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Legumin of Vicia faba major: accumulation in developing cotyledons, purification, mRNA characterization and chromosomal location of coding genes

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Summary. Experiments were carried out on *Vicia faba major* involving (1) determination of the pattern of legumin accumulation during seed development, (2) protein purification from mature cotyledons, (3) the characterization of legumin mRNA, and (4) the chromosomal localization of the genes coding for legumins. In developing cotyledons the synthesis of legumin begins 28 days after petal desiccation (DAPD), and 4 days after initiation of vicilin synthesis. The two subunits (α_A and β_A) of legumin A appear 2 days earlier than those (α_B and β_B) of legumin B. While the accumulation of vicilin peaks on the 30th DAPD, that of legumin continues during further seed development, and the synthesis of legumin mRNA peaks on the 37th DAPD. Northern blot hybridizations using two DNA plasmids containing cDNA inserts with sequence homology to the A- and B-type legumin genes, respectively, indicated that legumin mRNAs extracted from cotyledons 36 DAPD band below the 18S RNA band. In addition, a faint band below that of the 25S RNA band can be observed in legumin mRNAs extracted from cotyledons at an earlier developmental stage (30 DAPD). By means of polyacrylamide gel electrophoresis in the presence or absence of SDS and 2-mercaptoethanol, two fractions could be eluted after zonal isoelectric precipitation of the globulins from mature seeds: one fraction contains mainly vicilin, the other, legumin. In situ hybridization showed that legumin genes are arranged in two clusters: the genes coding for legumin A are located in the longer arm of the one between the two shortest subtelocentric chromosome pairs whose centromere is in a less terminal position; those coding for legumin B are located in the non-satellited arm of the longer submetacentric pair.

Key words: Chromosomal location of genes – Legumin – mRNA characterization – Seed storage proteins – *Vicia faba* – Broad bean

Introduction

Mature faba bean (*Vicia faba* L.) seeds consist of 25–30% protein, of which 75% is accounted for by the storage globulins legumin and vicilin, with the former being two- to three-fold more abundant than the latter. Legumins, or the 11S family, thus are the main storage proteins of *V. faba* seeds (Wright and Boulter 1974; Hebblethwaite 1983). They are preferentially synthesized at membrane-bound polysomes (Müntz 1978; Müntz et al. 1978) and then stored in protein bodies formed by the swelling of cisternae and the evagination of membranes of the endoplasmic reticulum (Neumann and Weber 1978).

Each structural unit of legumin in *V. faba* and *Pisum sativum* is synthesized in vitro as a single polypeptide chain precursor with a molecular mass (M_r) of 60,000–61,000 Da, which in vivo is cleaved into two components – an acidic component, α, M_r 36,200, and a basic component, β, M_r 22,000 (Croy et al. 1980; Bassüner et al. 1983). The native legumin molecule is composed of six $\alpha\beta$ units arranged as a trigonal antiprism, and the acidic and basic components of the $\alpha\beta$ unit are connected by disulphide bonds (Plietz et al. 1984). Horstmann (1983) showed that the six structural units (or $\alpha\beta$ pairs) can be grouped in at least two types of subunit pairs: A and B, with only the former containing methionine residues. The α_A and β_A subunits of legumin A are heavier than the corresponding subunits of legumin B. The same author also suggested that the mRNAs for the two legumin types are coded by two distinct gene subfamilies, and this was confirmed later by Wobus et al. (1986) and Bäumlein et al. (1986). As shown by Heim et al. (1989), in the field bean (*Vicia faba minor*), the B-type subfamily consists of 10 to 15 genes: some of these (at least 4) are highly homologous functional genes and others (at least 2) are highly diverged pseudogenes.

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No equivalent information exists, at present, in the case of the gene subfamily that codes for legumin A, although small cDNA clones that cover a limited portion of the nucleotide sequence of the genomic A-type gene are available (Wobus et al. 1986).

Most of the available information on the synthesis of legumin and mRNA and protein accumulation during seed development comes from studies on the field bean (*V. faba minor*); equivalent data is not available on the broad bean (*V. faba major*). There is no data in the literature on the chromosomal location of legumin genes in either *V. faba minor* or *V. faba major*. In this paper we report the results of research carried out on the latter: (1) the determination of the pattern of legumin accumulation during seed development, (2) protein purification from mature seeds, (3) the characterization of legumin mRNA, and (4) the chromosomal localization of the genes coding for legumins.

