Theor Appl Genet (1992) 84:617–623

Variability and inheritance of histone genes H3 and H4 in *Vicia faba*

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Received July 24, 1991; Accepted January 10, 1992
Communicated by G. Wenzel

Summary. We have compared copy numbers and blot-hybridization patterns of histone genes (H3 plus H4) between and within individuals of broad bean (*Vicia faba*). Copy number differences among individuals in the population of 200 individuals were as great as 27 fold, and as much as 3.2 fold among separate leaves of the same plant. Among F_2 progeny from genetic crosses, up to a 5.4-fold range was seen (mean = 3.5 fold), and among F_1 progeny of self-pollinated plants, up to a 5.9-fold range was observed (mean = 2.3 fold). Histone gene blot-hybridization patterns for *Eco*RI and *Hin*dIII were also variable among individuals and indicated that the genes are probably clustered in only a few chromosomal loci. The degree of variation in histone gene copy number per haploid genome (2–55 copies, or 27 fold) was similar to that found previously for ribosomal RNA genes (230–22000, or 95 fold) of *V. faba*. However, the two gene families change independently, since individuals with a high or low copy number for one gene can have either a high or low copy number for the other. The mechanisms(s) for rapid gene copy number change may be similar for these gene families.

Key words: Histone genes – H3 – H4 – *Vicia faba*

Introduction

The arrangement of histone genes within eukaryotic genomes varies greatly among species (Chaubet et al. 1987, 1989; Cool et al. 1988; Fitch et al. 1990; Gigot et al. 1987; Hentschel and Birnstiel 1981; Maxon et al. 1983; Old and Woodland 1984; Roberts et al. 1987; Wells et al. 1987). All five genes (H1, H2A, H2B, H3 and H4) may be tightly clustered within a few kilobases (kb) as in many echinoderms, or the genes may be dispersed to varying degrees as seen in a variety of other eukaryotes (e.g., Chaubet et al. 1986; Hentschel and Birnstiel 1981; Old and Woodland 1984). When clustered, copies of the gene cluster units may occur in tandem or may be dispersed. In many cases, tandemly repeating simple sequences (satellite DNA) occur between the gene clusters (Childs et al. 1981; Hentschel and Birnstiel 1981). The direction of transcription for each gene in the cluster is not always the same. In addition, copy numbers vary widely, from 1 copy of each gene per haploid genome in yeast to 2700 in axolotyl (Hilder et al. 1981). Even among closely related species there is no apparent correlation between histone gene copy number and genome size, growth rate or duration of development (Chernyshev et al. 1980; Fitch et al. 1990). Therefore, it appears that there are no general rules of arrangement or multiplicity of histone genes among a broad range of eukaryotes.

In plants, histone genes have been characterized for only a few species: alfalfa (Wu et al. 1988), *Arabidopsis thaliana* (Chaboute et al. 1987), maize (Gigot et al. 1987; Phillips et al. 1986), rice (Thomas and Padayatty 1983, 1984) and wheat (Tabata et al. 1983, 1984; Vakhitov and Kulikov 1986). The H3 and H4 genes have been studied most often and are well-conserved (from 72% to 97% nucleotide sequence homology (Chaubet et al. 1986, 1989)), thereby facilitating species comparisons. Generally, several versions of each of these genes are present in most plant species, with members varying more in DNA sequence than in amino acid sequence. The H3 genes are about 400 bp in length, while the H4 genes are about 300 bp long. Typically, 30–120 copies of each gene are present per haploid genome, although some tetraploid and hexaploid wheat and *Aegilops* species possess close

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to 400 copies per haploid genome. *Arabidopsis thaliana*, which has one of the smallest known plant genomes, has only 5–7 copies of each gene per haploid genome (Chaboute et al. 1987). Generally, the genes are dispersed in the genome, except in rice where the H2A, H2B and H4 genes are clustered.

