Expression of Anti-Atrazine scFv and Atrazine Chlorohydrolase TrzN in planta for Potential Phytoremediation of Atrazine Contamination

By
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ABSTRACT

EXPRESSION OF ANTI-ATRAZINE SCFV AND ATRAZINE CHLOROHYDROLASE TRZN IN PLANTA FOR POTENTIAL PHYTOREMEDIATION OF ATRAZINE CONTAMINATION

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University of Guelph, 2014

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Atrazine is extensively used in agricultural production to improve crop yield throughout North America. Due to contamination of surface water with atrazine, which is an environmental concern, tools for mitigating such contamination are needed. In this study, the potential use of genetically modified plants for remediation of atrazine contamination was investigated. Plants were genetically modified to express proteins of interest using Agrobacterium-mediated transformation. First, an anti-atrazine single chain variable fragment (scFv) was expressed in Lemna minor. In a hydroponic system, transgenic L. minor expressing the scFv showed higher tolerance to atrazine than wild-type plants. Absorption of $^{14}$C-atrazine in transgenic L. minor was greater than wild-type L. minor. Metabolites of atrazine were not found in transgenic L. minor. Second, L. minor was transformed to express atrazine chlorohydrolase (TrzN), but the protein could not be detected. Resistance to atrazine was found in the transgenic plants at low concentrations. Finally, the gene of atrazine chlorohydrolase TrzN was tested in Nicotiana Benthamiana by agroinfiltration. Transient Expression of atrazine
chlorohydrolase TrzN and tolerance to atrazine in transgenic *N. benthamiana* were evaluated through agroinfiltration followed by simulation of a field spray of atrazine. Tolerance to atrazine was induced by atrazine chlorohydrolase TrzN. The atrazine chlorohydrolase TrzN was produced in stable transgenic *N. benthamiana*. The protein was bound to cell membranes. The transgenic *N. benthamiana* were tolerant to atrazine when grown in both a hydroponic system and in soil with application of spray to the plants.
PREFACE

This thesis has been organized as manuscripts, which will be submitted for publication in scientific journals. Some repetition of introduction section and methods and materials section is unavoidable.
ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. J. C. Hall for his support throughout the course of my Ph.D. study. I am very grateful for the opportunity you have given me to learn and grow as an independent person. Thank you so much for your patience. I deeply thank Dr. Keith R. Solomon who is not only a mentor but also a father figure to me here in Canada. It meant a lot to me as an international student who lives far away from home. I would like to thank my advisory committee member, Dr. Richard Manderville and Dr. Derek Muir for your advice and time.

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I am so grateful for the financial support by the Royal Thai government, which gave me the opportunity to pursue my studies in Canada.

Finally, I would like to thank my parents, my uncles, my aunts, my sisters, my brother, and my extended family for your unconditional love and support. Thank you for
believing in me that I can do this and being available via video call anytime I needed someone to talk to.
DEDICATION

I would like to dedicate this work to my parents, uncles, aunts, sisters, brother, and Tumnoon Charaslertrangsi.
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementary determining region</td>
</tr>
<tr>
<td>C_H</td>
<td>Constant domain of the heavy chain</td>
</tr>
<tr>
<td>C_L</td>
<td>Constant domain of the light chain</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post infiltration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>6×His</td>
<td>6×Histidine tag</td>
</tr>
<tr>
<td>HC</td>
<td>Heavy chain</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IRED</td>
<td>Interim Registration Eligibility Decision</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LC</td>
<td>Light chain</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MES</td>
<td>1-(N-morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog Medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NBT/BCIP</td>
<td>Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation (400-700 nm)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain variable fragment</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer-DNA</td>
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<tr>
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1 LITERATURE REVIEW

1.1 Atrazine

Atrazine (Figure 1.1) is a triazine herbicide used for control of weeds in a number of crops. It is an inhibitor of Photosystem II (Giddings 2005). Atrazine has been commercially available since 1958 (United States Environmental Protection Agency), and it is registered under the Pesticide Control Product Act in Canada as a herbicide to be used as the main active ingredient for broadleaf weed control in crops (Pest Management Regulatory Agency 2007).

![Figure 1.1 Atrazine](image)

1.1.1 Usage

In Canada as well as the US, atrazine is primarily applied to resistant crops, e.g., corn (Zea mays), sugarcane (Saccharum officinarum), sorghum (Sorghum vulgare), and wheat (Triticum aestivum) (Heri et al. 2008). Atrazine formulations are usually suspensions, with a common concentration of the active ingredient being 480 g/L. Atrazine is often formulated as a mixture with other herbicides such as dicamba and metolachlor, to increase efficacy. In Canada, atrazine may be applied as a preplant, preemergent, or postemergent herbicide at recommended application rate of 1 kg a.i./ha

In North America, use of atrazine occurs in the mid-western United States, Southern Ontario, and British Colombia. During 2007, atrazine was the second most commonly used pesticide in the United States. Approximate 33-35 million kilograms of active ingredient were used in USA; the majority in the Corn Belt, which comprises Illinois, Indiana, Iowa, Minnesota, Wisconsin, Nebraska, Missouri, and Ohio (United States Environmental Protection Agency 2013, Larson et al. 1997).

1.1.2 Physical and chemical properties

Atrazine is a member of the s-triazine herbicide family (and belongs to the chlorotriazine subfamily). Key physicochemical properties of atrazine are: dissociation constant (pKa) of 1.7 at 21°C, and moderately soluble (33 mg/L in water at 27°C and pH 7). The octanol/water partition coefficient (log K_{OW}) is 2.68 at 25°C. Atrazine has low potential to sorb onto soil (K_{OC} is a range of 40 to 394 L/kg). Atrazine has low vapour pressure at $2.89 \times 10^{-7}$ mm Hg at 25°C (Giddings 2005, Humburg 1989). Because of these physicochemical properties and extensively use, atrazine is one of the most commonly detected pesticides in surface water and groundwater (Graymore et al. 2001, Stone et al. 2014).

1.1.3 Mechanism of action of atrazine

Atrazine is absorbed through roots and foliage, and is translocated to accumulate in the apical meristems and leaves (Humburg 1989). Translocation of atrazine is pseudoapoplastic, in which the herbicide is translocated through apoplast (non-living
continuum) but it enters symplast (living continuum) and then the target organ, the chloroplast. Atrazine inhibits photosynthesis through blocking the electron transfer pathway to photosystem II (PSII), which is essential for the production of photosynthetic energy. It competes with plastoquinone for binding to the QB-binding niche (Figure 1.2), the electron carrier, on the D1 protein of the photosystem II complex in chloroplast thylakoid membranes. This results ultimately in blocking of carbon dioxide fixation, ATP synthesis, and NADPH production. These are essential components in growth of plants (Shimabukuro and Swanson 1969, Humburg 1989). However, if plants are in the light, the chlorophyll continue to harvest light. Without electrons transferred from plastoquinone ($Q_B$) to $Q_A$, free radicals are formed. Free radicals are destructive to membranes of the chloroplasts and damage to chlorophyll of PSII as well as PSI. This results in chlorosis (loss of chlorophyll) and cell death (Stephenson and Solomon 2007).

![Diagram of Atrazine binding to D1 protein](image)

**Figure 1.2** Atrazine blocks the binding of plastoquinone ($Q_B$) on D1 protein.
1.1.4 Environmental fate

Atrazine is persistent in soils with a half-life of 20 to 146 d (mean = 44 d) (Giddings 2005). Atrazine is moderately soluble and adsorbs slightly onto soil colloids; it runs off into surface waters in areas of use as a result of irrigation or rainfall (Giddings 2005). With a relatively low octanol-water partitioning coefficient, atrazine is not likely to bioconcentrate or biomagnify in aquatic organisms (Giddings 2005). Moreover, it is not likely that atrazine will volatilize from an aquatic system under normal conditions, as it has a low vapour pressure (Giddings 2005). Given the wide use of atrazine in the Corn Belt and its physico-chemical properties, concerns of atrazine contamination of surface water and groundwater due to runoff have been raised.

1.1.5 Biotransformation

Atrazine released into the environment is degraded through biological and non-biological mechanisms. Certain plants, certain microorganisms, and animals are prominent in the degradation of atrazine. Atrazine is degraded by major enzymatic reactions in Phase I (conversion) and Phase II (conjugation). Reactions in Phase I metabolism include oxidation, reduction, and hydrolysis, in which a foreign compound is transformed to a less hydrophobic and generally less toxic compound. Cytochrome P450 in Phase I metabolism catalyzes oxidative N-dealkylation of atrazine and, as a result, the alkyl groups on the amino side-chains are removed (Kawahigashi et al. 2008). Phase II metabolism involves conjugation of atrazine and glutathione (GSH) by glutathione S-transferase, which results in formation of a water-soluble metabolite (Van Eerd et al. 2003). Three metabolic pathways are involved in the transformation of atrazine in plants (Figure 1.3). Glutathione conjugation is the displacement of chlorine atom at 2-carbon
atom of atrazine. Glutathione S-transferase catalyzes this reaction and this is the major pathway of detoxification of atrazine in corn and some resistant weeds (Lamoureux et al. 1998). An example of resistance of plants to atrazine is velvet leaf (*Abutilon theophrasti*). In this case, resistant plants over-produce glutathione S-transferase to metabolize atrazine (Lamoureux et al. 1998). The second mechanism is hydrolysis where the chlorine atom at the 2-carbon atom of s-triazine ring is replaced with a hydroxyl group (OH) (Lamoureux et al. 1998). In tolerant corn, 35-65% of atrazine taken up via roots is metabolized by hydroxylation (Lamoureux et al. 1998). The third pathway, N-dealkylation, is a metabolic reaction in which cytochrome P450 monooxygenases remove the side-chains; ethylamino and isopropyl amino groups (Lamoureux et al. 1998). In pea and sorghum, atrazine is degraded to less phytotoxic compounds such as desethylatrazine and desisopropylatrazine (Lamoureux et al. 1998).
Microbial degradation is the dominant degradation process of atrazine in soil (Mandelbaum et al. 2008). Soil microorganisms utilize atrazine as a source of energy and nitrogen (Humburg 1989). Atrazine is enzymatically detoxified to hydroxyatrazine, and subsequently cyanuric acid, carbon dioxide, and ammonia (Wackett et al. 2002). *Pseudomonas* sp. strain ADP, *Arthrobacter aurescens* TC1, and several isolated soil bacteria have been investigated for their ability to degrade atrazine into non-toxic metabolites (deSouza et al. 1996, Shapir et al. 2005, Shapir et al. 2007, Wackett et al. 2002).

Dissipation of atrazine in surface waters via photodegradation occurs by direct and indirect photolysis. Atrazine is degraded via direct photolysis, when it absorbs
sunlight at wavelengths >290 nm. Hydroxyatrazine; dealkylated products of hydroxyatrazine, e.g., desethylhydroxyatrazine, desisopropylhydroxyatrazine, ameline; and dealkylated products of atrazine, e.g., desethylatrazine, desisopropylatrazine, and diaminochloroatrazine have been reported as the metabolites from direct photolysis (Cessna 2008). However, as atrazine absorbs sunlight weakly, indirect photolysis in the presence of photosensitizers is the main pathway of dissipation of atrazine. Photosensitizers such as dissolved organic carbon, nitrate, and acetone absorb light and transfer energy to catalyze the degradation of atrazine. Cyanuric acid was the stable metabolite from indirect photolysis (Cessna 2008). Figure 1.4 illustrates the structure of major degradants of atrazine.
1.2 Bioassays to show resistance/tolerance to atrazine in plants

Resistance is defined by the Weed Science Society of America (1998) as the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type; whereas tolerance is defined as the inherent ability of a species to survive and reproduce after a herbicide treatment, implying that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant. Both are responsible for plants being able to ameliorate the effects of a herbicide.

Resistance/tolerance to a chemical occurs when living organisms are less affected or respond less to the chemical. Two major mechanisms for resistance/tolerance are: toxicokinetic, where a decreased amount of the toxic substance reaches the site of action.
where the adverse effect occurs and, toxicodynamic, where there is a reduced responsiveness of the target system to the chemical. Metabolic pathways in plants are capable of changing the structure of atrazine, which prevents atrazine from reaching or binding to the active site in the chloroplast. Conjugation with glutathione is the main mechanism responsible for selectivity (tolerance) to atrazine in corn and sugarcane versus susceptible weeds (Lamoureux et al. 1998). A mechanism of resistance has been reported in algae. A mutation in a chloroplast gene called psbA, which encodes for Q₈ protein, in Chlamydomonas reinhardtii causes a decreased affinity of atrazine to the D1 protein of photosystem II resulting in tolerance of atrazine in this alga (Erickson et al. 1984).

