Theor Appl Genet (1989) 77:805–808

Inheritance and mapping of seed lipoxygenase polypeptides in *Pisum*

H. North, R. Casey and C. Domoney

John Innes Institute and AFRC Institute of Plant Science Research, Colney Lane, Norwich, NR4 7UH, UK

Received November 21, 1988; Accepted December 22, 1988
Communicated by F. Salamini

Summary. Analysis of crosses of *Pisum* lines showing variation in the apparent molecular weight of seed lipoxygenase polypeptides indicates that the genes encoding the two major pea seed lipoxygenase polypeptides are closely linked. The lipoxygenase locus, designated *Lox,* maps to a position on linkage group 4 between *Np* and *le*.

Key words: Inheritance – Linkage – Lipoxygenase – *Pisum*

Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase, E.C. 1.13.11.12) (LOXs) are a group of monomeric non-haem-iron-containing enzymes which oxidise fatty acids containing cis,cis pentadiene systems to yield cis, trans-conjugated hydroperoxides and a range of secondary products and derivatives, often with desirable or undesirable tastes and odours (Galliard and Chan 1980). LOX activity is particularly high in legume seeds (Pinsky et al. 1971) and can present problems both in seed storage and as a result of the off-flavours and odours that it generates. However, LOX also catalyses a co-oxidation reaction which has been exploited by the breadmaking industry, where improved dough rheology and lower staling rates are obtained when high LOX activity soya-bean flour is added to wheat flour (Frazier 1979). This high LOX activity results from the comparatively large amount of LOX, 1% of the total seed protein (Kitamura 1984), present in mature soya-bean seeds.

Three major isozymes have been characterized in soya-bean seeds (Theorell et al. 1947; Christopher et al. 1970, 1972); LOX-1 has a higher pH optimum than LOX-2 and LOX-3 and has a different substrate preference. Complete or partial sequences for all three isozymes have been reported (Start et al. 1986; Shibata et al. 1987; Yenofsky et al. 1988). In pea seeds there is very little LOX-1; the two major isozymes are analogous to LOX-2 and LOX-3 (Reynolds and Klein 1982). A pea seed LOX sequence predicted from a full-length cDNA, corresponding to the upper of two polypeptides observed on SDS/polyacrylamide gels (SDS/PAGE) (Casey et al. 1985), shows strong homology to soya-bean LOX-3 (Ealing and Casey 1988).

The polypeptides of the three soya-bean isozymes can be separated by SDS/PAGE (Kitamura et al. 1983; Kitamura 1984). Using this method, genetic variants lacking one of LOX-1 (Hildebrand and Hymowitz 1981), LOX-2 (Davies et al. 1983) or LOX-3 (Kitamura et al. 1983) have been identified. Analyses of the inheritance of these null phenotypes revealed tight linkage of LOX-1 and LOX-2 genes but independent segregation of LOX-1 and LOX-3 genes; the absence of an isozyme was attributed to a single recessive allele lx_1, lx_2 or lx_3 (Hildebrand and Hymowitz 1982; Davies et al. 1983; Kitamura et al. 1983; Davies and Nielsen 1986).

The two major pea seed LOX polypeptides are more easily separated by SDS/PAGE than are the soya-bean polypeptides (Casey et al. 1985). We have identified apparent molecular weight variants of both pea polypeptides and used these for an analysis of their pattern of inheritance and for locating the genes encoding them on the genetic map.

Materials and methods

Chemicals

Chemicals were of AnalaR grade or of highest available purity from Sigma or BDH. Nitrocellulose filters were from Schleicher

and Schüll. Antiserum raised against soya-bean LOX-1 was a gift from Dr. N. C. Nielsen, Purdue University, U.S.A.; this antiserum also cross-reacts with LOX-2 and LOX-3.

Plant material

Two hundred and six different *Pisum* lines from the John Innes Germplasm Collection and F_1, F_2 and F_3 seed from selected crosses were analysed for apparent molecular weight variants of LOX (as described below). In the cross JI 407 (*Pisum sativum*) × JI 261 (*Pisum elatius*), F_2 seed/plants were scored for *Le/le, A/a, Kp/kp, Np/np, Gty/gty* and *M/m* phenotypes and also as homozygous or heterozygous for *I, R* and *D* (for details of phenotypes see Blixt 1974); JI 261 was dominant for all characteristics.

F_2 seed/plants from JI 64 (*Pisum elatius*) × JI 92 (*Pisum sativum* accession from Afghanistan) and its reciprocal were scored for *Gty/gty, Pl/pl, Np/np, Td/td,* and *M/m;* JI 64 was dominant for the first three characteristics and JI 92 was dominant for the last two.

