EMSP Project (DE-FG07-96ER20256)
"Chlorinated Hydrocarbon Degradation in Plants: Mechanisms and Enhancement of Phytoremediation of Groundwater Contamination"

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Final Report 2003

1. Research Objectives

Our research objectives are as follows:

- Transform poplar and other tree species to extend and optimize chlorinated hydrocarbon (CHC) oxidative activities.
- Determine the mechanisms of CHC oxidation in plants.
- Isolate the genes responsible for CHC oxidation in plants.

2. Research Progress and Implications

We have made significant progress toward an understanding of the biochemical mechanism of CHC transformation native to wild-type poplar. We have identified chloral, trichloroethanol, trichloroacetic acid, and dichloroacetic acid as products of TCE metabolism in poplar plants and in tissue cultures of poplar cells.(Newman et al. 1997; Newman et al. 1999) Use of radioactively labeled TCE showed that once taken up and transformed, most of the TCE was incorporated into plant tissue as a non-volatile, unextractable residue.(Shang et al. 2001; Shang and Gordon 2002) An assay for this transformation was developed and validated using TCE transformation by poplar suspension cells. Using this assay, it was shown that two different activities contribute to the fixation of TCE by poplar cells: one associated with cell walls and insoluble residues, the other associated with a high molecular weight, heat labile fraction of the cell extract, a fixation that was apparently catalyzed by plant enzymes.

The nature of the unextractable TCE metabolite was investigated.(Shang et al. 2001) We had previously shown that TCE metabolites in plants are similar to ones that result from cytochrome P450-mediated oxidation in mammals: trichloroethanol, trichloroacetate and dichloroacetate.(Newman et al. 1997; Newman et al. 1999) We have shown that one of these metabolites, trichloroethanol, is further glycosylated in several plant species. The glycoside was detected in all tissues (roots, stems, and leaves) in comparable levels, and is at least 10 fold more abundant than free trichloroethanol. The glycoside in tobacco was identified as the β-D-glucoside of trichloroethanol by comparison of the mass spectra and chromatographic retention time of its acetylation product to that of the synthesized standard. Trichloroethanol and its glucoside do not persist in plant tissue once plants are removed from TCE contaminated water, indicating further metabolism, following the pathway shown in **Figure 1**. We have found these products of TCE metabolism (trichloroethanol and its glucoside and di- and trichloroacetic acid) in all plants examined, i.e., poplar,(Newman et al. 1997; Newman et al. 1999) tobacco,(Doty et al. 2000) *Leuceana leucocephala*,(Doty et al. 2003) and *Atropa belledonna*.(Banerjee et al. 2002) They seem to be universal intermediates of plant metabolism of TCE.

We have also identified the intermediates of plant metabolism of carbon tetrachloride (CT).(Wang et al. 2002) Using cell cultures of poplar CT is transformed to chloroform and to CO_2, which are also products of CT transformation by CYP2E1 in mammals. These activities are inhibited by specific inhibitors of cytochrome P450s as developed in the study of mammalian

hepatic detoxification. The strongest inhibition was in the presence of specific inhibitors of CYP2E1, i.e., isoniazid, chlorzoxazone, 4-methylpyrazole, and 1-phenylimidazole (Table 1), supporting our hypothesis that the intrinsic transformations of CHCs are due to a plant cytochrome similar to CYP2E1. Inhibitors of enzymes involved in lignin synthesis (e.g., piperonylic acid) had no effect on CT transformations.

Molecular approaches to discovering the genes involved in plant metabolism of CHCs have also yielded promising results. We have acquired sufficient mRNA from tissue cultures of poplar cells to produce a cDNA expression library in yeast for screening for TCE transformation. We have also developed functional assays for TCE oxidation and for the oxidation of TCE intermediates in yeast. These methods are described in more detail below.

We have tested our transformed plants, both tobacco and poplar, with TCE, analyzing for production of trichloroethanol and chloride and bromide ion as indicators of CHC degradative activity. Experiments with transgenic tobacco compared to wild-type plants exposed to TCE have shown greatly increased levels of trichloroethanol, but chloride ion production by the transgenics was not enhanced over the wild-type, suggesting that total dechlorination of TCE was rate-limited by an enzymatic step downstream of the initial P450 oxidation of TCE to chloral and to trichloroethanol. This result is complicated by the requirement that plants have for chloride ion as a micronutrient. To measure the production of chloride, it is necessary to produce a low chloride background in the media, which can affect plant metabolism, and to analyze and sum the amounts in the plant tissues and in the medium. The cumulative errors from this procedure limit the sensitivity of our assay for dechlorination. We have recently shown that brominated hydrocarbons can act as surrogates, producing bromide that can easily be measured even in the presence of excess chloride. These methods are described in more detail below.

