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Somatic hybridization between the zinc accumulator *Thlaspi caerulescens* and *Brassica napus*

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Abstract Somatic hybrids between the zinc hyperaccumulator *Thlaspi caerulescens* and *Brassica napus* were produced by electrofusion of protoplasts isolated from each species. Optimization of electrofusion parameters yielded interspecies heteroplasmic fusion rates of up to 13%. Hybrids were selected by screening the growing calli for Zn tolerance. In addition, a second novel selection technique was developed based on the observation that a high proportion of hybrid microcalli grown in liquid media did not adhere to the wall of the culture vessel, while microcalli derived from parental cells did. Seventeen from a total of 64 regenerated plants were conclusively verified as hybrids by AFLP DNA analysis. The hybrid plants were grown in soil for up to 4 months, and at least five flowered. Several of these hybrids survived when grown on high-zinc media. These hybrids accumulated levels of zinc and cadmium that would have been toxic for *B. napus*. The data indicate that transfer of the trait for metal hyperaccumulation in plants is possible through somatic hybridization.

Key words Electrofusion · AFLP DNA analysis · Somatic fusion · Phytoremediation · Metal toxicity

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Introduction

Industrial practices such as mining, smelting, and the disposal of manufacturing wastes have increased the concentration of toxic metals in the environment (Nriagu 1979; Kabata-Pendias and Pendias 1989). At many zinc-mining and -smelting sites, levels of zinc and cadmium in soil have become so high that few plants survive, resulting in severe disruption of local ecosystems (Beyer 1988). A small number of plant species have the ability to grow in metal-contaminated soil and to actively accumulate heavy metals in their tissues. These plants may be used to extract toxic metals from soil, a process known as phytoextraction or phytoremediation (Chaney 1983; U.S. Department of Energy 1994, Chaney et al. 1997). *Thlaspi caerulescens* (alpine pennycress), a member of the Brassicaceae, is a Zn- and Cd-tolerant plant that can accumulate exceptionally high levels of both metals in its shoot tissue (Rascio 1977; Brown et al. 1995). The usefulness of *T. caerulescens* for soil remediation is limited, however, by its small size (approximately 15 cm high), slow growth rate, and rosette growth habit, which make mechanical harvesting difficult. The dry weight yield of *T. caerulescens* growing over a 6-month period produces approximately 3–4 t/ha (Baker et al. 1994). In their study these authors estimated that it would take 13 years to lower the Zn content of the soil in their study from 444 ppm to 300 ppm, which is the limit for Zn in agricultural land set by the Commission of the European Communities (CEC 1986). Most temperate-zone hyperaccumulators that have been identified are also small plants. Consequently, it would be desirable to have a larger and more vigorous hyperaccumulator plant that could remove potentially toxic metals from soil more efficiently and allow mechanical harvesting of the plants in order to improve the economics of phytoextraction.

The possibility of using molecular techniques to engineer a larger plant for hyperaccumulation is attractive; unfortunately, the number or types of genes responsible for metal tolerance and hyperaccumulation have not

been elucidated. Ingrouille and Smirnoff (1986) observed that tolerance and hyperaccumulation were genetically independent in *T. caerulescens*, and most genetic studies of metal tolerance in other species have concluded that tolerance is a polygenic trait (Antonovics et al. 1971). As an alternative to the isolation and characterization of all genes involved in metal tolerance, we attempted to hybridize *T. caerulescens* with a related species of higher biomass.

There have been numerous successful attempts at interspecific and intergeneric hybridization, both sexual and somatic, within the Brassicaceae. Some of these studies involve one of the high-biomass crop species in the genus *Brassica*, such as *Brassica napus* (canola, rapeseed), *Brassica oleracea* (cabbage, broccoli, etc.), or *Brassica juncea* (Indian mustard) (Prakash and Hinata 1980; Glimelius et al. 1991). Interspecific sexual hybridization may require the use of techniques to overcome initial sexual incompatibility, such as in vitro fertilization or embryo rescue. Somatic hybridization, which is accomplished by hybrid regeneration from tissue culture after protoplast fusion, encompasses species with complete sexual incompatibilities. Somatic hybrids from parents of a widely divergent genetic background may have low fertility and viability. Repeated backcrossing to one parent, while screening for a desired trait, may provide an opportunity to transfer genes from one genome to another via non-homologous recombination (Chevre et al. 1994). Alternatively, restoration of the fertility of asymmetric hybrids via spontaneous loss of some chromosomes of one parent has been reported (Gleba et al. 1988).

Most species in the Brassicaceae are amenable to protoplast-culture and plant-regeneration techniques. *B. napus* grows well in tissue culture, and has been fused with plants in many other genera, including *Diplotaxis* (Klimaszewska and Keller 1988), *Eruca* (Fahleson et al. 1988), *Arabidopsis* (Bauer-Weston et al. 1993), *Raphanus* (Sundberg and Glimelius 1991), *Sinapis* (Primard et al. 1988), *Lesquerella* (Skarzhinskaya et al. 1996), and *Moricandia* (O'Neil et al. 1996). Several of these somatic hybrids have proven fertile. *Thlaspi caerulescens* belongs to the Lepidieae tribe of the Brassicaceae, while crop Brassicas are in the Brassiceae tribe. Intertribal sexual hybridization in the Brassicaceae has not been reported, but intertribal somatic hybridization has been accomplished. Fahleson et al. (1994) produced somatic hybrids between *B. napus* and *Thlaspi perfoliatum*, a species that does not hyperaccumulate metals. Some of these *Brassica+Thlaspi* somatic hybrids were fertile when backcrossed to *B. napus*.

Electrofusion of plant protoplasts is an effective procedure for somatic hybridization (Bates 1985; Fish et al. 1988; Saunders et al. 1989a,b). In the present paper, we describe the use of protoplast electrofusion to produce somatic hybrids between *T. caerulescens* and *B. napus*. Such hybrids may serve as genetic "bridges" (Glimelius et al. 1991) for transferring *Thlaspi*'s metal tolerance and hyperaccumulation traits to a larger plant. We also report the first use of AFLP DNA analysis for somatic hybrid confirmation, and a novel method of hybrid selection.

Materials and methods

Plant material and culture

Seed of *T. caerulescens* J.&C. Presl, derived from seed collected from a Zn/Cd smelter site in Prayon, Belgium, was kindly supplied by Y. Li (USDA-ARS, Beltsville, Md). Seed of *B. napus* cv Westar was obtained from G. Bañuelos (USDA-ARS, Fresno, Calif.). Seeds of both species were surface-sterilized in 10% (v/v) commercial bleach (0.525% w/v sodium hypochlorite), containing 3 drops of Tween 20 (Sigma, St. Louis, Mo.) per 100 ml, for 15 min and rinsed in sterile water. Seeds were germinated on half-strength MS medium (Murashige and Skoog 1962) containing 0.7% (w/v) agar. *Thlaspi* plants were grown on MS medium in a growth chamber at 25°C with continuous fluorescent light at an intensity of 100 µE m^{-2} s^{-1}. *Brassica* seedlings were grown in the dark for 5 days immediately prior to protoplast isolation.