Materials and methods

Plant material

Plants of *Vicia faba major* line 'MGBF250' were grown in the Experimental Field Station of the University of Tuscia, Viterbo. They were spaced 50 × 30 cm, and neither nitrogen fertilizers nor weedicides or pesticides were applied; hand-weeding was performed when necessary. The two basal florets on the inflorescence at the 2nd and 3rd flowering nodes on the main stem were tagged and hand-tripped to favour self-fertilization. Starting from the 20th day after petal desiccation (DAPD; petal desiccation is indicative of ovule fertilization), pods were collected every day, the size and weight of developing seeds were recorded and the cotyledons were immediately stored in liquid nitrogen.

Mature seeds were germinated as already described (Cionini et al. 1985), and root tips were treated with a 0.05% aqueous solution of colchicine (Sigma) for 4 h at room temperature and fixed in ethanol-acetic acid 3:1 (v/v).

cDNA clones

The DNA plasmids pVfc70 and pVfc77, kindly provided by Dr. U. Wobus (Zentralinstitut für Genetik und Kulturpflanzenforschung der Akademie der Wissenschaften der DDR, Gatersleben), were used. pVfc70 contains a cDNA insert with sequence homology to approximately 40 nucleotides of the α region and approximately 600 nucleotides of the β region of the B-type legumin gene; the cDNA insert of pVfc77 shares homology with approximately 200 nucleotides of the α region and approximately 500 nucleotides of the β region of the A-type legumin gene.

Protein extraction and fractionation

Proteins were extracted according to the method devised by Casey (1979) with modifications. The cotyledons were finely ground in a Cyclotec mill with a 1-mm screen, and the powder was mixed with 0.05 *M* sodium borate buffer (pH 8.0; 1 g meal/20 ml buffer). After stirring for 2 h at 4 °C the material was centrifuged at 19,000 *g* for 10 min at 4 °C, and the supernatant was filtered through glass-wool and dialysed against 0.033 *M* sodium acetate buffer (pH 4.8).

To obtain a globulin-enriched fraction, the extract was centrifuged at 19,000 *g* for 10 min at 4 °C and resuspended in 0.05 *M* sodium borate buffer; finely ground, solid $(NH_4)_2SO_4$ was then added to final saturations of 40% or 70%. The precipitated proteins were centrifuged at 19,000 *g* for 10 min at 4 °C, resuspended in a small volume of sodium borate freed from ammonium sulphate by a passage through a Sephadex G-25 column and then purified and fractionated by gradient zonal iso-focusing as described by Wright and Boulter (1974).

Gel electrophoresis

Proteins for PAGE were prepared in a sample buffer containing either SDS or 2-mercaptoethanol (2-ME), both SDS and 2-ME, or in the absence of both. In addition, SDS was either added or not added to the gel components and the tray buffer. Sample buffers and polyacrylamide gels were prepared, and electrophoresis was carried out according to Montebove et al. (1987). RNA electrophoresis in agarose (1.5%) gels was performed in denaturing conditions by using a 1 × MOPS buffer (0.2 *M* 3-*N*-morpholino propane-sulphonic acid, 0.05 *M* Na-acetate pH 7, and 0.01 *M* Na_2-EDTA) and 40% formaldehyde. One microgram of mRNA was loaded on each slot.

RNA extraction and mRNA separation

Developing cotyledons frozen in liquid nitrogen were homogenized in a mortar at 4 °C in 50 m*M* TRIS-HCl buffer, pH 7.5, supplemented with 10 m*M* Na_2-EDTA and 150 m*M* NaCl (10 ml buffer: 1 g cotyledons), and the homogenate was centrifuged at 10,000 *g* for 10 min at 2 °C. An equal part of a solution of phenol, chloroform and isoamyl alcohol (50:50:1 v/v/v) containing 1.6% sarkosyl was added to the supernatant, and the mixture was shaken for 10 min at room temperature, cooled and centrifuged at 5,000 *g* for 10 min at 4 °C. The supernatant was re-extracted again as above, and the RNA was precipitated and stored as described by Sambrook et al. (1989). All the buffers and glassware were made free of RNase by the addition of or washing with dietyl-pyrocarbonate and autoclaving.

