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Intertribal somatic hybrids between *Brassica napus* and *Thlaspi perfoliatum* with high content of the *T. perfoliatum*-specific nervonic acid

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Abstract. Protoplast fusions were performed between hypocotyl protoplasts of *Brassica napus* and mesophyll protoplasts of *Thlaspi perfoliatum*. The two species are members of the *Lepidieae* and *Brassiceae* tribes, respectively, in the family of *Brassicaceae*. Seeds of *T. perfoliatum* are rich in the fatty acid $C_{24:1}$ (nervonic acid), an oil valuable for technical purposes. In the search for renewable oils to replace the mineral oils, plant breeders have been trying to develop oil crops with a high content of long-chain fatty acids. After fusion of *B. napus* protoplasts with non-irradiated as well as irradiated protoplasts of *T. perfoliatum* selection was carried out by flow cytometry and cell sorting. Of the shoots regenerated from different calli 27 were verified as hybrids or partial hybrids using the isoenzyme phosphoglucose isomerase (PGI) as a marker. Another 6 plants were identified as partial hybrids using a *T. perfoliatum*-specific repetitive DNA sequence. Slot blot experiments were performed to estimate the copy number of the repetitive DNA sequence in the parental species and in the hybrids. In *T. perfoliatum* there were approximately 10^5 copies per haploid genome, and the range in the hybrids was 1–37% of the value in *T. perfoliatum*. When the nuclear DNA content of the regenerated shoots was analysed we found partial as well as symmetric hybrids. Even though the rooting and establishment of hybrid shoots in the greenhouse were difficult, resulting in the death of many plants, 19 plants were cultured to full maturity. Seeds obtained from 15 plants were analysed to determine whether they contained nervonic acid, and 5 of the hybrids were found to contain significantly greater amounts of nervonic acid than *B. napus*.

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Introduction

As a result of efficient and successful plant breeding, rapeseed has become one of the major crops used for producing vegetable oils. The fatty acids from the oil differ in their properties – and thus in their potential uses – depending on the length and structure of their carbon chains (review Murphy 1992). The great versatility of the oil has stimulated commercial interest in modifying the fatty acid composition to enhance its nutritional value as well as its value for industrial applications. Several species in the *Brassicaceae* family contain unusual fatty acids (Appelquist 1976), some of which are considered valuable enough to warrant their inclusion in cultivated oil crops. Since the genes for most of these traits have yet to be identified, interspecific hybridization is presently the most suitable method for gene transfer. However, because various types of barriers preventing sexual hybridization exist, hybrids usually cannot be recovered through sexual crossing. One way to overcome these barriers is to use somatic hybridization.

In the *Brassicaceae*, somatic hybridization has recently developed into a promising technique for the introgression of alien genes into a domesticated crop. Hybrids have been produced between species belonging to the same genera (Schenk and Röbbelen 1982; Sundberg and Glimelius 1986; Sundberg et al. 1987; Rosén et al. 1988. Jourdan et al. 1989a, b; Sjödin and Glimelius 1989a, b; Kirti et al. 1991) and to those of different genera (Toriyama et al. 1987a, b; Chatterjee

et al. 1988; Fahleson et al. 1988a; Klimaszewska and Keller 1988; Primard et al. 1988; Sikdar et al. 1990; Hagimori et al. 1992). Several of the hybrids produced have been fertile, which has enabled backcrossing to the cultivated crop and thus created possibilities to utilize the hybrids in crop breeding programmes. In fact, several of the hybrids produced in our laboratory are included in breeding programmes in Sweden.

Much less has been reported on the production of intertribal somatic hybrids. Although Gleba and Hoffman (1980) combined *Arabidopsis thaliana* and *Brassica campestris*, no fertile hybrids were obtained. However, fertile intertribal somatic hybrids between *B. napus* and *A. thaliana* (Forsberg et al. 1994) and between *Nicotiana tabacum* and *Atropa belladonna* (Babiychuk et al. 1992) have recently been reported. Seeds were obtained in both cases, after selfing as well as after backcrossing to one of the parental species. These encouraging results indicate that somatic hybridization could be a functional method for transferring traits between species that are more distantly related than those previously presented.

The work presented here describes the production and characterization of intertribal somatic hybrids between *Brassica napus* and *Thlaspi perfoliatum*. In *T. perfoliatum* seeds the content of nervonic acid (*cis*-tetracos-15-enoic acid, $C_{24:1}$) is 19–20% (Miller et al. 1965). Since nervonic acid could be a valuable ingrediant in technical oils, but is present in very low amounts in *B. napus*, there is interest in transferring the gene(s) regulating the production of this fatty acid to rapeseed. By transferring the gene(s) responsible for the conversion of erucic acid to nervonic acid, it would be possible to breed for a rapeseed variety having high amounts of long-chain fatty acids. To our knowledge no successful sexual hybridization has been reported between *B. napus* and *T. perfoliatum*, which makes this report the first describing the combination of the genomes of these two species.