In the present article we report on gene copy variability and gene arrangement within the genome of broad bean (*Vicia faba*). We previously reported that the copy number of genes for ribosomal RNA (rDNA) varied by nearly 100 fold among individuals in a population of *V. faba*, from 230 to 22,000 copies per haploid genome (Rogers and Bendich 1987a). The blot-hybridization patterns for these genes also showed great individual variation (Rogers et al. 1986). The objective of the study presented here was to determine whether protein-coding genes also exhibit these within-population differences. We have analyzed the histone genes H3 and H4 from some of the same *V. faba* individuals previously used for the rDNA experiments.

Materials and methods

Sample selection

Of the over 450 DNA preparations from *Vicia faba* L. (broad Winsor bean) individuals that were used in our previous study of ribosomal DNA (Rogers and Bendich 1987a), 200 were chosen, for this study, so as to include those individuals near the extremes of the rDNA copy number distribution as well as some from the middle of the distribution. As such, this is not a random sample. The numbers used to designate individuals correspond to those in our previous paper on rDNA (Rogers and Bendich 1987a).

Restriction pattern assays

Up to 40 µg of total DNA was digested with 20–50 units of either *Eco*RI or *Hin*dIII at 37 °C for 8–24 h and with ribonucleases A (100 µg/ml) and T_1 (10 units/ml) for 30 min. The DNA was then subjected to electrophoresis on 0.4% agarose gels (30 cm long) at 60–90 V for 30 h. The "1-kb ladder" (Bethesda Research Laboratories) was used for molecular weight estimates. Gels were blotted onto either Nytran (Schleicher and Schuell) or Gene Screen Plus (New England Nuclear) and dried at room temperature. The blots were then hybridized to radioactively-labeled DNA probes as described below.

Gene copy number determinations

After digestion with ribonucleases (see above), DNA amounts were measured by a fluorometric method (Moore and Sutherland 1985). Since RNA oligonucleotide fluorescence can sometimes cause inaccurate estimation of the DNA concentrations, we tested its effects on DNA quantitation using varying amounts of lambda DNA and yeast tRNA. We determined that the amount of fluorescence contributed by the RNA oligonucleotides after RNase treatment (as above) was much less than 50% when DNA was between 5 and 60 ng/µl and RNA was below 1 µg/µl (data not shown). The ratio of RNA to DNA in the *V. faba* samples was always much lower than 20, as measured by ethidium fluorescence of agarose gels in which the DNA and RNA were well separated (data not shown). Therefore, for the concentration range in which we were working (between 5 and 50 ng/µl), the amount of total RNA in each of the samples was always below 1 µg/µl and would not appreciably affect DNA quantitation.

Following DNA quantitation, 2.5 µg of each DNA sample was treated with 0.5 *N* HCl for 1 min (at room temperature), denatured in 0.75 *N* NaOH for 3–5 min, then neutralized in 0.5 *M* TRIS (pH 7.5) and 1.5 *M* NaCl. Known amounts (200 fg–100 pg) of YpMS212, a plasmid containing the H3 and H4 histone genes of the yeast *Saccharomyces cerevisiae* (Meeks-Wagner and Hartwell 1986), or an equimolar mixture of plasmids H3C4 (coding sequence (Chaboute et al. 1987; Chaubet et al. 1986)) and H4C14 (coding sequence (Phillips et al. 1986)) from maize were prepared in the same way to construct a set of standards. Samples were blotted onto Nytran filters using a slot-blotting apparatus (Schleicher and Schuell) and hybridized to a radioactive probe as described below. The resulting autoradiographs were analyzed densitometrically, and standard curves were constructed using the values obtained from the plasmid standards. The amounts of hybridization for the plant DNAs were determined using these curves.

To determine the reproducibility of the slot-blotting assay of gene copy number, aliquots of DNA samples were loaded into different slots or onto different blots, followed by hybridization, autoradiography and densitometry. The variability averaged 1.24 fold ± 0.10 fold for 11 samples. Therefore, we used a value of 1.5 fold as the threshold for measurable differences between samples.

