Theor Appl Genet (1992) 84:403–410

Isozyme analysis as a tool for introgression of *Sinapis alba* germ plasm into *Brassica napus*

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Received November 9, 1991; Accepted November 15, 1991
Communicated by H. F. Linskens

Summary. Isozyme analysis of *Brassica napus* cv 'Topas' and CGRC5006 as well as of *Sinapis alba* cv 'Emergo' revealed significant polymorphism between the two species for the isozymes, aconitate hydratase, glucose phosphate isomerase, and diaphorase. F_1 hybrids between *B. napus* '5006' and *S. alba* cv 'Emergo' were backcrossed to *B. napus* cv 'Topas', and the S_1 progeny of the first two backcrosses were studied isozymically. At the backcross one level the frequency of *S. alba* or *S. alba* plus *B. napus* patterns observed ranged from 18% to 87% across the four lines studied. There were differences between lines for the frequency of *S. alba* patterns, which could have an impact on the efficiency of selection for subsequent backcrossing. By the backcross two generation in one of the two lines studied, GR86-24, the *S. alba* patterns for GPI and DIA had been lost, while in the other line, GR86-28, the *S. alba* pattern for ACO had been lost, resulting in lost opportunity for *S. alba* gene transfer. In a wide cross such as *S. alba* × *B. napus*, which requires an intensive effort to accomplish, the isozymes ACO, GPI, and DIA may serve as useful markers to ensure gene transfer between the two species has occurred. In addition, the identification of lines with divergent isozyme patterns from *B. napus* will provide the basis for establishing linkages between *S. alba* traits of interest and isozyme markers.

Key words: Interspecific breeding – *Sinapis alba* – *Brassica napus* – Isozymes

Introduction

Sinapis alba L. (white mustard) represents a potentially useful germ plasm source for *Brassica napus* L. (oilseed rape) improvement. Genes for resistance to blackspot (*Alternaria brassicae* Berk. Sacc.) (Brun et al. 1987) or flea beetle (*Phyllotetra cruciferae* Goeze) (Bodnaryk and Lamb 1991) could be transferred from *S. alba* into *B. napus*. The two species have been successfully hybridized (Ripley and Arnison 1990).

Isozymes can be useful markers in genetic studies. Suarez et al. (1991) used isozyme markers to facilitate gene transfer between species; other researchers (Quiros et al. 1988; Chen et al. 1989) have utilized isozymes to confirm interspecific hybridization. Phylogenetic studies frequently use isozyme markers (Schenck and Wolf 1986; Quiros et al. 1988). Isozymes can serve as useful markers for interspecific gene transfer if the parental material used in the crosses exhibits electrophoretically distinct banding patterns. The relative efficiency of isozymes, compared to morphological characters, may be quite high. In a study examining the products of interspecific hybridization between *Lycopersicon esculentum* and *Solanum pennilli* Tanksley et al. (1981) reported that isozyme markers were as efficient as a combination of three morphological markers. Expression at the seedling stage was an added advantage.

In the study reported here the degree of isozyme pattern divergence between *B. napus* and *S. alba* lines used in interspecific crosses was investigated, and the usefulness of isozymes in monitoring genetic transfer between *S. alba* and *B. napus* was assessed. The frequency of occurrence of the *S. alba* (donor parent) and *B. napus* (recurrent parent) isozyme patterns in six interspecific backcross-derived S_1 lines is reported.

Materials and methods

Plant material used in this study included *B. napus* cv 'Topas' and CrGC5006, and *S. alba* cv 'Emergo'. Four BC_1S_1 lines were analyzed: S6B2, S9B1, S15B1, and S16B1, all S_1 progeny of the cross [('Emergo' × CrGC5006) × 'Topas']. In addition, two BC_2S_1 lines, GR86-24 and GR86-28 (S_1 progeny of S9B1 × 'Topas' and S15B1 × 'Topas', respectively), were included.

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Table 1. Staining recipes for the three enzymes

