Stereospecific Analyses of Seed Triacylglycerols from High-Erucic Acid Brassicaceae: Detection of Erucic Acid at the *sn*-2 Position in *Brassica oleracea* L. Genotypes

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Stereospecific analyses of triacylglycerols from selected high-erucic acid breeding lines or cultivars of *Brassica napus* L. and *B. oleracea* L. have been performed. Initial lipase screening revealed that while all *B. napus* lines contained little or no erucic acid at the *sn*-2 position, several of the *B. oleracea* lines had significant proportions of erucic acid at this position. Detailed stereospecific analyses were performed on the triacylglycerols from these lines by using a Grignard-based deacylation, conversion of the *sn*-1, *sn*-2 and *sn*-3 monoacylglycerols to their di-dinitrophenyl urethane (DNPU) derivatives, resolution of the di-DNPU-monoacylglycerols (MAGs) by high-performance liquid chromatography on a chiral column, transmethylation of each *sn*-di-DNPU MAG fraction and analysis of the resulting fatty acid methyl esters by gas chromatography. The findings unequivocally demonstrate for the first time that, within the Brassicaceae, there exists *B. oleracea* germplasm containing seed oils with substantial erucic acid (30–35 mol%) at the *sn*-2 position. This has important implications for biotechnology and breeding efforts designed to increase the levels of erucic acid in rapeseed beyond 66 mol% to supply strategic industrial feedstocks. In the first instance, the germplasm will be of direct use in retrieving a gene encoding a *Brassica lyso*-phosphatidic acid acyltransferase with an affinity for erucoyl-CoA. In a breeding program, the germplasm offers promise for the introduction of this trait into *B. napus* by interspecific hybridization and embryo rescue.

KEY WORDS: ***Brassica napus*, *Brassica oleracea*, erucic acid, *lyso*-phosphatidic acid acyltransferase, *sn*-2 position, stereospecific analysis, triacylglycerols.**

The ability to modify the acyl composition of triacylglycerols (TAGs) in oilseed crops has important commercial implications. Specifically, there is considerable interest in developing rapeseed (*Brassica napus*) cultivars containing ultra-high levels of erucic acid and trierucin (trierucoylglycerol) in the seed oil (1–6). Fatty acids and their hydrogenation or cleavage products obtained from high-erucic acid oils are useful feedstocks in the manufacture of plastic films, plasticizers and surface coatings (7,8). There are now more than 1000 patents governing applications for the use of C_{22} oleochemicals and their derivatives (9). Trierucin is an excellent high-temperature lubricant, and it is utilized as a novel treatment for adrenoleukodystrophy (5,6). TAGs with a uniform acyl composition yield homogeneous fatty acid fractions and require minimal purification upon hydrolysis, thereby reducing processing costs.

In general, stereospecific analyses have revealed that erucic acid (22:1 Δ13) is virtually excluded from the *sn*-2 position of TAGs in seed oils of the Brassicaceae (this family now includes former members of the Cruciferae). Although, traditionally, the major high-erucic acid oilseeds studied have been *B. napus* and *B. campestris*, researchers have also analyzed selected, sometimes poorly-defined, germplasm of *B. juncea*, *B. oleracea*, *Lunaria annua*, *Sinapis alba*, *Crambe abyssinicia* and other wild Cruciferae, all of which have been shown to possess seed oils with a similar stereochemical distribution of erucic acid, with the *sn*-2 position essentially devoid of 22:1 (10–18). TAGs in high-erucic acid cultivars of *B. napus* do not contain trierucin (5,6,19,20). Thus far, no *Brassica* genotype has been found to contain more than 66 mol% 22:1 in its seed oil (21,22), presumably due to the exclusive *sn*-1 and *sn*-3 esterification. More recently, the exclusion of 22:1 from the *sn*-2 position has been shown to be due to the limited acyl specificity of the *lyso*-phosphatidic acid acyltransferase (LPAT) in *B. napus*, which lacks the capacity to utilize 22:1-CoA during TAG biosynthesis *via* the Kennedy pathway (1,23–25). Given this biochemical limitation, it has been suggested that the theoretical breeding limit for increasing the 22:1 content in *B. napus* is 66 mol% (*sn*-1 + 3) (1,2,5,6,13). Current high-erucic acid, low-glucosinolate rapeseed cultivars released by the University of Manitoba (Winnipeg, Manitoba, Canada), Hero in 1990 (26) and Mercury in 1992, have erucic acid contents of about 50–55 mol%.

