Plant Cell Reports (1999) 18: 412–417

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Regeneration of plants from protoplasts of *Capsella bursa-pastoris* and somatic hybridization with rapid cycling *Brassica oleracea*

Received: 13 April 1998 / Revision received: 25 August 1998 / Accepted: 31 August 1998

Abstract Fertile rooted plantlets were recovered from leaf mesophyll protoplasts of *Capsella bursa-pastoris*. Protoplasts cultured over a feeder layer of *Brassica napus* cells produced 221 colonies, 7 of which regenerated multiple plantlets. The nuclear DNA content of most regenerates varied from 0.89 to 1.0 pg/nucleus, close to the value for seed-grown *C. bursa-pastoris* (0.94 ± 0.03 pg/nucleus). Two regenerants had a tetraploid DNA content (1.8–2.0 pg). Plants with a DNA content close to *Capsella* produced seeds, both in vitro and in soil. Intertribal somatic hybrids were obtained by polyethylene glycol-mediated fusion of untreated *C. bursa-pastoris* protoplasts with iodoacetate-treated protoplasts of rapid-cycling *B. oleracea*. Plants were confirmed as somatic hybrids by isozyme and RAPD analysis. The nuclear DNA content of the hybrids ranged from 3.2 to 6.4 pg, higher than the sum of the parental genomes. One of two hybrids tested was resistant to *Alternaria brassicicola*, like the *Capsella* fusion partner. Hybrids rooted easily and produced sterile flowers when transplanted to soil.

Key words *Capsella bursa-pastoris* · Rapid-cycling *Brassica oleracea* · Protoplasts · Intertribal somatic hybrids · *Alternaria brassicicola*

Abbreviation *r.c.* Rapid cycling

Communicated by C. F. Quiros

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Introduction

Somatic hybridization can circumvent barriers of sexual incompatibility to allow utilization of wild species germplasm for crop improvement. In the family Brassicaceae, protoplasts from cultivated *Brassica* partners have been fused with various wild crucifers, including *Arabidopsis thaliana* (Gleba and Hoffmann 1980; Bauer-Weston et al. 1993; Forsberg et al. 1994), *Barbarea vulgaris* (Fahleson et al. 1994a), *Camelina sativa* (Narasimhulu et al. 1994), *Lesquerella fendleri* (Skarzhinskaya et al. 1996), and *Thlaspi perfoliatum* (Fahleson et al. 1994b). *Capsella bursa-pastoris* is another wild species with agronomically important traits potentially useful for introgression into crop Brassicas.

C. bursa-pastoris L. Medic (shepherd's purse, 2n = 4x = 32) is a small plant, widespread as a common weed. It is resistant to flea beetles (*Phyllotreta cruciferae* and *P. striolata*) (Feeny et al. 1970), cold tolerant, and has a short life cycle (Bonfils et al. 1992). In addition, it is highly resistant to *Alternaria brassicae* (Tewari 1991).

C. bursa-pastoris has been a model species for the study of embryogenesis since the work of Hanstein (1870). *Capsella* embryos have also been cultured in vitro (Rijven 1952; Raghavan and Torrey 1963; Monnier and Lagriffol 1986). The species has not been used for genetic manipulations, although Bonfils et al. (1992, 1995) reported plant regeneration from protoplasts of an embryogenic cell suspension of *C. bursa-pastoris*.

C. bursa-pastoris is sexually incompatible with crop crucifers, so somatic hybridization may be a useful approach for transfer of its agriculturally important traits. In this paper we describe the regeneration of plants from leaf protoplasts of *C. bursa-pastoris* (tribe Lepidiae) and production of intertribal somatic hybrids between it and rapid-cycling (r.c.) *Brassica oleracea* (tribe Brassiceae). The rapid-cycling material was selected as a fusion partner because it readily regenerates from leaf protoplasts (Hansen and Earle 1994).