1.2.1 Dose-response bioassay

In toxicological studies, test organisms are exposed to a substance through an exposure medium. The characteristic graph illustrating the relationship between the magnitude of the response of the biological system and the amount of toxicant administered is referred to as the dose–response relationship. The regulation of pesticides such as insecticides, herbicides, and fungicides requires experimental studies to investigate the toxicity of a particular pesticide in living organisms. Like other herbicides, atrazine is one of the most scrutinized compounds; the toxicity of atrazine has been widely studied in many organisms. In addition, dose-response bioassays are used for comparison studies of susceptibility between susceptible and resistance/tolerant plants. For example, a resistant biotype of Poligonum lapathifolium was 35.5-times more resistant to atrazine than the susceptible biotype (Deprado et al. 1995). In the same study, an atrazine dose-response assay was also conducted to compare two groups of Panicum
which were harvested from an untreated cornfield, and one repeatedly treated with atrazine. The EC\textsubscript{50} values from the assay were 2.3 (±0.50) kg a.i./ha (untreated cornfield) and 3.2 (±0.65) kg a.i./ha (treated cornfield) (Deprado et al. 1995). This indicates that they were not selected for resistance to atrazine.

Along with this dose-response data of atrazine in plants, uptake via foliar and root and metabolism bioassays can be conducted to elucidate the mechanism in more detail. In the study of terrestrial plants, application of the test material to the plant can be achieved in a number of ways, for example as droplets applied to the leaves from a syringe or micro-pipette, by dipping the plant material in a solution, or painting or spraying. The advantage of using droplets is that the test material can be placed at a particular location; however, it is time-consuming with large-scale studies. The most practical procedure and most likely to simulate the application method used by the farmer is spraying. The sprayer is able to deliver the solution evenly in a very short time (Roberts 2000).

1.2.2 Uptake bioassay

To evaluate the amount of pesticides that are taken up via roots, two procedures can be used: 1) the uptake can be monitored from a hydroponic solution; 2) the pesticide can be applied to soil. In all these studies, the use of radiolabelled material allows researchers to trace and quantify the substances absorbed into plants (Roberts 2000).

The route of application is different for studies to determine the fate of a compound in plants for phytoremediation purpose. Root uptake assays best represent the process of absorption of contaminants from soil in plant. Plant roots absorb contaminants along with absorption of water and nutrient. Many pesticides in soil can enter the roots
of plants by diffusion or with the flow of water. Limitations of the bioaccumulation of atrazine in *Lemna gibba* were studied using a root-uptake assay (Guimarães et al. 2011). At small concentrations of atrazine, *L. gibba* had low potential to remove the herbicide from the exposure solution, but at higher concentrations of atrazine, the removal rate increased. However, a decrease in rate of production of biomass was observed due to damage/death of plant, which was caused by the herbicide (Guimarães et al. 2011).

### 1.2.3 Metabolism bioassay

The objectives of studies of plant metabolism are to provide an estimate of total residues in the relevant portions of the treated plants, to identify the major components of the terminal residues, and to indicate the distribution of residues (Roberts 2000). They provide in-depth explanations of how organisms detoxify substances and provide an explanation for how they might survive without showing an adverse effect.

Metabolism of substances in plants also is important for phytoremediation of contaminants. Oxidation and conjugation are main mechanisms for degradation of contaminants in plants. Cytochrome P450s are involved in oxidation of pesticides. Conjugation of pesticides converts them to more water-soluble metabolite(s). Most plants metabolize a small percentage of absorbed pesticides. Hybrid poplar trees (*Populus deltoids*) were able to absorb atrazine from soil and metabolize atrazine. Hydroxylation and dealkylation were the main metabolic pathways of detoxification of atrazine in poplar trees. The transformation of atrazine was observed in roots, stems, and leaves of poplar (Burken and Schnoor 1997). The mechanism conferring atrazine resistance in weeds; *Poligonum lapathifolium* and *Panicum dichotomiflorum* was investigated in a metabolism assay followed by a dose-response assay (Deprado et al.
1995). Concentrations of glutathione- and cysteine-conjugated atrazine in the resistant (R) strain were 11% greater than susceptible *P. dichotomiflorum*. In study of *P. lapathifolium*, there was no difference in terms of metabolites; however, the resistance was due to less sensitivity of the active site as supported by study of the electron transport in isolated chloroplast from plant cells using Hill reaction (Deprado et al. 1995).

### 1.3 Risk assessment of atrazine

Risk assessment of a chemical is a process to determine a risk based on four main components. First, hazard identification is a step to identify adverse effects caused by a substance. Second, dose-response assessment is a step that evaluates the relationship between the adverse effect and the dose that causes the adverse effect. Third, exposure evaluation is the estimation of how much of a substance is taken into the body such as residues in food and the rate of consumption. Last but not least, risk characterization is the analysis of the latter two processes to evaluate the risk (Cassarett et al. 2001).

#### 1.3.1 Toxicity of atrazine

Acute toxicity studies of animals exposed to atrazine have been used by the regulatory agencies to assess the risk of exposure. Acute response is usually based on observations for 96 h after a single exposure. In mammalian studies, the acute oral LD$_{50}$ for rats is 1,869-3,090 mg/kg, acute dermal LD$_{50}$ is >2,000 mg/kg, and acute inhalation LC$_{50}$ is >5.8 mg/L (Breckenridge et al. 2008). The USEPA uses a chronic NOEL (no observable effect level) of 1.8 mg/kg/d of atrazine from a study of luteinizing hormone (LH) surge in female Sprague-Dawley rats to calculate a reference dose (RfD) (United States Environmental Protection Agency 2006). The USEPA has established a maximum
contaminant level (MCL) of atrazine at 0.003 mg/L in drinking water. In Canada, the
guideline for regulation of atrazine is that the interim maximum acceptable concentration
(IMAC) in drinking water be no more than 0.005 mg/L (Health Canada 1993).

Atrazine has shown negative effects in many aquatic organisms. As atrazine is an
inhibitor of photosynthesis, phytoplankton are the most sensitive, followed by
macrophytes, benthos, zooplankton, and fish, respectively in laboratory settings
(Giddings 2005, Solomon et al. 1996). $EC_{50}$ values of phytoplankton vary from 0.004
mg/L ($Selenastrum capricornutum$) to >3 mg/L ($Anabaena flos-aquae$). In zooplankton,
the range of $LC_{50s}$ varies from 1 mg/L ($Chironomus riparius$) to 150 mg/L ($Daphnia
magna$). $EC_{50}$ values in fish ranged from 0.22 mg/L ($Ictalurus punctatus$) to >100 mg/L
($Oryzias latipes$). Acute toxicity of atrazine for selected freshwater plants has a range
from an $EC_{50}$ of 0.021 mg/L ($Elodea canadensis$) to 8.7 mg/L ($Lemna minor$) (Giddings
2005). A short-term exposure (up to 14 d) to atrazine ranging from 0.005 to 0.16 mg/L
did not have a negative effect on either structure or function of periphyton communities
(Brain et al. 2012). In another study, Prosser et al. (2013) observed a rapid physiological
recovery of periphyton communities after exposure to 0.01-0.32 mg atrazine/L for 2, 6,
and 24 hr followed by transfer to clean medium (Prosser et al. 2013). In amphibian
toxicity studies, atrazine has been reported to demasculinize male American clawed frogs
($Xenopus laevis$) and cause gonadal dysgenesis and hermaphroditism in American
leopard frogs at concentrations as small as 0.0001 mg/L (Hayes et al. 2003, Hayes et al.
2010). However, other authors showed that there was no correlation of atrazine exposure
with sexual abnormality in laboratory settings (Coady et al. 2005, Hecker et al. 2004). A
weight of evidence shows that there are very few potential adverse effects of atrazine
exposure, at environmentally-relevant concentrations, to fish, amphibians, and reptiles (Van Der Kraak et al. 2014).

1.3.2 Exposure assessment

Runoff from agricultural fields is the major source of environmental contamination, particularly to water resources, from atrazine applied to corn and sorghum (Giddings 2005). In certain rivers in the central United States, atrazine concentrations exceeded 0.003 mg/L for days to weeks (United States Environmental Protection Agency 2003). Atrazine in surface water has been continuously monitored, and peak atrazine concentrations have been found to co-occur with field application and rainfall. During the period of 2002-2011, atrazine was one of the most frequently detected herbicides in streams and rivers. Concentrations of atrazine exceeded the human health benchmark (maximum contaminant level) of 0.003 mg/L between 2002-2011 (Stone et al. 2014). In Canada, of the 224 water samples tested from southern Ontario, atrazine was detected in 93% of the samples. The range of detection was 0.000006 – 0.015 mg/L in 2004 (Environment Canada 2011).

1.3.3 Risk management

Figure 1.5 illustrates risk management strategies to reduce the effect of agrochemicals (Ivan et al. 2007). The reduction in exposure to atrazine of non-target organisms is the focus these strategies. Risk mitigation (reduction) of toxicants in the environment is one of the strategies to clean up pollutants (United States Environmental Protection Agency 2012b). One of the techniques that have been approved by the USEPA is the use of buffer strips. Setback areas around farmland that contain crops or
natural flora are able to reduce the runoff of atrazine into water reservoirs (Giddings 2005). Moore et al. (2000) found that a buffer strip 100-280 m wide in agricultural areas proved to be more effective in atrazine mitigation than a constructed wetland in agricultural areas. Runes et al. (2001) found that atrazine could be degraded in constructed wetland microcosms, and also suggested that the bioaccumulation of atrazine in plant may be a major mechanism for dissipation of atrazine.

Figure 1.5     Risk management scheme of agrochemicals. Diagram modified from Ivan et al. 2007.
1.3.3.1 Phytoremediation

Conventional strategies have been developed to remove contaminants affecting aquatic plants from the aquatic environment, which include isolation and containment and removal of soil to landfill (Arthur et al. 2005). Despite their effectiveness, these techniques are very expensive, making widespread use of these strategies unfeasible (Cunningham and Ow 1996). The idea of using phytoremediation to clean up contaminated soil and water has been employed since the 1980s (Maestri and Marmiroli 2011). Phytoremediation is defined as the use of plants to clean up polluted soils and water in the environment. This technology offers a cost-effective and environment-friendly alternative or complementary technology for conventional measures using physical and chemical mitigation (Salt et al. 1995). As a result of absorption of nitrogen and other micronutrients from soil and water through root system, plants take up other contaminants, which they and their associated microbes have evolved mechanisms for detoxification of small molecules such as pesticides (Abhilash et al. 2009).

Plants decontaminate pollutants in environments via several processes. First, phytostabilization is the process whereby plants form a complex with metal pollutants in soil. Second, phytoextraction is the process through which plants accumulate and remove contaminants from soil followed by removal of plants biomass. Third, rhizofiltration is the process whereby roots of plants remove toxicants from contaminated water. The plants are then harvested. Fourth, phytodegradation is the process, in which a contaminant is transformed to a (less toxic) metabolite (Abhilash et al. 2009, Salt et al. 1995, Arthur et al. 2005, Kramer and Chardonnens 2001). Although phytoremediation is an effective means of mitigating a wide range of organic and inorganic pollutants, there
are limitations of this technology such as exudation from plant and lack of metabolic
degradation (Pilon-Smits 2005, Abhilash et al. 2009, Schnoor et al. 1995). In addition,
plants may be damaged by contaminants at high concentrations, when plants must
tolerate to the contaminants in order to remove them (Raskin and Ensley 2000).

With the concerns of atrazine contamination in soil and water from the use in
agriculture, researchers have focussed on the best strategy of using plants to
decontaminate atrazine. However, generally speaking, the phytoremediation approach
requires selection of suitable plants to clean up an environmental pollutant or mixtures of
environmental pollutants (Raskin and Ensley 2000). The atrazine-tolerant plant, *Lolium
multiflorum*, was found to degrade atrazine via monooxygenase (Cytochrome P450), and
has the potential to be exploited as a phytoremediating organism for atrazine (Merini et
al. 2009). Similarly, roots, leaves, and stems of poplar trees were shown to metabolize
atrazine to non-herbicidal compounds (Schnoor et al. 1995, Chang et al. 2005). *Panicum
virgatum* was reported to have the ability to degrade atrazine to cyanuric acid, when the
plants were treated with only low concentration of atrazine as 0.004 mg/kg (Albright and
Coats 2012).

1.4 Plant genetic engineering for phytoremediation

With the idea to make the use of plants for remediation more effective,
enhancement of the properties of plants for remediation using genetic biotechnology
started around 20 years ago (Maestri and Marmioli 2011). Genetic engineering, the
ability to manipulate genes in an organism, might allow us to expand phytoremediation
capabilities by enabling the transfer of phytoremediative traits from various organisms to
candidate plant species. Alternatively, endogenous phytoremediative traits can be
overexpressed (Maestri and Marmiroli 2011) and thereby made more effective. Plant biotechnology may increase the ability of candidate plants to degrade contaminants. Transgenic *Brassica jancea*, which was genetically modified to overexpress γ-glutamylcysteine synthetase (GCS) or glutathione synthetase (GS), showed increased tolerance toward atrazine (Flocco et al. 2004).

### 1.5 Transformation of plants and protein expression systems

The efficacy of plants to remediate contaminants can be improved by genetic transformation. Genetic transformation can modify plants to process the contaminants, i.e., by capturing them via expression of an antibody specific for the contaminant, or by metabolizing via an insertion of a new enzyme or enhancement of the expression of existing enzymes.