Protoplast isolation

Mesophyll protoplasts from *T. caerulescens* or *B. napus* were isolated from in vitro-grown plants. *Thlaspi* leaves were torn while peeling off the lower epidermis, and *Brassica* hypocotyls were cut into 1–2-mm segments. Approximately 1 g of plant tissue was plasmolysed for 1 h in 0.3 M sorbitol and 0.05 M $CaCl_2 \cdot 2H_2O$, then incubated in 12–15 ml of filter-sterilized digestion solution. The digestion solution contained 1% (w/v) Cellulysin (Calbiochem, San Diego, Calif.), 0.1% (w/v) Macerase (Calbiochem, San Diego, Calif.), and 5 mM of MES (2-N-morpholino-ethanesulfonic acid)-NaOH, pH 5.6, in Kao's medium #3 (Kao et al. 1975) as modified by Nagy and Maliga (1976). Digestion proceeded for 16–18 h in the dark at 25°C and 40 rpm. Released protoplasts were filtered through a 200-mesh stainless-steel screen with 74-µm pores. The digestion plate was rinsed with sufficient volume of W5 salt solution (Menczel et al. 1981) to *q.s.* the suspension to 50 ml. The protoplasts were pelleted by centrifugation at 100×*g* for 7 min, then resuspended in a salt solution containing 16% (w/v) sucrose (Banks and Evans 1976) and transferred to a 15-ml centrifuge tube. One milliliter of W5 solution was layered on top, and the protoplasts were centrifuged at 60×*g* for 13 min. The protoplast layer at the interface was collected and rinsed in 12 ml of W5 solution, pelleted at 50×*g* for 5 min, then resuspended in 0.5 M mannitol to cell densities of 1.5×10^5 *Brassica* protoplasts per ml and 3.0×10^5 *Thlaspi* protoplasts per ml. Equal volumes of the two suspensions were mixed in preparation for electrofusion.

Protoplast Electrofusion

Protoplasts were fused using one of two square-wave pulse generators from BTX Inc., (Model 200 or Model 2001, BTX, Inc., San Diego, Calif.). A 0.4-ml aliquot of protoplast suspension was placed in a 2-mm-gap electrofusion cuvette (Model 620, BTX, Inc., San Diego, Calif.), and the protoplasts were aligned using AC current with a frequency of 1 MHz and a field strength of 0.125 kV/cm for 25 s. These cells were fused by one or more 100-µs DC pulses. Heterokaryon fusion rates, the post-fusion number of intact cells, and protoplast viability were measured at various ranges of DC pulse-field strengths between 0 and 1.75 kV/cm with three replicates at each setting. Cell density was determined on a Bright Line hemacytometer (American Optical, Buffalo, N.Y.) by counting nine replicate areas for each sample. Hybrid protoplasts were visible as cells containing green chloroplasts from *Thlaspi* mesophyll cells and colorless granular cytoplasmic strands characteristic of dark-grown *Brassica* hypocotyl

cells (see Fig. 3A). Protoplast viability was determined 1–2 h after fusion by staining with fluoroscein diacetate (FDA), as previously described (Saunders et al. 1995). After electrofusion, the protoplasts remained undisturbed for 30 min and were then transferred to a microfuge tube and pelleted at 50×*g* for 5 min. The pellet was resuspended in 1.3 ml of filter-sterilized culture medium A (Glimelius et al. 1986), a modified 8p medium (Kao and Michayluk 1975) containing 4.5 µM of 2,4-dichlorophenoxy-acetic acid (2,4-D), 0.5 µM of 1-naphthaleneacetic acid (NAA), and 2.2 µM of 6-benzylamino-purine (BAP).

Protoplast culture and plant regeneration

Cell lines developed from protoplast-fusion experiments were cultured either without selection, with selection for Zn tolerance, or with selection on the basis of differential adhesion of microcalli to the culture vessel wall when grown in a liquid medium. Without selection, protoplasts were grown in liquid media for 19 days, at which time microcalli were plated on a semi-solid medium. After electrofusion, protoplasts suspended in medium A were transferred in 0.3-ml aliquots to wells of a 24-well flat-bottomed tissue-culture plate (Corning Glass Works, Corning, N.Y.) and grown in the dark at 25°C. On day 5, after cell division had begun, the medium was diluted with 0.9 ml of medium C, which was identical to medium A except that it did not contain 2,4-D, NAA, or BAP. On day 10, 1.0 ml of medium D (identical to medium A, but with one-fourth the concentrations of 2,4-D, NAA, and BAP) were added to each well, and two 0.7-ml aliquots were taken from each well and transferred to new wells. On day 19, 1.0 ml of medium D was added to each well, and microcalli were collected in a wide-bore pipette and plated on medium F, consisting of K3 medium (Nagy and Maliga 1976) with 0.1 M sucrose, 0.13% (w/v) agarose and the same hormone concentrations as medium D. The plated microcalli were cultured at 25°C in cool white fluorescent light with an intensity of 30 µE m^{-2} s^{-1} and a daylength of 16 h. Two-weeks later, 1–2 mm calli were transferred to fresh medium F and the light intensity was increased to 100 µE m^{-2} s^{-1}. On day 47, calli were transferred to medium G, consisting of K3 medium with 0.03 M sucrose, 0.7% (w/v) purified agar (Sigma Chemical Co., St. Louis, Mo.), 0.6 µM of indole-3-acetic acid (IAA), 2.2 µM BAP and 4.6 µM of zeatin, for shoot induction. Calli were transferred to fresh medium G at 2-week intervals. One regenerated shoot from each callus was transferred to hormone-free MS medium. If roots did not develop, shoots were transferred to MS medium containing 0.5 µM of NAA. Regenerated plants were propagated by rooting nodal cuttings in the same MS+0.5 µM NAA medium. Rooted plants were transplanted to soil and grown in a growth chamber at 25°C under cool white fluorescent light supplemented with 52 W tungsten lamps for a combined light intensity of 200 µE m^{-2} s^{-1} over a 16-h daylength.

Putative hybrid selection

To select for Zn-tolerance, the following changes were made in the cell-culture protocol. After electrofusion, the media used for protoplast culture contained basal nutrient Zn supplied as 7 µM of zinc sulfate. Appropriate Zn levels for screenings were identified in preliminary experiments. The Zn concentration in the liquid media was 275 µM. On day 24, microcalli were plated on a modified medium F containing 3160 µM of total Zn. On day 42, surviving green calli were transferred to standard medium F. Two-weeks later they were transferred to medium G, following the protocol for the unselected calli.

A second hybrid selection technique was developed based on calli that did not adhere to the wall of the culture vessel when grown in a liquid medium. After electro-fusion and resuspension in medium A, the protoplasts from three fusions were combined in a 60-mm Petri dish, with a total suspension volume of approximately 3.5 ml. On day 5, 3 vol of medium C were added. On day 10, half of the suspension was transferred to a new Petri dish, and a 0.8 vol of medium D was added to each dish. On day 19, the suspensions were transferred in 3-ml aliquots to 100-mm Petri dishes containing 15 ml of medium D, and were cultured at 25°C under cool white fluorescent light with an intensity of 30 µE m^{-2} s^{-1} and a daylength of 16 h. On day 38, floating calli 1–2 mm in diameter were plated on medium F. Two-weeks later, they were transferred to medium G, after which the protocol was the same as for the unselected calli.

DNA isolation

DNA was isolated with modifications from Doyle and Doyle (1987). Young leaf tissue was collected, frozen in liquid nitrogen, and ground to a powder. One-gram samples were combined with 3 ml of CTAB buffer [100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) hexadecyltrimethylammonium bromide (CTAB), and freshly prepared 1% (v/v) 2-mercaptoethanol]. The mixture was incubated at 60°C for 1 h, then phased extracted with an equal vol of phenol:chloroform:isoamyl alcohol (25:24:1). The emulsion was centrifuged at 10,000×*g* for 20 min at 4°C, and the upper phase collected. DNA was precipitated with 0.1 vol of 3 M sodium acetate-NaOH, pH 5.2, and 0.75 vol of cold isopropanol at –20°C for 1 h. The DNA pellet was collected by centrifugation at 8000×*g* for 15 min at 4°C, washed twice in 70% (v/v) ethanol, air-dried for 20 min, and resuspended in 300 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA). Insoluble debris was removed by further centrifugation at 14,000 *g* for 10 min at 4°C, and the supernatant was treated with 18 µg of RNase (Calbiochem, San Diego, Calif.) at 37°C for 1 h. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the emulsion was centrifuged at 14,000 *g* for 3 min at 4°C. The upper phase was transferred to a new tube, and 0.1 vol of 3 M sodium acetate-NaOH, pH 5.2, and 2.5 vol of cold 95% (v/v) ethanol were added. After a 10-min incubation on ice, the tube was centrifuged at 11,000 *g* for 5 min. The DNA was washed twice in 70% (v/v) ethanol, air-dried for 30 min, and resuspended in 50–300 µl of TE.

