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Peroxidase and Leucine-aminopeptidase in Diploid *Medicago* Species Closely Related to Alfalfa: Multiple Gene Loci, Multiple Allelism, and Linkage*

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Summary. The genetics of peroxidase and leucine-aminopeptidase isozymes was studied utilizing starch gel electrophoresis in the diploids *Medicago sativa* L. (*M. coerulea* Less.) and *M. falcata* L. Three anodal and one cathodal sets of peroxidase isozymes identify four linked loci. In addition, two anodal sets of leucine-aminopeptidase isozymes identify two loci that may be linked. The allozymes at each of the loci segregated as expected for monomeric enzymes. However in several crosses there were deficiencies in the number of progeny in particular genotypic classes. This could result from the segregation of recessive deleterious genes linked to some of the allozyme alleles. This is the first report of multiple loci and multiple alleles determining isozymes in *Medicago*.

Key words: Allozymes – Peroxidase – Leucine-aminopeptidase – Linkage – Lucerne – Medicago

Introduction

There are several reports on the inheritance of characteristics in alfalfa controlled by single genes that could be useful genetic markers (Barnes and Hanson 1967; Busbice et al. 1972).

Although some of these traits have been studied in diploids, the majority have been studied in tetraploids. Despite the importance of alfalfa as a forage crop, only a limited amount of genetic data has been accumulated. Only a few genes have been assigned to their respective chromosomes by trisomic analysis (Kasha and McLennan 1967; Stanford et al. 1972) and information on linkage groups is meagre. There is a need to identify simply inherited genetic markers in both diploid and tetraploid alfalfa. Inheritance patterns and linkage relationships can be elucidated first in diploids and subsequently studied in tetraploids. These genetic markers will be useful in alfalfa breeding programs, and in studies of development and taxonomic relationships (Quiros 1979; 1980a, 1980b).

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The research presented here is a first step toward such an objective. Allozymes determined by peroxidase (*Prx*) and leucine-aminopeptidase (*Lap*) genes are described. Evidence is presented for multiple allelism at six loci (four *Prx*, two *Lap*) in diploid alfalfa. There is additional peroxidase isozyme variation which has not been satisfactorily resolved. The four *Prx* loci comprise a cluster with moderately close linkage, while the data only suggest loose linkage between the two *Lap* loci. Allozyme variability at the *Prx* loci segregates independently of that at the *Lap* loci. Several accessions of diploid *Medicago sativa* L. (*Medicago coerulea* Less.) and *Medicago falcata* L. were studied. These species intercross freely with each other and also with tetraploid cultivated alfalfa (Lesins and Lesins 1979). Furthermore, the patterns of allozyme variability identified in diploid alfalfa, and perhaps many of the same allozymes, are found in cultivated alfalfa (Quiros 1979).

Materials and Methods

Accessions

Three accessions of diploid *Medicago coerulea* and four of diploid *Medicago falcata* are included in this study (Table 1). They are part of the *Medicago* germplasm collection established by Professor Karl Lesins at the University of Alberta.

Electrophoresis

Standard starch gel electrophoresis was carried out on crude extracts of leaf and root tissue of seedlings one to three months old. The techniques described by Quiros (1980a) were modified as follows: up to 35 samples per gel were applied with filter paper wicks, Whatman no. 3, measuring 2 mm × 9 mm. The wicks were soaked in the plant extract and inserted in the gel 3.5 cm from the anodal end. Extracts of at least three different plants of known genotypes were included as reference. The gels were run until the anodal front migrated 10 cm and the cathodal front 3 cm from the origin.

The symbols *Prx* and *Lap*, which designate peroxidase and leucine-aminopeptidase loci, respectively, are followed by a number

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Table 1. Sources of the peroxidase and leucine-aminopeptidase allozymes

Accession number	Species	Alleles						Origin
		Prx-1	*Prx-2*	*Prx-3*	*Prx-4*	*Lap-1*	*Lap-2*	
UAG116	*M. falcata*	$1^1\ 1^4\ 1^7\ 1^{15}$	$2^1\ 2^5$	–	–	$1^1\ 1^3$	–	Leningrad, USSR
UAG127	*M. falcata*	$1^1\ 1^2\ 1^4\ 1^n$	$2^1\ 2^2\ 2^5$	$3^1\ 3^3$	$4^1\ 4^n$	$1^1\ 1^3$	$2^3\ 2^4\ 2^5\ 2^6$	Kubarskatz, Bulgaria
UAG128	*M. falcata*	$1^2\ 1^4$	$2^1\ 2^2$	$3^1\ 3^3$	–	–	$2^3\ 2^6$	Versailles, France
UAG1827	*M. falcata*	–	–	–	–	–	$2^3\ 2^4$	Sweden
UAG506	*M. coerulea*	$1^1\ 1^4$	$2^1\ 2^2$	–	–	–	$2^1\ 2^2$	Iran
UAG509	*M. coerulea*	$1^1\ 1^4$	$2^1\ 2^2$	–	–	–	–	Georgia, USSR
–	'Saskatchewan White'[a]	$1^2\ 1^4$	$2^1\ 2^2$	–	–	$1^1\ 1^5$	–	Unknown

[a] Possibly *M. coerulea* or *M. hemicycla*

with either a superscript number or a letter – the former designating the locus and the latter the allele at that locus. For example, *Prx-1*^{2} is the designation for allele 2 at peroxidase locus 1; while *Prx-1*^{n} designates the null allele with no apparent enzyme activity. The genotypes are inferred from the electrophoretic patterns (zymograms) and all allozymes of the same electrophoretic class are assumed to be determined by same allele. The symbols PRX and LAP will be used when referring to the enzyme phenotypes.