ACO:	
Presoak the gel for 45–60 min at 35 °C in:	
0.2 *M* TRIS-HCl buffer, pH 8.0	30.0 ml
aconitic acid (10 mg/ml, pH 7.5)	5.0 ml
$MgCl_2$ (100 mg/ml)	1.0 ml
Add:	
0.2 *M* TRIS-HCl buffer, pH 8.0	20.0 ml
NADP (10 mg/ml)	1.0 ml
MTT (10 mg/ml)	1.0 ml
PMS (10 mg/ml)	0.3 ml
IDH (20 U/ml, Sigma Type IV or VI)	2.0 ml
Incubate at 35 °C for 30–60 min	
GPI:	
0.1 *M* TRIS-HCl buffer, pH 8.5	50.0 ml
$MgCl_2$ (100 mg/ml)	1.0 ml
fructose-6-phosphate (13 mg/ml)	1.0 ml
NADP (10 mg/ml)	1.0 ml
MTT (10 mg/ml)	1.0 ml
PMS (10 mg/ml)	0.3 ml
G6PDH (50 U/ml)	1.0 ml
Incubate at 35 °C for 30–60 min.	
DIA:	
0.2 *M* TRIS-HCl buffer, pH 8.0	50.0 ml
$MgCl_2$ (100 mg/ml)	1.0 ml
DCPIP (1 mg/ml)	0.3 ml
NADH	20.0 mg
MTT (10 mg/ml)	1.5 ml
Incubated at 35 °C for 45–60 min.	

Sample preparation

Sample preparation, electrophoretic separation, and staining followed the procedures of Thorpe et al. (1987). Sixty seeds of each line were germinated in continuous light on moistened germination paper for 5 days. One cotyledon was removed from each seedling and homogenized in 30 µl of extraction buffer (5 g sucrose, 2.5 g sodium ascorbate, 22.5 ml water, pH 7.4). Samples were stored at −23 °C until use.

Two continuous buffer systems were used for electrophoresis. Acid phosphatase (ACPH), malate dehydrogenase (MDH), diaphorae (DIA), and leucine aminopeptidase (LAP) were separated on the A system (tank buffer: 40.4 g L-histidine, 6.0 g citric acid, 4.0 l distilled water, pH 6.5; gel buffer: 3:1 dilution). Aconitate hydratase (ACO), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), and shikimate dehydrogenase (SKDH) were separated on the B system (tank buffer: 40.4 g L-histidine, 4.0 l distilled water, pH 7.5; gel buffer: 3:1 dilution). Gels for both systems were made using 64.0 g hydrolyzed potato starch (StarchArt, Smithville, Tex.), 15 g sucrose, and 525 ml gel buffer.

Samples were loaded for electrophoresis by inserting filter paper wicks saturated with supernatant from homogenized tissue samples into a slit cut 2.9 cm from the cathodal end of the gel. Thirty-two wicks, including controls (*B. napus* cv 'Westar'-W on Figs. 1–4) were loaded onto each gel.

Electrophoresis was conducted anodally at 180 V for 16 h at 4 °C. The gels were further cooled by icepacks. After electrophoresis, the gels were sliced horizontally, and each slice stained for a separate enzyme. Staining recipes for the three enzymes which proved informative in this study are given in Table 1; others can be found in Thorpe et al. (1987).

Results

Significant polymorphism between *B. napus* (cv 'Topas' and CrGC5006) and *S. alba* (cv 'Emergo') was observed in three of the eight enzymes. ACO, DIA, and GPI exhibited distinct differences in number and position of isozyme bands when zymograms from each species were compared.

ACO

In *B. napus*, ACO has at least three zones, each resulting from a separate isozyme locus, with the most distinct bands in zone 3 (Fig. 1 a and c). The patterns designated as ACO-3-1 (Fig. 1 c, single arrow) and ACO-3-2 (Fig. 1 a, single arrow), and the putative heterozygote ACO-3-1/2 (Fig. 1 c, double arrow) predominate; in the 'Topas' samples, out of 60 plants 46 were ACO-3-2, 5 were ACO-3-1, and 9 had the hybrid pattern. All CrGC5006 plants were ACO-3-2. The ACO bands in *S. alba* were slightly more anodal than the ACO-3 bands in *B. napus*. The *S. alba* isozyme appears to be monomeric and its activity is strong. The zone of activity corresponds to the ACO-3 locus in *B. napus* but is designated ACO-1_{sal} since there are no other bands expressed. The most anodal band is designated ACO-1_{sal}-1 (Fig. 1 b, from left single arrow 1). Out of 60 seedling samples 'Emergo' exhibited the ACO-1_{sal}-1 band in 31 seedlings, ACO-1_{sal}-2 (Fig. 1 b from single arrow 2) in 12, and ACO-1_{sal}-1/2 (Fig. 1 b, double arrow) in 17.

In the S_1 progeny of the BC_1 generation, the number of seedlings observed with ACO patterns consisting of the *S. alba* bands alone or in combination with *B. napus* bands were: S6B2, 29/60 (48%) (Fig. 1 d), S9B1, 14/60 (23%); S15B1, 32/60 (53%) (Fig. 1 e), and S16B1, 52/60 (87%). A small proportion of the seedlings with each cross exhibited both *S. alba* bands (ACO-1_{sal}-1/2) in addition to weakly expressed ACO-4 bands from *B. napus*. The remaining seedling from each S_1 line exhibited *B. napus* bands only.