Materials and methods

Plant material

Since erucic acid is the precursor to nervonic acid, the high erucic acid (average content 47%) variety of *Brassica napus* L. ssp. *oleifera*, cv 'WW 696', was used as one parent in the fusion experiments. Two accessions of *Thlaspi perfoliatum* L. (A and B) were used as the other parent, both having a nervonic acid content of about 19%. Seeds were kindly provided by W. Weibulls AB and the Botanical Garden, Uppsala, Sweden.

Protoplast isolation, fusion selection and culture of hybrid cells

The culture of plant material and isolation of protoplasts was conducted according to Glimelius (1984). Freshly isolated hypocotyl protoplasts of *B. napus* were suspended in W5 (Menczel et al. 1981) and stained with 250-500 µl of 5(6)-carboxyfluorescein-diacetate (CFDA) per milliliter of protoplast suspension (stock solution of CFDA 0.22 m*M* dissolved in W5) for 10 min prior to fusion. Mesophyll protoplasts from *T. perfoliatum* were isolated from 3-week-old, in vitro-cultured plants. Protoplasts were fused using 40% polyethylene glycol (PEG) (w/v, mol wt 1500) in 0.3 *M* glucose and 50 m*M* $CaCl_2$. $2H_2O$. The methods and conditions used for protoplast fusion were as described by Sundberg and Glimelius (1986). In one experiment the mesophyll protoplasts to *T. perfoliatum* (accession B), suspended in W5, were X-ray irradiated with a dose of 70 Gy before fusion. The treatment of cells before selection, carried out by means of flow cytometry and cell sorting, as well as the culturing of hybrid cells and regeneration of plantlets were carried out according to Sundberg et al. (1991). Regenerated plantlets were transferred to MS medium (Murashige and Skoog 1962) supplemented with 5.4 µ*M* NAA (1-naphtyl acetic acid) to stimulate root growth. After rooting, the plants were transferred to the greenhouse.

Isoenzyme analysis of regenerated plants

Isoenzyme analysis using phosphoglucose isomerase (PGI) was performed as described by Sundberg and Glimelius (1986).

Cloning of Thlaspi perfoliatum-specific repetitive DNA sequences, Southern and slot-blot analysis and estimation of copy number

Fragments from partially digested total DNA of *T. perfoliatum* were cloned into pUC18. Plasmid clones containing *T. perfoliatum*-specific repetitive sequences as inserts were obtained after differential screening of transformed *E. coli* XL 1 cells. Plasmid DNA was isolated according to Maniatis et al. (1982) and cut with *Hin*dIII + *Eco*RI. After electrophoresis the fragments were eluted from the gel using a Geluter III (E-C Apparatus Corp Fla, USA) according to the manufacturer's instructions.

Total DNA from the putative hybrid plants was isolated according to a modified, Landgren and Glimelius (1990) method of Bernatzky and Tanksley (1986) and completely digested with *Bam*HI. Southern blot analysis was performed using a *T. perfoliatum*-specific repetitive DNA sequence, a heterologous chloroplast sequence, (the *Sac*I fragment no. 13, 4.6 kb, Jansen and Palmer 1987), and eight mitochondrial heterologous gene probes (Landgren and Glimelius 1993).

For the slot-blot experiments a 48-well slot-blot apparatus (Bio-Rad) was used according to the manufacturer's instructions. To estimate the copy number in *T. perfoliatum*, serial dilutions from 50 to 2 ng/slot of plasmid DNA with and without insert and *T. perfoliatum* DNA were applied. To estimate intraspecific variation in *T. perfoliatum*, DNA samples from a number of different individuals were applied together with a serial dilution of a reference individual. The samples investigated in the above-mentioned experiments as well as the samples from the regenerated plants were replicated three times. The same two individuals of *T. perfoliatum* (A2 from accession A and B1 from accession B) were used as references in all experiments. Hybridization and washing conditions were as described by Landgren and Glimelius (1990).

Radioactivity of the hybridized DNA sequences was measured in a scintillation counter (LKB Wallac 1209 Rackbeta, LKB, Sweden) or indirectly by densitometer scanning (Ultroscan XL laser densitometer, LKB, Sweden). Comparisons of radioactivity between known amounts of plasmid DNA and genomic DNA were used to estimate copy number per haploid genome. Data were evaluated using the computer programmes Gelscan XL™ (Pharmacia, Sweden) and JMP 2.0 (SAS institute, N.C.,

Table 1. Results from protoplast fusion experiments between *B. napus* and *T. perfoliatum*

Species combined	Irradiation of mesophyll protoplasts (Gray)	Number of experiments	Hybrid cell frequency (percentage of living cells that were hybrids)		Number of calli	Number of calli that regenerated shoots	Total number of shoots obtained	Regeneration frequency (%). [Number of calli that regenerated shoots (A) or total number of shoots (B) divided by total number of calli		Number of flowering shoots
			Before sorting	After sorting				A	B	
Bn(+)Tp	70	1	7	87	192	10	34	5	18	30 from 3 calli
Bn(+)Tp	0	6	7	76	1735	50	100	3	6	38 from 3 calli

Bn, *B. napus*; Tp, *T. perfoliatum*

USA). The statistical tests included analysis of variance, regression analysis and chi-square.