Plant Culture

This study was initiated with about 50 plants per wild accession which were grown in pots in growth chambers at 21°C under 16 hrs of light, (300 μ sec^{-1} m^{-2}) and at 16°C under 8 hrs of darkness. Plants were selected on the basis of PRX and LAP electrophoretic variability, re-rooted and subsequently selfed or crossed following the techniques of Lesins (1955). Since different degrees of self-incompatibility were evident in these plants, selfing was continued until an adequate amount of seed for progeny tests was secured. The seed was scarified and germinated in Petri dishes lined with moist filter paper. When the cotyledons appeared, the seedlings were transplanted to wooden flats which had plexiglass covers to prevent dehydration.

Recombination and Segregation Analyses

The log-likelihood ratio test statistic G (Sokal and Rohlf 1969) was used to assess the goodness of fit of progeny distribution to expected Mendelian segregation ratios. The data for pairs of loci were tested for goodness of fit to expected segregation ratios at each locus separately and to the hypothesis of independent assortment (or free recombination) of the two loci. The maximum likelihood estimates of the recombination fraction were determined in those data which gave evidence of satisfactory segregation at each locus separately, but statistically significant deviations from the null hypothesis of free recombination. For the methodology of maximum likelihood estimation of recombination fractions, see for example, Allard (1956) and Morton (1962).

Definitions: Let $P(f_i \mid \theta)$ be the probability of occurrence of the progeny distribution, f_i, from cross i, given that the two loci are linked with recombination fraction θ. The probability of occurrence assuming free recombination is given by $P(f_i \mid ½)$.

The likelihood ratio $\dfrac{P(f_i \mid \theta)}{P(f_i \mid ½)}$

is the odds in favour of linkage versus no linkage at a specified value of θ. The maximum likelihood estimate of the recombination fraction $\hat{\theta}$, is that value which has the greatest odds in favor of linkage relative to free recombination.

The lod score (lod = logarithm of the odds) is defined by

$$z_i(\theta) = \log_{10} \frac{P(f_i \mid \theta)}{P(f_i \mid ½)} .$$

The maximum likelihood estimate of the recombination fraction was determined by an iterative procedure as that value in the interval $0 < \theta < 0.5$ which maximized the function $z(\theta)$. Maximum likelihood estimates were obtained for the progeny distribution of each informative cross and for the pooled results of the crosses for a particular pair of loci.

The lod scores of several crosses involving the same pair of loci can be summed to provide a pooled estimate of the odds in favor of linkage. Thus,

$$Z(\theta) = \sum_{i=1}^{n} z_i(\theta)$$

where n is the number of independent crosses.

Results

Peroxidase

Segregation of the band phenotypes was studied in several crosses to determine the number of loci and alleles. The alleles are listed in Table 1 and their allozymes are shown diagrammatically in Fig. 1. When root tissue was used for the assay, it was possible to differentiate among three sets of anodal and one set of cathodal allozymes. It is inferred that there are four structural loci. It is likely that additional loci are involved in the expression of cathodal peroxidases, but these have not been studied so far.

The allozymes of locus *Prx-1* were located closest to the anodal front. They are expressed as closely spaced double bands (Figs. 1, 3). In leaf tissue the alleles of locus *Prx-1* appear not to be expressed (Fig. 4). Low peroxidase activity was found in ovules and seeds for both anodal and cathodal isozymes. Leaf and root tissue were crushed to-

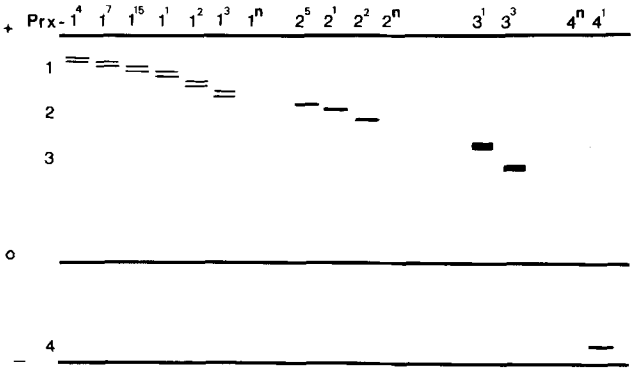


Fig. 1. Banding phenotypes for peroxidases. (*Prx-1*^{3} and *Prx-2*^{n} are not included in this study)

