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Expression of the C_3–C_4 intermediate character in somatic hybrids between *Brassica napus* and the C_3–C_4 species *Moricandia arvensis*

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Abstract The wild crucifer *Moricandia arvensis* is a potential source of alien genes for the genetic improvement of related Brassica crops. In particular *M. arvensis* has a C_3–C_4 intermediate photosynthetic mechanism which results in enhanced recapture of photorespired CO_2 and may increase plant water-use efficiency. In order to transfer this trait into *Brassica napus*, somatic hybridisations were made between leaf mesophyll protoplasts from cultured *M. arvensis* shoot tips and hypocotyl protoplasts from three *Brassica napus* cultivars, 'Ariana', 'Cobra' and 'Westar'. A total of 23 plants were recovered from fusion experiments and established in the greenhouse. A wide range of chromosome numbers were observed among the regenerated plants, including some apparent mixoploids. Thirteen of the regenerated plants were identified as nuclear hybrids between *B. napus* and *M. arvensis* on the basis of isozyme analysis. The phenotypes of these hybrids were typically rather *B. napus*-like, but much variability was observed, including variation in flower colour, leaf shape and colour, leaf waxiness, fertility and plant vigour. CO_2 compensation point measurements on the regenerated plants demonstrated that 3 of the hybrids express the *M. arvensis* C_3–C_4 intermediate character at the physiological level. Semi-thin sections through leaf tissues of these 3 plants revealed the presence of a Kranz-like leaf anatomy characteristic of *M. arvensis* but not found in *B. napus*. This is the first report of the expression of this potentially important agronomic trait, transferred from Moricandia, in *M. arvensis* × *B. napus* hybrids.

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Introduction

Moricandia arvensis is a member of the Brassiceae and a relative of the *Brassica* crop group. *M. arvensis* possesses several traits which are of biological, and potential agronomic, interest, including resistance to *Albugo candida*, moderate resistance to *Alternaria brassicae* (Kirti et al. 1992) and resistance to *Phyllotreta cruciferae* and *Plasmodiophora brassicae* (Takahata et al. 1993). Of particular interest is the photosynthetic/photorespiratory activity of *M. arvensis*, which is intermediate between that of C_3 and C_4 plants (Holaday and Chollet 1984). In common with other C_3–C_4 species, *M. arvensis* has a CO_2 compensation point intermediate between C_3 and C_4 plants (Holaday et al. 1981, 1982) and a Kranz-like leaf anatomy (Holaday et al. 1981). Rawsthorne et al. (1988) demonstrated that the C_3–C_4 intermediate physiology of *M. arvensis* is largely determined by the differential expression of active glycine decarboxylase (GDC) in the cells of the mature leaf. The combination of the Kranz-like leaf anatomy with the differential distribution of GDC, and consequent localisation of glycine decarboxylation, results in efficient recapture of photorespired CO_2 and accounts for the low CO_2 compensation point of this species (Rawsthorne et al. 1988). It has been suggested that the greater efficiency of CO_2 recapture observed in C_3–C_4 intermediates could, if introduced into crop species, improve their water-use efficiency in comparison to C_3 forms of the same crop and result in a yield advantage, especially under conditions of water stress (McVetty et al. 1989).

Essential to any attempt to exploit C_3–C_4 intermediacy is an understanding of the genetic control of the biochemical and anatomical traits associated with it. The generation of both cytoplasmic substitution lines and nuclear hybrids between *M. arvensis* and C_3 *Mori-*

candia species, or C_3 crop plant species of the Brassiceae, would provide the opportunity to study aspects of the genetic control of C_3–C_4 metabolism and its associated anatomy. Access to lines differing in CO_2 compensation point, Kranz-like leaf anatomy and the differential expression of GDC would permit an assessment of the relative importance and interdependence of the different characters. Sexual hybrids have been produced, at low frequencies, both within the genus *Moricandia* (S. Rawsthorne personal communication) and between *M. arvensis* and several *Brassica* species, including *B. alboglabra, B. rapa, B. nigra, B. napus* and *B. juncea* (Apel et al. 1984; Takahata et al. 1993; Takahata and Takeda 1990).

Somatic hybridisation has several potential advantages over sexual hybridisation. Firstly, by avoidance of sexual incompatability barriers wide hybrids may be recovered at higher frequencies than in sexual crosses. Secondly, protoplasts can be pre-treated prior to fusion to potentially increase gene introgression. Thirdly, sexual hybridisations have been successful when *M. arvensis* is the female parent, and this restricts the opportunities for making cytoplasmic substitution lines. Somatic hybridisations have been reported between *M. arvensis* and *B. oleracea* (Toriyama et al. 1987) and *M. arvensis* and *B. juncea* (Kirti et al. 1992). This report describes, for the first time, both the production and characterisation of somatic hybrids between *B. napus* and *M. arvensis* and the expression of *M. arvensis* C_3–C_4 metabolism and anatomy in *B. napus* × *M. arvensis* hybrids.

