Mercury pollution is a worldwide environmental problem with tens of thousands of contaminated sites. Phytoremediation is an environmentally friendly solution relying on long-lived plants like trees to clean contaminated soil, water and air. To explore the potential of transgenic hybrid hardwood trees to clean mercury pollutants, we first established efficient somatic embryogenesis systems for hybrid yellow-poplar (Liriodendron tulipifera × L. chinense) and hybrid sweetgum (Liquidambar styraciflua × L. formosana). Embryogenic cultures of both hybrids, consisting of proembryogenic masses (PEMs), were initiated from immature hybrid seeds on induction-maintenance medium (IMM) supplemented with plant growth regulators (PGRs) and casein hydrolysate (CH). For hybrid yellow-poplar, a high number of germinable embryos were produced when PEMs were grown in liquid IMM lacking CH, followed by size fractionation and plating on semisolid embryo development medium without CH, but with abscisic acid. For hybrid sweetgum, a high number of germinable embryos were produced on development medium without any PGRs or other supplements after PEMs
were grown in liquid IMM without CH, but with glutamine, asparagine and arginine. Using *Agrobacterium*-mediated gene transfer, transgenic hybrid sweetgum lines were generated either overexpressing a \( \gamma \)-glutamylcysteine synthetase gene (\( \gamma \)-ECS), or expressing modified mercuric ion reductase genes (\( \textit{merA} \)). Hybrid sweetgum PEMs transformed with \( \gamma \)-ECS driven by an actin promoter (ACT2) were able to grow in the presence of 50 \( \mu \)M HgCl\(_2\), which inhibited wild-type PEMs. However, abnormal plantlets were regenerated from the \( \gamma \)-ECS PEMs and they did not survive for more than a few weeks following germination. Mature somatic embryos generated from \( 35S: \textit{merA}9 \) and \( 35S: \textit{merA}18 \) PEMs were capable of conversion to normal plantlets on germination medium containing 25 \( \mu \)M HgCl\(_2\), by converting ionic mercury into its volatile, elemental form, Hg(0). Somatic embryos generated from \( \textit{ACT2:merA}77 \) PEMs converted to plantlets, but these remained unhealthy on Hg(II)-medium. Transgenic \( \textit{merA} \) plantlets released Hg(0) 2-3 times more efficiently than the wild-type plantlets. These results indicate that the \( \gamma \)-ECS gene may significantly affect the regeneration capacity of hybrid sweetgum PEMs and the growth of the plants. Hybrid sweetgum plants expressing modified \( \textit{merA} \) genes driven by a 35S promoter may provide a means for phytoremediation of mercury pollution.

INDEX WORDS: Phytoremediation, Somatic embryogenesis, Hybrid yellow-poplar, Hybrid sweetgum, Transgenic plants, Ionic mercury, Phytochelatin, Gamma glutamylcysteine synthetase, \( \gamma \)-ECS, Mercuric ion reductase, \( \textit{MerA} \), Sweetgum, Yellow-poplar
ENHANCING THE PRODUCTIVITY OF HYBRID HARDWOOD EMBRYOGENIC
CULTURES AND GENETIC TRANSFORMATION OF HYBRID SWEETGUM FOR
MERCURY PHYTOREMEDIATION

by

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To my beloved parents and wife
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW
The potential of tree biotechnology to increase the productivity of forestry industry and to provide solutions to environmental problems is tremendous, ranging from in vitro mass clonal propagation of the top commercial trees to detoxification of industrial waste (Mullin and Bertrand, 1998). Hybrid vigor (defined as the increase in size, yield, vigor or survival advantage of offspring over parents) has been widely used as a means of improving crop productivity (Shull, 1952; Cantrell, 1998). For most forest species, the major obstacle to the use of hybrids in operational forestry has been an inability to produce them easily and economically (Zobel and Talbert, 1984). Although the efficiency of in vitro propagation for many species still needs to be increased for genetic transformation and commercial mass propagation, in vitro propagation is under development and employed in some species for mass propagation of superior hybrids (Ahuja, 1987). The use of recombinant DNA technologies to introduce novel traits raises many possibilities to greatly expand the available gene pool and enhance genetic gain. To date, the number of actual field releases of genetically transformed trees is still quite small (Mullin and Bertrand, 1998), mainly because of the lack of efficient in vitro manipulation and efficient transformation systems, the limited availability of suitable target genes and the long life cycles of tree species. However, efficient genetic transformation systems have been developed in the past decade for some conifers and hardwoods, such as spruce and poplar (Wenck et al., 1999; Han et al., 2000). Phytoremediation, the use of plants to clean up toxic wastes, is an idea attracting increasing attention of scientists, industry and government (Stomp et al., 1993). Recently, it was reported that transgenic Indian mustard seedlings (Zhu et al., 1999b) and hybrid poplar trees (Gullner et al., 2001) expressing the phytoremediation gene gama glutamyl
cysteine synthetase (γ-ECS) increased biosynthesis of glutathione (GSH) and phytochelatins (PCs), which in turn enhanced cadmium (Cd) tolerance and accumulation. The objectives of this research were to improve somatic embryogenesis systems for hybrid yellow-poplar (*Liriodendron tulipifera* X *L. chinense*) and hybrid sweetgum (*Liquidambar styraciflua* X *L. formosana*) and to introduce the phytoremediation genes γ-ECS and merA into hybrid sweetgum using genetic engineering techniques.

**Hybrid yellow-poplar**

The genus *Liriodendron* comprises two species, *L. tulipifera* (yellow-poplar) and *L. chinense* (Chinese tuliptree). Yellow-poplar is one of the largest and most valuable hardwood trees in the eastern U.S., characterized by straight form, rapid height growth and desirable wood quality (Harlow et al., 1996; Russell, 1977). Tree improvement programs for yellow-poplar have lagged, since few seed orchards have been established in the southeastern U.S.. Chinese tuliptree is a medium to large tree native to China and Vietnam. Although the size, shape and coloration of the flower can be used to distinguish between the two species (Santamour, 1972a), Chinese tuliptree is morphologically similar to yellow-poplar. The two species have been separated for 10-16 million years by continental drift (Parks and Wendel, 1990).

The Chinese and American *Liriodendron* are still interfertile. Interspecific crosses between these two species were first reported in 1972 (Santamour, 1972a). Parks et al. (1983) reported that seedling-derived interspecific hybrids had significantly greater biomass than intraspecific hybrids or open pollinated seedlings. Although *Liriodendron* hybrid seedlings have been produced, large-scale production of hybrid seedlings is impossible because very few reproductively mature *L. chinense* parents were available in
North America (Merkle et al., 1993). Several vegetative propagation methods have been reported for yellow-poplar, including rooted cuttings, root cuttings and grafting. Root cuttings and grafting are labor-intensive and therefore expensive for operational use (Merkle, 1995). While stem cuttings were very difficult to root, 70% success can be obtained using cuttings taken from stump sprouts or epicormic shoots (Kormanik and Porterfield, 1966; McAlpine, 1964). However, the number of propagules produced by this method will be limited by the availability of the source hybrids because they must be sacrificed to obtain the stump sprouts or epicormic shoots.

Yellow-poplar has been efficiently regenerated in vitro via somatic embryogenesis (Merkle and Sommer, 1991; Merkle, 1995), an asexual developmental process leading to the differentiation of embryo-like structures from somatic cells. In this system, immature zygotic embryos (8 weeks post-pollination) are the most effective tissue for initiation of cultures. Proembryogenic masses (PEMs) are produced after culturing for 1-2 months on induction-maintainance medium (IMM) containing 2,4-dichlorophenoxyacetic acid (2,4-D), benzyladenine (BA) and casein hydrolysate (CH). PEMs can be grown in liquid IMM (suspension culture), size-fractionated and plated on basal medium (IMM without 2,4-D and BA) for large-scale production of mature embryos. Mature embryos convert to plantlets at a high frequency on basal medium lacking CH.

The application of this system to hybrid *Liriodendron* has also been tried (Merkle et al., 1993). The hybrid embryos produce PEMs and globular stage embryos on semisolid IMM. However, PEMs or globular stage embryos in liquid IMM grew slowly and proliferated as clusters of globular embryos. Collecting and plating them on basal medium only resulted in the production of callus that could not form mature embryos.
Since the hybrid cultures were not amenable to suspension culture and plating manipulation, the multiplication rate of hybrid cultures could not approach the level reported for embryogenic yellow-poplar cultures (up to 950 somatic embryos g\(^{-1}\) PEMs and 710 plantlets g\(^{-1}\) PEMs) (Merkle and Sommer, 1991). Therefore, the system needed to be improved for hybrid *Liriodendron*. Even with these problems, over 100 hybrid *Liriodendron* plantlets have already been regenerated using hybrid cultures on semisolid IMM. Plantlets showed rapid early growth, with leaves 2-3 times greater than the diameter of typical *L. tulipifera* leaves (Merkle et al., 1993).

**Hybrid sweetgum**

A fast-growing tree, sweetgum (*Liquidambar styraciflua L.*) is a widespread hardwood that develops best on rich, moisture alluvial soils in the southeastern U.S., where it is an important commercial species used for furniture, plywood and pulp. The tree possesses ornamental value and is an important source of storax gum, used in perfume and in the pharmaceutical industry (Harlow et al., 1996; Johnson, 1985). Like yellow-poplar, sweetgum also has counterparts native to eastern Asia that have been separated by continental drift for at least 10 million years (Parks and Wendel, 1990). The Formosan sweetgum (*L. formosana* Hance) has leaves with 3 lobes, whereas *L. styraciflua* has leaves with 5 lobes. Interspecific hybridization between the two species was first reported by Santamour (Santamour, 1972b) and the hybrids grew well in a planting (Vendrame et al., 2001). As has been the case with yellow-poplar, genetic improvement programs for sweetgum have lagged. The creation of hybrid sweetgum may generate superior hybrids for rapid fiber and biomass production.
There are more published reports on in vitro propagation of sweetgum than any other major southern hardwood. Sweetgum has been regenerated in vitro via adventitious buds from hypocotyl segment (Sommer, 1981), juvenile shoot tips, and mature leaves and petiole segments (Brand and Lineberger, 1988). An axillary shoot multiplication method was successfully applied to propagate selected genotypes for operational production of sweetgum at Union Camp Corporation. In this method, shoot cultures developed from axillary buds in on woody plant medium (WPM, Lloyd and McCown, 1980) containing BA and naphthaleneacetic acid (NAA Sutter and Baker, 1985). Nodule culture systems have been described for sweetgum. In one system, nodule cultures were established from young leaf discs on WPM with BA and NAA (Chen and Stomp, 1991). Shoot formation only occurred in medium with BA alone and root formation was best in medium containing only NAA. In another system, nodule cultures were initiated from seedling hypocotyls and proliferated in liquid modified Blaydes’ medium (Witham et al., 1971) containing thidiazuron (TDZ) and 2,4-D. Shoots differentiated in medium with TDZ and were rooted ex vitro (Kim et al., 1999).

Sweetgum was first regenerated via somatic embryogenesis from seedling hypocotyl-derived callus (Sommer and Brown, 1980). Sweetgum has also been regenerated from seed embryos (Merkle et al., 1998) following the same protocol described for yellow-poplar, except that no liquid medium, size fractionation and plating manipulation were involved. Regeneration of sweetgum from staminate and pistillate inflorescences via somatic embryogenesis has also been achieved (Merkle et al., 1998; Merkle and Battle, 2000). TDZ was found to be effective in inducing embryogenic cultures from both seed explants and inflorescences, while 2,4-D was only effective in
inducing cultures from seed explants. Recently, following the same protocol, hybrid sweetgum embryogenic cultures were regenerated from hybrid seed embryos (Vendrame et al., 2001). In these studies, sweetgum and hybrid sweetgum embryogenic cultures were proliferated via repetitive somatic embryogenesis, in which secondary embryos were generated from primary embryos. For germination, extra work was required to separate the clusters of repetitive embryos. Damage may occur during the separation operation, which in turn affects embryo germination and conversion. Therefore, in order to further explore the potential value of embryogenic cultures for hybrid sweetgum propagation or to use the hybrid sweetgum embryogenic cultures as target tissue for genetic transformation, it is highly desirable to improve the somatic embryogenesis system to produce singularized and synchronous embryos.