However, screening for total erucic acid content alone may not be sufficient to identify germplasm which possesses the capacity to place erucic acid into the *sn*-2 position of TAGs. Species of meadowfoam (*Limnanthes douglasii*, *L. alba*) have only 10–17 mol% total 22:1 Δ13 in seed TAGs, yet 67–100% of this is esterified to the *sn*-2 position (27,28). Furthermore, such positional specificity has been attributed to the selectivity of the meadowfoam LPAT, which is capable of inserting 22:1 into the *sn*-2 position of *sn*-1 acyl-LPA to give *sn*-1 acyl, *sn*-2 erucoyl-PA in the Kennedy pathway for TAG biosynthesis (1,5,6,23,24,29). It has also been demonstrated that *B. napus* can convert *sn*-1,2-dierucin to trierucin *in vitro*, suggesting that transgenic expression of an erucoyl-CoA:LPAT would indeed result in the desired product (30).

We have, for the first time, identified *B. oleracea* lines that have TAGs containing 22:1 at the *sn*-2 position in proportions that are uncharacteristically high for members of the Brassicaceae. Here we report the first detailed stereospecific analyses of these lines and, for comparison, those of the high-erucic acid *B. napus* cultivars Golden, Reston, Hero and Mercury.

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EXPERIMENTAL PROCEDURES

Seed material. Seeds of *B. napus* L. high-erucic acid cultivars Golden, Reston, Hero and Mercury were obtained from the University of Manitoba. Seeds of *B. oleracea* L. were selected from listings in Reference 22 and obtained, courtesy of Dr. J.R. McFerson, USDA, ARS Plant Genetic Resources Unit (Cornell University, Geneva, NY). All stereospecific analyses were performed with S_2 seed of the *B. oleracea* lines, produced in the greenhouse at the University of Manitoba through bud pollination techniques. Nasturtium (*Tropaeolum majus*) seeds (cv. Dwarf Double Golden Jewel) were purchased from Early's Farm & Garden Centre, Inc. (Saskatoon, Saskatchewan, Canada).

Reagents. All solvents were of high-performance liquid chromatography (HPLC) grade (Omni-Solv) and were obtained from BDH Inc. (Toronto, Ontario, Canada). Silica 60 G (Kiesel Gel 60 G) was obtained from E. Merck (Darmstadt, Germany), and 0.25 mm silica plates, with or without 10% boric acid, were prepared as described by Christie (31). Commercially prepared Silica 60 G plates were purchased from Whatman Inc. (Clifton, NJ). Neutral lipid standards were obtained from Nu-Chek-Prep, Inc. (Elysian, MN), and porcine pancreatic lipase was purchased from Sigma (St. Louis, MO). 3,5-Dinitro-phenyl isocyanate was custom-synthesized by Rose Scientific Ltd. (Edmonton, Alberta, Canada).

Preparation of a total lipid extract (TLE). The following protocol was suitable for extracting total lipids from 100 mg to 1 g of seed. The procedures described are based on 1 g of seed, but for lower quantities, the protocol was scaled down proportionally. To a test tube containing the seed, 2 mL isopropanol containing 0.2% wt/vol butylated hydroxytoluene (BHT) was added, the tube was sealed with a Teflon-lined cap and the mixture was boiled for 5 min on a water bath. The seed mixture was then homogenized using a polytron at high speed for 45 s. Another 2 mL isopropanol/BHT were added, the grinding was repeated, the mixture was capped and boiled once again for 5 min. To the boiled homogenate, 2 mL dichloromethane and 1.2 mL water were added, and the mixture was allowed to stand at room temperature for 1 h with occasional vortexing. The solution was then filtered through a glass-fiber filter in a Buchner funnel (Fisher Scientific, Nepean, Ontario, Canada) into a round-bottom flask. At this point, the lipid extract was usually monophasic; if more than one phase was present, additional isopropanol was added dropwise to obtain a single phase. To the mixture, 2 mL dichloromethane and 2 mL 1 M KCl in 0.2 M H_3PO_4 were added to separate the phases. After centrifugation, the lower organic phase was transferred to a tared tube. The upper aqueous phase was backwashed twice with dichloromethane, the organic phases were combined and blown to dryness under nitrogen. The total lipid content (in mg/g dry wt) was recorded. The TLE was adjusted to a concentration of 50 mg/mL and stored in dichloromethane at -20°C.