We now have several plants genetically modified to incorporate mammalian CYP2E1. We have moved from the potentially more controversial use of human genes to the gene for rabbit CYP2E1. This gene has been successfully inserted into tobacco(Doty et al. 2000), *A. belledonna* (Banerjee et al. 2002), and poplar (Doty, in preparation). Improved transformation of TCE to trichloroethanol has been demonstrated in all three species. For example, Figure 2 shows the free trichloroethanol production by poplar transformed with CYP2E1.

We have produced rabbit CYP2E1 transformants in two hybrid poplars: *Populus trichocarpa* x *P. deltoides* 184-402 and *Populus tremula* x *P. alba* N717 1-B4 developed at the INRA (Institut National de la Recherche Agronomique, France). The former is a fast-growing Pacific Northwest hybrid that has been successfully transformed at the experimental station at Oregon State University as well as in our lab, while the latter is a European hybrid that has been reported to be particularly easy to transform.

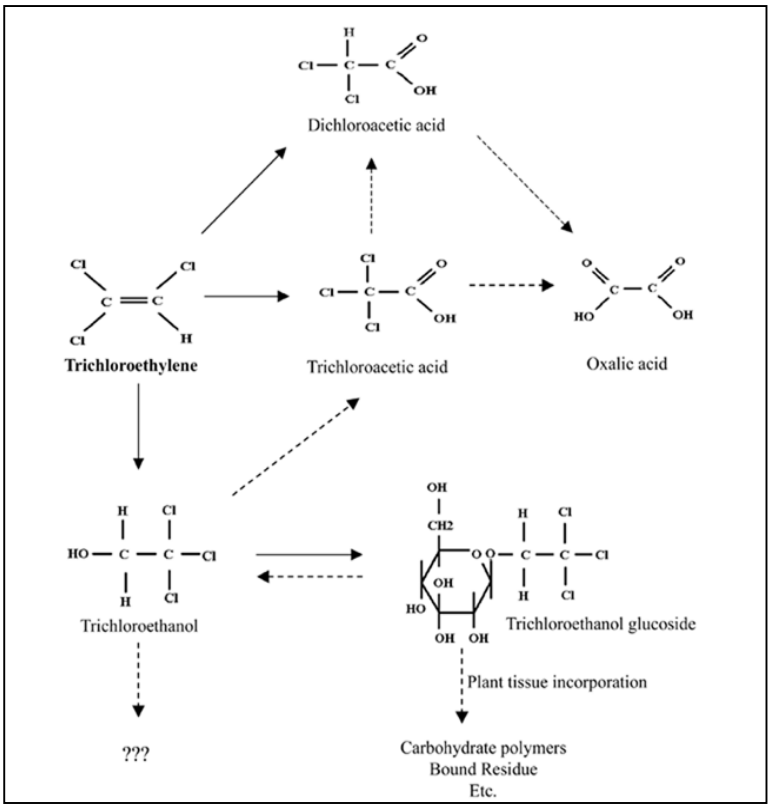

Figure 1 – The trichloroethanol pathway for TCE metabolism in poplar

Genetic transformation of poplar is relatively inefficient compared to that of tobacco, and the regeneration of plants from the callus is a slow and uncertain process. Several months are needed to produce shoots and roots from callus of poplar. We have conducted two experiments with techniques that were intended to improve transformation efficiency. *Agrobacterium fogenes* transformations were successful in producing rabbit CYP2E1 transformants of *A. belledonna*, but we have been unable to obtain hairy root cultures from poplar yet. Addition of the adenine biosynthesis inhibitor, azaserine, which has been reported by others to enhance transformation efficiency (Matt Metz, R Roberts, and EW Nester, submitted), had no effect on production of transformants from poplar.

Improvements to the rate and efficiency of plant regeneration of poplar as well as other plants useful to phytoremediation have been more difficult to achieve. We are experimenting with methods used to promote differentiation in mammalian stem cell cultures, such as optimizing the effects of diffusible factors involved in differentiation and embryogenesis and organogenesis by adjustments to the densities of callus cultures.

