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Bioremediation of Trichloroethylene:

Analysis of the Plant Gene Response to TCE and

Characterization of a Novel TCE-Degrading Endophyte

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Abstract

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Trichloroethylene is a common environmental contaminant. High levels of TCE have the potential to cause liver damage and malfunctions in the central nervous system, and it is considered a likely human carcinogen. More than 54% of Superfund sites in the United States are contaminated with TCE. This research explored two approaches for enhancing the phytoremediation of TCE, which includes analysis of transgenic plants and isolating and characterizing natural TCE-degrading bacteria.

The hypothesis of the first study was that certain detoxification genes such as cytochrome P450s (CYP), glutathione-S-transferases (GST), glycosyltransferases (UGT), and ATP-binding cassette (ABC) transporters are involved in TCE metabolism in poplar. The study found that poplar trees do have a genetic response to TCE and that many putative

detoxification genes are differentially regulated. Previous studies demonstrated that transgenic hybrid poplar plants expressing mammalian cytochrome P450 2E1 had greatly increased metabolism of TCE. In this research, the expression of poplar genes that may be involved in TCE metabolism between wild-type poplar and transgenic poplar were compared by microarray analysis. Through this analysis, many putative detoxification genes have been found and proposed to be involved in TCE metabolism. An important finding of the study was that transgenic poplar automatically upregulated many of these putative genes, reducing the need for further genetic manipulation.

The second study hypothesized that endophytes in poplar can degrade TCE more effectively than those currently used in bioremediation. Plants associate with various microbes which live around the plant's root or intercellular spaces of the aerial part of the plant. The research was aimed at isolating and investigating a novel endophyte having the ability to degrade TCE. A unique endophyte, identified as *Enterobacter sp*. PDN3, was isolated from hybrid poplar and shown to confer high tolerance to TCE. Without the addition of inducers such as toluene or phenol, PDN3 rapidly reduced TCE levels in media. Nearly 80% of TCE (55.3 μM) was dechlorinated by PDN3 in 5 days with 166 μM chloride ion production, suggesting TCE degradation.

Overall, the results of this dissertation research supported the hypotheses that specific plant genes and endophytes have strong potential for degrading pollutants.

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To my wife

CHAPTER 1
INTRODUCTION

1.1 Pollution problem

The widespread use of hazardous compounds due to rapid industrialization, military activities, and changed agricultural practices has led to increased contaminant concentrations in the environment. In the United States, contaminated sites are managed under the Superfund and brownfield programs. The Superfund sites are the nation's worst toxic waste sites which pose a significant threat to human health and the environment. The Environmental Protection Agency (EPA) designated the National Priorities List (NPL) for these sites and supervises remediation processes through a federal program. Brownfields are sites that were previously used for industrial or commercial purposes that have the potential for re-use after they are cleaned up. Both Superfund sites and brownfield sites are polluted with hazardous inorganic and organic contaminants.

Inorganic compounds include heavy metals such as mercury (Hg), lead (Pb), and cadmium (Cd), as well as nonmetallic compounds such as arsenic (As) and radionuclides like uranium (Ur) (Cherian & Oliveira, 2005). They are released into the environment by mining, industrial, and agricultural activities (LeDuc & Terry, 2005). Considering the fact that heavy metals have significant effects on human health, the contamination of soil with heavy metal is a worldwide environmental problem.

Organic compounds, which are mostly man-made, include chlorinated hydrocarbons (CHCs), polycyclic aromatic hydrocarbons (PAHs), explosives, and pesticides. These compounds are extremely toxic and persistent in the environment. For example, chlorinated

hydrocarbon solvents such as poly-chlorinated biphenyls (PCBs), trichloroethylene (TCE), perchloroethylene (PCE), chloroform (CF), and carbon tetrachloride (CT) are suspected or known carcinogens and neurotoxicants (Tabrez & Ahmad, 2009). They have been widely used as industrial solvents and fuel components and as intermediates. Many manufactured products such as paints, adhesives, gasoline, and plastics contain harmful organic compounds (Squillace, *et al.*, 1999, Collins, *et al.*, 2002).

Polycyclic aromatic hydrocarbons (PAHs) including naphthalene, phenanthrene, and anthracene are listed as priority pollutants by the U.S. EPA and the European Community (Samanta, *et al.*, 2002). The majority of these substances are highly recalcitrant molecules that can persist in the environment due to their hydrophobicity and low water solubility. These hydrophobic compounds occur in soil, sediment, water, air, and plants resulting from the incomplete combustion of coal, oil, petrol, wood, and petrochemical products (Andreoni & Gianfreda, 2007).

Explosives are highly reactive chemicals. They are released into the environment by manufacturing, storage, military activity and inappropriate waste disposal of explosive chemicals. In particular, explosive residues can affect various ecological and human receptors (Crocker, *et al.*, 2006) since they have the potential to reach surface water and groundwater (Clausen, *et al.*, 2004). The primary explosives at hazardous waste sites are 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine or royal demolition explosive (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine or high-melting-point explosive (HMX) (Van Aken, *et al.*, 2004).

Synthetic pesticides are most commonly used for pest control in agriculture and at the same time, have been a substantial environmental issue. Pesticides have been linked to a

wide range of human health hazards such as cancer, reproductive harm, and endocrine disruption (Gilden*, et al.*, 2010). The most commonly used in both agriculture and forestry for diseases and insect pests control are organochlorine dichlorodiphenyltrichloroethane (DDT) and organophosphorus compounds like chlorpyrifos (Mauriz*, et al.*, 2006).

All these metals and synthetic organic substances have caused undesirable consequences in the environment, which have been recognized as an environmental contamination problem of worldwide magnitude.

1.2 Traditional methods for remediating pollutants

In order to remove contaminants *in situ*, various engineering techniques have been applied such as excavation, transport, soil washing, extraction, pumping, aeration, air stripping and treating of contaminated water, addition of oxidants such as hydrogen peroxide or potassium permanganate, or incineration (Scullion, 2006, Doty, 2008). These applications can also cause secondary contamination and damage to the environment (Vidali, 2001).

In addition, applying these conventional methods is highly expensive, with the estimated costs of between $50 and $500 per ton of soil (Cunningham & Ow, 1996). In the United States alone, $6-8 billion is spent annually in remediation efforts, with global levels in the range of $25-50 billion (Glass, 1999, Tsao, 2003, Doty, 2008). In this regard, it is highly desirable to develop more cost-effective remediation methods. Advanced benign decontamination technologies such as phytoremediation using plants and bioremediation using microorganisms are continuing to emerge.

1.3 Bioremediation

The method using microorganisms or their enzymes to degrade or transform contaminants to less toxic or nontoxic products on a contaminated site is generally called bioremediation (Gianfreda & Rao, 2004). With the discovery of a number of soil microorganisms that are capable of degrading organic pollutants, microbial bioremediation is increasingly being considered as a reasonable and effective method for removing environmental contaminants. Since this approach has a relatively low cost and entails low-technology techniques, it is highly acceptable to the public and can be implemented on site (Kuritz & Wolk, 1995, Vidali, 2001, Glic, 2010).

The use of microorganisms to mediate inorganic contaminants like heavy metals is still under scientific investigations since metals cannot be degraded (Sinha et al. 2009). Some microorganisms can accumulate and translocate metals. Trotta et al. (2006) reported arbuscular mycorrhizae (AM) fungi increased the arsenic translocation in the As hyper-accumulating fern *Pteris vittata* (Minganti*, et al.*, 2004, Trotta*, et al.*, 2006). A wide range of microbes have also been found to have chromium (Cr) tolerance, resistance and reducing ability (Francisco*, et al.*, 2002, Dhal*, et al.*, 2010, He*, et al.*, 2010, Ibrahim*, et al.*, 2011).

Bioremediation is a promising technology for the removal of organic soil contaminants. Many laboratory and field studies have shown that microorganisms degrade synthetic organic compounds. Remediation of chlorinated solvents by microorganisms is discussed in Chapter 3.

Several studies have found that there are two distinct biological processes capable of biotransforming PCBs under aerobic oxidative processes and anaerobic reductive processes.

Chung and colleagues (1994) isolated *Rhodococcus erythropolis* from the wood-feeding termite and found that these bacteria aerobically degraded poly-chlorinated biphenyl (PCB) compounds (Chung*, et al.*, 1994). Some microorganisms were found to be capable of transforming and degrading PAHs and the biodegradative pathways have been reported in bacteria and fungi (Cerniglia, 1984, Neilson & Allard, 1998, Arun*, et al.*, 2008). Also, several bacterial species in the genera of *Agrobacterium*, *Rhizobium*, and *Sinorhizobium* are able to utilize both polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Damaj & Ahmad, 1996, Aitken*, et al.*, 1998, Frassinetti*, et al.*, 1998). Generally, contaminated sites are co-polluted with metal and organic compounds. Atagana reported that fungi in contaminated-soil with metals such as cadmium and nickel ions could biodegrade PAHs (Atagana, 2009). These abilities can be useful for removing PAHs and metals from the environment (Xia*, et al.*, 2006).

Studies reported several microorganisms including aerobic bacteria, aerobic fungi, and anaerobic bacteria which can degrade explosives (Duque*, et al.*, 1993, Esteve-Núñez*, et al.*, 2001, Zhang & Hughes, 2003, Sherburne*, et al.*, 2005). For example, Boopathy and Kulpa isolated *Methanococcus sp*. from a lake sediment that could transform 100 ppm of 2,4,6-trinitrotoluene (TNT) within 40-60 days (Boopathy & Kulpa, 1994).

In their pesticides studies, Singh et al. (2004) reported chlorpyrifos degradation by an *Enterobacter* strain (Singh*, et al.*, 2004). Qureshi and colleagues showed the bioremediation of organochlorine contaminated soil with combined biostimulation and bioaugmentation strategies (Qureshi*, et al.*, 2009). Newcombe and Crowley also reported that atrazine-contaminated soil could be remediated by repeated applications of certain bacteria which are able to degrade atrazine (Newcombe & Crowley, 1999).

To summarize, a variety of microbes have been identified that can degrade pollutants. Bioremediation technologies have been implemented at many contaminated sites and demonstrated the capability to degrade organic pollutants. Yet a wide-range of their application is limited since the environmental conditions such as temperature, pH, soil properties, and humidity are hard to control for the optimal biodegradation results. Even distribution of the microbes in the site, competition with native microbes, and monitoring all continue to be challenges with the use of this technology.

1.4 Phytoremediation

Phytoremediation is a highly promising new method which uses various plants and their associated microbes to degrade, accumulate, or immobilize contaminants from soil and water (Dietz & Schnoor, 2001). Recently, it has been adopted widely to clean up metals, pesticides, petroleum, explosives, PAHs, and organic compounds (Meagher, 2000). Phytoremediation has many obvious advantages compared to traditional physical or chemical approaches. First, it is relatively inexpensive to apply and simple to manage (Meagher, 2000, LeDuc & Terry, 2005). Second, it is less intrusive and provides an aesthetically pleasing environment, wildlife habitats, carbon sequestration, and soil stabilization while cleaning up the environment. Third, plant materials used for phytoremediation can be reprocessed into woodchips, pulp, or bioenergy resources (Stanton*, et al.*, 2002). There are other benefits to phytoremediation. Phytoremediation is likely to attract public support and the plants can be easily monitored for effective performance.

Phytoremediation techniques can be categorized according to the ways in which the contaminant is processed. Phytoextraction or phytoaccumulation uses plants to transfer

inorganic pollutants and accumulate them in plant tissues. Since metals cannot be degraded, harvest of the biomass must be done, followed by storage or “phytomining”.

Phytodegradation or phytotransformation is mostly suitable for organic pollutants since plants can break down or degrade the contaminants by metabolic processes with enzymatic modification within the plants. When degradation happens in the rhizosphere, this method is called rhizodegradation which utilizes the symbiotic relationship with soil organisms such as bacteria, yeast, and fungi. Phytostabilization uses certain plant species to immobilize contaminants in the soil and groundwater through absorption and accumulation by roots. Rhizofiltration is a water remediation technique that involves the uptake of contaminants by plant roots either by adsorption or precipitation of contaminants onto plant roots or the absorption of contaminants into the roots when contaminants are in solution surrounding the root zone. Phytovolatilization refers to the evapotranspiration of pollutants through stomata in its leaves. Sometimes the combination of these processes can be found in nature (Vidali, 2001, McGuinness & Dowling, 2009).

Plants can take up organic xenobiotic compounds with an octanol/water partition coefficient (Log K_{ow}) between 0.5 and 3.5. Contaminants with a Log $K_{ow} <1$ are very water-soluble and roots do not generally accumulate them since they can sparsely penetrate the lipid-containing root epidermis. Compounds with a log $K_{ow} > 3.5$ are hydrophobic and will absorb to the root tissues and not enter the plants (Schröder & Collins, 2011). In the case of PCBs, they have very high log K_{ow} values than other chemicals. They are in the range from 4.5 for monochlorobiphenyls to >8 for higher chlorinated PCBs. However, Chekol and colleagues reported several plants for phytoremediation of poly-chlorinated biphenyls (PCB) contaminated soil and all of the plant species treatments showed significantly greater PCB

biodegradation compared to the unplanted controls (Chekol*, et al.*, 2004).

There are several studies which have shown that explosives were taken up and degraded by plants (Thompson, 1997). For example, Flokstra et al. (2008) reported detoxification of TNT and RDX contaminated solutions by poplar tissue cultures and identified their metabolites (Flokstra*, et al.*, 2008). A similar study was conducted by Yoon et al. (2006), which showed the uptake and fate of TNT, RDX, and HMX by hybrid poplars in hydroponic systems (Yoon*, et al.*, 2006). In another study, Vila and others reported that several agronomic plants (maize, soybean, wheat, and rice) could grow on soils containing RDX and TNT and they were able to uptake these compounds (Vila*, et al.*, 2007).

Belden and colleagues (2003) have conducted a series of experiments to investigate the potential of using plants as tools for the remediation of pesticide-contaminated soil. They have demonstrated that a blend of prairie grasses increases dissipation rates of several pesticides including metolachlor, trifluralin, and pendimethalin (Belden*, et al.*, 2003). Lunney et al. (2004) also reported uptake of dichlorodiphenyltrichloroethane (DDT) in vascular plants for phytoremediation (Lunney*, et al.*, 2004).

Although phytoremediation is considered to be an environmentally friendly and multi-functional method of remediation, its efficiency can be limited due to seasonal effects and insufficient abilities to tolerate, detoxify, and accumulate pollutants in the highly contaminated area. Another limitation is that successful phytoremediation takes time, sometimes more than one decade, which makes it difficult to evaluate success in the early stage (Karlson, 2001, Vidali, 2001).

1.5 Enhancing phytoremediation

1.5.1 Phytoremediation with transgenic plants

Regarding the drawbacks mentioned above, plant genetic engineering, through inserting or overexpressing specific genes in the genome of the plants, provides an efficient method to enhance the phytoremediation capacity of plants (James & Strand, 2009). Most of the selected genes are involved in metabolism, uptake, or transport of specific pollutants. The overexpression of various enzymes such as cytochrome P450, pentaerythritol tetranitrate reductase (PETN), nitroreductase, peroxidases (PX), glutathione S-transferases (GST I), glycosyltransferases (UGTs), and organophosphorus hydrolase (OPH) have been achieved for many plant species and dramatically improved the capacity of plants to remediate environmental pollutants (Stomp*, et al.*, 1994, Dean*, et al.*, 1997, Cherian & Oliveira, 2005, Peuke & Rennenberg, 2005, Doty, 2008, Macek*, et al.*, 2008, James & Strand, 2009). These enzymes have been used to develop ideal plants for environmental cleanup. Especially, the use of plants expressing high activity of cytochrome P450s is considered as a potential strategy and has become an area of great interest for phytoremediation of xenobiotics (Kawahigashi*, et al.*, 2006). Processes for remediation by P450s are discussed in Chapter 2.

Phytoremediation of toxic metals has been successfully improved with transgenic plants. Bennett et al. (2003) developed transgenic Indian mustard plants overproducing the enzymes gamma-glutamylcysteine synthetase (ECS) or glutathione synthetase (GS) that increased levels of the metal-binding thiol peptides phytochelatins and glutathione, and resulted in Cd tolerance and accumulation (Bennett*, et al.*, 2003). Kawashima et al. (2004) also showed that transgenic tobacco plants over-expressing cysteine synthase were significantly more tolerant than wild-type plants in agar medium containing Cd, Se and Ni,

and the plants could enhance accumulation of Cd in shoots (Kawashima*, et al.*, 2004). Hussein and his colleagues developed transgenic tobacco plants engineered with bacterial *mer*A and *mer*B genes. The transgenic plants had enhanced uptake of mercury and organomercurials and improved translocation to shoots and volatilization (Hussein*, et al.*, 2007).

In another recent study, Rylott and colleagues (2011) developed transgenic *Arabidopsis thaliana* (Arabidopsis) including bacterial RDX-degrading xplA, and associated reductase xplB, from *Rhodococcus rhodochrous*, in combination with the TNT-detoxifying nitroreductase (NR), nfsI, from *Enterobacter cloacae*. The plants expressing xplA, xplB and NR prevented release of RDX in soil leachate, and grew on soil contaminated with phytoxic levels of RDX and TNT (Rylott*, et al.*, 2011).

Using transgenic plants for enhancing phytoremediation is highly effective, but it is not always possible to use transgenic plants in the field due to strict regulations governing the release of transgenic plants.

1.5.2 **Phytoremediation with endophytes**

Approaches for improving the phytoremediation of organic compounds include adding natural pollutant-degrading bacteria as well as applying growth promoting bacteria to enhance degradation and plant growth (Singh & Ward, 2004, de-Bashan*, et al.*, 2011, do Carmo*, et al.*, 2011, Hong*, et al.*, 2011, Ma*, et al.*, 2011, Myresiotis*, et al.*, 2011, Trejo*, et al.*, 2012). Most plants associate with various microbes which live around the plant's root or intercellular space of the plant. The term endophyte refers to microorganisms, bacteria or fungi, which live within plant tissues without causing any disease (Verma, 1991, Dobereiner,

1992, Gnanamanickam, 2006).

The mechanisms by which endophytes enter plant cells without inducing host defense responses are not yet understood, but several studies have shown that endophytes appear to enter in an opportunistic way (Doty, 2008). The common entry points are at a wound, or at disturbed cells at lateral root junctions or the root cap and it seems that low levels of cell wall degrading enzymes are released for entry (Doty, 2011, Maheshwari, 2011). These endophytes can also assist plant growth through nutrient acquisition and phytohormone production and increase plant resistance to aggressive factors such as pathogens, drought, and herbivores (Glick, 2003, Selosse*, et al.*, 2004, Welbaum*, et al.*, 2004, Doty, 2011).

Recently, several studies have examined the possibilities of using endophytes for assisting the phytoremediation of contaminated soil and groundwater. In their review of bacterial endophytes, Moore and his colleagues (2006) described 121 endophytes from hybrid poplar at a site contaminated with benzene, toluene, ethylbenzene, and xylenes (BTEX). Some of the isolates demonstrated tolerance to heavy metals, BTEX, and TCE (Moore*, et al.*, 2006). Walton and Anderson also reported that TCE degradation by microbial organisms was enhanced in the rhizosphere of various plant and tree species (Walton & Anderson, 1990). Germaine and colleagues described the inoculation of a model plant, the pea (*Pisum sativum*), with a genetically tagged bacterial endophyte that naturally possessed the ability to degrade 2,4-dichlorophenoxyacetic acid. The results showed that the inoculated plants had a higher capacity for 2,4-dichlorophenoxyacetic acid removal from soil and showed no 2,4-dichlorophenoxyacetic acid accumulation in their aerial tissues (Germaine*, et al.*, 2006). In another study, van Aken and his colleagues reported a methylotrophic bacterium, *Methylobacterium* sp. strain BJ001, isolated from hybrid poplar

(*P. deltoides* × *P. nigra* DN34), that was capable of degrading the explosives 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine or royal demolition explosive (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine or high-melting-point explosive (HMX) (VanAken*, et al.*, 2004).

Many recent studies have shown that natural endophytes have a capacity for xenobiotic degradation (Germaine*, et al.*, 2006, Ryan*, et al.*, 2007) in addition to providing other benefits to the plants such as nitrogen fixation, phosphate solubilization and stress tolerance (Rosenblueth & Martinez-Romero, 2006).

1.6 Research objectives

The objectives of this research are summarized into two major aims: 1) to study trichloroethylene (TCE) detoxification mechanisms in poplar trees by a genomic approach and 2) to isolate naturally occurring endophytes that degrade TCE.

The hypothesis of the first study is that there is a plant genetic response to TCE.

The second study hypothesizes that some endophytes in poplar can degrade TCE more effectively than those currently used.

This research will help us to find advanced ways to remove toxic organic chemicals economically, efficiently and in an eco-friendly manner by using plants and bacteria.

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CHAPTER 2

Differential Gene Regulation in Response to Trichloroethylene (TCE) in Hybrid Poplar and Transgenic Poplar

2.1 Introduction

2.1.1 Trichloroethylene

One of the most common VOC pollutants is trichloroethylene (TCE). This chlorinated solvent has been broadly used as a cleaning agent and solvent for many commercial, military, and industrial applications. The widespread use of TCE, along with its improper handling, storage, and disposal, has resulted in frequent detection of TCE in groundwater (Shang & Gordon, 2002, Doty*, et al.*, 2007). In the United States, 54% of the U.S. Environmental Protection Agency's Superfund sites contain TCE (Lee*, et al.*, 2006).

TCE is a nonflammable, colorless liquid at room temperature with a somewhat sweet odor and a sweet, burning taste. It is a dense non-aqueous phase liquid (DNAPL), and thus tends to sink into aquifers below the water table when spilled on the soil, which can result in long term secondary pollution (Davis*, et al.*, 2002). Several studies reported that TCE has the potential to cause liver damage and malfunctions in the central nervous system, and it is considered a possible human carcinogen (Waller*, et al.*, 1992, Milton*, et al.*, 1998). The Environmental Protection Agency (EPA) regulates TCE to a maximum contaminant level (MCL) in drinking water of 5µg/L (Table 1).

The uptake of TCE and several other organics by hybrid poplar have been reported by several studies (Newman*, et al.*, 1997, Gordon*, et al.*, 1998, Orchard*, et al.*, 2000, Komives*, et al.*, 2003). Trichloroethylene is relatively hydrophobic, with a log K_{ow} of approximately

2.5. TCE was observed to be taken up by plant roots via the vapor or water phases of soil and absorbed through a passive diffusion process (Collins*, et al.*, 2006).

Generally, metabolic processing of foreign chemicals (xenobiotics) by plants can be divided into three phases. The organic compounds taken up by plants are transformed, conjugated and transferred by various enzymes including cytochrome P450 monooxygenases (CYP450s), glutathione-S-transferases (GSTs), glycosyl transferases (UGTs), and ATP-binding cassette transporters (ABC transporters) that play essential roles in environmental interactions and defense against natural and synthetic toxic chemicals (Van Aken*, et al.*, 2004, Verkleij*, et al.*, 2009). The following sections will discuss these enzymes in more detail.

Once foreign organic compounds enter plants, various plant enzymes metabolize them. In the case of TCE, several oxidative metabolites of TCE, such as trichloroethanol (TCEOH), dichloroacetic acid (DCAA), and trichloroacetic acid (TCAA), have been observed, which are transformed by certain detoxifying enzymes in plant tissues (Figure 1) (Shang*, et al.*, 2001, Strycharz & Newman, 2009, Strycharz & Newman, 2009).

Most research attempts so far have focused on identifying the TCE degradation pathway by chemical observation (Newman*, et al.*, 1997, Gordon*, et al.*, 1998, Shang*, et al.*, 2001, James*, et al.*, 2008); molecular approaches to discover the genetic responses to TCE are needed for a further understanding of the process.

There is a lot of interest specifically in the use of poplar trees for phytoremediation since they can grow rapidly and have extensive root systems. *Populus* species have a small genome, ease of vegetative propagation, availability of genetic transformation systems, and genetic resources, which is very useful for molecular and genetic studies of tree biology (Tuskan*, et al.*, 2006).

Populus trichocarpa became the first tree to have its genome sequenced (Tuskan*, et al.*, 2006). With this regard, the analysis of the *Populus* transcriptome of TCE responses will provide evidence of what genes are expressed throughout the genome and which genes may play a role in detoxification.

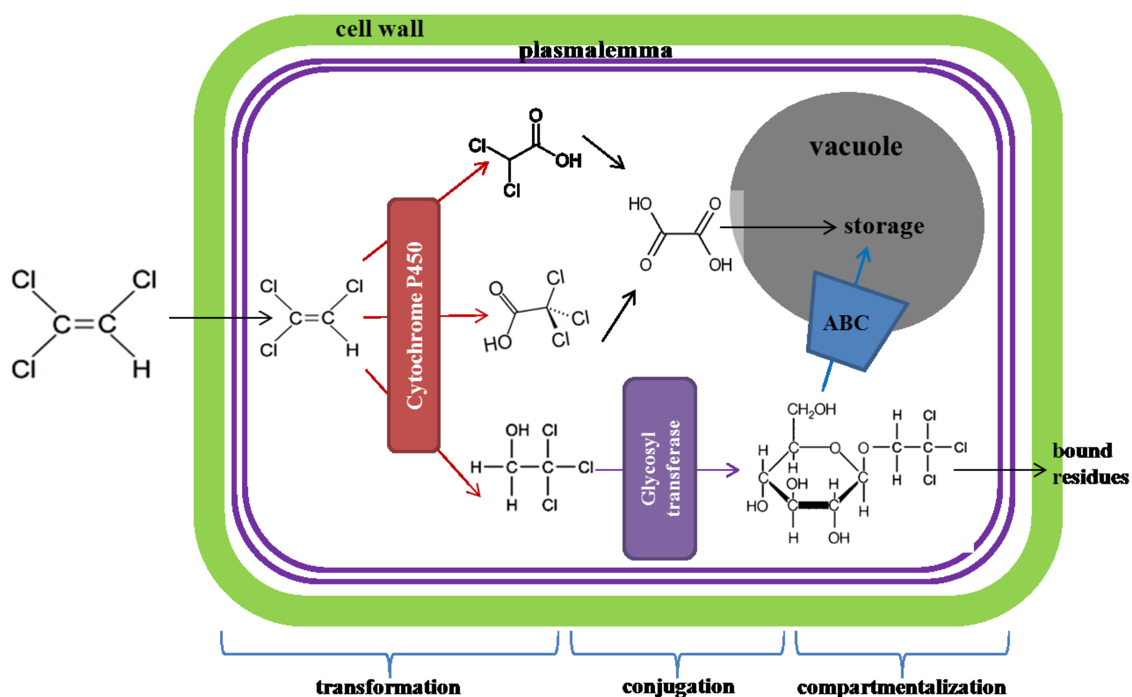
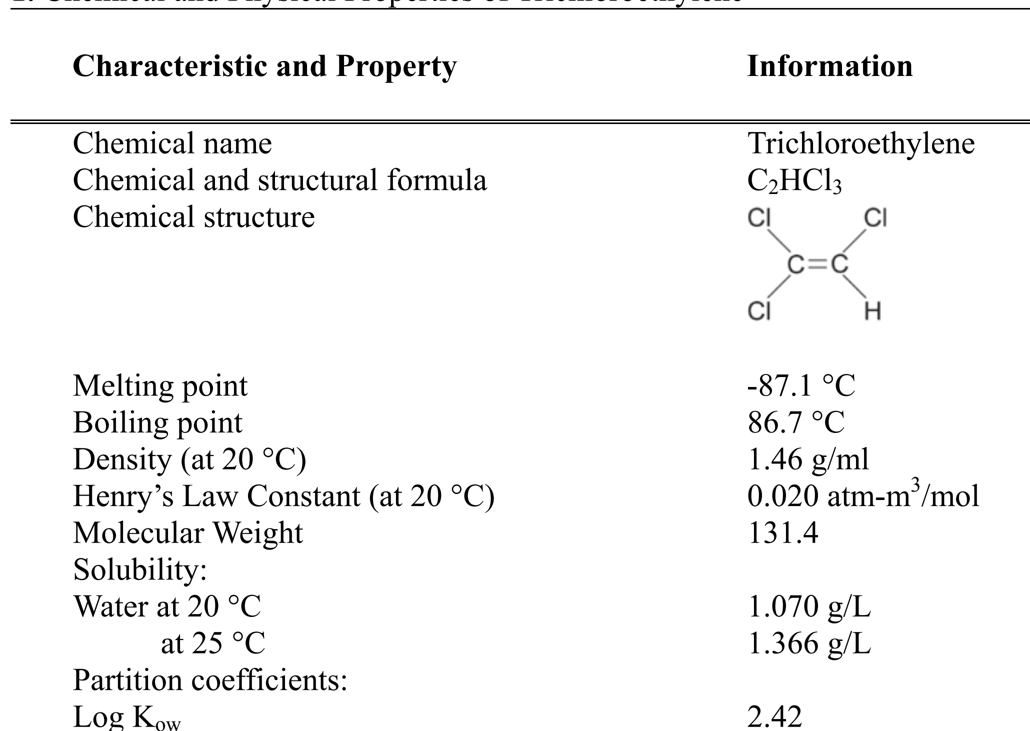


Figure 1. Proposed scheme of TCE metabolism in the plant cell according to the "green liver concept" (Shang*, et al.*, 2001; Reichenauer & Germida, 2008).

