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Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*

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Summary. Segregating allozyme and DNA polymorphisms were used to construct a preliminary linkage map for faba bean. Two F_2 populations were analyzed, the most informative of which was segregating for 66 markers. Eleven independently assorting linkage groups were identified in this population. One of the groups contained the 45*s* ribosomal array and could be assigned to the large metacentric chromosome I on which the nucleolar organizer region is located. This linkage group also contained two isozyme loci, *Est* and *Tpi-p*, suggesting that it may share some homology with chromosome 4 of garden pea on which three similar markers are syntenic. Additional aspects of the map and the extent of coverage of the total nuclear genome are discussed.

Key words: *Vicia faba* – Allozyme, RFLP and RAPD markers – Linkage group

Introduction

The faba bean (*Vicia faba*) is one of the oldest crops grown by man and is used as a source of protein and carbohydrate for both human and animals. Its critical role in crop rotation, in reducing energy cost, improving soil physical conditions, and decreasing the amount of diseases and weed populations has long been recognized. In spite of this potential, the total area of faba bean cultivation has decreased over the last century. The main reason given for this decline has been the low and unstable yields associated with the crop. In comparison with other pulse crops, faba bean has been the focus of relatively little research (including breeding), and few genetic markers have been identified. Sirks (1931) found that 19 genetic factors formed four linkage groups, but Sirks' material was lost during the Second World war. Recently, several morphological traits have been mapped to chromosomes using trisomics (Cabrera and Martín 1989; Cabrera et al. 1989) or translocation lines (Sjödin 1971), and a few isozyme systems have also been studied (Suso and Moreno 1982; Mancini et al. 1989). As yet, however, no extended linkage groups are recognised for this species.

The development of a detailed linkage map for *V. faba* will greatly increase the efficiency of genetic and breeding studies. Marker-assisted selection for quantitative traits (Tanksley et al. 1982) or for disease resistance (Weeden and Provvidenti 1988) has proven useful in different species. Molecular markers such as isozymes, restriction fragment length polymorphisms (RFLPs), and the more recently described random amplified polymorphic DNA (RAPD), all have excellent potential for being used as tools for gene mapping (Tanksley and Orton 1983; Helentjaris 1989; Weeden 1989; Welsh and McClelland 1990; Williams et al. 1990). In the present study, we report the linkage relationships of nine isozymes, one RFLP marker and 43 RAPD markers in *V. faba* as a first step towards the development of a complete gene map for this species.

Materials and methods

Three parental lines were involved in the crosses. Line Vf6 was used as female parent for each cross while the two lines used as pollen parents were Vf35 and Vf173 (from the collection of genetic variants at the Escuela Técnica Superior de Ingenieros

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Agrónomos in Córdoba). The parental lines were selected to maximize the number of segregating isozyme loci in the F_2 progeny. Plants were grown, and crosses made, in the greenhouse with supplemental lighting used when needed to obtain a 16-h day. The populations used for linkage mapping consisted of 20 F_2 individuals derived from the cross Vf6 Vf173 and 44 F_2 plants from the cross Vf6 Vf35.

Isozymes

Isozyme analysis was performed on extracts from young leaf tissue, except for peroxidase for which extracts from young, healthy root tissue was used. Two different extraction buffers were employed (Weeden and Emmo 1985). Horizontal starch-gel electrophoresis was performed according to Gottlieb (1973). Samples extracted in Tris-maleate (pH 8.0) buffer were placed on a pH 8.1 Tris-citrate/lithium borate system (Selander et al. 1971). Potassium phosphate extraction buffer (pH 7.0) was used for samples placed on either a pH 6.5 histidine (Cardy et al. 1980) or a pH 6.1 citrate/N-(3-aminopropyl)-morpholine gel system (Clayton and Tretiak 1972). Slices from the Tris-citrate/lithium borate-buffered gel where stained for peroxidase (PRX; EC 1.11.1.7), aspartate aminotransferase (AAT; EC 2.6.1.1) and triosephosphate isomerase (TPI; EC 5.3.1.1). Slices from the histidine-buffered gel were assayed for 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44). Methylumbelliferyl esterase (EST; EC 3.1.1.–) and acid phosphatase (ACP; EC 3.1.3.2) were resolved in citrate-gel system slices. Assay solutions were mixed according to standard recipes (Wendel and Weeden 1990).

DNA extraction

DNA was extracted from unexpanded or partially expanded leaflets using a procedure based on, but considerably modified from, the method of Lassner et al. (1989). About 100 mg of tissue was crushed in 1 ml of liquid N_2. Before the tissue thawed, 1 ml of CTAB buffer (Murray and Thompson 1980) containing 0.4% 2-mercaptoethanol and 0.5 mM sodium bisulphite was added and the grinding briefly continued. Approximately 0.5 ml of this aqueous slurry was poured into a 1.5 ml Eppendorf microcentrifuge tube containing 100 µl of 24:1 chloroform/octanol. The tube was briefly shaken and incubated at 65 °C for 30 min. The solution was allowed to cool to room temperature and sufficient 24:1 chloroform/octanol was added to nearly fill the tube. The mixture was shaken vigorously to form an emulsion, then centrifuged at 5 °C for 4 min (7,000 g) to separate the phases. The aqueous phase was transferred to a clean microcentrifuge tube. An equal volume of cold (– 20 °C) 95% ethanol was added to precipitate the DNA which was spooled out, washed in 76% ethanol 0.2 M sodium acetate for 5 min and dissolved in 100–200 µl of TE (pH 8.0). Coprecipitated RNA was eliminated by the addition of 0.7 units of RNase A. The DNA stock solution was directly used for the RFLP analysis. For generation of RAPD fragments, the solution was diluted with three volumes of sterile distilled water.

