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INVESTIGATIONS OF ISOZYMES IN SUGARBEET (*BETA VULGARIS* L.)

By

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ABSTRACT

INVESTIGATIONS OF ISOZYMES IN SUGARBEET

By

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In this study, eight new isozyme loci were identified (Mdh-1, Mdh-2, Mdh-3, Mdh-4, Mdh-5, Pgm-1, Pgi-1 and Skdh-1) and the inheritance and linkage relationships between four of these loci and two marker loci was determined. The Pgi-1 locus had normal F_1, but skewed F_2 segregation ratios. It was hypothesized that linkage of the Pgi locus to a self-incompatibility locus caused these deviations from monogenic segregation. Two additional loci, ME-1 and ME-2, were proposed, however, more segregation data will be necessary to confirm their inheritance. Isozyme electrophoresis was also effective in almost completely distinguishing thirty-two plants using only seven marker enzymes. A new dwarf sugarbeet mutant was also observed in the progeny of the crosses examined for isozyme analysis. This mutant, named spinach leaf appeared to be due to a single recessive gene which could not be overcome by application of gibberellic acid.

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CHAPTER I

INHERITANCE AND LINKAGE OF SOME ISOZYME LOCI IN SUGARBEET

INTRODUCTION

In the past 200 years, the sugarbeet (Beta vulgaris L.) has been developed from the mangel beet. Over this period great improvements have been made in many important characters such as yield, sugar percentage and disease resistance. As a result of these and other improvements, sugarbeet production in 1980 accounted for 42% of the world's sugar supply (Smith, 1987). Despite the enormous progress in breeding, study of the basic genetics of the sugarbeet has been limited in comparison with other crops.

Smith (1980) described 44 loci in sugarbeet. Although inheritance studies have been conducted on most of these loci, only four of them, hypocotyl color (R), yellow pigment (Y), annual growth habit (B) and monogerm (m), have been studied intensely. Of the loci which have been studied for linkage, ten are associated with the Y-R-B linkage group. Half of these ten loci encode betacyanin pigmentation patterns.

Four other linkage groups have also been proposed, however; three of these have only one marker gene (Theurer, 1968). This accounts for possibly five of the nine possible linkage groups in the diploid (n=18) sugarbeet. None of these linkage groups have been mapped to specific chromosomes. This can be contrasted with other plant species such as maize or tomato, in which hundreds of loci have been identified representing all of their chromosomes (Goodman, Stuber and Newton, 1982; Bernatzky and Tanksley, 1986).

There are many reasons why progress in increasing genetic

information in sugarbeets has been slow. One reason is a long generation time, due to the biennial nature of most sugarbeet lines. Many loci also show variable penetrance or expressivity. The segregation ratios obtained for these loci can be inconsistent from one environment to another. In addition to this, most of the marker loci which have been studied represent pigmentation, chlorophyll deficient or foliar mutants. Due to lethality of some mutants and problems distinguishing phenotypes of some of the foliar mutants, it has been difficult to obtain new information on the linkage of multiple loci.

An increase in genetic and linkage information could be of great value in sugarbeet research. Therefore, it would be highly desirable to identify new loci which are easy to distinguish and represent all of the linkage groups. These loci would also be helpful as markers in breeding and genetic studies. A method whereby this could be accomplished is isozyme electrophoresis.

Isozymes, first defined in 1959, are recognized as multiple molecular forms of enzymes with the same catalytic function (Markert and Moller, 1959). Although isozymes can be distinguished by many techniques, starch gel electrophoresis (SGE) is by far the most common and practical method used to acquire genetic and linkage information in plant species. One of many advantages of SGE is that genotypes can be identified without progeny testing since isozymes are generally codominant. This is valuable in F_2 linkage analysis since much smaller numbers of progeny are necessary when all genotypic classes are identified (Allard, 1956).

Another advantage of SGE is that horizontal slicing of the starch gel permits quantification of the same samples for several different enzymes. This allows the study of many different isozyme loci at one

time. The status of isozyme research in over 24 plant species has been recently reviewed (Tanksley and Orton, 1983).

Although a large number of plant species have been studied using isozymes, very little isozyme research has been conducted in sugarbeets. The research which has been done tends to fall into 4 categories: (1) attempts to correlate a tissue culture trait or an adult plant character, such as ploidy, % sucrose, or fresh weight, with an isozyme pattern (Spettoli, Cacco and Ferrari, 1976; Spettoli, Bottacin and Cacco, 1980; Lehnhardt, Wiedmann and Gunther, 1981; Kevers, Coumans, Degreef, Hoffinger and Gaspar, 1981; Kevers, Coumans, Degreef, Jacobs and Gaspar, 1981), (2) A general description of variability and banding patterns for different enzymes (Van Geyt and Smed, 1984, Monastreva, Reimers and Levites, 1982), (3) Use of banding patterns to distinguish cultivars (Itenov and Kristensen, 1985), interspecific hybrids (Oleo, Van Geyt, Lange and DeBock, 1986), and monosomic addition lines (Jung, Wehling and Loptein, 1986), (4) Inheritance of isozyme loci (Maletskii and Konalov, 1985).

We initiated a research project to assess the potential of increasing the number of marker loci and linkage relationships in sugarbeet using isozymes, and to obtain a general idea of their usefulness in identifying individual clones and lines. Other objectives of this research included an assessment of the genetic diversity of the East Lansing breeding population and the creation of markers for tissue culture and somaclonal variation studies. The present study was conducted to elucidate the inheritance of isozyme variability in six different enzyme systems and to probe possible linkage relationships between the isozymes and with two other marker loci.

MATERIALS AND METHODS

PRODUCTION OF A DOUBLED HAPLOID

A diploid plant, presumably completely homozygous, was produced from a haploid via an in vitro colchicine technique which was adapted from Hussey and Hepher, 1978. The haploid plant, n50 X 34, was detected in F_1 seedlings of a cross between a monogerm biennial plant and an extremely multigerm, annual, male sterile individual from the cytoplasmic male sterile Owens Annual (OA) line. The haploid plant resembled the female parent in every aspect except that it was smaller.

Shoot cultures of n50 X 34 were established from axillary buds on the flower stalk and were multiplied on Murashige-Skoog (MS) medium with 1.1 uM 6-benzyladenine (BA) (Saunders, 1982). Fifty small shoots, 4 mm in length, were soaked in a 1% autoclaved colchicine solution for 50 minutes and another 50 shoots were soaked in the same for 100 minutes. After soaking, the shoots were rinsed in sterile water and returned to the multiplication medium. When the shoots were approximately 3 cm tall they were placed on MS medium supplemented with 16.0 uM 1-naphthaleneacetic acid (NAA) to be rooted. Rooted shoots were transplanted into peat pots and grown in the greenhouse.

All of the plants which resulted from this technique were male-sterile annuals which bolted under incandescent lights. The bolting plants were exposed to pollen shedding plants nearby to see if any plants or branches set plentiful seed. This tested for chromosome doubling due to the colchicine treatment, since only a doubled haploid,

(functionally a diploid) would have been able to set plentiful seed.

Two functional diploids were detected and then multiplied by in vitro shoot culture from axillary buds as described earlier. These two plants were given the clonal designations doubled haploid A and B (DH-A and DH-B). They were homozygous for the green hypocotyl color (rr), annualism (BB), male fertility maintainer (xx,zz) and self-fertility (S^fS^f) alleles and also had the sterile (S) cytoplasm.

CROSSES

Six crosses were made in Fall, 1984, to produce F_1 seed for an inheritance and linkage study of several isozyme loci (Table 1.1). DH-A and DH-B were the female parents of three of these crosses. The male parents of these crosses, 138C, 138D and 138H, were diploid, biennial plants which were at least partial male fertility restorers. These plants had their female parents in common and were also carriers of a dwarf crinkly leaf mutant character called spinach leaf. The male parents were crossed with the DH-A and DH-B females and were also pair crossed (Table 1.1). These crosses were labeled with the letters E.C. (enzyme cross) followed by a number. In Spring, 1985, seed was sown from each of the crosses and F_1 families were obtained.

In Fall, 1985, plants from three F_1 families were grown in the greenhouse under artificial long days (incandescent lights on a 18:6 LD cycle) where they bolted, were scored for pollen fertility and were then bagged to produce F_2 seed. F_2 progeny from selected self-fertile F_1 individuals and many of the plants from the F_1 families were examined for isozyme segregation ratios via starch gel electrophoresis. Data on segregation at the annualism and hypocotyl color loci was also recorded for all of the F_1 and F_2 progenies.

Table 1.1. Description of crosses used for isozyme segregation analysis

EC #	Cross	Genotypes*
EC1	DH-A X 138D	rrBB X RRbb
EC4	DH-A X 138C	rrBB X Rrbb
EC6	138C X 138D	Rrbb X RRbb
EC8	138D X 138C	RRbb X Rrbb
EC10	138H X 138C	RRbb X Rrbb
EC12	DH-B X 138D	rrBB X RRbb

* genotypes were hypocotyl color: RR or Rr-red and rr-green; annualism: BB or Bb-annual and bb-biennial.

STARCH GEL ELECTROPHORESIS

Sample preparation

Young healthy leaves, approximately 5 cm long, were collected from greenhouse plants immediately before they were used. Three 1 cm diameter leaf discs were excised from each leaf blade, excluding the midvein, using a #5 cork borer. The discs were homogenized using a glass pestle in porcelain spot plates containing 4 drops of extraction buffer. The extraction buffer consisted of 0.1 M tris-HCl, pH 7.0 buffer, containing 18% glycerol, 5% soluble polyvinylpyrrolidone (PVP-40T), 0.5% Triton X-100 and 1.0% 2-mercaptoethanol which was added just before use. The spot plates were placed on blocks of ice in trays and kept covered to maintain temperatures near 0°C throughout sample preparation. Pollen from individual plants was also used for samples. The pollen was collected when the plants were in flower and frozen in glass vials until needed. A mound of pollen, approximately 3 mm in diameter, was placed in the spot plate, and ground in three drops of extraction buffer in the same manner as the leaf discs.

Systems

The apparatus was a slight modification of that described by O'Malley, Wheeler and Guries (1980). Two buffer systems were used to resolve all of the enzyme systems examined. System I consisted of a 0.065 M L-histidine, 0.02 M citric acid monohydrate, pH 5.7 electrode buffer and a gel buffer that was a 1:6 dilution of the electrode buffer (Cardy, Stuber and Goodman, 1980). System II consisted of a 0.125 M tris-citric acid, pH 7.0 electrode buffer modified from Cheliak and Pitel (1984). The gel buffer was a 0.125 M DL-histidine·HCl, 1.4 mM Na_2EDTA buffer adjusted to pH 7.0 with tris.

Gels were prepared with 500 ml gel buffer, 62.5 g potato starch (Sigma Chemical Company) and for system I gels only, 15 g sucrose. The heated and aspirated gel solution was poured into a plexiglass frame which had these dimensions: 21.7 cm long x 14.0 cm wide x 1.5 cm deep. The gels were allowed to cool for 2 hours, and were then covered with 2 layers of plastic wrap and refrigerated overnight.

Twenty paper wicks (Whatman Electrophoresis Paper No. 3MM), 4X15 mm in size, were soaked in leaf extract, blotted on paper toweling, and inserted into a slice in the starch gel either 10 or 6 cm from the cathodal side of the gel, for systems I and II, respectively. After loading the samples, the gel was placed in a refrigerator at 4°C, ice trays were placed on top of the gels and the gels were run at 20 watts constant power. After 25 minutes the wicks were removed and electrophoresis resumed for a total of 4 1/2 hours for system I and 6 hours for system II. The enzymes which were resolvable using system I were malate dehydrogenase (Mdh), phosphoglucomutase (Pgm), phosphoglucose isomerase (Pgi) and 6-phosphogluconic acid dehydrogenase (6-Pgdh). The enzymes which were resolvable using system II were Mdh,

Malic enzyme (ME), Shikimate dehydrogenase (Skdh) and Pgm. A summary of these methods can be found in Table 1.2.

Staining

After electrophoresis, gels were sliced into 1/16" thick slices using plastic spacers and a fine steel guitar string drawn taut on a hacksaw. Although 9 or 10 thin slices were obtained from a single gel, the top and bottom slices were discarded because they gave poor band resolution. The remaining 7 or 8 slices were immersed in stain solutions in the order given in Table 1.2, for 1 hour at 37°C.