Hybrid confirmation

AFLP DNA analysis was done on four individuals of each parent and on 26 regenerated plants. This procedure generates bands by PCR-amplification of selected genomic restriction fragments that are separated by electrophoresis on a denaturing sequencing acrylamide gel. AFLP DNA Analysis System-I kits (Life Technologies, Inc., Gaithersburg, Md.) were used according to the manufacturer's recommendations and as described by Lin et al. (1996). Unless indicated, all reagents for restriction-enzyme digestion, adapter ligation, and polymerase chain reaction (PCR) were supplied with the kit. Approximately 500 ng of genomic DNA was digested with 2 µl of *Eco*RI/*Mse*I restriction-enzyme stock solution [1.25 U/µl of both *Eco*RI and *Mse*I, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 50%(v/v) glycerol, 0.1% Triton X-100] and 5 µl of 50 mM Tris-HCl pH 7.5 containing 50 mM of Mg-acetate and 250 mM of K-acetate, *q.s.* to 25 µl with H_2O. The digestion proceeded for 2 h at 37°C, and the reaction was stopped at 70°C for 15 min. PCR adapters were ligated to the digested DNA using 24 µl of *Eco*RI and *Mse*I adapter/ligation solution containing 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate and 1 µl of T4 DNA ligase solution (1 U/µl in 10 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM KCl, and 50% glycerol). The reaction mixture was incubated at 20°C for 2 h, then a 10-µl aliquot was diluted 10-fold with TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). A selective PCR-amplification was performed on this sample with a Gene Amp 2400 thermal cycler (Perkin Elmer Corp., Norwalk, Conn.) with a temperature profile of 20 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. The reaction mixture contained 5 µl of the diluted sample, 5 µl of PCR buffer (100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, and 500 mM KCl), 1 unit of *Taq* DNA polymerase (Life Technologies, Inc., Gaithers-

burg, Md.), and 40 µl of a mixture containing *Eco*RI+A and *Mse*I+C primers and dNTPs. A 3-µl aliquot of the reaction mixture was diluted 50-fold in TE buffer, and the diluted sample was used as the template in a second selective PCR reaction. To prepare sufficient labeled primer for the amplification of 25 samples, 4.5 µl of *Eco*RI+ACC primer solution (27.8 ng/µl) was mixed with 2.5 µl of kinase buffer (350 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 500 mM KCl, and 5 mM 2-mercaptoethanol), 5 µl of [γ-^{32}P]ATP solution (3,000 Ci/mmol, Amersham, Inc., Arlington Heights, Ill.) containing 69 µCi, and 0.5 µL of T4 polynucleotide kinase (10 U/µl in 50 mM Tris-HCl pH 7.6, 25 mM KCl, 1 mM 2-mercaptoethanol, 0.1 µM ATP, 50% glycerol). The reaction proceeded at 37°C for 1 h and was stopped by enzyme inactivation at 70°C for 10 min. Two reagent mixtures, Mix 1 and Mix 2, were used in the PCR reaction. To make sufficient Mix 1 for 12 reactions, 6 µl of the ^{32}P-labeled EcoRI-ACC primer was mixed with 54 µl of unlabeled *Mse*I+3 primer (6.7 ng/µl) and dNTPs. Sufficient Mix 2 for 12 reactions contained 6 units *Taq* polymerase, 24 µl PCR buffer, *q.s.* to 120 µl. Each PCR reaction contained 5 µl of diluted preamplified sample, 5 µl of Mix 1, and 10 µl of Mix 2. The PCR profile was 1 cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, followed by 12 cycles with the same extension and denaturation steps but with the second temperature (annealing) lowered by 0.7°C in each cycle, and finally 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. After PCR-amplification, an equal volume of formamide dye [98% (w/v) formamide, 10 mM Na_2 EDTA, bromophenol blue, xylene cyanol] was added, and the mixture was heated at 90°C for 3 min and stored at 4°C. A 5% (w/v) polyacrylamide gel containing 19:1 acrylamide: bisacrylamide, 7.5 M urea, and 1×TBE buffer [0.1 M Tris(hydroxymethyl) aminomethane, 0.09 M boric acid, 0.001 M EDTA] was poured with 0.4-mm spacers and a sharks-tooth comb, and the gel was pre-electrophoresed at 50 W for 20 min. A 2-µl aliquot of the sample was loaded on the gel, and electrophoresis was performed at 50 W until the slower dye (xylene cyanol) was two-thirds down the length of the gel. The gel was dried and exposed to X-ray film, without an intensifying screen, overnight at –70°C.

Results

Protoplast Electrofusion

The pulse field-strength of a 100-µs DC square-wave electrofusion pulse was optimized for electrofusion of *B. napus* and *T. caerulescens* protoplasts by varying a single pulse from 0 to 1.75 kV/cm. The rate of *Brassica+Thlaspi* fusion (heterofusions) and the number of surviving intact protoplasts following the electrofusion pulse were determined by counting fused heterospecies pairs using bright-field microscopy 1–2 h after fusion. Fusion of protoplast membranes with complete circularization of the cell membrane was usually complete within 10–15 min, but hybrid protoplasts could be identified as long as 2 h after fusion because of the distinctive cytoplasmic traits of each parent. *Thlaspi* mesophyll protoplasts had clear cytoplasm containing green chloroplasts, while protoplasts obtained from dark-grown *B. napus* hypocotyls were colorless and contained distinctly granular cytoplasmic strands. Intact protoplasts were defined as those that appeared to have a complete, circular outer membrane.

As shown previously, increasing the field strength of the pulse resulted in a decrease in the number of intact protoplasts that survived the pulse (Saunders et al. 1989a; Lin et al. 1997). The heterofusion rate increased with the pulse field-strength from 0 to 1.25 kV/cm (Fig. 1). The viability of intact protoplasts 1–2 h post-fusion, as measured by fluorescein diacetate (FDA) staining, was high (80–90%) in those cells that remained intact throughout the range of field strengths used (data not shown). However, the total viability of the protoplast population in the suspension decreased significantly with increasing pulse field-strength compared to untreated controls, as has been reported previously (Saunders et al. 1995). From these results, it was determined that the optimal pulse field-strength for heterofusion was 1.25 kV/cm, which yielded a predictable fusion rate in the range of 10%.