In theory, expression of anti-pesticide antibody fragments in plant should enhance a plant’s ability to sequester a specific pesticide. The gene coding for antibody fragments could be engineered into the plant genome. The single chain variable fragment (scFv), which contains the binding site to the pesticide, may be able to enhance the capability of bioaccumulation (Chaudhry et al. 2002). The study showed the affinity of purified antibody against atrazine using ELISA (Longstaff et al. 1998). Another example of expressing an antibody fragment in plant was studied by (Almquist et al. 2004). Transgenic tobacco expressing anti-picloram scFv showed tolerance to this herbicide (Almquist et al. 2004).
1.5.1 *Agrobacterium tumefaciens*-mediated stable transformation

*Agrobacterium tumefaciens* is a soil microorganism that has the natural capacity of introducing DNA of interest into plant genomes. The tumor-inducing (Ti) plasmid contains several genes that encode proteins that are involved with producing galls in infected plant tissue. Thus, *A. tumefaciens* has been genetically manipulated to produce stable introduction of genes of interest into the host plant’s genome. *A. tumefaciens* inserts the transfer DNA (T-DNA) into the plant genome in a covalent form (Gelvin 2003). The binary vector approach has been widely used in plant transformation (Bevan 1984, Gelvin 2003). The concept of this approach is the *Agrobacterium* contains two plasmids, a *vir* helper vector and a binary vector (Gelvin 2003). The *vir* helper plasmid retains the *vir* region but the on the T-DNA, oncogenes and opine genes are removed so that *Agrobacterium* cannot produce tumors (disarmed). In the binary vector, only the left and right border and the origin of replication region remain. One or two selectable marker genes are incorporated into the binary vector for identification of transformed tissues (Gelvin 2003).

The process to produce stable transgenic plants using *Agrobacterium*-mediated plant transformation starts with co-cultivation of plant tissues, usually wounded leaf discs with *A. tumefaciens*. Then, selective media selects the transformed tissues followed by the regeneration of transformed plants (Chawla 2009). *Agrobacterium*-mediated transformation of plant has been studied widely in both basic and applied research. Stable levels of expression from stable transformation come with a trade-off of time consumption due to prolonged steps of selection and regeneration.
1.5.2  Agroinfiltration for transient expression

Before investing time in stable transformation, which may take months to years to produce stable transgenic plants, the gene construct can be tested using agroinfiltration (Kohli et al. 2003). Agroinfiltration is the insertion of a transient system for expression of foreign protein in plants. A suspension of *A. tumefaciens* carrying the gene of interest is prepared in infiltration buffer and is directly forced into the intercellular space of intact plant tissues using either a syringe, known as spot infiltrated, or vacuum for whole leaf/plant infiltration (Kapila et al. 1997, Voinnet et al. 2003, Wydro et al. 2006). Once the gene of interest harboured in the *Agrobacterium* is transferred into the nucleus, it can function as extrachromosomal entity without integrating into the plant chromosome (Finer 2010). The advantage of agroinfiltration is immediacy; plants can produce the desired protein within a week after being agroinfiltrated. Co-infiltration with p19, a suppressor of gene silencing, increases transient protein expression (Voinnet et al. 2003). However, a disadvantage of agroinfiltration is the decline in expression of the transgene after a short period of time.

*Nicotiana benthamiana* has been used as a model in plant genetic transformation technology. Agroinfiltration is highly efficient in *N. benthamiana*, and permits the protein of interest to be produced transiently in plant cells. This species has been used to screen and/or test if the construct of the gene of interest is efficient (Goodin et al. 2008, Kapila et al. 1997) and was used in part of the work described in this thesis.

1.6  *Lemna minor*

*Lemna minor* L. (Figure 1.6) is a member of the family Lemnacea and sub-family Lemnoideae (Mkandawire and Dudel 2007). It is commonly known as duckweed, and is
a small free-floating freshwater macrophyte (Hillman 1961). It is ubiquitously present in slow moving freshwater found in temperate climatic zones (Hillman 1961). *L. minor* grows as a small cluster (3 – 5) of leaf-like fronds with a single root at its center (Hillman 1961, Les et al. 1997, Newmaster SG 1997, Godfrey 1981). Usually, *L. minor* reproduces by vegetative budding, where new fronds arise from pockets or pouches on the margins of mother fronds. As a result, they grow rapidly, covering open areas of surface waters (Hillman 1961). Under ideal enriched nutritient conditions, the growth rate of *L. minor* is ca. 0.6 fronds per day (Mkandawire and Dudel 2007).
Figure 1.6  A photo of *Lemna minor* in modified Hoagland’s E+ medium.

Because of the advantageous properties of *Lemna*, i.e. small size, simple structure, rapid growth, and ease of manipulation under laboratory condition, it is used for *in vivo* toxicity tests in freshwater systems. It is a standard test organism in aquatic ecotoxicology to test chemical toxicity (Mkandawire and Dudel 2007). To register a new pesticide with most regulatory agencies, it is recommended that they be tested in an aquatic system containing *Lemna*. *Lemna* fronds are placed into vessels containing liquid medium and test chemical. After exposure to the chemical of interest, colonies are examined for changes in growth.

*L. minor* has the potential for phytoremediation of heavy metals in wastewater. The accumulation of heavy metals in *L. minor* occurs via biosorption by surface cells (Mkandawire and Dudel 2007). The mechanism includes transformation of substances into less toxic compounds and sequestration, by which foreign compounds are deposited into non-living compartments of the cell, such as the vacuole and/or the cell wall (Mkandawire and Dudel 2007). Small concentrations of zinc, mercury, arsenic,
chromium, and lead (<0.5 mg/L) in water resources were removed effectively by \textit{L. minor} within one to two weeks (Parra et al. 2012). \textit{L. minor} and \textit{L. gibba} are capable of fixing metals in the vacuole and in the cellulose of cells (Mkandawire and Dudel 2007). These two aquatic species are also capable of reusing organic wastes that are rich in nitrogen and phosphorus into plant protein (Mkandawire and Dudel 2007). These studies show that these aquatic species can utilize organic wastes throughout the year in tropical and temperate regions.

\textit{L. minor} contains large quantities of protein (35\% dry weight of biomass) and it is used as livestock feed for fish and cattle. Protein in \textit{Lemna} has high lysine and methionine content and is comparable to animal protein (Mkandawire and Dudel 2007). \textit{Lemna} species are also a good source of biogas (Leng et al. 1995). \textit{Lemna} has the potential to reduce algae blooms, because the mat of plants on the surface decreases penetration of light and takes up nutrients (Mkandawire and Dudel 2007).

Limitations of using \textit{Lemna} for phytoremediation have been discussed. They are sensitive to contaminants such as herbicides and heavy metals, which cause reduction in growth (Mkandawire and Dudel 2007). Also, they are vulnerable to surface-active hydrophobic substances (Taraldsen and Norbergking 1990). Since the bioaccumulation of contaminants using aquatic plants is generally based on excess biomass, as small plants, \textit{Lemna} species may not be ideal for high-volume phytoextraction (Mkandawire and Dudel 2007). However, up to this point, macrophytes have not been studied in terms of genetic transformation to sequester or detoxify environmental contaminants (Mkandawire and Dudel 2007) and provide a model organism that is easily grown in the laboratory. A
better understanding of ways to enhance mechanisms of uptake and detoxification might provide useful techniques to expand this approach to other macrophytes.

1.7 Antibodies

Antibodies (Abs) or immunoglobulins (Igs) are products of the immune response in mammals. They selectively function in recognizing new foreign molecules, known as antigens, such as toxins, viruses, and microbes that might be harmful. They bind to foreign antigens and stimulating phagocytosis, a process of engulfing a foreign particle in cells (2003), that mediates the ultimate destruction of the antigens.

In humans, there are five types of immunoglobulins: IgA, IgD, IgE, IgG, and IgM. IgGs are the most abundant immunoglobulin circulating in blood (Lydyard 2004). IgGs are comprised of two identical pairs of κ or λ light chains (LC) and γ heavy chains (HC) that are linked together by disulphide bonds at the hinge region. Each pair contains Fab fragment and Fc fragment. Each light chain has a constant region (C_L) and a variable region (V_L). Similarly, each heavy chain has a constant region (C_H) and a variable region (V_H) and three constant domains, CH1, CH2, and CH3. Variable regions of antibodies make the antibody specific to a particular antigen. Each variable region can be further divided into different complementarity-determining regions (CDRs). The amino acid sequences of CDRs determine the specificity of an antibody to an antigen. A pair of variable heavy and variable light chains is called single chain variable fragment (scFv).

Since antibodies have specific binding properties, they have been used as tools for the detection of contaminants in the environment such as herbicides (Yau et al. 1998, Longstaff et al. 1998). Antibodies could also be used to provide new characteristics to plants, such as herbicide resistance (Almquist et al. 2004, Eto et al. 2003).
1.8 Single-chain variable fragment (scFv)

The antigen binding sites of immunoglobulins are located in the variable heavy and light chain domains (Figure 1.7), and are separated from the constant domain fragments of the heavy and light chains. The variable domains of an immunoglobulin, by themselves, are called single-chain variable fragments (scFv). In producing recombinant protein, the scFvs can be created without the constant regions. Since scFvs lack an Fc region, they are much less immunogenic than full-length antibodies. The VH and VL are held together by non-covalent interactions only, which results in the dissociation of the structure under some conditions. A fusion of the variable regions of the heavy and light chains with a polypeptide linker increases stability. A typical polypeptide (((Glycine)$_4$Serine)$_3$) is often used as a linker in engineering of scFvs. In genetic engineering, the affinity of scFv can be improved by performing phage display. An anti-atrazine scFv can express a binding site specific to atrazine. The equilibrium dissociation constant of the scFv is $7.46 \times 10^{-10}$ and indicates that it has excellent affinity to atrazine (Kramer 2002).
Figure 1.7  Structure of immunoglobulin G and scFv. A) Immunoglobulin G: variable heavy domain (V\textsubscript{H}), variable light domain (V\textsubscript{L}), constant light domain (C\textsubscript{L}), constant heavy domains (CH\textsubscript{1}, CH\textsubscript{2}, CH\textsubscript{3}), and S-S represents disulphide bond. B) scFv: variable heavy domain (V\textsubscript{H}), variable light domain (V\textsubscript{L}), and black line represents linker.

1.9  **Enzymes for degradation of atrazine**

A number of pathways for degradation of atrazine into non-herbicidal derivatives are found in plants, microorganisms, and humans, (Maestri and Marmiroli 2011). Three main metabolic enzymes catalyzing the reaction are cytochrome P450, glutathione S-transferase, and atrazine chlorohydrolase (Ralebitso et al. 2002, Shapir et al. 2006). Microorganisms metabolise atrazine to hydroxyatrazine (Figure 1.8) via the enzyme atrazine chlorohydrolase (Mandelbaum et al. 2008).
Figure 1.8  Hydrolysis of atrazine by atrazine chlorohydrolase TrzN. Diagram modified from Shapir et al. 2007.

1.10 Genetic transformation in \textit{Lemna minor}

The first discovery of the capability of \textit{A. tumefaciens} to transfer genetic material to plants was in 1970s (Zaenen et al. 1974). Genetic transformation of plants has provided tremendous benefits in several ways, such as environmental protection and in the productions of drugs and precursors for the pharmaceutical industry (Fischer and Emans 2000, Giddings et al. 2000). As mentioned in Section 2.4, genetically modified plants that are resistant to the toxicants may be useful to remediate contaminated environments (Maestri and Marmiroli 2011). This statement is based on the fact that in the production of pharmaceuticals \textit{in planta}, plants provide cost-effective systems for the large-scale production of Abs, and their genetic resources are easily manipulated (Fischer and Emans 2000, Kirakosyan 2009).

Even though genetic transformation in plants has had a long history, the knowledge of genetic transformation in duckweeds; \textit{L. gibba} and \textit{L. minor} was only recently reported by Yamamoto et al. in 2001 using β-glucuronidase GUS, a reporter gene, as a model protein for selection (Yamamoto et al. 2001). In 2006, because of the advantages of high protein content and lack of zoonotic pathogens, expression of therapeutic protein was investigated in \textit{L. minor} (Cox et al. 2006). These authors
reported that \textit{L. minor} could be used to produce recombinant monoclonal antibodies (Cox et al. 2006).

Aquatic floating macrophytes such as \textit{L. minor} are good candidates for phytoremediation to remove atrazine from surface waters. In order to increase the capability of \textit{L. minor} to remove atrazine through uptake and degradation, expression of certain proteins via genetic transformation would need to be applied. A gene encoding an antibody able to target a herbicide may improve rates of uptake (Olea-Popelka et al. 2005). A gene encoding an atrazine-targeting catabolytic enzyme such as trzN (atrazine chlorohydrolase enzyme) may also increase rates of degradation of atrazine.

The objective of this research was to study a model for phytoremediation using plants expressing antibodies and/or enzymes. Three transgenic plants were included in this study; \textit{L. minor} expressing an anti-atrazine single chain variable fragment; \textit{L. minor} expressing the atrazine chlorohydrolase gene TrzN; and \textit{N. benthamiana} expressing TrzN. Transgenic \textit{L. minor} plants were characterized for tolerance to atrazine using a dose-response bioassay. Thereafter, the following hypotheses and research objectives were proposed. The hypotheses are expressed as null hypothesis.

\textbf{Hypothesis 1}: Genetic modification in \textit{L. minor} to express anti-atrazine scFv is not effective as a tool for phytoremediation of atrazine contamination. The hypothesis was tested in Chapter 2.