The separation of mRNAs with a poly(A)+ tail was performed by loading 10 mg total RNA onto 1 ml of packed oligo(dT) cellulose in a disposable glass chromatography column (Sambrook et al. 1989). Six fractions containing poly(A)+ RNA were eluted: the third and the fourth ones were pooled, and the mRNA was precipitated and resuspended in water at a final concentration of 0.3 µg/ml.

A rapid purification of mRNA from developing cotyledons and its immobilization on nitrocellulose membranes was performed using the Quick-blot kit (Schleicher and Schuell).

Northern blot hybridization

The transfer of RNA to nitrocellulose membranes (Hybond-C, Amersham) was performed over a period of 12 h using 20 × SSPE as the transfer buffer. pVfc70 and pVfc77 cDNA clones were ^{32}P-labeled using a nick-translation kit (Amersham) and 100 µCi of (α-^{32}P)dCTP; specific activities of 2×10^8 and 0.8×10^8 cpm/µg were obtained, respectively. Labeled DNAs were denatured by heating at 95 °C for 10 min and hybridized at a concentration of 6 ng/ml.

Dot blots were performed by spotting 5 µl of a mRNA solution prepared using the Quick-blot protocol, and the filters were hybridized as above.

In situ hybridization

pVfc70 and pVfc77 cDNA clones were labeled by the nick-translation method (Rigby et al. 1977) using ^{3}H-dATP and ^{3}H-dCTP (Amersham); the specific activities were 6.2×10^6 and 6.4×10^6 cpm/µg, respectively. Tips of colchicine-treated and fixed roots were squashed after treatment with a 5% aqueous

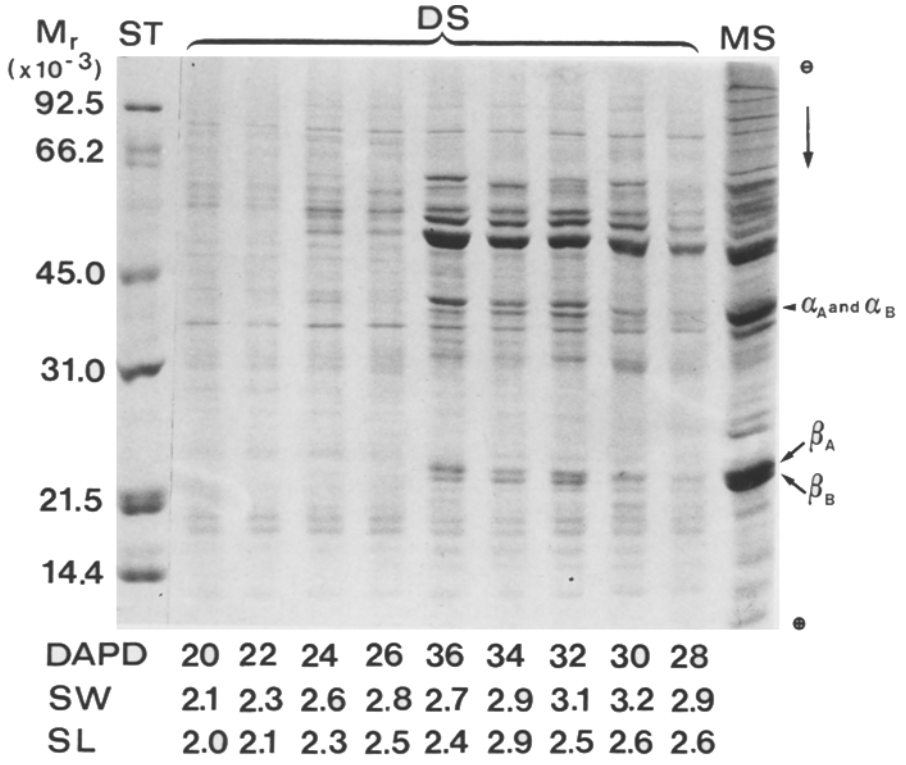

Fig. 1. SDS-PAGE of total proteins from cotyledons in developing (*DS*) and mature (*MS*) seeds. *ST* Standard M_r markers, *DAPD* days after petal desiccation; *SW* mean seed weight (g), *SL* mean seed length (cm); α and β acidic and basic subunits of A- and B-type legumin, respectively

