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Pisum lipoxygenase genes

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Summary. The copy number, genomic arrangement and linkage relationships of two classes of lipoxygenase gene have been investigated in *Pisum*(pea) lines. Each of the two classes contained two to three members in *P. sativum* lines. RFLPs associated with genomic fragments containing the 5′ sequences of one gene class permitted its correlation in genetical analyses with a lipoxygenase locus on linkage group 4, which was previously identified through polypeptide variation. Genetical analyses of RFLPs associated with other fragments identified by low- and medium-stringency hybridization to lipoxygenase cDNAs indicate the existence of other unlinked lipoxygenase gene loci.

Key words: Lipoxygenase – Mapping – *Pisum*

Introduction

Lipoxygenases are a widespread group of enzymes that are potentially of commercial importance due to their implication both in animal processes, leading to hypersensitive and inflammatory responses, and in plant processes, leading to flavour and odour formation (Galliard and Chan 1980; Hildebrand 1989). The enzymes have been studied intensively in plants and are relatively abundant in legume seeds. In soya bean seeds, three distinct lipoxygenases have been identified (Galliard and Chan 1980); *Pisum* (pea) seeds contain only two of these major enzymes, but also contain additional minor enzymes, some of which appear to be present in organs other than seeds (Domoney et al. 1990).

Flavour improvement of soya bean preparations has been achieved through the identification of a mutant lipoxygenase-2-less line and its use in breeding programmes (Davies et al. 1987). As yet, no lipoxygenase-less pea line has been identified. In the absence of such naturally occurring mutants, antisense gene transfer offers the possibility of gene product manipulation and improved plant products. However, attempts at manipulating the biological processes that are a consequence of lipoxygenase activity will demand a detailed knowledge of the genes that encode different lipoxygenase enzymes, their abundance, organization and genetics. We have previously isolated two lipoxygenase cDNAs representative of the two major pea seed lipoxygenases; these two sequences share approximately 85% homology at the amino acid level with each other and with soya bean lipoxygenase DNA sequences (Ealing and Casey 1988, 1989). None of the protein N-terminal sequences derived from pea seed lipoxygenase polypeptides (Domoney et al. 1990) corresponds exactly to the deduced sequences from the cDNAs, indicative of a pea seed lipoxygenase multigene family. This paper analyses the complexity of this family in terms of its organization and genetics.

Materials and methods

Plant material

The *Pisum* lines used were from the John Innes Germplasm Collection. Crosses between selected lines were established, and F_2 to F_7 seed and plants were used for DNA preparation and in scoring segregating characters. The lines JI 181 and JI 281 were homozygous dominant for *le*, determining internode length, whereas all other lines used were homozygous recessive for *le*.

Analysis of DNA

Pea genomic DNA was prepared, restricted and hybridized as described previously (Domoney et al. 1986; Ellis et al. 1986). The probes used in hybridizations were derived from the two near-full-length lipoxygenase cDNAs, pPE1036 [a longer ver-

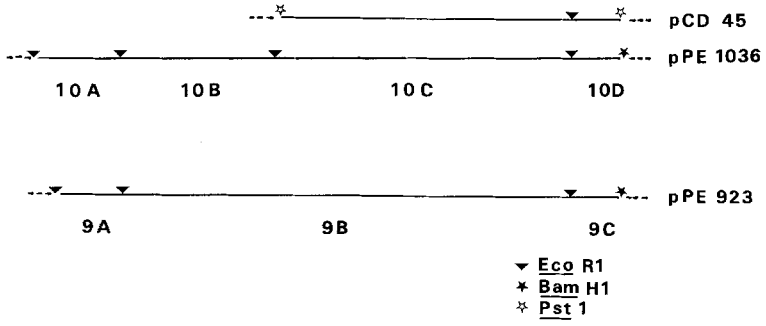

Fig. 1. Restriction maps of the near-full-length cDNA inserts, pPE1036 [corresponding to pCD45 (Casey et al. 1985)] and pPE923, showing the relative positions of the fragments used in hybridization analyses. The insert fragments 10A, 10B, 10C, 10D, 9A, 9B and 9C are 400, 760, 1,460, 230, 300, 2,220 and 230 bp, respectively