Materials and methods

Plant material

C. bursa-pastoris (wild collection from Geneva, N.Y.) (Fig. 1A) and r.c. *B. oleracea* (Crucifer Genetics Cooperative no. 3-1) (Fig. 1B) were used for protoplast experiments. Seeds were sterilized for 5 min with 70% ethanol, then for 15 min in 10% Clorox and rinsed three times in sterile water before transfer to Magenta boxes. Seeds were germinated on LS medium (Linsmaier and Skoog 1965) containing 1% sucrose and no growth regulators, solidified with 6 g/l Phytagar (Gibco BRL, Gaithersburg, M.D.) (LS-0). The plants were grown on the same medium at 25 °C under cool white fluorescent lights (60 μmol m^{-2} sec^{-1}) with a 16-h photoperiod.

Protoplast isolation, fusion and culture

Leaves of 1-month-old plants were used for protoplast experiments. R.c. *B. oleracea* protoplasts were isolated as previously described (Hansen and Earle 1994). The enzyme mixture used for *Capsella*

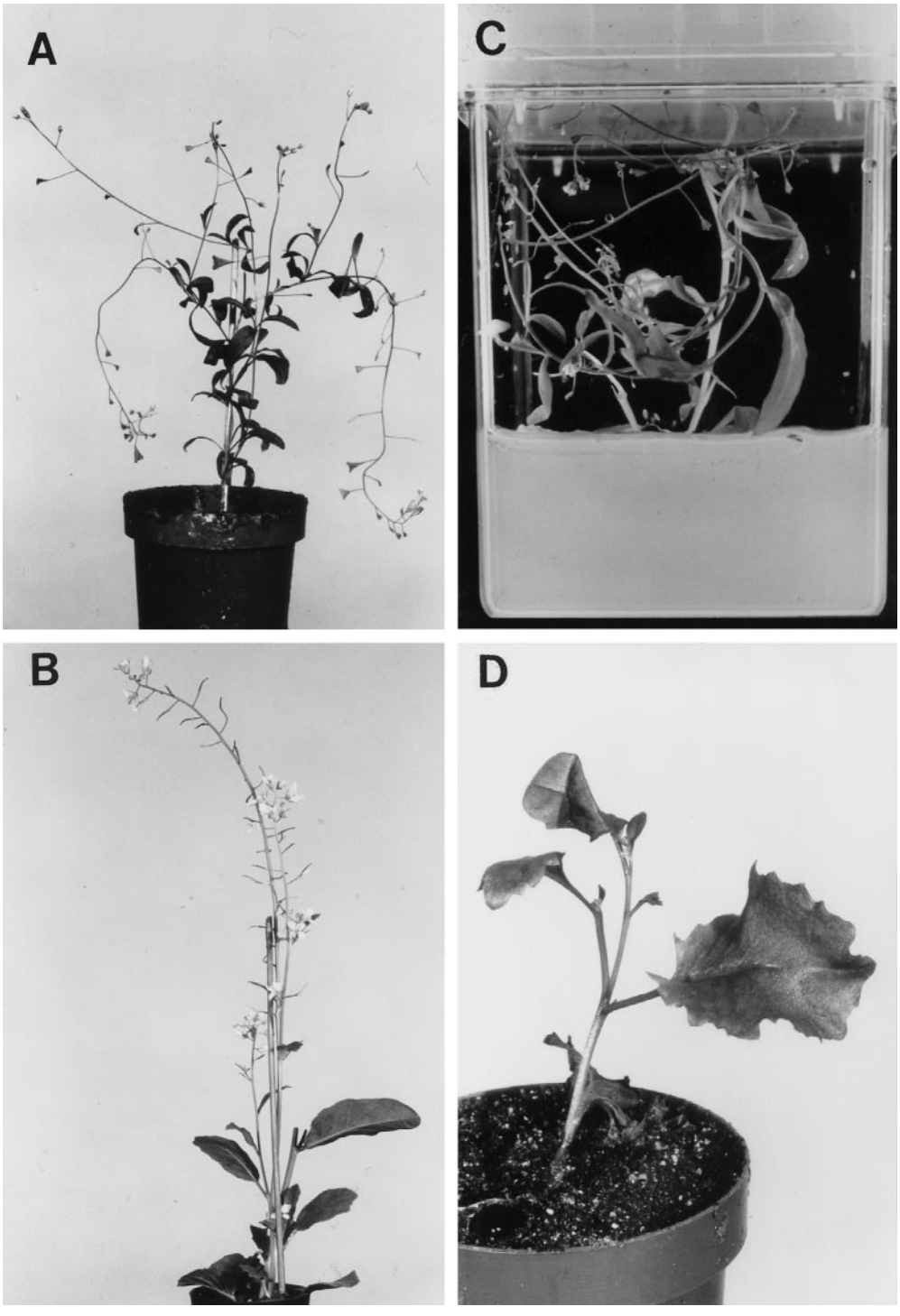

Fig. 1 **A** *Capsella bursa-pastoris* grown from seeds. **B** R.c. *Brassica oleracea*. **C** *C. bursa-pastoris* regenerated from leaf protoplasts. **D** Somatic hybrid

protoplasts contained 0.2% (wt/vol) Cellulase Onozuka RS (Yakult Honsha, Japan), 0.1% (wt/vol) Cellulysin Cellulase (Calbiochem, La Jolla, Calif.), and 0.2% (wt/vol) Macerozyme R-10 (Yakult Honsha, Japan) dissolved in W5 medium (Medgyesy et al. 1980)

Protoplasts of r.c. *B. oleracea* were treated with 3 mM iodoacetate in W5 solution (Medgyesy et al. 1980) for 15 min immediately before purification to prevent division of unfused *B. oleracea* protoplasts. Protoplasts of both partners were mixed in a 1:1 ratio and fused as described in Sigareva and Earle (1997). After fusion, protoplasts were cultured in the dark for 