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Measurement of gene number for seed storage proteins in *Pisum*

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ABSTRACT

We have measured the numbers of genes coding for the three seed storage proteins, vicilin, convicilin and legumin, in a number of Pisum genotypes of variant protein composition. No difference in gene number existed among P. sativum genotypes for any of the proteins. There were differences in the number of genes coding for individual proteins with approximately 11 genes (per haploid genome) for vicilin, 8 genes for legumin and 1 gene for convicilin.

INTRODUCTION

Two main classes of globulin storage proteins have been defined on the basis of solubility[1] and sedimentation coefficient[2]: the 7S and the 11S fractions. In Pisum, the 7S, or vicilin, fraction contains polypeptides in the M_r range 12,000 to 70,000[3-5]. All except the polypeptides of 70,000-M_r are derived from a protein known as vicilin and are closely related to each other in that the smaller polypeptides are derived from a class of precursor polypeptides of 47,000-M_r by post-translational proteolytic cleavage[6]. Vicilin precursors of 50,000-M_r are not processed in this way[6,7]; partial sequences of cDNAs for 47,000-M_r and 50,000-M_r polypeptides have shown a high degree of homology between these two polypeptide classes[7]. Polypeptides of 70,000-M_r are derived from a protein known as convicilin[4] and a partial sequence comparison of a cDNA for convicilin[8] with that for a vicilin has shown that a high degree of homology also exists between these polypeptides[9].

The 11S fraction of Pisum is known as legumin, which is composed of two subunit classes of M_r approximately 40,000 (acidic subunits) and 20,000 (basic subunits). Heterogeneity has been found for both subunit classes by one- and two-dimensional gel electrophoresis. Two-dimensional gel analyses have revealed a major and a minor class of subunits of M_r approximately 40,000[10]. In addition, there are minor acidic subunits of lower M_r than

the major subunits; these are of M_r approximately 24,000[11,12]. The majority of legumin precursors are synthesized as molecules of M_r approximately 60,000[13,14], each containing a covalently linked acidic and basic subunit[15,16]. The construction of cDNA clones corresponding to different legumin precursor polypeptides has been reported[15,17].

In Pisum, genetic variation in storage protein composition has been reported by a number of authors[3,10,18-23]. Of particular interest is the observation that, in a screen of near-isogenic round- and wrinkled-seeded lines, the round-seeded forms contained a higher proportion of legumin than the corresponding wrinkled-seeded form[23]. In addition, the levels of convicilin were found to be greatly elevated in some P. fulvum genotypes compared with P. sativum genotypes[22]. As a first step in elucidating the basis of such quantitative variability, we have measured gene copy number in a number of the extreme variant genotypes. In this study, we report the results obtained for five Pisum genotypes, using cDNA clones representing convicilin, two classes of vicilin and three classes of legumin polypeptides. The gene numbers obtained can, however, only be correlated with amounts of 7S and 11S proteins. These amounts are relatively easily defined by a number of methods[24], but the contribution made by the genes for each of the two classes of vicilin polypeptide and each of the three classes of legumin polypeptide, referred to in this study, is at present unknown. Although there are many cases in animal systems of a correlation between gene number and product (the enzyme dihydrofolate reductase in methotrexate-resistant cell lines is an example[25]), few cases are known in plants. A gene dosage effect has been observed in maize endosperms carrying 0-3 doses of an allele for a particular zein polypeptide[26]. Increased amounts of product have also been observed in addition lines of wheat when the number of chromosomes carrying particular genes has been increased[27]. A lack of detectable product has been shown to be due to a lack of functional genes in a soybean line containing an insertion in the seed lectin gene[28]. In a Riso mutant of barley containing very reduced levels of B hordein, a deletion of DNA from the Hor-2 locus has been demonstrated[29].