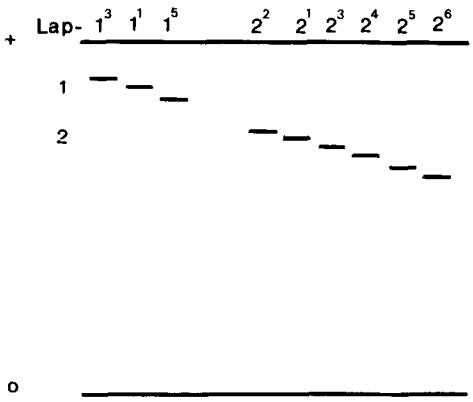
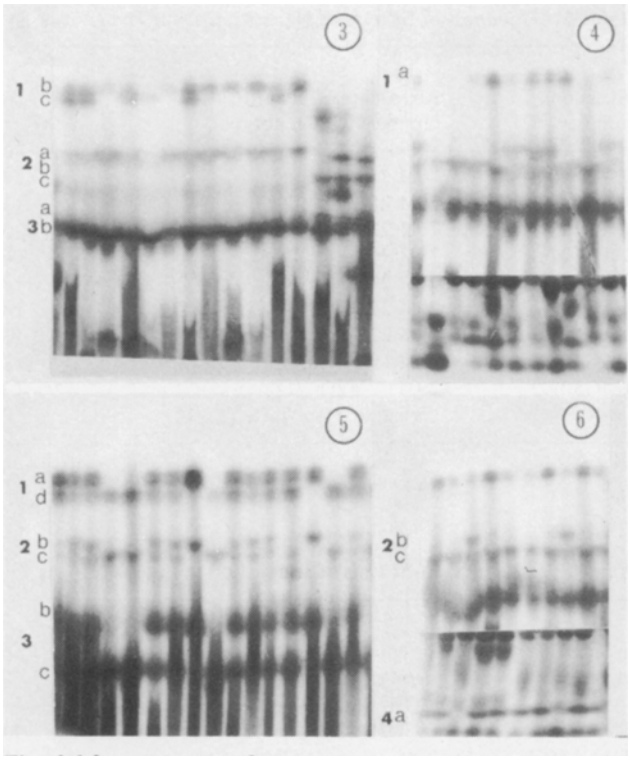


Fig. 2. Banding phenotypes for leucine-aminopeptidases

gether to maximize enzyme activity. Phenotypes that could not be determined on a first attempt were subsequently determined by re-rooting the seedlings and assaying newly developed adventitious roots.

The allozymes of alleles *Prx-1*^{7} and *Prx-1*^{15} migrate so close together that they overlap for one of the bands. Thus, the heterozygote displays a three-band phenotype (Fig. 3). A similar situation may occur with respect to the pairs of alleles *Prx-1*^{4}, *Prx-1*^{7} and *Prx-1*^{15}, *Prx-1*^{1} although crosses were not done for these particular combinations. In general, the *Prx-1* alleles segregated as single multiple-allelic series with the expected phenotypic ratios for monomeric enzymes (Table 2). The exceptions were the progeny of the plants 79M 2-42 and 'Saskatchewan White' where there was a deficiency in or absence of a homozygote class.

Interestingly, the null PRX-1^n phenotype is missing not only a double band in the PRX-1 region but also the constant, cathodal band (Fig. 4). This latter band appears in all of the species studied. The *Prx-1*^{n} allele is recessive at least with respect to *Prx-1*^{4} (Table 2). *Prx-1* is the most genetically variable of the four loci. At least four additional variants at this locus are under study.



Figs. 3-6.3 Alleles *Prx-1*^{7} (band 1b) and *Prx-1*^{15} (band 1c) segregating in the progeny of plant 79M 25-33. These alleles overlap their lower and upper band, respectively, resulting in a three banded phenotype for the heterozygote *Prx-1*^{7}/*Prx-1*^{15} (for example, plant two from the left). In addition all the plants in this progeny have the alleles *Prx-2*^{5} (band 2a) and *Prx-3*^{1} (band 3b). The last three plants from the left are reference plants, with the alleles *Prx-2*^{1} (band 2b) and *Prx-2*^{2} (band 2c). The second plant from the right has *Prx-3*^{2} (band 3a). 4 Gel showing plants with the null allele *Prx-1*^{n} (plants three and ten from the left). Note the absence of the end cathodal band in the *Prx-1*^{n} homozygous plants. In leaf tissue, locus *Prx-1* is inactive but the locus determining the end cathodal band is active (plant two from the left). Allele *Prx-1*^{4} (band 1a) is used as a reference. **5** Linkage between loci *Prx-1*, *Prx-2* and *Prx-3*, in the progeny of plant 79M 22-11 (genotype *Prx-1*^{4}, *Prx-2*^{1}, *Prx-3*^{1}/*Prx-1*^{2}, *Prx-2*^{2}, *Prx-3*^{3}). Homozygous plants for *Prx-1*^{4} (band 1a) are also homozygous for *Prx-2*^{1} (band 2b) and *Prx-3*^{1} (band 3b) (plants eight and 14 from the left). Homozygous plants for *Prx-1*^{2} (band 1d) are homozygous for *Prx-2*^{2} (band 2c) and for *Prx-3*^{3} (band 3c) (plants four, five, nine, and 15 from the left). The heterozygotes for one locus are heterozygous for the other two loci. **6** Linkage between the anodal locus *Prx-2* and the cathodal locus *Prx-4*. Progeny resulting from crossing plant 79M 20-6 (homozygous for *Prx-2*^{2} (band 2c) and *Prx-4*^{n}) by 79M 20-15 (*Prx-2*^{1} (band 2b), *Prx-4*^{n}/*Prx-2*^{2}, *Prx-4*^{1} (band 4a)). Heterozygotes *Prx-2*^{1}/*Prx-2*^{2} are null for *Prx-4* (plants three and eight from the left)

The allozymes of locus *Prx-2* migrate about half the distance from the origin to the region immediately below the PRX-1 allozymes. Three alleles were studied that were expressed as single bands (Figs. 3, 5). The alleles *Prx-2*^{1}, *Prx-2*^{2} and *Prx-2*^{5} segregated as expected for a single locus. However, the homozygotes *Prx-2*^{2}/*Prx-2*^{2} were absent