**Genetic transformation of sweetgum**

Although the production of transgenic plants has become a routine for many agricultural crops, research on the genetic transformation of trees has still focused on the gene transfer and regeneration techniques. After a decade’s efforts, efficient gene transfer systems have been developed for a number of conifers (especially *Picea, Pinus* and *Larix*) and hardwoods (especially *Populus, Populus* hybrids and *Eucalyptus*).

While many transformation reports in conifers involve studying the expression of reporter gene (GUS, β-glucuronidase) to optimize the systems either through biolistic or *Agrobacterium*-mediated gene transfer, a few genes conferring enhanced fitness traits have been transferred into several conifers. Using embryogenic cultures and biolistic gene transfer, *Picea glauca* was stably transformed with a *Bacillus thuringiensis* (B.t.) endotoxin gene conferring insect resistance (Ellis et al., 1993). *Pinus radiata* (’Bishop-
Hurley et al., 2001) and *Picea abies* (Brukin et al., 2000; Bishop-Hurley et al., 2001) were engineered with a herbicide (phosphinothricin/glufosinate)-resistance gene (*bar*). Whereas *Larix decidua* was transformed with an insect-resistance gene (B.t. gene) and herbicide (glyphosate)-resistance gene (*aroA*) through co-cultivation of seedling hypocotyls with *Agrobacterium rhizogenes* (Shin et al., 1994).

Among hardwoods, *Populus* species and hybrids provide models for molecular biology and genetic transformation studies. While biolistic gene transfer approaches have been developed for some poplars, leaf disc transformation and regeneration via *Agrobacterium* infection have become the most common gene transfer method (Mullin and Bertrand, 1998). With their ease of transformation, *Populus* species and hybrids have been transformed with a variety of genes conferring different fitness functions, such as pest resistance [e.g. B.t. gene (McCown et al., 1991); Proteinase inhibitors (Klopfenstein et al., 1993)], air-pollutant resistance [glutathione reductase (Saori Endo, 1997)], herbicide [e.g. glyphosate (Fillatti et al., 1987); glufosinate (DeBlock, 1990)]-resistance and crown gall disease-resistance (Ebinuma et al., 1991). More strikingly, modification of wood quality has been reported by genetic manipulation of the lignin biosynthesis pathway. Hybrid poplar genetically transformed with antisense cinnamyl alcohol dehydrogenase (CAD) showed a modest reduction of Klason lignin and superior pulping characteristics (Lapierre et al., 1999). Repression of lignin biosynthesis promoting cellulose accumulation and growth was observed in transgenic hybrid quaking aspen expressing antisense a 4-coumarate:CoA ligase gene (Hu et al., 1999). Another top commercial hardwood genus, *Eucalyptus*, has also been transformed with the GUS gene.
via co-cultivation with *Agrobacterium* by two groups (Mullins et al., 1997; Ho et al., 1998).

Both biological and biolistic gene transformation have been reported for sweetgum to date. The first transgenic sweetgum plants were produced using *Agrobacterium tumefaciens*-mediated gene transfer (Chen and Stomp, 1991). Transformation was accomplished by co-cultivation of leaf pieces or nodules with disarmed binary *Agrobacterium tumefaciens* (pBI121 in C58z707), harboring sequences encoding GUS and neomycin phosphotransferase (NPT II). Selection of transformed cells was carried out in liquid selective WPM containing 40 mg/L kanamycin sulfate in addition to 500mg/L each of carbenecillin and cefotaxime. Also using *Agrobacterium tumefaciens*, NPT II and either a chimeric B.t. toxin gene, a chimeric GUS, or a chimeric tobacco anionic peroxidase gene were introduced into sweetgum. Co-cultivated leaf tissues growing on non-selective WPM containing only cefotaxime were allowed to generate callus before transfer to selective medium containing 25 mg/L kanamycin (Sullivan and Lagrimini, 1993). Leaves of transgenic sweetgum expressing a tobacco anionic peroxidase were generally more resistant to feeding by caterpillars and beetles than wild-type leaves (Dowd et al., 1998). Transformation of sweetgum has also been achieved by microprojectile bombardment of nodule cultures (Kim et al., 1999). Shoots differentiated from nodules transformed with CaMV35S-HPH and CaMV35-GUS gene fusion in the presence of hygromycin B. On medium lacking hygromycin B, shoots elongated and displayed GUS activity in their expanding leaves and the stems.
**Improvement of somatic embryogenesis**

Somatic embryogenesis has been described as having the best potential to rapidly capture the benefits of breeding programs and to improve raw material uniformity. Embryogenic cultures are also regarded as ideal target materials for genetic transformation. Therefore, many forest products companies in the world recognize that somatic embryogenesis will be a key feature of future tree improvement programs. Large-scale production of somatic embryos for operational use is occurring for some spruce and firs (Merkle and Trigiano, 1994; Sutton et al., 1997).

Somatic embryogenesis is affected by a number of factors, including explant type (materials used for culture initiation), genotype, culture medium, and culture conditions. Auxin is the most important factor for regulation of induction and development of embryogenesis (Bhojwani et al., 1999). Many somatic embryogenic systems use a synthetic auxin for induction of somatic embryogenesis, followed by transfer to an auxin-free medium for embryo differentiation. Nitrogen source, polyamines, amino acids and other supplements, carbon source, osmotic treatment, abscisic acid, medium pH, synchronization of embryo development and gelling agent, have also been described as important factors in embryogenesis (George, 1993; George, 1993/1996). Because embryogenic competence varies with explant and genotype, specific requirements for medium composition and culture conditions are genotype-dependent and explant-dependent (Bhojwani et al., 1999). For many tree species, the efficiency of somatic embryogenesis is still too low for commercial application or genetic engineering. This is the case for hybrid yellow-poplar and hybrid sweetgum using the *L. tulipifera* embryogenic protocol. Process improvement for tissue culture systems is often the result
of literature-based empirical trial and error (Pullman et al., 1998). All the culture variables mentioned above could be tested and modified to optimize the somatic embryogenesis for hybrid yellow-poplar and hybrid sweetgum. Studying natural embryo development to mimic the gene expression patterns (Pullman et al., 1998) and the hormonal (Kapik, 1995), nutritional and physical (Pullman, 1997) conditions found in vivo or understanding how medium changes over time, such as activated carbon adsorption and pH effects, can further protocol development.

**Phytoremediation of toxic metals**

Agriculture, mining, smelting, electroplating and other industrial activities have resulted in the disposition of undesirable concentrations of metals such as aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), selenium (Se) and zinc (Zn) and radio-nuclides such cesium (Ce), strontium (Sm) and uranium (U), which poses a major environmental and human problem (Salt et al., 1998). Current clean-up methods involving the relocation or intensive on-site treatment of large quantity of soils and sediment, costing $150-$350 per ton (Ensley, 2000), are expensive and environmentally destructive. Phytoremediation, the new evolving technology of using plants to remove, sequester, or detoxify pollutants, is considered to be a cost-effective and environmentally friendly remediation approach for various pollutants. The consensus estimate for the cost of phytoremediation of soil at $25-$100 per ton, is much cheaper than current methods. The cost of phytoremediation of water is likely to be in the range of $0.6-$6.00 per 1000 gal treated (Glass, 2000). Furthermore, this approach can be a permanent treatment solution (i.e., permanently removing the contaminants from the sites) and is ecologically responsible, because it reclaims the soil *in situ* without unduly
disturbing the sites or burying the contaminants somewhere else. These advantages make phytoremediation attract the interest of site owners, regulators and the environmental engineering community (Stomp, 1993).

Phytoremediation involves multiple biological processes in the plant, including metal uptake by the roots, metal transport and translocation from roots to shoots, and accumulation or transformation in the plant cells. Based on which biological process plays a pivotal role, phytoremediation of toxic elemental pollutants can be classified into three strategies (Meagher, 2000). The first strategy is phytoextraction, in which plants are used to transport and concentrate metals to above-ground shoots for later harvest; the second is phytotransformation, in which plants transform metals to less toxic species or to volatile species and volatize them from the foliage; the last strategy is phyto-stabilization, in which plants stabilize metals in roots to prevent leaching from the site.

The efficiency of these biological processes can be elevated either by silvicultural practices, plant species screening or by genetic transformation. Factors in soil such as pH, redox potential, soil type, cation exchange capacity, metal concentration, microbial flora, and mycorrhizal status, influence metal bioavailability and uptake by roots. The solubility and transport of many heavy metals into roots is increased in acidic soils. Most organic or synthetic chelators increase metal ion uptake and translocation. For instance, application of ethylene diamine tetra-acetic acid (EDTA) to lead contaminated soil resulted in rapid accumulation of the lead-EDTA complexes in shoot tissues (Vassil et al., 1998). Plants with large root surface area take up toxins from soil and water more efficiently. Recent studies indicated that metal uptake is controlled by root transport systems. Putative plasma membrane copper (COPT1), iron (IRT1), and zinc (ZIP1, ZIP2, ZIP3)
transporters have been cloned from *Arabidopsis thaliana* (Kampfenkel et al., 1995; Edie et al., 1996; Gortz et al., 1998). The data suggest that it is possible to genetically manipulate metal ion transport systems in order to promote phytoremediation of heavy metals (Meagher, 2000).

The transformation of metals into relatively harmless form is one of the molecular mechanisms of phytoremediation. Elements such as As, Hg, Fe, Se and Cr can exist as either cationic or oxyanionic species. They also can exist as thio- or organo-metallics. These forms vary widely in their toxicity to human and animals (Meagher, 2000). Selenium is most toxic when incorporated into amino acid analogues and least toxic as volatile dimethylselenide. In *Astragalus*, selenium was hyperaccumulated with incorporation in proteins (Pilon-Smits et al., 1999). Alternatively, selenate can be converted into dimethylselenide, volatilizing from leaves and roots (Terry and Zayed, 1994). Methylmercury (MeHg) is the most toxic natural form of mercury and is biomagnified efficiently in the food chain. Mercury is less toxic as ionic mercury and least toxic as reduced, volatile metallic mercury. The bacterial enzymes *merB* and *merA* catalyze the detoxification of methyl and ionic mercury. Diverse plant species expressing *merA* were resistant to at least 10 times greater concentrations of Hg(II) than those that kill wild-type controls (Rugh et al., 1998; Rugh et al., 2000). Plant expressing both *merA* and *merB* showed significantly higher tolerance of MeHg than controls by converting MeHg to volatile mercury, and significantly higher tolerance of MeHg than *merB* plants (Bizily et al., 1999; Bizily et al., 2000).

Another very important molecular mechanism of phytoremediation is the chelation and sequestration of heavy metals by particular ligands. Two major classes of heavy
metal chelating peptides are known to exist in plants—metallothioneins (MTs) and phytochelatins (PCs) (Cobbett and Goldsbrough, 2002). Although the role of MTs in protection against Cd and Zn is unequivocally established in mammals, the function of MTs in plants is still inconclusive. The role of PCs in detoxification of some heavy metals, particularly Cd, is clearly established (Cobbett and Goldsbrough, 2002). Metals such as Ag(I), AsO$_3$(-III), Cd(II), Zn(II), Cu(II), Hg(II), and Ni(II) are sequestered by bonding with organic-sulfur (R-SH) on the cysteine residues of these peptides (Meagher, 2000). And these complexes might be confined in the vacuoles (Cobbett and Goldsbrough, 2002). While MTs are gene-encoded polypeptides, PCs, with the structure $(\gamma$-Glu-Cys)$_n$-Gly, are enzymatically synthesized peptides, from glutathione (GSH). GSH plays several roles in heavy metal tolerance and detoxification. It protects cells from oxidative stress damage such as that caused by heavy metals, and more importantly, it is the direct precursor of PCs (Noctor et al., 1998). GSH is synthesized from glutamate (Glu) and cysteine (Cys) in two sequential, ATP-dependent enzymatic reactions, catalyzed by gamma glutamyl cysteine synthetase ($\gamma$-ECS) and glutathione synthetase (GS), respectively. PC synthase (PS) catalyzes the elongation of $(\gamma$-Glu-Cys)$_n$ to form PCs. Studies showed that the $\gamma$-ECS is the rate-limiting for synthesis of GSH and PCs (Noctor et al., 1998; Zhu et al., 1999b), and that overexpression of $\gamma$-ECS or GS increased the production of GSH and PCs, which in turn enhanced the Cd tolerance and accumulation in Indian mustard (Zhu et al., 1999a; Zhu et al., 1999b). Other than MTs and PCs, plants also use organic acids for metal homeostasis and tolerance. For instance, the free amino acid histidine was responsible for nickel (Ni) tolerance and
hyperaccumulation in the genus *Alyssum* (Krämer et al., 1996). It was suggested that the manipulation of GSH/PC or other chelators’ concentrations through overexpression of these genes has significant potential for increasing the accumulation of toxic metals by plants (Noctor et al., 1998; Meagher, 2000).