The Pgi, Pgm and ME enzyme stains were adapted from Cardy, Stuber and Goodman (1980). The Pgi assay solution contained 50 mg $MgCl_2$, 100 mg D-fructose-6-phosphate, 40 units G6PDH (glucose-6-phosphate dehydrogenase), 10 mg NADP (β-nicotinamide adenine dinucleotide phosphate), 10 mg NBT (nitro blue tetrazolium) and 1.5 mg PMS (phenazine methosulfate) in 50 ml 0.1 M tris-HCl pH 8.0 buffer. The Pgm stain was made with 50 ml 0.1 M tris-HCl pH 8.5 buffer, 100 mg $MgCl_2$, 50 mg Na_2EDTA ((ethylenedinitrilo)-tetraacetic acid disodium salt), 250 mg glucose-1-phosphate, 40 units G6PDH, 10 mg NADP, 10 mg NBT and 1 mg PMS. The ME stain contained 60 ml 0.1 M tris-HCl pH 8.5 buffer plus 40 ml 1.2 M DL-malate pH 8.0 solution, 200 mg $MgCl_2$, 52 mg NADP, 40 mg NBT and 4 mg PMS.

The Mdh, Skdh and 6-Pgdh enzyme stains were adapted from Cheliak and Pitel (1984). Mdh was stained using 50 ml 0.5 M DL-malate pH 7.0 solution, 25 mg NAD (β-nicotinamide adenine dinucleotide), 20 mg NBT and 2 mg PMS in 50 ml 0.1 M tris-HCl pH 8.0 buffer. Skdh was stained with 40 mg shikimic acid, 10 mg NADP, 10 mg NBT and 1 mg PMS in 50 ml 0.1 M tris-HCl pH 8.0 buffer. The 6-Pgdh stain was made with 50 mg $MgCl_2$,

Table 1.2. Summary of electrophoretic materials and methods

	System I[a]	System II[b]
pH	5.7	7.0
Electrode Buffer (EB)	0.065 M L-histidine 0.020 M citric acid adjust pH with citrate	0.125 M tris adjusted to pH 7.0 with citric acid anhydrous
Gel Buffer (GB)	0.009 M L-histidine 0.003 M citric acid (1:6 dilution of EB)	0.05 M DL-histidine·HCl 0.0014 M EDTA to pH 7.0 with tris ~~(dilute 3 H_2O: 2 stock)~~
Gel	62.5 g potato starch and 15 g sucrose in 500 ml GB	62.5 g potato starch in 500 ml GB
Origin	10 cm	6 cm
Power	20 watts (50 mAmps, 400 V)	20 watts (75 mAmps, 270 V)
Duration	4.5 hours	6 hours
Enzymes[c]	1) Mdh 2) Pgm 3) Pgi 4) 6Pgdh	1) Mdh 2) ME 3) Skdh 4) Pgm

a Cardy, Stuber and Goodman, 1980.
b adapted from Cheliak and Pitel, 1984.
c gel slices stained in this order, starting from bottom slice.

20 mg 6-phosphogluconic acid, 10 mg NADP, 10 mg NBT and 1 mg PMS in 50 ml 0.1 M tris-HCl pH 8.0 buffer.

Gels were kept in the dark during staining to prevent excess background staining and to protect light sensitive reagents. Mdh, ME and 6-Pgdh stained gels were left overnight at room temperature to darken bands. Zymograms were scored and photographed for later reference.

NOMENCLATURE OF ISOZYMES

Loci and alleles, determined by band segregation, were designated using the IUPAC-IUB guidelines (1978). Each locus was named using the enzyme name followed by a number. The loci were numbered consecutively with the lowest number given to the locus which encoded the band with the highest mobility toward the anode. The designations slow (SS) and fast (FF) represented the two major bands or alleles seen at a locus and their mobilities relative to one another. The most anodal band was considered the fast isozyme. Banding patterns which showed both SS and FF bands and sometimes an intermediate band, were designated SF.

STATISTICS

Chi-square values were calculated to determine the inheritance of individual loci and linkage between loci. Data from two or more crosses was only combined if it was determined to be homogeneous by the chi-square homogeneity test (Little and Hills, 1978). Independence of loci was also determined by calculating the recombination frequencies and standard errors of F_2 progeny data using the maximum liklihood method (Allard, 1956).

RESULTS AND DISCUSSION

CROSSES

Crosses were made in Fall, 1984, to produce F_1 seed for genetic analysis of isozyme and marker loci. The seed from six F_1 crosses was sown in Summer, 1985. Where possible, the hypocotyl color locus was used as a marker to determine the fidelity of the F_1 crosses. At this locus, red hypocotyl color (RR, Rr) is dominant to green hypocotyl color (rr) (Kajanus, 1917; Keller, 1936). This marker was chosen because the phenotypes can be characterized a few days after germination.

Since no green progeny were observed, it was determined that no selfing or contamination by (r) pollen had occurred in the crosses labeled EC1, EC6 or EC12 (Table 1.3). An 11:9 segregation ratio of green to red seedlings implied fidelity of the EC4 cross. Self-incompatibility was relied on to control pollination in EC6, EC8 and EC10; since, when 138C and 138D, the respective female parents in the crosses, were individually bagged they did not set self seed. One could not detect contamination of the EC8 and EC10 crosses using the hypocotyl color marker because the female parents were homozygous dominant red. Further generations of EC6, EC8 and EC10 were not produced because all of the progeny were biennial.

Seed germination percentages of the lines ranged from 29-100% (Table 1.3). There were two problems, however, with these calculated values. First, they represented the number of seedlings which arose from multigerm seed balls and were not accurate measurements of the viability

Table 1.3. Percent seed germination and segregation of the F_1 progeny at the hypocotyl color marker locus

EC #	Parents	% germ	cross*	observed		expected	
				pink	green	pink	green
EC1	DH-A X 138D	58%	rr X RR	29	0	29	0
EC4	DH-A X 138C	40%	rr X Rr	11	9	10	10
EC6	138C X 138D	94%	Rr X RR	47	0	47	0
EC8	138D X 138C	>100%	RR X Rr	56	0	56	0
EC10	138H X 138C	100%	RR X Rr	50	0	50	0
EC12	DH-B X 138D	>100%	rr X RR	49	0	49	0

* hypocotyl color phenotypes are RR or Rr:red and rr:green.

of all individual embryos. Second, it was impossible to determine the extent to which they were affected by unfavorable environmental conditions during seed set. These conditions included low light intensity levels and an infestation of spider mites and aphids on the plants in the crossing bags.

In Fall, 1985, the F_1 plants which bolted under artificial long days (annuals) were isolated in selfing bags for F_2 seed production. All of the annual F_1's had inherited one S^f allele at the self-incompatibility (S) locus from DH-A or DH-B. Pollen which contains this allele is compatible on any stigma, thereby allowing plentiful self seed production (Owen, 1942).

These F_1 plants had also inherited the male sterile cytoplasm from DH-A or DH-B. This cytoplasm causes plants to be male sterile unless they have a dominant allele at either the X or Z fertility restorer locus (Owen, 1945). The number of fertile F_1's produced and the degree of fertility restoration were dependent upon the genotype of the male parents at the X and Z restorer loci. Therefore, the ratio of male

sterile to male fertile F_1's should have indicated the genotypes of the male parents.

Approximately half of the F_1's put in selfing bags in Fall, 1985, appeared to be male sterile. The other half of the F_1's set varying amounts of self seed. When the F_1's were bagged again in Fall, 1986, some plants which had set self seed in 1985 appeared sterile, and some plants which had not set seed in 1985 set plentiful seed (Table 1.4).

Table 1.4. The number of F_1 individuals which set self seed over two consecutive seasons

EC #	Total # progeny	# which set self seed				Total % plants setting seed
		Fall 85+86	Fall 85	Fall 86	Total	
EC1	20	6	1	2	9	45%
EC4	19	10	0	1	11	58%
EC12	21	7	2	4	13	62%

During both growing seasons many plants suffered from a severe insect infestation which interfered with pollen production and seed development. Many of the EC1, EC4 and EC12 F_1's died because of the infestation. Due to conflicting results over seasons, severe aphid infestations and the death of many F_1 plants, no conclusions were drawn about the genotypes of the male parents at the X and Z loci.

Segregation data for several isozyme and marker loci were collected from the surviving F_1 progeny and F_2 progeny of EC1, EC4, and EC12 individuals. Some F_1 and F_2 progenies were also analyzed for linkage between loci. It is important to note that the death of many F_1's during bagging as well as possible linkage of isozyme loci to recessive alleles at X and Z might have skewed the F_1 segregation ratios.

PRELIMINARY ISOZYME STUDIES

Before the isozyme banding patterns were defined for any plant, all of the isozymes were examined under a variety of conditions. Clones of the plants 138C, 138D, 138H and DH-A were examined extensively to insure their banding patterns were the same when different extraction buffers, gel and electrode buffers and enzyme stains were used. Samples from plants grown in the greenhouse over different seasons were also compared for consistency. Leaves of different sizes and ages were sampled over time to insure that banding patterns were not affected by plant growth conditions, plant age or leaf age. Only those enzymes which gave consistent banding patterns under all of these conditions were considered for inheritance studies.

ISOZYME INHERITANCE

Malate dehydrogenase

Gels stained for Mdh showed 3 distinct regions of banding activity. (Figure 1.1). The region which contained the fastest migrating bands showed three isozyme banding patterns: SS, FF and a three banded, SF pattern (Figure 1.2a). It was hypothesized that these bands were the products of a single locus, Mdh-1. The inheritance of these bands was determined by examining the segregation ratios of F_1 and F_2 progeny.

Crosses between two FF individuals resulted in all FF progeny (Table 1.5). Three SS X FF crosses resulted in progeny which uniformly expressed the SF genotype. F_2 progeny from five selfed SF individuals segregated in the 1SS:2SF:1FF expected ratio with a greater than .7 probability (Table 1.5). The segregation data of one F_2 line fit this ratio with a probability of .2-.3. The segregation data confirmed that the bands were the result of two alleles from a single locus, Mdh-1.

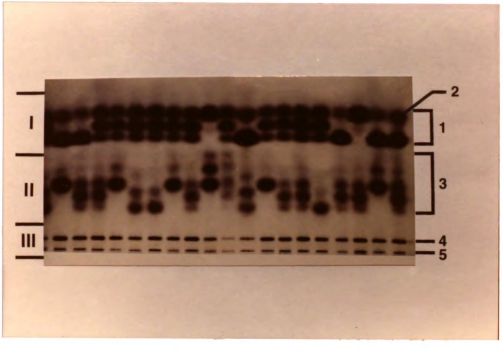


Figure 1.1. The three zones of activity (left) and five (right) loci of Mdh. This gel was resolved with system II and shows SS, SF, and FF types for both Mdh-1 and Mdh-3. Using this system the Mdh-2 band comigrated with the slow (SS) Mdh-1 band. When system I was used the single band of Mdh-2 comigrated with the fast (FF) Mdh-1 band.

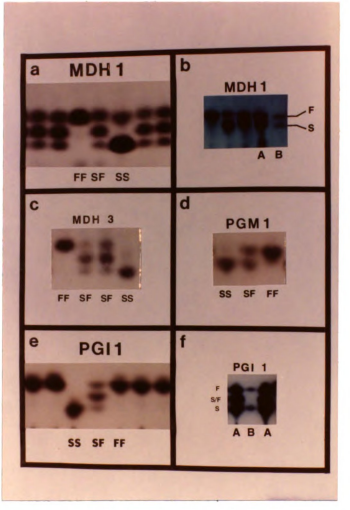


Figure 1.2. Starch gel zymograms showing the banding genotypes at 4 isozyme loci: a) FF, 3-banded SF and SS banding genotypes at Mdh-1, b) Comparison of leaf (A) and pollen (B) samples from an Mdh-1 SF plant, c) FF, 3-banded SF and SS banding genotypes at Mdh-3, d) SS, 2-banded SF and FF banding genotypes at Pgm-1, e) SS, 3-banded SF and FF banding genotypes at Pgi-1, f) Comparison of leaf (A) and pollen (B) samples from a Pgi-1 SF plant.

Table 1.5. Chi-square analysis of allelic segregation of Mdh-1 in F_1 and F_2 progenies of Beta vulgaris

EC #	Cross	Gen[@]	SS	SF	FF	N	X^2	P
EC1[&]	SS X FF	F1	--	20	--	20	---	--
EC12	SS X FF	F1	--	20	--	20	---	--
EC4	SS X FF	F1	--	18	--	18	---	--
EC6	FF X FF	F1	--	--	52	52	---	--
EC10	FF X FF	F1	--	--	25	25	---	--
EC4-9[*]	SF X SF	F2	2	13	4	19	3.000	.2-.3
EC1-22	SF X SF	F2	10	23	14	47	0.702	.7-.8
EC4-5	SF X SF	F2	23	53	25	101	0.326	.8-.9
EC4-18	SF X SF	F2	19	38	15	72	0.670	.7-.8
EC4-19	SF X SF	F2	11	23	14	48	0.458	.7-.8
EC4-20	SF X SF	F2	10	17	7	34	0.529	.7-.8

@ Gen: Generation, N: Total progeny, P: Probability,
SS: slow homozygote, SF: heterozygote, FF: fast homozygote.
* only spinach leaf mutant segregates were examined
& EC1: DHA X 138D, EC4: DHA X 138C, EC6: 138D X 138C,
EC10: 138H X 138C, EC12: DHB X 138D.

