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Analysis of a lipoxygenase pseudogene in *Pisum*

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Abstract Lipoxygenase (LOX) enzymes play important roles in plant biology, and in the quality of plant-derived foods, through the production of fatty acid hydroperoxides that are metabolized either to jasmonate, or to volatile aldehydes that are part of plant defence systems and/or impart tastes and aromas to fruits and vegetables. We have identified a lipoxygenase pseudogene in peas that is composed of three elements: rearranged LOX-2 and LOX-3 genes and an unidentified tract of DNA. We present evidence that such an arrangement is normally present in the genome of *Pisum sativum*, but is absent from *Pisum fulvum* lines, including a mutant line that lacks LOX-2 polypeptides. The absence of LOX-2 polypeptides and the pseudogene co-segregate. The pseudogene therefore has utility as a molecular marker for the introgression of the LOX-2-null phenotype into commercial *Pisum sativum* genotypes.

Key words Lipoxygenase · Pseudogene · *Pisum* · Marker · Mutant

Introduction

Lipoxygenases (LOX; E.C. 1.13.11.12) catalyze the formation of hydroperoxides from polyunsaturated fatty acids and molecular oxygen. The hydroperoxides are converted into a number of compounds that have biological activity. These include jasmonate, a potent activator of defence genes (Farmer and Ryan 1992) produced from the 13-hydroperoxide of linolenic acid by a sequence of reactions that begins with allene oxide synthase (Vick and Zimmerman 1983, 1984). Alternatively, hydroperoxides may be catalytically split by the action of hydroperoxide lyases to produce volatile aldehydes that can act as bacteriocides (Croft et al. 1993). Thus, the formation and subsequent metabolism of fatty acid hydroperoxides are part of important plant-defence mechanisms. Many of the volatile aldehydes produced through the lipid oxidation pathway also impart characteristic aromas and flavours to fruits, vegetables and foods derived from them. Some of these aromas are perceived as unpleasant, a good example being *n*-hexanal (produced by lyase action on the 13-hydroperoxide of linoleic acid), which is the underlying basis of the so-called "grassy-beany" flavour of soybean (*Glycine max*) seed products. Removal of a specific LOX (isoform-2) from soybean seeds improves their consumer acceptability (Davies et al. 1987).

Peas (*Pisum sativum* and its wild relative *Pisum fulvum*) contain two major seed LOX polypeptides (Casey et al. 1985). Analysis of cDNAs corresponding to these polypeptides has shown them to be similar to LOX-2 and LOX-3 from soybean seeds (Ealing and Casey 1988, 1989) and they are therefore referred to as pea LOX-2 and LOX-3. Genes corresponding to LOX-2 and LOX-3 from peas are present at a single genetic locus designated *lox* (North et al. 1989; Domoney et al. 1991). As part of a programme to study the structure and expression of pea LOX genes, we have cloned and sequenced individual LOX-2 and LOX-3 genes, designated *lox1 : Ps : 2* and *lox1 : Ps : 3*, respectively (Forster et al. 1994 b; Knox et al. 1994). During the course of investigating the organization of these two genes, we observed a 5-kb *Eco*RI fragment of pea genomic DNA that hybridized with LOX-2 and LOX-3 probes and appeared to be absent from *P. fulvum*. We describe here the structure of this 5-kb *Eco*RI fragment, its physical relationship with other pea LOX genes and its value as a molecular marker in pea.

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Materials and methods

Plant material

The *P. fulvum* lines JI 1006 and JI 1009, the *P. sativum* ssp. *abyssinicum* line JI 695 and the *P. sativum* cultivar 'Birte', examined in detail in this work, were from the John Innes Centre *Pisum* germplasm collection. Other lines from the John Innes collection, used for the analysis of LOX genes, included JI 224 Br, JI 224 Sp, JI 849 (all *P. fulvum*) and the *P. sativum* lines JI 181, JI 281, JI 399, JI 430, JI 813, JI 1201 and cultivars 'Alaska', 'Dark Skinned Perfection' and 'Scotch'. Dry, mature seeds were harvested from plants grown in a soil-based compost in a greenhouse at a minimum temperature of 15°C (day) or 10°C (night), with supplementary lighting in the winter to provide a 16-h photoperiod. For the analysis of segregating populations, small amounts (1–5 mg) of meal were removed for protein analysis from mature seeds by drilling with the point of a scalpel blade opposite the embryonic axis, and the remainder of the seed was planted to produce leaf material for DNA extraction and the next generation of seed. Leaves were harvested from seedlings that were approximately 6-weeks old, frozen in liquid nitrogen and stored at −70°C.