Table 1 -- Effects of P-450 inhibitors on carbon tetrachloride transformation

Inhibitors	Doses	CT transformation products measured as ^{14}C (dpm per bottle)	
		CO_2	Cell-bound ^{14}C
CO	0%	4480 ± 158	12400 ± 2210
	10%	3310 ± 132[a]	9260 ± 117
	40%	2570 ± 155[a]	7080 ± 358[a]
	70%	1660 ± 166[a]	6100 ± 331[a]
Isoniazid [b]	0 mM	3810 ± 231	8130 ± 190
	1 mM	2890 ± 221[a]	6170 ± 163[a]
	5 mM	2160 ± 145[a]	5910 ± 62[a]
	10 mM	1630 ± 127[a]	5320 ± 77[a]
Chlorzoxazone [b]	0 mM	1240 ± 115	3140 ± 57
	0.1 mM	1140 ± 16	2740 ± 132[a]
	0.2 mM	871 ± 170[a]	1840 ± 200[a]
	0.3 mM	969 ± 52[a]	1730 ± 112[a]
4-methylpyrazole [b]	0 mM	1230 ± 61	2550 ± 96
	0.1 mM	1080 ± 34[a]	1990 ± 48[a]
	0.2 mM	977 ± 35[a]	1450 ± 68[a]
	0.4 mM	776 ± 15[a]	1390 ± 80[a]
1-phenylimidazole [b]	0 mM	1700 ± 33	6980 ± 180
	0.1 mM	1270 ± 90[a]	4760 ± 226[a]
	0.2 mM	1020 ± 35[a]	2500 ± 418[a]
	0.3 mM	1070 ± 35[a]	2220 ± 139[a]
SKF-525	0 mM	3730 ± 175	7650 ± 769
	0.1 mM	3530 ± 630	8380 ± 1250
	0.2 mM	3770 ± 276	6820 ± 778
Piperonyl butoxide	0 mM	3190 ± 196	7740 ± 778
	0.01 mM	2250 ± 332[a]	9040 ± 1160
	0.05 mM	1840 ± 123[a]	7350 ± 668
Piperonylic acid [c]	0 mM	2767 ± 255	2871 ± 313
	0.25 mM	2066 ± 62	4207 ± 206[a]
	0.5 mM	2295 ± 201	4574 ± 639

Mean ± standard deviation of the mean

[a] Significantly different from response with no inhibitor ($p = 0.05$)

[b] Specific inhibitors of mammalian cytochrome P-450 2E1

[c] Specific inhibitor of plant cytochrome P-450 C4H

Inhibitor concentrations limited to levels that did not inhibit cell growth.