Materials and methods

Plant material

The *M. arvensis* seeds were supplied by Dr. S. Rawsthorne, John Innes Centre, Norwich, from an inbreeding population. The seeds of the three *B. napus* cultivars, 'Ariana', 'Cobra' and 'Westar', were supplied by Dr. A. E. Arthur, also of the John Innes Centre, Norwich. Seeds of both species were surface-sterilised for 1 min in 70% ethanol, then for 30 min in a 10% sodium hypochlorite solution containing 2–3 drops of Tween 20 per 100 ml and finally washed three times in sterile distilled water. The seeds were germinated aseptically in 100 ml powder round bottles on MS medium (Murashige and Skoog 1962) with 3% sucrose and 0.8% agarose (Sigma type I, Sigma Chemical Co, Poole, UK). *B. napus* seedlings were used when the hypocotyls were 3–4 cm long, 5–7 days after germination. *M. arvensis* shoot-tip cultures were established by removing the shoot tips (the top 2 cm) from 5- to 7-cm-tall seedlings and transferring them to seed germination medium in 1-2 Kilner bottles (FSA Lab Supplies, Loughborough, UK). Shoot tips from cultured shoots were transferred to fresh medium every 40–50 days. Both seedlings and shoot tips were maintained under continuous light at 24° ± 1°C.

Protoplast isolation

M. arvensis protoplasts were isolated from fully expanded leaves of 40-day-old shoot cultures, as described by Murata and Mathias (1992). *B. napus* protoplasts were isolated from hypocotyls of 5- to 7-day-old seedlings. These were cut into transverse segments, approximately 5 mm long, and pre-plasmolysed in 0.3 *M* sorbitol and 0.05 *M* calcium chloride. After 1 h the pre-plasmolysis solution was replaced with enzyme solution, 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co, Tokyo, Japan) and 1% Cellulase Onozuka RS (Yakult Honsha Co, Tokyo, Japan) in K_3 medium (Kao and Michayluk 1975) as modified by Nagy and Maliga (1976) to pH 5.6 and supplemented with 0.4 *M* sucrose. Following overnight digestion the enzyme/tissue was incubated on an orbital shaker at 60 rpm for 1 h. Purification of *B. napus* hypocotyl protoplasts from the crude digests followed the method described for *M. arvensis*, with the exception that the initial sieving was through an 80-μm mesh.

Protoplast inactivation treatments

Freshly prepared solutions of 0.25, 0.5, 0.75, 1.0 and 1.5 m*M* iodoacetic acid (Sigma Chemical Co, Poole, UK) in W5 salts were filter-sterilised. Equal volumes (2 ml) of IOA and freshly isolated protoplasts (5×10^5/ml) in W5 salts solution were mixed and incubated at room temperature for 30 min. The protoplasts were then washed three times in W5 solution before fusion. Protoplasts were UV-irradiated by spreading a thin film of protoplast suspension (1×10^5/ml), in W5 solution over the base of a 3.5-cm petri dish. The UV source had an output, at the level of the protoplasts, of 0.5 J/s per square meter at 360 nm. The protoplasts were irradiated for 2 h, then incubated in the dark for 2 h to eliminate photo-repair and finally washed three times in W5 solution before fusion.

Protoplast fusion

In separate experiments hypocotyl protoplasts of the three *B. napus* cultivars were fused with mesophyll protoplasts of *M. arvensis* using a method modified from that of Menczel and Wolfe (1984). The density of each of the purified protoplast suspensions was adjusted to $1–2 \times 10^5$/ml, and equal volumes were mixed ready for fusion treatments. The polyethylene glycol (PEG) fusion solution was prepared immediately before use by mixing 7 ml of 14% (w/v) PEG 8000 (Sigma Chemical Co, Poole, UK) with 1 ml of 0.53 *M* glycine-sodium hydroxide buffer, pH 10.0, and 1 ml of dimethylsulfoxide. Four 50 μl droplets of PEG solution were then placed on the base of a 6-cm petri dish to form the corners of a square with 1–2 mm separating each drop. A 200 μl drop of the mixed protoplast suspension was then placed in the centre so as to cause the drops to partially coalesce. After 5 min the dish was tilted to thoroughly mix the drops. After a further 10 min this mixture was diluted with 6 ml of a post-fusion wash solution containing W5 salts and 0.05 *M* MES and left undisturbed at room temperature for 2 h. The fusion mixture was then pipetted into a centrifuge tube and centrifuged at 50 *g* for 2 min before being resuspended in culture medium.