Protein analysis

Extracts of ground, mature pea seed meal in SDS-polyacrylamide gel (PAGE) sample buffer were prepared according to Domoney and Casey (1985) and analyzed by SDS-PAGE/Western blotting as described by Casey et al. (1985), using 10% separating gels and an antibody raised against pea seed LOX fraction 'B' (Domoney et al. 1990).

Nucleic-acids analysis

Plant DNA was extracted from leaves according to Dellaporta et al. (1983). Restriction digestion, Southern blotting, plasmid DNA isolation, labelling of probes with [^{32}P]-αdCTP by random-primed synthesis, and separation of DNA by electrophoresis in agarose gels in Tris-acetate buffer all followed standard techniques (Sambrook et al. 1989). 5′,3′ and internal cDNA probes from LOX-2 and LOX-3 cDNAs were obtained by plasmid digestion with *Eco*RI and *Bam*HI, followed by gel purification, as described by Domoney et al. (1991). Screening of a genomic library of cv 'Birte' DNA, constructed in λGEM12 using half-filled *Xho*I arms and pea DNA partially digested with *Sau*3A, was as described before (Forster et al. 1994 b; Knox et al. 1994). Sequencing of subcloned genomic DNA fragments and intact phage DNA was as described by Knox et al. (1994).

The genomic clone λLOX-5 DNA, digested with *Eco*RI, was blotted and probed with 5′,3′ and internal LOX cDNA probes (as above). For mapping, λLOX-5 DNA was digested with *Bam*HI, *Eco*RI, *Sac*I and *Hin*dIII, alone or in various combinations, and the fragments separated by agarose-gel electrophoresis and capillary blotted bi-directionally to nitrocellulose. Duplicate blots were hybridized with the entire λLOX-5 DNA, or a 1.1-kb *Eco*RI/*Xba*I subfragment from the 5-kb *Eco*RI fragment (see Fig. 2 A, later). To confirm the relative position of the 5 kb *Eco*RI fragment, λLOX-5 DNA was digested with *Bam*HI and the resultant 7.5-kb and 9.5-kb fragments purified, digested with *Sac*I or *Eco*RI, blotted and probed with entire λLOX-5 DNA and the 3′ probe from LOX-2. Blots were washed in 0.1 × SSC/0.1% SDS at 50°C or 65°C and exposed to pre-flashed X-ray film at −70°C.

Results and discussion

Identification and characteristics of the 5-kb *Eco*RI fragment

Southern-blot analysis of *Eco*RI-digested DNA from selected *P. sativum* and *P. fulvum* genotypes, using internal fragments from seed LOX-2 and LOX-3 cDNAs (Domoney et al. 1991), showed hybridization to fragments known to derive from the corresponding genes *lox1 : Ps : 2* (Forster et al. 1994 b) and *lox1 : Ps : 3* (Knox et al. 1994) (and see also Domoney et al. 1991), plus a fragment of approximately 5-kb (Fig. 1 A, B) that had two unusual characteristics:

(1) it was present in the 11 *P. sativum*, but absent from the five *P. fulvum*, genotypes examined (see also Domoney et al. 1991), and
(2) it hybridized to LOX-2 and LOX-3 probes at high stringency; the probes themselves do not, however, cross-hybridize at high stringency.

In order to clone the approximately 5-kb *Eco*RI fragment, a pea genomic library, from cv 'Birte', in λGEM-12 (Forster et al. 1994b) was screened with LOX-2 and LOX-3 internal cDNA probes. A number of positive phages were purified, four of which were found to contain an approximately 5-kb *Eco*RI fragment.