Histone gene copy numbers were calculated as in the following example. The average histone hybridization signal was 2.80 pg per 2.5 µg of total DNA loaded, or 1.12×10^{-6}. This value was then multiplied by the haploid genome size of 13.2 pg (Bennett and Smith 1976) and by 9.11×10^5 kb (which equals 6.03×10^{11} Da/pg divided by 6.62×10^5 Da/kb) to obtain the number of kilobases of histone DNA per haploid genome, in this case 13.5 kb. Finally, this number was divided by 0.72 kb [the sum of the lengths of H3 (410 bp) and H4 (310 bp); (Chaboute et al. 1987)] to obtain the copy number of each gene per haploid genome of 18.7 (rounded to 19) in *V. faba* leaves (Table 1). For Fig. 3, the percentage rDNA from Rogers and Bendich (1987a) has been converted to gene copy number by using a calculation as above, except that 10.5 kb was used in the last step as the average length of the rDNA repeat.

Radioactive probing

Blots were prehybridized at 65 °C for 4–12 h in hybridization solution [50% formamide, 1% sodium dodecyl sulfate (SDS) and 1 *M* NaCl]. The probes used were [^{32}P]-labeled DNA plasmids (YpMS212 from yeast or a mixture of H3C4 and H4C14 from maize, see above). For each 250 ng of plasmid DNA 40–70% of 50 µCi of [α-^{32}P]-dCTP (3,000 Ci/mmole) was incorporated using a random primer labeling kit (Boehringer Mannheim). The [^{32}P]-labeled plasmid was denatured (in hybridization solution) at 95 °C for 10 min and then added to each blot at a concentration of about 3–5 ng/ml. The blots were then incubated at 38 °C (for YpMS212) or 40 °C (for H3C4 plus H4C14) for 16–24 h, rinsed twice with 2 × SSC (0.3 *M* NaCl, 0.03 *M* sodium citrate) at room temperature followed by two 30-min washes in 2 × SSC and 1% SDS at 60 °C (for YpMS212) or 63 °C (for H3C4 plus H4C14) and two 30-min washes in 0.1 × SSC at room temperature. The blots were then placed onto X-ray film (Kodak XAR-2) for autoradiography with intensification screens (Du Pont lightning plus) for the Southern blots and without screens for the slot-blots.

To determine the lower limits of the slot-blot assays, hybridizations were carried out with and without nonhomologous

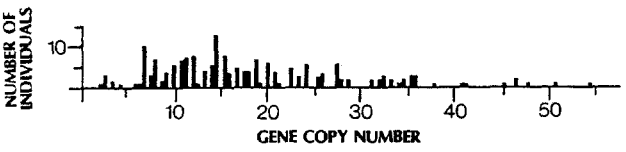

Fig. 1. Variation among *V. faba* individuals in histone gene amounts for the leaves from 193 individuals. The histone gene amounts are given as estimated copy number

Table 1. Summary of histone gene copy numbers in *Vicia faba* tissues

Tissue	Haploid copy number			
	Variation	Mean	Range	*N*
Leaves	27	19	2–55	193
Cotyledon	3.0	4	2–6	5
Embryo	2.0	5	3–7	2
Various tissues (1 plant)[a]	3.2	30	14–45	18
Leaves (single line of self-pollinated)[b]	5.9	18	6–35	39
Leaves (single line of cross-pollinated)[c]	5.4	20	7–36	121

Average copy number and copy number range were calculated as described in Materials and methods using the data on histone gene H3 and H4 amounts (Figs. 1 and 2). The maximum amount of variation is also presented. *N* = number of individuals

[a] The values for variation, average and range are from separate leaves from plant 4–2 from one of the 1 × 8 crosses. For all within-individual tissue comparisons the average variation was 1.7 fold

[b] The values for variation, average and range are from the F_1 progeny of plant 33, which exhibited the widest variation. For all F_1 progeny from all plant lines the average variation was 2.3 fold

[c] The values for variation, average and range are from the F_2 progeny from the 1 × 11 cross, which exhibited the widest variation. For all F_2 progeny from all crosses the average variation was 3.5 fold

DNA (sheared salmon sperm DNA). At signal levels below 1 pg, the amounts of nonspecific hybridization sometimes lead to inconclusive results. Therefore, when salmon sperm DNA was not included in the hybridization solution, signals below 1 pg were excluded from the data sets. However, for most of these samples it was clear that fewer than 5 copies of each gene (H3 and H4) per haploid genome were indicated.