Table 2. Goodness-of-fit tests of segregation at the *Prx-1* locus

Acession number	Parental plant identification number (with genotype)	Distribution of progeny			Expected phenotypic ratio	G
UAG116	79M 25-46($1^1/1^7$)	54(1^1)	97($1^1/1^7$)	69(1^7)	1:2:1	4.91
UAG116	79M 25-33($1^7/1^{15}$)	11(1^7)	26($1^7/1^{15}$)	11(1^{15})	1:2:1	0.33
UAG127	79M 20-51($1^1/1^4$)	5(1^1)	15($1^1/1^4$)	3(1^4)	1:2:1	2.67
UAG127	79M 20-6 ($1^n/1^4$)	6(1^n)		31(1^4)	1:3	1.67
UAG127	79M 20-6 ($1^n/1^4$) × 79M 20-51($1^1/1^4$)	14(1^1)	8($1^1/1^4$)	20(1^4)	1:1:2	1.75
UAG127	79M 20-50($1^n/1^4$) × 79M 20-51($1^1/1^4$)	14(1^1)	8($1^1/1^4$)	27(1^4)	1:1:2	2.38
UAG128	79M 22-11($1^2/1^4$)	13(1^2)	43($1^2/1^4$)	17(1^4)	1:2:1	2.86
UAG506	79M 18-13($1^1/1^4$)	3(1^1)	17($1^1/1^4$)	9(1^4)	1:2:1	4.01
UAG509	79M 2-42($1^1/1^4$)	13(1^1)	30($1^1/1^4$)	2(1^4)	1:2:1	14.11***
–	'Saskatchewan White'($1^2/1^4$)	0(1^2)	20($1^2/1^4$)	11(1^4)	1:2:1	17.90***

*** $P < 0.001$

Table 3. Goodness-of-fit tests of segregation at the *Prx-2* locus

Accession number	Parental plant identification number (with genotype)	Distribution of progeny			Expected phenotypic ratio	G
UAG116	79M 25-33($2^1/2^5$)	11(2^1)	26($2^1/2^5$)	11(2^5)	1:2:1	0.33
UAG116	79M 25-46($2^1/2^5$)[a]	49(2^1)	167($2^1/2^5 + 2^5$)		1:3	0.63
UAG127	79M 20-51($2^1/2^5$)	7(2^1)	14($2^1/2^5$)	2(2^5)	1:2:1	4.04
UAG127	79M 20-15($2^1/2^2$) × 79M 20-6($2^2/2^2$)	31(2^2)	24($2^1/2^2$)		1:1	0.89
UAG 127	79M 20-2 ($2^1/2^2$) × 79M 20-6($2^2/2^2$)	18(2^2)	12($2^1/2^2$)		1:1	1.21
UAG127	79M 20-51($2^1/2^5$) × 79M 20-50($2^1/2^1$)	26(2^1)	24($2^1/2^5$)		1:1	0.08
UAG128	79M 22-11($2^1/2^2$)	15(2^1)	41($2^1/2^2$)	17(2^2)	1:2:1	1.24
UAG506	79M 18-13($2^1/2^2$)	9(2^1)	17($2^1/2^2$)	4(2^2)	1:2:1	2.50
UAG509	79M 2-42($2^1/2^2$)	7(2^1)	37($2^1/2^2$)	0(2^2)	1:2:1	32.14***
–	'Saskatchewan White'($2^1/2^2$)	2(2^1)	20($2^1/2^2$)	9(2^2)	1:2:1	7.47*

* $P < 0.05$
*** $P < 0.001$
[a] The progeny were classified into two groups by including plants of genotypes *Prx-2*^{1}/*Prx-2*^{5} and *Prx-2*^{5}/*Prx-2*^{5} in the same class. The resolution of the bands was not sufficient for further discrimination of genotypes among these progeny

Table 4. Goodness-of-fit tests of segregation at the *Prx-3* and *Prx-4* loci

Accession number	Parental plant identification number (with genotype)	Distribution of progeny			Expected phenotypic ratio	G
UAG127	79M 20-51($3^1/3^3$)	4(3^1)	14($3^1/3^3$)	6(3^3)	1:2:1	1.07
UAG127	79M 20-50($3^1/3^3$) × 79M 20-51($3^1/3^3$)	12(3^1)	28($3^1/3^3$)	9(3^3)	1:2:1	1.43
UAG127	79M 20-15($4^n/4^1$) × 79M 20-6 ($4^n/4^n$)	26(4^n)		31(4^1)	1:1	0.44
UAG128	79M 22-11($3^1/3^3$)	14(3^1)	43($3^1/3^3$)	19(3^3)	1:2:1	2.08

and *Prx-2*^{1}/*Prx-2*^{1} were deficient among the progeny of plant 79M 2-42 (Table 3).

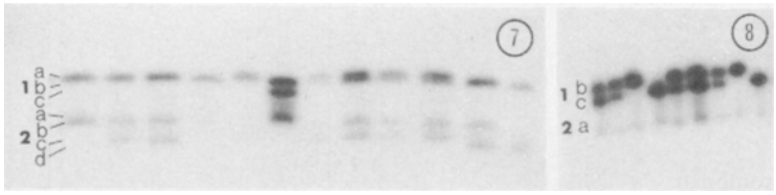
Two alleles of locus *Prx-3*, *Prx-3*^{1} and *Prx-3*^{3}, were studied. The allozymes migrate to positions between the origin and the PRX-2 region (Figs. 3, 5). Crosses involving these alleles also segregated according to expectation (Table 4).

