Genetics and Molecular Biology, 25, 1, 65-72 (2002)

www.sbg.org.br

Variability among inbred lines and RFLP mapping of sunflower isozymes

Alicia D. Carrera[1], G. Pizarro[1], M. Poverene[1], S. Feingold[2,4], A.J. León[2] and S.T. Berry[3]

[1]*Universidad Nacional del Sur, Departamento de Agronomía y CERZOS (CONICET) Bahía Blanca, Argentina.*
[2]*Advanta Semillas, Centro de Biotecnología, Balcarce. Argentina.*
[3]*SES-Europe NV/SA, Advanta Biotechnology Laboratory, Tienen, Belgium.*
[4]*Present address: Laboratorio de Biotecnología Agrícola, INTA Balcarce, Argentina.*

Abstract

Eight isozyme systems were used in this study: acid phosphatase (ACP), alcohol dehydrogenase (ADH), esterase (EST), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), 6-phosphogluconate dehydrogenase (PGD), and phosphoglucomutase (PGM). The polymorphism of these enzyme systems was studied in 25 elite inbred lines. A total of 19 loci were identified, but only eight of them were polymorphic in the germplasm tested. The polymorphic index for the eight informative markers ranged from 0.08 to 0.57, with a mean value of 0.36.

Five isozyme loci were mapped in $F_{2:3}$ populations with existing RFLP data. *Est-1*, *Gdh-2* and *Pgi-2* were mapped to linkage groups 3, 14 and 9, respectively. As in previous reports, an ACP locus and a PGD locus were found to be linked, both located in linkage group 2 of the public sunflower map.

Key words: isozyme, polymorphisms, RFLP markers, sunflower, linkage map, *Helianthus annuus*.

Received: March 5, 2002; accepted: March 25, 2002.

Introduction

Sunflower (*Helianthus annuus* L.) is a diploid (2n = 34) species, which is second only to soybean in its importance as an annual oil seed crop. Despite its economic value, the number of simply inherited genes identified in sunflower is relatively small (Miller, 1992), and there is no classical genetic map for this species. Several sunflower RFLP linkage maps were published recently (Berry *et al*., 1995; Gentzbittel *et al*., 1995, 1999; Jan *et al*. 1998), but unfortunately the relationship between these different maps is unknown, because the vast majority of the markers are not publicly available. The mapping of major gene loci, such as the *Rf1* locus (Gentzbittel *et al*., 1995; Berry *et al*., 1997; Jan *et al*., 1998), the downy mildew resistance gene cluster (Vear *et al*., 1997), *Orobanche* resistance (Lu *et al*., 2000) and the H*yp-1* locus (León *et al*., 1996), as well as protein markers, such as seed storage proteins (Serre *et al*., 2001) and isozymes, will provide landmarks allowing the alignment of some linkage groups.

Isozymes have been used to assess genetic variation in both domesticated and wild sunflower populations (Dry and Burdon, 1986; Rieseberg *et al*., 1990; Cronn *et al*., 1997), as well as to establish phylogenetic relationships and speciation mechanisms within the genus *Helianthus* (Rieseberg *et al*., 1991; 1998). They have also been used to study intra specific variation in both wild (Carrera and Poverene, 1995; Carrera *et al*., 1996) and cultivated sunflowers (Carrera and Poverene, 1991; Quillet *et al*., 1992), as well as to identify interspecific hybrids (Carrera *et al*., 1996). In cultivated sunflower, Quillet *et al*. (1992) demonstrated that eight polymorphic isozyme loci could discriminate between distantly related inbred lines, whereas Tersac *et al*. (1994) were able to group cultivated populations according to their geographic origin by using isozyme data. Unfortunately, the relatively small number of isozyme systems limits their use in genetic mapping studies. However, isozymes are still routinely used by seed companies for seed purity testing of both inbred and hybrid seed lots, because, in comparison with RFLP markers, the assays are relatively cheap and quick to run. Therefore, knowledge about the linkage arrangement of isozyme loci would allow a more even sampling of the sunflower genome.

Berry *et al*., (1997) released a RFLP sunflower map for public research, comprising 81 loci covering all 17 linkage groups. To date, two classical genetic markers, the CMS restoration *Rf1* gene (Kinman, 1970) and the *HAG-5*

Send correspondence to Alicia D. Carrera. Departamento de Agronomía , Universidad Nacional del Sur, San Andrés 800, 8000 Bahía Blanca, Argentina. E-mail: acarrera@criba.edu.ar.

gene controlling 2S albumin (Allen *et al*., 1987), were included in this public map. The objectives of this study were: i) to investigate the level of genetic variation of isozymes in 25 elite sunflower inbred lines, and ii) to map polymorphic isozyme loci onto the public RFLP linkage map of cultivated sunflower.

Materials and Methods

Plant material

Twenty-five inbred lines were studied by electrophoretic assay (Table I), including the parents of four $F_{2:3}$ populations, which had previously been used for RFLP mapping studies. Ten seeds from each inbred line were analysed, in order to test for uniformity within the lines. The genotype of each F_2 plant was re-created by bulking 8-9 seeds from their F_3 progenies obtained by selfing.