Table 1. Chemical and Physical Properties of Trichloroethylene



Characteristic and Property	Information
Chemical name	Trichloroethylene
Chemical and structural formula	C_2HCl_3
Chemical structure	Cl, Cl, C=C, Cl, H
Melting point	-87.1 °C
Boiling point	86.7 °C
Density (at 20 °C)	1.46 g/ml
Henry's Law Constant (at 20 °C)	0.020 atm-m^3/mol
Molecular Weight	131.4
Solubility:	
Water at 20 °C	1.070 g/L
at 25 °C	1.366 g/L
Partition coefficients:	
Log K_{ow}	2.42

2.1.2 Cytochrome P450s

Cytochrome P450 proteins are one of the largest superfamilies of enzymes and play significant roles in the genomes of all organisms from bacteria to plants and human (Morant, et al., 2003). It is named for the absorption band at 450nm of their carbon-monooxide-bound form. Cytochrome P450 monooxygenases (P450s) are heme dependent mixed function oxidases that utilize NADPH or NADH (Werck-Reichhart & Feyereisen, 2000). A diverse range of chemical reactions including hydroxylation, epoxidation, demethylation, dehalogenation, desaturation, and isomerization were catalyzed by cytochrome P450s (Jackson*, et al.*, 2007).

In plants, cytochrome P450s contribute to vital processes such as carbon source

assimilation and biosynthesis of hormones (Werck-Reichhart & Feyereisen, 2000, Hannemann*, et al.*, 2007). For example, synthesis of lignin phenolics, membrane sterols, phytoalexins, and terpenoids utilize CYP P450s. CYP P450s have also been implicated in structural components of living organisms such as lignin phenolics (Donaldson & Luster, 1991).

Cytochrome P450 proteins can be found in all cells. Yet these enzymes are expressed at low levels and the products of CYP 450 in plants are often hydrophobic, which made the study of plant P450s difficult. Moreover, the expression of many CYP 450s is only under certain circumstances and exclusively in a few cell types (Chapple, 1998). Due to these limitations, there are few reports of cloned plant P450s suitable for use in phytoremediation. For instance, CYP71A10 was the first plant enzyme from soybean shown to actively metabolize an herbicide. In another study, Diderjean et al. reported a successful transgenic approach with a gene for CYP 76B1 from Jerusalem artichoke. The transgenic plants with CYP71A10 and CYP76B1 both significantly enhanced tolerance to several herbicides (Siminszky*, et al.*, 1999, Didierjean*, et al.*, 2002).

However, the introduction of animal and bacteria cytochrome P450s into plants is considered to be the most useful technique for phytoremediation since xenobiotic detoxification and sterol biosynthesis are the major functions of cytochrome P450s in bacteria and animals (Ro*, et al.*, 2002). A bacterial P450 (*Xpl*A) successfully engineered into Arabidopsis plants was capable of degrading military explosive such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Rylott*, et al.*, 2006). Kawahigashi et al. introduced human cytochrome P450 genes CYP1A1, CYP2B6, and CYP2C19, into rice plants (Oryza sativa cv. ‘Nipponbare’). The transgenic rice plants have tolerance to different classes of herbicides

because of enhanced P450-mediated detoxification by introduced P450 species (Kawahigashi*, et al.*, 2003, Kawahigashi*, et al.*, 2005, Kawahigashi*, et al.*, 2005, Kawahigashi*, et al.*, 2006). Transgenic potato plants expressing either human or rat cytochrome P450 genes have enhanced metabolism and tolerance to various herbicides such atrazine, metolachlor, and acetochrolin in vitro environment (Selosse*, et al.*, 2004).

Mammalian cytochrome P450 2E1 oxidizes a wide range of important pollutants, including TCE, ethylene dibromide, carbon tetrachloride, chloroform, and vinyl chloride (Singh*, et al.*, 2011). Doty and colleagues developed transgenic tobacco plants having a dramatically enhanced ability in metabolizing TCE up to 640-fold as compared with null vector control plants. Regarding uptake and debromination of ethylene dibromide, the transgenic plants also demonstrated to have enhanced capability (Doty*, et al.*, 2000). The research team tested several low molecular weight volatile organic compounds (VOCs) with the same transgenic tobacco and demonstrated greatly increased rates of removal of vinyl chloride (VC), carbon tetrachloride (CT), benzene, toluene, chloroform, and bromodichloromethane (BDCM), compared to wild-type or vector control tobacco (James*, et al.*, 2008). The research team also reported the genetic transformation of hybrid poplar plants (*Populus tremula* × *Populus alba*) overexpressing mammalian cytochrome P450 2E1 (CYP2E1). The engineered trees were capable of the enhanced metabolism of five volatile toxic compounds: TCE, VC, CT, chloroform and benzene. In addition, these engineered plants demonstrated superior removal of the pollutants from the air (Doty*, et al.*, 2007).

The use of these bacterial and mammalian CYP450s in transgenic plants, however, could be involved in regulatory issues; therefore, it is becoming essential to identify additional, naturally-occurring plant cytochrome P450s that can be potentially used for

phyotoremediation such as herbicide detoxifications and TCE degradation.

Many cytochrome P450-encoding sequences in plants have already been found through genome and expressed sequence tags (ESTs) projects (Ro*, et al.*, 2001, Ro*, et al.*, 2002). Annotations in the completely sequenced genomes of Arabidopsis, rice and poplar have indicated that 272 CYP450 genes are contained in the Arabidopsis genome and that 457 genes are contained in the Oryza genome and 312 genes in the *Poplus*. However, it is difficult to assign a catalytic function to the cloned genes and only a limited number of CYP450s have been identified and characterized so far. In the case of Arabidopsis, more than 200 CYP450 genes in the genome still await functional characterization. With regard to this issue, this study hypothesizes that certain CYP 450s in plants could play an essential role in metabolizing TCE.

2.1.3 **Glutathione S-transferases (GSTs)**

Enzymes of the glutathione S-transferase (GST) family can be found in all organisms, where they catalyze a variety of reactions and accept endogenous and xenobiotic substrates (Allocati*, et al.*, 2009). They have a critical role in the detoxification of xenobiotics by conjugating glutathione (GSH) to a hydrophobic substrate such as pesticides and herbicides (Rennenberg, 2005, èOztetik, 2008, Brazier-Hicks*, et al.*, 2008, Schroder*, et al.*, 2008). There are many reports that carcinogens including PAHs are detoxified and conjugated by glutathione S-transferases (GSTs) and_glucosyltransferases (GTs) (Dixon*, et al.*, 1998, Edwards*, et al.*, 2000, Xiang*, et al.*, 2001, Ada*, et al.*, 2007). Rawls (1996) showed that CYP 450s are involved in epoxide formation on the dechlorination pathway, whereas glutathione S-transferases catalyze reactions with glutathione. Recently, Zhang et al. (2011) developed

transgenic alfalfa plants simultaneously expressing both human CYP2E1 and glutathione S-transferase (GST). The plants showed enhanced resistance to mixed contaminants of heavy metals and organic pollutants (Zhang & Liu, 2011). DeRidder et al.(2002) reported that GSTs of Arabidopsis were induced by herbicides and high levels of glutathione S-transferase (GST) activity were detected in poplar leaves after exposure to herbicides (Komives*, et al.*, 2003).

In plants, GSTs are divided into seven classes: theta, zeta, phi, tau, lambda, glutathione-dependent dehydroascorbate reductase (DHAR), and tetrachlorohydroquinone (TCHQ) dehalogenase (Basantani & Srivastava, 2007). In Arabidopsis, a total of 47 GST members have been identified, with 14 belonging to the phi class, 3 in the theta class, 2 in the zeta class, and 28 in the tau class (Gong*, et al.*, 2005). In this study, I hypothesized that GSTs are involved in the plant response to TCE.

2.1.4 **Glycosyltransferases**

Glycosyltransferases enzymes work as a catalyst for the transfer of a sugar residue from a donor to an acceptor molecule which is often one of the last steps in the biosynthesis of plant phenolic compounds including anthocyanins and flavonoids (Li*, et al.*, 2007). These enzymes perform various functions such as the regulation of hormone homeostasis and the biosynthesis and storage of secondary compounds (Gachon*, et al.*, 2005). In particular, these enzymes detoxify reactive compounds and increase their solubility by transferring sugars from uridine 5'- diphosphate-glycosides (UDP-glycosides) to a variety of small lipophilic aglycones (Hefner*, et al.*, 2002, Bowles*, et al.*, 2006). The glucosylation of xenobiotics, notably chlorinated phenols, and the associated GT activities have been studied in some detail, notably in soybean and wheat (Brazier*, et al.*, 2002). The glycosylation of

trichloroethanol is responsible for the removal of approximately 90% of the free trichloroethanol in TCE-exposed plants (Shang*, et al.*, 2001). Therefore, it likely constitutes an important survival mechanism for plant cells. Plant glycosyltransferases are part of a multigene superfamily with 47 distinct families (Ross*, et al.*, 2001). In the completed genome sequence of *Arabidopsis thaliana*, there are 117 sequences containing the consensus, scattered across all of the five chromosomes (Li, et al., 2001, Paquette, et al., 2003)

2.1.5 ATP binding cassette (ABC) transporter

The ATP binding cassette (ABC) transporter is one of the active transport systems of the cell, which plays a central role in detoxification processes (Rea, 1999) and is widespread in archaea, eubacteria, and eukaryotes (Higgins, 1992). The ATP-binding cassette (ABC) transporters are one of the largest protein families in plants. The majority of ABC proteins are transporters of mineral ions, lipids, and peptides as well as involved in the regulation of channels and other primary pumps (Rea, 2007). Importantly to this study, they can transport substrates such as conjugated organic compounds and xenobiotics out of cells and into vacuoles (Meagher, 2000). Insoluble compounds are generally assumed to be stored in the cell wall by ATP-binding cassette (ABC) transporters. More than 120 genes have been annotated in the Arabidopsis genome based on specific nucleotide binding motifs and multiple transmembrane domains (Sánchez-Fernández*, et al.*, 2001, Kaneda*, et al.*, 2011). Various transport substrates such as fatty acids, cuticular lipids, auxin, heavy metals, xenobiotics, and secondary metabolites are known to be present in plant ABC transporters (Kaneda*, et al.*, 2011).

2.2 Research objectives

The hypothesis of this research is that there is a plant genetic response to trichloroethylene (TCE) both in hybrid poplar *Populus tremula* × *P. alba* N717 1-B4 (INRA) and in transgenic hybrid poplar.

Aim 1) Identify and compare the poplar gene expression in response to TCE between hybrid poplar and transgenic hybrid poplar by microarray analysis

Aim 2) Verify and quantify the expression of specific genes putatively involved in TCE metabolism by quantitative reverse transcriptase *real time PCR* (*qRT-PCR*)

Aim 3) Determine specific cytochrome P 450s which can degrade TCE by using a yeast system

Aim 4) Propose deduced amino acid sequences of the detoxification genes cytochrome P450s, glutathione S-transfereases and glycosyltransferases from INRA and Nisqually-1.

2.3 Materials and Methods

2.3.1 Plant materials and TCE treatments

Plants were grown on Murashige and Skoog (MS) tissue culture medium (Murashige & Skoog, 1962) in vitro at 20℃ with a photoperiod of 16h light and 8h dark. About twelve week-old and approximately the same size plants were used for the experiment. To determine if any gene regulation is changed in response to TCE, triplicate wild type hybrid poplar *Populus. tremula x P. alba* N717 1-B4 (INRA) and INRA transgenic poplar, either the null vector control plant KH200 or the rabbit CYP2E1 line 78 (Doty*, et al.*, 2007), were dosed with trichloroethylene for 2 and 24 hours and compared with undosed control plants. All samples were treated in sterile 40ml Volatile Organics Analysis (VOA) vials, sealed with Teflon-lined septum valve caps, and then the MS medium was dosed to a level of 50µg/ml TCE (99.5% purity; Sigma-Aldrich). After each time exposure, plants were frozen with liquid nitrogen.

2.3.2 RNA extraction

Total RNA was extracted from the leaves of TCE-treated and untreated plants of wild type hybrid poplar clone INRA 717 1-B4, null vector control plant KH200, and CYP2E1 line 78. Frozen leaves (approximately 0.5g) were homogenized with a mortar and pestle in liquid N_2. RNA was purified from each sample, using the RNeasy Plant Mini Kit (Qiagen) and stored at –70°C. The quality of total RNA was evaluated using a model 2100 Bio-analyzer (Agilent Technologies, Santa Clara, Calif., USA). RNA yields were quantified by measuring

the absorbance of a 1: 50 dilution of the samples with an ultraviolet spectrophotometer at 260 and 280 nm. Only total RNA and subsequent cRNA samples with appropriate size distribution and an A260:A280 ratio of 1.8–2.1 were used for microarray analysis.

2.3.3 Microarray analysis

Microarray analysis was carried out under two different conditions. The first microarray analysis generated gene expression data from twelve Affymetrix Gene Chip Poplar Genome Arrays (Affymetrix, Santa Clara, CA, USA) (Lockhart*, et al.*, 1996). Triplicate RNA samples of TCE-treated and untreated wild type INRA 717-1B4 (TCE 2 hours treated and 24 hours treated) were used. The second experiment generated gene expression measurements from four Affymetrix Poplar Genome Arrays. Triplicate RNA samples from null vector KH200 and transgenic CYP2E1 line 78 (TCE treated, untreated) were pooled and investigated by microarray analysis (Peng*, et al.*, 2003).

Total RNA was processed for use on the Affymetrix Poplar Genome GeneChip arrays according to the manufacturer's protocol. In brief, 10 µg of total RNA was used in a reverse transcription reaction to generate first-strand cDNA, using the SuperScript choice system (Invitrogen) with oligo(dT) 24 primer fused to T7 RNA polymerase promoter. After second-strand synthesis, biotin-labeled target complementary RNA (cRNA) was prepared using the BioArray high-yield RNA transcript labeling kit (Enzo Biochem, New York) in the presence of biotinylated UTP and CTP. After purification and fragmentation, 15 µg of cRNA was used in a 300-ul hybridization mixture containing added hybridization controls. A total of 200 µl of the mixture was hybridized on arrays for 16 hours at 45°C. Standard post-hybridization wash and double-stain protocols were used on an Affymetrix Gene Chip fluidics station 450.

Arrays were scanned on an Affymetrix Gene Chip scanner 2500.

2.3.4 Gene ontology annotataion of expressed genes

The gene Ontology functional classification was obtained from the Arabidopsis GO database of the Arabidopsis Information Resource (TAIR, http://arabidopsis.org). Arabidopsis annotations were taken from BLASTX- hits against the NCBI protein database with an E-value of 1.0E-20 or score >80.

2.3.5 Quantitative reverse transcriptase *real time PCR* (*qRT-PCR*) Analysis

Total RNA was isolated as described above. Total RNA used for qRT-PCR verification was obtained from the leaves of wild type hybrid poplar INRA 717 1-B4, the null vector control plant KH200, and the CYP2E1 line 78. The mRNA was converted to cDNA using the BioRad iScript kit. Primers to each of the upregulated genes were designed (Table 2). Selected putative genes were compared to an internal reference gene (18S rRNA) that served to normalize expression levels and to give unit-less, realistic values of candidate gene expression. Probes with a fluorescent tag (Taqman) specifically designed to complement an internal region of the 18S rRNA and the genes of interest were used to quantify gene expression.

Table 2. Primer pairs and fluorescently labeled probes for detoxification gene analysis

Gene	Type of oligo	Sequence (5'-3')
Cytochrome P450		
	Primer (forward)	AATTGCCACCGTGGAGCTTT
	Primer (reverse)	TATGCATAGCAAGACCGGGTTG
	Probe	FAM-5'-CCCACCAGGCATTTCCCAGTCGAAT-3'-3BHQ_1
Glutathione		
	Primer (forward)	CATCCTTGCAGAACTTGAGATATTTAGAA
	Primer (reverse)	TACCAGTTTCAGACCTGCTACCTCC
	Probe	FAM-5'-AAGGTCTGCTAGTCCAATTGCCTCCCCA-3'-3BHQ_1
Glycosyltransferase		
	Primer (forward)	GAACGCCAAGAAATGGAAAGATTTA
	Primer (reverse)	CAACCAAGCAAATATGGCATATCG
	Probe	FAM-5'-CTTATCCGAGGAACCACCGTCCTTGACA-3'-TAMRA
ABC- transporter		
	Primer (forward)	TGCCCTGGTTGGAGAAAGTG
	Primer (reverse)	TGGAGACTCTGAATGTCAATTCCAT
	Probe	FAM-5'-TTGCAATAATGAGATCACCGTTGATTTCCCA-3'-TAMRA
poplar 18S rRNA		
	Primer (forward)	AAT TGT TGG TCT TCA ACG AGG AA
	Primer (reverse)	AAA GGG CAG GGA CGT AGT CAA
	Probe	FAM-5'-TCC TAG TAA GCG CGA GTC ATC AG-3'-TAMRA

2.3.6 Plant cytochrome P 450s gene expression in yeast

To develop a faster way to screen the putative P450 genes for involvement in TCE metabolism than using transgenic plants, a yeast expression system was established. Dr. Mary Schuler at the University of Illinois kindly gave us yeast *Saccharomyces cerevisiae* strain WAT11 and the yeast expression vector pYeDP60 which has been shown to work well in CYP 450 expression (Pompon*, et al.*, 1996, Herman*, et al.*, 2007). To verify this system for TCE metabolism, CYP 2E1 cDNA was cloned as a positive control. Based on microarray results, several putative cytochrome P450 probes have been found. Primers were designed based on putative P450 genes from NCBI and JGI data base and PCR was performed directly on *Populus trichocarpa* (Nisqually) and *Populus tremula x P. alba* N717 1-B4 (INRA) cDNA.

The PCR products were cloned into pGEM T Easy Vector (Promega). The presence of cDNA inserts was verified in the transformants, and sequenced using SP6 and T7 primers that bind to the vector to check for sequence errors introduced during PCR. Full-length cDNAs encoding poplar CYP450s were cloned into the yeast expression vector pYEDP 60. The rabbit CYP2E1 and several candidate plant CYP450 cDNAs were cloned into the yeast expression vector and introduced them into yeast strain WAT11 (*Saccharomyces cerevisiae*).

The transformed yeast was grown in galactose-containing minimal medium to induce expression of the cDNAs, and then the cultures were dosed with TCE in sealed 250ml flasks. The supernatants and cell extracts were then analyzed for TCE metabolites using gas chromatography-electron captor detection (GC-ECD).

2.3.7 Phylogenetic analysis

DNA sequences were assembled using the Seqman software (DNA STAR Inc.) and analyzed using BLAST. Sequence comparisons with public databases were performed via the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/), by employing the BLASTN algorithm. Phylogenetic analysis was done using CLUSTAL W software. Evolutionary distance matrix were constructed using the algorithm of Jukes and Cantor (Thompson*, et al.*, 1994) and the evolutionary trees for the data sets were inferred from the neighbor joining method (Saitou & Nei, 1987) by using MEGA version 4.0.1 (Tamura*, et al.*, 2007).

2.4 Results

2.4.1 Microarray analysis

The first microarray experiment was designed to differentiate gene responses of wild type poplar between 2 hours and 24 hours TCE treatment; the second microarray analysis was performed to compare the null vector control plant KH200 with vector containing mammalian CYP2E1 line 78.

Based on the TCE dosed INRA hybrid aspen microarray results, differentially expressed genes were selected based on $p<0.05$ and more than 2-fold change (FC) in gene expression. The results showed that 857 genes were up- or down regulated. 379 probe sets were differentially expressed only at 2 hours (214 upregulated and 165 downregulated); 376 probe sets were changed at 24 hours (141 upregulated and 235 downregulated). A number of probe sets (102) were changed at both time comparisons (Figure 2). In the 2 hour dosed TCE plants, 290 genes were upregulated and 191 genes were down regulated over 2 fold. Twenty-four hours following TCE dosing, 167 genes were upregulated and 311 genes were down regulated (Figure 4).

In the TCE dosed transgenic plants, the vector control KH200 and the CYP2E1 line 78 differentially expressed genes were selected based on greater than 2-fold change (FC) in gene expression. The results revealed that a total of 4,095 genes were differentially regulated in response to TCE. As displayed in the Venn diagram, Seven hundred ninety genes were categorized in the vector control plant; 2,928 genes were categorized in the CYP2E1 transgenic plant; 377 genes were in the intersection (Figure 3). In the vector control KH200,

517 genes were upregulated and 650 genes were down regulated. Interestingly, in the transgenic line 78, a much larger number of genes were differentially expressed, with 1601 genes being upregulated and 1705 genes down regulated (Figure 5).

Since the poplar genome had not yet been well annotated, upregulated poplar genes were assigned to the corresponding *Arabidopsis* annotation with BLASTX- hits against the NCBI protein database with an E-value of 1.0E-20 or score >80. From the BLASTX results, 2 hour TCE (175/290) genes, 24 hour TCE (102/167) genes, KH200 (276/517) genes and line 78 (674/1601) genes were similar with *Arabidopsis* genes and the rest of genes were unknown or had low similarities with *Arabidopsis*.

To access biological process, cellular components, and molecular function in gene expression patterns of TCE-exposed plants, the upregulated genes in 2 hours and 24 hours TCE exposed plants, KH200 plants, and CYP2E1 plants were classified into functional categories.

Figure 6 and 9 show the categorization of the major biological functions of these genes. Figure 7 and 10 show the categorization of the major cellular components of the genes and Figure 8 and 11 show the categorization of the major molecular functions of the genes.

The biological functions of most genes in 2 hour TCE-exposed plants were associated with metabolic processes (73 genes, 55%) and cellular processes (62 genes, 47%). However, after 24 hours, metabolic processes (26 genes, 32%) and cellular processes (24 genes, 29%) were remarkably declined. Other responses to stress, abiotic or biotic stimuli, and other biological processes were also declined after 24 hours. Interestingly, the number of unknown biological process genes of the 2 hours and 24 hours TCE exposed plants was 22 and 28, respectively, but the proportion of these unknown processes was 17% and 33% (Figure 6). In

the proportion of cellular components of functional category, chloroplast, cytoplasmic components, intercellular components, membranes and plastid related genes were up-regulated almost three times higher than 24 hours plants (Figure 7). Also, genes involved in binding activity, enzyme activity, transfersase and transport activities were higher than 24 hour TCE-exposed plants (Figure 8).

For the null vector control plant KH200, the proportion of genes involved in metabolic processes (89 of 194 genes, 46%) and protein metabolism (27 of 194 genes, 14%) that were differentially regulated in response to TCE was similar to that of the transgenic CYP2E1 plant in metabolic processes (216 of 468 genes, 46%) and protein metabolism (59 of 468 genes, 14%). However, the proportion of cellular processes, response to stress, and abiotic or biotic stimulus related genes in the null vector control plant KH200 was lower than the CYP2E1 plant. Although the proportion of each process for the vector control KH200 response was similar to that of the CYP2E1 plant, a larger number of CYP2E1 plant genes were expressed in each category. Also, the number of unknown biological process genes of KH200 plants and CYP2E1 plants was 51 out of 194 (26%) and 78 out of 468 (17%), respectively (Figure 9).

Regarding the proportion of cellular components in each Gene Ontology functional category, mitochondria, cytoplasmic components, intercellular components, and plasma membrane related genes in the transgenic CYP2E1 plant were up-regulated higher than KH200. However, only other cellular components and nucleus related genes in KH200 vector control plants were higher than CYP2E1 line 78 (Figure 10). Also, other binding and enzyme activity genes of CYP2E1 line 78 in molecular function category were higher than KH200 (Figure 11).

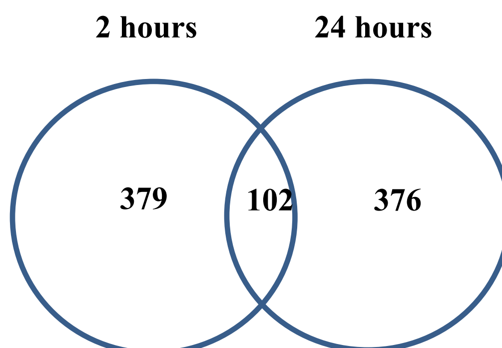


Figure 2. Venn diagram representing 857 differentially expressed probes in wild hybrid poplar after TCE treatment at 2, and 24 hours. The circles represent a time point with the numbers representing the probes (fold change ≥ |2.0| and p value ≤0.05) at two time comparisons.

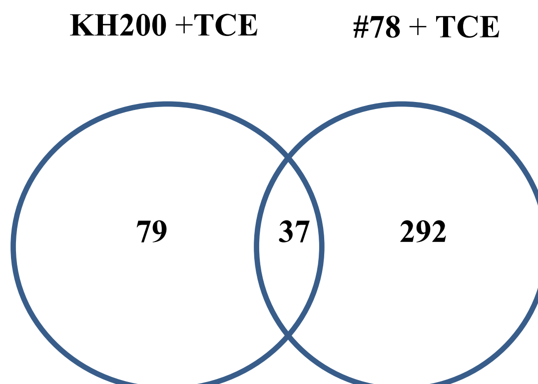


Figure 3. Venn diagram representing 4095 differentially expressed probes in transgenic poplar after TCE treatment at 24 hours. The circles represent vector control-KH200 (left) and CYP2E1-#78 (right) with the numbers representing the probes (fold change ≥ |2.0|).

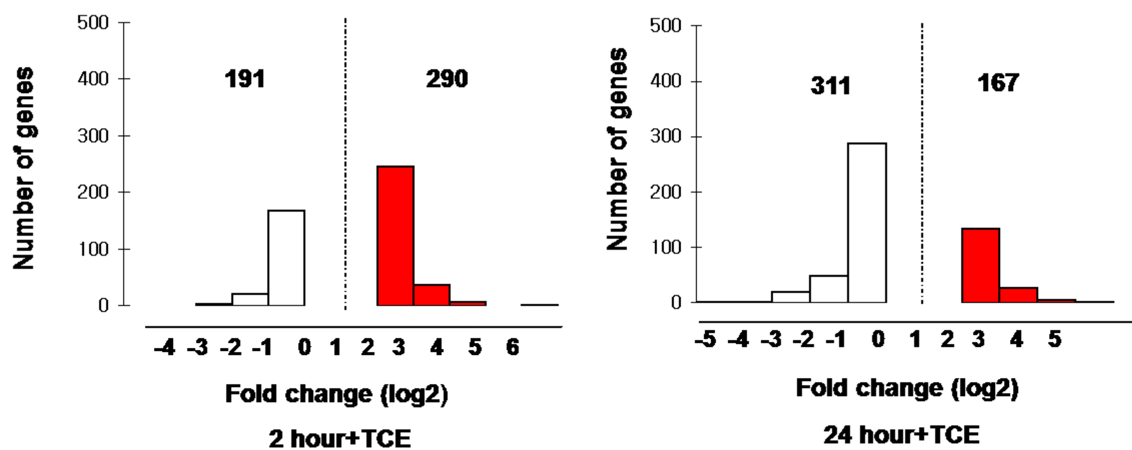


Figure 4. Histograms of the fold change (log2) distribution for all genes in wild hybrid poplar after TCE treatment at 2 (left), and 24 hours (right). The histograms show the distribution of fold changes for up-regulated genes (red bars), down-regulated genes (open bars) and the numbers representing total genes with p values less than 0.05.

