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Bioengineering of a Phytoremediation Plant by Means of Somatic Hybridization

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ABSTRACT

Phytoremediation is a technology that exploits a plant's ability to remove contaminants from the environment or render toxic compounds harmless. An efficient metal phytoremediating plant must combine high biomass production and established cultivation methods with high tolerance to a specific contaminant and ability for root uptake, translocation, and compartmentalization of contaminants in the above-ground biomass. Symmetric and asymmetric somatic hybridizations were used to introduce toxic metal-resistant traits from *Thlaspi caerulescens* into *Brassica juncea*. *B. juncea* hypocotyl protoplasts were fused with *T. caerulescens* mesophyll protoplasts. The hypocotyl protoplasts of *B. juncea* were stained with CFDA before fusion and thus fluoresced green under UV, whereas the mesophyll protoplasts of *T. caerulescens* had red autofluorescense. Heteroplasmic fusion products were identified and selected by flow cytometry and cell sorting. All putative hybrids grown in the greenhouse had morphological characteristics of *B. juncea*. A *Thlaspi*-specific repetitive sequence was hybridized to total DNA of plants, including the parental species. All plants from both symmetric and asymmetric fusions showed *Thlaspi*-specific hybridization patterns while *B. juncea* did not exhibit any hybridization signal. Hybrid plants, produced by asymmetric somatic hybridization between the two species, demonstrated high metal accumulation potential, tolerance to toxic metals, and good biomass production.

I. INTRODUCTION

Phytoremediation has been attracting attention as a rapidly developing, inexpensive plant-based remediation technology (Carbisu and Alkorta 2001; Blaylock *et al.*, 1999; Salt *et al.*, 1998; Raskin *et al.*, 1997). This technology exploits the natural ability of a green plant to accumulate a variety of chemical elements and transport them from the substrate to above ground parts (Dushenkov *et al.*, 1997). The ability to accumulate heavy metals to unusually high levels and to tolerate elevated levels of toxic metals has been reported in a number of plants (Baker and Brooks 1989; Ernst *et al.*, 1992; Ma *et al.*, 2001). A plant with an abnormally high level of metal accumulation is called a hyperaccumulator (Jaffre, 1976; Baker and Brooks, 1989). A large number of hyperaccumulators species belong to the Brassicaceae family, in particular, to the *Thlaspi* genus (Reeves and Baker, 2000). The wild *Thlaspi* species possess the highest hyperaccumulating abilities of Ni (Baker and Brooks, 1989) and Zn (Reeves and Brooks, 1983). However, the phytoremediation potential of *Thlaspi* species, including *T. caerulescens* J & C Presl, is limited by low biomass and slow plant growth. Among the high biomass crops, *Brassica juncea* (L.) Czern. (Indian mustard) was identified as the best accumulating species (Kumar *et al.*, 1995). However, *B. juncea* is less efficient in extracting metals from soil compared with *Thlaspi*. Moreover, *B. juncea* is sensitive to high concentrations of heavy metal in soil. Enhancing desirable traits in phytoremediation plants, such as high biomass, high metal uptake, or overcoming toxicity limitations, is an important step in the development of phytoremediation technology.

The introgression of beneficial traits from wild hyperaccumulators to the genome of cultivated crops can be achieved through hybridization. Because *T. caerulescens*

and *B. juncea* belong to different tribes of the Brassicaceae family and are sexually incompatible, technology of somatic cell hybridization constitutes the ultimate solution to producing hybrids. It was demonstrated that somatic hybrids created by electrofusion of isolates from hyperaccumulator *T. caerulescens* and crop plant *Brassica napus* L. had metal-tolerant levels compared with *T. caerulescens* (Brewer *et al.*, 1999). A number of intertribal hybrids of Brassicaceae have been produced successfully using somatic cell hybridization technology (Gleba and Hoffman, 1980; Skarzhinskaya *et al.*, 1996) and in most experiments fertile plants were obtained.

The objective of this study was to bioengineer hybrid plants that combine robust biomass production with maximum metal accumulation and tolerance to high toxic metal concentrations in soil.

