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Using Trees to Remediate Groundwaters Contaminated with Chlorinated Hydrocarbons

MECHANISMS AND GENETIC ENHANCEMENT OF PLANT METABOLISM OF CHLORINATED HYDROCARBONS

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Abstract

Full scale field trials have shown that poplar trees can fully degrade trichloroethylene (TCE), perchloroethylene, and carbon tetrachloride (CT). To understand the mechanism of this unusual biological attack on fully chlorinated hydrocarbons, we have studied the biotransformation of CT by pure cultures of poplar cells. CT metabolism was inhibited by CO and by isonizid, inhibitors of cytochrome P450 2E1 enzymes in mammals. Along with CO2, chloroform was produced during the transformation of CT. These results support the hypothesis that an enzyme similar to mammalian cytochrome P450 2E1 is responsible for the degradation of chlorinated hydrocarbons in plants.

In other experiments we have introduced the genes for mammalian cytochrome P450 2E1 into tobacco and poplar. Transformed tobacco exposed to TCE produced 10 to 37 times the amount of trichloroethanol, a TCE metabolite, compared to control plants. Transformed poplar plants also produced greater amounts of trichloroethanol but to a lesser degree than tobacco. New poplar constructs with the potential for greater expression of P450 2E1 are in preparation. Such genetically engineered trees could be used to economically treat contaminated vadose zones directly or to treat vapors extracted from contaminated soils, in addition to treating contaminated groundwaters either through direct contact with shallow aquifers or by treating groundwater pumped from deep aquifers.

Need

- Chlorinated hydrocarbon (CHC) contamination of vadose zone soils and groundwaters is widespread at DOE sites
  - Carbon tetrachloride (CT) contaminates the vadose zone and aquifer under large areas of the 200W area at the Hanford Reservation threatening the Columbia River.
  - Perchloroethylene (PCE) and trichloroethylene (TCE) contaminates the groundwater of the Savannah River site.
- Phytoremediation meets STCG Technology Needs Statements from Savannah River and Hanford,
  - "Deploy Phytoremediation or a Passive Technology for Attenuation of VOCs" SR99-3024 (http://www.srs.gov/general/srtech/stcg/Needs/sr-3024.htm).
  - "Innovative Technologies to Replace Pump and Treat Technologies for Groundwater Remediation" SR99-3018 (http://www.srs.gov/general/srtech/stcg/Needs/sr-3018.htm)
    - "Cost-Effective, In-Situ Remediation of Carbon Tetrachloride in the Vadose Zone and Groundwater" RL-SS01 (http://www.pnl.gov/stcg/fy99/sc/rlss01.stm)
- Currently available cleanup technologies characterized by excessive costs, increased risks, long schedules, or the production of secondary waste streams

Background

- Anderson and Walton (1992) reported enhanced degradation of TCE in the rhizosphere of various plants.
- Narayanan et al. (1995) described the use of grasses to extract TCE from shallow contaminated soils
- Schnabel et al. (1997) described the uptake of TCE by crop plants.
- Previous work from our laboratory has shown that hybrid poplars have the ability to degrade CHCs (Newman et al, 1997).
  - Compounds which some of the trees have been shown to take up and degrade include PCE, TCE, and CT.
  - Plant species tested include black locust, three varieties of poplar, both hybrid and native, willow, eucalyptus, and tobacco -- all showing some capacity for CHC transformation.

Poplar Plantings Oxidize Fully Chlorinated Hydrocarbons

- Remarkable in view of the inability of known aerobic bacteria to attack these important pollutants.
- Field tests of poplar in pilot scale experiments treating artificial polluted groundwater have shown that TCE, CT, and PCE were not transpired to any significant extent; rather they were oxidized as shown by the accumulation of chloride ion in the rhizosphere soil
- Tests of TCE mineralization in rhizosphere soils suggested that most of the TCE degradation occurred in tree tissue, rather than in the soil microbial community
- Chlorinated organics did not accumulate in plant tissues, avoiding contamination of the above-ground ecosystem with chlorinated organics

Chloride Balance for Field Test of Phytoremediation of TCE

Chlorine added in the form of TCE-chlorine was balanced against the amount of TCE-chlorine, metabolite -- chlorine & free chloride ion recovered from the system. Masses given cover the 3 years the experiment ran.

	1995	1996	1997	3-year total loss	3-yr total recovered
	Moles of chlorine or chloride ion.				
TCE-chlorine lost from the irrigation water	2.75	13.4	11.32	27.47	
TCE-chlorine recovered from transpiration	0.28	0.87	0		1.15
TCE-chlorine recovered as metabolites in.	0.03×10^{-3}	0.006	0.002		0.008
.. leaves					
branches	0.03×10^{-3}	0.005	0.002		0.007
trunk	0.05×10^{-3}	0.01	0.003		0.013
roots	0.03×10^{-3}	0.006	0.002		0.008
Excess chloride ion in soil	ND	ND	18		18
		Chloride balance		27.5	19.2
		Recovery efficiency			70%