**Use of trees as for phytoremediation**

Compared to the small biomass production and slow growth of natural hyper-accumulator plants, trees possess many physiological and structural characteristics that make them good candidates for phytoremediation (Stomp, 1993). For example, their perennial habit allows for prolonged service and their extensive root masses and high transpiration rates permit efficient metal ion absorption and translocation. Massive nonliving woody tissue allows for pollutant storage. More living tissues and higher energy in the tree could be used to deal with metal ions. Easy establishment and maintenance make the cost of phytoremediation low. The best scenario is that trees could be used in combination with herbaceous plants to construct a dynamic community for phytoremediation.

Several tree species have been examined for the potential for phytoremediation. Plantations of hybrid poplar, sweetgum, sycamore, cottonwood, willow, and loblolly pine have been studied for municipal wastewater treatment (Stomp, 1993). As a model species in tree biotechnology, poplar trees have been studied most intensively among trees for remediation of various pollutants, such as TCE, the explosive TNT, atrazine, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), aniline and selenium (Che, 2000; Meagher, 2000). Data showed that hybrid poplar (*P. trichocarpa X P. deltoides*) were capable of degrading TCE to trichloroethanol, chlorinated acetates, and finally CO₂ (Newman et al.,
1997; Newman et al., 1999). Poplar tree plantations have also been established in South Dakota and Kansas to stabilize Zn, As, Cd, and Pb at the contaminated sites (Berti and Cunningham, 2000).

The potential of transgenic trees for phytoremediation is attracting increasing efforts. Transgenic hybrid poplars overexpressing $\gamma$-ECS in the chloroplasts or in the cytosols exposed to Cd, contained higher amounts of PCs and accumulated higher amounts of Cd in their roots than wild-type plants. They also showed increased tolerance to chloroacetanilide herbicides, probably due to their elevated thiols after herbicide exposure (Gullner et al., 2001). Yellow-poplar (Rugh et al., 1998) and eastern cottonwood (Che, 2000) genetically engineered with *merA* were resistant to Hg(II), which it detoxified volatile metallic Hg(0). Eastern cottonwood trees expressing *merB* showed higher resistance to phenylmercuric acetate (PMA) than wild-type plants, which could be explained by the conversion of PMA to less toxic Hg(II) (Che, 2000). Although studies of the potential of transgenic trees for phytoremediation are still confined to the laboratory and much research is needed before their application to the field, it is generally believed that trees engineered with appropriate sets of genes may offer a viable means of restoration of heavy metal contaminated sites around the world (Rugh et al., 2000; Stomp et al., 1993).

Sweetgum, together with deep-rooted trees (poplar, cottonwood, willow) has been highly recommended for remediating inundated bottomlands, mainly because they are particularly water tolerant (Rugh et al., 2000). With a wide-spreading root system, sweetgum is an aggressive pioneer in disturbed sites. Sweetgum stumps sprout prolifically and can produce an abundance of fast-growing root suckers (Harlow et al.,
1996). These characteristics make sweetgum a very good candidate for phytoremediation, potentially with a very low maintenance cost for a very long service. In order to use sweetgum for phytoremediation of toxic metals, the remedial capacity of sweetgum needs to be significantly enhanced by genetic transfer.

All the reported transgenic sweetgum trees described to date were transformed and regenerated via organogenesis. Since somatic embryogenic cultures proliferate as thousands and thousands of cells or cell clumps, it is expected that embryogenic cultures may be the better targets for either Agrobacterium infection or biolistic transformation. Facilitated by suspension cultures, thousands of somatic embryos can be produced at one time and are ready to germinate, without the multiple steps needed to induce shoots and roots required in organogenesis (Merkle and Trigiano, 1994). Thus somatic embryogenesis holds many advantages over organogenesis in terms of frequency, labor cost and final products. Sweetgum has already been tested for waste treatment and regenerated via somatic embryogenesis. Therefore, genetic transformation of sweetgum or hybrid sweetgum embryogenic cultures with phytoremediation genes such as γ-ECS or merA may be promising strategies for the production of plants with superior heavy metal remediation capacity.
LITERATURE CITED


CHAPTER 2

ENHANCING THE PRODUCTIVITY OF HYBRID YELLOW-POPLAR

(LIRIODENDRON TULIPIFERA × L.CHINENSE) AND HYBRID SWEETGUM

(LIQUIDAMBAR STYRACIFLUA × L. FORMOSANA) EMBRYOGENIC

CULTURES

1Dai, J., L. Wang, W.A., Vendrame and S.A., Merkle,. to be submitted to In Vitro

Cellular and Developmental Biology—Plant
ABSTRACT

High-frequency embryogenesis systems were established for hybrid yellow-poplar (*Liriodendron tulipifera* × *L. chinense*) and hybrid sweetgum (*Liquidambar styraciflua* × *L. formosana*) by modifying a medium originally developed for embryogenic yellow-poplar cultures. Embryogenic cultures of both hybrids, consisting of proembryogenic masses (PEMs), were initiated from immature hybrid seeds on an induction-maintenance medium (IMM) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), benzyladenine (BA) and casein hydrolysate (CH). For hybrid yellow-poplar, as many as 2100 germinable embryos/4000 cells or cell clumps were produced when PEMs were grown in liquid IMM lacking CH, at a pH that varied with genotype (3.5 or 5.6), followed by size fractionation and plating on semisolid embryo development medium (DM; IMM lacking 2,4-D and BA) without CH, but supplemented with 4.0 mg/L abscisic acid (ABA). For hybrid sweetgum, up to 1650 germinable embryos/4000 cells or cell clumps were produced when PEMs were grown in liquid IMM without CH, but with 550 mg/L L-glutamine, 510 mg/L asparagine and 170 mg/L arginine. Embryos developed from cell clumps on IMM without any plant growth regulators (PGRs) or other supplements. Somatic embryos of both hybrids germinated at a high frequency on IMM without any PGRs or other supplements.

Keywords *Liriodendron tulipifera, Liriodendron chinense, Liquidambar styraciflua, Liquidambar formosana, Somatic embryogenesis*
INTRODUCTION

Long growing seasons, abundant moisture, and relatively inexpensive land make the southern U.S. a prime location for plantations of fast-growing forest tree species to be used for such purposes as biomass energy or pulp and paper production. Two well-known fast-growing hardwood trees native to this region are sweetgum (*Liquidambar styraciflua*) and yellow-poplar (*Liriodendron tulipifera*). Yellow-poplar is one of the most distinctive and valuable hardwoods in the eastern U.S. Large volumes of yellow-poplar wood are used for furniture, plywood, and other light construction lumber. It is also used for pulping (Russell 1977). Sweetgum is an important commercial species in the southeastern U.S. used for furniture and plywood, and its use by the southern paper industry increases annually (Harlow et al. 1996). Both yellow-poplar and sweetgum have counterparts native to eastern Asia, which have been separated from the North American species for at least 10 million years by continental drift (Parks and Wendel 1990). Chinese tuliptree (*Liriodendron chinense*), native to China and Vietnam, is morphologically similar to yellow-poplar. Sexual hybridization between yellow-poplar and Chinese tuliptree has been reported (Santamour 1972b, Parks et al. 1983) and heterotic grow rates were observed for the hybrid seedlings (Parks et al. 1983). Formosan sweetgum (*Liquidambar formosana*) is found in the temperate forests of eastern Asia. *L. formosana* is interfertile with *L. styraciflua* and hybrids between the two species have continued to grow well in a test planting (personal communication, F.S. Santamour, National Arboretum, Washington, D.C.). No intensive genetic improvement programs exist for either yellow-poplar or sweetgum. However, both are already known as fast-growing trees and hybrids between the North American and Asian species may
exhibit even faster growth and thus may be desirable for rapid production of forest products, biomass for energy or for phytoremediation purposes.

Hybrid yellow-poplar and hybrid sweetgum have both been regenerated via somatic embryogenesis (Merkle et al. 1993, Vendrame et al. 2001). Hybrid yellow-poplar plantlets regenerated from somatic embryos also showed heterotic growth in the greenhouse (Merkle et al. 1993). In both studies, somatic embryos were developed following transfer of proembryogenic masses (PEMs) from semisolid induction maintenance medium (IMM) to embryo development medium (DM). However, hybrid yellow-poplar embryos were not synchronized and only a limited number of germinable embryos were produced (Merkle et al. 1993). Hybrid sweetgum cultures mainly proliferated by repetitive embryogenesis (Vendrame et al. 2001), resulting in fused clusters of embryos. For germination, clustered embryos needed to be manually separated and were often damaged during the separation operation. Therefore, for both hybrids, the process was inefficient and needed to be improved for the cultures to be suitable for either mass propagation or genetic transformation. Suspension culture and size fractionation manipulation were considered as critical factors in mass production of synchronized and singularized yellow-poplar somatic embryos (Merkle et al. 1990). However, after growth in liquid IMM, fractionated hybrid yellow-poplar PEMs only produced callus, rather than embryos, following plating on semisolid DM (Merkle et al. 1993). The capacity of hybrid sweetgum suspension cultures for embryo production has not been tested. We hypothesized that the yellow-poplar suspension culture system described in Merkle et al. (1990) could be adapted for mass production of both hybrids. Therefore, the objective of this study was to improve the somatic embryogenic systems
for hybrid yellow-poplar and hybrid sweetgum through modifying the compositions or pH values of liquid IMM and semisolid DM.

**MATERIALS AND METHODS**

**Plant materials, culture initiation and experimental approach**

Controlled crosses between yellow-poplar and Chinese tuliptree parents were carried out by Dr. Clifford Parks using trees in his collection at the University of North Carolina, Chapel Hill, NC. Immature fruits (8 weeks post-pollination) were collected in July, 1999 and shipped via overnight mail to our laboratory at the University of Georgia. Cultures were initiated following procedures described previously (Merkle and Sommer 1991). Briefly, after surface sterilization, seeds were dissected under aseptic conditions and embryos and surrounding endosperm were placed on semisolid yellow-poplar IMM, which contained 9.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 1.1 µM benzyladenine (BA), 1 g/L casein hydrolysate (enzymatic; CH), and 40 g/L sucrose, and was gelled with 8 g/L Phytagar (Gibco). Three embryos were explanted per 60 × 15 mm plastic Petri dish and incubated in the dark at 25°C. Hybrid embryogenic cultures produced from these explants following 1-2 months of culture (Fig. 1A) were maintained by transferring approximately 0.5 g of material to fresh semisolid IMM every three weeks. Hybrid sweetgum cultures were initiated and maintained using a similar procedure and the same semisolid IMM, as described in Vendrame et al. (2001).

Based on the literature, several potentially important culture variables were tested in a series of empirical experiments, using the following general protocol: embryogenic suspension cultures were initiated by inoculating approximately 0.5 g PEMs into 125 ml
Erlenmeyer flasks containing 50 ml liquid yellow-poplar IMM or IMM variants (described below). Flasks were incubated in the dark at 25° C on a rotary shaker at 120 rpm. After two culture cycles (2 weeks/cycle for hybrid yellow-poplar, 3 weeks /cycle for hybrid sweetgum), PEMs (38-140 µm in diameter) were collected using stainless steel CELLECTOR® sieves (Bellco Glass), as described previously (Merkle et al. 1990). The collected PEMs were re-suspended in liquid IMM and the cell/cell clump density was estimated under a microscope. The re-suspended cultures were then collected on filter paper (4.25 cm in diameter) under mild vacuum using a Buchner funnel, and cultured along with the filters on semisolid yellow-poplar DM [IMM without plant growth regulators (PGRs)] or DM variants (described below) in the dark at 25° C to produce somatic embryos. The pH of DM was adjusted to 5.6 before autoclaving. Mature somatic embryos with well-formed cotyledons were germinated on IMM without PGRs or other supplements under cool white fluorescent light (100 µmol·m⁻²·s⁻¹) at 25° C. Samples of germinants were transferred to potting mix (Fafard #3) and grown in a humidifying chamber prior to transfer to the greenhouse.