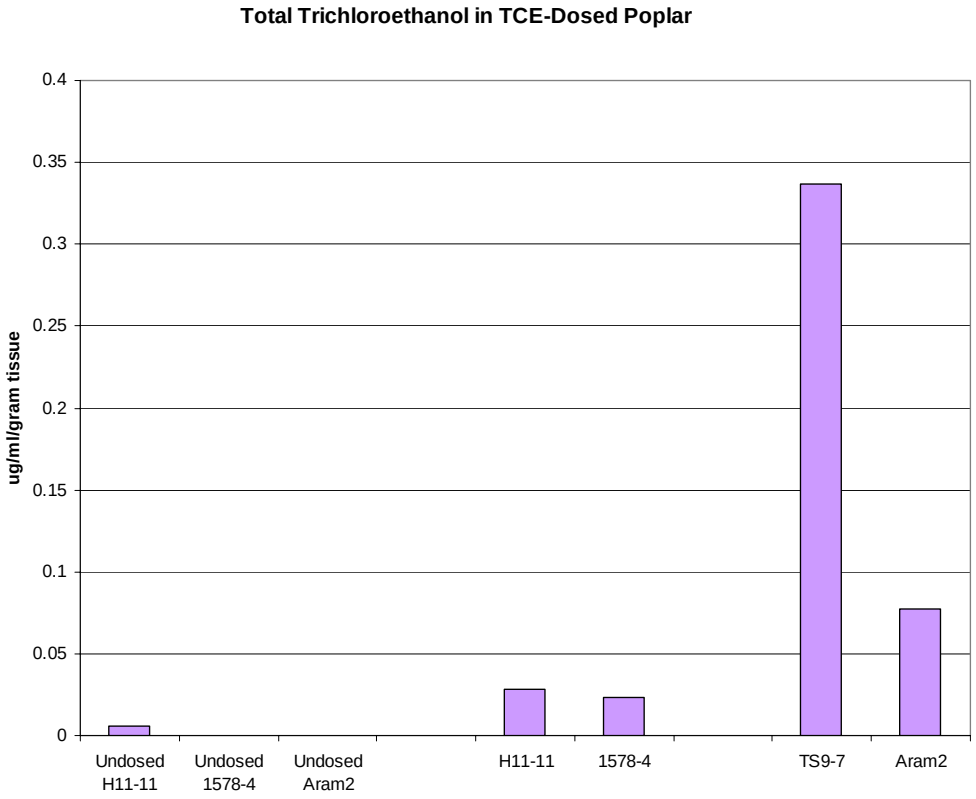

Figure 2 -- Oxidation of TCE by transgenic poplar
TS9-7 is poplar hybrid H11-11 transformed with Pmac-human CYP2E1-Tmas. Aram2 is H11-11 transformed with human CYP2E1, cytochrome b5, & oxidoreductase, each driven by the Mac promoter. H11-11 & 1578-4 are controls without CYP2E1. 1578-4 is the H11-11 vector control. Plants were exposed to 50 µg/ml TCE for 5 days. The total free (unconjugated) trichloroethanol per plant is shown.

We are also involved in collaborations with Steve Strauss at OSU to increase our ability to handle large numbers of transformed poplar callus cultures, and Ron Sederoff at North Carolina State University to produce CYP2E1 transformants of pine.

An obvious deficiency of phytoremediation that uses deciduous trees such as poplars is the annual loss of leaves and the almost complete cessation of phytoremediation during the winter months. This effect can be partially ameliorated by planting deciduous plants in an area far beyond the plume of the toxin (Matthews et al. 2003). Gymnosperms are expected to function for a greater fraction of the year. To this end we have carried out preliminary experiments with loblolly pine in collaboration with Dr. Ronald Sederoff at North Carolina State Department of Forest Biotechnology. Dr. Sederoff has had experience with the transformation of gymnosperms and is working with us on the incorporation of various cytochrome p450s into the loblolly pines. Thus, we will not only have normal loblolly pines for use in our phytoremediation experiments, but those that contain a more active rabbit CYP2E1. These are very preliminary experiments that are underway, but they show promise.

TCE Metabolism Pathway in Plants

Trichloroethanol Disappearance in Poplar

We have shown that trichloroethanol is not the final TCE metabolite in poplar and tobacco plants. When the TCE-exposed plants were removed from TCE, the levels of trichloroethanol declined. To better quantify this degradation, we used poplar cell cultures. We exposed eight flasks of poplar culture cells to TCE for three days. Three flasks of cells were then analyzed for trichloroethanol levels, while the remaining flasks of cells were collected and resuspended in fresh medium without TCE. These cultures were allowed further growth for three or six more days. The levels of trichloroethanol dropped dramatically after three days (Figure 3). Therefore, the effect was not limited to whole plants, suggesting that the genes involved in trichloroethanol metabolism are expressed in poplar culture cells as well as in the whole plants.