2 days in liquid medium B (Pelletier et al. 1983) and then transferred to Millipore filters over a *Brassica napus* feeder layer as in Walters and Earle (1990). Calli were transferred sequentially from medium B to C to E and to F of Pelletier et al. (1983). Regenerated shoots were excised and placed in Magenta boxes containing LS-0 medium solidified with either 6 g/l Phytagar or 2.2 g/l Gelrite (Sigma, St. Louis, Mo.).

Nuclear DNA content

The nuclear DNA content of parental and regenerated plants was determined by flow cytometry of leaf tissue (Arumuganathan and Earle 1991). Nuclei from rice (cv. Taipei 309, 2C = 0.87 pg) were used as a standard. Samples were analyzed on an EPICS PROFILE cell cytometer (Coulter Electronics, Hialeah, Fla.).

Isozyme analysis

Leaf tissue (50–100 mg) from in vitro-grown plantlets was ground in 200 µl of extraction buffer (0.1 M K_2HPO_4, 10% glycerol, 10% PVP-40, 0.1% β-mercaptoethanol and 0.5% Triton-X 100). Extracts were run and stained in cellulose acetate plates as described by Hebert and Beaton (1989). After electrophoresis (20–30 min, 200 V), plates were stained for the following enzymes: leucine amino peptidase, phosphoglucose isomerase, phosphoglucomutase, alcohol dehydrogenase, α-amylase, aspartate amino transferase, isocitrate dehydrogenase, and malate dehydrogenase (MDH I). The stain solution for MDH I was modified from that recommended by Hebert and Beaton (1989). It contained 14 mM $MgCl_2$, 5.7 mM Tris (pH 8.0), 2.2 mM NADP, 23 mM malic acid, 1.6 mM 3-(4,5-dimethylthiazolyl-z)-2-5 diphenyltetrazolium bromide (Sigma) and 0.4 mM phenazine methosulfate (Sigma). Four milliliters of a 16 mg/ml solution of Seaplaque agarose (FMC BioProducts, Rockland, Me.) were added to 5 ml of the stain solution.