Protoplast culture and plant regeneration

The protoplast culture method was modified from that of Poulsen and Nielsen (1989). The protoplasts were plated in 6-cm plastic petri dishes, at a density of 1×10^5 protoplasts per milliliter, in 1 ml of liquid 8P medium (Kao and Michayluk 1975) with 0.4 *M* glucose, 0.5 mg/l BAP, 0.1 mg/l NAA and 1 mg/l 2, 4-D. After 3–5 days the cultures were diluted with 3 ml 8P medium without 2, 4-D. Three weeks after protoplast isolation, the regenerated cell colonies were plated onto 25 ml K3 medium (Nagy and Maliga 1976) supplemented with 0.1 *M* sucrose, 0.5 mg/l BAP, 0.1 mg/l NAA, 0.25 mg/l 2, 4-D, 1 mg/l of ABA and 0.25% agarose (Sigma type I) in 9-cm plastic petri dishes. The cultures were maintained at 24° ± 1°C in the dark.

After a further 3 weeks calli were transferred to shoot regeneration medium, K3 medium with 1% sucrose, 0.4% agarose (Sigma type I) and BAP (0.1–2.0 mg/l) or zeatin (1.0–5.0 mg/l). The first regenerating shoots were seen after 2-3 weeks. Shoots longer than 5 cm were transferred to MS medium with 3% sucrose and 1% agarose (Sigma type I) for root development and subsequently to 'Jiffy 7s' (a/s Jiffy Products, Ryomgaard, Norway) in Magenta containers (Sigma Chemical Co Poole, UK). Within 2-3 weeks of transfer most plantlets had become well established, and the 'Jiffy 7s' were then potted into John Innes N°1 compost and transferred to the glasshouse. Initially,

the pots were covered with plastic bags to ensure high humidity. After 4–5 days the bags were cut open to wean the plants to normal glasshouse conditions.

All media were sterilised by autoclaving. The 2, 4-D was added prior to sterilisation, all other growth regulators were filter-sterilised and added to the media after autoclaving.

Isozyme analysis

The isozyme composition of regenerated plants was analysed on isoelectric focussing gels using the method described by O'Neill and Mathias (1995). Four leaf enzyme activities were assayed. Peroxidase activity was resolved on a gel containing a 1:1 mix of pH 4–6 and pH 5–7 ampholytes. Phosphoglucoisomerase activity was resolved on a gel containing a 2:1 mix of pH 3–5 and pH 4–8 ampholytes. Esterase and acid phosphatase activities were resolved on pH 3–10 gels.

Characterisation of cytoplasmic organelles

The cytoplasmic DNA content of the regenerated plants was determined by probing restriction digests of total leaf DNA with mitochondria- and chloroplast-specific probes. DNA was prepared from approximately 2 g of freeze-dried leaf material as described by Gawel and Jarret (1991) and digested with *Eco*RI and *Bam*HI endorestriction nucleases. Filters were probed with two mitochondrial genome-specific probes from maize, 8-3F2 and 2c13 (Lonsdale et al. 1984) and a chloroplast genome-specific probe from wheat, B(10–18) (Bowman and Dyer 1986).

Determination of chromosome numbers

Root tips were collected from plants established in soil. Mitotic chromosome spreads were prepared using a modification of the method described by Ijima and Fukui (1991). The roots were collected into freshly prepared ice cold 3:1 ethanol:acetic acid and stored at −20°C until use. Root tips were digested for 45 min at 37°C in an enzyme mixture containing 4% Cellulase Onozuka RS (Yakult Honsha Co, Tokyo, Japan), 0.3% Pectolyase Y23 (Seishin Pharmaceutical Co, Tokyo, Japan), 1.5% Macerozyme R200 (Yakult Honsha Co, Tokyo, Japan) and 1 m*M* EDTA, pH 4.2. Air-dried chromosome spreads were stained in 1% Wright's stain (Sigma Chemical Co, Poole, UK) at room temperature for 1 h before counting.

CO_2 compensation point measurements

Plants were replicated by taking cuttings from axillary shoots. The CO_2 compensation point (Γ) measurements were made on attached, young, fully-expanded leaves of healthy plants grown from cuttings in a controlled environment. The value of Γ (in ppm of CO_2) was measured in a closed-circuit gas-exchange system as described by Hunt et al. (1987). Γ was measured at high (1250 $\mu m/m^2/s$), medium (780 $\mu m/m^2/s$) and low (360 $\mu m/m^2/s$) light intensities at 25°C. The ratio of Γ at low/Γ at high light intensity was calculated. Replicated measurements were compared using a standard X^2 test.

Tissue sectioning

Pieces of leaf tissue from fully expanded leaves were fixed and embedded as described by Rawsthorne et al. (1988) except that all manipulations were carried out at 4°C and the resin was cured at 65°C for 48 h. Sections of 2 μm were cut for microscopy.