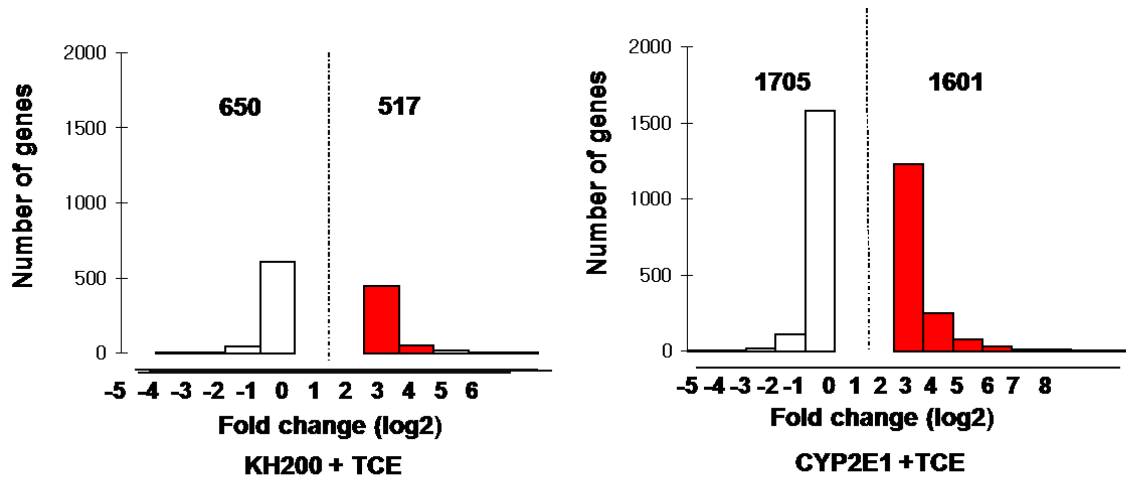


Figure 5. Histograms of the fold change (log2) distribution for all genes in transgenic hybrid poplar after TCE treatment at 24 hours. Vector control-KH200 (left) and CYP2E1-#78 (right). The histograms show the distribution of fold changes for up-regulated genes (red bars), down-regulated genes (open bars) and the numbers representing total genes with p values less than 0.05.

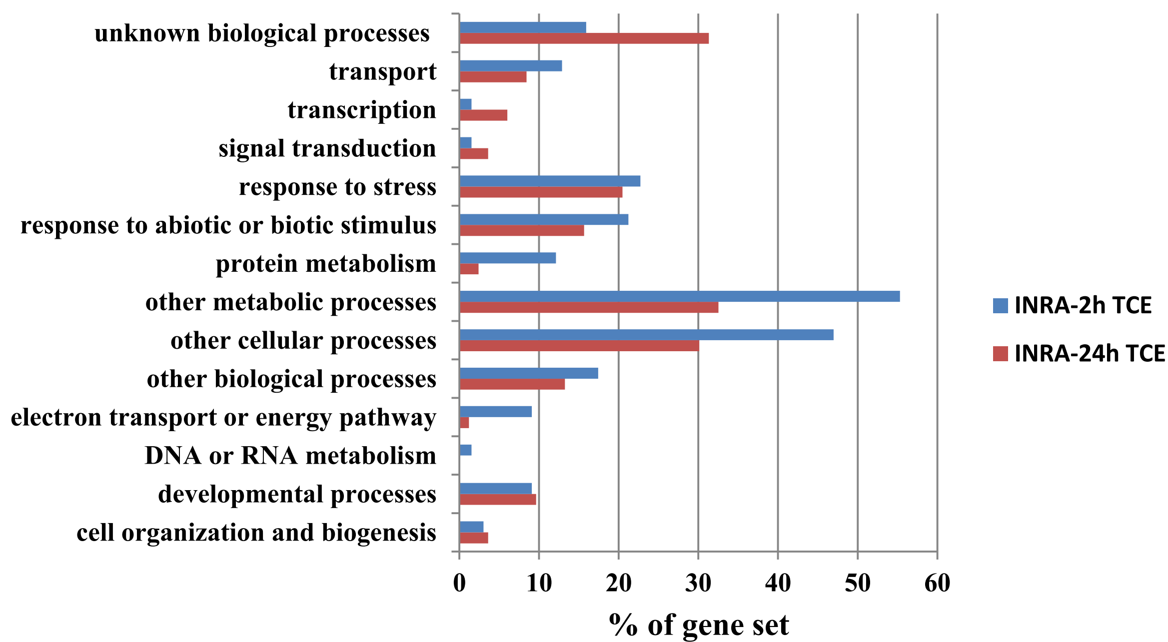


Figure 6. Proportion of biological processes in each Gene Ontology functional category. The percentage is based on the number of transcripts in the set. The 2 hour TCE dosed plants revealed much higher proportion of biological processes than 24 hour TCE dosed plants.

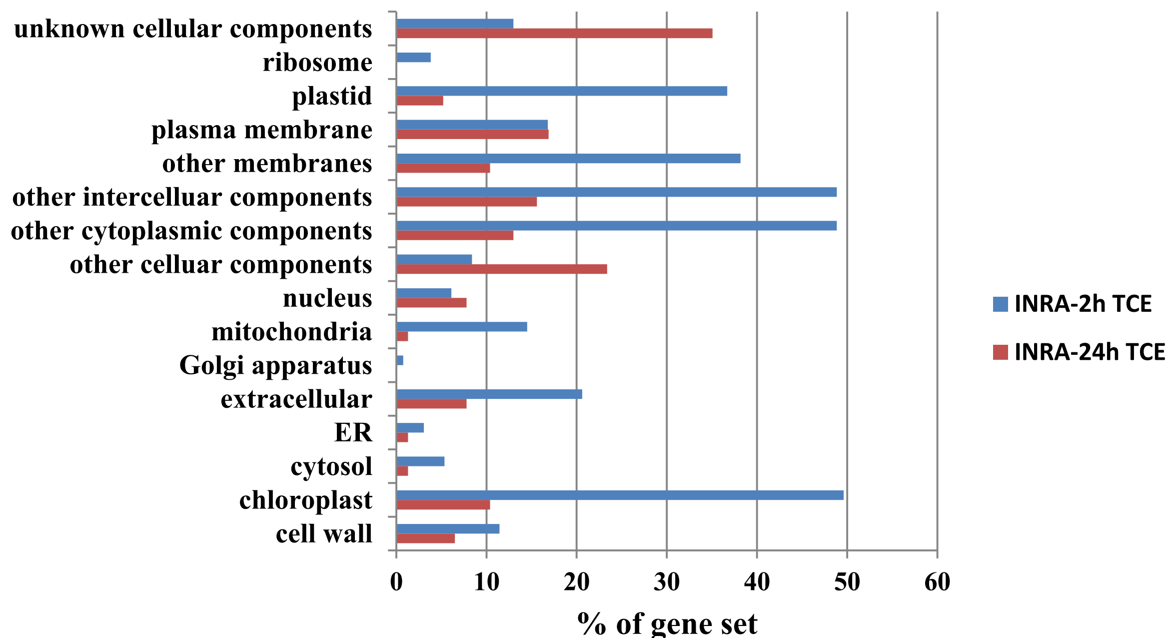


Figure 7. Proportion of cellular components in each Gene Ontology functional category. The percentage is based on the number of transcripts in the set. The 2 hour TCE dosed plants revealed much higher proportion of cellular components than 24 hour TCE dosed plants.

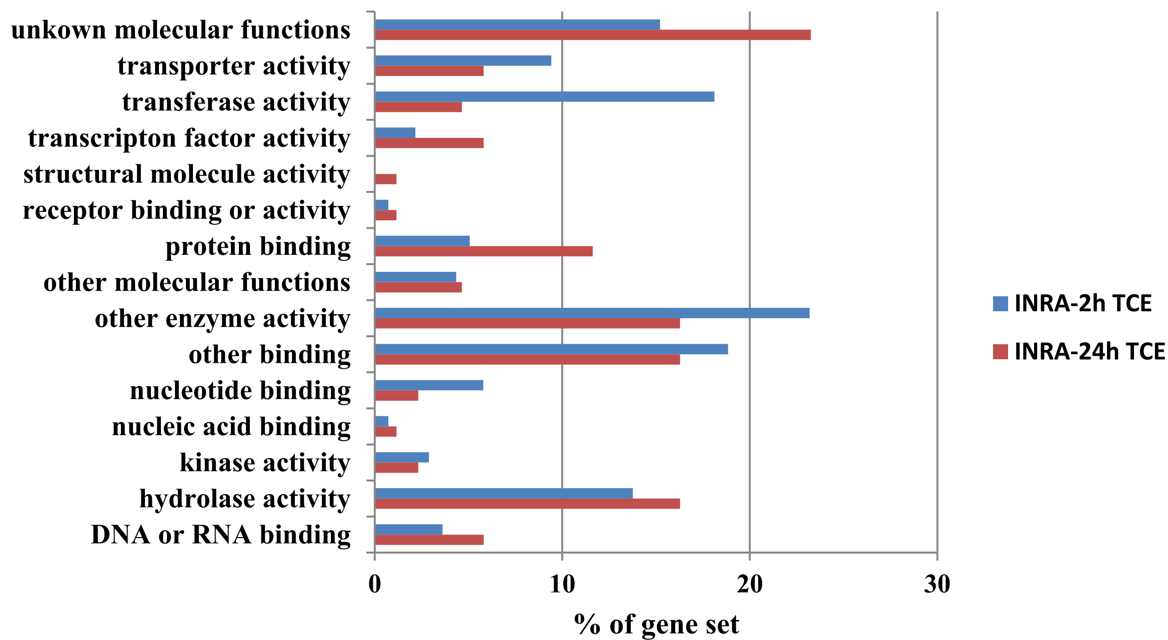


Figure 8. Proportion of molecular functions in each Gene Ontology functional category. The percentage is based on the number of transcripts in the set. The 2 hour TCE dosed plants revealed much higher proportion of molecular functions than 24 hour TCE dosed plants.

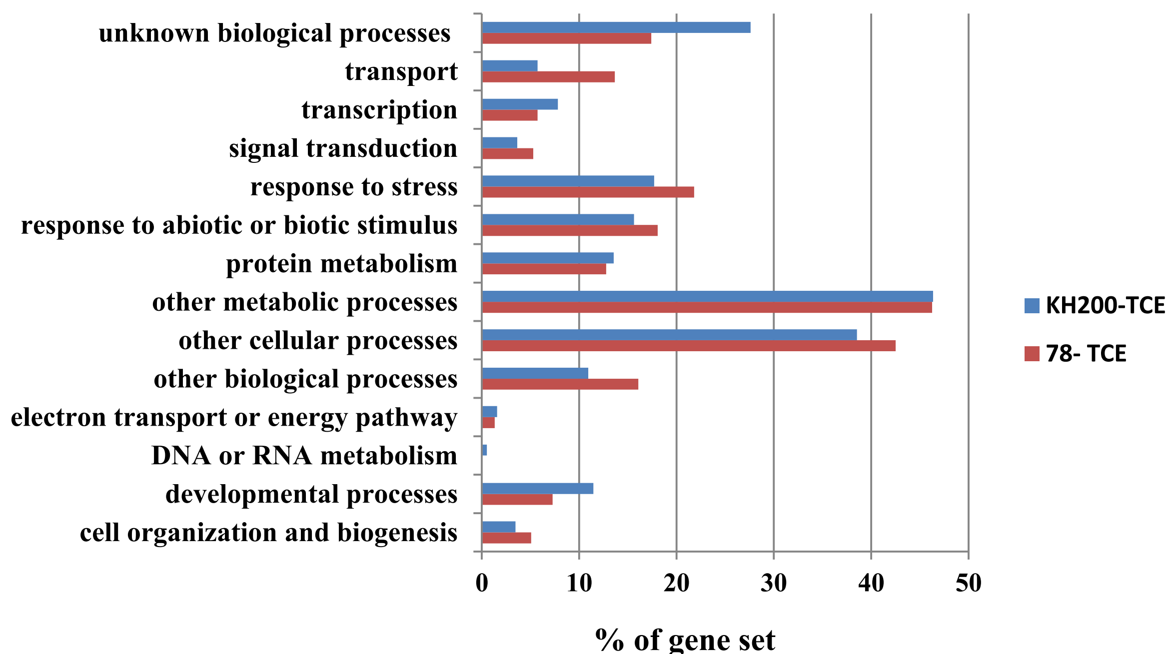


Figure 9. Proportion of biological processes in each Gene Ontology functional category. The percentage is based on the number of transcripts in the set. The proportion of each process of vector control KH200 was similar with CYP2E1 #78. However, the number of CYP2E1 #78 genes in each category was actually displayed a larger set of genes expressed.

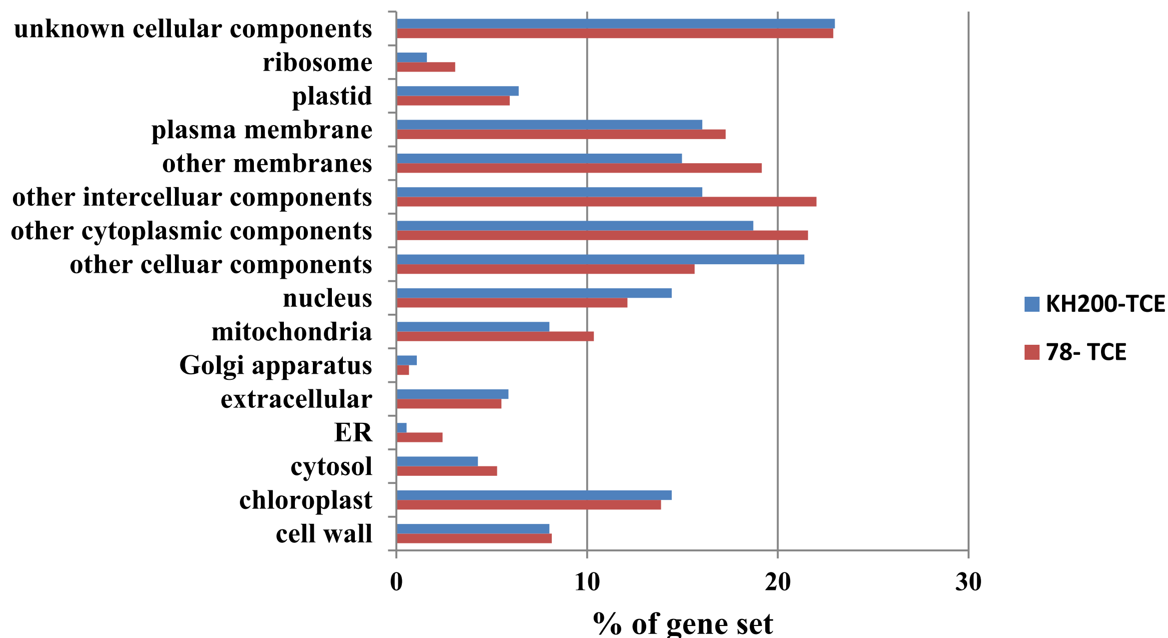


Figure 10. Proportion of cellular components in each Gene Ontology functional category. The percentage is based on the number of transcripts in the set. The proportion of each process of vector control KH200 was similar with CYP2E1 #78. However, the number of CYP2E1 #78 genes in each category was actually displayed a larger set of genes expressed.

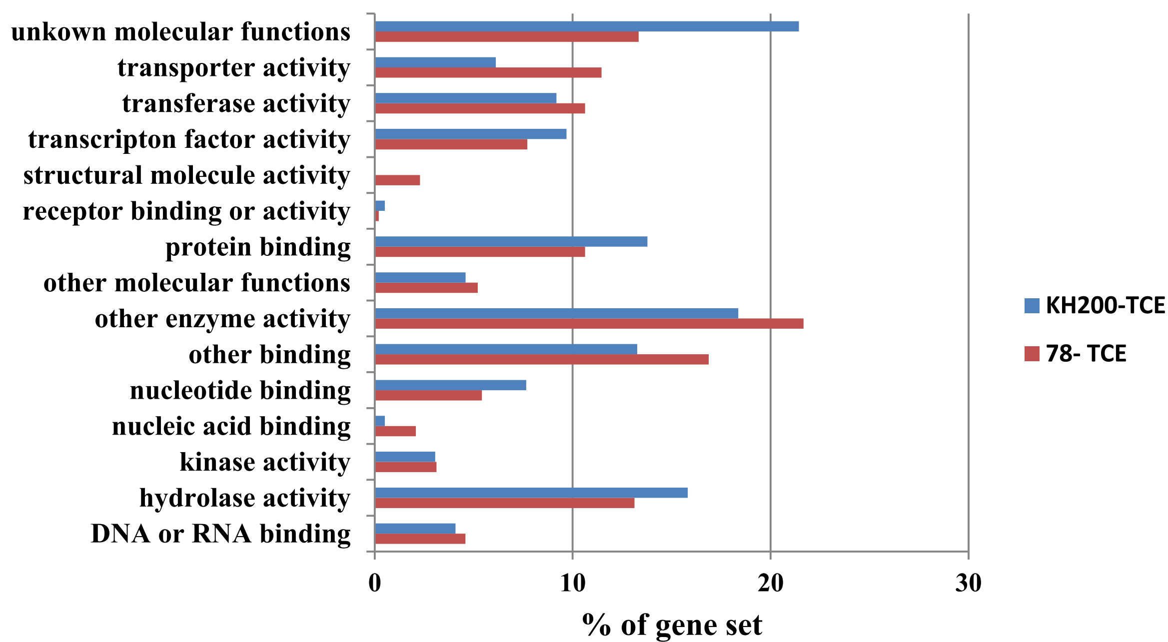


Figure 11. Proportion of molecular functions in each Gene Ontology functional category. The percentage is based on the number of transcripts in the set. The proportion of each process of vector control KH200 was similar with CYP2E1 #78. However, the number of CYP2E1 #78 genes in each category was actually displayed a larger set of genes expressed.

2.4.2 qRT-PCR analysis of targeted genes of TCE degradation

To further support the microarray results, and to confirm the expression of TCE metabolism- related genes, qRT-PCR was carried out on 4 selected genes including cytochrome P450, glutathione S-transferase, UDP-glycosyltransferase and ABC transporter that are putatively involved in the xenobiotic detoxification metabolic pathway. The results of the qRT-PCR analysis are presented in Figure 12 and 13. Assuming equal annealing and amplification properties of the PCR primers, the relative amounts of individual mRNAs were normalized to the expression of 18S rRNA. The expression patterns somewhat differed in 2 hours TCE and 24 hours TCE treatments. Overall, the levels of expression of the four targeted in the CYP2E1 line 78 with TCE treatment were highly expressed compared to control plants and these differences were statistically significant except cytochrome P450.

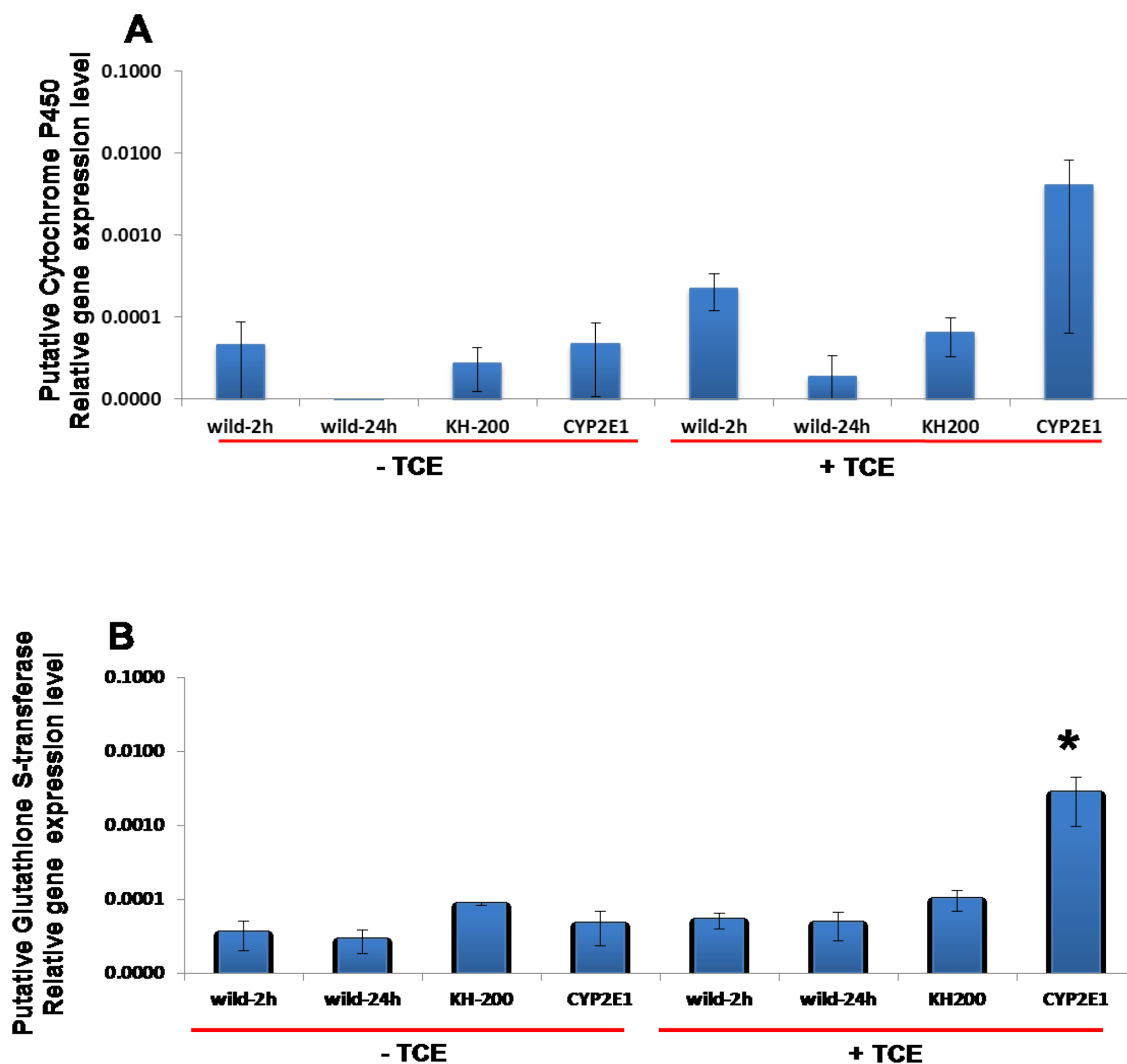


Figure 12. Relative mRNA gene expression levels in the transgenic poplar. The qRT-PCR analysis of putative CYP450 and glutathione S-transferase is presented. Values represent the mean ± SE for n = 3 plants for each treatment. The asterisk indicates significant difference from other treatments ($P<0.05$)

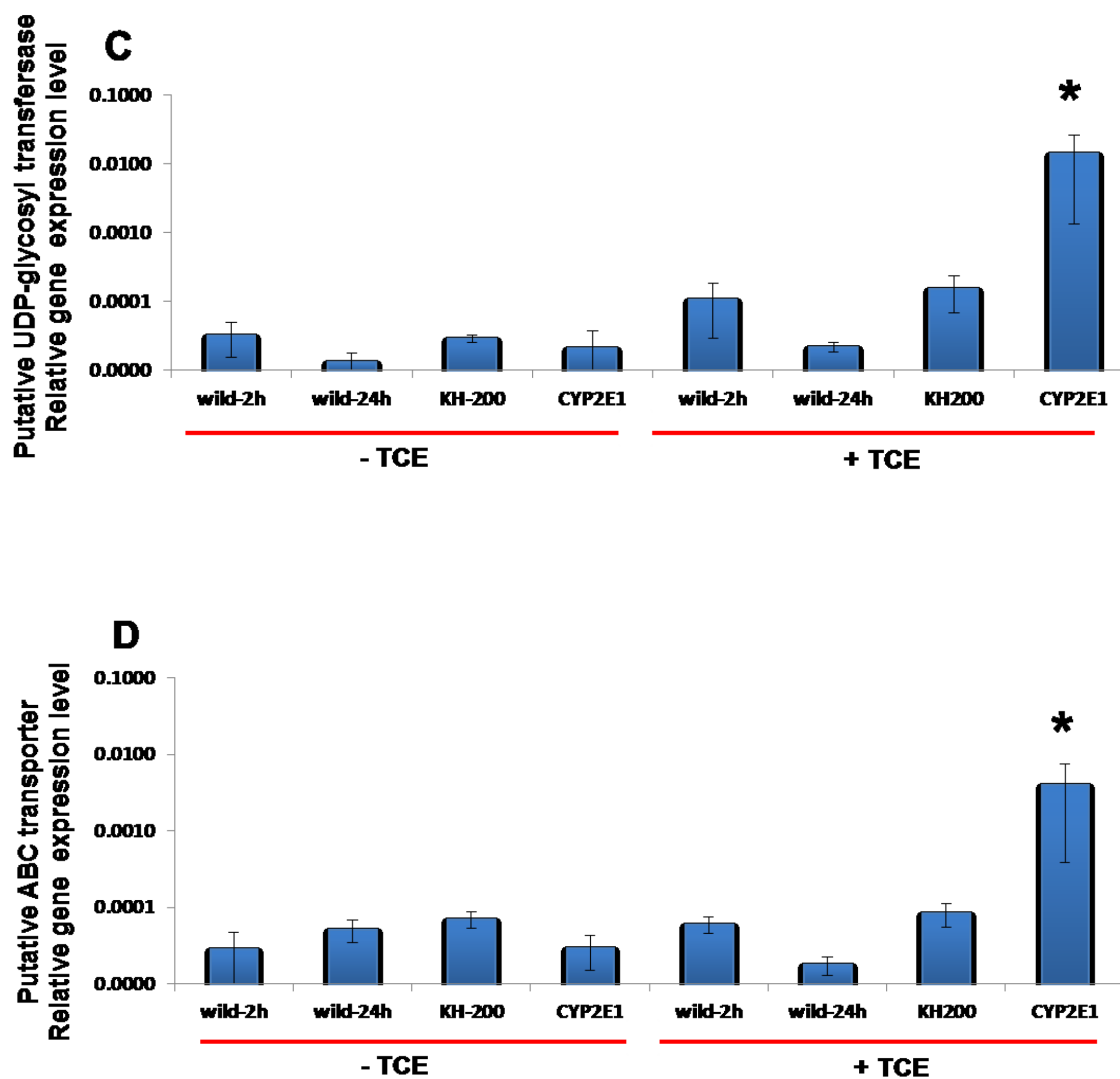


Figure 13. Relative mRNA gene expression levels in the transgenic poplar. The qRT-PCR analysis of putative UDP glucosyltransferase and ABC transporter is presented. Values represent the mean ± SE for n = 3 plants for each treatment. The asterisk indicates significant difference from other treatments (P<0.05)

2.4.3 Correlation of gene expression data between microarray and qRT-PCR

The mRNA levels of four probes including cytochrome P450, glutathione S-transferase, UDP-glycosyltransferase and ABC transporter were quantified in leaves by qRT-PCR. The quantitative data were normalized as a ratio to 18S rRNA expression and then calculated as a ratio of expression from the TCE dosed plants to the control plants. As shown in Figure 14, the data correlated to expression indicated that there was strong correlation between qRT-PCR and the microarray data with a R^2 of 0.98.

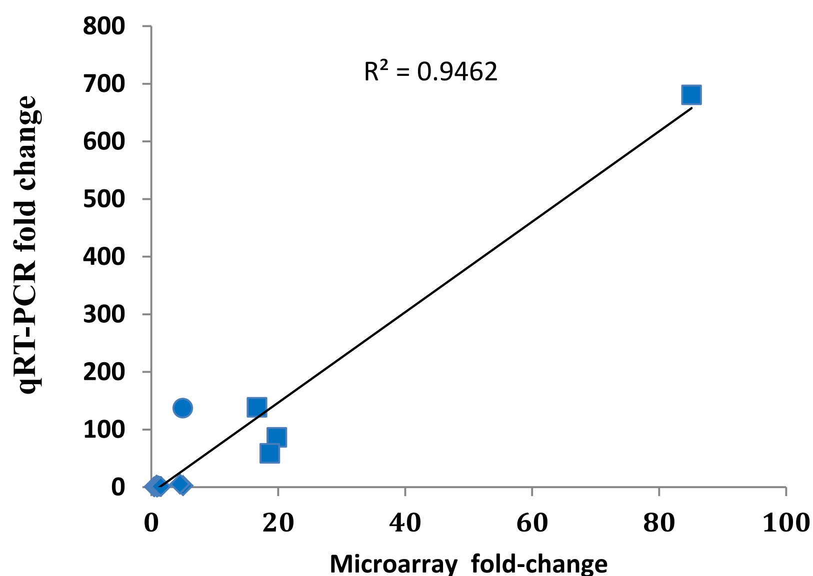


Figure 14. Correlation among gene expression data obtained by microarray analysis and qRT-PCR. Values represent the mean mRNA expression values for CYP450, glutathione, glucosyltransferase and ABC transporter normalized to the expression of 18S rRNA for all individuals with the microarray data on the X-axis and the qRT-PCR data on the Y-axis.

2.4.4 Plant Cytochrome P 450s gene expression in yeast

In the first study to find the mammalian CYP2E1 ortholog in plants, TCE metabolism experiments were performed with Arabidopsis since it is widely used as a model organism for molecular genetic studies in plants. Several Arabidopsis cytochrome P450 genes including 71B29, 77A7, 77A9, 94D2, and 96A13 that were up-regulated in response to TCE from the microarray analysis were tested for TCE degradation in a yeast expression system. Then, putative poplar cytochrome P450 genes (INRA CYP450 and Nisqually CYP450) were tested for TCE degradation in a yeast expression system.