RFLP analysis

DNA (500 ng/sample) was digested with *Eco*RI as per supplier's recommendations, separated on 0.8% agarose gels, and blotted according to Southern (1975). Plasmid pGmr-1 containing a complete 18*s*-25*s* rDNA repeat from soybean (Doyle and Beachy 1985) was used as a probe. Labelling was carried out with dioxigenin-11-dUTP by nick translation (Maniatis et al. 1982). Hybridization and detection of chemiluminescence was performed according to instructions supplied with the Genisus™ system (Boeringer Mannheim Corporation, Indianapolis).

Amplification of DNA fragments using single primers

A total of 53 oligonucleotide primers were surveyed (see Table 1). Conditions for DNA amplification were standardized for all primers. Each 25 µl amplification reaction contained: 20–40 ng of plant genomic DNA, buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin], 100 µm of each dNTP, 2–4 µM of primer, and 1U of *Taq* DNA polymerase (Promega). The mix was overlayed with mineral oil. Amplification was performed in a Coy TempCycler Model 50 programmed for 40 cycles with the following temperature profile: 1 min at 94 °C, 2 min at 35 °C, 2 min at 72 °C. Maximum ramping speed was used; however, the Coy instrument still required approximately 3 min to change from 94 °C to 35 °C. Thus the annealing time was significantly longer than 2 min, albeit at temperatures higher than 35 °C. Cycling was concluded with a final extension at 72 °C for 8 min. Controls lacking template DNA were included in an initial test with parental DNA and occasionally with the segregating progeny DNA. Reaction products were resolved by electrophoresis on gels consisting of 1% agarose, 1% Nu-Sieve agarose, and 1 TBE buffer. Gels were stained with ethidium bromide and photographed. Amplified fragments were designated using the

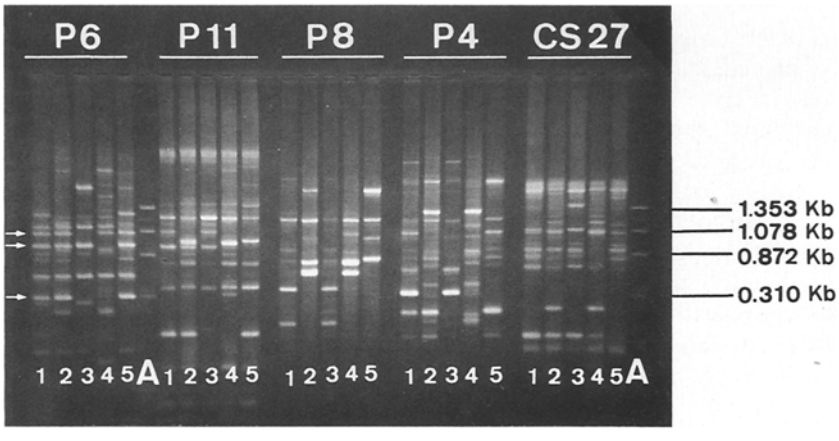

Fig. 1. Amplification of *V. faba* DNA using the following primers: P6, P11, P8, P4 and CS27 (see Table 1). *Lane A*, ϕx 174/*Hae* III digest; *lane 1*, F_2 plant (cross Vf6 Vf173); *lane 2*, F_2 plant (cross Vf6 Vf35); *lane 3*, Vf173; *lane 4*, Vf35; *lane 5*, Vf6. As one of the numerous examples, the *arrows* point to the three polymorphic bands detected in the cross Vf6 Vf35 using the primer P6

primer ID number, and numbering from higher to lower molecular weight (Fig. 1).

Linkage analysis

Goodness-of-fit to the expected F_2 segregation of 1:2:1 (RFLP and most isozyme markers) or 3:1 (RAPD markers) was tested by Chi-square analysis. Linkage between all the considered markers was estimated from F_2 segregation using maximum-likelihood formulae as applied by the LINKAGE-1 program (Suiter et al. 1983). Multilocus linkage groups were constructed using MAPMAKER (Lander et al. 1987). In general, linkages were reported only when recombination frequency was ≤ 0.20 and LOD values were greater than 3. In some cases, especially in the cross Vf6 Vf13, higher values than those mentioned were taken into account. The relatively small size of the F_2 populations, as well as the prevalence of dominant/recessive markers, required stringent limits on the reporting of linkage. Results from the cross Vf6 Vf173 were used primarily to support those obtained in the Vf6 Vf35 F_2, rather than to identify new linkages.