\textbf{Objective 1}: To examine the ability of transgenic \textit{L. minor} expressing anti-atrazine scFv for potential of phytoremediation of atrazine contamination.
Hypothesis 2: Genetic modification in *L. minor* to express atrazine chlorohydrolase TrzN is not effective as a tool for phytoremediation of atrazine contamination. The hypothesis was tested in Chapter 3.

Objective 2: To examine the ability of transgenic *L. minor* expressing atrazine chlorohydrolase TrzN for potential of phytoremediation of atrazine contamination.

Hypothesis 3: Transient and stable expression of atrazine chlorohydrolase TrzN in *N. benthamiana* do not have a potential for atrazine phytoremediation. The hypothesis was tested in Chapter 4.

Objective 3: To determine whether transient and stable expression of atrazine chlorohydrolase TrzN in *N. benthamiana* have a potential to use as a tool for phytoremediation of atrazine contamination.
2. EXPRESSION OF AN ANTI-ATRAZINE SCFV ANTIBODY FRAGMENT IN TRANSGENIC DUCKWEED (LEMNA MINOR) FOR POTENTIAL USE IN PHYTOREMEDIATION

ABSTRACT

Lemna minor, an aquatic plant, is used for phytoremediation of environmental contaminants such as heavy metals and herbicides, in aquatic systems. Even though plants can remediate toxic chemicals naturally, they can be genetically modified to sequester toxic compounds. To enhance the capabilities of L. minor to sequester atrazine, we stably expressed an anti-atrazine scFv in L. minor using an Agrobacterium-mediated transformation protocol. RT-PCR analysis and Western blotting confirmed the transcription as well as the expression of the scFv gene in the transgenic lines. The concentration of scFv expressed in line # 8 (LMscFv8) was approximately 3.7 mg scFv/kg fresh weight. After a 14-d exposure to atrazine at concentrations of 0.001 to 1.0 mg/L, LMscFv8 had significantly better growth when measured in terms of frond number, chlorophyll content, and dry weight. Using 14C-atrazine, an increased uptake was observed in transgenic L. minor after 10 d. No major metabolites of atrazine were found in either transgenic or wild-type L. minor.

2.1 Introduction

Atrazine is widely used as a herbicide for broadleaf weed control. It is used for pre- and post-emergent weed control in corn, sugarcane and sorghum and other crops all across North America. According to a report by US EPA, in 2007, 33-35 million kilograms of atrazine was used in the United States (United States Environmental Protection Agency 2013). Atrazine has shown negative effects in many aquatic
organisms. In laboratory toxicity testing for freshwater organisms, phytoplankton is the most sensitive species followed by macrophytes, benthos, zooplankton, and fish, respectively (Giddings 2005).

Runoff is the main route of environmental contamination, particularly to surface water of atrazine used in agricultural activities. It is a concern as water resources exposed to atrazine are contaminated by runoff water emanating from corn and sorghum agricultural area treated with atrazine. Furthermore, it is one of the most frequently detected herbicide in water resources (Stone et al. 2014). Atrazine in surface water has been continously monitored, and peak atrazine concentrations have been found to co-occur with field application and rainfall. Concentrations of atrazine exceeded the human health benchmark (maximum contaminant level) of 0.003 mg/L between 2002-2011 (Stone et al. 2014).

Dissipation of atrazine in surface waters via photodegradation occurs by direct and indirect photolysis. Atrazine is degraded via direct photolysis, when it absorbs sunlight at wavelengths >290 nm. Hydroxyatrazine; dealkylated products of hydroxyatrazine, i.e., desethylhydroxyatrazine, desisopropylhydroxyatrazine, and ameline; dealkylated products of atrazine, i.e., desethylatrazine, desisopropylatrazine, and diaminochloroatrazine were the metabolites produced via direct photolysis. However, since atrazine absorbs sunlight weakly, indirect photolysis in the presence of photosensitizers was the main dissipation route for atrazine. Photosensitizers such as dissolved organic carbon, nitrate, and acetone absorb light and transfer energy to catalyze the degradation of atrazine. Cyanuric acid was the stable metabolite from indirect photolytic reaction (Cessna 2008). Figure 2.1 shows atrazine and its metabolites.
Figure 2.1  Atrazine and its metabolites found in surface water.

The idea of using phytoremediation to clean up contaminated soil and water was established in the 1980s (Maestri and Marmiroli 2011). Phytoremediation, the use of plants to remediate polluted soils and water in the environment, offers a cost-effective and environment-friendly alternative or complementary technology along with conventional methods which use physical and chemical mitigation (Salt et al. 1995). With the idea of using plants for remediation, genetic engineering, the ability to manipulate genes in an organism, will allow us to expand capabilities for phytoremediation by enabling the transfer of phytoremediative traits from various organisms to candidate plant species (Maestri and Marmiroli 2011). Aquatic macrophytes are good candidates for remediation of water as they are environmentally friendly and cost-effective. *L. minor* (common duckweed) belonging to the family *Lemnaceae* is one of the smallest aquatic macrophytes and free-floating,
monocotyledonous angiosperms. This species of duckweed normally consists of a small cluster (three to five) of flat leaves or fronds. It is able to grow rapidly, covering open areas of surface water. It is found in slow moving fresh water throughout the temperate regions of the globe. This aquatic plant is used in standard toxicity testing for pesticides (OECD, 1998, 2002).

Aquatic plants have great potential to function as on-site biosinks and biofilters of aquatic pollutants because of their abundance and limited mobility (Olette et al. 2008, Dosnon-Olette et al. 2010, Taraldsen and Norbergking 1990). They have been successfully used to sequester selected heavy metals following uptake through their root systems and movement to the rest of the plant. *L. minor* has been used for the phytoremediation of water contaminated with heavy metals. Low level of zinc, mercury, arsenic, chromium, and lead (as less than 0.5 mg/L) contaminated in water resources were removed effectively by atrazine within one to two weeks (Parra et al. 2012). Advantageous properties of *L. minor* include; small size, simple structure, and rapid growth, hence it has been used for *in vitro* toxicity testing of freshwater system. However, it is vulnerable to surface-active and hydrophobic substances (Tel-Or and Forni 2011).

Almquist et al. (2004) studied the expression of an anti-picoloram single chain variable fragment (scFv) in tobacco and showed that the scFv could sequester picloram without harm to the plant at doses substantially greater than the wild type. With this research in mind, the objective of present work was to investigate whether genetic modification in *L. minor* to express anti-atrazine scFv fragment is effective as a tool for phytoremediation of atrazine-contaminated water. In order to test the hypothesis an scFv
gene for atrazine binding was inserted and expressed in *L. minor* using *Agrobacterium*-mediated transformation. The *L. minor* expressing the scFv against atrazine was studied using biological assays, i.e., dose-response assay and uptake assay, and compared with wild type *L. minor*.

2.2 Materials and Methods

2.2.1 Plant materials

Sterile flasks of *L. minor* L were maintained in liquid modified Hoagland’s E+ medium (Environment Canada, 2007) and subcultured every three to four weeks. Culture conditions were 25°C with a 16-h photoperiod and a light intensity of 100 µmol of photons/m²/sec.

2.2.2 Uptake assay in wild-type *Lemna minor*

A preliminary assay was done to determine absorption of atrazine in wild-type *L. minor*. Seven d prior to the start of the study, *L. minor*, which was cultured in sterile conditions, was moved to modified Hoagland’s E+ medium to acclimatize. Three 3-frond-stage *L. minor* plants were placed in 1.5 mL of modified Hoagland’s E+ medium containing 4 concentrations of ^14^C-atrazine. The specific activity of ^14^C-atrazine 50 mCi/mmol was used to convert disintegration per minute (dpm) to µg/L. The concentrations of ^14^C-atrazine spiked into samples were 13, 25, 37, 49 µg/L. After 7 d of treatment, the plant samples were quickly rinsed with wash solution containing 20% ethanol, 75% water, and 5% Tween 20 (v: v: v). ^14^C in 1 to 5 mL samples (^14^C radioactive adhered outside fronds) was determined by adding approximately 10 mL of Ecolite (MP Biomedicals, cat # 88247504, Fisher Scientific, Ottawa, ON, Canada) to each of the rinsate and medium, after which radioactivity was measured using a Beckman
liquid scintillation counter (LSS; Model LS6K Liquid Scintillation Analyzer, Beckman Instruments, Inc., Fullerton, CA). Plant samples were lyophilized and weighed. Biomass <500 mg) was combusted (3 min at 89°C) to $^{14}\text{CO}_2$ with a biological oxidizer (OX-500, R.J. Harvey Instrument Co., Hillsdale, NJ). The $^{14}\text{CO}_2$ was trapped in 10 mL of Carbon-14 Cocktail (OX-161, R.J. Harvey Instrument Co., Hillsdale, NJ) and the radioactivity of each sample was measured using a liquid scintillation counter. The recovery of $^{14}\text{C}$ by the biological oxidizer was 82.5%. The medium containing $^{14}\text{C}$-atrazine was also collected and analyzed using scintillation spectrophotometry. Data were analyzed by linear regression performed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc. CA, USA).

2.2.3 Plasmid and Agrobacterium strain

Based on its coding sequences Figure 2.2, which was developed by Kramer (2002), the scFv fragment was codon optimized for expression in planta. The gene was synthesized and inserted into binary vector pBISN1 using XbaI and SacI restriction sites by GENEART®. The expression of scFv was under the control of CaMV35S promoter. Figure 2.3 shows a schematic diagram of the heavy and light chains, which were connected by linker (((Glycine)$_4$Serine)$_3$) fused to a 6× Histidine tag and KDEL endoplasmic reticulum retrieval signal. Binary vector containing the anti-atrazine scFv gene was introduced into A. tumefaciens strain C58 via electroporation. The Agrobacterium containing the helper plasmid pmP90 was grown in YEP medium with 50 mg/L kanamycin and 25 mg/L gentamycin.
Figure 2.2  Sequence alignment of anti-atrazine scFv codon optimized for *Lemna minor.*
Figure 2.3  Schematic representation of anti-atrazine scFv. (A), binary vector pBISN1 (B), and pBISN1-scFv (C) for expression of anti-atrazine scFv in *Lemna minor*. 35S, CaMV35S promoter; V\textsubscript{H}, L, polypeptide linker (\((\text{Glycine})\text{Serine})_3; V\textsubscript{L}, H, 6\times \text{Histidine tag}; K, KDEL.
2.2.4 Plant transformation and regeneration

Nodular callus was produced from the fronds (Figure 2.5) using the protocol described in Yamamoto et al. (2002). A starter Agrobacterium culture (5 mL) was initiated from the frozen glycerol stock and grown in YEP medium with 50 mg/L kanamycin and 25 mg/L gentamycin. The culture was grown in a 50-mL centrifuge tube on a shaker at 200 rpm, 28°C for 2 d. After 2 d, one mL of the Agrobacterium culture was transferred to nine mL of YEP medium with 50 mg/L kanamycin and 25 mg/L gentamycin in a 50-mL tube and grown overnight on the shaker until the OD$_{600}$ measurement was approximately 1.0.

For transformation of *L. minor*, one mL of the overnight *Agrobacterium* culture was added to 20 mL of Hoagland’s solution in a deep Petri dish. *L. minor* nodules were cut into small pieces (0.5 cm) and allowed to soak in the *Agrobacterium* suspension for 30 minutes. The nodule pieces were blotted on filter paper and placed on solid nodule production medium and kept in the dark for 3 d. For selection, the nodules were rinsed with water, blotted on filter paper, and transferred directly to solid frond regeneration media consisting of ½ strength SH salts and SH vitamins (Schenk and Hildebrandt, 1972), 0.5% sucrose and 0.8% agar plus 100 mg/L kanamycin and 300 mg/L timentin. The plates containing the nodules were placed in a culture room at 23°C ± 2°C and a 16-h photoperiod (PAR fluence rate = 60 µmol/m$^2$/sec) provided by cool-white fluorescent lights. The nodules were subcultured every 3 weeks to fresh selection media.

Over the next few weeks, regenerating fronds produced on the transformed nodules were transferred to solid multiplication media (SH salts and vitamins, 1.0% sucrose and 0.8% agar) with 100 mg/L kanamycin and 300 mg/L timentin. Subsequently,
the mature transgenic fronds were multiplied and maintained in liquid medium consisting of modified Hoagland’s E+ medium with 10 mg/L kanamycin and 300 mg/L timentin.