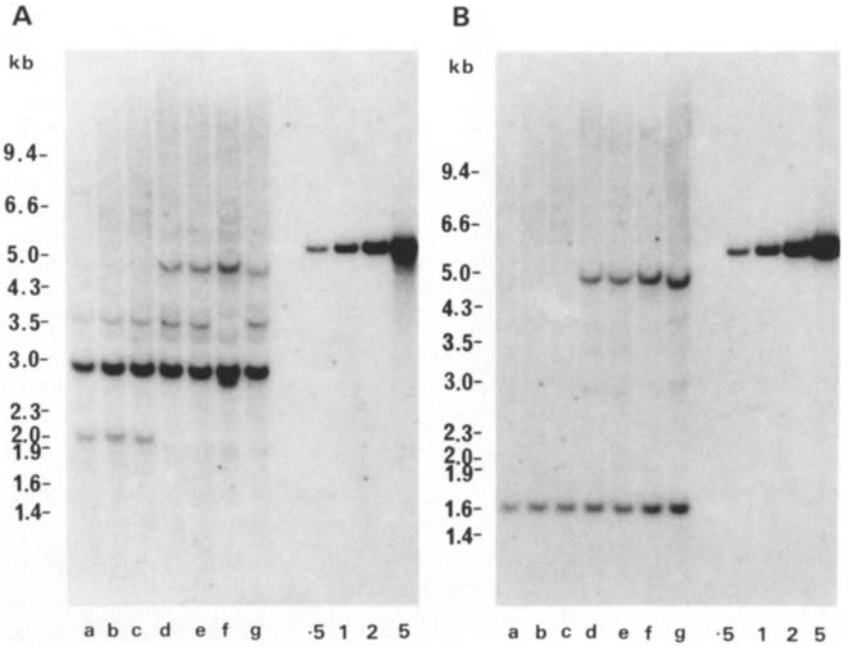

Fig. 2A, B. Hybridization of cDNA insert subfragments 9B (**A**) and 10C (**B**) to 5 µg EcoRI-digested genomic DNA from three *P. fulvum* [JI 224Sp (*a*), JI 224Br (*b*), JI 849 (*c*)] and four *P. sativum* [cv Alaska (*d*), cv Scotch (*e*), cv Birte (*f*), JI 181 (*g*)] genotypes. Amounts of Aat2-linearized pPE923 (*A*) and pPE1036 (*B*), corresponding to 0.5, 1, 2 and 5 gene copy equivalents per haploid genome per 5 µg pea DNA were included in each hybridization. Filters were washed in 0.1 × SSC, 0.1% (w/v) SDS at 65 °C. The positions adopted by DNA marker (λ/EcoRI/HindIII) are shown in kilobases

sion of pCD45 (Casey et al. 1985)] and pPE923, which were isolated from a library constructed using RNA from cv Birte (Ealing and Casey 1988, 1989); the probes 10A-D and 9A-C were derived from pPE1036 and pPE923, using three and two internal EcoRI sites, respectively, and polylinker EcoRI and BamHI sites (Fig. 1), and were labelled by Nick-translation. Following hybridization, filters were washed for 4 h in 2 × SSC, 0.1% (w/v) SDS at 50 °C (low stringency), or in 0.1 × SSC, 0.1% (w/v) SDS at 50 °C (medium stringency), 60 °C or at 65 °C (high stringency). Filters were exposed to preflashed film at −70 °C.

For copy number analyses, amounts of Aat2-linearized plasmids equivalent to 0.5, 1, 2 and 5 copies per haploid genome in 5 µg pea DNA [assuming a pea haploid genome size of 4.6 pg (Murray et al. 1978)] were loaded on gels alongside EcoRI-digested pea DNAs; after electrophoresis, DNAs were blotted directly to nitrocellulose without staining. Blots were hybridized to the internal cDNA fragments 9B or 10C, washed in 0.1 × SSC, 0.1% (w/v) SDS at 65 °C and exposed to preflashed film at −70 °C. Appropriate exposures of autoradiographs were scanned using a Joyce-Loebl Chromoscan 3, and amounts of hybridization to pea DNA fragments were quantified relative to those of the copy number equivalents in the linear range.