MATERIALS AND METHODS

Plant Material

Four genotypes of Pisum sativum L. were used in this study: the round-seeded and wrinkled-seeded near-isogenic lines, BCI/4R and BCI/4W,

respectively, and the cultivars (cvs.) Dark Skinned Perfection (a wrinkled-seeded line) and Birte (a round-seeded genotype). One Pisum fulvum L. genotype, JI 224 Sp, was included.

Dried, mature seeds from each genotype were used for the preparation of total protein and young leaves from healthy plants derived from the same seed batches were frozen in liquid nitrogen and stored at -70°C for DNA preparation.

Protein preparation and quantitation

Dried mature seeds were ground to a fine powder, sieved and extracted with buffered salt solution as previously described[24], except that dithiothreitol was omitted. The concentration of protein in the extracts was measured by the dye-binding[30] and Lowry[31] methods, using bovine serum albumin as a standard; the results from each method were closely concordant and the means of the two methods were used to calculate salt-extractable protein concentrations. Salt extracts were examined for possible selective extraction of particular protein fractions by comparison of their SDS-gel profiles with those of SDS extracts of ground seed material[24]; there was no evidence of selective extraction of any major protein component.

The ratio of 11S (legumin) to 7S (vicilin plus convicilin) proteins in the extracts were measured by analytical centrifugation as outlined elsewhere[24], using an M.S.E. centriscan operated at 50,000 r.p.m. and 20°C. Estimation of legumin:vicilin plus convicilin ratios by cellulose acetate electrophoresis was as described[23,32].

DNA preparation and digestion

Leaves were frozen in liquid nitrogen, ground to a fine powder and thawed in 3xSSC (SSC is 0.15M sodium chloride; 0.015M sodium citrate; pH7.0); 0.1M sodium diethyldithiocarbamate; 0.1M EDTA, pH 8.0. The resulting paste was made to 0.1% (w/v) SDS and vigorously mixed with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was separated by centrifugation and DNA precipitated with two volumes of ethanol. The DNA was dissolved in 10mM Tris; 1mM EDTA; pH 8.0 and purified on caesium chloride/ethidium bromide gradients. DNA recovered from gradients was phenol-extracted, ethanol-precipitated and dissolved in water.

DNAs were digested with EcoRI (The Boehringer Corp.), BamHI (BRL) or HindIII (BRL) as recommended by the manufacturers, except that 10 (EcoRI) or 5 (BamHI and HindIII) units of enzyme were used per μg DNA and digestions were performed for 4h. Digested samples were ethanol-precipitated and dissolved in 10mM Tris; 1mM EDTA; pH 8.0. The DNA

concentration of each sample was determined by optical density measurements. Ten μg of digested DNAs were subjected to electrophoresis on 0.8% agarose gels overnight and transferred to nitrocellulose filters[33].

Gene copy number determination

The construction and characterization of cDNA clones for all the major seed storage proteins has been described elsewhere[8,17]. The cv. Birte was used in the construction of the cDNA library.

The vicilin cDNA clones, pCD4 and pCD48 (corresponding to the 47,000-M_r and the 50,000-M_r vicilin precursor polypeptides, respectively[8]), a convicilin cDNA clone, pCD75 (containing a longer insert (of approximately 1Kb) than pCD59[8]) and the legumin cDNA clones, pCD32, pCD40 and pCD43 (corresponding to the legumin polypeptides of 80,000-M_r, 63,000 to 65,000-M_r and 60,000-M_r, respectively[17]) were used in gene copy number determinations. The six recombinant plasmids were linearized with BamHI (none of the cDNA inserts from these plasmids contain BamHI restriction sites). Assuming a pea haploid genome size of 4.6pg[34], amounts of linearized plasmid equivalent to 0.5, 1, 2, 5 and 8 copies per haploid genome in 10μg pea DNA were subjected to electrophoresis on gels alongside digested pea DNAs and concomitantly transferred to nitrocellulose filters.