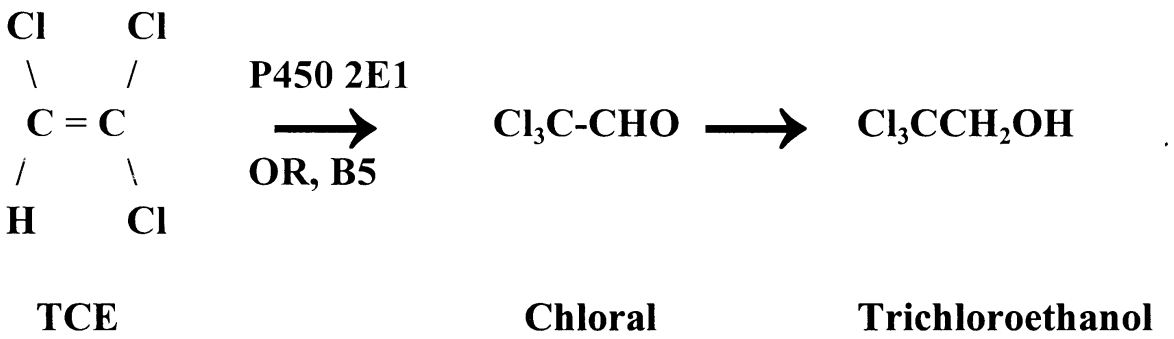
ND: Not determined

Genetic Engineering to Enhance CHC Metabolism (Doty, et al, accepted by *Proc. Nat. Acad. Sci.*)

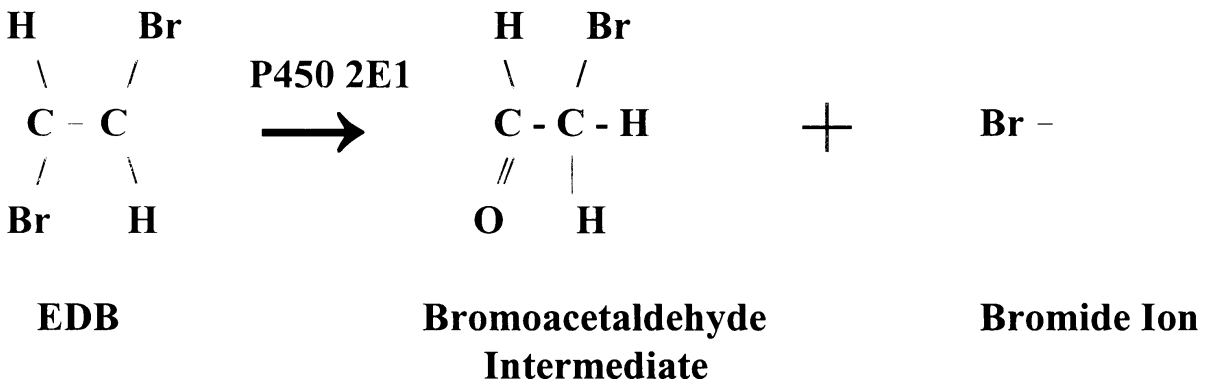
- P450 2E1 shown to oxidize a wide variety of substances, including TCE, ethylene dibromide (EDB), and CT in mammals
- We cloned the P450 2E1 gene under the control of the strong, constitutive, MAC promoter and introduced it into tobacco and poplar using *Agrobacterium*-mediated transformation.
- Transgenic tobacco and poplar plants were regenerated and verified to contain the transgenes using PCR.

Mammalian Metabolic Pathways, Initiated by P450 2E1, for Trichlorothylene (TCE) and Ethylene Dibromide (EDB)

