and, at intervals thereafter, cotyledons were collected, labeled with ^{14}C-amino acids for 2 h, and the proportion of legumin synthesis determined using Sepharose-IgG-legumin. An increased level of legumin synthesis was detectable 24 h after transfer, and the contribution of legumin to total protein synthesis increased at an almost linear rate for the next 5 d (Fig. 2). Cotyledons from plants maintained on S-deficient medium during this period showed little increase in legumin synthesis (Fig. 2).

A similar recovery experiment used detached pods from S-deficient plants, rather than the whole plant, so that the influence of other plant tissues on the recovery of legumin synthesis in the cotyledons was minimized. The pods were cultured from 20 DAF in nutrient with or without S. An increase in legumin as a proportion of total protein synthesis was again observed following the increase in S supply (Fig. 2), although the rate of recovery of legumin synthesis was slower than in pods remaining attached to the plant. Some variability in the trends may reflect different degrees of S deficiency in individual pods used at different harvest times.

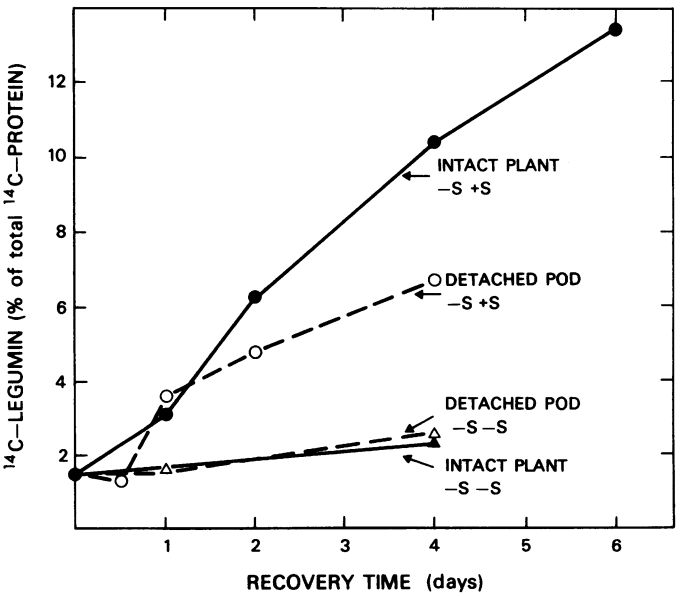

FIG. 2. Legumin synthesis in cotyledons from pea plants recovering from S deficiency. S-deficient plants (or detached pods from same) were transferred to an adequate S supply at 20 DAF (–S+S). At various times after transfer, cotyledons from these plants (or detached pods) were incubated with ^{14}C-amino acids for 2 h and the proportion of [^{14}C]legumin in the radioactive proteins was estimated by immunoaffinity chromatography. Other plants (or detached pods) remained in S-deficient nutrient prior to pulse-labeling of their cotyledons (–S–S).

EFFECT OF S STATUS ON LEGUMIN mRNA LEVELS

The reduced level of legumin synthesis in S-deficient cotyledons could be due either to reduced levels of legumin mRNA, or to less efficient translation of control levels of legumin mRNA. Two approaches were used to estimate levels of legumin mRNA: by *in vitro* translation of polysomal RNA and by hybridization of a legumin cDNA plasmid to filter-bound polysomal or total RNA.

Legumin mRNA Levels Assessed by *in vitro* Translation of Polysomal RNA. Polysomal RNA was isolated from developing cotyledons of plants of contrasting S status and translated in a cell-free system from wheat germ. Preparations derived from S-deficient cotyledons (22 and 25 DAF) directed less synthesis (32% and 40%, respectively) of legumin than RNA from control cotyledons. When S-deficient plants (20 DAF) were transferred to adequate S for 6 d prior to harvest, their cotyledonary polysomal RNA directed the synthesis of more legumin (134%) than that from the corresponding S-adequate, control plants. These results suggested that there are changes in the level of legumin mRNA in response to S supply. Such an interpretation depends, however, on the assumption that the level of legumin-related *in vitro* products is a true measure of legumin mRNA abundance. A more direct and complete measure of changes in legumin mRNA levels in response to S supply was therefore made using a legumin cDNA probe.

Legumin mRNA Levels Assessed by Hybridization with cDNA Probes.