Linkage analysis

Fisher's exact test (Fisher 1954) was used to detect nonrandomness in the segregation patterns of the F_2 and F_7 populations, where the population size prohibited the use of a χ^2 analysis. The segregation patterns of the F_6 population were analysed by χ^2 analysis. Recombination frequencies for data where nonrandomness was detected were calculated according to Stevens (1940) (F_2 data) or Haldane and Waddington (1931) (F_6 data). Recombination frequencies were converted to map units according to Haldane's mapping function (Haldane 1919).

Results

Copy number

The internal cDNA fragments 9B and 10C were hybridized to EcoRI-digested pea DNA, and the hybridization intensities were compared to those given by amounts of linearized plasmid equivalent to 0.5–5 copies per haploid genome. Figure 2 shows that marked differences in hybridization patterns were observed between *P. fulvum* (a–c) and *P. sativum* (d–g) genotypes; in particular, one class of EcoRI fragments was absent from *P. fulvum* DNA hybridized to the insert fragment 10C (Fig. 2B). Quantitation of the hybridization signals indicated a copy number of 2–3 in *P. sativum* genotypes for both

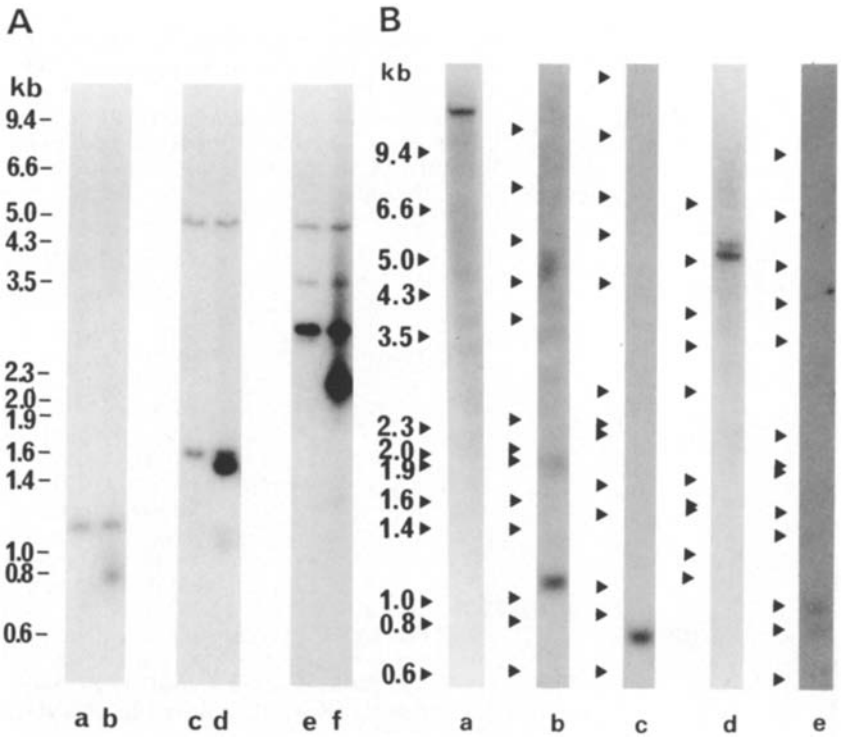

Fig. 3. **A** Hybridization of the cDNA insert internal subfragments 10B (*a, b*), 10C (*c, d*) and 9B (*e, f*) to EcoRI-digested genomic DNA from cv Birte. Insert subfragments 10B, 10C and 9B (ca. 2–5 pg) were added to the genomic DNA in *b, d, f,* respectively. The positions adopted by DNA markers are shown in kilobases. **B** Hybridization of the cDNA insert subfragments 10A (*a*), 10B (*b*), 10D (*c*), 9A (*d*) and 9C (*e*) to EcoRI-digested genomic DNA from cv Birte. Filters were washed in 0.1 × SSC, 0.1% (w/v) SDS at 60 °C. The positions adopted by DNA markers of given size are indicated (kb) for *a*. The positions of the same markers are shown by *arrows* for *b*, *c*, and *e*. In *d*, the positions of all except the 1.0-, 0.8- and 0.6-kb markers are shown. (Tracks *a*–*e* are from different gels)

probes (2.60 ± 0.47 for 9B; 2.27 ± 0.52 for 10C). It is likely that the fragments of lower intensity in Fig. 2A represent genes with reduced homology to the probe DNA, rather than lower copy numbers, and thus gene copy estimates represent a minimum possible gene number.