Isozyme electrophoresis

Samples were prepared from seeds soaked for 24 h (48 h for PGM) (using) in a 0.1 M Tris-HCl-mercaptoethanol buffer (pH 7.5). The following enzymes were assayed: acid phosphatase (ACP), alcohol dehydrogenase (ADH), esterase (EST), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), 6-phosphogluconate dehydrogenase (PGD), and phosphoglucomutase (PGM). The isozymes were resolved on 12% horizontal starch gel; the buffer systems and staining methods are described in Carrera and Poverene (1995), after Soltis *et al*. (1983). The number of loci and alleles were interpreted according to Torres (1983), Kahler and Lay (1985), Rieseberg and Soltis (1989), and Carrera and Poverene (1991, 1995). Loci were designated by giving the number 1 to the most anodally migrating isozyme and sequentially numbering the additional loci in a decreasing order of electrophoretic mobility. The most anodally migrating allozyme was designated by the letter `a'.

Data analysis and map construction

The genetic similarity between two inbred lines was estimated according to Nei (1987), using the BIOSYS-1 program (Swofford and Selander, 1981) The genetic distance matrix was used to construct a phenogram by the UPGMA clustering method. The reliability of the UPGMA tree was examined using bootstrap resampling analysis, through a new version of the same BIOSYS-2 program, to construct a consensus tree through Phylip3.5C (Felsenstein, 1995). Various standard measures of genetic variation were also calculated, including the proportion of polymorphic loci (P), the mean number of alleles across all loci (A), the mean number of alleles per polymorphic locus (Ap), and the polymorphic index (PI; defined as $1 - \Sigma p_i^2$, where "pi" is the frequency of the i^{th} allele).

Table I - Origin of the sunflower inbred lines used in this study.

Inbred	Version	Origin	Source
ZENB1	Maintainer	Argentina	ADVANTA
ZENB8	Maintainer	Argentina	ADVANTA
ZENB9	Maintainer	Argentina	ADVANTA
ZENB10	Maintainer	Argentina	ADVANTA
ZENB11	Maintainer	Argentina	ADVANTA
ZENB12	Maintainer	Argentina	ADVANTA
ZENB13	Maintainer	Argentina	ADVANTA
0043	Maintainer	-	Edirne Institute, Turkey
HA300	Maintainer	Peredovik 301 (USA re-selection from the Russian Peredovik population)	USDA
HA302	Maintainer	Peredovik 304 (USA re-selection from the Russian Peredovik population)	USDA
HA89	Maintainer	Russian VNIIMK 8931 population	USDA
HA89DM	Maintainer	Russian VNIIMK 8931 population	ADVANTA
SD	Maintainer	-	INRA
ZENR1	Restorer	Argentina	ADVANTA
ZENR6	Restorer	Australia	ADVANTA
ZENR7	Restorer	Argentina	ADVANTA
ZENR8	Restorer	Argentina	ADVANTA
ZENR10	Restorer	Argentina	ADVANTA
ZENR11	Restorer	Argentina	ADVANTA
ZENR12	Restorer	Argentina	ADVANTA
ZENR13	Restorer	Australia	ADVANTA
ZENR14	Restorer	Argentina	ADVANTA
ZENR15 (HA2)	Restorer	Australia	ADVANTA
ZENR15 (W21)	Restorer	Australia	ADVANTA
P1380	Restorer	Romanian re-selection from the Russian Peredovik population	Fundulea Institute, Romania

The isozyme loci were mapped onto the public RFLP sunflower map. To construct this map, 81 genomic and cDNA probes were selected, and detected loci covering approximately 1200 cM of the sunflower genome, arranged into 17 linkage groups, as was demonstrated by segregation data from nine different F_2 populations (Berry *et al*., 1997). Isozyme linkage maps were constructed using data from four out of nine F_2 populations. The F_2 plants were self-pollinated to produce $F_{2:3}$ families, which were used as mapping populations (Table III).

The segregation of the alleles at each locus was checked against the expected ratios for a codominant marker in a F_2 population, using a chisquare test with a significance level of 5%. The linkage analyses were (performed with) made using Mapmaker version 3.0 (Lander *et al.*, 1987). A constant LOD score of 3.0 was used. The Haldane function was used to obtain the cM values. The isozyme loci were placed in the map by using the "try" command. The "error detection" command was used to check for mistakes in scoring and data entry, and for double crossover events. Linkage group and RFLP loci are named according to Berry *et al*. (1997).

