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Phytoremediation of Chlorpyrifos Insecticide: The Use of Woody Plants and Transgenics to Enhance and Understand the Uptake, Translocation, and Transformation of Chlorpyrifos

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Abstract

Phytoremediation of Chlorpyrifos Insecticide: The Use of Woody Plants and Transgenics to Enhance and Understand the Uptake, Translocation, and Transformation of Chlorpyrifos

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Poplar and willow trees have a significant ability to take up CPS and to translocate/degrade it within the plants and thus, assist in removing it from the hydroponic solution. To our knowledge, the poplar and willow species presented in this study were the first woody plants to be tested for potential phytoremediation applications for CPS. Since poplar and willow are able to efficiently take up CPS, there is the possibility of enhancing the CPS degradative potential by genetic manipulation of metabolism *in planta*. The results presented here indicate that phytoremediation of CPS and other OP insecticides should be a fertile area for future development.

In order to test for this potential, two types of transgenic plants were developed. Transgenic poplar plants ('PON1-34') expressing rabbit *pon1* gene, which is known to be involved in OP insecticide degradation pathways, were produced and investigated for increased tolerance and removal of CPS. However, there was no change in phytotoxicity of CPS or uptake of CPS in the

transgenic lines. The his-tagged PON1 protein was not detected by the western blot (protein immunoblot), when the his-tag antibody was used to detect his-tagged PON1 protein in bacterial and plant total protein. In order to increase the expression level of PON1 protein in plants, a tobacco plant was transformed with rabbit PON1 using the chloroplast transformation method. The activity of plant-derived PON1 was analyzed and CPS tolerance and uptake were investigated in chloroplast transformed tobacco to assess the function of rabbit PON1 enzyme in plants. However, there was no response in the PON1 transgenic tobacco as compared to wild type tobacco in terms of phytotoxicity, CPS/CPO removal, and PON1 enzyme activity assay. In addition, PON1 protein expressed in *E. coli* was not detected by the western blot analysis, when the his-tag antibody was used to detect his-tagged PON1 protein in bacterial total protein. Through these two transgenic approaches, three possibilities exist for this failure (i) the PON1 protein is not folding properly and is targeted for destruction, (ii) the addition of the his-tag to the N-terminal did not work for the detection by the western blotting, or (iii) the expression level of the PON1 protein was very low. Since the PON1 enzyme activity assay did give positive results, it suggests that the constructs (pART27-PON1 and pLD2-CtV-PON1) was functional and that the enzyme was active for at least a short period of time under specific conditions.

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Chapter 1 Introduction

1.1 Chlorpyrifos (CPS)

1.1.1 Organophosphorus insecticide

Chlorpyrifos (CPS), [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] (Figure 1), is one of the most widely used organophosphorus (OP) insecticides worldwide, both in crop production and in non-agricultural applications. It has been used to control foliage- and soil-borne insect pests on a variety of food and feed crops since its first introduction in 1965. The greatest crop use is corn, and other major crops treated with CPS are cotton, apples, alfalfa, citrus, peanuts, pecans and wheat. Non-agricultural uses of CPS include termiticide, turf, golf courses, cattle ear tags, ornamental sites (nursery/landscapers), indoor pest control, etc. Similar to other organophosphates, its insecticidal action is due to the inhibition of butyrylcholinesterase (BChE) and acetylcholinesterase (AChE), which are key enzymes necessary for the proper functioning of the nervous system, including the brain, thus resulting death of the target pest (ATSDR, 1997; USEPA, 2000, 2002).

Among the 1287 sites listed on the U.S. Environmental Protection Agency (EPA) Superfund final National Priority List (NPL) as of August 04, 2011, 387 sites were reported to have pesticide contamination. The OP pesticides, including CPS, account for about 50 % of insecticides used in the US (USEPA, 2011). Crops in the US are sprayed with between eight and ten million pounds of this toxic chemical every year. The states of heaviest use are Nebraska, Illinois, Indiana, and California's San Joaquin Valley. Among the crops that carry its residue are all manner of common foods, such as apples, bell peppers, kale, and asparagus (USGS, 2002). This widespread contamination of CPS is a potential health and environmental concern.

1.1.2 Degradation pathways of CPS

CPS is a degradable compound, and it undergoes chemical changes in the environment as well as in the human body. The biotransformation pathways of CPS are shown in Figure 1 (adapted from Eaton *et al*., 2008). One metabolic reaction is the desulfuration, which occurs by transformation at the double bond of the central phosphorus atom from sulfur to oxygen. This metabolic reaction results in activation of the CPS forming chlorpyrifos-oxon (CPO), a more potent inhibitor of cholinesterase enzymes, since this metabolite must be formed to express potent anticholinesterase activity. CPS by itself is not toxic, so it is unable to inhibit cholinesterase to any significant extent (Chambers, 1992). However, it creates a toxic metabolite when it is transformed by the environment or *in vivo* by cytochrome P450 (CYP) to CPO in mammals, which is about 3000 times as potent than CPS itself (Williamson, Terry, and Bartlett, 2006).

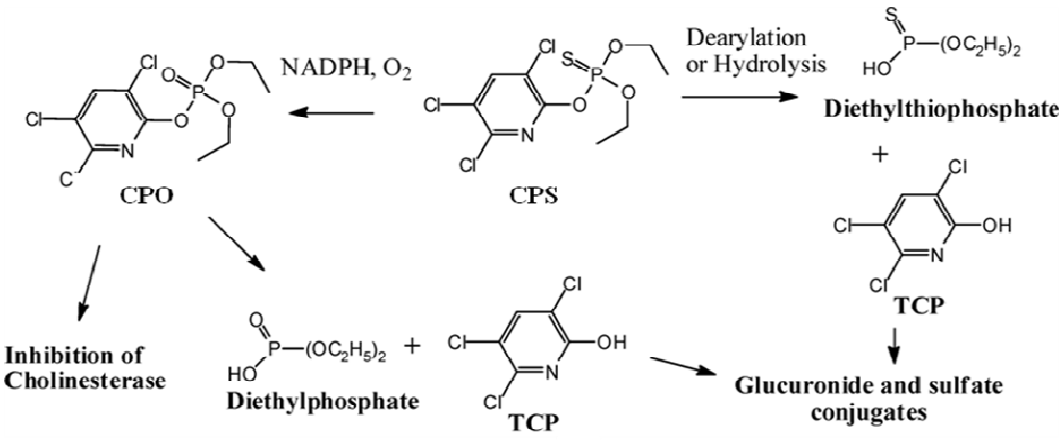

Figure 1 Biotransformation of CPS in experimental animals (adapted from Eaton *et al*., 2008)

Another important chemical reaction is hydrolysis of the organophosphate, which occurs by cleavage of the phosphorus ester bond to yield the breakdown products, a dialkylphosphate and the leaving group. This reaction results in detoxification/inactivation of the organophosphate, as

the leaving group and dialkylphosphate metabolites do not inhibit cholinesterase enzymes and are considered toxicologically insignificant by regulatory authorities. In this reaction, CPS is metabolized to diethylthiophosphate and leaving group trichloropyridinol (TCP) through CYP-mediated dearylation, and CPO breaks down to diethylphosphate and TCP by the enzyme paraoxonase (Figure 1). A detailed discussion of enzyme-related metabolic reactions will be provided in Chapter 4. TCP is further degraded via microbial activity and photolysis to carbon dioxide and organic matter in soil and water, and it may be excreted directly or following glucuronide and sulfate conjugation *in vivo* in animals.

The metabolic fate of CPS has also been investigated in plants (Smith, Watson and Fischer, 1967a, 1967b), but little is known about the fate of this contaminant and its metabolites within a plant system, compared to its metabolic pathways in the environment and in mammals. Although most of the CPS applied to plants is lost through volatilization prior to plant uptake, conversion to TCP after taken up by plant tissue and desulfuration to CPO on plant surfaces have been reported (Roberts & Hutson, 1999).

1.1.3 Adverse impacts caused by CPS and its metabolites

Although CPS is a degradable compound, it can take weeks to years for all of the CPS to break down once it has applied. While it remains in the environment, it can affect human health and the environment in many ways. As a result of its broad-spectrum effects on non-target organisms, CPS adversely affects other organisms besides the pests it is designed to kill. CPS/CPO exposures kill beneficial arthropods including bees, ladybird beetles and parasitic wasps, and other animals including fish and other aquatic organisms, birds, cats, pigs, monkeys, and humans (Cox, 1995).

As an insecticide, CPS is not expected to be toxic to plants. Surprisingly, it has been shown that a number of different plants are damaged by exposure to CPS. Delayed or reduced seedling

emergence in both carrot and onion (Sinclair, Neeson, and Williams 1992), fruit deformities in grapefruit (Beck *et al*., 1991), and abnormal cell division in vetch (Amer and Farah, 1983) have all resulted from CPS treatment.

According to the US-EPA, exposure of the U.S. population to CPS and its metabolites is widespread and has been related to a variety of nerve disorders in humans. Symptoms of acute poisoning include headache, nausea, dizziness, confusion, and in some extreme cases even respiratory paralysis and death. Human birth defects and male infertility have also been associated with exposure to CPS and its products.

More recently, the potentially greater risks of infants and children to pesticides were comprehensively discussed. Exposures of Children to pesticide residues may be relatively higher than those of most adults. Pound for pound, children generally eat more than adults, and they may be exposed more heavily to certain pesticides because they consume a diet different from that of adults (National Research Council, 1993). For instance, children typically consume larger quantities of applesauce, milk, and orange juice per pound of body weight. Exposure to CPS is associated with early childhood developmental delays, according to a study by researchers at Columbia University's Mailman School of Public Health (Lovasi *et al*., 2011).

Public attention on pesticides tends to focus more on consumer exposure-which is the levels of pesticides left on the foods we eat at the time of purchase, rather than worker or environmental concerns. However, those who actually work with the chemical face far more intense exposure to the pesticides, so especially agricultural workers and their children are at extreme risk (Winter, 2000). Numerous cases of worker illness and injuries that result from pesticide exposure have been reported and epidemiological evidence linking occupational exposure to pesticides with an increase in the incidence of cancers is overwhelming. In addition, a study with pregnant migrant workers and their children showed that CPS caused an increased risk of brain-dead babies, low

birth weights, severe and unusual birth defects, and attention deficit hyperactivity disorder (ADHD). (Hoar *et al*., 1986; Council on Scientific Affairs, 1988; California Department of Pesticide Regulation, 1999).

Concerns about related health effects had led to restrictions on its use. To mitigate risks from exposure, the use of CPS has been restricted for nearly all residential uses and even for some agricultural purposes in the US and in some European countries (USEPA, 2002). However, it is still widely used throughout the agricultural industry in the US, and it continues to be used in developing countries such as India, to control crop damage from insects (Eaton *et al*., 2008).

1.2 Phytoremediation: using green plants to clean up the environment

Phytoremediation is an emerging technology which uses plants for the cleanup of environmental contaminants located in the soil, sediments, groundwater, surface water; and even the atmosphere. This technology has been extensively reviewed by many research groups (Cunningham & Berti, 1993; Salt, Smith and Raskin, 1998; Meagher, 2000; Dietz & Schnoor, 2001; McCutcheon & Schnoor, 2003; Newman & Reynolds, 2004; Suresh & Ravishankar, 2004; Pilon-Smits, 2005; Pilon-Smits & Freeman, 2006).

Phytoremediation covers a broad spectrum of pollutants and remediation techniques which include many treatment strategies. Researchers have found that plants can be used to treat a variety of contaminants including petroleum hydrocarbons, solvents, pesticides, metals, radionuclides, explosives, and other organic compounds (Chappell, 1998). Depending on the type of pollutant and processes, various phytoremediation techniques have been developed: Phytoextraction, rhizofiltration, phytostabilization, rhizodegradation, phytodegradation, phytovolatilization, hydraulic control, vegetation cover and buffer stripes (Trapp & Karlson, 2001; Schröder, Harvey and Schwitzguébel, 2002; Suresh & Ravishankar, 2004; Pilon-Smits, 2005). Phytoextraction refers to removal of contaminants from soil and translocation to harvestable

tissues (stems and leaves). This technique is preferably used for heavy metals like nickel, zinc, copper, lead (Blaylock *et al*., 1997), chromium and cadmium. Rhizofiltration is the sorption of contaminants by plant roots or other plant parts. In this technique, plants can be used as filters in a hydroponic setup and heavy metals or lipophilic compounds can be extracted from water and aqueous waste streams. Phytostabilization is the use of plants to stabilize pollutants in soil. This treatment immobilizes pollutants either simply by stabilizing soil itself to prevent erosion, leaching, or runoff, or by converting pollutants from a soluble form to less bioavailable forms. Unlike phytoextraction, phytostabilization focuses mainly on precipitation of pollutants in the rhizosphere but not sequestration in plants. In phytodegradation, plants can break down organic pollutants directly through their own enzymatic activities. Plants can facilitate biodegradation of organic pollutants by soil microbes in the rhizosphere of plants, a process called rhizodegradation or phytostimulation. Phyto- and rhizodegradation are used for the remediation of organic contaminations, among them petroleum, polycyclic aromatic hydrocarbons (PAH), explosives including trinitrotoluene (TNT) and Royal Demolition Explosive (RDX), chlorinated solvents and pesticides. In phytovolatilization, certain contaminants such as mercury and selenium taken up by the plant roots pass through the plant and are released in volatile form to the atmosphere (Kotrba *et al*., 2009). These various phytoremediation techniques are not mutually exclusive. Because pollutant distribution and concentration are heterogeneous for many sites, the most efficient phytoremediation solution may be a combination of different technologies. For instance, Hansen *et al*. (1998) has shown that accumulation, stabilization and volatilization can occur simultaneously in a constructed wetland for selenium removal.

Plant species are selected for phytoremediation based on factors such as ability to extract, degrade or bioaccumulate contaminants, the degradative enzymes they produce, adaptation to local climates, high biomass, growth rate, depth of root structure, compatibility with soils, ease of planting and maintenance, and ability to take up large quantities of water through the roots

(Chappell, 1998). The probability of a plant being an effective phytoremediator depends on the type of pollutant; therefore, a variety of plant species and individual clones within that species should be tested for the best detoxification of a specific pollutant.

1.2.1 Benefits over traditional methods

Although various engineering–based methods such as soil excavation, transport, soil washing or burning, pump and treat systems and addition of reactants such as hydrogen peroxide or potassium permanganate have been applied to remediate contaminated soils (Doty, 2008), there are many advantages of phytoremediation over traditional methods for the environmental cleanup (Table 1).

Its primary advantage is that phytoremediation is on average 10 times cheaper than engineering-based remediation methods (Chappell, 1998; Glass, 1999), since its biological processes are ultimately solar-driven. Unlike other conventional remediation strategies, phytoremediation which utilizes the natural ability of plants to extract chemicals from water, soil, and air uses energy from sunlight. Another benefit of phytoremediation involves public and environmental safety issues. The fact that phytoremediation is usually carried out *in situ* and that plants act as soil stabilizers may reduce exposure of the pollutants to humans, wildlfe, and the environment by minimizing the amount of contaminants that could leave the site and enter the surrounding systems (Pilon-Smits, 2005; Doty, 2008). It is also low impact and public acceptance of phytoremediation is expected to be high. Phytoremediation as a green technology is more environmentally friendly, more aestherically pleasing and more widely accepted in a community than conventional methods involving unsightly excavations and heavy equipment such as chemical plants and bulldozers (Paz-Alberto & Sigua, 2013). The performance of remediation using plants is also easier to be kept track of, because plants can be visually monitored. The condition of the plants is easily observed and collecting and testing

of plant samples for the presence of the pollutant over time is simple and easy, unlike bioremediation with microorganisms (Doty, 2008). Other advantages of phytoremediation include: reprocessing of plant materials used for phyremediation into a useful product such as wood, pulp, or bioenergy (Stanton *et al*., 2002), providing wildlife habitat (Moser *et al*., 2002; Kuzovkina &Quigley, 2005), and carbon sequestration to reduce global warming potential.

Table 1 Advantages and disadvantages of phytoremediation (Chappell, 1998)

Advantages	Disadvantages
Cost-effective	
Passive, solar-driven	Slower than conventional methods
High public acceptance	Phytotoxicity effects of pollutants
Prevention of further contamination	Limited by bioavailability of pollutants
Easy to monitor	Limited to shallow contaminants
Recycling plant materials	Unknown effects of biodegradation products
Providing wildlife habitat	Potential for contaminants to enter the food chain

1.2.2 Challenges

Phytoremediation has advantages but also limitations (Table 1). Its major disadvantage is its relatively slow pace compared to the conventional methods like excavation, incineration, or pump-and-treat systems, as it often takes years or even decades, limiting applicability (Elizabeth Pilon-Smits, 2005). Another disadvantage is that phytoremediation can only work at sites that are well suited for plant growth (Chappell, 1998). This means that the concentration of pollutants cannot be toxic to the plants and the environmental and climatic conditions of the contaminated sites cannot be too harsh for successful plant survival and growth. Phytoremediation is also limited by root depth; a pollutant may be located in a deep aquifer, well below the maximum rooting depth. Phytoremediation may also be limited by the bioavailability of the pollutants. If a pollutant is tightly bound to the soil particles, then it may not be available to

plants (Chappell, 1998). Also, potential danger might exist as contaminants enter the food chain. This problem may occur when contaminants, especially heavy metals, are bio-accumulated in plants which then pass into the food chain by being eaten by animals (Kirchner, 2001). For these reasons, the challenge for researchers is to both understand and enhance the plants' performance in removing toxicants from the environment. Recently, there has been an increase in research on improving phytoremediation capability of plants through the use of transgenic plants and symbiotic endophytic microorganisms within plant tissues.

1.2.3 Enhancing phytoremediation

1.2.3.1 Phytoremediation with transgenic plants

The obvious approach to address these limitations is the application of plant genetic engineering as an efficient tool to enhance the phytoremediation capacity of plants. In this plant biotechnology, selected genes from various organisms such as bacteria, plants, and mammals are being inserted or overexpressed in plants to increase plant tolerance or metabolism of organic chemicals or heavy metals. Transgenic plants for enhancing phytoremediation has been extensively reviewed (Eapen, Singh, and D'Souza, 2007; Macek *et al*., 2008; Van Aken, 2008; Abhilash, Jamil, and Singh, 2009; Dowling & Doty, 2009; Kotrba *et al*., 2009; Dhankher *et al*., 2011).

The first transgenic plants for phytoremediation were developed for remediating heavy metal in soil. Tobacco plants (*Nicotiana tabaccum*) expressing a yeast metallothionein gene were able to tolerate high levels of cadmium (Misra & Gedamu, 1989) and *Arabidopsis thaliana* overexpressing mercuric ion reductase showed higher tolerance to mercury (Rugh *et al*., 1996). Phytoremediation of organic pollutants using engineered plants was first attempted for explosives (French *et al*., 1999). French *et al*. (1999) expressed the bacterial gene, pentaerythritol tetranitrate reductase, in tobacco, resulting in increased removal and tolerance of

nitroaromatic explosives. In recent years, the efficiency of transgenic plants to degrade a variety of organic pollutants such as chlorinated solvents, explosives, phenolics, herbcide etc. has been widely acknowledged. For instance, overexpression of cytochrome P450s (CYP), involved in the metabolism of xenobiotics, led to increased removal rates of a variety of organic pollutants and herbicides. Transgenic plants overexpressing *CYP2E1* gene produced hundreds of times more metabolite of trichloroethylene (TCE), one of the most common ground water pollutants, compared with nontransgenics (Doty *et al*., 2000, 2007). Transgenic potato and rice plants overexpressing the gene *CYP1A1*, *CYP2B6*, or *CYP2C19* metabolize a wide range of herbicides (Inui *et al*., 2001; Kawahigashi *et al*., 2006, 2007). Genes involved in the uptake or detoxification of toxic metals were also used to create transgenic plants to remediate a variety of metals such as mercury, selenium, arsenic, lead, and cadmium (Dhankher *et al*., 2002, 2003; Eapen & D'Souza, 2005; Hsieh *et al*., 2009; Kotrba *et al*., 2009; Ruiz & Daniell, 2009). Although numerous examples of research has shown that the use of genetically modified plants carrying a suitable gene is highly effective for enhancing phytoremediation, the use of such plants is very limited to field application due to strict regulations governing the release of transgenic plants to the environment.

1.2.3.2 Phytoremediation with endophytes

In addition to transgenic plant approaches, the use of endophytes also led to improved phytoremediation. The term 'endophyte' refers to microorganisms, often bacteria and fungi, that live within a plant without causing apparent disease (Clay & Schardl, 2002), unlike rhizospheric bacteria living on or around the plant roots.

The use of endophytes for enhancing phytoremediation has been reviewed (Newman & Reynolds, 2005; Zhuang *et al*., 2007; Doty, 2008; Khan & Doty, 2011). Methylotrophic bacterium isolated from hybrid poplar was capable of degrading the explosives TNT, RDX, and HMX (Van

Aken *et al*., 2004; Van Aken, Yoon, and Schnoor, 2004), endphytic isolates from hybrid cottonwood at a BTEX-contaminated site were able to tolerate heavy metals, BTEX and TCE (Moore *et al*., 2006), and the endophytes of the nickel hyperaccumulator were tolerant to higher nickel concentrations than the rhizospheric bacteria (Idris *et al*., 2004). Intentional inoculations of plants with endophytic strains which degrade pollutants are also known to enhance its phytoremediation capability. For examples, inoculated pea plants with bacteria that degraded a herbicide 2,4-D increased resistance to the herbicide (Germaine *et al*., 2006) and yellow lupine plants inoculated with engineered endophytes had higher tolerance of toluene (Barac *et al*., 2004).

1.3 Phytoremediation of CPS

1.3.1 Previous research and limitations

Phytoremediation of pesticides has been reviewed (Hussain *et al*., 2009). Phytoremediation has been shown to be useful in the removal of CPS (Moore *et al*., 2002). However, CPS remediation studies have been limited to plant-associated microorganisms (Singh *et al*., 2004; Yang *et al*., 2005; Yu *et al*., 2006). It remains to be seen whether plants are able to take up and transform OP pesticides without the participation of associated microbes. Removal of CPS by plants is based on the ability of plants to take up CPS into tissues and to degrade it. Although the fate of CPS has been documented in the environment, little is known about the fate of this contaminant and its metabolites within a plant system (Smith, Watson and Fischer, 1967a, 1967b; Rouchaud *et al*., 1991). In addition, most plant species that have been tested so far for CPS uptake are herbaceous plants. Compared to herbaceous plants, woody plants such as poplar and willow offer some distinct advantages for treatment of contaminated sites. These species are perennial, long-lived (80-100 years), hardy, fast growing, and easily propagated, providing high biomass and an extensive root system. Recent findings which suggest that they can take up and degrade

organic contaminants also support this plant family as the one of choice for the phytoremediation studies (Burken & Schnoor, 1997; Newman *et al*., 1997; Pilon-Smits *et al*., 1998; Robinson *et al*., 2000). It is expected that these types of plants will be significant in phytoremediation research in the future.