Ploidy level analysis

The nuclear DNA contents of the hybrids and parental material were determined in a flow cytometer as described by Fahleson et al. (1988b). The weight of one *B. napus* and one *T. perfoliatum* genome was estimated to be 2.1 and 0.9 pg, respectively. The hybrids were assigned to one of five different classes as follows: (1) plants with a nuclear DNA content corresponding to the DNA content of *B. napus*, (2) plants with a DNA content greater than that of *B. napus* but less than the sum of *B. napus* and *T. perfoliatum*, (3) plants with values equal to this sum, (4) plants with a DNA content greater than the sum of the parental content and (5) plants whose DNA contents differed between cells. The intervals of each class corresponded to a 95% confidence interval of the DNA content of that class with a standard deviation of 3.5% (Sundberg et al. 1991). Hybrids from the same callus were represented by a single, randomly chosen, individual. Nine hybrids (from 3 different calli) from the non-irradiated and 7 hybrids (from 2 different calli) from the irradiated material were tested for correlation between nuclear DNA content and amount of the *T. perfoliatum* repetitive sequence.

Fertility and embryo rescue

The obtained hybrids were self-fertilized and backcrossed with pollen from *B. napus*. Fertility was measured as the number of seeds obtained per pollinated flower and expressed as a percentage of *B. napus* fertility. The fertility of *B. napus* was estimated from crosses between different individuals. Embryo rescue was carried out as described by Mathias et al. (1990) with some modifications. The MS medium used in the double-layer culture method was supplemented with 10 g/l sucrose. In the solid phase 0.8% agar was used.

Fatty acid analysis

Seeds from backcrossed hybrids were germinated on a moistened filter paper in a petri dish. One of the emerging cotyledons was dissected from the embryo and directly methylated. We followed the methylation procedure described by Wiberg et al. (1991) in all but one respect; i.e. 2 ml of 4% HCl in methanol was added. The methyl esters were extracted by adding 1 ml *n*-hexane and 0.5 ml distilled water. The hexane phase was removed and stored at −20 °C. Before analysis, each sample was evaporated and dissolved in 100 µl hexane. From each sample 2–3 µl was analysed by gas liquid chromatography with a glass column (2m × 2 mm internal diameter) containing 10% BDS on Chromosorb W (HP 80–100 mesh) on a Shimadzu GC-9A gas chromatograph equipped with a FID-detector and a 50-sample automatic injection rack. The *B. napus* control consisted of seeds from plants regenerated from protoplasts and grown to maturity in the greenhouse. These plants were kept as an isolated population in the greenhouse.

Results

Fusion, selection, regeneration and verification of somatic hybrids

Fusion and regeneration frequencies are summarized in Table 1. The frequency of calli that differentiated into shoots averaged 3%. Of the 27 shoots, each from a different callus analysed in the experiments using non-irradiated mesophyll protoplasts, 19 had a biparental isoenzyme pattern for PGI (Table 2, Fig. 1). The corresponding figure for 9 shoots analysed in the experiment with irradiated mesophyll protoplasts was 8. All of the other analysed plants expressed the pattern of *B. napus*.

Table 2. Results from isoenzyme analysis and analysis of presence of *T. perfoliatum*-specific repetitive DNA sequence in regenerated plants obtained after protoplast fusion between *B. napus* and *T. perfoliatum*

Number of individuals analysed from non-irradiated protoplasts (0) and irradiated protoplasts (70). Each individual was from a different callus	Frequency (%) of plants shown to contain *T. perfoliatum* DNA using		
	Isozyme marker (PGI)	Repetivitive sequence (1:19)	Repetitive sequence and isozyme marker
27 (0)	70		
9 (70)	88		
20 (0)		90	65
6 (70)		100	83

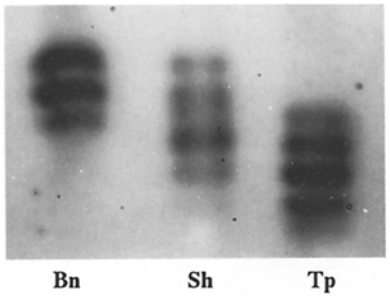

Fig. 1. Part of an isoenzyme gel stained for PGI. *Bn B. napus, Sh* Somatic hybrid, *Tp T. perfoliatum*

Cloning and characterization of T. perfoliatum-specific repetitive DNA sequences

Three hundred transformed *E. coli* colonies were selected. After differential screening 26 were further evaluated using Southern blot analysis. These analyses revealed two recombinant plasmids containing inserts that were repetitive and species specific (Figs. 2, 3a). The 2 clones gave identical patterns that were typical of tandemly repeated sequences. Only one was used in this study. The length of the DNA fragment (1:19) was estimated to be 800 bp.