Results

Copy number variation

For the 200 *V. faba* individuals surveyed, the average gene copy number was 19 per haploid genome for the H3 gene and the H4 gene (the hybridization probe contained both genes). There was a 27-fold range in copy number, from 2 to 55 copies (Table 1, Fig. 1). The coefficient of variation was 56.7%, indicating a broad distribution. For the same individuals, the range for rDNA was 65 fold, with a coefficient of variation of 72.9%; data from Rogers and Bendich (1987a). Because we used a heterologous probe, the hybridization signal, and therefore the calculated copy numbers, may be somewhat lower than actual values. Within a group of siblings obtained from the self-pollination of plants that had come from at least two previous rounds of self-pollination, the largest range in copy number was 5.9 fold (Table 2, siblings from parent plant 33), the average range was 2.3 fold. Seven lines showed a variation of less than 2 fold (Table 2, siblings of parent plants 17, 19, 21, 28, 41, 44 and 46), three lines showed a moderate amount of variation, between 2 and 3 fold (siblings of parent plants 18, 22 and 24) and two lines showed an amount of variation greater than 3 fold (siblings of parent plants 16 and 33).

Among the F_2 siblings from genetic crosses the mean range in copy number was 3.5 fold. The largest range was 5.4 fold (Fig. 2, cross 1 × 11). Nine crosses exhibited

Table 2. Comparison of histone and ribosomal gene copy numbers among *Vicia faba* siblings (produced from self-pollination)

Parent plant	Histone genes H3 and H4 Haploid copy number				Ribosomal RNA genes[a] Haploid copy number			
	Variation	Mean	Range	*N*	Variation	Mean	Range	*N*
16	3.6	14	7–24	4	1.5	1100	900–1400	6
17	1.9	32	25–47	3	6.8	2800	700–4700	7
18	2.5	17	8–20	4	2.9	5200	3700–10600	6
19	1.2	47	41–51	3	3.8	5200	1900–7300	3
21	1.1	33	33–35	3	2.0	4500	3600–7000	4
22	2.2	27	17–36	5	5.3	5600	2200–11500	5
24	2.4	24	15–35	3	1.5	7000	6000–8700	5
28	1.9	15	12–23	3	5.8	4400	1600–9300	4
33	5.9	18	6–35	4	4.1	4500	1800–7400	7
41	1.6	45	35–55	2	3.1	2700	1600–5000	5
44	1.7	23	17–28	4	4.6	2100	800–3700	9
46	1.5	30	24–36	2	1.8	1900	1400–2500	3

[a] Data from Rogers and Bendich (1987a).