The positive control, CYP2E1, produced a strong peak of TCEOH (Figure 15); however, none of the *Arabidopsis* P450 genes tested were able to convert TCE to TCEOH.

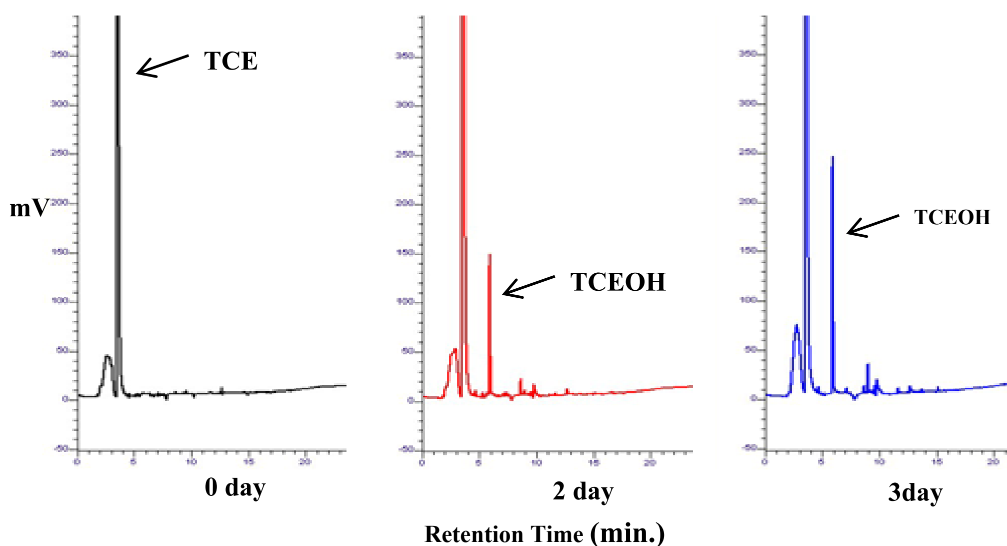


Figure 15. Samples of gas chromatograph (GC–ECD) of the trichloroethylene assay. The positive control CYP2E1 overexpressed yeast strain dosed with TCE was extracted. A, 0 day. B, 2 day. C, 3 day.

2.4.5 Phylogenietic analysis

2.4.5.1 Cytochrome P450

A 1515-bp cytochrome p450 gene fragment was amplified from Nisqually-1 and INRA genomic DNA with CYP450 INRA and NISQ primers and then sequenced. Phylogenetic analysis of the cytochrome P450 amino sequence showed that both cytochrome P450s belong the cytochrome P450 83F family; the Nisqually CYP450 gene was most closely related to the CYP83F1 and INRA CYP450 gene was closely related to the CYP83F4 (Figure 16).

2.4.5.2 Glutathione

A 678-bp glutathione gene fragment was amplified from Nisqually-1 and INRA genomic DNA with GST INRA and NISQ primers and then sequenced. Phylogenetic analysis of the glutathione sequence showed that both INRA and Nisqually glutathione genes were belonged to the GST 41family (Figure 17).

2.4.5.3 UDP-glycosyltransferase

A 1428 bp glycosyltransferase family gene fragment was amplified from Nisqually-1 and INRA genomic DNA with UDP INRA and NISQ primers and then sequenced. Phylogenetic analysis of the glycosyltransferase family sequence showed that both genes were belonged to the UGT 75L family (Figure 18).

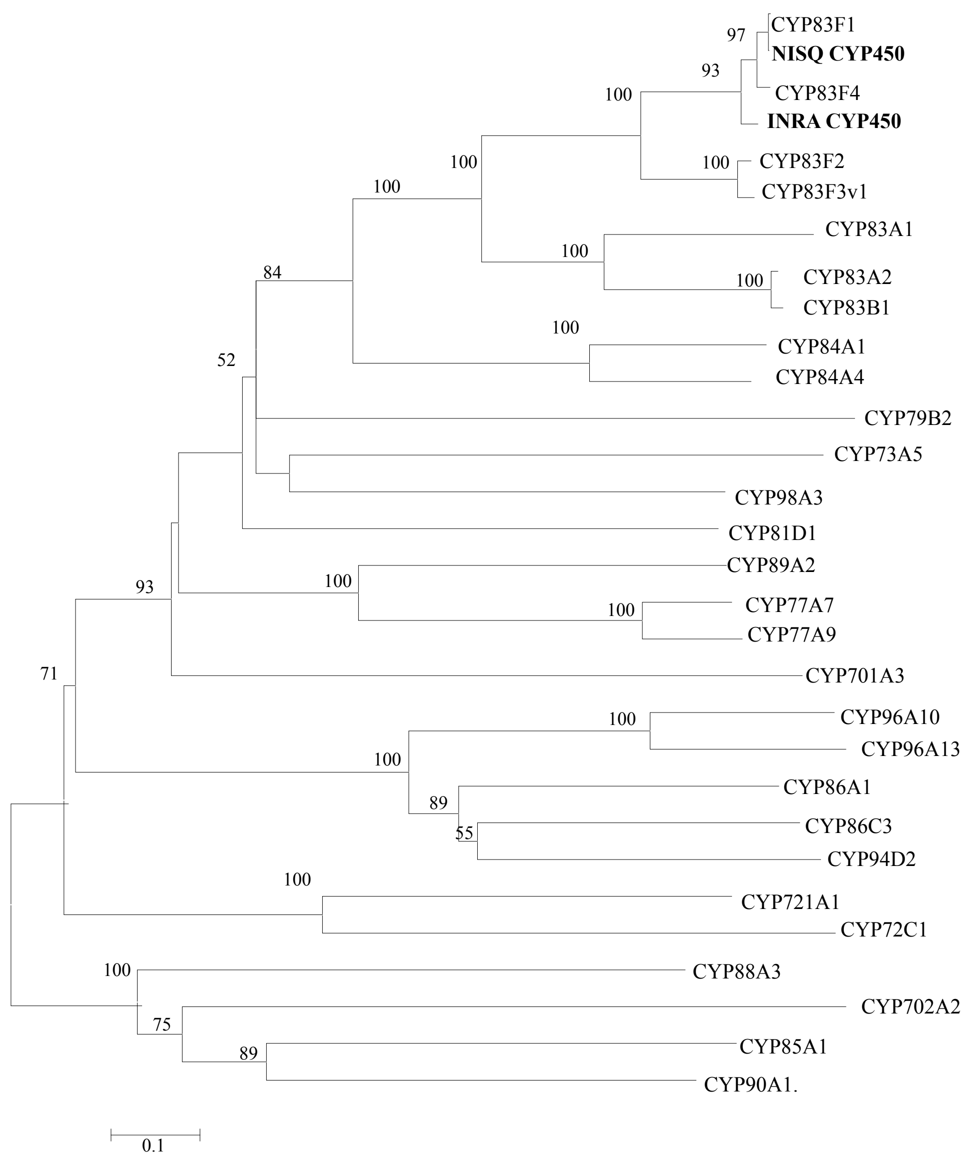


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Figure 16. Phylogenetic tree of the plant deduced amino sequences of cytochrome. The phylogenetic relationship of Nisqually -1 (*Populus trichocarpa*) and INRA CYPs was analyzed with ClustalW and NJ tree. Poplar CYP 450 sequences were retrieved from the DOE Joint Genome Institute website.

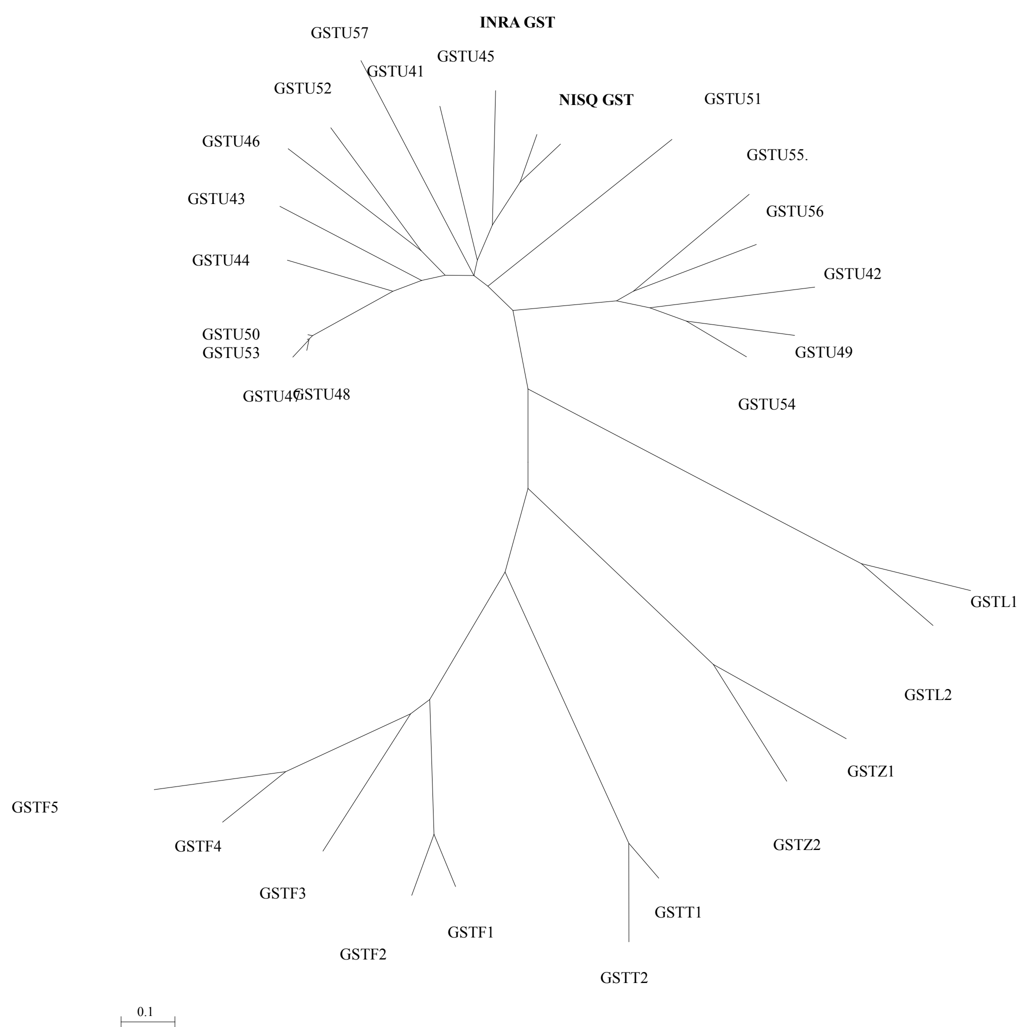

Figure 17. An unrooted Neighbor-joining tree of glutathione plant amino sequences. Sequences were aligned using the program Clustal W and the NJ tree was comouted using the DNAstar package

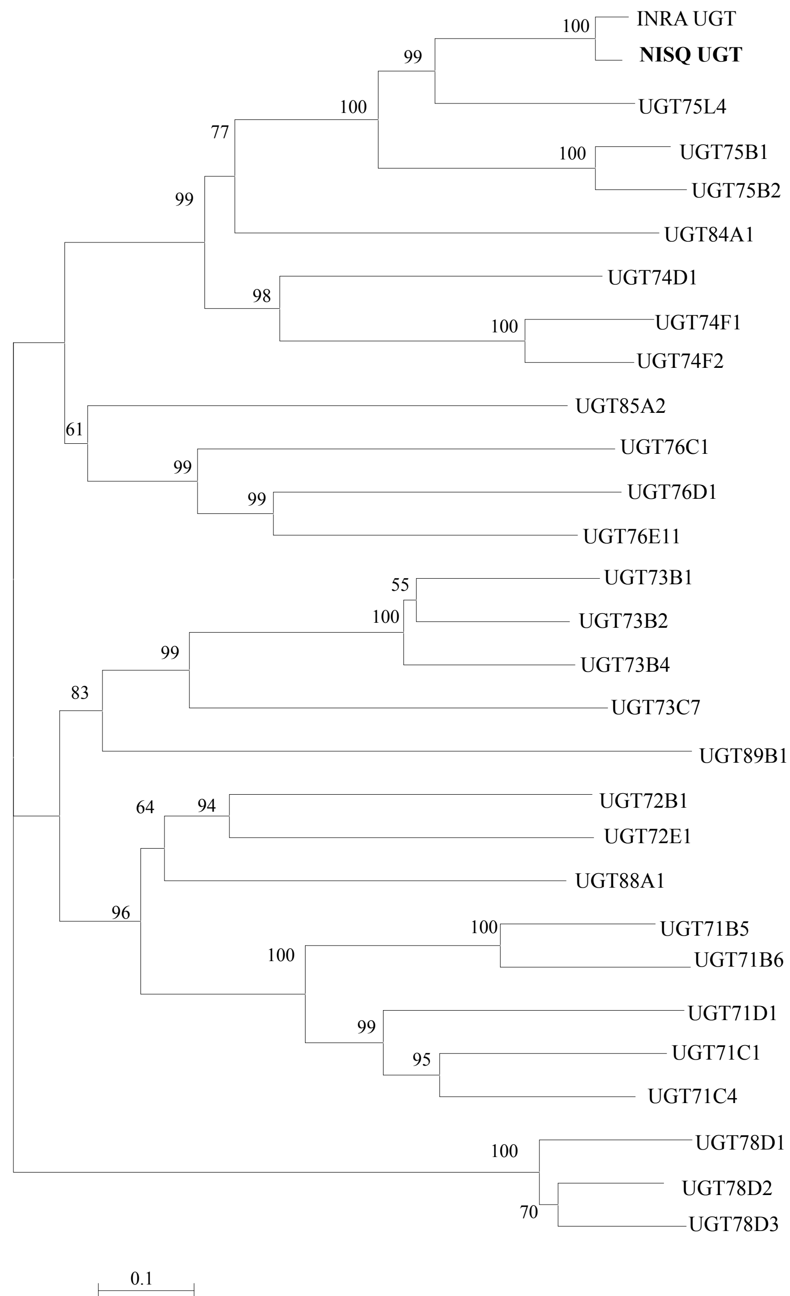

Figure 18. Phylogenetic tree of the poplar glycosyltransferase family. The phylogenetic relationship of Populus trichocarpa GTs was analyzed with ClustalW and NJ tree. Poplar GT sequences were retrieved from the DOE Joint Genome Institute website.

2.5 Discussion

This research aimed at examining the genetic response of plants to trichloroethylene (TCE), with an emphasis on putative detoxifying enzymes. There are few published reports about cloned plant P450s suitable for use in phytoremediation. In the first studies, the responses of TCE in Arabidopsis and wild-type hybrid poplar at two time points were compared using microarrays. However, there were neither highly up- or down regulated genes, nor were differentially expressed genes significantly related to detoxification genes. To gain a better understanding of how TCE is further metabolized by plants, in this work, the transgenic plants with an increased metabolism of TCE were compared using a microarray analysis.

In the TCE dosed hybrid aspen microarray results, six cytochrome P450s were differentially expressed at 24 hours TCE exposure in the vector control plants, and 23 P450 genes were differentially expressed at 24 hours TCE exposure in the CYP2E1 line 78 (Table A-1). At 2 hours, Ptp.5109.1.S1_at probe had the highest fold changes. It had homology with cytochrome P450 of *Populus trichocarpa* (E-value 5e-44, score 179). At 24 hours TCE exposure in the vector control plants, Ptp.6116.1. S1_at probe had the highest fold changes (5-fold changes). However in the transgenic line 78, Ptp.6116.1.S1_at probe had the highest differential expression up to 20-fold change. Ptp.6116.1.S1_at is homologous with cytochrome P450 of *P. trichocarpa* (E-value 1e-85, score 318).Ptp.5109.1.S1_at probe was shown 2-fold changes in the CYP2E1 line 78. Three genes out of seven cytochrome P450s are involved in cellular processes, four genes out of seven metabolic processes, and one gene is related to stress responses in the GO biological processes. However, the remaining 17

genes have not been annotated in Arabidopsis GO functional categorization.

According to the microarray results, expression of glutathione S-transferases was not differentially affected in the vector control plants after 24 hours exposure to TCE, however, 18 glutathione transferase genes were differentially expressed in the CYP2E1 transgenic line 78 after 24 hours TCE exposure (Table A-2). Glutathione S-transferases of the same family were defined as having >80% amino acid sequence identity. Interestingly, most glutathione S-transferases were highly upregulated compared with other genes following TCE treatment. For example, PtpAffx.43231.1.A1_a_at probe had 168-fold upregulation. PtpAffx.43231.1.A1_a_at is a predicted protein of *P. trichocarpa* (E-value 1e-123, score 446), and, it has homology with glutathione S-transferase (class tau) of *Arabidopsis thaliana* (E-value 1e-69, score 266). Most glutathione S-transferases are involved in cellular processes (6/6, 100%), metabolic processes (6/6, 100%), stress responses (2/6, 33.3%), and other biological processes (4/6, 66.7%) in GO biological processes. The remaining 12 genes were not annotated in Arabidopsis GO functional categorization.

According to the microarray results of glucosyltransferases, five glucosyltransferase genes were differentially regulated at 24 hours TCE exposure in the vector control plants. Glucosyltransferases were defined as belonging to the same family based on having >68% amino acid sequence identity. However, 21 glucosyltransferases were differentially regulated in the CYP2E1 transgenic line 78 after 24 hours TCE exposure (Table A-3). All five genes were upregulated approximately 2-fold in the vector control plants. But in the CYP2E1 line 78, probe Ptp.6958.1. S1_s_at and PtpAffx.31211.1.A1_at were upregulated 98-and 85-fold, respectively. In the BLASTX result, most probes were predicted proteins in *P. trichocarpa*; also, they had high similarity with other plants glucosyltransferases. Most

glucosyltransferases are involved in metabolic processes (15/16, 93.8%) in the GO biological processes. The remaining ten genes have not been annotated in Arabidopsis GO functional categorization.

The microarray results showed that ABC transporter genes were not differentially expressed in the vector control plants after exposure to TCE for 24 hours. However, 15 ABC transporter genes were upregulated in the CYP2E1 line 78 after 24 hours TCE exposure (Table A-4). ABC transporters of the same family were defined as having >80% amino acid sequence identity. PtpAffx.141628.1.S1_at probe showed 17-fold upregulation in CYP2E1 plants exposed to TCE for 24 hours. In the BLASTX results, PtpAffx.141628.1.S1_at was identified as a multidrug/pheromone exporter, MDR family, ABC transporter family of *P. trichocarpa* (E-value: 1e-98, score 362). It was also a putative ABC transporter in *A. thaliana* (E-value: 6e-85, score 316). All ABC transporters are involved in transport (8/11, 73%) in the GO biological processes. The remaining six genes were not annotated in Arabidopsis GO functional categorization.

Based on the results from microarray analysis of TCE-dosed hybrid aspen, the number of upregulated genes was two times higher than downregulated genes after 2 hours of TCE exposure. However, after 24 hours, the number of down regulated genes was two times higher than upregulated genes. Also, the biological process of the 2 hours TCE dosed plants revealed a much higher proportion in the biological processes category. But after 24 hours, all biological processes except unknown biological processes were remarkably declined. At 2 hours, 20 genes were putatively related to one of xenobiotic metabolizing genes such as cytochrome P450, glutathione, glycosyltransferase and ABC transporter.

The KH200-vector control plants dosed with TCE upregulated 517 genes and

downregulated 650 genes. In contrast, the CYP2E1 transgenic plants dosed with TCE upregulated 1601 genes and downregulated 1705 genes. The induction of genes is most likely important for transformation, conjugation, and compartmentation of toxic metabolites in response to the increased metabolism of TCE in the transgenic lines. The level of fold change was also higher than that of the control plants.

Four selected probes from microarray results corresponding to genes involved in phase I, II and III drug metabolism, which were chosen for RT-PCR analysis to verify and quantify the expression of detoxification genes, were highly expressed. The Ptp.6116.1.S1_at probe corresponds to a CYP450 gene (95% similarity) that is upregulated 87-fold in the CYP2E1 line. Also, PtpAffx.25444.1.S1_x_at probe, which corresponds to a glutathione S-transferase (98% similarity), had a 59 times greater change in the CYP2E1 line 78 than control poplar. Particularly, PtpAffx.31211.1.A1_at probe which was glycosyltransferase (69% similarity) showed a 681-fold change in the CYP2E1 line 78. PtpAffx.141628.1.S1_at probe, an ABC transporter (95% similarity), showed a 139-fold change in the CYP2E1 line 78.

In addition, there are several other genes that may be involved in detoxification, such as laccases (Bollag*, et al.*, 1988), peroxidases (Kawano, 2003), and dehalogenases (Wang & Chen, 2007) (Table A-5).

The expression levels of each treatment showed a large genetic response in the transgenic CYP2E1 line 78, based on the qRT-PCR results. Glutathiones S-transferase, glycosyltransferase, and ABC transporter genes showed significantly increased expression. The microarray and qRT-PCR strongly correlated (r^2=0.98). Detoxification genes in CYP 2E1 line 78 appeared to be expressed in both microarray and qRT-PCR data.

One explanation of the lesser response in the vector control plants is that

trichloroethylene is not a natural substrate for these endogenous poplar genes and, therefore, might not induce the expression of the relevant detoxification genes. Mammalian CYP450s can degrade diverse xenobiotics (Williams*, et al.*, 2003) and specially mammalian CYP2E1 is known to take part in the biotransformation not only of TCE but also of ethanol and acetone and of many small molecule substrates such as halogenated hydrocarbons (Anzenbacher & Anzenbacherová, 2001).

It appeared that the genetic response of wild type hybrid poplar INRA 717 1-B4 was in response to the toxicity of TCE. However, the transgenic poplar plants over expressing mammalian cytochrome P450 2E1 showed a stronger genetic response than wild-type hybrid poplar INRA 717-1B4, most likely because the metabolic pathway of TCE had already been initiated by CYP450 2E1 enzyme.

In summary, the responses of gene expression of hybrid poplar and transgenic poplar exposed to TCE were measured. Many detoxification genes including cytochrome P450s, transferases, and transporters that appear to be involved in TCE metabolism were identified. The presence and expression of the mammalian CYP2E1 gene initiated the metabolic pathway for TCE and the abundance of TCE metabolites triggered a strong expression of glutathione-S-transferases, glucosyltransferases, and ABC transporters. In addition, their mRNA expression levels were remarkably higher than those of wild-type hybrid poplar plants. To find the CYP2E1 ortholog in Arabidopsis and poplar, several putative plant cytochrome P450 genes from Arabidopsis (71B29, 77A7, 77A9, 94D2, and 96A13) and poplar (INRA CYP450 and Nisqually CYP450) were tested for TCE degradation in a yeast expression system. Yet none of the putative plant cytochrome P450 genes tested were able to convert TCE to TCEOH. It is difficult to assign a catalytic function to the cloned genes since

312 CYP450s genes are estimated to exist in poplar and only a limited number of P450s have been identified and characterized so far. In the case of Arabidopsis, the functions of more than 200 out of the 272 P450 genes have yet to be identified (Nelson, 2006).

Recent studies reported that CYP77 and CYP93 families are responsible for hydroxylation at terminal and internal points on fatty acids. CYP94 family is mediating fatty acid hydroxylation and CYP96 with several Arabidopsis members in fatty acid synthesis (Nelson & Werck-Reichhart, 2011). Therefore, the research aim to identify the CYP2E1 ortholog in plants was not achieved.

Overall, this study indicated that the rate-limiting step in TCE degradation was the initial activation by a CYP450. When this was overexpressed in transgenic plants, the subsequent genes in the TCE metabolic pathway seemed to be activated automatically. Recently, James et al. performed a field study to evaluate the effectiveness of the transgenic poplars expressing cytochrome P450 2E1 in a field setting. The transgenic trees had significantly greater concentrations of known TCE metabolites than wild plants and increased removal of TCE (James et al, unpublished)

2.6 References

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Table A-1. Cytochrome P450s upregulated genes

Probe set ID	Fold Change (Log2)	UnitProt ID	BLASTX vs. *Arabidopsis*	Score	E-value	AGI number
Wild 2 hours dosed TCE						
Ptp.5109.1.S1_at	3.42	BU810061	cytochrome P450 monooxygenase	81.6	7e-14	AT4G31500
PtpAffx.308.5.S1_s_at	2.97	CN523454	cytochrome P450 [Populus trichocarpa]	464	1e-129	n.a.
Ptp.6116.1.S1_at	2.31	BU811435	putative cytochrome P450 protein [Arabidopsis ...	187	5e-46	At2g02580
PtpAffx.27268.1.S1_at	1.59	CA924374	cytochrome P450 like protein [Arabidopsis tha...	181	1e-44	AT5G10600
Ptp.4154.1.S1_s_at	1.24	CV277475	putative cytochrome P450	217	1e-54	AT2G45570
PtpAffx.103190.1.S1_at	1.18	CN524584	cytochrome P450 [Populus trichocarpa]	456	4e-127	n.a.
PtpAffx.145798.1.S1_at	1.15	CX182212	cytochrome P450, putative [Arabidop...	217	7e-55	At1g31800
PtpAffx.139165.1.S1_at	1.06	BP922839	cytochrome P450-like protein [Arabidopsis thal...	161	3e-38	AT2G23190
PtpAffx.113940.1.A1_at	1.02	CV277947	ref|NP_196694.1| monooxygenase family protein [Arabidopsis th...	293	4e-77	At5g11330
CYP2E1 #78 dosed TCE						
Ptp.6116.1.S1_at	4.31	BU811435	putative cytochrome P450 protein [Arabidopsis ...	187	7e-46	At2g02580
PtpAffx.54125.1.A1_s_at	4.01	CK095265	cytochrome P450 [Arabidopsis thaliana] >gb|AAP...	108	4e-22	AT3G26210
PtpAffx.23537.2.A1_a_at	2.24	CV260178	CYP82G1 (cytochrome P450, family 82, subfamily G, polypeptide	160	3e-38	AT3G25180
Ptp.4154.1.S1_s_at	2.08	CV277475	CYP76C2 (cytochrome P450, family 76, subfamily C	217	3e-55	AT2G45570
PtpAffx.3847.3.A1_at	1.94	CV258896	cytochrome P450 [Populus trichocarpa]	105	2e-21	n.a.
PtpAffx.19676.1.S1_at	1.85	CX184740	cytochrome P450-like protein [Arabidopsis thal...	178	1e-43	AT4G37370
PtpAffx.83404.1.A1_at	1.65	CA933449	cytochrome P450 flavonoid 3',5'-hydroxylase [Populus trichocarpa]	463	7e-129	n.a.
PtpAffx.74008.1.S1_s_at	1.64	BU893238	cytochrome P450 [Arabidopsis thaliana]	134	1e-29	AT3G19270
Ptp.2193.1.A1_s_at	1.58	CK112033	cytochrome P450-like protein [Arabidopsis thal...	171	2e-41	At3g48520
PtpAffx.32849.1.S1_at	1.53	BU813715	cytochrome P450 [Arabidopsis thaliana]	102	2e-20	AT2G29090
PtpAffx.21215.1.S1_at	1.50	DN487798	cytochrome P450-like protein [Arabidopsis thal...	133	9e-30	AT4G37360
PtpAffx.417.3.A1_a_at	1.46	CV259584	cytochrome P450 family protein [Arabidopsis ...	159	1e-37	At1g12740
PtpAffx.100032.1.A1_at	1.38	BP934526	cytochrome P450-like protein [Arabidopsis thal...	204	4e-51	AT4G37360
PtpAffx.84185.1.S1_a_at	1.34	CV266140	cytochrome P450 family [Arabidopsis thaliana...	127	3e-28	AT5G36130
PtpAffx.33787.1.A1_at	1.25	CV277411	cytochrome p450	279	7e-74	AT3G25180
PtpAffx.36102.1.A1_at	1.17	BP936961	cytochrome P450-like protein [Arabidopsis tha...	103	2e-20	AT3G20090
Ptp.5109.1.S1_at	1.16	BU810061	putative cytochrome P450 monooxygenase [Arabid...	81.6	5e-14	AT4G31500
Ptp.2726.1.S1_at	1.15	DN492953	cytochrome P450 [Arabidopsis thaliana]	282	1e-74	At1g64900
PtpAffx.103190.1.S1_at	1.14	CN524584	cytochrome P450 [Populus trichocarpa]	456	3e-127	n.a.
PtpAffx.80069.1.S1_s_at	1.12	DN501927	cytochrome P450 [Arabidopsis thaliana]	371	3e-101	AT3G19270
PtpAffx.151850.1.A1_at	1.07	BP933761	cytochrome like protein [Arabidopsis thaliana]	177	3e-43	AT4G37360
PtpAffx.22222.1.A1_at	1.04	CV240869	cytochrome p450 (CYP78A9)	228	2e-58	AT5G09970
Ptp.325.1.A1_s_at	1.04	CK088352	cytochrome p450 (CYP78A9) [Arabidopsis thaliana]	278	1e-73	At3g61880