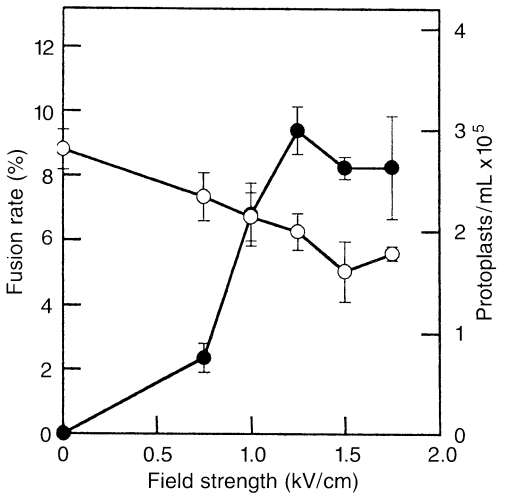

Fig. 1 Effect of pulse field-strength on protoplast electrofusion. *B. napus+T. caerulescens* protoplast fusion rate (●) and the post-fusion number of intact protoplasts (○) measured after the application of one electrofusion pulse at a range of field strengths. Mean of three replications with standard error as bars

Table 1 Frequency of *T. caerulescens+B. napus* somatic hybrid plants produced via electrofusion of protoplasts using a range of fusion pulse-field strengths

Field strength (kV/cm)	Number of		Hybrid frequency (%)
	Plants	Hybrids	
0	5	0	0
0.75	4	0	0
1.00	8	1	13
1.25	7	3	43
1.50	6	2	33
1.75	6	2	33

Table 1 shows the number of hybrid plants regenerated from cultured protoplasts subjected to the range of electrofusion pulse field-strengths used in this study. The highest number of hybrids grew from protoplasts that were electrofused at 1.25 kV/cm. Hybrid confirmation was accomplished by AFLP DNA analysis.

To determine the effect of multiple electrofusion pulses on the rate of somatic hybridization, the number of electrofusion pulses was varied from 0 to 5, and fusion rate and the number of surviving intact protoplasts were

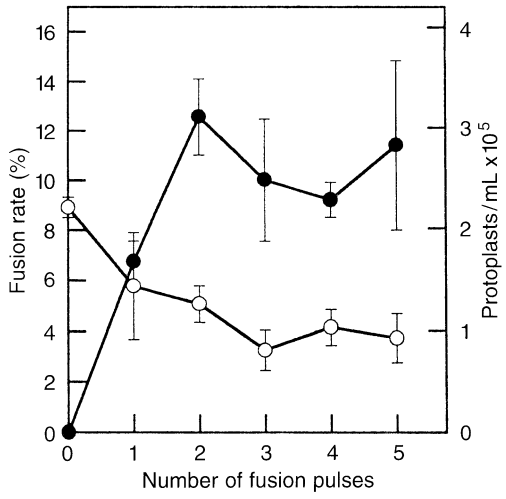

Fig. 2 Effect of pulse number on protoplast electrofusion. *B. napus*+*T. caerulescens* protoplast fusion rate (●) and post-fusion number of intact protoplasts (○) measured after the application of from 0 to 5 electrofusion pulses with a field strength of 1.25 kV/cm. Mean of three replications with standard error as *bars*

measured. All pulses were 100-µs DC square-wave pulses with a field strength of 1.25 kV/cm, and with an interval of 1 s between consecutive pulses. As shown in Fig. 2, the fusion rate increased as the number of pulses increased from 0 to 2. Beyond this range the number of intact cells after electrofusion declined significantly. Viability of those protoplasts that remained intact following the electrofusion pulse remained high as measured by FDA staining throughout the range of pulse numbers employed (data not shown). Figure 2 shows that two electrofusion pulses at 1.25 kV/cm produced the highest heterofusion rate of 13%. The rates of fusion between protoplasts from the same parents were not critically determined, but roughly corresponded to those of heterofusions.

Protoplast culture and putative hybrid selection

Protoplasts of unfused or self-fused *T. caerulescens* did not grow into cell clusters or divide successfully under the culture conditions used in this study. This characteristic eliminated the regeneration of any *T. caerulescens* cells that did not contain some genetic component of *Brassica*. Of the plants that were regenerated without an active selection mechanism from protoplasts subjected to electrofusion, 11% were confirmed as somatic hybrids (Table 2).

Further refinements in selection techniques to reduce unfused or self-fused *B. napus* cell lines in vitro were accomplished by two methods. In one technique, calli from electrofused protoplasts were screened for zinc tolerance by culturing them for 18 days on a medium containing 3,160 µM of total zinc. The zinc concentration in the control medium was 7 µM. Of 2800 calli that received the high-zinc treatment, approximately 6% survived the screen and were able to produce chlorophyll. Five plants, all confirmed hybrids, were regenerated from these zinc-tolerant calli (Table 2).

The second method of hybrid selection was based on a difference observed between the behavior of suspension cell clusters from *B. napus* and those from the electrofusion treatment. When the cells were grown in a liquid medium for longer than 1 month, over 95% of the *B. napus* calli adhered to the bottom of the Petri dish, while approximately 30% of the calli from the fusion experiment of both parents remained floating in the medium. It was hypothesized that this difference might be related to the hybrid character of some of the cells, so providing a mechanism for hybrid selection. Adhering and non-adhering suspension cell clusters were separated and cultured separately. Plants regenerated from non-adhering cells had a confirmed somatic hybrid frequency of 67%, while only 25% of plants regenerated from adhering calli were confirmed as somatic hybrids (Table 2).

Phenotypes of regenerated plants

A total of 64 plants were regenerated from *B. napus* and *T. caerulescens* cells subjected to electrofusion. Of these, none had the *T. caerulescens* phenotype, 47 had the *B. napus* phenotype, and the remaining 17 plants had an intermediate or a unique phenotype of a putative hybrid (Table 3). Since only one shoot was cultured from each callus, each putative hybrid was derived from an independent fusion event. Genomic DNA from the 17 putative hybrids was subjected to AFLP DNA analysis and all 17 plants were confirmed as hybrids. Thirteen of these hybrid plants were successfully transplanted to soil and grown to maturity.

Most hybrids were intermediate in size between the parental species (shown in Fig. 3B) and they displayed a

Table 2 Frequency of somatic hybrid plants produced using four methods of in vitro selection

Selection method	Number of			Frequency of calli that produced plants (%)	Frequency of plants that were hybrid (%)
	Calli	Plants	Hybrids		
Zn screen	182	5	5	2.7	100
Non-adhering calli	541	9	996	1.7	67
Adhering calli	108	4	1	3.7	25
Partial selection (against *Thlaspi*)	3193	46	5	1.4	11
Totals	4024	64	17	1.6	27