The S_1 progeny of the two BC_2 lines Gr86-24 and Gr86-28, derived as backcrosses to 'Topas' of S9B1 and S15B1, respectively, were analyzed. Gr86-24 expressed the *S. alba* or *S. alba* plus *B. napus* (Fig. 1 f, double arrow) patterns in 51/60 seedlings (85%). Gr86-28 expressed only the ACO-3-2 pattern from *B. napus* (Fig. 1 g, arrow).

PGM

PGM in *B. napus* is characterized by the expression of loci (PGM-1 through PGM-4) from both *B. oleracea* and *B. campestris*, resulting in a multiple-banded pattern. PGM-1, the most anodal, is a plastid-encoded isozyme and is highly conserved across the Brassica and related genera (M. L. Thorpe, personal communication). PGM-1 in *S. alba* corresponds to PGM-1 in *B. napus* (Fig. 2). PGM-2 in *B. napus* consists of two bands: PGM-2-1 is

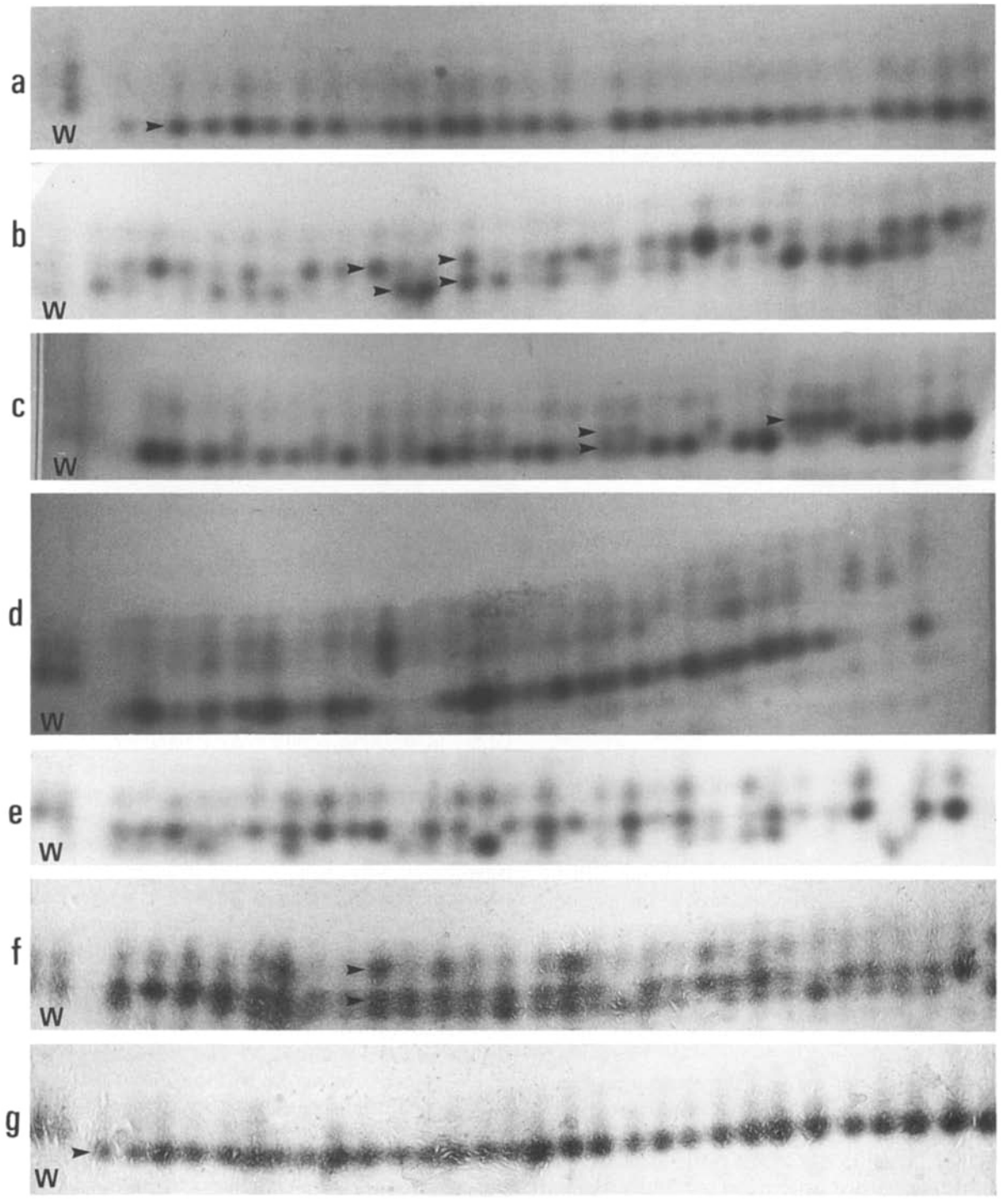

Fig. 1a–g. ACO zymograms for **a** *B. napus* '5006', **b** *S. alba* cv 'Emergo', **c** *B. napus* cv 'Topas', **d** S_1 of S6B2, **e** S_1 of S15B1, **f** S_1 of GR86-24, and **g** S_1 of GR86-28. *W B. napus* cv 'Westar' (control)