1.3.2 Three approaches to enhanced phytoremediation of CPS

Three approaches to enhanced phytoremediation of CPS are proposed in this study.

a) Uptake and degradation of CPS by poplar and willow: Woody plants of the Salicaceae family, the genus *Populus* – which includes poplars and cottonwoods – and the genus *Salix* – which includes willows – were investigated for removal of CPS from hydroponic solution (Chapter 3).

b) Engineering transgenic poplar overexpressing paraoxonase 1 (PON1) for improved degradation of CPS: Poplar was overexpressed with a mammalian gene involved in CPS metabolism for enhancing phytoremediation and this transgenic poplar was studied for removal and degradation of CPS compared to nontransgenics (Chapter 4).

c) Engineering transgenic tobacco overexpressing PON1 through chloroplast transformation for high-level transgene expression: Tobacco's chloroplast was modified using chloroplast genetic engineering method and the transgenic tobacco plants were investigated for PON1 protein activity, CPS uptake, and degradation (Chapter 5).

1.4 Research objectives

The objectives of this research included:

a) to investigate plants' ability to take up CPS from hydroponic solution,

b) to overexpress mammalian genes, which are involved in CPS metabolism, in transgenic plants,

c) to test the transgenic plants for increased tolerance and CPS removal, compared to wild-type plants, and

d) to test if chloroplast transformation can increase expression of foreign genes in regenerated plants.

These four objectives are explained in Chapter 3, 4, and 5. Chapter 2 is a detailed description of the materials and methods used.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chlorpyrifos Pestanal® (CPS, 99.2 % purity) and 3,5,6-trichloro-2-pyridinol (TCP, 99.5 % purity) were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and Chem Service Inc. (West Chester, PA, USA), respectively. Both CPS and TCP solutions were prepared by dissolving the appropriate volumes in methanol (99.9 % purity) for 10 mg/ml stock solutions which were diluted serially in methyl *tert*-butyl ether (MTBE, ≥ 99.8 %, Sigma Aldrich) for standard solutions. Unless otherwise mention, All other chemicals were purchased from Fisher Scientific Inc. (Hudson, NH, USA) and all the enzymes from New England BioLabs Inc. (NEB; Ipswich, MA, USA).

2.1.2 Media

Two types of media were used for the plant growth: MS (Murashige and Skoog) medium and Hoagland solution, which are examples of the most popular nutrient solutions in plant tissue culture (Hoagland & Arnon, 1950; Murashige & Skoog, 1962). They are made from MS with vitamins mixture (Caisson Labs) and Hoagland's No. 2 basal salt mixture (Caisson Labs), respectively in general accordance with the manufacturer's instructions.

LB (Luria-Bertani) medium is a nutritionally rich medium that is primarily used for the growth of *E. coli* (Bertani, 1951). It is a mixture of 10 g/L Bacto-tryptone (BD Biosciences), 5 g/L Bacto-yeast extract (BD Biosciences), and 10 g/L sodium chloride (Sigma-Aldrich). The addition of 14 g/L Bacto-agar (BD Biosciences) to LB results in the formation of a gel that bacteria can grow on.

MGL medium is used for the growth of agrobacteria. It is prepared from 5 g/L Bacto-tryptone, 2.5 g/L Bacto-yeast extract, 5.2 g/L sodium chloride, 10 g/L mannitol , 2.32 g/L sodium

glutamate, 500 mg/L KH_2PO_4, 200 mg/L $MgSO_4 \cdot 7H_2O$, and 2 ug/L biotin. For MGL agar plates, 15 g/L Bacto-agar was added.

2.2 Laboratory protocols

2.2.1 Extraction

2.2.1.1 Plant genomic DNA extraction

Plant genomic DNA was isolated from plant leaf tissue using the MasterPure Plant Leaf DNA Purification Kit (Epicentre). Frozen leaf samples (about 100 mg) were ground using a handheld micro-grinder (Research Products International Crop.) and followed by the Kit protocol. Purified DNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and was kept in the freezer at -20 °C until further analysis of PCR, restriction enzyme digestion, or ligation.

2.2.1.2 Plant RNA extraction

Total RNA was extracted from plant leaf tissue using Trizol reagent (Invitrogen) according to Chomczynski & Mackey (1995). Snap-frozen leaf samples (approximately 100 mg) were ground under liquid nitrogen, homogenized in 1 ml Trizol per 100 mg tissue, incubated for 5 min at room temperature, mixed with 200 µl chloroform (Invitrogen) per 1 ml Trizol, vortexed and incubated for 2-3 min, and centrifuged for 15 min at 2-8 °C to separate the mixture into three phases. The upper aqueous phase was transferred carefully and mixed with 500 µl isopropanol (Invitrogen) per 1 ml of Trizol to precipitate the RNA, incubated for 10 min at 15-30 °C, and centrifuged for 10 min at 2-8 °C. For RNA washing, the supernatant was removed and the RNA pellet was washed with 1 ml 75 % ethanol, vortexed and centrifuged for 5 min at 2-8 °C. After repeating the washing procedure, the RNA pellet was air-dried for 2-3 min, and the RNA was dissolved in DEPC-treated water (Life Technologies). RNA yields were quantified using the

spectrophotometer and the RNA was kept in the freezer at -80 °C until further analysis of RT-PCR.

2.2.1.3 Plant protein extraction

Total soluble plant protein was extracted according to Singh, Ding, & Daniell (2009). Leaf material (100 mg) was ground in liquid nitrogen and 200 µl of freshly prepared plant extraction buffer (PEB) -100 mM NaCl, 10 mM EDTA (pH 8), 200 mM Tris-HCl, pH 8, 0.05% Tween-20, 0.1% SDS, 14 mM β-mercaptoethanol (BME), 200 mM sucrose, and 2 mM phenyl methyl sulfonyl fluoride (PMSF)- was added on ice. The leaf tissue was homogenized using a handheld micro-grinder for 5 min by keeping the samples on ice to prevent overheating, centrifuged at 10,000 ×g for 5 min at 4℃, and proceeded the super natant directly to protein quantitation using the spectrophotometer and was kept in the freezer at -80 °C until further analysis of Western blot or protein activity assay.

2.2.1.4 Bacterial protein extraction

Bacterial protein was extracted using B-PER II bacterial protein extraction reagent (Thermo Scientific) in general accordance with the manufacturer's instructions. Total 20 ml of bacterial culture in LB broth was centrifuged at 5,000 ×g for 10 min, 2 ml reagent per gram of cell pellet was added, homogenized by pipetting, incubated 10-15 min, and centrifuged at 15,000 xg for 5 min to separate soluble proteins from the insoluble proteins. The supernatant was then used for the protein quantitation and was kept in the freezer at -80 °C until further analysis of Western blot or protein activity assay.

2.2.2 Molecular work

2.2.2.1 Polymerase chain reaction (PCR)

PCR was performed on DNA extracts in a final volume of 25 µl contained final concentrations of 1X PCR Pre-Mix buffer G (Epicentre), 160 ng of forward and reverse primers, 5 units of Taq DNA polymerase (NEB), and 1 µl of template DNA. The reaction mixture was held at 98 °C for 5 min followed by 34 cycles of amplification at 94 °C for 1 min (denaturation), 50 °C for 1 min (annealing), and 72 °C for 1 min (extension), with a final step of 72 °C for 10 min (final extension) in Thermal Cycler (Bio-rad Labs). The size of PCR products is determined by comparison with a DNA ladder (Invitrogen), which contains DNA fragments of known size, run on the 0.7 % agarose gel (1X TAE buffer) alongside the PCR products.

2.2.2.2 Reverse Transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a sensitive method for the detection of mRNA expression levels. RT-PCR was performed using OneStep RT-PCR Kit (Qiagen) to detect RNA expression levels. Using this kit, total RNA extracts were used as a template for first-strand cDNA synthesis by reverse transcription and subsequently, the newly synthesized cDNA was amplified using traditional PCR with gene-specific primers. A final volume of 50 µl contained 200 ng of total RNA, 240 ng of forward and reverse primers, 2 µl OneStep RT-PCR Enzyme Mix, 1X OneStep RT-PCR Buffer, 2 µl dNTP Mix, and RNase-Free water. Thermal cycler conditions used were: 30 min at 50 ℃ (reverse transcription), 15 min at 95 ℃ (ini tial PCR activation) followed by 40 cycles of amplification 40 s at 94 °C (denaturation), 40 s at 50 °C (annealing), 1 min at 72 °C (extension), and finally extending for 10 min at 72 °C. The size of RT-PCR products was determined by gel electrophoresis and RNA expression levels were determined relatively by band intensity.

2.2.2.3 Molecular cloning

The DNA fragments were cloned into desired expression vectors as follows. The size-confirmed PCR products were digested with restriction enzymes (NEB) for which the recognition sequences flank the gene of interest at 37 ℃ for 1 -16 hours ("Restriction enzyme digestion"). The digestion products were run on an agarose gel and the DNA was extracted from the target band on the gel using the QIAquick Gel Extraction Kit (Qiagen) ("Purification"). The DNA was then incubated at 16 ℃ overnight with T4 DNA ligas e (NEB) and the desired dephosphorylated vectors that were also cleaved by the same restriction enzymes used for the digestion of insert genes ("Ligation"). The ligation mix was used to transform DH5α competent cells (Invitrogen) by heat shock ("*E. coli* transformation"), and plated on selective medium. Plasmid DNA was extracted from *E. Coli* using QIAprep Spin Miniprep Kit (Qiagen) ("Plasmid DNA extraction"). Restriction enzyme digestion of newly extracted plasmid DNA was performed in order to check the success of ligation, and insert-confirmed plasmid DNA was processed for sequencing.

2.2.2.4 Sample sequencing

The inserted genes were sequenced using gene-specific primers by the University of Washington Biochemistry Department Sequencing Facility (BDSF) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI3730 XL sequencer (Applied Biosystems).

Since BDSF shut down on April, 2013, sequencing has been conducted by Genewiz Inc. A mixture of DNA template and gene-specific primers were prepared according to its guidelines. DNA sequences obtained were assembled using the FinchTV v1.4 (Geospiza Inc.) as a chromatogram viewing software and analyzed for homology based on known DNA sequence using BLAST (Basic Local Alignment Search Tool). When 100 % identity of the inserted genes

with the original DNA sequences was confirmed, 33 % glycerol stocks were prepared and stored in the freezer at -80 °C for long-term storage.

2.2.2.5 Western blot

SDS-polyacrylamide gel electrophoresis (PAGE) was used to separate the recombinant proteins, followed by transferring the proteins onto nitrocellulose membranes. Prior to loading on a 12 % precast polyacrylamide gel (Bio-Rad), protein samples were denatured in a loading buffer (Laemmli sample buffer, Bio-Rad) containing SDS 2.1 % and glycerol 26.3 % (w/v). The premixed electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3) (Bio-Rad) was used as a running buffer. Prestained molecular weight markers (Precision Plus Protein All Blue Standards, Bio-Rad) were loaded on each gel. SDS-PAGE was performed at room temperature at 200 V for 35 min using Mini-PROTEAN electrophoresis cell system (Bio-Rad). One gel was stained with Imperial^{TM} Protein Stain (Thermo Scientific) by a procedure suggested by the manufacturer, and another was further processed for the Western blot.

When the gel for the Western blot was done, the protein profile was electrophoresed and blotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at constant 100 V for 1 h with transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) using the transfer box (Mini Trans-Blot Module, Bio-Rad). Electro-transfer onto a membrane was carried out at room temperature, but the ice pack was used to prevent it from overheating.

When finished transferring to membrane, the membrane was washed (2x) with pure water for 5 m each, followed by Invitrogen Western Breeze protocol. The membrane was first incubated in Blocking solution for 30 m and then incubated with mouse anti-His (1:250, Invitrogen) for 1 h as primary antibody. Following wash with Antibody Wash solution, the membrane was incubated with secondary antibody solution (anti-mouse) for 30 m as secondary antibody. After a final step

washing in Antibody Wash solution, BCIP/NBP chromogenic substrate from the kit was used for protein visualization.

2.2.3 Plant transformation

2.2.3.1 Agrobacterium-mediated plant transformation

Agrobacterium-mediated transformation is the easiest and most common plant transformation. In this method, *Agrobacterium* transfers a specific piece of its DNA into plant cells. The hybrid poplar clone INRA 717-1B4 (*P. tremula* X *P. alba*) was transformed with *Agrobacterium tumefaciens* using standard protocols (Han *et al*., 2000) as follows: plant tissue was cut into small pieces and co-cultured with *Agrobacterium* containing the gene of interest, the co-cultured poplar leaves were placed on callus induction medium (CIM; MS medium supplemented with 10 µM NAA and 5 µM 2-iP) with 100 µM acetosyringone for 2 days in the dark, the adhering bacteria were removed by washing with sterile water (4x) and washing solution (1x) (half-strength MS broth supplemented with 250 mg/L cefotaxime, 500 mg/L vancomycin, and 500 mg/L timentin), the washed leaf disks were transferred to CIM with appropriate antibiotics for 2 weeks in the dark, to shoot induction medium (SIM; MS medium supplemented with 0.1 µM thidiazuron) with kanamycin (100 mg/L) for 2-3 months in the light, to shoot elongation medium (SEM; MS medium supplemented with 114 µg/L BAP) with appropriate antibiotics until stems are long enough for removal from disk, and to rooting medium (RM; MS medium supplemented with 1 µM IBA and 0.1 % PPM) with appropriate antibiotics. After the antibiotic-resistant shoots were regenerated, the shoots were analyzed for transgene integration.

2.2.3.2 Plant transformation by particle bombardment

Tobacco chloroplasts were transformed by particle bombardment, which is a commonly used method for genetic transformation of plants and other organisms. In this technique, millions of

DNA-coated metal particles are shot at target cells or tissues using a biolistic device or gene gun (Kikkert, Vidal, & Reisch, 2004). Particle delivery system (PDS) 1000 He (Bio-Rad) was used to deliver the DNA-coated gold particles to tobacco leaf tissue. The exact protocol was adopted from Singh, Ding, & Daniell (2009).

2.2.3.3 *Agrobacterium*-mediated infiltration

Transient gene expression (a temporary expression of a gene by the non-permanent presence of a foreign gene) was performed using *Agrobacterium*-mediated infiltration method. The electro-competent *Agrobacterium* cells GV3101 harboring the target vectors were used for *Agrobacterium*-mediated infiltration with leaf explants of *Nicotiana benthamiana*, a close relative of tobacco. GV3101 cells harboring p19, the binary pART27 plasmid (null vector control), and the pART27 derivative pART27-hisPON1 (his-tagged PON1 in a plant expression vector) respectively were cultured overnight in 4 ml LB media with antibiotics. 500 µl of the overnight cultured cells were transferred to 10 ml LB, 10 mM MES and 20 µM acetosyringone were added, and cultured overnight again. The cell was centrifuged, supernatant was removed, the pellet was re-suspended in 11 ml infiltration solution (10 mM MES, 0.1 mM acetosyringone, and 10 mM $MgCl_2$), the final cultures were diluted to a final OD600 of 1.0, two 10 ml solutions (5 ml GV/p19 + 5 ml GV/pART27, 5 ml GV/p19 + 5 ml GV/pART27-hisPON1) were used for the infiltration into the leaf tissue. Three days after infiltration, agro-infiltrated leafs were harvested and were treated for the protein extraction.

2.2.4 Extraction of chlorinated compounds from samples

2.2.4.1 Extraction for GC analysis

To extract CPS from the hydroponic solution, 4 ml of the solution were sampled and added deep into a 15-ml amber vial containing 2 ml 10 % NaCl and 5 ml MTBE. The vials were

inverted repeatedly for one minute, and 1 ml of the MTBE layer was removed and analyzed by gas chromatography (GC). For the analysis of TCP, an additional 1 ml of the solution was filtered using a 0.22 µm porosity nylon syringe filter (Restek, Bellefonte, PA) and analyzed by HPLC (high performance liquid chromatography) instead, since TCP was not detectable by GC.

Plants were subjected to extraction of the chlorinated compounds as described by Shang *et al*. (2001). All plant samples were washed out with sterile water to remove CPS remaining on the surfaces of tissues. Plants were ground in liquid nitrogen, and transferred to chilled 25-ml glass centrifuge tubes (Corex). Two ml of 1 N H_2SO_4/10 % NaCl solution was added and the tubes were shaken vigorously for 1 min. After 5 ml of MTBE was added, the aqueous extract was clarified by centrifugation at 8,000 rpm for 10 min. The 3.5 ml of MTBE layer was transferred to 15-ml amber vials containing 2 g Na_2SO_4 and incubated at room temperature for 1 h. 1 ml of the MTBE layer was removed and run on the GC with a set of CPS standard solutions. An additional 1ml of the MTBE was concentrated using a nitrogen evaporator (Organomation, Berlin, MA). The residue was redissolved in methanol and analyzed with a set of TCP standard solutions by HPLC.

2.2.4.2 Extraction for HPLC analysis

For the analysis of chlorinated compounds by HPLC, certain amount of acetonitrile was added to samples (liquid, soil, and plant), vortexed 1 min, centrifuged at 10,000 rpm for 5 min (2x), filtered using a 0.2 µm porosity PTFE syringe filter (VWR), and analyzed by HPLC. For liquid samples, the equal volume of acetonitrile was mixed with the hydroponic solution. Plant samples were separated into leaf, stem, root tissue followed by grinding with liquid nitrogen, and 5 ml acetonitrile was added to ground plant tissue. 5 ml acetonitrile was added to dried soil samples (approximately 200 mg) in triplicate. Spike sample analysis (or known addition) was used to test the method at varying concentrations of analyte.

2.3 Data analysis and statistical methods

2.3.1 GC (gas chromatography) analysis

The concentrations of CPS were analyzed using gas chromatography (Perkin-Elmer Clarus 500) with an electron capture detector (GC-ECD). The method for GC described below was modified from Rogers, Clark, and DiVincenzo (2006). Chromatographic separations were achieved on a PTE-5 capillary column (30 m × 0.32 mm x 0.32 μm film thickness) from Supelco (Bellefonte, PA). The temperature of injector and detector were maintained at 230 ℃ and 300 ℃, respectively. Helium was used as the carrier gas with a flow rate of 2.0 ml/min. The argon/methane (95/5) was used as the make-up gas with a flow rate of 60 ml/min. Initial oven temperature was 80 ℃ for 6.5 min, then programmed at 30 ℃/min to 250 ℃, then 15 ℃/min to 280 ℃, and lastly 30 ℃/min to 300 ℃ and held for 5 min. Under these conditions, the total run time was 19.8 min, and the retention time for CPS was about 14 min. The standard curves of pure CPS in MTBE were used to quantify the compounds.

2.3.2 HPLC (high performance liquid chromatography) analysis

HPLC analysis I (used for Chapter 3): HPLC was used to quantify TCP concentrations in cultures. The Waters modular HPLC system (Waters, Milford, MA) consisted of a Waters 717+ autosampler, two Waters 515 HPLC pumps, and a Waters 9926 photodiode array detector. The mobile phase consisted of 40:60 water acetonitrile mix run at an isocratic flow rate of 1ml/min, on a Waters C18 column (4.6 x 250 mm) set, with concentration determined based on absorbance at 220 nm. The chromatographic data was collected in Waters Millennium32 software and peak integration and analysis were conducted using the software. Under these run conditions, the TCP peak retention time was 3.4 min. and the detection limit was 0.1 mg/L.

HPLC analysis II (used for Chapter 4 & 5): Shimadzu HPLC binary system (Shimadzu, Columbia, MD) equipped with UV was used on Kinetex C18 column (150 x 4.6 mm) set, which is specific for pesticide analysis. The exact chromatographic conditions were adopted from Abu-Qare & Abou-Donia (2001).

2.3.3 Statistics

All statistical analyses were performed by using one-way Analysis of Variance (ANOVA) with Tukey's HSD (honestly significant difference) post hoc analysis. An ANOVA p-value greater than 0.05 (i.e., 95% confidence interval) was used to indicate that the sample sets were not significantly different from each other.

Chapter 3[1]

Uptake and Degradation of Chlorpyrifos by Poplar and Willow

3.1 Introduction

Phytoremediation has been shown to be useful in the removal of CPS. However, CPS remediation studies have been limited to plant-associated microorganisms or herbaceous plants (See section 1.3.1). The hypothesis of this study was that CPS may be removed and degraded by woody plants, which offer some distinct advantages for treatment of contaminated sites compared to herbaceous plants, such as high biomass, an extensive root system, long-loved (80-100 years), and so on.

There were two research questions. Because the removal of CPS by plants is based on the ability of plants to take up CPS into tissues, the first question was whether the woody plants such as poplar and willow are able to take up CPS from hydroponic solution. The second question was what happens to CPS once it is taken up by plants. In this study, woody plants with phytoremediation capabilities for removing CPS from hydroponic solutions were reported. To this end, the ability of *Populus* and *Salix* spp. to take up CPS, the levels of CPS accumulated in plant tissues, as well as plant metabolism of CPS were examined in detail. This work represents the first report for phytoremediation of CPS using poplar and willow plants.

[1] Material in this Chapter has been published (Lee, Strand, & Doty, 2012).

3.2 Experimental design

3.2.1 Plant materials

Seven clones of *Populus* and *Salix* spp. were selected for the study of CPS uptake from hydroponic solution. The full lists of plants are shown in **Table 2**. *In vitro*-grown, 50- to 60-day-old plantlets maintained on MS medium served as explants sources, and they were washed with sterile water to aseptically remove traces of agar before being transferred for the CPS treatment. Among the seven clones, the hybrid poplar clone 'INRA 717-1B4' was chosen for the rest of the experiments because of its fast and easy propagation through tissue culture and its successful utilization in previous phytoremediation experiments (Pilon-Smits *et al*., 1998; Doty *et al*., 2007). All of the plant materials were incubated at 24 ℃ with a 14-h photoperiod in a growth chamber (Percival).

Table 2 The plants tested for CPS uptake study

Plant line	Scientific name	Country of origin
INRA 717-1B4	Populus tremula x P. alba	France
Nisqually-1	P. trichocarpa	USA
SX61	Salix sachalinensis	Asia
SX64	S. miyabeana	Asia
SX67	S. miyabeana	Asia
SV1	S. dasyclados	Europe
94006	S. purpurea	Europe

3.2.2 Phytotoxicity of CPS

To evaluate the toxicity of CPS, poplar clones 'INRA 717-1B4', in triplicate, were exposed to various concentrations of CPS (Figure 2). Plant cuttings were placed in sterile 40-ml clear Volatile Organics Analysis (VOA) vials containing 10 ml of MS broth. The liquid concentrations

of CPS were 0, 25, 50, 75, 100, 150, 200, 250, 300, and 350 mg/L (0, 0.07, 0.14, 0.21, 0.29, 0.43, 0.57, 0.71, 0.86, and 1.00 mM), respectively. Health of the plants was monitored visually.

3.2.3 CPS uptake by plants

To evaluate plant potential for uptake of CPS, three plants of each species were place in sterile 40-ml clear VOA vials containing 17 ml of MS broth and capped with septum valve caps (Mininert). For controls, there were additional six vials containing MS broth without plants. CPS was added to the solution to a final concentration of 25 mg/L through the mini-nert valves using a glass gastight syringe (Hamilton, Reno, NV). All vials were incubated under a 14-h photoperiod for 7 days, but three unplanted vials were covered with aluminum foil to prevent light penetration in order to determine if there is a significant difference in the abiotic CPS degradation between under light and dark treatment. The 4 ml of liquid samples were removed from the hydroponic solution in each vial at intervals of 0, 3, and 7 days for analysis of the concentrations of CPS.