TCE Metabolism



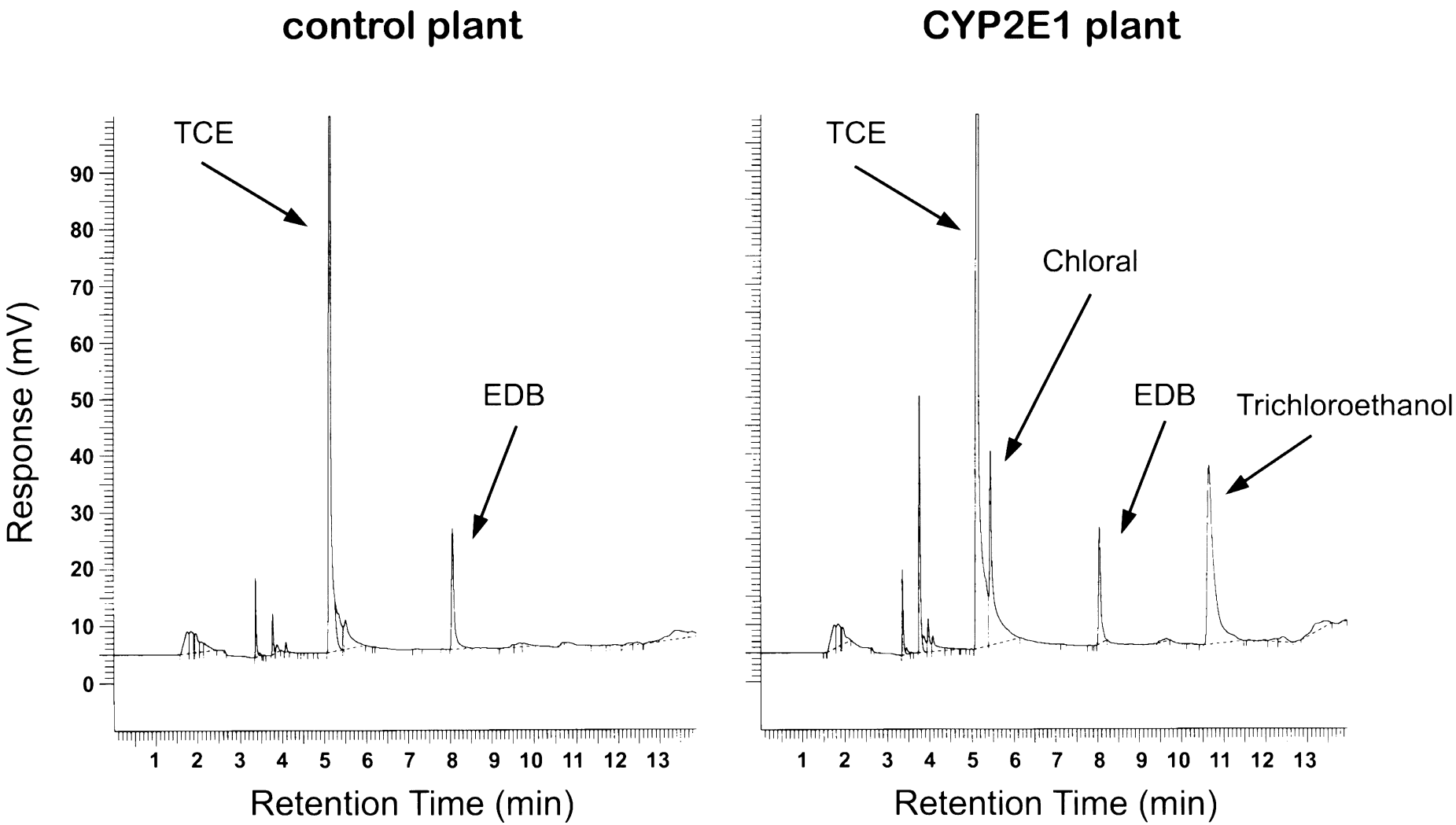
EDB Metabolism



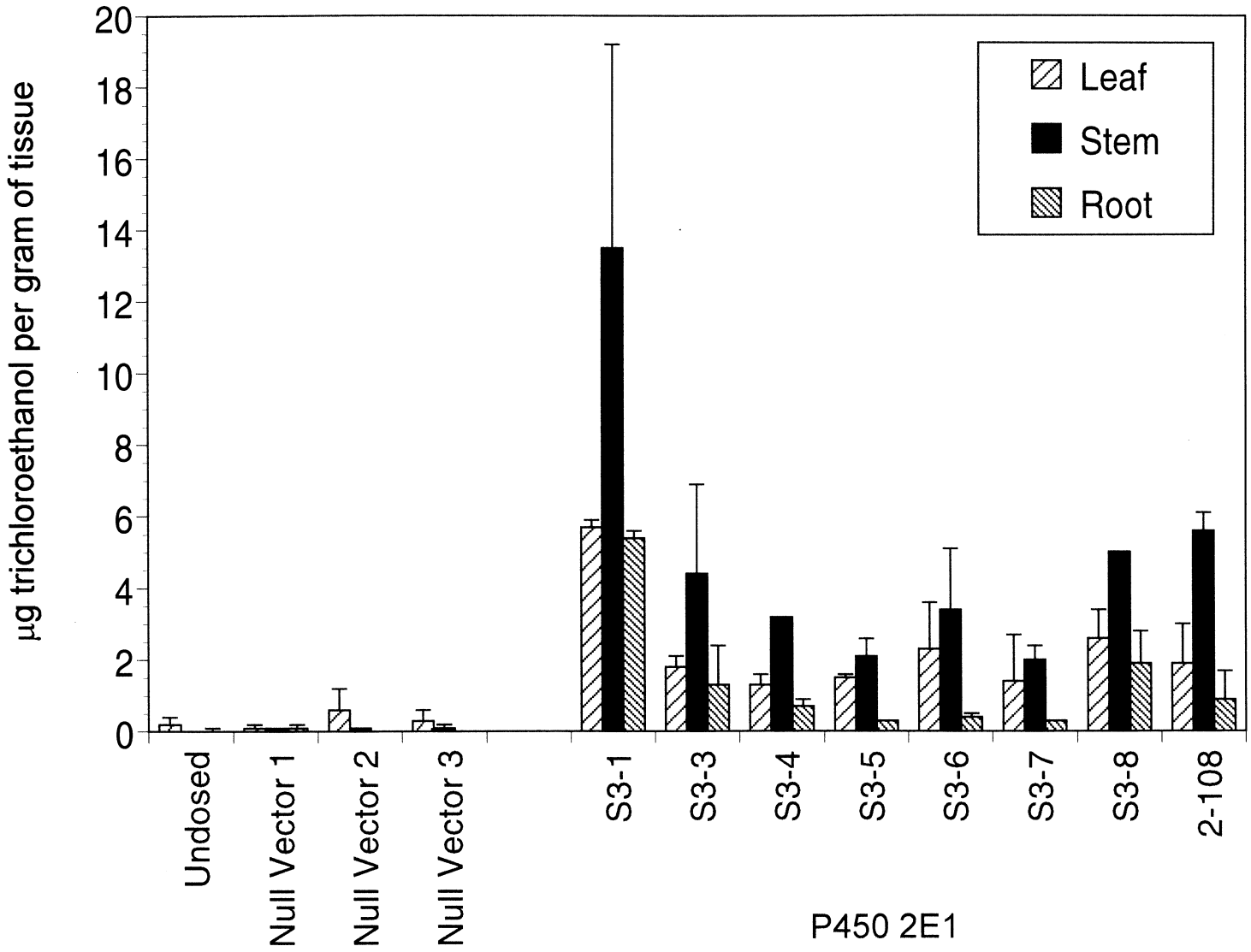
TCE Metabolism in P450 2E1 Tobacco.