Results

Polymorphism was present for nine of the 15 isozymes resolved in six enzyme systems. The results obtained for each enzyme system are explained below:

Peroxidase. Several zones of peroxidase activity were present in both anodal and cathodal slices. Genetic analysis was performed on the two most-anodal peroxidases (PRX-1 and PRX-2). PRX-1 showed electrophoretic variation in both crosses and produced an intense, sharp band near the front (Fig. 2a), whereas PRX-2 segregated only in the cross Vf6 Vf35 and displayed paler and broader bands. Both PRX-1 and PRX-2 gave segregation ratios not significantly different from the 1:2:1 ratio expected for a codominantly expressed locus. In both cases the heterozygous phenotype was two-banded, indicating that the enzymes were monomers.

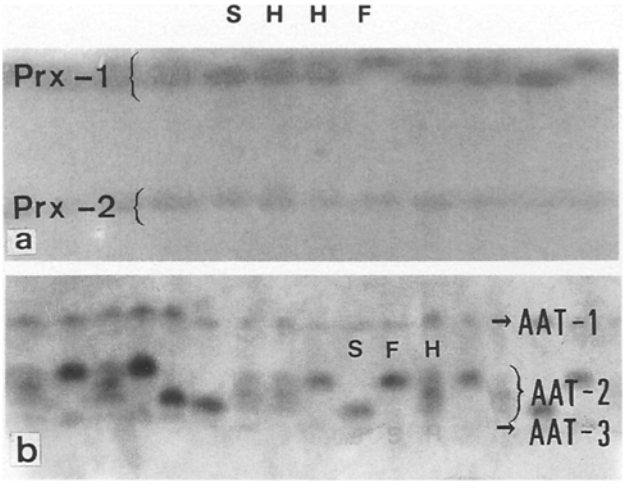

Fig. 2. **a** Phenotypes of the anodal peroxidases in root extracts from cross Vf6 Vf173 segregating at PRX-1. Phenotype designations are: *H*, heterozygous; *S*, slow; *F*, fast. **b** Aspartate aminotransferase phenotypes obtained from an F_2 population. Phenotype designation are the same as given in Fig. 2a. Heterozygous individuals at ATT-2 has three bands, suggesting that the isozyme is dimeric. The anode is at top of the figure

Aspartate aminotransferase. Three isozymes were detected in gel slices stained for AAT. In the cross Vf6 Vf173 all the zones of activity were monomorphic. In Vf6 Vf35 the fastest migrating isozyme (AAT-1) was monomorphic while AAT-2 and AAT-3 were variable. AAT-1 and AAT-3 stained very faintly while AAT-2 produced the darkest band on the gel. The AAT-2 slow allozyme overlapped the fast allozyme of AAT-3, making AAT-3 segregation difficult to score. The hybrid phenotype for AAT-2 displayed three bands, suggesting that the isozyme is dimeric (Fig. 2b). Although no organelle isolation was performed, the intense appearance of the AAT-2 bands in leaf extracts suggested that this isozyme was specific to the plastid organelle, as are the most intensely staining isozymes in pea and lentil leaf tissue (Weeden and Marx 1987; Muehlbauer et al. 1989). We have tentatively designated the locus encoding the AAT-2 subunits as *Aat-p*, reflecting its probable homology to *Aat-p* in pea and lentil. The subcellular location of AAT-3 could not be inferred from either its position or its relative intensity. Both loci fit the expected 1:2:1 ratio in the F_2 analysed.

Triosephosphate isomerase. Only one clear region of TPI activity was resolved, this displaying a mobility of approximately 0.4 relative to the borate front. Polymorphism for this TPI was apparent in the cross Vf6 Vf173 but not in Vf6 Vf35. The TPI-1 segregation pattern approximated the 1:2:1 ratio expected for a single nuclear gene. Heterozygous individuals exhibited a three-banded pattern, indicating that TPI-1 is functionally dimeric.

6-Phosphogluconate dehydrogenase. Of the two zones of activity detected for this enzyme system, electrophoretic variation was found only for the more-anodal one, 6PGD-1, which segregated in the expected 1:2:1 ratio. The 6PGD-2 allozyme overlapped the slow allozyme of 6PGD-1. The polymorphism in 6PGD-1 was detected only in the 6 × 173 progeny.

Esterase. Methylumbelliferyl esterase was assayed on the three systems previously mentioned. The pH 6.1 gel displayed the best resolution, and four zones of activity were present in the anodal slice. All the isozymes were monomorphic in the cross Vf6 Vf173, whereas in Vf6 Vf35 polymorphisms was observed for the most-anodal isozyme (EST-1). Heterozygous plants exhibited only the two parental bands indicating that the enzyme is monomeric. This isozyme locus exhibited normal F_2

codominant single-gene segregation. Alpha-esterase was also assayed on three systems but in all cases was difficult to resolve. Again, the alphaisozymes were best resolved in the pH 6.1 gel. The alpha-esterase co-segregated with the more easily resolved EST-1, and both isozyme were considered to be produced by the same locus.

