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Further genetic analysis and linkage relationships of isozyme loci in the pea

Confirmation of the diploid nature of the genome

ABSTRACT: Allozyme polymorphism is described and the mode of inheritance determined for 15 loci in *Pisum sativum*. The approximate position on the pea linkage map is described for 12 of these loci. The remaining three loci formed a single group that assorted independently of morphological markers on each of the seven currently recognized linkage groups. Possible cases of gene duplication are identified in the alcohol dehydrogenase and esterase enzyme systems, but in neither case was polyploidy a likely explanation for the paired loci. Nor did the arrangement of the isozyme loci on the linkage map give any indication that the pea genome is of polyploid derivation. We conclude that the "polymeric" genes that have been described in the pea are probably not homologous pairs and do not reflect a polyploid ancestry.

Norman F. Weeden

Gerald A. Marx

A LINKAGE MAP provides a summary of the arrangement of genes on chromosomes and constitutes a valuable genetic tool for breeders, population biologists, and cytogeneticists. The analysis of loci coding biochemically identifiable components such as proteins or RNAs further augments the information value of linkage maps because homologous loci can often be recognized in other species. In plants, interspecific comparisons of the number of isozymes present and their intracellular compartmentation have revealed consistent patterns of expression in many plant species[11]. Deviations from the expected pattern of isozyme expression have helped to identify cases of gene duplication[9,28] or to confirm[12], or reject[10] the polyploid nature of a genome.

The garden pea is generally treated as a diploid, for it possesses a relatively low chromosome number ($n = 7$), and the chromosomes exhibit normal pairing at meiosis. However, systematic considerations have led Goldblatt[7] to suggest that the subfamily Papilionoideae, which includes the genus *Pisum*, is of polyploid derivation. Furthermore, at least five cases of "polymeric" genes, pairs of genes that appear to produce the same phenotypic effects, have been identified in the pea. Lamprecht[15,16] suggested that such a large number of duplicate genes demonstrated the polyploid nature of the pea genome.

Despite the availability of an extensive linkage map for the pea[2], only 20 genes or multigene clusters, specifying identifiable proteins, have been located on this map[1,5,13,17,18,21,24,38] precluding evaluation of the ploidy level of the pea genome through the use of such biochemical markers. The purpose of the study reported here was to increase the number of isozyme loci available on the pea linkage map. The data were used to examine the question of polyploid derivation.

Materials and Methods

Seed were obtained from the collection of genetic variants at the New York State Agricultural Experiment Station, Geneva, NY; from PI accessions maintained at the Northeast Regional Plant Introduction Station, Geneva, NY; and from the collection at the John Innes Institution, Norwich, England. The parental lines used for the described

The authors are affiliated with the Department of Horticultural Sciences, NYS Agricultural Experiment Station, Cornell University, Geneva, NY 14456. Special thanks are due to A. C. Emmo for valuable technical assistance and to K. A. Suiter for the use of an improved version of his LINKAGE-1 program. This research was supported in part by an appropriation of funds under the Hatch Act (project no. 33491).
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crosses and their phenotypes for marker genes are presented in Table I. Plants were grown and crosses made in the greenhouse with supplemental lighting used when needed to obtain a 16-hour day.

Linkage studies were performed on F_2 populations growing in the greenhouse. Morphological characters were scored at various stages during the development of the plant. Segregation and linkage analysis was performed using the computer program LINKAGE-1[31].

Most isozyme systems were assayed on extracts from young leaves, obtained as described previously[35]. For several isozymes, however, minor modifications of the procedure were necessary for optimal results. For peroxidase isozymes, young healthy root tissue was extracted in buffer that did not contain 2-mercaptoethanol. In order to induce alcohol dehydrogenase isozymes, roots were submerged in water for 15 hours before sampling. This anaerobic treatment did not significantly affect the expression of the other enzymes we assayed. Peptidase phenotypes were determined using extracts from seed allowed to imbibe overnight.

Horizontal starch gel electrophoresis on gels of 10 percent starch was performed as described by Gottlieb[8]. Samples extracted in the tris-HCl buffer were placed on a p*H* 8.1 tris-citrate/lithium borate system[29]. The phosphate extraction buffer was used for samples subjected to electrophoresis on either a p*H* 6.5 histidine[3] or a p*H* 6.1 citrate/N-(3-aminopropyl)-morpholine[4] gel system.

