Genetic Modification of Wetland Grasses for Phytoremediation

Mihály Czakó[a], Xianzhong Feng[b], Yuke He[b], Dali Liang[a], and László Márton[a,*]

[a] Department of Biological Sciences, University of South Carolina, 700 Sumter St, Columbia, SC 29208, USA. Fax: 803-777-4002. E-mail: marton@mail.biol.sc.edu

[b] National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, People's Republic of China

* Author for correspondence and reprint requests

Z. Naturforsch. **60 c**, 285–291 (2005)

Wetland grasses and grass-like monocots are very important natural remediators of pollutants. Their genetic improvement is an important task because introduction of key transgenes can dramatically improve their remediation potential. Tissue culture is prerequisite for genetic manipulation, and methods are reported here for *in vitro* culture and micropropagation of a number of wetland plants of various ecological requirements such as salt marsh, brackish water, riverbanks, and various zones of lakes and ponds, and bogs. The monocots represent numerous genera in various families such as Poaceae, Cyperaceae, Juncaceae, and Typhaceae. The reported species are in various stages of micropropagation and *Arundo donax* is scaled for mass propagation for selecting elite lines for pytoremediation.

Transfer of key genes for mercury phytoremediation into the salt marsh cordgrass (*Spartina alterniflora)* is also reported here. All but one transgenic lines contained both the organomercurial lyase (*merB*) and mercuric reductase (*merA*) sequences showing that co-introduction into *Spartina* of two genes from separate *Agrobacterium* strains is possible.

Key words: Cell Culture, Mercury, Phytoremediation, *Spartina alterniflora*

Introduction

"Phytoremediation is the use of plants to partially or substantially remediate pollutants in contaminated soil, sludge, sediment ground water, surface water and waste water" according to the Environmental Protection Agency of the USA, encompassing a number of methods for the degradation (phyto- and rhizodegradation), removal (phytoextraction, rhizofiltration and phytovolatilization), or immobilization (hydraulic control and phytostabilization) of contaminants.

Phytoremediation of wetlands is a sensitive issue because the ecological balance and species diversity is supposed to be retained. Remediation cannot be left to one all-purpose wetland species. Instead, tissue culture and transgenic technology needs to be developed for several species from each habitat concerned. Our laboratory has initiated a program for developing such technologies for select species from various wetlands, including salt marshes, brackish water, riverbanks, and various zones of lakes and ponds, and bogs.

The monocots represent numerous genera in various families such as Poaceae, Cyperaceae, Juncaceae, and Typhaceae. Whether used as ornamentals, sources of energy, or as useful vehicles to carry out industrial processes such as phytoremediation, such grass-like plants are important. Accordingly, it would be useful to be able to provide a method by which such plants could be propagated even in areas in which plants of these genera are sterile and in a manner that would require shorter time, less effort and less area than conventional methods. It would also be useful if the method is independent of seasons and sustainable at a high rate of propagation. Furthermore, it would be useful if a method could be provided that permitted better genetic manipulation of wetland grasses for enhanced remediation ability.

Environmentally important plants were neglected by plant breeders, however, their remediation potential has also to be explored. Introduction of key genes to increase the remediation ability is an obvious approach and significant progress has been made in recent years in producing genetically modified plants from several plant species. However, a dependable procedure for tissue culture and plant regeneration is a prerequisite for gene transfer.

Plant regeneration from cultured cells of the great majority of monocot (mostly graminaceous) species that have been reported so far, is achieved

0939–5075/2005/0300–0285 $ 06.00

from callus initiated on high concentrations of a strong auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Conger, 1995). Induction of regenerable tissue cultures from monocot species is conventionally attempted from immature embryos or immature inflorescences. Both approaches have been shown to work on *Typha glauca, T. angustifolia* and *T. latifolia* (Rogers *et al.*, 1998). No report of tissue culturing of the fourth North American species, *T. dominguensis*, has been found. Tissue culture of only one *Juncus* species has been reported (Sarma and Rogers, 1998), and no reports of regenerable tissue cultures have been found for species of the grass, *Erianthus giganteus*, sedges of the genera *Cyperus* and *Carex*, and bulrushes of the genus *Scirpus* and *Schoenoplectus*.