The existence of a three banded SF type provided strong evidence that the locus encoded a dimeric enzyme. In addition, the pollen of these SF individuals showed activity for the slow and fast bands, but not for the intermediate band (Figure 1.2b). When gels which contained pollen samples from SF plants were stained for a variety of dimeric enzyme systems, they did not show activity for the intermediate band (Tanksley, Zamir and Rick, 1981). It was determined that Mdh-1 was a single locus with 2 alleles which encoded a dimeric enzyme.

A band was observed which always comigrated with one of the Mdh-1 bands (Figure 1.1). When Mdh was resolved using system I, this band migrated to the same position as the intermediate band of Mdh-1. When system II was used, this band migrated to the same position as the fast band of Mdh-1. This band appeared regardless of the Mdh-1 genotype of

the plant from which the sample was taken; however, it was obscured in SF and FF types when system II was used. All parents and progeny of the crosses showed activity for this band, but pollen did not. It was determined that this band is produced by a locus distinct from Mdh-1. This locus was designated Mdh-2 and was represented by only one allele.

The second region of banding activity was only resolvable with system II. This region contained 3 banding types, SS, FF and a three banded, SF type (Figure 1.1). It was hypothesized that these bands were the products of a single locus, Mdh-3. Each of the banding types was often coordinately expressed with several faint, faster migrating bands (Figure 1.1). Before determining if the major bands represented a new locus, it was necessary to examine the nature of the faint bands.

The faint bands showed variability in activity in different samplings of the same individual. In some instances the bands were inactive in individuals which had previously shown activity for these bands. In addition, the bands that were associated with the FF band, never appeared with the SS band, and vice versa. Since they did not associate independently it was unlikely that the faint bands and the major bands were the products of different loci.

It was also unlikely that the faint bands were due to intra- or interlocus heterodimers, since they were not located in a position intermediate to any two bands or loci. The phenomenon of associated Mdh bands was also observed in maize and was assumed to be due to secondary modification (McMillin and Scandalios, 1980). Other researchers were able to eliminate faint, trailing, Mdh bands in Maize by purification of the soluble Mdhs (Goodman and Stuber, 1983). The faint bands were determined to be artifactual, therefore they were ignored in the later collection of data for this study.

A cross between two SS plants resulted in all SS progeny (Table 1.6). Two SS X FF crosses produced progeny which gave a three banded, SF phenotype. This three banded pattern was similar to that observed in the Mdh-1, SF types (Figure 1.2c). F_2 progeny from two, selfed SF types segregated in the expected 1SS:2SF:1FF ratio with a greater than 0.7 probability (Table 1.6). Since pollen was not active at this locus, comparisons of pollen and leaf tissue could not be used to confirm the dimeric nature of this enzyme. Nonetheless, the existence of an intermediate band was adequate to conclude that the Mdh-3 locus encoded a dimeric enzyme for which two alleles existed.

The third region of Mdh activity (observed only when system II was used) showed less activity than the other two regions. This region consisted of two, thin (less active) bands located close to the origin on the gel (Figure 1.1). These isozymes were always active except in pollen samples. When cross progeny were examined the bands never segregated or interacted.

In many species, MDH is known to be compartmentalized in microbodies, mitochondria and the cytosol (Ting, Fuhr, Curry and Zschoche, 1975). The activity of microbody Mdh in maize was shown to be much lower then in the activity of the cytosolic and mitochondrial isozymes (Yang and Scandalios, 1975b). The similarities between the Mdh zymograms in maize and sugarbeet imply that the thin bands are the products of isozymes localized in microbodies. It was determined that these bands were the products of two loci, Mdh-4 and Mdh-5 each of which had only one allele.

Table 1.6. Chi-square analysis of allelic segregation of Mdh-3 in F_1 and F_2 progenies of Beta vulgaris

EC #	Cross	Gen@	SS	SF	FF	N	X^2	P
EC1&	SS X SS	F1	16	--	--	16	---	--
EC6	SS X FF	F1	--	17	--	17	---	--
EC4	SS X FF	F1	--	7	--	7	---	--
EC4-20	SF X SF	F2	10	15	9	34	0.529	.7-.8
EC4-9*	SF X SF	F2	6	9	5	20	0.300	.8-.9

@ Gen: Generation, N: Total progeny, P: Probability,
SS: slow homozygote, SF: heterozygote, FF: fast homozygote.
* only spinach leaf mutant segregates were examined
& EC1: DHA X 138D, EC4: DHA X 138C, EC6: 138D X 138C.

Phosphoglucomutase

Gels stained for Pgm showed two regions of activity; however, only the more anodal (faster) region was resolvable. In gels resolved using system I, the slower region virtually disappeared. The bands in this region never varied and also appeared to comigrate with the bands of the faster region. Stuber and Goodman (1983) observed a similar phenomenon and concluded that each allele of the Pgm locus was associated with a pair of isozyme bands. Since system I was primarily used in this study, the slower region of activity was simply ignored. The faster region contained three isozyme banding patterns: SS, FF and a two banded SF pattern (Figure 1.2d). It was hypothesized that these bands were the products of a single locus, Pgm-1.

Three FF X SF crosses segregated in the expected 1SF:1FF segregation ratio (Table 1.7). The data from two crosses between SF types and five selfs of SF individuals fit the 1SS:2SF:1FF expected ratio. The SF individuals showed activity for the S and F bands but did not

show activity for an intermediate band (Figure 1.2d). The banding patterns from pollen versus leaf extracts were identical for SS, SF and FF individuals. The lack of an intermediate band in the heterozygotes indicated that the locus encoded a monomeric enzyme. It was determined that the Pgm-1 locus encoded a monomeric enzyme for which two alleles were observed.

Table 1.7 Chi-square analysis of allelic segregation of Pgm-1 in F_1 and F_2 progenies of Beta vulgaris

EC #	Cross	Gen@	SS	SF	FF	N	X2	P
EC1&	FF X SF	F1	--	6	13	19	2.579	.1-.2
EC12	FF X SF	F1	--	9	12	21	0.429	.5-.6
EC4	FF X SF	F1	--	11	4	15	3.267	.05-.1
EC10	SF X SF	F1	9	12	4	25	2.040	.3-.4
EC6	SF X SF	F1	15	27	10	52	1.039	.5-.6
EC4-9*	SF X SF	F2	3	11	4	18	1.000	.6-.7
EC1-22	SF X SF	F2	11	31	5	47	6.319	.05-.1
EC4-5	SF X SF	F2	28	49	24	101	0.406	.8-.9
EC4-18	SF X SF	F2	13	35	24	72	3.420	.1-.2
EC4-20	SF X SF	F2	10	14	10	34	1.059	.5-.6

@ Gen: Generation, N: Total progeny, P: Probability,
SS: slow homozygote, SF: heterozygote, FF: fast homozygote.
* only spinach leaf mutant segregates were examined
& EC1: DHA X 138D, EC4: DHA X 138C, EC6: 138D X 138C,
EC10: 138H X 138C, EC12: DHB X 138D.

Phosphoglucose isomerase

After separation with system II, gels stained for Pgi showed two distinctly stained regions, neither of which could be clearly resolved into bands. Pollen was active only in the slower of the 2 regions. Gels separated with system I showed only one clearly resolvable region of activity. This region showed pollen activity and contained three isozyme banding patterns: SS, FF and a three banded SF pattern. It was

hypothesized that these bands were encoded by a single locus, Pgi-1 (Figure 1.2e).

Three FF X SF crosses resulted in F1 progeny which segregated in the expected 1SF:1FF ratio. F1 progeny, from a SS X SF cross, segregated in the 1SS:1SF expected ratio (Table 1.8). Pollen from SF individuals showed activity for the slow and fast bands but not the intermediate band. (Figure 1.2f). This is the kind of data that one would expect for a locus with two alleles, each encoding a subunit of a dimeric enzyme.

Table 1.8. Chi-square analysis of allelic segregation of Pgi-1 in F_1 and F_2 progenies of Beta vulgaris

EC #	Cross	Gen@	SS	SF	FF	N	X^2	P
EC1&	FF X SF	F1	--	9	10	19	0.052	.8-.9
EC12	FF X SF	F1	--	13	8	21	1.190	.2-.3
EC4	FF X SF	F1	--	10	6	16	1.000	.3-.4
EC10	SS X SF	F1	11	14	--	25	0.360	.5-.6
EC6	SF X SF	F1	6	28	18	52	5.846	.05-.1
EC1-22	SF X SF	F2	2	22	23	47	18.957	<.05
EC4-5	SF X SF	F2	10	50	41	101	19.039	<.05
EC4-18	SF X SF	F2	5	39	28	72	15.190	<.05
EC4-19	SF X SF	F2	4	21	23	48	15.792	<.05

@ Gen: Generation, N: Total progeny, P: Probability,
SS: slow homozygote, SF: heterozygote, FF: fast homozygote
* only spinach leaf mutant segregates were examined
& EC1: DHA X 138D, EC4: DHA X 138C, EC6: 138D X C, EC10: 138H X C, EC12: DHB X 138D.

In contrast to this data, F_1 progeny from a cross between two SF plants and F_2 progeny from selfs of four SF individuals did not segregate in the expected 1SS:2SF:1FF ratio (Table 1.8). The ratios were consistently skewed such that when the data was averaged, 8.4% of

the progeny were of the SS type, and 41.6% of the progeny were of the FF type (Table 1.9). Only the SF type, found in a total of 50.0% of the progeny, was close to its expected value.

Table 1.9. Frequency of Pgi banding types in segregating F_1 and F_2 progeny

Family	SS	SF	FF	N
EC4-5	.099	.495	.406	101
EC4-18	.069	.542	.389	72
EC4-19	.083	.438	.479	48
EC1-22	.043	.468	.489	47
EC6	.115	.539	.346	52
-All data combined	.084	.500	.416	320
-EC 4 F_2 data only	.086	.498	.416	221

All of the F_1 plants from EC1, EC4 and EC12 had received one normal self-incompatibilty allele from the original male parent and one S^f allele from the female parent. Pollen which contains the S^f allele is compatible on any stigma; however, pollen containing any other S allele may or may not be compatible. Overall the Beta vulgaris incompatibility system is extremely complex and contains either 2 or 4 loci with multiple alleles (Owen, 1942, Larsen, 1977).

A simplified model was developed to explain the skewed Pgi ratio. This model was based on the premise that the self-compatability system involved was monogenically inherited. Assuming this premise to be true, the following was also true: (1) Pollen which contained the (S) allele contributed by the male parent was self-incompatible in the F1's, and (2) The segregation ratios of any genes linked to the (S) locus were skewed in favor of the allele contributed by the female parent.

Expected Pgi segregation ratios were calculated for a range of recombination percentages. An example which was calculated following this model, using a value of 17% recombination between the loci, can be found in Table 8.