Selection of Legumin Clone. One of the major families of cross-hybridizing inserts identified amongst the cDNA clones constructed from the poly(A) RNA fraction of cotyledon RNA (see "Materials and Methods") had properties consistent with it representing legumin sequences. The plasmid containing the largest insert in this family, pPS15-75, hybridized to an abundant mRNA with a length of approximately 1,700 nucleotides (the size expected to code for a legumin precursor polypeptide of mol wt = 60,000–65,000) (23). The abundance of this mRNA species in cotyledons of different ages followed the *in vivo* developmental pattern of legumin accumulation which has been described by Thomson *et al.* (25, data not shown).

Direct evidence that pPS15-75 contains legumin sequences was provided by hybrid release translation. For these experiments, the insert in pPS15-75 was subcloned into the Pst I site of phage fd103. This insert contains an internal Pst I site which results in fragments of approximately 1380 and 300 base pairs after Pst I digestion. One resultant phage (fd103-75L) contained the strand of the 1380 base pair fragment which is complementary to an abundant mRNA species in cotyledons (22 DAF). Hybridization of fd103-75L single-stranded DNA with total poly(A) RNA from 22 DAF cotyledons and subsequent size fractionation separated mRNA species which hybridize with the insert of this phage DNA from nonhybridizing mRNA species (4). When the hybridized mRNA in the excluded peak was used to program an *in vitro* translation system before and after denaturation of the hybrids, a group of polypeptides at mol wt = 60,000 to 65,000 was synthesized by the denatured sample (Fig. 3). These polypeptides are of a similar size to legumin precursor polypeptides synthesized *in vivo* and *in vitro*, and were selectively retained by Sepharose-IgG-legumin (Fig. 3). Some *in vitro* products of lower mol wt also bound selectively to the immunoaffinity gel (Fig. 3, track 5). Since this affinity gel has been shown to be specific for legumin-related proteins synthesized *in vivo* (23, 24), these are presumably legumin-related products resulting from premature chain termination or from partly degraded legumin mRNA.

The sequence of 29 amino acids at the amino terminus of the basic subunit of legumin has been determined by Casey *et al.* (3). Partial sequencing of the insert in pPS15-75 therefore was carried out with a view to further validating the legumin cDNA clone. Ten plasmids which hybridize with the insert in pPS15-75 have been examined, and all but one contained an internal Pst I site resulting in a fragment of 300 base pairs when the plasmid was digested with Pst I. The length of the other fragment varied between the plasmids. This finding suggests that the 300 base pair fragment represents sequences at the 3′ end of the mRNA. Approximately 550 bases are required to code for the legumin basic subunit (mol wt = 20,000) which, in the equivalent soybean storage protein (glycinin), is encoded at the 3′ end of the mRNA, the acidic subunit being at the 5′ end (27). Initial sequencing studies indicated that a Sau 3a site, located approximately 320 base pairs from the internal Pst I site of the insert, was approximately 54 amino acid residues into the basic subunit. Both strands were sequenced towards the 5′ end of the mRNA from the Sau 3a site, and a portion of this sequence is shown in Figure 4.

A stretch of 30 amino acids in the only open reading frame of the sequence (Fig. 4) is closely related to the published amino terminal sequence of the legumin basic subunit (3). The minor differences between the two sequences probably reflect the heter-