3.2.4 CPS metabolism in plants

In order to determine whether plants metabolize CPS, poplar clones ‘INRA 717-1B4’ were treated with CPS, and the amount of CPS remaining in plants, in quintuplicate, was monitored over a time course (5 weeks). A total of 25 plants were placed in sterile 125-ml clear glass jars containing 20 ml of MS broth. CPS was added to the solution to a final concentration of 20 mg/L. At the end of 7 days, one set (5 plants) of plant samples were analyzed for the CPS extraction, and the rest of the plants were moved to medium devoid of CPS. CPS was extracted from the other set of plant samples every week. In order to localize the CPS within different plant tissues, stem, leaf, and root tissues were extracted separately.

3.3 Results

3.3.1 Phytotoxicity of CPS

Cuttings of hybrid poplar clone ‘INRA 717-1B4’ were exposed to a range of concentrations (25-350 mg/L) of CPS. After seven days of exposure, cuttings exhibited significant blackening of the mesophyllic (non-vein) leaf tissue, and mortality at CPS concentrations of 150 mg/L and higher (Figure 2). After another seven days, the plant cuttings at 100 mg/L also showed mortality and the cuttings at 75 mg/L were dead after another four weeks. The highest non-lethal dose of CPS was 50 mg/L for six-week exposure.

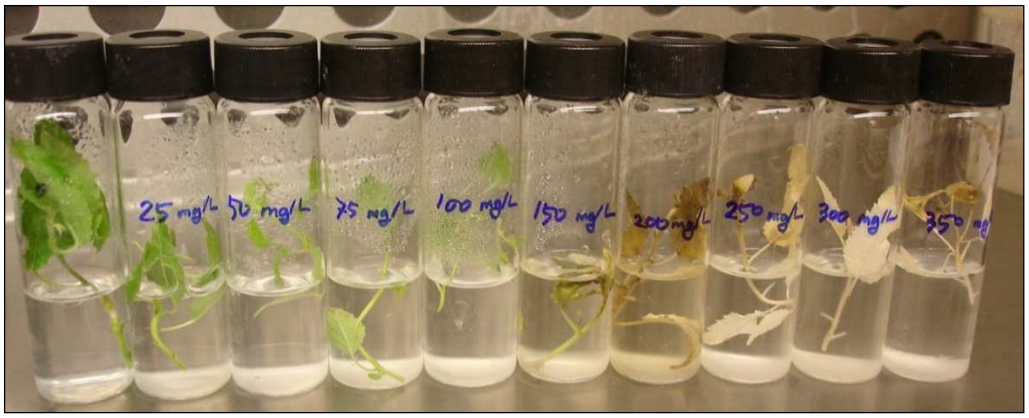

Figure 2 Small unrooted cuttings, in triplicate, taken from the apical stem of hybrid poplar clone ‘INRA 717-1B4’, were exposed to various concentrations of CPS: 0, 25, 50, 75, 100, 150, 200, 250, 300, and 350 mg/L (from left to right). The highest non-lethal dose of CPS was 50 mg/L for six-week exposure. Shown is representative photograph of plants for each treatment.

3.3.2 CPS removal

To study whether CPS was taken up by plants from a nutrient solution, the concentration of CPS in the liquid growth medium was monitored. During the 1-week growth, there were no adverse effects of CPS exposure on plant growth and appearance. The results indicated that CPS was taken up from the nutrient solution by the plants (Figure 3 and Table 3), and the

uptake of CPS varies among different plants. While a trace amount of CPS was lost from the unplanted vials, removal of CPS from the planted vials was 2-10-fold greater. Of the initial dose, 46.0 ± 2.9 % and 34.4 ± 0.0 % was removed by SX61 and 94006 clones, respectively. The best performing line, SX64, removed CPS with the highest uptake rate of 21.3 ± 2.1 µg of CPS·day^{-1}·g^{-1} plant wet weight. There was a significant difference between three clones (INRA 717-1B4, Nisqually-1, and SX61) and SX64 at the 5% significance level ($p = 0.011$). There was no significant difference between light and dark controls. From the analysis of the nutrient solution, it was apparent that the total quantity of CPS in the nutrient solution decreased with time.

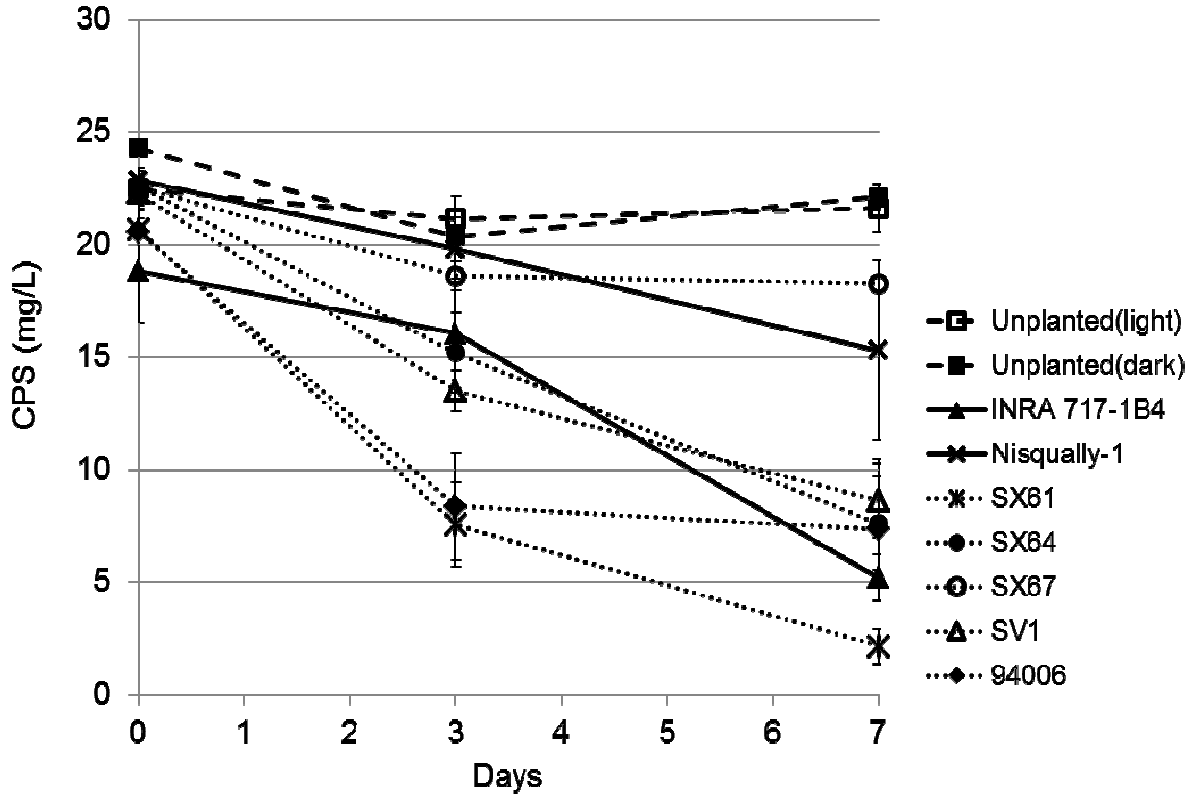

Figure 3 The decrease of CPS in hydroponic solution. The concentrations of CPS in the hydroponic solution were monitored for seven days. CPS was taken up from the nutrient solution as the plants were being grown. The data are shown as the mean ± SEM from three samples. The solid lines correspond to two poplar clones, dashed lines unplanted vials, and dotted lines five willow clones.

Table 3 Removal of CPS from hydroponic solution by plants for 7 days

Plant line	% removal*	Removal rate
Unplanted (under light)	8.4 ± 3.4	**5.08 ± 2.07
Unplanted (under dark)	5.6 ± 0.0	**3.38 ± 0.00
INRA 717-1B4	25.7 ± 2.2	***11.44 ± 1.53[a]
Nisqually-1	16.1 ± 7.1	***10.65 ± 1.85[a]
SX61	46.0 ± 2.9	***11.58 ± 1.96[a]
SX64	33.4 ± 5.7	***21.27 ± 2.09[b]
SX67	11.8 ± 1.7	***15.17 ± 2.16[ab]
SV1	32.8 ± 3.2	***15.45 ± 1.40[ab]
94006	34.4 ± 0.0	***18.86 ± 2.26[ab]

* Data is not normalized to plant mass.

** Micrograms of CPS per day ± SEM (standard error of the mean)

*** Micrograms of CPS per day per gram of fresh weight ± SEM

The data are shown as the mean ± SEM from three samples.

Different letters denote significant differences at $P < 0.05$.

3.3.3 CPS accumulation in plant tissue

After 1 week of exposure to CPS, a total of twenty one plant samples (seven clones in triplicate) were analyzed for the uptake or removal of CPS from hydroponic solution. The results show that considerable amounts of CPS accumulated in all plants (Figure 4), and the bioaccumulation of CPS varied among different plants. Higher (17 %) CPS accumulation was measured in SX64, which removed CPS with the highest uptake rate, than in Nisqually-1 ($P < 0.05$). The clone that accumulated the most CPS per gram of tissue was SX67, and higher (68-83 %) CPS accumulation occurred in SX67 compared to INRA 717-1B4, SX61, and SV1. The mean % of CPS accumulation in plants was about 57 to 208 % of the total removal (Figure 5). 57 % of the CPS removed from hydroponic solution was accumulated in SX61, suggesting that SX61 showed the most degradation capability of CPS.

To determine whether plants accumulate CPS in shoot or in root, the distribution of CPS among individual tissues was investigated for the poplar clone 'INRA 717-1B4'. After 7 days of CPS exposure, the plants were harvested, and shoots and roots were analyzed separately. CPS was nearly equally divided between shoots and roots (Table 4). However, levels of CPS normalized to gram of tissue showed that roots have 155.1 % higher CPS contents than shoots and the difference was significant (P=0.029) (Figure 6). We propose, therefore, that some amounts of CPS taken up by roots were translocated to upper plant biomass in poplar and that a much higher concentration of CPS was shown in roots than in shoots.

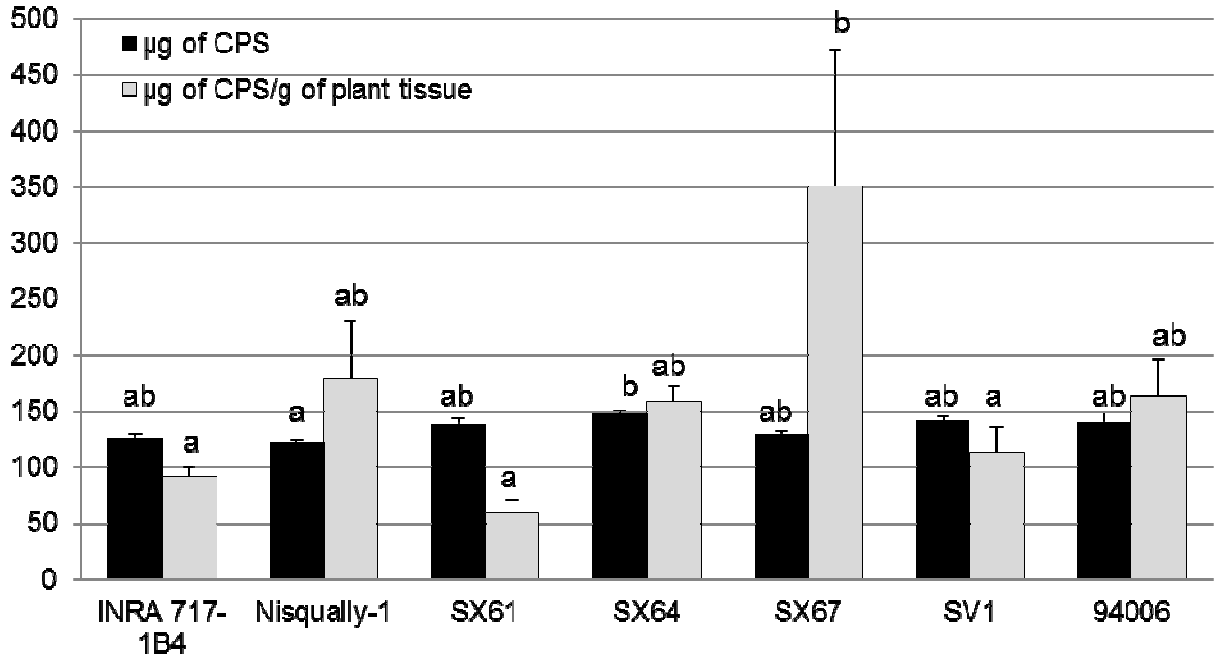

Figure 4 The amounts of CPS accumulated in plant tissue. After 1 week of exposure to CPS, all of the plant explants were ground and treated for the CPS extraction from plant tissue. The results show that considerable amounts of CPS were accumulated in plant tissue. Dark-colored column: microgram of CPS per plant; light-colored column: microgram of CPS normalized to plant weight. The data are shown as the mean ± SEM from three samples. Different letters denote significant differences at $P < 0.05$. P values for the microgram of CPS and microgram of CPS$\cdot g^{-1}$ are 0.03 and 0.01, respectively.

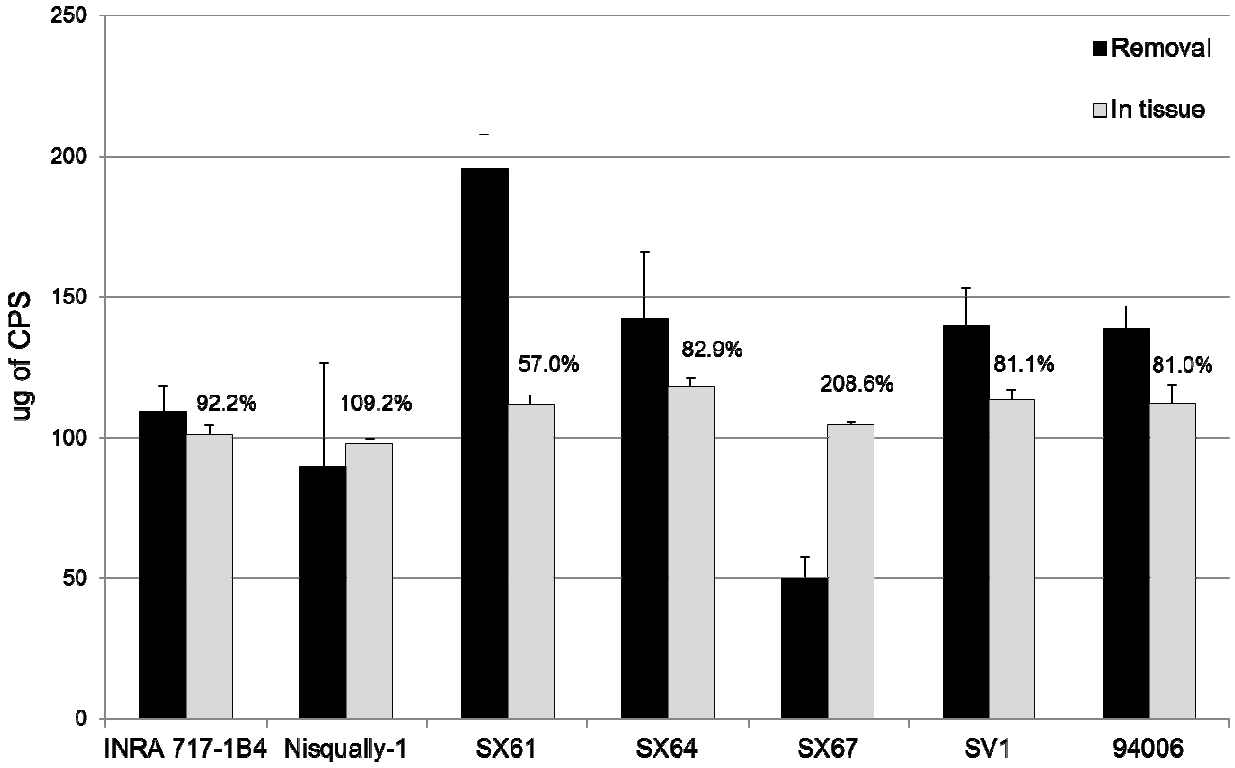

Figure 5 Removal vs accumulation in plant tissue. The mean % of CPS accumulation in plants was about 57 to 208 % of the total removal. The data are shown as the mean ± SEM from three samples. * %= accumulation of CPS (µg) in plant tissue / removal of CPS (µg) from hydroponic solution * 100

Table 4 The distribution of CPS (µg) in shoots and roots of hydroponic poplar clone 'INRA 717-1B4', in quintuplicate, exposed to CPS for 1 week

Plant number	Shoot	Root
1	19.28	17.48
2	25.57	24.36
3	22.12	23.46
4	28.42	17.42
5	19.18	24.26

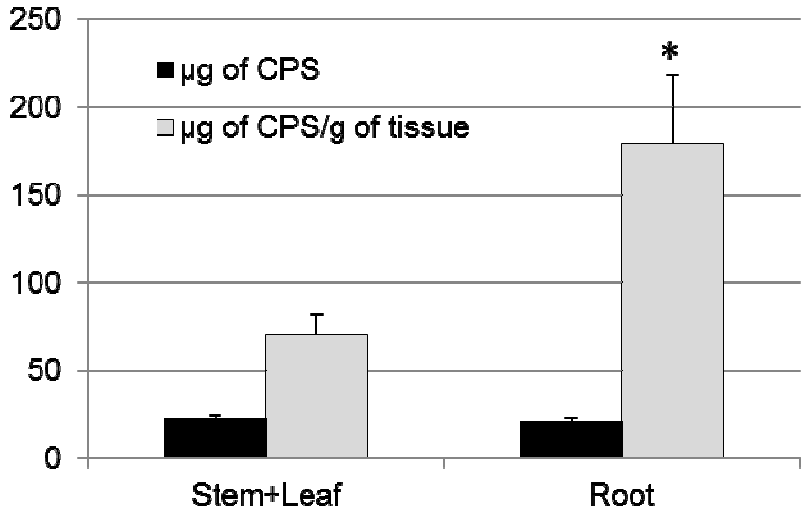

Figure 6 The distribution of CPS among plant tissues. After 1 week of exposure to CPS, the amount of CPS accumulated in plant tissue was investigated from shoots and roots separately from a poplar clone 'INRA 717-1B4'. Roots accumulated much higher concentrations of CPS than did shoots. Dark-colored column: microgram of CPS per plant tissue; light-colored column: microgram of CPS normalized to plant weight. The data are shown as the mean ± SEM from five plant samples. Significant difference ($P < 0.05$) is indicated by asterisk (*).

3.3.4 The fate of CPS in plant tissue

During the 1-week growth, no visual symptoms of CPS toxicity were observed for the poplar plants growing in media with 25 mg/L CPS. During the following 4-week growth, after the plants were transferred to media devoid of CPS, most plants showed enhanced plant growth with auxiliary shoot proliferation (data not shown). Figure 7 shows that the amount of CPS decreased dramatically within the hydroponic poplar plants. The total amounts of CPS (µg) decreased at a rate of 40.1 % over the first week, 61.7 % over the second week, 55.2 % over the third week, and 84.3 % over the fourth week. Overall, the results indicated that 97.7 % (R), 94.8 % (S), and 99.1 % (L) of CPS accumulated in plants were metabolized within the plant tissue. Comparing the five time periods for a given tissue, the difference in all three plant tissues was statistically significant ($P < 0.000$). Comparing the three different plant tissues for a given time period, the difference between each time point decreased with time, and there was no

significant difference in week 5 (W5) when compared CPS remaining in root, stem, and leaf (P = 0.276). Importantly, there was no CPS released back to the nutrient solution from plants (data not shown).

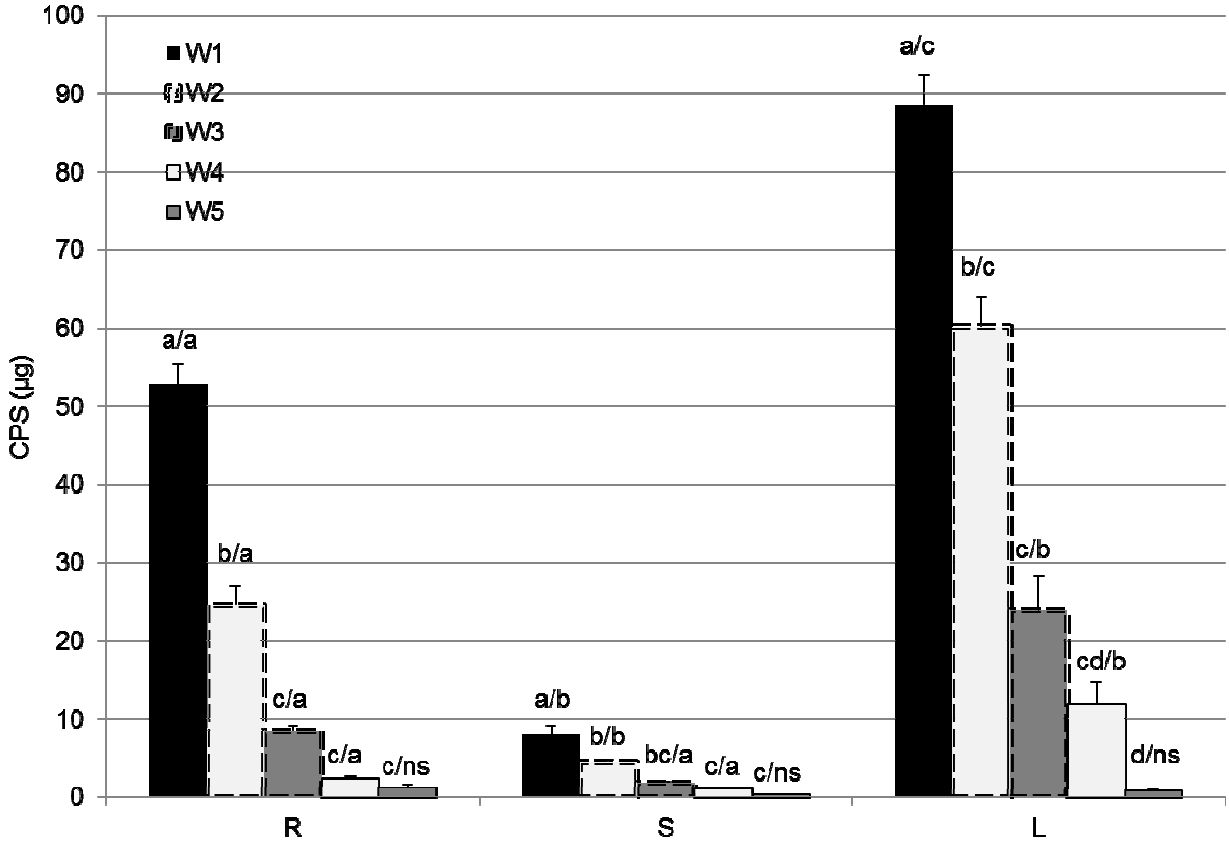

Figure 7 The amounts of CPS remaining in hydroponic poplar plants 1 (W1), 2 (W2), 3 (W3), 4 (W4), and 5 weeks (W5) after CPS dose. Plants 'INRA 717-1B4' were treated with CPS for 7 days, then moved to media devoid of CPS for 4 more weeks. CPS was extracted from root (R), stem (S), and leaf (L), separately at each time point. As the results show, CPS did not persist in the plants, suggesting further metabolism of CPS in plant tissue. The data are shown as the mean ± SEM from five plant samples. Different letters denote significant differences at $P < 0.05$ (first letter: five time periods for a given plant tissue; second letter: three parts of plant for a given time period); ns denotes non-significant difference at the 0.05 level. P values for the microgram of CPS are 0.000 (R, S, L, W1, and W2), 0.001 (W3 and W4), and 0.276 (W5).

