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RE: First-year progress report on DOE Grant Entitled: Phytoremediation of Ionic and Methyl Mercury Pollution

Dear Dr. Massey:

The long-term goal of our research is to manipulate single-gene traits into plants, enabling them to process heavy metals and remediate heavy-metal pollution by resistance, sequestration, removal, and management of these contaminants (Meagher and Rugh, 1996; Meagher et al., 1997). The working hypothesis behind this proposal was that “transgenic plants expressing both the bacterial organo mercury lyase (*merB*) and the mercuric ion reductase gene (*merA*) will A) remove the mercury from polluted sites and B) prevent methyl mercury from entering the food chain.” We have had a very successful first year either testing aspects of this hypothesis directly or preparing material needed for future experiments. Our results are outlined below under goals A and B, which are explicit in this hypothesis. There were less than 10% of the funds remaining in any category as projected in our first 12 month budget at the end of the first year, with the exception of the equipment category which had 25% of the funds remaining (~\$8,000). Much of this remaining equipment money is being spent this week on a mercury vapor analyzer.

It might be useful to remember that at the time this grant was awarded, we had successfully engineered a small model plant, *Arabidopsis thaliana*, to use a highly modified bacterial mercuric ion reductase gene, *merA9*, to detoxify ionic mercury (Hg(II)), reducing it to Hg(0) (Rugh et al., 1996). Seeds from these plants germinate, grow, and set seed at normal growth rates on levels of Hg(II) that are lethal to normal plants. In assays on transgenic seedlings suspended in a solution of Hg(II), 10 ng of Hg(0) was evolved per min per mg wet weight of plant tissue. However? at that time, we had no information on expression of *merA* in any other plant species, nor had we expressed *merB* in any plant.

Goal A: To remove the mercury from polluted sites.

Initial physiological studies were performed on the mercuric ion reductase gene, *merA*, expressed in *Arabidopsis* plant lines. When grown on highly mercury-contaminated soil as shown in **Figure 1A**, transgenic plants accumulated undetectable levels of mercury, while controls accumulated substantial levels (**Figure 1B**). Evidently expression of the MerA protein catalyzes Hg(0) volatilization and loss from the system and prevents Hg(II) from becoming strongly bound to plant tissues. This is an important result, because it suggests that the MerA-expressing plants will be safer for herbivores to consume than native plants growing at a contaminated site. It should be noted that in this experiment, a sulfur- and humic acid-rich soil was used so that control plants could grow, albeit poorly, up to 1000

ppm mercury. In poor soil, sand, or laboratory media, the control plants do not grow at concentrations above 5 ppm mercury.

The small size of *Arabidopsis* plants prevented us from carrying out more complex physiological studies, so transgenic tobacco, *Liriodendron tulipifera* (yellow poplar), and *Brassica napus* (rape seed) plant lines expressing *merA* were constructed. Monoclonal antibodies were made to the MerA protein and we developed simple ELISA and western blot assays for MerA expression. These immunological assays confirmed the expression of MerA in these two new macrophytes. Using transgenic tobacco, a number of experiments have examined the effectiveness of this system in the phytoremediation of mercury, and novel experiments are being set up to learn more about the mechanisms of mercury processing by these plants. For example, we demonstrated that when *merA9* tobacco plants are grown in hydroponic media with as little as 1 ppm or 5 μM Hg(II) as shown in **Figure 2A**, they volatilize 80% of the mercury from the system in a week (**Figure 2B**), whereas the control plants have little impact. In this experiment, 1000 μg of Hg(II) was added to 1 liter of media at $t = 0$ and the roots, tops, and media were all assayed at the times indicated. All the Hg(II) is bound to roots of both control and *merA9* plants in the first 24 hrs, but only the transgenic plants efficiently eliminated mercury from the roots over the next few days. As in the above experiments with *Arabidopsis* no significant amount of mercury is ever found in stems or leaves of the *merA9* tobacco plants. Similar results are obtained for the transgenic plants at much higher concentrations of Hg(II) in the media (not shown). In contrast, in this defined media, where Hg(II) is readily available, control plants do not survive with significantly higher Hg(II) concentrations. These data suggest that MerA-expressing transgenic plants (1) can be used to process industrial waste streams contaminated with mercury and (2) work efficiently even at very low levels of Hg(II) (i.e., the EPA limit for water is typically about 1 ppm). For the immediate future, our physiological experiments with transgenic tobacco are focused on determining if metallic Hg(0) vapor is transpired up through the vascular system and out of leaves, like H_2O vapor and waste gasses like CO_2 .

In an experiment designed to monitor mercury volatilization from the media similar to that published for *Arabidopsis*, transgenic and wild-type yellow poplar plantlets were transplanted to media containing 5 ppm Hg(II), as shown in **Figure 3A** (Rugh, 1997; Rugh et al., in prep.-b). A new generation construct expressing the mercuric ion reductase gene, *merA18*, was used in place of *merA9*. The air in head-space above each plant was sampled for Hg(0) content every 12 hours. The total cumulative Hg(0) vapor released from the system vs time is plotted in **Figure 3B**. Plantlets of various yellow poplar lines expressing MerA volatilized Hg(0) 10-20 times more efficiently than control plantlets. These data demonstrate how efficient and flexible this transgenic system is and how easily a variety of transgenic plant species may be used in the phytoremediation of mercury.

