

**Report for 2000-2002**

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**“Chlorinated Hydrocarbon Degradation in Plants: Mechanisms and Enhancement of Phytoremediation of Groundwater Contamination”**

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**Summary**

Several varieties of transgenic poplar containing cytochrome P-450 2E1 have been constructed and are undergoing tests. Strategies for improving public acceptance and safety of transgenic poplar for chlorinated hydrocarbon phytoremediation are being developed. We have discovered a unique rhizobium species that lives within the stems of poplar and we are investigating whether this bacterium contributes nitrogen fixed from the air to the plant and whether this endophyte could be used to introduce genes into poplar. Studies of the production of chloride ion from TCE have shown that our present P-450 constructs did not produce chloride more rapidly than wild type plants. Follow-up studies will determine if there are other rate limiting downstream steps in TCE metabolism in plants. Studies of the metabolism of carbon tetrachloride in poplar cells have provided evidence that the native plant metabolism is due to the activity of oxidative enzymes similar to the mammalian cytochrome P-450 2E1.

**Metabolism of TCE and EDB by Transgenic Poplar**

We have developed several varieties of transgenic poplar containing the human cytochrome 2E1 gene under the control of different promoters and some plants with NADPH P450 oxidoreductase and cytochrome b5 in addition to 2E1. To screen the transgenic plants for enhanced metabolism of halogenated hydrocarbons, we have grown cuttings in sterile hydroponics and dosed the solution with ethylene dibromide (EDB). Two plants with both the greatest uptake of EDB and the highest release of bromide ion into the hydroponic solution were TS9-7, an H11-11 (*P. trichocarpa* x *P. deltoides*) clone with the h2E1 gene driven by a root-specific promoter, and Aram2, an H11-11 clone with the h2E1, OR, and b5 all driven by Mac promoters that are expressed throughout the plant but most highly in roots. More plants were screened for enhanced metabolism of TCE. Free (unconjugated) trichloroethanol, an early metabolite of TCE, was extracted from tissues of the dosed plants. Again, plant TS9-7 was superior to the other plants, having six times more of the metabolite in leaves and eight times more in roots, compared to levels in the control plant. We are currently propagating this plant and more control plants for a more thorough analysis.

**Poplar Transformation with Cytochrome P450 2E1 of Rabbit**

In an effort to increase the expression of the transgene, we developed another binary vector containing the rabbit 2E1 gene under the control of the widely and successfully utilized

CaMV 35S promoter, and flanked the DNA with matrix attachment regions that increase the expression of transgenes in poplar. We obtained a more easily transformable poplar clone, 717-1B4, a *P. tremula* x *P. alba* cross developed by the INRA of France. After propagating the clone in tissue culture, we did one transformation experiment and shoots are now visible. When they are sufficiently grown, we will extract DNA from a leaf of each shoot and use PCR to verify if the shoots are transgenic. We will continue doing more transformation experiments as soon as sufficient tissue becomes available. We are also using several different techniques to transform our more recalcitrant varieties of poplar (*P. trichocarpa* x *P. deltoides* clones).

### **Development of a Binary Vector Conferring Salt Tolerance**

We are developing an alternative selection for transgenic poplar cells that would utilize salt resistance rather than antibiotic resistance. The release of antibiotic resistance genes into the environment has been a concern of those opposed to genetically modified plants. By making our plants salt tolerant instead, the plants will also have the advantage of being able to grow in less than favorable environmental conditions. We obtained a cDNA clone from aspen from the lab of Arie Altman. We have purified the gene and will clone it into a binary vector to test if the gene will confer salt tolerance on our poplar varieties.