Results

Polymorphism and genetic distance

The eight enzyme systems assayed revealed 19 loci, with a total of 28 alleles (A = 1.47). The average number of alleles per polymorphic locus (Ap) was 2.125. All polymorphic loci were bi-allelic, except for *Est-1*, which was tri-allelic. Eleven loci were monomorphic in the tested lines *(Adh-1, Adh-2, Gdh-1, Mdh-2, Mdh-3, Pgd-1, Pgd-2*, *Pgi-1*, *Pgm-2*, *Pgm3*, *Pgm-4*), and eight loci were found to be polymorphic (*Acp-1*, *Est-1*, *Gdh-2*, *Mdh-1*, *Mdh-4*, *Pgd-3*, *Pgi-2*, *Pgm-1*). The proportion of polymorphic loci (P) was therefore 0.42. There were 17 different genotypes, 12 (48%) of which were unique within this set of material (Table II). The PI ranged from 0.08 for *Pgi-2* to 0.57 for *Est-1*, the only system revealing three alleles, with a mean value of 0.36. No intra-line heterogeneity was observed, except for ZENR15 (W21), which had two *Acp-1* genotypes. The isozyme data were used according to Nei (1987), to calculate the genetic distance between all possible pairs of lines, and the result was used to construct the phenogram shown in Figure 1. The consensus tree, obtained by 100 bootstrap resampling of loci (not shown), resembled the original topology showed in Figure 1. Major groups of restorer and maintainer lines were similar in both the UPGMA and the consensus tree, although groups were resolved with low bootstrap values.

Table II - Allozyme genotypes observed in 25 sunflower lines and the polymorphic index (PI) value for each of the isozyme marker loci.

Inbred	*Acp-1*	*Est-1*	*Gdh-2*	*Pgd-3*	*Pgi-2*	*Pgm-1*	*Mdh-1*	*Mdh-4*
ZENB1	bb	cc	bb	Bb	aa	bb	bb	bb
ZENB8	bb	cc	bb	Bb	aa	aa	bb	bb
ZENB9	bb	cc	bb	bb	aa	bb	bb	bb
ZENB10	bb	cc	bb	bb	aa	bb	bb	bb
ZENB11	bb	cc	bb	bb	aa	bb	bb	bb
ZENB12	bb	cc	bb	bb	aa	bb	aa	bb
ZENB13	bb	cc	bb	bb	aa	bb	bb	bb
HA89	bb	cc	bb	aa	aa	bb	bb	bb
HA89DM	bb	cc	bb	aa	aa	bb	bb	bb
HA300	bb	aa	bb	bb	aa	bb	bb	bb
HA302	bb	aa	bb	bb	aa	bb	aa	aa
0043	bb	bb	aa	bb	aa	bb	aa	aa
SD	bb	cc	aa	aa	aa	bb	aa	aa
ZENR1	bb	bb	bb	bb	bb	bb	bb	bb
ZENR6	bb	bb	aa	bb	aa	aa	aa	bb
ZENR7	bb	bb	bb	aa	aa	bb	aa	aa
ZENR8	bb	cc	bb	bb	aa	bb	aa	aa
ZENR10	bb	cc	bb	bb	aa	bb	aa	aa
ZENR11	bb	bb	bb	aa	aa	bb	aa	bb
ZENR12	bb	bb	aa	aa	aa	bb	aa	aa
ZENR13	aa	bb	bb	bb	aa	aa	aa	aa
ZENR14	bb	bb	bb	aa	aa	bb	aa	aa
ZENR15(HA2)	bb	bb	bb	bb	aa	aa	aa	aa
ZENR15(W21)	aa/bb	bb	bb	bb	aa	aa	aa	aa
P1380	aa	bb	aa	bb	aa	bb	bb	bb
PI	0.18	0.57	0.32	0.40	0.08	0.32	0.49	0.49

Table III - The segregation ratio of the five polymorphic isozyme marker loci in the $F_{2:3}$ mapping populations

Locus	$F_{2:3}$ populations	N. of plants	Observed ratios			P
			aa	ab	bb	
Acp-1	ZENR11 x ZENR13	121	22	71	28	0,10
Est-1	ZENR1 x ZENR8	137	28	70	39	0,30
	ZENB13 x SD	77	12	43	22	0,10
Gdh-2	ZENB13 x SD	77	20	40	17	0,80
Pgd-3	HA89 x ZENB8	150	34	82	34	0,50
	ZENR11 x ZENR13	113	26	62	25	0,50
Pgi-2	ZENR1 x ZENR8	138	34	69	35	0,99

Chi-square probability compared to the expected 1 :2: 1 segregation ratio.

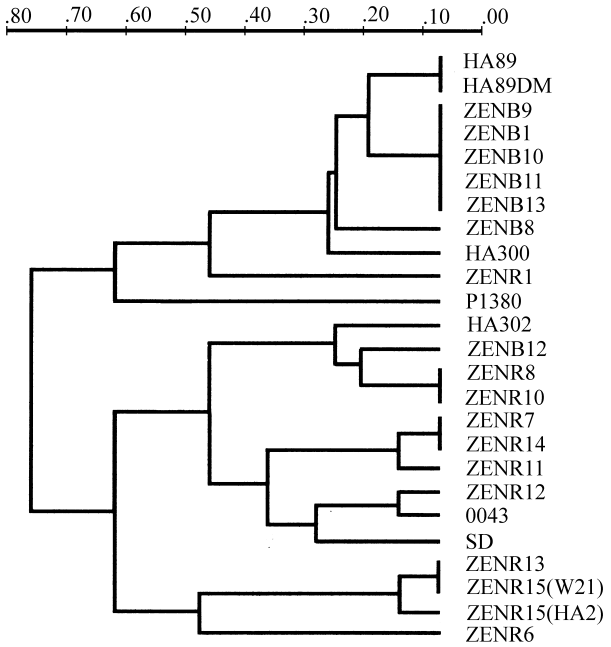

Figure 1 - Dendrogram of Neil distances constructed using UPGMA method for the 8 polymorphic isozyme systems.