Many of the largest mercury contaminated sites with the most serious environmental impact are aquatic or marine ecosystems. Aquatic and marine grasses engineered to express MerA would be the most suitable for remediation of such environments. Expression of transgenes in monocots will take some extra effort, however, because the standard 35S CaMV plant promoter does not work efficiently or reliably in monocots. Monocots require special promoters, and relatively little is known about the few promoters that have been studied. New *merA* gene constructs were made for monocot expression using a variety of promoters and these are being tried out in rice, needle grass (salt tolerant estuarian grass), and *Spartina* (an extremely productive grass that dominates many estuarian environments). Only the rice transformation and regeneration system is at all characterized. We are setting up the rice transformation system to model monocot gene expression in our own laboratory and have sent these novel *merA* constructs to a collaborators laboratories to transform needle grass and *Spartina*.

Goal B: To prevent methyl mercury from entering the food chain.

At industrial, agricultural, and government sites polluted with mercury, endogenous bacteria produce an organic form of mercury, called methyl mercury, which is the most serious threat to the environment. Methyl mercury is biomagnified up the food chain, poses an immediate threat to wildlife and human populations, and is the most common source of mercury poisoning. As part of an operon of genes dealing with mercury, gram-negative bacteria living in these environments often encode an organomercury lyase. MerB, which protonolyzes the carbon mercury bond in a variety of organic

mercury compounds to produce the less toxic Hg(II) and a reduced carbon compound. We have constructed a modified bacterial gene, *merBpe*, for optimal bacterial and plant expression. Expression of the partially synthetic *merBpe* gene was confirmed in *E. coli* and in the small model plant, *Arabidopsis thaliana*. Monoclonal antibodies were prepared to the MerB protein and used to develop MerB assays which demonstrated that several lines of transgenic plants expressed the protein at high levels. Notably, seeds from the *merBpe* plants germinate and seedlings grow on 1-2 uM phenyl mercury acetate (PMA) or methyl mercury chloride (MeHg) as shown in **Figures 4A and 4B (left lane)** (Bizily et al., in prep.). These levels are lethal to normal plants (not shown) or *merA9*-expressing plants (**Figures 4A and 4B, right lane**). The most resistant *merBpe* transgenic lines grow on 1-2 uM of organic mercury at rates typical of unchallenged controls. Our work suggests that native macrophytes (e.g., trees, shrubs, grasses) engineered to express *merBpe* derivatives could be used to clean methyl mercury from polluted sites and prevent it from entering the food chain.

Using classical genetic crosses we constructed doubly transgenic plant lines expressing *merBpe* and the mercuric ion reductase, *merA9* (**Figure 4A-D, central lane**). With the two enzymes coupled together, MerB protonolyzes methyl mercury to Hg(II) and MerA electrochemically reduces Hg(II) to the relatively nontoxic metallic mercury, Hg(0), which is volatilized from the plants and the environment (Rugh et al., in prep.-a). Shoots from these plants grow at normal rates on much higher levels of organic mercury compounds (**Figure 4C and 4D, central lane**) than plants expressing *merBpe* or *merA9* alone (left and right lanes) or wild-type controls (not shown).

Our next step will be to engineer *merBpe*-expressing tobacco and yellow poplar lines, which can be used in physiological studies on the metabolism of organic mercury compounds. In addition, gene constructs suitable for *merBpe* expression in monocots are being made.

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If any additional information would be useful to document our progress please let me know.

Sincerely,

Richard B. Meagher
Professor and Head

Figure 1. Transgenic *merA9* *Arabidopsis* shoots do not accumulate mercury.

A. Transgenic *merA9* (left column) and wild type RLD (right column) *Arabidopsis* seeds were germinated on soil containing 0 (top row), 100 (middle row), 250 (not shown) and 1000 (bottom row) ppm Hg(II). **B.** Mercury accumulated in *Arabidopsis* shoot tissues after growth on mercury contaminated soil with various levels of mercury. Leaves were harvested from 4 week old plants and subjected to ICP analysis.

Figure 2. Transgenic *merA9* tobacco efficiently remove mercury from hydroponic media. **A.** Transgenic *merA9* tobacco and control plants were grown in hydroponic media. **B.** Mercury content of the media + root tissues at the times indicated was determined by ICP analysis. Insignificant levels of mercury were found in the leaf and stem tissues at all time points, and little mercury remained in the media by 24 hrs. 1000 ug of mercury was added to 1 liter of media at $t = 0$ (i.e., the initial concentration was 5 uM or 1 ppm).

Figure 3. Transgenic *merA9* yellow poplar plantlets vaporize significantly more metallic mercury from defined media than control plantlets. **A.** Transgenic *merA18* and control yellow poplar (*Liriodendron tulipifera*) plantlets were grown in defined agar media containing 5 μ M (1 ppm) Hg(II). **B.** Mercury vapor [Hg(0)] was assayed from the head space in each tube every 12 hours and is reported as a cumulative value normalized to the weight of the plantlet at the end of the experiment. Values shown are the average from several transgenic and several control plants. After six days all the controls had evolved little Hg(0) (107- 1020 pg/mg) and all the transgenic plant lines had evolved mercury efficiently (3500-11,670 pg/mg).

Figure 4. Germination of transgenic *Arabidopsis* seeds on phenyl mercury acetate (PMA): A. 0 uM; B. 1 uM; C. 3 uM; and D. 5 uM. The three lanes on each plate contain transgenic seeds expressing *merBpe* (left), both *merBpe* and *merA9* (center) and *merA9* alone (right).