Inserts were obtained from cDNA clones by polyacrylamide gel electrophoresis of PstI (BRL)-restricted recombinant plasmids and recovery of the inserts from gel slices by shaking in 10mM Tris; 1mM EDTA; pH8.0. The cDNA inserts were nick-translated[35] with [α-^{32}P] dCTP to a specific activity of 1 to 5×10^8 c.p.m. per μg.

Nitrocellulose filters were pre-hybridized in 0.6M NaCl; 0.12M Tris; 8mM EDTA; pH 8.0 containing 0.1% (w/v) SDS and 10x Denhardt's[36] at 65°C for 3h. Hybridization was in the above containing 0.1% (w/v) $Na_4P_2O_7$ and 10% (w/v) dextran sulphate, with 1 to 3×10^6 c.p.m. of nick-translated cDNA insert per ml of hybridization solution, at 65°C for 18h. Filters were washed at least twice in 0.1 x SSC; 0.1% (w/v) SDS at 65°C, dried and autoradiographed. Autoradiographs were scanned using a Joyce-Loebl microdensitometer and each gel track was scanned at least twice. Amounts of hybridization were quantified relative to that calculated for the copy number equivalents in the linear range.

RESULTS

Protein quantitation

The relative merits of different techniques for the measurement of pea seed storage proteins have been outlined previously[24]. Possible artefacts

Table 1. The amounts of 7S and 11S proteins in five genotypes of Pisum, estimated by analytical centrifugation (AC) and cellulose acetate electrophoresis (CAE). Each value (±S.D.) represents the mean of 2 (AC) or 4 (CAE) determinations.

Genotype	µg 7S protein per 100µg extractable protein		µg 11S protein per 100µg extractable protein	
	AC	CAE	AC	CAE
224 Sp	73.4±2.0	62.9±5.9	13.6±1.3	27.2±3.7
BCI/4R	48.5±3.3	58.0±1.8	34.9±1.6	32.8±1.3
BCI/4W	62.0±0.7	60.2±2.6	23.1±1.8	23.1±0.6
DSP	82.8±2.7	67.5±4.4	10.4±1.1	16.6±3.3
BIRTE	49.1±1.2	58.4±1.7	32.6±0.3	31.8±0.9

involved in immuno-electrophoretic measurements were avoided in the present study and only results obtained by analytical ultra-centrifugation and cellulose acetate electrophoresis are used in genotypic comparisons. Table 1 shows that a wide range of 7S and 11S protein values exists for the five genotypes studied. More legumin per unit protein is present in seeds of BCI/4R than in those of BCI/4W, in agreement with the observations of Davies[23], using other near-isogenic round and wrinkled lines. The values obtained for cv. Birte are very similar to those of BCI/4R. The lowest legumin-containing genotype is cv. DSP . The P. fulvum genotype, JI 224 Sp, is also a low legumin-containing line. There is a discrepancy, however, between the values obtained for this genotype by the two methods; the presence of an atypical form of legumin in this genotype has been observed to cause differences in values obtained using different methods[24]. We have shown that JI 224 Sp contains an elevated level of convicilin[8].

Measurement of gene number

Only EcoRI digests are shown (Figs. 1 and 2) for comparison with copy number equivalents of linearized plasmid; similar results were obtained with BamHI and HindIII digests in all cases, except with pCD48, where hybridization was to BamHI fragments of approximately 20Kb and the extent of hybridization was markedly lower than that observed in EcoRI or HindIII digests. Since gels stained after transfer showed the retention of high molecular weight DNA, the poorer transfer of large DNA fragments to nitrocellulose was deemed to be responsible for the lower amount of