Acid phosphatase. This enzyme system exhibited two isozymes (ACP-1 and ACP-2). The more-anodal isozyme (ACP-1) was polymorphic in Vf6 Vf173 while ACP-2 was monomorphic. Only two phenotypes could be distinguished in the F_2, one with ACP-1 activity present (as in Vf173) and one in which it appeared to be lacking (as in Vf6). The segregation for ACP-1 deviated significantly ($P > 0.05$) from the expected 3:1 ratio. In Vf6 Vf35, ACP-1 was monomorphic and ACP-2 polymorphic. Three distinct ACP-2 phenotypes were observed in the F_2 progeny, and F_2 segregation fitted the expected 1:2:1 ratio. The two-banded phenotype of the heterozygotes suggested that ACP-2 is functionally monomeric.

RFLP

The phenotypes for *Eco*RI-digested DNA hybridized with pGmr-1 are presented in Fig. 3. Only three phenotypes were observed in the F_2 suggesting that the rDNA genes were segregating as a single Mendelian unit. The molecular lengths of the predominant polymorphic fragments were 7.3 kb in both pollen parents, Vf35 and Vf173, and 6.7 kb in the female parent, Vf6 (Fig. 3). The polymorphism appears to be generated in the larger spacer region because the other *Eco*RI fragment was very similar in size to the more conserved 3.7-kb *Eco*RI fragment in pea that contains the 18 and 5.8 *s* genes (Polans et al. 1986; Rogers et al. 1986; Rogers and Bendich 1987). In both crosses the 3.7-kb *Eco*RI fragment displayed a darker, broader, but monomorphic band. A faint 3.4-kb band was observed in the digest of Vf35 DNA but not in the digest of Vf6 DNA. This band was also observed in some, but not all, of the progeny possesing the Vf35 genotype (Fig. 3). It was not seen in any of the heterozygous or Vf6 genotypes. The basis of this polymorphism has yet to be thoroughly investigated, but our current data suggest that the band is produced by cleavage of an *Eco*RI site rarely cut during normal digestion of genomic DNA, and thus is visible only in lanes heavily loaded with DNA of the Vf35 genotype.

Random amplified polymorphic DNA

Table 1 presents the results of the 53 primers surveyed. Seventeen primers (32%) out of the total had consistent amplification products that resolved 59 scorable polymorphic loci in Vf6 Vf35 and 47 in Vf6 Vf173. Most primers showed polymorphism in both crosses. Primers P13, P31, and CS30 were polymorphic only in the cross between Vf6 and Vf35, and CS14 only detected differences in the cross Vf6 Vf173. An average of 3.2 fragments per primer were amplified. The average of RAPD markers described in Vf6 Vf35 was higher (3.5) than in Vf6 Vf173 (2.8). Only seven primers failed to amplify faba been genomic DNA. About 10% of the control samples lacking template DNA occasionally gave amplification products. However, in no instances were these 'artifactual' products observed in samples containing faba bean template DNA that were run simultaneously using the same reaction mix. Spurious amplification products (bands not present in either parent) were sometimes observed in amplifications of progeny DNA. Such bands were usually very faint and were not considered in the genetic analysis. It must be emphasized that 21 of the primers [noted as Y(b) in Table 1], although not displaying polymorphism in the crosses assayed, must be considered potentially useful primers for the

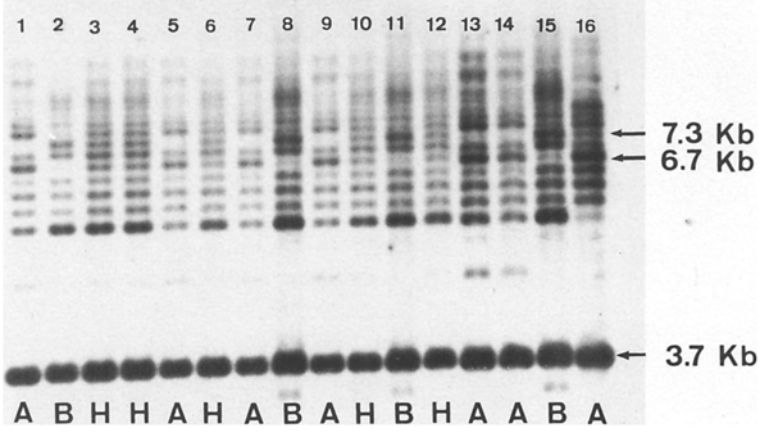

Fig. 3. Hybridization patterns of Rrn genes to *Eco*RI digests of *V. faba* genomic DNA probed with the plasmid pGmr-1 which contains a complete 18*s*–25*s* rDNA repeat from soybean. *Lanes 1–14* represent F_2 phenotypes from the cross Vf6 Vf35. *Lanes 15 and 16* represent the parental lines Vf35 and Vf6, respectively. 7.3 kb and 6.7 kb were the molecular lengths of the predominant polymorphic fragments. *A*, maternal phenotype Vf6; *B*, parental phenotype Vf35; *H*, heterozygous