The assays for aspartate aminotransferase (AAT; EC 2.6.1.1), methylumbelliferyl esterase (EST; EC 3.1.1.-), 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) and phosphoglucomutase (PGM; EC 2.7.5.1) have been described previously[38]. The peroxidase (PRX; EC 1.11.1.7) assay also was based on a standard recipe: 25 ml of 0.1 M acetate p*H* 5.0 buffer containing 1 drop 30 percent hydrogen peroxide and 25 mg 3-amino-9-ethylcarbazole (dissolved in 2 ml N,N-dimethylformamide). Alcohol dehydrogenase (ADH; EC 1.1.1.1) was assayed according to Shaw and Prasad[30]. The beta-galactosidase (GAL; EC 3.2.1.23) assay consisted of 0.5 mM methylumbellyferyl-beta-D-galactoside in 0.1 M sodium citrate, p*H* 4.5. The beta N-acetyl-glucosaminidase (NAG; EC 3.2.1.30) assay also involved the methylumbellyferyl substrate, consisting of 0.1 M sodium citrate p*H* 4.5, containing 5 mg 4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide. Mannose 6-phosphate isomerase (MPI; EC 5.3.8.1) was assayed by the double-coupled technique described in Nichols and Ruddle[23]. Peptidase (PEP; EC 3.4.15.-) was assayed using an agarose overlay in which 5 ml of a 1.4 percent SeaPlaque (FMC Corporation, Rockland, Maine) solution at 37°C was combined with 5 ml of assay mix and the resulting solution poured onto the cut surface of the gel. The assay mix was modified from Nichols and Ruddle[23] and included 4 ml 0.1 M tris-HCl p*H* 8.0, 1.0 ml 0.1 M $MgCl_2$, 6 mg L-glycyl-L-leucine, 10 mg L-phenylalanyl-L-proline, 5 mg dianisidine, 2 units peroxidase, and 0.5 mg snake venom.

Except where noted, reagents were obtained from Sigma Chemical Company, St. Louis, Missouri. The N-(3-aminopropyl) morpholine used in the p*H* 6.1 gel buffer was

Table I. Relevant morphological marker genes in parental lines

Line	Abbreviation used in present paper	Marker traits*
83-11	*a*	—
83-12	*b*	*a, i, le*
Wellensiek's tester	*c*	*d, i, f, st, b, fa, gp, cr, fs, fl, wsp, r, tl*
PI 179449	*d*	—
B78-288	*e*	*d*
A1078-236	*f*	*i, k, M, st, f, b, le, fs, wlo*
A1078-238	*g*	*i, wb, M, f, le, gp, pl, r*
PI 358612	*h*	—
B77-257	*i*	*f, fs, pl*
A1078-235	*j*	*f, le, ce, fs*
C879-344	*k*	*i, Pu, Pur, st, b*
A683-168	*l*	*f, le, ce, fs, pl*
22-11	*m*	*i, s, wb, st, f, b, le, pl, tl, r*
J136	*n*	*a, wa*
A1078-239	*o*	*i, st, f, le, gp, cr, wlo, pl, r, bt*
PI 343997	*p*	*M*
J121	*q*	*lf, M, f, ce, cr, fs*
A683-222	*r*	*wb, s, cp*

* For description of characters see Blixt[2]

Table II. Segregation of isozyme phenotypes in *Pisum sativum*

Isozyme	Cross	Isozyme phenotype† F(+)	H	S(−)	χ^2 (1:2:1)‡	Isozyme	Cross	Isozyme phenotype† F(+)	H	S(−)	χ^2 (1:2:1)‡
ADH-1	l × k	11	26	14	0.40	GAL-2	a × b	38	63	26	2.3
	m × n	20	24	16	2.9		c × d	40		21	2.9
AMY-1	a × b	28	67	35	0.88		k × c	28		12	0.53
	c × d	19	26	19	2.3	GAL-3	a × b	35	68	24	2.54
	e × f	7	28	5	6.66*		l × k	12	21	11	0.10
AAT-2	a × b	32	69	29	0.63	MPI	c × d	17	28	17	0.58
	c × d	13	26	26	7.8 *		k × c	7	21	5	2.7
	e × f	12	20	9	0.46	NAG-1	a × b	7	28	11	2.9
	g × h	6	10	4	0.40		k × c	14	23	6	3.18
AAT-4	c × d	13	32	20	1.5		l × d	3	18	5	4.15
	l × d	14	16	15	4.8	PEP-3	e × f	9	15	9	0.27
EST-1	e × f	13	19	8	1.4		k × r	8	17	9	0.06
	i × j	12	17	15	2.7	PRX-1	m × q	14	26	14	0.07
EST-2	a × b	27	61	22	1.8		o × p	4	13	4	1.2
	c × d	5	23	7	3.7	PGM-1	e × f	11	22	8	0.76
	i × j	18	24	12	2.0		k × r	8	12	11	2.16
	m × q	10	17	11	0.47	6PGD-1	e × f	8	19	14	1.98
EST-3	e × f	9	21	10	0.17		k × r	9	15	10	0.61
	g × h	6	8	6	0.80						

† Phenotype designations: F(+) = fast variant (or, for GAL-2 in crosses c × d and k × c, activity present); H = heterozygous; S(−) = slow variant (or, for GAL-2 in crosses c × d, and k × c, activity absent)
‡ Chi square for GAL-2 in crosses c × d and k × c was calculated on the basis of an expected 3:1 ratio
* Significant at $P = 0.05$

purchased from Aldrich Chemical Company, Milwaukee, Wisconsin.