- Tobacco plants containing exogenous P450 2E1 and containing only the null vector were dosed with TCE
- We used hydroponically-grown plants in order to study the metabolism of the plants apart from that of soil microbes
- Amounts of TCE metabolites compared
  - The initial detectable product of TCE oxidation by P450 2E1 is chloral, which is further metabolized to trichloroethanol or trichloroacetic acid
- Surface-sterilized transgenic plants were grown hydroponically
  - sealed into flasks
  - dosed with 118 μg/ml TCE for five days
- Plant tissues were collected, extracted, and analyzed by a gas chromatograph equipped with an electron capture detector (GC-ECD)
- The amount of the TCE metabolite, trichloroethanol, was greatly increased in all the P450 2E1 plants compared to that in the vector control plants
- Free trichloroethanol in the highest expressing plant, 3-1, was elevated 642X in roots, 171X in stems, and 140X in leaves, compared to the null vector plants.
- The average increase in the other P450 2E1 plants was 149X in roots, 44X in stem, and 72X in leaves

Gas Chromatographs of Root Extracts from a Vector Control Plant (left) and from a Transgenic P450 2E1 Plant (right)

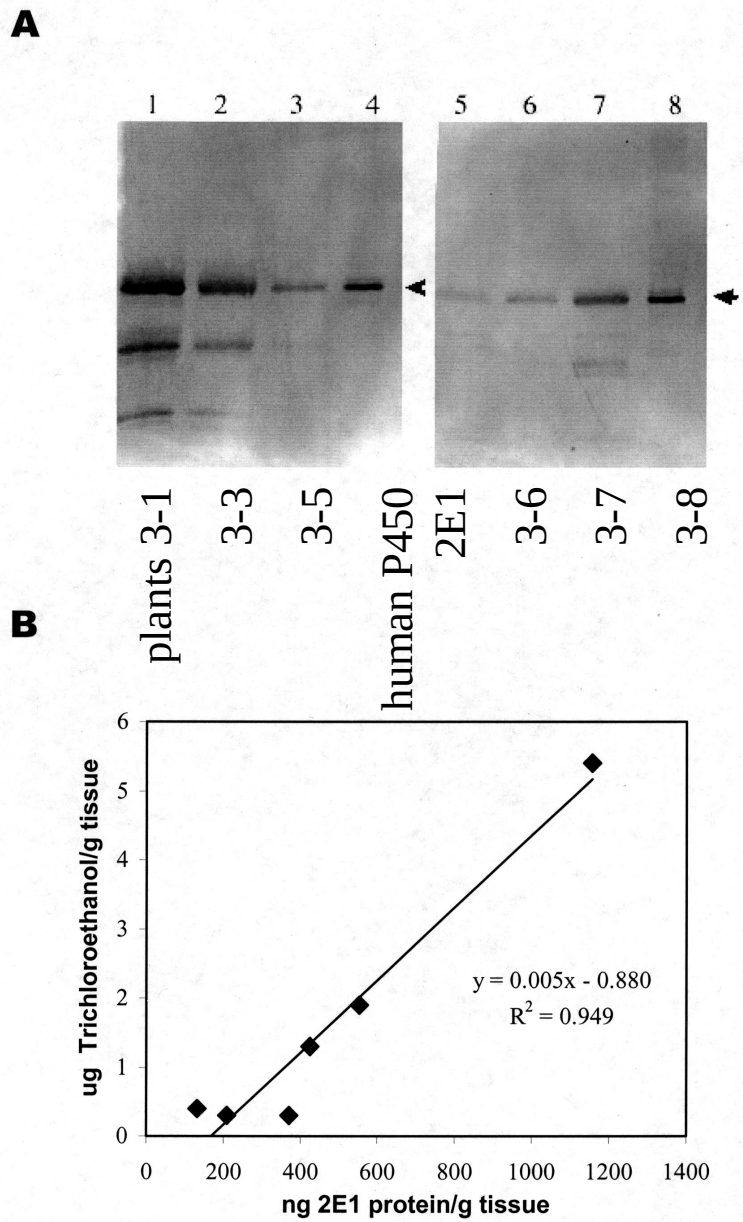


Trichloroethanol Production in Transgenic Plants (right) Compared to Null Vector Controls



Quantitative Western Blot Analysis for P450 2E1

- Performed on the same root tissue as the hydroponic experiment.
- A). Western blot of root protein extracts from transgenic tobacco plants.
  - Rabbit polyclonal anti-P450 2E1 used as the primary antibody.
- B). Correlation between trichloroethanol and P450 2E1 concentrations in transgenic tobacco roots.