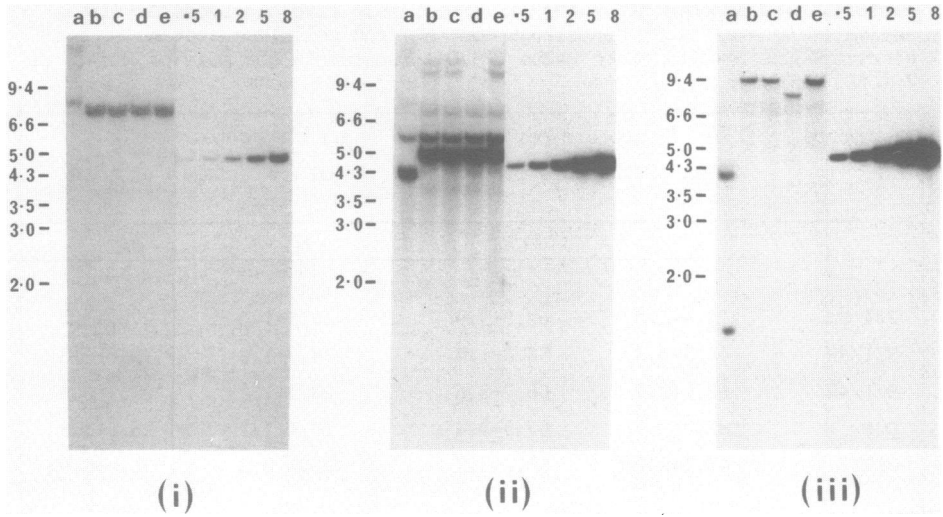

Fig. 1. Hybridization of nick-translated inserts from pCD4 (i), pCD48 (ii) and pCD75 (iii) to EcoRI-digested DNA from five Pisum genotypes (a-e). The hybridization intensities given by 10µg DNA from JI 224 Sp (a), BCI/4R (b), BCI/4W (c), cv. DSP (d) and cv. Birte (e) were compared with those given by amounts of linearized pCD4 (i), pCD48 (ii) and pCD75 (iii), corresponding to 0.5, 1, 2, 5 and 8 gene copy equivalents per haploid genome per 10µg Pisum DNA.

hybridization observed for these fragments. A discrepancy observed in hybridizations with the insert from pCD75 is discussed in b) below.

None of the cDNA inserts used contained restriction sites for EcoRI or BamHI; the inserts from pCD48 and pCD75 contained HindIII sites.

All the hybridizations using probes for 7S proteins were performed at least twice, using independently prepared pea DNA and independently determined copy number equivalents. There was good consistency between experiments.

Gene number for 7S proteins

a) Vicilin: Hybridization of the insert from pCD4 (for the 47,000-M_r polypeptide) to digested DNAs (Fig. 1 (i)) suggested a copy number of five to seven for all genotypes except JI 224 Sp which had a copy number of approximately three in all digests. When the DNA loaded per track was reduced to 5µg (data not shown), the apparently single band of approximately 7.5Kb which hybridized in 4 genotypes (Fig. 1 (i) b-e) was seen to be a doublet, in which the lower band was of greater intensity than the upper. The weaker hybridization to JI 224 Sp (Fig. 1 (i) a) than to