Here we report on *in vitro* culture methods for forty-eight wetland monocot species. One species in particular, *Spartina alterniflora* of the salt marshes of the eastern United States has successfully been transformed with genes of phytoremediation importance. Most pollutants are accumulated in wetlands and the plants there act as 'pumps' for nutrients and metal ions by removing stored elements from the sediment pools. They also can degrade organic pollutants *in planta* or in the rhizosphere by releasing exoenzymes and/or by enhancing the microbial degradation processes. Fast and efficient breeding of these plant species requires dependable procedures for tissue culture and plant regeneration, allowing somaclonal selection and transfer of key genes.

Plants do not have efficient enzymes for breaking down organomercurials and for detoxification of ionic mercury, therefore introduction of organomercurial lyase and mercuric reductase genes into major wetland plants from a bacterial source appears feasible to increase their mercury remediation ability. The function of the mercuric reductase (*merA*) and organomercurial lyase (*merB*) gene constructs has been demonstrated in model species as well as in rice and trees (Rugh *et al.*, 1995, 1998a, 1998b; Heaton *et al.*, 1998, 2003; Bizily *et al.*, 1999, 2000).

Spartina alterniflora is an attractive target species because it is a globally widespread, large, perennial grass, which forms monocultural stands in salt marshes (Mobberley, 1956). Here we report on transferring and co-expression of the *merA* and *merB* genes in *S. alterniflora*. Both genes have been transferred simultaneously to create transgenic lines that are resistant to both organomercurials and ionic mercury salts.

Materials and Methods

Immature inflorescences were collected in Columbia, Charleston, and Hartsville, South Carolina. Explants were disinfected by dilute bleach solution then cultured on primary explant medium DM-8 in the dark at 26–28 °C. DM-8 medium (Czakó and Márton, 2001) contained (in mg l^{-1}, unless indicated otherwise): Murashige and Skoog salts [MS (Murashige and Skoog, 1962), Sigma Fine Chemicals] 4,300; Miller's salt solution [6% (w/v) KH_2PO_4], 3 ml; *myo*-inositol, 100; Vitamix (Márton and Browse, 1991), 2 ml; sucrose, 30,000; supplemented with the plant growth regulators adenine hemisulfate, 400 μM; picloram, 0.12; indole-3-butyric acid, 1; 2,4-dichlorophenoxyacetic acid, 0.5; isopentenyladenine, 0.5; *trans*-zeatin, 0.5; thidiazuron (TDZ) 3.0; and solidified with Phytagel (Sigma Fine Chemicals), 2 g l^{-1}.

Medium II_1-S contained (in mg l^{-1}, unless indicated otherwise): MS salts, 4,300; $(NH_4)_2SO_4$, 200; Miller's salt solution, 3 ml; *myo*-inositol, 200; Vitamix, 2 ml; L-glutamine, 200; sucrose, 30,000; supplemented with the plant growth regulator 2,4-dichlorophenoxyacetic acid, 1; solidified with agar (granulated, Fisher Scientific), 7 g l^{-1}. The pH of all tissue culture media was adjusted to 5.8 before sterilization in a pressure cooker (109 °C, 35 kPa, 25 min).

The secondary medium (*0201*) contained fewer plant growth regulators at reduced levels: 0.2 mg l^{-1} 2,4-D and 0.1 μM TDZ of the plant growth regulators. The tertiary, shoot induction/multiplication medium contained no vitamins or *myo*-inositol and only 0.1 μM TDZ as plant growth regulator. Embryogenic callus culture of *S. alterniflora* was maintained on R4 medium (Utomo *et al.*, 2001). Callus was subcultured every four weeks for maintenance. Regenerated and rooted plants were separated, potted in the greenhouse, and initially kept under plastic wrap cover to help acclimatization.