Results

Alcohol dehydrogenase

A single ADH band was observed in dry and imbibed seed extracts. Root and leaf material lacked significant ADH activity unless the tissue was placed under anaerobic conditions for approximately 15 hours, whereupon three activity bands could be seen after electrophoresis. The most anodal of these bands, ADH-1, corresponded to the form found in seed extracts. Polymorphism was observed in ADH-1 but not in the most slowly migrating form, ADH-2 (Figure 1*A*). Segregation analysis demonstrated that the polymorphism in ADH-1 behaved as a monogenic trait in segregating populations (Table II). The mobility of the band intermediate between ADH-1 and ADH-2 shadowed that of ADH-1 (Figure 1*A*), suggesting that it was the hybrid dimer between ADH-1 and ADH-2 subunits. The ADH isozymes in pea thus appear to be specified by two loci, *Adh-1* and *Adh-2*.

Amylase

A single amylase band was observed after electrophoresis of leaf extracts from inbred lines. This amylase was not activated by $CaCl_2$, was inhibited by 1×10^{-5} M $CuCl_2$ and was active at low p*H*. It therefore appears to be a beta-amylase. Two variants were observed, and crosses between plants which possessed the alternate phenotypes gave hybrids showing both forms (Figure 1*B*). Self-pollination of these hybrids produced an F_2 population with the 1:2:1 ratio for amylase phenotypes expected for diallelic inheritance (Table II).

Aspartate aminotransferase

Four zones of AAT activity could be seen on gels[38]. Cell fractionation studies demonstrated that each zone represented the AAT from a separate subcellular compartment, and the genetic basis of the plastid isozyme (AAT-2) and the mitochondrial isozyme (AAT-3) was discussed previously[37,38]. Here we report the identification of a rare variant in AAT-4, the cytosolic form (Figure 1*C*). Segregation analysis of the polymorphism in AAT-4 (Table II) indicated monogenic control. This locus, *Aat-c*, assorted independently of *Aat-m* and *Aat-p*.

Esterase

Four leaf esterases can be observed on the p*H* 6.1 gel (Figure 2*A*). Electrophoresis on a histidine, p*H* 6.5 gel gave better resolution of the EST-2 bands as well as adequate resolution of the EST-3 allozymes (Figure 2*B*), but EST-1 variation was difficult to interpret and the EST-4 isozymes could not be observed on this gel. EST-2 behaved as a monomeric enzyme, for the hybrid phenotype exhibited only the two parental bands. In contrast, EST-3 appeared to be a dimeric enzyme because the hybrid phenotype was 3-banded (Figure 2*A*). The variants of EST-3 showed simple segregation ratios (Table II), indicating control by a single locus, *Est-3*, which assorted independently of *Est-2* and *Est-4*.

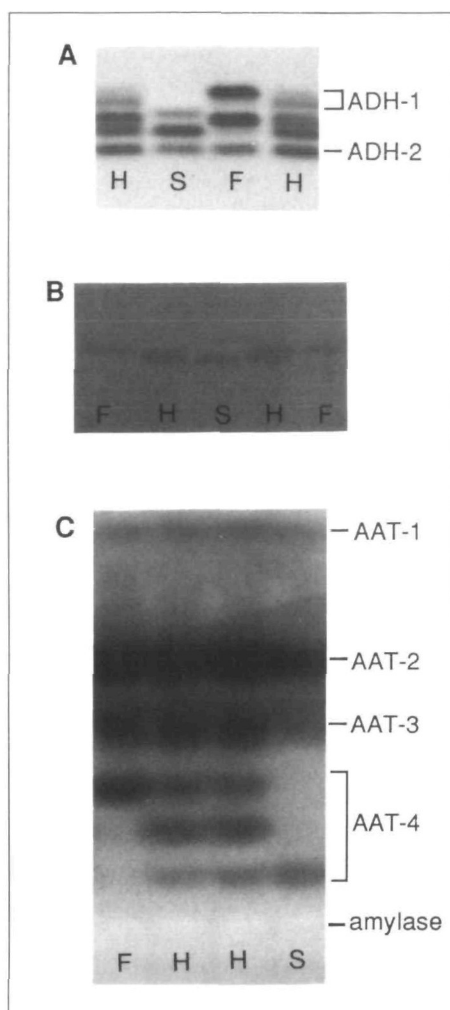

FIGURE 1 *A* shows alcohol dehydrogenase phenotypes obtained from extracts of pea roots submerged in water for 15 hours previous to extraction. Phenotype designations are the same as given in Table II with H = heterozygous, S = slow, and F = fast. *B*—amylase phenotypes obtained from an F_2 population. Parental phenotypes are single-banded. Both bands are expressed in plants heterozygous at *Amy-1*. *C*—pea aspartate aminotransferase patterns, demonstrating variation in the cytosolic isozyme, AAT-4. The 3-banded AAT-4 phenotype observed in heterozygous plants indicates that this isozyme possesses a dimeric structure. Anode is at top of figure.