An isozyme of locus *Prx-4* represents the only band so far genetically differentiated. Most plants have a band which migrates at the cathodal front; the isozyme of locus

Prx-4 migrates to a position immediately above this band (Figs. 1, 6). Two alleles were inferred to be segregating at this locus: allele $Prx\text{-}4^1$ (band 4a, Fig. 6) and a null allele $Prx\text{-}4^n$. Segregation was as expected in the progeny of a single cross (Table 4).

Leucine-Aminopeptidase

Strong enzyme activity was found in crude extracts of tissues from root, leaf, ovules, and seed. In all cases two loci, *Lap-1* and *Lap-2*, determining anodal allozymes could be differentiated (Figs. 2, 7). LAP-1 allozymes migrate closer to the anodal front and display higher enzyme activity than LAP-2 allozymes (Figs. 7, 8). This locus has to be scored while the gels are in the stain before the LAP-2 bands have completed the reaction. Three alleles were studied for *Lap-1* and six alleles for *Lap-2* (Fig. 2). Additional alleles at each locus, including null alleles, are inferred from isozyme patterns. The allozymes of each locus are single bands and in most cases segregate as ex-



Figs. 7 and 8. 7 Alleles $Lap\text{-}1^1$ (band 1b), $Lap\text{-}1^3$ (band 1a), $Lap\text{-}2^3$ (band 2b), $Lap\text{-}2^4$ (band 2c), and $Lap\text{-}2^5$ (band 2d) segregating in the progeny of plants 79M 20-2 ($Lap\text{-}1^1, Lap\text{-}2^3/Lap\text{-}2^4$) by 79M 20-6 ($Lap\text{-}1^1/Lap\text{-}1^3, Lap\text{-}2^3/Lap\text{-}2^5$). Plant one from the left is homozygous for $Lap\text{-}1^1$ and $Lap\text{-}2^3$, while plant eight is heterozygous for $Lap\text{-}1^1/Lap\text{-}1^3$ and $Lap\text{-}2^3/Lap\text{-}2^4$. Plant 11 is homozygous for $Lap\text{-}1^1$ and heterozygous for $Lap\text{-}2^3/Lap\text{-}2^5$, while plant 12 is heterozygous for $Lap\text{-}2^4/Lap\text{-}2^5$. Plant six from the left, used as a reference ('Saskatchewan White'), has the genotype $Lap\text{-}1^1/Lap\text{-}1^5$ (band 1c), $Lap\text{-}2^1$ (band 2a). 8 Progeny of plant 'Saskatchewan White' segregating for $Lap\text{-}1^1$ (band 1b) and $Lap\text{-}1^5$ (band 1c)

Table 5. Goodness-of-fit tests of segregation at the *Lap-1* locus

Accession number	Parental plant identification number (with genotype)	Distribution of progeny			Expected phenotypic ratio	G
UAG116	79M 25-46($1^1/1^3$)	51(1^1)	115($1^1/1^3$)	42(1^3)	1:2:1	3.20
UAG127	79M 20-6 ($1^1/1^3$)	13(1^1)	24($1^1/1^3$)	2(1^3)	1:2:1	11.11**
UAG127	79M 20-51($1^1/1^3$)	3(1^1)	15($1^1/1^3$)	5(1^3)	1:2:1	2.67
UAG127	79M 20-6 ($1^1/1^3$) × 79M 20-15($1^1/1^1$)	26(1^1)	23($1^1/1^3$)		1:1	0.18
UAG127	79M 20-51($1^1/1^3$) × 79M 20-50($1^1/1^1$)	23(1^1)	27($1^1/1^3$)		1:1	0.32
UAG127	79M 20-6 ($1^1/1^3$) × 79M 20-51($1^1/1^3$)[a]	12(1^3)	29($1^1/1^3 + 1^1$)		1:3	0.39
–	'Saskatchewan White'($1^1/1^5$)	3(1^1)	24($1^1/1^5$)	4(1^5)	1:2:1	10.00**

** $P < 0.01$

[a] The progeny were classified into two groups because of overstaining of the bands. The first class included $Lap\text{-}1^3/Lap\text{-}1^3$ and the second class included $Lap\text{-}1^1/Lap\text{-}1^3$ and $Lap\text{-}1^1/Lap\text{-}1^1$