The average copy number of the *T. perfoliatum*-specific repetitive DNA sequence in *T. perfoliatum* was estimated to be 10^5 copies per haploid genome. To study intraspecific variation in *T. perfoliatum*, we tested 13 plants from two accessions. A significant difference in hybridization to the repetitive DNA sequence was

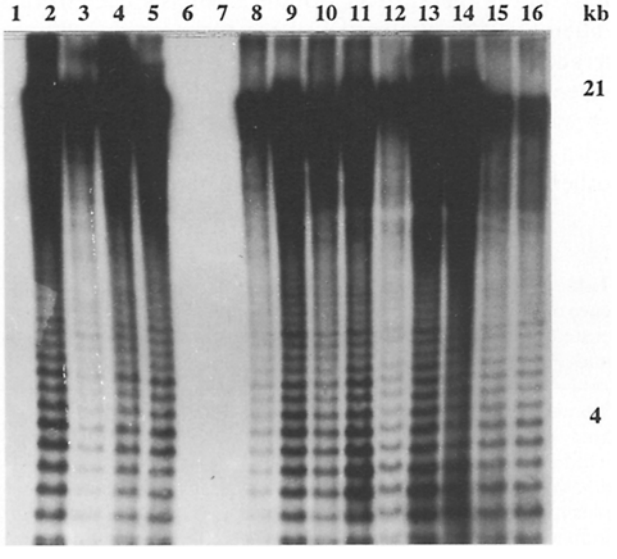

Fig. 2. Southern blot hybridization of total DNA after digestion with *Bam*HI and hybridization with a *T. perfoliatum*-specific repetitive DNA sequence. *Lane 1 B. napus, lane 2 T. perfoliatum, lanes 3–16* regenerated shoots from protoplast fusion experiments between *B. napus* and *T. perfoliatum*. Approximate molecular weights are indicated

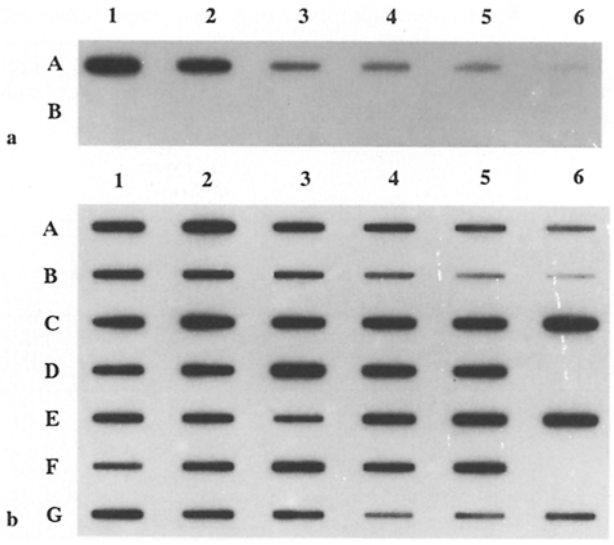

Fig. 3a–b. Slot-blot experiments with total DNA hybridized with a *T. perfoliatum*-specific repetitive DNA sequence. **a** *Slots A1-A6* Serial dilutions of *T. perfoliatum* DNA where the concentration is halved for each step starting with 36 ng/slot in slot A1, *slots B1-B6* serial dilutions of *B. napus* DNA halved for each step starting with 100 ng/slot at slot B1. **b** *Slots A1-A6* serial dilutions of *T. perfoliatum* (accession A) halved for each step starting with 50 ng/slot at A1, *slots B1-B6* serial dilutions as in slots A1-A6 of *T. perfoliatum* DNA (accession B), *slots C1-D5* somatic hybrids from callus 67/2, *slot E1* hybrid 78/6, *slot E2* hybrid 78/7, *slot E3* hybrid 78/24, *slot E4* hybrid 86/3, *slot E5* hybrid 86/5, *slot E6* hybrid 86/8, *slot F1* hybrid 86/9, *slots F2-G2* (except slot F6) hybrids from callus 86/10, *slot G3* hybrid 86/11, *slot G4-G6* hybrids from callus 86/24. A 100 ng amount of DNA from somatic hybrids between *B. napus* and *T. perfoliatum* was applied

observed between the two accessions ($t = 3.4764$, $P = 0.0013$), with accession A giving on average a higher value. Significant variation in hybridization to the repetitive sequence was also observed between plants within the two accessions (Fig. 4a) ($F = 16.9761$, $P < 0.001$).