usually very weakly expressed; PGM-2-2 is strongly expressed. In *S. alba*, PGM-2 is characterized by two bands, both of which are strongly expressed.

PGM-3 in the *B. napus* material used in this study consists of a single band. In *S. alba*, the PGM-3 locus exhibits either as a single band or is not expressed, suggesting the presence of two alleles, one of which is null (Fig. 2b, arrow). These alleles are designated as PGM-3_{sal}-1 and PGM-3_{sal}-$\emptyset$. In 'Emergo', the pattern expressed is almost exclusively PGM-3_{sal}-1. PGM-4 has two bands in the *B. napus* material analyzed in this study (Fig. 2a). In *S. alba*, only one band is expressed in PGM-4.

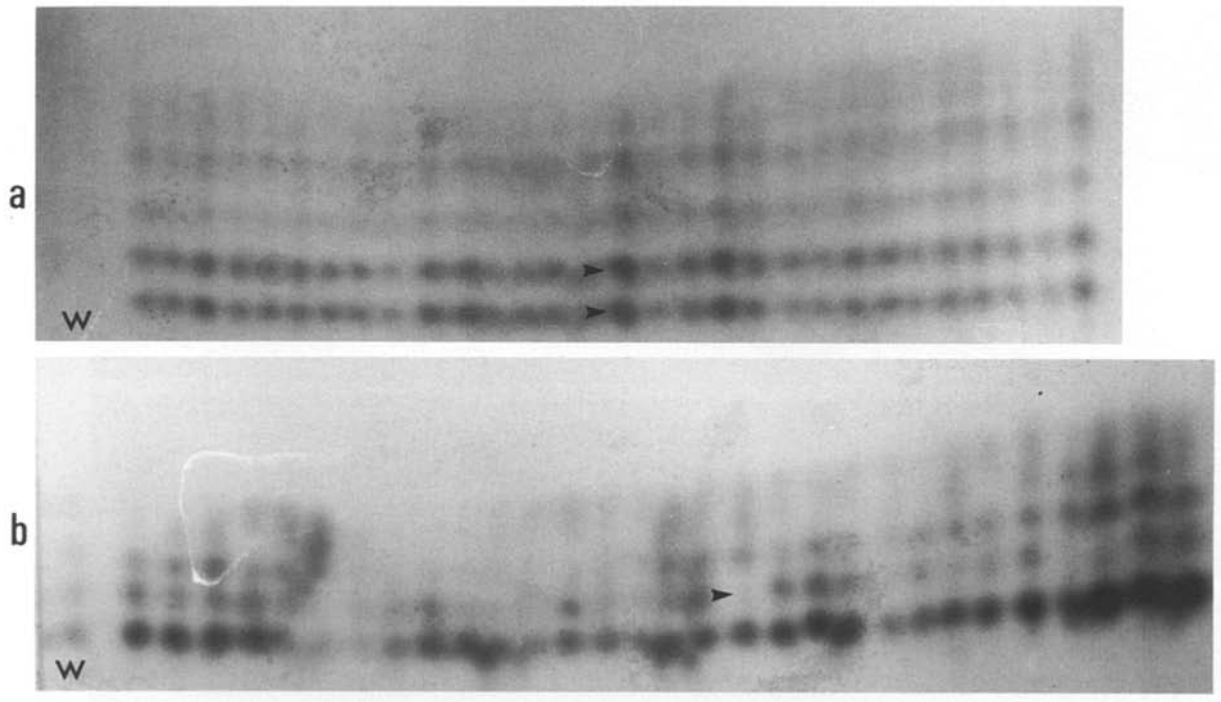

Fig. 2a, b. PGM zymograms for **a** *B. napus* cv 'Topas', **b** *S. alba* cv 'Emergo'. *W B. napus* cv 'Westar' (control)

Due to the complex nature of the PGM banding patterns, this enzyme was not examined in subsequent generations.