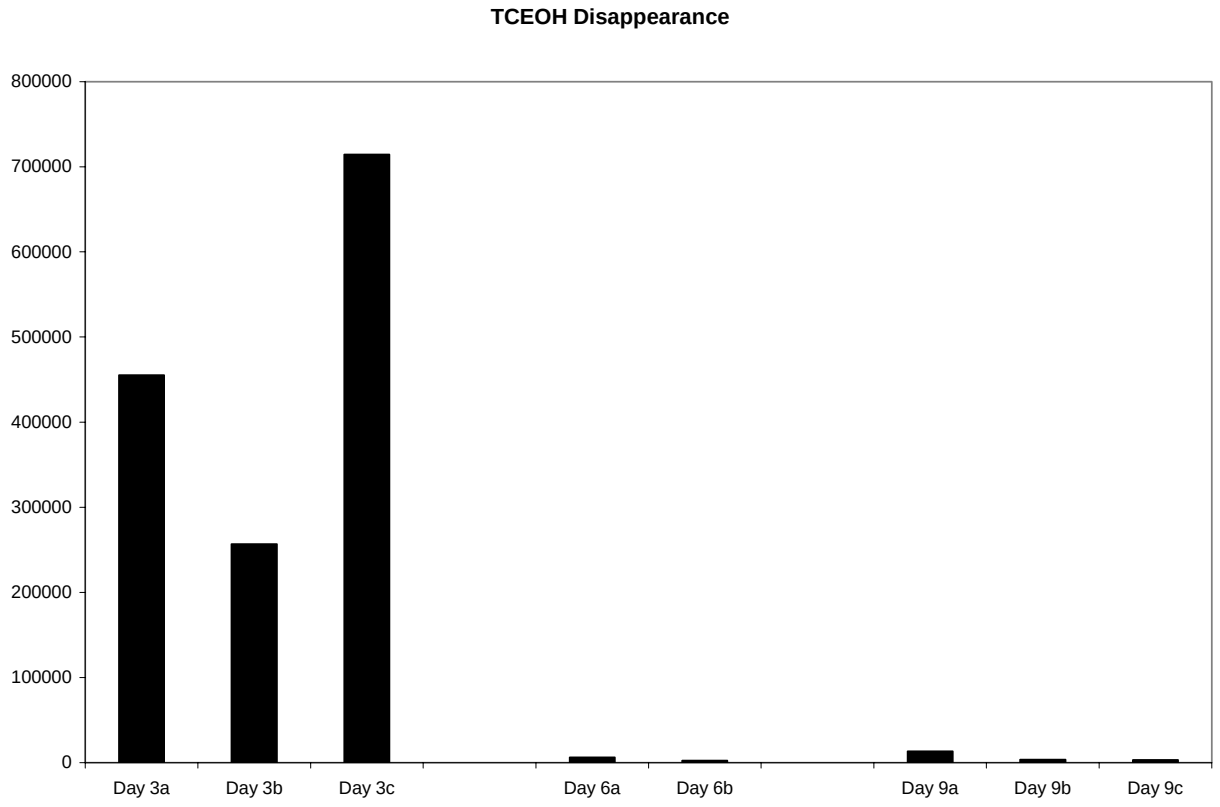

Figure 3 – Trichloroethanol disappearance following dosage of poplar tissue cultures with trichloroethylene

Bromide Ion Release from Tribromoethanol-dosed Poplar

Due to the difficulties in quantifying chloride ion release from trichloroethanol metabolism, we decided to use tribromoethanol as a surrogate for trichloroethanol and to then measure bromide ion release to quantify its degradation by plant cells. We dosed poplar culture cells to a level of 50 µg/ml tribromoethanol and collected the cells and supernatants after four days. We dried and extracted the cells in water and analyzed these samples as well as samples of the supernatants on the ion chromatograph for bromide ion. We had also done a series of controls including undosed

cells, media without cells, cell filtrates, and killed cells. There was strong production of bromide ion from the living poplar cells but not from any of the negative controls (Figure 2).

We also conducted a time course experiment of bromide production from tribromoethanol. There was a steady increase in bromide ion production from day one to day seven when we ended the experiment (Figure 5).