In the binary vectors pVSTI*merA*pe9 (Rugh *et al.*, 1996) and pVSTI*merB* (Bizily *et al.*, 1999) the coding sequence of *merA* and *merB*, respectively, is under the control of the cauliflower mosaic virus 35S RNA promoter (p35S::*merA*pe9::ter and p35S::*merB*::ter, respectively, Fig. 1), and there is also a kanamycin resistance gene under the control of the nopaline synthase promoter

(pNOS::NptII::ter) in the T-region. Further experimental details have been published elsewhere.

Results and Discussion

In vitro culture and micropropagation

Developing cell culture and micropropagation protocols for a diverse group of wetland monocot species was facilitated by the availability of a universal cell culture initiation medium, which had successfully been used for a great number of species (Czakó and Márton, 2001). The method comprised using immature inflorescences as explants and cultivating the tissue on a primary medium to produce totipotent embryogenic tissue culture, cultivating the embryogenic tissue on a secondary medium to produce regenerating cultures, then sustaining shoot multiplication and production of complete plantlets having roots and shoots of tertiary medium before acclimating the plantlets in soil. Table I lists the species, and the stage of cell culture they are advanced to.

Spartina alterniflora was the first species considered and which has been the most problematic as none of the published protocols for *in vitro* regeneration of *Spartina* species (Croughan *et al.*, 1993; Li and Gallagher, 1995; Li *et al.*, 1996; Wang *et al.*, 2003) afforded a fast growing and friable cell culture from the local, South Carolina ecotype. Immature embryos and seedlings were cultured on Murashige and Skoog salts with 2,4-D from 0.1–1.0 mg/l without success. Amendments were made to the nitrogen, sulfur, and chloride concentrations and to the organic supplements, and the effect of glucose vs. sucrose as carbon sources were compared (data not shown). Only the medium designated as II_1-S supported callus formation. There was no essential difference between callus originated from embryo or seedling because it is the mesocotyl in each case, which formed any callus. The resulting hard, yellow callus grew slowly, doubled its fresh mass in about two months, and often segregated sectors that extruded copious amounts of a clear and very viscous gel. Suspension cultures established from embryo-derived callus invariably turned thick with the viscous substance. Only root regeneration has so far been possible from this callus (not shown).

Immature inflorescences were also used as explants but none of the above media containing only 2,4-D as plant growth regulator supported primary callus formation, and the few clones that were established were similar in nature to those obtained from embryos. A more complex medium, designated DM-8 was tested since it proved very efficient in inducing embryogenic callus from a variety of plants (Czakó and Márton, 2001; Márton and Czakó, 2004). DM-8 supported the formation of primary callus on about 0.05% of immature inflorescence explants. The resulting hard, nodular callus turned green under light, as opposed to callus induced from embryos. Despite its very slow growth (doubled its fresh mass in about three months) it did advance to plantlet regeneration. The plantlets elongated and became vigorous plants after transfer to pots.

Establishment of primary callus from the 47 other species listed in Table I was straightforward. It was preferred that the explant be obtained from

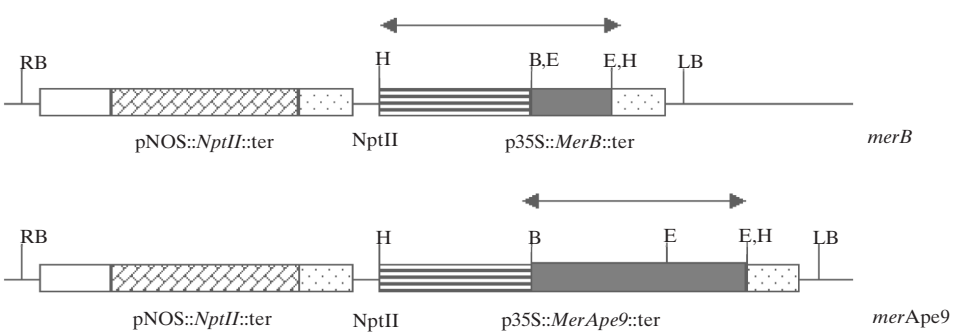

Fig. 1. Functional map of the T-region of the binary vectors used to introduce the bacterial mercury pathway into *Spartina*. Arrows indicate DNA fragments used for nucleic acid hybridizations. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; LB, T-region left border; *merA*pe9, mercuric reductase gene version 9, modified codons (Rugh *et al.*, 1996); *merB*, organomercurial lyase gene coding region; p35S, CAMV 35S RNA promoter; pNOS, nopaline synthase promoter; RB, T-region right border; ter, nopaline synthase gene terminator region.