Results

Fusion and regeneration

Five concentrations of IOA (0.25, 0.5, 0.75, 1.0 and 1.5 m*M*) were tested for their effects on freshly isolated protoplasts of *B. napus* and *M. arvensis*. A 30-min treatment with IOA at a final concentration of 0.25 m*M* was sufficient to prevent subsequent division of *M. arvensis* protoplasts, and a 30-min treatment with 0.5 m*M* IOA prevented division in *B. napus*. In both species all of the treated protoplasts collapsed during the first 48 h of culture following treatment. The UV treatment used in these experiments (4080 J/m^2 at 360 nm) did not prevent protoplast division or the subsequent development of cell colonies in either species. It did, however, delay the onset of division in treated protoplasts by 3–4 days compared with untreated controls. Subsequently there was no difference between the treated and control protoplasts (data not presented).

M. arvensis mesophyll protoplasts were fused with hypocotyl protoplasts of the *B. napus* varieties, 'Ariana', 'Cobra' and 'Westar', as described above. The protoplasts of both species were stable during the fusion treatment, and after 12–18 h in culture many had regained a normal appearance. However, in cultures where protoplasts had been treated with UV and IOA a large proportion collapsed or became brown over the first 2–10 days in culture. The mean number of cell colonies regenerated per dish was determined after 3 weeks, when the colonies were transferred to solid K3 medium, and expressed as a percentage of total protoplasts plated (Table 1). Under the described culture conditions the protoplast regeneration frequency of *M. arvensis* is so low that its failure to regenerate operates as

Table 1 Mean plating efficiency (P.E. = number of colonies regenerated expressed as a percentage of the total protoplasts plated) of protoplast fusion experiments between *M. arvensis* (P.E. 0.5%) and *B. napus* cvs 'Ariana' (P.E. 2.8%), 'Cobra' (P.E. 2.4%) and 'Westar' (P.E. 2.0%) (*n/d* not determined)

Protoplast pretreatments[a]	Ariana × *M. arvensis*	Cobra × *M. arvensis*	Wester × *M. arvensis*
Untreated × untreated	1.3%[2b]	2.7%[2]	2.5%[1]
UV × IOA	1.5%[4]	2.9%[4]	0.7%[4]
UV + IOA	*n/d*	2.3%[4]	0.7%[1]
IOA × UV	0.5%[4]	0.1%[2]	0.0%[3]

[a] The order of UV and IOA in the first column indicates which parent was treated with UV and which with IOA eg. in the bottom row the *B. napus* parent was treated with IOA and the *M. arvensis* parent with UV. × indicates that protoplasts were fused, + indicates protoplasts were mixed but not fused

[b] The superscript next to the PE refers to the number of experiments contributing to mean PE

a partial selection in fusion treatments. In fusions where it was the *B. napus* parent that was treated with IOA very low plating efficiencies were observed. In contrast, when the *B. napus* partner was UV-treated and *M. arvensis* was IOA-treated, there was little effect on colony formation in comparison to the untreated controls (Table 1). Thus, *B. napus* makes the major contribution to regeneration from fusions, while the contribution from *M. arvensis* is negligible. The experiments with different *B. napus* cultivars were not replicated sufficiently to be confident of apparent varietial differences in plating efficiencies (Table 2).

The shoot regeneration frequencies from callus were very variable (Table 2). Shoot regeneration from the 'Cobra' × *M. arvensis* fusion was similar to that from the untreated *B. napus* parent, but in the 'Ariana' × *M. arvensis* and 'Westar' × *M. arvensis* fusions the regeneration was approximately 25–30% of that of the untreated *B. napus* partner. In the 'Cobra' × *M. arvensis* and 'Westar' × *M. arvensis* combinations, where the *B. napus* parent was treated with IOA, the absence of shoot regeneration may partly reflect the very low frequency of callus regeneration from protoplasts (see Table 1). Many calli produced multiple shoots, and in total 170 shoots were regenerated from fusion experiments. Many of these died when attempts were made to root them *in vitro* or upon transfer to soil. Twenty-three shoots, from independent calli, survived to the stage where they could be used in further analysis.