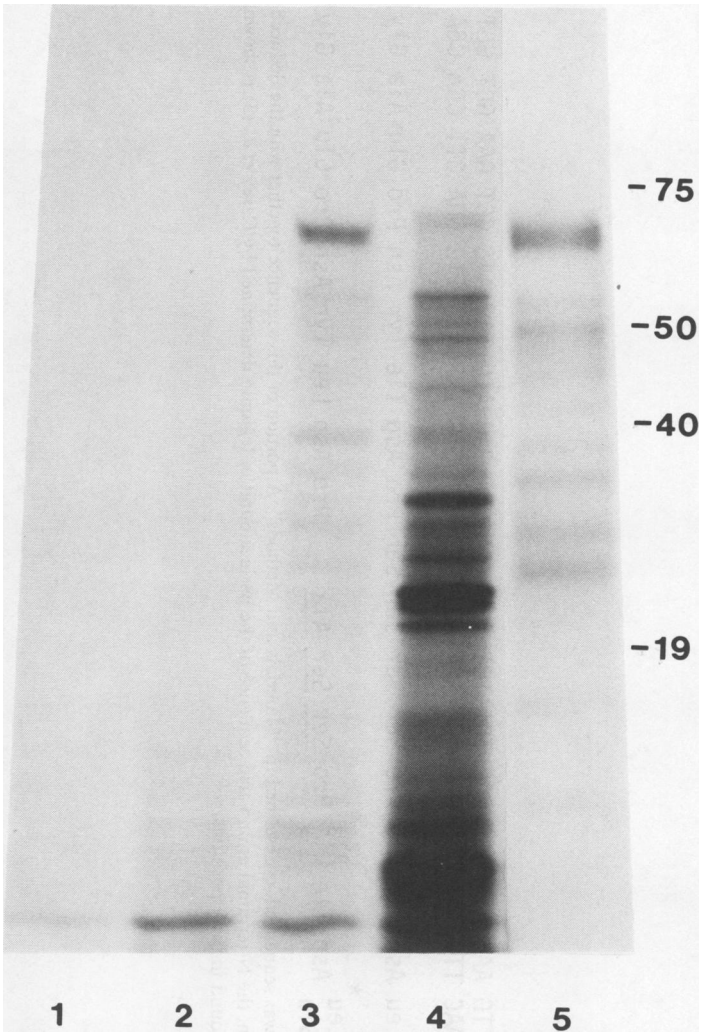

FIG. 3. Validation of legumin cDNA clone by hybrid release translation and immunoaffinity-chromatography of translation products. RNA preparations were translated *in vitro* in the wheat germ system and the products were fractionated by SDS-PAGE and detected by fluorography. Lane 1, no added RNA: lane 2, hybrids between fd103-75L and poly(A) RNA, fractionated as described in "Materials and Methods," added to translation mixture without methylmercuric hydroxide (MMH); lane 3, hybrids added with MMH (7 mM); lane 4, total poly(A) RNA (22 DAF); lane 5, hybrid selected products which bind to Sepharose-IgG-legumin. Numbers indicate the mol wt $\times 10^{-3}$ of reference polypeptides.

```
GGG CTT GAG GAA ACA GTT TGC ACT GCT AAA CTT CGA TTG AAC ATT GGC CCG TCT TCT TCA TCA CCA GAC ATC TAC AAC CCT GAA GCT GGT
CCC GAA CTC CTT TGT CAA ACG TGA CGA TTT GAA GCT AAC TTG TAA CCG GGC AGA AGA AGT AGT GGT CTG TAG ATG TTG GGA CTT CGA CCA

(a) Gly Leu Glu Glu Thr Val Cys Thr Ala Lys Leu Arg Leu Asn Ile Gly Pro Ser Ser Ser Ser Pro Asp Ile Tyr Asn Pro Glu Ala Gly
                                          *
(b) Gly Leu Glu Glu Thr Ile Cys Thr Ala Lys Leu Arg Leu Asn Ile Ala Pro Ser Ser Ala    Pro Asp Leu Tyr Asn Pro Glu Ala Gly
                    Val                   Glu                 Gly
```

FIG. 4. Nucleotide sequence of a portion of pPS15-75. A Sau 3a-Hha I fragment was sequenced as described in "Materials and Methods". A portion of this sequence together with the deduced amino acid sequence of the only open reading frame is shown in (a). For comparison, the N-terminal amino acid sequence of the basic subunit of legumin determined by Casey *et al.* (3) is shown in (b). Amino acid residues underlined indicate uncertainty. (*), Two residues were found in these positions.

ogeneity known to exist in the basic subunits of legumin (3), and both acidic and basic subunits of glycinin (17).

Therefore, on the basis of sequence information and hybrid release translation, we conclude that the insert in pPS15-75 represents legumin mRNA sequences. This plasmid was then used to quantify legumin mRNA levels in cotyledons from pea plants of contrasting S status.

Legumin mRNA Levels. In the first set of experiments, polysomal RNA was extracted from cotyledons of control and S-deficient plants, as well as from S-deficient plants which had received adequate S for 6 d prior to harvest. Hybridization of the ^{33}P-labeled legumin plasmid to these RNA samples revealed major changes in the relative levels of legumin mRNA (Fig. 5a). Hybridization of a radioactive cDNA probe prepared from 25S rRNA to the same filter shows that approximately equal amounts of 25S rRNA were loaded in each lane (Fig. 5b). When levels of legumin mRNA (determined from densitometer tracings of the original fluorograph represented in Fig. 5a) were normalized with respect to 25S rRNA (determined from densitometer tracings of fluorograph represented in Fig. 5b), samples from S-deficient plants were found to contain 8% (22 DAF) or 12% (25 DAF) of the control level of legumin mRNA. The sample from S-deficient plants which received adequate S for 6 d prior to harvest had 110% of the control level of legumin mRNA. These direct estimates of legumin mRNA were thus consistent with the observed reduction in translatable legumin mRNA using the wheat germ system as described above.