Table I. Stages of *in vitro* culture of wetland monocots.

Species	Family	Primary callus	Embryogenic callus	Multishoot culture	Acclimatized plants
	Cyperaceae				
Carex acutiformis Ehrh.		+	+	+	+
Carex divisa Huds.		+	+	+	
Carex gracilis Curt.		+	+	+	
Carex hyalinolepis Steud.		+	+		
Carex melanostachya Willd.		+	+	+	
Carex nigra Reichard		+	+	+	
Carex pendula Huds.		+	+	+	
Carex riparia Curtis		+	+	+	
Carex spicata Huds.					
Carex vulpina L.		+	+	+	+
Cladium jamaicense Crantz		+	+	+	
Cyperus giganteus Vahl.		+	+	+	
Cyperus haspan L.		+	+	+	
Cyperus iria L.		+	+	+	
Cyperus pseudovegetus Steud.		+	+		
Cyperus retrorsus Chapm.		+	+	+	+
Eleocharis palustris Roem. & Schult.		+	+		
Eleocharis vivipara Link		+	+	+	+
Lepironia articulata Domin.		+	+	+	
Schoenoplectus californicus Soják		+	+	+	
Schoenoplectus tabernaemontani Palla		+	+	+	+
Scirpus americanus Pers.		+	+		
	Juncaceae				
Juncus articulatus L.		+	+		
Juncus compressus Jacq.		+	+		
Juncus dichotomus Elliott		+	+		
Juncus effusus L.		+	+	+	+
Juncus roemerianus Scheele		+	+	+	
Juncus tenuis Willd.		*	*		
	Poaceae				
Arundo donax L.		+	+	+	+
Erianthus giganteus P. Beauv.		+	+		
Erianthus strictus Baldwin		+	+		
Miscanthus sinensis Andersson		+	+	+	+
Panicum dichotomum L.		+	+		
Paspalum urvillei Steud.		+	+	+	+
Phragmites australis Steud.		+	+	+	+
Setaria gigantea Makino		+	+		
Sorghum halepense Pers.		+			
Spartina alterniflora Loisel		+	+	+	+
Spartina cynosuroides Roth.		+			
Spartina patens Muhl.		+			
Spartina pectinata Link		+			
Spartina spartinae Hitchc.		+			
Vetiveria zizanioides Nash		+	+		
	Typhaceae				
Typha angustifolia L.		+	+		
Typha dominguensis Pers.		+	+		
Typha × *glauca* Godr.		+			
Typha latifolia L.		+	+	+	

an immature inflorescence. The tips of field-grown or greenhouse-grown pre-flowering shoots of Poaceae and Typhaceae with leaf sheaths completely enclosing a developing but yet unemerged immature inflorescence, whose surface has been sterilized, were stripped of the leaves and the inflorescences were cut into cross-sectional pieces, which were then cultivated on a solid-type primary medium containing plant hormones. Primary callus and, in certain species, shoot formation, but not elongation, occur on the primary medium, and so the method is therefore suitable for sustained maintenance and propagation of the totipotent tissue culture.

Gene transfer

Spartina was considered furthermore for *Agrobacterium*-mediated transformation. Six stable geneticin resistant lines were recovered from about 170 g callus. The presence of *merA* and *merB* genes was verified by PCR (not shown) and Southern blotting. All but one (#9) geneticin resistant lines contained both the *merA* and the *merB* sequences as verified by Southern blotting (data not shown). Northern blotting showed that there are differences between levels of individual gene expression between individual transformants. While the exposure levels cannot be used for evaluating relative expression of *merA* and *merB* in an individual transformant, it is clear that #3 showed high expression of *merB* relative to *merA*, whereas #7 shows relatively higher *merA* expression.