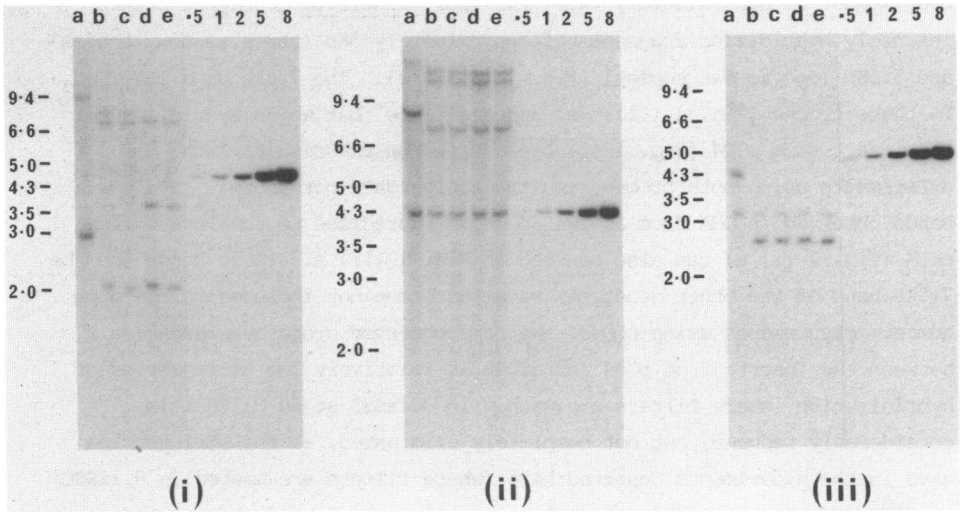

Fig. 2. Hybridization of nick-translated inserts from pCD40 (i), pCD43 (ii) and pCD32 (iii) to EcoRI-digested DNA from five Pisum genotypes (a-e). The hybridization intensities given by 10µg DNA from JI 224 Sp (a), BCI/4R (b), BCI/4W (c), cv. DSP (d) and cv. Birte (e) were compared with those given by amounts of linearized pCD40 (i), pCD43 (ii) and pCD32 (iii), corresponding to 0.5, 1, 2, 5 and 8 gene copy equivalents per haploid genome per 10µg Pisum DNA.

the other genotypes could be a result of fewer genes coding for the 47,000-M_r polypeptide in JI 224 Sp or it could be a result of sufficient sequence difference to cause lower homology with the probe DNA (from cv. Birte). The latter possibility is favoured by experiments in which filters were washed at a range of temperatures between 50°C and 70°C. While the intensity of hybridization to DNA from cv. Birte remained relatively unchanged from 50°C to 65°C, there was a marked reduction in the extent of hybridization to DNA from JI 224 Sp, as the stringency of the wash conditions increased from 55°C to 65°C (data not shown). There was no difference between the four P. sativum genotypes in the hybridization pattern obtained with EcoRI (Fig. 1 (i) b-e), BamHI or HindIII digests. The hybridization pattern of DNA from JI 224 Sp was more similar to that of DNA from the P.sativum genotypes in BamHI and HindIII digests (data not shown).

Hybridization of the insert from pCD48 (for the 50,000-M_r polypeptide) to digested DNA from the five genotypes (Fig. 1 (ii)) suggested a copy number of four to six for all genotypes except JI 224 Sp, which had a lower

copy number of approximately two. All four P. sativum genotypes showed intensely hybridizing fragments of approximately 5Kb (three to four copies) and 5.5Kb (one to two copies) (Fig. 1 (ii) b-e). The 7.5Kb band observed in these tracks (Fig. 1 (ii) b-e) appears to be that which hybridizes to the insert from pCD4 (Fig. 1 (i) b-e), based on the results from experiments using both probes simultaneously (data not shown). The two bands observed in DNA from JI 224 Sp which hybridized to the insert from pCD4 (Fig. 1 (i) a) can also be seen in Fig. 1 (ii) a. These bands and the 7.5Kb band of the other genotypes were therefore not included in the gene number measurements using pCD48. We have observed cross-hybridization between the inserts from pCD4 and pCD48 at relatively low stringencies of hybridization (where filters are washed in 0.1xSSC at 50°C) that is considerably reduced, but not completely eliminated, at the stringencies used in the experiments depicted here (where filters are washed in 0.1xSSC at 65°C) (data not shown); thus the visualization of bands corresponding to one probe in hybridizations to the other probably represents this residual cross-hybridization to genomic DNA. The stringency of wash employed in this study is a compromise between cross-reaction using different probes at lower temperatures and loss of signal at higher temperatures. Lower stringency washes (data not shown) did not increase the number of fragments which hybridized but merely increased the prominence of the fragments corresponding to the other vicilin probe.

A doublet of bands of approximately 10 and 10.5Kb hybridized to the insert from pCD48 in DNA from BCI/4R, BCI/4W and cv. Birte (Fig. 1 (ii) b,c,e) but was absent from cv. DSP (Fig. 1 (ii) d). The low intensity of hybridization to these fragments (0.2 to 0.3 copy equivalent) suggests that they contain only part of the probe sequence or that they contain sequences related to, but not completely homologous to, the probe sequence.