Structure of the 5-kb fragment

The sequence of the approximately 5-kb fragment from the clone λLOX-5, named *lox1 : Ps : 6* and deposited in the GenEMBL database as Y15423, showed (Fig. 2 A) three features:

(1) 2.89 kb of sequence that has no database homologue and contains no extensive open reading frame;
(2) 0.87 kb of a LOX-2 gene that is 97% identical to *lox1 : Ps : 2* (Forster et al. 1994 b) nucleotides 3116–3990; and
(3) 1.08 kb of a LOX-3 gene that is 99% identical to nucleotides 8216–9300 of *lox1 : Ps : 3* (Knox et al. 1994) and in opposite orientation to the LOX-2 gene sequence.

There are several lines of evidence to suggest that such a structure exists in genomic DNA and is not a cloning artifact. First, the 5-kb *Eco*RI fragment is detected in 11 lines of *P. sativum* by hybridization of *Eco*RI digests to LOX-2 and LOX-3 probes and to the cloned approximately 5-kb fragment. Second, the fragment is present in four independent λ phage clones; in each of these the fragment has been shown to be the same by restriction mapping and Southern blotting/probing of *Eco*RI, *Pst*I and *Xba*I digests. Third, the arrangement of LOX-2- and LOX-3-homologous

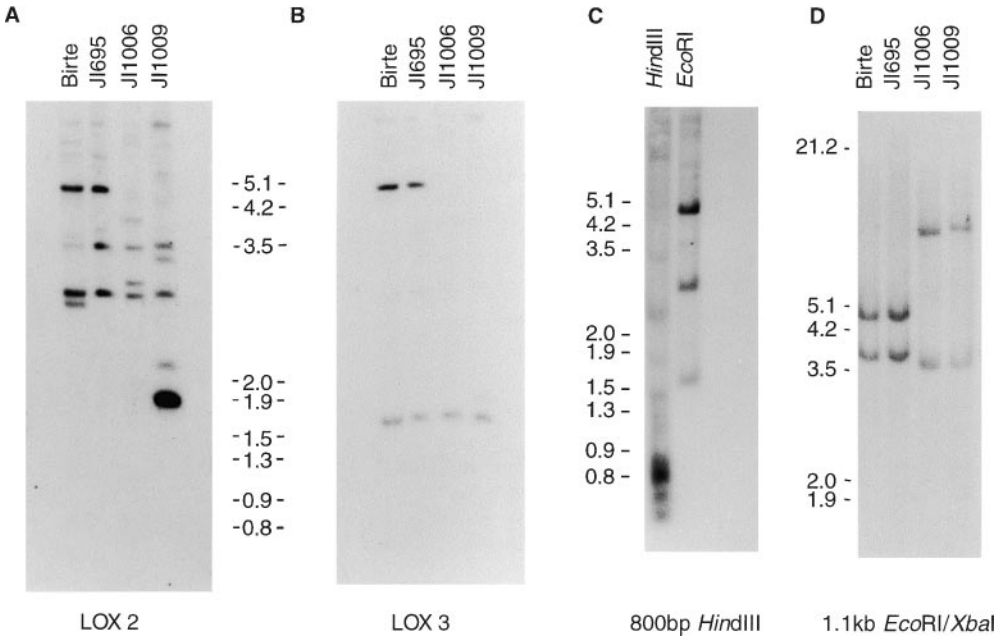

Fig. 1A–D Southern hybridization analysis of: **A, B** *P. sativum* (cv 'Birte' and JI 695) and *P. fulvum* (JI 1006 and JI 1009) DNA. Genomic DNA was digested with *Eco*RI and subjected to Southern-hybridization analysis using internal LOX-2 (**A**) and LOX-3 (**B**) cDNA probes. Filters were washed in 0.1 × SSC at 65°C and exposed to pre-flashed X-ray film at − 70°C for 6 days. **C** DNA from *P. sativum* cv 'Birte'. DNA was digested with *Hin*dIII or *Eco*RI and probed with the 800-bp *Hin*dIII fragment shown in Fig. 2 A. Filters were washed in 0.1 × SSC at 50°C and exposed to pre-flashed X-ray film at −70°C for 6 days. **D** DNA from *P. sativum* (cv 'Birte' and JI 695) and *P. fulvum* (JI 1006 and JI 1009). DNA was digested with *Eco*RI and probed with the 1.1-kb *Eco*RI/*Xba*I fragment (Fig. 2 A). Filters were washed in 0.1 × SSC at 65°C and exposed to pre-flashed X-ray film at − 70°C for 3 days. The estimated sizes of hybridizing fragments are indicated in kilobase pairs