Protein gel electrophoresis and 'Western' blotting

Samples were prepared for electrophoresis using 1–2 mg of dry seed meal (1 mg meal/67 µl sample solution) which was removed from the seed non-destructively; seeds were then sown and the plants scored for their physical phenotypes. Proteins were extracted either under partially denaturing conditions using urea/SDS extracts of samples as described by Kitamura et al. (1983) and Kitamura (1984) or under fully denaturing conditions (Laemmli 1970). Both preparations gave identical resolution on gel electrophoresis. SDS/PAGE was carried out using a 10% (w/v) separating gel and a 5% (w/v) stacking gel (Laemmli 1970) at 100 V for 17 h (1 × 150 × 140 mm gels) or 120 mA for 3 h (1 × 105 × 120 mm gels).

Polypeptides were electro-blotted onto nitrocellulose and LOX polypeptides visualized as described by Casey et al. (1985).

Linkage analysis

A χ^2-analysis or Fisher's exact test (Fisher 1954) was used to detect non-randomness in the segregation patterns of F_2 populations. For this purpose it was necessary to regroup F_2 data into a 2 × 2 table (in which the four classes were AXBX, aaBX, AXbb or aabb, where X is unknown), or a 2 × 1 table (by defining phenotypes where one character was recessive as class A, and phenotypes where none or both characters were recessive as class B). When non-randomness was detected it was attributed to linkage, and recombination frequencies were estimated using the maximum likelihood equations of Allard (1956). Recombination frequencies were converted to map units using Haldane's mapping function (Haldane 1919), with confidence limits based on the errors of the recombination frequencies.

In the cross JI 407 × JI 261, F_3 plants were scored, enabling F_2 plants to be classed as homozygous or heterozygous for *Np* and *le*. Fisher's exact test was performed on the nine F_2 classes, which were regrouped into four classes: parental homozygotes, recombinant homozygotes, single heterozygotes and double heterozygotes. Recombination frequencies and map units were calculated from the data as nine classes. All repetitive calculations were carried out using a simple BASIC programme.

Results and discussion

Selected *Pisum* lines (206) were screened for variation in apparent molecular weight of the two major LOX polypeptides observed by SDS/PAGE and 'Western' blotting. Four lines were selected as being appropriate for studying the inheritance of LOX polypeptides: JI 407 and JI 261, where variation in the lower molecular weight polypeptide was observed, and JI 92 and JI 64, where variation in both polypeptides was apparent. Analysis of F_1 seed from the cross JI 92 × JI 64 showed co-dominant inheritance of polypeptide patterns. This indicates that these polypeptide variants are due to variation in the structural genes; variation at a genetic locus responsible for processing or post-translational modification would be expected to yield F_1 phenotypes that resembled the parent with the most extreme activity, giving a dominant characteristic (Thomson and Schroeder 1978). Analysis of F_2 seed from both crosses (Fig. 1) confirmed simple Mendelian co-dominant inheritance for both polypeptides. F_3 seeds from both populations were examined for their LOX polypeptide pattern and verified the F_2 data. We have designated the pea lipoxygenase locus *Lox*. In the JI 407 × JI 261 cross, linkage was detected between *Lox* and both *le* (determining internode length) and *Np* (controlling the presence of neoplastic growths on pods) (Table 1), which are in linkage group 4 (Blixt 1974). Linkage between *le* and *Np* was also apparent.

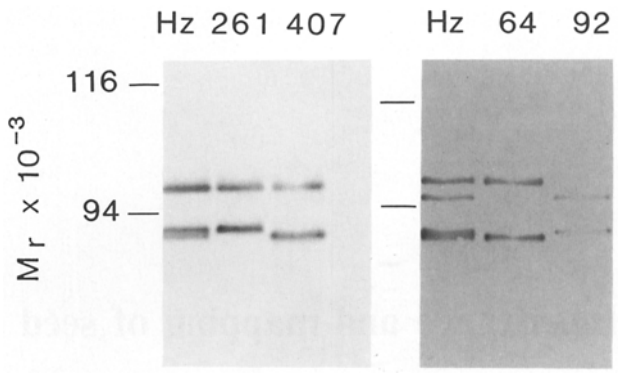

Fig. 1. Partially denaturing electrophoresis/'Western' blot analyses of F_2 seeds from the crosses JI 407 × JI 261 and JI 92 × JI 64 probed with anti-lipoxygenase. Lanes labelled 261, 407, 64, 92 indicate F_2 homozygotes equivalent to the parents JI 261, JI 407, JI 64 and JI 92, respectively. Lanes labelled Hz indicate F_2 heterozygotes. The positions of molecular weight markers are indicated

