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Comparative and genetic studies of isozymes in resynthesized and cultivated *Brassica napus* L., *B. campestris* L. and *B. alboglabra* Bailey

B. Y. Chen [1], W. K. Heneen [1]* and V. Simonsen [2]

[1] Department of Crop Genetics and Breeding, The Swedish University of Agricultural Sciences, S-268 00 Svalöv, Sweden
[2] Department of Ecology and Genetics, University of Aarhus, Ny Munkegade, DK-8000 Aarhus C, Denmark

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Summary. Enzyme electrophoresis was used to compare newly resynthesized *Brassica napus* with its actual parental diploid species, *B. campestris* and *B. alboglabra*. Comparisons were also made with cultivated *B. napus*. Of the eight enzyme systems assayed, four were monomorphic (hexokinase, malate dehydrogenase, mannose phosphate isomerase and peroxidase), whereas the remaining four were polymorphic (glucosephosphate isomerase, leucine aminopeptidase, phosphoglucomutase and shikimate dehydrogenase), when comparisons were made within or between species. The polymorphic enzyme patterns observed in the newly resynthesized *B. napus* disclosed that the homoeologous loci contributed by the parental species were expressed in the amphiploid. Analysis of the glucosephosphate isomerase enzyme in a breeding line (Sv 02372) of *B. napus* indicated that, in this case, the gene originating from *B. campestris* was switched off whereas that of *B. oleracea* was expressed. Duplicated enzyme loci were observed in *B. campestris* and *B. alboglabra*, thus providing additional evidence to support the hypothesis that these species are actually secondary polyploids derived from an unknown archetype of $x=6$.

Key words: *Brassica* – Enzyme electrophoresis – Inheritance – Silent gene – Duplicate loci

Introduction

Proteins or isozymes, being the ultimate products of gene expression, serve as excellent genetic markers. There has been an increasing use of isozymes in *Brassica* research when dealing with aspects such as taxonomic or phylogenetic relationship of different species (Vaughan and Denford 1968; Vaughan et al. 1970; Khalatkar 1976; Vaughan 1977; Uchimiya and Wildman 1978; Yadava et al. 1979; Iwasaki 1983; Arús et al. 1987), identification and characterization of interspecific hybrids (Coulthart and Denford 1982; Schenck and Wolf 1986; Sundberg and Glimelius 1986; Terada et al. 1987) and determination of genome evolution patterns (Quiros et al. 1985, 1987). Isozymes are very useful tools for sib-determination in hybrid seed production, if there are appropriate differences in isozyme zymograms between the parental inbred lines (Nijenhuis 1971).

The cultivated oilseed rape (*Brassica napus* L., genomes AACC) is an amphiploid derived from *B. campestris* L. (AA) and *B. oleracea* L. (CC) (U 1935). The spontaneous formation of this species had occurred in an unknown historical time. Once formed, it must have undergone an evolutionary process largely independent from its parental diploid species, in spite of the possibility of introgression through backcrossing. From an evolutionary viewpoint, it is of interest to compare newly resynthesized material of *B. napus* with cultivated accessions which are of natural origin. Isozymes are very suitable characters for such comparisons since they may imply eventual changes in parental homoeologous genes that have occurred during the evolution of the amphiploid.

Newly resynthesized *B. napus* has been obtained in a breeding program for introducing the yellow-seeded character from the parental diploids into this species (Chen et al. 1988). Part of this material has been studied using isozyme analysis. It is the purpose of this paper to present the results obtained from comparisons made on resynthesized and cultivated forms of *B. napus*, also in

* To whom correspondence and reprint requests should be addressed

relation to the parental species. Isozyme inheritance of glucosephosphate isomerase was also studied in a cross between resynthesized and cultivated oilseed rape lines.

Materials and methods

Plant material

The following accessions were used: *Brassica alboglabra* Bailey (a form of *B. oleracea,* Prakash and Hinata 1980) (No 4003, No 6510), *B. campestris* L. (Sv 85-38301, Sv 741008), *B. campestris* L. var. yellow sarson (K-151, K-88, T-42, RS-24), resynthesized *B. napus* L. (No 7075, No 7076, No 2305, No 3401, No 7406, No 7407, No 7408, No 7409), and *B. napus* L. of natural origin (Topas, Global, Puma, Sv 02372). Actual parents of the resynthesized *B. napus* lines are indicated in Table 1 (under Results) and further details on the origin of this material can be found in Chen et al. (1988).