**Experiments with hybrid yellow-poplar**

**Effect of low pH of liquid IMM on somatic embryo production.** Treatments in this experiment were liquid yellow-poplar IMM at pH 3.5, 4.0, 4.5, 5.0 or 5.6 (control) prior to autoclaving. Following culture in liquid medium and size fractionation as described above, approximately two thousand cells and cell clumps per filter paper were cultured on semisolid yellow-poplar DM to produce somatic embryos.

**Effect of supplemental amino acids in liquid IMM on somatic embryo production.** In this experiment, the pH of all IMM variants was adjusted to 3.5 prior to autoclaving. The
four tested liquid IMM were: IMM1 (1g/L CH), IMM2 (lacking CH), IMM3 (lacking CH but with 550 mg/L glutamine) and IMM4 (lacking CH but with 550 mg/L glutamine, 510 mg/L asparagine and 170 mg/L arginine). All amino acids were filter-sterilized and added to cooled, autoclaved medium. Approximately two thousand cells and cell clumps per filter paper were cultured on semisolid yellow-poplar DM to produce somatic embryos.

**Effects of CH and pH in liquid IMM on somatic embryo production.**

Two factors (CH and pH) were tested in this factorial experiment. Liquid media were IMM1 and IMM2 (see above), IMM5 (1g/L CH at pH5.6) and IMM6 (lacking CH at pH5.6). Approximately four thousand cells and cell clumps per filter paper were cultured on semisolid yellow-poplar DM or on DM without CH.

**Effect of supplemental ABA in semisolid DM on somatic embryo production.**

This experiment was tested with two types of liquid media: IMM2 and IMM6. After size fractionation, approximately four thousand cells or cell clumps per filter paper were cultured on semisolid DM without CH or DM lacking CH but with 4 mg/L ABA. ABA was filter-sterilized and added to cooled, autoclaved medium.

**Experiments with hybrid sweetgum**

**Effects of CH and pH in liquid IMM and ABA in DM on somatic embryo production.** The same four liquid IMM used in the yellow-poplar CH and pH experiment described above (IMM1, IMM2, IMM5, IMM6) were used here. Approximately four thousand cells or cell clumps per filter paper were cultured on semisolid DM or on DM without CH, but with 4.0 mg/L ABA.

**Effect of supplemental amino acids in liquid IMM on somatic embryo production.**

The liquid IMM used here were the same as in the yellow-poplar amino acid experiment
(IMM1-4), except that the pH of all media was adjusted to 5.6 prior to autoclaving. Approximately four thousand cells or cell clumps per filter paper were cultured on semisolid yellow-poplar DM without CH or on DM supplemented with amino acids or CH, corresponding to the liquid IMM in which the PEMs were grown.

**Data collection, experimental design and statistical analysis**

Four hybrid yellow-poplar PEM lines and either 3 or 4 hybrid sweetgum lines were used in the experiments. All the experiments above were repeated at least two times and each treatment had three flasks and corresponding Petri plates. Germinated somatic embryos were counted and data were plotted using Microsoft Excel Chart Wizard (Microsoft® Excel 2000). The data from the hybrid yellow-poplar CH and pH experiment, ABA experiment, and the hybrid sweetgum amino acid experiment were tested with Poisson Regression using the Generative Model (GENMOD) procedure (Stockes et al. 2000).

**RESULTS AND DISCUSSION**

**Experiments with hybrid yellow-poplar**

**pH and amino acid experiments.** A medium pH of 4.0-5.0 has shown to be associated with the maintenance of pre-globular stage embryos in carrot (Smith and Krikorian 1990). The pH experiment tested the effect of the pH of liquid IMM on embryo production of hybrid *Liriodendron*. All four hybrid yellow-poplar lines produced the highest numbers of germinable somatic embryos [16 embryos/2000 cells or cell clumps, on average (Fig. 2)] following growth in liquid IMM at pH 3.5, compared to other pH values. Casein hydrolysate (CH) was included in all IMM and DM in the pH experiment. However, individual amino acids or mixtures of them proved superior to CH for embryo
production in orchardgrass (Trigiano et al. 1992). Therefore, media at pH 3.5 and supplemented with different amino acids were tested. Glutamine alone or a mixture of the three amino acids had no promotive effect on embryo production under the conditions tested (Fig. 3). However, the hybrid cultures grown in liquid IMM without any organic nitrogen supplements produced similar numbers of somatic embryos to those grown in liquid IMM with CH (Fig. 3), suggesting that CH could be omitted from the hybrid suspension medium.

**CH and pH experiment.** The effects of CH and pH and their possible interactions were examined in this experiment. When grown in liquid IMM without CH at pH 3.5 or 5.6, the hybrid suspension cultures proliferated as relatively homogeneous clumps of small cells and appeared clear and yellow (Fig. 1C), whereas the control cultures (pH 5.6 with CH) appeared cloudy due to the production of numerous free, elongated, vacuolate cells (Fig. 1B). The latter cultures also darkened more quickly. Tissue darkening may be the result of the production of phenolic compounds when tissues are wounded and/or senescent (George 1993). The hybrid cultures produced significantly more somatic embryos when plated on DM without CH than on DM with CH (Fig. 4A-B; Table 1), and the number of germinable embryos produced was significantly higher following growth in liquid IMM without CH than in liquid IMM with CH (Table 1, profile plot not shown). Interestingly, while three of the lines produced normal embryos on DM lacking CH (Fig. 1D), line D suspensions produced embryos that germinated precociously while still on embryo development medium (Fig. 1E).

Obtained from enzymatic digestion of milk, CH is a mixture of up to 18 amino acids and other unknown substances, with glutamine the most common (George 1993).
Because it showed promotive effects, CH has been used in many embryogenesis systems. However, CH apparently inhibited somatic embryo development in our hybrid yellow-poplar cultures. This was also the case with *Picea mariana* cultures (Tautorus et al. 1990). CH might include toxic substances or induce the production of these substances during autoclaving (Anstis and Northcote 1973) or under the conditions used.

**ABA experiment.**

This experiment was initially designed to test the ability of ABA in semisolid DM to prevent line D embryos from germinating precociously. Unexpectedly, after growth in liquid IMM lacking CH at pH 3.5 or 5.6, all the hybrid lines produced a significantly higher number of germinable somatic embryos when developed on DM without CH, but containing 4.0 mg/L ABA, than on DM without ABA (Fig. 5A, Table 2). When cultured on DM supplemented with 4.0 mg/L ABA, hybrid lines A and B produced a higher number of embryos following growth in liquid IMM without CH at pH 3.5 (2100 (Fig. 1G) and 1470 embryos/4000 cells, respectively) compared to pH 5.6, while lines C and D produced more embryos following growth in liquid IMM without CH at pH 5.6 (900 and 1370 embryos/4000 cells (Fig. 1H), respectively) compared to pH 3.5 (Table 2). Statistical analysis indicated that culture line and pH had a significant interaction (Table 2, profile plot not shown). Using this protocol, after two cycles of growth (2 weeks/cycle), 0.5 g PEMs could produce more than 48,000 cells and cell clumps of 38-140 µm in diameter, which could potentially produce 10,800-24,600 synchronized, germinable embryos three weeks following plating. Mature somatic embryos converted to plantlets in another four weeks, and after hardening-off for six weeks in potting mix, somatic seedlings were ready to transfer to the greenhouse (Fig. 1I-J). ABA is known to
prevent precocious germination and promote embryo maturation in several species (Ammirato 1988). Previously, ABA was tested for synchronization of yellow-poplar embryos, but the embryos produced failed to convert (Merkle et al. 1990).

**Experiments with hybrid sweetgum**

**CH, pH and ABA experiment.** In the presence of CH, sweetgum suspensions appeared yellow and clear. Hybrid sweetgum cultures grew much more slowly in liquid IMM without CH than in IMM with CH. When the size-fractionated cells and cell clumps were cultured on DM without CH, but with ABA, few embryos were produced by any of the hybrid lines for any treatment (data not shown). In contrast, when cultured on DM with CH, line 2 produced a very high number of singularized embryos (940 embryos / 4000 cells) after growth in liquid IMM with CH at pH 5.6 (Fig. 7), although the embryos remained attached to cell aggregates (Fig. 6A). Line 1 produced higher numbers of embryos following growth in liquid IMM with CH at pH 3.5 and in IMM without CH at pH 5.6 than following growth in the other two IMMs, although the numbers were much lower (250, 130 embryos/4000 cells, respectively). Line 3 did not produce embryos under any of the tested conditions (Fig. 7). The data suggested that some components of CH might be required for the growth of the cultures and that the standard pH of 5.6 may be the most appropriate for these cultures.

**Amino acid experiment.** The effects of substitution of CH with amino acids on embryo production were examined in this experiment. The numbers of embryos produced were boosted for three of the four tested hybrid lines after the cultures, grown in liquid IMM containing glutamine, asparagine and arginine or glutamine alone, were cultured on DM without CH. In addition, fewer embryos developed with cell aggregates attached to them.
(Fig. 6 D-E). Lines 1, 2 and 4 produced the highest numbers of singularized, germinable embryos (1650, 1064 and 1000 / 4000 cell clumps on average, respectively; Fig. 8), after growth in IMM with the mixture of 3 amino acids. Line 3 still failed to produce any embryos with any treatment. Statistical analysis indicated that following growth in the 3 amino acid mixture, the number of embryos that developed was significantly higher than for the other three IMM treatments (Table 3, profile plot not shown). These 3 lines also produced a high number of embryos when the cultures were plated on DM supplemented with amino acids or CH corresponding to the preceding liquid IMM (data not shown). However, DM without CH was preferred since more embryos were produced on it.

After two cycles of growth (3 weeks/cycle) in liquid IMM containing the 3 amino acid mixture, 0.5 gram hybrid sweetgum PEMs could produce more than 16,000 cells or cell clumps of 38-140 µm in diameter, which in turn could potentially produce 4,000-330,000 singularized, germinable embryos after developing for eight weeks (Fig. 6C). Mature embryos converted to somatic seedlings in another four weeks, and these were ready to transfer to the greenhouse after hardening-off for 8-10 weeks in potting mix (Fig. 6G).

Our results indicate that empirical testing of some fundamental culture variables can make it possible to efficiently produce propagules of some potentially valuable clones of hybrid yellow-poplar and hybrid sweetgum for biomass production and other applications. However, it is also clear that interactions between treatments and genotypes may necessitate optimization of cultural treatments for the most highly desirable clones on a clone-by-clone basis. These optimized regeneration systems should also make possible the production of transgenic hybrid trees with novel traits, since similar systems
have proven to make excellent target material for genetic transformation (e.g. Rugh et al. 1998). We are particularly interested in employing some of the more productive hybrid clones for transformation with heavy metal resistance genes. The combination of rapid biomass accumulation resulting from heterotic growth rates with the ability to accumulate or otherwise handle heavy metal pollution should make these trees powerful tools for remediation of heavy metal-contaminated soil and water. Some of the same hybrid sweetgum clones used in this study have already been engineered with heavy metal resistance genes and are being tested for possible phytoremediation applications (Dai et al., in preparation).
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Table 1. Results of Poisson regression analysis for effects of CH and pH in IMM on somatic embryo production of hybrid yellow-poplar cultures on development medium with and without CH

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Table 2. Results of Poisson regression analysis for effect of ABA in development medium on somatic embryo production of hybrid yellow-poplar cultures

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Table 3. Results of Poisson regression analysis for effect of amino acids in liquid IMM on somatic embryo production of hybrid sweetgum cultures on development medium without CH

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Figure 1. Embryogenic hybrid yellow-poplar cultures, somatic embryos and plantlets. 

A. Hybrid yellow-poplar proembryogenic masses (PEMs) arising from immature zygotic embryo explant on semisolid induction-maintenance medium (IMM). Bar=500 µm. 

B. Hybrid yellow-poplar embryogenic cultures growing in liquid IMM proliferated as large cell clumps and free vacuolate cells. Bar=550 µm. 

C. Hybrid PEMs growing in liquid IMM without CH proliferated as relatively homogeneous, small clumps of cells without the presence of free, vacuolate cells. Bar=550 µm. 

D. Mature hybrid somatic embryos produced on embryo development medium (DM) without CH after growth in liquid IMM lacking CH. Bar=1.75 mm. 

E. Precociously germinating somatic embryos of line D after 3 weeks on DM without CH following growth in liquid IMM without CH. Bar=3.0 mm. 

F. Mature somatic embryos were transferred to germination medium. Bar=5.0 mm. 

G. After growth in liquid IMM without CH at pH 3.5, hybrid line A produced 2140 somatic embryos / 4000 cells or cell clumps when cultured on filter paper overlaid on DM without CH but with 4 mg/L ABA. Filter is 4.25 cm in diameter. 