Zymograms

Pgd-3 zymograms obtained from artificial bulks showed that a given genotype could be detected even in samples with a 1:8 ratio. The sensitivity of the method was lower for the other enzymes, and so three bulks of three seeds for each $F_{2:3}$ family were used. The patterns of the *Mdh-1*, *Mdh-4* and *Pgm-1* loci could not be resolved in the $F_{2:3}$ populations. These enzymes are under the genetic control of several loci encoding for cytoplasmic and organelle variants. Moreover, enzyme monomers coded by different genes can combine to produce intergenic heterodimers, making the identification of homo- and heterozygous individuals uncertain. The following five loci were selected for linkage analysis: *Acp-1*, *Est-1*, *Gdh-2*, *Pgd-3*, and *Pgi-2*. Examples of zymograms obtained in the $F_{2:3}$ mapping populations are shown in Figures 2 and 3. The number, spacing and relative intensity of bands of heterozygous individuals were in agreement with the expected quaternary structure of active enzymes and the ploidy level. *Acp-1*, *Pgd-3* and *Pgi-2* presented either a single band or a three-banded pattern consistent with a dimeric structure. *Gdh-2* patterns had one or multiple bands according to a tetrameric structure, but low resolution was observed in heterozygotes at this locus, due to the small distance between the bands. *Est-1* patterns were particularly complex, with single alleles coding for multiple bands, but allelic variants could be unambiguously identified.

Mapping

The five polymorphic loci (*Acp-1*, *Est-1*, *Gdh-2*, *Pgd-3*, and *Pgi-2*) showed co-dominant expression, and their segregation in the F_2 populations fitted the expected 1:2:1 ratio (Table III). These isozyme loci were mapped to linkage groups 2, 3, 9, 14 of the public RFLP map (Figure 4). The *Acp-1* and *Pgd-3* loci were found linked to the marker ZVG0005 on group 2, and the distance between these loci was estimated to be 11.3 cM. The *Est-1* locus mapped to group 3, in the interval flanked by ZVG0009 and ZVG0010, and this position was confirmed in two separate mapping populations (Table III). *Gdh-2* was located 2 cM above ZVG0062 in group 14, and *Pgi-2* mapped to group 9, between ZVG0040 and ZVG0041.

Discussion

Genetic variation at sunflower isozyme loci

The following 15 isozyme systems have been reported to reveal useful polymorphism in cultivated sunflower germplasm: aconitase (ACO), acid phosphatase (ACP), alcohol dehydrogenase (ADH), esterase (EST), glutamate dehydrogenase (GDH), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), peroxidase (PRX), phosphogluconate dehydrogenase (PGD), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and shikimate dehydrogenase (SKDH) (Lay *et al*., 1988; Quillet *et al*., 1992; Tersac *et al*., 1994; Cronn *et al*., 1997; Mestries *et al*., 1998). Eight of these systems were screened in this study, revealing nineteen loci, eight of which were polymorphic. The observed values of P (0.42), A (1.47) and Ap (2.125) were very similar to those reported by Cronn *et al*. (1997), who studied isozyme polymorphism in 700 plants from both oilseed and confectionary sunflower accessions. Except for *Mdh-3*, the loci that were found to be monomorphic in the 25 elite inbred lines had previously also been found to be monomorphic in cultivated sunflower germplasm (Carrera and Poverene, 1991, 1995; Quillet *et al.*, 1992).