II. MATERIAL AND METHODS

A. Plant Material

Brassica juncea seeds cv. 173874 were obtained from the USDA/ARS Plant Introduction Station of Iowa State University. Dr. A. Baker kindly provided seeds of the natural hyperaccumulator *Thlaspi caerulescens*.

Mesophyll protoplasts were isolated from 1-month-old leaves of *in vitro* grown *T. caerulescens* plants and etiolated 5-day-old hypocotyls of *B. juncea*. Protoplasts were isolated according to Glimelius (Glimelius 1984). Freshly isolated hypocotyl protoplasts were stained with 250 to 500 ml/ml of 5(6) carboxyfluorescein diacetate (CFDA) per ml of protoplast suspension for 10 min prior to fusion (Sjodin and Glimelius, 1989). Protoplast fusions were performed using polyethylene glycol (PEG, 40%, w/v, MW1500) as described by Sundberg and Glimelius (Sundberg and Glimelius, 1986). Eight thousand heteroplasmic fusion products were sorted into each well of a multivial plate in 120 ml of 8 pm medium (Sundberg and Glimelius, 1991). After 6 days, cell colonies were embedded into 0.6% Sea plaque agarose (Sundberg and Glimelius, 1986). Two weeks later, colonies were transferred to liquid modified K3 medium (Nagy and Maliga, 1976) for further growth. After 3 to 4 weeks, colonies were placed on solid K3 medium for regeneration. Newly regenerated plants were transferred to hormone-free MS medium (Murashige and Skoog, 1962) and cultured under conditions according to Sjodin and Glimelius (Sjodin and Glimelius, 1989).

In the symmetric fusion experiment, protoplasts of two species were fused without any pretreatments. In the asymmetric fusion experiment, *T. caerulescens* mesophyll protoplasts were irradiated before fusion. Irradiation was performed in W5 medium (Menczel *et al.*, 1981) by using an X-ray Siemens Stabilipan 200 apparatus with the dose of 60 Krad. Nonfused *T. caerulescens* protoplasts were cultured as a control in each fusion experiment.

B. Morphological Characterization

Morphological characterization was performed on greenhouse grown plants, which included leaves and flowers as well as plant growth habits. The viability of pollen was determined by acetoorsein staining of 1000 pollen grains from three flowers of

each plant and calculating the average. Seed set for each plant was calculated after self-pollination and backcrossing to *B. juncea*. Fertility was expressed as the number of seeds produced per plant.

Total DNA from putative hybrids and parental species was isolated from greenhouse plants according to Dellaporta *et al.* (1983) with modifications (Forsberg *et al.*, 1994). Four to six milligrams of DNA were digested with Hind III, separated by gel electrophoresis and transferred to nylon filters. The filters were hybridized to the *Thlaspi*-specific repetitive DNA sequence isolated by Fahleson et al. (Fahleson *et al.*, 1994). The nuclear DNA probe was ^{32}P-dCTP- labeled by Random Primers Labeling System according to manufacturer's instructions (Life Technologies). The incubation, washing, and exposure of filters were carried out according to Landgren and Glimelius (Landgren and Glimelius, 1990).

C. Tolerance and Metal Uptake Experiments

For sterile culture experiments, regenerated somatic hybrids were grown on hormone-free MS agarose medium (Murashige and Skoog, 1962). For shoot multiplication, plants were transferred to MS media + 0.2 mg/l Kinetin, and to MS medium with 0.1 mg/l NAA root induction. For metal uptake/resistance $Ni(NO_3)_2$ and $Zn(NO_3)_2$ were added to media. Plants were cultivated for 5 weeks, harvested, dried, and analyzed for metal content. Experiments were conducted in an environmentally controlled growth chamber at 25°C, 75% relative humidity, and 16 h photoperiod (600 μmol $m^{-2}.sec^{-1}$) provided by a combination of incandescent and cool-white fluorescent lights.

Selected sterile rooted plants were transferred to 10-cm pots containing 350 g Standard Farm Soil artificially contaminated with 600 mg kg^{-1} Pb, or soil contaminated with Zn and Ni and grown for 5 weeks. Plants were fed weekly with nutrient solution (1 g L^{-1} Hydrosol™ supplemented with 0.6 g L^{-1} $Ca(NO_3)_2$). After 5 weeks plants were harvested, rinsed with distilled water, dried, and analyzed for metal content. Experiments were conducted in the greenhouse at 24°C and 16 h photoperiod.