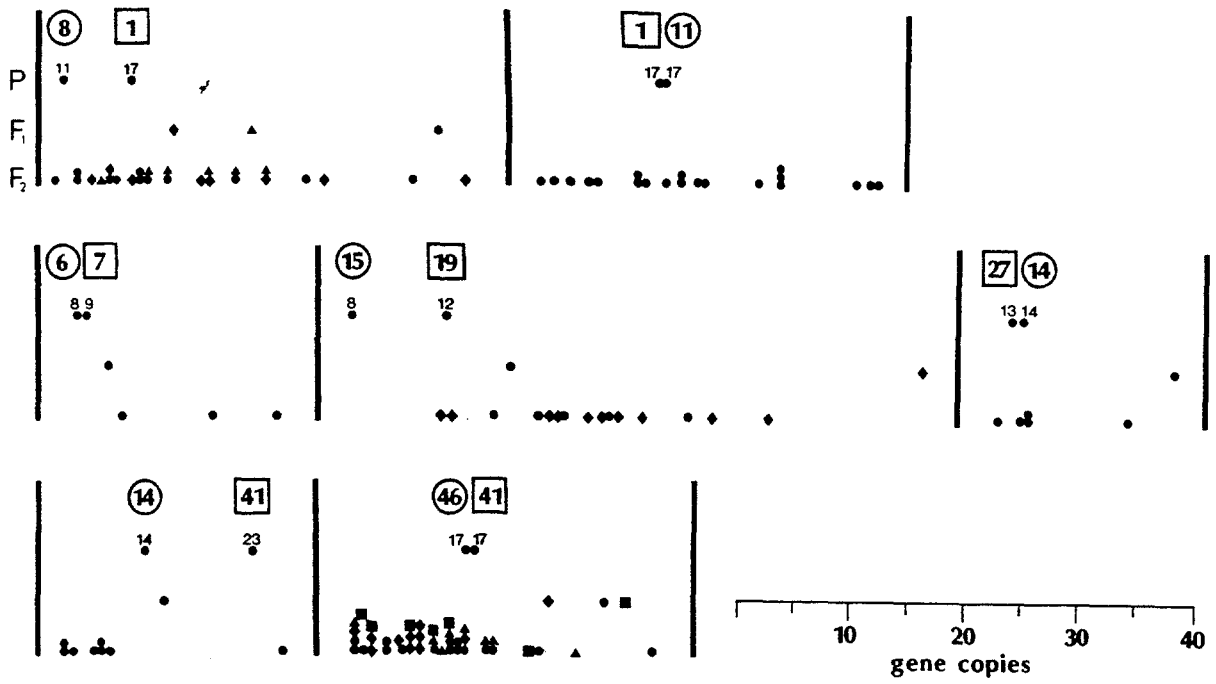

Fig. 2. Distribution of histone gene amounts for parents and progeny produced from cross-pollination. The two *data points* at the *top* in each comparison represent the amounts of histone genes measured in the leaves of the two parents. The *data point(s)* in the *middle rows* represent the value(s) for the leaves of the F_1 heterozygote(s). The *lower row* of *data points* are the amounts for the leaves of the F_2 progeny produced from self-pollination of the F_1 heterozygote(s). The scale is in calculated histone gene copy number. Copy number values appear *above the data points* for each of the parents. Parent plant numbers are either in a *circle* (indicating female parent) or *square* (indicating male parent)

moderate to high variation, where the variation in gene copy number was greater than 2 fold (Fig. 2, F_2 siblings from the crosses 1×8 (dots), 1×8 (diamonds), 1×11, 19×15 (diamonds), 41×14, 41×46 (dots), 41×46 (triangles) and 41×46 (squares)). Four crosses exhibited little variation [Fig. 2, crosses 1×8 (triangles), 7×6, 19×15 (dots), 27×14 and 41×46 (diamonds)].

In most cases, assays of tissues from individual plants gave no variability in copy number. However, in one case there was a 3.2 fold range in copy number (separate leaves of the same individual, see Table 1), and in a second a 1.7-fold range (embryo and cotyledon from one seed) was found.

The histone gene copy number was compared to rDNA copy number for the same individual DNAs (Table 2 and Fig. 3). In a few distributions the histone gene and rDNA copy numbers appear to be somewhat correlated (as indicated by parallel lines in cross 1 for 19×15, for example). For most distributions, however, the two gene copy values appear to change independently (as indicated by non-parallel and crossing lines). The correlation between histone gene copy number and rDNA copy number for these 200 individuals was poor ($r = 0.20$), indicating that copy numbers are changing independently for the two gene families (Fig. 4).