Table 6. Goodness-of-fit tests of segregation at the *Lap-2* locus

Accession number	Parental plant identification number (with genotype)	Distribution of progeny				Expected phenotypic ratio	G
UAG127	79M 20-6 ($2^3/2^5$)	1(2^3)	28($2^3/2^5$)	10(2^5)		1:2:1	16.21***
UAG127	79M 20-51($2^3/2^6$)	6(2^3)	12($2^3/2^6$)	5(2^6)		1:2:1	0.13
UAG127	79M 20-2 ($2^3/2^4$) × 79M 20-15($2^3/2^3$)	10(2^3)	6($2^3/2^4$)			1:1	1.01
UAG127	79M 20-6 ($2^3/2^5$) × 79M 20-15($2^3/2^3$)	22(2^3)	27($2^3/2^5$)			1:1	0.51
UAG127	79M 20-2 ($2^3/2^4$) × 79M 20-6 ($2^3/2^5$)	8(2^3)	11($2^3/2^5$)	8($2^3/2^4$)	3($2^4/2^5$)	1:1:1:1	4.99
UAG127	79M 20-6 ($2^3/2^5$) × 79M 20-51($2^3/2^6$)	6(2^3)	11($2^3/2^6$)	11($2^3/2^5$)	14($2^5/2^6$)	1:1:1:1	3.39
UAG127	79M 20-51($2^3/2^6$) × 79M 20-50($2^5/2^6$)	4(2^6)	17($2^3/2^5$)	18($2^3/2^6$)	5($2^5/2^6$)	1:1:1:1	16.55***
UAG128	79M 22-11($2^3/2^6$)	3(2^3)	54($2^3/2^6$)	20(2^6)		1:2:1	26.92***
UAG1827	79M 45-12($2^3/2^4$)	4(2^3)	50($2^3/2^4$)	20(2^4)		1:2:1	20.98***
UAG506	79M 18-13($2^1/2^2$)	7(2^1)	14($2^1/2^2$)	8(2^2)		1:2:1	0.10

*** $P < 0.001$

pected for monomeric enzymes (Tables 5, 6 and Figs. 7, 8). Several exceptions to expected segregation ratios were found, which generally involved a deficiency in or absence of homozygotes (Tables 5, 6).

Linkage

Examination of the peroxidase segregation patterns revealed linkage between the four loci. Figure 5 shows the segregating progeny of plant 79M 22-11 with genotype *Prx-1*^{4}, *Prx-2*^{1}, *Prx-3*^{1}/*Prx-1*^{2}, *Prx-2*^{2}, *Prx-3*^{3}. The linkage between *Prx-2* and *Prx-4* loci is illustrated in Fig. 6 for the segregating progeny resulting from the cross 79M 20-6 x 79M 20-15 of genotypes *Prx-2*^{2}, *Prx-4*^{n}/*Prx-2*^{2}, *Prx-4*^{n} x *Prx-2*^{1}, *Prx-4*^{n}/*Prx-2*^{2}, *Prx-4*^{1}.

In all crosses which where informative for recombination analysis, the phase of linkage in each parent was determined by inspection of the progeny distribution. Maximum likelihood estimates of the recombination fraction were obtained as outlined in the section on methods. The results of the linkage analysis are given in Table 7. Since the heterogeneity of the estimates of the recombination fraction for each interval was not statistically significant, pooled estimates were calculated. There is strong evidence for a peroxidase linkage group with recombination fractions of 5.2% between loci *Prx-1* and *Prx-2* (Z = 57.5, 5 crosses), 9.1% between loci *Prx-1* and *Prx-3* (Z = 25.5, 3 crosses), and 15.9% between loci *Prx-2* and *Prx-3* (Z = 13.7, 3 crosses). The estimate of the recombination fraction between loci *Prx-2* and *Prx-4* is 10.5% (Z = 8.8). An ordering of the *Prx-1*, *Prx-2* and *Prx-3* loci consistent with these results is:

Prx-2 – 5.2% – *Prx-1* – 9.1% – *Prx-3*.

The position of *Prx-4* locus relative to *Prx-2* and *Prx-3* has not been determined. It should be noted that the implied ordering of the *Prx* intervals is not consistent from family to family (Table 7). Thus, there is some uncertainty about the sequence of the peroxidase loci.

The evidence in favor of linkage between the two *Lap* loci is not as strong as that for the *Prx* loci. Conditional on the phases of linkage of the two crosses given in Table 8, the pooled estimate of the recombination fraction is 31% (Z = 2.0) for the *Lap-1*, *Lap-2* interval. However, if it is assumed that coupling and repulsion phases for either cross are equally likely *a priori*, then the estimate of the recombination fraction remains the same, but the lod score is decreased (Z = 1.7). Tests of independent assortment of PRX and LAP allozymes show satisfactory fit for those crosses which gave expected segregation ratios at each locus separately. Therefore, the *Lap* loci are either in a different linkage group than the cluster of *Prx* loci or are sufficiently far apart on the same chromosome to allow for free recombination.

Table 7. Maximum likelihood estimation of recombination fractions for the *Prx* linkage group

Accession number	Parental plant identification number (with genotype)	Recombination fraction [$\hat{\theta}$] and lod score [$z(\hat{\theta})$]							
		Prx-1, Prx-2		*Prx-1, Prx-3*		*Prx-2, Prx-3*		*Prx-2, Prx-4*	
		$\hat{\theta}$	$z(\hat{\theta})$	$\hat{\theta}$	$z(\hat{\theta})$	$\hat{\theta}\theta$	$z(\hat{\theta})$	$\hat{\theta}$	$z(\hat{\theta})$
UAG116	79M 25-33(1^7, 2^5/1^{15}, 2^1)	0.021	17.46	–	–	–	–	–	–
UAG127	79M 20-51(1^4, 2^5/1^1, 2^1)	0.068	5.15	0.045	6.37	0.068	5.45	–	–
UAG127	79M 20-51(1^4, 2^5, 3^1/1^1, 2^1, 3^3) x 79M 20-50(1^4, 2^1, 3^3/1^n, 2^1, 3^1)	0.080	9.00	0.131	4.73	0.095	3.45	–	–
UAG127	79M 20-6 (2^2, 4^n/2^2, 4^n) x 79M 20-15(2^1, 4^n/2^2, 4^1)	–	–	–	–	–	–	0.105	8.83
UAG128	79M 22-11(1^4, 2^1, 3^1/1^2, 2^2, 3^3)	0.072	16.77	0.091	14.89	0.203	5.91	–	–
UAG506	79M 18-13(1^4, 2^1/1^1, 2^2)	0.017	10.45	–	–	–	–	–	–
Pooled Estimates		0.052	57.53	0.091	25.51	0.159	13.67	–	–