GPI

GPI-1 in *S. alba* corresponds exactly with the GPI-1 locus in *B. napus* (Fig. 3, arrow above W). This plastid locus is highly conserved over the Brassica genus; for this reason, the locus is designated as GPI-1 with no subscript. GPI-2 in *S. alba* (Fig. 3b, arrow) is slightly more anodal than GPI-2 in *B. napus*. Only one pattern was observed in *S. alba*. The locus and allele designation is GPI-2_{sal}-1.

In the S_1 progeny of the BC_1 the *S. alba* isozyme, alone or in combination with *B. napus* isozymes, was observed as follows: S6B2, 22/60 (37%); S9B1, 30/60 (50%); S15B1, 32/60 (53%), and S16B1, 31/60 (52%) (Fig. 3d). A four-banded pattern not previously seen in either species was observed in a small number of seedlings in each line: S6B2, 5/60 (8%); S9B1, 2/60 (3%); S15B1, 3/60 (5%), and S16B1, 4/60 (7%) (Fig. 3d, arrow).

GR86-24 exhibited only the *B. napus* isozymes (Fig. 3e). GR86-28 expressed *S. alba* isozymes alone or in combination with *B. napus* isozymes in 11/60 seedlings (18%). Twenty-seven of the 60 seedlings (45%) of GR86-28 had the four-banded pattern (Fig. 3f, arrow).

DIA

DIA-1 in *B. napus* is putatively a tetramer which may exhibit some post-translational modification (M. L. Thorpe, personal communication). The homozygotic patterns exhibit a strong single band with several less intense bands above or below the primary band. The heterozygote is five-banded with a stronger central band, typical of a tetramer (Fig. 4a, arrow). The DIA-2 locus is rarely seen in *B. napus*. DIA in *S. alba* appears also to be a tetramer which migrates to the same zone as the *B. napus* isozymes (Fig. 4b).

The S_1 progeny of the BC_1 lines also exhibit a multiple-banded pattern. Migration of D1A-1_{sal}-1 is only slightly cathodal to DIA-1_{bn}-1, and the expression of the *S. alba* isozyme in the S_1 is distinguishable from the DIA-1_{bn}-1/2 heterozygote by the position of the most intensely stained band: in material expressing *S. alba* isozymes the most intensely stained band is the most cathodal (Fig. 4c, arrow), whereas in the *B. napus* heterozygote it is the centre band (Fig. 4d, arrow). Based on this assessment, the following frequencies of *S. alba* or *S. alba* plus *B. napus* isozyme expression were observed: S6B2, 19/60 (32%) (Fig. 4c); S9B1, 11/60 (18%); S15B1, 16/60 (27%), and S16B1, 16/60 (27%). In the S_1 progeny of the subsequent backcross of S9B1 to 'Topas', Gr86-24, only the *B. napus* isozyme was present (Fig. 4d). Of the 60 Gr86-28 seedlings, 8 (13%) expressed the *S. alba* isozyme.