Table 1.10. Possible F_2 genotypes using 17% recombination between the Pgi and self-incompatibility loci

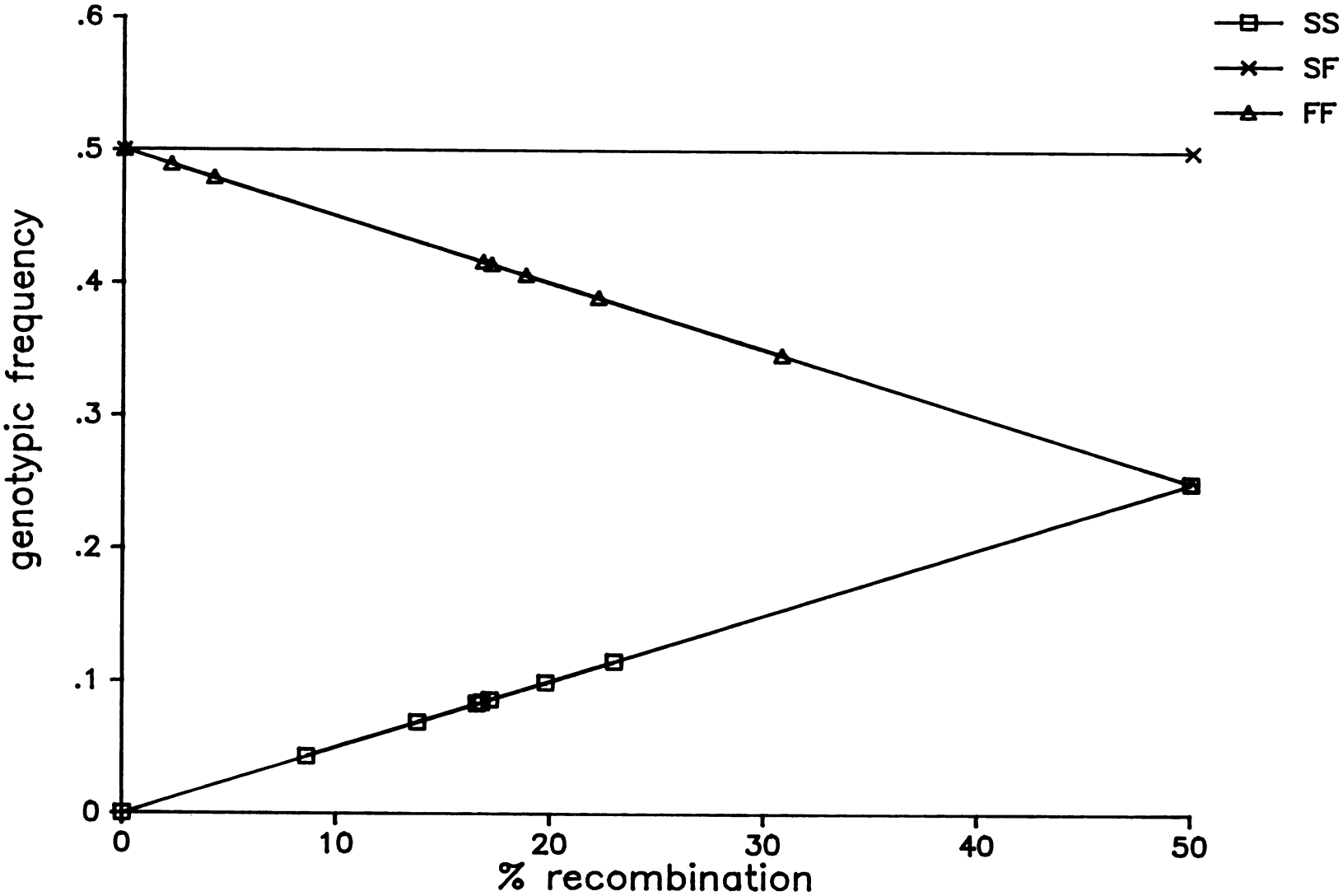
		Male Gametes			
		Parental types		Recombinant types	
		F Sf	S S1	F S1	S Sf
Female Gametes		.83	incompatible		.17
	.415 F Sf	.3444 FF SfSf	----	----	.0706 SF SfS1
P	.415 S S1	.3444 SF SfS1	----	----	.0706 SS SfS1
	.085 F S1	.0706 FF SfS1	----	----	.0144 SF SfS1
R	.085 S Sf	.0706 SF SfSf	----	----	.0144 SS SfSf
Expected Pgi ratio		.085 SS	.500 SF	.415 FF	

When the calculated values in Table 1.10 were compared to the combined values in Table 1.9, it was obvious that the frequencies of the banding types were very similar. Equations for lines describing the three banding types at percent recombinations (%R) from 0 to 50 percent were calculated using the incompatibility/linkage model as a basis. These equations, with Y equal to the frequency of the banding types and X equal to the %R, are:

$$SS: \quad Y = .005X$$
$$SF: \quad Y = .500$$
$$FF: \quad Y = -.005X + .5$$

Figure 1.3 is a graph of these lines. If the %R was 0%, the ratio

Figure 1.3. Graph of lines describing the SS, SF and FF banding types from 0% to 50% recombination. Combined data and data from each of the F_2 families in Table 1.9 are plotted on the SS and FF lines.


SS
SF
FF
genotypic frequency
.6
.5
.4
.3
.2
.1
0
0
10
20
30
40
50
% recombination

of the banding frequencies would be .5SF:.5FF and there would be no SS types. If the %R was 50%, the loci would be independent and the ratio of the banding frequencies would be .25SS:.50SF:.25FF. When the SS and FF data from each of the crosses were plotted on these lines the data tended to cluster between 15 and 25% recombination. When the SS and FF equations were calculated using the banding frequency values from the combined data, the resulting %R for both equations was 16.8%.

It was also possible that the skewed Pgi-1 ratio observed in this study was entirely or partially due to linkage between Pgi-1 and an embryonic lethal locus. A similar situation was observed in Beta vulgaris in conjuction with the alcohol dehydrogenase (ADH) locus (Maletskii and Konovalov, 1986). Segregating progeny from the self-pollinations of 13 sister lines were examined. Some lines segregated in the expected 1:2:1 ratio while others deviated from this ratio. They concluded that the ADH locus was linked to an incompatibility locus and a locus which behaved like an embryonic lethal.

In our study, 138C and 138D were carriers of a dwarf crinkly leafed mutant phenotype named spinach leaf. Approximately 9% of the progeny from crosses between 138C and 138D are of the spinach leaf phenotype. Spinach leaf seedlings have shortened hypocotyls, are slow to germinate and are often deformed and die (see Chapter 3). Assuming that this mutant is caused by a single gene, there may have been lethality of some of the embryos which would have been spinach leaf types. If this were the case, any locus linked to the spinach leaf allele would have a skewed segregation ratio. Partial lethality of pollen carrying the mutant allele would have had a similar effect, even in the F_1 progeny.

The evidence does not support this theory for the following

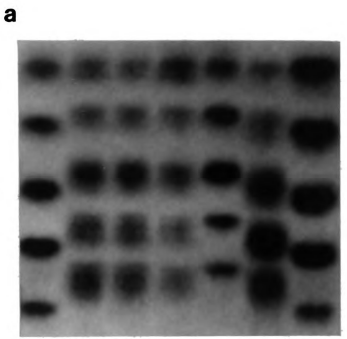
reasons: 1) F_1 progeny from the original EC1, 4, 10 and 12 crosses did not segregate in the skewed ratios that they should have if there was gametophytic lethality involved (Table 1.8) and 2) Lines which did not produce F_2 spinach segregates still gave skewed F_2 Pgi ratios. Although the spinach mutant may have had an effect, the skewed Pgi ratio was probably due to linkage between the Pgi-1 and self-incompatibility loci.

The map distance between the two loci was estimated to be 16.8 map units. It was determined that the Pgi-1 locus encoded a dimeric enzyme for which two alleles were observed. Nonetheless, some additional crosses should be analyzed to confirm these conclusions and to insure that any lethal affects of the mutant did not confound the segregation data. More information about the spinach leaf mutant can be found in Chapter 3.

Malic enzyme

Two distinct ME banding types were observed in the parental lines, each composed of 5 equally spaced bands. When the two types were compared, the most anodal bands of both were located at the same migration distance on the gel. The other four bands, however, were in different positions (Figure 1.4). These banding types were identified using the Roman numerals I and II. Pollen was active for only the most anodal band.

A cross between between two type I individuals produced progeny which exhibited only banding type I (Table 1.11). All of the progeny from a type II X type I cross produced progeny which showed activity of both of the banding types plus some additional bands for a total of 15 bands. Individuals showing this pattern were labeled a hybrid (H) banding type (Figure 1.4a). A self of a hybrid type gave progeny which



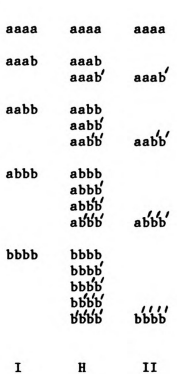


Figure 1.4. a) This Malic enzyme zymogram was resolved using system II and shows the parents (Types I + II) and segregating F_2 progeny of a type H, F1 individual. b) diagram showing the structures of the tetramers composing the three banding patterns. a, b and b′ represent the polypeptides produced by the alleles from the proposed loci ME-1, and ME-2.

segregated in a 1:2:1 (type I : hybrid : type II) ratio with a greater than 0.8 probability (Table 1.11).

Table 1.11. Results of crosses for the tentative locus ME-2

EC #	Cross*	Gen@	II	H	I	N	X^2	P
EC1&	II X I	F1	--	16	--	16	---	--
EC6	I X I	F1	--	--	17	17	---	--
EC4-20	H X H	F2	8	16	10	34	0.353	.8-.9

* Type I and II represent banding patterns and the fast and slow alleles respectively of the hypothesized ME-2 locus.
@ Gen: Generation, N: Total progeny, P: Probability, SS: slow homozygote, SF: heterozygote, FF: fast homozygote.
& EC1: DHA X 138D, EC4: DHA X 138C, EC6: 138D X 138C.

From this data it was hypothesized that malic enzyme was controlled by two loci which interacted to form a five banded pattern, the result of a tetrameric enzyme. Based on this hypothesis the observed data could be explained by the following: 1) The fastest band of both type I and II patterns was the result of the same allele at the invariant ME-1 locus, 2) The slowest band of both type I and II patterns represented two different alleles at the ME-2 locus, slow (type II) and fast (type I), 3) The three intermediate bands of both patterns were a result of interlocus heterotetramers between ME-1 and ME-2, 4) The hybrid banding pattern was caused by inter- and intralocus heterotetramers between the ME-1 locus and both alleles of the ME-2 locus. The result of all of these bands was a 15 banded pattern, and 5) The 1:2:1 (type I : hybrid : type II) ratio was actually a 1SS:2SF:1FF segregation of the two alleles of the ME-2 locus. Figure 1.4b is a diagram of this hypothesis.

Evidence in support of this hypothesis is provided by many other

species (maize for example) which have tetrameric malic enzyme isozymes (Goodman and Stuber, 1983). Researchers working with many other species have chosen not to study ME because of its complex banding patterns. In sugarbeets, Van Geyt and Smed (1984) described an article by Levites (1979) which proposed that the five ME isozymes observed in his study were the result of a tetrameric enzyme, controlled by one gene. Van Geyt and Smed felt that the starch gel electrophoresis method used by Levites did not adequately resolve the bands, therefore they disagreed with his proposal. Since neither author examined segregation data they could not establish the genetic basis of the observed zymograms. Although the segregation data (in our study) seemed to fit our hypothesis, more progeny should be analyzed before a definitive statement of ME inheritance is made.

Shikimate dehydrogenase

Only one Skdh band was observed in the parents of all the crosses. All of the progeny of four different F_1 crosses had activity for this band (Table 1.12). Pollen was not active for the Skdh enzyme. The band was tentatively designated as a product of the Skdh-1 locus. A slightly faster band was discovered in a genotype which was not used in the crosses. This will be discussed in Chapter 2.

6-Phosphogluconate dehydrogenase

When parents of the crosses were stained for 6-Pgdh, they all exhibited the same four banded pattern. This pattern was composed of a single fast band followed by three evenly spaced, slower bands. All cross progeny showed the same four banded pattern.

Table 1.12. Results of crosses for Skdh-1

EC #	Cross*	Gen@	I	N	X^2	P
EC6&	I X I	F1	17	17	---	--
EC8	I X I	F1	2	2	---	--
EC10	I X I	F1	1	1	---	--
EC1	I X I	F1	2	2	---	--

* Type I represents the single band of the hypothesized Skdh-1 locus.
@ Gen: Generation, N: Total progeny, P: Probability.
& EC1: DHA X 138D, EC6: 138D X 138C, EC 8: 138C X 138D, EC10: 138H X 138C.

There are several possible explanations for this banding pattern. 1) Each band was caused by a different locus. 2) Some of the bands were caused by different loci and some were artifactual. 3) The pattern was caused by the three invariant loci, two of which interacted to form an interlocus heterodimer. In many plant species 6-Pgdh isozymes have been shown to be dimers encoded by two loci, one cytoplasmic and one associated with plastids (Gottlieb, 1981). Maize 6-Pgdh isozymes are encoded by two cytosolic loci which interact to form heterodimers (Goodman, Stuber, Newton and Weissinger, 1980b). Heterodimers are not formed between enzymes located in different cellular compartments.

Since most species have been shown to have only two 6-Pgdh loci it is unlikely, but not impossible, that there are four different loci in sugarbeet. To confirm any of these explanations, however, it will be necessary to find an individual which has a different 6-Pgdh banding pattern and use it as a parent in crosses. Analysis of the ensuing segregating progeny can then be used to determine the correct explanation.

Hypocotyl color and annualism

The hypocotyl color and annualism loci were used as marker loci in crosses and were tested with the isozyme loci for linkage. These loci encode monogenically inherited traits and are known to be found on the same linkage group (Kajanus, 1917; Keller, 1936; Munerati, 1931; Abegg, 1936). Red hypocotyl color is dominant to green, and annual flowering habit is dominant to biennial. The expected segregation of the alleles of one or both of these loci was observed in most of the lines which were also examined for isozyme segregation (Table 1.13). The data did not fit segregation ratios for one line (Table 1.13). This was probably due to poor penetrance of the (B) allele under adverse environmental conditions.