3.4 Discussion

Since CPS is an insecticide, it was not expected to be toxic to plants. Surprisingly, CPS has been shown to adversely affect a variety of plants including alfalfa (*Medicago sativa*), clover (*Melilotus alba* and *Trifolium pratense*) (Smith, Funke, & Schultz, 1978), *Pinus halepensis* (Olofinboba & Kozlowski, 1982), and *Arabidopsis thaliana* (Aben, Houx, & Leistra, 1992). However, information is limited on CPS toxicity to plants compared to a number of animal toxicity studies. It is well known that CPS and CPO are directly toxic to the nervous system in insects and other animals primarily by inhibition of cholinesterase (ChE). Interestingly, ChE has also been found in plants. ChE has been isolated from mung bean roots (Riov & Jaffe, 1973), and the ChE activity assay in 118 plant species confirmed widespread distribution of ChE in the plant kingdom (Gupta & Gupta, 1997). The ChE from plant tissues is not identical with any animal ChE but shows similarity in several important properties to ChE. In addition, CPS exposure has also been shown to inhibit enzymes other than ChE in the laboratory animals (Cox, 1995). ChE may participate in several neurophysiological-like processes such as modulating hormone levels in plants, or binding to certain plant proteins. In addition, plants may be affected by CPS indirectly. Sardar and Kole (2005) conducted a laboratory experiment to evaluate the effect of CPS on the availability of the major plant nutrients (N, P, and K) in soil. Their results show that there was a significant decrease in the available N and P content in soil treated with CPS in comparison to the control set. Decrease in availability of these nutrients might be due to the inhibitory effect of CPS or its metabolites indirectly on microorganisms involved in mobilizing these elements, such as di-nitrogen fixing bacteria and phosphate solubilizing microorganisms or directly on the soil nitrification and the phosphatase enzyme in soil. We hypothesize, therefore, that CPS and its metabolites adversely affect plants directly by inhibition of ChE and other enzymes in plant tissues or soil, and also affects plants indirectly by limiting the uptake of nutrients through inhibiting microbial growth.

The uptake of CPS by herbaceous plants has been investigated previously. In general, it has been shown that negligible levels of CPS enter the plant via the roots, indicating its non-systemic nature. However, most studies have focused on CPS uptake by plants in soil (Smith *et al*., 1967a; Bauriedel, McKellar, & Miller, 1976; Bauriedel & Miller, 1986a, 1986b), and the results were similar (i.e., negligible uptake) to the results obtained in nutrient solution (Smith & Watson, 1964). While previous study have shown its uptake by plants to be insignificant, our results show that significant amounts of CPS were taken up from the nutrient solution by woody plants, poplar and willow. The fact that only living plants were capable of taking up CPS into its tissue, while CPS was released back from tissues to the media when plants were dead also supports the idea of CPS uptake by plants (data not shown). Further, not only rooted poplar cuttings but unrooted poplar cuttings removed CPS from nutrient solution to similar degrees (µg/g) (data not shown). Since pesticides' bioavailability and translocation in plant was found to be octanol-water partition coefficient (K_{ow})-dependent (Bromilow & Chamberlain, 1995), CPS, which is a very lipophilic compound with a higher K_{ow} (4.7~5.3), is expected to quickly cross biomembranes and then sorb to the roots (Burken & Schnoor, 1996; Trapp, 2000; Trapp, 2004). Once introduced inside the plant, CPS was distributed throughout the plant in this work as well as in other studies (Smith *et al*., 1967b). Further, in poplar plants dosed with CPS, accumulation of CPS was more pronounced in roots than in shoots on a per gram basis (Figure 6) and this finding corresponds to that of Azmat, Haider, and Riaz (2009). It has been observed that roots were important in accumulating compounds due to their direct exposure of toxic chemicals as underground parts, and transporting the compounds to aboveground organs (shoots) (Azmat, Haider, and Riaz, 2009).

The dramatic decline of CPS accumulated in plants (Figure 7) indicates further metabolism of CPS in plant tissue. It also demonstrates that the poplar clone 'INRA 717-1B4' is a very efficient plant material to use for phytoremediation due to the ability to biodegrade CPS. This

characteristic is an advantage since the harvested plants would not be considered hazardous waste as is the case for phytoremediation of non-degradable pollutants such as heavy metals. Judging by the increased growth of the plants following removal from CPS (data not shown), it can be assumed that the degradative products were not phytotoxic. It is important to know what happens to CPS when it enters the plant. Since pesticides taken up from soil or water are usually metabolized into less toxic or non-toxic products by several metabolic processes in plants (Laurent, Debrauwer, & Pascal-Lorber, 2006; Peuke & Rennenberg, 2005), we first hypothesized that 3,5,6,-trichloro-2-pyridinol (TCP), a primary metabolite of CPS (Racke, 1993) (Figure 1), might be detected in both hydroponic solution and plant tissue as CPS is taken up by plants and its concentration decreases in tissue with time. However, no TCP was found as CPS levels declined in hydroponic poplar plants. CPS may have degraded to TCP within the plant, and then further rapidly degraded either partially or completely in plant tissues to form unidentified metabolites. Interestingly, TCP is further mineralized in animals to carbon dioxide and water (Eaton *et al*., 2008), and plants fix this pesticide-derived carbon as natural, cellular materials (e.g., starch and cellulose) through anabolic activity (Racke, 1993). Metabolism also includes liberation of chloride and the formation of trivial amounts of several unidentified water soluble decomposition products (Smith *et al*., 1967b). Thus, further metabolism and mineralization of TCP may have occurred in plant tissue via unknown mechanisms. Another possible pathway for CPS degradation in poplar could result in unextractable compounds. Bauriedel and Miller (1986a, 1986b) conducted a series of studies with sugar beets and corn in CPS-treated soil and found that there were only traces of CPS and TCP (1-3 %) whereas the major residues (62-79 %) were unextractable products, indicating that the metabolites were in a conjugated form. Conjugated compounds are then usually sequestered into the vacuole or become part of cell wall material (Dietz & Schnoor, 2001).

3.5 Summary

This report demonstrates that poplar and willow trees, unlike the herbaceous plants in previous studies, have a significant ability to take up CPS and to translocate it within the plants and thus, assist in removing it from the hydroponic solution. CPS did not persist in the plants, suggesting further metabolism of CPS in plant tissue. To our knowledge, the poplar and willow species presented in this study were the first woody plants to be tested for potential phytoremediation applications for CPS. Since poplar and willow are able to efficiently take up CPS, there is the possibility of enhancing the CPS degradative potential by genetic manipulation of metabolism *in planta*. The research of enhancing phytoremediation of CPS using transgenic plants expressing enzymes, which are known to be involved in OP insecticide degradation pathways, are addressed in Chapter 4 and 5. The results presented here indicate that phytoremediation of CPS and other OP insecticides should be a fertile area for future development.

Chapter 4

Engineering Transgenic Poplar Overexpressing Paraoxonase 1 for Improved Degradation of CPS

4.1 Introduction

The majority of polluted sites have mixed pollutants, and mixtures of agricultural pesticides pose a potential threat to wetland and aquatic plants (Lytle & Lytle, 2001). CPS is commonly used in combination with other pesticides such as diazinon, atrazine, or carbaryl, which are phytotoxic. It may be more toxic in combination with other chemicals than individual use (Abou-Donia *et al*., 1996; Lee *et al*., 2004; Lytle & Lytle, 2002). The treatment of mixed contamination poses a challenge to environmental remediation technologies because of the vast difference in the physicochemical properties of the contaminants to be treated. Lytle and Lytle (2002) conducted a study showing that the presence of a pesticide mixture affected uptake of CPS by plants due to a higher variability in CPS. Plants might be tolerant to one type but killed by another type of pollutant. In addition, since the CPS is moved to foliage, the insecticide may have unintended consequences. Therefore there is a need to improve the tolerance of and degradation of CPS in the plants to make it degrade faster, preferably with multiple tolerances of mixed pollutants for the phytoremediation of CPS.

CPS is a degradable compound and there are two enzymes involved in CPS metabolism in mammals: cytochrome P450 (CYP) and paraoxonase 1 (PON1) (Figure 8). CPS can be activated by CYP through a desulfuration reaction to form a more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO), in mammals. CPS can also be detoxified by CYP through a dearylation reaction that degrades the parent compound, forming TCP, which in non-toxic (Chambers, 1992; Tang *et al*., 2001). CPO can be detoxified by PON1 through a hydrolysis

reaction to form TCP, and CPS can also be detoxified by PON1 through a hydrolysis reaction to form TCP (NPIC, 2013). Thus, both oxidative dearylation of chlorpyrifos by CYP enzymes and hydrolysis of CPS/CPO by paraoxonase contribute to the formation of TCP, which is the major chlorpyrifos metabolite. The hypothesis is that transgenic expression of genes involved in CPS metabolism is expected to increase tolerance, removal, and degradation of CPS. In other words, transgenic plants overexpressing CYP & PON1 would take up CPS and degrade CPS to CPO and TCP.

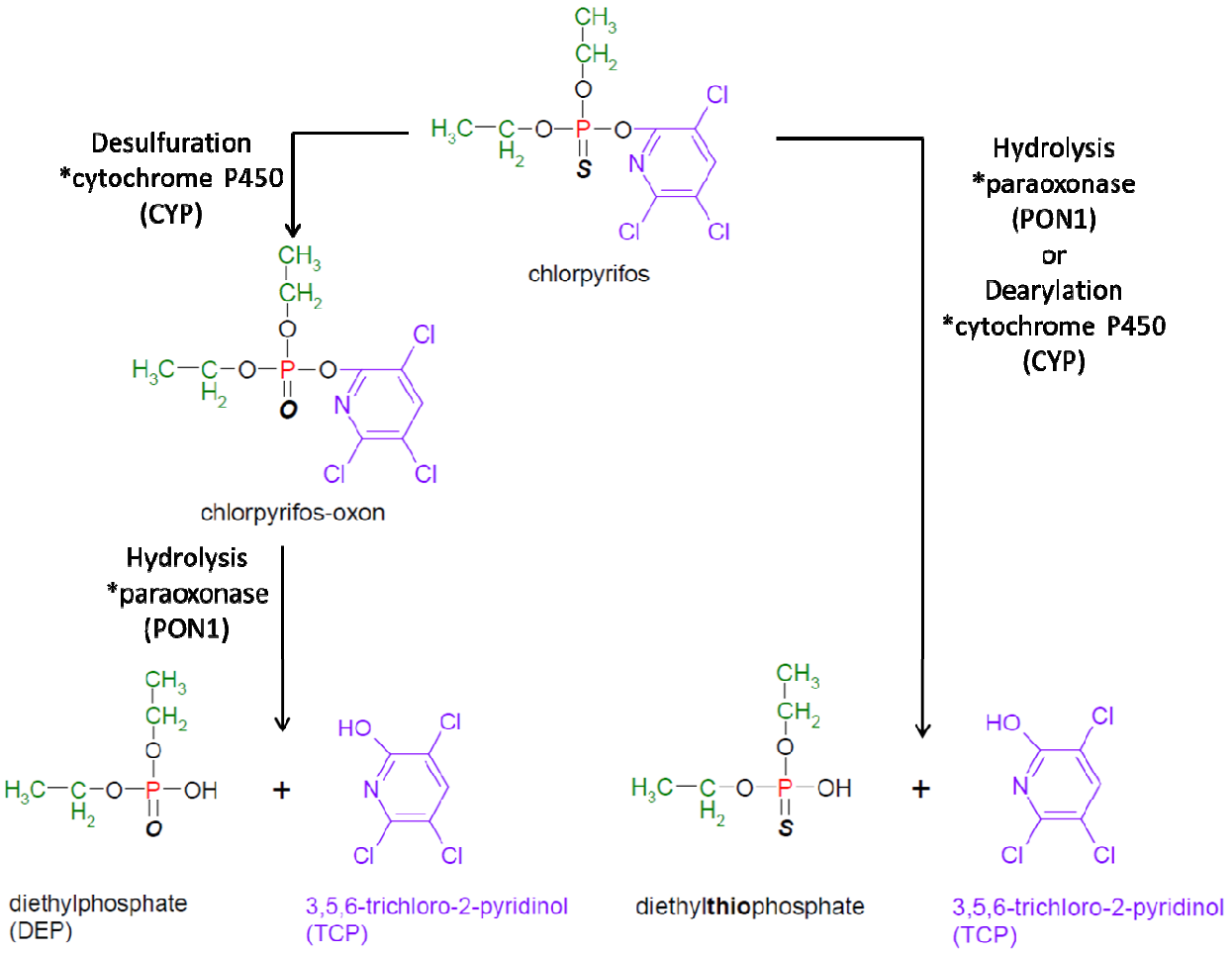

Figure 8 CPS metabolism by two enzymes, CYP and PON1, in mammals

4.1.1 CYP2B6 and phytoremediation

CYP superfamily is a large and diverse group of enzymes and there are hundreds of CYP genes in mammals and plants. A screen of several human CYP isoforms demonstrated that CYP 1A2, 2B6, 2C9, 2C19, and 3A4 were involved in CPS metabolism (Tang *et al*., 2001). Among them CYP2B6 was chosen for this study since CYP2B6 has the highest desulfuration activity, producing the oxon metabolite from CPS 3- to 5-fold more efficient than other isoforms (Buratti *et al*., 2003; Sams, Cocker, & Lennard, 2004; Tang *et al*., 2001). Recent studies have documented an important role for CYP2B6 in the metabolism of several pesticides and other environmental xenobiotics (Hodgson & Rose, 2007).

CYP2B6 has been used for phytoremediation studies with rice and herbicides (Hirose *et al*., 2005; Kawahigashi *et al*., 2005, 2006), potato and herbicides (Inui *et al*., 2000, 2001). When metolachlor, a herbicide, was added as twice (1.76 mg) or 4 times (3.52 mg) higher doses than those in practical use in cornfields, CYP2B6 rice plants showed healthy growth in the presence of high conc. of metolachlor, while the growth of control plants was inhibited (Kawahigashi, Hirose, Ohkawa, et al., 2005). When plants were incubated with metolachlor for 1 or 6 days, the mean amount of metolachlor in the CYP2B6 plants was 25% that of the control plants after both 1 day and 6 days of incubation. While the control plants were able to reduce the amount of metolachlor in the medium by 31%, the CYP2B6 rice plants reduced metolachlor in the medium by 51%. Therefore, the CYP2B6 rice plants removed metolachlor 1.65 times more effectively from the culture medium than nontransgenic plants for 6 days.

4.1.2 Paraoxonase 1 (PON1)

PON1 is a member of a family of proteins that also includes PON2 and PON3. PON1 hydrolyzes a wide range of substrates, including the active metabolites of a variety of organophosphorus (OP) insecticides, paraoxon, the substrate that provides its name, diazoxon

(DZO), and chlorpyrifos-oxon, as well as the nerve agents sarin, soman, and VX (Davies *et al*., 1996; Eaton *et al*., 2008). It has been suggested that high levels of paraoxonase may be protective against the neurotoxic effects of organophosphate substrates of this enzyme in several studies (Costa *et al*., 1987; Furlong *et al*., 1988, 1989; LaDu & Eckerson, 1984) and this hypothesis was also supported by experiments with animal systems in which exogenous administration of PON1 provided protection against acute OP poisoning in rodents (Costa *et al*., 1990; Li, Costa, & Furlong, 1993; Li, Furlong, & Costa, 1995; Main, 1956). More details of PON1 protein in plant systems are discussed in Chapter 5.

Hassett *et al*. (1991) demonstrated striking nucleotide and deduced amino acid similarities (greater than 85 %) between rabbit and human cDNA clones by comparison of rabbit and human PON1 sequences. Alignment of rabbit and human cDNA coding regions revealed an 86% identity and the protein sequences deduced from these clones indicated an 85% identity, suggesting an important metabolic role and constraints on the evolution of this protein.

4.2 Experimental design

4.2.1 Construction of transformation vector and plant transformation

Human CYP2B6 cDNA was provided by Dr. Waziers (University of René Descartes, Paris, France). A 1476-bp fragment was isolated from the original cDNA clone by digestion with *Kpn* I. The vector pART7 and pART27 used in this study are a set of plant expression vectors constructed by Gleave (1992). This CYP2B6 coding sequence first cloned into the *Kpn* I-digested pART7, containing the CaMV 35S promoter and OCS 3′-terminator. The fusion gene fragments containing promoter and terminator were then cleaved by *Not* I and inserted into the correspondent *Not* I site of T-DNA region in pART27 expression vector, forming pART27-CYP2B6 (Figure 9).

Rabbit PON1 cDNA was provided by Dr. Furlong (University of Washington, WA). A 1080-bp fragment was isolated from the original cDNA clone by PCR using appropriate primers. This PCR product of rabbit PON1 coding sequence was cloned to pGEM-T Easy vector (Promega Corporation, Madison, WI), forming pGEM-T Easy-PON1 vector. The 1080-bp fragment was isolated again from the pGEM-T Easy-PON1 vector by digestion with *Eco* RI, and first cloned into the *Eco* RI-digested pART7. The fusion gene fragments containing promoter and terminator were then cloned into pART27 vector as described above, forming pART27-PON1 (Figure 9). The resulting plasmids (pART27-CYP2B6 and pART27-PON1) were the recombinant plant expression vectors of these exogenous genes and were used to transform a hybrid poplar clone INRA 717-1B4 using *Agrobacterium*-mediated plant transformation.

The *Agrobacterium tumefaciens* strains C58C1 (pGV3850, pToK47) harboring the binary pART27 plasmid (null vector control) or the pART27 derivatives (pART27-CYP2B6 and pART27-PON1) were used for co-cultivation experiments with leaf explants of hybrid poplar clone ‘INRA 717-1B4’ (*Populus tremula* x *P. alba*) as described previously (Figure 10). Regenerated kanamycin (100 mg/L)-resistant shoots were selected and analyzed for transgene integration.

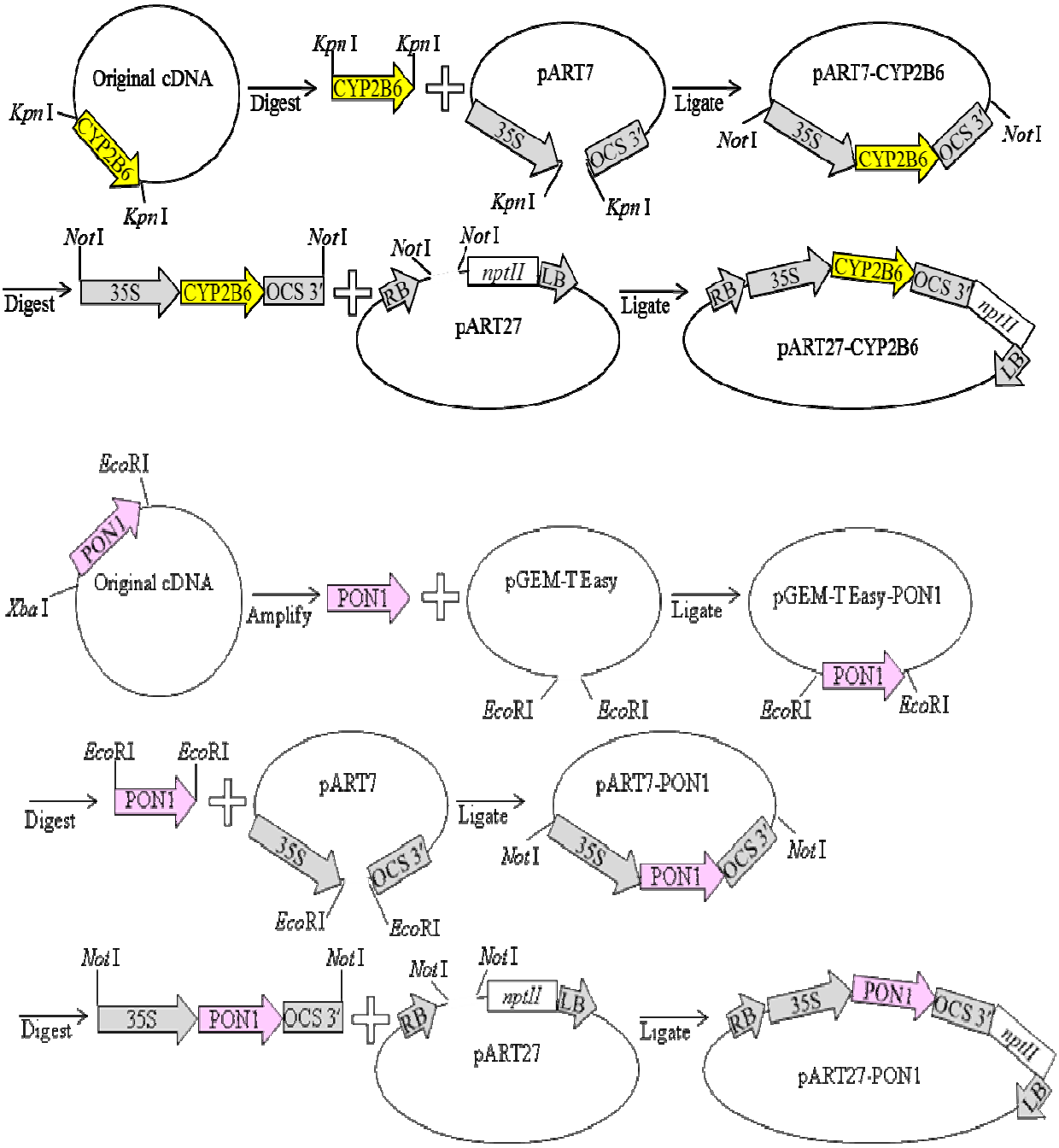

Figure 9 Schematic representation of the cloning process to obtain plant transformation vectors. **Up**: Steps involved in the construction of plant expression vector containing CYP2B6 (pART27-CYP2B6). CYP2B6 is indicated by the yellow arrow. **Down**: Steps involved in the construction of plant expression vector containing PON1 (pART27-PON1). PON1 is indicated by the pink arrow.