RAPD analysis

Genomic DNA was extracted from young leaves according to Hu and Quiros (1991). Ten-base oligonucleotide primers (Operon Technologies, Alameda, Calif.) were used to test parental and fusion-derived plants. Amplification conditions were those of Hu and Quiros (1991): 2 min 30 s of denaturation at 94 °C followed by 45 cycles of 92 °C/30 s, 35 °C/1 min, and 72 °C/1 min. PCR products were separated on a 2% agarose gel and photographed with Polaroid 667 film.

Analysis of resistance to *Alternaria brassicicola*

A. brassicicola was grown on potato dextrose agar (39 g/l) at 25 °C with a 16-h photoperiod (50–60 µmol m^{-2} sec^{-1}). Two to three weeks after subculturing, conidia were washed off the plates with 10 ml of distilled water. To screen for susceptibility to *A. brassicicola*, detached leaves were placed into 100×15 mm glass Petri dishes containing Phytagar (6.6 g/l, pH 5.8). Plates were sealed with Parafilm to retain moisture and incubated for 4–5 days. Drops (15 µl) of conidial suspensions (25,000 or 37,000 conidia/ml) were placed on the leaves of *C. bursa-pastoris* and *B. oleracea*. Leaves of two somatic hybrids were inoculated with 37,000 conidia/ml. Three leaves were inoculated for each combination. Drops of distilled water were placed on leaves as controls.

Disease severity was evaluated 5 days after inoculation using a visual rating scale (King 1994). Disease severity ranged from 1 to 10, where 1 = no disease, 2 = a few small flecks, 3 = small flecks but no large lesions, 4 = small flecks and a few lesions, 5–9 = increasing number and size of lesions, and 10 = dead. Plants with ratings of 3 or less were classified as resistant, a rating of 4 indicated slight or borderline resistance, and ratings of 5 and above indicated varying degrees of susceptibility.

Results and discussion

Tests of resistance to *A. brassicicola*

Tewari et al. (1991) reported that *C. bursa-pastoris* was virtually immune to *A. brassicae*. Studies by King (1994) indicated that it is also resistant to *A. brassicicola*. In our tests, detached leaves of plants from the wild collection showed no signs of disease when inoculated with 25,000 or 37,000 conidia/ml of *A. brassicicola* while *B. oleracea* leaves developed disease. Disease severity was rated 1 for *C. bursa-pastoris* and 7 for *B. oleracea*. This accession of *C. bursa-pastoris* was used for further protoplast regeneration and fusion experiments.

Regeneration of plants from protoplasts of *C. bursa-pastoris*

Control *C. bursa-pastoris* protoplasts in the fusion experiment produced 221 colonies from 5×10^4 plated protoplasts. Seven colonies (3.1%) developed multiple shoots from which plants were regenerated (Fig. 1C). The plants rooted normally and flowered in Magenta boxes. One plant was transferred to soil, where it flowered and produced seeds.

C. bursa-pastoris plants grown from seeds had a nuclear DNA content of 0.94±0.03 pg/nucleus. The nuclear DNA content of five plants regenerated from protoplasts varied from 0.89 to 1.0 pg. Two other regenerated plants showed a tetraploid DNA content (1.8 or 2.0 pg/nucleus).

All protoplast-derived plants looked normal. Regeneration of plants from leaf mesophyll protoplasts has several advantages over the procedure described by Bonfils et al. (1992, 1995), in which an embryogenic cell suspension was the source of protoplasts. It is difficult to establish suspensions suitable for protoplast isolation. Moreover, many of the plants recovered by Bonfils et al. showed morphological abnormalities; all were male sterile and did not produce seeds. Our experiments used more easily obtained plant material: 1-month-old seedlings germinated in vitro. Except for modification of the enzyme solution, the protoplast procedures were those previously used for *Brassica* species in our laboratory. Mature plants were recovered within 5 months after protoplast isolation. All plants with DNA content close to diploid *Capsella* produced fertile flowers and seeds either in vitro (Fig. 1C) or in soil.