Gene organization

The sizes of the cDNA internal EcoRI fragments 10B, 10C and 9B are smaller than the corresponding EcoRI fragment sizes in genomic DNA from cv Birte, indicating the presence of introns in these genomic fragments (Fig. 3A, compare a, c, e, with b, d, f). Although two different EcoRI fragments hybridize to the internal probe 10C, only one EcoRI fragment hybridizes to 10B (Fig. 3A, a); likewise, only one EcoRI fragment hybridizes to the cDNA 5′ and 3′ end fragments, 10A and 10D (Fig. 3B, a, c, respectively). These data suggest that the 5-kb fragments that hybridize to 10C do not contain sequences closely homologous to the remainder of the cDNA; the 5-kb fragments may contain very divergent regions corresponding to the adjacent cDNA fragments and also lack the EcoRI sites present in the cDNA or, alternatively, they may differ from the 1.6-kb EcoRI fragments only in the number and/or size of introns. If the latter is the case, then both the 5-kb and 1.6-kb fragments homologous to 10C must have adjacent EcoRI fragments of apparently identical size. The absence of 5-kb fragments homologous to 10C in *P. fulvum* genomic DNA, and the apparent conservation of the 1.6-kb fragment (Fig. 2B) among all genotypes examined, suggest that genes containing the 5-kb fragment may have arisen more recently in *P. sativum* genotypes by duplication and divergence of an ancestral gene.

In genomic DNA from cv Birte, two EcoRI fragments hybridized to the 9A and 9C probes, which correspond to the 5′ and 3′ ends, respectively (Fig. 3B, d, e); the two fragments were of a similar size with a given probe, but it is not known how these relate to the three or four classes of internal fragment corresponding to 9B (Fig. 3A, e). Both 3′ probes (10D, 9C) hybridized to small EcoRI fragments of approximately 0.7–1 kb (Fig. 3B, c, e), whereas both 5′ probes (10A, 9A) hybridized to larger EcoRI fragments (Fig. 3B, a, d); these results suggest a possible conservation of an EcoRI site to the 3′ end of both classes of gene. The results further suggest that sequences 5′ to both classes of gene can be studied by analysis of EcoRI fragments of ca. 7 and 15 kb from cv Birte.

RFLP analyses

A selection of pea lines was screened with the seven probes and polymorphisms sought in the corresponding genomic fragments for use in genetical analyses. In EcoRI digests, no polymorphism could be detected for fragments hybridizing to 9A, and the 10C probe detected only the polymorphism described above between *P. fulvum* and *P. sativum* genotypes. Polymorphisms were de-