Table 8. Maximum likelihood estimation of recombination fractions for the *Lap* linkage group

Accession number	Parental plant identification number (genotype with inferred linkage phase)	Recombination Fraction [$\hat{\theta}$] and lod score [$z(\hat{\theta})$] *Lap-1, Lap-2* $\hat{\theta}$	$z(\hat{\theta})$
UAG127	79M 20-51($1^1, 2^6/1^3, 2^3$)	0.170	2.47
UAG127	79M 20-6 ($1^1, 2^5/1^3, 2^3$) X 79M 20-15($1^1, 2^3/1^1, 2^3$)	0.388	0.54
Pooled Estimate		0.309	2.04

Discussion

The inheritance of allozyme variation at each of four peroxidase loci and two leucine-aminopeptidase loci, with the exception of the PRX-1^n phenotype (see below), is consistent with independence of gene expression of the isozyme structural loci. The deficiency in or absence of segregating classes in several of the crosses might be explained by the close linkage of balanced lethals and semi-lethals to the enzyme loci. Hidden deleterious genetic variation would not be unexpected in *Medicago* species that reproduce almost exclusively by outcrossing. If inbred, they suffer a severe reduction in fitness as a consequence of homozygosity for recessive lethals and semi-lethals. Low seed production was observed in many progenies from selfed or sibling crosses. Often the products of such crosses were empty pods or pods containing just one seed whereas at least three seeds per pod are expected. The seeds from such crosses generally failed to germinate or produced abnormal seedlings that were, for example, dwarfed, chlorophyll deficient or lacked growing points.

The PRX-1^n phenotype, in which certain peroxidase bands at both anodal and cathodal positions are missing, is interesting. An explanation for the null phenotype is that it results from a chromosomal deletion which includes the *Prx-1* locus and the structural locus of the particular cathodal isozyme, assuming that both loci are closely linked. This two-locus model is consistent with the observation that PRX-1 allozymes are not expressed in leaf tissue although cathodal bands are present (Fig. 4). This suggests that expression of the *Prx-1* locus can be regulated independently of that of the *Prx* structural loci that determine cathodal isozymes.

Almost all of the data for the analyses of linkage (Tables 7, 8) have been obtained from crosses of *Medicago falcata* because they were more genetically variable (Quiros 1979). One additional set of linkage data for the *Prx-1*, *Prx-2* interval came from a cross of *Medicago coerulea*, the progeny of 79M 18-13. The estimate of the recombination fraction, 1.7% in this family, is the smallest value for this interval among all progenies. It is, however, close to that estimated in the family of the *Medicago falcata* plant 79M 25-33, which is 2.1% (Table 7). The chi-square test of heterogeneity of the estimates of the recombination fraction was not statistically significant ($X^2 = 5.99$, 4 df). Therefore, there is no compelling evidence that *Medicago coerulea* and *Medicago falcata* have different recombination values for the *Prx-1*, *Prx-2* interval. This is not unexpected, since they appear to be different varieties of a single species rather than two different species (Lesins and Lesins 1979).

The finding that the four peroxidase loci comprise a linkage group in *Medicago* is similar to that in *Lycopersicon* (Rick et al. 1979). In *Lycopersicon* there is a pair of linked loci on each of chromosomes two and three. In rice, however, there are two unlinked loci determining leaf peroxidase and the enzyme is dimeric (Pai et al. 1973).

The evidence for loose linkage between the *Lap-1* and *Lap-2* loci in *Medicago* is weak. The pooled estimate of the recombination fraction for the *Lap-1*, *Lap-2* interval is 31%, coupling and repulsion phases considered equally likely. The test cross 79M 20-6 x 79M 20-15 gives an estimate of the recombination fraction of 38.8%, assuming the phase is known. The odds in favor of such loose linkage relative to free recombination is only 3.5:1 (Table 8). The family of plant 79M 20-51, which is a progeny test and hence more informative for this type of analysis, provides evidence of tighter linkage, assuming phase is known. The estimate of the recombination fraction is 17%, with odds of approximately 300:1, which is very close to the value found in maize. In maize the estimated recombination for the *Lap* interval is 16% (Beckman et al. 1964). In pine, however, the two leucine-aminopeptidase loci show no linkage (Adams and Joly 1980).

It is common to find linkage between loci determining isozymes in other plants. For instance, two loci for glutamate oxaloacetate are linked and four esterase loci are linked in tomatoes (Tanksley and Rick 1980). The loci for the respiratory enzymes alcohol dehydrogenase and phosphoglucomutase are also linked in these species (Tanksley 1979). A similar situation pertains to barley, where three of seven esterase loci are linked (Kahler and Allard 1970). An explanation of these common observations of linked

loci is that they have originated by gene duplication (see Roose and Gottlieb 1980; Tanksley and Rick 1980).