sequences shown in Fig. 2 A would predict the existence of a *Hin*dIII fragment of approximately 0.8 kb in genomic DNA; this has been demonstrated by probing *Hin*dIII digests of 'Birte' genomic DNA with the approximately 0.8-kb fragment (Fig. 1 C). The 0.8-kb *Hin*dIII probe also detects fragments in *Eco*RI-digested 'Birte' DNA (Fig. 1 C) that correspond to the 5-kb *Eco*RI fragment and to internal 3-kb LOX-2- and 1.6-kb LOX-3- homologous fragments (Domoney et al. 1991), thereby confirming its origin and composition. Together, these data suggest that such an arrangement of LOX gene fragments within the pseudogene is a normal feature of the genomic DNA of 'Birte', JI 695 and nine other *P. sativum* lines.

The 1.1-kb *Eco*RI/*Xba*I fragment from the 5′ end of the unidentified 2.89-kb sequence (Fig. 2) also hybridized to the 5-kb *Eco*RI fragment in genomic DNA from 'Birte', plus a fragment of 4 kb (Fig. 1 D). The hybridization intensity of the two fragments was comparable and suggested that the unidentified DNA was not highly repeated within the pea genome. In those λ clones that contained the 5-kb *Eco*RI fragment, it was the only fragment to hybridize to the 1.1-kb *Eco*RI/*Xba*I probe. Thus, the homologous 4-kb fragment in 'Birte' genomic DNA may be present, at low copy number, at a genetic locus other than *lox*, where the approximately 5-kb fragment resides (see below). The 2.89-kb region of the sequence provided no evidence of a transposon 'footprint'.

Inheritance of the 5-kb fragment

All five *P. fulvum* lines so far examined (Fig. 1 A, B, and Domoney et al. 1991) have lacked the 5-kb *Eco*RI fragment when probed with LOX cDNA internal fragments. Hybridization of the 1.1-kb *Eco*RI/*Xba*I subfragment of the 5-kb pseudogene (Fig. 2 A) to selected *P. sativum* and *P. fulvum* lines (Fig. 1 D) shows that the latter lines lack the 5-kb fragment but contain an approximately 8-kb fragment that is absent from the *P. sativum* lines. In addition to lacking the 5-kb fragment, JI 1006 also lacks detectable LOX-2 polypeptide (North 1990). The lack of the 5-kb *Eco*RI fragment might therefore prove to be a useful marker for the absence of LOX-2 in a population segregating for this LOX-2-null phenotype. To investigate such a possibility, and because crosses between JI 1006 and 'Birte' had proved unsuccessful, JI 1006 was initially crossed to the *P. sativum* ssp. *abyssinicum* line (JI 695) to generate an F_2 line lacking LOX-2. This, in turn, was crossed to 'Birte' and individual F_2 progeny lacking LOX-2 were selected by Western blotting (Forster et al. 1999). These were repeatedly back-crossed to 'Birte', selecting the LOX-2-null F_2 lines at each stage. At the fourth backcross generation, segregation of the LOX-2 polypeptide and the 5-kb pseudogene was analyzed;