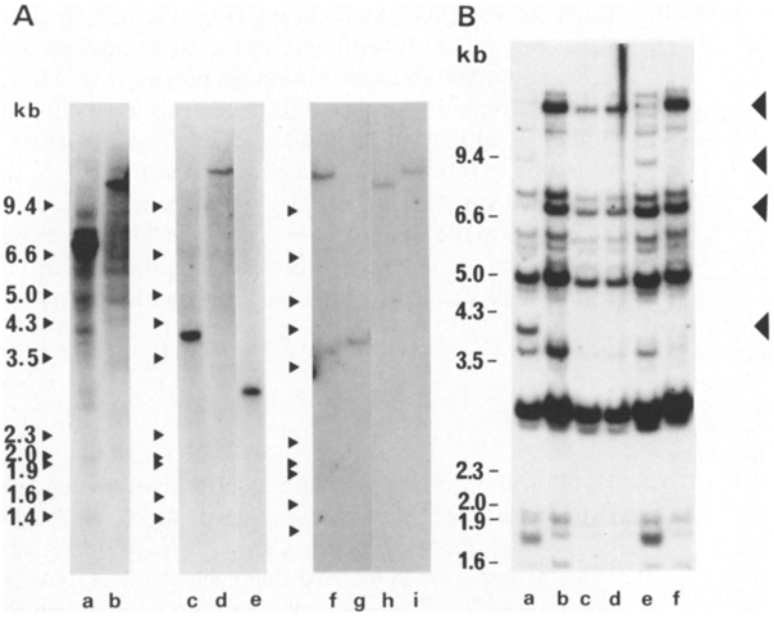

Fig. 4. **A** Hybridization of the cDNA insert subfragment 10A to EcoRI-digested genomic DNA from JI 181 (*a*, *c*), JI 430 (*b*, *d*), JI 224Br (*e*), JI 399 (*f*), JI 281 (*g*), JI 813 (*h*) and JI 1201 (*i*). Filters were washed in 2 × SSC, 0.1% (w/v) SDS at 50 °C (*a*, *b*) or in 0.1 × SSC, 0.1% (w/v) SDS at 50 °C (*c*–*i*). (The same pattern was observed for *c*–*i* whether filters were washed in 0.1 × SSC, 0.1% (w/v) SDS at 50 °C or at 65 °C). **B** Hybridization of the cDNA insert subfragment 9B to EcoRI-digested genomic DNA from JI 813 (*a*), JI 1201 (*b*) and four F_7 plants (*e*–*f*) derived from the cross JI 813 × JI 1201. Filters were washed in 0.1 × SSC, 0.1% (w/v) SDS at 50 °C. *Arrows* show the polymorphisms discussed in the text

tected for fragments hybridizing to 10B and 9C, but only between a *P. fulvum* and *P. sativum* genotype and, for the 10D probe, a small size difference was detected in one *P. fulvum* genotype (data not shown). These lines were not used for genetical analyses, as interspecific crosses often lead to distorted segregation ratios or are unsuccessful.

By contrast, the approximately 15-kb fragment that hybridized to 10A in genomic DNA from cv Birte was found to be polymorphic among several *Pisum* lines. Figure 4A (c–i) shows that, at high stringency, four different fragment sizes were evident, one of which (Fig. 4A, e) was found only in *P. fulvum* lines. These fragments are homologous to the 5′ end of the cDNA, and it is likely that they are predisposed to being polymorphic in that they contain a few kilobases of DNA (apart from 400 bp of coding sequence and the upstream control sequences), which are unrelated to the structural gene corresponding to the probe. However, after low stringency washes (Fig. 4A, a, b), the probe 10A detects several additional fragments, at least one of which appears more highly reiterated than the single fragment remaining after high stringency washing; these 'low homology' fragments are also polymorphic between some lines.

Although there was no polymorphism associated with the predominant fragments that hybridized to 9B, longer exposures of blots, washed at medium stringency, revealed polymorphisms in other DNA fragments (Fig. 4B).

Analyses of crosses

An F_2 population from the cross JI 430 × JI 181 and F_6 and F_7 populations derived from the crosses JI 281 × JI 399 and JI 813 × JI 1201, respectively, were used. DNA from the F_2 population (JI 430 × JI 181) was hybridized to probe 10A under conditions of low and high stringency; DNA from the F_6 population (JI 281 × JI 399) was hybridized to probe 10A under conditions of high stringency, whereas DNA from the F_7 population (JI 813 × 1201) was hybridized to probe 10A under conditions of high stringency (there was no obvious polymorphism at low stringency) and to probe 9B under conditions of medium stringency.

The segregation of the 4-kb/15-kb fragments (Fig. 4A, f, g) corresponding to probe 10A in the F_6 plants derived from the cross JI 281 × JI 399 closely followed the segregation of *le*, determining internode length, giving a map distance of 2.17 (Table 1). Linkage to other RFLPs in this linkage group was consistent with this map distance for this population (data not shown). Although linkage to *le* was also detected in the data obtained from the F_2 JI 430 × JI 181 segregants, the map distance between these two markers appeared to be larger in this cross (Table 1).