Table 1. Sequence of the 5′ oligonucleotides used in, and general results from, the crosses Vf6 Vf35 and Vf6 and Vf173

Primer ID	Sequence (5′–3′)	Amplified product*	No. of scorable RAPDs	
			Vf6 Vf35	Vf6 Vf173
P1	CCTGTAGTGG	Y	4	1
P2	TACCTTCCGT	N	–	–
P3	GTCCGTTGGG	Y (a)	–	–
P4	GTTAGGTCGT	Y	6	6
P5	TCTCTGTCCC	Y (b)	–	–
P6	TCGCCCCATT	Y	3	3
P7	CGTGGTTCCC	Y (b)	–	–
P8	GTCCCGTTAC	Y	8	5
P9	ACGCCCTAGT	Y (b)	–	–
P10	TTTCACATGG	N	–	–
P11	CTGTGCTGTG	Y	4	4
P12	TGGTGGATGTA	Y (b)	–	–
P13	GGTGATGTCC	Y	4	–
P14	TGCCTTCCAT	Y (a)	–	–
P15	GGGTTGCCGT	Y	2	3
P16	GGATATTCGG	Y (a)	–	–
P17	CTATTTTGCC	Y (b)	–	–
P18	AGTTCGTCTG	Y (b)	–	–
P19	GAGTATAGAG	N	–	–
P20	TGCTGGATTG	Y (b)	–	–
P21	CCCTGTCTCT	Y (b)	–	–
P22	TTTGAGGATG	Y (a)	–	–
P23	CGTTAACCTT	Y (a)	–	–
P24	AGCACTGTCA	Y (b)	–	–
P25	AATGAAGCCA	Y (b)	–	–
P26	TACTGCTGGG	Y (b)	–	–
P27	ACCTCGAGCA	Y (a)	–	–
P28	TCGTAGCCAA	Y (a)	–	–
P29	TCCTGGAGCCCG	N	–	–
P30	CAACTGGTAATG	Y (b)	–	–
P31	TGTGATATCG	Y	2	–
P105	CAGTCGCGTG	Y	5	3
P137	ATCTGCGACA	Y	6	5
P145	TAGCGGCTAC	Y (b)	–	–
P146	TGACATGCGA	Y (b)	–	–
P157	GTCATGTCGA	Y (b)	–	–
CS11	CAGTGGAATG	N	–	–
CS12	GCGACGCCTA	Y	4	5
CS14	GTCACCCGGA	Y	–	3
CS16	CGTTGGATGC	Y	2	1
CS17	TACGCTGATC	N	–	–
CS19	TACGGCTGGC	Y (b)	–	–
CS20	TGAACCGCCG	Y (b)	–	–
CS21	CCCTACCGAC	Y (b)	–	–
CS22	CGTCGTGGAA	Y (b)	–	–
CS23	CCACGCTATA	Y (b)	–	–
CS24	GCGGCATTGT	Y	2	5
CS25	GTGTAATCGC	Y (b)	–	–
CS26	TTGGTCGGAA	Y (b)	–	–
CS27	AGTGGTCGCG	Y	4	2
CS28	TCCATGCCGG	N	–	–
CS29	CCAGACAAGC	Y	2	1
CS30	GCGTAGAGAC	Y	1	–
Total			59	47

* Y, presence; N, absence; Y (a), presence of amplified product but bands are faint and not consistent; Y (b), presence of amplified product but polymorphism was not detected

detection of variation within the species. The segregation of the 'present' and 'absent' phenotypes usually provided a good fit to the expected 3:1 ratio.

Linkage analysis

Each cross was analyzed separately as few loci were segregating in both (see Discussion). Joint segregation analysis identified linkage between 32 pair-combinations of markers in the cross Vf6 Vf35 and 16 in the cross Vf6 Vf173 (Tables 2 and 3). The number of RAPD markers that assorted independently was 36 in Vf6 Vf35 and 33 in Vf6 Vf173 but only 28 and 15 of them, respectively, could be clearly assigned to linkage groups.

Cross Vf6 Vf35. The 32 pair-combinations were assembled into ten linkage groups, one of which could be associated with a known chromosomal marker. Linkage group 1 included four loci (Table 2) among which was the rDNA array Rrn, identifying this group as part of chromosomes I (following the Michaelis and Rieger 1959 designation). The map length of this linkage group was shorter than that expected for the largest chromosome in *V. faba*. Thus, we assume only part of the total chromosome is represented.

Prx-1 and four more RAPD markers formed the second linkage group. Distances between markers were reasonably additive (Fig. 4). No recombination was detected between P105-1 and CS29-1 because these RAPD markers were in repulsion. The position of CS29-1 was determined based on its linkage with the co-dominant marker, *Prx-1*. The map distance included in this sequence is approximately 60 cM.