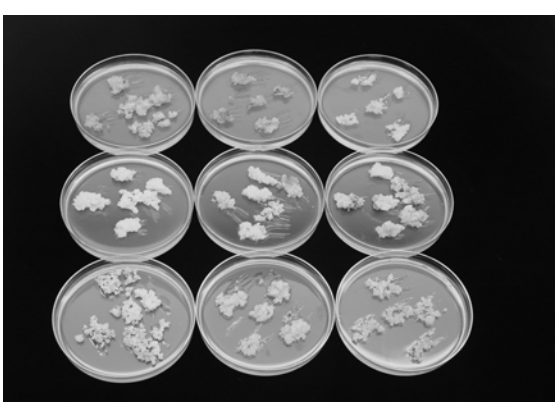

Fig. 2. Growth response of *Spartina* callus on various levels of phenylmercuric acetate. Top row: WT; middle row: #3; bottom row: #7. First column: no PMA; 2nd column: 50 μM; 3rd column: 150 μM.

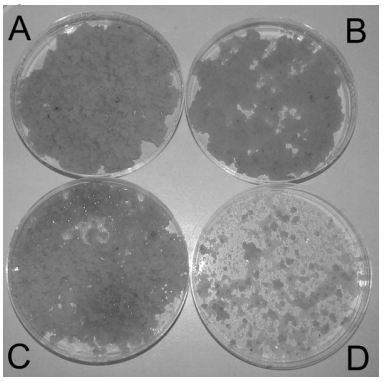

Fig. 3. Hygromycin selection of primary transformants of *Spartina alterniflora* cocultivation with following *Agrobacterium tumefaciens* LBA4404 carrying pCAMBIA1301 (GenBank accession AF234297). (A) Vigorously growing untreated colonies on hygromycin-free medium after four weeks of culture. (B) Untreated cell cultures swell but fail to grow on medium containing hygromycin at 25 mg/l. (C) The cell cultures retain its viability after cocultivation treatment as evidenced by abundant growth on hygromycin-free medium. (D) Selective growth of putative transformants on selective medium.

The steady-state RNA levels appeared to correlate with the phenotype. Line #7 showed the highest resistance to $HgCl_2$ (up to 500 μM), whereas line #3 was the most resistant to phenylmercuric acetate (PMA) (*e.g.* Fig. 2). Wild-type (WT) callus is sensitive to PMA at 50 μM and to $HgCl_2$ at 225 μM.

The suitability of this cell culture for hygromycin selection was also demonstrated. Hygromycin at 25 mg/l provided stringent selection against normal cells (Fig. 3) after cocultivation with *Agrobacterium* harboring a binary vector carrying a hygromycin selectable marker gene.

It was established that it is feasible to create transgenic *S. alterniflora*. All but one geneticin resistant line contained both the *merA* and the *merB* genes proving that co-introduction of two genes from separate *Agrobacterium* strains is possible although the overall frequency of transformation is low. The significance of this fact is that essentially the complete mercury detoxification pathway is incorporated into an ecologically important species in one step. As organic mercury is likely to form in inorganic mercury contaminated sites it is very advantageous to have a transgenic plant that have the capacity to deal with not only mercuric salts but organic forms as well. Organomercurials are converted to much less toxic inorganic mercury,

which in turn is volatilized by the transgenic plant (Meagher, 2000).

S. alterniflora's natural capacities for pollutant remediation have been evaluated before. The chemical similarity of selenium to sulfur allows Se to enter the DMSP pathway and this is the basis of *S. alterniflora's* potential use for abatement of selenium pollution by phytovolatilization (Ansede *et al.*, 1999; Meagher, 2000). *S. alterniflora* also has tolerance to certain man-made organic pollutants. It is uniquely adapted to the salt marsh which is impacted both by halocompounds elaborated by marine organisms and also man-made halogenated organics (Sanger *et al.*, 1999).