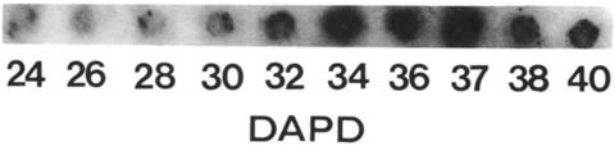

Fig. 2. Northern dot blot hybridization of mRNA from developing cotyledons at different days after petal desiccation (*DAPD*)

solution of pectinase (Sigma), as already described by Cionini et al. (1985), and in situ hybridization was performed according to Macgregor and Mizuno (1976). Chromosomal DNA was denatured in 0.07 *N* NaOH for 3 min at room temperature, and each ^{3}H-labeled cDNA clone was hybridized at a concentration of 1 µg/ml. Cross-competition experiments were performed by hybridizing each labeled cDNA probe in the presence of a 100-fold excess of the other unlabeled one. After incubation, unbound DNA was removed by stringent washings including hot SSC and trichloroacetic acid (Hennen et al. 1975). The slides were then covered with Ilford L_4 emulsion and developed after exposure times ranging from 1 week to 6 months.

Results

Globulin accumulation in developing cotyledons

The banding pattern after SDS-PAGE of the total proteins in cotyledons at different developmental stages is shown in Fig. 1. Bands corresponding to those of vicilin (Fig. 5d) began to appear on the 24th day after petal desiccation (DAPD). Their staining intensity reached a maximum on the 30th DAPD and then remained practically unchanged. At this stage of seed development, vicilin bands dominated the banding pattern of the proteins; from this stage onwards, however, legumin bands also began to become prominent. Bands in the gel regions where legumin subunits were expected (see Introduction) started to appear on the 28th DAPD, 4 days after the appearance of vicilin, when, on average, the seeds weighed 2.9 g and were 2.6 cm in length. The synthesis of legumin A started before that of legumin B, since both the basic (α) and acidic (β) subunits of the former appeared 2 days before the lighter, faster moving subunits of the latter. Figure 1 shows results obtained when developing cotyledons were analysed up to the 36th DAPD; no changes were observed during further seed development. Both seed weight and length increased steadily up to the 30th DAPD, then decreased as a result of the seed starting to dry out.

Dot blot hybridizations of mRNAs extracted from seeds at different developmental stages were performed using the ^{32}P-labeled pVfc77 cDNA clone. The results showed that the synthesis of A-type legumin mRNA increased steadily up to the 37th DAPD and then decreased (Fig. 2).

mRNA characterization

The results of experiments characterizing legumin mRNA are shown in Fig. 3. The total RNA extracted from

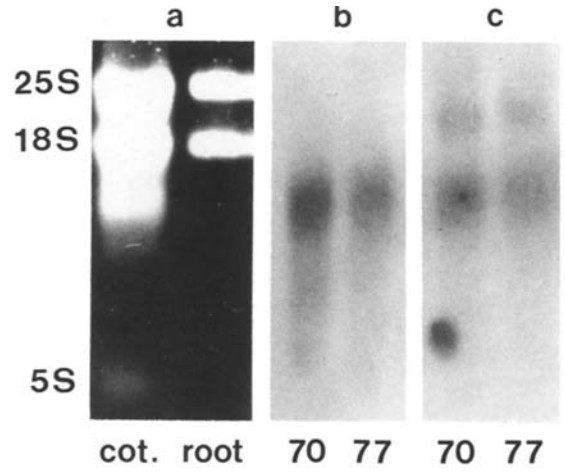

Fig. 3. Agarose gel electrophoresis of total RNA from developing cotyledons (36 DAPD) and roots (**a**), and Northern blot hybridization of ^{32}P-labeled pVfc70 or pVfc77 cDNA clones to RNA from cotyledons at the 36th (**b**) or 30th (**c**) DAPD

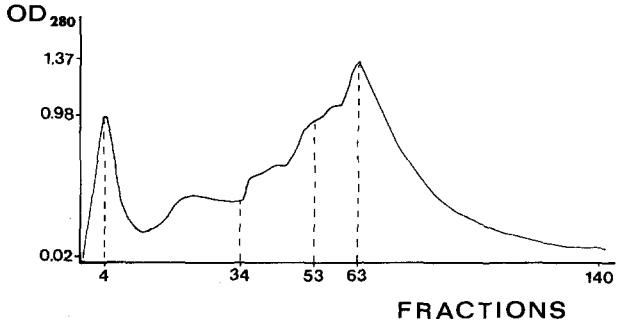

Fig. 4. Fractions eluted from gradient zonal isoelectric precipitation of the proteins from cotyledons in mature seeds in the presence of $(NH_4)_2SO_4$

developing cotyledons 36 DAPD was studied and compared by agarose gel electrophoresis with that extracted from roots. Two bands below those of 25S and 18S RNAs, respectively, were present in the former and not in the latter (Fig. 3a). Both pVfc70 and pVfc77 cDNA clones, which were tested separately, hybridized to the RNA band below that of 18S RNA (Fig. 3b). In repetitions of the Northern blots using RNAs extracted from cotyledons at a different developmental stage (30 DAPD), a faint label was seen after hybridization with both DNA probes also below the 25S RNA band (Fig. 3c).