Recovery of fusion products

After fusion of protoplasts from *B. oleracea* and *C. bursa-pastoris*, 1,360 individual colonies were transferred from the filters to medium E and then to medium F. Twenty-five colonies (1.8%) developed shoots. These shoots, together with the callus at their base, were transferred to fresh medium F. Thirteen colonies regenerated shoots large enough for transfer to Magenta boxes containing LS-0 medium (Table 1).

Shoots from seven colonies rooted easily. Their morphology resembled *Capsella* plants grown from seeds in vitro. The DNA content of four of these plants was 0.9–1.0 pg/nucleus; for the others, it was 1.8–2.0 pg/nucleus. Since no inactivation procedure was used to prevent division of unfused *Capsella* protoplasts, these seven plants were probably regenerated diploid or tetraploid *Capsella* escapes. The diploid Capsella escapes all produced flowers, and some formed seeds.

The DNA content of five other plants recovered from the fusion experiment ranged from 3.2 to 6.4 pg/nucleus, more than the sum of the parental lines (1.3 pg for *B. oleracea*, 0.9–1.0 pg for *Capsella*). This suggests that fusions of more than two protoplasts were involved.

Isozyme analysis confirmed that these five plants were somatic hybrids. Of the eight isozymes tested, only MDH showed a differential banding pattern for the parental lines (Fig. 2). (Resolution of bands is less clear in the cellulose acetate system than on starch or acrylamide gels, but is still useful in combination with other types of analyses.) Plants regenerated from the fusion experiment were tested for the presence of the MDH bands. Plants with a DNA content of 3.2–6.4 pg and an additional line not tested for DNA content had the MDH banding pattern of *B. oleracea* plus additional bands not found in either parent. Regenerants with a DNA content close to (or twice) that of seed-grown

Table 1 Analysis of plants recovered after fusion of *Capsella bursa-pastoris* and r.c. *Brassica oleracea* protoplasts (*B Brassica* bands, *C Capsella* bands, *H* hybrid bands; *nt* not tested)

Plant	DNA content (pg/nucleus)	Malate dehydrogenase isozyme analysis	RAPD analysis	Interpretation
B. oleracea	1.3	B	B	Fusion partner
C. bursa-pastoris	0.9	C	C	Fusion partner
H1	3.6	B + H	B + C	Somatic hybrid
H2	0.9	C	C	*Capsella* escape
H3	4.2	B + H	B + C	Somatic hybrid
H4	1.8	C	C	*Capsella* escape
H5	nt	B + H	nt	Somatic hybrid
H7	3.2	B + H	nt	Somatic hybrid
H8	6.4	B + H	B + C	Somatic hybrid
H9	1.0	C	nt	*Capsella* escape
H10	2.0	C	nt	*Capsella* escape
H11	1.8	C	nt	*Capsella* escape
H12	0.9	C	nt	*Capsella* escape
H13	1.0	C	C	*Capsella* escape
H25	3.6	B + H	B + C	Somatic hybrid

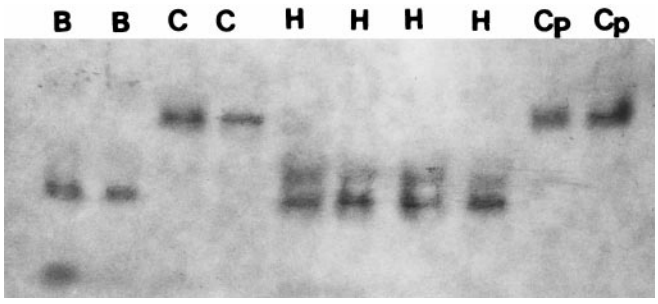

Fig. 2 Malate dehydrogenase banding profiles of leaves of parental lines and somatic hybrids (*B B. oleracea*, *C C. bursa-pastoris* grown from seeds, *H* somatic hybrids, *Cp C. bursa-pastoris* regenerated from protoplasts)