In a second experiment, total cotyledon RNA, rather than polysomal RNA, was used to check the possibility that the reduced level of legumin mRNA found in S-deficient plants simply reflected failure of normal levels of legumin mRNA to bind quantitatively to polysomes. Similar results were obtained (Fig. 5c). Densitometric analysis indicated that cotyledons from S-deficient plants contained only 7% of the amount of legumin mRNA in control cotyledons.

In this experiment, a more detailed study was also made of the effect of restoring S on the time course of recovery of legumin mRNA. In S-deficient plants changed to control nutrient, legumin mRNA levels returned to near-normal values within 48 h (Fig. 6a). Plants left on S-deficient medium maintained low levels of legumin mRNA. Similarly, pods from S-deficient plants showed marked increases in legumin mRNA levels when cultured in the presence of S, and no increase in its absence (Fig. 6b).

DISCUSSION

An earlier finding (19), that legumin accumulation was markedly reduced in seeds of pea plants grown at suboptimal S levels, raised the question as to the mechanisms responsible for this effect. Experiments described here indicate that the reduced legumin content of mature seeds is largely a consequence of reduced legumin synthesis during seed formation resulting from low levels of legumin mRNA in cotyledons from S-deficient plants.

Legumin, like the homologous soybean protein, glycinin, is a complex of 4 or 5 closely related proteins each consisting of acidic and basic subunits (mol wt approximately 40,000 and 20,000, respectively). These arise by post translational processing of a set of primary translation products which also reflect this microheterogeneity (6, 23). Data on legumin (3) and glycinin (17) indicate that this microheterogeneity is due to small differences in amino acid sequence, implying the existence of a family of closely related legumin mRNAs. The immunological methods used to assess legumin synthesis *in vivo* and *in vitro* have been shown to take account of all members of the legumin family (13, 23). Similarly, the results of the hybrid release translation experiment reported here (Fig. 3) and of hybrid arrest translation (data not shown) indicate that the cDNA clone used to quantify legumin mRNA hybridizes with most, if not all legumin mRNA species. Thus, the