Southern and slot-blot analyses

From the experiments with non-irradiated mesophyll protoplasts, 20 shoots, each from a different callus were analysed with the *T. perfoliatum*-specific repetitive DNA sequence (Figs. 2, 3b). Among these, 18 tested positive for the repetitive sequence and 13 tested positive for the repetitive sequence while also showing a biparental pattern for PGI. The corresponding figures for the 6 shoots from the experiment with irradiated mesophyll protoplasts were 6 and 5 (Table 2). No shoots that had a biparental pattern for PGI tested negative for the repetitive sequence. Regarding hybridization to the repetitive sequence, no significant difference was observed between hybrids derived from irradiated parental material and those derived from non-irradiated parental material ($P = 0.3158$) On the other

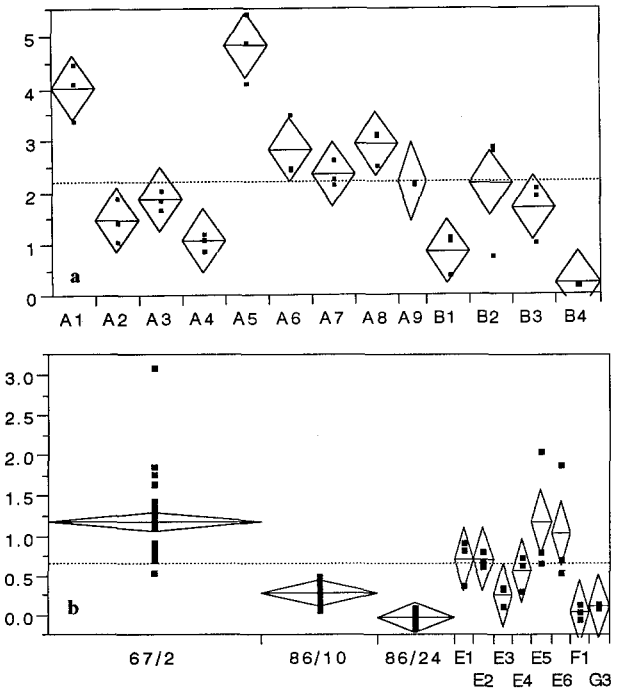

Fig. 4a–b. Diagrams showing hybridization of the *T. perfoliatum*-specific repetitive DNA sequence (*y axis*) in different individuals, relative to a reference *T. perfoliatum* individual **(a)** or relative to the average value in *T. perfoliatum* **(b)**. The diagrams were obtained after evaluation of the slot-blot autoradiograms using densitometer scanning and computer analysis. The height of the squares represents a 95% confidence interval. **a** Hybridization of the *T. perfoliatum*-specific repetitive DNA sequence in different individuals of accessions A and B of *T. perfoliatum*. The *horizontal line* denotes the average value. **b** Hybridization of the *T. perfoliatum*-specific repetitive DNA sequence in different *B. napus* (+) *T. perfoliatum* somatic hybrids. Each hybrid is denoted by its respective slot number (see text to Fig. 3b) or, in the cases of several hybrids from the same callus, the hybrids were grouped (hybrids from callus 67/2, 86/10 and 86/24)

hand, a statistically significant variation was observed between different hybrids. (Fig. 4b). Among hybrid shoots from callus 67/2 1 shoot 67/2 J, (slot D3, Fig. 3b) showed a higher value than the others in this group ($t = 4.6975$, $P = 0.0001$).

Organelle type

All 13 hybrids, each from a different callus analysed for chloroplast (cp) type, showed the *B. napus* cpDNA pattern (Fig. 5). Twelve hybrids from the non-irradiated material were subjected to a mtDNA analysis. Seven hybrids were investigated with two different restriction enzymes and eight mitochondrial heterologous gene probes (Landgren and Glimelius 1994). Of these 7 hybrids 4 were found to contain rearranged mtDNA. The other 5 were subjected to mtDNA analysis using all of the heterologous gene probes but only

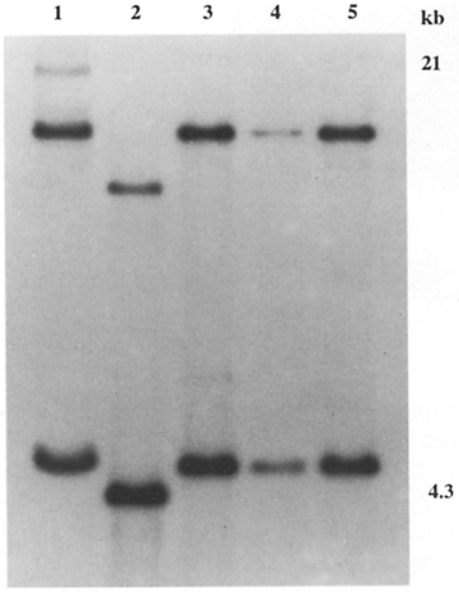

Fig. 5. Southern blot hybridization pattern of total DNA hybridized with a chloroplast-specific probe. *Lane 1 B. napus, lane 2 T. perfoliatum, lanes 3–5* regenerated plants from protoplast fusion experiments between *B. napus* and *T. perfoliatum*. Approximate molecular weights are indicated

one of the restriction enzymes (*Bam*HI). Among these, 3 were found to have a rearranged mt genome. All hybrids lacking a rearranged mtDNA pattern showed the *B. napus* pattern.