Table A-2. Glutathione S-transferase upregulated genes

Probe set ID	Fold Change (Log2)	UnitProt ID	BLASTX vs. *Arabidopsis*	Score	E-value	AGI number
CYP2E1 #78 dosed TCE						
PtpAffx.43231.1.A1_a_at	7.39	CV242243	Glutathione S-transferase	266	1e-69	AT1G17180
PtpAffx.43231.1.A1_x_at	6.58	CV242243	ATGSTU25 (Arabidopsis thaliana Glutathione S...	266	1e-69	AT1G17180
PtpAffx.25444.1.S1_x_at	5.29	CV274263	glutathione S-transferase GST 18 [Populus alb...	244	7e-63	n.a.
PtpAffx.4246.2.S1_a_at	4.90	DN488053	ATGSTU25 (Arabidopsis thaliana Glutathione S...	246	2e-63	AT1G17180
PtpAffx.25444.1.S1_s_at	4.83	CV274263	glutathione S-transferase GST 18 [Populus alb...	244	7e-63	n.a.
PtpAffx.121024.1.A1_at	4.22	CV240122	ATGSTU8 (Arabidopsis thaliana Glutathione S-...	135	3e-30	At3g09270
Ptp.3168.1.S1_at	3.97	CX659317	glutathione S-transferase GST 18 [Populus alb...	72.8	1e-11	n.a.
Ptp.7096.1.S1_x_at	3.11	AB190439.1	glutathione-s-transferase 7 [Arabidopsis lyrat...	184	2e-45	At2g29420
PtpAffx.23427.1.S1_s_at	3.07	CV280572	glutathione S-transferase	192	2e-47	At2g29420
Ptp.5347.1.S1_s_at	2.80	DN486427	glutathione S-transferase [Arabidopsis thaliana]	236	2e-60	At1g02930
PtpAffx.77335.1.S1_at	2.65	DN493413	glutathione-S-transferase [Arabidopsis cebenne...	129	1e-28	At2g29420
Ptp.5347.1.S1_x_at	2.36	DN486427	glutathione S-transferase [Arabidopsis thaliana]	236	2e-60	At1g02930
Ptp.3920.1.S1_at	2.25	CX168810	Arabidopsis thaliana Glutathione S-transferase			At3g09270
Ptp.5536.1.S1_at	1.87	CV254150	glutathione-s-transferase 7 [Arabidopsis lyrat...	134	1e-33	At3g09270
Ptp.7441.1.S1_at	1.82	CV255885	glutathione transferase, putative [Arabidopsis...	286	4e-76	At1g78380
PtpAffx.50247.1.A1_at	1.13	CV253145	glutathione transferase	227	8e-58	AT1G17180
Ptp.6936.1.S1_at	1.05	BU888078	glutathione-S-transferase 3 [Arabidopsis lyrat...	196	2e-48	AT2G29460
PtpAffx.43231.1.A1_a_at	7.39	CV242243	Glutathione S-transferase	266	1e-69	AT1G17180

Table A-3. Glucosyltransferase upregulated genes

Probe set ID	Fold Change (Log2)	UnitProt ID	BLASTX vs. *Arabidopsis* Score E-value	AGI number
Wild 2 hours dosed TCE				
PtpAffx.157099.1.S1_at	1.47	CF236245	dbj|BAF75897.1| glucosyltransferase [Cyclamen persicum] 265 1e-68	AT4G01070
PtpAffx.158710.1.S1_s_at	1.34	CX178903	dbj|BAG80556.1| UDP-glucose:glucosyltransferase [Lycium barba... 295 1e-77	AT1G01420
PtpAffx.283.2.A1_x_at	1.26	CV227694	ref|NP_566865.2| SUS4; UDP-glycosyltransferase/ sucrose synth... 140 4e-31	AT3G43190
PtpAffx.53565.2.A1_a_at	1.01	CV283727	ref|NP_192016.1| GT72B1; UDP-glucosyltransferase/ UDP-glycosy... 307 2e-81	AT4G01070
PtpAffx.70907.1.S1_at	1.19	CN520005	gb|EEF29506.1| UDP-glucuronosyltransferase, putative [Ricinus... 384 2e-104	AT1G22360
CYP2E1 #78 dosed TCE				
Ptp.6958.1.S1_s_at	6.62	DN488765	glucosyltransferase like protein [Arabidopsis... 199 2e-49	AT4g15550
PtpAffx.31211.1.A1_at	6.41	CV243415	putative glucosyltransferase [Arabidopsis thal... 236 2e-60	At4g14090
Ptp.973.1.A1_at	4.25	CK089832	putative glucosyltransferase [Arabidopsis thal... 88.2 5e-16	AT2G31790
PtpAffx.143539.1.S1_at	3.45	CX184307	putative glucosyltransferase [Arabidopsis thal... 209 2e-52	AT1G05670
PtpAffx.89781.1.A1_at	3.22	CV254856	glucosyltransferase like protein [Arabidopsis... 234 4e-60	At1g05560
PtpAffx.19634.1.S1_at	3.03	CN521144	glucosyltransferase like protein [Arabidopsis... 186 8e-46	AT4G15550
PtpAffx.33827.1.S1_at	2.61	DN486397	glucosyltransferase-related [Arabidopsis tha... 129 1e-28	AT3G21750
PtpAffx.139063.1.S1_at	2.14	BP926381	UDP-glycosyltransferase/ transferase 132 1e-33	AT4G15550
PtpAffx.158231.1.A1_at	2.05	CV279584	glucosyltransferase like protein [Arabidopsis... 166 6e-40	At1g05560
Ptp.2154.1.A1_at	2.05	CK093723	UTP-glucose glucosyltransferase [Arabidopsis ... 205 2e-54	AT3G21750
Ptp.6569.1.S1_at	2.05	BP924568	UTP-glucose glucosyltransferase [Arabidopsis ... 121 2e-26	AT3G21750
PtpAffx.84967.1.A1_at	2.00	CV257201	UDP-glucuronosyltransferase [Arabidopsis thali... 290 7e-77	At1g22370
PtpAffx.45875.1.S1_at	1.31	CK090979	UDP-glucoronosyl/UDP-glucosyl transferase family protein 186 6e-46	AT2G30150
Ptp.6228.1.S1_at	1.27	DN490570	putative glucosyl transferase [Arabidopsis th... 63.2 5e-16	AT2G36780
PtpAffx.146922.2.A1_at	1.22	CV253471	glucosyltransferase-like protein [Arabidopsis... 212 1e-56	AT4G34135
PtpAffx.211.1.A1_at	1.19	DN495737	UDP-glucoronosyl/UDP-glucosyl transferase family protein 82 3E-15	AT2G30150
PtpAffx.211.1.A1_a_at	1.15	DN495737	UDP-glucoronosyl/UDP-glucosyl transferase family protein 82 7e-15	AT2G30150
PtpAffx.42772.1.S1_at	1.08	CV254866	UTP-glucose glucosyltransferase [Arabidopsis ... 263 8e-69	AT3g21760
PtpAffx.2665.1.S1_at	1.05	BU869257	putative glucosyltransferase [Arabidopsis thal... 119 2e-25	At2g15490
PtpAffx.13160.1.A1_at	1.04	CV254230	UDP-glucoronosyl/UDP-glucosyl transferase fa... 117 1e-24	AT2G30150
Ptp.2808.1.S1_at	1.04	DN485934	UTP-glucose glucosyltransferase [Arabidopsis ... 203 1e-63	AT3g21760

Table A-4. ABC transporter upregulated genes

Probe set ID	Fold Change (Log2)	UnitProt ID	BLASTX vs. *Arabidopsis*	Score	E-value	AGI number
Wild 2 hours dosed TCE						
PtpAffx.16868.1.S1_at	1.88	CV253653	ref|XP_002318606.1| ABC transporter family, cholesterol/phosp...	379	4e-103	AT5G61700
PtpAffx.158977.1.S1_at	1.18	BU866894	ref|NP_200733.2| sugar transporter family protein [Arabidopsi...	205	1e-50	At5g59250
CYP2E1 #78 dosed TCE						
PtpAffx.141628.1.S1_at	4.06	BU861542	Putative ABC transporter [Arabidopsi...	309	1e-82	AT1G02520
PtpAffx.16622.1.A1_at	1.53	BP931445	transferase family protein [Arabidopsis thal...	79.7	1e-13	AT3G26040
Ptp.2310.1.A1_at	1.52	CK088838	putative ABC transporter [Arabidopsis thaliana]	142	7e-53	AT1G15520
PtpAffx.134772.1.S1_s_at	1.49	DN483641	ABC-type transport-like protein [Arabidopsis ...	77.0	1e-12	AT3G47730
PtpAffx.47344.1.S1_at	1.44	CK097216	ABC transporter, putative [Arabidop...	196	8e-49	AT1G31770
PtpAffx.608.4.S1_a_at	1.38	CA825625	transferase family protein [Arabidopsis thal...	225	2e-57	AT5g01210
Ptp.5594.1.S1_at	1.28	CK113725	Putative ABC transporter [Arabidops...	286	1e-75	AT1G15520
PtpAffx.78518.1.A1_at	1.23	CV263509	sugar transporter, putative [Arabidopsis tha...	298	8e-80	At5g18840
PtpAffx.144197.1.A1_a_at	1.22	DN490048	putative ABC transporter [Arabidopsis thaliana]	206	1E-51	AT1G15520
PtpAffx.89823.2.A1_a_at	1.20	DN487789	putative ABC transporter [Arabidopsis thaliana]	286	4e-75	AT1G59870
PtpAffx.608.3.S1_at	1.19	AJ771418	transferase family protein	174	2E-42	AT5g01210
Ptp.2310.1.A1_s_at	1.15	CK088838	putative ABC transporter [Arabidopsis thaliana]	167	1e-63	AT1G15520
PtpAffx.71022.1.S1_at	1.14	DN503518	putative sugar transporter [Arabidopsis thaliana]	179	9e-44	AT2G48020
Ptp.7087.1.S1_at	1.08	DN488195	ABC transporter family protein	295	2e-78	AT3G28345
Ptp.1775.1.S1_at	1.07	AY935502.1	transferring glycosyl groups	779	0.0	AT3G18660

Table A-5. Detoxification upregulated genes

Probe set ID	Fold Change (Log2)	UnitProt ID	BLASTX vs. *Arabidopsis*	Score	E-value	AGI number
CYP2E1 #78 dosed TCE						
putative laccase						
PtpAffx.16362.1.S1_at	2.59	DN487816	putative laccase (diphenol oxidase) family protein	187	3e-46	AT5G48100
Putative peroxidase						
PtpAffx.18226.1.A1_a_at	2.20	CV263828	peroxidase 72 (PER72) (P72) (PRXR8) [Arabido...	261	3e-68	At5g66390
PtpAffx.10445.1.S1_at	5.54	CV256716	peroxidase, putative [Arabidopsis thaliana] ...	256	2e-66	AT5G06720
PtpAffx.54628.1.S1_at	1.74	CV254688	peroxidase, putative [Arabidopsis thaliana] ...	191	3e-47	AT5G05340
PtpAffx.16117.1.A1_a_at	1.51	DN488185	peroxidase, putative [Arabidopsis thaliana] ...	164	4e-52	AT5g14130
PtpAffx.86604.1.S1_at	1.29	BP935860	peroxidase, putative [Arabidopsis thaliana] ...	150	4e-35	AT5G39580
Ptp.5821.1.S1_at	1.16	CV240646	glutathione peroxidase [Arabidopsis thaliana]	278	4e-73	At2g31570
PtpAffx.48031.1.S1_at	1.16	DN490972	peroxidase 64 (PER64) (P64) (PRXR4) [Arabido...	316	1e-84	AT5G42180
PtpAffx.148046.1.S1_at	1.16	BU896059	peroxidase 17 (PER17) (P17) [Arabidopsis tha...	281	2e-74	AT2G22420
PtpAffx.249.187.S1_at	1.15	BP933230	peroxidase ATP4a [Arabidopsis thaliana]	122	3e-26	At1g71695
Ptp.2344.1.A1_x_at	1.10	CV243674	peroxidase ATP4a [Arabidopsis thaliana]	292	7e-78	AT1G71695
Putative dehalogenase						
Ptp.6210.1.S1_at	2.02	CV267239	haloacid dehalogenase-like hydrolase family protein [Arabidop	297	4e-79	AT2G38740
Ptp.6210.1.S1_s_at	1.55	CV267239	haloacid dehalogenase-like hydrolase family protein	297	3e-79	AT2G38740
PtpAffx.4862.1.S1_a_at	1.43	BP936752	haloacid dehalogenase-like hydrolase family protein	246	2e-63	At5g44730
PtpAffx.2655.3.S1_a_at	1.25	CN524159	haloacid dehalogenase-like hydrolase family protein	319	1e-85	AT5G02230

BLASTX vs. Arabidopsis putative annotation of array elements assigned according to The Arabidopsis Information Resources protein set using BLASTX

(a)

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ATGGCATTACTCATATTCGTAATTCTCTTCCTCTCCATCATTTTCTTGTTTCTTCTCAAGAAAAACAAAATCTC
TAAAAGAGCTTGTTTCCCTCCTGGCCCCAACGGTCTTCCCTTGATAGGTAACTTGCACCAGCTTGATAGCTCAA
ACCTTCAAACGCAATTATGGAAACTATCTCAAAAATATGGCCCCCTCATGTCCTTAAAGCTAGGTTTCAAGCGA
ACCCTCGTAGTCTCTTCAGCCAAAATGGCTGAAGAGGTACTGAAAACCCATGATCTTGAATTTTGTAGCAGGCC
TCTCTTGACTGGCCAGCAAAAATTTTCCTACAATGGTTTAGACGTGGCCTTTTCACCATATGGTGCTTATTGGA
GGGAGATGAAAAAAATATGTGTCGTTCATCTCCTCAACTCGACACGAGTGCAAAGTTTTCGTACCAACAGAGAA
GATGAGGTATCGCATATGACTGAAAAAATTTCCAAAGCAGCTCTTGCTTCTAAACCCTTCAACTTGACTGAAGG
AATGCTGTCTCTTACAAGCACAGCCATATGCAGAACTGCCTTCGGAAAAAGGTACGAGGATGGAGGAATTGAAG
GAAGTAGGTTCCTTGCGTTGCTTAATGAAACCGAAGCCCTGTTTACGATGTTTTTTCTTTCTGATTATTTTCCA
TACATGGGATGGGTTGATAGACTCACGGGACGCGCCCATCGTCTTGAAAAAAATTTCAGAGAGTTTGATGTTTT
CTACCAACAAATTATTGATGAACATCTTGATCCAGAGCGACCAAAGCCAGACCATGAGGACATACTTGACGTTT
TGCTTCAAATATACAAGGATCGCACTTTTAAAGTTCAACTAACGCTTGATCACATCAAAGCAATTCTAATGAAC
ATATTTGTTGGTGGGACTGATACAGCTGCTGCTACTGTGATCTGGGCCATGAGCCTTCTAATGAAAAACCCCGA
AGCAATGAGAAAAGCTCAAGAAGAAGTTCGAAAGGTGATAGGAGATAAAGGTTTTGTGTACGAAGATGATGTTC
AACAATTGCCTTACCTTAAAGCTGTGGTTAAAGAGACCATGAGATTGCAACCAACAGCACCACTACTAGTCCCA
AGAGAAACAACTACGGAGTGTAACATAGGTGGGTACGAAATACCAGCCAAGACCTTAGTTTACGTCAATGCGTG
GGCTATTGGAAGGGACACAGAAGTTTGGGAGAACCCGTATGTGTTCATTCCTGATAGGTTCTTGGGCAGTTCTA
TTGATTTGAAAGGACAAGATTTTGAGCTGATACCATTTGGTGCGGGTCGAAGAATTTGTCCTGGTATATATATG
GGAATTGCCACCGTGGAGCTTTCACTTTCTAATCTTCTCTACAAATTCGACTGGGAAATGCCTGGTGGGATGAA
GAGGGAAGACATAGACGTTGATCATACGCAACCCGGTCTTGCTATGCATACGAGGGACGCTCTCTGCCTCGTGC
CTAAGGCGTATGCTGTGATGGGCAATGATGCGTAA
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(b)

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  1 MALLIFVILF LSIIFLFLLK KNKISKRACF PPGPNGLPLI GNLHQLDSSN
 51 LQTQLWKLSQ KYGPLMSLKL GFKRTLVVSS AKMAEEVLKT HDLEFCSRPL
101 LTGQQKFSYN GLDVAFSPYG AYWREMKKIC VVHLLNSTRV QSFRTNREDE
151 VSHMTEKISK AALASKPFNL TEGMLSLTST AICRTAFGKR YEDGGIEGSR
201 FLALLNETEA LFTMFFLSDY FPYMGWVDRL TGRAHRLEKN FREFDVFYQQ
251 IIDEHLDPER PKPDHEDILD VLLQIYKDRT FKVQLTLDHI KAILMNIFVG
301 GTDTAAATVI WAMSLLMKNP EAMRKAQEEV RKVIGDKGFV YEDDVQQLPY
351 LKAVVKETMR LQPTAPLLVP RETTTECNIG GYEIPAKTLV YVNAWAIGRD
401 TEVWENPYVF IPDRFLGSSI DLKGQDFELI PFGAGRRICP GIYMGIATVE
451 LSLSNLLYKF DWEMPGGMKR EDIDVDHTQP GLAMHTRDAL CLVPKAYAVM
501 GNDA*
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Figure 19. Nucleotide sequences (a) and amino acid sequence (b) of cytochrome P450 from Nisqually-1.

(a)

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ATGGCATTACTCATATTCGTAATTCTCTTCCTCTCCATCATTTTCTTGTTTCTTCTCAATAAAAACAAAATCTC
TAAAAGAGCTCGTTTCCCTCCTGGCCCCAACGGTCTTCCCTTGATAGGTAACTTGCACCAGCTTGATAGCTCAA
ACCTTCAAACGCATTTATGGAAACTATCTCAAAAATATGGCCCCCTCATGTCCTTAAAGCTAGGTTTCAAGCGA
ACCCTCGTAGTCTCTTCAGCCAAAATGGCTGAAGAGGTACTGAAAACCCATGATCTTGAATTTTGTAGCAGGCC
TCTCTTGACTGGCCAGCAAAAATTTTCCTACAATGGTTTAGACTTGGCCTTTTCACCATATGGTGCTTATTGGA
GGGAGATGAAAAAAATATGTGTCGTTCATCTCCTCAACTCGACACGAGTGCAAAGTTTTCGTACCAACAGAGAA
GATGAGGCATCGCATATGATTGAAAAAATTTCCAAAGCAGCTCTTGCTTCTAAACCCTTCAACTTGACTGAAGC
AATGCTGTCTCTTACAAGCACAGCCATATGCAGAACTGCCTTTGGAAAAAGGTACGAGGATGGAGGAATTGAAG
GAAGTAGGTTCCATGCGTTGCTTAAGGAAACCCAAGCCCTGTTTACGATGTTTTATCTTTCTGATTGTTTTCCA
TACATGGGATGGGTTGATAGACTCACGGGACTTGCCCATCGTCTTGAAAAAAATTTCAGAGAGTTTGATGTTTT
CTACCAAGAAATCATTGATGAACATCTTGATCCAGAGCGACCAAAGCCCGACCATGAGGACATACTTGACGTTT
TGCTTCAAATATACAAGGATCGCACTTTTAAAGTTCAACTAACGTTTGATCACATCAAAGCAATTCTAATGGAC
ATATTTGTTGCTGGGACTGATACAGCTGCTGCTACTGTGATTTGGACCATGAGCCTTCTAATGAAAAACCCTGA
AGCAATGAGAAAAGCTCAAGAAGAAGTTCGAAAGGTGATAGGAGATAAAGGTTTTGTGTTCGAAGATGATGTTA
CACAATTGCCCTACCTTAAAGCTGTGGTTAAAGAGACCATGAGATTGCAACCAACAGCACCACTACTACTCCCA
AGAGAAACAACGACGGAGTGTAACATAGGTGGGTACGAAATACCAGCCAAGACCTTAGTTTACGTGAATGCGTG
GGCTATTGGAAGGGACACAGAAGCTTGGGAGAACCCATATGTGTTCGATCCTGAAAGGTTCTTGGGCAGTTCTA
TTGATCTTAAAGGAAATGATTTTGAGCTCATACCGTTTGGTGCTGGTCGAAGAATTTGTCCAGGGATATTTATG
GGAATTGCCACCGTGGAGCTTTCACTTTCTAATCTTCTCTACAAATTCGACTGGGAAATGCCTGGTGGGATGAA
GAGGGAAGACATAGACATCGATCATACGCAACCCGGTCTTGCTATGCATACGAGGGACGCTCTCTGCCTCGTGC
CTAAGGCCTATGCTGTGATGGGCAATGATGCGTAA
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(b)

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  1 MALLIFVILF LSIIFLFLLN KNKISKRARF PPGPNGLPLI GNLHQLDSSN
 51 LQTHLWKLSQ KYGPLMSLKL GFKRTLVVSS AKMAEEVLKT HDLEFCSRPL
101 LTGQQKFSYN GLDLAFSPYG AYWREMKKIC VVHLLNSTRV QSFRTNREDE
151 ASHMIEKISK AALASKPFNL TEAMLSLTST AICRTAFGKR YEDGGIEGSR
201 FHALLKETQA LFTMFYLSDC FPYMGWVDRL TGLAHRLEKN FREFDVFYQE
251 IIDEHLDPER PKPDHEDILD VLLQIYKDRT FKVQLTFDHI KAILMDIFVA
301 GTDTAAATVI WTMSLLMKNP EAMRKAQEEV RKVIGDKGFV FEDDVTQLPY
351 LKAVVKETMR LQPTAPLLLP RETTTECNIG GYEIPAKTLV YVNAWAIGRD
401 TEAWENPYVF DPERFLGSSI DLKGNDFELI PFGAGRRICP GIFMGIATVE
451 LSLSNLLYKF DWEMPGGMKR EDIDIDHTQP GLAMHTRDAL CLVPKAYAVM
501 GNDA*
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Figure 20. Nucleotide sequences (a) and amino acid sequence (b) of cytochrome P450 from INRA.

(a)

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ATGGCAGAGGAAGTGAAGGTTTTTAGGTCATGGTCAAGTCCATTTCCTTTGAGAGTCATCTGGGCACTGAGATT
GAAGGGTGTTAAGTTTGATGTAATATGCGAAGATCTCTTCAACAAGAGCCCTTTGTTCTTGCAATATAATCCTG
TCGAAAAGAAGGTTCCTGTGCTTGTCCACAACGGTAAAGCCATCTGTGAATCGCTAGTCATTCTTGAATACATT
GAAGAGACATGGAAGCAAACTCCTTTGTTGCCTGAAGATCCTTACCAGAAAGCCAATGCTCGTTTCTGGGCCAA
ATTTAGTGATGACAAGGTCTTCCAGTCAATTAAGTGGGATGTGCTCTTGAAGGAGGGAAAAGAGCAAGAAGAAG
GGATACTTGCATCCTTGCAGAACTTGAGATATTTAGAAGAAGAGCTAAGAGGAAAGAAATTCTTCGGTGGGGAG
GCAATTGGACTAGCAGACCTTGCACTAGGATGGCTTGCTTATTACCTTAATATCTTTGAGGAGGTAGCAGGTCT
GAAACTGGTAGACCAGGAAAGTTTTCCATCCTTGGTGGCATGGATGCAAGAATTTGCAAATGCTCCAGTCGTCC
ATGGAAGCTGGCCCGATAGAGACAAGCTGGCTGAGAAGCTTGTTGCCATGCGCGAGGCCAGTCTTGGAAAAGAA
ACACCTAAATGA
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(b)

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  1 MAEEVKVFRS WSSPFPLRVI WALRLKGVKF DVICEDLFNK SPLFLQYNPV
 51 EKKVPVLVHN GKAICESLVI LEYIEETWKQ TPLLPEDPYQ KANARFWAKF
101 SDDKVFQSIK WDVLLKEGKE QEEGILASLQ NLRYLEEELR GKKFFGGEAI
151 GLADLALGWL AYYLNIFEEV AGLKLVDQES FPSLVAWMQE FANAPVVHGS
201 WPDRDKLAEK LVAMREASLG KETPK*
```

Figure 21. Nucleotide sequences (a) and amino acid sequence (b) of glutathione from INRA.

(a)

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ATGGCAGAGGAAGTGAAGGTTTTTAGGTCATGGTCAAGTCCATTTGCTCTGAGAGTCATCTGGGCACTGAAATT
GAAGGGTGTTGAGTTTGATACCATATATGAAGATCTCTCCAACAAGAGCCCTTTACTCTTGCAATACAATCCTA
TCCACAAGAAGGTTCCCGTGCTTGTCCACAATGGTAAAGTCATCTGTGAATCACTTGTCATTCTAGAATACATC
GACGAGACATGGAAGCAAAATCCTCTGTTGCCTGAAGATCCTCACCAGCAAGCCAATGCTCGTTTCTGGGCCAA
GTTTGGTGATGATAAGGTTTTGCAATCAATTGTGTGGGGTGTGCTTATGAAGGAGGGAAAAGAGCTAGAAGAAG
GGGTCCTTGCATCCTTGGAGAACTTGAAATATTTAGAAGAAGAGATAAGAGGAAAGAAATTCTTTGGTGGGGAG
ACTATTGGGCTAGCGGATATTGCATTAGGATGGCTTGCTTATTACCTTGATATCTTTGAGGAGATACTAGGTCT
AAAACTGATAGACCAGGAAAAGTTTCCATCCTTAGCGGCATGGAAGCAGGAATTTGCAAATGCTCCAATCATCC
ATGAGAACTGGCCGGATAGGGACAAGCTGGTTAACAAGTTTGTTGCCATGCGCGAGGCCAGTCTTGGAAAAGAA
ACACCTAAATGA
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(b)

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  1 MAEEVKVFRS WSSPFALRVI WALKLKGVEF DTIYEDLSNK SPLLLQYNPI
 51 HKKVPVLVHN GKVICESLVI LEYIDETWKQ NPLLPEDPHQ QANARFWAKF
101 GDDKVLQSIV WGVLMKEGKE LEEGVLASLE NLKYLEEEIR GKKFFGGETI
151 GLADIALGWL AYYLDIFEEI LGLKLIDQEK FPSLAAWKQE FANAPIIHEN
201 WPDRDKLVNK FVAMREASLG KETPK*
```

Figure 22. Nucleotide sequences (a) and amino acid sequence (b) of glutathione from Nisqually-1.

(a)

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ATGATCAAGCAACCTCACTTCCTTCTGGTGACCTATCCCGCACAAGGCCACATAAATCCTACCCTCCAATTCGC
CAAGGGTCTGACACGTATTGGTGTTCTCGTCACCCTTGTTACCTCTCTCTCTGCCGGCCGCCGCATGTCCAAAA
CTTTGTTTCCTGACGGTTTGTCATTTGTTACCTTTTCAGATGGATATGACGACGGGTTAAAAGCTGAAGATGAC
AAAGATCATTTCATGTCTGAGCTCAAACGTCGAGGCTCACAGACTCTCAATGAGCTTATCGTAGATAGTGCAAA
AGAAGGAAAGCCCGTCACTTGTTTGGTTTACACGATGCTTCTACACTGGGCAACCGAGGTGGCACGCGCACAGC
ATCTCCCAGCAGCACTTCTTTGGACCCAGCCTGCAACTGTCTTTGACATCTATTACTATTACTTCAATGGTTAT
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AGCTCAGCCAAGAAACCAACCCAAAAGTGCTTGTCAACTCTTTCGATGCATTGGAATTGGTAGCTATGAATGCC
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TAAATCCTTTGGAGGGGATCTTTTCCAAGGCTCCGAGGATTACACTGAATGGTTGAACTCAAAGACTAAATCAT
CAGTGGTTTATGTATCTTTTGGGAGCATTTTGGTGTTACCGAAGCGACAAATCAAGGAGATTGCTCGTGGATTG
GTAGATAGTGGTCTTCCATTCCTGTGGGTCATAAGGGATGAACAGAACAAGAACGAGGTGAAAGAAGAAGAAGA
GGAGGAGGACGACCATCTGAGTGCCTGCAGGGAGGCGATATTGGAAAGGCAAGGGATGATAGTGCCATGGTGCT
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AGCTTGGTCTCTGAGGTTCCAGTTGTGGCATTTCCTCACTGGACAGATCAAGGAACAAATGCCAAGCTGATTGC
AGACGTGTGGAAGACAGGAGTGAGGGTGGTGGCTAATGAAGAAGGGATAGTCGAAGGCGATGAGATTAAGAGGT
GCTTGGATCTGGTTATGGCACCTGGAAAGACGGGAGAAGACATCAGAAAGAACGCCAAGAAATGGAAAGATCTA
GCAAGAGATGCTGTCAGGGAAGGAGGTTCCTCGGATAAGAATCTAAAGGCTTTTGTGGATGAGGTTGGCAAGGG
CTGCTTCTAGGGTACCGCTGTA
```

(b)

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  1 MIKQPHFLLV TYPAQGHINP TLQFAKGLTR IGVLVTLVTS LSAGRRMSKT
 51 LFPDGLSFVT FSDGYDDGLK AEDDKDHFMS ELKRRGSQTL NELIVDSAKE
101 GKPVTCLVYT MLLHWATEVA RAQHLPAALL WTQPATVFDI YYYYFNGYGD
151 IFNNCKDTSY AIELPGLPPF ASRDLPSFVL PSNTYTFALQ LFQEQLEQLS
201 QETNPKVLVN SFDALELVAM NAIEKLNLVG IGPLIPSAFL DGKDPLDKSF
251 GGDLFQGSED YTEWLNSKTK SSVVYVSFGS ILVLPKRQIK EIARGLVDSG
301 LPFLWVIRDE QNKNEVKEEE EEEDDHLSAC REAILERQGM IVPWCSQVEV
351 LSHPSIGCFV THCGWNSTME SLVSEVPVVA FPHWTDQGTN AKLIADVWKT
401 GVRVVANEEG IVEGDEIKRC LDLVMAPGKT GEDIRKNAKK WKDLARDAVR
451 EGGSSDKNLK AFVDEVGKGC F*
```

Figure 23. Nucleotide sequences (a) and amino acid sequence (b) of glycosyltransferase from INRA.