LINKAGE

Mdh-1 was found to be unlinked to the Pgm-1, B and R loci when chi-square goodness of fit and recombination values were calculated (Table 1.14). Pgm-1 was also found to be independent of the R and B loci. Mdh-3, the proposed loci and all of the non-segregating loci were not included in the linkage tests.

The data from the EC 6 and F_2 progeny (see Table 1.8) which segregated for Pgi-1 gave highly significant chi-square values when linkage of Pgi-1 to the the Mdh-1, Pgm-1, hypocotyl color and annualism loci was tested (Tables 1.15 and 1.16). When the recombination percentages were calculated using Allard's maximum likelihood formulas, however, they were all very close to 50%, which indicated independence. These conflicting results were most likely a result of the skewed segregation of the alleles at the Pgi-1 locus. The calculations done using Allard's formulas were not affected by the skewed Pgi-1 ratios, whereas the chi-

Table 1.13. Chi-square analysis of allelic segregation of R and B in F_1 and F_2 progenies of Beta vulgaris

EC #	Cross	Gen@	A_	aa	N	X^2	P
R: Hypocotyl color							
EC1	rr X RR	F1	29	0	29	--	--
EC12	"	F1	49	0	49	--	--
EC4	rr X Rr	F1	11	9	20	0.200	.6-.7
EC6	Rr X RR	F1	52	0	52	--	--
EC8	RR X Rr	F1	56	0	56	--	--
EC10	"	F1	50	0	50	--	--
EC4-5	rr X rr	F2	0	101	101	--	--
EC4-18	Rr X Rr	F2	56	16	72	0.296	.5-.6
EC4-19	"	F2	36	12	48	0.000	1.00
COMBINE	F2's 4-18,4-19		92	28	120	0.178	.6-.7
EC4-9*	rr X rr	F2	0	32	32	--	--
EC4-20	"	F2	0	34	34	--	--
EC1-22	Rr X Rr	F2	31	16	47	2.050	.1-.2
B: Annualism							
EC1	BB X bb	F1	29	0	29	--	--
EC4	"	F1	20	0	20	--	--
EC12	"	F1	49	0	49	--	--
EC6	bb X bb	F1	0	52	52	--	--
EC8	"	F1	0	56	56	--	--
EC10	"	F1	0	50	50	--	--
EC4-5	Bb X Bb	F2	72	29	101	0.740	.3-.4
EC4-9*	"	F2	22	10	32	0.667	.4-.5
EC1-22	"	F2	26	21	47	9.709	<.05

@ Gen: Generation, N: Total progeny, P: Probability,
A_ : dominant phenotype, aa: recessive phenotype.
* Only spinach leaf mutant progeny were examined.

Table 1.14. F_2 segregation ratios of Mdhl, Pgml, R and B, paired for linkage analysis

Loci	N	S,A[@]	S,a		H,A	H,a		F,A	F,a		X^2	P	p
		S,S	S,H	S,F	H,S	H,H	H,F	F,S	F,H	F,F			
Mdhl-B													
EC 4-5	101	17*	7*		37	15		18	7		0.86	.9-.95	50+/- 6.1%
Pgml-B													
EC 4-5	101	21	7		31	18		20	4		4.75	.4-.5	46+/- 6.1%
Mdhl-R													
EC 1-22	47	7	3		15	8		9	5		2.99	.7-.8	
EC 4-18	72	17	2		30	8		9	6		4.74	.4-.5	
EC 4-19	48	5	6		20	3		11	3		6.94	.2-.3	
EC 4*	120	22	8		50	11		20	9		2.24	.8-.9	52+/- 5.6%
Pgml-R													
EC 1-22	47	10	1		18	13		3	2		14.22	<.05	
EC 4-18	72	8	5		30	5		18	6		3.30	.6-.7	45+/- 7.2%
Mdhl-Pgml													
EC 1-22	47	2	8	0	7	12	4	2	11	1	10.87	.2-.3	
EC 4-5	101	6	14	3	14	24	15	8	11	6	3.24	.9-.95	
EC 4-18	72	1	12	6	8	16	14	4	7	4	7.89	.4-.5	
EC 4-20	34	3	4	3	5	6	6	2	4	1	2.94	.9-.95	
EC 4*	207	10	30	12	27	46	35	14	22	11	6.26	.6-.7	48+/- 3.5%

@ S: slow homozygote, H: heterozygote, F: fast homozygote, A: dominant locus phenotype, a: recessive locus phenotypes, P: probability of independence, p: recombination percent +/- standard error determined using Allard's maximum likelihood formulas.

* pooled data was shown to be homogeneous using homogeneity chi-square test.

Table 1.15. Analysis of linkage between Pgi-1, B and R using recombination frequencies and adjusted chi-square values

Loci	N	S,A[@]	S,a	H,A	H,a	F,A	F,a	X^2	P	p
Pgi1-B										
EC 4-5	101	7	3	33	17	32	9	21.60	<.05	47+/- 6.1%
adjust exp^		6.4	2.1	37.9	12.6	31.5	10.5	2.80	.7-.8	
Pgi1-R										
EC 1-22	47	2	0	17	5	12	11	31.64	<.05	
EC 4-18	72	3	2	31	8	22	6	16.11	<.05	
EC 4-19	48	3	1	18	3	15	8	19.16	<.05	
EC 4*	110	6	3	49	11	37	14*	34.88	<.05	49+/- 5.8%
adjust exp		6.93	2.31	41.25	13.75	34.32	11.44	3.12	.6-.7	

@ S: slow homozygote, H: heterozygote, F: fast homozygote, A: dominant locus phenotype, a: recessive locus phenotypes, P: probability of independence, p: recombination +/- standard error determined using Allard's maximum likelihood formulas.

* pooled data was shown to be homogeneous using homogeneity chi-square test.

^ expected chi-square values adjusted for skewed Pgi-1 segregation ratios.

Table 1.16. Analysis of linkage between Pgi-1, Mdh-1 and Pgm-1 using recombination frequencies and adjusted chi-square values

Loci	N	S,S@	S,H	S,F	H,S	H,H	H,F	F,S	F,H	F,F	X^2	P	p
Pgi1-Mdh1													
EC 1-22	47	0	0	2	6	8	8	4	15	4	24.02	<.05	
EC 4-5	101	2	6	2	15	24	11	6	23	12	23.36	<.05	
EC 4-18	72	1	3	1	12	22	5	6	13	9	19.89	<.05	
EC 4-19	48	0	4	0	3	9	9	8	10	5	24.25	<.05	
EC 4*	221	3	13	3	30	55	25	20	46	26	50.92	<.05	53+/- 3.4%
adj exp^		4.6	9.3	4.6	27.6	55.2	27.6	23.0	46.0	23.0	3.83	.8-.9	
Pgi1-Pgm1													
EC 6	52	3	1	2	9	14	5	3	12	3	11.23	.1-.2	
EC 1-22	47	0	2	0	4	16	2	7	13	3	27.38	<.05	
EC 4-5	101	3	4	3	16	25	9	9	20	12	21.89	<.05	
EC 4-18	72	0	2	3	7	21	11	6	12	10	20.06	<.05	
EC 4*	173	3	6	6	23	46	20	15	32	22	37.68	<.05	52+/- 3.8%
adj exp		3.6	7.3	3.6	21.6	43.2	21.6	18.0	36.0	18.0	4.15	.8-.9	

@ S: slow homozygote, H: heterozygote, F: fast homozygote, P: probability of independence, p: recombination percent +/- standard error determined using Allard's maximum likelihood formulas.

* pooled data was shown to be homogeneous using homogeneity chi-square test.

^ expected chi-square values adjusted for skewed Pgi-1 segregation ratios.

square calculations were.

To confirm if skewed Pgi-1 ratio was the cause of the high chi-square values in all of the pairs of loci which were tested, expected values were calculated which accounted for the skewed Pgi ratio. These adjusted expected values were calculated by multiplying the observed Pgi class frequency (O) by the expected class frequency of the non-skewed locus (E), times the number of progeny (n). Formulas used in these calculations, using the combined data values from Table 1.9 (as an example) are as follows:

expected ?,? = 0 (E) X n
expected S,S = .084 (.25) X n
expected H,S = .500 (.25) X n
expected F,H = .416 (.50) X n

When the adjusted chi-square values were used to calculate the independence between the Pgi-1 and Mdh-1, Pgm-1, R and B loci, the probability of independence was above 0.60 in every case (Tables 1.15 and 1.16). These values were in agreement with the recombination percentages determined using Allard's equations on unadjusted data.

Chi-square values for independence between the Pgi-1 and Pgm-1 loci were also calculated for three F_1 lines (Table 1.17). These lines were significant since they did not have a skewed Pgi-1 ratio. The chi-square values for all three lines confirmed the independence of the Pgi-1 and Pgm-1 loci. It can be implied that the high chi-square values which were observed in the EC6 and F_2 data in Tables 1.15 and 1.16 were a result of the skewed Pgi ratio. It was determined that there was no linkage between Pgi-1 and any of the loci examined. As discussed earlier these crosses should be reproduced using parents which do not carry the spinach leaf mutant.

Table 1.17. Analysis of linkage between Pgi and Pgm using F_1 segregation data

Line	N	H;H[@] S,S	H,S	H;F S,H	F;H H,H	F;F S,F	H,F	X^2	P
EC1[&]	19		7	6	3	3		2.68	.4-.5
EC12	21		4	8	4	5		2.05	.5-.6
EC1+12[*]	40		11	14	7	8		3.00	.3-.4
EC10	25	3	6	7	5	1	3	4.44	.4-.5

@ S: slow homozygote, H: heterozygote, F: fast homozygote, P: probability of independence.
* pooled data was shown to be homogeneous.
& EC1: DHA X 138D, EC10: 138H X 138C, EC12: DHB X 138D.

SUMMARY

In summary, eight new loci were identified, four of which had more than one allele and segregated as expected (Table 1.18). None of these loci were found to be linked to one another or to the annualism or hypocotyl color loci. Two additional loci were proposed, however, more progeny must be analyzed to determine their inheritance. Pollen samples showed activity at only three of the eight loci. Of the segregating loci, three encoded dimeric enzymes and one encoded a monomeric enzyme.

Table 1.18. Summary of the identified isozyme loci and their important characteristics

Locus	# subunits[&]	# alleles	pollen	system
Mdh-1	2	2	active	I or II
Mdh-2	-	1	inactive	I or II
Mdh-3	2	2	inactive	II
Mdh-4	-	1	inactive	II
Mdh-5	-	1	inactive	II
Pgm-1	1	2	active	I or II
Pgi-1	2	2	active	I
Skdh-1	-	1	inactive	II
ME-1*	5	1	active	II
ME-2*	5	2	inactive	II

& - means # of subunits not known
* these loci are only proposed

CHAPTER II

FINGERPRINTING OF GENOTYPES USING ISOZYMES

CHAPTER II

FINGERPRINTING OF GENOTYPES USING ISOZYMES

INTRODUCTION

Isozyme electrophoresis has been used to distinguish cultivars and assess the genetic variation in a number of crops (Ignart and Weeden, 1984; Wu, Harivandi, Harding and Davis, 1984). Horizontal starch gel electrophoresis is the most common method used since a large number of samples and enzymes can be assayed quickly and inexpensively. Even heterozygous lines can be distinguished using allelic frequencies of isozyme loci.

Breeding lines and hybrids of many crops (maize for example) are highly inbred and homogeneous and have been readily distinguished using genotypic differences at isozyme loci (Cardy and Kannenberg, 1982). The relative ease by which cultivars of any species can be identified depends upon the isozyme uniformity within each line. Cultivars of species which have variable alleles at isozyme loci have been distinguished by comparing the allozyme frequencies of the cultivars (Nielsen, Ostergaard and Johansen, 1983).

Of the few papers written about sugarbeet isozymes, only one dealt specifically with the use of isozymes for distinguishing cultivars (Itenov and Kristensen, 1985). There have also been papers about using isozymes to distinguish interspecific hybrids and monosomic addition lines (Oleo, Van Geyt, Lange and DeBock, 1986; Jung, Wehling and Loptein, 1986).

Most sugarbeet breeding lines and cultivars are heterogeneous due to their modes of pollination and hybrid seed production. This

heterogeneity should permit a group of unrelated plants to be easily distinguished if enough marker loci are examined. The ability to distinguish individuals could have a variety of uses in a breeding program. Some examples of these uses are: detecting pollen contamination in crosses, checking seed lot integrity, detecting mislabeling of seed, plants or tissue culture plates, and assessing the genetic diversity of a breeding population. The objective of this research was to determine if individual plants from seed or tissue culture could be distinguished by isozyme phenotypes or "fingerprinting".