Fractionation of the globulin components

The protein fraction of cotyledons in mature seeds precipitated in the presence of 40% $(NH_4)_2SO_4$, which was larger than that precipitated when 70% ammonium sulphate was used but which did not differ in SDS-PAGE banding pattern, was loaded on a Sephadex G-50 column and subjected to gradient zonal isoelectric precipitation. The elution pattern showed two major groups of globulins with absorbance profiles peaking in fractions 4 and 63, respectively (Fig. 4).

These two fractions and, in addition, unfractionated proteins and fractions 34 and 53 were electrophoresed on polyacrylamide gels in the presence or absence of SDS in samples, gels and tray buffer and in the presence or absence of the reducing agent 2-mercaptoethanol (2-ME, Fig. 5) in sample buffers. Fraction 34, which corresponds to a 'valley' in the absorbance profile found in Fig. 4, did not contain any significant amount of proteins. Proteins with lower M_r than the proteins in fraction 63 were observed in fraction 4 and 53 in the absence of SDS; moreover, fraction 4 contained low M_r proteins not found in fraction 53 (Fig. 5a). The addition of 2-ME to the extraction buffer did not change these patterns, but did make separation possible in the unfractionated proteins (Fig. 5b, lane 0) of lower M_r components not contained in fraction 63. After SDS-PAGE of the protein fractions extracted in the absence of 2-ME, one large band (L) was observed in fractions 0 and 63, and five bands (1–5) at M_r 31,000–65,000 appeared in fraction 4 (Fig. 5c). In the presence of 2-ME (Fig. 5d) the proteins in these latter bands did not change their migration distances: therefore, they were prevalently non-associated, single polypeptides of vicilin. In fraction 63, band L disappeared, and in its place three prominent bands containing polypeptides with M_r 38,000 (α_A and α_B), 24,000 (β_A) and 22,000 (β_B), respectively, were observed. Therefore, this fraction mainly contained polypeptides associated by disulphide bonds, and their estimated M_r indicated that they were legumin subunits. Two other associated legumin-like polypeptides were detected (bands 6 and 7), which were abundant only in fraction 63.

Chromosomal localization of legumin genes

Nick-translated pVfc70 and pVfc77 cDNA clones were hybridized separately to squashes of colchicine-treated root tips. After hybridization with pVfc77 cDNA (homologous to nucleotides of legumin A genes), labeling was seen over one region of the non-satellited arm of the longer submetacentric chromosome pair. In addition, a few silver grains were observed over the long arm of a subtelocentric pair (Fig. 6a), even though they were not present in all the metaphase plates. When pVfc70 cDNA (homologous to nucleotides of legumin B genes) was hybridized, the labeling was specular in relation to that already described: a few silver grains were often seen over the non-satellited arm of the submetacentric pair and one main labeling location appeared, at the same position as above, over the longer arm of a subtelocentric pair (Fig. 6b). After cross-competition hybridizations, silver grains were detected, though fewer in number, only at the main labeling sites.