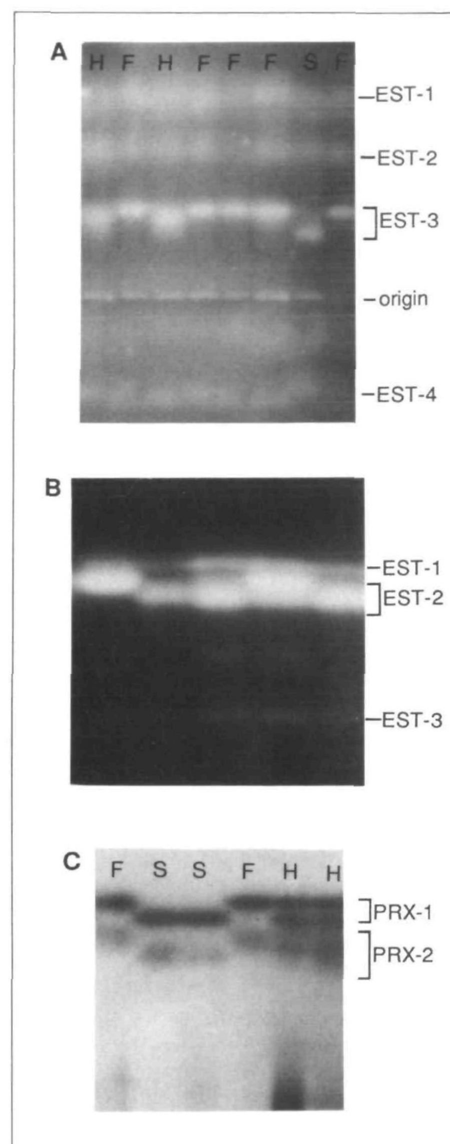

FIGURE 2 *A* shows esterase patterns on a p*H* 6.1 gel system. Segregation for EST-3 is apparent with H = heterozygous, S = slow, and F = fast phenotypes. *B*—esterase phenotypes on a histidine, p*H* 6.5 gel. Segregation can be seen for EST-2. The esterase activity between EST-2 and EST-3 is produced by an unnamed esterase that is not always observable in leaf extracts. *C*—phenotypes of the anodal peroxidases in root extracts from an F_2 population segregating at *Px-1*. The fainter, more diffuse PRX-2 bands exhibit the same segregation pattern as the PRX-1 bands and may be a product of the same gene. Anode is at top of figure.

Galactosidase

There are three galactosidase isozymes observable in most pea leaf extracts[36]. Only two, which migrate cathodally on the p*H* 6.1

gel, were investigated in the studies reported here. Several cathodal variants showed very low activity and were treated as GAL 'minus' mutants. When such variants were crossed with lines exhibiting normal expression of beta galactosidase it was often difficult to distinguish the heterozygous phenotype from the normal in segregating populations. In such cases we only differentiated GAL 'plus' and GAL 'minus' phenotypes. Both cathodal isozymes exhibited simple Mendelian inheritance (Table II). Joint segregation analysis demonstrated that the two isozymes were coded by distinct loci, designated *Gal-2* and *Gal-3*[36].

Mannose phosphate isomerase

One MPI isozyme could be seen near the front ($Rf = 0.85$) on the p*H* 8.1 gel. The two MPI variants observed were too similar in mobility to resolve the individual bands in the heterozygous phenotype; thus we could not establish a quaternary structure for the enzyme. Genetic analysis demonstrated that a single locus, *Mpi*, was responsible for the observed polymorphism (Table II).

Beta-N-acetyl-glucosaminidase

One clearly defined zone of activity, NAG-1, was observed in root or leaf extracts from homozygous lines. Only two forms of NAG-1 were found among the pea lines tested, and genetic studies indicated that these were allozymic variants (Table II). The hybrid phenotype exhibited a 3-banded phenotype, suggesting that the active enzyme is a dimer. A second zone of NAG activity occasionally was seen near the origin; however, we could not clearly resolve variants or consistently obtain activity in this region.

Peptidases

A number of peptidases were observed in seed extracts using the dipeptides phenylala-