TCE Metabolism Downstream of the Trichloroethanol Step

- A possible metabolic route for trichloroethanol would be a glucoside conjugate.
- TCE-exposed tobacco examined for the presence of trichloroethanol-glucoside.
- Aqueous extract of tobacco tissue incubated with either β-glucosidase or bovine serum albumin (BSA, as a protein control).
- At the end of the incubation, the mixtures were extracted and analyzed on GC-ECD for trichloroethanol.
- Trichloroethanol extracted from the glucosidase incubation represents total trichloroethanol, free and conjugated; the BSA incubation represents only the amount of free trichloroethanol.
- No difference in transgenic tobacco ability to form glucoside, (data not shown).
- In P450 2E1 transgenic tobacco, most trichloroethanol present as glucoside conjugate :
  - 94±4% of total trichloroethanol as glucoside in leaves,
  - 87±8% in roots and 75±15% in stems.
- Identity of trichloroethanol glucoside confirmed by GC-MS of its acetylated derivative
- When tobacco plants were allowed to recover for 9 days after exposure to TCE, free trichloroethanol & the glucoside decreased
- Thus, trichloroethanol does not accumulate in the plants.

Trichloroethanol Concentration in P450 2E1 Transgenic Tobacco Leaves (µg/mL)

	plant	Free TriClEtOH	Conjugated TriClEtOH	Total TriClEtOH
TCE-dosed [a]	#1	0.093	1.94	2.03
	#2	0.099	1.82	1.92
TCE-dosed & recovered [b]	#1	0.013	0.67	0.68
	#2	0.014	0.90	0.91

[a] Hydroponic tobacco plants dosed with TCE for 5 days.

[b] Hydroponic tobacco plants dosed with TCE for 5 days, then moved to media without TCE for 9 days.

Ethylene Dibromide Metabolism by Transgenic Tobacco

- To determine the relative rates of metabolism, the amount of the bromide ion released from EDB was determined.
- Free chloride ion released upon metabolism of TCE was was rapidly taken up by the plants from the hydroponic solution (data not shown)
- Bromide ion, however, was not taken up and there was no background level of bromide in the hydroponic solution of undosed plants.
- Thus, the metabolism of EDB, a known substrate of P450 2E1, was studied.
- Cuttings of the P450 2E1 plant, 3-1, and null vector control plants were grown in hydroponics and then dosed to a level of 2.2 μg EDB/ml.
- After five days, the hydroponic solution was analyzed by ion chromatography

Enhanced Uptake and Degradation of Ethylene Dibromide (EDB) by Transgenic Tobacco Containing P450 2E1

Plant	Percent EDB Loss	Final Concentration of EDB remaining in hydroponic solution, μg/mL	Br^- Released, μg/ml
Null Vector Controls	63.2 ± 13.3	1.14 ± 0.45	0.054 ± 0.058
Transgenic 3-1	98.1 ± 1.1	0.024 ± 0.025	0.243 ± 0.142

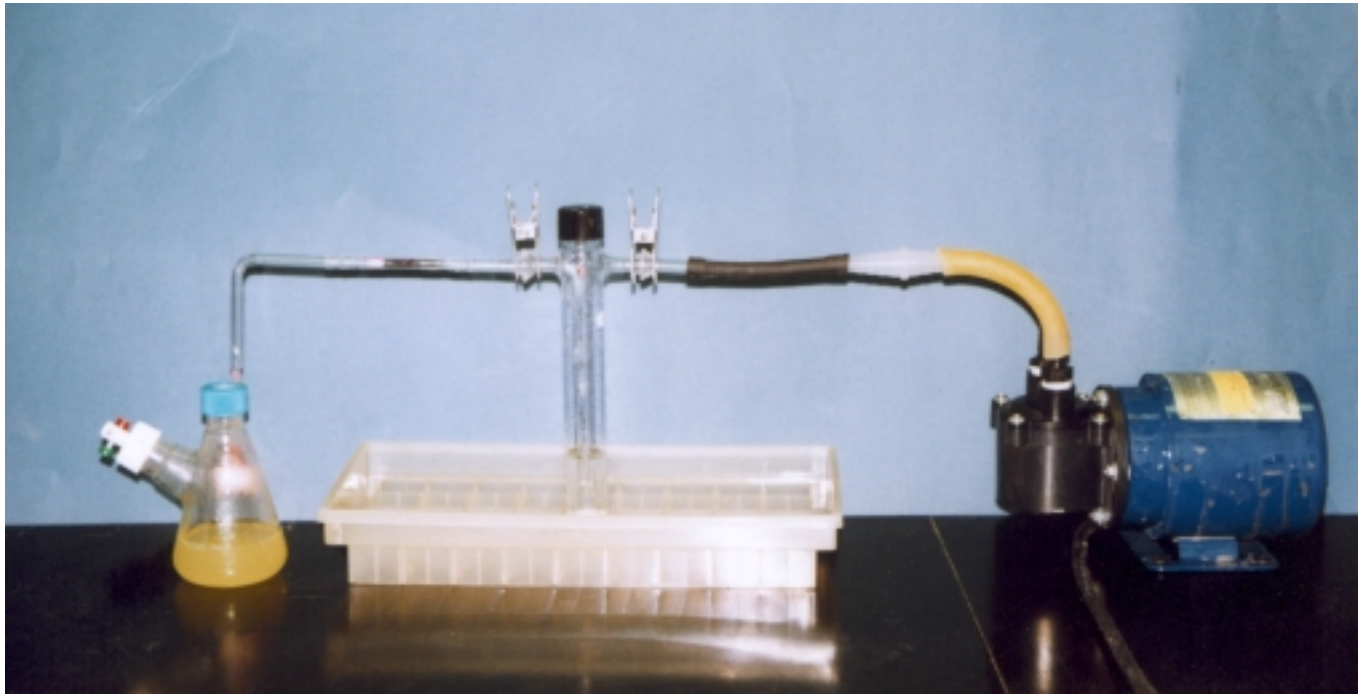
- Bromide ion released in the solution by the P450 2E1 plant 3-1 increased 5X compared to the vector control plants.
- Average EDB loss of 98% from hydroponic solution of the 3-1 plants and 63% from hydroponic solution of the vector control plants.