H. After growth in liquid IMM without CH at pH 5.6, hybrid line D produced 1370 somatic embryos / 4000 cells or cell clumps cultured on filter paper overlaid on DM without CH but with 4.0 mg/L ABA. Filter paper is 4.25 cm in diameter. 

I. Germinating somatic embryos on germination medium after 3 weeks. Bar=7.0 mm. 

J. Acclimated plantlets in potting mix were ready for transfer to greenhouse after 6 weeks. Bar=5.0 cm. 

Figure 2. Effect of pH of liquid IMM on somatic embryo production from hybrid yellow-poplar cultures four weeks following size-fractionation and plating of PEMs on DM.
Figure 3. Effect of CH and supplemental amino acids in liquid IMM on somatic embryo production of hybrid yellow-poplar cultures four weeks following size-fractionation and plating of PEMs on DM. The concentrations of the amino acids were 550 mg/L glutamine, 510 mg/L asparagine and 170 mg/L arginine.

Figure 4. Effects of CH in liquid IMM and pH of liquid IMM on hybrid yellow-poplar somatic embryo production. A. Numbers of germinable somatic embryos produced four weeks following size-fractionation and plating of PEMs on DM. B. Numbers of germinable somatic embryos produced four weeks following size-fractionation and plating of PEMs on DM lacking CH. Results of statistical analysis are shown in Table 1.

Figure 5. A. Effect of ABA in DM lacking CH on hybrid yellow-poplar somatic embryo production. Numbers shown are the average numbers of somatic embryos from 4 (ABA) or 3 (No ABA) hybrid lines. B. Numbers of somatic embryos produced by four hybrid lines on medium with 4.0 mg/l ABA after the cultures were grown in liquid IMM without CH at two pH values. Results of statistical analysis are shown in Table 2.

Figure 6. Embryogenic hybrid sweetgum cultures, somatic embryos and plantlets. A. After growth in liquid IMM, hybrid line 2 produced 940 somatic embryos / 4000 cells or cell clumps cultured on filter paper on DM. Filter is 4.25 cm in diameter. B. After growth in liquid IMM lacking CH but supplemented with 550 mg/l glutamine, hybrid line 1 produced 1240 somatic embryos / 4000 cells or cell clumps cultured on filter paper on
DM. Filter is 4.25 cm in diameter. C. After growth in liquid IMM without CH but with 550 mg/l glutamine, 510 mg/l asparagine and 170 mg/l arginine, hybrid line 1 produced 1760 germinable embryos / 4000 cells or cell clumps cultured on filter paper on DM. Filter is 4.25 cm in diameter. D. After growth in liquid IMM with CH and culture on DM, fractionated cells produced more cell aggregates that were attached to the embryos. Bar=1.0 mm E. After growth in liquid IMM without CH but with amino acid mixture and culture on DM, fractionated cells produced less cell aggregates that were attached to the embryos. Bar=1.0 mm F. Germinating somatic embryos after 4 weeks on IMM without any PGRs or supplements. Petri dish is 60 mm in diameter. G. Acclimated plantlets in potting mix were ready for transfer to greenhouse after 10 weeks. Bar=5.0 cm.

**Figure 7. Effects of CH and pH in liquid IMM on somatic embryo production of hybrid sweetgum cultures.**

**Figure 8. Effect of supplemental amino acids in liquid IMM on somatic embryo production of hybrid sweetgum cultures.** Germinable somatic embryos were produced eight weeks following size-fractionation and plating of PEMs on DM. The concentrations of three amino acids were 550 mg/L glutamine, 510 mg/L asparagine and 170 mg/L arginine. Results of statistical analysis are shown in Table 3.
Number of germinable embryos/2000 cell clumps

Liquid IMM pH
Number of germinable embryos/4000 cell clumps

- Line
- A
- B
- C
- D
- CH&glu
- glutamine
- CH
- NCH
- 3 amino acids

Number of germinable embryos/4000 cell clumps

- 0
- 50
- 100
- 150
- 200
- 250
- 300
- 350
- 400
A

B
A

DM lacking CH

Number of germinable embryos/4000 cell clumps

pH3.5

pH5.6

ABA No ABA

B

Number of germinable embryos/4000 cell clumps

pH3.5

pH5.6

A B C D

Line
CHAPTER 3

DEVELOPMENT OF TRANSGENIC HYBRID SWEETGUM (*LIQUIDAMBAR. STYRACIFLUA* × *L. FORMOSANA*) EXPRESSING $\gamma$-GLUTAMYL-CYSTEINE SYNTHETASE OR MERURIC REDUCTASE FOR PHYTOREMEDIATION OF MERCURY POLLUTION$^1$

$^1$Dai, J., R.B. Meagher and S.A. Merkle, to be submitted to New Phytologist
ABSTRACT

Mercury pollution is a major environmental problem. Transgenic plants expressing a modified bacterial mercuric ion reductase gene (merA) can detoxify ionic mercury [Hg(II)] by converting it to volatile, less toxic, elementary mercury [Hg(0)]. An alternative strategy for mercury phytoremediation is the use of plants to sequester ionic mercury with phytochelatins in harvestable tissues. Using Agrobacterium-mediated gene transfer, we generated transgenic hybrid sweetgum (Liquidambar styraciflua × L. formosana). These plants overexpressed a bacterial γ-glutamylcysteine synthetase gene (γ-ECS), which is the first and most important enzyme in the phytochelatin synthesis pathway, or one of three genes encoding a mercuric ion reductase (merA9, merA18, merA77). Hybrid sweetgum proembryogenic masses (PEMs) transformed with γ-ECS driven by a strong, constitutive actin promoter (ACT2) and terminator were able to grow in the presence of 50 µM HgCl2, which inhibited wild-type PEMs. Plantlets were regenerated from five ACT2:ECS PEM lines and two SRS1:ECS (light-induced soybean rubisco promoter, SRS1) PEM lines under non-selective conditions. Plantlets that were PCR- and western-positive for γ-ECS were regenerated from one ACT2:ECS PEM line and both SRS1:ECS PEM lines, but the plantlets had an abnormal plant type and did not survive for more than a few weeks following germination. In contrast, mature somatic embryos generated from 35S:merA9, 35S:merA18 PEMs under non-selective conditions converted to normal plantlets on germination medium containing 25 µM HgCl2, while control embryos were killed on this medium. Somatic embryos generated from ACT2:merA77 PEMs produced plantlets that grew poorly on Hg(II)-medium. Transgenic merA plantlets released Hg(0) 2-3 times more efficiently than the wild-type plantlets.
These results indicate that the $\gamma$-ECS gene may negatively affect the development of hybrid sweetgum PEMs and plantlets. However, hybrid sweetgum plants expressing modified $merA$ genes driven by the 35S promoter may provide a means for phytoremediation of mercury pollution.

**Key Words:** phytoremediation, mercury, *Liquidambar styraciflua × L. formosana*, transgenic plants, $\gamma$-glutamylcysteine synthetase gene ($\gamma$-ECS), mercuric reductase gene ($merA$)

**Abbreviations** IMM: induction-maintenance medium, PEM: proembryogenic mass, COMT: bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase
INTRODUCTION

Mercury is one of the most hazardous heavy metals. Industrial and agricultural activities have released tremendous amounts of mercury into the biosphere during the past century. Phytoremediation, the use of plants to remove, sequester, or detoxify pollutants, is considered to be a cost-effective and environmentally friendly remediation approach for toxic metals (Meagher 2000). Since there are no known plant species that naturally accumulate or detoxify mercury and other mercurial compounds, recent mercury phytoremediation research has focused on genetically engineering plants with the bacterial mercuric reductase gene (*merA*) and/or the organomercurial lyase gene (*merB*) to convert ionic mercury into a volatile and less toxic elemental form, Hg(0), which rapidly transpires from the plant (Rugh et al., 1996; Rugh et al., 1998; Che, et al. in press; Bizily et al., 1999; Bizily et al., 2000). In the laboratory, *merA* transgenic plants grew in the presence of otherwise lethal levels of ionic mercury by reducing it to elemental mercury, which was volatilized from *merA* plants. This remediation approach is justified at locations where the accelerated volatilization and atmospheric dilution of Hg(0) back to low environmental levels is acceptable.

An alternative strategy for mercury phytoremediation is the use of plants to sequester ionic mercury into harvestable tissues. The principle mechanism by which plants sequester heavy metals involves the formation of complexes with cysteine-rich peptides called phytochelatins (PCs), and the confinement of these complexes to the vacuole (Cobbett 2000). PCs can form complexes with metals such as Cd, Pb, Ag, Hg and As in vitro. With the general structure \((\gamma\text{-Glu-Cys})_{2-11}\text{-Gly}\), PCs are synthesized from the precursor glutathione (GSH), which is synthesized from glutamate (Glu), cysteine (Cys)
and glycine (Gly) in two sequential, ATP-dependent enzymatic reactions, catalyzed by gamma glutamyl cysteine synthetase (γ-ECS) and glutathione synthetase (GS), respectively. PC synthase (PS) catalyzes the elongation of the GSH to form PCs. The reaction catalyzed by γ-ECS is believed to be the rate-limiting step for the synthesis of GSH and PCs (Noctor et al. 1998, Zhu et al. 1999). GSH serves as a major antioxidant in plant cells, protecting the cells from oxidative stress damage caused by various factors such as heavy metals. GSH is also a substrate of GSH S-transferase, which catalyzes the conjugation of GSH with potentially dangerous xenobiotics such as herbicides. Over-expression of γ-ECS in Indian mustard increased the production of GSH and PCs, which in turn enhanced Cd tolerance and accumulation (Zhu et al. 1999). Transgenic hybrid poplars overexpressing γ-ECS showed increased tolerance to Cd and chloroacetanilide herbicides, probably due to their elevated thiols after Cd/herbicide exposure (Gullner et al., 2001). Recently it was shown that Arabidopsis expressing the bacterial γ-ECS under control of a strong constitutive actin promoter (ACT2) were moderately resistant to mercury and arsenic (Dhankher et al. 2002, Li et al. submitted). In addition, GSH has intrinsic value as a flavor precursor in food and as an anti-carcinogen. Therefore, genetic manipulation of GSH metabolism provides many possibilities for environmental as well as industrial applications (Noctor et al. 1998).

Sweetgum (Liquidambar styraciflua) is a major hardwood tree of economic importance in the southern United States. Sweetgum trees grow best on rich, moisture alluvial soils. With its wide-spreading root system, sweetgum is an aggressive pioneer on disturbed sites. Sweetgum stump-sprouts prolifically and can produce an abundance of fast growing root suckers, which may provide prolonged service with low maintenance
cost (Harlow et al. 1996). Sweetgum, like cottonwoods and willows, is particularly water-tolerant and has been tested for waste treatment (Stomp 1993). These characteristics make sweetgum a very good candidate for phytoremediation of wetlands. In order to use sweetgum for phytoremediation purposes, however, the remedial capacity of the tree needs to be significantly enhanced by genetic transformation. Genetic transformation of sweetgum has been reported previously. Young leaf explants (Chen and Stomp 1992) and leaf tissue derived from aseptic shoot tip cultures (Sullivan and Largrimini 1993) were transformed through *Agrobacterium*-mediated gene transfer and transgenic sweetgum trees were regenerated via adventitious shoot production. Transgenic sweetgum trees were also produced via adventitious shoot production following microprojectile bombardment of nodule cultures (Kim et al. 1999). Genetic transformation of sweetgum embryogenic cultures has not been reported to date. Recently, an efficient somatic embryogenesis system was established for hybrids between sweetgum and its Asian counterpart, *L. formosana* (Dai et al., submitted). Hybrid sweetgum may exhibit even faster growth than the parent species and thus may be even more desirable for phytoremediation. The objective of this study was to develop transgenic hybrid sweetgum trees overexpressing either a modified *merA* or a bacterial γ- *ECS* for phytoremediation of ionic mercury and potentially other heavy metal pollutants as well.

**MATERIALS AND METHODS**

**Plant material and suspension cultures**

Hybrid sweetgum embryogenic cultures were initiated from immature hybrid seeds in mid-June and mid-July 1999, as previously described (Vendrame et al. 2001). Briefly,
following fruit sterilization, immature seeds were removed aseptically, nicked with a scalpel and cultured, 3 seeds per 60 mm plastic petri dish, on induction-maintenance medium (IMM), which contained 9.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 1.1 μM benzyladenine (BA), 1 g/l casein hydrolysate (CH) and 40 g/l sucrose. The medium was gelled with 0.8 % Phytagar (Gibco). Explants were transferred to fresh semisolid IMM monthly until proembryogenic masses (PEMs) started to form. Once embryogenic cultures were established, PEMs were maintained by transferring approximately 0.5 g material to fresh semisolid IMM every three weeks.