Purification of TAG fraction. Silicic acid (Bio-Sil HA, minus 325 mesh size; Bio-Rad Laboratories Ltd., Richmond, CA) (3 g) was slurried in 5 mL hexane, and 2 mL of this slurry was applied to a Poly-Prep column (Bio-Rad) fitted with a 2.5-inch #22 needle. The 2-mL silica bed was washed with 20 mL hexane. To separate the TAGs from the free fatty acids (FFA), diacylglycerols (DAGs) and polar lipids, a 50-mg equivalent of the TLE was applied to the silica column and washed in with a further 2 mL hexane. TAGs were eluted with 2 × 10 mL aliquots of 10% diethyl ether in hexane. From experiments with standard mixtures, >80% of trierucin was recovered in this fraction; DAGs did not elute in this fraction. After transferring the eluant to a tared tube and blowing the mixture to dryness under nitrogen, the weight of TAGs was recorded.

Lipase screen of TAGs from high-erucic acid germplasm. A portion (3 mg) of the TAG fraction from each line was analyzed for total *vs. sn*-2 acyl composition by using pancreatic lipase as described by Christie (31). Those TAGs from germplasm/accession lines showing significant erucic acid at the *sn*-2 position in the initial lipase screen, were subjected to detailed stereospecific analyses designed to determine unequivocally the proportions of fatty acids, and erucic acid in particular, at each *sn*-position.

Grignard-based stereospecific analysis of TAGs. The method was adapted from that described by Takagi and Ando (32) and has been optimized for the resolution of TAGs containing long-chain fatty acids. All ether extractions were performed with ether saturated with 10% (wt/vol) boric acid to minimize acyl migration. TAG (100 mg) was dissolved in 3 mL dry diethyl ether, 330 μL ethyl magnesium bromide was added and the Grignard reaction was allowed to proceed with continual vortexing for 1 min. To stop the reaction, 100 μL glacial acetic acid was slowly added, followed by 3.3 mL 10% (wt/vol) boric acid solution. After adding a further 1 mL diethyl ether, the organic phase was removed, and the lower aqueous phase was backwashed three times with diethyl ether. The combined ether fractions were washed with 2% (wt/vol) sodium bicarbonate (5 × 1 mL), followed by distilled water (3 × 1 mL). To the washed ether fraction, 0.5 mL dichloromethane/benzene/methanol (1:1:1, vol/vol/vol) was added, and the mixture was dried under nitrogen. The Grignard products were dissolved in 500 μL chloroform and applied to a 0.5 mm 20 × 20 cm Kiesel Gel 60 G plate containing 10% boric acid to minimize acyl migration. The [*sn*-1 + 3] monoacylglycerols (MAGs) were resolved from the *sn*-2 MAGs by developing the plate in chloroform/methanol (98:2, vol/vol). The two MAG fractions were recovered from the silica by extraction with borate-saturated diethyl ether. The *sn*-2 MAG fraction, which was contaminated by trailing DAGs, was purified further by a second thin-layer chromatography (TLC) on 0.25 mm Whatman Silica G plates developed in chloroform/methanol (98:2, vol/vol).

Resolution of MAGs as the di-dinitrophenylurethane (DNPU)-derivatives. The [*sn*-1 + 3] MAGs were resolved as follows: The [*sn*-1 + 3] MAGs were converted to their corresponding di-DNPU derivatives with 3,5-dinitrophenyl isocyanate as described by Takagi and Ando (32). The DNPU derivatives were pre-purified on Kiesel Gel 60 G TLC plates, developed in *n*-hexane/dichloroethane/ethanol (40:15:6, vol/vol/vol), and after recovery from the silica with water-saturated diethyl ether, the di-DNPU MAG mixture was dissolved in 1 mL *n*-hexane/dichloroethane/ethanol (40:12:3, vol/vol/vol) and filtered through a 0.2 μm Spin-X nylon membrane (Costar, Cambridge, MA).