Table III. Joint segregation analysis in crosses of *Pisum sativum*

Locus	Marker locus	Original cross	*N*	No. progeny with designated phenotype†									χ^2	Recomb. fraction	SE
				1/1	1/H	1/2	H/1	H/H	H/2	2/1	2/H	2/2			
Chromosome 1															
Aat-p	*A*	a × b	129	15	—	14	20	—	48	3	—	29	13	32	5
	D	c × d	65	7	—	19	10	—	16	5	—	8	0.9	n.s.*	—
	Idh	a × b	128	8	16	4	15	38	15	8	13	11	4.2	n.s.	—
Est-3	*Aat-p*	e × f	40	8	4	0	1	14	4	0	3	6	27	16	5
		g × h	20	3	0	1	2	8	0	1	0	5	20	17	7
Chromosome 2															
Est-1	*Est-2*	i × j	44	13	2	0	2	15	0	0	0	12	69	5	2
	Skdh	e × f	39	6	1	1	2	14	3	0	4	8	27	17	5
	Aat-m	e × f	40	6	7	0	3	14	2	1	1	6	23	21	5
Est-2	*Skdh*	a × b	106	22	5	0	6	47	7	0	7	12	83	13	2
	Aat-m	a × b	104	21	2	2	9	42	9	0	8	11	64	17	3
		c × d	35	6	2	0	1	20	0	0	1	5	47	6	3
	Aldo-p	a × b	86	11	10	1	8	28	10	2	6	10	21	27	4
		i × j	45	5	3	0	1	17	3	1	5	10	29	17	4
	S	m × q	38	5	—	6	2	—	15	0	—	10	8	21	8
Amy-1	*Aat-m*	e × f	36	4	1	0	2	12	9	2	4	1	13	38	8
		a × b	122	15	11	0	10	41	12	0	10	23	59	19	3
		c × d	64	11	8	0	0	20	6	0	6	13	47	17	4
	Aldo-p	a × b	86	16	2	0	5	40	3	0	3	17	102	8	2
	Est-2	a × b	108	8	13	1	11	35	13	2	12	13	16	31	4
		c × d	37	5	4	0	0	13	1	0	5	6	26	16	5
Gal-2	*Aldo-p*	a × b	86	16	7	2	5	35	4	0	2	15	73	14	3
		k × c	39	6	18	4	—	—	—	2	3	6	7	30	8
	Amy-1	a × b	124	21	3	0	4	52	7	1	11	25	121	12	2
		c × d	60	17	17	5	—	—	—	1	6	14	20	20	6
Chromosome 3															
Aat-c	*Lap-2*	l × d	34	8	2	0	1	8	2	0	3	10	31	12	4
		c × d	50	4	4	0	4	15	4	0	5	14	25	18	4
	Acp-3	c × d	39	3	5	1	5	8	5	3	3	6	40	n.s.	—
Gal-3	*Lap-2*	a × b	121	12	9	1	6	46	11	2	12	22	52	21	3
	Acp-3	a × b	98	19	9	0	10	31	7	1	3	18	66	17	3
		l × k	37	7	17	5	—	—	—	0	2	6	10	20	7
	St	l × k	41	26	—	7	—	—	—	2	—	6	8.6	28	13
Adh-1	*St*	l × k	51	13	—	1	20	—	6	3	—	8	14	22	6
		m × n	59	11	4	0	—	—	—	8	20	16	17	21	6
	Acp-3	l × k	46	9	4	0	2	17	3	0	1	10	44	11	3
	Gal-3	l × k	41	9	—	1	20	—	1	4	—	6	14	19	7

† 1 = phenotype of female parent, 2 = phenotype of male parent, H = phenotype of F_1 plants if different than parents

* n.s. = not significant at $P = 0.05$

→

nine-proline and glycyl-leucine. The most anodal, weakly staining isozyme, PEP-1, possessed the same mobility as the previously defined[27,38] LAP-1 isozyme and appeared to be the same protein. We did not observe a peptidase corresponding to LAP-2; however, PEP-3 also possessed weak LAP activity and would occasionally appear on gels assayed for LAP. This isozyme exhibited significantly greater activity when the substrate alanyl beta-naphthylamide was substituted for the leucyl derivative. Variants of PEP-3 segregated as allelic forms (Table II). This segregation was independent of segregation at either *Lap-1* or *Lap-2* and was ascribed to a third locus, *Pep-3*.

Peroxidase

Several zones of PRX activity could be seen when root extracts were subjected to electrophoresis (Figure 2*C*). Genetic analysis was performed on only the two most anodal peroxidases, PRX-1 and PRX-2. PRX-1 produced an intense, sharp band near the front ($Rf = 0.7$–0.8) whereas PRX-2 formed a more lightly staining broader zone with a mobility of approximately 0.6. Both zones gave identical segregation patterns in F_2 populations; thus we could not determine if the two zones were products of distinct but tightly linked loci or if one was a post-translation-