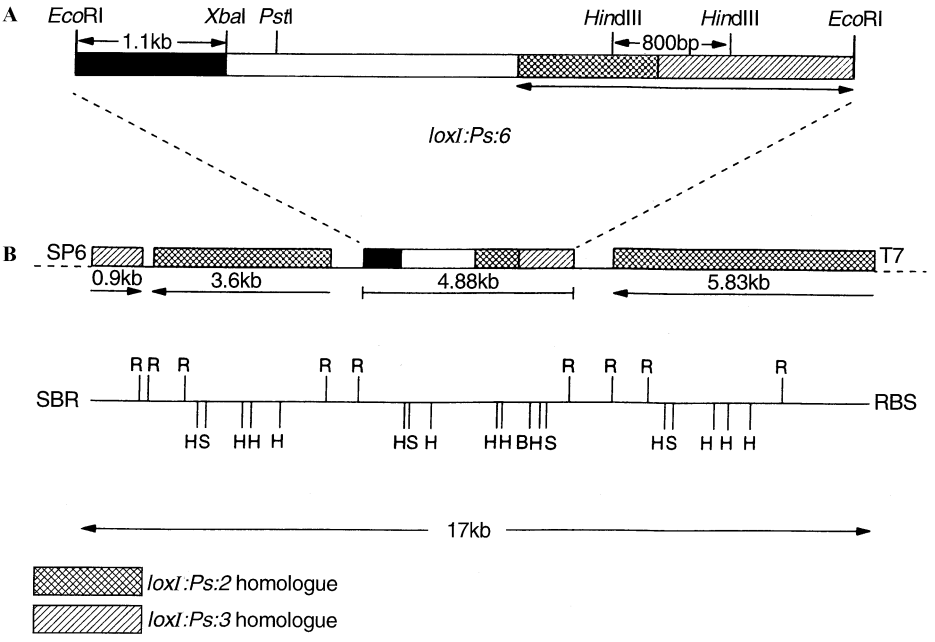

Fig. 2 Diagrammatic representation of: **A** The structure of *lox1 : Ps : 6* within the genomic clone λLOX-5. The 1.1-kb *Eco*RI/*Xba*I and 800-bp *Hin*dIII probes are shown. **B** the organization of LOX genes within the genomic clone λLOX-5. Restriction sites are shown as: R, *Eco*RI; H, *Hin*dIII; S, *Sac*I; B, *Bam*HI. The SP6 and T7 promoters are part of the λGEM-12 cloning vector. *Arrows* below the hatched areas represent the directionality of the clone in the mRNA sense (5′ → 3′)

Table 1 Segregation analysis of the LOX-2 polypeptide and the 5-kb pseudogene

LOX-2$^+$/5 kb$^+$	LOX-2$^+$/5 kb$^-$	LOX-2$^-$/5 kb$^+$	LOX-2$^-$/5 kb$^-$	$\chi^2_{(3)}$	*P*
65	0	0	23	92.6	< 0.001

portions of F_2 seeds were screened by Western blotting for the presence or absence of LOX-2 and the remainder of each seed grown-on to provide plant material for DNA, which was then screened for the presence or absence of the 5-kb *Eco*RI fragment using the 1.1-kb *Eco*RI/*Xba*I probe (Fig. 2 A). The analysis of 88 seeds and plants (Table 1) showed that the absence of the 5-kb fragment co-segregated completely with the LOX-2-null phenotype. The inheritance of the 5-kb fragment appeared to be unlinked to that of the 4-kb fragment (Fig. 1 D). By the fourth backcross, however, the 8-kb fragment, originally detected in JI 1006 (using the 1.1-kb *Eco*RI/*Xba*I probe) as a possible allelic alternative to the 5-kb fragment (see Fig. 1 D), had been lost.

Gene organization and the location of the 5-kb pseudogene

Three genomic clones (λLOX-1, -5 and -6) contained apparently normal seed LOX genes as well as the 5-kb *Eco*RI pseudogene; the longest (λLOX-5) was examined in more detail to gain information on the arrangement of genes at the *lox* locus.

λLOX-5 (Fig. 2 B) contains an insert of approximately 17 kb, and 2 kb of sequence at the T7 end of the λLOX-5 insert is identical to part of the promoter region of *lox1 : Ps : 2* (Forster et al. 1994 b)[1]. This *lox1 : Ps : 2* promoter can drive the expression of a β-glucuronidase reporter gene in transgenic tobacco (Forster et al. 1994 a); it is likely then that the *lox1 : Ps : 2*-homologous gene at the T7 end of λLOX-5 is functional, since all the other *Eco*RI fragments of this *lox1 : Ps : 2*- homologue are of normal size. The other (SP6) end consists of a small amount of *lox1 : Ps : 3*-homologous sequence beginning at the *Sau*3A site at position 2370 in *lox1 : Ps : 3* (Knox et al. 1994). The *lox1 : Ps : 2*- and *lox1 : Ps : 3*-homologous sequences in λLOX-5 are in opposite orientation.