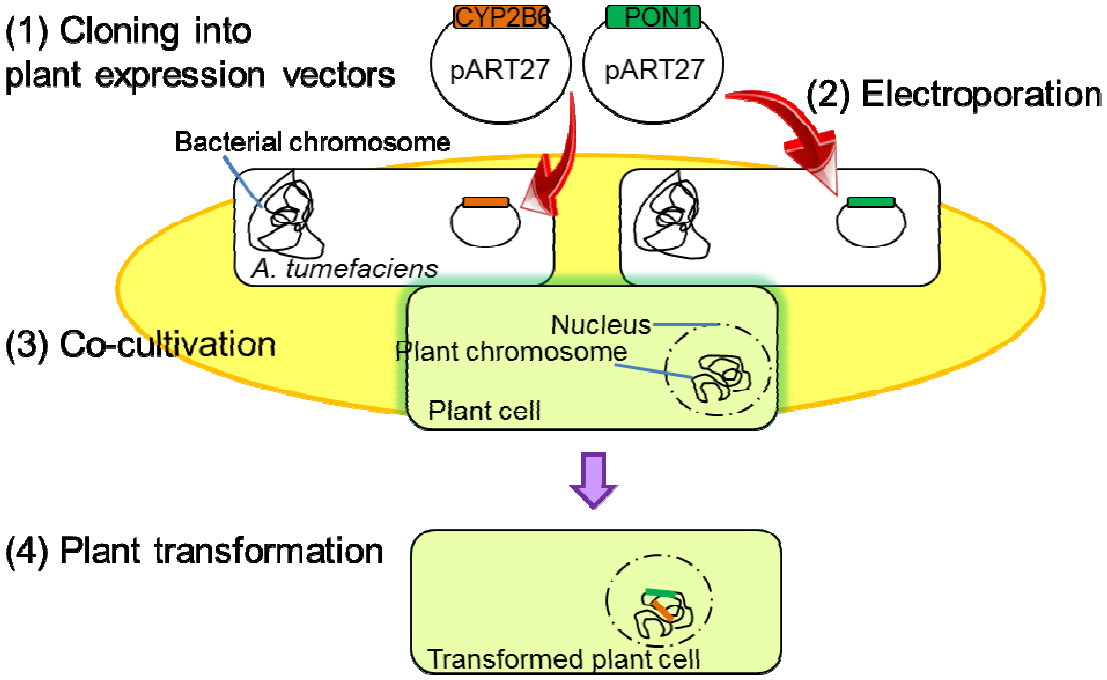

Figure 10 Schematic representation of plant transformation process using *Agrobacterium*-mediated method. **(1)** Cloning into plant expression vectors: Human CYP2B6 and rabbit PON1 were cloned into plant expression vector pART27, forming pART27-CYP2B6 and pART27-PON1. CYP2B6 and PON1 are indicated by the orange and green box, respectively. **(2)** Electroporation: The binary plasmids were transferred into the *Agrobacterium tumefaciens* using electroporation. **(3)** Co-cultivation: *Agrobacterium* harboring the binary vectors were co-cultivated with plant leaf. **(4)** Plant transformation: *Agrobacterium* transfers its DNA into plant cell.

4.2.2 PCR analysis of putative transgenic plants

Genomic DNA prepared from each of the kanamycin-resistant shoots was used for PCR (polymerase chain reaction) analysis. PCR primers specific for CYP2B6, FP 5′-CACACAGTGAATTCAGCCACCAGA-3′ and RP 5′-CAAATCCGCTTCCCTAAGGAGAAG-3′ and for PON1, FP 5′-ATCAACATCTCACCAGACGGCAAG-3′ and RP 5′-CTGCATAAGCCACTGTCACTTTGG-3′, were prepared and confirmed using plasmid DNAs, pART27-CYP2B6 and pART27-PON1, as positive controls (Figure 11). The denaturation, annealing, and extension temperatures were 94, 50, and 72 ℃, respectively.

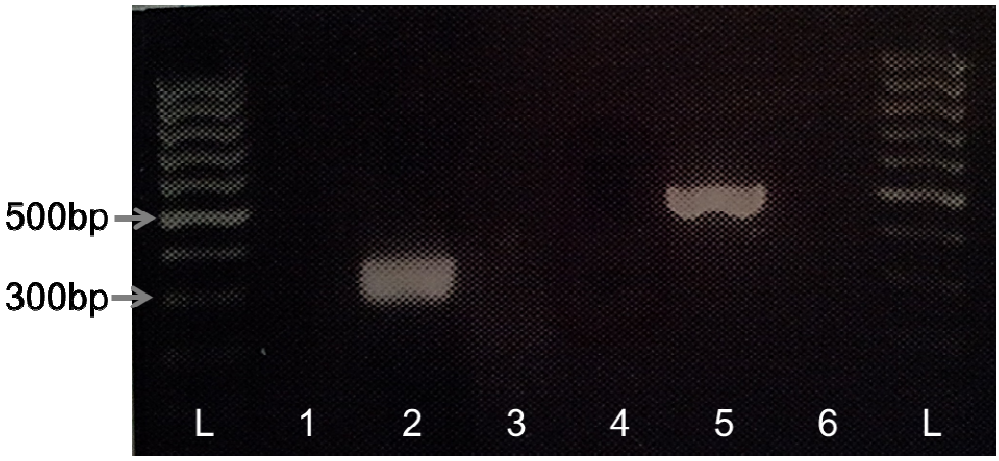

Figure 11 Preparation of gene specific primers and confirmation with plasmid controls. PON1-specific primers are expected to produce 300-bp PCR fragment and CYP2B6-specific primers are expected to produce 500-bp PCR fragment; lane 1-3, PCR products with PON1-specific primers; lane 4-6, PCR products with CYP2B6-specific primers; 1 and 4, negative (no DNA) controls; 2 and 5, positive (pART27-PON1 and pART27-CYP2B6, respectively) controls; 3 and 6, DNAs extracted from non-transgenic poplar; L, 100 bp DNA ladder (Fermentas).

4.2.3 RT-PCR for transgene expression analysis

Transgene expression in plants was analyzed by RT-PCR. RNA extractions from fresh leaf samples of all transgenic lines were performed using the TRIzol (Invitrogen, CA) method. The same amounts of RNA (200 ng) were then used to synthesize cDNA by using the OneStep RT-PCR kit (Qiagen, CA) using a pair of CYP2B6- and PON1-specific primers used in section 4.2.2.1.

4.2.4 Tolerance and removal of CPS and CPO

To evaluate the transgenic plants for reduced phytotoxicity of CPO, PON1 transgenic poplar and wild-type poplar, in triplicate, were exposed to 100 mg/L of CPO. Plant cuttings were placed in sterile 8-oz clear jars containing 20 ml of half strength MS broth. Health of the plants was monitored visually.

To investigate CPS/CPO uptake by PON1 transgenic poplar plants, a transgenic line ‘PON1-34’ and wild-type poplar ‘INRA 717-1B4’ were placed in sterile 8-oz clear jars containing 40 ml of half strength MS broth in triplicate. For controls, there were an additional three jars containing MS broth without plants. CPS and CPO were added to the solution to a final concentration of 20 mg/L and 10 mg/L, respectively. All jars were incubated under a 14-h photoperiod for 7 days. The 500 µl of liquid samples were removed from the hydroponic solution in each vial at intervals of 0, 3, and 7 days for HPLC analysis of the concentrations of CPS/CPO. At the end of 7 days, plant samples were analyzed for CPO accumulation.

4.2.5 Western blot analysis of PON1 protein expression

To investigate whether the *pon1* gene was making active PON1 protein in transgenic plants, PON1 protein expression needs to be confirmed by western blot. However, due to the lack of commercially available antibodies against rabbit PON1, a protein tag was needed, so a his-tag, a widely-use protein tag, was chosen for this study. The protein expression is easily checked using a his-tag since there are a lot of commercial anti-his tag antibodies. This was accomplished using PCR with modified primers (Figure 12) to fuse the his-tag in-frame to the N-terminal of the *pon1* gene so that his-tagged PON1 protein is synthesized. The his-tagged PON1 was inserted in a plant expression vector (pART27) as described in section 4.2.1 (Figure 9) and the resulting plasmid pART27-hisPON1 was expressed in XL10-Gold ultracompetent cells by *E. coli* transformation and in plant (*Nicotiana benthamiana*) by *Agrobacterium*-mediated infiltration method as described previously in Chapter 2. Protein was extracted from (1) *E. coli* harboring pART27-hisPON1 and (2) agro-infiltrated plant leaf tissue with *Agrobacterium* cells GV3101 harboring pART27-hisPON1, and hisPON1 protein expression levels were analyzed by western blotting.

FP 5′ TACAGCATTATGCACCATCACCACCATCACGCTAAACTGACAGCGC 3′

RP 5′ TACAGCCTAATTGGCCTGTGAGAGCTCACAGTAGAGAGCTTTGTGG 3′

Figure 12 PCR primer sequences. Black lines indicate partial sequences matched with PON1 cDNA. The his-tag is indicated by the red.

4.3 Results

4.3.1 PCR analysis of putative transgenic plants

The presence of transgenes in kanamycin-resistant putative transgenic lines was confirmed by PCR using CYP2B6- and PON1-specific PCR primers. A total of 45 transgenic plants were obtained. Among them, 35 plants (PON1-1~35) were PCR-positive for PON1 by showing the expected 300-bp PCR fragment, and 10 plants (PON12B6-1~10) were PCR-positive for both CYP2B6 and PON1 by showing both expected 300-bp and 500-bp fragments. However, there were no transgenic plants which were PCR-positive for CYP2B6 alone. All the transgenic poplar plants were subcultured on MS medium for further analysis, but one PON1/2B6 transgenic line, PON12B6-3, was lost due to fungal contamination. The PON1 transgenic poplar showed normal growth and morphology including plant height and leaf color compared with non-transformed poplar, but there was a morphological difference observed between the double transformed and non-transformed poplar plants in that, the double transgenic plants were very unhealthy at the end and did not survive for unknown reasons.

4.3.2 RT-PCR for transgene expression analysis

High PON1 mRNA expression in the transgenic poplar was confirmed by RT-PCR. RNAs extracted from 9 transgenic plants expressing two genes, CYP2B6 and PON1, showed different expression levels of PON1 (Figure 13A) and CYP2B6 (Figure 13B). RNAs extracted from 35 transgenic poplars expressing PON1 only, showed different expression levels of PON1 (Figure

13C). A transgenic line, PON1-34, showing higher level of expression compared to other transgenic lines was chosen for further study. A no-RT control was done by regular PCR using the RNA samples to verify that there was no DNA in the RNA samples, and no band was obtained (data not shown).

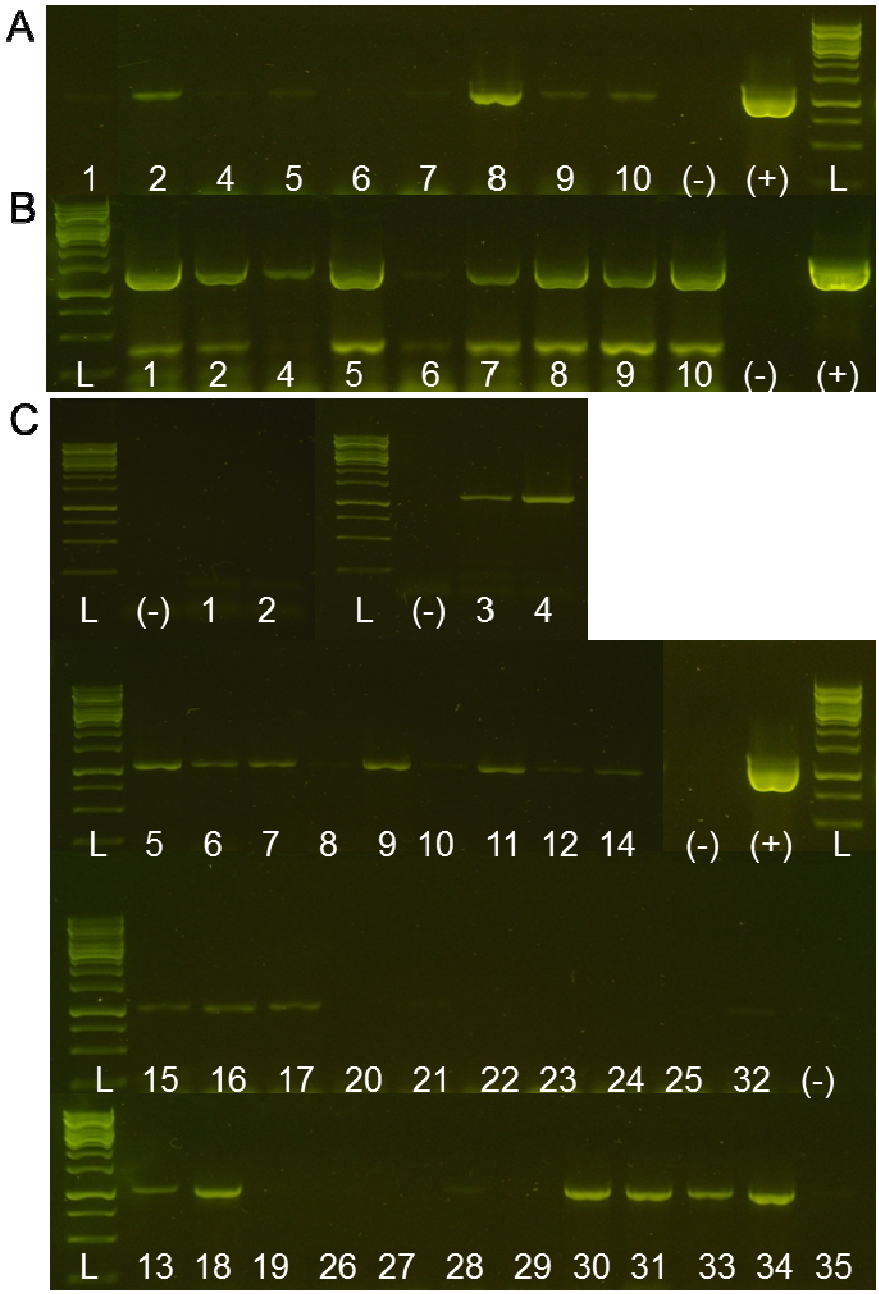

Figure 13 Analysis of transgene expression in plants by RT-PCR. cDNA synthesis from RNAs extracted from 9 transgenic lines expressing two genes, CYP2B6 and PON1, using PON1-specific primers **(A)** and CYP2B6-specific primers **(B)**. **(C)**: cDNA synthesis from RNAs extracted from 35 transgenic lines expressing one gene, PON1, using PON1-specific primers.

4.3.3 Tolerance and removal of CPS and CPO

There was no significant difference between wild type and PON1 transgenic poplar plants in terms of phytotoxicity (Figure 14) and CPS/CPO removal (Figure 15). When plants were treated with 100 mg/L of CPO, they started showing symptoms about 4 days after treatment and they were gradually dying for another month (Figure 14). PON1 transgenic poplar was not able to tolerate CPO any better than wild-type poplar.

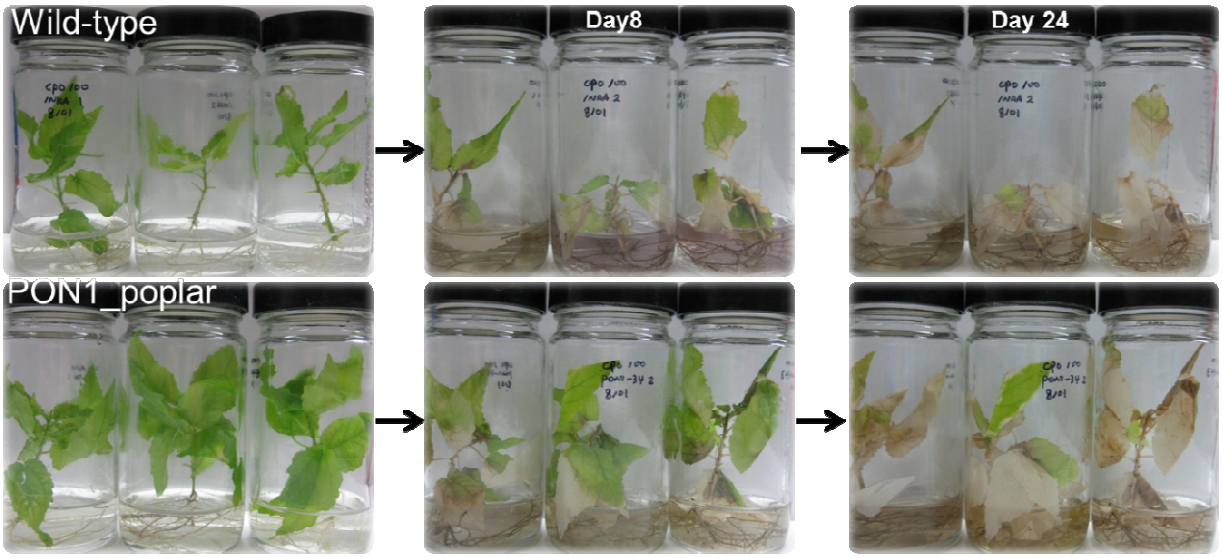

Figure 14 Phytotoxicity of CPO. Wild-type and PON1 transgenic poplar were exposed to 100 mg/L CPO for 24 days and the health of the plants was monitored visually.

Both wild-type and PON1 transgenic poplar were able to take up CPS and CPO, but there was no difference between wild-type and transgenic as PON1 transgenic poplar took up CPO as much as wild-type did (Figure 15). The amounts (µg) of CPO accumulated in plant tissue were 1.53 ± 0.2 and 1.21 ± 0.11 for PON1 transgenic and wild-type, respectively, and the difference was statistically insignificant.

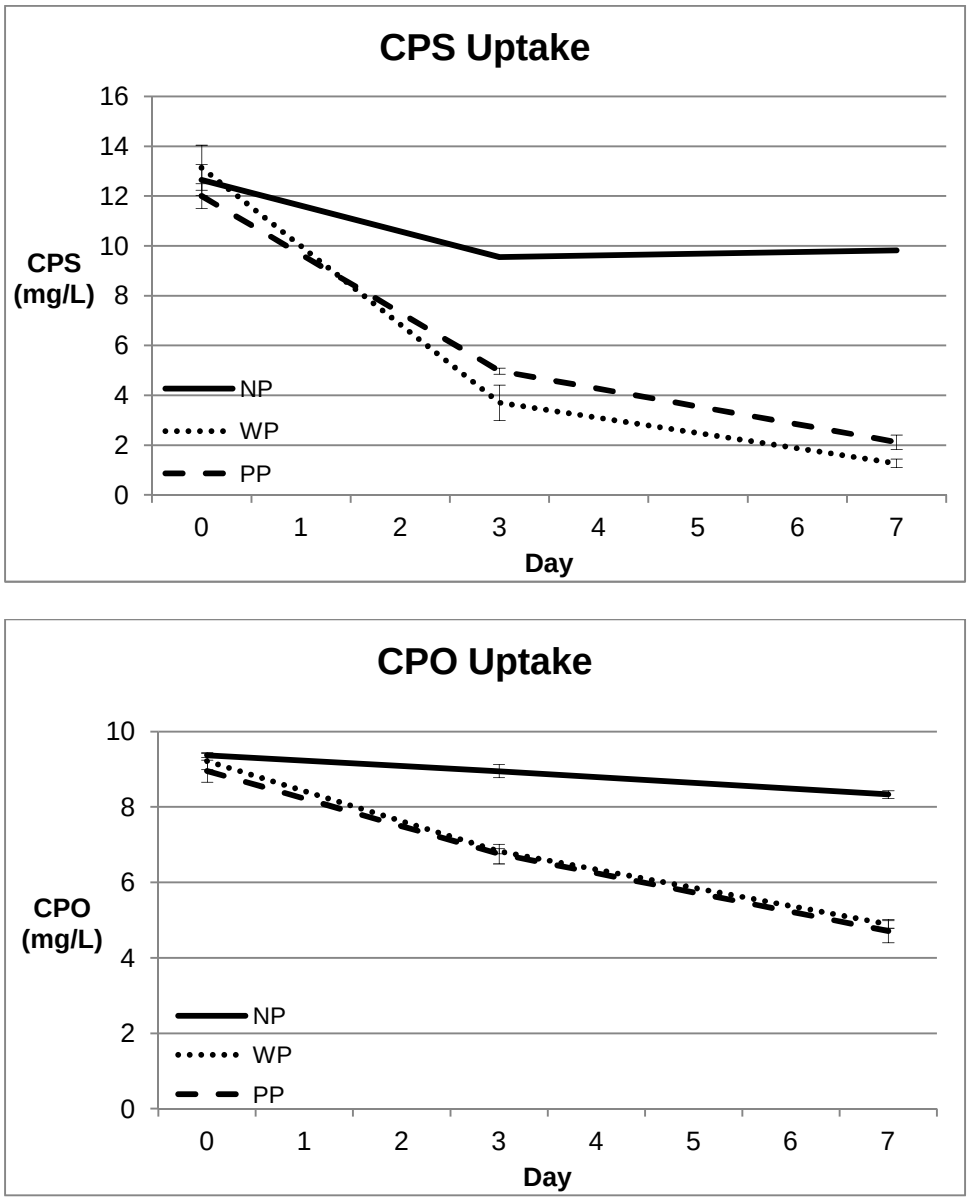

Figure 15 The decrease of CPS and CPO in hydroponic solution. The concentrations of CPS/CPO in the hydroponic solution were monitored for seven days. The data are shown as the mean ± SEM from three samples. NP: no plant control, PP: PON1 transgenic poplar, WP: wild-type poplar.

4.3.4 Western blot analysis of PON1 protein expression

Western blot analysis was performed using WesternBreeze Chromogenic Western Blot Immunodetection Kit (Invitrogen) to detect rabbit PON1 protein expression levels. It was expected that PON1 protein would be found as a single protein band close to 40 kDa on SDS-

PAGE and Western blot (Bayrak *et al*., 2010). However, the his-tagged PON1 protein was not detected by the western blot (protein immunoblot), when his-tag antibody was used to detect his-tagged fusion protein in bacterial (**Figure 16**, lane 2) and plant total protein (**Figure 16**, lane 4). In this study, a his-tag was added to the N-terminal of the *pon1* gene, since Dr. Furlong's lab had tried adding a his-tag to the C-terminal of the protein and it did not work very well. It seems either the addition of the his-tag to the N-terminal did not work for the detection by the western blotting, or the expression level of the PON1 protein was very low.

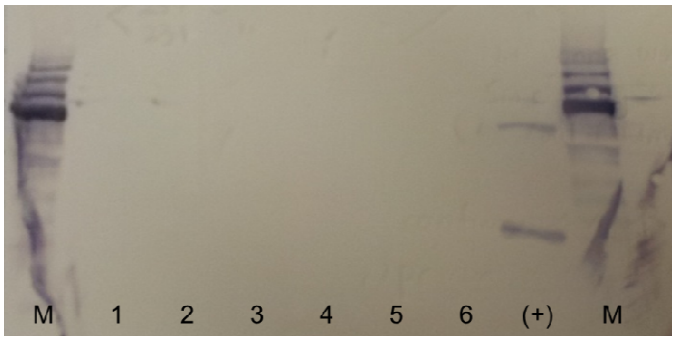

Figure 16 Western blot analysis of the protein samples. **M**: molecular weight markers, **1**: protein extracted from XL10-Gold harboring pART27, **2**: protein extracted from XL10-Gold harboring pART27-hisPON1, **3**: protein extracted from agro-infiltrated plant with GV3101 harboring pART27, **4**: protein extracted from agro-infiltrated plant with GV3101 harboring pART27-hisPON1, **5**: protein extracted from XL10-Gold harboring pLD2-CtV, **6**: protein extracted from XL10-Gold harboring pLD2-CtV-hisPON1, **(+)**: a his fusion protein as a positive control provided from Dr. Furlong

4.4 Discussion

4.4.1 Transgenic poplar overexpressing CYP2B6

We hypothesized that there would be three types of transgenic poplar plants when plant materials were co-cultivated with two *Agrobacterium* strains containing CYP2B6 and PON1 respectively for the plant transformation: (1) transgenic poplar overexpressing PON1 alone, (2) transgenic poplar overexpressing CYP2B6 alone, and (3) double transgenic poplar

overexpressing PON1 and CYP2B6. However, there was no transgenic poplar overexpressing CYP2B6 alone obtained. In addition, there was a morphological difference observed between the double transformed and non-transformed poplar plants, and the double transgenic plants were very unhealthy at the end and did not survive for unknown reasons. Although CYP2B6 was successfully expressed in transgenic rice and potato plants (Hirose *et al*., 2005; Inui *et al*., 2000, 2001; Kawahigashi *et al*., 2005, 2006), overexpression in poplar seems to be deleterious. It may be that the introduced human CYPs including CYP2B6 disturb the endogenous P450 system in plants or the secondary metabolic pathways in plants by their competing enzymatic actions, because human CYPs have broad substrate specificities (Hirose *et al*., 2005; Kawahigashi *et al*., 2005; Kawahigashi *et al*., 2007).