To determine the relative order of the *Np, le* and *Lox* loci, the F_2 segregants were classified into those homozygous or heterozygous at the *le* and *Np* loci by analysis of F_3 plants. At least ten F_3 plants from an F_2 parent were required to ensure at the 95% confidence level that a given F_2 plant was homozygous dominant at a given locus; as a result, data for only 28 members of the F_2 population were used. When a Fisher's exact test was performed on these data (Table 2), linkage between *Lox* and *le,* and between *Lox* and *Np,* was indicated. Non-randomness was not evident for the *le/Np* data here,

Table 1. Data obtained from the F_2 segregants from the cross JI 407 × JI 261. The classes A, B, C, D, E and F refer to the following: 3 × 2 table: A – AABX; B – AaBX; C – aaBX; D – AAbb; E – Aabb; F – aabb; 2 × 2 table: A – AXBX; B – aaBX; C – AXbb; D – aabb; 2 × 1 table: A – aaBX, AXbb; B – AXBX, aabb where X is an unspecified allele and A and B represent dominant genes. Regrouping to a 2 × 1 table for the *Lox/le* classes was necessary for a valid χ^2-analysis. Values in brackets indicate regrouped data

Character pair	A	B	C	D	E	F	*P*	R.f.%	m.u.
Lox/le	14	30	4	0	8	12	–	19.0± 7.8	23.9 (>12.7, <38.4)
	(12	56	–	–	–	–	0.0003)	–	–
Lox/Np	14	30	2	0	11	14	0.001	19.0± 7.8	23.9 (>12.7, <38.4)
le/Np	36	7	12	13	–	–	0.01	28.5±13.7	42.2 (>17.5, <92.9)

P Probability by χ^2-analysis
R.f. recombination frequencies (Allard 1956)
m.u. equivalent map units (centimorgans). Confidence limits of map units were calculated on the basis of recombination frequency errors

Table 2. Data obtained from 28 of the F_2 segregants from the cross JI 407 × JI 261 for which homozygosity/heterozygosity status was determined for *le* and *Np* by analysis of F_3 plants. The classes P, R, S and D refer to parental homozygotes, recombinant homozygotes, single heterozygotes and double heterozygotes, respectively, regrouped from the nine classes scored. Recombination frequencies (Allard 1956) (R.f.) and equivalent map units (m.u.) (centimorgans) were calculated from the data of the nine classes. Confidence limits of map units were calculated on the basis of recombination frequency errors

Character pair	P	R	S	D	*P*	R.f. %	m.u.
Lox/le	7	0	12	9	0.043	24.5±7	33.7 (>21.5, <49.7)
Lox/Np	7	0	8	13	0.005	15.5±5	18.6 (>11,8, <26.4)
le/Np	6	1	12	9	0.157	29.0±8	43.4 (>27.2, <67.4)

P probability (Fisher 1954)

Table 3. Data obtained from the F_2 segregants from the cross of JI 92 × JI 64 and reciprocal. The classes A, B, C, D, E and F refer to the following: 3 × 2 table: A – AABX; B – AaBX; C – aaBX; D – AAbb; E – Aabb; F – aabb; 2 × 2 table: A – AXBX; B – aaBX; C – AXbb; D – aabb where X is an unspecified allele and A and B represent dominant genes. Regrouping to a 2 × 2 table was necessary for a valid χ^2-analysis. Values in brackets indicate regrouped data

Character pair	A	B	C	D	E	F	*P*	R.f.%	m.u.
Lox/Np	6	26	4	0	8	19	–	15.5±7.2	18.6 (>9.1, <30.3)
	(32	4	8	19	–	–	<0.002)	–	–
Lox/Np	15	24	8	0	9	13	–	23.0±7.9	30.8 (>17.9, <48.1)
reciprocal	(39	8	9	13	–	–	<0.0001)	–	–

P Probability by χ^2-analysis
R.f. recombination frequencies (Allard 1956)
m.u. equivalent map units (centimorgans). Confidence limits of map units were calculated on the basis of recombination frequency errors

presumably because of the smaller number of plants analysed. The available F_3 data allowed the assignment of all nine possible genotypic classes, providing the maximum amount of information from the segregation. These data (Table 2) were used for calculation of recombination frequencies and map units, according to Allard (1956). The order of the loci, determined from the data in Table 2, is *Np – Lox – le*. The map distance for *le* to *Np* of 42–43 centimorgans (Tables 1 and 2) compares reasonably well with the 28 centimorgan value reported by Blixt (1974). However, the values have high errors due to the relatively small population size.

In the crosses JI 92 × JI 64 and its reciprocal, linkage of *Lox* to *Np* was also detected (Table 3). (There was no variation at the *le* locus in this cross). There was reasonable agreement of map distances obtained in all the crosses.