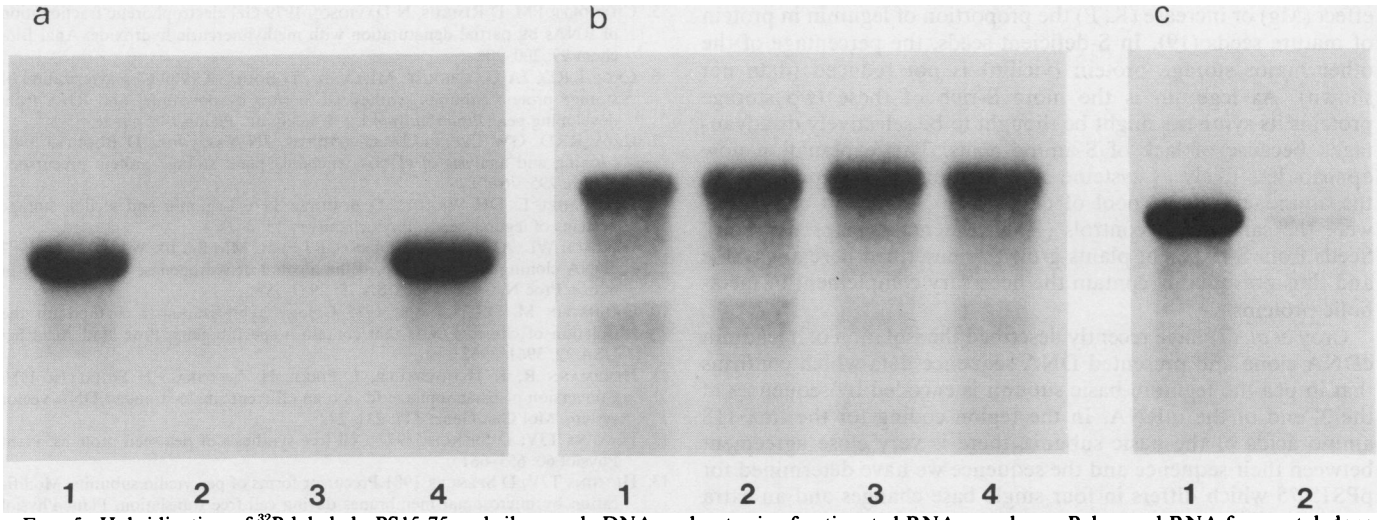

FIG. 5. Hybridization of ^{32}P-labeled pPS15-75 and ribosomal cDNA probes to size-fractionated RNA samples. a, Polysomal RNA from cotyledons as indicated hybridized with pPS15-75. Lane 1, 22 DAF control; lane 2, 22 DAF −S; lane 3, 25 DAF −S; lane 4, 26 DAF, −S until 20 DAF, +S for next 6 d. b, Same filter as in 'a' but hybridized with cDNA to 25S rRNA. c, Total RNA from control cotyledons 24 DAF (lane 1) and S-deficient cotyledons 24 DAF (lane 2) hybridized with pPS15-75.

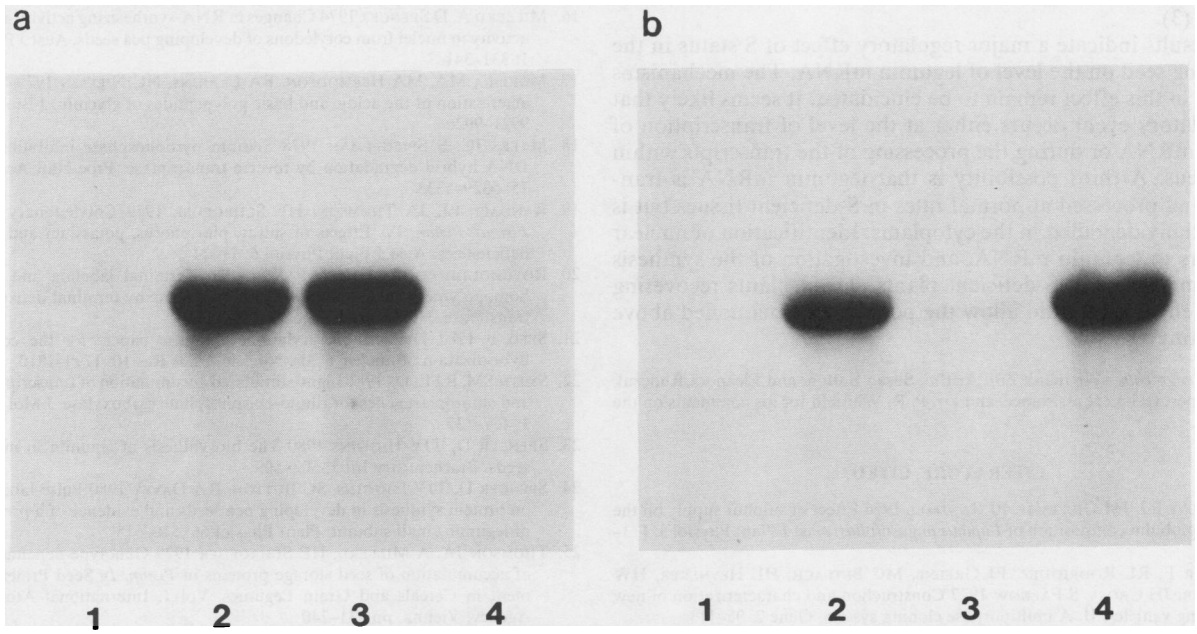

FIG. 6. Hybridization of pPS15-75 to size-fractionated RNA samples. a, Plant recovery. Total RNA from cotyledons as indicated. Lane 1, 20 DAF −S; lane 2, 22 DAF, −S until 20 DAF, +S for 2 d; lane 3, 26 DAF, −S until 20 DAF, +S for 6 d; lane 4, 24 DAF −S. b, Pod recovery. Total RNA from cotyledons as indicated. Lane 1, 20 DAF −S; lane 2, as in lane 1 but pods cultured an additional 4 d +S; lane 3, as in lane 1 but pods cultured an additional 4 d −S; lane 4, 20 DAF +S, then pods cultured an additional d +S.

methods used should give a true indication of variations in the level of legumin synthesis and of total legumin mRNAs. The possibility remains, however, that minor representatives of the legumin family may not respond to S deficiency in the same manner.