Transgenic Poplar

- Poor expresssion of P450 2E1 gene in first transformation experiments with poplar
  - Barely detectable expression by RT-PCR analysis and western blot analysis
- Increase in TCE metabolism by about two-fold
- We need to boost expression level in poplar
  - Rabbit cDNA encoding cytochrome P450 2E1
  - CaMV 35S promoter and Matrix attachment regions, shown to promote strong expression in poplar
  - "supervirulent" Agrobacterium tumefaciens strain, EHA105
- Control binary vectors include a 35S-gus-mas construct in place of the 35S-r2E1-mas construct and an antisense r2E1 cDNA construct
- Used fast-growing poplar clone, 50-197

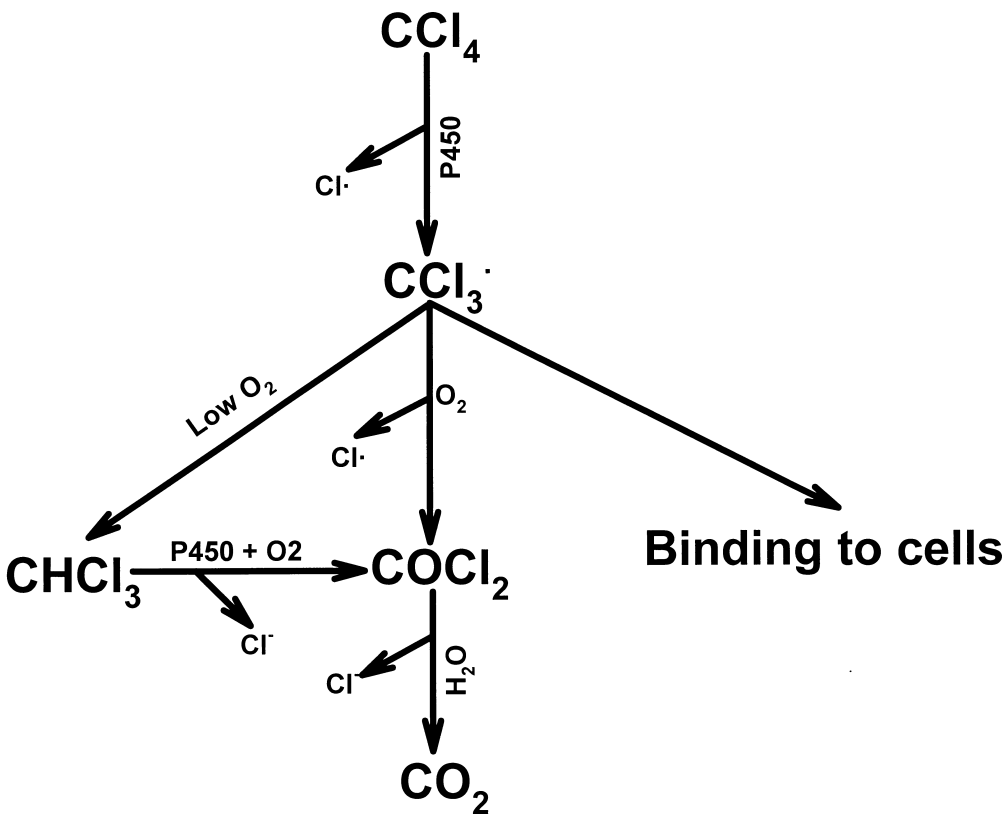
Biochemical Mechanism of Carbon Tetrachloride Degradation by Poplar Cells (Wang et al, submitted)

- *in vitro* system to study the mechanism of CT transformation by poplar H11-11 cells
- Two-necked flasks
- Dissolved oxygen maintained above 2 mg/L
- At the end of the experiment the flask was flushed with CT-free air
- Effluent gas passed through activated carbon tube to trap CT and NaOH trap for CO_2
- CT metabolites bound to the cell tissue filtered and quantified by combustion and LSC