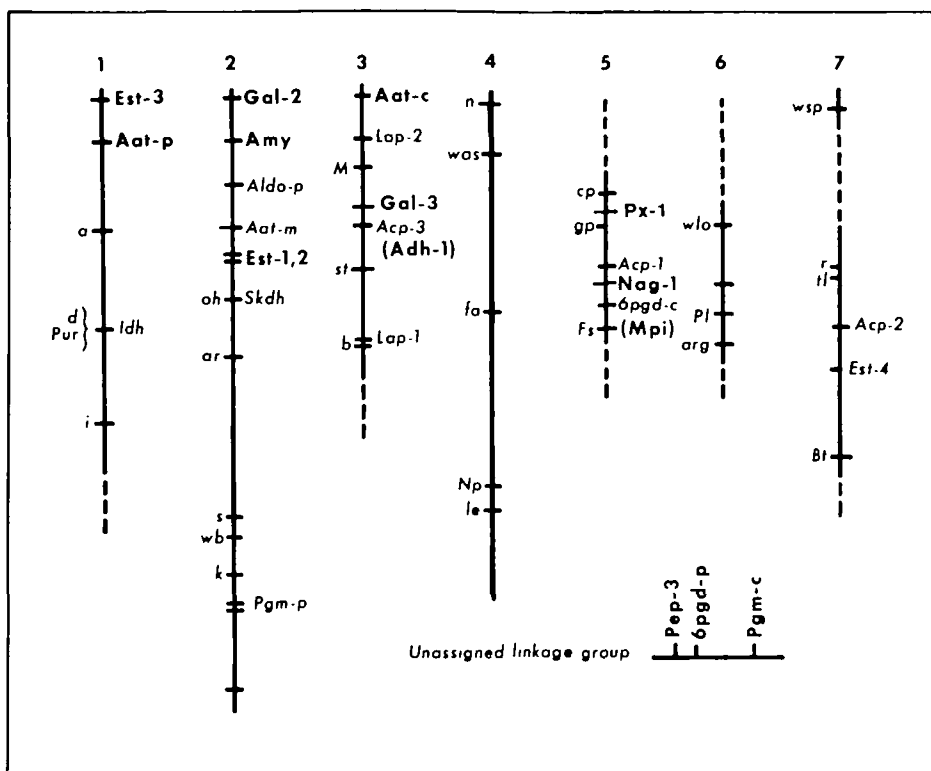

FIGURE 3 Linkage relationships of 15 isozyme loci (shown in bold) with previously mapped morphological and isozyme markers. The seven vertical bars represent the seven chromosomal linkage groups as presented in Blixt[2]. The horizontal bar represents an eighth linkage group that assorts independently of all other markers shown. This eighth linkage group may be associated with chromosome 7 on the basis of its linkage to a ribosomal DNA cluster (see discussion in text). Dashed lines at ends of certain linkage groups indicate the positions of other markers not used in our studies.

Table III. Continued

Locus	Marker locus	Original cross	*N*	No. progeny with designated phenotype†									χ^2	Recomb. fraction	SE
				1/1	1/H	1/2	H/1	H/H	H/2	2/1	2/H	2/2			
Chromosome 5															
Px-1	*Gp*	o × p	16	4	—	0	0	—	10	0	—	2	16	0.0	—
	Acp-1	o × p	19	1	3	0	2	7	2	0	0	4	11	21	7
		m × q	53	8	5	1	5	14	6	2	4	8	14	28	5
	Cr	m × q	49	13	—	1	16	—	6	1	—	12	23	17	6
	Ce	m × q	50	12	—	2	15	—	9	5	—	7	5.5	34	8
	6pgd-c	m × q	53	7	6	11	7	13	5	33	3	8	11	32	6
	Fs	m × q	51	12	—	2	15	—	9	7	—	6	3.4	n.s.	—
Nag-1	*Acp-1*	a × b	46	9	2	0	1	27	0	0	4	3	48	8	3
		k × c	37	3	3	0	1	17	0	2	2	8	25	17	5
	6pgd-c	k × c	43	5	0	1	0	21	2	0	1	13	64	6	3
		a × b	46	11	0	0	0	25	3	0	3	4	56	7	3
		l × d	26	4	2	0	1	15	1	0	1	2	21	10	4
	Ce	l × d	26	3	—	2	2	—	16	0	—	3	6.8	17	8
Mpi	*Gp*	c × d	59	6	—	10	7	—	19	3	—	15	2.9	n.s.	—
	Cr	c × d	61	6	—	11	5	—	22	1	—	16	4.7	34 (n.s.)	—
	Fs	c × d	59	16	—	0	24	—	3	1	—	15	42	7	3
	6pgd-c	k × c	31	2	2	0	3	9	2	0	8	5	8.0	28	7
Unmapped linkage group															
Pgm-c	*6pgd-c*	e × f	41	10	1	0	4	16	2	0	2	6	38	12	4
		l × r	27	6	3	0	2	9	1	1	1	4	18	18	6
	Pep-3	e × f	33	2	3	0	6	10	3	1	2	6	10	28	7
		l × r	27	5	4	0	2	9	1	1	1	4	16	21	6
Pep-3	*6pgd-c*	e × f	33	2	5	2	1	12	2	1	0	8	18	25	6
		l × r	34	9	0	0	1	15	1	0	0	8	57	3	2

al modification of the other. The intensely staining peroxidase appears to be the one previously investigated by Matthews and Williams[22]. We have therefore used their locus designation, *Px-1*.

Phosphoglucomutase and 6-phosphogluconate dehydrogenase

The phenotypes and segregation data for the PGM and 6PGD isozymes in the pea have been described previously[34,37,38]. The results presented in Table II confirm that both the cytosolic isozyme of PGM and the plastid isozyme of 6PGD show simple segregation ratios.