(a)

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ATGATCAAGCAACCTCACTTCCTTCTGGTGACCTATCCCGCACAAGGCCACATAAATCCTGCCCTCCAATTCGC
CAAGGGTCTGACACGTATTGGTGCTCTCGTCACCCTTGTTACCTCTCTCTCTGCCGGCCGCCGCATGTCCAAAA
CTTTGTTTCCTGACGGTTTGTCATTCGTTACCTTTTCAGATGGATATGACGACGGGTTTAAACCTGAAGATGAC
AGAGATCATTTCACGTCTGAGCTCAAACGTCGAGGCTCACAGACTCTCAATGAGCTTATCGTAGATAGTGCAAA
AGAAGGAAAGCCCGTCACTTGTTTGGTTTACACGATGCTTCTACATTGGGCATCTGAGGTGGCACGCGCACAGC
ATCTCCCAGCAGCACTTCTTTGGATCCAGCCTGCAACTGTCTTTGACATCTATTACTATTACTTCAATGGTTAT
GGTGATATCTTCAATAATTGCAAGGACACTTCGTATGCTATTGAATTACCAGGGCTCCCACCACTTGCTAGCCG
TGACCTTCCCTCGTTTGTACTTCCTTCAAATACTTATACTTTTGCACTCCAGATGTTTCAAGAGCAGCTGGAGC
AGCTCAGCCAAGAAACCAACCCAAAAGTGCTTGTCAACTCTTTCGATGCATTGGAATTGGGAGCTATGAATGCC
ACTGAAAAGTTCAACTTGATTGGAATCGGGCCATTGATCCCATCTGCTTTCTTGGATGGAAAAgATCCGTTGGA
TAAATCCTTTGGAGGGGATATTTTCCACGGCTCCGAGGATTACACTGAATGGTTGAACTCAAAGACTAAATCAT
CAGTTGTTTATGTATCTTTTGGGAGCATTTTGGTGTTATCGAAGCGACAAATTGAGGAGATTGCTCGTGGATTG
GTAGATAGTGGTCTTACATTCCTGTGGGTCATAAGGGATGAACAGAAGAAGAACGAAGTGAAAGAAGAAGAAGA
AGAAGAAGAAGAAGAGGATCATCTGAGGGCCTGTAGAGAGGCGATATTGGAAAGGCAAGGGATGATAGTGCCAT
GGTGTTGTCAAGTGGGGGTTTTGTCCCATCCTTCGATAGGATGTTTTGTGACACACTGTGGGTGGAACTCAACT
CTGGAGAGCTTGGTTTGTGAGGTTCCAGTTGTGGCATTTCCTCACTGGACAGATCAAGGAACAAATGCCAAGCT
GATTGCAGACGTGTGGAAGACAGGAGTGAGGGTGGTGGCTAATGAAGAAGGGATAGTCGAAGGCGATGAGATTA
AGAGGTGCTTGGATCTGGTTATGGCACATGGAAAGACGGGAGAAGACATCAGAAAGAACGCCAAGAAATGGAAA
GATTTAGCAAGAGATGCTGTCAAGGAAGGAGGTTCCTCGGATAAGAATCTAAAGGCTTTTGTGGATGAGGTTGG
CAAGGGCTGCTTCTAGGGTACCGCTGTA
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(b)

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  1 MIKQPHFLLV TYPAQGHINP ALQFAKGLTR IGALVTLVTS LSAGRRMSKT
 51 LFPDGLSFVT FSDGYDDGFK PEDDRDHFTS ELKRRGSQTL NELIVDSAKE
101 GKPVTCLVYT MLLHWASEVA RAQHLPAALL WIQPATVFDI YYYYFNGYGD
151 IFNNCKDTSY AIELPGLPPL ASRDLPSFVL PSNTYTFALQ MFQEQLEQLS
201 QETNPKVLVN SFDALELGAM NATEKFNLIG IGPLIPSAFL DGKDPLDKSF
251 GGDIFHGSED YTEWLNSKTK SSVVYVSFGS ILVLSKRQIE EIARGLVDSG
301 LTFLWVIRDE QKKNEVKEEE EEEEEEDHLR ACREAILERQ GMIVPWCCQV
351 GVLSHPSIGC FVTHCGWNST LESLVCEVPV VAFPHWTDQG TNAKLIADVW
401 KTGVRVVANE EGIVEGDEIK RCLDLVMAHG KTGEDIRKNA KKWKDLARDA
451 VKEGGSSDKN LKAFVDEVGK GCF*
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Figure 24. Nucleotide sequences (a) and amino acid sequence (b) of glycosyltransferase from Nisqually-1.

CHAPTER 3

Biodegradation of Trichloroethylene (TCE) by an Endophyte of Hybrid Poplar

3.1 Introduction

As discussed in Chapter 2, many studies have revealed that biological degradation of chlorinated compounds generally occurs by two different metabolic processes, anaerobically and aerobically (Lee*, et al.*, 1998, Chomsurin*, et al.*, 2008).

Under anaerobic conditions, the bioremediation of chlorinated solvents involves a reductive dechlorination process in which chlorinated ethenes are used as electron acceptors. This has been used as the most common method in bioremediation. TCE degradation generates products such as dichloroethane (cis-DCE and trans-DCE), vinyl chloride (VC), and ethane from microbial reductive dechlorination (Figure 25). Yet these chemicals are more toxic to the environment than TCE and can be accumulated in the environment (Middeldorp*, et al.*, 1999, Kao*, et al.*, 2003, Griffin*, et al.*, 2004, Zhang & Bennett, 2005).

Bacteria of the genus *Dehalococcoides* have been shown to completely dechlorinate TCE to harmless byproducts under anaerobic conditions (Löffler*, et al.*, 2003). However, members of this genus are strict anaerobes, slow-growing and pH-sensitive, thereby limiting their application at contaminated sites. Moreover, not all "*Dehalococcoides*" spp. contain the full set of required genes for complete reduction to non-toxic ethene (Ernst, 2009) and sometimes the presence of co-contaminants such as chloroform (Duhamel*, et al.*, 2002) and chlorinated ethanes (Grostern & Edwards, 2006) may inhibit some dehalogenating cultures.

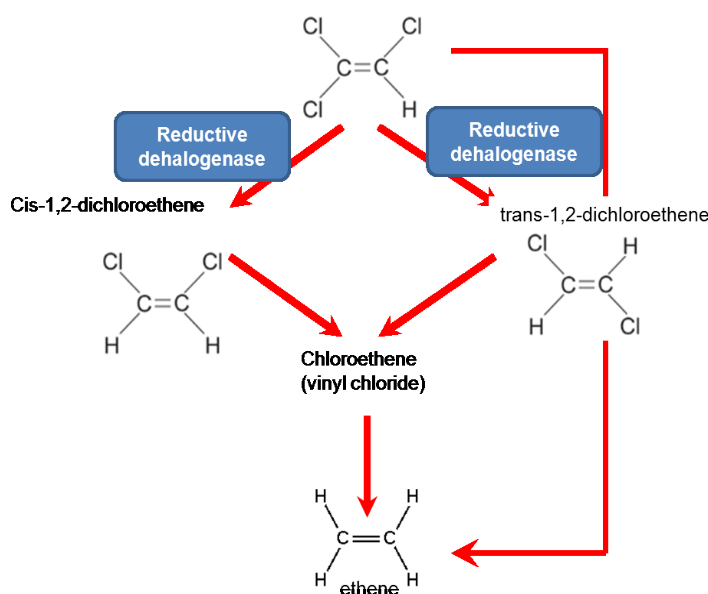

Figure 25. Reductive dechlorination pathway under anaerobic conditions (Zhang & Bennett, 2005).

Although reductive dechorination by anaerobes is the most commonly used method in bioremediation, aerobic microbes have also been utilized. Several classes of aerobic microorganisms express broad-specificity catabolic enzymes that may degrade chlorinated solvents without any direct linkage to their carbon and energy metabolism. This process is called cometabolism (Wackett et al., 1995). *Methanotrophs* (methane oxidizers) and *Pseudomonads* (phenol and toluene oxidizers) are known to perform such a process (Semprini, 1997). *Methanotrophs*, a group of gram-negative bacteria which use methane as a sole source of carbon and energy, have two types of oxygenases including soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO). Both forms of the MMO play a significant role in oxidizing methane (Oremland & Culbertson, 1992) and can also be used for remediating contaminated sites with trichloroethylene (TCE), congeners of dichloroethylene (DCE), and polychlorinated biphenyls, via cometabolism (Lontoh &

Semrau, 1998, Morton*, et al.*, 2000) (Figure 26).

Members of the genus *Pseudomonas* are one of the most well-studied aromatic hydrocarbon degrading bacteria which have toluene oxygenase. *Burkholderia* (*Pseudomonas*) *cepacia* strain G4 has a toluene *ortho* monooxygenase (TOM) and is also the best trichloroethene (TCE) co-oxidizing strain yet discovered (Fries*, et al.*, 1997) (Figure 27 A). After Nelson et al. first reported this bacteria (Nelson*, et al.*, 1986), many researchers have conducted studies of it (Folsom*, et al.*, 1990, Krumme*, et al.*, 1993, Landa*, et al.*, 1994, Yeager*, et al.*, 2001). *Pseudomonas putida* F1 also has the ability to degrade benzene, toluene, and ethylbenzene, but it has a toluene dioxygenase (Zylstra*, et al.*, 1988, Zylstra & Gibson, 1989) (Figure 27B). Recently, Ryoo et al. (2000) reported *Pseudomonas stutzeri* OX1 which degrades tetrachloroethylene with toluene–o-xylene monooxygenase (ToMO). All three toluene oxygenases described above oxidize TCE, DCEs, and VC primarily into CO_2 and Cl (Shields and Francesconi, 1996; Shields et al., 1994).

Using these microbes for degradation of TCE in bioremediation studies has been attempted where the microbes utilize methane, phenol or toluene as growth substrates and produce enzymes that can degrade TCE as well as its degradation products (Chang & Alvarez-Cohen, 1995). Since there is no gain of energy from this process, field application of co-metabolism for TCE degradation can be expensive. Another limitation is that the main substrates such as phenol or toluene are toxic and introducing them into the soil can result in secondary contamination problems.

Therefore, researchers have tried to engineer microbes to degrade TCE constitutively without addition of co-substrates like toluene and phenol. Shields and Reagin developed a mutant strain, *Burkholderia* (*Pseudomonas*) *cepacia* G4 5223 PR1 that constitutively

produces the toluene ortho-monooxygenase responsible for degradation of TCE without addition of co-substrates such as toluene and phenol. Martin et al. used the enzyme complex of *Escherichia coli* starvation promoters to control toluene monooxygenase synthesis, which could degrade trichloroethylene (TCE) and phenol (Shields & Reagin, 1992, Matin*, et al.*, 1995, Pflugmacher*, et al.*, 1996). Engineered bacteria, however, may have limitations to field applications since it requires approval under Toxic Substance Control Act (TSCA) regulations before environmental release (Ezezika & Singer, 2010).

More recently, several studies have tried to find natural substitutes for toxic substrates as the inducers of cometabolic degradation of TCE under aerobic conditions, and phytochemicals have been attracting much interest as natural alternatives to synthetic compounds (Wu-Yuan*, et al.*, 1988, Sato*, et al.*, 1996). Brown et al. (2009) reported plant terpenoids can be used to enhance biotransformation of TCE. In another study, Suttinun et al (2000) reported that plant essential oils and their components such as cumene, limonene, carvone, and pinene can be used as alternative inducers for TCE cometabolic degradation in a toluene-degrading bacterium, *Rhodococcus* sp. L4 (Suttinun*, et al.*, 2009, Suttinun*, et al.*, 2010). Also, Dey and Roy reported that *Bacillus sp*. 2479 isolated from a hazardous waste site has the ability to degrade TCE in the absence organic compounds (Dey & Roy, 2009), but the degradation of TCE is not well documented.

The poplar homogenate containing natural phenolic compounds was tested for the ability to induce growth and TCE degradation of *Burkholderia cepacia* strain G4. Also, the ability of plant homogenate as an inducer of the toluene-ortho-monooxygeanse (TOM) gene was evaluated with real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

In the second study, endophytes isolated from poplar trees were tested for the ability to grow with TCE as an energy source and aerobically metabolize TCE to chloride without the addition of inducing aromatic compounds.

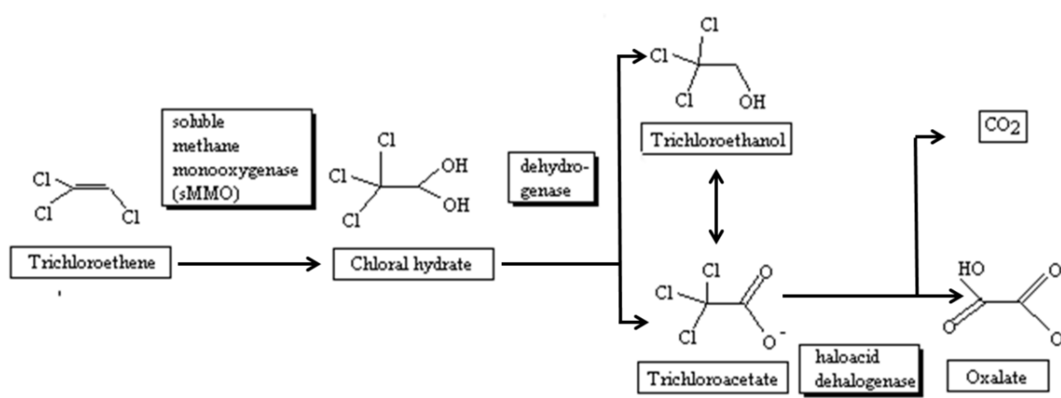

Figure 26. TCE degradation process by soluble methane monooxygenase (sMMO) of *Methanotrophs* (Figures from University of Minnesota, http://umbbd.msi.umn.edu/tce/tce_image_map.html)

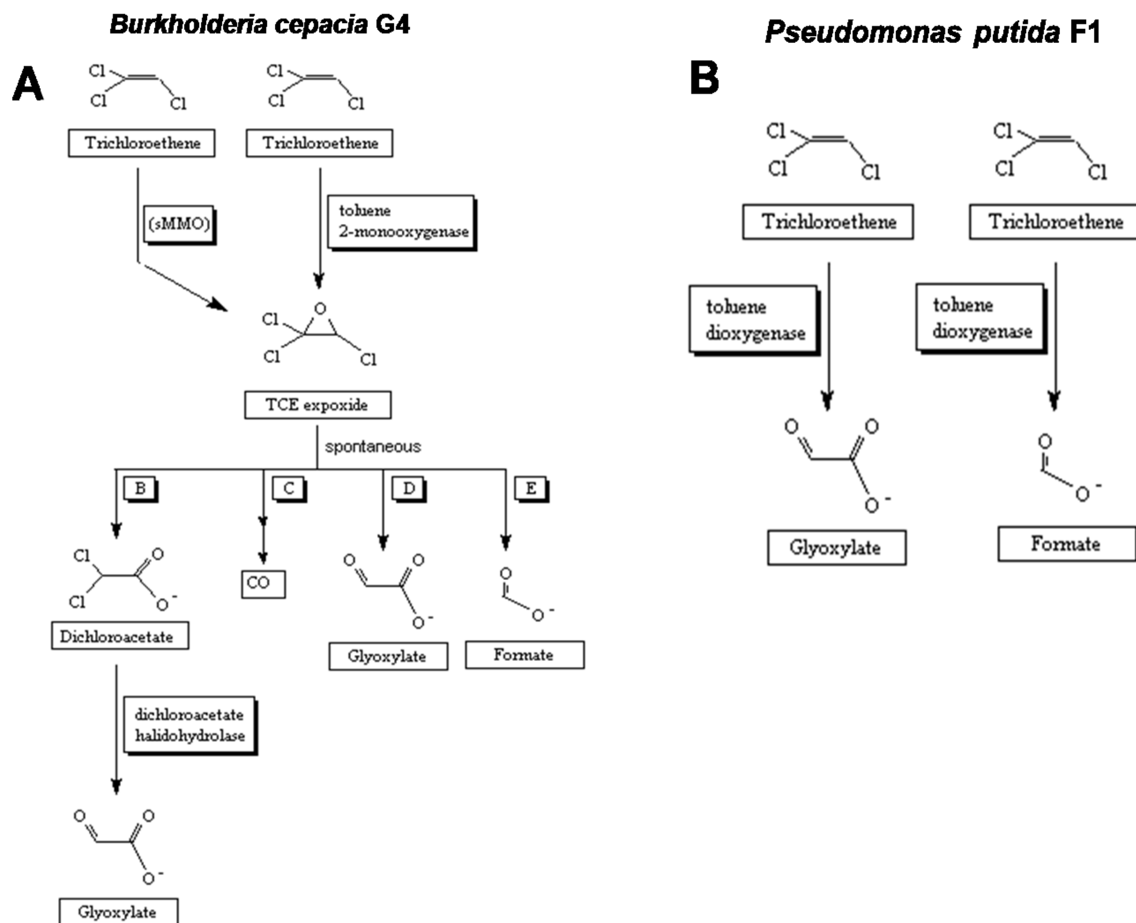

Figure 27. TCE- degradation by toluene-*ortho*- monoxygenase of *Burkholderia* (*Pseudomonas*) *cepacia* strain G4 (A) and toluene dioxygenase of *Pseudomonas putida* F1 (B) (Figures from University of Minnesota, http://umbbd.msi.umn.edu/tce/tce_image_map.html)

3.2 Research objectives

The specific hypotheses were:

- Plant homogenate can induce toluene-ortho-monooxygeanse (TOM) gene of *Burkholderia cepacia* strain G4 to degrade trichloroethylene (TCE)
- Certain endophytes in poplar and willow can degrade TCE

Aim 1) Test the resistance of *Burkholderia cepacia* strain G4 from phytochemicals of poplar homogenate

Aim 2) Test the ability of plant homogenate to act as the inducer of the toluene-ortho-monooxygeanse (TOM) gene of *Burkholderia cepacia* strain G4

A**im 3)** Isolate a naturally occurring endophyte that can degrade TCE

Aim 4) Identify and characterize the isolate by genetic approaches

3.3 Materials and Methods

3.3.1 Plant homogenate

Five grams of fresh poplar leaves with 30ml water were ground with a mortar and pestle, and passed through a 150µm screen sieve, then sterilized by filtration with a 0.2-µm syringe filter (Figure 28).

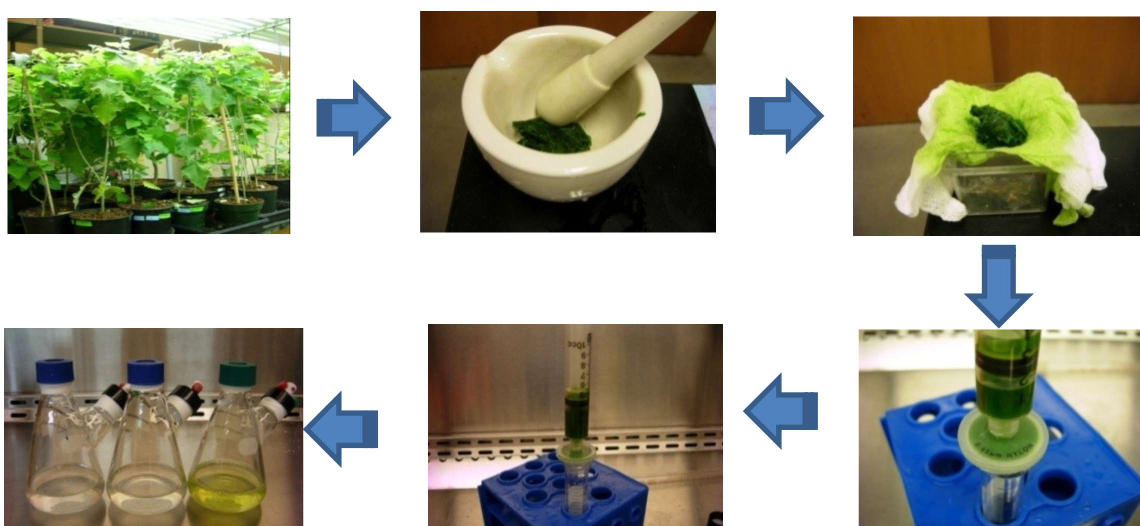

Figure 28. Process of making plant homogenate.

3.3.2 Growth experiments of G4

G4 was grown in 25ml of M9 minimal media without glucose, containing 0, 250 µl, 500 µl, and 1000 µl of plant homogenate. Cultures were grown in 125ml flasks and shaken for 2 days at 200rpm at 30°C. Growth was monitored every 24 hours using a Biochrom WPA Biowave spectrophotometer by measuring the optical density at 600 nm. Five µl of toluene was added as a positive control and G4 without toluene and homogenate was used as negative control.

3.3.3 TCE degradation of G4

G4 was grown overnight in LB media, harvested and washed three times with DI water and then adjusted to an OD 600 nm of 0.1 using a spectrophotometer in M9 minimal media without glucose. 500µl plant homogenate and 30 µl of toluene were added to 15ml media. The 25ml serum bottles were sealed immediately with aluminum crimp caps with Teflon-lined septa. Each three bottles, containing TCE with toluene and TCE with plant homogenate and controls containing TCE only were prepared for each sampling time period at every four hours. Samples were shaken at 150 rpm at 30°C for 5 days.

3.3.4 RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was prepared by using an RNeasy Protect Bacteria Mini kit (Qiagen) according to the instructions of the manufacturer. Once the sort was complete, samples were stored at −80°C for further processing. The quality of each RNA sample was assessed using either the RNA 6000 Pico or Nano LabChip Kit with a 2100 Bioanalyzer (Agilent Technologies). Approximately 20 ng total RNA from each sample was used for labeling and hybridization. Approximately 2 µg of total RNA was reverse transcribed into cDNA using the BioRad iScript kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Primers to each of the upregulated genes were designed (Table 1). Selected putative genes were compared to an internal reference gene (18S rRNA) that served to normalize expression levels and to give unit-less, realistic values of candidate gene expression. Probes with a fluorescent tag (Taqman) specifically designed to complement an internal region of the 16S rRNA and the genes of interest were used to quantify gene expression.

3.3.5 Endophyte isolation and enrichment

Dormant cuttings of several varieties of young poplars were collected from TCE contaminated sites in Illinois (kindly provided by Dr. Jud Isebrands (USDA, IL, USA)) and Washington State. To isolate endophytes, the cuttings (approx. 8 cm) were surface-sterilized with 0.3 % sodium hypochlorite for 10 minutes and then 1 % iodophor for 5 minutes and finally rinsed three times with sterile water. Surface-sterile cuttings were chopped aseptically into 0.5 cm pieces and suspended in 30ml of sterile distilled water and incubated at 30°C for 24h with shaking (Figure 29). To enrich for potential TCE degraders, one milliliter of this sample was added into 25 ml of synthetic medium containing (g/l): Peptone, 5; Beef extract, 3; Sodium chloride, 5; pH 6.9 (Dey & Roy, 2009). Cultures that grew well were enriched by transfer to fresh medium containing 730µg/ml of TCE. Endophytes were isolated from the samples growing in TCE using the dilution plate technique. Pure colonies were obtained by streak purification and frozen in 33 % glycerol and stored at -80°C until further studies.

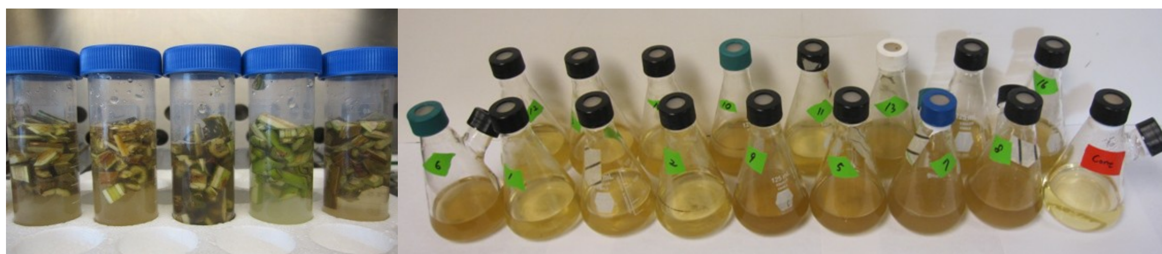

Figure 29. Isolation of endophytes from poplar.

3.3.6 Fujiwara assay for TCE degradeation products

The Fujiwara test is an analytical colorimetric method that detects the presence of certain trichloro-compounds (Leibman & Hindman, 1964, Moss & Rylance, 1966, Reith*, et al.*, 1974) and it was used to screen all the endophytes for TCE degradation potential. Briefly, to 2ml of the test sample grown 7 days in M9 medium containing (g/l) Na_2HPO_4, 6; NaCl, 0.5; KH_2PO_4, 3; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.5; Peptone. 2; pH 7.2, 2ml of pyridine and 4ml of 10M KOH was added and mixed well. The sample was placed in a boiling water bath for 3 minutes and transferred immediately into an ice bath. A positive reaction was indicated by a deep red color.

3.3.7 Growth experiments

The isolate that gave a positive reaction in the Fujiwara test was grown in M9 minimal media with 0.2% peptone, containing 730µg/ml of TCE. Cultures were grown in 125ml flasks and shaken for 3 days at 200 rpm at 30°C. Growth was monitored every 24hours using a Biochrom WPA Biowave spectrophotometer by measuring the optical density at 600 nm. *Burkholderia cepacia* G4 (Landa*, et al.*, 1994) was included as a positive control and *Agrobacterium tumefaciens* C58 (Goodner*, et al.*, 2001) and *Escherichia coli* DH5α (Dower*, et al.*, 1988) were used as negative controls.

3.3.8 TCE degradation, chloride, and nitrite production

PDN3 was grown overnight in M9 medium with 0.2% peptone, harvested and washed three times with chloride-free medium (5ml of 1M $Ca(NO_3)_2$; 5ml of 1M KNO_3; 2ml of 1M $MgSO_4$ $7H_2O$; 1ml 1M $(NH_4)H_2PO_4$; 200ul 1M Na_2NO_3; 20ml 1.65g of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$; 1.4g of boric acid in 1L DI water) and then adjusted to an OD 600 of 1.0 using a spectrophotometer. For the TCE degradation and chloride production studies, we used chloride-free medium. Trichloroethylene was then added at a concentration of 10µg/ml to 125ml Erlenmeyer flasks with screw caps with Teflon mininert valves to prevent evaporation of TCE. Five replicates, both with and without PDN3, were shaken at 150 rpm at 30°C for 5 days. At 24hr intervals, 2ml aliquots were removed from each flask for measuring TCE removal and chloride production. For TCE quantification, 1ml sample was extracted into hexane and sodium chloride (Doty*, et al.*, 2007) and analyzed by the Clarus 500 gas chromatography equipped with an electron capture detector (GC-ECD) and a PTE-5 (5% phenyl methyl siloxane) fused-silica capillary column (30 m × 0.32 mm ID; thickness, 0.32 µm). The gas chromatography conditions were as follows: injector temperature 150°C, detector temperature 250°C, initial column temperature 40°C (1.80 min) then, programmed at 40–55°C at a rate of 45°C min^{-1}, and 55–135°C at a rate of 10°C min^{-1}. The carrier gas (hydrogen) flow rate was 14 ml min^{-1}. Trichloroethylene concentration was quantified by using standards in hexane.