Evidence for multiple loci and multiple allelism determining isozyme variation in *Medicago* is presented. Although multiple allelism has been assumed for a long time in alfalfa (Busbice et al. 1972; Dunbier and Bingham 1975) direct evidence had not been presented. Earlier evidence of gene clusters determining a biochemical trait in *Medicago* was suggested by Lesins (1961) for anthocyanin production. The genetic polymorphisms of the *Prx* and *Lap* loci in alfalfa suggest that a continued search for genetic variability of other biochemical markers will be successful in tetraploids as well as diploids. These biochemical markers can then be utilized to elucidate the genetic basis of tetraploid alfalfa. For example, there is lack of agreement as to whether alfalfa is an autotetraploid or an allotetraploid, although most of the evidence supports the former hypothesis (Busbice et al. 1972; Stanford 1951). In addition, these markers can be used in marked chromosome substitution in the tetraploid crop and for detection of natural cross pollination (see Quiros 1980b). They can also be utilized in the study of the genetic structure of *Medicago* populations. Additionally, biochemical markers for various chromosome segments might prove to be an adequate system to test the hypothesis of maximum heterozygosity in tetraploids (Dunbier and Bingham 1975), that is, tetrallelic plants for the loci determining yield are more productive than those which are tri-, di- or mono-allelic for those loci.

There is differential expression of PRX isozymes in differentiated tissues of diploid *Medicago*. It is of fundamental importance to understand how multiple loci are regulated in the development of diploid and tetraploid alfalfa.

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Literature

Adams, W.T.; Joly, R.J. (1980): Genetics of allozyme variants in loblolly pine. J. Hered. **71**, 33-40

Allard, R.W. (1956): Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia **24**, 235-278

Barnes, D.K.; Hanson, C.H. (1967): An illustrated summary of genetic traits in tetraploid and diploid alfalfa. US Dept. Agr. Techn. Bull. 1370

Beckman, L.; Scandalios, J.G.; Brewbaker, J.L. (1964): Genetics of leucine aminopeptidase isozymes in maize. Genetics **50**, 899-904

Busbice, T.H.; Hill Jr., R.R.; Carnahan, H.L. (1972): Genetics and breeding procedures. In: Alfalfa Science and Technology, Monograph, No. 15 (ed.: Hanson, C.H.), pp. 283-318. Madison, Wis.: Am. Soc. Agron.

Dunbier, M.W.; Bingham, E.T. (1975): Maximum heterozygosity in alfalfa: Results using haploid-derived autotetraploids. Crop Sci. **15**, 527-531

Kahler, A.L.; Allard, R.W. (1970): Genetics of isozyme variants in barley. 1. Esterases. Crop Sci. **10**, 444-448

Kasha, K.J.; McLennan, H.A. (1967): Trisomics in diploid alfalfa. 1. Production, fertility and transmission. Chromosoma **21**, 232-242

Lesins, K. (1955): Techniques for rooting cuttings, chromosome doubling and flower emasculation in alfalfa. Can. J. Agr. Sci. **35**, 58-67

Lesins, K. (1961): Interspecific crosses involving alfalfa. I. *Medicago dzhawakhetica* (Bordz.) Vass. × *M. sativa* L. and its peculiarities. Can. J. Genet. Cytol. **3**, 135-152

Lesins, K.A.; Lesins, I. (1979): Genus *Medicago* (Leguminosae): A Taxogenetic Study, The Hague: W. Junk

Morton, N.E. (1962): Segegration and linkage. In: Methodology in Human Genetics (ed. Burdette, W.J.), pp. 17-52. San Fransisco: Holden-Day:

Pai, C.; Endo, T.; Oka, H.I. (1973): Genic analysis for peroxidase isozymes and their organ specificity in *Oryza perennis* and *O. sativa*. Can. J. Genet. Cytol. **15**, 845-853

Quiros, C.F. (1979): Origin of alfalfa cultivars as revealed by closely related diploids. Forage Notes **24**(2), 18-24

Quiros, C.F. (1980a): Identification of alfalfa plants by enzyme electrophoresis. Crop Sci. **20**, 262-264

Quiros, C.F. (1980b): Enzyme electrophoresis as an aid for alfalfa breeding. 27th Natl. Alfalfa Improvement Conference Report (in press)

Rick, C.M.; Tanksley, S.D.; Fobes, J.F. (1979): A pseudoduplication in *Lycopersicon pimpinellifolium*. Proc. Nat. Acad. Sci. (USA) **76**, 3435-3439

Roose, M.L.; Gottlieb, L.D. (1980): Alcohol dehydrogenase in the diploid plant *Stephanomeria exigua* (Compositae): Gene duplication, mode of inheritance and linkage. Genetics **95**, 171-186

Sokal, R.R.; Rohlf, F.J. (1969): Biometry. San Fransisco: Freeman

Stanford, E.H. (1951): Tetrasomic inheritance in alfalfa. Agron. J. **43**, 222-225

Stanford, E.H.; Clement Jr., W.M.; Bingham, E.T. (1972): Cytology and evolution of the *Medicago sativa-falcata* complex. In: Alfalfa Science and Technology, Monograph No. 15 (ed. Hanson, C.H.), pp. 87-101. Madison, Wis. Soc. Agron.

Tanksley, S.D. (1979): Linkage, chromosomal association, and expression of *Adh-1* and *Pgm-2* in tomato. Biochem. Genet. **17**, 1159-1167

Tanksley, S.D.; Rick, C.M. (1980): Genetics of esterases in species of *Lycopersicon*. Theor. Appl. Genet. **56**, 209-219

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