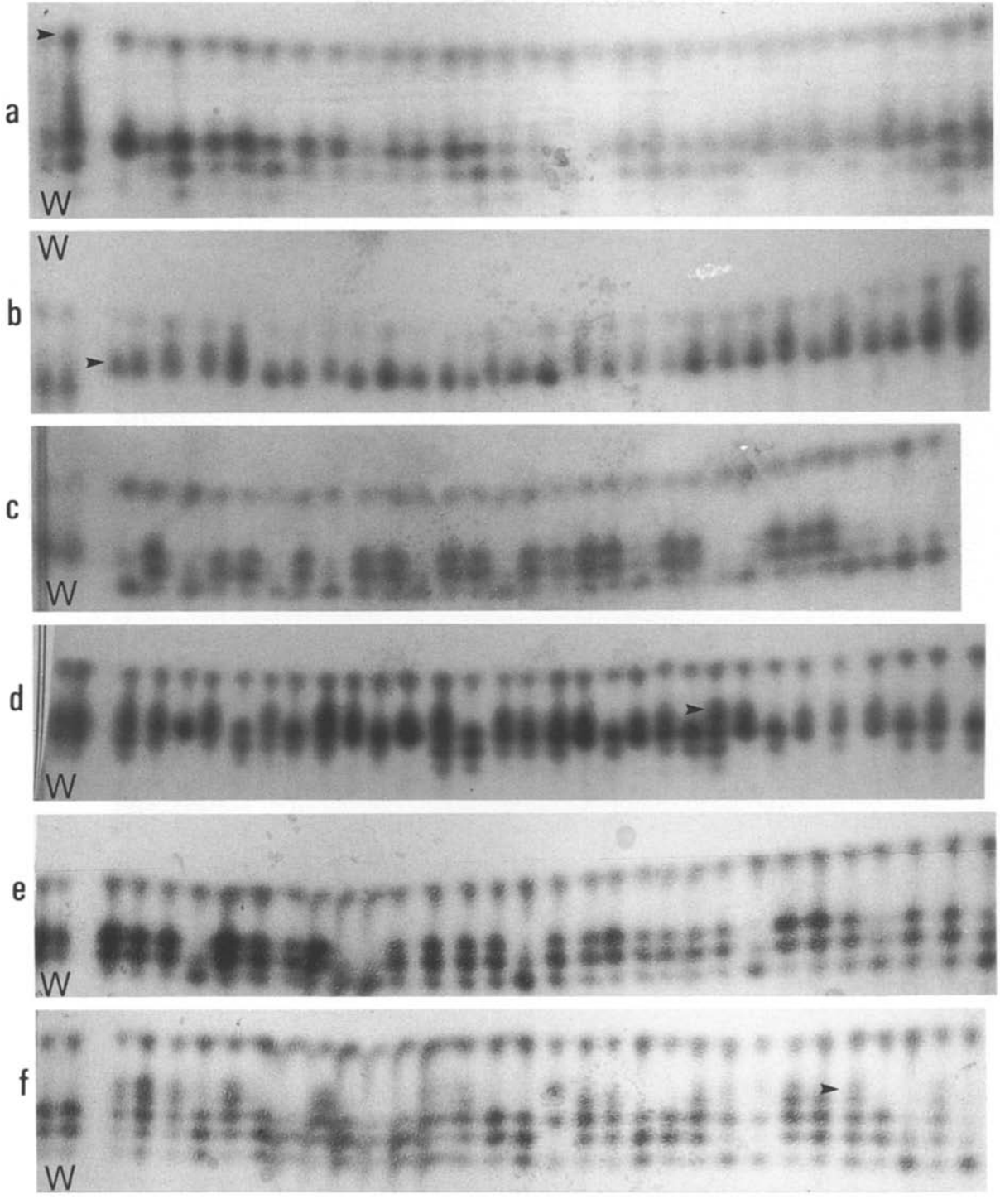

Fig. 3a–f. GPI zymograms for **a** *B. napus* '5006', **b** *S. alba* cv 'Emergo', **c** *B. napus* cv 'Topas', **d** S_1 of S16B1, **e** S_1 of GR86-24, **f** S_1 of GR86-28. *W B. napus* cv 'Westar' (control)

Discussion

Isozymes can be useful markers for the introgression of genes from one species to another. Their relatively inexpensive nature makes them preferable to RFLP analysis for preliminary studies (Suarez et al. 1991). Genes conferring resistance to flea beetle and black spot could be transferred from *S. alba* to *B. napus* following the successful interspecific cross (Ripley and Arnison 1990). Black spot resistance may be controlled by a small number of genes; flea beetle resistance is most likely a quantitative trait. Suarez et al. (1991) have demonstrated the utility of isozymes as markers for the introgression of quantitative trait loci.