Verification of somatic hybrids

Of the 23 regenerated plants 13 had hybrid isozyme patterns for one or more of the isoenzymes assayed (Table 3). Of the remainder, 8 had *B. napus*-like patterns,

Table 2 Shoot regeneration frequencies (percentage of calli producing one or more shoots) from calli regenerated from fusion experiments

	Ariana × *M. arvensis*	Cobra × *M. arvensis*	Wester × *M. arvensis*
Untreated	2.1%	20.2%	1.2%
UV × IOA	7.4%	14.3%	1.7%
IOA × UV	7.6%	0.0%	0.0%
B. napus	22.6%	16.7%	8.3%
M. arvensis	0.0%	7.1%	0.0%

Table 3 Summary of isozyme, cytoplasm, CO_2 compensation point, chromosome number and fertility data from putative somatic hybrid plants

Protoplast fusion combination	Plant number	Isozyme pattern	Cytoplasm	CO_2 compensation points[a]		Chromosome number (range)	Seed set[b]
				Γ	Γ Ratio		
Ariana × *M. arvensis*							
UV × IOA	5–5	Hybrid	ND	ND	ND	ND	ND
	5–14	*B. napus*	ND	47.0	0.99	62	None
	5–15	*B. napus*	*B. napus*	43.1	0.93	60–67	None
	5–18	Hybrid	ND	46.2	0.98	56	S
	5–19	Hybrid	*B. napus*	47.7	0.93	62–65	S
	5–28	Hybrid	*B. napus*	52.0	0.91	68	None
	5–30	Hybrid	*B. napus*	47.0	0.96	67–73	S
	6–13	*M. arvensis*	*M. arvensis*	19.6	0.59	44	S
Cobra × *M. arvensis*							
none × none	2–2	*B. napus*	*B. napus*	42.0	0.99	58–69	S
	2–8	Hybrid	*B. napus*	ND	ND	60	None
	2–13	Hybrid	*B. napus*	40.2	0.96	70	BC
	2–14	ND	ND	42.0	0.95	58	BC
	3–1	*B. napus*	*B. napus*	39.6	1.0	76	S
	9–83	Hybrid	*B. napus*	46.0	0.93	58–64	ND
UV × IOA	9–7	Hybrid	*B. napus*	ND	ND	62–65	ND
	9–12	Hybrid	*B. napus*	35.6	0.92	74	BC
	9–14	Hybrid	*B. napus*	33.7	0.80	64 & 71–73	None
	9–16	Hybrid	*B. napus*	33.8	0.92	72	None
	9–17	*B. napus*	*B. napus*	41.1	0.94	56–76	S
	9–24	Hybrid	*B. napus*	ND	ND	72	S
	9–30	*B. napus*	*B. napus*	46.0	0.97	38	S
	9–74	*B. napus*	*B. napus*	47.0	0.95	ND	S
Westar × *M. arvensis*							
none × none	1–1	*B. napus*	*B. napus*	40.0	0.96	70–72	ND

[a] Γ measured at 1250 μE/m²/s (high light intensity), ratio = Γ at high light intensity/Γ at low light intensity (360 μE/m²/s). *M. arvensis* Γ = 15.94 and Γ ratio = 0.5. *B. napus* Γ = 41.4 and Γ ratio = 0.96

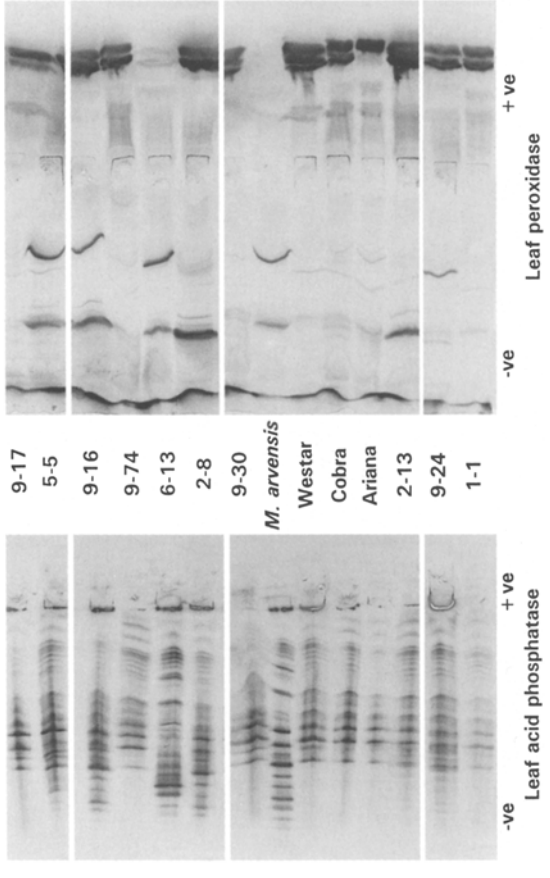
[b] ND, Not determined; S, plants set selfed seed; BC, seed set when plant backcrossed to *B. napus* parent

and 1 had a pattern similar to that of *M. arvensis*. In some hybrids a combined *B. napus* + *M. arvensis* pattern was observed; in others there were single bands from *M. arvensis* against a *B. napus* background; and occasionally novel "hybrid" bands occurred (Fig. 1). Hybrids typically showed hybrid isozyme patterns for more than one enzyme system. In all plants the restriction fragments detected by the chloroplast- and mitochondria-specific probes were identical to those found in *B. napus*, with the exception of plant 6–13 where the fragments resembled those in *M. arvensis* (data not presented).