We began to assess *Spartina's* natural biodegradation activity towards 2,4,6-trichlorophenol (TCP) and trichloroethene (TCE) (Márton *et al.*, 2000), respectively, in hydroponic experiments. It is able to break down and detoxify various halogenated organic contaminants among them the notorious TCE and halogenated phenols (Márton *et al.*, 2000). Data for TCE collected in a stationary (*i.e.* zero flow rate) model with *Spartina* (Bejarano, 2000) showed that as high as 0.88 mM TCE was tolerated after an initial decrease in transpiration rate for 4 d followed by a complete recovery. Repeated later in 40 l containers, the initial 'shock' effect was confirmed, and it was shown that the microflora has an important contribution, which needs to be assessed.

Hydroponic experiments with TCP showed that *Spartina* tolerates as much as 10 mg/l, the overall rate was 0.8 mg TCP/(day plant) sufficient to reduce TCP concentration to zero by day 30. However, initial rates can be as high as 135 mg/l d suggesting that a flow through bioreactor would be very efficient in TCP consumption. Apparently,

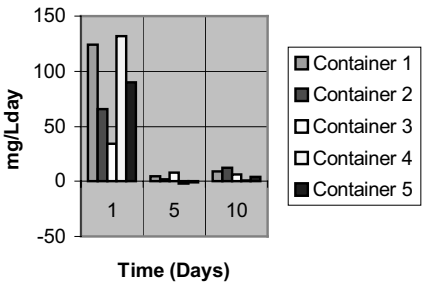

Fig. 4. Conversion rate of 2,4,6-trichlorophenol in hydroponic cultures of *Spartina alterniflora*.

the rate of metabolism peaks again 10 d later indicating induction of *de novo* gene activity (Fig. 4).

While *Spartina alterniflora* is native to the salt marshes of the eastern coasts of the Americas, it grows best under non-saline conditions when competition is absent. It has successfully been introduced into temperate and subtropical (sometimes tropical) regions of all continents (Chung, 1989). Therefore, the potential areas of application include nearly all wetlands globally.

With the availability of embryogenic cell cultures and plant regeneration systems for a great number of other wetland species, it is expected that transgenic plants of other species listed in Table I can be generated.

Acknowledgements

The Louisiana ecotype of *S. alterniflora* was kindly provided by Drs. H. Utomo and T. Croughan (Rice Research Station, Crowley, LA). This research was supported by grants from the South Carolina Sea Grant Consortium. The *merA* and *merB* gene constructs were kindly provided by Dr. R. Meagher (University of Georgia, Athens, GA).

Ansede J. H., Pellechia P. J., and Yoch D. C. (1999), Selenium biotransformation by the salt marsh cordgrass *Spartina alterniflora*: Evidence for dimethylselenoniopropionate formation. Environ. Sci. Technol. **33**, 2064–2069.

Bejarano A. (2000), Uptake and fate of trichloroethylene (TCE) in *Spartina alterniflora* Loisel. MS Thesis. The University of South Carolina, Columbia.

Bizily S. P., Rugh C. L., Summers A. O., and Meagher R. B. (1999), Phytoremediation of methylmercury pollution: merB expression in *Arabidopsis thaliana* confers resistance to organomercurials. Proc. Natl. Acad. Sci. USA **96**, 6808–6813.

Bizily S. P., Rugh C. L., and Meagher R. B. (2000), Phytodetoxification of hazardous organomercurials by genetically engineered plants. Nat. Biotechnol. **18**, 213–217.

Chung C. H. (1989), Ecological engineering of coastlines with saltmarsh plantations. In: Ecological Engineering: an Introduction to Ecotechnology (Mitsch W. J. and Jorgensen S. E., eds.). Wiley and Sons, New York, pp. 255–289.

Conger B. V. and Kuklin A. I. (1995), *In vitro* culture and plant regeneration in gramineous crops. In: Current Issues in Plant Molecular and Cellular Biology (Terzi M., Cella R., and Falavigna A., eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 59–68.