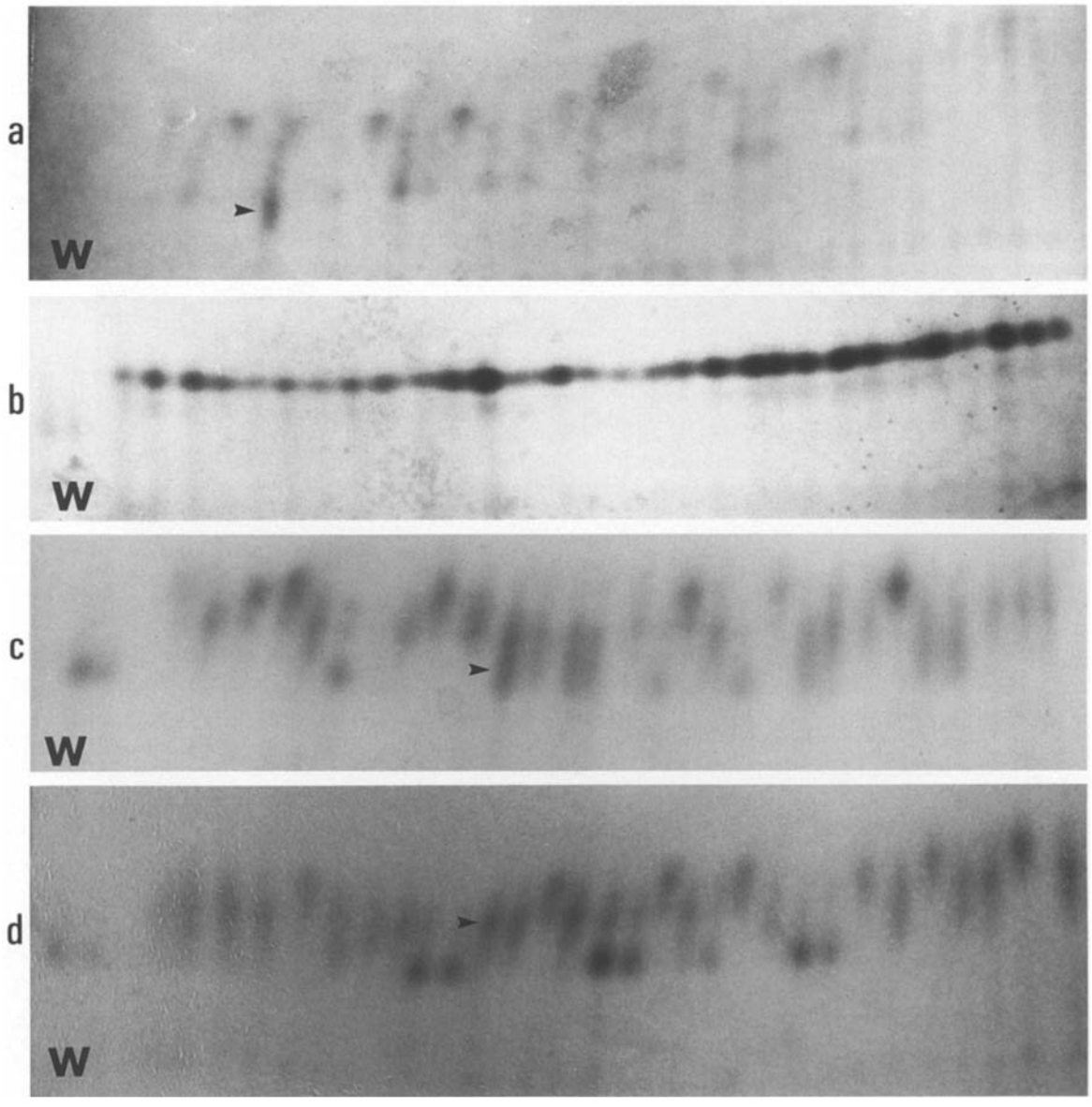

Fig. 4a–d. DIA zymograms for **a** *B. napus* cv 'Topas', **b** *S. alba* cv 'Emergo', **c** S_1 of S6B2, **d** S_1 of GR86-24. *W B. napus* cv 'Westar' (control)

Results from this study suggest sufficient polymorphism exists in at least three isozymes to provide markers to follow the introgression of genes from *S. alba* to *B. napus*.

ACO

In the S_1 progeny of the BC_1 lines, no interaction between the *S. alba* ACO bands and the *B. napus* bands was observed, with a few exceptions. In some seedlings the putatively heterozygotic ACO-1_{sal}-1,2 pattern or ACO-1_{sal}-2 pattern was recorded alone or in combination with ACO_{bn}-4. This may indicate replacement of *B. napus* genes with *S. alba* genes; alternatively, the *S. alba* locus may be more strongly expressed due to a superior ability to compete for substrate.

ACO-1_{sal}-1 and ACO-1_{sal}-2 may represent duplicate loci (ACO-1_{sal}-1 and ACO-1_{sal}-1′), rather than alleles at the same locus. Both bands have similar mobilities, and the ACO-1_{sal}-1,2 pattern was observed at a low frequency in each BC_1S_1 line examined. The BC_1 plants used to produce the S_1 lines were found to have 50 chromosomes, consisting of 38 from *B. napus* and 12 from *S. alba* (Ripley and Arnison 1990). It seems unlikely that the ACO-1_{sal}-1,2 pattern could arise from two alleles at the same locus; duplicate loci are therefore more probable.

Single- and double-banded patterns would arise if a null allele were present at one or both of the duplicated loci. Further investigation of this isozyme is needed.

In the BC_2S_1 lines ACO could be used to separate the two lines. One line examined, GR-86-24, continued to exhibit a modified pattern, while the other, GR-86-28, expressed the *B. napus* ACO-3-2 pattern exclusively. The divergence between the two lines for *S. alba* isozyme expression could prove useful in line selection for subsequent backcrossing to *B. napus*. GR86-24 and GR86-28 also could be useful in a study of linkage of ACO genes to other traits. RFLP or PCR techniques could be used in a detailed analysis of lines with a common pedigree but divergent *S. alba* ACO patterns.