The *sn*-1 and *sn*-3 di-DPNU-MAGs were separated by HPLC. The HPLC system consisted of 2 Sumichiral OA-4100 5 μm (4 mm ϕ × 25 cm) columns (YMC Inc., Morris Plains, NJ) linked in series to a Spectra-Physics SP8700XR liquid chromatograph (San Jose, CA) fitted

with an SP8780XR autosampler. A 100-μL aliquot of the [*sn*-1+3] di-DNPU-MAG mixture was injected into the HPLC. The di-DNPU-MAGs were eluted isocratically with *n*-hexane/1,2-dichloroethane/ethanol (40:12:3, vol/vol/vol). The elution profile for the di-DNPU-MAG derivatives was monitored at 254 nm with a Spectroflow 773 Absorbance Detector (Kratos Analytical Instruments, Ramsey, NJ). The peak fractions were collected, and the solvent was removed under nitrogen. The two chiral columns linked in series improved the separation of di-DNPU mono-erucoyl-glycerides.

Transmethylation. All fatty acids present in the TLE, TAG, *sn*-2 MAG, *sn*-1 or *sn*-3 di-DNPU-MAG and [*sn*-1+3] FFA fractions were converted to the corresponding fatty acid methyl esters with 1 N methanolic HCl and analyzed by gas chromatography on a DB-23 column, as described previously (25).

Direct probe mass spectrometry. Analysis of intact TAG fractions by direct-probe mass spectrometry was performed as described previously (20).

RESULTS AND DISCUSSION

An initial screen of the selected *B. oleracea* and *B. napus* germplasm indicated that erucic acid was virtually excluded from the *sn*-2 position of TAGs from all high-erucic acid *B. napus* germplasm. However, three *B. oleracea* accessions did contain TAGs with a substantial proportion (>20 mol%) of erucic acid at the *sn*-2 position (Table 1). It is clear from the initial lipase screen that there is no correlation between total 22:1 content and that proportion found at the *sn*-2 position. Only when the total 22:1 content is >66 mol% is it safe to assume that there must absolutely be some proportion at the *sn*-2 position (as in nasturtium; see Table 2).

TABLE 1

Total Erucic Acid Content and Proportion Found at the *sn*-2 Position by Lipase Analysis of Total Lipid Extracts from High-Erucic Acid Lines of *Brassica napus* and *B. oleracea*

Germplasm	Cultivar or accession number[a]	Total erucic acid content (wt%)	*sn*-2 Erucic acid content (wt%)
B. napus	cv. Golden	40.6	3.0
B. napus	cv. Reston	30.0	0.5
B. napus	cv. Hero	49.0	1.0
B. napus	cv. Mercury	52.0	0.8
B. oleracea	PI285061b	62.4[b]	23.3
B. oleracea	PI372890	62.1[b]	23.7
B. oleracea	PI372897	60.0[b]	23.9

[a]USDA, ARS Plant Genetic Resources Unit, Cornell University, Geneva, NY, collection; plant introduction number.
[b]As reported in Reference 22.

The TAGs from two *B. oleracea* lines shown by the lipase screen to contain >20 mol% 22:1 in the *sn*-2 position (Table 1), were subjected to a further Grignard-based stereospecific analysis in which the compositions of all three *sn*-positions were determined. These analyses confirmed the unusually high level of 22:1 at the *sn*-2 position in the *B. oleracea* lines (Table 2). As a control for these detailed analyses, a TAG fraction from nasturtium seed oil was

TABLE 2

Stereospecific Analysis of *Brassica napus, B. oleracea* and *Tropaeolum majus* Genotypes