Linkages observed

Analysis of joint segregation ratios for deviations from random assortment revealed six linkage groups, five of which could be associated with chromosomal markers.

Chromosome 1: Two isozyme loci, *Aat-p* and *Est-3*, were added to the linkage map for chromosome 1; *Aat-p* appeared to be about 30 map units from *A*. *Est-3* was linked to *Aat-p* but assorted independently of *A* (Table III). Two other loci, *Idh* and *D*, located about 35 map units towards the centromere from *A*, also segregated in at least some of the crosses used to map *Aat-p* and *Est-3*. However, linkage was not observed between the two pairs of loci (Table III). Therefore *Aat-p* and *Est-3* were placed on the opposite side of *A* from *Idh* and *D* (Figure 3).

Chromosome 2: Four isozyme markers, *Est-1*, *Est-2*, *Amy-1*, and *Gal-2*, were added to the region of chromosome 2 distal to *Oh* (Figure 3). The two esterase loci were tightly linked and appeared to be located between two previously mapped isozyme loci, *Skdh* and *Aat-m* (Table III). *Amy-1* and *Gal-2* were placed distal to *Aldo-p* so that the entire sequence of loci was: (*Skdh*, *Oh*)—(*Est-1*, *Est-2*)—*Aat-m*—*Aldo-p*—*Amy-1*—*Gal-2*.

Chromosome 3: The loci *Aat-c*, *Adh-1*, and *Gal-3* displayed linkage with markers on chromosome 3. *Aat-c* was placed distal to *M* and *Lap-2* on the basis of relative linkage distances (Table III). Both *Adh-1* and *Gal-3* mapped between *M* and *St*, *Gal-3* being closer to *M*. *Adh-1* was tightly linked with *Acp-3* (Table III), but we could not determine whether the former locus was on the *M* or the *St* side of *Acp-3*. Thus, we have tentatively placed the two loci together on the linkage map (Figure 3) in spite of the significant recombination frequency observed between them.

Chromosome 5: Three isozyme loci were added to the linkage map of this chromosome (Figure 3). In a 6-point cross, *Px-1* showed closest linkage to *Acp-1* and no linkage to *Fs* (Table III). These results place *Px-1* on the *Gp* side of *Acp-1*. The second cross (o × p) confirmed that *Px-1* was very near to *Gp*, for no recombinants were observed among the 16 plants analyzed.

Nag-1 mapped close to *6pgd-c*, showing only 6–10 percent recombination with this locus. Three-point crosses involving either *Acp-1* or *Ce* and the two tightly linked loci indicated that *Nag-1* was between *Acp-1* and *6pgd-c*. *Mpi* also exhibited linkage with *6pgd-c;* however, *Mpi* appeared to be closer to *Fs* than *6pgd-c*, possibly on the far side of *Fs* from *6pgd-c*. The 4-point cross involving the morphological markers *Gp*, *Cr*, and *Fs* placed *Mpi* only seven recombinant units from *Fs* (Table III).

Unmapped linkage group: A set of three isozyme loci showed linkage among themselves but not with any of the markers shown in Figure 3. This group included *6pgd-p*, *Pgm-c*, and *Pep-3*. The group spans some 20–25 recombinant units, the order of the loci being: *Pep-3*—*6pgd-p*—*Pgm-c*.

Discussion

The linkage data we generated during our mapping of isozyme loci provided a rigorous test of the linkage map published by Blixt[2] and confirmed its overall accuracy. The map for chromosome 1 required extension to include *Aat-p* and *Est-3* but otherwise was not modified. However, conflicting data now exist in the literature regarding the proper position of *Aat-p* relative to *A*. Mahmoud et al.[18] reported linkage between *A* and an aspartate aminotransferase locus (*Got*). Although the investigators did not determine the intracellular compartmentation of the AAT isozyme, their report of linkage with *A* indicates that the isozyme was the plastid-specific form encoded by *Aat-p*. Mahmoud et al.[18] placed the locus between *A* and *D* on the basis of a statistically nonsignificant deviation from random assortment involving *Aat-p* and *D*. *Aat-p* lies a significant distance from *A*, and we would have expected to see linkage between it and both *D* and *Idh* if it were located between *A* and *D*. As no such linkage was observed (Table III), we have placed *Aat-p* and *Est-3* distal to *A*; however, further studies will be necessary to confirm this location.

The majority of the chromosome 2 linkage group was corroborated by the results of the isozyme mapping. The *Ar*—*Oh*—*Aldo-p* linkage has been extended to permit space for *Amy-1* and *Gal-2*. Relative map distances indicate that *Gal-2* is farther from *Oh* than is *Chi-5*, the most distal locus on Blixt's map. *Chi-5* was not included among the markers we used, so that we were unable to directly test this possibility. Linkage between isozyme loci in the *Oh* region and markers toward the opposite end of the chromosome was observed only in cross m × q (Table III) in which *Est-2* displayed linkage with *S*.