To determine the chromosome numbers of regenerated plants we counted four to eight chromosome spreads per plant. Counting was made difficult by the typically large numbers of chromosomes in regenerated plants and by the fact that some plants appear to be mixoploid. Thus, in some cases a single figure could be established with confidence, but in others a range of numbers was a more accurate reflection of chromosome content. Only 1 plant (9-30) had a chromosome number ($2n = 38$) equal to that of either parent (*B. napus* $2n = 38$, *M. arvensis* $2n = 28$). Even those plants with non-variant isozyme patterns typically had non-standard chromosome numbers, greater than 38, suggesting they had originated from homofusions followed by chromosome loss.

The regenerated plants that were established in the glasshouse were generally *B. napus*-like in appearance. Observed phenotypic variations included lack of vigour, "intermediate" leaf shapes, degree of leaf waxiness, leaf colour, flower colour and the presence/absence and degree of male sterility. Very small and distorted plants were also recovered from fusion experiments, at low frequencies, but these did not survive under glasshouse conditions.

Fig. 1 Selected zymograms of putative hybrids and parents which illustrate the isozyme patterns observed among regenerated plants. The leaf peroxidase gel was a 1:1 mix of pH 4–6: pH 5–7 ampholytes. In the plants 5–5 and 9–16 both strong bands from the *M. arvensis* parent can be seen against the *B. napus* pattern. In 2–8, 2–13 and 9–24 single bands from *M. arvensis* are present in the *B. napus* background. The leaf acid phosphatase gel was a pH 3–10 gel. The 5 or 6 most acidic isoforms of this enzyme are diagnostic for *M. arvensis* and, combined with information from other zymograms, enables the detection of genetic material from *M. arvensis* in hybrid plants



CO_2 compensation points

The compensation points (Γ) of the regenerated plants were measured as described above. In 3 of the hybrid plants, 9–12 ($\Gamma = 35.6$) 9–14 ($\Gamma = 33.7$) and 9–16 ($\Gamma = 33.8$), the Γ values were significantly different (at the 5% level) from the parental controls (*B. napus* $\Gamma = 41.4$, *M. arvensis* $\Gamma = 15.9$). These three hybrids are expressing the C_3–C_4 character, although it is expressed less efficiently than in *M. arvensis*. The Γ of *M. arvensis* decreases with increasing light intensity, while the Γ of related C_3 species is unchanged. Thus, variation in Γ in response to high and low light levels is also an indicator of C_3–C_4 intermediate photosynthesis. In hybrids 9–12 and 9–16 this ratio was 0.93, not significantly different from the C_3 parent (0.96) but significantly different from *M. arvensis* (0.50) at the 5% level. In 9–14 this ratio was 0.80, significantly different from both parents at the 5% level. This confirms that in 9–14 the C_3–C_4 character is being expressed at the physiological level, although expression is not so efficient as in *M. arvensis*. While in plants 9–12 and 9–16 the C_3–C_4 character is expressed as a modified Γ, it does not result in a *M. arvensis*-like response to changing light intensities. Only 1 plant (6–13) had a Γ value ($\Gamma = 19.6$) and high/low light Γ ratio (0.59) equivalent to that of the *M. arvensis* control. Other characteristics of this plant indicated that it was *M. arvensis*, and there was no evidence of hybridity.

The C_3–C_4 character in *M. arvensis* is associated with the presence of a Kranz-like ring of bundle sheath cells around the vascular tissues of the leaf. Within these cells are large numbers of mitochondria arranged centripetally adjacent to the vascular bundle and overlain by an outer layer of chloroplasts. Such an anatomy is not found in *B. napus*. Thin sections through leaves from plants 9–12, 9–14 and 9–16 revealed a distribution of mitochondria and chloroplasts, within the bundle sheath cells, that resembled, but was not as distinct as, that in leaves of *M. arvensis* (Fig. 2). Sections through leaves of a "non-hybrid" control (plant 9–17) had an internal anatomy like that of the *B. napus* control.