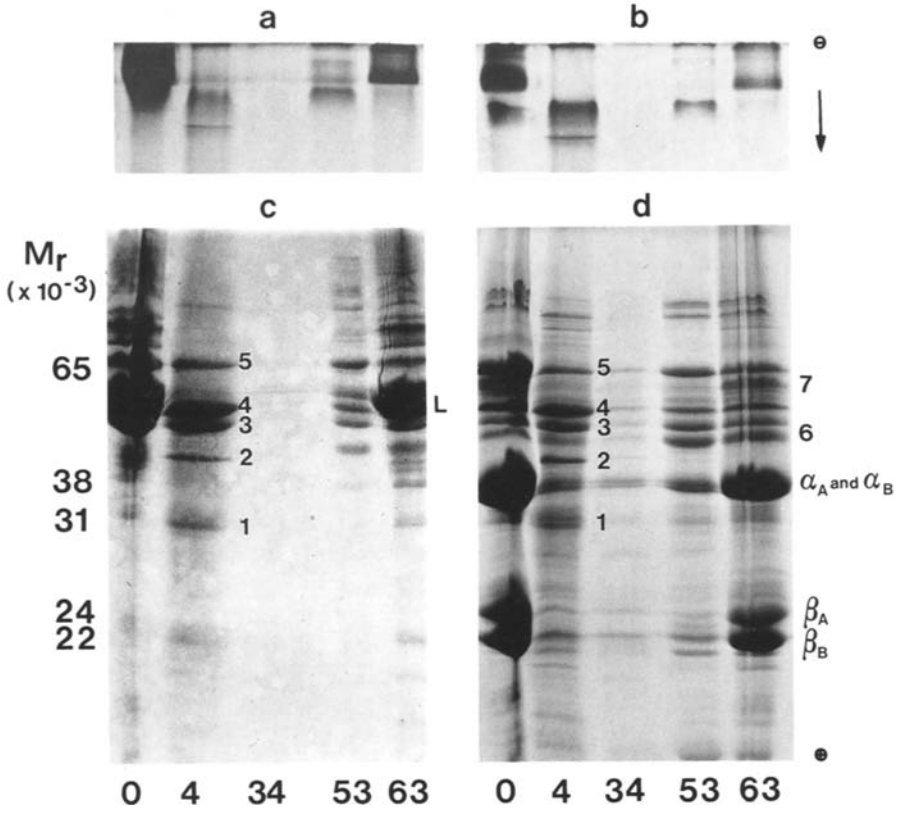

Fig. 5a–d. Polyacrylamide gel electrophoresis, in the presence or absence of SDS and 2-mercaptoethanol (2-ME), of the unfractionated proteins from cotyledons in mature seeds precipitated using 40% $(NH_4)_2SO_4$ (*lane 0*) and of fractions 4, 34, 53 and 63 of Fig. 4. The M_r scale is derived from the standard markers in Fig. 1 (not shown). **a** no SDS, no 2-ME; **b** no SDS, +2-ME; **c** +SDS, no 2-ME; **d** +SDS, +2-ME

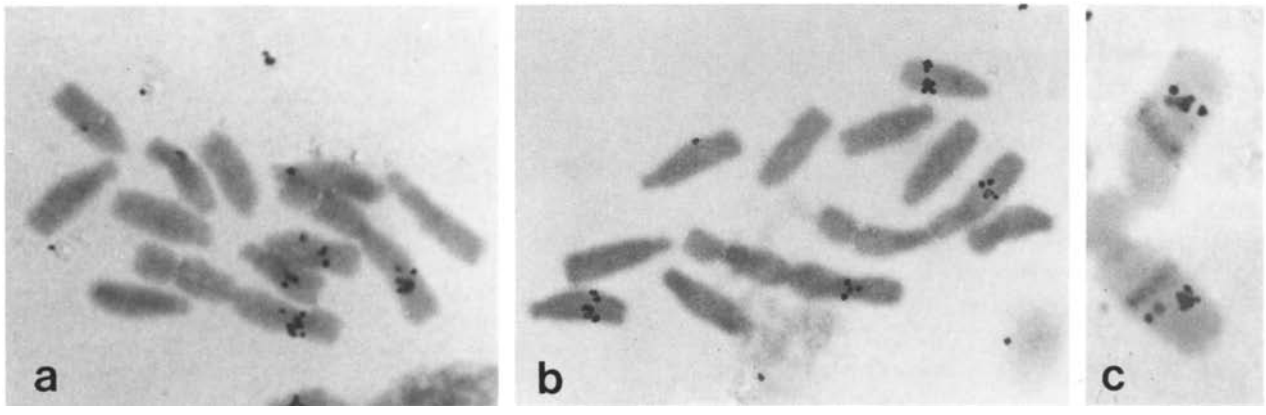

Fig. 6a–c. Autoradiographs after in situ hybridization with ^3H-labeled pVfc77 (**a**) or pVfc70 (**b** and **c**) cDNA clones. Giemsa. **a** and **b**, ×1,500; **c** ×2,600. Note in **c** the large Giemsa band that characterizes the subtelocentric chromosome pair over which labeling is mainly located after hybridization with pVfc70

It was not easy to recognize the labeled chromosome among the five subtelocentric chromosome pairs, since they are very similar and chromosome morphology is unavoidably affected by the hybridization procedure. However, on the basis of (1) careful analyses of the chromosome length carried out with the aid of a nonius-equipped micrometric ocular, (2) the observation of the centromere position and (3) the Giemsa banding (see Vosa and Marchi 1972) that occasionally occurs in preparations subjected to in situ hybridization (Fig. 6c) we can state that the genes for legumin B are located in the one between the two shortest subtelocentric chromosome pairs whose centromere is in a less terminal position.