Rhizofiltration experiments were conducted in the greenhouse at 24°C and 16 h photoperiod. The cuttings from the somatic hybrids were grown hydroponically in the greenhouse and rooted using 1% gibberelic acid. Miniature batch rhizofiltration systems were used to determine metal uptake. Plants were put into individual plastic cylinders containing 1 L of continuously aerated tap water with different concentrations of $Ni(N0_3)_2$. After 5 weeks of exposure, the roots and shoots were harvested separately, rinsed in distilled water, dried, and analyzed for Ni content.

Plant tissue samples were dried in a forced-air oven at 60°C, ground to 20 mesh using a stainless steel Wiley Mill and digested using nitric and perchloric acids by placing 0.25 g dried plant material into a 50-mL Folin digestion tube. Five mL of concentrated nitric acid (15.9 *M*) was added to the material and allowed to stand for a minimum of 6 h. The samples were placed on a heating block at 190 ± 10°C in a perchloric acid hood for 15 min or until the volume was reduced by 50%. The tubes were then removed from the heating block and allowed to cool to room temperature. One milliliter of concentrated perchloric acid (11.9 *M*) was added to each sample and

the tubes returned to the heating block for 1 h or until the digestate was clear. The digested sample was diluted to 25 mL and analyzed for total metals by ICP using EPA SW-846 Method 6010 (USEPA 1986). Appropriate duplicates and spikes were carried through the digestion procedure as well as the NIST Peach Leaf Standard (SRM 1547) as part of the QA/QC plan.

III. RESULTS AND DISCUSSION

A. Morphological Characterization

In the two sets of experiments *B. juncea* hypocotyl protoplasts were fused with *T. caerulescens* mesophyll protoplasts in a final concentration of 9 x 10^5 of both cell types. The hypocotyl protoplasts of *B. juncea* were stained with CFDA before fusion and thus fluoresced green in the UV, while the mesophyll protoplasts of *T. caerulescens* had red autofluorescence. Hence, it was possible to identify and select the heteroplasmic fusion products by flow cytometry and cell sorting. The selection efficiency of this procedure was about 70%. The plating efficiency of the hybrid cells was about 2.5% for the symmetric fusions and just 0.6% for the asymmetric fusions. Sixty-seven calli from the symmetric fusions and 18 from asymmetric fusions were transferred to the regeneration media and 12 and 4 shoots were regenerated, respectively.

All putative hybrids for both symmetric and asymmetric fusions were rooted and subsequently transferred to the greenhouse. The putative hybrids displayed unique, hybrid-specific leaf morphology with predominance of *Brassica* traits (Table 1). Some plants from the symmetric fusions had leaves that were more elongated and dissected than those of *B. juncea*. The growth habit and leaf shape of all plants from the asymmetric fusions were similar to *B. juncea.*

All plants from the symmetric fusions produced *B. juncea*-like flowers with deviations from the regular morphology. Those deviations included the color and size of the flowers, reduced petals or absence of anthers, distortion of pistils (Table 1). Three plants from the asymmetric fusions developed *B. juncea*-like flowers with reduced number of stamens.

All plants from primary regenerants were grown to maturity. The symmetric fusion hybrids produced only a few viable pollen grains and thus were male sterile. They were female sterile as well because they did not produce seeds after pollination with *B. juncea.* Two plants from the asymmetric fusions produced pollen with a range of viability (78%, 52%, 29% when compared with 100% viability of *B. juncea* pollen) and after self-pollination two lines (60/24 and 60/31) produced seeds. However, seed set was rather low (Table 1).

The presence of *Brassica* genetic material in hybrids was evident because all putative hybrids clearly demonstrated morphological characteristics of *B. juncea*. To check the presence of *T. caerulescens* genetic material, *Thlaspi*-specific repetitive sequence was hybridized to total DNA of hybrids and the parental species. All plants from both symmetric and asymmetric fusions were analyzed and showed *Thlaspi*-specific hybridization patterns, whereas *B. juncea* did not exhibit any hybridization signal (Table 1).