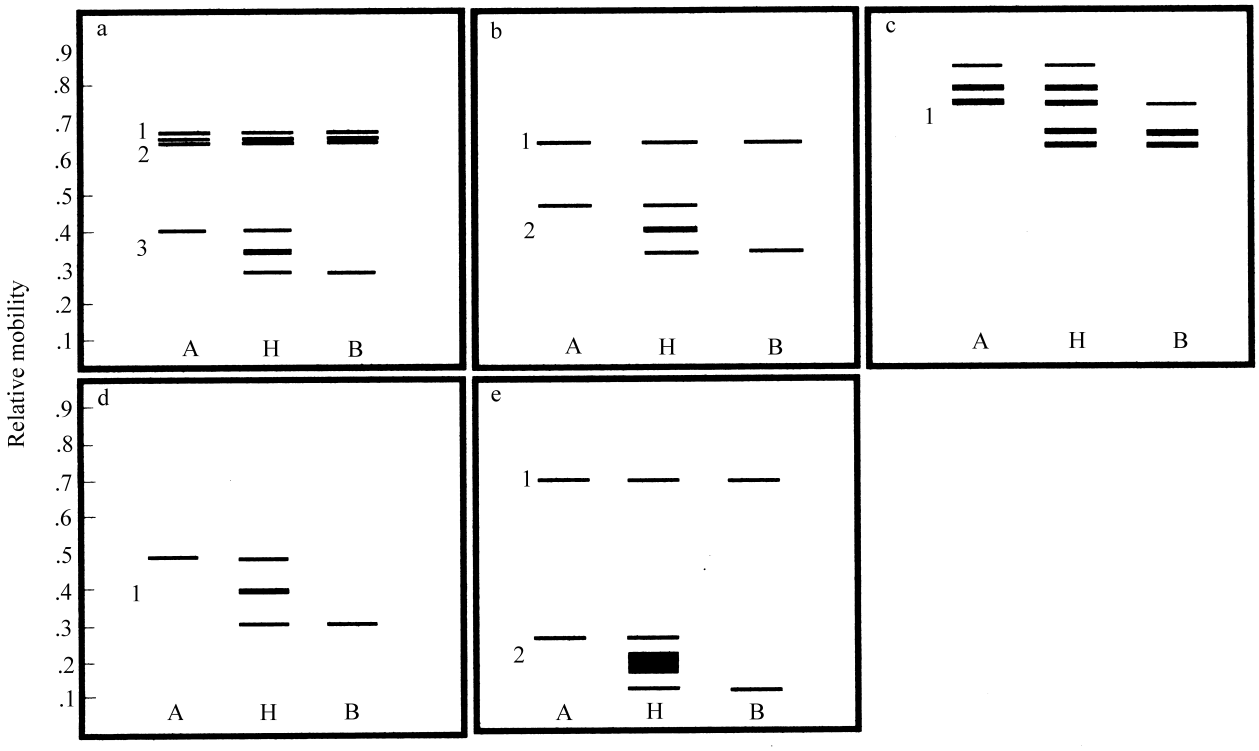

Figure 2 - Isozyme banding paterns and the genotypes assigned in the segregating F2:3 populations. A and B = the parental homozygotes, H = heterozygote. a. PGD; b. PGL; c. Est; d. ACP; e. GDH.

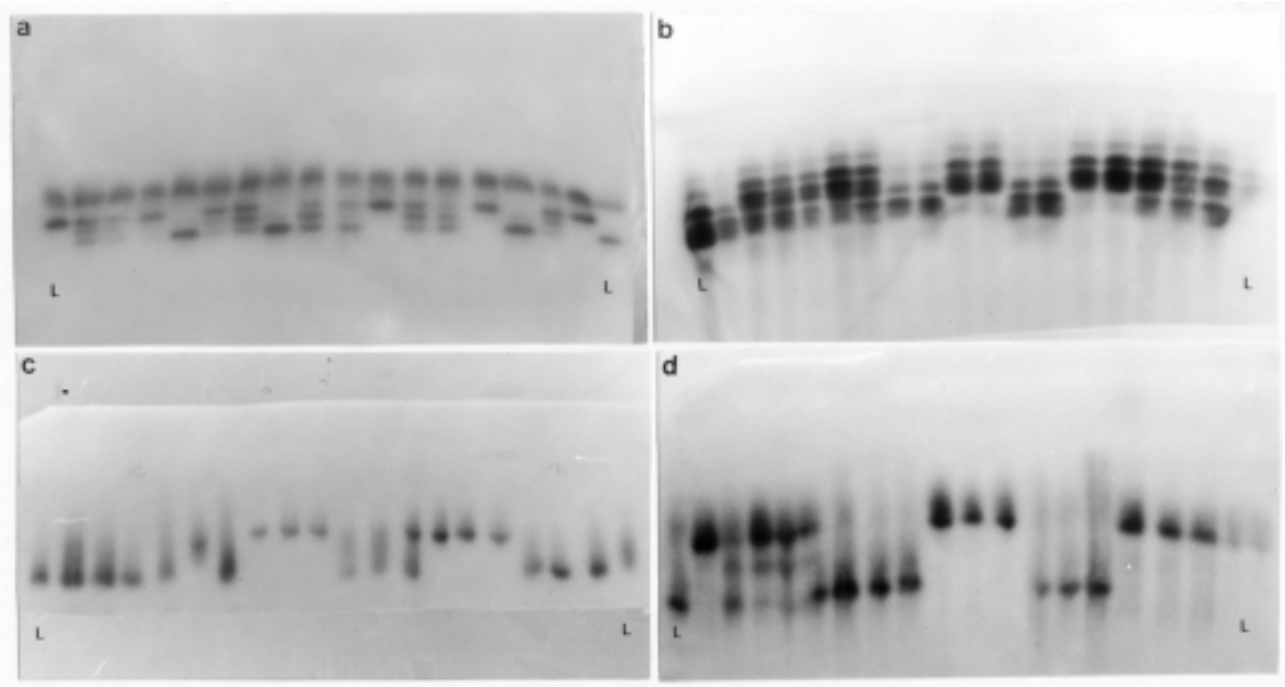

Figure 3 - Segregating F2:3 populations. L = parental inbred lines. a. PGD; b. EST; c. GDH; d. ACP.