Table 3. Summary of hybridization bands (refer to Fig. 5)

Lane	Enzyme	Hybridization bands (kb)				*Eco*RI pattern type
1	*Eco*RI		3.5	1.9	1.6	I
2	*Hin*dIII		6.8	1.0		
3	*Eco*RI		3.7	2.1	1.8	II
4	*Hin*dIII		7.5	1.1		
5	*Eco*RI		4.3	4.0	2.2	III
6	*Eco*RI		3.4	1.9	1.6	IV
7	*Eco*RI	5.8	3.5	1.9	1.6	I
8	*Eco*RI	5.9	3.5	1.9	1.6	V
9	*Eco*RI	5.8	3.5	1.9	1.6	I
10	*Eco*RI	5.8	3.5	1.9	1.6	I

Blot-hybridization pattern variability

Figure 5 shows the histone gene hybridization patterns for 8 individuals after digestion with *Eco*RI and for 2 individuals after digestion with *Hin*dIII. The 2 *Hin*dIII patterns obtained were different, as were 5 of the 8 *Eco*RI patterns (Table 3 and Fig. 5). The most common *Eco*RI pattern (Table 3, type I) included bands at 1.6, 1.9 and 3.5 kb (as well as an additional band at 5.8 kb when the maize probes were used; Fig. 5, lanes 1, 7, 9 and 10). Three of the other *Eco*RI patterns (Table 3, types II, IV

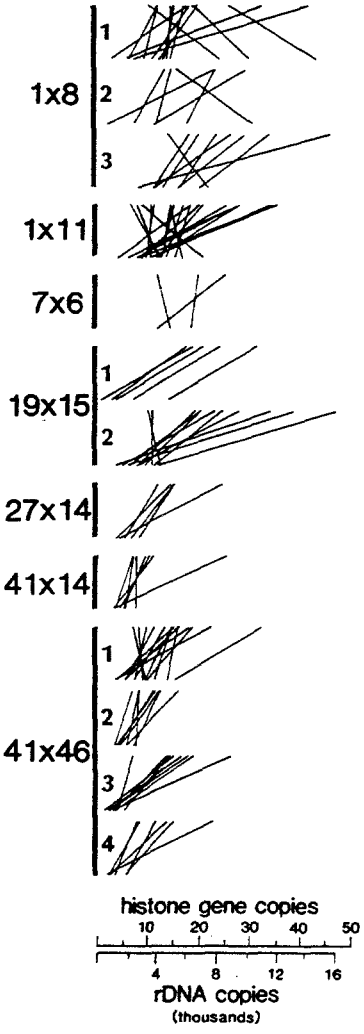

Fig. 3. Comparison of histone gene and rDNA copy numbers for F_2 individuals from cross-pollinated parents. For each comparison the *upper values* are histone gene copy numbers whereas the *lower values* are rDNA copy numbers. *Lines* join values for the same DNA preparation for each individual. The *large numbers (left)* correspond to the plant numbers in Fig. 2. *Numbers* to the *right* of the *heavy vertical lines* indicate seperate crosses of the same two parents. *Data points* in Fig. 2 correspond to the following numbers in this figure: *1* dots, *2* diamonds, *3* triangles, *4* squares. Copy number ranges from 5 to 50 for histone genes and from 200 to 15000 for rDNA