Croughan T. P., Cao H. X., Regan R. P., and Meche M. M. (1993), Biotechnology applications to coastal erosion control. Annu. Res. Rep. LA State Univ. Baton Rouge **85**, 157–160.

Czakó M. and Márton L. (2001), A heartwood pigment in *Dalbergia* cell cultures. Phytochemistry **57**, 1013–1022.

Heaton A. C. P., Rugh C. L., Wang N. J., and Meagher R. B. (1998), Phytoremediation of mercury- and methylmercury-polluted soils using genetically engineered plants. J. Soil Contam. **7**, 497–509.

Heaton A. C. P., Rugh C. L., Kim T., Wang N. J., and Meagher R. B. (2003), Toward detoxifying mercury-polluted aquatic sediments with rice genetically engineered for mercury resistance. Environ. Toxicol. Chem. **22**, 2940–2947.

Li X. G. and Gallagher J. L. (1996), Tissue culture and plant regeneration of big cordgrass, *Spartina cynosuroides*: Implications for wetland restoration. Wetlands **16**, 410–415.

Li X. G., Seliskar D. M., Moga J. A., and Gallagher J. L. (1995), Plant regeneration from callus-cultures of saltmarsh hay, *Spartina patens*, and its cellular-based salt tolerance. Aquat. Bot. **51**, 103–113.

Márton L. and Browse J. (1991), Facile transformation of *Arabidopsis*. Plant Cell Rep. **10**, 235–239.

Márton L. and Czakó M. (2004), Sustained totipotent culture of selected monocot genera. U.S. Patent No. 6,821,782 B2.

Márton L., Chen Y. P., and Czakó M. (2000), Method for decomposing toxic organic pollutants. US Patent No 6,087,547.

Meagher R. B. (2000), Phytoremediation of toxic elemental and organic pollutants. Curr. Opin. Plant Biol. **3**, 153–162.

Mobberley D. (1956), Taxonomy and distribution of the genus *Spartina*. Iowa State Coll. J. Sci. **30**, 471–574.

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

Rogers S. D., Beech J., and Sarma K. S. (1998), Shoot regeneration and plant acclimatization of the wetland monocot cattail (*Typha latifolia*). Plant Cell Rep. **18**, 71–75.

Rugh C. L., Wilde H. D., Merkle S. A., and Meagher R. B. (1995), Genetic transformation of *Arabidopsis thaliana* with an engineered bacterial gene for mercuric reductase. Plant Physiol. **108**, 23–23.

Rugh C. L., Wilde H. D., Stack N. M., Thompson D. M., Summers A. O., and Meagher R. B. (1996), Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial merA gene. Proc. Natl. Acad. Sci. USA **93**, 3182–3187.

Rugh C. L., Gragson G. M., Meagher R. B., and Merkle S. A. (1998a), Toxic mercury reduction and remediation using transgenic plants with a modified bacterial gene. Hortscience **33**, 618–621.

Rugh C. L., Senecoff J. F., Meagher R. B., and Merkle S. A. (1998b), Development of transgenic yellow poplar for mercury phytoremediation. Nat. Biotechnol. **16**, 925–928.

Sanger D. M., Holland A. E., and Scott G. I. (1999), Tidal creek and salt marsh sediments in South Carolina coastal estuaries: I. Distribution of trace metals. Arch. Environ. Contam. Tox. **37**, 445–457.

Sarma K. S. and Rogers S. M. D. (1998), Plant regeneration and multiplication of the emergent wetland monocot *Juncus acuminatus*. Plant Cell Rep. **17**, 656–660.

Utomo H. S., Wenefrida I., and Croughan T. P. (2001), Smooth cordgrass synthetic seeds: Production, storage and potential use for coastal erosion controls. TurfGrass Trends **10**, 12–15.

Wang J. B., Seliskar D. M., and Gallagher J. L. (2003), Tissue culture and plant regeneration of *Spartina alterniflora*: Implications for wetland restoration. Wetlands **23**, 386–393.