MATERIALS AND METHODS

Germplasm

A germplasm collection of individual plants was chosen which represented a diverse range of Beta vulgaris germplasm. This collection included plants sampled from sugarbeet breeding lines, mutant stocks, hybrid parents and a variety of Beta cultivars (Table 2.1). Plants were obtained from tissue culture stocks, or from random selections of single seedlings from a line. These plants were grown and maintained in the greenhouse where leaves were always available for sampling.

A total of 32 different lines and 39 individuals were examined by starch gel electrophoresis. Only 1 individual was sampled for 30 of the lines, whereas, several individuals were sampled from the remaining 2 lines (EL 44 and FC 701/5). Isozyme phenotypes, for 5 loci and 2 enzyme systems, were recorded for almost every individual.

Starch gel electrophoresis

The banding patterns of all of the individuals were determined by gel electrophoresis using the procedures and stains described in Chapter 1. In addition to the enzyme stains which were previously described, two enzyme systems, isocitrate dehydrogenase (Idh) and glutamic dehydrogenase (Gdh), were also used. The stain for isocitrate dehydrogenase contained 100 ml 0.1 M tris-HCl pH 8.0 buffer, 100 mg $MgCl_2$, 600 mg DL-isocitrate acid, 40 mg NADP, 40 mg NBT and 2 mg PMS. Glutamic dehydrogenase was stained with 2 g L-glutamic acid, 20 mg NAD,

20 mg NBT and 1 mg PMS in 50 ml 0.1 M tris-HCl pH 8.0.

Table 2.1. Germplasm used in fingerprinting study

Clone or Population	Characteristics	Source
T4E57-4	trout leaf mutant	J.C. Theurer, E. Lansing, MI
84MS-20	trout leaf mutant	J.C. Theurer, E. Lansing, MI
4C22-3	chlorina mutant	J.C. Theurer, E. Lansing, MI
M816-1	yellow root mutant	J.C. Theurer, E. Lansing, MI
4E25-3	semi-dwarf mutant	J.C. Theurer, E. Lansing, MI
MO78-48	plaintain leaf mutant	J.C. Theurer, E. Lansing, MI
J-3	stigmoid mutant	T. Kinoshita, Japan
EL 36	Type O, monogerm	USDA stock, E. Lansing, MI
EL 40	multigerm	USDA stock, E. Lansing, MI
EL 44	Type O, monogerm	USDA stock, E. Lansing, MI
EL 45	Type O, monogerm	USDA stock, E. Lansing, MI
EL 48	monogerm	USDA stock, E. Lansing, MI
FC 506	Type O, monogerm	R. Zielke, Carrollton, MI
FC 607	Type O, monogerm	G.A. Smith, Ft. Collins, CO
FC 708	Type O, monogerm	R. Hecker, Ft. Collins, CO
L53	some mangel background	J.C. Theurer, E. Lansing, MI
SP6822	multigerm	USDA stock, E. Lansing, MI
SP6926	Type O	G. Coe, Beltsville, MD
CMS B	male sterile	J.W. Saunders, E. Lansing, MI
80-111	Type O	J.W. Saunders, E. Lansing, MI
80-60	Type O	J.W. Saunders, E. Lansing, MI
138	spinach leaf source	J.W. Saunders, E. Lansing, MI
I436-3	Type O	J.W. Saunders, E. Lansing, MI
OA-1	Owens Annual Tester	J.W. Saunders, E. Lansing, MI
FC 701/5	rhizoctonia resistance	R. Hecker, Ft. Collins, CO
F1003	low respiration	D. Cole, Fargo, ND
GWK	mangel	Gartons White Knight
CHARD	chard	Fordhook Giant (Northrup King Seeds)
EW	table beet	Early Wonder (Northrup King Seeds)
WB 222	B. lomatogona	M.H.Yu, Salinas, CA

RESULTS AND DISCUSSION

Preliminary experiments

A variety of isozyme stains and electrophoresis buffer systems were examined to determine which ones fulfilled the criteria necessary for fingerprinting of individual plants. The criteria were: (1) the system and stain provided adequate resolution of the observed banding patterns, (2) the patterns contained enough variability to distinguish at least 2 individuals, and (3) the banding pattern of an individual was the same over a range of environmental conditions, leaf sizes and plant ages. The systems and enzyme stains which were examined are summarized in Table 2.2. Of all the systems and enzymes examined, only seven enzymes and two buffer systems fulfilled the outlined criteria.

A summary of the results of an isozyme survey of 32 diverse Beta genotypes is found in Table 2.3. As discussed in Chapter 1, crosses between a few of these genotypes were analyzed and loci were identified for four of the seven enzyme systems. These loci were Mdh-1, Mdh-3, Pgm-1, Pgi-1 and Skdh-1. In addition to these loci, the enzymes ME, Idh and Gdh gave consistent, repeatable patterns which were used to distinguish several individuals.

The frequencies of the banding genotypes among the 32 individuals were calculated for the four loci with more than one allele (Table 2.4). When these frequencies were calculated only one individual was randomly chosen from EL 44 and FC 701/5 to avoid possible skewing of the data. Since the 32 plants were not randomly selected, the frequencies in Table

Table 2.2. Summary of all the isozymes and systems examined

System*	pH	Gpd@	B-Glu	Akp	Skdh	Adh	Sdh	Per	Est	Gdh	Acp	Got	Hk	Glr	Lap	G6p	G2d	6pg	Mdh	Pgm	Pgi	Me	Idh
CS-B	5.7				X			X			X							X	X	X	X	X	X
CPH	7.0				X			X		X	X	X			X	X	X	X	X	X	X	X	X
CS-A	5.0								X	X	X	X	X	X		X	X		X	X	X	X	X
CS-C	6.5							X	X		X					X			X	X	X		X
Ash	7.0	X	X		X	X			X		X					X	X	X	X		X	X	X
CS-D	8.3			X		X	X	X	X	X	X	X	X	X	X			X	X	X	X	X	X

* CS-A,-B,-C and -D: Cardy, Stuber and Goodman, 1980.
CPH: Cheliak and Pitel,1984.
Ash: Ashton and Braden, 1961.

@ Abbreviations of enzymes

Gpd	glycerol-3-phosphate dehydrogenase	Hk	hexokinase
B-Glu	Beta glucosidase	Glr	glutathione reductase
Akp	alkaline phosphatase	Lap	leucine amino peptidase
Skdh	shikimate dehydrogenase	G6P	glucose-6-phosphate dehydrogenase
Adh	alcohol dehydrogenase	G2d	glycerate dehydrogenase
Sdh	sorbitol dehydrogenase	6pg	6-phosphogluconate dehydrogenase
Per	peroxidase	Mdh	malate dehydrogenase
Est	esterase	Pgm	phosphoglucomutase
Gdh	glutamate dehydrogenase	Pgi	phosphoglucose isomerase
Acp	acid phosphatase	Me	malic enzyme
Got	glutamate oxalate transaminase	Idh	isocitrate dehydrogenase

Table 2.3. Isozyme genotypes for clonal fingerprints

Clone	Source[@]	Isozyme system							
		MDH1	PGM1	PGI1	ME	IDH	MDH3	SKDH1	GDH
T4E57-4	MU-trout leaf	S*	S	F	II&	II	S	F	S
84MS-20-93	MU-trout leaf	S	S	F	I	II	S	S	-
4C22-3-1	MU-chlorina	S	SF	SF	I	II	S	S	S
EL 44-405	HP	S	F	S	II	I	S	S	S
EL 44-93	HP	S	F	S	II	I	S	-	-
EL 44-76	HP	S	-	S	II	I	S	S	-
EL 44-92	HP	S	-	S	II	I	S	S	-
EL 45-206	HP	S	F	S	II	I	S	-	S
M816-1-1	MU-yellow root	S	F	S	I	I	S	S	S
EL 48-91	HP	S	F	SF	H	I	S	-	-
EL 36-93	HP	S	F	F	U	I	F	-	-
OA-1	Owens annual	S	F	F	II	II	S	S	S
L53-90	HP	S	F	F	II	I	S	-	-
EL 40-92	HP	SF	SF	SF	U	I	SF	S	-
J-3	MU-stigmoid	SF	F	SF	I	I	S	S	F
4E25-3-1	MU-semi-dwarf	F	S	SF	I	II	S	S	S
6822-33	HP	F	S	F	H	I	F	S	S
FC 708-96	HP	F	S	F	I	I	S	-	-
FC 701/5-90	GR	F	S	F	I	I	S	-	-
FC 701/5-99	GR	F	SF	F	U	I	S	S	-
FC 701/5-98	GR	F	SF	F	U	I	S	S	-
FC 701/5-95	GR	F	S	F	I	I	S	S	-
FC 701/5-94	GR	SF	-	-	I	I	S	S	-
138H	BP	F	SF	S	I	I	SF	S	S
138C	BP	F	SF	SF	I	I	F	S	S
138D	BP	F	SF	SF	I	I	S	S	S
I436-3	BP	F	SF	SF	I	I	S	S	S
GWK-2	CV-mangel	F	SF	SF	I	II	S	S	S
F1003-7	GR-USSR origin	F	SF	F	II	I	S	S	S
CMS B	BP-male sterile	F	SF	F	I	I	S	S	S
FC 506-5	HP	F	F	S	I	I	S	S	S
FC 607-0-20	HP	F	F	S	U	I	S	-	S
6926-0-3	HP	F	F	SF	U	I	S	-	S
80-111	BP	F	F	SF	I	I	S	-	S
80-60	BP	F	F	F	I	I	S	S	S
CHARD-2	CV-chard	F	F	F	III	I	S	S	S
M078-48-1	MU-plaintain lf	F	F	F	II	I	S	S	S
EW-53	CV-table beet	U	F	SF	U	I	S	S	S
WB 222	B. lomotogona	U	SF	U	IV	I	S	S	-

@ The following are abbreviations used for the source: MU-mutant, HP-hybrid parent, CV-cultivar, GR-germplasm release and BP-breeding population.

* S, F and SF represent the homozygous slow, homozygous fast and heterozygous banding genotypes, - means undetermined.

& The following represent banding patterns: U-unique and H-both I+II.

2.4 may not be representative of a random survey of the Beta vulgaris germplasm population. Nonetheless, these values are presented to provide an estimate of the possible genotypic frequencies.

Table 2.4. Frequencies of genotypes at each isozyme locus

Locus	Banding genotype			
	SS	SF	FF	other
Mdh-1	.31	.06	.56	.06
Mdh-3	.84	.06	.09	0
Pgm-1	.19	.31	.50	0
Pgi-1	.19	.38	.41	.03

Malate dehydrogenase

Most genotypes exhibited one of the SS, SF or FF, Mdh-1 banding genotypes which were discussed in Chapter 1 (Table 2.4). It is interesting to note that only 2 out of 32 individuals were of the SF, or heterozygous type. It is difficult, however, to speculate as to why this frequency is so low because the sample was not random.

Two individuals, EW-53 and WB 222-1, did not show any of the three previously mentioned Mdh-1 banding genotypes. EW-53 was a selection from the commercial table beet cultivar, Early Wonder. The band produced by this individual was slightly slower than the fast band of Mdh-1 and slightly faster than the Mdh-2 band (Figure 2.1a). These results were observed when system I was used; however, when system II was used the EW-53 band could not be distinguished from the Mdh-1 fast band.

WB 222 was the accession number for the seed lot of a Beta

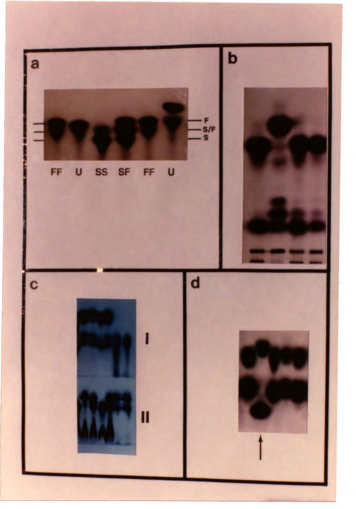


Figure 2.1. a) Mdh banding patterns using system I: (L to R) FF; band from EW-53; SS; SF; FF and band from WB 222-1. b) Mdh banding patterns using system II: (L to R) Mdh-1 SS; bands from WB 222-1; and two Mdh-1 SS types. Note Mdh-5 band is missing in WB 222-1. c) Comparison of Pgi zymograms using systems I (bottom) and II (top). d) Pgi zymogram (system II) showing WB 222-1 in lane 2.

lomatogona wild individual. The individual that was examined in this study was a seedling from that seed lot. WB 222-1 gave two bands which were active in the region of Mdh-1. When system I was used, there was a faint band which comigrated with the fast band of Mdh-1 as well as an even faster migrating dark band (Figure 2.1a). When system II was used, the faster migrating band was in the same position, but the slower migrating band appeared to have become two faint bands (Figure 2.1b). It was not determined whether the alleles which encoded the unique Mdh isozymes of these two individuals were from any of the previously described loci, or if they represented alleles of new Mdh loci.