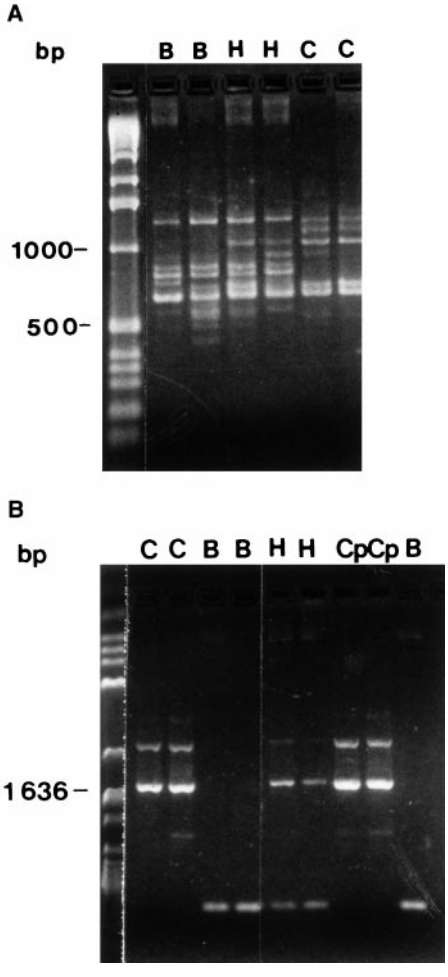

Fig. 3A,B RAPD profiles of parental lines and somatic hybrids generated using primer OPA-18 (**A**) and primer OPA-19 (**B**) (*B B. oleracea*, *H* somatic hybrids, *C C. bursa-pastoris* grown from seeds, *Cp C. bursa-pastoris* regenerated from protoplasts)

Capsella always had the MDH banding profile of *Capsella* alone (Fig. 2, Cp lanes).

Hybridity of the regenerated plants was confirmed by RAPD analysis. Primers OPA-18 and OPA-19 (Fig. 3A and 3B, respectively) showed consistent polymorphism

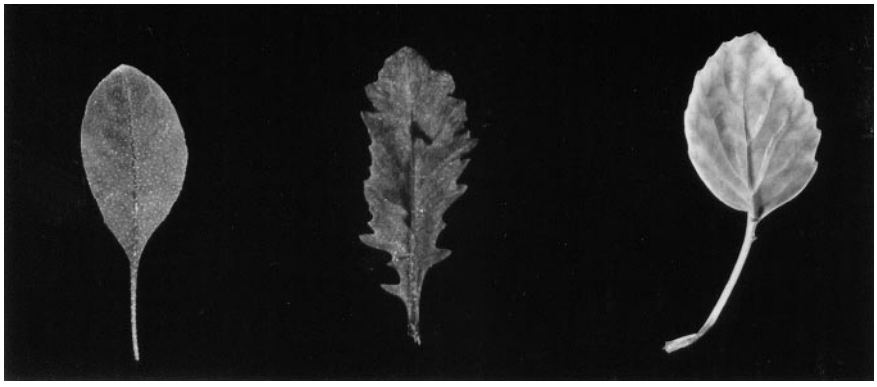

Fig. 4 Leaves of young *C. bursa-pastoris* (*left*), somatic hybrid (*center*), and r.c. *B. oleracea* (*right*)

between the fusion partners. Primer OPA-18 generated multiple banding profiles in both *B. oleracea* and *C. bursa-pastoris*. Some bands (approximately 600 bp and 1,500 bp) were present in both parents, but two (about 800 bp and 820 bp) were present only in *B. oleracea* and two (about 1,000 bp and 1,200 bp) were present only in *C. bursa-pastoris*. Somatic hybrids showed both *B. oleracea*-specific bands plus the 1,000-bp band specific to *C. bursa-pastoris*. Although all hybrids contained bands specific to each parent, their banding profiles were not identical. One hybrid (H3, on the right) has a distinct band of approximately 900 bp which was less pronounced in both parents and was not present in another hybrid (H1, on the left). H1 and H3 originated from different fusion-derived calli, so their different RAPD banding patterns may result from different combinations of parental genomes.