Discussion

The SDS-PAGE banding pattern of the polypeptides forming the vicilin and legumin fractions of the proteins in the seeds of *Vicia faba major* (Fig. 5) does not differ, either qualitatively or quantitatively, from that reported in *Vicia faba minor* (Wright and Boulter 1972; Horstmann 1983). Our findings indicate that in *V. faba major* legumin synthesis in developing cotyledons starts 4 days after that of vicilin – 2 days before the seed begins to dry out (Fig. 1). This pattern is similar to that found in *V. faba minor* because the accumulation of vicilin precedes that of legumin. On the other hand, in *V. faba minor*, the two syntheses start simultaneously (Bassüner et al. 1983). Another difference between field and broad beans concerns the timing in the accumulation of seed storage proteins: in the former, both globulins are accumulated when the cotyledons are growing rapidly by cell elongation (Bassüner et al. 1983), whereas in the latter this is not true for legumin, which is predominantly synthesized at a later stage of seed development. In *Phaseolus coccineus* too the accumulation of vicilin does not begin at the same time as that of legumin, but in this species the synthesis of legumin starts before that of vicilin (Bernardi et al. 1990a). It may be worth noting that in the genus *Phaseolus*, unlike *V. faba*, the most abundant seed storage protein is vicilin (see Bernardi et al. 1990b). However, the relatively delayed synthesis of the most abundant legumin cannot be considered a general rule: indeed, in *Pisum sativum*, vicilin predominates (Weber et al. 1981) and, during seed development, its synthesis peaks earlier than that of legumin (Croy et al. 1988).

No changes were observed in the accumulation pattern of legumin after the 36th DAPD, and the synthesis of the mRNA was seen to peak at the 37th DAPD (Fig. 2). This supports the view already put forward on the basis of the low storage protein turnover during cotyledon development in other legumin seeds that the rate of globulin accumulation reflects the rate of its biosynthesis (Bassüner et al. 1983). Moreover, our findings may be added to others indicating that the regulation of the rate of globulin synthesis is linked to that of its mRNA (Püchel et al. 1979).

The mRNAs that direct the synthesis of both A- and B-type legumins in seeds on the 36th DAPD band below 18S RNA (Fig. 3). However, a larger mRNA for legumin can also be detected, although in a small quantity and at an earlier stage of seed development (30 DAPD; Fig. 3c). This suggests that in *V. faba major* genes are present that code for polypeptides other than the A- and B-type legumin but with a similar amino acid composition and that they undergo stringent developmental regulation. As a matter of fact, the presence of legumin-like polypeptides whose M_r are larger than those of the α and β subunits can be observed in fraction 63 in Fig. 5d. In addition Bassüner et al. (1983) reported that in *V. faba minor* high molecular weight legumin polypeptides are synthesized in vitro. In *Pisum sativum*, immunoprecipitation of the cell-free products of mRNA from developing cotyledons using an antibody to legumin shows three classes of precursors; their relative amounts vary throughout seed development, with the largest being more prevalent in the early stages (see Casey et al. 1988). Work is in progress to elucidate this point.

As to the chromosomal location of the legumin genes, our results indicate that they are arranged in two clusters that are located in different chromosomes pairs: one cluster comprises the genes coding for legumin A; the other is made up of those coding for legumin B. Indeed, the appearance of a minor labeling site in addition to the main one after hybridization with each cDNA probe (Fig. 6a, b) may be due to the substantial homology (ca. 50%; Wobus et al. 1986) of the nucleotide sequence existing between the two gene subfamilies. This view is supported by the specular appearence of the labeling pattern after hybridization with pVfc70 or pVfc77 cDNA clones and by the results of the cross-competition experiments. In *Pisum sativum* too, the genes coding for legA and legJ, which share sequence homology between them and with the genes for *V. faba* A- and B-type legumin, form two clusters that are located in two different chromosome pairs (see Casey et al. 1988).

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