Of the remaining Mdh loci examined, only Mdh-3 had more than one active allele. The only bands observed were the SS, SF and FF types which were described in Chapter 1. Eighty-four percent of the 32 individuals examined were of the slow, or SS banding type (Table 2.4). The only other notable occurance was the absence of the Mdh-5 band in the WB 222-1 individual (Figure 2.1b).

Phosphoglucose isomerase

When system I was used, most plants surveyed exhibited one of the SS, SF or FF, Pgi-1 banding genotypes which were described in Chapter 1. None of the banding genotypes were rare; however, there were considerably more individuals with the SF and FF banding types than there were with the SS banding type (Table 2.4). System II did not adequately resolve Pgi-1 into its individual bands, however, two darkly stained regions were observed (Fig 2.1c). The slower of these two regions was shown to be Pgi-1 (see Chapter 1).

One individual (WB 222-1) did not exhibit an SS, SF or FF banding type when system I was used. In fact, WB 222-1 did not show any

activity in the region of the gel occupied by Pgi-1. When system II was used to resolve group of genotypes for Pgi, WB 222-1 showed activity for a unique, slow migrating band (Figure 1d). All of the other plants were active for the Pgi-1 region and for a faster unidentified region. WB 222-1 also showed activity for a band which was faster than this unidentified region. Interspecific crosses and improved gel resolution will be needed to determine if the WB 222-1 band is allelic to Pgi-1, or if it is a different locus.

Malic enzyme

When stained for ME, the majority of the genotypes gave banding patterns I and II (Figure 2.2a). Patterns I, II, and the hybrid (H) pattern which showed both the I + II banding patterns, were described in Chapter 1. Patterns I, II and III consisted of 5 regularly spaced bands. The fastest migrating band of all three patterns was in the same position, however, the slowest bands were in different positions. The remaining bands were evenly spaced between the fastest and slowest bands.

WB 222-1 also gave a pattern (IV) which consisted of five evenly spaced bands; however, the fastest band was slower migrating than the fastest band of the other patterns. A few other individuals exhibited consistent, repeatable banding patterns which were different from each other and the other types (Figure 2.2a). These 7 unique (U) types differed from I, II, H and III by having either missing or additional bands.

Shikimate dehydrogenase

When 26 surveyed plants were stained for Skdh, 25 of these individuals showed the same single band. This band was defined in

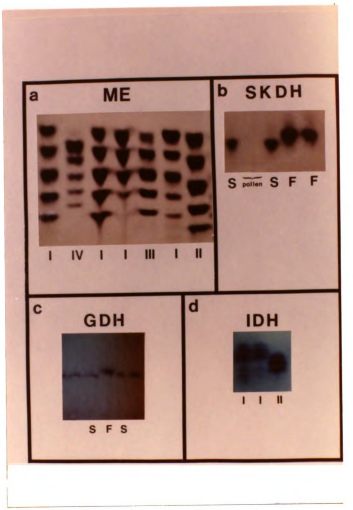


Figure 2.2. a) Malic enzyme banding patterns: I, II, III and IV. b) Shikimate dehydrogenase banding patterns: S and F. c) Glutamic dehydrogenase banding patterns: S and F. d) Idh banding patterns: I and II.

Chapter 1 as belonging to Skdh-1. One individual, a trout leaf mutant, showed a different band which was faster migrating than the band shown by the others (Figure 2.2b). Additional crosses involving this individual will be needed to determine whether or not this band was allelic to the Skdh-1 band.

Glutamate dehydrogenase

When 24 plants were stained for Gdh, 23 of these individuals showed the same single band which was labeled slow (S). One individual, a stigmoid mutant, showed a different band which was faster migrating than the band shown by the others (Figure 2.2c). Additional crosses involving this individual will be necessary to determine whether or not this band is allelic to the slow Gdh band.

Phosphoglucomutase

All individuals exhibited one of the SS, SF or FF Pgm-1 types which were described in Chapter 1. Only 19% of the individuals were SS types and 50% were FF types (Table 2.4).

Isocitrate dehydrogenase

The isocitrate dehydrogenase system could not be resolved adequately enough to identify loci, however, repeatable banding patterns were observed. In particular, several individuals exhibited a three banded pattern which was labeled pattern II. All other banding patterns were labeled pattern I. Although some of the pattern I individuals appeared different from each other, they were not consistently distinguishable and were therefore combined into one group. (Figure 2.2d).

Fingerprinting of lines

To obtain an estimate of the isozyme variability within sugarbeet lines, several plants from two of the lines were examined. There was no variability among the 4 plants examined from the line EL-44; however, there was variability for two of the enzymes among the 5 plants examined from FC-701/5 (Table 2.3). This variability indicated that comparisons of allelic frequencies would probably be necessary to distinguish sugarbeet cultivars. Allelic frquencies were recently used to distinguish 18 cultivars of monogerm, triploid sugarbeet (Itenov and Kristensen, 1985).

Summary

The main objective of this study was to determine if individual plants from seed or tissue cultures could be distinguished by fingerprinting using isozyme loci. This was shown to be very effective since very few marker enzymes were necessary to almost completely distinguish 32 different plants. Of the 32 different clones examined, 28 clones (87.5%) could be distinguished using isozymes alone. Only two pairs of clones (one pair closely related) could not be distinguished. These pairs of lines were EL 44/EL 45-206 and 138D/I436-3 (Table 2.3)

CHAPTER III

DESCRIPTION OF A SPINACH LEAF MUTANT

CHAPTER III

DESCRIPTION OF A SPINACH LEAF MUTANT

INTRODUCTION

Dwarf mutants are very common in many plant species and have provided a valuable contribution to breeding and the study of genetics and physiology (Pelton, 1964; Liu and Loy, 1972). Several dwarf sugarbeet mutants have been previously described. Abegg (1940) described 4 dwarf mutants. Crinkled foliage (Cr) had a reduced plant size and was linked to the R linkage group. The flaccid leaf mutant (f) had reduced vigor and was encoded by a locus which appeared to be independent of the R linkage group. A lethal mutant, miniature (m), had a greatly reduced plant size and was probably independant of the R linkage group. There was no linkage data available for the nana (n) mutant which had thich leathery leaves. All of these dwarf phenotypes were encoded by single recessive genes.

In addition, Theurer recently described a sugarbeet mutant called dwarf (d) which was also a single gene recessive (Theurer, 1968). The seedlings of this mutant had shortened hypocotyls and mature plants produced 10 cm tall seedstalks which could be elongated by GA treatment for improved seed production. It is impossible to determine if the dwarf (d) mutant is unique, since the mutants described by Abegg are no longer available.

A potentially new dwarf sugarbeet mutant was discovered in progeny of a cross between two half-sibs (138C and 138D). These half-sibs had a common female parentage. The leaves of the dwarf mutant plant were dark green and crinkled like those of a spinach plant, thus the mutant was

named spinach leaf (sl). The purpose of this study was to describe the spinach leaf mutant and to elucidate its mode of inheritance.

MATERIALS AND METHODS

Crosses

The parentage used to study the spinach leaf mutant was described in detail in Chapter 1. Reciprocal crosses were made in the greenhouse between the self-incompatible plants, 138C and 138D. F_1 crosses between male-sterile plants DH-A or DH-B and 138C or D were selfed and F_2 lines were produced. All of the above crosses were examined for segregation of spinach leaf types.

Gibberellic acid experiments

Experiment I - Eight week old, non-flowering, annual and biennial spinach leaf plants, which were full sib progeny of the F_1 plant EC4-9, were tested for "normalization" by gibberelic acid (GA_3) treatment. As controls, non-spinach leaf progeny of EC4-9 were also treated with GA. Three treatment levels of 200ug, 800ug and 1500ug GA_3 in/50ul 50%(v/v) acetone:water were used. 50ul of solution was applied to the apex every five days for a total of four applications.

Experiment II - Self seed of the F_1 plant EC4-5 were treated with GA_3 to see if it would improve the germination of spinach leaf seedlings. In two replications of 50 seed balls, seed was soaked for 30 minutes in either 1mg GA_3/150 ml water or 150 ml water. The seed was placed on moistened cotton wadding at room temperature for two days until they germinated. They were then transplanted into potting soil and placed in the greenhouse. Data was collected on percent seed balls showing emergence from the soil after 2, 3, 4 and 5 days.

RESULTS AND DISCUSSION

Description of a dwarf mutant

The spinach leaf mutant had a phenotype which was distinctly different from a normal sugar beet plant. Emergence of the spinach leaf seedlings took a mean of 4.6 days longer than normal seedlings from the same seed ball (Table 3.1). The cotyledons of the mutant seedlings opened at the soil surface compared to a height of 3-4 cm above the soil surface for a normal seedling (Figure 3.1a). On many occasions, the cotyledons even opened below the soil surface and were only observed when the surface of the soil was probed. The cotyledons of the mutant seedling were also much smaller than the normal seedlings. When the seedlings were removed from the soil, the difference in seedling height was due to the shorter hypocotyl of the spinach leaf seedling (Figure 3.1b).

The mature plant phenotypes were also very different. At every stage of the growth cycle the overall size of the mutant plant was about one third of the size of a normal plant. The leaf blade was also about one third the size of a normal leaf and was darker green, thicker, crinkled and brittle (Figure 3.1c). This description of the spinach leaf mutant is very similar to that of many single gene recessive dwarf mutants in other species (Pelton, 1964).

When F_2 progeny of an F_1 spinach carrier were exposed to artificial long days, the annual mutant and normal plants both produced flower stalks. The normal type segregated for male-fertile and male-sterile plants, however, all of the spinach leaf mutants appeared male-sterile.

The male-sterile anthers were creamy-white in color and showed no dehiscence. When the flower stalks of several mutant plants and a control DH-A plant were dusted regularly with pollen, the control plant set prolific seed but the mutant plants set no seed. Unfortunately the spinach leaf plant could not be selfed or backcrossed because it was both male and female sterile.

Table 3.1. Emergence date differences between normal and spinach leaf progeny of the F_1 line 4-7

seed ball #	normal days to emerge	spinach days to emerge	difference
1	3	8	5
2	4	7	3
3	4	8	4
4	4	19	15
5	4	8	4
6	4	7	3
7	4	7	3
8	4	7	3
9	4	8	4
10	5	7	2
Mean	4	8.6	4.6

Gibberellic acid experiments

In many plant species, the phenotype of single-gene dwarf plants has been altered to that of a normal plant by exogenous application of hormones. One of the most commonly used hormones is gibberellic acid (GA), especially GA_3 (Phinney, 1956a). Two experiments were conducted to determine if the spinach leaf mutant would respond to treatment with GA.

In the first experiment, the apices of spinach leaf and normal

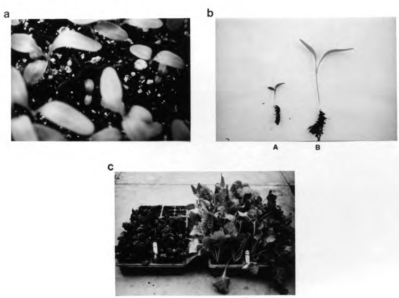


Figure 3.1. a) Germinating normal and spinach leaf seedlings. b) Comparison of spinach leaf (A) and normal (B) seedlings of the same age. c) Comparison of six week old spinach leaf (A) and normal (B) plants.

plants were treated with GA_3 to see the new growth response. New leaves which emerged on the mutant plant after GA treatment were somewhat yellowish and damaged, however, they were still of the spinach phenotype. New leaves of the GA-treated normal plants were also damaged and yellowish and were somewhat narrower than normal. Bolting of the annual segregates occured, however, this was probably due to the long day conditions, not the GA treatment. The flowers on the seed stalks of the spinach leaf mutant still appeared male and female sterile by visual inspection and lack of seed set. After four applications, the treatments were stopped because there was no apparent treatment effect except toxicity to the leaves. The damage to the leaves was probably due to the acetone in the GA treatment. None of the GA treatment levels caused any normalization of the spinach leaf phenotype, however, seed stalk height appeared to increase.

Other dwarf mutants have been found which were not responsive to GA. In tomatoes, the height of certain dwarfs was increased, but their phenotypes were not normalized (Soost, 1959). GA treatment also increased the seedstalk height in the sugarbeet dwarf (d) mutant, however, it did not normalize the mutant phenotype (Theurer, 1983). It is possible that a different form of GA might be effective on this mutant or it may be simply a GA insensitve dwarf.