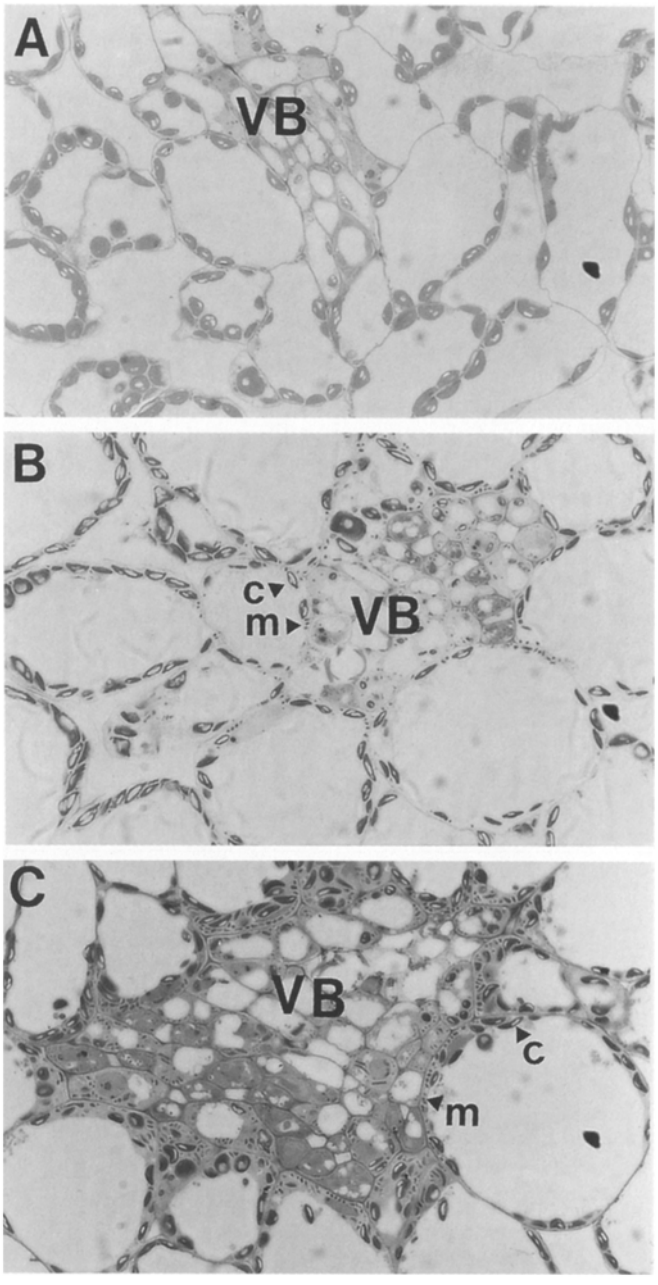


Fig. 2A–C Sections (2 μm) through leaves of **A** *B. napus* cv 'Westar', **B** hybrid plant 9–16 and **C** *M. arvensis*. The Kranz-like anatomy of *M. arvensis* is visible in the bundle sheath cells, with mitochondria (*m*) and chloroplasts (*c*) clearly preferentially associated with the cell walls adjacent to the vascular bundle (*VB*). A similar, although less distinct, arrangement can be seen in the section of hybrid leaf, but in the *B. napus* sections no such arrangement is evident

The regenerated plants were bagged prior to flowering. Initially, plants were allowed to self-pollinate, and those that did not set selfed seed were hand-pollinated and treated with carbon dioxide. Plants that could not be selfed were backcrossed with pollen of the *B. napus* parent line. Among the plants regenerated from the 'Cobra' × *M. arvensis* fusions, those which had *B. napus*-like isozyme patterns all set selfed seed. The hybrid 9–24 was self-fertile, and seed was recovered from hybrids 2–13, 2–14 and 9–12 on backcrossing to 'Cobra'. In the 'Ariana' × *M. arvensis* fusions 3 of the hybrids (5–18, 5–19 and 5–30) set selfed seed. The 2 plants with *B. napus*-like isozyme patterns (5–14 and 5–15) and the hybrid 5–28 did not set selfed seed and have not yet been backcrossed to 'Ariana'. Plant 6–13, which appears to be a *M. arvensis* homofusion, set selfed seed.

Discussion

This paper reports the production of somatic hybrids between *M. arvensis* and *B. napus* and the expression of potentially important agronomic traits in the regenerated plants.

Ultra-violet irradiation has been suggested as an alternative to X- and γ-radiation for the inactivation of protoplasts and for inducing chromosome damage and elimination in order to generate asymmetric somatic hybrids (Atanassov et al. 1991; Hall et al. 1992a, b). A 254-nm UV source has been reported to inhibit protoplast division, but the source used in these experiments ($\lambda = 360$ nm) did not irreversibly damage treated protoplasts, despite extended exposure times that resulted in overall radiation doses equivalent to those reported as effective in other systems (Hall et al. 1992a, b). However, the treatment was retained as radiation damage increases transformation frequencies in protoplasts following DNA uptake (Kohler et al. 1990), and the delay in the onset of cell division in treated protoplasts indicated that there was some damage following UV irradiation. Iodoacetic acid and iodoacetamide have been reported to be an effective means of inhibiting the regeneration of protoplasts (Sidorov et al. 1981; Kemble et al. 1986). The IOA treatments used here were effective in preventing the development of both *B. napus* and *M. arvensis* protoplasts. The dominant role of *B. napus* in protoplast regeneration can be seen in Table 1, where treatment of the *B. napus* partner with IOA dramatically reduces colony formation. The plating efficiency of *M. arvensis* protoplasts, under the culture conditions described above, was so low as to operate as a partial selection, and therefore the most stringent hybrid selection conditions were when the *B. napus* partner was treated with IOA.