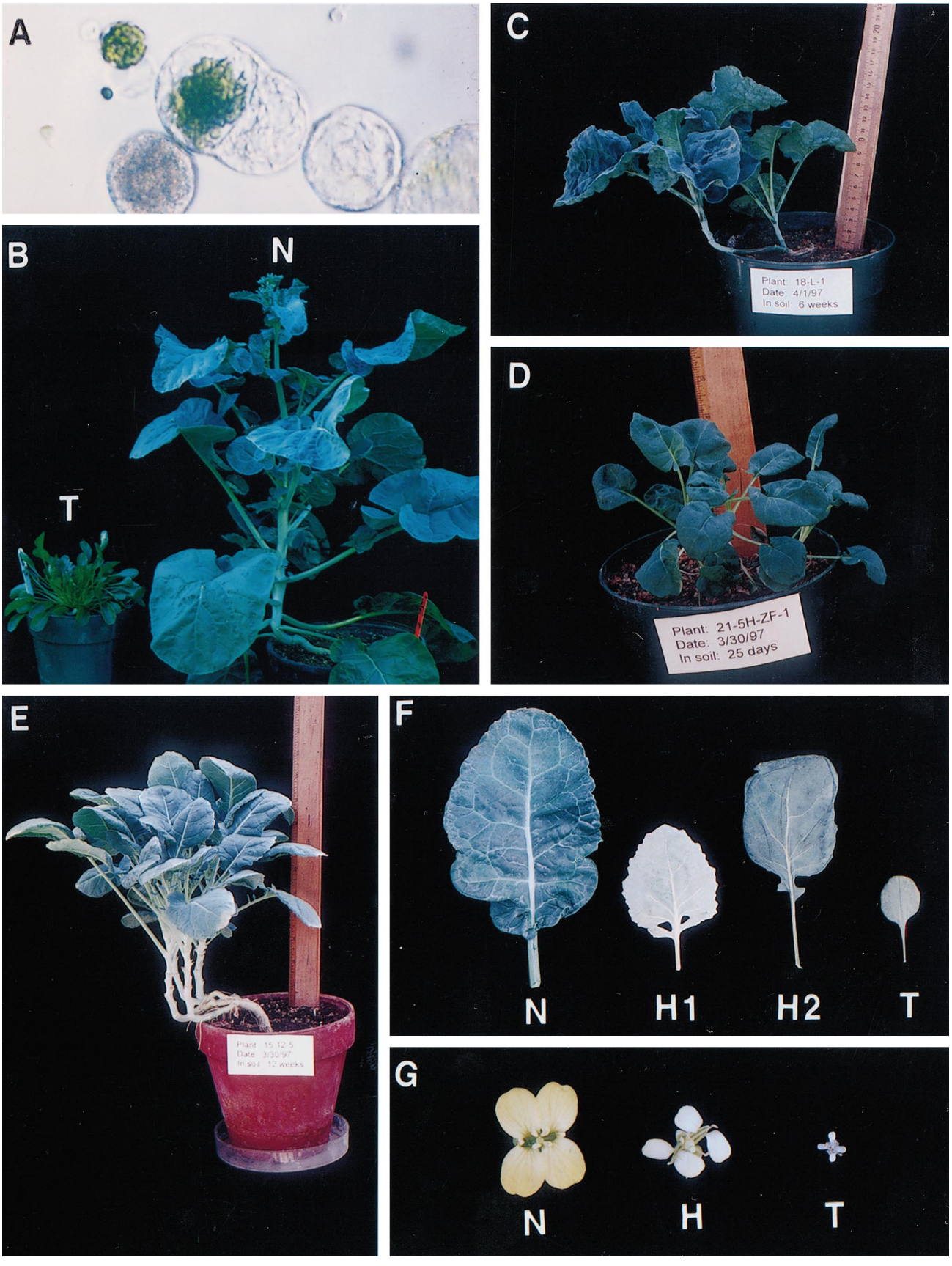
A
B
N
T
C
Plant: 18-L-1
Date: 4/1/97
In soil: 6 weeks
D
Plant: 21-5H-ZF-1
Date: 3/30/97
In soil: 25 days
E
Plant: 15-12-5
Date: 3/30/97
In soil: 12 weeks
F
N
H1
H2
T
G
N
H
T

Table 3 Phenotype and hybrid character of plants regenerated from protoplast fusion experiments between *B. napus* and *T. caerulescens*

Phenotype	Number of plants	Number analyzed by AFLP	Number of verified hybrids
B. napus-like	47	9	0
T. caerulescens-like	0	–	–
Intermediate or unique	17	17	17
Totals	64	26	17

range of morphologies (Fig. 3C–E). None had the rosette growth habit characteristic of *T. caerulescens*. The largest hybrid grew to a height of approximately 30 cm in a 15-cm pot prior to flowering. Leaf size and shape were also intermediate in most hybrids (Fig. 3F), although two had leaves that were approximately the same size as those of *Thlaspi*. Five confirmed hybrids flowered, producing flowers intermediate in size between those of the parental species (Fig. 3G). Regenerated plants of the *B. napus*-type flowered as soon as 38 days after transplantation to soil, while the hybrids took 92–127 days to flower. *T. caerulescens* requires a vernalization period in order to flower. Flowers of four plants had pale-yellow petals and pistils, while the fifth plant had bright yellow petals and a green pistil similar to *B. napus*. *T. caerulescens* flowers have white petals and a pale yellow pistil. Stamen and anther size were variable among the hybrid plants, and in some flowers these organs were rudimentary. None of the flowers produced viable pollen.

Using pollen from *B. napus*, several flowers were pollinated and siliques developed. At least one of the hybrid plants produced seed although the seed appears slightly smaller than normal for *B. napus*.

Hybrid confirmation

The production of somatic hybrids was confirmed by AFLP DNA analysis. In this procedure, selected restriction fragments are amplified by PCR and then separated by electrophoresis on a denaturing polyacrylamide sequencing gel. AFLP assays of parental genomic DNA produced banding patterns characteristic of each species. A different banding pattern was obtained for each of four primer pairs used. Of the four primer pairs selected for this analysis, *Eco*RI-ACC/*Mse*I-CAA produced the greatest number of discriminating bands (Fig. 4). Approximately 20 bands are present in *T. caerulescens* samples

◀ **Fig. 3A–G** Protoplast fusion and plant phenotypes of *B. napus*+*T. caerulescens* somatic hybrids and parental species. **A** Fusion taking place between a *T. caerulescens* mesophyll protoplast (left) and a *B. napus* hypocotyl protoplast (right). **B** Fifteen-week-old *T. caerulescens* (plant T) and eight-week-old *B. napus* (plant N). **C–E** Somatic hybrids. **F** Leaf morphology of *B. napus* (N), *T. caerulescens* (T), and two somatic hybrids (H1, H2). **G** Flower morphology of *B. napus* (N), *T. caerulescens* (T), and a somatic hybrid (H)

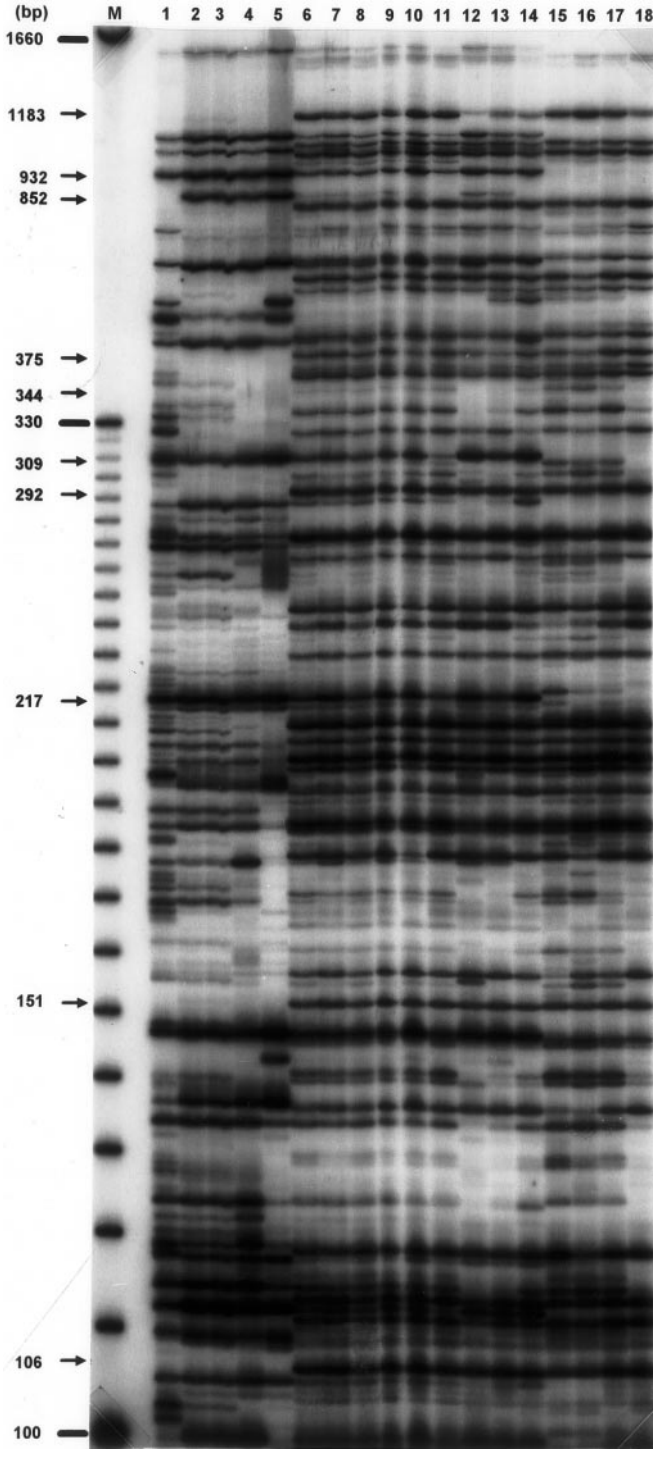

Fig. 4 AFLP banding patterns using the primer pair E-ACC/M-CAA of *T. caerulescens* plants (l*anes 1–5*), *B. napus* plants (*lanes 15–18*), and *B. napus*+*T. caerulescens* somatic hybrids (*lanes 6–14*). Lane M contains molecular-weight markers. Three of the approximately 20 bands characteristic of *T. caerulescens* are indicated with *arrows* at 217, 309, and 932 bp. Five of the approximately 30 bands characteristic of *B. napus* 'Westar' are indicated at 106, 151, 292, 375, and 1183 bp. An example of a polymorphic band in *T. caerulescens* is shown at 852 bp. Polymorphism, as expected, is also seen in the hybrids at 344 bp