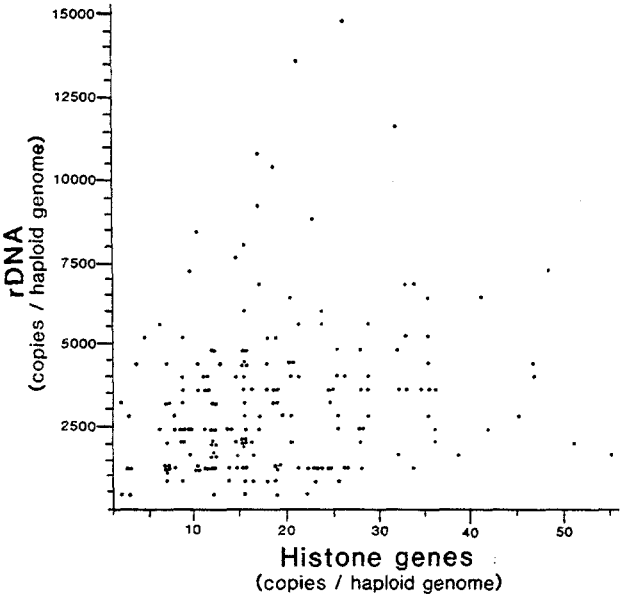

Fig. 4. Plot of the histone gene copy values (*abscisa*) and rDNA copy values (*ordinate*) for each of the 200 *V. faba* individuals. Regression analysis produced an r value of 0.20

and V; Fig. 5, lanes 3, 6 and 8, respectively) were slight variations of the common pattern, while 1 (Table 3, type III; Fig. 5, lane 5) exhibited an entirely novel pattern.

Discussion

We report that histone genes H3 and H4 vary among individuals of *Vicia faba*, both in copy number and in blot-hybridization pattern. While the 27-fold range in copy number found in this study may seem large, it is less than the 65-fold range found for ribosomal RNA genes

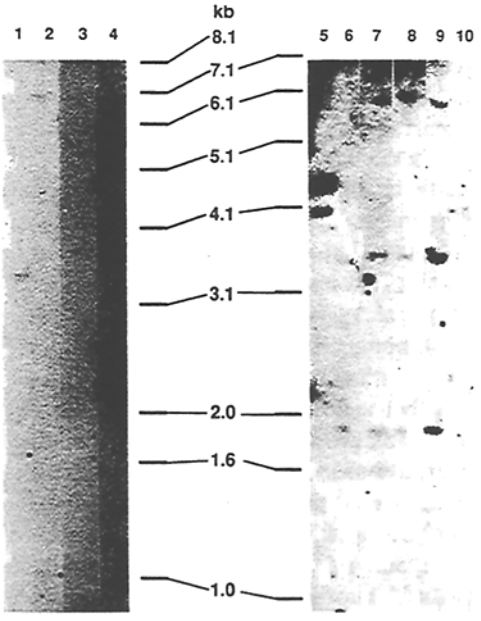

Fig. 5. Autoradiograph of blot-hybridization patterns for 10 *V. faba* individuals. Approximately 20 µg of total DNA was loaded onto each lane. *Lanes 2* and *4* are *Hin*dIII digests, whereas the remainder are *Eco*RI digests. The hybridization probe was YpMS212 (from *Saccharomyces cerevisiae*) in *lanes 1–4* and a combination of H3C4 and H3C14 (from *Zea mays*) in *lanes 5–10*. Faint bands (that were evident on the original autoradiogram) are marked by *dots* in lanes 1–4. See Table 3 for a list of the molecular weights of the bands in each lane and categorization of the *Eco*RI patterns.

(rDNA) among the same individuals. The 5.9-fold range among siblings is also less than the 19-fold range we found for rDNA (Rogers and Bendich 1987a). The average copy number is about 20 for each of the histone genes and about 2,500 for rDNA (Rogers and Bendich 1987a). This difference should represent an increased probability for change both in gene arrangement and copy number for rDNA, but the mechanism of change may be similar for both gene families.

We concluded previously that frequent DNA exchanges (including those in somatic cells) lead to the variation seen for the *V. faba* rDNA genes (Rogers and Bendich 1987a, b, 1988). Furthermore, we found that such exchanges occurred among the tandem repeats of a 325-bp sequence within the intergenic spacer of *V. faba* rDNA after this DNA was used to transform *E. coli* (Rogers and Bendich 1988). Satellite DNA, composed of sequences in tandem array, is common in regions that undergo unequal recombination (Pont et al. 1987; Smith 1976) and is often found between histone gene clusters (Childs et al. 1981; Hentschel and Birnstiel 1981). Here we find that not only are the histone genes changing in number among individuals, their flanking sequences are also variable as inferred from blot-hybridization patterns. Also, since restriction fragment sizes changed without any change in the total number of fragments, exchanges among tandemly arrayed sequences may be responsible for the variability in *V. faba* histone genes. Since we detected some copy number changes in these genes among different tissues of individual plants, it appears that the rearrangements can occur during somatic cell divisions, as is the case with rDNA.