In comparison with the results obtained by Quillet *et al*. (1992), this set of isozyme markers was far less informative. Quillet *et al*., (1992) were able to distinguish 45 out of 52 lines (86.5%) by using eight systems, whereas, in this survey, only 12 out of 25 lines (48%) could be uniquely distinguished. This apparent reduction in discriminatory power was probably due to a narrower sampling of the sunflower germplasm represented by this small set of inbred lines (Table I).

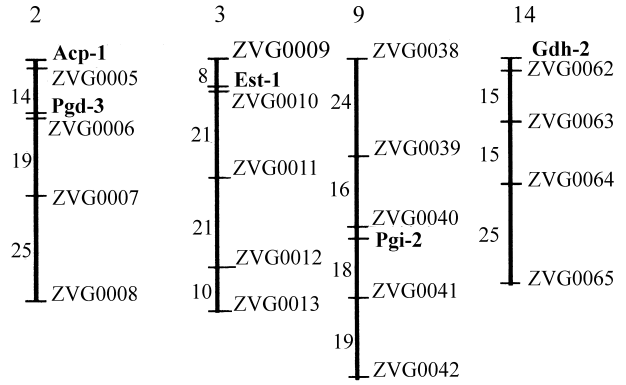

Figure 4 - The map position of five isoenzyme loci on four sunflower RPFL linkage groups. Linkage group and RFLP locus nomenclature is according to Berry *et al.* (1997) and map distances are given in centi Morgans. Isozyme loci are indicated in bold.

Comparison between isozyme and RFLP markers in sunflower

The mean number of isozyme alleles per polymorphic locus (Ap = 2.125) was lower than the values reported for RFLP markers (Berry *et al*., 1994; Zhang *et al*., 1995). A number of closely related lines was used in this study (*e.g.*, all the B lines in the cluster ZENB8 to HA89 are sister lines derived from a cross between these two females). This narrow sampling of germplasm (would) could reduce the level of polymorphism at the isozyme loci studied. One allelic form was found to predominate at each isozyme locus (Table II), as reported by Cronn *et al*. (1997) in a much larger germplasm survey. This is reflected by the low PI values for each isozyme marker (Table II), which made it necessary to use a number of different $F_{2:3}$ populations, in order to map as many loci as possible (Table III).

Cluster analysis (both UPGMA and bootstrap) of the isozyme fingerprinting data (Figure 1) revealed that the eight polymorphic loci were capable of separating the majority of CMS maintainer (B) lines from the restorer (R) lines. What makes the statistical separation possible are the differences in allele frequency between the B and R germplasm pools. For example, the *Est-1a* allele was only found in the B lines. However, the discrimination between males and females was not absolute. For (example) instance, the males ZENR8 and ZENR10 clustered more closely with the females, (and) whereas SD and 0043 clustered with the males. This undoubtedly reflects the narrowness of the germplasm used, the low level of isozyme polymorphism, and the poor genome coverage offered by the eight polymorphic isozyme systems. Studies with large numbers of molecular markers (Berry *et al*., 1994, Zhang *et al*., 1995; Hongtrakul *et al*., 1997) give a better resolution and tend to reflect the pedigree more closely.

Genetic mapping

There have been several reports on the inheritance of isozyme loci in populations of both wild and cultivated sunflower (Kahler and Lay, 1985; Torres, 1983; Rieseberg *et al*., 1993 and 1995; Quillet *et al*., 1995), and several pairs of loci have been found to be linked (Table IV). Mestries *et al*. (1998) were the first authors who reported the mapping of a number of isozyme loci onto a proprietary RFLP linkage map. These authors mapped the following 6 isozyme loci to five different linkage groups in a single F_2 population: *Got-1*, *Idh-I*, *Me*, *Pgd-2*, *Pgi-3* and *Sdh*. *Pgi-3* and *Idh-1* were found to be linked (Table IV). However, the present report is the first one on the mapping of sunflower isozyme loci onto a public linkage map, comprising 17 linkage groups. The five polymorphic isozyme loci, *Acp-1*, *Est-1*, *Gdh-2*, *Pgd-3*, and *Pgi-2,* mapped to four different linkage groups, with *Acp-1* and *Pgd-3* linked to group 2. Genetic linkage between an acid phosphatase locus (*Acp-1*) and a phosphogluconate dehydrogenase locus (*Pgd-1*) was originally reported by Lay *et al*. (1988), with a recombination fraction of 0.05. This value of 0.05 equates to approximately 5 cM, using either Kosambi or Haldane mapping functions (Lynch and Walsh, 1998), and is similar to the figure of 11.3 cM determined in this study. However, Lay *et al*. (1988) used a different nomenclature system to code their isozyme loci, which is an important consideration when comparing loci in different publications. Thus, it is possible, for example, that the PGD and PGI loci mapped by Mestries *et al*. (1998) are the same as those reported here.

Several isozyme systems revealed duplicated loci (Figure 2) in the sunflower genome, but unfortunately none of these duplications could be mapped in the $F_{2:3}$ populations. RFLP markers also detect duplicated loci (Berry *et al*., 1996), and these observations are consistent with the hypothesis that sunflower is an ancient polyploid (Jackson and Murray, 1983).