Primer OPA-19 produced one band of 100 bp specific to *B. oleracea* and two bands of 1,636 bp and 2,036 bp specific to *C. bursa-pastoris* (Fig. 3B). Hybrids combined the banding profiles of both parents. Protoplast-derived plants of *C. bursa-pastoris* always showed a banding pattern similar to seed-grown *Capsella* plants, when tested with primer OPA-19. It was therefore easy to distinguish plants regenerated from *Capsella* protoplasts from the somatic hybrids. No *B. oleracea* escapes were seen, indicating that the pretreatment with iodoacetate was effective.

Three somatic hybrid lines produced rooted plants which were transferred to soil. The hybrid plants grown in soil (Fig. 1D) combined morphological characteristics of both parents (Fig. 1A, B), but their height was approximately 10 cm compared to 30–40 cm for r.c. *B. oleracea* and 25–30 cm for *C. bursa-pastoris*. Leaves of the hybrids were lanceolate with serrated margins and trichomes (Fig. 4). *Capsella* leaves are lanceolate with trichomes, and *B. oleracea* leaves are waxy and oval with serrated margins. Plants of two lines produced flowers both in vitro and in soil. These flowers had intermediate morphology and were male sterile. When pollinated with pollen of r.c. *B. oleracea*, they failed to produce seeds. Poor fertility of the hybrids may be due to the phylogenetic distance between the parental species and/or to the fact that the hybrid nuclei combined more than two parental genomes.

Although some of the plants transferred to soil reached the flowering stage, it was impossible to maintain them in the greenhouse. Despite much effort testing different soil mixtures and humidity conditions, the plants failed to survive more than 5–6 weeks. In contrast, the somatic hybrids grew vigorously in vitro and were easily subcultured for more than a year. The plants also grew well in pots of vermiculite supplemented with liquid MS salts.

Intertribal somatic hybrids are known to be difficult to culture in soil, although success has been achieved in some intertribal crucifer somatic hybrids [e.g., *A. thaliana* + *B. napus* (Forsberg et al. 1994)]. Skarzhinskaya et al. (1996) reported that asymmetric hybrids obtained after fusion of irradiated *L. fendleri* protoplasts with *B. napus* grew better in soil and also had better fertility than symmetric hybrids.

Two *C. bursa-pastoris* + *B. oleracea* somatic hybrids (H1 and H3) were tested for resistance to *A. brassicicola*. H1 was as susceptible as the *B. oleracea* parent (rating of 7). H3 was considered resistant (rating of 3). Thus, resistance to *Alternaria* can be expressed in the somatic hybrids.

In conclusion, normal fertile plants were easily regenerated from leaf mesophyll protoplasts of *C. bursa-pastoris* with the feeder layer technique. Such plants were recovered both from control *Capsella* protoplasts and from the untreated *Capsella* protoplasts that were part of the fusion mixture. Intertribal somatic hybrid plants between *C. bursa-pastoris* and r.c. *B. oleracea* were obtained and grown to flowering. These plants failed to survive long term in the greenhouse and had poor fertility, which hampers their use in further breeding for disease resistance. Somatic hybrids whose DNA content was the sum of the parental genomes may be more useful. Production of asymmetric hybrids may also improve both the survival of hybrid plants in soil and their fertility.

Acknowledgements This work was supported by the Vegetable and Flower Seed Permanent Research Fund of the American Seed Trade Association and by the New York State Integrated Pest Management Program.

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