In the second experiment, F_2 seed from an F_1 plant (known to produce spinach leaf progeny) was treated with GA_3 to determine if GA would improve germination and recovery of spinach leaf progeny. This was attempted because the recovery of some tomato dwarf mutants from seed has been vastly improved by application of GA solution to the seed (J. Zeevaart, personal communication). The rate of emergence of the beet seed treated with GA appeared faster than that of the untreated

seed (Fig 3.2). Germination of the spinach types may also have improved. In the 2 replications of the GA treatment, 1/110 and 2/136 seedlings were spinach leaf types. In the 2 replications which had no GA treatment there were no spinach leaf seedlings out of a total of 104 and 120 seedlings (Table 3.2).

In a similar experiment, F_2 seed from eight F_1's which were Pgi SF were treated with GA to attempt to improve spinach type germination. Only one of the eight lines produced any spinach progeny. The ratio of normal:spinach progeny from the treated seed of this line was 21:1 as compared to 47:1 for an untreated control. Although the GA treatment appeared to improve recovery of the spinach leaf mutant, the number of mutant progeny was not increased to a frequency of one quarter of the progeny. This is the frequency that would be expected if the mutant is controlled by a single recessive gene.

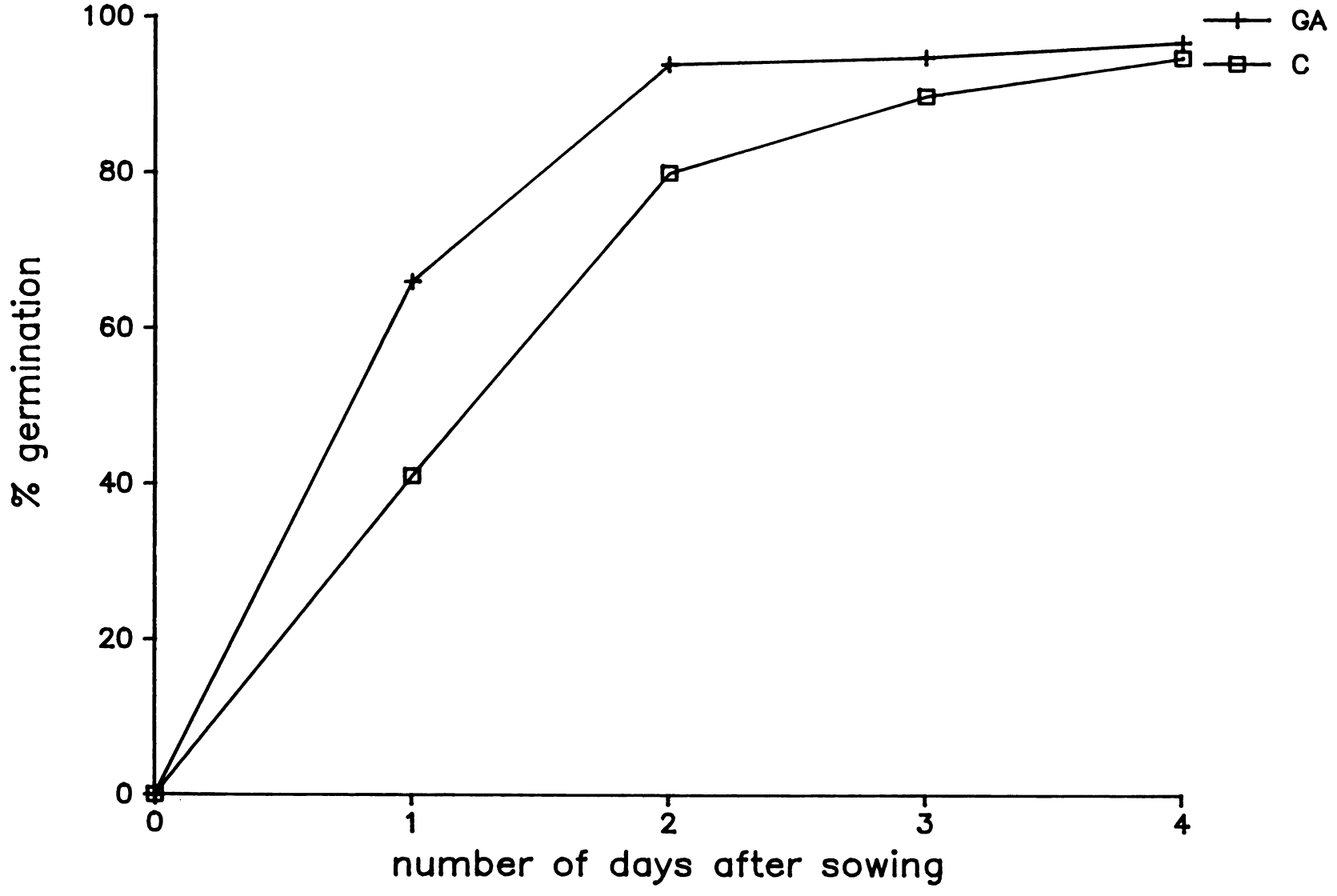
Table 3.2. Comparison of the number of spinach leaf seedlings from untreated and GA treated seed balls

treatment	# seed sown	# seedlings	# spinach leaf
Control I	50	104	0
Control II	49	120	0
GA I	50	110	1
GA II	50	136	2

Inheritance of the spinach leaf mutant

Under ideal circumstances, the inheritance of the spinach leaf mutant would have been determined by selfing the mutant or backcrossing it to one of its carrier parents. Unfortunately, female sterility of the mutant precluded this possibility. Examination of segregation

Figure 3.2. Comparison of the percent germination (averaged over 2 reps) of untreated and gibberelic acid treated seed of the F_1 spinach carrier EC4-5.


100
80
60
40
20
0
% germination
0
1
2
3
4
number of days after sowing
GA
C

ratios of F_1 and F_2 progenies from spinach carriers was the only way by which the inheritance could be hypothesized.

Progeny from reciprocal 138C and 138D crosses segregated for normal and spinach seedlings. In two separate sowings of EC6 F_1 seed, the normal to spinach ratio was 15:1 and 6:1 (Table 3.3). Progeny from EC8 segregated in a 10:1 normal:spinach ratio. There may have been more spinach progeny from this cross except for the fact that only the first 56 seedlings to germinate were transplanted. This happened before it was known that the spinach leaf types germinate slowly. The 6:1, 10:1 and 15:1 F_1 segregation ratios might have actually been 3:1 ratios which were skewed by poor germination and viability of the spinach leaf mutant seeds. More progeny were examined to determine if this explanation was correct.

Table 3.3. Summary of initial F_1 crosses which gave spinach progeny

EC #	Cross	# sown	# germ	% germ	Sp	Sp ratio
EC6	138C X 138D	50	47	94%	3	15:1
EC8	138D X 138C	50	59@	>100%	5	10:1
EC6*	138C X 138D	49	47	96%	7	6:1

@ Only 56 were transplanted. of the last 5 EC8 seedlings transplanted, 4 were spinach.
* second sowing

F_2 Progeny from 27 F_1 individuals were also examined for spinach leaf segregation (Table 3.4). Only 7 out of 27 lines produced spinach leaf progeny. If the spinach leaf mutant is controlled by a single recessive gene, half of the F_1 lines should have been carriers of the spinach leaf allele. For five of the 27 lines, however, less than 80

Table 3.4. Spinach leaf segregation in three separate sowings of F_2 seed

EC #	sown on 4.29.86 # sown	# germ	# sp	sown on 6.29.86 # sown	# germ	# sp	total # of progeny
1-1	50	43	0	70	*	0	43*
1-6	50	43	0	0	-	-	43
1-7	50	60	0	160	*	0	60*
1-20	50	51	0	0	-	-	65
1-20@	54	14	0	-	-	-	-
1-22	50	51	0	155	116	0	252
1-22@	105	85	0	-	-	-	-
1-24	50	71	1	244	266	1	337
1-28	50	58	0	124	120	0	178
4-5	50	52	0	54	*	1	52*
4-6	50	63	0	87	126	0	189
4-7	50	79	0	136	184	4	486
4-7@	104	223	10	0	-	-	-
4-7@	50	96	2	0	-	-	-
4-8	50	62	0	79	108	1	170
4-9	50	59	0	220	326	32	385
4-10	50	57	0	230	519	14	576
4-14	50	74	0	97	*	0	74*
4-16	50	47	0	168	*	0	47*
4-18@	110	255	0	0	-	-	255
4-19	50	33	0	144	191	0	382
4-19@	103	158	0	-	-	-	-
4-20	50	24	0	0	-	-	192
4-20@	108	168	0	-	-	-	-
12-9	50	55	0	86	131	0	317
12-9@	109	131	0	-	-	-	-
12-15	50	60	0	133	*	0	60*
12-16@	100	128	0	0	-	-	128
12-17	50	50	0	259	*	0	50*
12-21	50	51	0	96	*	0	51*
12-22	50	40	0	0	-	-	40
12-25	50	75	0	0	-	-	75
12-36	50	53	0	0	-	-	53
12-37	50	46	2	53	184	6	230

@ seed from these lines were treated with GA_3 before they were sown on 12.26.86

* complete data not available

seedlings were available for spinach leaf segregation counts. This was not an adequate number to determine if the parent F_1 was a mutant carrier. In addition, many of the F_2 seedlings were deformed, died and could not be classified. This evidence indicated that although half of the lines may have been spinach carriers, underrepresentation of the spinach leaf seedlings caused misclassification of some lines.

For all of the F_1 and F_2 progeny which segregated for spinach leaf, the normal to spinach ratio ranged from 6:1 to 264:1 (Table 3.5). It is difficult to explain why there was such a wide range of segregation ratios. Although the spinach leaf seedlings were definately weak and often inviable, this does not explain how one line could give an 8:1 ratio and another give a 264:1 ratio.

Table 3.5. Summary of spinach ratios from the F_1's and F_2's

Line	spinach	normal	germ %	ratio	fraction
EC6*	3	44	94%	15:1	.0638
EC6	7	41	96%	6:1	.1429
EC8	5	51	118%	10:1	.0893
EC1-24	1	70	142%	69:1	.0143
EC1-24	1	265	109%	264:1	.0038
EC4-5	1	53	--	52:1	.0189
EC4-5@	3	243	246%	81:1	.0123
EC4-7	4	180	135%	44:1	.0222
EC4-7	2	94	98%	47:1	.0208
EC4-7@	10	213	96%	21:1	.0448
EC4-8	1	107	137%	106:1	.0093
EC4-9	32	294	148%	8:1	.1111
EC4-10	15	504	226%	33:1	.0294
EC12-37	2	44	92%	21:1	.0455
EC12-37	8	176	120%	21:1	.0455
Total	92	2136	--	23:1	.0413

* lines listed more than once indicate different sowings
@ treated with GA

The percent germination of all the lines was greater than 90%, however, this was not a reliable indicator since all of the seed was multigerm. The health of the plant may have contributed to the variability of the segregation ratios. Some plants were infested with aphids and spider mites during seed set, therefore weak seeds may have been aborted in an effort to conserve energy for the healthier seed. If this were the case, the spinach leaf embryos may have been preferentially aborted causing skewed progeny ratios.

The skewed ratio might also be a result of linkage of the spinach leaf locus to the self-incompatibility locus. A 20% recombination frequency between the loci would result in a 9:1, normal to spinach leaf ratio (Table 3.6). This coupled with poor germination and variable health of the F_1's during seed set might explain the observed range of skewed ratios.

Table 3.6. Possible F_2 phenotypes given 20% recombination between the spinach leaf and self-incompatibility loci

		Male gametes			
		Parental types		Recombinant types	
		Sl Sf	sl Sl	Sl Sl	sl Sf
Female Gametes		.8	incompatible		.2
P	.4 Sl Sf*	.32 SlSl SfSf	----	----	.08 Slsl SfSf
	.4 sl Sl	.32 Slsl SfSl	----	----	.08 slsl SfSl
R	.1 Sl Sl	.08 SlSl SfSl	----	----	.02 Slsl SfSl
	.1 sl Sf	.08 Slsl SfSf	----	----	.02 slsl SfSf

* Sl,sl: spinach leaf types; Sf,Sl: self-incompatibility types
Expected spinach ratio: .9 normal leaf : .1 spinach leaf

Summary

Although the results were inconclusive, the spinach leaf dwarf mutant was probably caused by a recessive allele at one locus. This is the mode of inheritance of the five known sugarbeet dwarf mutants as well as most of the dwarf mutants in other species (Abegg, 1940; Owen and Ryser, 1942; Theurer, 1983; Pelton, 1964). None of the evidence argues against this possibility and much of it supports a recessive single locus mode of inheritance. The spinach leaf mutant also appears to be GA insensitive although GA treatment may slightly improve seed germination.

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