Table IV - Isozyme loci mapped in both intra- and inter-specific *Helianthus* populations.

Reference	Mapped isozyme loci	Linked isozyme loci
Torres and Diedenhoffen (1976)	*Adh-1, Adh-2, Acp-1*	
Kahler and Lay (1985) Lay *et al*. (1988)	*Prx-3, Mdh-1, Idh-2, Gpi -2, Pgm-4, Pgd-1, Acp-1, Acp-2, Sdh-3*	*Prx-3 & Pgm-4 Acp-1 & Pgd-1*
Rieseberg *et al*. (1993)	*Acp-1*	
Quillet *et al*. (1995)	*Mdh-1*, *Mdh-2*, *Pgm-1*, *Sdh-1*, *Acp-1*, *Me-1*	*Mdh-1 & Sdh-1*
Mestries *et al*. (1998)	*Got-1*, *Idh-I*, *Me*, *Pgd-2*, *Pgi-3*, *Sdh*	*Idh-1 & Pgi-3*
This study	*Acp-1*, *Pgd-3*, *Est-1*, *Pgi-2*, *Gdh-2*	*Acp-1 & Pgd-3*

The adoption of a standard nomenclature system for isozyme loci in sunflower, and the continued mapping of additional loci onto existing RFLP linkage maps, will provide an additional set of freely available public markers. Mapped isozyme loci, along with classical genetic markers and public microsatellites, will certainly help to integrate the various published sunflower maps.

References

Allen RD, Cohen EA, Von der Haar RA, Adams CA, Ma, D.P., Nessler CL and Thomas TL (1987) Sequence and expression of a gene encoding an albumin storage protein in sunflower. Mol. Gen. Genet. 210:211-218.

Berry ST, Allen RJ, Barnes SR and Caligari PDS (1994) Molecular analysis of *Helianthus annuus* L. 1. Restriction fragment length polymorphism between inbred lines of cultivated sunflower. Theor. Appl. Genet. 89:435-441.

Berry ST, León AJ, Hanfrey CC, Challis P, Burkholz A, Barnes SR, Rufener GK, Lee M and Caligari PDS (1995) Molecular marker analysis of *Helianthus annuus* L. 2. Construction of an RFLP linkage map for cultivated sunflower. Theor. Appl. Genet. 91:195-199.

Berry ST, Leon AJ, Challis P, Livini C, Jones R, Hanfrey CC, Griffiths S and Roberts A (1996) Construction of a high density, composite RFLP linkage map for cultivated sunflower (*Helianthus annuus* L.). Proceedings: 1155-1160. 14TH Int. Sunflower Conf. v. 2, Beijing/Shenyang, China.

Berry ST, León AJ, Peerbolte R, Challis P, Livini C, Jones R and Feingold S (1997) Presentation of the Advanta sunflower RFLP linkage map for public research. Proceedings: 113-118. 19TH Sunflower Res. Workshop, Fargo, USA.

Carrera A, and Poverene M (1991) Variabilidad genética en girasol mediante marcadores isoenzimáticos. Investigación Agraria (Spain) 6:281-293.

Carrera A, and Poverene M (1995) Isozyme variation in *Helianthus petiolaris* and sunflower, *H. annuus*. Euphytica 81: 251-257.

Carrera A, Poverene M and Rodriguez RH (1996) Isozyme variability *in Helianthus argophyllus*. Its application in crosses with cultivated sunflower. Helia 19:19-28.

Cronn R, Brothers M, Klier K, Bretting PK, and Wendel JF (1997) Allozyme variation in domesticated annual sunflower and its wild relatives. Theor. Appl. Genet. 95:532.545.

Dry PJ and Burdon JJ (1986) Genetic structure of natural populations of wild sunflowers (*Helianthus annuus* L.) in Australia. Aust. J. Biol. Sci. 39:255-270.

Felsenstein J (1995) PHYLIP (Phylogeny Inference Package) Version 3.5c. University of Washington. http://evolution.genetics.washington.edu/phylip/getme.html.

Gentzbittel L, Vear F, Zhang Y-X, Bervillé A and Nicolas P (1995) Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.) Theor. Appl. Genet. 90:1079-1086.

Gentzbittel L, Mestries E, Mouzeyar S, Mazeyrat F, Badaoui S, Vear F, Tourvieille de Labrouhe D, and Nicolas P (1999) A composite map of expressed sequences and phenotypic traits of the sunflower (*Helianthus annuus* L.) genome. Theor. Appl. Genet. 99:218-234.

Hongtrakul V, Huestis GM, and Knapp SJ (1997) Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germplasm: genetic diversity among oilseed inbred lines. Theor. Appl. Genet. 95:400-407.

Jan CC, Vick BA, Miller JF, Kahler AL and Butler ET (1998) Construction of an RFLP linkage map for cultivated sunflower. Theor. Appl. Genet. 96:15-22.

Jackson RC and Murray B (1983) Colchicine induced quadrivalent formation in *Helianthus*: evidence for ancient polyploidy. Theor. Appl. Genet. 64:219-222

Kahler A and Lay L (1985) Genetics of electrophoretic variants in the annual sunflower. J. Hered. 76:335-340.