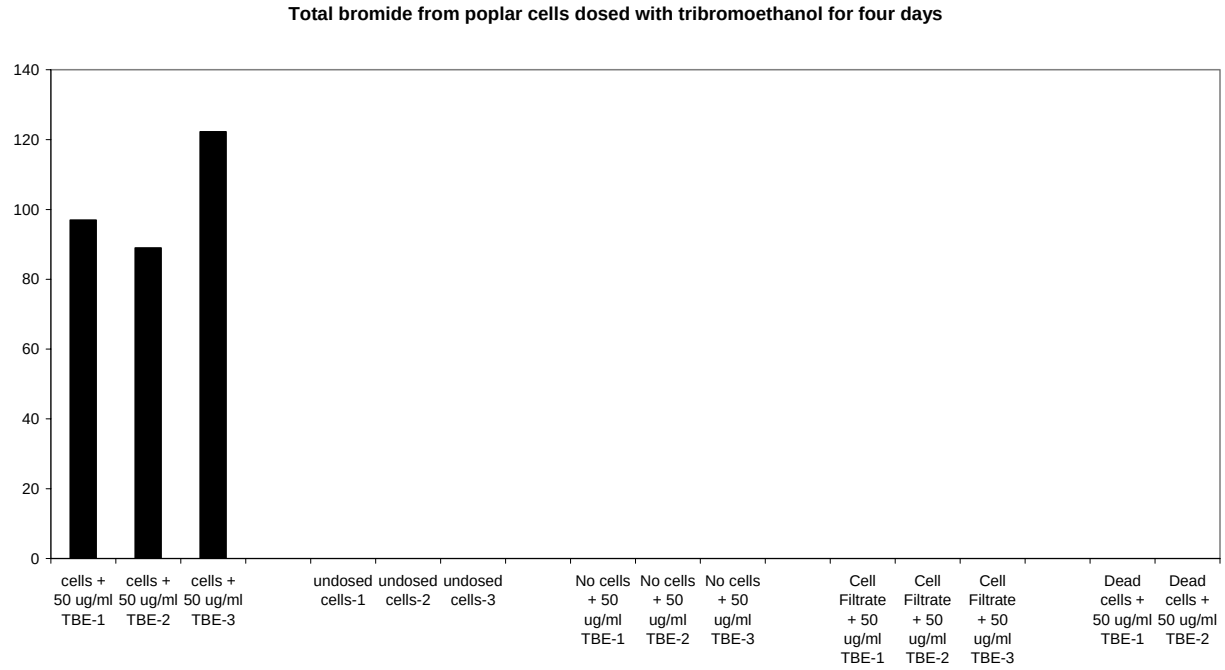

Figure 4 – Bromide ion production in poplar cell cultures exposed to tribromoethanol

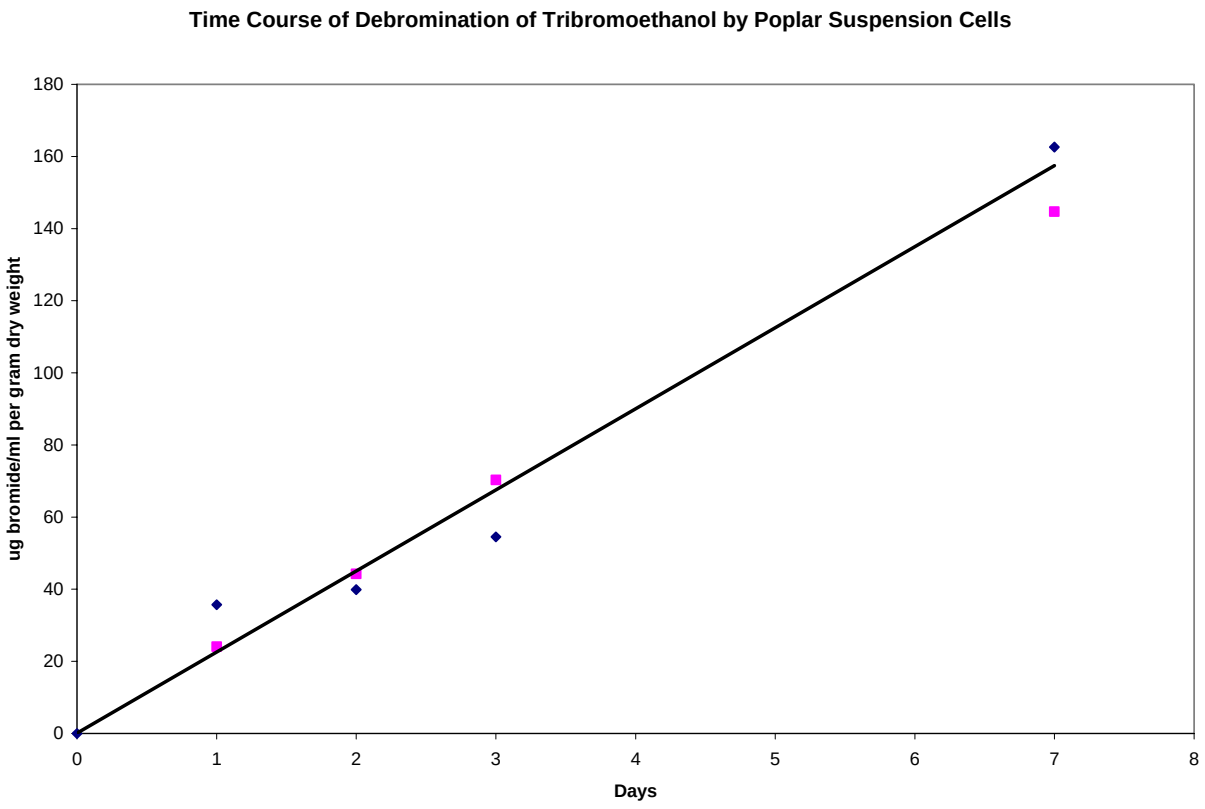

Figure 5 – The time course of bromide ion production by poplar cell cultures exposed to tribromoethanol

Arabidopsis

We obtained Arabidopsis cell culture stock from the laboratory of Luca Comai (UW Biology) and grew the cells in a series of flasks for metabolism experiments. We dosed the cell culture with TCE and analyzed the cells and media for metabolites. Arabidopsis, like poplar, was able to produce trichloroethanol from TCE. Therefore, we will be able to use Arabidopsis as a model plant for the genetic study of TCE metabolism in plants.