4.4.2 The lack of phenotype of the PON1 transgenic poplar

Transgenic poplar plants overexpressing the *pon1* gene, which is known to be involved in CPS metabolism, was successfully developed, but there was no change in phytotoxicity of CPS or uptake of CPS in the transgenic lines, and no PON1 protein was detected by western blotting. Since transcription was confirmed by RT-PCR analysis, a likely explanation for the lack of PON1 protein and phenotype is that the protein is not folding properly and is targeted for destruction. Work in Dr. Furlong's lab has shown that strong expression of functional PON1 enzyme was only achieved using a modified *E. coli* strain for production of foreign proteins.

In further support of this hypothesis, the Mor lab at Arizona State University had attempted PON1 expression in plants, but it was not successful. They advised not to pursue using PON1 in transgenic plants since PON1 is a "temperamental enzyme" (T Mor, personal communication).

Sometimes the gene needs to be re-synthesized in order to get expression in plants. A *cry* gene from the soil bacterium *Bacillus thuringiensis* (Bt), which encodes insecticidal Cry proteins, was expressed in tobacco plants (Vaeck *et al*., 1987). However, despite the use of strong promoters,

the first transformed plants produced too little toxin protein (less than 0.001 % of the leaf soluble proteins). The genes of *Bt*, which have a large proportion of A and T bases (66 %), are not consistent with optimum codon usage in plants and the resulting transcripts may be unstable. In order to optimize the codon usage, *Bt* toxin genes were resynthesized partially or completely, resulting in much higher expression in plants (0.02 to 0.5 % of leaf total soluble proteins). Rugh *et al*. (1998) and others (Rugh *et al*., 1996; Yang, Nairn, & Ozias-Akins, 2003) also demonstrated that nuclear transgenic plants were failed to express the bacterial *merA* (mercuric ion reductase) gene, but when the coding sequence of the *merA* gene was modified to plant preferred codons, transgenic *Arabidopsis thaliana*, yellow poplar, peanut and Eastern cottonwood plants exhibited high levels of resistance (up to 25-100 µM) against $HgCl_2$. Therefore, the modification of the coding sequence to plant preferred codons might be needed in order to enhance the expression levels of *pon1* gene, and the expression of modified *pon1* gene in plants needs to be investigated.

4.4.3 Mutation

In the process of the sequence confirmation, multiple nucleotide changes that would result in amino acid changes were found. First, there was a single nucleotide change in the construct of PON1-inserted plant expression vector used in this work, compared to the DNA sequence of rabbit PON1 constructed and provided by Dr. Furlong that causes a single amino acid change: Serine (S) was replaced by asparagine (N) (Table 5). Secondly, there were four more nucleotide changes when it compares to the original DNA sequence of rabbit PON1, but the new codon specifies the same amino acid, resulting in an unmutated protein. In summary, there were five nucleotide changes but only a single amino acid change. This change in amino acid sequence may affect protein function and could be a cause for the lack of phenotype of the transgenic poplar. In order to test if this mutation affects proper PON1 protein synthesis, *pon1* gene without

the mutation was expressed in *E. coli* and the PON1 enzyme activity towards phenyl acetate was measured, compared to the mutated *pon1* gene in *E. coli* using spectrophotometric methods as described in section 5.2.6 (Richter & Furlong, 1999). Since Dr. Furlong's construct showed very strong PON1 enzyme activity towards phenyl acetate, a single nucleotide sequence was changed (a**a**t → a**g**t) to specify the same amino acid (S). The results showed that the enzyme activity of the unmutated PON1 was nearly identical to the mutated PON1 (Figure 17). Therefore this mutation was not the reason for the lack of phenotype of the transgenic poplar. The result also supported that the transformed *E. coli* produced a certain amount of PON1 protein which was able to hydrolyze phenyl acetate, forming phenol (Figure 17).

Table 5 PON1 DNA sequences and its deduced protein sequences. **(1)** Original PON1 sequence, **(2)** PON1 sequence which was given to me by Dr. Furlong. It has four mutated DNA sequences, but no change in protein sequence, **(3)** PON1 sequence from this work. It has five mutated DNA sequences and one change in protein sequence compared to the original. Mutated sequences are indicated by the pink.

Position	484-486		487-489		496-498		505-507		508-510	
Sequence	DNA	Protein	DNA	Protein	DNA	Protein	DNA	Protein	DNA	Protein
(1) Original	aag	K	ctt	L	agt	S	gac	D	att	I
(2) Dr. Furlong's	aa**a**	K	ct**c**	L	agt	S	ga**t**	D	at**c**	I
(3) This work	aa**a**	K	ct**c**	L	a**a**t	**N**	ga**t**	D	at**c**	I

(1) DNA sequence (1080bp) of paraoxonase 1 [*Oryctolagus cuniculus*] rabbit

```
   1 atggctaaac tgacagcgct cacgctcttg gggctgggat tggcactctt cgatggacag
  61 aagtcttctt tccaaacacg atttaatgtt caccgtgaag taactccagt ggaacttcct
 121 aactgtaatt tagttaaagg gattgacaat ggttctgaag acttggaaat actgcccaat
 181 ggactggctt tcatcagcgc cggattaaaa tatcctggaa taatgagctt tgaccctgat
 241 aagcctggaa agatacttct aatggacctg aatgagaaag acccagtagt attggaactg
 301 agcattactg gaagtacatt tgatttatct tcatttaacc ctcatgggat tagcacattc
 361 acagatgaag ataatatcgt ctacctgatg gtggtgaacc atccagattc caagtccaca
 421 gtggagttgt ttaaattcca agaaaaagaa aaatcacttt tgcatctgaa aaccatcaga
 481 cacaagcttc tgcctagtgt gaatgacatt gtcgctgtgg gacctgaaca cttttatgct
 541 accaatgatc actattttat tgacccttac ttaaaatcct gggaaatgca tttgggatta
 601 gcgtggtcat ttgttactta ttatagtccc aatgatgttc gagtagtggc agaaggattt
 661 gattttgcta acggaatcaa catctcacca gacggcaagt atgtctatat agctgaactg
 721 ctggctcata agatccatgt gtatgaaaag cacgctaatt ggactttaac tccattgaag
```

```
      781 tccctcgact ttaacactct tgtggacaac atatccgtgg atcctgtgac aggggacctt
      841 tgggttggtt gtcatcccaa tggcatgcga atcttctact atgacccaaa gaatcctcct
      901 gcatcagagg tgcttcgaat ccaggacatt ttatccaaag agcccaaagt gacagtggct
      961 tatgcagaaa atggcactgt gttacagggc agcacggtgg ccgctgtgta caaagggaaa
     1021 atgctggttg gcaccgtgtt ccacaaagct ctctactgtg agctctcaca ggccaattag
Protein sequence (359)
        1 makltaltll glglalfdgq kssfqtrfnv hrevtpvelp ncnlvkgidn gsedleilpn
       61 glafisaglk ypgimsfdpd kpgkillmdl nekdpvvlel sitgstfdls sfnphgistf
      121 tdednivylm vvnhpdskst velfkfqeke ksllhlktir hkllpsvndi vavgpehfya
      181 tndhyfidpy lkswemhlgl awsfvtyysp ndvrvvaegf dfanginisp dgkyvyiael
      241 lahkihvyek hanwtltplk sldfntlvdn isvdpvtgdl wvgchpngmr ifyydpknpp
      301 asevlriqdi lskepkvtva yaengtvlqg stvaavykgk mlvgtvfhka lycelsqan
```

(2) DNA sequence in Dr. Furlong's cDNA, pSCodon-RaPON1-cytB

```
        1 atggctaaac tgacagcgct cacgctcttg gggctgggat tggcactctt cgatggacag
       61 aagtcttctt tccaaacacg atttaatgtt caccgtgaag taactccagt ggaacttcct
      121 aactgtaatt tagttaaagg gattgacaat ggttctgaag acttggaaat actgcccaat
      181 ggactggctt tcatcagcgc cggattaaaa tatcctggaa taatgagctt tgaccctgat
      241 aagcctggaa agatacttct aatggacctg aatgagaaag acccagtagt attggaactg
      301 agcattactg gaagtacatt tgatttatct tcatttaacc ctcatgggat tagcacattc
      361 acagatgaag ataatatcgt ctacctgatg gtggtgaacc atccagattc caagtccaca
      421 gtggagttgt ttaaattcca agaaaaagaa aaatcacttt tgcatctgaa aaccatcaga
      481 cacaaactcc tgcctagtgt gaatgatatc gtcgctgtgg gacctgaaca cttttatgct
      541 accaatgatc actattttat tgacccttac ttaaaatcct gggaaatgca tttgggatta
      601 gcgtggtcat ttgttactta ttatagtccc aatgatgttc gagtagtggc agaaggattt
      661 gattttgcta acggaatcaa catctcacca gacggcaagt atgtctatat agctgaactg
      721 ctggctcata agatccatgt gtatgaaaag cacgctaatt ggactttaac tccattgaag
      781 tccctcgact ttaacactct tgtggacaac atatccgtgg atcctgtgac aggggacctt
      841 tgggttggtt gtcatcccaa tggcatgcga atcttctact atgacccaaa gaatcctcct
      901 gcatcagagg tgcttcgaat ccaggacatt ttatccaaag agcccaaagt gacagtggct
      961 tatgcagaaa atggcactgt gttacagggc agcacggtgg ccgctgtgta caaagggaaa
     1021 atgctggttg gcaccgtgtt ccacaaagct ctctactgtg agctctcaca ggccaattag
Protein sequence (359)
        1 makltaltll glglalfdgq kssfqtrfnv hrevtpvelp ncnlvkgidn gsedleilpn
       61 glafisaglk ypgimsfdpd kpgkillmdl nekdpvvlel sitgstfdls sfnphgistf
      121 tdednivylm vvnhpdskst velfkfqeke ksllhlktir hkllpsvndi vavgpehfya
      181 tndhyfidpy lkswemhlgl awsfvtyysp ndvrvvaegf dfanginisp dgkyvyiael
      241 lahkihvyek hanwtltplk sldfntlvdn isvdpvtgdl wvgchpngmr ifyydpknpp
      301 asevlriqdi lskepkvtva yaengtvlqg stvaavykgk mlvgtvfhka lycelsqan
```