Hybrid sweetgum suspension cultures were initiated by inoculating approximately 0.5 g PEMs from 10 selected lines into 125 ml Erlenmeyer flasks containing 50 ml liquid IMM. Suspensions were grown in the dark at 25°C and agitated on a rotary shaker at 120 rpm. After two cycles of growth of 2 weeks each, the suspensions were passed through 380 μm stainless steel CELLECTOR® sieves (Bellco Glass, Inc.) to remove large cell clumps. Suspension cultures that grew in large clumps were broken up by forcing them through the sieve with a spoonula. Fractionated cells and small clumps were transferred to fresh medium after 2 days and grown for another 2 weeks prior to transformation experiments.

**Agrobacterium and vectors**

*Agrobacterium tumefaciens* strain C58 was used for γ-ECS gene transformation. The vectors used were pBINACT2:ECS and pBINSRS1:ECS, both pBIN19 derivatives containing one copy of the *nptII* gene conferring kanamycin-resistance, and the coding region of Gamma glutamylcysteine synthetase from *E. coli* (γ-ECS, accession no. X03954). The γ-ECS gene was driven by either a constitutive actin promoter (ACT2) or a
light-induced soybean rubisco promoter (SRS1) (Dhankher, et al., 2002). The 35S:merA9 and 35S:merA18 gene constructs were both derived from pVSTI, as described earlier by Rugh et al. (1996, 1998). They contained the bacterial merA gene with either 9% or 18% of the sequence modified for improved plant expression by overlap extension PCR, and the nptII gene. A third version of the merA gene, merA77, had 77% of the sequence modified and was driven by the ACT2 promoter and terminator.

*Agrobacterium* was grown overnight in yeast extraction medium (YEP) supplemented with appropriate antibiotics to an OD₆₀₀ of 1.5, centrifuged at 1500 g (3600 rpm) for 10 minutes and re-suspended in liquid IMM at final OD₆₀₀ of 0.2 for transformation.

**Transformation and selection**

Co-cultivation of hybrid sweetgum PEMs with *Agrobacterium* was carried out following a protocol modified from one provided by Dr. Daniel Carraway (International Paper Co., Bainbridge, GA). Kanamycin was chosen as the selection agent since it was previously used to select transformed sweetgum nodule cultures and leaf explants (Chen and Stomp 1991, Sullivan and Lagrimini 1993). Following sensitivity testing, 50 mg/l kan was chosen for selection. Approximately 3 ml settled cell volume (SCV) of cells and cell clumps in 30 ml liquid IMM were transferred to a 50 ml sterile Falcon tube with 500 mg sterile sand and vortexed for 5 seconds at maximum speed. Cells were then transferred with 50 ml liquid IMM into 125 ml flasks, and acetosyringone (final concentration 50 µM) and 5 ml *Agrobacterium* suspension were added. After co-cultivation for 16-20 hours on a shaker at 120 rpm in the dark at 25°C, cultures were transferred to Falcon tubes and centrifuged at 1500 g (3600 rpm) for 3 minutes to settle cells into a loose pellet.
The supernatant was discarded and the cultures were incubated in the tube without shaking for 48 hours in the dark. Following 3 washes with liquid IMM with 300 mg/L Timentin (ticarcillin disodium and clavulanate potassium), the cultures were grown in flasks containing 50 ml of the same medium on a shaker for another 48 hours. To obtain more stringent selection in one experiment, suspension cultures were further size-fractionated at this point and only cell clumps 38-140 µm in diameter were used for selection. The cultures were again transferred to Falcon tubes and rinsed twice with the same medium. The settled cells were re-suspended in 15 ml IMM containing 50 mg/L kan, 300 mg/L Timentin and 5 g/L low-melting-pointing agarose (Bio-Rad), held at 40 °C. Approximately 3 ml of these suspensions was spread on 100 mm plastic Petri dishes containing semisolid IMM (selection medium) with 50 mg/L kan and 300 mg/L Timentin. After 4-6 weeks, kanamycin-resistant (Kan^R) colonies were isolated and transferred to fresh semisolid selection medium at 3-week intervals. Colonies showing rapid growth were used to initiate suspension cultures in liquid IMM with 50 mg/L kan.

**PCR analysis**

DNA template was isolated from Kan^R PEM lines using an E.Z.N.A® Plant DNA Isolation Kit (Omega Bio-Tek, Doraville, GA). For the putative γ-ECS PEMs, multiplex polymerase chain reaction (PCR) was performed to amplify the γ-ECS and nptII genes, and a sweetgum internal gene, bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT, accession no. AF139533), using three sets of primers in a single reaction. The multiplex PCR primers used were as follows:

- **Forward γ-ECS primer:** ACTGACGCACAAATGGATTAC
- **Reverse γ-ECS primer:** GCACCAAACAGATAAGGAATGAC
Forward *nptII* primer:  GATGATTGAACAAGATGGATTGC

Reverse *nptII* primer:  CTCAGAAGAACTCGTCAAGAAGG

Forward COMT primer:  GCAAGATCGAGAGGCTTTAC

Reverse COMT primer:  CACCACCAACATCAACCAC

Standard PCR was performed to detect γ-ECS among the plantlets developed from the putative *ACT2:ECS* PEMs. Fifty ng DNA was PCR-amplified in a 25 µl reaction mix containing 200 µM dNTP, 0.3 µM of each primer, 2U Taq DNA polymerase, 2 mM MgCl₂ and 2.5 µl 10× buffer. After a one-minute treatment at 94° C in a thermocycler, the reaction was incubated for 35 cycles (denaturation at 94 °C for 30 s, annealing at 53° C for 30 s, and extension at 72° C for 30 s). The final cycle included 94° C for 30 s, 53° C for 30 s, and 72° C for 10 min. PCR products were separated on 1% agarose gel, stained with ethidium bromide, and detected and photographed under UV light.

For putative *merA* plantlets, regular PCR reactions were performed with the following primers.

Forward (merAp1294S, *merA*): AGTGACCATTCTTGCACGCTCCACTCTCTT


Reverse (merApl380, *merA*77): ACAATAGGCACCTTTGAGTGAGTAAGGGTCA

After a five-minute treatment at 94° C in a thermocycler, the reaction was incubated for 40 cycles (denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72° C for 30 s). The final cycle included 94° C for 30 s, 62° C for 30 s, and 72° C for 7 min. PCR products were separated on 2% agarose gel, stained with ethidium bromide, and detected and photographed under UV light.
Southern analysis

DNA was isolated from approximately 200 mg of *ACT2:ECS* PEMs from PCR-positive lines using the plant DNA isolation kit described above. Isolated DNA (10 µg/line) was digested with 100 units of *BamHI* or a combination of *BamHI* and *NcoI* (100 units each). Restriction digested DNA was concentrated through ethanol precipitation and electrophoresed through a 0.8 % agarose gel. Resolved DNAs were transferred onto a Hybond™-N membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) and cross-linked with UV for 30 seconds. Southern hybridizations were performed as described by Sambrook et al. (1989) using gel-purified, random primed probe for *γ-ECS*. The probe, a *BamHI-NcoI* fragment from pBIN19-derived pBIN*ACT2:ECS*, encompassed the entire *γ-ECS* gene. Following hybridization, the membrane was washed in 2X SSC for 15 min at 65º C, followed by a wash in 0.1X SSC for 15 min. The membrane was then exposed to Kodak XAR-5 film at -70º C for 2 days.

Western analysis

Western blot analysis was used to examine the expression of *ACT2:ECS* in the transgenic hybrid sweetgum PEMs and the regenerated plantlets. Wild-type and transgenic hybrid sweetgum PEMs (200 mg) were ground in liquid nitrogen and transferred to microcentrifuge tubes to which 100 µl of 2x Laemmli’s SDS sample buffer (Laemmli 1970) per tube was added. Culture extracts were spun down (14,000 rpm) for 15 min at room temperature and the supernatants were stored at -20 ºC. The samples were boiled for 5 min and centrifuged for 1 min before loading the gel. Equal volumes of the protein samples (8 µl) were loaded into each lane and separated on a 10 % SDS-PAGE gel. Resolved proteins were electroblotted onto an Immobilon-P membrane (Millipore Inc.,
Bedford, MA). The blot was probed with the $\gamma$-ECS specific monoclonal antibody mAbECS (Li et al. 2001), followed by a secondary polyclonal sheep antimouse IgG conjugated with horseradish peroxidase. The blot was exposed for 10 min to X-ray film (Hyperfilm, Amersham) and signals were visualized using chemiluminescence (Amersham Lifescience, Cleveland, OH).

The same western analysis protocol was used to analyze the expression of merA in transgenic merA plantlets with the following modification: 100 µl protein extraction buffer per sample was added to the ground tissues and the protein concentration was determined by Bradford assay before 100 µl 2x Laemmli’s SDS sample buffer per sample was added. Equal amounts of protein were loaded on each lane, separated on a 10 % SDS-PAGE gel and blotted. The blots were probed with anti-MerA monoclonal antibody 11F9 (Rugh et al. 1998), labeled with sheep antimouse IgG conjugated with horseradish peroxidase, exposed for 2 min to X-ray film and visualized with chemiluminescence.

Toxicity experiments and regeneration

ACT2:ECS PEM lines were assayed for resistance on Hg-selection medium, which was semisolid IMM containing 50 µM HgCl$_2$. Approximately 2-3 mm$^3$ of PEMs per line were transferred to 100 mm plastic Petri plates containing 25 ml of semisolid Hg-selection medium and compared to wild-type PEMs for growth and survival after 4 weeks. The treatment was replicated three times for each line.

PEMs were grown on semisolid kanamycin selection medium for at least 3 months following Agrobacterium co-cultivation, followed by 3 weeks in liquid kanamycin-selection medium. Then, Hg(II) resistant (Hg$^R$) ACT2:ECS PEM and putative SRS1:ECS PEM lines were used to produce somatic embryos under non-selective conditions
according to the protocol described previously (Dai et al., submitted). For transgenic PEM lines that proliferated as large cell clumps, cell clumps were broken apart by forcing them through stainless steel sieves with a spoonula to produce cell clumps of a size (38-140 µm in diameter) that allowed development of singularized embryos. Mature somatic embryos with well-formed cotyledons were selected and germinated on germination medium (semisolid IMM without any other plant growth regulators or any supplements but with 5 mg/l gibberellic acid) under cool white fluorescent light (100 µmol m\(^{-2}\)s\(^{-1}\)) at 25° C. After culturing on germination medium for 2-3 weeks, germinants were used for resistance assays on Hg-selection medium containing 25 µM HgCl\(_2\).

Somatic embryos were generated from PEMs of nine putative merA lines using the same procedures. Mature embryos were used for resistance assays on Hg-germination medium, which contained 25 µM HgCl\(_2\). Eight wild-type somatic embryos and eight putative merA embryos per line were transferred to 100 mm plastic Petri dish plates containing 30 ml semisolid Hg-germination medium and evaluated for germination and survival after 2-3 weeks. The treatment was replicated three times for each line.

All other mature embryos were germinated on nonselective-medium. To screen out non-transgenic plantlets, these plantlets were transferred to 25 µM HgCl\(_2\) medium. Only Hg(II)-resistant plantlets were used for further analysis.

**Mercury volatilization assays**

Hg(II)-resistant plantlets were grown on non-Hg(II) selection medium for 10 days before the assay. Three of each merA9, merA18, merA77 and wild-type (WT) hybrid sweetgum plantlets were weighed and incubated in 1.5 ml of 25 µM HgCl\(_2\) solution in mercury sampling tubes. Hg(0) released by the hybrid sweetgum plantlets was measured on a
Jerome 431 mercury vapor analyzer (Arizona Instrument, Phoenix, AZ) as described by Rugh et al. (1996). The samples were assayed every minute for 10 minutes. The amount of Hg(0) volatilization was standardized by dividing the amount of Hg(0) evolved by the fresh weight of the plantlets in the assay.