### **Analysis of a Poplar Endophyte**

We have recently discovered a rhizobium species within the stems of poplars (cottonwood trees). Surface-sterilized poplar stems from both greenhouse and outdoors were incubated on plant growth medium. Bacteria with prodigious exopolysaccharide production grew from the cut sites, and surrounded the explants without affecting plant growth. Sequencing of 16S and 23S ribosomal RNA genes of the poplar endophyte revealed that the bacteria is *Rhizobium tropici*. This species is known for its ability to nodulate a wide range of legumes, although its endophytic nature has not been reported. We reasoned that if this poplar endophyte can fix dinitrogen within the stems of poplar, a nonlegume, then the plants might grow in nitrogen-free medium. Indeed, surface-sterilized cuttings of poplar grew vigorously in medium lacking fixed nitrogen (nitrate and ammonium), unlike control plants of tobacco, tomato, and unnodulated clover. Sterile, *in vitro*-grown poplar plantlets inoculated with the bacteria grew vigorously in nitrogen-free medium while uninoculated plants failed to grow. We are continuing to study this important interaction between endophytic rhizobium and our poplar clones.

An alternate approach to transforming poplar with genes for the metabolism of halogenated hydrocarbons is to instead transform the endophyte with the genes, and then introduce the bacteria into the plants. This method offers a number of advantages. Whole plasmids from detoxifying bacteria could be introduced into the poplar endophyte. Multi-subunit genes from bacteria are often encoded by several genes in an operon which cannot be transcribed by plants. Another advantage would be the rapid production of a large number of plants with enhanced ability to remediate. Rather than regenerate transgenic plants from single transformed cells, hundreds of plants could simply be dipped into the bacterial inoculum. With the ability of poplars to take up large volumes of contaminated water and the ability of the bacteria to degrade the pollutant, we believe that significant reductions of the contaminants could be attained. A

third advantage to this method is the removal of genetic engineering from the process. Whole plasmids from bacteria that already exist in the environment would be introduced into endophytic bacteria that naturally exist in poplars. There would be no selection for maintenance of the plasmid other than the presence of TCE in the environment that would favor bacteria that contained the plasmid. We have already determined that we can mobilize plasmids into the poplar endophyte.

### **Chloride Production from TCE, Transgenic Tobacco Compared to Wild-Type**

We have compared the ability of transgenic tobacco plants to completely dechlorinate TCE against the ability of wild-type control plants. The transgenic plants were grown from seed from first generation clones and the presence of antibiotic resistance genes, was confirmed by survival of leaf disks on agar plates with kanamycin. The plants were grown in hydroponic culture. After substantial root systems had formed, the plants were transferred to flasks that could be sealed around the stem and dosed with TCE through a septum valve. Plants were grown in a fume hood in flasks sealed with glass plates and plumber's putty around the stems.

In order to accurately determine the production of chloride, the concentration of chloride in the media during the TCE exposure was limited. Eight plants of each variety (wild type or transgenic) received solution with only trace chloride (0.016 ppm), and the other eight received 1/2 strength Hoagland's solution with chloride added (3.7 ppm). Each group of eight was further split into two groups of four: one exposed to TCE, and the other undosed. At the same time, six flasks containing glass rods instead of plants were set up to control for abiotic losses of TCE and chloride production.

Aqueous samples were taken on days 0, 7, and 14. After 14 days, all plants were removed from their flasks, rinsed, weighed, and frozen at  $-80^{\circ}\text{C}$ . Plant tissues were extracted for Cl<sup>-</sup> analysis by ion chromatography.

TCE concentrations in the growth solutions fell from about 95 ppm on day zero to 40 ppm on the seventh day in the glass rod controls. Planted flasks had lower TCE concentrations in the media after 7-14 days, but the differences were not significantly different due to large variation between replicates.

The concentration of chloride in the solution of the glass rod controls did not significantly change over the course of the experiment.

Since chloride is an essential plant micronutrient, we expected chloride in the solutions to be taken up to supply the chloride requirements of new plant growth. Indeed chloride in the nutrient solutions of plants was lower than in the controls. In order to use chloride production as a measure of the total destruction of TCE we determined the mass of chloride in the media and in the plant tissue using the concentrations of chloride in each multiplied by the masses, and summed them to obtain the total chloride mass in each flask at the end of the experiment.

