GENETICALLY ENGINEERING PLANTS WITH A GENE FOR MERCURIC ION REDUCTION AND RESISTANCE

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Abstract. We are engineering a bacterial gene encoding mercuric reductase for the production of plants with the ability to electrochemically reduce toxic, ionic mercury. Bacterial mercury resistance operons have been described in detail (Summers, 1986). One gene contained in the polycistron is *merA*, which codes for mercuric ion reductase. This enzyme catalyzes the reduction of toxic Hg++ to far less toxic Hg^{o}. The coding sequence of the bacterial gene is very GC-rich, possibly preventing observable expression in transgenic plants. We designed a *merA* sequence that contained codons more typical of highly expressed plant genes. Overlap Extension-PCR (OE-PCR) was used to modify the codons in a 9% block of coding sequence and to alter the non-coding regulatory sequences. *Agrobacterium-mediated* transformation was used to produce *Arabidopsis* having the modified gene, *merA9*. These plants are capable of germinating and growing to seed set on medium containing up to 100 mercuric chloride, whereas control seeds fail to grow on 25 µ.M Hg++. Full length mRNA was detected in mercury resistant plants using northern blot hybridization. Mercury-resistant transgenic *Arabidopsis* seedlings were observed to evolve Hg^{o} vapor from buffered $HgC1_2$ at up to three times the rate of control seedlings. We are continuing to investigate the effect of further sequence modification for the optimization of *merA* gene expression in transgenic plants. Ultimately, we intend to develop transgenic tree species transformed with modified *merA* constructs. We have developed reliable and efficient protocols for tissue culture propagation, plant regeneration and gene transfer for yellow-poplar *(Liriodendron tulipifera).* Our progress towards the production of *merA* transgenic yellow-poplar is discussed.

Keywords: phytoremediation, genetic engineering, mercury resistance, *Arabidopsis thaliana, Liriodendron tulipifera*

INTRODUCTION

The use of plants to stabilize, reduce or detoxify aquatic and terrestrial pollution is known as phytoremediation. Many terrestrial environments are naturally high in phytotoxic metallic compounds (Alloway, 1990) and when combined with sites contaminated with heavy metals from man-made sources this creates pollution hazards that are very difficult and expensive to remediate (Nriagu and Pacyna, 1988). Some plant species have developed the ability to thrive on many of these sites by hyperaccumulating the metals and sequestering them away from sensitive physiological processes (Baker, 1989). These plant taxa have been suggested as possible phytoremediation "crops" (Baker et al., 1994), though their implementation in the near term is hampered by inadequate understanding of the physiological, biochemical and genetic complexity of hyperaccumulation. Molecular genetic techniques have been used to characterize and manipulate plant genes involved in heavy metal tolerance or uptake and should aid in their eventual application

(Zhou and Goldsbrough, 1994). Biotechnology may also allow the utilization of novel, foreign genes from organisms having metabolic pathways for the processing of toxic heavy metals (Stomp et al., 1993). Our laboratory has been involved in this latter approach for the development of potentially phytoremediative species using a bacterial gene for ionic mercury detoxification.

Bacterial mercury resistance operons have been described in detail and one gene of this operon , *merA* , codes for mercuric ion reductase (Summers and Sugarman, 1974; Summers, 1986). This enzyme catalyzes the reduction of toxic, ionic mercury to volatile, elemental mercury having far lower toxicity. Early attempts to confer Hg++ resistance to plants using the wildtype *merA* gene were unsuccessful (Thompson, 1990). Petunia plants were transformed with plant expression vectors containing the *merA* gene. These plants remained mercury sensitive and no full length gene product could be detected. Analysis of the *merA* DNA sequence revealed a high GC (up to 75%) nucleotide bias (Barrineau et al., 1984), in contrast to the –50% GC content of common, highly expressed plant genes (Murray et al., 1989). We hypothesized that the highly GC-skewed codon usage was extremely unfavorable for plant gene expression machinery, and therefore sequence modification would confer *merA* gene activity and ionic mercury resistance in plants. This strategy has been successful for improving the expression other foreign genes in transgenic plants (Fischoff et al., 1987). We have designed an altered DNA coding sequence for the *merA* gene that retains the coded amino acid sequence of the enzyme, but optimizes the codon usage, lowers the GC-bias to 45-55%, and alters the noncoding regulatory regions of the gene for efficient expression in plants. A directed mutagenesis strategy is being used to develop stepwise versions of modified *merA* gene constructs for transformation and analysis in plants species.