**RESULTS**

**Transformation with γ-ECS genes**

A total of 10 embryogenic hybrid sweetgum suspension lines, representing 6 crosses between the two *Liquidambar* species, were established and co-cultivated with *Agrobacterium* containing either *ACT2:ECS* or *SRS1:ECS* in a binary vector. After culture on selection medium for 4 weeks, one hybrid sweetgum line produced kanamycin-resistant (Kan\(^R\)) PEM colonies. When cell clumps <380 µm in diameter were used as target material for transformation, light yellow PEM colonies arose from a background of watery, dark yellow cell clumps (Fig. 1A). An average of 82 Kan\(^R\) colonies was isolated and transferred to fresh selection medium per plate (approximately 0.5 ml SCV of sieved cells) for each construct. When cell clumps between 38-140 µm in diameter were used as target material, kanamycin-resistant PEM colonies grew much faster than other cell clumps, which grew very slowly and remained small (Fig. 1B). Using this approach, an average of 17 and 30 Kan\(^R\) colonies per plate (0.3 ml SCV of 38-140 µm cells or cell clumps) were obtained for the *ACT2:ECS* and *SRS1:ECS* constructs, respectively.

Putatively transformed PEM colonies were maintained by transfer to fresh semisolid selection medium at 3-week intervals. The colonies showing fastest growth were used to
initiate suspension cultures in liquid IMM with 50 mg/L kan for stringent selection and to accelerate proliferation. After several rounds of kanamycin selection in liquid medium, the presence of the ACT2/SRS1:ECs transgenes in hybrid sweetgum cells was assayed by multiplex genomic DNA-PCR. A subset of ten independent, fast-growing KanR PEM lines showed both the expected 447 bp γ-ECs and 780 bp nptII PCR products (7 samples shown in Fig. 2). Both wild-type PEMs and transformed lines showed a 750 bp, COMT (internal control) PCR product. As shown in Figure 3, neither γ-ECs nor nptII PCR products were observed with DNA from wild-type PEMs.

DNA was isolated from three γ-ECs PCR-positive PEM lines and analyzed by Southern hybridization for the presence of integrated copies of the γ-ECs gene. Hybridization of a 1.6 kilobase BamHI-NcoI fragment of pBINACT2:ECs containing the entire γ-ECs gene to genomic DNA demonstrated the presence of full–length copies of the γ-ECs expression unit in these three lines (Fig. 3). Hybridization of the γ-ECs probe to BamHI-digested genomic DNA showed that multiple γ-ECs genes had integrated into the sweetgum genome. No hybridization was detected in genomic DNA from non-transformed PEMs (Fig. 3).

Western blot analysis was performed to examine γ-ECs protein expression in PCR positive hybrid sweetgum PEMs. Since the SRS1 promoter only allows expression in green (i.e. regenerated plant) tissues (Dhankher, et al., 2002), only PEMs from ACT2:ECs lines were analyzed. Six KanR, PCR-positive PEM lines were used for western blot analysis. Western blotting indicated that all the ACT2:ECs PEM lines examined contained a protein with the same molecular mass (57 kDa) as that from confirmed ACT2:ECs Arabidopsis thaliana plants (provided by Dr. Yujing Li, Genetics
Department, University of Georgia), which reacted with the antibody (Fig. 4). No band was detected in the wild-type extract (Fig. 4).

*ACT2:ECS* lines were assayed for ionic mercury resistance 4 months following cocultivation. On medium without HgCl$_2$, both wild-type hybrid sweetgum PEMs and transgenic *ACT2:ECS* hybrid sweetgum PEMs grew normally (Fig. 1C). All *ACT2:ECS* PEM lines tested were resistant to 50 µM HgCl$_2$, while wild-type hybrid sweetgum PEMs darkened at this concentration after 4 weeks (Fig. 1D).

After the PEMs were grown on semisolid kanamycin selection medium followed by 3 weeks in liquid kanamycin-selection medium, Hg(II)-resistant (Hg$^R$) *ACT2:ECS* PEM and putative *SRS1:ECS* PEM lines were transferred to liquid medium without kanamycin for proliferation prior to size fractionation and plating for somatic embryo development and plantlet production. Large numbers of globular embryos were produced after 3 weeks of culturing on IMM without any PGRs or other supplements. However, only a few dozen of these continued development to the cotyledonary stage after 6 weeks (Fig. 1F, 1G). As a result, the number of mature somatic embryos produced from *Agrobacterium* co-cultivated Kan$^R$ PEMs was significantly lower than the number of somatic embryos produced from their non-transformed counterparts (Fig. 1E, 1F). The embryos were transferred to fresh basal medium and grown for another 4 weeks for maturation. Mature somatic embryos were germinated on the same medium under cool white fluorescent light (Fig. 1H). Germination rate of the embryos was greatly enhanced when 5 mg/L gibberellic acid was included in the medium. Somatic seedlings were regenerated from five *ACT2:ECS* PEM lines and two *SRS1:ECS* PEM lines under non-selection conditions. Plantlets of one *ACT2:ECS* line and both *SRS1:ECS* lines were abnormal, with no
epicotyl shoots and a rosetted appearance (Fig. 1H). These plants failed to continue growth on fresh germination medium and did not survive transfer to potting mix. Thus, they were not tested for mercuric ion resistance. While normal plantlets developed from the three remaining ACT2:ECS PEM lines, none of these showed any greater resistance to 25 \( \mu \text{M} \) ionic mercury in the germination medium than did wild-type controls (Fig. 1J). DNA isolated from the abnormal plantlets developed from ACT2:ECS PEM lines and assayed by PCR showed the expected band (Fig. 5A), but DNA from normal plantlets produced from other ACT2:ECS PEM lines failed to show this band. Western blot analysis showed that protein samples from the abnormal plantlets contained a protein with the same molecular mass (57 kDa) as that from confirmed ACT2:ECS A. thaliana, which reacted with the antibody (Fig. 5B). No band was detected in the wild-type extract (Fig. 5B).

**Transformation with merA genes**

Using the same Kan selection protocol as was used with the \( \gamma \)-ECS material, averages of 20, 25 and 17 Kan\(^R\) colonies per plate (0.5 ml SCV cell clumps of <380 \( \mu \text{m} \) in diameter) were obtained for constructs merA9, merA18 (Fig. 6A) and merA77 (Fig. 6B), respectively. Mature embryos and normal plantlets were produced from three putative transgenic PEM lines each of merA9, merA18 and merA77, for a total of 9 merA lines. Unlike the \( \gamma \)-ECS material, many merA embryos continued to develop beyond the globular stage (Fig. 6C, 6D), and normal plantlets were produced when these somatic embryos were germinated on germination medium (Fig. 6E).

Mature somatic embryos generated from putative 35S:merA9 and 35S:merA18 PEMs were capable of conversion to normal plantlets on germination medium containing 25 \( \mu \text{M} \)
HgCl\textsubscript{2}, while control embryos were killed on this level of mercuric ion (Fig. 6F, 6G). However, somatic embryos generated from \textit{ACT2:merA77} PEMs produced unhealthy plantlets that resembled wild-type controls on Hg(II)-medium (Fig. 6H).

Plantlets from all putative \textit{merA} transgenic lines were assayed by genomic DNA-PCR. Of these, three \textit{merA9} and three \textit{merA18} lines showed the expected 210 bp \textit{merA} PCR product, and three \textit{merA77} lines showed the expected 258 bp PCR product, confirming the presence of the \textit{merA} transgene. No product was observed with DNA from wild-type plants, as shown in Figure 7. Western blotting indicated that all the plantlets from \textit{merA} lines examined contained a protein with the same molecular mass (66 kDa) as that from confirmed \textit{merA} \textit{A. thaliana} plants (provided by Dr. Rebecca Balish, Genetics Department, University of Georgia), which reacted with the antibody 11F9 (Fig. 8). No band was detected in the wild-type extract (Fig. 8). No samples from \textit{merA77} lines were used for this analysis due to the very low protein concentration of the samples.

Approximately 3-cm tall wild-type and transgenic \textit{merA} plantlets (30-60 mg) were placed in mercury sampling tubes and exposed to Hg(II). Transgenic \textit{merA} plantlets released 2-3 times more Hg(0) than wild-type plantlets (Fig. 9A). Furthermore, \textit{35S:merA18} plantlets released relatively higher Hg(0) amounts than \textit{35S:merA9} and \textit{ACT2:merA77} plantlets (Fig. 9B).

**DISCUSSION**

The tested \textit{Agrobacterium}-mediated transformation protocol worked well for hybrid sweetgum PEMs, but for only one of the ten lines tested. Hundreds of independently
transformed PEM lines were obtained from this genotype, but no other genotypes tested
gave rise to kanamycin-resistant colonies. The protocol needs to be optimized for other
genotypes to broaden the genetic base of transgenic hybrid sweetgum clones.

Transformed hybrid sweetgum PEMs grown on kanamycin medium appeared very
similar to the initial PEMs grown on kanamycin-free medium. The transgenic hybrid
sweetgum embryogenic cultures expressing the bacterial γ-glutamylcysteine synthetase
gene driven by the ACT2 promoter were able to grow in the presence of an ionic mercury
concentration toxic to wild-type cultures. Although somatic embryos and plantlets were
regenerated from these mercuric ion-resistant PEM lines on non-selective medium, a
large number of globular embryos from these PEM lines failed to develop into
cotyledonary-stage embryos. Also, while plantlets developed normally from some lines,
abnormal plantlets were produced from one ACT2:ECS PEM line and two SRS1:ECS
PEM lines. Unfortunately, the normal plantlets showed no resistance to 25 µM ionic
mercury. PCR and western blot analysis suggested that normal plantlets were not
transformed while the abnormal plantlets were transgenic. These results indicated that
either the ACT2:ECS PEMs were chimeric or the transgenes were lost in some cultures
during the proliferation and embryo development stages. Furthermore, most of the
globular embryos stopped development and the regenerated transgenic plantlets were
abnormal, possibly because the γ-ECS siphoned off too much glutamine or because γ-
ECS reacted with Zn or Cu, causing rosetting. Another possibility is that the ACT2
promoter caused co-suppression of host genes.

In contrast, mature somatic embryos generated from putative 35S:merA9 and
35S:merA18 PEMs were capable of conversion to normal plantlets on germination
medium containing 25 µM HgCl₂, and all of these merA plantlet lines tested were PCR and western-positive. MerA9 expression levels were higher than MerA18, which is consistent with the result reported by Rugh et al. (1998). Transgenic merA hybrid sweetgum plantlets released Hg(0) 2-3 times more efficiently than did the wild-type plantlets. However, MerA expression levels did not appear to be related to mercury evolution rate, which is consistent with the results reported by Bizily et al. (2000), but contrary to the results reported by Che (2000). The different performance of transgenic γ-ECS plantlets and transgenic merA plantlets suggested that the 35S:merA genes could be expressed in hybrid sweetgum without hindering the normal growth of the plants while the γ-ECS could not. Compared to 35S:merA9/18 plantlets, ACT2:merA77 germinants appeared unhealthy on Hg(II) supplemented-germination medium. This difference might be explained by the differences in the promoter and terminator used or the percentage of coding sequence modified.

Based on these results, we believe that these 35S:merA plants offer great promise for enhancing the efficiency of mercury phytovolatilization from mercury-contaminated soils and wastewater. Further research, however, is required to quantify mercury volatilization of these transgenic plants in greenhouse tests and ultimately in the field.
LITERATURE CITED


Dai, J. Vendrame, V.A. and Merkle, S.A. Enhancing the productivity of hybrid yellow-poplar (Liridendron tulipifera × L. chinense) and hybrid sweetgum (Liquidambar styraciflua × L. formosana) embryogenic cultures (in preparation).


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Figure 1. Transgenic γ-ECS hybrid sweetgum proembryogenic masses (PEMs) and regenerated plantlets.  

A. Kanamycin-resistant PEM colonies (arrows) arose from cell clumps (originally <380 µm in diameter) embedded in agarose selection medium with 50 mg/L kanamycin, 5 weeks following co-cultivation with *Agrobacterium* containing *ACT2:ECS*. Bar=1.0 mm.  

B. A kanamycin-resistant PEM colony that arose from cell clumps (originally 38-140 µm in diameter) embedded in selection medium with 50 mg/L kanamycin, 5 weeks after *Agrobacterium*-co-cultivation. Bar=0.8 mm.  