2.2.5 Analysis of transgenic *Lemna minor* lines

2.2.5.1 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to confirm the transcription of the inserted scFv gene. After subcultured for 10 days in modified Hoagland’s E+ medium containing 10 mg/L kanamycin and 300 mg/L timentin for transgenic *L. minor*, while the regular modified Hoagland’s E+ medium was used for wild-type *L. minor*, total RNA was extracted from whole kanamycin-resistant plants using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instruction. To synthesize cDNA, 2 μg of RNA was added with 1 μL oligo dT primer and water to bring the volume to 11 μL. A master mix containing 4 μL 5 × First strand Buffer, 2 μL 0.1 M DTT, 1 μL RNase OUT™, and 1 μL 10 mM dNTP was prepared. Reactions were run in a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) at 65°C for 5 min, samples was placed on ice for 2 min, after which the master mix was added. The reaction was run at 42°C for 2 min, then 1 μL SuperScript™ II Reverse Transcriptase (Invitrogen, Canada) was added. The reaction was run at 42°C for 60 min followed by 70°C for 15 min. cDNA from reverse transcription product was amplified using PCR with DNA primers 5’-AAAAGAGGGTCTGACATGG-3’ and 5’-GCTTGATCTCGAGCTTGTTG-3’. All primers were synthesized by the Laboratory Services Division, University of Guelph. A master mix consisted of 2.5 μL 10 × Buffer, 2.5 μL 2 mM dNTPs, 1 μL 50 mM MgCl2, and 0.13 μL 5U/ μL Platinum® Taq Polymerase. It was added to the reactions containing
1 µL of sample cDNA, 0.5 µL of 20 pmol/µL forward and reverse primers. Total volume was adjusted to 25 µL with water. The reaction was run in the PTC-100 thermal cycler with 1 cycle of 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, and 1 cycle of 5 min at 72°C. The reaction product was run on a 1% agarose gel, using EZ-Vision® Two 6× dye to visualize DNA.

### 2.2.5.2 SDS-PAGE and Western blot analysis

Fresh plants were harvested, and homogenized by Mixer Mill MM 300 (Qiagen, Mississauga, ON, Canada.) with three volume of cold phosphate buffered saline. Total soluble protein (TSP) was isolated from whole lysate and clarified by centrifugation at 15,000 rpm for 30 minutes at 4°C. TSP was determined using the Bio-Rad Protein Assay (Mississauga, Canada). Bovine serum albumin (BSA; Thermo Scientific, Nepean, Canada) was used as the protein standard solution. In order to denature the proteins, 30 µg of TSP of each supernatant was diluted with an equal volume of 5× reducing buffer, boiled, and separated on a 10.0% SDS–polyacrylamide gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA) for 1 h at 100 V, 4°C. The membrane was blocked with 4% w/v skim milk in water overnight at 4°C. The protein of interest, i.e., scFv tagged with hexahistidine was detected by probing with an anti-polyhistidine monoclonal antibody (mAb) (Penta-His™, Qiagen) followed by goat anti mouse antibody conjugated to alkaline phosphatase (Pierce Chemical Co., Rockford, IL) and developed in 1-Step™ NBT/BCIP Developing Solution (Pierce, Canada).
2.2.5.3 Growth and transgene expression under different growing conditions

To determine if the transgene expression was affected by the growing conditions, the transgenic L. minor was subcultured in modified Hoagland’s E+ medium and grown under four treatment conditions: 18°C, PAR fluence rate = 100 µmol/m²/sec and PAR fluence rate = 500 µmol/m²/sec; 25°C, 100 µmol/m²/sec; 25°C and 500 µmol/m²/sec.; n = 4. Light source was supplied by cool-white fluorescent lamps. Plants were cultured for 14 d. The fresh weights were determined. The expression of the anti-atrazine scFv was examined using Western blot analysis of crude plant extracts. The blot was probed with an anti-polyhistidine monoclonal antibody (mAb) (Penta-His™, Qiagen) (details of the protocol are described in 2.2.5.2).

2.2.5.4 Dose-response bioassay

Wild-type and transgenic L. minor scFv # 8 (LMscFv8) were subcultured in sterile Hoagland’s E+ media without sucrose for 14 d prior to the experiment (Brain and Solomon 2007). Atrazine was dissolved in acetone. Working solutions of atrazine treated medium were prepared. Final concentrations of treatments were 0.001, 0.01, 0.1, 0.5, 1.0, 5.0, and 10.0 mg/L. Nine treatment levels (including two controls and seven concentrations) were employed with three replicates. Two L. minor plants, each with three fronds were transferred to each 125-mL Erlenmeyer flasks containing 50 mL medium aliquot from working solutions.

Once the 14-d exposure was complete, end points based on the adverse effects of atrazine on the growth of L. minor were evaluated. Three biological endpoints for the test included number of fronds, dry weight, and chlorophyll a and b content. Plant samples were lyophilized at -50°C for 24 hours. The weight of dry samples was
determined. The chlorophylls were extracted from plant samples with 20 mL N, N-dimethylformamide. Supernatant was collected and analyzed using a spectrophotometer at 643.8, 663.8 and 750.0 nm; the amount of chlorophyll a and b content was determined using the equations below.

Chlorophyll a content: \[13.43 \times (A_{663.8} - A_{750})] - [3.47 \times (A_{646.8} - A_{750})]\]

Chlorophyll b content: \[22.90 \times (A_{646.8} - A_{750})] - [5.38 \times (A_{663.8} - A_{750})]\]

Chlorophyll a +b content: \[19.43 \times (A_{646.8} - A_{750})] - [8.05 \times (A_{663.8} - A_{750})]\]

Number of fronds for each sample (FS) at each exposure concentration and number of fronds of the control (FC) were used to calculate percent of control (% control). Dry weight and chlorophyll a + b content were also calculated as percent of control.

\[\% \text{ control} = \frac{F_S}{F_C} \times 100\]

Data were analyzed using a nonlinear regression model from GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc. CA, USA). The model used was: \(Y = \text{Bottom} + (\text{Top-Bottom})/(1+10^{(X-\text{LogEC}_{50})})\); where \(Y\) is the response from control of the endpoint, and \(X\) is log concentration. Confidence intervals (95% CI) were determined for the responses generated from wild-type \textit{L. minor} and transgenic LMscFv8.

2.2.5.5 \textit{Comparison of absorption of }^{14}\text{C-}\textit{atrazine in wild-type and transgenic \textit{Lemna minor}}\n
Seven d prior to the start of the study, \textit{L. minor fronds} were placed in modified Hoagland’s E+ medium without sucrose for preconditioning. Three-frond stage \textit{L. minor} plants were added to 1.5 mL modified Hoagland’s E+ medium containing ca. 1.3 µg/L of
\(^{14}\text{C}\)-atrazine. The specific activity of the \(^{14}\text{C}\)-atrazine was 50 mCu/mmol, which was used to convert disintegration per minute (dpm) to µg/L. After 7, 10, and 14 d of treatment, plant samples were rinsed with wash solution containing 20% ethanol, 75% water, and 5% Tween 20 (v: v: v). Plant samples were rinsed to remove any \(^{14}\text{C}\)-atrazine that adhered to the outside of the fronds. Ten mL of Ecolite (MP Biomedicals, cat # 88247504, Fisher Scientific, Ottawa, ON, Canada) was added to each of the rinsate and medium samples and the radioactivity was determined using a Beckman liquid scintillation counter (LSS; Model LS6K Liquid Scintillation Analyzer, Beckman Instruments, Inc., Fullerton, CA). Plant samples were dried lyophilized and weighed. Biomass was combusted (3 min at 84°C) to \(^{14}\text{CO}_2\) with a biological oxidizer (OX-500, R.J. Harvey Instrument Co., Hillsdale, NJ). The \(^{14}\text{CO}_2\) was trapped in 10 mL of Carbon-14 Cocktail (OX-161, R.J. Harvey Instrument Co., Hillsdale, NJ). The radioactivity of each sample was measured by liquid scintillation spectrophotometry. The recovery of \(^{14}\text{C}\) by the biological oxidizer was 95%. The untreated medium and atrazine treated medium were also collected and analyzed on the scintillation counter.

The data were analyzed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc. CA, USA). The trend of overall uptake was presented as a bar graph. The percent total uptake of radioactivity data was plotted with standard error. The comparisons between wild-type and each transgenic \(L.\ minor\) harvested at each time point were analyzed by multiple t-test with 95% confidence and presented in a table.

**2.2.5.6 Metabolism assay**

The 0.5 g of wild-type and LMscFv8 plants were treated in 1.2 mL Modified Hoagland’s E+ medium containing ca. \(2 \times 10^6\) dpm of \(^{14}\text{C}\)-atrazine. Plants were
harvested at d 7 and stored in dark at -20°C. ¹⁴C-atrazine and its metabolites were extracted as follows. The frozen plant material was ground with a homogenizer (Mixer Mill MM 300) containing 1 mL of water and methanol (1:4). The pulverized tissue added to microcentrifuge tubes. Each microcentrifuge tube was spun at 14,000 rpm for 30 minutes and the supernatant transferred to microfuge tube. Supernatant was stored at -20°C until it was analyzed by high-pressure liquid chromatography (HPLC). Plant extract (20 µL) was analyzed by reverse phase HPLC on an Agilent 1100 series HPLC (Agilent Technologies Inc., Alpharetta, GA, USA) equipped with a Thermo Scientific Hypersil BDS 3 µm C18 HPLC column (3 µm particle size; 4.6- x 100 mm; cat# 03-050-116, Fisher Scientific Company, Ottawa, ON). The chromatographic condition is shown in Table 2.1 below. The flow rate was 0.5 mL/minute. The column temperature was maintained at ambient room temperature. ¹⁴C-atrazine and its radiolabeled metabolites were detected and quantified using a radioflow detector (LB 508, Berthold Technologies GmbH & Co. KG, 75323 Bad Wildbad, Germany) equipped with a type Z cell having a volume of 1000 µL. The counting efficiency was 90%.
Table 2.1  High performance liquid chromatography conditions for separation of atrazine and its metabolites.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention Time of standards (min)</th>
<th>Gradient conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desisopropylatrazine</td>
<td>8.8</td>
<td>The column was adjusted to pH (6.8) with 50mM Phosphate buffer prior to sample injection</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>10.6</td>
<td>Water : acetonitrile [90 : 10] (min: 0-6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water : acetonitrile [75 : 25] (min: 6-21)</td>
</tr>
<tr>
<td>Desethylatrazine</td>
<td>11.2</td>
<td>Water : acetonitrile [35 : 65] (min: 21-23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetonitrile [100] (min: 23-25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol [100] (min: 25-27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water : acetonitrile [90 : 10] (min: 30-35)</td>
</tr>
</tbody>
</table>
2.3 Results

2.3.1 Uptake assay in wild-type *Lemna minor*

Uptake of $^{14}$C-atrazine after 7 d of treatment, determined as the percent total plant uptake, ranged from 1.03 to 3.58%. The higher the concentration of atrazine applied, the higher the total percent of $^{14}$C-atrazine taken up by wild-type *L. minor*.

![Graph showing linear regression of $^{14}$C-atrazine uptake in wild-type *Lemna minor*. Atrazine concentrations in part per billion (µg/L) are shown on the X-axis. Percent total uptake of total radioactivity is on Y-axis. Linear regression equation was $Y = 0.068X + 0.21$, and $R^2 = 0.91$.](image)

Figure 2.4 Linear regression of $^{14}$C-labeled atrazine uptake in wild-type *Lemna minor*. Atrazine concentrations in part per billion (µg/L) are shown on the X-axis. Percent total uptake of total radioactivity is on Y-axis. Linear regression equation was $Y = 0.068X + 0.21$, and $R^2 = 0.91$. 
2.3.2 Production of transgenic *Lemna minor* lines

Small nodular calli (Figure 2.5 A) were produced from fronds of *L. minor* and maintained on nodule production medium and were used as a source of tissue for *Agrobacterium* transformation. Transformed nodules were transferred directly from co-cultivation medium to solid regeneration medium with selective agent (Figure 2.5 B) in some cases as early as one week after the initial transfer. These sections became dark green and regenerated into fronds (Figure 2.5 C-D). Negative controls did not produce any dark green spots on the nodules. When transgenic fronds developed to the three-frond stage, they were transferred to modified liquid Hoagland’s medium containing kanamycin and timentin for multiplication. Only one regenerating frond was selected per nodule and identified as a transgenic event. Transgenic lines were vegetatively propagated in modified Hoagland’s E+ medium and were maintained for four years without losing the ability to express the anti-atrazine scFv (Figure 2.8).
Figure 2.5 Various stages of *Lemna minor* transformation. A) nodules used in co-cultivation with *Agrobacterium* containing an anti-atrazine scFv gene. B) transformed nodules on frond regeneration medium containing kanamycin. C) a dark green transformed nodule. D) a regenerated frond.
Transgene *L. minor* lines were maintained without kanamycin in the medium. However, kanamycin was introduced to the medium before testing. After 14 d in kanamycin, all transgenic *L. minor* lines survived in the presence of kanamycin; wild-type *L. minor* was used as a negative control (Figure 2.6).

![Figure 2.6](image)

Figure 2.6 Appearance of wild-type and transgenic *Lemna minor* lines after they were grown in modified Hoagland’s E+ medium containing 10 mg/L kanamycin for 14 d. A) wild-type *L. minor*, B) transgenic LMscFv4, C) transgenic LMscFv6, D) transgenic LMscFv8, E) transgenic LMscFv10, F) transgenic LMscFv11, G) transgenic LMscFv15, and H) transgenic LMscFv16.
2.3.3 Reverse transcription polymerase chain reaction

Three out of ten transformed *L. minor* lines were used to determine whether there was expression of mRNA. mRNA could be seen in all the three lines, i.e., # 6, # 8 and # 16, while there was no expression of scFv in wild-type *L. minor*. Figure 2.7 shows the size of cDNA as a PCR product run on 10% agarose gel.