For chloride and nitrite analysis, a 1ml of the aliquot was injected into an ion chromatograph (Dionex, model DX-120) equipped with a *Dionex AS40* Auto-sampler with a Dionex Ionpac AG14/AS14 (250 mm × 4.0 mm) column. Chloride concentration was

determined by external standards.

3.3.9 16S rDNA and rpoB gene sequence analysis

Genomic DNA was prepared from PDN3, and PCR was performed using the universal 16S rRNA primers 8F and 1492R, as described previously (Doty, 2005, Doty, 2009). RNA polymerase beta-subunit encoding gene (rpoB) primers were CM_7 and CM31b (Mollet*, et al.*, 1997). The 1.5 Kb of 16s rRNA and 1.0Kb of rpoB PCR products were purified using a gel extraction kit (Qiagen) and subcloned into pGEM T Easy (Promega). The inserted genes were sequenced using the T7 and SP6 primer sites on the vector by the University of Washington Biochemistry Department Sequencing Facility using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI3730 XL sequencer (Applied Biosystems). DNA sequences were assembled using the Seqman software (DNA STAR Inc.) and analyzed using BLAST. Sequence comparisons with public databases were performed via the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/), by employing the BLASTN algorithm. Phylogenetic analysis was done using CLUSTAL W software. Evolutionary distance matrix were constructed using the algorithm of Jukes and Cantor (Thompson*, et al.*, 1994) and the evolutionary trees for the data sets were inferred from the neighbor joining method (Saitou & Nei, 1987) by using MEGA version 4.0.1 (Tamura*, et al.*, 2007).

3.3.10 Functional gene PCR amplification.

PCR primers were used to test for the presence of the oxygenase genes associated with methane-oxidizing bacteria. Primers that targeted particulate methane monooxygenase (pMMO) and a soluble methane monooxygenase (sMMO) gene specifically were used (Table

1). The amplification of pmoA (encoding a subunit of pMMO) genes was performed via a semi-nested PCR approach using the 5′ primer A189 and the 3′ primer A682 (Holmes*, et al.*, 1995). The aliquots of the first round of PCR (2.25μ l) were used as the template in the second round of PCR using the primer pairs A189f/mb601r and A189f/mb661R. The primer set of mmoxA/mmoxB was used for amplifying mmoX gene that encodes for sMMO. All PCR products were verified on 1% agarose gels stained with SYBR and visualized by using UV light. Images were captured and recorded by using Kodak software. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa) with standard desalting. The cloning and sequencing were carried out as described previously.

3.3.11 **Colorimetric assay to detect mononoxygenase activity**

The activity of oxygenase enzymes was evaluated by naphthalene assays modified from the method described by Brusseau et al (Brusseau*, et al.*, 1990). and Wachett et al. (1983). The assay is based on the assumption that an oxygenase can oxidize naphthalene to 1- or 2-naphthol which is measured by reaction with tetrazotized o-dianisidine to form a purple naphthol diazo complex. The intensity of the naphthol diazo complex is measured at A530. Briefly, to 1 ml of cell suspension (OD_{600}= 0.2) 1 ml of naphthalene stock solution (234 µM) was added into a 3-ml vial sealed with 13-mm Teflon-lined rubber septa (Alltech Co.) and the mixture was incubated at 30°C on a shaker at 150 rpm for 60 min before addition of 100 µl of freshly made 0.2% (wt/vol) tetrazotized o-dianisidine. The A_{530} of naphthol diazo dye was measured by a Biochrom WPA Biowave spectrophotometer within 2 min, since the absorbance starts to increase after 2 min. All samples were measured in triplicate.

3.3.12 **IAA production of PDN3**

PDN3 endophytic strain was analyzed for indole-3-acetic acid (IAA) production. For rapid quantitative estimation in broth culture, the colorimetric method of Gordon and Weber (1951) was used. The cultures were grown in the dark for 7 days, sampled daily, centrifuged at 13000 X g for 10 min, and the production of IAA was assayed in duplicated supernatant samples. The presence of IAA in each supernatant was measured colorimetrically by adding two parts of 0.01 M $FeCl_3$ in 35% $HClO_4$ to one part of supernatant followed by reading the optical density at 530 nm after 25 min. The recorded absorbances were read off a standard curve prepared from pure IAA (Sigma-Aldrich, St.Louis, MO).

3.4 Results

3.4.1 Growth experiments of G4 with plant homogenate

The experiment tested whether *Burkholderia cepacia* strain G4 could grow in plant homogenate since plant – derived compounds (phytochemicals) inhibit the growth of some bacteria (Jayaraman*, et al.*, 2010). As shown in Figure 30, it was found that higher concentrations of plant homogenate caused increased growth of *Burkholderia cepacia* strain G4. This shows that *Burkholderia cepacia* strain G4 can use plant homogenate as an energy source and it has tolerance to phytochemicals.

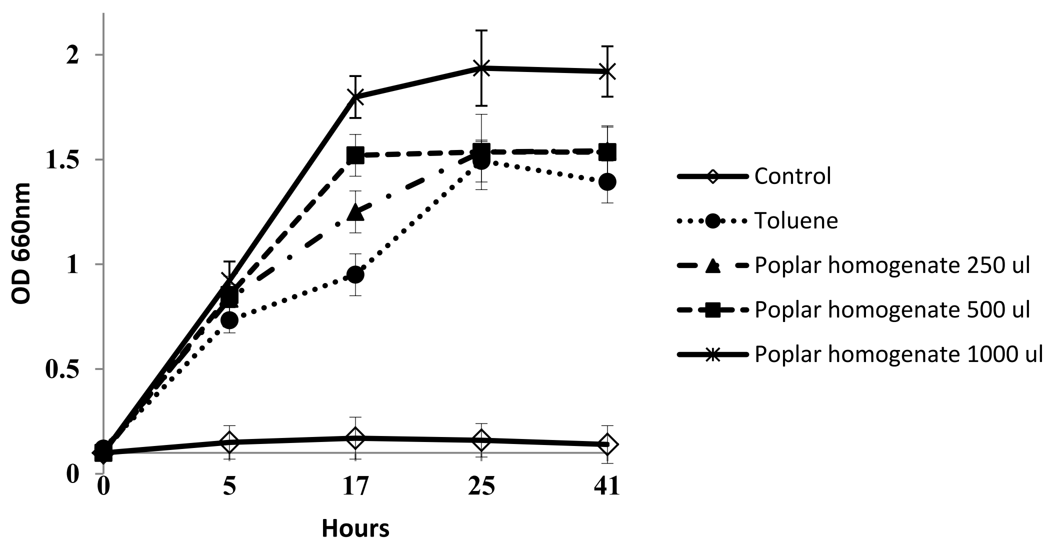

Figure 30. Growth curves of *Burkholderia cepacia* strain G4 in presence of poplar homogenate.

3.4.2 TCE degradation of G4 with plant homogenate

The ability of *Burkholderia cepacia* G4 to degrade TCE with plant homogenate as an inducer of toluene-ortho-monooxygeanse (TOM) gene was tested. First, in order to find the optimum concentration of plant homogenate for TCE degradation, the TCE degradation with different plant homogenate concentrations was monitored. As shown in the Figure 31, almost 80% of TCE was degraded by *Burkholderia cepacia* strain G4 with 500µl of plant homogenate in a week, indicating that this level of homogenate was as good as toluene as the inducer of TCE degradation.

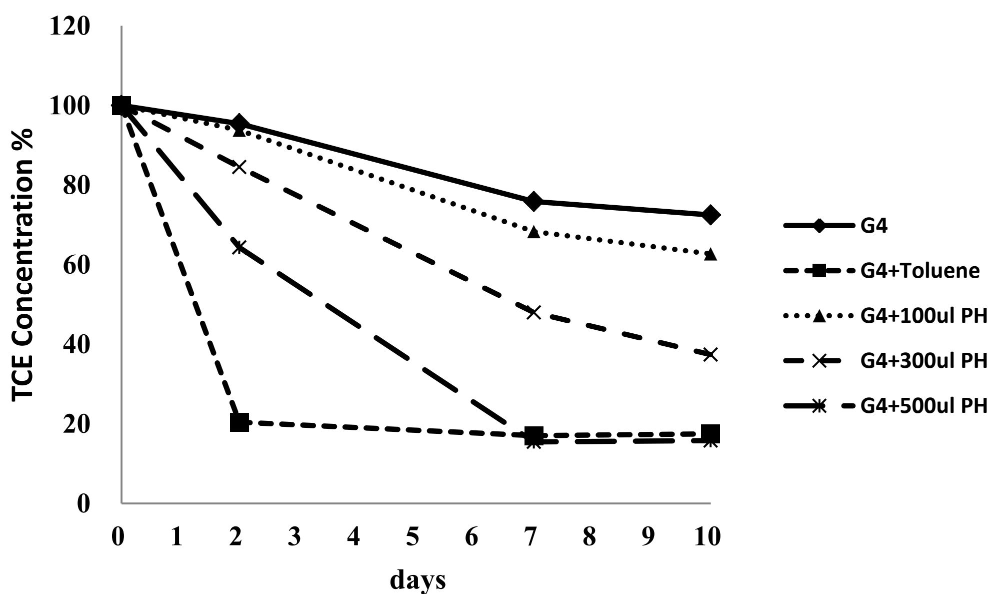

Figure 31. TCE removal by *Burkholderia cepacia* G4 with different concentration of poplar homogenate

As shown in the Figure 32, the gas phase of TCE concentration was increased from the beginning of time for TCE saturation. The level of TCE concentration was rapidly degraded after four hours in both samples each added with toluene and poplar homogenate and the ratio of TCE degradation was almost same in both samples. The degradation of TCE in *Burkholderia cepacia* strain G4 with toluene was declined after 20 hours and almost 40% of TCE was degraded. However, *Burkholderia cepacia* strain G4 with 500µl of plant homogenate was consistently degraded and 80% of TCE was removed.

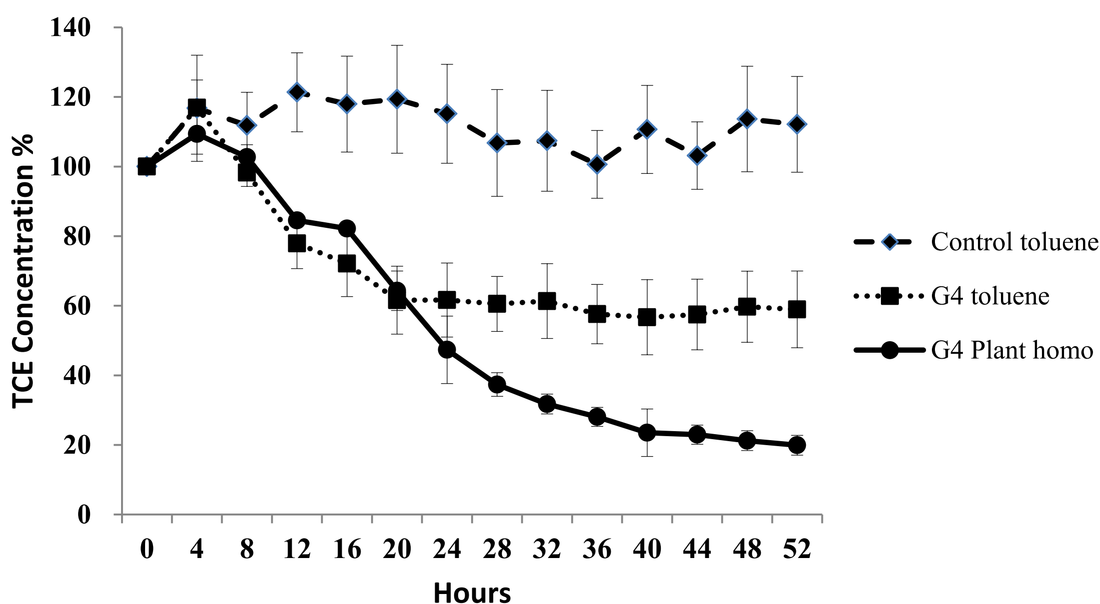

Figure 32. Time course of TCE degradation by *Burkholderia cepacia* G4 with plant homogenate compared to with toluene.

3.4.3 RNA isolation and reverse transcription-polymerase chain reaction

To further support the TCE degradation of *Burkholderia cepacia* G4 with plant homogenate, and to confirm the expression of the toluene-ortho-monooxygeanse (TOM) gene, RT-PCR and qRT-PCR was carried out. The results of the RT-PCR and qRT-PCR analysis are presented in Figures 33 and 34. Assuming equal annealing and amplification

properties of the PCR primers (Table 3), the relative amounts of individual mRNAs were normalized to the expression of 16S rRNA. The levels of expression of targeted TOM genes with plant homogenate were highly expressed compared to others and these differences were statistically significant.

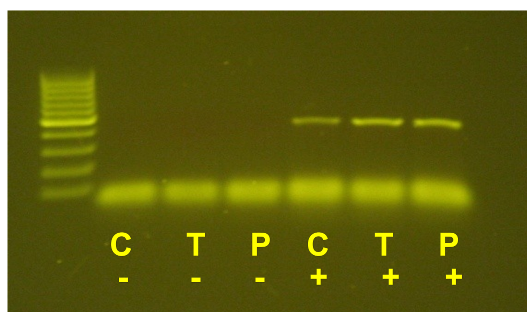

Figure 33. RT-PCR analysis TOM gene expression of *Burkholderia cepacia* G4. (-) negative control, (+) positive control, (C) TCE, (T) TCE+toluene, (P) TCE+plant homogenate

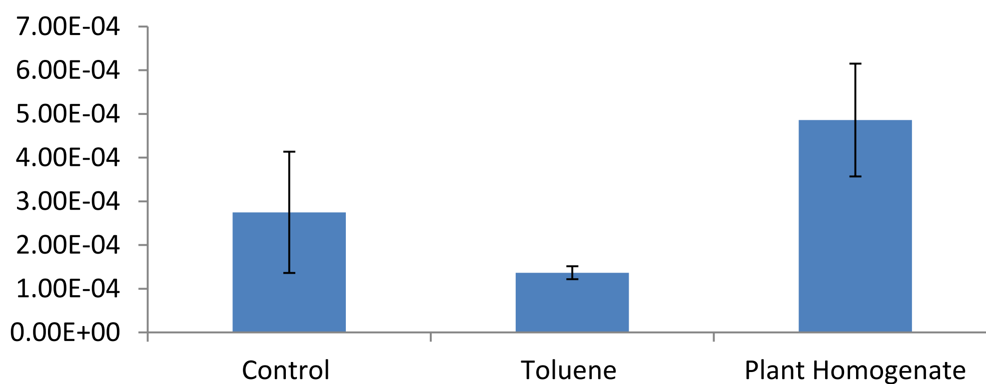

Figure 34. qRT-PCR analysis of TOM gene expression of *Burkholderia cepacia* G4. Values represent the mean

Table 3. Primer pairs and fluorescently labeled probes for TOM gene analysis

Gene	Type of oligo	Sequence (5'-3')
Burkholderia cepacia		
TOMA5		
	Primer (forward)	AAGTTCATTTCCGCAGCC
	Primer (reverse)	AGTAGTCACGATCACACCC
	Probe	6FAM-5'-AAAGCGGACTGCGTACTTGTTGG-3'-TAMRA
16S rRNA		
	Primer (forward)	CGGAAAGAAAACCTTGGCTC
	Primer (reverse)	GTTAGCCGGTGCTTATTCTTC
	Probe	6FAM-5'- TACCGTCATCCCCCGGCTGTATTA -3'-TAMRA

3.4.4 Identification of TCE-degrading endophytes

Out of the several hundreds of endophytes that were isolated from the plant samples, very few were resistant to high levels of TCE. These TCE-resistant strains were further screened for TCE degradation activity by the Fujiwara test. As seen in Figure 35, one culture, PDN, from hybrid poplar DN177 (*Populus deltoides* × *P. nigra*) demonstrated TCE degradation since it changed the color of the aqueous phase to pale red. When this culture was plated using the dilution plate technique, it was found to be a mixed consortium which was further purified into four individual colonies-PDN1, PDN2, PDN3 and PDN4. To test if the PDN isolates actually degraded TCE, the four isolates were grown in M9 with peptone (0.2%) containing 10 µg/ml of TCE and the removal was monitored using a gas chromatograph. As seen in Figure 36, one isolate-PDN3-removed all of the TCE from the media within 7 days.

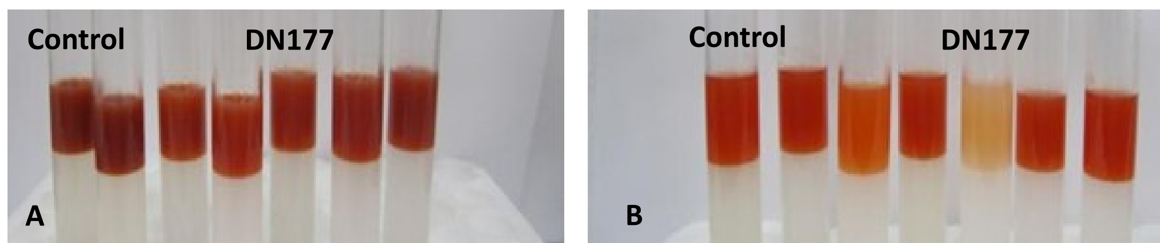

Figure 35. Fujiwara test (A) 0 day. (B) after 7 days. The cultures were grown in M9 minimal media containing 10 μ g/ml TCE with 0.2% peptone at 150 rpm at 30 °C. Note the reduction in the deep red color by an endophytic isolate from poplar clone DN177.

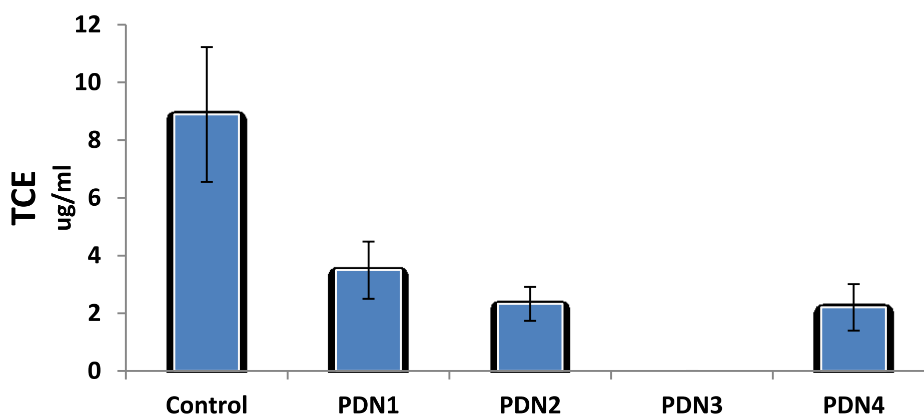

Figure 36. The concentration of TCE in cultures after 7 days TCE exposure. The cultures were grown in M9 minimal media with 0.2% peptone containing 10 μ g/ml of TCE. The experiments were performed in triplicate and the bars indicate standard deviations.

3.4.5 TCE degradation, chloride, and nitrite production

To confirm PDN3 degradation activity, the chloride release in the media coincident with TCE removal was monitored. As seen in the Figure 37, nearly 80% of TCE was degraded by PDN3 in 5 days with an increase in chloride ion concentration. On the first day,

full conversion of TCE to chloride ion was achieved, releasing 127.7 μM of chloride ions from 42.3 μM of TCE, indicating that the strain PDN3 completely dechlorinated TCE. The TCE removal was highest in the first 48 hours before leveling out. Neither chloride release nor TCE removal was observed in samples without any PDN3. In a majority of the bioremediation systems, TCE degradation stops at dichloroethenes (DCEs) or vinyl chloride (VC) (Hwu & Lu, 2006). Unlike these systems, PDN3 fully dechlorinated TCE. Several studies on the degradation of TCE by various oxygenases have shown that TCE is initially converted to TCE epoxide or chloral, and the intermediate formed is enzyme-dependent (Newman & Wackett, 1997, Ishida & Nakamura, 2000). To identify any metabolites of TCE in PDN3, GC-MS and GC-TOF was performed to analyze headspace samples and hexane extracts from liquid samples of cells. However, these known intermediates of TCE degradation were not detected by GC-MS and GC-TOF.

Another interesting result was the short term production of nitrite by PDN3 in response to TCE addition (Figure 38), which could be an oxidation product of ammonia, thereby indicating the presence of ammonia oxidizing genes or some unknown mechanism that was induced upon addition of TCE.

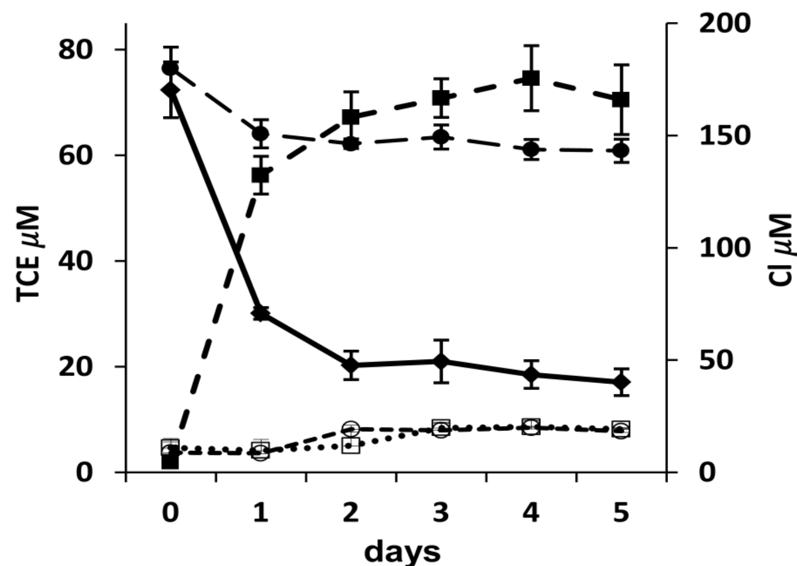

Figure 37. Time course of TCE degradation and chloride production by PDN3 in chloride free medium. Trichloroethylene was added at a concentration of 76.3 µM to 20ml chloride free medium in 125 ml Erlenmeyer Flasks with screw caps with Teflon mininert valves to prevent evaporation of TCE. The cultures were incubated at 150 rpm at 30 °C. The closed diamonds indicate TCE removal by strain PDN3, and closed circles indicate TCE in the uninoculated control, closed squares indicate chloride production from TCE by PDN3, open circles indicate chloride in the uninoculated control with TCE and open squares indicate chloride in the uninoculated control without TCE.

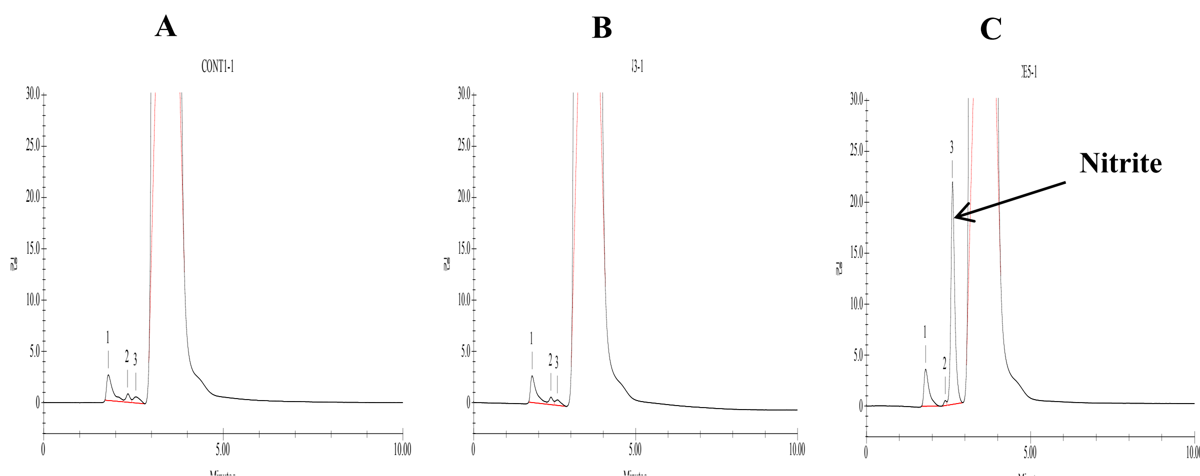

Figure 38. Chromatograms of nitrite production in cultures from (A) Blank with TCE, (B) PDN3, (C) PDN3 with TCE. The cultures were grown in chloride free medium and incubated at 150 rpm at 30 °C.

3.4.6 Growth measurements

The first selection screen used in this study was for tolerance to high levels of TCE. To compare the performance of PDN3 with other strains, growth curves were performed using PDN3, *Burkholderia cepacia* strain G4, a known TCE-degrading bacteria, *Escherichia coli* DH5α and *Agrobacterium tumefaciens* strain C58 as negative controls. As seen in Figure 39, PDN3 grew better than the other cultures in the presence of 5.5mM of TCE. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* strain C58 did grow, but were slightly inhibited in the presence of TCE (Figure 39A), and all of the cultures grew well in the absence of TCE, reaching saturation within 24 hours in M9 minimal media with 0.2% peptone instead of glucose (Figure 39B). Since PDN3 grew better with TCE than without TCE, these results suggest that PDN3 uses TCE as an additional carbon source.

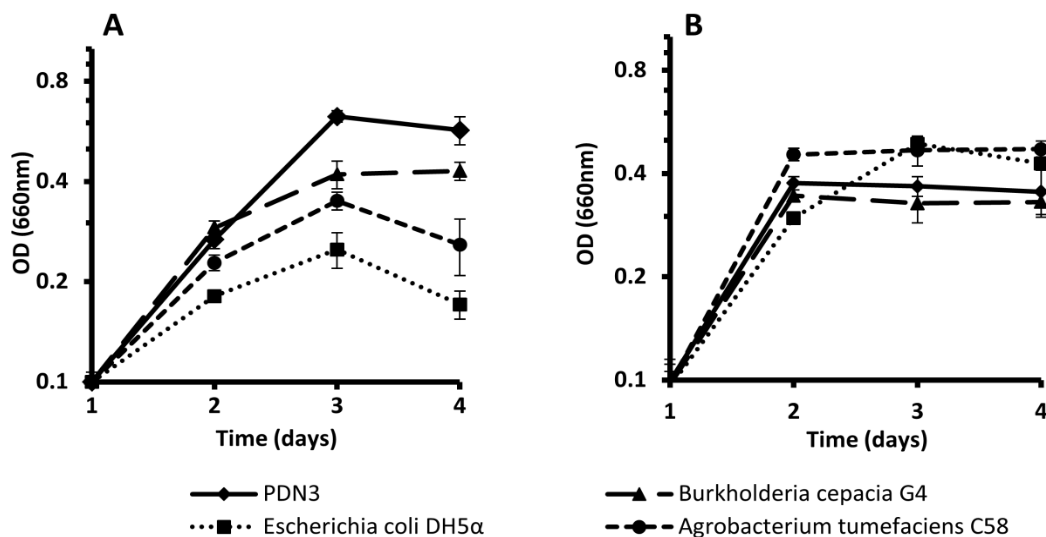

Figure 39. Growth curves of PDN3 in presence of TCE (A) and absence of TCE (B) in M9 minimal media with 0.2% peptone instead of glucose containing 5.5mM of TCE. *Burkholderia cepacia*-G4 was included as a positive control and *Agrobacterium tumefaciens*-C58 and *Escherichia coli*-DH5α served as negative control. The experiments were performed in triplicate; error bars indicate standard deviation.

3.4.7 Naphthalene oxidation assay

Naphthalene oxidation assay that is based upon naphthol production was performed to assay for monooxygenase activity. High naphthol production indicates high activity and this was shown by PDN3 (Figure 40) which produced 2.5-3 times higher naphthol when compared with *Escherichia coli* DH5α and *Agrobacterium tumefaciens* strain C58 (used as negative controls). Interestingly, naphthol production was also higher than *Burkholderia cepacia* G4 in the presence of toluene. However, *Burkholderia cepacia* G4 without toluene was similar with other negative controls. The strong naphthol production by PDN3 confirms monooxygenase activity. Further genetic characterization is required to completely understand the mechanism of TCE degradation by this isolate.