Table 2. Segregation data for pairs of loci exhibiting significant deviations from random assortment in the cross Vf6 Vf35

Loci	N	Chi-square	*P*	r ± SE
Linkage group 1				
Rrn/P8-5	39	9.29	0.010	23 ± 7
Rrn/(*EST*)	22	12.82	0.012	24 ± 7
(*EST*)/P15-1	23	9.64	0.008	25 ± 8
Linkage group 2				
P105-5/P8-6	36	22.50	<0.001	7 ± 17
P105-5/*Prx-1*	33	10.31	0.006	23 ± 8
P105-5/P105-1	36	14.40	<0.001	19 ± 16
Prx-1/P105-1	30	22.05	<0.001	5 ± 4
Prx-1/CS29-1	31	12.03	0.002	28 ± 9
P105-1/CS29-1	32	7.44	0.006	0 ± 2
Linkage group 3				
CS30-1/CS24-1	31	15.06	<0.001	13 ± 18
CS30-1/P8-4	34	9.67	0.002	21 ± 16
CS24-1/P8-4	31	26.36	<0.001	3 ± 18
CS24-1/*Prx-2*	24	24.00	<0.001	0 ± 2
P8-4/*Prx-2*	28	21.64	<0.001	4 ± 4
Linkage group 4				
P105-2/*Acp-2*	38	36.13	<0.001	2 ± 2
Linkage group 5				
Aat-2/P105-4	43	21.24	<0.001	14 ± 6
P105-4/P4-6	38	9.28	0.002	22 ± 15
Linkage group 6				
P1-3/P8-1	27	5.08	0.024	25 ± 18
P1-3/P8-3	27	9.61	0.002	17 ± 19
P1-3/P6-2	26	11.74	0.001	14 ± 19
P8-1/P8-3	39	29.75	<0.001	5 ± 16
P8-1/P6-2	38	18.78	<0.001	13 ± 16
P8-3/P6-2	38	26.10	<0.001	8 ± 16
Linkage group 7				
P137-5/CS27-3	37	13.14	<0.001	17 ± 16
P137-5/P13-1	37	8.83	0.003	22 ± 15
P27-3/P13-1	33	17.19	<0.001	12 ± 17
P13-1/P13-2	37	7.51	0.006	0 ± 3
P13-2/P13-3	37	16.92	<0.001	15 ± 16
Linkage group 8				
CS16-1/CS12-4	38	14.03	<0.001	16 ± 16
CS12-4/CS16-2	38	10.25	0.001	22 ± 15
Linkage group 9				
CS29-2/P11-4	35	15.33	<0.001	14 ± 16
Linkage group 10				
CS27-2/CS27-4	37	12.07	0.001	19 ± 15

N, number of plants
P, probability
r, recombination frequency

Table 3. Segregation data for pairs of loci exhibiting significant deviations from random assortment in the cross Vf6 Vf173

Loci	N	Chi-square	*P*	r ± SE
Linkage group 1				
Tpi-1/*Rrn*	20	13.28	0.010	23 ± 8
Tpi-1/P11-2	15	11.14	0.004	6 ± 6
Tpi-1/CS27-1	16	12.01	0.002	6 ± 6
Rrn/CS12-1	15	8.74	0.013	22 ± 11
Rrn/CS24-2	19	10.75	0.005	12 ± 7
CS12-1/CS24-2	15	6.86	0.009	18 ± 24
Linkage group 2				
Prx-1/*Acp-1*	18	7.71	0.021	17 ± 9
Acp-1/P4-6	15	6.74	0.009	0 ± 3
P4-6/*Prx-1*	15	6.78	0.034	14 ± 9
Linkage group 3				
P105-1/CS14-1	16	5.56	0.018	15 ± 24
CS14-1/CS14-2	16	6.15	0.013	0 ± 2
CS14-1/CS14-3	15	5.10	0.024	16 ± 25
CS14-2/CS24-3	15	5.63	0.018	0 ± 3
Linkage group 4				
CS14-3/CS29-1	16	9.90	0.002	7.5 ± 25
Linkage group 5				
P4-1/P6-2	13	10.37	0.001	6 ± 27
Linkage group 6				
P8-1/P8-2	15	7.35	0.007	13 ± 25

N, number of plants
P, probability
r, recombination frequency

Linkage group 3 included an isozyme (*Prx-2*) and three RAPD markers. This sequence covered only 25 cM of the genome and the order proposed is shown in Fig. 4. P8-4 was so close to *Prx-2* that the sequence of these relative to the other two RAPD markers could not be resolved. Two other linkage groups included isozyme loci. In group 4, *Acp-2* displayed a tight linkage with P105-2 (2 ± 2 cM), and *Aat-2* and P105-4 were both placed in group 5 (Table 2, Fig. 4). P105-4 also was linked with P4-6, but no linkage between this last marker and *Aat-2* was detected. Approximately 35 cM of the genome is represented in group 5.

The remaining linkage groups included only dominant markers. Linkage group 6 consisted of four RAPD markers and was approximately 25 cM in length. Linkage group 7 contained five RAPD markers. P13-1 showed a tight linkage with P13-2 and, because no recombinants were recovered, we consider that both markers might be allelic. The sequence of markers belonging to this group covered approximately 40 cM of the *V. faba* map. Finally, linkage groups 8, 9 and 10 included three, two and two RAPD loci respectively (Table 2, Fig. 4).