E. coli

For cDNA library screening (see section below), we needed to know if bacteria could also produce the same metabolites as the plant cells. When dosed with tribromoethanol, the bacteria released bromide ion. However, when dosed with TCE, the cells did not produce trichloroethanol. Therefore they are blocked at the early steps in this pathway and can be used to functionally clone plant genes involved in this early step of TCE degradation.

cDNA Library Construction

Poplar cDNA

We successfully purified poplar mRNA from cell cultures dosed with TCE. We isolated cDNA

but were unable to clone it into the pRS316GAL cloning vector with high efficiency using the method of (Liu et al. 1992). We are turning to a different vector used successfully for similar work as ours for the identification of plant genes. From Leon Kochian=s laboratory, we obtained the yeast expression vector, pFL61. We purified it and are beginning to clone control genes (P450 and oxidoreductase) into it to verify that this vector system will work for our purposes.

Arabidopsis cDNA Library

While we continue constructing a poplar cDNA expression vector for use in yeast, we also purchased from Stratagene an Arabidopsis cDNA library for expression in bacteria. The library is in a phagemid and the genes are expressed with the *lac* promoter. We amplified the library and did a mass excision to collect the library as phagemids. We transfected SOLR cells with very high efficiency and purified the phagemid DNA from several colonies and verified the integrity of the library.

To create a positive control for designing a screen in bacteria for genes involved in the early steps of TCE metabolism, we subcloned the rabbit cytochrome P450 2E1 cDNA into the same cloning site in the phagemid as the cDNA library but using blunt ligation. Following sequence verification, we will introduce this vector into E. coli and assay for early TCE metabolites. If the gene does function in E. coli, we will design tests for visually screening the Arabidopsis library.

Poplar Transformations

Poplar Transformed with Human Cytochrome P450 2E1

We have introduced the human CYP2E1 gene into the poplar hybrid, H11-11, a P. trichocarpa x P. deltoides cross. We screened over a dozen transgenic plants, and have now focused on the best two plants. TS9-7 contains the CYP2E1 gene under the control of the root-specific glutamine synthetase gene froms soybean, while Aram2 contains the CYP2E1 gene, oxidoreductase gene, and cytochrome b5 gene, all under the control of Mac promoters. These two plants consistently perform well in assays for TCE metabolism and EDB metabolism. Figure 6 shows the contrast in trichloroethanol levels between TCE-dosed control plants, non-transgenic H11-11 and transgenic H11-11 containing the null binary vector, and the two CYP2E1 plants dosed with TCE. Further work is being done to study the enhanced metabolism of halogenated hydrocarbons on these two clones.