Carbon Tetrachloride Metabolism in Mammalian Hepatic System

- Transformation of CT in mammalian systems has been extensively investigated
- The first step cytochrome P450-dependent reductive dechlorination of CT to trichloromethyl radical ($\bullet CCl_3$)
- Trichloromethyl radical may undergo three different reactions:
  - $\bullet CCl_3$ oxidized to phosgene ($COCl_2$), which then to produce CO_2
  - With low oxygen, $\bullet CCl_3$ abstracts a hydrogen atom from its environment to form chloroform ($CHCl_3$)
  - $\bullet CCl_3$ also binds to lipids and proteins



Transformation of CT by Poplar Cells Analogous to Pathway in Mammalian systems

- Both CO_2 and CF identified as metabolites of CT transformation by poplar cells
- Significant binding of CT-intermediates to poplar cells

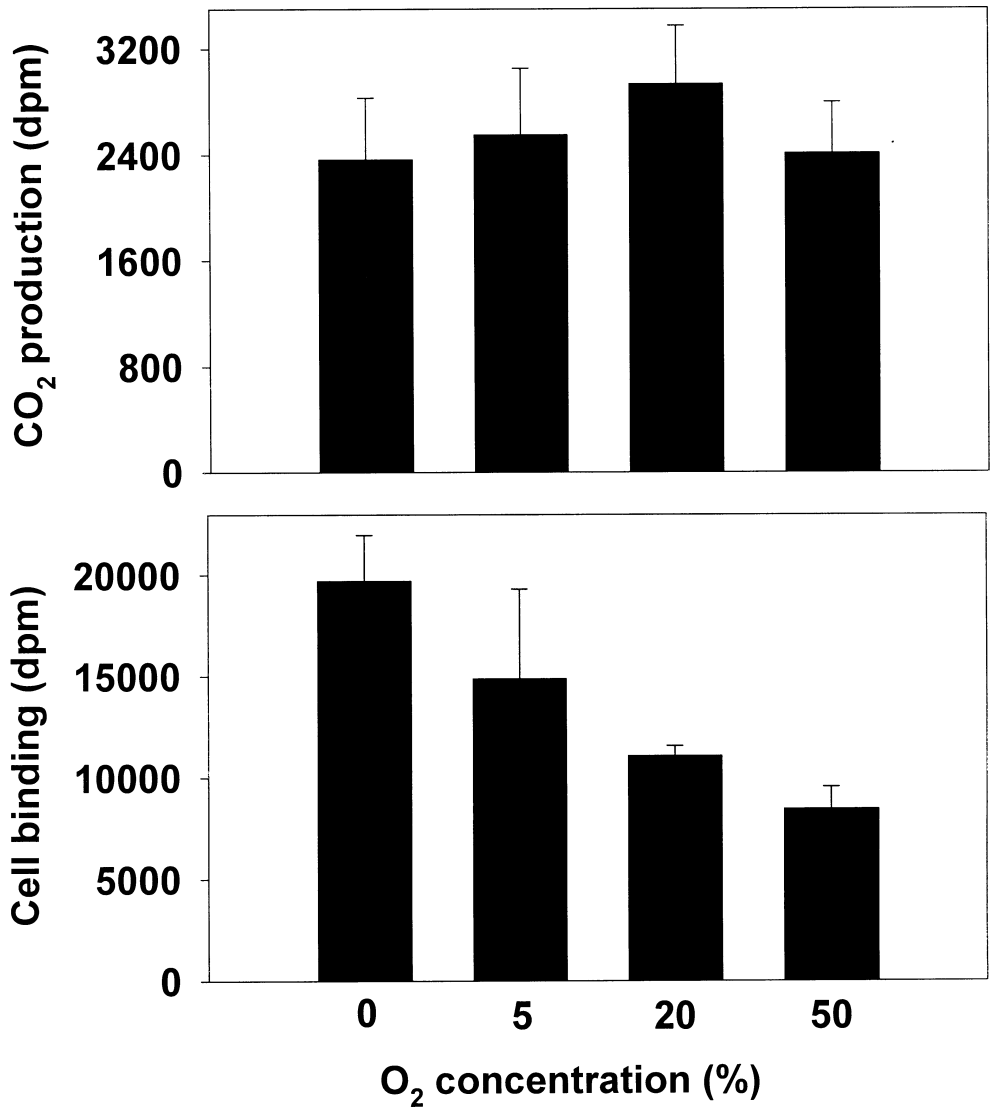
Products of carbon tetrachloride transformation by poplar cells

	CO_2 (dpm)	Cell binding (dpm)	Chloroform (µg)
MS	2490 ± 160	0	0.09 ± 0.02
Dead	1730 ± 90	3360 ± 140	0.07 ± 0.07
Living	4450 ± 200	12700 ± 2400	0.93 ± 0.20

Effects of Oxygen on Metabolism of CT by Poplar Cells

- At low oxygen levels, more chloroform (CF) produced or CT carbon bound to poplar cells because less oxygen available to compete for $\bullet CCl_3$ to produce CO_2
- With more oxygen, more $\bullet CCl_3$ could be oxidized to CO_2
- Less $\bullet CCl_3$ available to form CF or bind to the cells.