![Agarose gel electrophoresis of RT-PCR with mRNA isolated from transgenic plants containing anti-atrazine scFv gene. Primers used amplified an 800 bp product Lane 1) 1 Kb DNA ladder, lane 2) blank, lanes 3, 4 and 5 transgenic LMsFv6, transgenic LMsFv8, transgenic LMsFv16, lane 6) blank, lane 7) wild-type *Lemna minor*, lane 8) blank, and lane 9) cDNA from *Agrobacterium tumefaciens* (positive control) containing the scFv gene of interest.](image)
2.3.4 SDS-PAGE and Western blot analysis

A 29 kDa anti-atrazine scFv fragment was detected in all the transgenic plant lines tested. A series of reducing SDS-PAGE gels and immunoblots probed with primary anti-histidine antibody followed by secondary goat anti-mouse antibody-alkaline phosphatase conjugated revealed that line # 8 had slightly greater protein expression among the three lines (Figure 2.8).

Figure 2.8  Immunoblot probed with anti 6×Histidine antibody to detect anti-atrazine scFv bands. Immunoblot compares the expression of the anti-atrazine scFv in transgenic Lemna minor. Lane 1) standard protein ladder, lane 2) standard 6×Histidine ladder, land 3) transgenic LMscFv4, lane 4) transgenic LMscFv6, lane 5) transgenic LMscFv8, lane 6) transgenic LMscFv10, lane 7) transgenic LMscFv11, lane 8) transgenic LMscFv15, lane 9) transgenic LMscFv16, and lane 10) wild-type Lemna minor. Along the left side, protein standard indicates migration of molecular weight.
2.3.5  **Optimal growth conditions for *Lemna minor***

According to the result from previous Western blotting experiment, transgenic *L. minor* # 8, hereafter known as LMscFv8, had a slightly greater protein expression. This line was used to determine the optimal growth condition for biomass production and the protein expression (Figure 2.9). *L. minor* growth was better at 25°C than at 18°C. Changes in the light intensity did not improve the growth at 18°C. At 25°C, plant growth or biomass was twice as great at light intensity at 100 than at 500 µmol/m²/sec.

![Biomass Bar Graph](image)

**Figure 2.9**  Bar graph represents a comparison of fresh weight of transgenic *Lemna minor* grown under four different conditions; 18°C, 100 µmol/m²/sec; 18°C, 500 µmol/m²/sec; 25°C, 100 µmol/m²/sec; 25°C, 500 µmol/m²/sec. The light intensities are shown below the X-axis. The fresh weight in grams is shown on the Y-axis. The error bars are standard error of mean, n = 4.
Anti-atrazine scFv expression in LMscFv8 was similar under all four growth regimes. However, the expression of anti-atrazine scFv was slightly lower when plants grown in culture room conditions (30°C, 60 μmol/m²/sec) (Figure 2.10). On the basis of these experiments 18°C, 100 μmol/m²/sec was selected for growing and maintaining the transgenic *L. minor* lines since biomass yield was greatest. Under these conditions LMscFv8 produced 3.77 mg scFv/kg fresh weight.

**Figure 2.10** Western blot comparing the expression of the anti-atrazine scFv in transgenic LMscFv8. Lane 1) standard protein ladder, lane 2) and 3) 6×His ladder, lane 4) blank, lane 5) transgenic LMscFv8 grown at 18°C, 100 μmol/m²/sec, lane 6) transgenic LMscFv8 grown at 18°C, 500 μmol/m²/sec, lane 7) transgenic LMscFv8 grown at 25°C, 100 μmol/m²/sec, lane 8) transgenic LMscFv8 grown at 25°C, 500 μmol/m²/sec, lane 9) 30°C, 60 μmol/m²/sec, lane 10) Wild-type *Lemna minor*
2.3.6 *In vivo* atrazine tolerance bioassay

The tolerance levels of the best expression line among the other expressers of active scFv were determined by an atrazine dose-response bioassay (data for other lines not shown). LMSCFv8 was propagated in selective modified Hoagland’s E+ medium and were treated in liquid modified Hoagland’s E+ medium without sucrose containing seven different atrazine concentrations. Figure 2.11–2.13 show that when LMSCFv8 and wild type *L. minor* were compared using the mean of frond number, dry weight, and chlorophyll a and b content as the percent of control, LMSCFv8 was significantly more tolerant (*P* <0.05) to atrazine. The EC$_{50}$ of LMSCFv 8 is twice as great as that of wild-type *L. minor*. 
Figure 2.11  Dose-response curves of wild type and transformed *Lemna minor* (Wild-type vs. LMscFv8). Wild-type (“○” with solid green line) and LMscFv8 (“●” with solid red line) represent dose-response curve of the mean frond number (as percent of untreated control) with 95% confidence interval bands. The EC$_{50}$ values are indicated on the right side.
Figure 2.12  Dose-response curves of wild type and transformed *Lemna minor* (Wild-type vs. LMscFv8). Wild-type (‘○’ with solid green line) and LMscFv8 (‘●’ with solid red line) represent dose-response curve of the mean dry weight (as percent of untreated control) with 95% confidence interval bands. The EC$_{50}$ values are indicated on the right side.
Figure 2.13  Dose-response curves of wild type and transformed *Lemna minor* (Wild-type vs. LMscFv8). Wild-type ("○" with solid green line) and LMscFv8 ("●" with solid red line) represent dose-response curve of the mean chlorophyll a+b content (as percent of untreated control) with 95% confidence interval bands. The EC$_{50}$ values are indicated on the right side.
2.3.7 Comparison of absorption of $^{14}$C-atrazine in wild-type and transgenic *Lemna minor*

To determine if the anti-atrazine scFv produced in LMscFv8 results in greater sequestration of atrazine than wild-type *L. minor*. Wild-type *L. minor* and two lines of transgenic *L. minor* expressing anti-atrazine scFv were treated with $^{14}$C-atrazine. Whole plants were harvested 7, 10, and 14 d initiation of the experiment. A two-sample t-test was performed to compare percent total uptake of wild-type versus each transgenic *L. minor* line on days 7, 10, and 14.

Trends of absorption in selected *L. minor* lines shown in Figure 2.14 illustrate that absorption of $^{14}$C in wild-type *L. minor* increased from day 7 to day 14. The similar trend was found in LMscFv6, with the exception of d 10 where there was less absorption than in the control. LMscFv8 had greater absorption of $^{14}$C at days 7 and 10, however the percent of uptake at day 14 decreased. By the end of the experiment (day 14), transgenic LMscFv8 was over grown in the containers (picture not shown), which may have caused plant stress. Consequently, LMscFv may have redistributed $^{14}$C back into the nutrient solution.

According to the data in Table 2.2, LMscFv8 had higher absorption of $^{14}$C at day 10 than the control group ($p$-value = 0.038).
Absorption of $^{14}$C-atrazine presented as percent of total uptake. Mean percentage $^{14}$C-atrazine uptake by wild-type *Lemna minor* was represented by a green bar with standard error of mean ($n = 4$). Mean percentage $^{14}$C-atrazine uptake by two transgenic *Lemna minor* lines, LMsFv6 and LMsFv8, were represented by blue and red bars, respectively, with standard error of mean ($n = 4$).
Table 2.2  Whole plant absorption of radioactivity, expressed as percent of total uptake. $^{14}$C-atrazine was spiked into liquid medium contained plant samples. Each well (experimental unit) was counted as one replication with the total of 4 replications.

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean$^a$ (SEM)</th>
<th>Difference S6-WT $p$-value</th>
<th>Difference S8-WT $p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>LMscFv6</td>
<td>LMscFv8</td>
</tr>
<tr>
<td>7</td>
<td>4.99 (1.76)</td>
<td>4.89 (1.32)</td>
<td>12.16 (2.46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.10 0.983</td>
<td>7.17 0.194</td>
</tr>
<tr>
<td>10</td>
<td>8.99 (2.87)</td>
<td>5.01 (1.59)</td>
<td>20.88 (5.73)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-3.98 0.416</td>
<td>11.89 0.038</td>
</tr>
<tr>
<td>14</td>
<td>14.21 (5.82)</td>
<td>11.81 (4.37)</td>
<td>8.50 (0.79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-2.40 0.621</td>
<td>-5.71 0.296</td>
</tr>
</tbody>
</table>

$^a$ Data from one experiment with 4 replicates and presented as means with standard error in parentheses.


2.3.8 Metabolism assay

The parent compound atrazine and three metabolites were detected in medium and plant extract. Sample chromatogram of standards is presented in Figure 2.15. Results are presented as the percent of $^{14}$C activity that was extracted from liquid medium and plant samples. The percent of $^{14}$C activity of atrazine and metabolites from medium, and plants after 7 d exposure are shown in Figure 2.16 and 2.17, respectively. Atrazine was the major compound in both medium and plants from wild-type and LMscFv8 with the percent of atrazine content in the medium being 87.9 and 86.1, respectively, while in the plant it was 86.8 and 89.0%, respectively. The three metabolites were found at concentrations less than 4.0% in both the wild type and LMscFv8. In wild-type, hydroxyatrazine was found at, 1.2% in the plant, and 1.7% in the medium. In LMscFv8, hydroxyatrazine was found at 0.6% in the plant and 2.7% in the medium. Desisopropylatrazine was found at 1.2% in the wild-type plant, and 1.0% in the medium, while 0.7% was found in LMscFv8 plants and 0.8% in the medium. Desethylatrazine was, 1.6% in the wild type plant, and, 3.7% in the medium, while it was 2.0% in LMscFv8 and 2.4% in the medium.

Atrazine content in plant tissue of LMscFv8 was statistically higher than in the wild-type ($p$-value <0.05). The contents of metabolites were not different between wild-type and LMscFv8.
Figure 2.15  Chromatograph of UV detector output for atrazine and its metabolites. DIA, desisopropylatrazine; HA, hydroxyatrazine; DEA, desethylatrazine; and AT, atrazine.
Figure 2.16  Mean percentage of atrazine and metabolites in medium. Atrazine is represented as a black bar. Hydroxyatrazine, desisopropylatrazine, and desethylatrazine are represented as green, grey and red bars, respectively. Bars represent standard errors of mean (n = 3)

Table 2.3  Percentage of atrazine and its metabolites in medium.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean (SEM)</th>
<th>Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>LMscFv8</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>87.9 (0.3)</td>
<td>86.1 (0.3)</td>
<td>-1.9</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>1.7 (0.9)</td>
<td>2.7 (0.2)</td>
<td>1.0</td>
</tr>
<tr>
<td>Desisopropylatrazine</td>
<td>1.0 (0.4)</td>
<td>0.8 (0.1)</td>
<td>-2.3</td>
</tr>
<tr>
<td>Desethylatrazine</td>
<td>3.7 (0.8)</td>
<td>2.4 (0.2)</td>
<td>-1.3</td>
</tr>
</tbody>
</table>
Figure 2.17  Mean percentage of atrazine and metabolites from whole plants. Atrazine is represented as a black bar. Hydroxyatrazine, desisopropylatrazine, and desethylatrazine are represented as green, grey and red bars, respectively. Bars represent standard errors of mean (n = 3).

Table 2.4 Percentage of atrazine and its metabolites in plant tissues.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean (SEM)</th>
<th>Difference S8-WT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>LMsFv8</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>86.7 (0.3)</td>
<td>89.0 (0.4)</td>
<td>2.3</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>1.2 (0.1)</td>
<td>0.6 (0.3)</td>
<td>-0.6</td>
</tr>
<tr>
<td>Desisopropylatrazine</td>
<td>1.2 (0.1)</td>
<td>0.7 (0.4)</td>
<td>-0.5</td>
</tr>
<tr>
<td>Desethylatrazine</td>
<td>1.6 (0.2)</td>
<td>2.0 (0.4)</td>
<td>0.4</td>
</tr>
</tbody>
</table>
2.4 Discussion

The purpose of the research presented in this Chapter was to express a recombinant anti-atrazine scFv in transgenic *L. minor* to improve herbicide uptake and sequester atrazine so that *L. minor* could be used for phytoremediation of atrazine contaminated water. Furthermore, a short, straightforward and easy to handle protocol for *Agrobacterium*-mediated transformation in *L. minor* was developed to produce recombinant protein. This protocol was much simpler than that developed by Yamamoto’s group where individual callus was maintained separately in liquid medium containing 10 mg/L antibiotic. The steps for clone selection were accomplished using simpler medium plus a higher stringency for selection by using a 10 fold higher concentration of antibiotic. Unlike in Yamamoto’s protocol, transformed nodules could be placed directly on solid selection medium, which improved the selection process, as individual nodules did not need to be grown in different flasks of liquid medium. All the transgenic lines produced expressed the scFv. Furthermore, these stable transformants remained stable for at least four years after the initial transformation (i.e., none lost gene at time of publication). This stability of the transgene may be due to the reproductive system of duckweed. *L. minor* has vegetative reproduction in which all the genetic material gets passed on from mother to daughter fonds via meristematic tissue (Lemon 2000).