Sample preparation

Electrophoresis assays were made on individual seedlings. Six to nine seeds were used from each accession to detect the allelic variation within accession. Seeds were germinated on moist filter paper for about 1 week. Cotyledons and hypocotyls were sampled together for electrophoresis. The sample buffer composition was the Tris-HCl grinding buffer-PVP solution as given by Soltis et al. (1983). For extraction of enzyme, 30 µl sample buffer was used.

Electrophoresis and enzyme detection

The following eight enzymes were assayed – 1: glucosephosphate isomerase [GPI, EC 5.3.1.9, preferential enzyme name and enzyme commission number as given by Dixon and Webb (1979)]; 2: hexokinase (HK, EC 2.7.1.1); 3: leucine aminopeptidase (LAP, EC 3.4.11.1); 4: malate dehydrogenase (MDH, EC 1.1.1.37); 5: mannose phosphate isomerase MPI, EC 5.3.1.8); 6: peroxidase (PER, EC 1.11.1.7); 7: phosphoglucomutase (PGM, EC 2.7.5.1); 8: shikimate dehydrogenase (SDH, EC 1.1.1.25).

Electrophoresis was performed in starch gels (12% Connaught) using three different buffer systems: (1) Gottlieb IV buffer (Gottlieb 1981) for HK, MPI and LAP; (2) Histidine buffer ph = 8.0 (Brown 1983) for GPI, PGM and SDH; (3) Tris-citrate buffer (Nielsen and Chapman 1977) for MDH and PER. The gels were run at 100 mA for 3 h for all buffer systems.

Examples of assays made on these enzymes can be found in: Yndgård (1972; GPI), Jelnes (1971; HK), Shaw and Prasad (1970; LAP), Frydenberg and Simonsen (1973; MDH), Harris and Hopkinson (1976; MPI and PGM), Nielsen and Johansen (1986; PER), Weeden and Gottlieb (1980, SDH).

Isozyme and allozyme nomenclature

Isozymes controlled by different loci in diploid species (e.g. *B. alboglabra* and *B. campestris*) are expressed as bands at different zones of the zymogram. Different isozymes/loci and their corresponding banded zones are numbered, starting from the zone closest to the anode as number '1'. The abbreviation of the enzyme is followed by this numerical code (e.g. Gpi-2 indicating the locus expressed by the second banded zone from the anode). Different bands within a zone, expressing allozymes, are designated by a superscripted alphabetical code starting by 'a' for the most anodal band. The genomic location of the locus is indicated by the genome symbol which in the present material would be A or C. Thus, Gpi-2C^{a} would indicate the allele of the locus coding the allozyme 'a' of the second isozyme of GPI, located in the C genome. Homozygote and heterozygote genotypes can be expressed as Gpi-2C^{aa} and Gpi-2C^{ab}, respectively. An inactive or silent allele would be designated by the superscription 'null'. In a monomorphic enzyme system, the same zymogram characterizes different individuals. A variation of zymogram patterns on the other hand indicates a polymorphic enzyme system.

Results and discussion

Starch gel electrophoresis yielded resolvable zymograms which were consistent for each of the eight enzyme systems and for the different *Brassica* accessions studied. Four of the eight enzyme systems (HK, MDH, MPI and PER) were found to be monomorphic. The zymograms of HK, MPI and PER were single-banded, but three-banded for MDH. Apparently, there is more than one locus for MDH in the diploid species *B. alboglabra* and *B. campestris.* The remaining four systems (GPI, LAP, PGM, and SDH) showed polymorphisms within species (due to allelic variants) and/or between the different species (due to differences between homoeologous loci). In one *B. napus* accession of natural origin, the polymorphisms also comprised a null (silent) locus for GPI isozyme which is located in the A genome (see below).

Polymorphic isozymes and zymogram comparisons

The inheritance of the three polymorphic isozyme systems, GPI, LAP and PGM, was studied in *B. oleracea* by Arús and Orton (1983). The genetics of SDH has not yet been reported. The designations given here to the different SDH loci are tentative and need to be genetically confirmed. The occurrence of different alleles for the same locus and/or different loci for various isozymes in all *Brassica* accessions is presented in Table 1. A detailed description and zymogram comparisons between accessions are presented below.