Nearly the entire sequence of morphological markers on chromosome 3 was confirmed by isozyme studies. We have previously reported that *Aat-c* is on the opposite side of *Tac* relative to *St*[20]. New evidence indicates that *uni*, the most distal marker on the standard linkage map for one arm of chromosome 3, is allelic to *tac* (Marx, unpub. data). *Aat-c* thus becomes the most distal marker on this arm of chromosome 3.

The linkage map for chromosome 5, at least between *Cp* and *Fs*, also was supported by isozyme results. Unfortunately, isozyme loci were not found beyond *Cp*, which precluded the use of isozyme loci to test the hypothesis of Lamm[14] that the chromosome 7 linkage group actually belongs with chromosome 5. Evidence suggesting that the *Pep-3*—*6pgd-p*—*Pgm-c* linkage group is on chromosome 7 has recently been reported[25]. The group was found to be linked with *Rrn-2*, one of the two clusters of rRNA genes in the pea genome. The RNA gene clusters probably correspond to the two nucleolar organizer regions on chromosomes 4 and 7, with *Rrn-2* being the array on chromosome 7. The absence of linkage between this group and the classical chromosome 7 markers is compatible with Lamm's hypothesis, but also can be explained by assuming that the isozyme loci are on the satellite portion of the long arm of the chromosome and thus are too far from the other markers to show linkage.

Other investigators have presented evidence for additional isozymes in the systems we have examined. Przybylska et al.[26] reported two amylase loci, *Amy-1* and *Amy-2*, were expressed in seed extracts. The single amylase we observed in leaf extracts apparently is identical to the more anodal of the two seed amylases (Przybylska, pers. commun.), and we have used her nomenclature (*Amy-1*) for this locus. The chromosomal location of a locus coding a seed amylase recently has been reported by Mahmoud et al.[18]. This locus was designated *Amy* and was found to be linked with *K* on the opposite end of chromosome 2 from *Amy-1*. At present we do not know if *Amy* is the same as *Amy-2* of Przybylska or if it represents a third isozyme in seed.

Hunt and Barnes[13] demonstrated that a seed esterase was linked to *Fw*, a gene for *Fusarium* wilt resistance located on chromosome 4. Mahmoud et al.[18] using the chromo-

some 4 marker, *Br*, confirmed this location of the esterase locus. This esterase does not correspond to any of the four major esterases we have observed in leaf extracts, although we have verified its presence in seed using polyacrylamide gel electrophoresis.

Most of the enzyme systems examined contained more than one isozyme; however, these multiple loci do not necessarily represent duplication of genetic material as a result of polyploidy. In the AAT, ALDO, PGM, and 6PGD enzyme systems each isozyme is located in a different subcellular compartment. The origin of such forms may predate the existence of the typical plant cell[33] and therefore cannot be attributed to polyploidy within the Leguminosae. The remaining polygenic systems (ACP, ADH, EST, GAL, LAP, NAG, and PRX) are more difficult to evaluate for duplicate genes. One characteristic of a duplicate gene system specifying multimeric enzymes is the intergenic combination of subunits to produce a fixed heterozygous pattern. Of the multimeric enzymes examined (IDH, AAT, ALDO, 6PGD, EST-3, NAG-1, and ADH) only ADH displayed an intergenic hybrid band (Figure 1*A*). Thus, ADH-1 and ADH-2 could be isozymes coded by duplicate loci. Yet similar ADH phenotypes have been described in many diploid species[6,19,32], and it is doubtful that the two loci in pea were formed as a consequence of polyploidy.

The remaining enzyme systems (ACP, EST, GAL, LAP, and PRX) are not particularly useful for investigating the ploidy level of a species because diploid plants vary in the number of isozymes present per cell. Nor does a consensus exist regarding the number of isozymes expected in each system. The *Est-1*—*Est-2* cluster could represent a duplication resulting from unequal crossing over, but not from polyploidy.

Examples of duplicated linkage patterns, which would be expected even in allopolyploids[12], were not observed in the pea. One pair of polymeric genes identified by Lamprecht consists of *f* (on chromosome 3) and *fs* (on chromosome 5). *F* is linked to *Acp-3* and *Gal-3*, whereas *Fs* is not closely linked to any of the remaining *Acp* or *Gal* loci. *Acp-1* is over 20 recombinant units from *Fs*[38]. *Acp-2* is on chromosome 7, and *Gal-2* is on chromosome 2 (Figure 3). Similar analysis demonstrates that linkage relationships with other polymeric loci (e.g., *Pu* and *Pur*, *P* and *V*) give no indication of duplicated linkage groups. We conclude that these polymeric genes are not vestiges of a previous polyploidy event. If the Papilionoideae are derived from a polyploid ancestor as suggested by Goldblatt[7], at least *Pisum* has undergone diploidization to such an extent that, with regard to expressed isozyme loci, its genome no longer retains any evidence of a polyploid heritage.

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