Ploidy level analysis

Plant included in Fig. 6. had been regenerated from 21 different calli and verified as hybrids or partial hybrids based on the presence of genetic markers from both parents. The material consisted of 16 hybrids from

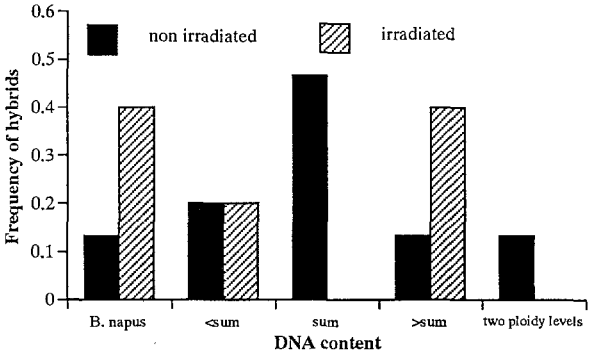

Fig. 6. Frequency distribution of *B. napus* (+) *T. perfoliatum* somatic hybrids in different classes according to their nuclear DNA content. The class limits were set as a 95% confidence interval of the DNA content of the class with a standard deviation of 3.5% (Sundberg and Glimelius 1991b). The DNA content of the class "sum" is the sum of the parental genomes

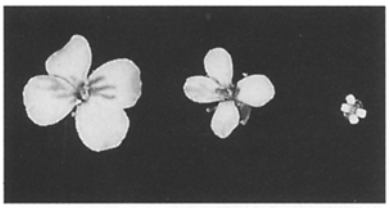

Fig. 7. Flower morphology of *B. napus* (*Bn*), somatic hybrid (*Sh*) and *T. perfoliatum* (*Tp*)

non-irradiated material and 5 hybrids from irradiated material. Of the non-irradiated material included in Fig. 6, 5 hybrids had a nuclear DNA content below the parental sum, 7 hybrids had a content equal to the sum and 2 had a content above the sum. The corresponding figures for the irradiated material were 3, 0 and 2, respectively. Two hybrids were found that contained two levels of DNA content. No correlation between copy number of the *T. perfoliatum*-specific repetitive sequence and nuclear DNA content was found.

Fertility determination and flower morphology

Relative to *B. napus* and *T. perfoliatum* flowers, the hybrid flowers had an intermediate morphology (Fig. 7). Their anthers were smaller than those of the *B. napus* parent. In one hybrid (86/24 A) the flowers did not develop any petals at all or had only two or three petals. *B. napus* produced 10.7 seeds per flower when crossed. The fertility of the different hybrids averaged 0.6% of that of *B. napus* when backcrossed to the latter (Table 3). No selfed seed could be obtained owing to a lack of pollen production or, in two cases (hybrids 67/2 A and 86/24 A), to a lack of functional pollen. No difference in fertility was found between the hybrids derived from irradiated material and those derived from non-irradiated material.

Embryo rescue of progeny from the hybrids

To increase the number of progeny obtained from the initial hybrids we used a method for embryo rescue that had been described by Mathias et al. (1990). Five hybrids from different calli (24 plants in total) were fertilized with pollen from *B. napus*. On average, 69 flowers per plant (range 2–377) were pollinated. Three hybrid plants, 67/2 A, 67/2 D and 67/2 H derived from callus 67/2 produced embryos that developed into normal looking plants after being transferred to MS medium. Embryo rescue failed for the other hybrids investigated.

Fatty acid analysis

The nervonic acid content was measured in seeds obtained from 12 hybrids backcrossed to rapeseed. These hybrids were derived from 4 calli. For 5 of the hybrids the average values differed significantly ($P < 0.01$) from those of the control (Table 3). Some

Table 3. Results from fertility and fatty acids analyses of 12 somatic hybrids (from 4 different calli) between *B. napus* and *T. perfoliatum*