TABLE 1
Characteristics of the Somatic Hybrid Plants (*B. juncea* + *T. caerulescens*)

Hybrid lines	Morphology		Fertility	Seeds	Presence of
	Leaves	Flowers			*Thlaspi*-specific DNA
Symmetric fusion somatic hybrids					
0/1-1	elongated	Abn	MS	0	(+)
0/4	B	Abn	MS	0	(+)
0/5	B	B/N	MS	0	(+)
0/6	B	B/N	MS	0	(+)
0/7	elongated	Abn	MS	0	(+)
0/9	B	B/N	MS	0	(+)
0/15	B	B/T	MS	0	(+)
0/16	B	B/N	MS	0	(+)
0/19	B	Abn	MS	0	(+)
0/20	B	B/T	MS	0	(+)
0/31	round	Abn	MS	0	(+)
Asymmetric fusion somatic hybrids					
60/22	B	B/N	F	78	(+)
60/24	B	B/N	F	52	(+)
60/31	B	B/small	F	29	(+)
Parent forms					
B. juncea	B	B	F	100	(-)
T. caerulescens	T	N/A	F	N/A	(+)

Note: B —*B. juncea*-Specific Morphology; T —*T. caerulescens* Specific Morphology; Abn — abnormal flower morphology (rudimentary stamens, distorted pistils, small or rudimentary petals); B/T — flowers displayed Brassica or *Thlaspi*-specific morphological features; MS — male sterile; F — fertile; (+) — presence of *Thlaspi*-specific repetitive sequences; (-) — absence of *Thlaspi*-specific repetitive sequences; N/A — not applicable

B. Tolerance and Metal Uptake Experiments

Vegetatively propagated plants have been subjected to metal uptake studies. Metal uptake has been evaluated in *in vitro* culture, hydroponics, and soil. Although the initial analysis had involved all regenerants, subsequent studies focused on fertile plants.

A dilution series of experiments in sterile media were conducted to determine the LC_{90} (concentration of metal lethal for 90% of plant population) for Ni and Zn. Preliminary studies showed that somatic hybrids grew well on media in the presence of Ni and Zn and had excellent shooting ability, particularly in the presence of Zn, while rooting was not observed for any hybrid line or *Thlaspi.* Zinc concentration in excess of 5 m*M* negatively affected the solidification of agarose media. At the highest Zn concentration, plants grew similar to the control. The most visible indication of heavy metal stress was changes in leaf coloration. Hence, LC_{90} for Zn was not reached. Hybrid line 60/31 grown in media containing 5 m*M* Zn had Zn concentration at 48.8 g kg^{-1} compared with 78.1 g kg^{-1} in natural Zn hyperaccumulator *T. caerulescens,* and 10.6 g kg^{-1} in *B. juncea*. Plants were affected and stressed by high metal concentrations only in leaf coloration. A threshold concentration for testing Ni resistant lines was 1.7 m*M* Ni in the media. Asymmetric hybrids demonstrated tolerance to Zn and Ni present in the media (Plate 1*). Also, the asymmetric hybrids accumulated significantly higher amounts of Ni compared with *Brassica* plants or symmetric hybrids, while remaining physiologically unaffected. For example, asymmetric hybrid line 60/31 had Ni concentration at 4.3 g kg^{-1} compared with 1.0 g kg^{-1} in *T. caerulescens,* and 1.5 g kg^{-1} in *B. juncea*.

Similar results were obtained during the hydroponics experiments. Asymmetric somatic hybrids demonstrated high Zn concentrations in the shoots compared with *B. juncea* plants. In the plants treated with 5 m*M* Zn, concentration of Zn in hybrid 60/31 shoots was 23 g kg^{-1}, 7.5-fold higher than in *B. juncea* shoots.

The experiments with cloned plants grown in heavy metal-contaminated soil confirmed the asymmetric somatic hybrids tolerance to high metal concentrations in the soil and the hybrid's ability to accumulate heavy metals in the aboveground biomass (Plate 2*). In the greenhouse experiments with the soil containing 26 mg kg^{-1} Ni and 463 mg kg^{-1} Zn, the asymmetric hybrid 60/31 above-ground biomass reached dried weight of 1.40 g compared with 0.21 g for *B. juncea* and only 0.07 g for slowly growing *T. caerulescens.* The hybrid had the highest metal concentration compared with the parent forms. The concentration of Ni and Zn in hybrid shoots was 0.4 g kg^{-1} and 19.1 g kg^{-1}, respectively.