Hybridization of bi-directional blots of various digests of λLOX-5 DNA to probes corresponding to the 5′ and 3′ ends and internal parts of LOX-2 and LOX-3 cDNA clones (Domoney et al. 1991) and to the 1.1-kb *Eco*RI/*Xba*I fragment indicated the organization shown in Fig. 2 B, which includes a further 3.6 kb of a *lox1 : Ps : 2*-homologous gene within the interior of the clone. The *lox1 : Ps : 6* pseudogene (5-kb *Eco*RI fragment) appears to have displaced the promoter region of this *lox1 : Ps : 2* homologue, which presumably then is not functional. The 3′ part of this *lox1 : Ps : 2* homologue is 0.77 kb instead of the usual 0.91 kb. This

[1] *lox1 : Ps : 2* was from another λ clone, λLOX-2, which differs from λLOX-5; it contains a second *lox1 : Ps : 2* homologue and does not contain the *lox1 : Ps : 6* pseudogene (unpublished data)

polymorphism has been attributed in other λLOX clones to a T → C base change that generates a new *Eco*RI site (data not shown).

λLOX-1 and λLOX-6 both contained sequences that were almost identical to *lox1 : Ps : 2*, plus the *lox1 : Ps : 6* pseudogene. λLOX-6 was virtually identical to λLOX-5, apart from the length of the 5′-noncoding sequence of the *lox1 : Ps : 2*-homologous gene at the T7 end of the inserts. The relative arrangement of LOX genes within λLOX-1 and λLOX-5/6 makes it clear that the *lox1 : Ps : 2* homologues in λLOX1 and λLOX5/6 are not the same. This in turn means that there is more than one copy of the *lox1 : Ps : 6* pseudogene in 'Birte' DNA.

The absence of the pseudogene appears to be a characteristic of *P. fulvum* that is a useful marker for the introgression of the LOX-2-null phenotype into elite breeding populations. The absence of the pseudogene from LOX-2 null lines probably reflects the origin of the null mutation in a *P. fulvum* line; there is no evidence to suggest that the two are necessarily causally related. A number of other *P. fulvum* lines contain LOX-2 polypeptides (Casey et al. 1985).

It is not unknown for pseudogenes to be closely associated with active homologues within the pea genome; the major storage protein gene *leg*A, for instance, is found on the same genomic clone as a corresponding pseudogene, *leg*D (Lycett et al. 1984). It is to be expected that duplication and divergence of multiple genes at a locus may in some cases lead to changes that render a gene inactive and thereby release it from selection; such genes tend to accumulate mutations in the form of small deletions/insertions and single base substitutions, as in the case of *leg*D. *lox1 : Ps : 6* is more extreme in this respect, showing rearrangements to form a chimaeric gene that cannot produce an active enzyme and to introduce a tract of unidentified DNA. The evidence to support its existence in genomic DNA is strong and comes from several lines of investigation. It is not obvious from a consideration of its structure how *lox1 : Ps : 6* has been formed, but it appears from the gene arrangements within λLOX-5 and -6 that there may be instances within the pea genome of functional LOX-2 and -3 genes in inverted orientation and close proximity (see Fig. 2 B). These may provide the substrate for the formation of structures such as that found in *lox1 : Ps : 6*.

In conclusion, *lox1 : Ps : 6* is a rearranged seed lipoxygenase pseudogene that is present in all accessions of *P. sativum* so far examined and may have arisen since the divergence of *P. sativum* and *P. fulvum*. Backcross lines that lack LOX-2 have been generated in which the absence of *lox1 : Ps : 6* seems to be a reliable molecular marker associated with the null phenotype. Since the removal of LOX-2 may lead to improved seed quality through the reduction in volatile aldehydes that cause off-flavours in frozen peas (Casey et al. 1995), such a marker could prove to be of practical utility in pea improvement.

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