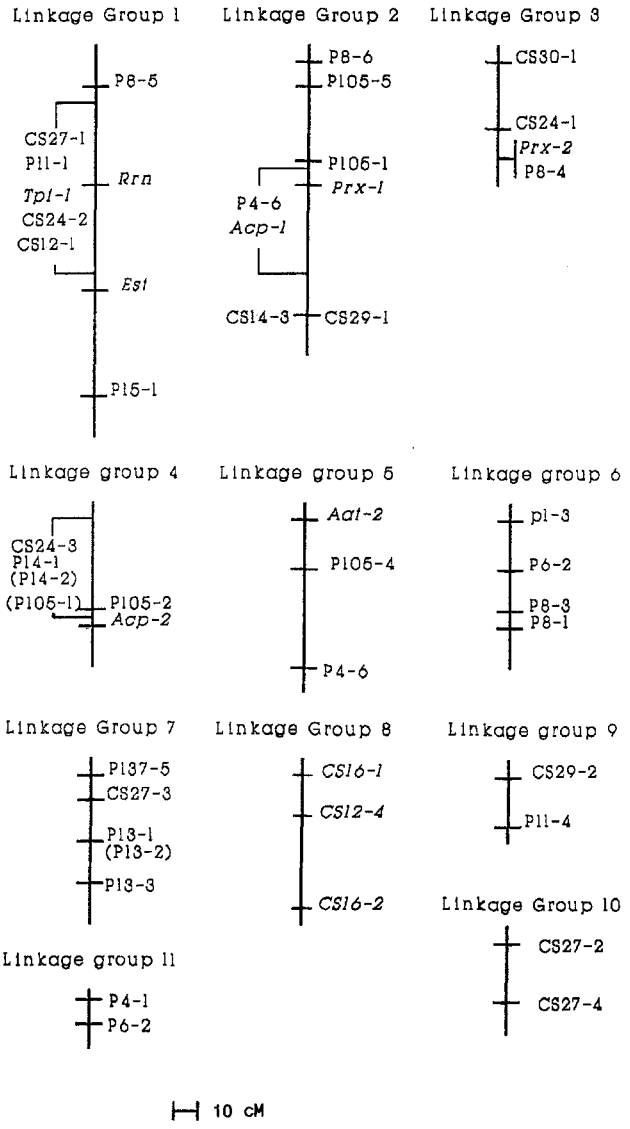

Fig. 4. Linkage relationships of seven isozyme loci (shown in *italics*), one RFLP locus and 43 RAPD loci in *V. faba*. The 11 *vertical bars* represent fragments of chromosomes. Only linkage group 1 is associated with Chromosome I on the basis of its linkage to a ribosomal DNA marker. Markers on the left were identified in cross Vf6 Vf173 (which was used primarily to confirm results from Vf6 Vf35). For this reason, these markers are not displayed in a linear order (see discussion in text)

Cross Vf6 Vf173. Six linkage groups could be defined, one of which contained the ribosomal array and was assigned to chromosome I. This group included one isozyme (*Tpi-1*), the 45*s* ribosomal array (*Rrn*), and four RAPD markers (Table 3). As was the case in the Vf6 Vf35 results, only a short distance of chromosome I is represented by this linkage group.

Linkage group 2 included three markers, *Prx-1*, *Acp-1* and P4-6. *Prx-1* was approximately 17 map units from *Acp-1* and also showed linkage with P4-6 (Table 3). P4-6 showed no recombination with *Acp-1*, but since these markers were in repulsion phase the distance between them could not be determined precisely.

Four RAPD markers constituted linkage group 3 (Table 3). The absence of recombinants between CS14-1 and CS14-2 suggested that these might be allelic. Relative map distances placed P14-1 (P14-2) between P105-1 and CS24-4 (Fig. 4). Each of the remaining groups contained two RAPD markers (Fig. 4).

Discussion

Analysis of segregating isozyme loci, the 45*s* ribosomal array, and RAPD markers in two *V. faba* F_2 populations has identified at least 11 linkage groups. One linkage group, reported in both populations, contains the 45*s* ribosomal gene array and can be associated with the large metacentric chromosome possessing the nucleolar organizer region. In *V. faba*, this region is located on the short arm of chromosome I. Two isozyme loci, *Tpi-1* and *Est*, were also placed in this linkage group. Unfortunately, neither of the F_2 populations analysed had all the three loci segregating simultaneously, preventing the determination of their relative order. The remaining linkage groups did not contain markers assigned to specific chromosomes. In addition, more linkage groups have been identified than there are chromosomes. At present, we are unable to determine which linkage groups are syntenic or

whether we have markers on every chromosome. Although a total of 66 markers were segregating in the Vf6 Vf35 population, the total map distance defined among linked markers was small (300–350 cM) relative to the expected size of the *V. faba* genome (700–1500 cM). These results reflect problems associated with using dominant/recessive markers in relatively small populations. In our analysis, only linkage between markers in coupling or with a co-dominant marker could be detected. Consequently, the number of RAPD markers was effectively reduced by a half.