Ultimately, we intend to develop transgenic tree species transformed with modified *merA* constructs. Trees possess many desirable characteristics for a putative phytoremediative species. Their long life, deeply mining root system, and large reservoir of non-living woody tissues seem optimal for the removal, storage and remediation of heavy metal contaminants from the soil. We have developed reliable and efficient protocols for tissue culture propagation, plant regeneration and gene transfer for yellow-poplar (Merlde and Sommer, 1986; Wilde et al., 1992). Our progress towards further *merA* modification and transgenic tree development will be reported. Additionally, the theoretical phytoremediative benefits and potential advantages of merA-expressing tree species will be discussed as part of our long term goals for this project.

MATERIALS AND METHODS

Overlap extension (OE)-PCR for modification of *merA* .

The OE-PCR procedure is essentially as described by Ho et al. (1989). Terminal primers were designed to alter the sequences up- and downstream of the coding sequences to optimize for gene regulation in plants. The internal primers were designed to change the nucleotide coding sequence of the wildtype *merA* gene to codons more common to plants genes. Two halves of the gene were amplified separately using internal/terminal sense/antisense primer pairs. The two products were then gel purified, annealed and primed by the two external oligonucleotides, 5'S and 3'N, to complete each successive, modified *merA* fragment. The fragments were cleaved at designed restriction ends, *Bam HI* and *Pst I,* and ligated into the same sites of the cloning vector pBluescriptSKII (Strategene, La Jolla, CA). To screen against PCR-error mutants, the pBS-merA molecules were transformed into a Hg++-supersensitive, merA-deficient *E.coli* strain. Only ligation products complementing *merA*- and conferring resistance to 200 µM Hg++ on replica plates were selected.

Agrobacterium-mediated transformation of modified *merApe.*

The *BamHI/PstI* fragment of *merA9* was subcloned into the compatible site in the binary plant expression vector, pVSTI (Malik and Wahab, 1993), and transformed into the

Agrobacterium strain LBA4404. This strain was then used to inoculate A. *thaliana* root explants and regenerate selected, genetically transformed plants as described in Marton and Browse (1991).

Determination of H22 evolution by *merA9-A.thaliana*

Transformed *merA-Arabidopsis* lines and control lines lacking the *merA* gene were sterilized and germinated on GB5 medium containing no growth regulators or selective reagents (e.g. Hg++ or kanamycin). Twelve to 15 day old seedlings were weighed and placed in the side-arm reaction tube connected to the Jerome 431 mercury vapor analyzer. This apparatus accurately measures and displays evolved, elemental mercury drawn from either standard curve or experimental samples. The plants were assayed in 2 ml of 25 p.M $HgC1_2$, 50 mM phosphate, pH 6.8, with Hg^o evolution sampled each minute over a 10 minute period.

Hg-±± resistance seed germination assays.

merA9-A. thaliana and control seeds were surface sterilized and transferred to modified GB5 medium plates containing 0, 25, 50, 75, or 100 1.tM $HgC1_2$; wrapped with Parafilm and incubated at 24^0C with 16 hr day cycle. Mercury resistance was defined as full expansion of true leaves and elongation of true roots by greater than half of the seeds sown upon medium containing Hg++.

Detection of *merA* mRNA in transgenic A. *thaliana* using northern blot analysis.

Total RNA was isolated from stem and leaf tissues of 15-20 day old seedlings using a phenol/SDS/LiC1 extraction protocol (Ausubel et al., 1987). The samples were run on an agarose-formaldehyde gel and blotted to Nitran filters. The filters were washed at moderate stringency in 1X RNA Hybridization Mix with 40% deionized formamide at 48^0C (Hightower and Meagher, 1985) using the *merA9* 1.7 kb insert as radioactively labeled probe. To quantify the sample loadings and the *merA* hybridized band, the blots were stripped and re-hybridized with a probe for 18s rRNA. The sample loading quantification and subsequent normalized adjustment factors for the positive *merA* hybridization bands were determined using Phospholmager analysis (Molecular Dynamics, Inc.).