Callus	Individual[a]	Ploidy level category (according to text)	Fertility. Seeds per pollinated flower (% of *B. napus*)	Number of seeds analysed	Content of nervonic acid (in %)[b]	
					Average	Highest value
67/2	67/2 A	2	3.1	34	1.9*	4.9
	67/2 B	nd	2.1	4	1.8*	3.7
	67/2 D	1	0.5	4	2.9*	4.2
	67/2 E	1	0.2	1	3.0	
	67/2 F	2	0.2	1	1.4	
	67/2 G	2	0.2	5	2.1*	2.9
86/10	86/10 C (I)	1	0.1	1	2.3	
	86/10 E (I)	1	0.2	1	1.5	
86/11	86/11 A (I)	1	1.4	3	2.2*	4.2
	86/11 B (I)	2	0.5	3	1.3	
86/24	86/24 A	nd	0.2	1	1.4	
	86/24 B	nd	0.2	1	1.2	
	Control:					
	B. napus cv. WW 696 plants regenerated from protoplast culture			45	1.1	1.8
	T. perfoliatum			14	19.5	22.2

nd, Not determined

[a] I, Irradiated protoplasts

[b] A significant difference compared with the control ($P < 0.01$) is denoted by an asterisk

individuals also showed an altered fatty acid pattern with unusually high amounts of saturated fatty acids, mainly palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$). In addition, the stearic acid content in a seed resulting from the selfing of a regenerated shoot, classified as a non-hybrid plant, was six fold higher than that found in the parental species (from 1.1% to 6.9%).

Discussion

Our investigation shows that rapeseed and *T. perfoliatum* can be combined into fully functional intertribal somatic hybrids displaying features from both parental species, including the presence of nervonic acid in some of the hybrids. Fatty acid analysis of seeds was performed on 12 plants from 4 calli. As a control, we used plants regenerated from hypocotyl protoplasts of *B. napus*. In seeds from these plants the nervonic acid ($C_{24:1}$) content was on averge 1.1%. In ordinary seeds the content was 0.6%, indicating that tissue culture conditions might have had a small influence on the nervonic acid content. Five of the hybrids had a nervonic acid content that was significantly higher than that of the control plants. However, despite the increase in nervonic acid content obtained in some of the hybrids, it was still much lower that the present in *T. perfoliatum* (20%). The reason for this is not known. There is a lack of knowledge regarding the number of genes that participate in the regulation of the elongation into nervonic acid and factors influencing their expression. In the hybrids investigated, some of the genes for the C_{22}–C_{24} elongation might be absent, or, if they are present, they may not function properly in a new genetic background.

An interesting result regarding the composition of other fatty acids was that some individuals showed a novel, altered, fatty acid pattern that did not resemble any of the parental patterns. All of them contained a high amount of saturated fatty acids. One individual, which could not be classified as a hybrid according to the available markers, contained six times more stearic acid ($C_{18:0}$) than the parental species. Since tissue culture per se can induce chromosomal rearrangements, this procedure cannot be discounted as being one cause of the altered fatty acid patterns even though we found that it did not markedly affect nervonic acid content. However, the seeds were shrunken in most of the cases, indicating that the novel fatty acid patttern was due to abnormal development and delayed fatty acid biosynthesis rather than to influences by tissue culture. Progeny from this plant will have to be analysed further before we can draw any conclusions.

The fusion and regeneration frequencies obtained in the present study are comparable to those reported from hybridization experiments between more closely related species (Sundberg et al. 1991). However, the growth and development of hybrid shoot culture both in vitro and in the greenhouse were poor compared with the growth and development of the interspecific and intergeneric hybrids produced. This was especially true for root production. NAA was shown to increase root production and was therefore included in the rooting medium. Nevertheless, many plants died at an early stage, and even the plants that produced roots, enabling them to be transferred to the greenhouse, had problems surviving the transfer. Furthermore, all of the transferred plants were male sterile, possibly owing to an incompatibility expressed at later stages of plant ontogenesis that prevented normal development into fully functional and fertile plants. Although difficult to compare, since one of the parental species (*A. thaliana*) consisted of a cell line, similar results concerning root formation and fertility were reported by Hoffman and Adachi (1981). Even more severe developmental problems have been reported for hybrids produced between *B. napus* and *Barbarea vulgaris* (Fahleson et al. 1994); i.e. the hybrids never reached maturity in the greenhouse. In contrast, the *A. thaliana* (+) *B. napus* hybrids obtained by Forsberg et al. (1994) and the *Nicotiana-Atropa* hybrids produced by Babiychuk et al. (1992) developed into fully mature plants that were able to set seeds. The differences in response may depend on different degrees of homology between the genomes of the particular species combined.