CONCLUSION

Modern biotechnological methods may be used successfully to bioengineer a phytoremediation plant with desired traits. In the near future it may be possible to control the rate of heavy metals uptake into the root and translocation to the aboveground biomass by manipulating the expression of metal transporter genes (Guerinot and Salt 2001; Pence *et al.,* 2000). Metal-binding proteins such as metallothioneins

* Plates 1 and 2 appear following page 168.

and phytochelatins play an important role in metal sequestration and to increase organisms' tolerance to heavy metals. Attempts to engineer the production of metallothioneins in model plants or to manipulate phytochelatins pathways and expression have been reported (Mejare and Bulow, 2001). The introduction of the two bacterial *mer* genes (*merA, merB*) is being used to produce transgenic high biomass plants capable of removing and detoxifying mercury (Rugh *et al.,* 2000). Somatic hybridization is often overlooked as a method of engineering plant for phytoremediation. Brewer *et al.,* (1999) established that somatic hybridization could be used to combine desirable traits from hyperaccumulator and crop plant. We successfully demonstrated the feasibility of using somatic hybridization for improvement of phytoremediation properties of plants when mechanisms of particular traits are still unknown. Asymmetric hybrids produced in this study combined valuable properties from different descents. The hybrid inherited high biomass production from *B. juncea* along with heavy metal tolerance and Zn and Ni accumulation potency from *T. caerulescens.* Further selection and field testing are required to introduce the new hybrid into phytoremediation technology.

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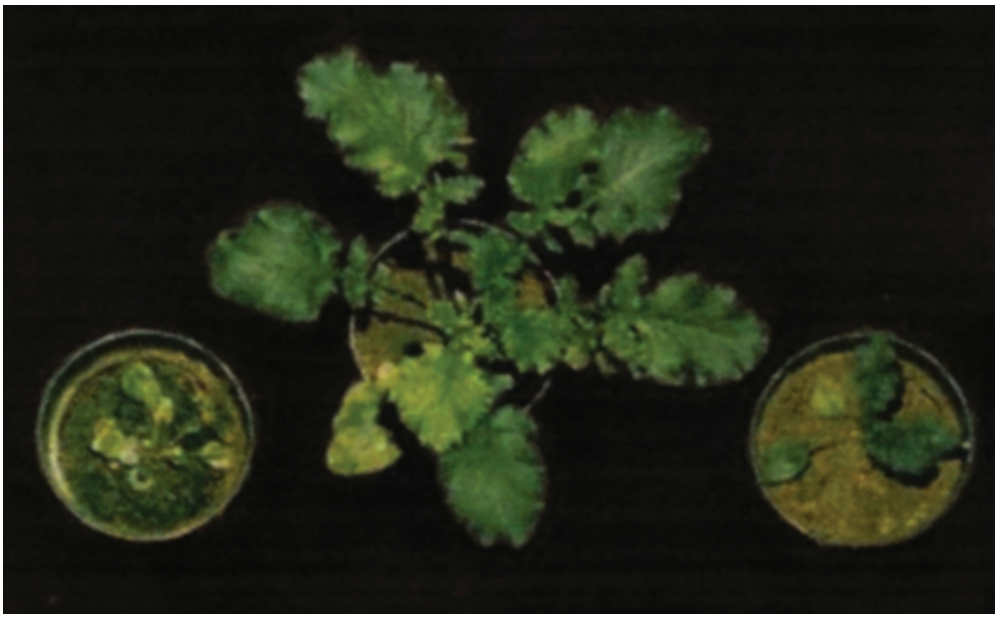
A B C

PLATE 1. *B. juncea* (A), asymmetric hybrid (B), and *T. caerulescens* (C) grown on sterile media containing 1m*M* Zn.



A B C

PLATE 2. *T. caerulescens* (A), asymmetric hybrid (B), and *B. juncea* in the soil containing 26 mg kg^{-1} Ni, 463 mg kg^{-1} and 277 mg kg^{-1} Pb