Variation in the level of *translatable* mRNAs for other plant proteins as a result of a variety of nongenetic factors has previously been reported, *e.g.* the effect of light on mRNA for the small subunit of ribulose bisphosphate carboxylase (26), and of GA_3 on the mRNA for α-amylase in aleurone layers (14). More recently, the use of cDNA clones as hybridization probes has made possible the measurement of changes in the total cellular level of specific mRNAs, *e.g.* in the mRNAs for both large and small subunit of ribulose bisphosphate carboxylase in response to light (22) and in alcohol dehydrogenase mRNA in maize in response to anaerobiosis (9). However, we are not aware of other situations in which the supply of an essential plant nutrient element has been shown to regulate the total cellular level of an mRNA as reported here for legumin mRNA. Similar changes in legumin mRNA levels were found in both total RNA preparations and polysomal RNA preparations.

The effects of S nutrition on legumin mRNA levels and legumin synthesis were seen most strikingly in developing seeds from S-deficient plants restored to adequate S nutrition as little as 24 h previously. Inasmuch as detached pods from such plants also responded to S supply in culture, the effect of S is presumably on the developing fruit itself, and not the result of a change in supply of a regulatory metabolite from elsewhere in the plant.

The effect of S deficiency in reducing legumin synthesis is relatively specific. Deficiencies of other nutrients either have no

effect (Mg) or increase (K, P) the proportion of legumin in protein of mature seeds (19). In S-deficient seeds, the percentage of the other major storage protein (vicilin) is not reduced (data not shown). As legumin is the more S-rich of these two storage proteins its synthesis might be thought to be selectively disadvantaged because of lack of S-amino acids. This explanation now appears less likely as cysteine and methionine concentrations in the aminoacyl-tRNA pool of developing S-deficient cotyledons were the same as in controls (P. K. Macnicol, in preparation). Seeds from S-deficient plants grown as described here are viable and thus presumably contain the necessary complement of metabolic proteins.

Croy *et al.* (7) have recently described the isolation of a legumin cDNA clone and presented DNA sequence data which confirms that in pea the legumin basic subunit is encoded by sequences at the 3′ end of the mRNA. In the region coding for the first 118 amino acids of the basic subunit, there is very close agreement between their sequence and the sequence we have determined for pPS15-75 which differs in four single base changes and an extra serine codon (Fig. 4, and P. Chandler, unpublished data). They suggest on the basis of Southern hybridizations that there are approximately four copies of this legumin gene per genome equivalent, which may relate to the heterogeneity observed in both the primary translation products of legumin (6, 23) and in the mature subunits (3).

Our results indicate a major regulatory effect of S status in the developing seed on the level of legumin mRNA. The mechanisms involved in this effect remain to be elucidated. It seems likely that the regulatory event occurs either at the level of transcription of legumin mRNA or during the processing of the transcripts within the nucleus. A third possibility is that legumin mRNA is transcribed and processed at normal rates in S-deficient tissues but is preferentially degraded in the cytoplams. Identification of nuclear precursors to legumin mRNA, and investigation of the synthesis of such molecules in S-deficient plants and in plants recovering from S deficiency should allow the possibilities mentioned above to be distinguished.

Acknowledgments—We thank Zufi Ariffin, Susan Button, and Eleanora Rudczuk for their expert technical assistance, and Dr. P. R. Whitfeld for his comments on the manuscript.