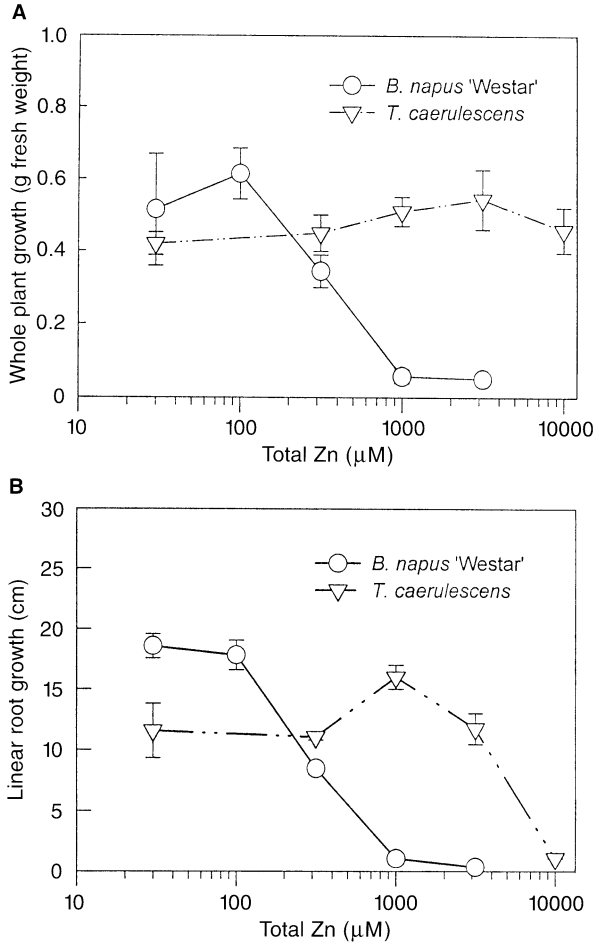

Fig. 5A, B In vitro growth of *B. napus* 'Westar', and *T. caerulescens* on an agar-based nutrient medium containing five levels of Zn. **A** Whole-plant growth (g fresh weight). **B** Increase in length of the longest root (cm)

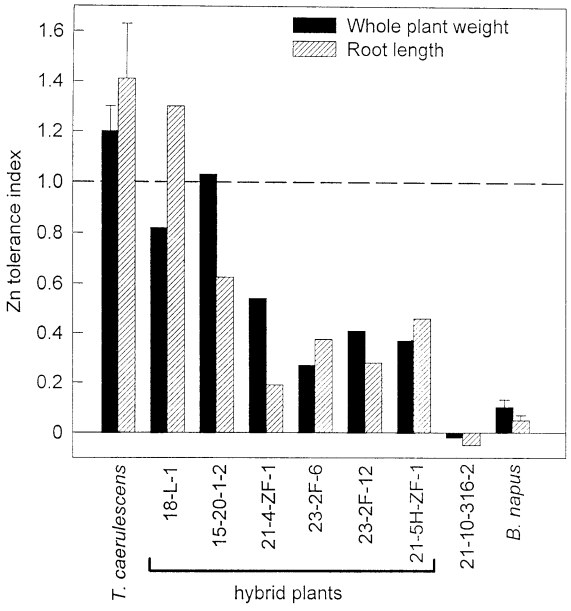

Fig. 6 In vitro Zn tolerance of seven regenerated plants compared with parental levels. Zn tolerance indices were calculated for two measures of growth: whole-plant fresh weight and length of the longest root. The Zn tolerance index=growth on a high-Zn medium (1000 μM of total Zn) divided by growth on a control medium (30 μM of total Zn)

(lanes 1–5) that are not present in *B. napus* (lanes 15–18), while *B. napus* plants contained almost 30 unique bands. Individual variation in polymorphic bands was higher in *T. caerulescens* than in *B. napus* reflecting the greater degree of heterogeneity in the collection. Of 26 regenerated plants analyzed, nine had *B. napus* AFLP DNA patterns and 17 had hybrid patterns, containing more than ten unique bands characteristic of each parent (Fig. 4). All of the 17 plants with hybrid banding patterns had previously been classified as putative hybrids based on their phenotypes (Table 3). Thus a close correlation existed between the morphology of the plant and the AFLP DNA banding patterns. Bands that were conserved within all individuals of a parent were rarely absent from a hybrid, indicating that most hybrids contained the full complement of chromosomes from each parent.

Zn tolerance of regenerated plants

B. napus 'Westar,' and *T. caerulescens* were grown from seed on media containing five levels of total Zn. Zn concentrations for *Brassica* were 30, 100, 316, 1000, and 3160 μM, and Zn concentrations used for *Thlaspi* were 30, 316, 1000, 3160, and 10,000 μM. Whole-plant growth and root growth are shown in Fig. 5 A and B. The 1000-μM Zn treatment was lethal to *Brassica* but not to *T. caerulescens*. Zn tolerance indices were defined as growth on 1000-μM Zn divided by growth on 30-μM Zn. The values of the index for whole plant growth were 0.11 for *B. napus* and 1.2 for *T. caerulescens*. For linear root growth, the index values were 0.06 for *B. napus* and 1.4 for *T. caerulescens*.

Based on this result, the Zn tolerance of regenerated plants was determined by vegetatively propagating multiple nodal cuttings of each plant and growing duplicate plants on both high (1000 μM) and low (30 μM) zinc media. Seven plants regenerated from *B. napus+T. caerulescens* electrofusion experiments were evaluated on both Zn media. Six plants had been verified as somatic hybrids by AFLP analysis. The seventh plant had a *B. napus*-type morphology and was included as a regenerated control. Zn tolerance indices for both parental species and the regenerated plants are shown in Fig. 6. Plant 21–10–316–2, which had a *B. napus* morphology, did not grow on the high-Zn medium. The Zn tolerance indices of all hybrid plants tested had values intermediate between those of the parents. All hybrids except 15–20–1-2 showed some chlorosis on the high-Zn medium, but survived for the length of the experiment (4 months). Plants 18-L-1, 21–4-ZF-1, and 21–5H-ZF-1, which had been regenerated from calli screened on high-Zn, were not as a group more Zn-tolerant than the other hybrids.

Leaves from plants 18-L-1 and 15–20–1-2 grown on the 1000-µM Zn medium were analyzed for Zn. The leaf Zn content of 18-L-1, on a dry weight basis, was 3600 mg kg^{-1}, and that of 15–20–1-2 was 3200 mg kg^{-1}, confirming the uptake and concentration of zinc in leaf tissue. These levels are considerably higher than the 500 mg kg^{-1} tolerated by most crop plants before toxicity occurs (Chaney 1993).