Glucosephosphate isomerase (GPI)

Two zones of enzymatic activity were detected. The first zone (i.e. closer to the anode, GPI-1) exhibited a single band and was monomorphic for all accessions. The second zone (i.e. closer to the cathode, GPI-2) was polymorphic. In *B. alboglabra,* No 4003 had two allelic variants, Gpi-2C^{a} and Gpi-2C^{d}, while No 6510 had the Gpi-2C^{a} allele only. In *B. campestris,* Sv 85-38301 had alleles Gpi-2A^{a} and Gpi-2A^{b}; Sv 741008 had the allele Gpi-2A^{b}; whereas the four yellow sarson accessions had the allelic variant Gpi-2A^{c}. When No 4003 and Sv 85-38301 were heterozygous (i.e. Gpi-2C^{ad} and Gpi-2A^{ab}), three-banded zymograms were observed. This indicates that GPI is functionally a dimeric enzyme, as reported by Arús and Orton (1983). GPI enzymes have been found to be functionally dimeric also in other plant species (Gottlieb 1982; Cheliak and Pitel 1984; Kahler

Table 1. The alleles and loci controlling allozymes and isozymes in *Brassica alboglabra, B. campestris* and *B. napus* of resynthesized and spontaneous origin (+ = present)

Accession	GPI				LAP			PGM				SDH			
	Gpi-2A[a] Gpi-2C[a]	Gpi-2A[b]	Gpi-2A[c] Gpi-2C[c]	Gpi-2C[d]	Lap-1A[a]	Lap-1A[b]	Lap-1C[c]	Pgm-1C[a]	Pgm-1A[b] Pgm-1C[b]	Pgm-2A[c]	Pgm-2C[d]	Sdh-1C[a]	Sdh-1A[b]	Sdh-2A[c]	Sdh-3A[d] Sdh-2C[d]
B. alboglabra Bailey															
No4003	(+			+)*			+	(+	+)		+	+			+
No6510	+						+		+		+	+			+
B. campestris L.															
Sv85-38301	(+	+)			(+	+)			+	+			+	+	
Sv741008		+			(+	+)			+	+			+	+	
B. campestris L. var yellow sarson															
K-151			+		+				+	+			+	+	+
K-88			+		+				+	+			+		+
T-42			+		+				+	+			+		+
RS-24			+		+				+	+			+	+	+
B. napus L. (resynthesized)															
No7075 (No6510 × Sv85-38301)	+	+				+	+		+	+	+	+	+	+	+
No7076 (Sv85-38301 × No6510)	+	+					+		+	+	+	+	+	+	+
No2305 (No4003 × Sv85-38301)		+		+	+		+		+	+	+	+	+	+	+
No3401 (No4003 × Sv741008)		+		+		+	+		+	+	+	+	+	+	+
No7406 (No4003 × K-151)			+	+	+		+		+	+	+	+	+	+	+
No7407 (No4003 × K-88)	+		+		+		+		+	+	+	+	+		+
No7408 (No4003 × T-42)			+	+	+		+		+	+	+	+	+		+
No7409 (No4003 × RS-24)			+	+	+		+		+	+	+	+	+	+	+
B. napus L. (natural)															
Topas			+			+	+		+	+	+	+	+	+	+
Global		+				+	+		+	+	+	+	+	+	+
Puma			+			+	+		+	+	+	+	+	+	+
Sv02372			+			+	+		+	+	+	+	+	+	+

* +located within brackets indicate alleles of the same locus

and Lay 1985; Østergaard et al. 1985; Quiros and McHale 1985; Suiter 1988).

In all accessions of resynthesized *B. napus*, only three-banded zymograms were observed. This could be explained by the presence of two GPI loci derived from the parental diploid species, Gpi-2A and Gpi-2C and the formation of interspecific heterodimers. Since A and C genomes apparently were derived from the same archetype of x = 6 (Prakash and Hinata 1980), the formation of such interspecific heterodimers could be expected. The homoeology between the A and C genomes was further indicated by the fact that the divergence between the homoeologous loci was within the intraspecific allelic divergence, on the basis of electrophoretic migration distance. On the other hand, all *B. napus* accessions of spontaneous origin exhibited single-banded zymograms only. This is most likely a consequence of the inactivation of one parental GPI-locus. This will be shown later on.