LITERATURE CITED

1. BLAGROVE RJ, JM GILLESPIE, PJ RANDALL 1976 Effect of sulphur supply on the seed globulin composition of *Lupinus angustifolius*. Aust J Plant Physiol 3: 173–184
2. BOLIVAR F, RL RODRIGUEZ, PJ GREENE, MC BETLACH, HL HEYNEKER, HW BOYER, JH CROSA, S FALKOW 1977 Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2: 95–113
3. CASEY R, JF MARCH, E SANGER 1981 *N*-Terminal sequence of β-subunits of legumin from *Pisum sativum*. Phytochemistry 20: 161–163
4. CHANDLER PM 1982 The use of single-stranded phage DNAs in hybrid arrest and release translation. Anal Biochem In press
5. CHANDLER PM, D RIMKUS, N DAVIDSON 1979 Gel electrophoretic fractionation of RNAs by partial denaturation with methylmercuric hydroxide. Anal Biochem 99: 200–206
6. CROY RRD, JA GATEHOUSE, MI EVANS, D BOULTER 1980 Characterization of storage protein subunits synthesized *in vitro* by polysomes and RNA from developing pea (*Pisum sativum* L.). I. Legumin. Planta 148: 49–56
7. CROY RRD, GW LYCETT, JA GATEHOUSE, JN YARWOOD, D BOULTER 1982 Cloning and analysis of cDNAs encoding plant storage protein precursors. Nature 295: 76–79
8. DERBYSHIRE E, DH WRIGHT, D BOULTER 1976 Legumin and vicilin, storage proteins of legume seeds. Phytochemistry 15: 3–24
9. GERLACH WL, AJ PRYOR, ES DENNIS, RJ FERL, MM SACHS, WJ PEACOCK 1982 cDNA cloning and induction of the alcohol dehydrogenase (*Adh-1*) gene in maize. Proc Natl Acad Sci USA 79: 2981–2985
10. GRUNSTEIN M, DS HOGNESS 1975 Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc Natl Acad Sci USA 72: 3961–3965
11. HERRMANN R, K NEUGEBAUER, E PIRKL, H ZENTGRAF, H SCHALLER 1980 Conversion of bacteriophage fd into an efficient single-stranded DNA vector system. Mol Gen Genet 177: 231–242
12. HIGGINS TJV, D SPENCER 1977 Cell-free synthesis of pea seed proteins. Plant Physiol 60: 655–661
13. HIGGINS TJV, D SPENCER 1981 Precursor forms of pea vicilin subunits. Modification by microsomal membranes during cell-free translation. Plant Physiol 67: 205–211
14. HIGGINS TJV, JA ZWAR, JV JACOBSEN 1977 Hormonal control of the level of translatable mRNA for α-amylase in barley aleurone layers. Colloq Int Cent Natl Rech Sci 261: 481–486
15. MAXAM AM, W GILBERT 1980 Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol 65: 499–560
16. MILLERD A, D SPENCER 1974 Changes in RNA-synthesizing activity and template activity in nuclei from cotyledons of developing pea seeds. Aust J Plant Physiol 1: 331–341
17. MOREIRA MA, MA HERMODSON, BA LARKINS, NC NIELSEN 1979 Partial characterization of the acidic and basic polypeptides of glycinin. J Biol Chem 254: 9921–9926
18. MYERS JC, S SPIEGELMAN 1978 Sodium pyrophosphate inhibition of RNA·DNA hybrid degradation by reverse transcriptase. Proc Natl Acad Sci USA 75: 5329–5333
19. RANDALL PJ, JA THOMSON, HE SCHROEDER 1979 Cotyledonary proteins in *Pisum sativum*. IV. Effects of sulfur, phosphorus, potassium and magnesium deficiencies. Aust J Plant Physiol 6: 11–24
20. ROYCHOUDHURY R, E JAY, R WU 1976 Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. Nucleic Acids Res 3: 101–116
21. SEED B 1982 Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids. Nucleic Acids Res 10: 1799–1810
22. SMITH SM, RJ ELLIS 1981 Light-stimulated accumulation of transcripts of nuclear and chloroplast genes for ribulosebisphosphate carboxylase. J Mol Appl Genet 1: 127–137
23. SPENCER D, TJV HIGGINS 1980 The biosynthesis of legumin in maturing pea seeds. Biochemistry Int 1: 502–509
24. SPENCER D, TJV HIGGINS, SC BUTTON, RA DAVEY 1980 Pulse-labeling studies on protein synthesis in developing pea seeds and evidence of a precursor form of legumin small subunit. Plant Physiol 66: 510–515
25. THOMSON JA, A MILLERD, HE SCHROEDER 1979 Genotype-dependent patterns of accumulation of seed storage proteins in *Pisum*. *In* Seed Protein Improvement in Cereals and Grain Legumes, Vol 1. International Atomic Energy Agency, Vienna, pp 231–240
26. TOBIN, EM, JL SUTTIE 1980 Light effects on the synthesis of ribulose-1,5-bisphosphate carboxylase in *Lemna gibba* L. G-3. Plant Physiol 65: 641–647
27. TUMER NE, JD RICHTER, NC NIELSEN 1982 Structural characterization of the glycinin precursors. J Biol Chem 257: 4016–4018