Transformation and regeneration of *merA-yellow-poplar.*

Embryogenic cultures of yellow-poplar *(Liriodendron tulipifera)* were established and transformed as described in Merkle and Sommer (1986) and Wilde et al.(1992). To summarize, immature seed explants were cultured on growth medium containing plant growth regulators with subculturing at three week intervals. After 1-2 months proembryogenic masses (PEMs) developed from zygotic embryos. PEMs will develop into somatic embryos when size fractionated (38-140 µm) and transferred to hormone-free medium. For transformation, the fractionated PEMs were collected on filter paper, transferred onto growth medium and bombarded with DNA-adsorbed microprojectiles for each of the *merA* constructs using the Biolistic transformation system as directed by the manufacturer (Du Pont, Wilmington, DE). After two days bombarded cultures were transferred to growth medium containing 100 mg/1 kanamycin to select for cells transformed with the selective marker gene also contained on the plant expression vector.

RESULTS AND DISCUSSION

merA Gene Modification

Overlap-Extension PCR (OE-PCR) was used to generate modified nucleotide sequences of the *merA* gene. In the initial *merA* modification, *merApe0,* only the flanking regions have been altered to include 5' and 3' untranslated plant and *E.coli* regulatory sequences. Each construct ID is shortened, for example *merApe0 to merA0,* when transformed into plants. *MerApe9* has had its

coding sequence changed to more typical plant codon usage within a 9% block of the reading frame. The *merApe0* through *merApe38* modifications have been completed. Six more OE-PCR rounds are required to complete *merApe47* to *merApe100.* Note that all these sequences encode a normal MerA protein, since only conserved changes are made to more typical *E.coli* and plant codons. *E.coli* transformants for each *merA* modification prepared to date were analyzed for their ability to reduce ionic mercury to volatile, elemental mercury. Each stepwise alteration of the *merA* sequence allowed increased Hg^o evolution by the transformed cells (data not shown).

Table 1. Comparative *merA9* gene transcription and whole plant expression. Shown are relative rates of *merA9* mRNA abundance, ability to evolve elemental Hg, and resistance to ionic Hg-containing growth medium by *merA* 9-transformed *A.thaliana* lines and control lines. The three *Arabidopsis* control lines: RLD, ACT7/GUS, and 35S/GUS; do not contain any form of *merA* construct. All others are independently transformed lines or sublines for the *merA9* gene.

Plant Line	Relative *merA* mRNA1	Relative ^{Hgo} Evolution2	Hg++ Resistance
1A	0.86	2.87	+++
1B	1.00	3.07	++++
1C	0.78	2.75	+++
5B	0.49	2.17	++++
2A	0.51	1.70	++++
4D	0.32	1.33	+
7A	0	0.93	
RLD	0	1.00	
ACT7/GUS	0	1.21	
35S/GUS	0	1.06	

[1] Relative *merA* mRNA values are based upon quantified northern blots of total RNA using *merA9* probe. *MerA* mRNA values were normalized by secondary labeling with a probe for 18s rRNA to account for uneven loading of the samples on the agarose gel.

[2] Relative Hg^o evolution rates by whole plants of total Hg^o for a 10 minute sampling period to determine ng Hg^o evolved/mg plant/min. Values are normalized against the wildtype strain, RLD, as equal to 1.

[3] Hg++resistance as determined by plant growth assays. Growth was defined as germination and production of true roots and true, expanded leaves. Symbols represent >50% germination and continued growth and development at up to the following [Hg++]: = 0 μM, + = 25 μM, ++ = 50 4M, +++ = 75 μM, ++++ = 100μ M Hg++.

Development and Characterization of *merA-Arabidopsis* plants

In an indirect assay for MerA enzymatic activity in transgenic *merA9-A.thaliana,* 12-15 day-old seedlings were examined for their ability to evolve Hg^o from a buffered 25 μM Hg++ solution. The assays were performed with 10-30 mg of plant material (6-10 seedlings), for ten minutes with mercury vapor analyzer sampling each minute. The *merA9* lines evolved Hg^o at

approximately three times the rate of the control *(merA-)* lines (see Table 1). These data demonstrate the substantial gain of function conferred by the *merA9* gene in many of the transformed lines. Several sublines derived from Kan ^{R}/Hg^{R} selected transformants display little or no evidence of *MerA* enzyme activity, probably due to Mendelian segregation of the gene from these sublines.

merA9-A.thaliana and control seeds were placed on Hg++-containing medium and assayed for resistance. The controls failed to germinate on [Hg++] > 50 pM, though seeds occasionally germinated and grew briefly at lower levels. The *merA9* seeds germinate and grow vigorously to flowering at up to 100 µ.M. Of special note is the slightly less vigorous growth by the *merA* plants on mercury-free (0 µM) medium. We suspect that this may be due to low-level affinity of the MerA enzyme for other divalent metallic cations, and may be disruptive of normal metabolic processes involving ions such as Zn2+, Cu^{2}+ or Mg2+.