Although these changes might be assumed to be important to the organism, they have no obvious phenotypic effect on the plants. Histone genes have been found to vary by at least 10 fold among members of the genus *Drosophila* (Fitch et al. 1990), 9 fold among *Aegilops* species and 15 fold among *Triticum* species (Vakhitov and Kulikov 1986). As with rDNA there does not appear to be a relationship between number of copies of histone genes and genome size, growth or developmental characteristics, and calculations indicate that 1 or 2 gene copies can produce amounts of histone proteins sufficient for most eukaryotes (Old and Woodland 1984). Therefore, as with rDNA, beyond a certain minimum copy number of histone genes, which for plants appears to be between 2 and 5 per haploid genome, additional copies appear to be superfluous, as has been inferred for other organisms (Chernyshev et al. 1980; Fitch et al. 1990; Hentschel and Birnstiel 1981; John and Miklos 1988). As long as an adequate number of functional gene copies is maintained, the plants can evidently tolerate these rapid changes.

The changes may create genetic diversity for adaptive purposes, as suggested for rDNA in wheat (Flavell et al. 1986) and other plants (Rogers and Bendich 1988; Walbot and Cullis 1985) and for histone genes in *Drosophila melanogaster* (Chernyshev et al. 1980). Another possibility is that certain steps in some required event in DNA metabolism incidentally lead to non-adaptive changes. Alternatively, these rapid changes may reflect a self-perpetuating tendency of multi-copy sequences irrespective of any benefit they may confer upon their host organism, as in the cases of the 2-micron plasmid in the nucleus of yeast (Futcher et al. 1988), the omega intron of yeast mitochondrial rDNA and other introns (Lambowitz 1989).

Two bands were consistently observed in the *Hin*dIII digests, while three to four bands were seen with *Eco*RI digests, indicating either variation in sequences immediately surrounding each histone gene or changes flanking histone gene clusters (Fig. 5, Table 3). Each of the bands was 1.0 kb or larger, so it is possible that all *Eco*RI bands represent separate loci. Whether there are several loci or only one locus, the observed bands probably represent tandem duplications of the histone genes or gene clusters, as indicated by the intensities of the bands relative to those from standards. A tandem arrangement of histone genes is uncommon among the few plant species that have been surveyed so far, where the genes appear to be generally dispersed. One exception is in rice where the H2A, H2B and H4 genes are clustered (Thomas and Padayatty 1983, 1984). Therefore, as in other eukaryotes, no general pattern of histone gene arrangement is evident for plants.

The change in copy number of ribosomal RNA genes is independent of that for histone genes (Table 2, Fig. 3). Consequently, the histone and ribosomal genes are probably not closely linked, although they may share a common mechanism for rapid change: unequal crossing-over, gene conversion or similar mechanisms (Pichersky 1990). Structural characterization of the gene sequences, including flanking regions and chromosomal locations, may lead to a clearer picture of the process by which gene copy number changes.

Individual variability in copy number for a protein-coding gene may seem surprising since such variability would be expected to disrupt the simple patterns of inheritance commonly found for phenotypic markers. Traits whose inheritance does not conform to a simple pattern are often considered as polygenic or may be excluded from further study. Some of the complex patterns of inheritance might, however, be attributable to gene copy variability among the individuals used in the genetic analysis. It will be of interest to assess copy number in a large number of individuals that exhibit simple inheritance patterns for a gene considered to be single copy in most of those individuals.

Acknowledgements. We thank Dr. Katerine LoBuglio and Ms. Lisa Simpson providing technical assistance.

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