Atmosphere	Chloroform production (µg)
Air	0.17 ± 0.03
Nitrogen gas	2.79 ± 0.11



- Recovery of dosed ^{14}C was about 100%
- Total amount of metabolites accounted for about 3% of dosed CT
- No chlorinated hydrocarbons other than CT were detected in the non-radiolabelled CT
- No radiolabelled volatile contaminants were found in radiolabelled CT
- Radiolabelled non-volatile residue accounted for only about 0.04% of total radioactivity
- Therefore, the metabolites detected in our experiments were produced from the metabolism of dosed CT by poplar cells

Effects of Inhibitors of P450s on CT Metabolism

- Chlorzoxazone, isoniazid, 4-methylpyrazole and 1-phenylimidazole, are specific inhibitors of the P-450 2E1 enzyme
  - Significantly inhibited both CO_2 production and ^{14}C binding ($P = 0.01$ or 0.05)
- Piperonyl butoxide markedly inhibited CO_2 production
  - The inhibitory effects of CO, isoniazid and piperonyl butoxide on CT metabolism have been observed in mammalian systems
- SKF-525 produced no inhibitory effect on the CT metabolism
  - SKF-525 has also been shown to be a weak inhibitor of covalent binding of ^{14}C-CT in mammalian systems
- Results consistent with at least a partial involvement of a P450-2E1-like enzyme in CT metabolism in plants.

Inhibitors	Doses	Products of CT metabolism, dpm	
		CO_2	Cell binding
CO	0%	4500 ± 160	12000 ± 2200
	10%	3300 ± 130	9300 ± 120
	40%	2600 ± 160	7100 ± 360
	70%	1700 ± 170	6100 ± 330
Isoniazid	0 mM	3800 ± 230	8100 ± 190
	1 mM	2900 ± 220	6200 ± 160
	5 mM	2200 ± 150	5900 ± 62
	10 mM	1600 ± 130	5300 ± 77
Chlorzoxazone	0 mM	1200 ± 120	3100 ± 57
	0.1 mM	1100 ± 16	2700 ± 130
	0.2 mM	870 ± 170	1800 ± 200
	0.3 mM	970 ± 52	1700 ± 110
4-methylpyrazole	0 mM	1200 ± 61	2500 ± 96
	0.1 mM	1100 ± 34	2000 ± 48
	0.2 mM	980 ± 35	1500 ± 68
	0.4 mM	780 ± 15	1400 ± 80
1-phenylimidazole	0 mM	1700 ± 33	7000 ± 180
	0.1 mM	1300 ± 90	4800 ± 230
	0.2 mM	1000 ± 35	2500 ± 420
	0.3 mM	1100 ± 35	2200 ± 140
SKF-525	0 mM	3700 ± 180	7700 ± 770
	0.1 mM	3500 ± 630	8400 ± 1300
	0.2 mM	3800 ± 280	6800 ± 780
Piperonyl butoxide	0 mM	3200 ± 200	7700 ± 780
	0.01 mM	2200 ± 330	9000 ± 1200
	0.05 mM	1800 ± 120	7300 ± 670

Comparison of CT Metabolism of Field-Grown Trees and Tissue Culture Cells

- % CT mineralization and fixation by poplar cell cultures was quite low compared to the high level of CT removal and dechlorination observed in field trials
  - about 0.4 μg/day/g dry weight cells in tissue culture
  - versus about 40 μg/day/g dry weight leaf and root tissue for field-grown poplar
- Similar depressed level of TCE transformation by cell tissue culture compared to field trials
- Possible explanations may be advanced to account for these discrepancies:
  - Field degradation of CT may be due primarily to microbial activity in the rhizosphere
  - Undifferentiated cell line used in our tissue culture experiments may have had limited detoxifying activity compared to full grown plants.
- Cell culture work is a useful approach for the study of the mechanism of chlorinated hydrocarbon degradation
  - However, the results cannot be quantitatively extrapolated to field applications because of the limitations of cell culture growth

Summary

- First report of a potentially useful biodegradative process for CT that operates under aerobic conditions
  - contrasts with the inability of microbial enzymatic systems to attack CT under aerobic conditions
- Transgenic plants can have 5X or more increased rate of metabolism of halogenated hydrocarbons
- Transgenic plants can reduce concentrations of halogenated hydrocarbons in water

Applications to Technology Needs at DOE Sites

- Sub-irrigate plantations of trees at Hanford and Savannah River with water pumped from the contaminated aquifers
  - Nitrate in the Hanford groundwater would also be removed by the trees.
- Comparison to pump and treat with activated carbon adsorption and regeneration suggested that 30 year costs for pump and irrigate phytoremediation will be 20% or less of conventional system cost
  - Applied to the estimated $50-70M costs for expanded pump and treat at Hanford, a cost savings of $40-65M can be anticipated
- Vapor treatment
  - Diffuse CT-contaminated gases from vapor extraction into the root zones of the plants
  - Vapor treatment practical only if plants used are able to reduce concentration of CHCs -- Transgenic plants
- Advantages of using transgenic plants
  - Less land area required -- less irrigation equipment
  - Water can be treated, rather than consumed