(3) DNA sequence in pART27/PON1

```
        1 atggctaaac tgacagcgct cacgctcttg gggctgggat tggcactctt cgatggacag
       61 aagtcttctt tccaaacacg atttaatgtt caccgtgaag taactccagt ggaacttcct
      121 aactgtaatt tagttaaagg gattgacaat ggttctgaag acttggaaat actgcccaat
      181 ggactggctt tcatcagcgc cggattaaaa tatcctggaa taatgagctt tgaccctgat
      241 aagcctggaa agatacttct aatggacctg aatgagaaag acccagtagt attggaactg
      301 agcattactg gaagtacatt tgatttatct tcatttaacc ctcatgggat tagcacattc
      361 acagatgaag ataatatcgt ctacctgatg gtggtgaacc atccagattc caagtccaca
      421 gtggagttgt ttaaattcca agaaaaagaa aaatcacttt tgcatctgaa aaccatcaga
      481 cacaaactcc tgcctaatgt gaatgatatc gtcgctgtgg gacctgaaca cttttatgct
      541 accaatgatc actattttat tgacccttac ttaaaatcct gggaaatgca tttgggatta
      601 gcgtggtcat ttgttactta ttatagtccc aatgatgttc gagtagtggc agaaggattt
      661 gattttgcta acggaatcaa catctcacca gacggcaagt atgtctatat agctgaactg
      721 ctggctcata agatccatgt gtatgaaaag cacgctaatt ggactttaac tccattgaag
      781 tccctcgact ttaacactct tgtggacaac atatccgtgg atcctgtgac aggggacctt
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      841 tgggttggtt gtcatcccaa tggcatgcga atcttctact atgacccaaa gaatcctcct
      901 gcatcagagg tgcttcgaat ccaggacatt ttatccaaag agcccaaagt gacagtggct
      961 tatgcagaaa atggcactgt gttacagggc agcacggtgg ccgctgtgta caaagggaaa
     1021 atgctggttg gcaccgtgtt ccacaaagct ctctactgtg agctctcaca ggccaattag
Protein sequence (359)
        1 makltaltll glglalfdgq kssfqtrfnv hrevtpvelp ncnlvkgidn gsedleilpn
       61 glafisaglk ypgimsfdpd kpgkillmdl nekdpvvlel sitgstfdls sfnphgistf
      121 tdednivylm vvnhpdskst velfkfqeke ksllhlktir hkllpnvndi vavgpehfya
      181 tndhyfidpy lkswemhlgl awsfvtyysp ndvrvvaegf dfanginisp dgkyvyiael
      241 lahkihvyek hanwtltplk sldfntlvdn isvdpvtgdl wvgchpngmr ifyydpknpp
      301 asevlriqdi lskepkvtva yaengtvlqg stvaavykgk mlvgtvfhka lycelsqan
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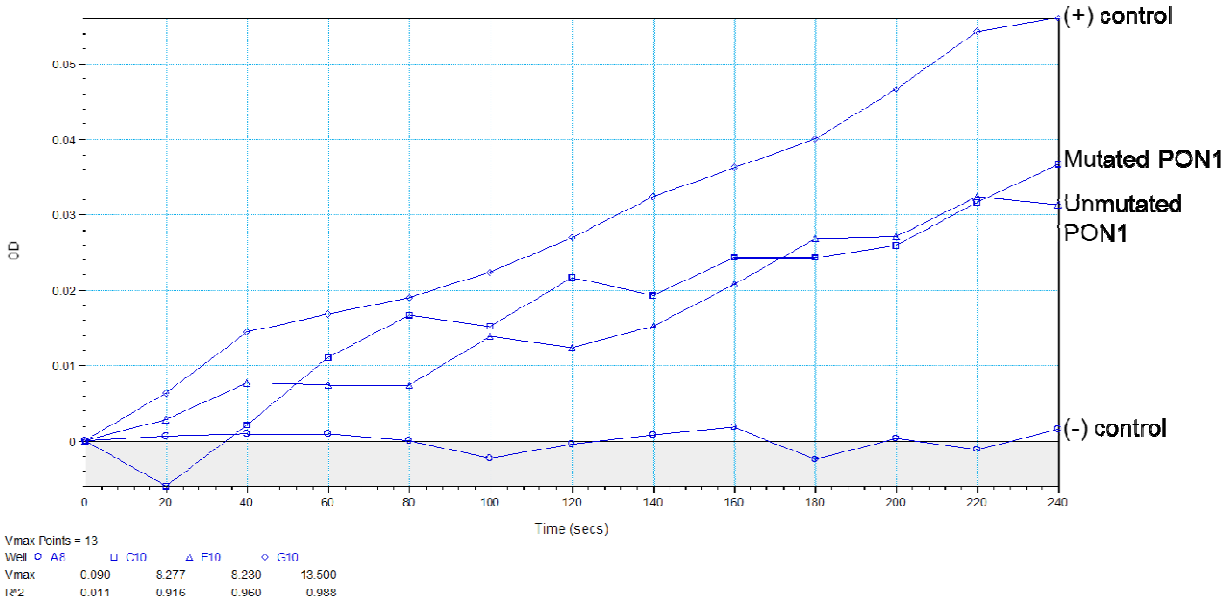

Figure 17 Rate of formation of phenol was measured by monitoring the increase in absorbance (270 nm, also called OD, optical density) as phenyl acetate is hydrolyzed by PON1, forming phenol (section 5.2.6). **(+) control**: positive control, bacterial protein extracted from SE1 *E. coli* harboring PON1-inserted pSCodon plasmid, which is known to have strong activity towards phenyl acetate, **Mutated PON1**: bacterial protein extracted from DH5α competent *E. coli* harboring mutated PON1-inserted plant expression vector (pART27-mtPON1), **Unmutated PON1**: bacterial protein extracted from DH5α competent *E. coli* harboring PON1-inserted plant expression vector (pART27-PON1), **(-) control**: null vector control, bacterial protein extracted from DH5α competent *E. coli* harboring plant expression vector (pART27) without the gene of interest. Shown is representative photograph of PON1 enzyme activity for four different vector constructs.

4.5 Summary

Transgenic expression of genes involved in CPS metabolism was expected to increase tolerance and removal of CPS. Transgenic poplar plants ('PON1-34') that express rabbit *pon1* gene, which is known to be involved in OP insecticide degradation pathways, were obtained by the *Agrobacterium*-mediated transformation method, and investigated for increased tolerance and removal of CPS. However, there was no change in phytotoxicity of CPS or uptake of CPS in the transgenic lines. The his-tagged PON1 protein was not detected by the western blot (protein immunoblot), when the his-tag antibody was used to detect his-tagged PON1 protein in bacterial and plant total protein. Three possibilities exist for this failure (i) the protein is not folding properly and is targeted for destruction, (ii) the addition of the his-tag to the N-terminal did not work for the detection by the western blotting, or (iii) the expression level of the PON1 protein was very low. However, the result of PON1 enzyme activity towards phenyl acetate supported that the transformed *E. coli* produced a certain amount of PON1 protein which was able to hydrolyze phenyl acetate, forming phenol. Since the PON1 enzyme activity assay did give positive results, it demonstrates that the construct (pART27-PON1) was functional and that the enzyme was active for at least a short period of time under specific conditions. In order to enhance the *pon1* gene expression, the modification of the coding sequence of *pon1* gene to plant preferred codons can be conducted, or the use of other protein tags such as GST (Glutathione S-transferase) can be tested to see if GST-tagged PON1 protein is produced and detected by the western blot.

Chapter 5

Engineering Transgenic Tobacco Overexpressing PON1 through Chloroplast Transformation for High-Level Transgene Expression

5.1 Introduction

The PON1 enzyme hydrolyzes and detoxifies chlorpyrifos oxon (CPO), the oxon form of CPS (Figure 8). Several investigators have examined the possibility of using PON1 protein capable of detoxifying OPs. An *In vivo* assay in mice injected with PON1 demonstrated that PON1 protects against CPS toxicity (Li *et al*., 1995; Stevens *et al*., 2008).

There are several available sources of PON1 protein such as human plasma (Li *et al*., 2000), rabbit serum (Li *et al*., 1995), or bacteria cell cultures (Stevens *et al*., 2008). However these sources are expensive, supply-limited, and may be contaminated with human pathogens. In an effort to avoid these problems, we propose transgenic plants as a PON1 source. However, one of the major concerns of plant nuclear transformation is low level of gene expression. Higher expression level of protein in plants can be accomplished through the chloroplast genetic engineering method. Instead of nuclear DNA, the DNA in the plant's chloroplasts is modified. A typical plant cell contains approximately 100 chloroplasts and each chloroplast contains about 100 copies of its genome. So, chloroplast genetically-engineered plants would have high levels of integration of transgenes-up to 10,000 copies per cell-which elevate expression levels of recombinant proteins (up to 46% of the plant's total soluble protein) (Daniell, Ruiz, & Dhingra, 2005). In addition to the high-level transgene expression, multi-gene engineering in a single transformation event and transgene containment by maternal inheritance, as well as a lack of gene silencing, position and pleiotropic effects and undesirable foreign DNA are the advantages of chloroplast genetic engineering over nuclear transformation (Daniell *et al*., 2005).

Chloroplast genetic engineering has been used for phytoremediation. In these studies, the native bacterial *merA* (mercuric ion reductase) and *merB* (organomercurial lyase) genes were integrated into the tobacco chloroplast genome (Hussein *et al*., 2007; Ruiz *et al*., 2003). The transgenic plants were resistant to very high concentrations of phenylmercuric acetate (PMA), up to 400 µM. This is the first report of chloroplast genetic engineering for phytoremediation (Ruiz *et al*., 2003). Transgenic plants grew exceedingly well in soil contaminated with 300 µM PMA or $HgCl_2$, accumulating Hg in roots to levels surpassing the concentration in soil, up to 2000 µg/g, and 100-fold more Hg in leaves than untransformed plants (Hussein *et al*., 2007).

The hypothesis is that PON1 protein expression level in plants is expected to increase by using the chloroplast engineering method and the plant-derived PON1 protein may be used for rapid on-site remediation of spills as a soil amendment or for clinical and therapeutic use. Tobacco (*Nicotiana tabacum*) has been used for plastid genetic engineering due to its easy genetic manipulation and its considerable growth rate-it can produce 40 tons of fresh leaf weight per acre and up to one million seeds per plant (Arlen *et al*., 2007). For these reasons, tobacco plants were chosen for this study. In order to see if PON1 could be overexpressed in plants as an ultimately less expensive source, a tobacco plant was transformed with rabbit PON1 using the chloroplast engineering method. The activity of plant-derived PON1 was analyzed and CPS tolerance and degradation tests were investigated in chloroplast genetically-engineered tobacco to assess the function of rabbit PON1 enzyme in plants.

5.2 Experimental design

5.2.1 Construction of chloroplast transformation vector

The tobacco chloroplast transformation vector, pLD2-CtV, was provided by Dr. Daniell (University of Central Florida, FL) who invented the chloroplast transformation technique (Figure 18). The vector has selectable marker gene (*aad*A1), regulatory sequences (Prrn and T*psb*A),

and chloroplast flanking sequences (trnI and trnA). What is so special about chloroplast transformation vector is that it is designed with two homologous flanking sequences with tobacco chloroplast genome. These two sites lead to site-specific integration of transgenes into the chloroplast genome and it is different from random nuclear integration because transgenes are integrated via homologous recombination. A 1080-bp fragment of rabbit PON1 was isolated from the original cDNA clone by PCR using appropriate primers (Figure 19). This PCR product of rabbit PON1 coding sequence was cloned into the double (*Not* I and *Xba* I)-digested pLD2-CtV (Figure 19). The inserted gene was confirmed by sequencing and this resulting plasmid (pLD2-CtV-PON1) was used to transform tobacco.

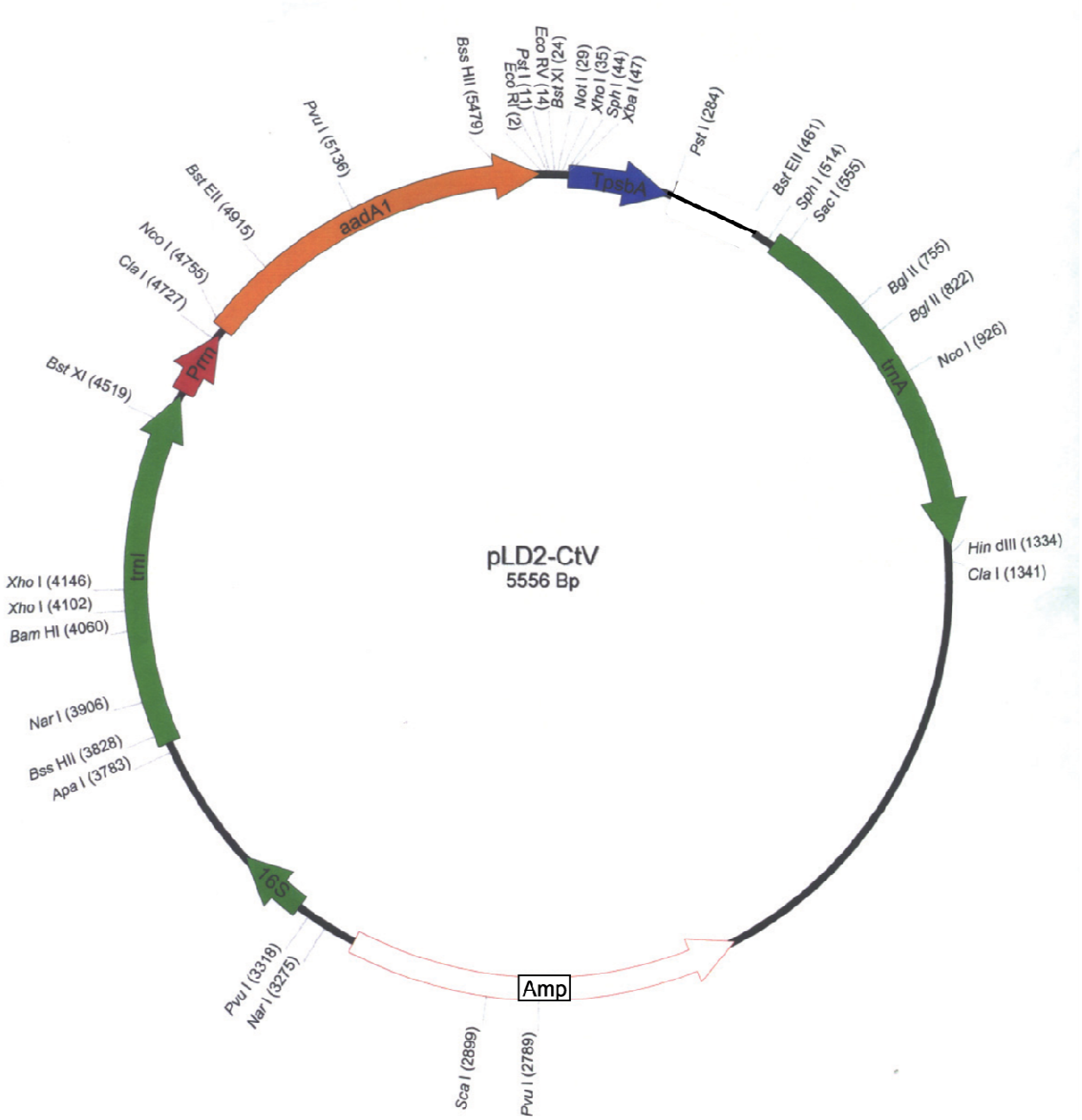

Figure 18 Tobacco chloroplast transformation vector, pLD2-CtV, with selectable marker gene (*aad*A1), regulatory sequences (Prrn and T*psb*A), chloroplast flanking sequences (trnI and trnA) for site-specific transgene integration. Among the multiple cloning sites, *Not* I/*Xba* I sites were used for the gene insertion.

FP 5′ TACAGCGCGGCCGCATTATGGCTAAACTGACA 3′
Not I

RP 5′ TACAGCTCTAGACTAATTGGCCTGTGAGAGCTC 3′
Xba I

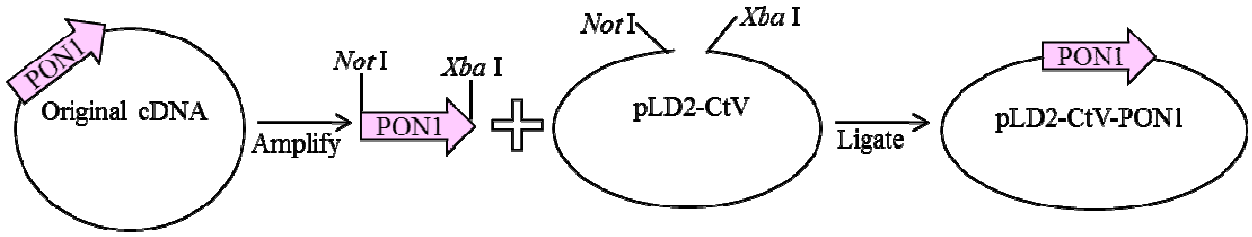

Figure 19 (**Up)**: PCR primer sequences. Black solid lines indicate restriction enzyme recognition sites, and black dashed lines indicate partial sequences matched with PON1 cDNA. (**Down)**: Schematic representation of the cloning process to obtain tobacco chloroplast transformation vector containing PON1 (pLD2-CtV-PON1). PON1 is indicated by the pink arrow.

5.2.2 Plant transformation

Tobacco (*Nicotiana tabacum*) was transformed using a particle delivery system, PDS-1000/He (Bio-Rad Labs, WA). Gold particles coated with the vector DNA pLD2-CtV (null vector control) or pLD2-CtV-PON1 (PON1-inserted vector) were bombarded onto tobacco leaf tissue as described previously (Singh *et al*., 2009). After 5 weeks of bombardment, a putative spectinomycin (500 mg/L)-resistant shoot was developed from 1st round of selection (Figure 20A~D) and analyzed transgene integration by PCR.

The leaves of the primary regenerated PCR-positive transplastomic shoot (Figure 20E) were cut into small pieces and subjected to a second round of selection to achieve homoplasmy (Figure 20F). Homoplasmy is the integration of transgenes into each chloroplast genome and elimination of untransformed chloroplast genome, and is easily accomplished via organogenesis, after several rounds of selection (Ruiz & Daniell, 2009). Initially, few copies of the chloroplast

genomes receive transgene cassette and after several rounds of selection, untransformed genome copies are replaced by transgenic copies, leading to complete homoplasmy. The regenerated shoots after second round of selection (Figure 20G) were separated and transferred to fresh selection medium containing 500 mg/L spectinomycin for third round of selection (Figure 20H). 3-4 weeks after the regenerated shoots after third round of selection was transferred to the agar vessels for rooting (Figure 20I), homoplasmic plants with roots were transferred to a pot containing soil (Figure 20J). When the seed pods were matured, the pods were harvested (Figure 20K) and these seeds were stored in airtight conical tubes at 4 ℃.

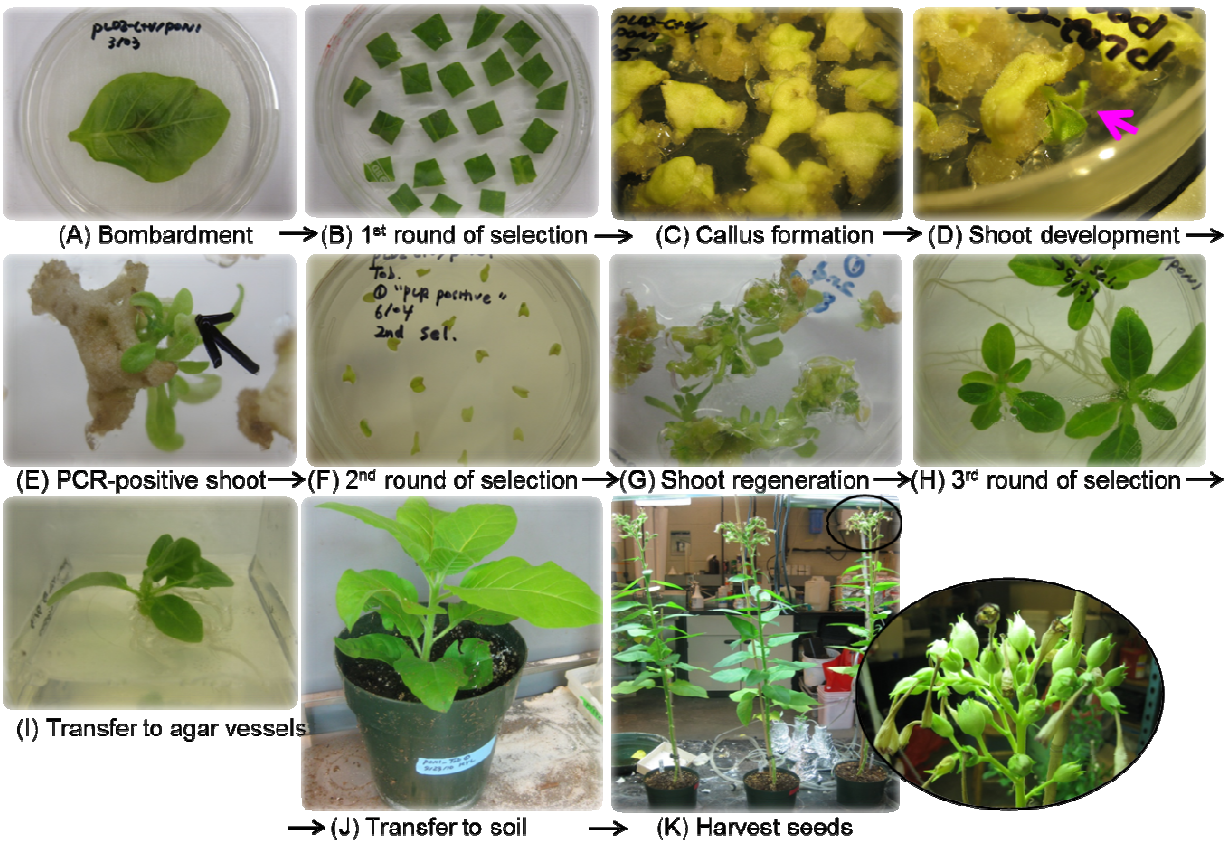

Figure 20 Generation of transgenic tobacco plants. **A-B**: Each of the bombarded leaf (**A**) was cut into small (5 mm^2) pieces and place the abaxial side (bombarded side) touching selection medium for first round of selection (**B**). **C-D**: After 5 weeks of bombardment, putative transplastomic shoot appeared (**D**). **E-G**: Several shoots appeared from the leaves of PCR-positive transformant transferred to fresh selection medium for second round of selection. **H-I**: Regenerated shoots (**G**) were transferred to fresh selection

medium for third round of selection where roots appear. **J-K**: Each transformant was transferred to soil and the pods were harvested when they were matured.

5.2.3 PCR analysis of putative transgenic plants

The transgene integration in putative transplastomic plants was confirmed by PCR. There are 3 pairs of PCR primer used. To confirm the site-specific integration of transgene cassette into chloroplast genome, PCR was performed using a primer pair, 3P and 3M (Singh *et al*., 2009), which anneals to the native chloroplast genome and *aad*A1 genes, respectively (Figure 21). This PCR product cannot be obtained in nuclear transgenic plants or spontaneous mutants, thus both possibilities could be eliminated. To confirm the integration of transgene, two sets of primers, 5P and 2M (Singh *et al*., 2009), which anneals to the *aad*A1 and *trn*A genes, respectively, and a PON1-specific primer pair (Figure 19), which anneals to the 5′ and 3′ end of *pon1* gene, respectively, were used (Figure 21). PCR program used as described previously (Singh *et al*., 2009).

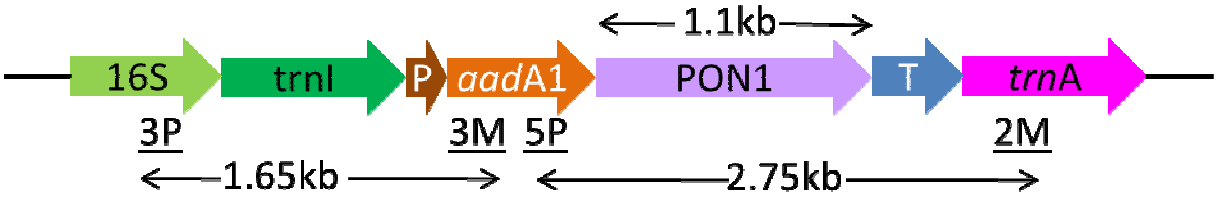

Figure 21 Schematic representation of tobacco chloroplast transformation vector with the gene of interest (PON1), selectable marker gene (*aad*A1), regulatory sequences, chloroplast flanking sequences (*trn*I and *trn*A) for site-specific transgene integration, along with the primer annealing sites.

5.2.4 RT-PCR for transgene expression analysis

Transgene expression in plants was analyzed by RT-PCR. RNA extraction from fresh leaf sample of the PON1 transgenic tobacco was performed using the TRIzol (Invitrogen, CA)

method. The 200 ng of RNA was then used to synthesize cDNA by using the OneStep RT-PCR kit (Qiagen, CA) using a pair of PON1-specific primers (Figure 19).

5.2.5 Germination test

Germination tests of seeds from chloroplast engineered plants were performed. Seeds from nontransgenic tobacco plants and seeds harvested from chloroplast engineered tobacco plants were surface-sterilized with 10 % commercial bleach and 1 % iodophor solution, and rinsed with sterile distilled water 3 times. The surface-sterilized seeds were transferred to half strength MS medium supplemented with 500 mg/L spectinomycin and germinated at 25 ℃ with a 14-h photoperiod in the growth chamber.

5.2.6 PON1 activity assay

To test the activity of plant-derived PON1 protein, total soluble plant protein was extracted from PON1 transgenic tobacco as described previously (Singh *et al*., 2009), but the plant extraction buffer was modified (Table 6) since SDS, EDTA, BME (β-mercaptoethanol), PMSF (phenyl methyl sulfonyl fluoride), and high Tris-HCl will inhibit PON1 activity. The purification of PON1 protein from the crude plant extract was achieved through DEAE (Diethylaminoethyl) chromatography method, which has previously proven useful for purifying PON1 from plasma (Golmanesh, Mehrani, & Tabei, 2008; Stevens *et al*., 2008). Both the crude plant extract and the purified PON1 through DEAE chromatography were tested for the PON1 activity assay.

Unlike the experiment described in Chapter 4, bacteria-derived PON1 enzyme activity towards phenyl acetate (Figure 22) was measured in bacterial protein extracted from DH5α competent *E. coli* harboring PON1-inserted chloroplast expression vector (pLD2-CtV-PON1), compared to a positive control, bacterial protein extracted from SE1 *E. coli* harboring PON1-inserted pSCodon plasmid.

Among several substrate-specific kinetic enzyme assays, arylesterase activity is considered a reliable measurement of PON1 enzyme levels (Huen *et al*., 2009). In this assay, PON1 enzyme activity towards phenyl acetate (Figure 22) was measured in plant total protein extracted from PON1 transgenic tobacco using spectrophotometric methods as described previously (Richter & Furlong, 1999). Rate of formation of phenol was measured every 20 s by monitoring the increase in absorbance (270 nm, at 25 ℃) for 4 m a fter the addition of 20 µl (1:10 dilution) of plant protein to 200 µl of a 3.26 mM phenyl acetate solution (9 mM Tris–HCl buffer, pH 8.0, 0.9 mM $CaCl_2$) in a SpectraMax *PLUS* Microplate Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). As a positive control, bacterial protein extracted from SE1 *E. coli* harboring PON1-inserted pSCodon plasmid, which is known to have strong activity towards phenyl acetate, was provided by Dr. Furlong (University of Washington, WA) and was used to compare the activity with plant protein extracted from PON1 transgenic tobacco.

Table 6 Plant extraction buffer

Singh's buffer (Singh *et al*., 2009)	Modified buffer
100 mM NaCl	100 mM NaCl