C. Wild-type (WT) hybrid sweetgum PEMs and transgenic *ACT2:ECS* hybrid sweetgum PEMs on medium without HgCl₂ (4 weeks). Bar=1.0 cm  

D. Transgenic *ACT2:ECS* hybrid sweetgum PEMs grew on medium with 50 µM HgCl₂, while wild-type (WT) hybrid sweetgum PEMs darkened at that level within 4 weeks. Bar=1.0 cm.  

E. Cotyledonary somatic embryos produced from wild-type PEMs on embryo development medium (8 weeks, filter paper is 4.25 cm in diameter).  

F. Fewer cotyledonary somatic embryos were produced from transgenic γ-ECS PEMs on embryo development medium (8 weeks, filter paper is 4.25 cm in diameter).  

G. Few γ-ECS globular embryos developed into cotyledonary somatic embryos while most (arrow) did not. Bar=3.0 mm.  

H. Abnormal plantlets without epicotyl shoots were produced from cotyledonary embryos of one *ACT2:ECS* PEM line and two *SRS1:ECS* PEM lines. Bar=0.5 cm.  

I. Normal plantlets produced from cotyledonary embryos of other *ACT2:ECS* PEM lines. Bar=1.0 cm.  

J. Neither wild-type plantlets nor normal plantlets produced from transgenic *ACT2:ECS* hybrid sweetgum PEMs were unable to survive on medium with 25 µM HgCl₂ (4 weeks). Bar=1.0 cm.
Figure 2. Multiplex PCR analysis of genomic DNA from wild-type (WT) and putative γ-ECS transformed hybrid sweetgum PEMs. Ethidium bromide-stained agarose gel of genomic DNA-PCR using three sets of oligo primers specific to γ-ECS, nptII and the sweetgum internal gene bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), respectively in a single reaction. The right-side arrows indicate the expected 780 bp nptII, 750 bp COMT and 447 bp γ-ECS PCR products respectively. The left three lanes contain molecular weight ladder (MWL), the positive DNA control [γ-ECS (+)] and wild-type (WT) negative control respectively. The positive DNA control lane shows the PCR product generated from the pBINACT2:ECS construct. The remaining lanes are products from independent putative γ-ECS transgenic lines (A-γ-ECS-1, 2 and 3 were transformed with pBINACT2:ECS and S-γ-ECS-1, 2, 3 and 4 were transformed with pBINSRS1:ECS.

Figure 3. Southern analysis of genomic DNA from wild-type (WT) and putative ACT2:ECS transformed hybrid sweetgum PEMs. Genomic DNA (10 µg/line) was digested with a combination of BamHI and NcoI (100 units each, lanes 1, 2, 3) or with 100 units of BamHI (lane 4, 5). Lanes 7 and 10 are wild-type control and positive control, respectively.

Figure 4. Western blot of extracts from wild-type (WT) and γ-ECS transformed hybrid sweetgum PEMs 6 independently transformed lines. Equal amounts of total protein were loaded on each lane, separated on a 10 % SDS-PAGE gel, blotted, probed with γ-ECS specific monoclonal antibody mAbECS, labeled with sheep antimouse IgG
conjugated with horseradish peroxidase, and visualized with chemiluminescence. Purified \( \gamma \)-ECS protein \([\gamma \text{-ECS (+)}]\) (57 kDa) isolated from \textit{Arabidopsis thaliana} plants was included as a positive control.

**Figure 5. Molecular characterization of plantlets developed from \( \gamma \text{-ECS} \) PEMs.**

A. DNA sample from abnormal plantlets (A-\( \gamma \)-ECS-2) showed the expected 447 bp band. No band was detected in the wild-type (WT) or the normal plantlet samples (line 1, 3, 4, 5). B. Protein sample from the abnormal plantlets developed from A-\( \gamma \)-ECS-2 contained a protein with the same molecular mass (57 kDa) as that from confirmed \textit{ACT2:ECS} \textit{Arabidopsis thaliana} plants. No band was detected in the wild-type (WT) extract or the normal plantlet extracts (line 3, 4, 5).

**Figure 6. Transgenic \textit{merA} hybrid sweetgum proembryogenic masses (PEMs) and regenerated plantlets.**

A. Kanamycin-resistant PEM colonies arose from cell clumps (originally <380 µm in diameter) embedded in selection medium with 50 mg/L kanamycin 5 weeks after co-cultivation with \textit{Agrobacterium} containing \textit{35S:merA9}. Bar=1.0 mm. B. Two kanamycin-resistant PEM colonies arose from cell clumps (originally <380 µm in diameter) embedded in selection medium with 50 mg/L kanamycin 5 weeks after co-cultivation with \textit{Agrobacterium} containing \textit{ACT2:merA77}. Bar=800 µm. C. Cotyledonary somatic embryos produced from putative \textit{merA9} PEMs on embryo development medium (8 weeks, filter paper is 4.25cm in diameter). D. Cotyledonary somatic embryos produced from putative \textit{merA77} PEMs on embryo development medium (8 weeks, filter paper is 4.25cm in diameter). E. Germinating
transgenic 35S:merA9 and wild-type hybrid sweetgum embryos on non-selective-medium. Bar=1.0cm. F. Germinating transgenic 35S:merA9 (right) and wild-type (left) hybrid sweetgum embryos on medium with 25 µM HgCl₂ at 2 weeks. Bar=1.0cm. G. Germinating transgenic 35S:merA18 (right) and wild-type (left) hybrid sweetgum embryos on medium with 25 µM HgCl₂ at 2 weeks. Bar=1.0cm. H. Germinating transgenic ACT2:merA77 (right) and wild-type (left) hybrid sweetgum embryos on medium with 25 µM HgCl₂ at 2 weeks. Bar=1.0cm.

**Figure 7.** PCR analysis of genomic DNA from transgenic merA9, merA18, merA77 and wild-type (WT) hybrid sweetgum plantlets. Plantlets from all the putative merA transgenic lines were assayed by genomic DNA-PCR. Of the merA lines examined, three 35S:merA9 and three 35S:merA18 lines showed the expected 210 bp merA PCR product, three ACT2:merA77 lines showed 258 bp PCR products. No product was observed with the water-control and the DNA from wild-type plants.

**Figure 8.** Western blot of plantlet extracts from transgenic merA9, merA18 and wild-type (WT) hybrid sweetgum plantlets. A. Equal amounts of protein were loaded on each lane, separated on a 10 % SDS-PAGE gel, blotted, probed with anti-MerA monoclonal antibody 11F9, labeled with sheep antimouse IgG conjugated with horseradish peroxidase, and visualized with chemiluminescence. Purified MerA protein (66 kDa) isolated from Arabidopsis thaliana plants was included as a positive control [merA (+)]. B. Coomassie staining of a 10 % SDS-PAGE gel loaded with the same amounts of protein each lane as the gel used in A.
Figure 9. Hg(0) volatilization assays of transgenic merA9, merA18, merA77 and wild-type (WT) hybrid sweetgum plantlets. A. Average values of Hg(0) released by wild-type and transgenic line 35S:merA18-2. Hg(0) was measured every minute for 10 min, and the amount of Hg(0) at each measurement was divided by the fresh weight (30-60 mg) of the plantlet to give the relative values. Data are the mean ± SE of 3 replicates. B. Total Hg(0) released by wild-type and transgenic merA9-3, merA18-2 and merA77-1 plantlets over 10 min. Data are the mean ± SE of 3 replicates.
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<th>MWL</th>
<th>γ-ECS(+)</th>
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- NPTII 780 bp
- COMT 750 bp
- γ-ECS 447 bp
1.6 kb
A

MWL(+)/WT ACT2:ECS lines

B

WT ACT2:ECS lines (+)

2 3 4 5
A

![Graph showing Hg(0) production over time for WT and merA18-2 plantlet lines. The x-axis represents time in minutes (0 to 10), and the y-axis represents ng Hg(0)/mg Plantlet Tissue. The graph shows a linear increase in Hg(0) production with time for both lines, with merA18-2 showing a slight increase over WT.]

B

![Bar graph comparing Hg(0) production for different plantlet lines. The x-axis represents Plantlet Lines (WT, 35S-merA9-3, 35S-merA18-2, Act2-merA77-1), and the y-axis represents ng Hg(0)/10 min/mg plant tissue. The graph shows a significant increase in Hg(0) production for 35S-merA18-2 compared to the other lines.]

CHAPTER 4

CONCLUSIONS
Mercury pollution is a worldwide environmental problem. Recently, mercury phytoremediation research has focused on testing the ability of genetically engineered plants with modified bacterial mercuric ionic reductase (merA) genes to convert ionic mercury into a volatile and less toxic elemental form, Hg(0), which rapidly transpires from the plant cells. However, phytovolatilization may not be suitable due to the hazards of releasing Hg(0) or the rapid redeposition of Hg(0) at certain locations. An alternative strategy is the use of plants to sequester mercury in harvestable tissues. Transgenic Arabidopsis thaliana plants over-expressing a bacterial phytochelatin gene—gamma glutamyl cysteine synthetase (γ-ECS) showed enhanced mercury tolerance and accumulation. Since trees possess physiological and structural characteristics such as their extensive root system, rapid growth, and large biomass production, that make them good candidates for phytoremediation, our objective is to produce transgenic trees overexpressing γ-ECS or expressing merA genes for mercury phytoremediation.

Two well-known fast-growing hardwood trees native to the southeastern U.S. are sweetgum (Liquidambar styraciflua) and yellow-poplar (Liriodendron tulipifera). Hybrid yellow-poplar (Liriodendron tulipifera × L. chinense) and hybrid sweetgum (Liquidambar styraciflua × L. formosana) may exhibit even faster growth and thus may be desirable for phytoremediation purpose or other forest products. Because no efficient tissue culture propagation systems are available for both hybrid hardwoods, systems for high frequency in vitro regeneration were first established. Embryogenic cultures of both hybrids, consisting of proembryogenic masses (PEMs), were initiated from immature hybrid seeds on induction-maintenance medium (IMM) supplemented with plant growth regulators (PGRs) and casein hydrolysate (CH). For hybrid yellow-poplar, as many as
2100 germinable embryos/4000 cells or cell clumps were produced when PEMs were grown in liquid IMM lacking CH at a pH that varied with genotype (3.5 or 5.6), followed by size fractionation and plating on semisolid embryo development medium without CH, but supplemented with 4.0 mg/L abscisic acid. For hybrid sweetgum, up to 1650 germinable embryos/4000 cells or cell clumps were produced when PEMs were grown in liquid IMM without CH, but with 550 mg/L glutamine, 510 mg/L asparagine and 170 mg/L arginine. Embryos developed from cell clumps on IMM without any PGRs or other supplements. Somatic embryos of both hybrids germinated at a high frequency on IMM without any PGRs or other supplements.

Mercury pollution is frequently deposited in lowland regions. Sweetgum trees are good candidates for mercury phytoremediation due to their water tolerance of wet soils. We introduced a $\gamma$-ECS gene into hybrid sweetgum through Agrobacterium-mediated gene transfer. Kanamycin resistant hybrid sweetgum PEMs were able to grow in the presence of 50 $\mu$M $\text{HgCl}_2$, which inhibited nontransformed PEMs. The increased resistance of transgenic $\gamma$-ECS PEMs to mercury could be explained by the chelation of mercury ions with phytochelatins and sequestration of the complexes into cell vacuoles. Abnormal (rosetted) plantlets were produced from the $\gamma$-ECS PEM lines, and these did not survive for more than a few weeks following germination. These abnormal plantlet lines were both PCR- and western-positive. Using the same protocol, we also generated $\text{merA}9$, $\text{merA}18$ and $\text{merA}77$ hybrid sweetgum. Mature somatic embryos generated from $35S:\text{merA}9$ and $35S:\text{merA}18$ PEMs under non-selection conditions were capable of conversion to normal plantlets on germination medium containing 25 $\mu$M HgCl$_2$, by converting ionic mercury into volatile and less toxic elemental form, Hg(0), while control
embryos did not grow and were killed on this medium. Somatic embryos generated from \textit{ACT2:merA77} PEMs converted to unhealthy plantlets on Hg(II)-medium. The Hg (II)-resistant plantlets tested were PCR and western-positive. Transgenic \textit{merA} plantlets released Hg(0) 2-3 times more efficiently than the wild-type plantlets. These results indicate that the \textit{γ-ECS} gene may significantly affect the regeneration capacity of hybrid sweetgum PEMs and the growth of the plantlets. Although further investigation is required to determine their ability to convert ionic mercury into elementary mercury in the field, we expected that hybrid sweetgum plants expressing modified \textit{merA} genes may provide a means for phytoremediation of mercury pollution.