Leucine aminopeptidase (LAP)

This enzyme exhibited two zones of enzymatic activity. LAP-1 was polymorphic but more studies are needed to clarify the nature of LAP-2. The *B. alboglabra* lines No 4003 and No 6510 had the allele Lap-1C^c. Two other allelic variants, Lap-1A^a and Lap-1A^b, were detected in the *B. campestris* lines Sv 85-38301 and Sv 741008. The four yellow sarson accessions had the same allele Lap-1A^a. With the exception of No 7076, all resynthesized *B. napus* accessions showed two-banded zymograms (Fig. 1 a), which was simple summation of the bands of the parental diploids. This fits well in that LAP is functionally a monomeric enzyme (Arús and Orton 1983). The zymogram of No 7076 exhibited a single band, indicating the activity of Lap-1C^c. Depending on the genotype of the *B. campestris* parent, such a one-banded zymogram could be a consequence of a concurring Lap-1A^c or due to the presence of a Lap-1A^{null}. These two Lap-1A alleles have not been found in the limited number of seedlings analysed. A two-banded phenotype for LAP-1 was observed in all *B. napus* accessions of spontaneous origin, thereby indicating the expression of both Lap-1A and Lap-1C loci.

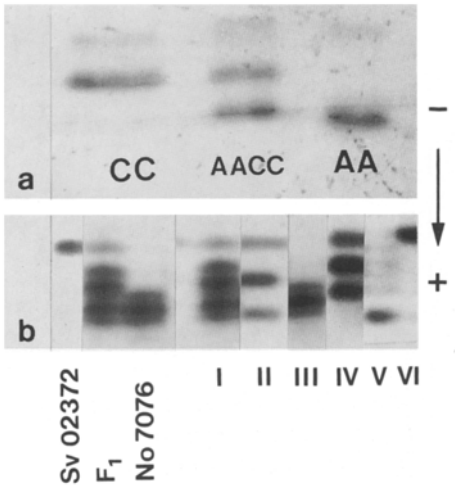

Fig. 1. **a** The expression of Lap-1A^a of *Brassica campestris* (AA) and Lap-1C^c of *B. alboglabra* (CC) in the resynthesized *B. napus* (AACC). **b** *B. napus* zymograms showing segregation patterns of GPI in the F_1 and F_2 of the cross Sv02372 × No7076 and the F'_2 of the backcross (Sv02372 × No7076) × Sv02372 (Table 2)

Phosphoglucomutase (PGM)

Two regions, one fast migrating (PGM-1) and one slow migrating (PGM-2), were observed in the zymograms of this enzyme. According to Arús and Shields (1983), PGM-1 in *B. oleracea* is coded by one locus Pgm-1C. Each allele of this locus is expressed by two bands (possibly due to post-translational modification). The faster-migrating band of each pair is less active than the slower-migrating band. The same was observed in this study in both *B. alboglabra* and *B. campestris* accessions. Two allelic variants, Pgm-1C^a and Pgm-1C^b, were found in No 4003. All *B. campestris* accessions were invariant by having the same allele Pgm-1A^b. In regard to PGM-2, the two loci Pgm-2A^c and Pgm-2C^d were distinguished by their different electrophoretic mobilities. No allelic variation was detected between intraspecific accessions. The zymograms of the resynthesized *B. napus* lines were an addition of the two parental patterns, which is an expected result for a monomeric enzyme. All cultivars of *B. napus* showed the same phenotype as the resynthesized lines.

Shikimate dehydrogenase (SDH)

SDH is functionally a monomeric enzyme (Brown 1983). The *B. alboglabra* accessions No 4003 and No 6510 had the same two-banded phenotype. The two *B. campestris* accessions Sv 85-38301 and Sv 741008 shared another type of two-banded phenotype. Interestingly, the four yellow sarson accessions showed three-banded (K-151, RS-24) and two-banded (K-88, T-42) zymograms, although these accessions have the same geographical origin and are morphologically very much alike. Since the self-compatible K-151 and RS-24 were inbred lines and showed no variation within accession, their three bands would be most likely coded by three loci. The two-banded phenotype of K-88 and T-42 was probably due to the null mutation of Sdh-2A^c. The phenotype of Sv 85-38301 and Sv 741008 could similarly be accounted for by the null mutation of Sdh-3A^d. The three loci of SDH in the A genome could be a result of gene duplication. Inheritance study is under way to test this hypothesis. The bands of resynthesized *B. napus* were in all cases a simple addition of those of the corresponding actual parental diploid species. All cultivated *B. napus* lines of

Table 2. Segregation of zymograms for GPI enzyme at the second zone in F_2 and F_2' (backcross) progenies of crosses between natural (Sv02372) and resynthesized (No7076) *Brassica napus* lines (Fig. 1 b) and the goodness-of-fit tests to the genetic model proposed