			Fatty acid (mol% of total)										
Species	Genotype	Fraction	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0	24:1
B. napus	Golden	TAG[a]	3.4	1.5	15.9	13.3	7.9	1.0	12.7	0.7	42.6	0.2	0.8
		sn-1	7.2	8.4	15.0	—	—	—	17.8	—	50.0	—	1.4
		sn-2	1.9	1.1	35.4	36.2	19.8	—	1.6	—	3.5	0.4	—
		sn-3	7.0	4.2	4.3	—	—	2.7	14.0	1.6	64.4	—	1.8
	Reston	TAG	4.1	1.8	33.0	12.5	4.6	1.0	11.8	0.7	29.5	—	1.0
		sn-1	40.7	26.2	9.2	2.5	—	—	4.0	—	8.2	—	—
		sn-2	2.1	1.7	34.7	45.2	20.7	—	1.1	—	3.7	1.2	—
		sn-3	15.9	8.2	16.4	3.3	—	2.3	11.1	—	42.8	—	—
	Hero	TAG	3.4	1.2	14.6	12.9	8.0	1.0	7.9	0.3	49.4	0.3	1.1
		sn-1	17.5	13.3	10.4	2.2	0.8	0.7	9.2	0.8	42.6	2.3	
		sn-2	1.0	0.4	37.5	35.5	21.8	—	0.7	0.2	2.1	0.5	0.2
		sn-3	12.1	7.7	4.8	1.1	—	2.6	5.9	2.3	61.0	2.0	0.6
	Mercury	TAG	3.0	1.2	14.1	11.4	7.7	0.9	7.6	0.8	51.7	0.3	1.1
		sn-1	10.1	4.7	7.1	2.1	2.1	0.5	13.7	—	58.0	—	1.7
		sn-2	1.7	2.2	35.6	31.4	20.7	0.6	1.1	0.4	4.4	—	1.8
		sn-3	10.9	6.7	5.3	1.1	—	3.4	7.9	2.8	59.6	1.4	1.1
B. oleracea	PI285061b	TAG	6.3	1.0	7.8	13.9	11.1	0.3	2.5	0.6	54.9	0.2	1.5
		sn-1	8.7	3.5	8.8	11.0	7.0	0.4	3.9	0.9	53.6	0.4	1.7
		sn-2	7.8	4.6	7.5	9.9	7.4	0.5	3.5	0.7	55.4	0.7	2.2
		sn-3	9.4	7.3	8.9	10.6	7.3	0.6	3.5	0.8	49.8	0.5	1.4
	PI372890	TAG	4.8	0.7	7.1	14.7	12.1	0.3	3.9	0.5	53.3	0.3	2.3
		sn-1	4.4	1.8	6.9	10.9	7.7	0.7	3.9	0.7	59.0	0.9	2.9
		sn-2	10.8	10.0	6.2	8.3	6.3	0.8	3.5	1.1	48.7	1.6	2.7
		sn-3	4.6	4.4	7.0	9.8	7.7	0.6	4.0	0.7	57.9	0.7	2.6
T. majus	Dwarf Double Golden Jewel	TAG	0.7	—	2.1	—	0.4	—	16.6	0.5	78.3	—	1.4
		sn-1	4.4	4.8	3.1	0.9	—	—	14.5	—	71.2	—	1.1
		sn-2	1.6	1.0	3.2	—	0.4	—	12.8	0.3	80.2	—	0.5
		sn-3	2.1	4.1	2.2	—	—	—	14.3	1.5	73.6	—	2.2

[a]TAG, triacylglycerol.

TABLE 3

Stereospecific Analysis[a] of *Brassica napus, B. oleracea* and *Tropaeolum majus* Genotypes

Species	Genotype	Fraction	Fatty acid (mol% of total)										
			16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0	24:1
B. napus	Golden	*sn*-1	44.7	61.3	27.4	—	—	—	53.3	—	42.4	—	43.8
		sn-2	11.8	8.0	64.7	100.0	100.0	—	4.8	—	3.0	100.0	—
		sn-3	43.5	30.7	7.9	—	—	100.0	41.9	100.0	54.6	—	56.2
	Reston	*sn*-1	69.3	72.6	15.3	4.9	—	—	24.7	—	15.0	—	—
		sn-1	3.6	4.7	57.5	88.6	100.0	—	6.8	—	6.8	100.0	—
		sn-3	27.1	22.7	27.2	6.5	—	100.0	68.5	—	78.2	—	—
	Hero	*sn*-1	57.2	62.1	19.7	5.7	3.5	21.2	58.2	24.2	40.3	47.9	—
		sn-2	3.3	1.9	71.2	91.5	96.5	78.8	4.4	6.1	2.0	10.4	25.0
		sn-3	39.5	36.0	9.1	2.8	—	—	37.3	69.7	57.7	41.7	75.0
	Mercury	*sn*-1	44.5	34.6	14.8	6.1	9.2	11.1	60.4	—	47.5	—	37.0
		sn-2	7.5	16.2	74.2	90.8	90.8	13.3	4.8	12.5	3.6	—	39.1
		sn-3	48.0	49.2	11.0	3.2	—	75.6	34.8	87.5	48.9	100.0	23.9
B. oleracea	PI285061b	*sn*-1	33.6	22.7	34.9	34.9	32.3	26.7	35.8	37.5	33.8	25.0	32.1
		sn-2	30.1	29.9	29.8	31.4	34.1	33.3	32.1	29.2	34.9	43.8	41.5
		sn-3	36.3	47.4	35.3	33.7	33.6	40.0	32.1	33.3	31.4	31.3	26.4
	PI372890	*sn*-1	22.2	11.1	34.3	37.6	35.5	33.3	34.2	28.0	35.6	28.1	35.4
		sn-2	54.5	61.7	30.8	28.6	29.0	38.1	30.7	44.0	29.4	50.0	32.9
		sn-3	23.2	27.2	34.8	33.8	35.5	28.6	35.1	28.0	35.0	21.9	31.7
T. majus	Dwarf Double Golden Jewel	*sn*-1	54.3	48.5	36.5	100.0	—	—	34.9	—	31.6	—	28.9
		sn-2	19.8	10.1	37.6	—	100.0	—	30.8	16.7	35.6	—	13.2
		sn-3	25.9	41.4	25.9	—	—	—	34.4	83.3	32.7	—	57.9