Kinman ML (1970) New developments in the USDA and state experimental station breeding programmes. Proceedings: 181-183. 4TH Int. Sunflower Conf., Int. Sunflower Assoc., Toowoomba, Qld, Australia.

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE and Newburg L (1987) MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181

Lay C, Gerdes J, Kahler A, Whalen R (1988) Inheritance and linkage of nine electrophoretic variants in *Helianthus annuus* L. Proceedings: 411. 12TH Int. Sunflower Conf., Novi Sad, Yugoslavia.

León AJ, Lee M, Rufener GK, Berry ST and Mowers RP (1996) Genetic mapping of a locus (hyp) affecting seed hypodermis color in sunflower. Crop Sci. 36:1666-1668.

Lu YH, Melero-Vara JM, Garcia-Tejada JA, Blanchard P (2000). Development of SCAR markers linked to the gene *Or5* conferring resistance to broomrape (*Orobanche cumana* Wallr.) in sunflower. Theor. Appl. Genet. 100:625-632.

Lynch M and Walsh B (1998) Genetics and Analysis of Quantitative Traits. Sinauer Associates, Inc. Publishers, MA, USA.

Mestries E, Gentzbittel L, Tourvieille de Labrouhe D, Nicolas P and Vear F (1998) Analyses of quantitative trait loci associated with resistance to *Sclerotinia sclerotiorum* in sunflower (*Helianthus annuus* L.) using molecular markers. Mol. Breed. 4:215-226.

Miller JF (1992) Update on inheritance of sunflower characteristics. Proceedings: 905-945. 13TH Int. Sunflower Conf. vol.2, Pisa, Italy.

Nei M (1987) Molecular and evolutionary genetics. Columbia University Press, New York.

Quillet MC, Vear F and Branlard G (1992) The use of isozyme polymorphism for identification of sunflower (*Helianthus annuus*) inbred lines. J. Genet. Breed. 46:795-804.

Quillet MC, Madjidian N, Griveau Y, Serieys H, Tersac M, Lorieux M and Bervillé A (1995) Mapping genetic factors controlling pollen viability in an interspecific cross in *Helianthus* sect. *Helianthus*. Theor. Appl. Genet. 91:1195-1202.

Rieseberg L (1991) Phylogenetic and systematic inferences from chloroplast DNA and isozyme variation in *Helianthus* sect. *Helianthus* (*Asteraceae*). Sys. Bot. 16:50-76.

Rieseberg L and Soltis D (1989) Assessing the utility of isozyme number for determining ploidal level: Evidence from *Helianthus* and *Heliomeris* (*Asteraceae*). Aliso 12:277-286.

Rieseberg L and Seiler G (1990) Molecular evidence and the origin and development of the domesticated sunflower (*Helianthus annuus, Asteraceae*). Econ. Bot. 44:79-91.

Rieseberg LH, Choi H, Chan R and Spore C (1993) Genomic map of a diploid hybrid species. Heredity 70:285-293.

Rieseberg L, Van Fossen C and Desrochers A (1995) Hybrid speciation accompanied by genomic reorganization in wild sunflowers. Nature 375:313-316.

Rieseberg L, Baird SJE and Desrochers AM (1998) Patterns of mating in wild sunflower hybrid zones. Evolution 52:713-726.

Serre M, Feingold S, Salaberry T, Leon A, and Berry S (2001). The genetic map position of the locus encoding the 2S albumin seed storage proteins in cultivated sunflower (*Helianthus annuus* L.). Euphytica (in press).

Swofford DL and Selander RB (1989) BIOSYS-1. A computer program for the analysis of allelic variation in population genetics and biochemical systematics. Illinois Natural History Survey.

Soltis D, Haufler C, Darrow D and Gastony G (1983) Starch gel electrophoresis of ferns: A compilation of grinding buffers, gel and electrode buffers, and staining schedules. Amer. Fern J. 73:9-27.

Tersac M, Blanchard P, Brunel D and Vincourt P (1994) Relationships between heterosis and enzymatic polymorphism in populations of cultivated sunflowers (*Helianthus annuus* L.). Theor. Appl. Genet. 88:49-55.

Torres A (1983) Sunflower (*Helianthus annuus* L.). In: Tanksley SD and Orton TJ (eds) Isozymes in Plant Genetics and Breeding, Part B. Elsevier Science Publishers B.V., Amsterdam, pp. 329-338.

Torres AM and Diedenhoffen U (1976) The genetic control of sunflower seed acid phosphatase. Canadian J. Genet. Cytol. 18:709-716.

Vear F, Gentzbittel L, Philippon J, Mouzeyar S, Mestries E, Roeckel-Drevet P, Tourvielle De Labrouhe D and Nicolas P (1997) The genetics of resistance to five races of downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 95:584-589.

Zhang YX, Gentzbittel L, Vear F and Nicolas N (1995) Assessment of inter- and intra-inbred line variability in sunflower (*Helianthus annuus*) by RFLPs. Genome 38:1040-1048.