Among calli recovered from fusions, the shoot regeneration frequencies (0–14%) were comparable with reports from previous *B. napus* intergeneric somatic hybridisation experiments (Fahleson et al. 1994b; Primard et al. 1988; Fahleson et al. 1988; Klimaszewska and Keller 1988; Fahleson et al. 1994a). The overall efficiency of hybrid production was high as 47% of the regenerated plants which were analysed were nuclear hybrids based on isozyme analysis.

Only 1 plant was recovered with a chromosome number equal to that of either parent (plant 9–30), demonstrating that the majority originate from either

hetero- or homo-fusions. The chromosomes of *M. arvensis* are on average smaller than those of *B. napus* but the similarity, and overlap, in size makes the chromosomes of the two species largely indistinguishable from one another. Thus, it was impossible to estimate the number of chromosomes from each parent contributing to the hybrid genomes. However, use of genomic in-situ hybridisation techniques would enable the number of chromosomes from each parental genome to be established. Techniques are being developed to enable identification of individual Brassiceae chromosomes (Ohmido et al. 1995; Kamisugi et al. 1995), and it will eventually be feasible to fully karyotype these hybrids. The observation that the majority of hybrids were *B. napus*-like in appearance indicates that the Brassica genome is either dominant, or simply predominates, in most hybrids. The ability to recognise the presence/absence of specific chromosomes coincident with the presence/absence of phenotypic characters will be a significant step in the genetic analysis of hybrid characters.

The C_3–C_4 intermediate character results from the combination of a Kranz-like anatomy with the cell-specific differential expression of the nuclear-encoded P-subunit of mitochondrial glycine decarboxylase (Rawsthorne 1992). The intermediate CO_2 compensation points of hybrids 9–12, 9–14 and 9–16 demonstrate that they are expressing the C_3–C_4 intermediate character of *M. arvensis*. Only 9–14 exhibited the significant light-sensitive photosynthetic/photorespiratory response that is also a characteristic of C_3–C_4 intermediate and true C_4 species. Thus, the efficiency with which the character is expressed differs among the hybrids. This may result from a number of factors including incompatability at the level of cellular systems and incomplete or chimaeric expression of the C_3–C_4 character within the leaves of hybrid plants. The observation of a Kranz-like anatomy in the leaves of 9–12, 9–14 and 9–16 confirms that the C_3–C_4 character is being expressed at both the physiological and anatomical levels. Inefficient or incomplete expression of either, or both, of these elements could account for the CO_2 compensation points observed in the hybrids that are intermediate between those of the C_3 and C_3–C_4 parents. The apparent differences between the hybrids provide the opportunity for further dissection of the intermediate character, including the use of gold-labelled antibodies to investigate cell-specific expression of the proteins of the glycine decarboxylase complex in leaf sections (Rawsthorne et al. 1988).

Seed is available from plant 9–12, and this provides the opportunity to extend our analysis to subsequent generations. However, it has not been possible to set seed on hybrids 9–14 and 9–16, either by selfing or backcrossing to *B. napus*, and thus genetic analysis of the character in the progeny of these hybrids is currently confounded. Attempts to produce seed are continuing as the recovery of progeny plants is essential if the hybrids are to be used in genetic analysis, as a starting point for introgression of the C_3–C_4 character into *B. napus* or as bridging species in assisted sexual crosses between *M. arvensis* and *B. napus*.

Several Moricandia × Brassica hybrids have been reported (Apel et al. 1984; Toriyama et al. 1987; Kirti et al. 1992; Takahata et al. 1993; Takahata and Takeda 1990), but there has been only one previous report of expression of the C_3–C_4 character in the hybrids (Apel et al. 1984). This report describes for the first time the expression of potentially useful agronomic characters in somatic hybrids between *B. napus* and *M. arvensis*. The CO_2 compensation point character is particularly significant because of its long-term potential impact on plant performance in the field. While the analysis of primary regenerants as described here is informative, the transmission of characters to subsequent generations is of greater significance and the production of selfed and backcrossed seed from the majority of hybrid plants will enable us to carry out more detailed phenotypic and genetic characterisation (nuclear and organellar) in subsequent generations. At the same time cytological and biochemical analysis of the primary hybrids will continue.

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