In the crosses using JI 92 and JI 64, no recombinants were observed between the two banding patterns among the 200 F_2 seeds tested, indicating close linkage of the genes encoding the two polypeptide classes. Alternatively, the polypeptides could be the products of the same locus or gene; one might, for instance, be a post-translational modification of the other. However, cell-free

translation of mRNA from developing pea seed of the cv 'Birte' produces two lipoxygenase polypeptides of similar size to those seen on 'Western' blots (Casey et al. 1985). cDNA clones corresponding to these cell-free products have different sequences (P. Ealing, personal communication), making it unlikely that the two major polypeptides are products of the same gene.

As LOX-1 appears to be a minor component in pea, the two polypeptides studied here (Fig. 1) are likely to correspond to LOX-2 and LOX-3, and their linkage is in contrast to the situation in soya-bean where tight linkage was detected between LOX-1 and LOX-2, but not between LOX-2 and LOX-3 (Davies and Nielsen 1986).

Acknowledgements. This work was supported by the Agricultural and Food Research Council New Initiatives Scheme via a grant-in-aid to the John Innes Institute. We thank Dr. P. Matthews for providing seed from the germplasm and for help and advice, Dr. N. Ellis for his help with linkage analyses, Prof. D. R. Davies for his comments on the manuscript and T. Dalzell for typing the manuscript.

References

Allard RW (1956) Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia 24: 235–278

Blixt S (1974) The pea. In: King RC (ed) Handbook of genetics, vol. 2. Plenum Press, New York, pp 181–221

Casey R, Domoney C, Nielsen NC (1985) Isolation of a cDNA clone for pea (*Pisum sativum*) seed lipoxygenase. Biochem J 232:79–85

Christopher J, Pistorius E, Axelrod B (1970) Isolation of an isozyme for soybean lipoxygenase. Biochim Biophys Acta 198: 12–19

Christopher J, Pistorius EK, Axelrod B (1972) Isolation of a third isozyme of soybean lipoxygenase. Biochim Biophys Acta 284:54–62

Davies CS, Nielsen NC (1986) Genetic analysis of a null-allele for lipoxygenase-2 in soybean. Crop Sci 26:460–463

Davies CS, Kitamura K, Nielsen NC (1983) Identification and characterization of soybean lipoxygenase isozyme genes. Agron Abstr American Society of Agronomy, Madison, pp 61

Ealing PM, Casey R (1988) The complete amino acid sequence of a pea (*Pisum sativum*) seed lipoxygenase predicted from a near full length cDNA. Biochem J 253:915–918

Fisher RA (1954) Statistical methods for research workers, 12th ed. Oliver and Boyd, Edinburgh, pp 96–97

Frazier PJ (1979) Lipoxygenase action and lipid binding in breadmaking. Bakers Dig 53:8–29

Galliard T, Chan HWS (1980) Lipoxygenases. In: Conn EE, Stumpf PK (eds) The biochemistry of plants, vol. 4. Academic Press, London New York, pp 131–161

Haldane JBS (1919) The combination of linkage values and the calculation of distances between the loci of linked factors. J Genet 8:299–309

Hildebrand DF, Hymowitz T (1981) Two soybean genotypes lacking lipoxygenase-1. J Amer Oil Chem Soc 58:583–586

Hildebrand DF, Hymowitz T (1982) Inheritance of lipoxygenase-1 activity in soybean seeds. Crop Sci 22:851–853

Kitamura K (1984) Biochemical characterization of lipoxygenase lacking mutants, L-1-less, L-2-less and L-3-less soybeans. Agric Biol Chem 48:2339–2346

Kitamura K, Davies CS, Kaizuma N, Nielsen NC (1983) Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds. Crop Sci 23:924–927

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Pinsky A, Grossman S, Trop M (1971) Lipoxygenase content and antioxidant activity of some fruit and vegetables. J Food Sci 36:571–572

Reynolds PA, Klein BP (1982) Calcium interaction with substrates of type-2 pea lipoxygenases. J Food Sci 47: 1999–2003

Shibata D, Steczko J, Dixon JE, Hermodson N, Yazdanparast R, Axelrod B (1987) Primary structure of soybean lipoxygenase 1. J Biol Chem 262:10080–10085

Start WG, Ma Y, Polacco JC, Hildebrand DF, Freyer GA, Altshuler M (1986) Two soybean seed lipoxygenase nulls accumulate reduced levels of lipoxygenase transcripts. Plant Mol Biol 7:11–23

Theorell H, Holman RT, Akeson A (1947) Crystalline lipoxidase. Acta Chem. Scand 1:571–576

Thomson JA, Schroeder HE (1978) Cotyledonary storage proteins in *Pisum sativum* II. Hereditary variation in components of the legumin and vicilin fractions. Aust J Plant Physiol 5:281–294

Yenofsky RL, Fine M, Liu C (1988) Isolation and characterisation of a soybean (*Glycine max*) lipoxygenase-3 gene. Mol Gen Genet 211:215–222