Given the greatly increased production of trichloroethanol in the transgenic tobacco, we expected the transgenics to produce more chloride. This was not the case. Although the plants clearly produced chloride in response to TCE addition, there were no significant differences in total chloride between transgenic versus wild type plants under any set of conditions. Plant tissues did exhibit chloride concentration differences. Root and stem tissues in plants growing with added chloride in the media had significantly higher chloride concentrations in TCE dosed

plants than in undosed plants.

These results suggest that TCE degradation in the transgenic plants is rate-limited by a step downstream from trichloroethanol. Although we have determined that trichloroethanol can be exchanged with a pool of glucoside-conjugated trichloroethanol, we have little understanding of subsequent steps in the dechlorination of TCE. We have observed trichloroacetate and dichloroacetate in plant tissues after exposure to TCE. If plant metabolism of TCE is similar to mammalian pathways we would expect that trichloroethanol would ultimately be degraded to unchlorinated products such as glyoxylate.

Based on these results we plan to repeat the experiment to confirm the results and to measure the accumulation of trichloroethanol in tobacco and in transgenic vs. wild-type poplar. The degradation of carbon tetrachloride (CT) in plant tissue is much simpler than the degradation of TCE, consisting of a single dechlorination to chloroform, followed by an abiotic degradation to phosgene and hydrolysis to chloride and carbon dioxide. Since the mammalian cytochrome P-450 2E1 introduced into our transgenic tobacco and poplar is also capable of metabolizing CT, we will repeat the comparison of chloride production from wild-type vs. transgenic tobacco and poplar using CT as the chlorinated hydrocarbon.

### **Carbon Tetrachloride Degradation in Poplar**

Previous work in our lab showed that oxidation of carbon tetrachloride in tissue cultures of poplar cells proceeded to CO<sub>2</sub> though the intermediate chloroform. More chloroform was produced under anaerobic conditions. No dichloro- or mono-chloromethane was detected. Significant <sup>14</sup>C was detected bound to cell tissues after exposure to radiolabeled CT and the amount of bound carbon increased under anaerobic conditions. These results are consistent with an oxidative activity being responsible for CT degradation.

CT oxidation to CO<sub>2</sub> and/or covalent cell binding of CT carbon was inhibited by carbon monoxide, a general inhibitor of cytochrome P-450 activity and by specific inhibitors of mammalian cytochrome P-450 2E1 (chlorzoxazone, isoniazid, 4-methylpyrazole, piperonyl butoxide and 1-phenylimidazole), but not by inhibitors of lignin peroxidases, NaVO<sub>3</sub> or 4-amino-1,2,4-triazole, or by piperonylic acid, a highly specific inhibitor of trans-cinnamate 4-hydroxylase, the most common cytochrome P-450 in plants. Taken together these results support the hypothesis that CT oxidation in poplar cells is due to plant monooxygenase activities closely related to that of the mammalian cytochrome P-450 2E1.

### **Publications**

1. Doty, S. L., Shang, T. Q., Wilson, A. M., Moore, A. L., Strand, S. E., Oda, C. and Gordon, M. P. Metabolism of the halogenated hydrocarbons, TCE and EDB, by the tropical leguminous tree, *Leuceana leucocephala* *Water Research*, in press.
2. Banerjee, S., T. Q. Shang, A. M. Wilson, A. L. Moore, S. E. Strand, M. P. Gordon, S. L. Doty. Expression of functional mammalian P450 2E1 in hairy root cultures. (2002) *Biotechnology and Bioengineering* 77: (4) 462-466.
3. Doty, S. L., Q.-T. Shang, A. M. Wilson, J. Tangen, A. D. Westergreen, L. A. Newman, S. E. Strand, and M. P. Gordon. Enhanced Metabolism of Halogenated Hydrocarbons in Transgenic Plants Containing Mammalian Cytochrome P450 2E1. (2000) *Proc. National Academy of Sciences*. 97, 6287-6291.

4. Wang, X., Gordon, M. P., Strand, S. E., Mechanism of Aerobic Transformation of Carbon Tetrachloride by Poplar Cells. *Environmental Toxicology and Chemistry*, submitted.

#### **Graduate Student theses**

Xiaoping Wang, Ph.D., June 2002  
Willy Welzenbach, M.S., June 2002