Isoenzyme analysis was used to verify hybrid character. However, the use a *T. perfoliatum*-specific repetitive DNA sequence as marker allowed a larger number of plants to be verified as containing DNA from *T. perfoliatum*, indicating that this tandemly repeated sequence is present on more than one location in the *T. perfoliatum* genome. However, if this sequence is located to a certain region of all the *T. perfoliatum* chromosomes, as has been shown for other tandemly repeated sequences (Jones and Flavell 1982; Iwabuchi et al. 1991; review Lapitan 1992), and/or if it is evenly distributed to its locations cannot be concluded at this stage. The lack of correlation between nuclear DNA content and amount of *T. perfoliatum*-specific repetitive sequence in the hybrids could therefore be due to an uneven distribution of this sequence on the chromosomes and/or that the sequence is not present on all chromosomes in the *T. perfoliatum* genome. Considerable variation in hybridization to the repetitive sequence was observed between plants within the two *T. perfoliatum* accessions. Significant variation was also found between different hybrids. Thus, some of the hybrids clearly differed in their genetic makeup. However, the variation registered among the parental individuals within accession A and B makes it difficult to interpret the variation between hybrids. Due to this intraspecific variation, it might be possible that hybrids

that differ in their copy number still contain the same chromosome complement from *T. perfoliatum*.

Of the *Thlaspobrassica* material produced in this study, 6 hybrids flowered. However, all of the plants were male sterile because their anthers either failed to produce pollen or the pollen produced was non-functional. Since anther and pollen development have been shown to involve nuclear-mitochondrial interactions (Lonsdale 1987) it is possible that an incompatibility between the hybrid nucleus and the mitochondria is responsible for the absence of pollen and the production of inviable pollen. It was found that 58% of the investigated hybrids had a rearranged mtDNA pattern, which can be associated with male sterility (Dewey et al. 1986; Köhler et al. 1991; Håkansson and Glimelius 1991). However, male sterility can also be due to incompatibility between nuclear genomes, which may explain why hybrid 67/2 A was male sterile even though it displayed the mtDNA pattern identical to *B. napus*. Further investigations of male sterility might lead to detection of a new alloplasmic cytoplasmic male-sterile (CMS)-system that could be of value in future breeding programmes. The chloroplast type was also investigated. Analysis of the cpDNA banding pattern showed that all hybrids displayed the *B. napus* pattern. Such biased segregation has also been found in other somatic hybrids produced within the *Brassicaceae* (Sundberg and Glimelius 1991; Forsberg et al. 1994).

Although seeds could be obtained from the hybrids after backcrossing, the seed set of the hybrids was very low. Investigations performed on other hybrid combinations have shown that the efficiency with which distantly related species can be combined into fertile hybrids can, at least in some cases, be enhanced by using "gamma-fusion" (review Hinnisdaels et al. 1988). To investigate this possibility we carried out an experiment where the *T. perfoliatum* protoplasts were irradiated prior to fusion. On the basis of the ploidy level analysis the hybrids derived from the irradiated material, in general, were found to be asymmetric, having a nuclear DNA content lower than the parental sum. In contrast, hybrids deriver from the non-irradiated material were generally symmetric, but even here asymmetric hybrids could be found. The asymmetric hybrids had lost genetic material from *T. perfoliatum*, as verified by the elimination of *T. perfoliatum*-specific markers. At first, results from the ploidy level analysis seemed to be contrasted by the fact that there was a higher frequency of complete hybrids from the irradiated material, as judged from the analyses regarding presence of the *T. perfoliatum*-specific markers, than from the non-irradiated material. However, when chi-square tests were applied no significant differences could be detected. According to the ploidy level analysis, the irradiation led to a transfer of a limited amount of *T. perfoliatum* DNA. However, since only a few hybrids were derived from the experiment where irradiation had been applied, it is difficult to draw any conclusions. The regeneration frequency and the frequency of flowering plants were higher in the material combining irradiated *T. perfoliatum* protoplasts with rapeseed than in plants obtained from non-irradiated material suggesting that hybrids could be more efficiently produced using irradiated *T. perfoliatum* protoplasts. Nevertheless, the fertility of the hybrids derived from the irradiated material was as low as that of the hybrids derived from the non-irradiated material, indicating that even if asymmetric hybrids had been produced to a higher degree from the irradiated material their fertility was not improved.

In summary, this investigation shows that it is possible to obtain hybrid plants between the distantly related species *B. napus* and *T. perfoliatum*. Some of these hybrids also contained significantly higher amounts of nervonic acid than *B. napus*, proving that somatic hybrids can be used as bridges for transferring traits of value from one species to another. The fact that somatic hybridization can be carried out successfully despite a lack of detailed knowledge regarding the genes responsible for a desirable trait makes it an attractive alternative to transformation. Like transformation, desirable traits from remote sources can be transferred to a cultivated crop, but unlike transformation the gene(s) coding for the trait do not have to be identified and cloned. The poor growth of the material, both in vitro and in the greenhouse, together with a high degree of sterility, indicate that it can be difficult to combine species from different tribes into fully functional hybrids. Nevertheless, the fact that we obtained hybrid plants that could be backcrossed to rapeseed and which also contained the desired trait confirmed that somatic hybridization has great potential in future breeding work.

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