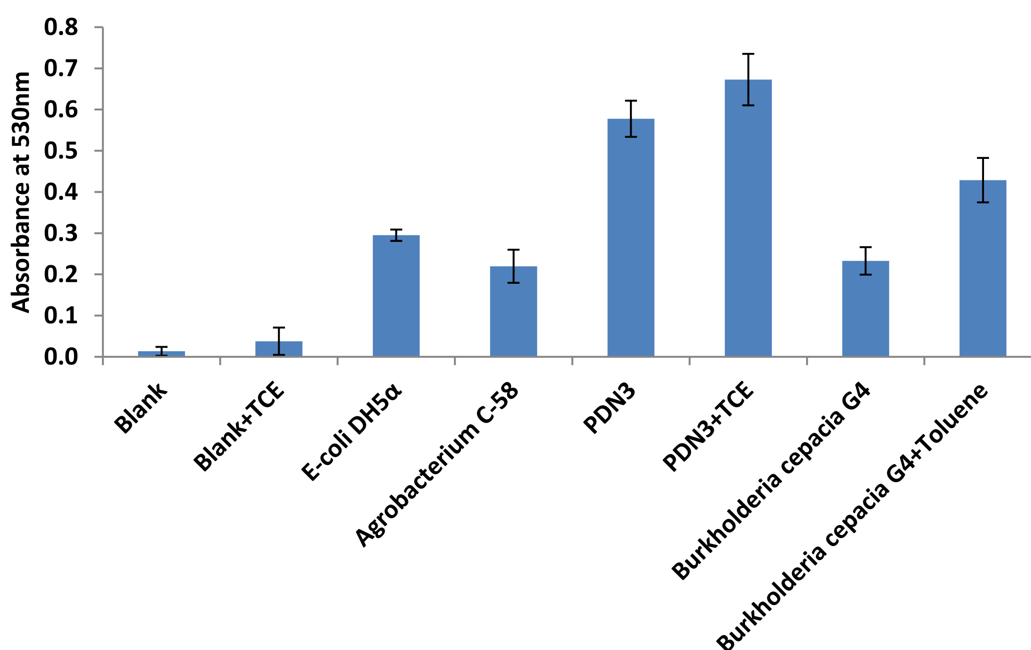

Figure 40. Naphthalene oxidation assay. Naphthol production was measured as A530 after 2 hours of exposure to naphthalene. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* strain C58 were used as negative controls and *Burkholderia cepacia* G4 with toluene was used as positive control.

3.4.8 **Phylogenetic analysis of PDN3**

For phylogenetic analysis, nearly the entire length of the 16s rRNA (1432 bp) gene and rpoB gene (1090 bp) of PDN3 were amplified and sequenced. The sequences for 16s rRNA gene and rpoB gene were deposited in GenBank under accession numbers JN634853 and JN634854, respectively. 16s rRNA (1425/1430) and rpoB (1000/1008) genes were 99% identical with the sequence of the *Enterobacter asburiae* (HQ242719). In addition, the comparison of the partial 16S rRNA (1366 bp) and rpoB gene sequence (512 bp) from the PDN3 with *Enterobacter* group sequences from the database showed that both sequences were closest to *Enterobacter asburiae.* A neighbor-joining analysis is shown in Figure 41 and 42.

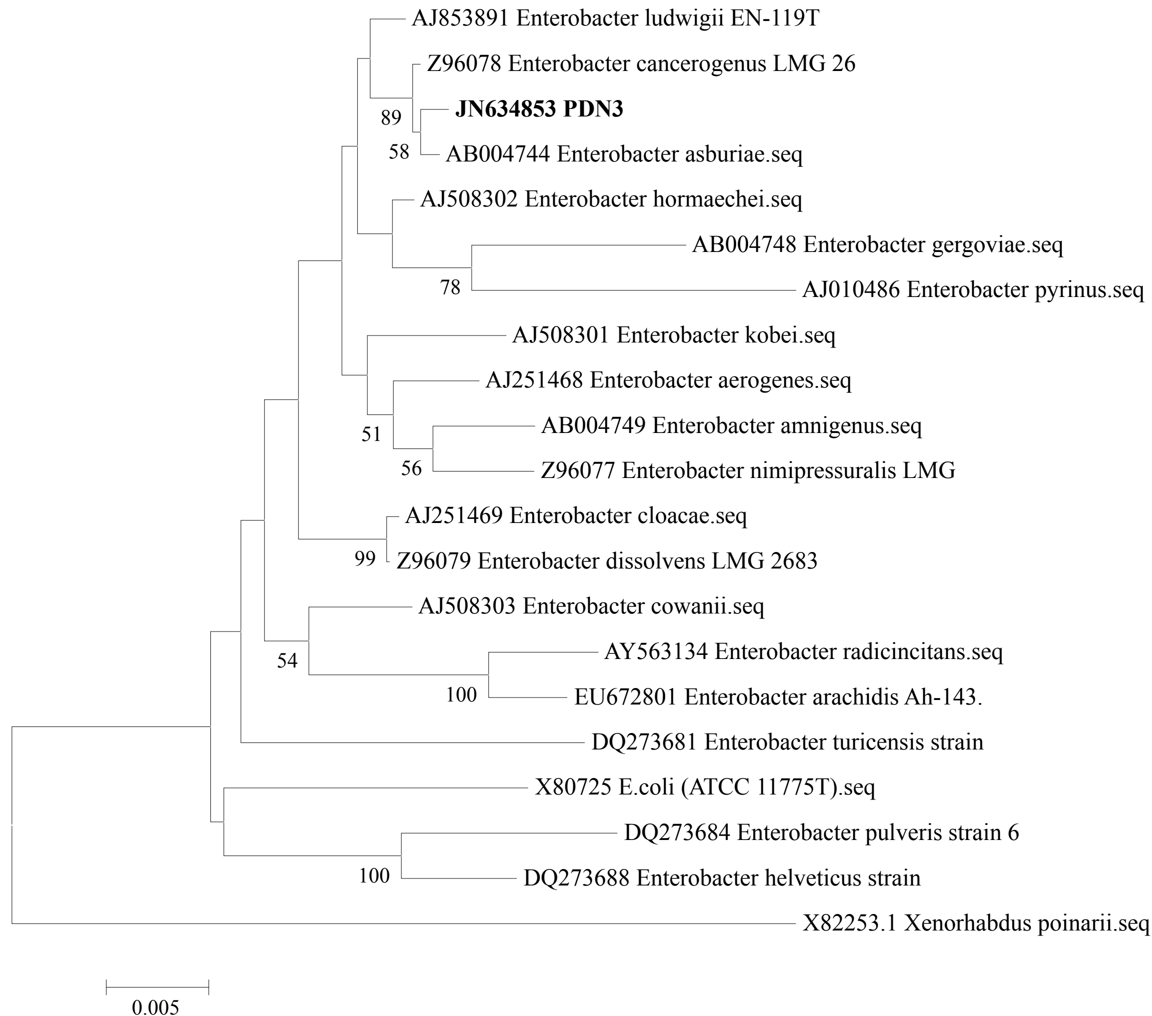

Figure 41. Phylogenetic relationships of endophyte PDN3 obtained in culture from poplar and the related bacterial species. The Neighbor-Joining dendogram was derived from a 16S rRNA sequence distance matrix (Jukes-Cantor). Bootstrap confidence levels (expressed as percentage of 1000 replications) greater than 50% are indicated at the internodes.

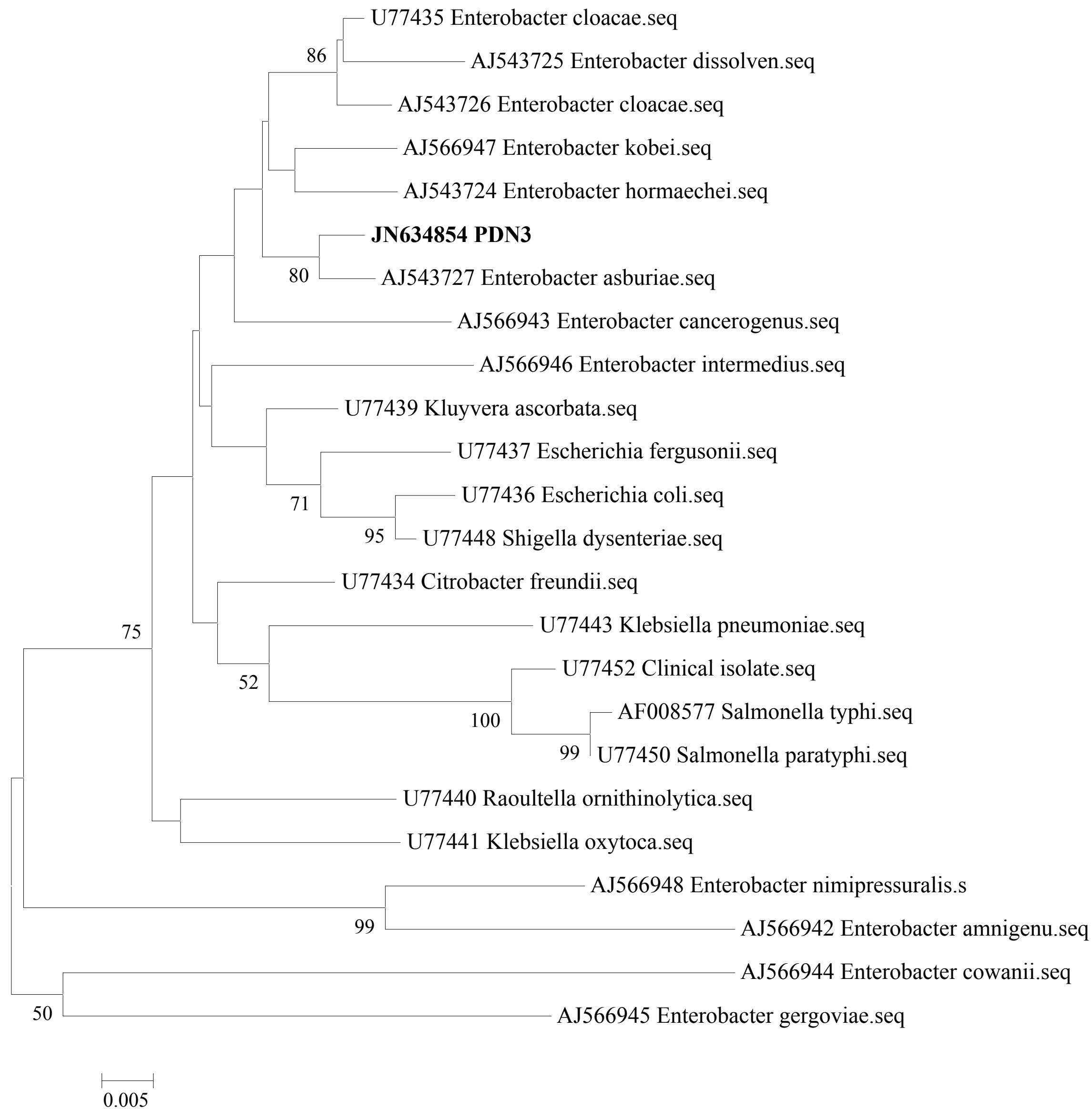

Figure 42. Phylogenetic relationships of endophyte PDN3 obtained in culture from poplar and the related bacterial species. The Neighbor-Joining dendogram was derived from RNA polymerase beta-subunit encoding gene (rpoB) sequence distance matrix (Jukes-Cantor). Bootstrap confidence levels greater than 50% are indicated at the internodes.

3.4.9 Molecular analysis for the MMO gene of PDN3

PCR amplification using specific primer pairs was performed to determine if PDN3 has known pMMO, sMMO, and AMO genes (Table 4). These primers have been used extensively in environmental studies to provide a molecular profile of the methane/ammonia-oxidizing community (Dunfield & Knowles, 1995, Henckel*, et al.*, 2000, Bourne*, et al.*, 2001). The amplification of pMMO/AMO genes was performed by the primer set (A189f-A682r) since the ammonia monooxygenase (AMO) and particulate methane monooxygenase (pMMO) share high sequence identity (Holmes*, et al.*, 1995). The second round of PCR (semi-nested PCR) used several specific primer sets (A189f/mb601r, A189f/A650r, A189f/Mb661r_nd, A189f/mb661R, and A189f/mb661) since pmoA (encoding a subunit of pMMO) primer, mb661, used in conjugation with the A189 primer, was designed and demonstrated specificity to amplify pmoA sequences (Bourne*, et al.*, 2001). Only two primer pairs (A189f/mb601r and A189f/mb661R) resulted in the presence of expected PCR product sizes of 432bp and 490bp fragments for pMMO respectively (Figure 43). Four other primer sets (mmoxA/mmoxB, A189f/mb661, A189f/A650r, and A189f/Mb661r_nd) failed to amplify a methane monooxygenase gene. The putative pmoA PCR products were cloned and sequenced and revealed no homology to known oxygenases.

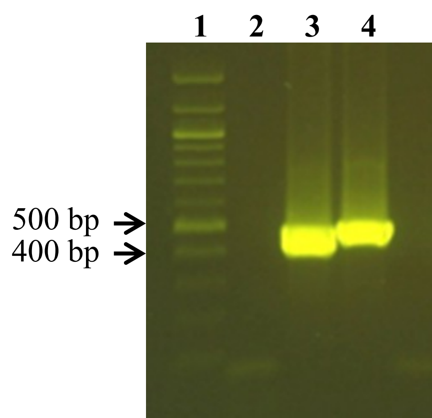

Figure 43. Agarose gel electrophoresis of pmoA PCR amplified products from genomic DNA of TCE degrading endophyte PDN3. Lane 1: Ladder of 1Kb; Lange 2: negative control of pmoA and lane 3-4: PCR products of pmoA gene showing amplification at 470bp.

Table 4. PCR primers used for amplification of pmoA and mmoX genes

Forward/reverse primer	Sequence (5'-3')	Product size (bp)	Reference
A189f/	GGNGACTGGGACTTCTGG/	525	(Holmes, *et al.*, 1995)
A682r	GAASGCNGAGAAGAASGC		
A189F/Mb601R	ACRTAGTGGTAACCTTGYAA	432	
A189F/Mb661R	GGTAARGACGTTGCNCCGG	491	(Kolb, *et al.*, 2003)
A189f/mb661	CCGGMGCAACGTCYTTACC	510	(Costello & Lidstrom, 1999)
A189f/A650r	ACGTCCTTACCGAAGGT	478	(Bourne, *et al.*, 2001)
A189f/Mb661r_nd	CCGGCGCAACGTCCTTACC	510	(Lin, *et al.*, 2005)
mmoXA/	ACCAAGGARCARTTCAAG/	1230	(Auman, *et al.*, 2000)
mmoXB	TGGCACTCRTARCGCTC		

3.4.10 IAA production of PDN3

The test of indole-3-acetic acid (IAA) production was conducted with several poplar and willow endophytes since some of the endophytes are able to promote plant growth by producing phytohormones (Figure 44). The strain PDN3 could produce the maximum IAA yield of 10.5 mg/ml.

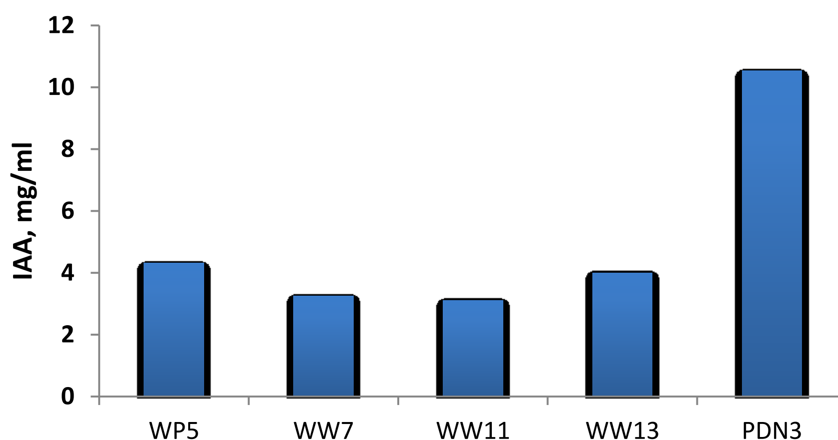

Figure 44. Production of IAA by poplar and willow endophytes. IAA reacts with $FeCl_3$ and $HClO_4$, and the absorbance of the color produced was measured at 530 nm on a spectrophotometer. The strain PDN3 produced the maximum IAA.

3.5 Discussion

First of all, this study found that *Burkholderia cepacia* strain G4 could grow on plant homogenate at the concentrations tested. The poplar homogenate also functions as an inducer of the toluene-ortho-monooxygeanse (TOM) gene in *Burkholderia cepacia* G4. These results showed that certain natural plant phenolic compounds stimulated TCE-degrading bacteria for TCE bioremediation.

Secondly, the results of this study demonstrated that a naturally occurring poplar endophyte can aerobically degrade trichloroethylene (TCE) without the need of any aromatic co-substrates. Generally under aerobic conditions, TCE is co-metabolized and thus requires substrates such as methane, phenol and toluene (Alvarez-Cohen & Speitel, 2001). However, this is the first report of a novel endophyte that biodegrades TCE aerobically in the absence of aromatic compounds. A TCE toxicity test followed by the Fujiwara test were an effective screening tool for predicting the TCE degradation activity of the several endophytes isolated from the different poplar clones. Out of two promising cultures that were resistant to high concentrations of TCE, one endophytic culture from a DN177 hybrid poplar indicated TCE degradation activity. When this culture was plated, it was found to be a mixed consortium. Streak purification resulted in one colony, identified as PDN3, which degraded TCE to chloride ion, as confirmed by the molar ratio of TCE removal to chloride generation of 1:3. In most other studies, microbial degradation of TCE has been demonstrated under a variety of restrictive conditions (Harker & Kim, 1990). When calculated, the initial rate of TCE consumption by PDN3 at 30°C was found to be 4.86 nmol min^{-1}mg of total cell $protein^{-1}$. Others have reported a maximum TCE degradation rates of 8-9 nmol min^{-1}mg of total cell

protein^{-1} at 25°C with phenol-grown *Burholderia cepacia* (Yeager*, et al.*, 2001). The ability of PDN3 to completely metabolize TCE without need of any aromatic substrates or production of any toxic byproducts is a very encouraging result. However, there is a possibility that if GC did not have the right column for chlorinated compounds, the metabolites may not have been detected.

The analytical results suggested that monooxygenase activity of PDN3 may be responsible for TCE degradation. There is a report that methane oxidizing bacteria oxidize ammonia to nitrite (Hutton & Zobell, 1953). Nitrite production was observed when TCE was being metabolized by PDN3 strongly suggesting the presence of TCE-inducible monooxygenases. Chu et al. (1998) reported that methane-oxidizing bacteria can degrade TCE as effectively as nitrate-supplied cultures by methane monooxygenase (Chu & Alvarez-Cohen, 1998) and since the analysis was performed with chloride-free medium which contains ammonium and nitrate, PDN3 seems have an ammonia/methane monooxygenase. Oxygenases are generally divided into two groups, monooxygenases and dioxygenases. Both classes of oxygenases are implicated in bacterial TCE degradation (Wackett*, et al.*, 1989). The expression of toluene mono/dioxygenase activity is required for aromatic compound degradation and methane monooxygenase in methanotrophs (Brusseau*, et al.*, 1990) and ammonia monooxygenase in *Nitrosomonas europaea* are also proposed to oxidize TCE in gaseous substrates (Rasche*, et al.*, 1991). All these oxygenases display relatively broad substrate specificity and they could be one of the potential oxygenases involved in TCE degradation in PDN3.

Since monoxygenase activity by genetic analysis could not be confirmed, a chemical assay was performed to monitor monooxygenase enzyme expression of this novel strain. A

colorimetric assay based upon naphthol production was used. This assay has previously been applied to detect the methane monooxygenase activity of *Methylosinus trichosporium* OB3b (Morton*, et al.*, 2000) and *Pseudomonas mendocina* KR1 (Tao*, et al.*, 2005). It was previously developed as a rapid method for detecting monooxygenase activity and demonstrated that dioxane degradation was positively correlated with monooxygenase activity (Mahendra & Alvarez-Cohen, 2006). In this case, strain PDN3 produced more naphthol (more monooxygenase activity) than the negative controls (*Escherichia coli* DH5α and *Agrobacterium tumefaciens* C58). A known TCE degrader *Burkholderia cepacia,* G4, was used as a positive control and it also produced naphthol in presence of toluene. This result strongly suggests that monoxygenases are involved in the metabolism of TCE.

In an attempt to identify PDN3, genetic analysis was done. The phylogenetic position of PDN3 was inferred from 16s rRNA gene and rpoB gene sequence comparison. Mollet et al (1998) introduced the usefulness of RNA polymerase beta subunit encoding gene (rpoB) sequences for the identification of members of the *Enterobacteriaceae* (Mollet*, et al.*, 1997). Results suggest that PDN3 belongs to the genus *Enterobacter*. 16s rRNA and rpoB gene sequence analysis indicated that their sequence was most related to the sequence of the *Enterobacter asburiae* (HQ242719, 99% similarity). In previous studies, *E. asburiae* strains have been reported as endophytes and rhizospheric microbes associated with sweet potato, cotton, bean and cucumber (Quadt-Hallamnn*, et al.*, 1997, Asis Jr & Adachi, 2004). Facultative anaerobes, *Enterobacter strain MS-1* and *E. agglomerans*, have been reported to reductively dechlorinate PCE, thereby converting it to DCE. This occurs only under strictly anoxic conditions (Sharma & McCarty, 1996, Holliger*, et al.*, 1998). Some species of *Enterobacter* have been reported to degrade phosphonate (Lee*, et al.*, 1992), glyphosate

(Dick & Quinn, 1995), pentaerythriotl tetranitrate (Binks*, et al.*, 1996), trinitrotoluene (French*, et al.*, 1998) and organophosphate (Singh*, et al.*, 2004).

This study showed that natural plant homogenate has a potential to be used as natural substitutes for toxic substrates to induce co-metabolic degradation of TCE under aerobic conditions. Overall, bioremediation of TCE contaminated sites using this novel strain PDN3 seems to be a promising cost effective remediation strategy. This strain could be further used to inoculate trees such as poplar and willows and be tested for increased TCE removal and degradation.

3.6 References

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CHAPTER 4

CONCLUSION

Bioremediation is an innovative decontamination technology which utilizes microorganisms, plants, or their enzymes to return the natural environment altered by contaminants to its original condition. The technology has been applied to a wide range of chemicals including chlorinated hydrocarbons, polycyclic aromatic hydrocarbons, explosives, and pesticides. This dissertation research explored two approaches for enhancing the bioremediation of a chlorinated solvent, trichloroethylene (TCE).

TCE has been widely used as an industrial solvent for many decades and has become one of the most common environmental contaminants of soil, groundwater, and air. Several studies have reported that TCE can trigger harmful biological effects of organic compounds, increasing their toxicity and mutagenicity. There have been a number of attempts using genetically modified plants or microorganisms to improve the degradation of TCE in bioremediation technology.

In particular, the use of poplar trees for degradation of TCE has attracted considerable attention since poplar trees can grow rapidly and have extensive root systems which can effectively remove TCE from soil, groundwater, and air. Many studies also reported that poplar trees have the ability to degrade TCE. As described in Chapter 2, the first research of this study hypothesized that there are characteristic plant genes whose expression is regulated in response to TCE in hybrid poplar. The *Populus* transcriptome of the TCE response could indicate of what genes were expressed and which genes might play a role in detoxification. These detoxification genes could also be exploited in transgenic plants for bioremediation of

TCE or other chlorinated solvents.

The natural poplar genes involved in TCE metabolism between hybrid poplar and CYP2E1 transgenic hybrid poplar were identified and compared by microarray analysis. The transgenic poplar overexpressing the mammalian CYP2E1 gene showed a large number of differentially expressed transcripts, suggesting that a metabolic pathway for degrading TCE to metabolites had been initiated by the activity of CYP2E1 on TCE. A number of up-regulated genes which responded to TCE in hybrid poplar were found to be involved in metabolic and cellular processes, and stress responses, but the functions of many genes were unknown. In addition, the expression levels of targeted specific detoxification genes, including cytochrome P450 (CYP450), glutathione S-transferase (GST), glycosyltransferase (UGT), and ABC transporter, in the CYP2E1 transgenic hybrid poplar with TCE treatment were very high compared to other plants and the differences were statistically significant.

Thus, this research indicated that the rate-limiting step in TCE degradation was the initial activation by a CYP450. When this gene was overexpressed in transgenic plants, the expression of the first enzyme in the pathway automatically triggered all the rest of the native poplar genes involved in detoxification. Further research needs to be done to verify the rest of the detoxification genes since these genes may have biotechnological potential for degradation of organic pollutants. Although none of the putative plant cytochrome P450 genes from Arabidopsis and poplar which were tested with a yeast expression system was able to convert TCE to TCEOH, there is a possibility that TCE could not induce the expression of the plant CYP450 genes since TCE is not a natural substrate to plants. If the TCE-degrading P450 gene is not up-regulated in response to TCE, it makes it difficult to predict which of the hundreds of P450s are involved in TCE degradation.

In Chapter 3, another approach using microorganisms was studied in order to enhance TCE degradation. The use of plant microbes, endophytes, to clean up environmental pollutants has gained momentum in past years. Recent studies reported that endophytic bacteria have the ability to degrade toxic chemicals, and several endophytic bacteria from poplar were able to degrade toxic chemicals such as benzene, toluene, ethylbenzene and xylene (BTEX).

The second aim of this dissertation research described in Chapter 3 focused on the use of TCE-degrading bacteria to test two hypotheses. First, plant homogenate (phytochemicals) can induce the toluene-ortho-monooxygeanse (TOM) gene of *Burkholderia cepacia* strain G4 to degrade trichloroethylene (TCE). Second, certain endophytes in poplar can degrade TCE without any inducer such as phenol and toluene.

Phytochemicals are compounds that are produced by natural plants. Polyphenolic compounds including flavonoids, anthocyanins, isoflavones and tannins are common chemicals in plants. These chemicals generally mediate chemical defense against microorganisms, viruses, and herbivores. Thus, the growth experiment of *Burkholderia cepacia* strain G4 was conducted with poplar homogenate to test whether strain G4 could grow in phytochemicals. This research showed that *Burkholderia cepacia* strain G4 is able to use poplar homogenate as an energy source and it has tolerance to phytochemicals. In addition, the levels of expression of targeted TOM genes of *Burkholderia cepacia* strain G4 were highly expressed by poplar homogenate. This study opens the prospect of applying plant homogenate, which is naturally safe and inexpensive, to stimulate TCE-degrading bacteria for TCE bioremediation. Conceivably, plant homogenate could be exploited to aid in

the removal of TCE and other organic contaminants to induce bacteria-assisted phytoremediation.

From this result, it was postulated that plant microbes (endophytes) which have already been adapted to phytochemicals and environmental pollutants within the plants growing on a contaminated site could degrade the pollutants since bacteria adapt and adjust quickly to different organic substances and environmental stress by several adaptive mechanisms. A novel endophyte *Enterobacter* sp. PDN3 was isolated from a poplar tree growing on a TCE contaminated site. In particular, bacteria of the species *Enterobacter* sp. have been shown to have the ability to degrade several toxic organic compounds as an endophyte. PDN3 grew well in high levels of TCE and aerobically metabolized TCE to chloride without the addition of inducing synthetic aromatic compounds. To our knowledge, this is the first report on a natural TCE-degrading bacteria isolated from a poplar tree. Regarding identification of the enzyme systems and the pathways involved in its degradation, however, further research needs to be done since monoxygenase activity by genetic analysis could not be confirmed. In addition, PDN3 may also be investigated for the ability of degrading other toxic chemicals.

Some of the endophytes are also proven to be able to promote plant growth and increase nutrient uptake by the synthesis of phytohormones such as indole-3-acetic acid (IAA), cytokinins, acetoin, and ethylene, as well as other plant growth-promoting substances such as siderophores. Especially, auxins (i.e., IAA) and acetoin are reported to be produced by endophytic strain of *Enterobacter*.

The study in Chapter 3 was conducted to assess functional potentialities in relation to plant growth promoting activities. The results showed that PDN3 was able to produce high

levels of IAA. Thus, this novel strain *Enterobacter sp*. PDN3 can be recommended as a bioinoculant which can help to enhance phytoremediation and promote plant growth in the promising bioenergy crops such as poplar and willow.

Overall, the results of this dissertation research supported the hypotheses that specific plant genes and endophytes have strong potential for degrading pollutants. Further research should be conducted to investigate and evaluate the effectiveness of genetic manipulation of putative plant detoxification genes and endophyte-assisted phytoremediation on TCE.

Natural resources such as plants and microorganisms have great potential to be exploited for bioremediation technologies. The findings of this dissertation research can be applied to develop a cost-effective and environmentally-friendly strategy to strongly reduce the amount of harmful pollutants in our environment.

VITA

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