[a]Distribution of each fatty acid across all three *sn*-positions.

analyzed at the same time and confirmed to contain a large proportion of 22:1 at the *sn*-2 position, as reported previously (6). The small proportions of 22:1 detected at the *sn*-2 position of the high-erucic acid *B. napus* lines (Table 2) are well within the range caused by limited acyl migration from the *sn*-1 and *sn*-3 positions to the *sn*-2 position on MAGs. Despite taking the usual precautions to avoid high temperatures, to limit exposure to polar, highly acidic or basic solvents, and to perform extractions and TLC separations in the presence of boric acid, some isomerization appears unavoidable (33,34). The detailed stereospecific analyses (Table 2) revealed that, while useful as an initial screen, the lipase analysis alone (Table 1) can significantly underestimate the proportion of 22:1 at the *sn*-2 position. This indicates the value of the Grignard-based stereospecific analysis to define more quantitatively the true distribution of erucic acid.

The distributions of each fatty acid across all three *sn*-positions is presented in Table 3. The same general conclusions regarding the presence of substantial proportions of 22:1 at the *sn*-2 position may be reached from presenting the information in this form. However, the data in Table 3 more clearly reflect the specificity of the Kennedy pathway acyltransferases and therefore impinge on strategies for modifying seed oil composition by molecular genetic or traditional breeding methods. We believe this form of presenting stereospecific data to be preferable. From these data, all three acyltransferases appear to be relatively nonspecific in both *B. oleracea* lines, although there is some apparent preference for placing saturated fatty acids into the *sn*-2 position in line 372890. While the total amount of saturates in this line is admittedly small (<7%), such specificity may be worth exploiting for other oilseed modifications.

Analyses of those *B. oleracea* TAGs containing significant 22:1 at the *sn*-2 position by direct-probe mass spectrometry did not show any significant trierucin. Trierucin was also absent from the seed oils of the high-erucic acid *B. napus* cultivars Hero and Mercury (data not shown). These findings are consistent with previous reports conducted in high-erucic acid *B. napus* cultivars Reston and Golden (5,6,20).

This study has shown, for the first time that, within the Brassicaceae, there is germplasm, specifically in lines of *B. oleracea*, that contains significant proportions of erucic acid at the *sn*-2 position of seed TAGs. This new finding has important implications for research designed to increase the levels of erucic acid in rapeseed beyond 66 mol% to supply strategic industrial oils. Using biotechnology, we hope to isolate and transgenically express a gene encoding an erucoyl-CoA:LPAT, in the best high-erucic acid *B. napus* germplasm available (5,6). Now, there is a target gene source identified within the Brassicaceae. Accordingly, in a parallel breeding approach, the *B. oleracea* germplasm offers promise for the introduction of this trait into *B. napus* by interspecific hybridization and embryo rescue.

ACKNOWLEDGMENTS

The authors thank Dr. J.R. McFerson, USDA, ARS Plant Genetic Resources Unit, Cornell University (Geneva, NY) for his assistance in supplying several hundred selected lines of *B. oleracea* germplasm from the USDA collection. We also thank L. Hogge, D. Olson and D. Reed for additional technical assistance in the mass spectrometry and GC analyses of the TAGs, and Drs. J-T. Zou and W. Keller for their critical evaluations of the manuscript. This is National Research Council of Canada Publication No. 37338; Dept. of Plant Science Contribution No. 948.

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[Received July 12, 1993; accepted October 7, 1993]