The antibody fragment expression in *L. minor* could be achieved by using *Agrobacterium*-mediated stable transformation. Our selection process with kanamycin was very effective, as we did not get any escapes. The transgene was transcribed to mRNA as the result shown by RT-PCR. Transgenic lines produced were analyzed for
expression of anti-atrazine antibody fragment. All of the seven lines analyzed showed the presence of this fragment in Western blot analysis. Under optimized growth conditions, i.e., 100 µmol/m²/sec and 25°C and in the presence of atrazine, transgenic LMscFv8 produced significantly more biomass than the wild type. In addition to the protein expression in *L. minor*, the scFv was found in liquid medium after being purified using Western blot analysis (data not shown).

To study tolerance of transgenic *L. minor*, plants were exposed to various concentrations of atrazine. Analysis of morphological and physiological parameters of transgenic lines exposed to atrazine-enriched medium had increased tolerance to atrazine, with line #8 (LMscFv8) having the greatest tolerance. The result from this study suggested that antibodies with specific antigen specificity improve tolerance to the environmental contaminant, i.e., the antigen, in plants. Based on an uptake assay, it was shown that wild-type *L. minor* has the ability to passively absorb atrazine within a range of 20-50 mg/L when spiked into the liquid medium. The concentration of atrazine taken up by *L. minor* correlated with the concentrations of atrazine in the medium as was reported by Burken and Schnoor (1996). To rule out any possible metabolic reaction causing tolerance in transgenic *L. minor*, the extent of atrazine metabolism in wild type and LMscFv8 was examined using HPLC. No major metabolites were found in wild-type and transgenic *L. minor*, suggesting that tolerance of LMscFv8 to atrazine was due to sequestration of anti-atrazine by the scFv. Tolerance to the herbicide picloram was previously found in transgenic tobacco expressing an anti-picloram scFv (Almquist et al. 2004). Under field application simulation, tobacco seedlings expressing the anti-picloram scFv could survive 10 µM picloram after 12 weeks of application, whereas as
major injury and death occurred to wild-type tobacco (Almquist et al. 2004). Almquist et al. (2004) suggested that this scFv induced tolerance to a herbicide correlates with two factors; the affinity of an antibody for the herbicide, and the expression level of Ab/scFv in the plant. With the idea that anti-atrazine scFv which has excellent affinity to atrazine (Equilibrium dissociation constant of scFv is \(7.46 \times 10^{-10} \text{ M}\)) (Kramer 2002), the transgenic \textit{L. minor} expressing the scFv should sequester more atrazine from a contaminated environment than was shown in this research. LMsFcFv8 absorbed more \(^{14}\text{C}-\text{atrazine}\) 7 and 10 d after exposure than did wild-type \textit{L. minor}. Furthermore, based Western blot analysis which showed expression of 3.77 mg scFv per kg fresh plant biomass in LMsFcFv8 and, increased tolerance to atrazine as demonstrated by a dose-response assay, it can be concluded that the main factor of the atrazine tolerance in transgenic \textit{L. minor} was influenced by a binding of atrazine to the anti-atrazine scFv.

Since greater antibody expression in mg scfv per kg plant biomass is the main factor for improving xenobiotic tolerance, it is clear that if we could have expressed greater concentration of the scFv tolerance to atrazine would have increased. In this study, the CaMV35S promoter originating from a dicotyledenous plant may not be the best promoter to induce gene expression a in monocot plant like \textit{L. minor}. Although the CaMV35S promoter has been used in expression of GUS in Indian \textit{L. minor}, the authors only showed RNA transcription but did not show GUS translation via protein expression (Chhabra et al. 2011).

Several authors have used wild type \textit{L. minor} for phytoremediation in water systems of contaminants such as heavy metals, and agricultural chemicals (Olette et al. 2008, Parra et al. 2012). The potential of using \textit{L. minor} for heavy metal remediation was
proposed by (Parra et al. 2012). After 21 d of treatment in liquid medium, the percent of removal of arsenic, mercury, and lead were 5, 30, and 30, respectively (Parra et al. 2012). *L. minor* was found to be able to remove herbicide flazasulfuron at the rate of $11 \pm 1 \mu g/g$ fresh weight under the laboratory setting (Olette et al. 2008). According to the potential of using wild type duckweed for remediation, the idea of increasing the ability of the plant to sequester a herbicide by recombinant expression of and scFv against it is warranted. The results of this study, support the hypothesis that affinity-absorption occurs in plants, resulting in the sequestration of atrazine by showing tolerance to atrazine in transgenic duckweeds. Therefore, immunomodulation-mediated tolerance will provide another tool for capture of low molecular weight contaminants in the environment to manage chemical exposure risks.
3 EXPRESSION OF ATRAZINE CHLOROHYDROLASE TRZN GENE IN TRANSGENIC DUCKWEED (*LEMNA MINOR*)

**ABSTRACT**

Atrazine is one of the most used herbicides in North America to control broadleaf weeds. With heavy usage mainly in the US and Canada, it is frequently detected in water systems nearby agricultural areas. Transgenic plants that can degrade atrazine may be useful for remediation. To investigate the potential use in atrazine remediation, *Lemna minor* was genetically modified to express atrazine chlorohydrolase TrzN gene using *Agrobacterium*-mediated transformation. Atrazine chlorohydrolase TrzN metabolizes atrazine by hydrolyzing atrazine to non-phytotoxic hydroxyatrazine. RT-PCR result showed that atrazine chlorohydrolase TrzN gene was inserted into *L. minor*. However, according to immunoblot analysis, protein expression could not be detected. Due to low expression, transgenic *L. minor* only showed increasing tolerance to atrazine at concentrations of 0.01, and 0.1 mg/L in hydroponic culture as compared to wild-type *L. minor*. Moreover, the concentration of hydroxyatrazine and other atrazine metabolites found in media and plant tissue were not significantly different between transgenic and wild-type *L. minor*.

3.1 **Introduction**

As mentioned in Chapter 2, atrazine is one of the most herbicides contaminated in environment. Previous studies have shown the potential of transgenic plants to remove or to detoxify atrazine from contaminated soil. The strategies were to express the genes encoding enzymatic proteins from other organisms such as mammalian monooxygenase cytochrome P450, plant glutathione S-transferase, and chlorohydrolase atzA from soil
microorganism (Flocco et al. 2004, Kawahigashi et al. 2006, Wang et al. 2005). Aquatic macrophytes are candidates of interest in genetic engineering as they can potentially decontaminate water resources.

The objective of this study was to investigate whether genetic modification in *L. minor* to express atrazine chlorohydrolase TrzN, an enzyme that metabolizes atrazine, is effective as a tool for phytoremediation of atrazine-contaminated water. In order to test the hypothesis, the atrazine chlorohydrolase TrzN gene was inserted and expressed in *L. minor* using *Agrobacterium*-mediated transformation. The efficiency of metabolism of atrazine to hydroxyatrazine by *L. minor* expressing atrazine chlorohydrolase TrzN was studied using biological assays: dose-response assay and metabolism assay.

3.2 Materials and methods

3.2.1 Plasmid and *Agrobacterium* strain

The atrazine chlorohydrolase TrzN was codon optimized for expression *in planta* Figure 3.1. C-Myc tag and 6×Histidine tag were incorporated for detection purpose. The gene was inserted into binary vector pBISN1 using SalI and SacI restriction sites by GENEART®. The expression of atrazine chlorohydrolase TrzN was under the control of CaMV35S promoter. Figure 3.2 shows a schematic diagram of the atrazine chlorohydrolase TrzN fused to C-Myc tag and 6×Histidine tag, the construct for expression in *L. minor*. The binary vector containing the atrazine chlorohydrolase TrzN gene was introduced into *A. tumefaciens* strain C58 via electroporation. The *Agrobacterium* containing the helper plasmid pmP90 was grown in YEP medium with 50 mg/L kanamycin and 25 mg/L gentamycin.
Figure 3.1  Sequence alignment of atrazine chlorohydrolase TrzN (EC 3.8.1.8) codon optimized for *Lemma minor*.
Figure 3.2  Schematic representation of atrazine chlorohydrolase TrzN (A), binary vector pBISN1 (B), and pBISN1-TrzN (C) for expression of atrazine chlorohydrolase TrzN in *Lemna minor*. 35S, CaMV35S promoter; TrzN, atrazine chlorohydrolase TrzN; C, C-Myc tag; H, 6×Histidine tag.
3.2.2 *Lemna minor* genetic transformation and regeneration

The procedure of plant transformation is as described in Chapter 2. Transgenic fronds were produced from transformed nodules, and were transferred to solid multiplication media (SH salts and vitamins, 1.0% sucrose and 0.8% agar) with 100 mg/L kanamycin and 300 mg/L timentin. Subsequently, the fronds from each transgenic line were also multiplied and maintained in liquid media consisting of modified Hoagland’s E+ medium with 10 mg/L kanamycin and 300 mg/L timentin.

3.2.3 Analysis of transgenic lines

3.2.3.1 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to confirm the transcription of the inserted atrazine chlorohydrolase TrzN gene. Transgenic lines were subcultured for 10 d in modified Hoagland’s E+ medium containing 10 mg/L kanamycin and 300 mg/L timentin and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). To synthesize cDNA, 2 µg of RNA was added with 1 µL oligo dT primer and water to bring the volume to 11 µL. A master mix containing 4 µL 5 × First Strand Buffer, 2 µL 0.1 M DTT, 1 µL RNase OUT™, and 1 µL 10 mM dNTP was prepared. Reactions were run in a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) at 65°C for 5 min, and samples was placed on ice for 2 min, after which the master mix was added. The reaction was run at 42°C for 2 min, then 1 µL SuperScript™ II Reverse Transcriptase (Invitrogen, Canada) was added. The reaction was run at 42°C for 60 min followed by 70°C for 15 min. cDNA from reverse transcription was amplified using PCR with DNA primers 5’-CCTTCGACGACCAAGAGAGA-3’ and 5’-
GTCTGAGGTCCCCGAGCA-3’. All primers were synthesized by the Laboratory Services Division, University of Guelph. A master mix consisted of 2.5 µL 10 × Buffer, 2.5 µL 2 mM dNTPs, 1 µL 50 mM MgCl₂, and 0.13 µL 5U/µL Platinum® Taq Polymerase. It was added to the reactions containing 1 µL of sample cDNA, 0.5 µL of 20 pmol/µL forward and reverse primers. Total volume was adjusted to 25 µL with water. The reaction was run in the PTC-100 thermal cycler. The cycles were 1 cycle of 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, and 1 cycle of 5 min at 72°C. The reaction product was run on a 1% agarose gel using EZ-Vision® Two 6× dye to visualize DNA.

3.2.3.2 Purification of atrazine chlorohydrolase TrzN and Immunoblot analysis

Frozen plant samples were ground in liquid nitrogen using a mortar and pestle. The samples were mixed with one volume of cold phosphate buffer saline. Total soluble protein (TSP) was clarified by centrifugation at 15,000 rpm for 30 minutes at 4°C. The supernatant was collected. The supernatant was applied to a His SpinTrap™ column containing Ni Sepharose medium (GE Healthcare) equilibrated with binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 20 mM imidazole, pH 7.4). The atrazine chlorohydrolase TrzN was eluted using elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 7.4). The eluates were stored at 4°C. Crude extract and purified atrazine chlorohydrolase TrzN were determined by SDS-PAGE and immunoblotting. Protein samples were diluted with an equal volume of 5× reducing buffer, boiled, and separated on a 10% SDS–polyacrylamide gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA) for 1 h at 100 V, 4°C. Membranes were blocked with 4% w/v skim milk in water overnight at 4°C. The protein
of interest atrazine chlorohydrolase TrzN tagged with hexahistidine was detected by probing with an anti-polyhistidine monoclonal antibody (mAb) (Penta-His™, Qiagen) followed by goat anti-mouse antibody conjugated to alkaline phosphatase (Pierce Chemical Co., Rockford, IL) and developed in 1-Step™ NBT/BCIP Developing Solution (Pierce, Canada).

3.2.3.3 Dose-response bioassay

Wild-type and two lines of transgenic duckweeds LMscFv8 (control; plant material from Chapter 2) and LMTrzN11 expressing atrazine chlorohydrolase TrzN were subcultured in sterile modified Hoagland’s E+ media without sucrose for 14 d prior to the experiment. Atrazine concentrations were 0.01, 0.1, 1.0, and 10.0 mg/L. Six treatments (including two controls and four concentrations) were employed in triplicate. Two L. minor plants, each with three fronds were transferred to a 125-mL Erlenmeyer flasks containing 50 mL medium.

Once the 7-d exposure was complete, endpoints based on the adverse effects of atrazine on the growth of L. minor were evaluated. Three biological endpoints measured were number of fronds, dry weight, and chlorophyll a and b content. Plant samples were lyophilized at -50°C for 24 h. The weight of dry samples was determined. Chlorophyll a and b were extracted from plant samples with 20 mL N, N-dimethylformamide as described in Chapter 2.

The data were analyzed with a log inhibitor concentration versus response model using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc. CA, USA).
The model was: \( Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1+10^{(X-\text{LogEC}_{50})}) \); where \( Y \) is the response from control of the endpoint, and \( X \) is the log concentration of atrazine.

3.2.3.4 Metabolism assay

The procedure for the metabolism assay was as described in Chapter 2.

3.3 Results

Plants were propagated in modified Hoagland’s E+ medium containing 10 mg/L kanamycin for 14 d. Wild-type \( L. \) minor was not able to grow and propagate. Transgenic lines all grew in the presence of kanamycin with LMTrzN10 and LMTrzN11 11 growing better than line LMTrzN12 (Figure 3.3).