In HindIII digests of DNA from all five genotypes (data not shown), a single fragment of 2Kb hybridized to the insert from pCD48. This fragment must contain most of the coding sequence for the 50,000-M_r polypeptide; the mRNA size for this protein has been previously shown to be in the range 1,550 to 1,700 nucleotides[8,38] while the complete phaseolin (the homologous protein of Phaseolus vulgaris[7]) gene is 1990 bp long[39]. Sequencing of the insert from pCD48 (unpublished data) shows that a HindIII site is present 21 nucleotides from the 5' end of the insert and comparison with the protein sequence of the 50,000-M_r vicilin polypeptide[7,40] shows that the pCD48 insert contains the N-terminal sequence plus 84 to 90 nucleotides 5'

to the N-terminus, probably corresponding to a signal peptide, containing 4 methionine and 1 cysteine residues, in agreement with the results of others[38,40] (the N-terminus of the 50,000-M_r polypeptide is one of three amino acids in the sequence Ser-Arg-Ser[7,40]). Thus it seems highly probable that a single HindIII fragment contains most of the gene sequence for this protein.

b) Convicilin: Hybridization of the insert from pCD75 to digested DNAs from the five genotypes (Fig. 1 (iii) showed that the copy number for convicilin was markedly lower than that derived for either of the two vicilin probes: approximately one for JI 224 Sp and 0.5 for the P. sativum genotypes in EcoRI digests (Fig. 1 (iii)). This apparent difference in copy number between JI 224 Sp and the other genotypes was not observed in BamHI or HindIII digests. In BamHI digests (data not shown), the fragments which hybridized were in the range 10 to 20Kb for all genotypes and an apparent copy number of approximately 0.5 was obtained for all genotypes. In HindIII digests (data not shown), 2 to 3Kb fragments hybridized in DNA from the P. sativum genotypes and a copy number of one was obtained. A copy number of approximately one was also apparent when the five genotypes were hybridized with the insert from pCD59, another convicilin cDNA clone[8,9]. Among the P. sativum genotypes, the cv. DSP displayed a variant hybridization pattern in EcoRI (Fig. 1 (iii)), BamHI and HindIII digests. Minor hybridizing bands were also apparent in hybridizations to convicilin cDNA inserts: in particular, fragments of approximately 7Kb and 14Kb were observed in EcoRI digests of DNA from the P. sativum genotypes (Fig. 1 (iii) b-e). These do not appear to correspond to fragments hybridizing to either of the vicilin probes and therefore probably do not represent cross-hybridization to vicilin sequences.

Gene number for 11S proteins

Despite considerable cross-reaction between pCD32 and pCD40 in hybrid-selection experiments and 'Northern' blot analyses[17], there was no evidence of cross-hybridization at the genomic level under the standard conditions used. A different set of fragments was found to hybridize to each of the three probes used. We have never observed cross-hybridization between the inserts from pCD43 and pCD32/pCD40 under any conditions.

Fig. 2(i) shows the hybridization of EcoRI-digested DNAs to the insert from pCD40 (corresponding to legumin polypeptides of 63,000 to 65,000-M_r). A copy number of one to three was obtained for all genotypes. In EcoRI and BamHI digests, the cvs. DSP and Birte exhibited identical hybridization

patterns which differed from those of the genotypes BCI/4R and BCI/4W. In HindIII digests, the cvs. DSP and Birte also differed from each other (the genotypes BCI/4R and BCI/4W were identical throughout).