Several somatic hybrid plants were transplanted into Zn- and Cd-contaminated soil from smelter emissions that contained approximately 4000–5000 mg kg^{-1} of Zn, pH 5.8, which was lethal to *B. napus* seedlings. Plant 15–12–5, a rooted hybrid cutting growing in vermiculite before it was transferred to the high-Zn soil, grew in the soil for 2 months. Its leaves were a light-green color for the 1st month, then they became increasingly chlorotic. Leaves from this plant contained 2900 mg Zn kg^{-1} and 170 mg Cd kg^{-1}, which was again higher than that tolerated by non Zn tolerant crop plants.

Discussion

Somatic hybrids in the Brassicaceae with some degree of fertility have been produced between a number of individual species such as *B. napus* or *B. juncea*, and sexually incompatible combinations such as *Diplotaxis harra*, *Lesquerella fendleri*, and *T. perfoliatum* (Fahleson et al. 1994; Begum et al. 1995; Skarzhinskaya et al. 1996). In our study, protoplast electrofusion was used to produce mature intertribal somatic hybrids between the oilseed crop species *B. napus* and *T. caerulescens*, a hyperaccumulator of zinc and cadmium. This is the first known report of hybridization between a metal hyperaccumulator and a larger species, providing a potential mechanism for practical phytoremediation of metal-contaminated soil.

Previous somatic hybridizations involving *B. napus* have commonly used polyethylene glycol (PEG) for protoplast fusion (e.g., Klimaszewska and Keller 1988; Sundberg and Glimelius 1991; Bauer-Weston et al. 1993; O'Neil et al. 1996). Although this technique is effective, there have been reports that higher fusion frequencies are obtainable with electrofusion for some protoplast combinations (Bates 1985; Fish et al. 1988; Saunders et al. 1989b). High fusion frequencies are especially important when selection methods for the heterokaryons are not available. We obtained a heterofusion rate of 13% (equivalent to a total fusion rate of about 26%) after optimization of the electrofusion parameters (Fig. 2). This result is consistent with previously reported total electrofusion rates of 20–35% (Kohn et al. 1985; Fish et al. 1988; Saunders et al. 1989b), and is higher than the heterofusion rates reported for PEG fusions in *B. napus* (Sjodin and Glimelius 1989; Lelivelt et al. 1993). Optimization of electrofusion parameters depends on several factors, including the size and origin of the protoplasts being fused (Tempelaar and Jones 1985; Saunders et al. 1993). Pulse field-strengths reported in studies using other species range from 0.5 to 2.1 kV/cm, with 1–4 electrofusion pulses (Chang et al. 1992; Pupilli et al. 1992; Cheng et al. 1995).

Although hybrid plants were obtained without a complete selection method, this required the culture of large numbers of non-hybrid calli and regenerated plants. Several techniques have been developed for selecting hybrid cell lines at early stages, including fluorescence-activated cell sorting, antibiotic resistance, and reversible metabolic inhibition of parental protoplasts with chemicals such as iodoacetate (Morikawa and Yamada 1992). In our study, screening calli on a high-Zn medium, coupled with the inability of *T. caerulescens* to grow under the tissue-culture conditions used, proved to be sufficient for obtaining hybrid plants (Table 2). Since only about 6% of the calli from fusion experiments survived the Zn screen, it is likely that some hybrid calli were not stable or did not express Zn tolerance well.

A second (previously unreported) selection method was developed based on the apparent differential adhesive capabilities of *B. napus* and hybrid cell colonies grown in liquid medium. Among fusion experiment-derived cell colonies, it was observed that some adhered to the bottom of the Petri dish (as did *B. napus* colonies), while others did not. AFLP DNA analysis of plants that were regenerated from the two populations indicated that a high proportion of plants derived from "floating" colonies were hybrids (Table 2). Most hybrid cell clusters eventually adhered to the vessel wall if grown long enough in the liquid medium, so the phenomenon may be a transient effect due to the hybridization.

In this study, AFLP DNA analysis has been used for the first time for somatic-hybrid identification. In previous reports, a variety of techniques have been employed to verify somatic hybrids, including plant morphologies, isozyme analysis, RFLP DNA markers, detection of species-specific repetitive DNA sequences, chromosome morphology, and measurement of DNA content by flow cytometry (Saul and Potrykus 1984; Morikawa and Yamada 1992; Cheng et al. 1995). Often multiple techniques are used because results of one method are not sufficient to draw unequivocal conclusions about hybridity. This is in part due to the tendency of somatic hybrids to lose chromosomes or parts of chromosomes during development (reviewed by Harms 1983). Using just one PCR primer pair, AFLP DNA analysis detected as many as 50 randomly located polymorphisms useful for distinguishing the DNA of the two parental species. By increasing the number of primer pairs to four, over 120 polymorphisms were detected on one gel. Of these 120, about 70 were present only in *B. napus* and about 50 were present only in *T. caerulescens*. Since *B. napus* has 19 pairs of chromosomes and *T. caerulescens* has seven pairs, it is likely that some portion of each of the chromosomes was represented by one or more polymorphism. Thus AFLP should be useful for detecting highly asymmetric hybrids in which most of one parent's genome has been lost. The hybrids produced in this study appeared to contain most of the DNA from each parent based on the AFLP DNA

analysis. AFLP should be useful for detecting chromosome losses that may occur in future somatic or sexual generations of these plants.

Several of the somatic hybrids produced in this study have characteristics that would make them superior to *T. caerulescens* for use in phytoremediation. These characteristics include larger above-ground and below-ground biomass, faster growth of the plant, and an erect growth habit to facilitate mechanical harvesting of the plants. The growth of the plant in tissue culture on high-zinc media (Fig. 5 A and B) as well as on high-Zn soil (Fig. 6), conclusively demonstrate the utility of somatic hybridization as a technique for the production of metal-tolerant plants. By combining the zinc-tolerant character of *T. caerulescens* with the large size of *B. napus*, a plant with the potential for phytoextraction of metals from toxic soils has been produced. We are currently investigating the production of viable seeds from backcrosses to *B. napus*.

References

Antonovics J, Bradshaw AD, Turner RG (1971) Heavy metal tolerance in plants. Adv Ecol Res 7:1–85

Baker AJM, McGrath SP, Sidoli CMD, Reeves RD (1994) The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. Resourc, Conser Recycling 11:41–49

Banks MS, Evans PK (1976) A comparison of the isolation and culture of mesophyll protoplasts from several *Nicotiana* species and their hybrids. Plant Sci Lett 7:409–416

Bates GW (1985) Electrical fusion for optimal formation of protoplast heterokaryons in *Nicotiana*. Planta 165:217–224

Bauer-Weston B, Keller W, Webb J, Gleddie S (1993) Production and characterization of asymmetric somatic hybrids between *Arabidopsis thaliana* and *Brassica napus.* Theor Appl Genet 86:150–158

Begum F, Paul S, Bag N, Sikdar SR, Sen SK (1995) Somatic hybrids between *Brassica juncea* (L). Czern and *Diplotaxis harra* (Forsk) Boiss and the generation of backcross progenies. Theor Appl Genet 91:1167–1172

Beyer WN (1988) Damage to the forest ecosystem on Blue Mountain from zinc smelting. In: Trace substances in environmental health XXII. University of Missouri, Columbia, Mo., pp 249–262

Brown SL, Chaney RL, Angle JS, Baker AJM (1995) Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. Soil Sci Soc Am J 59:125–133

CEC (Commission of the European Communities) (1986) Council directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Official J Europ Commun L181 (86/278/EEC):6–12

Chaney RL (1983) Plant uptake of inorganic waste constituents. In: Parr JF, Marsh PB, Kla JM (eds) Land treatment of hazardous wastes. Noyes Data Corporation, Park Ridge, New Jersey, pp 50–76