Northern blot analysis of *merA9-A.thaliana* lines was performed for detection and quantitative comparison of *merA9* mRNA. Total RNA extractions of T3 generation *merA9* plants were run on agarose-formaldehyde gels, transferred to filters and hybridized with radioactively labeled *merA9* insert. We obtained *merA9* -specific bands for most of the transgenic lines, with only nonspecific background staining visible in a few of the *merA9* sublines and the three control lines. Rehybridization with a probe for the 18S rRNA band served to quantify the loading of the samples for relative *merA9* mRNA abundance determination. As shown in Table 1, *merA* mRNA values are closely related to both level of mercury resistance and relative mercury evolution among the transgenic and control plants.

This research demonstrates that sequence modification of a region of the *merA* gene was sufficient to confer ionic mercury resistance to the plant species *Arabidopsis thaliana.* We intend to continue the analysis of each of the currently modified *merA* constructs in plant systems, especially in light of the increased Hg++-reducing ability observed in *E.coli* with each successive version of the gene. We will further characterize the genetic makeup of the *merA9* plant lines by genomic Southern blot analysis to assist in our understanding of the variation among lines and the segregation patterns between sib sublines. We are also preparing a series of metabolic uptake analyses to elucidate the effect that the gene may have upon normal metallic ion metabolism, especially in the absence of Hg++. We speculate the poorer growth of the *merA* lines may be due to effects upon the normal metabolism of other divalent metal cations, such as Mg-$^{++}$, Ca^{++}, and Zn++.

We are most interested in the development of *merA-Liriodendron.* We feel that phytoremediation may be most effectively performed in such a system due to specific characteristics of trees. Trees are deeply soil mining plants and would be more capable of penetrating a greater underground volume than most hyperaccumulator species. The majority of aerial mass in a tree is non-living tissues - perhaps ideal for accumulation and sequestration of hazardous substances. In addition to the long life span of trees, their prolonged juvenile period allows for 10 or more years before the issue of outcrossing by a genetically engineered species is a problem. We are confident that we will be capable of obtaining transgenic *Liriodendron* for the *merA* constructs using protocols developed in our lab for genetic transformation and regeneration of yellow-poplar. Our preliminary success with *Arabidopsis* has encouraged us to continue to develop *merA-Liriodendron* and to determine their effectiveness for the reduction of toxic, ionic mercury ion in laboratory studies. We feel that this research has great potential for the development of an effective pilot phytoremediative system.

As we continue to characterize our *merA* plant systems, issues relevant to the use of these organisms in pollution control and abatement projects become important for consideration. There remains concern about the introduction of transgenic plants into the environment, though much of this fear will probably diminish as their safety is demonstrated in field trials and the value added characteristics of genetically engineered products are realized. Additionally, genetic tools are becoming increasingly available to prevent the unwanted escape of transgenic germplasm into wild populations (Goldberg et al., 1993). We can apply the genetic control of pollen and seed sterility with our *merA* plants, as well as further ensure no escape occurs by harvesting of these trees prior to sexual maturity. Concerns may also be raised about the deliberate evolution of gaseous mercury from polluted sites into the atmosphere. Precautions should certainly be taken to avoid releasing excessive levels of elemental mercury near population centers, but the substantially greater hazard is to ignore the high concentrations of highly toxic, ionic mercury at polluted sites. Furthermore, mercury-contaminated areas are continually volatilizing elemental mercury due to soil chemical reactivity and biological processes by microbial and plant populations adapted to such sites (Barkay et al., 1992), and thus even an aggressive phytoremediation program on selected areas would not significantly effect the current levels of atmospheric mercury. Therefore, we propose that the use of *merA* trees could be used to accelerate this process on our most urgent and sensitive sites and alleviate the hazards to local habitats and adjacent water supplies.

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