Fig. 2(ii) shows the hybridization of EcoRI-digested DNAs to the insert from pCD43 (corresponding to a legumin polypeptide of 60,000-M_r) for which a copy number of four to six was obtained for all genotypes. All the P. sativum genotypes exhibited an identical hybridization pattern in EcoRI digests with hybridization to four major fragments and a weaker band of approximately 2Kb (Fig. 2(ii) b-e). The P. sativum genotypes were also identical in BamHI and HindIII digests. These hybridization patterns are also similar to those of Croy et al[15], using EcoRI and HindIII digests of DNA from cv. Feltham First.

Fig. 2(iii) shows the hybridization of EcoRI-digested DNAs to the insert from pCD32 (corresponding to the 80,000-M_r legumin polypeptide). A copy number of one to two was obtained for all genotypes. The four P. sativum genotypes gave identical hybridization patterns in EcoRI (Fig. 2 (iii) b-e), BamHI and HindIII digests.

DISCUSSION

There was no appreciable difference in the numbers of genes coding for the storage proteins legumin, vicilin and convicilin among the P. sativum genotypes used in this study, although these genotypes differed in seed protein composition (Table 1). There was no appreciable difference in gene numbers between the P. fulvum and the P. sativum genotypes, except in the case of vicilin, where the former appeared to have a lower gene number; sequence divergence from the probe DNA was suspected to be responsible for these differences. The results suggest that the factors responsible for the different amounts of proteins synthesized in different genotypes reside in the transcriptional/translational processes of the seed rather than simply in gene number.

The gene numbers obtained in the present study are in reasonable agreement with those available for other legume species. The combined vicilin gene number obtained agrees well with the report that there are 10 to 15 phaseolin gene copies per haploid P. vulgaris genome[41]. A gene copy number of approximately 4 per haploid genome has been obtained for 11S proteins in Glycine max[42]; however, the particular cDNA probe used by Goldberg et al[42] cannot be easily equated with any of the three 11S probes used here. In addition, the results presented here are in good agreement

with those published for a single genotype of pea (cv. Feltham First) using two vicilin[6] and one legumin[15] cDNA probes. As noted previously (see results section), the cDNA probe (for a 60,000-M_r polypeptide) used in the latter study is probably homologous to pCD43 and as such the gene number reported by Croy et al[15] represents the copy number of one class of legumin genes only. The hybridization pattern for the 47,000-M_r vicilin polypeptide[6] and the legumin 60,000-M_r polypeptide[15] are similar to those shown here. Croy et al[15] have noted that the gene number obtained by them cannot wholly account for the reported number of major and minor legumin species[10,11,43,44]. A legumin genomic clone having approximately 98% homology with the cDNA used by Croy et al[15] has been reported[16] as probably belonging to a major legumin family. The gene numbers for three classes of legumin genes are provided by the present study; the correlation between these three classes and major/minor species of legumin polypeptides must await further sequencing at the DNA and protein level. The gene numbers obtained for two of the classes (the 63,000 to 65,000-M_r and the 80,000-M_r polypeptides) were lower than that for the third (60,000-M_r polypeptide). It is possible that other classes of legumin gene exist which have limited homology to any of the three classes discussed here.

The overall numbers of genes for the three proteins bear a relationship to the gross proportions of these which exist in the seed; for all P. sativum genotypes examined, the amounts of the three protein classes can be graded as vicilin > legumin > convicilin and the average total gene number corresponding to these proteins are 11, 8 and 1, respectively. It has been reported[45] that the relative amounts of short-term nuclear transcripts relate closely to gene copy number in experiments using two vicilin and one legumin cDNA clones. Although the above protein pattern applies also to the P. fulvum genotype, the proportion of convicilin is much greater (approximately half the amount of legumin; see [8] and Table I) than in the P. sativum genotypes. These gene number data suggest that this elevated proportion of convicilin is the product of 1 gene compared with approximately 8 genes for legumin. However, it is possible that all the gene numbers obtained for P. fulvum are inaccurate due to lower homologies with the probes used, although this was suspected only in the case of vicilin. An additional limitation of such gene number measurement studies lies in the undefined nature of the genes being measured, that is, the number of pseudogenes or inactive genes being measured are unknown.

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