Chaney RL (1993) Zinc phytotoxicity. In: Robson AD (ed) Zinc in soils and plants. Kluwer Academic Publishers, Dordrecht, pp 135–150

Chaney RL, Malik M, Li YM, Brown SL, Brewer EP, Angle JS, Baker AJM (1997) Phytoremediation of soils metals. Curr Opin Biotechnol 8:279–284

Chang DC, Chassy BM, Saunders JA, Sowers AE (1992) Guide to electroporation and electrofusion, Library of Congress # GH585.5E48H36. Academic Press, San Diego, Calif., pp 1–581

Cheng J, Saunders JA, Sinden SL (1995) Colorado potato beetle-resistant somatic hybrid potato plants produced via protoplast electrofusion. In Vitro Cell Dev Biol 31:90–95

Chevre AM, Eber F, Margale E, Kerlan MC, Primard C, Vedel F, Delseny M, Pelletier G (1994) Comparison of somatic and sexual *Brassica napus-Sinapis alba* hybrids and their progeny by cytogenetic studies and molecular characterization. Genome 37:367–374

Doyle JJ„ Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Fahleson J, Rahlen L, Glimelius K (1988) Analysis of plants regenerated from protoplast fusion between *Brassica napus* and *Eruca sativa.* Theor Appl Genet 76:507–512

Fahleson J, Eriksson I, Landgren M, Stymme S, Glimelius K (1994) Intertribal somatic hybrids between *Brassica napus* and *Thlaspi perfoliatum* with a high content of the *T. perfoliatum*-specific nervonic acid. Theor Appl Genet 87:795–804

Fish N, Karp A, Jones MGK (1988) Production of somatic hybrids by electrofusion in *Solanum.* Theor Appl Genet 76:260–266

Gleba YY, Hinnisdaels S, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherep NN, Negrutiu I, Jacobs M (1988) Intergeneric asymmetric hybrids between *Nicotiana plumbaginifolia* and *Atropa belladonna* obtained by "gamma-fusion". Theor Appl Genet 76:760–766

Glimelius K, Djupsjöbacka M, Fellner-Feldegg H (1986) Selection and enrichment of plant protoplast heterokaryons of *Brassicaceae* by flow sorting. Plant Sci 45:133–141

Glimelius K, Fahleson J, Landgren M, Sjödin C, Sundberg E (1991) Gene transfer via somatic hybridization in plants. Trends Biotechnol 9:24–30

Harms CT (1983) Protoplasts 1983, lecture proceedings. Birkhäuser Verlag, Basel, pp 69–84

Ingrouille MJ, Smirnoff NW (1986) *Thlaspi caerulescens* J and C Presl (*Thlaspi alpestre* L.) in Britain. New Phytol 102: 219–233

Kabata-Pendias A, Pendias H (1989) Trace elements in the soil and plants. CRC Press, Boca Raton, Florida

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Planta 126:105–110

Klimaszewska K, Keller WA (1988) Regeneration and characterization of somatic hybrids between *Brassica napus* and *Diplotaxis harra.* Plant Sci 58:211–222

Kohn H, Schieder R and Schieder O (1985) Somatic hybrids in tobacco mediated by electrofusion. Plant Sci 38:121–128

Lelivelt CLC, Leunissen EHM, Frederiks HJ, Helsper JPFG, Krens FA (1993) Transfer of resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) from *Sinapis alba* L. (white mustard) to the *Brassica napus* L. gene pool by means of sexual and somatic hybridization. Theor Appl Genet 85:688–696

Lin CH, Xiao L, Hou BH, Ha SB, Saunders JA (1997) Optimization of electroporation conditions for expression of GUS activity in protoplasts and intact plant cells. Plant Physiol Biochem 35:959–968

Lin JJ, Kuo J, Ma J, Saunders JA, Beard HS, MacDonald MH, Kenworthy W, Ude GN, Matthews BF (1996) Identification of molecular markers in soybean comparing RFLP, RAPD and AFLP DNA mapping techniques. Plant Mol Biol Rep 14: 156–169

Menczel L, Nagy F, Kiss ZsR, Maliga P (1981) Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum+Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor Appl Genet 59:191–195

Morikawa H, Yamada Y (1992) Plant biotechnology: comprehensive biotechnology, 2nd Suppl., Pergamon Press, Oxford, pp 199–222

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nagy JJ, Maliga P (1976) Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris.* Z Pflanzenphysiol 78:453–455

Nriagu JO (1979) Global inventory of natural and anthropogenic emissions of trace metals to the atmosphere. Nature 279: 409–411

O'Neil CM, Murata T, Morgan CL, Mathias RJ (1996) Expression of the C_3–C_4 intermediate character in somatic hybrids between *Brassica napus* and the C_3–C_4 species *Moricandia arvensis.* Theor Appl Genet 93:1234–1241

Prakash S, Hinata K (1980) Taxonomy, cytogenetics and origin of crop *Brassicas*, a review. Opera Bot 55:1–57

Primard C, Vedel F, Mathieu C, Pelletier G, Chevre AM (1988) Interspecific somatic hybridization between *Brassica napus* and *Brassica hirta* (*Sinapis alba* L.). Theor Appl Genet 75: 546–552

Pupilli E, Scarpa GM, Damiani F, Arcioni S (1992) Production of interspecific somatic hybrid plants in the genus *Medicago* through protoplast fusion. Theor Appl Genet 84:792–797

Rascio W (1977) Metal accumulation by some plants growing on zinc mine deposits. Oikos 29:250–253

Saunders JA, CR Smith, Kaper JM (1989a) Effects of electroporation pulse wave on the incorporation of viral RNA into tobacco protoplasts. Biotechniques 7:1124–1131

Saunders JA, Matthews BF, Miller PD (1989b) Plant gene transfer using electrofusion and electroporation. In: Neumann E, Sowers AE, Jordan CA (eds). Electroporation and electrofusion in cell biology plenum press, New York, pp 343–354

Saunders JA, Smith CR, Cheng JP, Matthews BF, O'Neill NR (1993) Methods for gene transfer in plants using electrical manipulation, successes and failures. In: Shamaan NA, Mahmood M, Muse R, Ramli J, Syed MA, Arshad JH, Yusoff K, Abdullah N (eds) Applications of plant in vitro technology. SP-Muda Printing Sdn Bhd, Kuala Lumpur, Malaysia, pp 26–42

Saunders JA, Lin CH, Hou BH, Cheng JP, Tsengwa N, Lin JJ, Smith CR, McIntosh MS, Van Wert S (1995) Rapid optimization of electroporation conditions for plant cells, protoplasts, and pollen. Mol Biotechnol 3:181–190

Saul MW, Potrykus I (1984) Species-specific repetitive DNA used to identify interspecific somatic hybrids. Plant Cell Rep 3:65–67

Sjödin C, Glimelius K (1989) *Brassica naponigra*, a somatic hybrid resistant to *Phoma lingam*. Theor Appl Genet 77:651–656

Skarzhinskaya M, Landgren M, Glimelius K (1996) Production of intertribal somatic hybrids between *Brassica napus* L. and *Lesquerella fendlerii* (Gray) Wats. Theor Appl Genet 93: 1242–1250

Sundberg E, Glimelius K (1991) Effects of parental ploidy level and genetic divergence on chromosome elimination and chloroplast segregation in somatic hybrids within the *Brassicaceae*. Theor Appl Genet 83:81–88

Tempelaar MJ, Jones MGK (1985) Fusion characteristics of plant protoplasts in electric fields. Planta 165:205–216

US Department of Energy (1994) Summary report of a workshop on phytoremediation research needs, DOE/EM-0224, US Department of Commerce, Springfield, Virginia