One aspect of the mapping results that particularly interested us was whether linkages paralleling those found in pea and lentil existed in faba bean. In order for such 'conserved' linkage groups to be identified the position of homologous loci must be compared. In the current analysis we are limited to the isozyme loci and the 45*s* ribosomal array, because homologous relationships among RAPD markers are presently difficult to evaluate. Two possible conserved linkage groups were detected using this small set of loci. One of the 45*s* ribosomal arrays in pea is also syntenic with an esterase locus and *Tpi-p* (Weeden and Wolko 1990), suggesting that linkages on chromosomes 4 in pea may be conserved in faba bean. In pea the 45*s* array is over 50 map units from *Tpi-p*, and in lentil the ribosomal array appears to be linked to different markers. Thus there is reason to question whether the *Rrn – Est – Tip-p* linkage is conserved within the Viciae.

In the second linkage group, the isozyme loci *Prx-1* and *Acp-1* represent a stronger case for a conserved linkage group. In pea *Prx-1* is linked to *Acp-1* and *Nag* (Weeden and Marx 1987). In lentils *Prx-1* is linked to *Nag* (Weeden et al. 1988) indicating that this region is probably conserved between pea and lentil. The identification of linkage between *Prx-1* and *Acp-1* in faba bean suggests that the linkage has been conserved in *Vicia* as well. Both peroxidase and acid phosphatase are enzyme systems in which between-taxa homologies are often difficult to determine. However, the homology of *Prx-1* in pea, *Prx-1* in lentil, and *Prx-1* in faba bean is strongly supported by the unique phenotype shared by all three isozymes. In each case the isozyme is a sharp band migrating near the borate front (Rf = 0.8–0.9), is expressed primarily in root tissue, and is sensitive to reducing agents such as 2-mercaptoethanol (unpublished observations). There are no other peroxidases in any of the species that produce a similar phenotype. With respect to the acid phosphatase comparison, it is possible that *Acp-1* in pea is not homologous to Acp in faba bean. At present no compelling evidence exists to either establish or reject our hypothesis of homology.

In two instances a RAPD marker segregating in the Vf6 Vf35 population appeared to be identical to one segregating in the Vf6 Vf173 population. P105-1 in the former displayed the same size as P105-2 in the latter population. In addition a fragment of the same size was observed in Vf6 but not in Vf35 or Vf173. Consequently, linkage group 3 in the former cross must be equivalent to linkage group 4 in Vf6 Vf35. Linkage group 4 in Vf6 Vf173 and linkage group 2 in Vf6 Vf35 were also considered as a single group because P29-1 is the same marker in both crosses. A final comparison involves P8-1 and P8-3 in Vf6 Vf35 with P8-1 and P8-2 in Vf6 Vf173. P8-1 is the same marker in both crosses and P8-3 is identical to P8-2. Hence the linkage group 6 from each cross could be combined. Distances between the two considered loci were not exactly the same, but the small population size analyzed, the comparison between co-dominant markers, and consequently the high standard errors found, could justify this lack of additivity. In all cases, because only one or few loci were common in the joined linkage groups it was difficult to establish the correct gene sequence. We have to emphasize that nearly all our linkage data and conclusions are based on the Vf6 Vf35 progeny. We were conservative in our identification of linkage groups and the Vf6 Vf173 data was used primarily for confirmation. Nevertheless, results from this cross were needed to place *Tpi-p* on chromosome I, *Acp-1* on linkage group 2, and to identify linkage group 11 (see Fig. 4).

Two of the enzymes systems we analyzed, aspartate aminotransferase and esterase, were also investigated by Mancini et al. (1989). They demonstrated the presence of two polymorphic GOT (= AAT) isozymes in *V. faba*, which they designated GOT-2 and GOT-3. They also reported a monomorphic GOT-1. Our results agree with this previous work. We have chosen to use aspartate aminotransferase as the name for the enzyme because it is the name preferred by the International Union of Biochemistry (1984) and is used for homologous loci in closely related species such as pea and lentil (Weeden and Marx 1987). In the esterase system we resolved four zones of activity whereas Mancini et al. (1989) described three. We believe our polymorphic EST-1 may be identical to the polymorphic EST-2 described by Mancini and co-workers; however, further confirmatory tests are required to clarify the terminology of this system.

Altogether 11 independent segments of the faba bean genome were reported in this study. Although only one of the groups has been associated with a specific chromosome, we hope that the linkage data we have generated will provide a foundation for the development of a complete gene map of *V. faba*. A more extended map could be defined by studying additional allozymic variants in wider faba bean crosses. Moreover, the availability of a complete

trisomic series for the five acrocentric chromosomes (Martín and Barceló 1984) and a good collection of morphological mutants should greatly assist work in this area. The possible conservation of linkage groups identified in related genera, i.e., *Pisum*, *Lens* and *Cicer* (Gaur and Slinkard 1990; Weeden et al. 1992), may provide considerable information about linkage relationships in *Vicia*.

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