GPI

In the backcross lines examined, *S. alba* GPI bands were seen in addition to *B. napus* bands. GPI-2 in both *B. campestris* and *B. oleracea* is dimeric: when alleles from both genomes are present in *B. napus*, interlocus heterodimers are formed and expressed as a three-banded pattern. Interaction of this type was not noted between *B. napus* and *S. alba* isozymes, unless the band marked by an arrow in Fig. 3 f is the heterodimeric band. If no interaction exists, it suggests that either GPI-2 in *S. alba* is not a dimer or that the structures of the isozymes are not capable of forming heterodimers. A similar result was reported for *B. nigra* × *B. napus* interspecific hybrids, which exhibited no interlocus dimers (Chevre et al. 1991). The GPI-2 isozyme patterns of *S. alba* and *B. nigra* are very similar. Previous studies have indicated that *B. nigra* and *B. juncea* may be closely allied with *S. alba* or *S. arvensis*, based on examination of chromosome association during meiosis in interspecific hybrids (Mizushima 1980). The isozyme patterns recorded for *B. nigra* and *S. alba* provide further evidence for a close relationship between the *S. alba* genome and the B genome of *B. nigra*.

DIA

The difficulty in distinguishing the *S. alba* DIA pattern from the DIA-1_{bn}-1,2 heterozygotic pattern limits this isozymes usefulness as a marker. The results reported (Table 2) are tentative. DIA will be of limited use as a marker except in combination with either GPI or ACO.

The isozymes which provide the most information regarding the introgression of *S. alba* genes into *B. napus* are ACO and GPI. These two isozymes generally show a high degree of polymorphism in *B. napus*: investigation of the frequency and range of isozyme patterns seen in *B. napus* parent before introgression of *S. alba* genes is necessary to accurately identify the origin of individual isozyme bands.

The number of *S. alba* chromosomes remaining in the lines studied is of interest. The BC_1 lines used to produce the BC_1S_1 lines contained 50 chromosomes, 38 from *B. napus* and 12 from *S. alba*. At meiosis in the BC_1 lines, the *B. napus* chromosomes should pair normally, giving 19 bivalents, while the *S. alba* chromosomes would be seen as 12 univalents. After meiosis, a range of chromosome numbers would be expected. In this study, the presence of approximately equal frequencies of the *S. alba* ACO, GPI, and DIA isozymes may indicate that up to 3 *S. alba* chromosomes still remain in the lines examined. Alternatively, up to three recombination events may have occurred, or some combination of chromosome retention and recombination events. Retention of *S. alba* chromosomes would appear to be the most probable explanation, since the *S. alba* bands are often seen in combination with *B. napus* bands. Chien et al. (1982) report that in an interspecific cross the presence of isozymes of a given species indicates that the chromosomes which encode them are still present. Recombination, however, must be considered, due to the expression in some seedlings of modified *B. napus* plus *S. alba* patterns.

S. alba ACO bands were lost in the BC_2S_1 line GR86-28; GPI bands from *S. alba* were lost in the BC_2S_1 line GR86-24. The rapid loss of *S. alba* chromosomes, while expected, may limit the opportunity to transfer potentially useful genes from *S. alba* to *B. napus*. In this wide cross, isozymes may provide the breeder with a method to select backcross lines that still contain *S. alba* genes. This could be particularly important for selection in advanced generations, or in cases where the economic traits of interest are linked to the isozyme marker (Tanksley et al. 1981). Isozymes also may be useful in facilitating the rapid reconstitution of the recurrent parent genome during selection in backcross generations (Tanksley et al. 1981). For the interspecific cross between *S. alba* and *B. napus*, ACO and GPI isozymes have been identified as markers applicable to the monitoring of the introgression of *S. alba* genes into the *B. napus* genome, thus increasing the efficiency of selection.

Table 2. Frequency of non-*B. napus* isozyme patterns

Genotype	ACO	GPI	DIA
Emergo	60/60	60/60	60/60
S6B2[a]	29/60	22/60	19/60
S6B1	14/60	30/60	11/60
S15B1	32/60	32/60	15/60
S16B1	52/60	31/60	16/60
GR86-24	51/60	0	0
GR86-28	0	38/60	8/60

[a] This and all subsequent entries refer to the S_1 progeny of the line listed

Acknowledgements. The authors gratefully acknowledge the funding for this project, which included an Agriculture Canada/ Natural Science and Engineering Research Council grant to W. D. Beversdorf.

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