The segregation of the ca. 7-, 7.5- and 14-kb bands that hybridized to probe 10A under conditions of low stringency (Fig. 4A, a, b) in the cross JI 430 × JI 181 was random, relative to each other (Table 2). In addition, there was no correlation between the segregation of these bands and the 4-kb/15-kb bands (Fig. 4A, c, d) which hybridized under conditions of high stringency to 10A or *le* (Table 2). Thus, four distinct genetic loci are defined by probe 10A.

Analysis of F_7 homozygotes from the cross JI 813 × JI 1201 in medium stringency hybridizations to probe 9B showed that the ca. 7-kb and 4-kb bands (Fig. 4B, a, b) were allelic alternatives; the ca. 15-kb

Table 1. Segregation of the 4-kb/15-kb (A/a) and *Le/le* (B/b) alleles among the F_6 JI 281 × JI 399 homozygotes and the F_2 JI 430 × JI 181 segregants

Cross	AB	Ab	aB	ab	*P*	R.f.	m.u.
F_6 (281 × 399)	23	1	1	26	<0.0001	0.020 ±0.015	2.08 ±1.56
F_2 (430 × 181)	20	4	0	5	0.0011	0.149 ±0.073	17.7 ±8.7

The 4-kb/15-kb bands are detected at high stringency by probe 10A. The six F_2 classes scored are regrouped such that factors are in the coupling phase. *P*, probability, as determined from a χ^2 analysis (F_6) or a Fisher's (1954) exact test on a 2 × 2 contingency table (F_2); R.f., recombination frequency, determined for the F_6 population using the relationship of Haldane and Waddington (1931) and, for the F_2 population, according to Stevens (1940); m.u., equivalent map units (Haldane 1919)

Table 2. Segregation of the 14-kb (LS1), 7.5-kb (LS2) and 7-kb (LS3) fragments detected in low-stringency hybridizations to probe 10A (Fig. 4A, a, b), in relation to the segregation of the 4-kb/15-kb fragments detected by 10A at high stringency (HS) (Fig. 4A, c, d) and *le* in the F_2 population from the cross JI 430 × JI 181

Character pair	AB	Ab	aB	ab	*P*
HS/LS1	16	5	6	2	0.37
HS/LS2	18	6	4	1	0.43
HS/LS3	15	9	5	0	0.13
le/LS1	15	5	7	2	0.38
le/LS2	15	5	7	2	0.38
le/LS3	14	6	6	3	0.32
LS1/LS2	15	8	7	0	0.08
LS1/LS3	16	7	4	3	0.29
LS2/LS3	15	7	5	3	0.32

A and B refer to dominant first and second characters, respectively. The data obtained for HS (six classes scored) have been regrouped into four classes such that factors are in the coupling phase. *P*, probability, as determined from a Fisher's (1954) exact test on a 2 × 2 contingency table

Table 3. Segregation of 7-kb/4-kb bands (9B-1) and the 15-kb/9.4-kb bands (9B-2) that hybridized to probe 9B (Fig. 4B), in relation to the segregation of each other and the fragments that hybridized to probe 10A (Fig. 4A, h, i) in the F_7 JI 813 × JI 1201 population

Character pair	813/813	813/1201	1201/813	1201/1201	*P*
9B-1/10A	3	6	13	14	0.231
9B-2/10A	7	8	7	12	0.233
9B-1/9B-2	7	2	10	18	0.030

The class 813/1201 contains individuals that were JI 813-like for the first and JI 1201-like for the second character, respectively, etc. *P*, probability, as determined from a Fisher's (1954) exact test on a 2 × 2 contigency table

fragment was present as a heavy (Fig. 4B, b, c, d, f) or light (Fig. 4B, a, e) band and, in the latter case, an approximately 9.4-kb band was always present (Fig. 4B, a, e). The segregation of these four fragments was unlinked (Table 3) to that of the 14-kb/15-kb fragment corresponding to probe 10A in the same cross (Fig. 4A, h, i); these data suggest the existence of two genetic loci with homology to the second major class of seed lipoxygenase gene, which are unlinked to each other and are unlinked to the locus defined by high homology with probe 10A (Table 3).