Generation	Zymogram	I	II	III	IV	V	VI
	− cc	—	—		—		—
	bc	—			—		
	ac	—	—				
	bb	—		—	—		
	ab	—		—			
	↓ aa	—	—	—		—	
	+						
	Genotype	$2A^{bb}\ 2C^{ac}$ $2A^{b\,null}\ 2C^{ac}$	$2A^{null\,null}\ 2C^{ac}$	$2A^{bb}\ 2C^{aa}$ $2A^{b\,null}\ 2C^{aa}$	$2A^{bb}\ 2C^{cc}$ $2A^{b\,null}\ 2C^{cc}$	$2A^{null\,null}\ 2C^{aa}$	$2A^{null\,null}\ 2C^{cc}$
F_2	Exp	11.25	3.75	5.625	5.625	1.875	1.875
Sv02372 × No7076	Obs	13	4	2	8	1	2
$\chi^2 = 4.04$, DF = 5, P_{range}: 0.70 − 0.50							
F_2'	Exp	6.25	6.25		6.25		6.25
(Sv02372 × No7076)	Obs	5	9		4		7
× Sv02372							
$\chi^2 = 2.36$, DF = 3, P_{range}: 0.50 − 0.30							

spontaneous origin exhibited the four-banded zymograms observed in most accessions of the resynthesized *B. napus*.

Inheritance of GPI isozyme in B. napus

As mentioned above, all *B. napus* accessions of spontaneous origin exhibited a single-banded phenotype whereas the resynthesized ones had a three-banded phenotype. The inheritance was then studied in a cross between the breeding line Sv 02372 and the resynthesized accession No 7076.

From the pedigree of No 7076, it can be inferred that the genotype of No 7076 is Gpi-$2A^{bb}$ Gpi-$2C^{aa}$. Sv 02372 is a homozygous line because no allelic variation was detected. The F_1 between Sv 02372 and No 7076 showed a six-banded zymogram. The segregation of zymograms in F_2 and F_2' (backcross to Sv 02372) and the Chi-square test are given in Table 2 (Fig. 1 b). Due to the small sampling size, the observed frequencies for zymogram III, V and VI in the F_2 progeny were a little too low for a good Chi-square test. These three zymograms were therefore pooled and a new Chi-square test was calculated. A good fitness was again realized ($\chi^2 = 3.33$, DF = 3, P_{range}: 0.50 − 0.30).

These results conclusively indicated the presence of a null locus for GPI enzyme located in the A genome. The genotype of Sv 02372 would thus be Gpi-$2A^{null\,null}$ Gpi-$2C^{cc}$. Considering the great importance of GPI enzyme in the glycolytic pathway, it must be hardly bearable for a diploid species such as *B. campestris* to harbor a null mutation for this enzyme. No such allele has ever been reported in any diploid *Brassica* species before. Therefore, the null mutation or 'switching off' of this locus was probably an event which had occurred after the spontaneous formation of the amphiploid *B. napus*. The presence of an active Gpi-2C in the C genome of *B. napus* would compensate the null mutation in the A-locus. The 'switching off' of duplicate loci after polyploidization was reported in fish species (Ferris and Whitt 1977). In hexaploid wheat, it was shown that few of the duplicate loci have been silenced (Hart 1983), which is probably due to the recent origin of this species (Hart and Langston 1977). *B. napus* is believed to be of fairly recent origin (Olsson 1960). This may explain the high retention of homoeologous gene expression for three polymorphic isozyme systems among the four examined.

Evidence for the secondary polyploid nature of Brassica

Several researchers have advocated that the basic genome of *Brassica* is x = 6 (Sikka 1940; Röbbelen 1960; Kamala 1976; Prakash and Hinata 1980). From this basic number the genomes A = 10 and C = 9 as well as B = 8 (*B. nigra*) would have been derived by polyploidization, aneuploidy and chromosome rearrangements. In this study, the zymograms for MDH and SDH enzymes in *B. alboglabra* and *B. campestris* indicated the presence of duplicate loci, thereby supporting the above assumption. Similar and additional evidence from analyses made on these and other isozymes has also recently been presented by Quiros (1987) and Quiros et al. (1987). Studies

on maximum numbers of duplicate genes for isozymes will provide information for further testing the x = 6 as the basic *Brassica* genome.

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