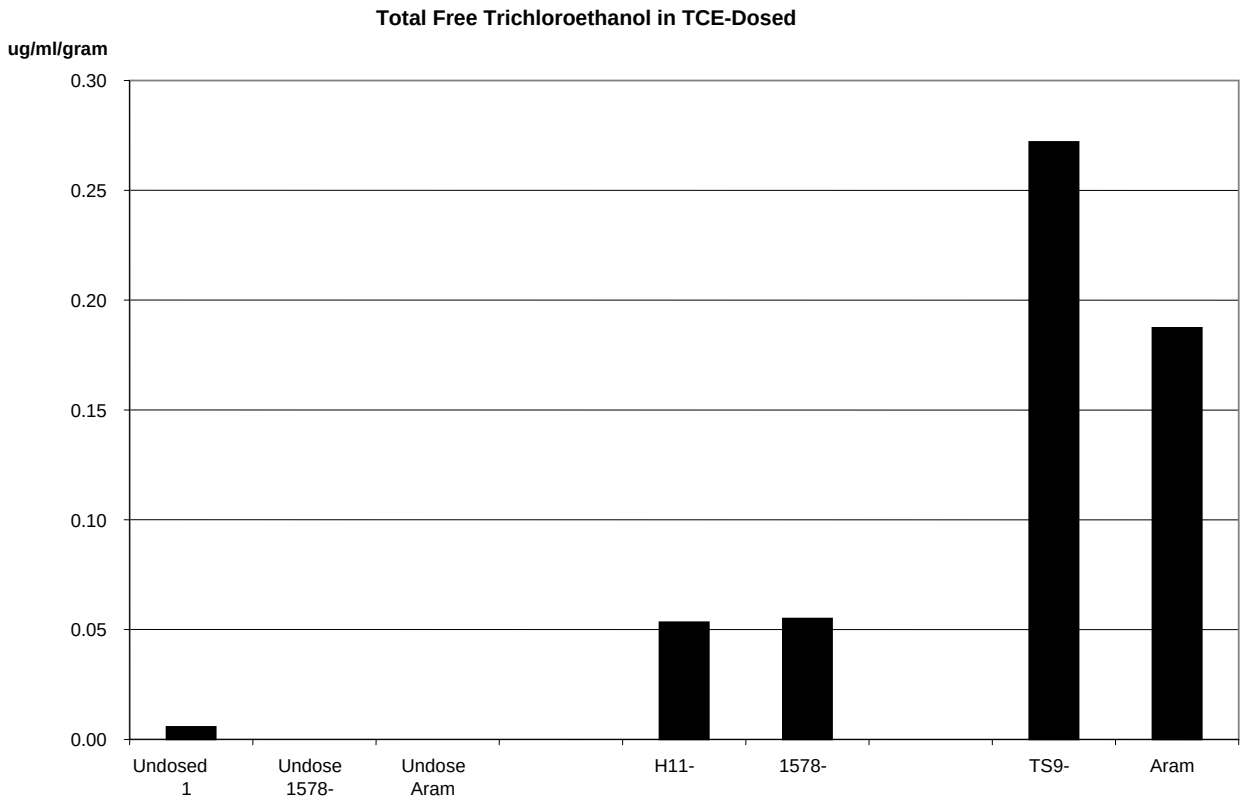

Figure 6 – Total free trichloroethanol in transformed poplar exposed to TCE

Rabbit CYP2E1 Constructs

We were not satisfied with the level of increased TCE metabolism in poplars by the CYP2E1 constructs, so we developed a new construct. We used the 35S promoter instead of the Mac promoter which is a hybrid of 35S and mas promoters. The 35S promoter has been proven to work very well in poplars. We also switched to a different binary vector that flanks the T-DNA genes of interest with matrix attachment regions that increase expression and stability of transgenes in poplar. We introduced the final constructs into both types of Agrobacterium for poplar transformations.

Transformation of Poplar by Agrobacterium rhizogenes containing Rabbit CYP2E1

There are no reports of the use of A. rhizogenes to transform the poplar hybrid, INRA 717-1B4. We constructed a supervirulent A. rhizogenes strain and infected sterile leaves. Hairy roots grew readily from the poplar. When transferred to hormone-free MS plates, the roots grew but at a much slower rate than Atropa belledonna or tobacco roots. We have several root lines containing the rabbit P450 2E1 gene. When we have propagated enough transgenic roots, we can begin TCE metabolism studies in this system.

Transformation of Poplar by Disarmed Agrobacterium tumefaciens

We and our colleague, Rick Meilan (Oregon State University), have developed over a hundred

transgenic poplar containing the rabbit P450 2E1 gene and null vector. The poplars include the P. tremula x P. alba clone 717-1B4, the P. tremula x P. tremuloides clone 353-38, and our triploid P. trichocarpa x P. deltoides clone. We have transferred the plants to soil successfully, and are continuing to propagate the plants prior to analysis for enhanced TCE metabolism.

Four graduate students (two doctorates and two MS) were supported on this project and graduated. Six peer-reviewed papers were published:

Wang, X., Dossett, M., Gordon, M. P., and Strand, S. E.. Fate of Carbon Tetrachloride during Phytoremediation with Poplar under Controlled Field Conditions. (in press) Accepted by Environmental Science & Technology.

Matthews, D. W., J. Massmann, and S. E. Strand. Influence of Aquifer Properties on Phytoremediation Effectiveness. (2003) Ground Water, 41(1)41-47.

Doty, S. L., T. Q. Shang, A. M. Wilson, A. L. Moore, L. A. Newman, S. E. Strand, and M. P. Gordon. Metabolism of the Halogenated Hydrocarbons, TCE and EDB, by the Tropical Leguminous Tree, Leuceana leucocephala. (2003) Water Research, 37 (2) 441-449.

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