Discussion

Hybridization of lipoxygenase cDNAs representing the two major seed lipoxygenase polypeptides of *Pisum* (Casey et al. 1985; Ealing and Casey 1988, 1989) to *P. sativum* genomic DNA showed that each of the cDNAs is homologous to a class of genes, each containing two to three members. The gene numbers reported here are likely to be a minimum estimate of the number of genes which are highly homologous to the individual probes and thus may not include genes for one or more of the sequence variants previously identified by N-terminal sequence analysis of seed lipoxygenase polypeptides. Three seed polypeptides, two of which displayed heterogeneity, were identified by N-terminal sequence analysis, and none of these showed identity to the N-terminal sequences predicted from pPE923 and pPE1036 (Domoney et al. 1990).

The two classes of gene (Fig. 2A, B) are distinguishable under conditions of medium-stringency hybridizations, reflecting the homology of the two isolated cDNAs (Ealing and Casey 1988, 1989). Two EcoRI fragments of different sizes homologous to an internal probe from pPE1036 were observed in hybridization to *P. sativum* DNA, but only one of these was present in *P. fulvum* DNA, indicating a lower copy number of this gene in the latter. In the absence of information on the expression of genes containing these two different internal fragments, however, it is not possible to attach any significance to these differences. Hybridizations of genomic DNA to cDNA fragments adjacent to the internal probe suggested only one fragment size class in each case; the variant internal gene fragments may thus contain flanking gene sequences which are indistinguishable or, on the other hand, which are nonhomologous to such an extent that only those corresponding to the cDNA are detected.

Polymorphisms corresponding to pPE1036 were detected among *P. sativum* genes only when the 'large' EcoRI fragments containing the 5′ 400 bp of coding sequence were examined (Fig. 4A). In two crosses, RFLPs associated with these fragments showed linkage to *le*, which has been assigned to linkage group 4 (Blixt 1974).

A lipoxygenase locus, designated *Lox*, has been previously mapped to linkage group 4 based on an analysis of seed polypeptide variants in two crosses; in one of these crosses, linkage to *le* indicated a distance of 13–38 map units between *Lox* and *le* (North et al. 1989). This range covers the linkage value obtained for the RFLP associated with pPE1036 and *le* in the cross JI 430 × JI 181 (Table 1), and indicates that the same locus may be involved. A significantly smaller map distance obtained for the same markers in JI 281 × JI 399 (Table 1) may indicate that crossover suppression has occurred in this cross, resulting in a reduction of the linkage map in this region.

In addition to the *Lox* locus, defined by high-stringency hybridization of pPE1036, three other loci were defined in one of the crosses (JI 430 × JI 181) by low-stringency hybridization, using the same probe, and these were unlinked to each other and to the *Lox* and *le* loci (Table 2). The nature of the relationship of these sequences to each other must await the isolation and sequence analysis of the corresponding genes. A further two loci were identified in medium-stringency hybridization, using an internal probe from pPE923, representing the second lipoxygenase cDNA class; these two loci were also unlinked to the *Lox* locus (defined by high-stringency hybridizations to pPE1036) in the same cross (Table 3).

The *Lox* locus, defined by polypeptide analysis, contains genes corresponding to both major seed lipoxygenase polypeptides (North et al. 1989) and, as such, functional gene sequences corresponding to the two cDNAs would be expected to display close linkage. The present data are not necessarily inconsistent with this expectation, in that no polymorphism was associated with the predominant 2.9-kb fragment corresponding to pPE923 and, hence, the genetics of these sequences was not examined. The variant fragments homologous to pPE923 (probe 9 B) that were scored (Fig. 4 B) are not among the predominant bands detected using this probe at high stringency (Fig. 2 A), and thus the loci containing these variant fragments may not contain the genes corresponding to pPE923.

The results presented here describe the lipoxygenase gene family in pea; the existence of six loci is indicated by hybridization analysis, using two probes and three crosses, but it is possible that some of the loci identified in different crosses may coincide. The data presented serve to illustrate the complexities associated with attempts to correlate gene loci, identified in hybridization analyses, with gene products in terms of both cDNAs and protein. The genetical analysis employed here has permitted one such correlation to be made.

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