200 mM Tris-HCl, pH 8	20 mM Tris-HCl, pH 8
0.05 % Tween-20	0.1 % Tween-20
200 mM sucrose	200 mM sucrose
10 mM EDTA	$CaCl_2$ 5 mM
0.1 % SDS	
14 mM BME	
2 mM PMSF	

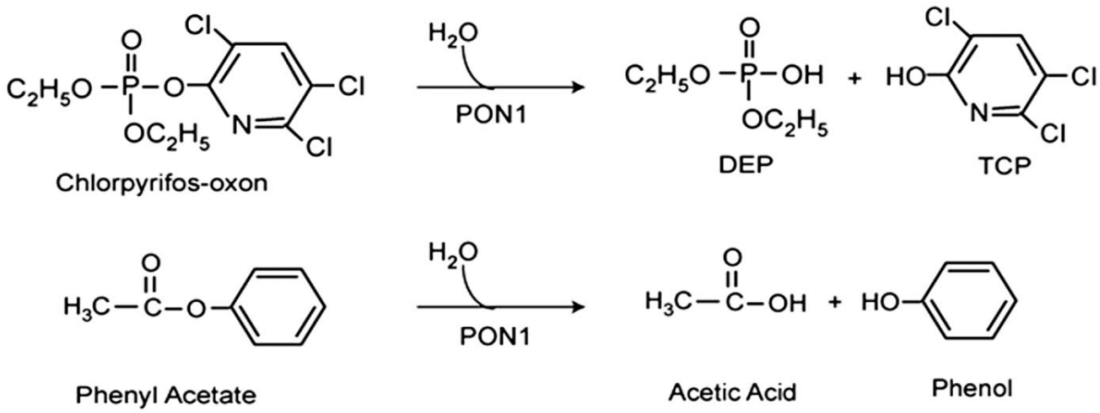

Figure 22 The hydrolysis mechanism of CPS-oxon and phenyl acetate by PON1 enzyme. Like CPS-oxon, phenyl acetate is hydrolyzed by PON1, forming phenol (adapted from Chambers, 2008).

5.2.7 Tolerance and removal of CPS and CPO

To evaluate the toxicity of CPS/CPO, PON1 transgenic poplar and wild-type poplar, in triplicate, were exposed to various concentrations of CPS and CPO. (1) Plant cuttings were exposed to 0, 50, 100, and 150 mg/L of CPS, and (2) leaf discs and (3) seeds were treated with 10 and 20 mg/L of CPS/CPO. Seed plates were incubated at 4 ℃ in dark for three days and then at 24 ℃ in light for 30 more days. Health of the plants was monitored visually.

To investigate CPS/CPO uptake by PON1 transgenic tobacco plants, PON1 transgenic tobacco and wild-type tobacco were placed in sterile 8-oz clear jars containing 40 ml of half strength MS broth in triplicate. For controls, there were an additional three jars containing MS broth without plants. CPS and CPO were added to the solution to a final concentration of 20 mg/L and 10 mg/L, respectively. All jars were incubated under a 14-h photoperiod for 7 days. The 500 µl of liquid samples were removed from the hydroponic solution in each vial at intervals of 0, 3, and 7 days for HPLC analysis of the concentrations of CPS/CPO. At the end of 7 days, plant samples were analyzed for CPO accumulation.

5.2.8 Western blot analysis of PON1 protein expression

To investigate whether the *pon1* gene was making active PON1 protein in transgenic plants, PON1 protein expression needs to be confirmed by western blot. However, due to lack of commercially available antibodies against rabbit PON1, a protein tag was needed and a his-tag was chosen for the PON1 expression in *E. coli*. The protein expression is easily checked using a his-tag since there are a lot of commercial anti-his tag antibodies. This was accomplished using PCR with modified primers (Figure 23) to fuse the his-tag in-frame to the N-terminal of the *pon1* gene so that his-tagged PON1 protein is synthesized. Unlike the experiment described in Chapter 4, the his-tagged *pon1* gene was inserted in a tobacco chloroplast transformation vector (pLD2-CtV) as described in section 5.2.1 (Figure 19) and was expressed in XL10-Gold ultracompetent cells by *E. coli* transformation. Protein was extracted from *E. coli* harboring pLD2-CtV-hisPON1 and hisPON1 protein expression levels were analyzed by western blotting.

FP 5′ TACAGC**GCGGCCGC**GGAGGATGCACCATCACCACCATCAC**GCTAAACTGACAGCGC** 3′

RP 5′ TACAGC**TCTAGA**CTAATTGGCCTGTGAGAGCTCACAGTAGAGAGCTTTGTGGAACA 3′

Figure 23 PCR primer sequences. Black lines indicate partial sequences matched with PON1 cDNA. His-tag is indicated by the red. Letters in bold indicate restriction enzyme recognition sites; NotI (GCGGCCGC) and XbaI (TCTAGA).

5.3 Results.

5.3.1 PCR analysis of putative transgenic plants

5 weeks after the particle bombardment, a putative spectinomycin-resistant shoot developed (Figure 20D) and integration of the foreign gene cassettes into the chloroplast genome was confirmed by PCR screening (Figure 24). This PCR analysis confirmed that rabbit PON1 was successfully integrated into the tobacco chloroplast genome, not into the nuclear genome.

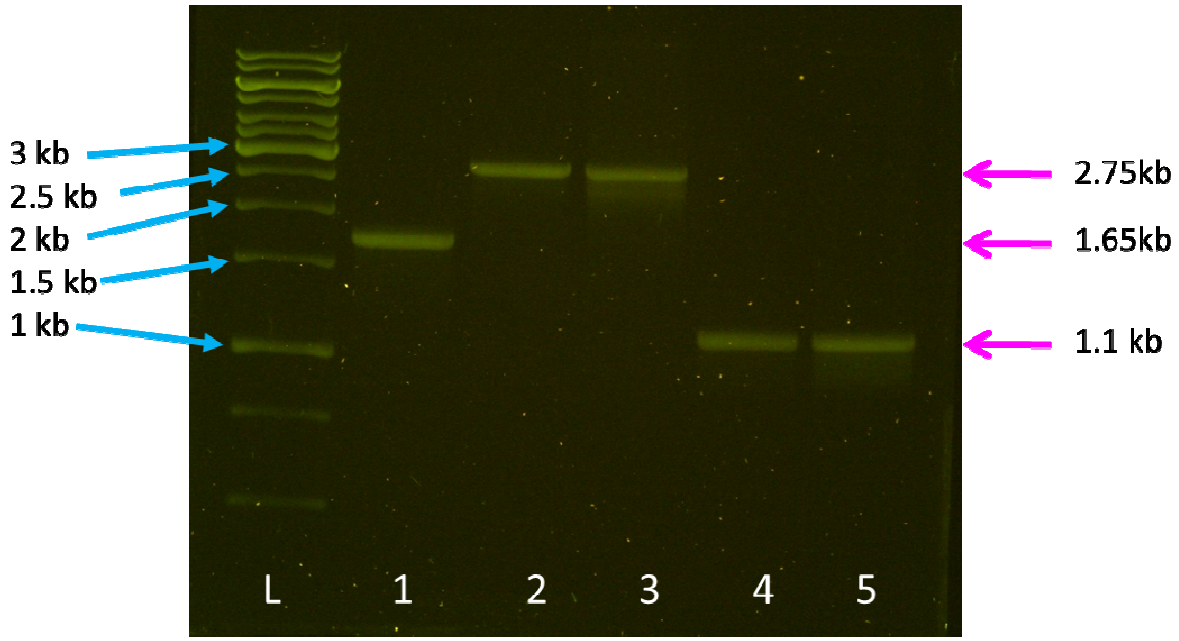

Figure 24 The confirmation of transgene integration by PCR analysis. 3P/3M, 5P/2M, and PON1-specific primer pairs were used (Figure 21); lane 1, PCR product of DNA from PON1 tobacco with 3P/3M primers (1.65 kb); lane 2, PCR product of DNA from PON1 tobacco with 5P/2M primers (2.75 kb); lane 3, A positive (plasmid pLD2-CtV-PON1) control with 5P/2M primers (2.75 kb); lane 4, PCR product of DNA from PON1 tobacco with PON1-specific primers (1.1 kb); lane 5, A positive (plasmid pLD2-CtV-PON1) control with PON1-specific primers (1.1 kb); L, 1kb DNA ladder (Fermentas). The negative (no DNA) controls were included but not shown here.

5.3.2 RT-PCR for transgene expression analysis

High PON1 mRNA expression in chloroplast transgenic tobacco has been confirmed by RT-PCR. RNA extracted from the chloroplast transgenic tobacco (Figure 25, lane 12) showed much higher expression level of PON1, compared to nuclear transgenic poplar (Figure 25, lane 1-8) as expected. A no-RT control was done by regular PCR using the RNA samples to verify that there was no DNA in the RNA samples, and no band was obtained (data not shown).

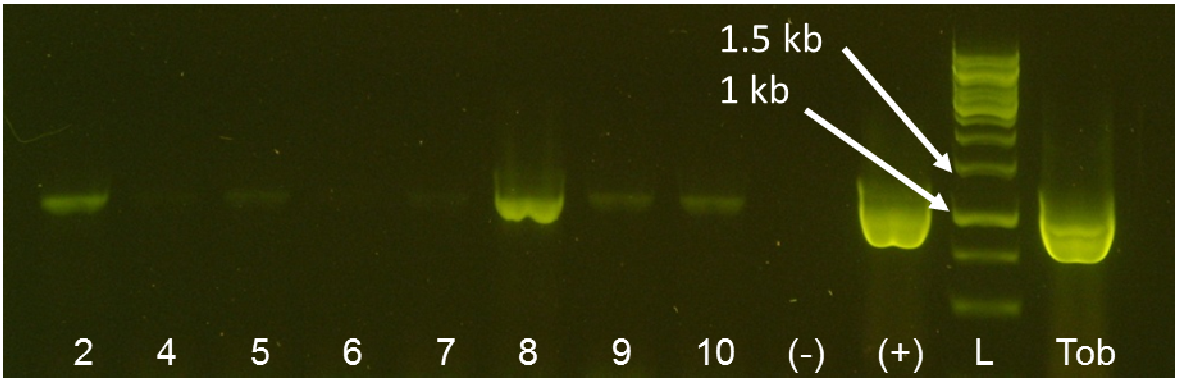

Figure 25 Analysis of transgene expression in chloroplast transformed tobacco by RT-PCR compared to that in nuclear transformed poplar; lane 1-8, cDNA synthesis from RNAs extracted from 8 nuclear transgenic poplar lines expressing CYP2B6 and PON1, using PON1-specific primers (Figure 13A, transgenic lines PON12B6-2,4,5,6,7,8,9,10); lane 12 (Tob), cDNA synthesis from RNA extracted from the chloroplast transgenic tobacco expressing PON1, using PON1-specific primers; L, 1kb DNA ladder (Fermentas); (-), A negative (no RNA) control; (+), A positive control. PCR product of plasmid DNA (pART27-PON1) using PON1-specific primers.

5.3.3 Germination test

Seeds from wild-type tobacco plants failed to grow on selection medium with the antibiotic spectinomycin, whereas all the seeds from self-pollinated chloroplast transgenic tobacco were germinated and grew well, producing copious roots and leaves (Figure 26). This confirms presence of transgene in chloroplast engineered tobacco. Antibiotic resistance of all seedlings confirms homoplasmy of plants regenerated from green seedling tissue.

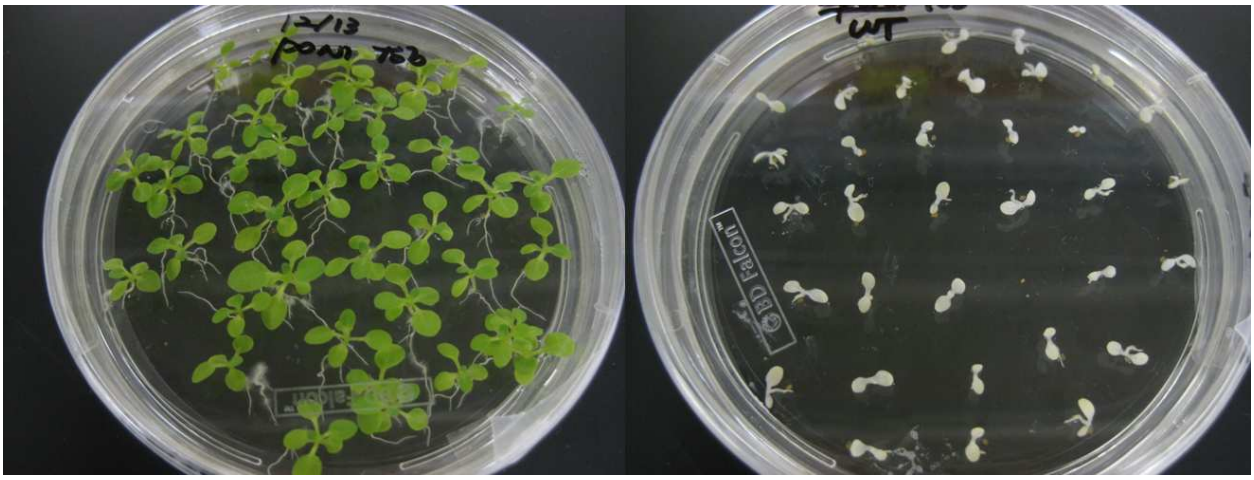

Figure 26 Germination assays of plants regenerated from seeds. The Petri dish on the left shows the progeny from self-pollinated chloroplast engineered tobacco with PON1 germinated on spectinomycin-containing medium showing antibiotic resistance of all seedlings. The Petri dish on the right displays the wild-type control, which is sensitive to the antibiotic. Selection agent had detrimental effect on the wild-type seeds (unable to germinate or bleach soon after germination) confirming absence of transgene.

5.3.4 Plant-derived PON1 activity assay

It is unclear whether there was *in vitro* PON1 activity. The activity of the plant-derived PON1 enzyme on phenyl acetate was investigated, but both the crude plant extract and the purified PON1 enzyme through DEAE (Diethylaminoethyl) chromatography method showed little (or no) activity (data not shown). Since the *in vitro* assay results were inconsistent and not repeatable, it is difficult to make solid conclusions about its activity. It seems like there were other proteins in the crude plant extract beside the PON1 protein. Additionally, the purification method through DEAE was originally for purifying PON1 from plasma, so the purification method for purifying PON1 from plants needs to be developed.

5.3.5 Tolerance and removal of CPS and CPO

To assess the *in vivo* PON1 activity in chloroplast genetically-engineered tobacco, tolerance and removal of CPS was investigated. There was no significant difference between wild type

and PON1 transgenic tobacco plants in terms of phytotoxicity (Figure 27) and CPS/CPO removal (Figure 28). When plant cuttings were treated with various concentrations of CPS, both transgenic and non-transgenic did not show any deleterious effects for 3 weeks (data not shown). When leaf discs were treated with two different concentrations of CPS/CPO, both transgenic and non-transgenic showed deleterious effects as the concentration of chemical gets higher, but there was no difference between two types of plants (Figure 27). The germination rates of seeds decreased when CPS/CPO were added, and seeds from wild-type and transgenic tobacco plants failed to grow on medium with CPO, Seedlings and leaf disks were more susceptible to CPO than to CPS. PON1 transgenic tobacco was not able to tolerate CPS/CPO any better than wild-type tobacco.

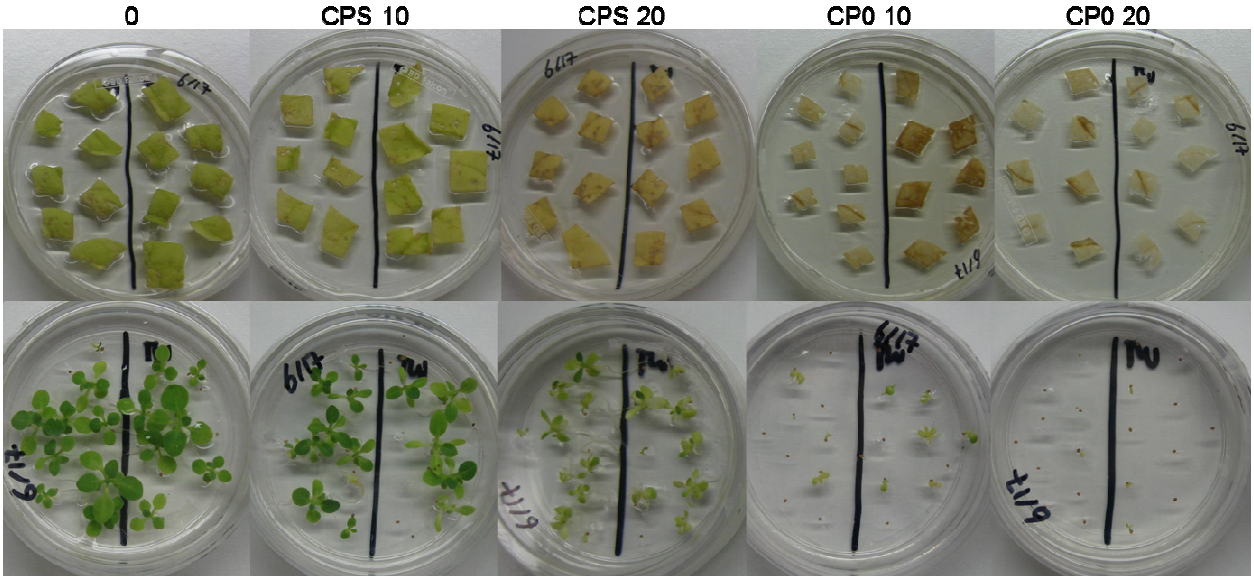

Figure 27 Phytotoxicity of CPS/CPO. Leaf discs and seeds of wild-type (right side of the dish) and PON1 transgenic tobacco (left side of the dish) were exposed to CPS and CPO and the health of the plants was monitored visually.

Both wild-type and PON1 transgenic tobacco were able to take up CPS and CPO, but there was no difference between wild-type (WT) and transgenic (PT) as PON1 transgenic tobacco took up

CPS and CPO as much as wild-type did (Figure 28). After one week exposure, the amounts (μg) of CPO accumulated in plant tissue were 2.60 ± 0.28 and 2.02 ± 0.04 for PON1 transgenic and wild-type, respectively, and the difference was statistically insignificant.

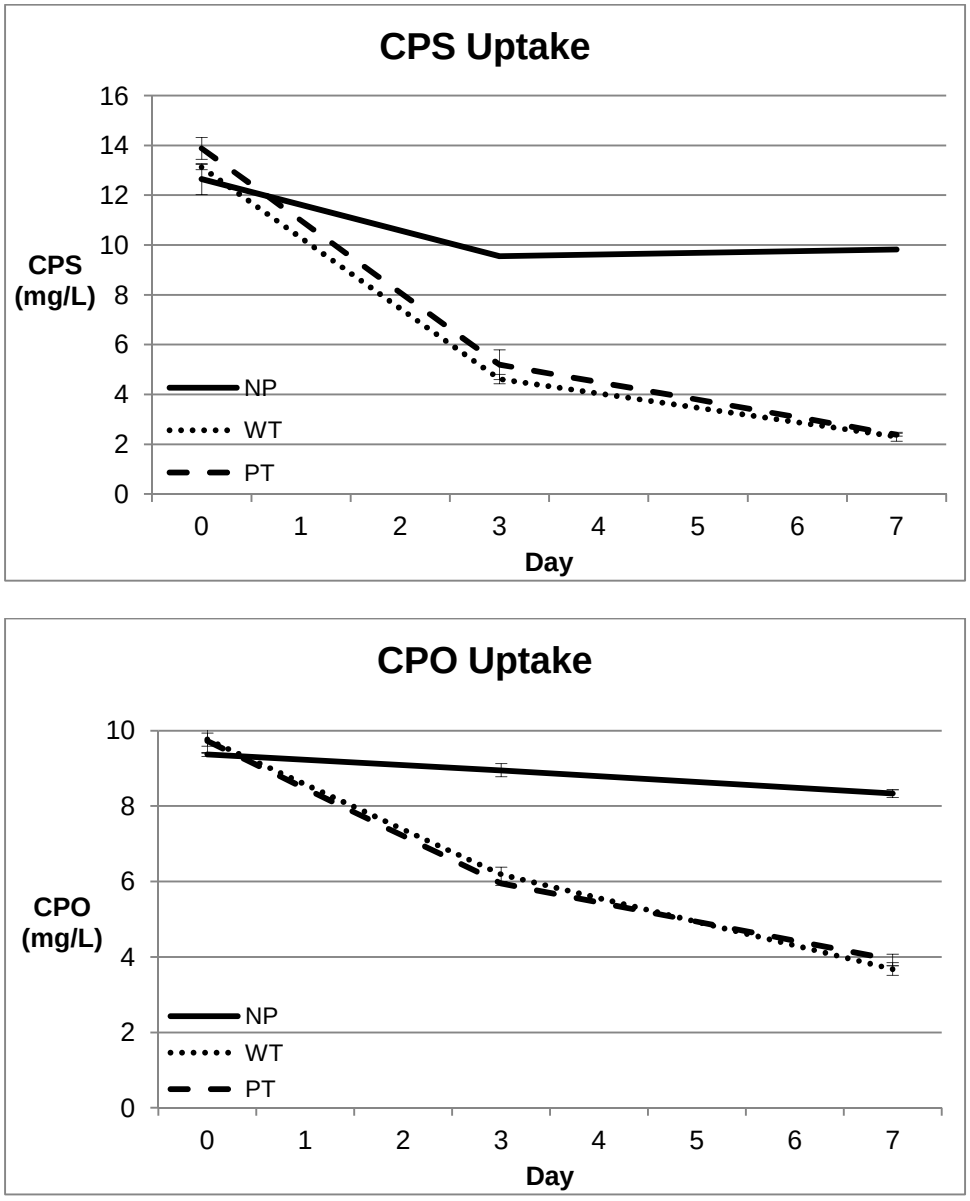

Figure 28 The decrease of CPS and CPO in hydroponic solution. The concentrations of CPS/CPO in the hydroponic solution were monitored for seven days. The data are shown as the mean ± SEM from three samples. NP: no plant control, PT: PON1 transgenic tobacco, WT: wild-type tobacco.

5.3.6 Bacteria-derived PON1 activity assay

The transcription of *pon1* gene in chloroplast expression vector was confirmed by RT-PCR showing high mRNA levels (Figure 25), but there was neither *in vitro* nor *in vivo* PON1 activity. To investigate levels of protein accumulation, the PON1-inserted chloroplast expression vector (pLD2-CtV-PON1) was tested in *E. coli* system because of the high similarity in the transcription and translation systems between *E. coli* and chloroplasts (Verma & Daniell, 2007). The growth of *E. coli* harboring the chloroplast transformation vector in the presence of spectinomycin (500 mg/L) confirmed expression of the selectable marker gene (*aad*A1), but bacterial protein showed very little phenyl acetate activity, compared to the positive control, suggesting that enzyme was active for at least a short period of time under specific conditions or the *pon1* gene may not make enough PON1 (data not shown).

5.3.7 Western blot analysis of PON1 protein expression

Western blot analysis was performed using WesternBreeze Chromogenic Western Blot Immunodetection Kit (Invitrogen) to detect rabbit PON1 protein expression levels. It was expected that PON1 protein may be found as a single protein band close to 40 kDa on SDS-PAGE and western blot (Bayrak *et al*., 2010). However, the his-tagged PON1 protein was not detected by the western blot (protein immunoblot), when a his-tag antibody was used to detect his-tagged fusion protein in bacterial protein (Figure 16, lane 6).

5.4 Discussion

The transgenic tobacco plants expressing rabbit PON1 have been obtained by the chloroplast engineering method and the level of gene expression at DNA and RNA levels was confirmed. However, the plant-derived PON1 enzyme showed very low activity on phenyl acetate and PON1 protein expressed in *E. coli* was not detected by the western blot analysis.

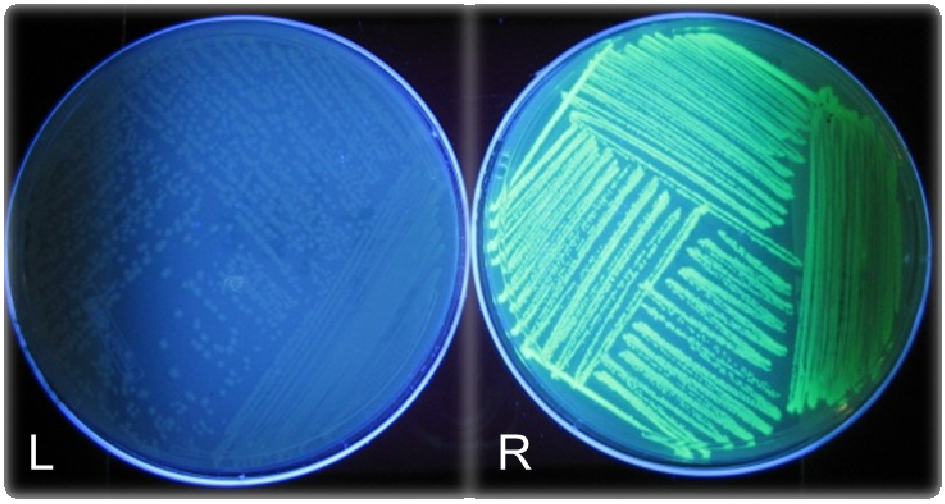

Figure 29 Green fluorescence under UV light, **L**: pLD2-CtV-(ggagg) GFP, **R**: pLD2-CtV-(g10L) GFP

There are many ways to increase protein expression when the chloroplast is engineered and phage T7 ‘gene *10* leader’ RBS (ribosome-binding sites) is known to enhance translation in chloroplasts up to 100-fold when fused to endogenous promoters (Dhingra, Portis, & Daniell, 2004; Kumar, Dhingra, & Daniell, 2004; Olins *et al*., 1988; Staub *et al*., 2000). To test whether the ‘gene *10* leader’ (g10L) RBS enhances PON1 expression, ‘ggagg’ RBS sequence was replaced to g10L sequence (5′-TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCC-3’), GFP (green fluorescent protein)-tag was added to the tobacco chloroplast expression vector (pLD2-CtV), and green fluorescence was observed, compared to GFP-tagged chloroplast transformation vector which contains ‘ggagg’ RBS. A 756-bp fragment of ‘sythetic construct modified green fluorescent protein GFP5 (mgfp5) mRNA (GenBank: U87973.1)' which cloned into the tobacco chloroplast expression vector was provided by Dr. Zhang (University of Washington, WA). The g10L RBS sequence was added to the N-terminal of the *mgfp5* gene by PCR using appropriate primers so that g10L fused mGFP5 protein is synthesized. The PCR product was cloned into the double (*Not* I and *Xba* I)-digested pLD2-CtV. The inserted gene was confirmed by sequencing. The results showed that there was very strong green fluorescence

when the RBS of the vector was replaced from “ggagg” to g10L sequence (Figure 29). However, there was no strong green fluorescence generated when the GFP-tagged chloroplast transformation vector containing ‘ggagg’ RBS (Figure 29), suggesting that the g10L enhances the expression of GFP in the chloroplast transformation vector. Therefore, inappropriate use of RBS may be the reason why the plant-derived PON1 enzyme showed very low activity on phenyl acetate and PON1 protein expressed in *E. coli* was not detected by the western blot analysis. Therefore RBS optimization may be a solution to increase gene expression.

5.5 Summary

In order to increase the expression level of PON1 protein in plants, a tobacco plant was transformed with rabbit PON1 using the chloroplast transformation method. The activity of plant-derived PON1 was analyzed and CPS tolerance and uptake test was investigated in chloroplast transformed tobacco to assess the function of rabbit PON1 enzyme in plants. However, there was no response in the PON1 transgenic tobacco as compared to wild type tobacco in terms of phytotoxicity, CPS/CPO removal, and PON1 enzyme activity assay. In addition, PON1 protein expressed in *E. coli* was not detected by the western blot analysis, when the his-tag antibody was used to detect his-tagged PON1 protein in bacterial total protein. Three possibilities exist for this failure (i) the PON1 protein is not folding properly and is targeted for destruction, (ii) the addition of the his-tag to the N-terminal did not work for the detection by the western blotting, or (iii) the expression level of the PON1 protein was very low. Since the PON1 enzyme activity assay did give positive results, it suggests that the enzyme was active for at least a short period of time under specific conditions. To enhance PON1 expression in chloroplast transformation vector, RBS optimization was suggested, but further investigation is needed such as construction of a PON1-inserted chloroplast transformation vector with g10L RBS and expression of the construct in plants.

Chapter 6 Conclusions

My dissertation contains three main studies. The results of the first study demonstrated that poplar and willow trees, unlike the herbaceous plants in previous studies, have a significant ability to take up CPS and to translocate/degrade it within the plants and thus, assist in removing it from the hydroponic solution. To our knowledge, the poplar and willow species presented in this study were the first woody plants to be tested for potential phytoremediation applications for CPS (Lee *et al*., 2012). Further study is necessary to determine the potential uptake mechanism and CPS metabolism pathway of plants not only in CPS-treated hydroponic solution but also in soil and soil-applied residues, since a number of soil physicochemical factors such as moisture, redox conditions, pH, temperature, organic matter, and nutrients, affect microbial activity, chemical diffusion in soils, and further the uptake and translocation of pesticide by plants in soil. The second study focused on the possibility of enhancing the CPS degradative potential of poplar and willow by genetic manipulation of metabolism *in planta*. However, the results of enhancing phytoremediation of CPS using transgenic plants expressing PON1, which is known to be involved in OP insecticide degradation pathways, using *Agrobacterium*-mediated nuclear transformation method, demonstrated that there was no enhanced ability of transgenic poplar to decrease the phytotoxicity and increase the removal of CPS and CPO. The modification of the coding sequence to plant preferred codons might be needed in order to enhance the expression levels of rabbit PON1 protein in plants. The results of the third study demonstrated that chloroplast transgenic tobacco overexpressing the *pon1* gene failed to express PON1 protein at detectable levels. Inappropriate use of RBS may be the reason why the plant-derived PON1 enzyme showed very low activity on phenyl acetate, and PON1 protein expressed in *E. coli* was not detected by the western blot analysis. RBS optimization may be a solution to increase gene expression. The results presented here indicate

that phytoremediation of CPS and other OP insecticides should be a fertile area for future development. Wild-type poplar and willow can degrade CPS but slowly. Genetic engineering has many challenges both in the research and with application outside considering all the restrictions to GMOs especially along the waterways where they would be most needed. Other research could be done such as screening more varieties of poplar and willow plants for natural abilities to rapidly degrade OP pesticides.

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