Figure 3.3 Appearance of wild-type and transgenic \( \text{Lemna minor} \) lines after they were grown in modified Hoagland’s E+ medium containing 10 mg/L kanamycin for 14 d. A) wild-type \( L. \) minor, B) transgenic LMTrzT10, C) transgenic LMTrzT11, D) transgenic LMTrzT12.
3.3.1 Reverse transcription polymerase chain reaction

Three out of twelve transformed *L. minor* lines were tested to detect the expression of mRNA from atrazine chlorohydrolase TrzN gene. Expression of mRNA was found in three transgenic *L. minor* lines LMTrzT10, LMTrzN11 and LMTrzN12 (Figure 3.4).

![Agarose gel electrophoresis of RT-PCR with mRNA isolated from transgenic plants containing atrazine chlorohydrolase TrzN gene. Expected amplicon size is 800 bp. Lane 1) 1 Kb DNA ladder, lane 2) blank, 3), 4), 5) transgenic LMTrzN10, transgenic LMTrzN11, transgenic LMTrzN12, respectively, lane 6) blank, lane 7) wild-type *Lemna minor*, lane 8) blank, and lane 9) cDNA from *Agrobacterium tumefaciens* (positive control) containing the gene of interest (TrzN).](image)
3.3.2 SDS-PAGE and Western blot analysis

Atrazine chlorohydrolase TrzN from crude extract and purified sample could not be detected in an immunoblot probed with anti-histidine antibody (Figure 3.5).

![SDS-PAGE and Western blot analysis](image)

**Figure 3.5** Immunoblot of crude extracts and purified atrazine chlorohydrolase TrzN from *Lemna minor*. TrzN was separated using 10% SDS-PAGE under reducing condition and probed with anti 6×Histidine antibody. scFv from transgenic LMscFv8 was used as a control. Lane 1) standard 6×Histidine ladder, lane 2) crude extract of wild-type *L. minor*, lane 3) crude extract of transgenic LMscFv8, lane 4) crude extract of transgenic LMTrzN10, lane 5) crude extract of transgenic LMTrzN11, lane 6) purified protein of wild-type *Lemna minor*, lane 7) purified protein of transgenic LMscFv8, lane 8) purified protein of transgenic LMTrzN10, and lane 9) purified protein of transgenic LMTrzN11. The arrows on the right indicate the expected size of the scFv and TrzN.

3.3.3 *In vivo* atrazine tolerance bioassay

The tolerance level of LMTrzN11 was determined by an atrazine dose-response bioassay. Transgenic LMTrzN11 that had been vegetatively propagated in selective
modified Hoagland’s E+ medium was treated in liquid modified Hoagland’s E+ medium without sucrose but with atrazine. Transgenic *L. minor* LMcFv8 was included in this experiment as a control. Concentration-response curves for test with atrazine are presented in Figure 3.6 – 3.8 as frond number, dry weight, and chlorophyll a+b content, respectively.

In hydroponic testing, transgenic LMTrzN11 was able to survive in the presence of 0.01 and 0.1 mg/L atrazine. Based on frond production, LMTrzN11 increased tolerance to atrazine, when compared to the wild-type by 25%, and 38% at 0.01, and 0.1 mg/L atrazine, respectively. Dry weight data shows that the growth of transgenic plant was 20% (0.01 mg/L) and 17% (0.1 mg/L) greater than wild-type plant. Chlorophyll a+b content indicates that tolerance in LMTrzN11 increased by 28% and 41% in the presence of 0.01 and 0.1 mg/L atrazine, respectively. The results suggested that the transgenic *L. minor* expressing atrazine chlorohydrolase TrzN were tolerant to atrazine, but only at low concentrations. This may due to very low expression of the atrazine chlorohydrolase TrzN gene in transgenic plants, as was indicated by low expression on SDS PAGE, i.e. no visible display of TrzN even upon concentration (Figure 3.5).
Figure 3.6  Dose-response curves of the two *Lemna minor* (Wild-type vs. LMscFv8 and LMTrzN11) after 7 d of exposure to atrazine. Wild-type (‘○’ with dotted green line), LMscFv8 (‘●’ with solid red line), and LMTrzN11 (‘□’ with dotted blue line). Error bars represent the standard error of mean of three replicates (n=3).
Figure 3.7 Dose-response curves of the two *L. minor* (Wild-type vs. LMscFv8 and LMTrzN11) after 7 d of exposure to atrazine. Wild-type (“○” with dotted green line), LMscFv8 (“■” with solid red line), and LMTrzN11 (“□” with dotted blue line). Error bars represent the standard error of mean of three replicates (n=3).
Figure 3.8  Dose-response curves of the two *Lemna minor* (Wild-type vs. LMcFv8 and LMTrzN11) after 7 d of exposure to atrazine. Wild-type (‘○’ with dotted green line), LMcFv8 (‘●’ with solid red line), and LMTrzN11 (‘■’ with dotted blue line). Error bars represent the standard error of mean of three replicates (n=3).
3.3.4 Metabolism assay

To compare and investigate the phytoremediation capacity of wild-type and transgenic *L. minor*, the metabolites of atrazine were evaluated from the supernatant of culture medium and the plant extract using HPLC as described in Chapter 2. Validation of the detection was as described and shown in Figure 2.15, where chromatogram of standards: atrazine, hydroxyatrazine, desisopropyl atrazine, and desethylatrazine were presented. In assessing the metabolism of atrazine by *L. minor*, Figure 3.9 and 3.10 shows the percent $^{14}$C activity in the liquid medium and plant extract, respectively, seven d after continuous exposure of the plants to atrazine. The results showed that atrazine was the major compound in the medium, and the percent of atrazine content in the medium from wild-type, and LMTrzN11 were 87.9, and 86.8, respectively. In the plant, percent of content found in both wild-type, and LMTrzN11 were similar at 86.7. However, three atrazine metabolites were detected at the negligible concentration in both wild-type and LMTrzN11. In wild-type, hydroxyatrazine was found at 1.7% in the medium, and 1.2% in the plants. In LMTrzN11, hydroxyatrazine was found at 3.1% in the medium, and 1.2% in the plants. The percent content of desisopropylatrazine was 1.0% in the medium, and 1.2% in the plants for wild-type. The percent content of desisopropylatrazine was 1.1% in the medium, and 1.1% in plants for LMTrzN11. The result of wild-type shows that desethylatrazine was 3.7% in the medium and 1.6% in the plant. The result of LMTrzN11 shows that desethylatrazine was 1.8% in the medium, and 1.9% in the plant.

The contents of atrazine and its metabolites were not statistically different ($\alpha$ $>$0.05) between wild-type and transgenic *L. minor*. The small amounts of metabolites
found in either the wild-type *L. minor* or transgenic *L. minor* LMTrzN11 suggested that metabolism was not a potential mechanism of tolerance.

![% Content in medium](chart.png)

**Figure 3.9** Mean percentage of atrazine and metabolites in medium with standard error of mean (n = 3).

**Table 3.1** Percentage of atrazine and metabolites in medium.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean (SEM)</th>
<th>Difference T11-WT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>LMTrzN 11</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>87.9 (0.3)</td>
<td>86.8 (0.2)</td>
<td>-1.1</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>1.7 (0.9)</td>
<td>3.1 (0.9)</td>
<td>1.3</td>
</tr>
<tr>
<td>Desisopropylatrazine</td>
<td>1.0 (0.4)</td>
<td>1.1 (0.3)</td>
<td>-0.3</td>
</tr>
<tr>
<td>Desethylatrazine</td>
<td>3.7 (0.8)</td>
<td>1.8 (0.9)</td>
<td>-1.9</td>
</tr>
</tbody>
</table>
Figure 3.10  Mean percentage of atrazine and metabolites extracted from whole plants with standard error of mean (n = 3).

Table 3.2  Percentage of atrazine and metabolites in plant tissues.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean (SEM)</th>
<th>Difference T11-WT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>LMTrzN 11</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>86.7 (0.3)</td>
<td>86.7 (0.6)</td>
<td>0.0</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>1.2 (0.1)</td>
<td>1.2 (0.1)</td>
<td>-0.1</td>
</tr>
<tr>
<td>Desisopropylatrazine</td>
<td>1.2 (0.1)</td>
<td>1.1 (0.1)</td>
<td>-0.1</td>
</tr>
<tr>
<td>Desethylatrazine</td>
<td>1.6 (0.2)</td>
<td>1.9 (0.2)</td>
<td>0.3</td>
</tr>
</tbody>
</table>
3.4 Discussion

In this Chapter, studies were conducted to determine if genetic modification in *L. minor* to express the atrazine chlorohydrolase TrzN gene is an effective tool for phytoremediation of atrazine-contaminated water. Genetic modification of *L. minor*, which is novel and challenging, was first revealed in 2001 by Yamamoto’s group (Yamamoto et al. 2001). In this study, the genetic modification in *L. minor* could be achieved by using *Agrobacterium*-mediated stable transformation to insert the atrazine chlorohydrolase TrzN gene.

The transgenic lines were able to grow in the presence of kanamycin, the selectable marker. The atrazine chlorohydrolase TrzN gene was transcribed to mRNA in the transgenic lines as shown by the result of RT-PCR. However, in terms of gene expression, the lines tested did not show the presence of the atrazine chlorohydrolase TrzN enzyme in immunoblot analysis even after concentration and purification using a Ni column. This might due to the atrazine chlorohydrolase TrzN level that was below the detection limit using the current methodology. Alternatively, it is also possible that the CaMV35S promoter used in this study may not be efficient enough to induce gene expression, resulting in low protein production in the genetic transformation of the monocot plant *L. minor*. Although the CaMV35S promoter has been used in genetic transformation in Indian *L. minor*, Chabra et al. (2011) only showed PCR amplification that indicates gene insertion and detection of marker protein GUS activity without reporting the efficacy of protein expression (Chhabra et al. 2011). Expression of genes may be improved by using promoters specific to monocots, such as the maize ubiquitin
promoter (Christensen and Quail 1996) and actin I promoter from rice (Mcelroy et al. 1990), which have been found to be more efficient than CaMV35S promoter.

To investigate the protective effect of atrazine chlorohydrolase TrzN in plants, dose-response bioassays were conducted. After 7 days of exposure to atrazine in hydroponic medium, greater atrazine tolerance in LMTzrN11 was observed at the two lowest concentrations. To test the efficacy of the introduced atrazine chlorohydrolase TrzN expressing transgenic *L. minor*, hydroxyatrazine was expected to be a major metabolite in transgenic plants. The metabolism assay using $^{14}$C-atrazine showed that the transgenic plants did not degrade atrazine to hydroxyatrazine more rapidly than wild-type *L. minor*, however atrazine in medium from transgenic group was less than wild-type. This could be due to the low expression of protein as shown from immunoblot result.

The efforts to develop transgenic plants for the application of remediation of atrazine in the environment have been reported previously (Maestri and Marmiroli 2011). Most of the plants selected for genetic modification were terrestrial plants such as tobacco, potato, and rice, and their goal was to remediate atrazine in soil (Yamada et al. 2002, Kawahigashi et al. 2008, Wang et al. 2005). Transgenic rice expressing mammalian cytochrome P450 that targets atrazine has also been reported (Kawahigashi et al. 2008). Kawahigashi et al. (2008) studied the effectiveness of atrazine remediation in soil by transgenic rice (pIKBACH). pIKBACH rice co-expressing three human CYP1A1, CYP2B6, and CYP2C19 genes could resist 2.18 mg of atrazine. The researchers also showed that growing pIKBACH and wild-type rice in soil containing 4.2 µM atrazine for 1 month of exposure could reduce the remaining atrazine in the soil at 45.7% and 60.8%, respectively, of the initial application (Kawahigashi et al. 2008). The
use of transgenic potato expressing rat CYP1A1 gene was also studied (Yamada et al. 2002). It was reported that with the expression of CYP1A1 gene, twice as much atrazine was degraded into N-dealkylated metabolites when compared to wild-type potato. Other studies also examined the effect of introducing atrazine chlorohydrolase TrzN from soil bacteria. Atrazine chlorohydrolase TrzN can metabolize atrazine to hydroxyatrazine and has been studied in plant transformation for phytoremediation purpose. (Wang et al. 2005) transferred this dechlorinating gene into three plant species: tobacco, alfalfa, and Arabidopsis. Under the hydroponic setting, transgenic tobacco, alfalfa, and Arabidopsis resisted 15, 10, and 5 µg/mL of atrazine, respectively, when compared to the wild-type (Wang et al. 2005). According to analyses using thin layer chromatography, hydroxyatrazine was the major metabolite in these transgenic plants after exposure to $^{14}$C-atrazine (Wang et al. 2005).

To our knowledge, the aim of this study is the first attempt to incorporate the atrazine chlorohydrolase TrzN gene from soil bacteria into a small aquatic plant (L. minor) for remediation purpose. Due to the intrinsic nature of enzymes, transgenic lines may not have produced quantities large enough to be detected. With improvements to the construct, there is potential for enhancing the expression of this gene and the efficacy of the resulting transgenic L. minor to remediate atrazine.
4. INDUCTION OF TOLERANCE TO ATRAZINE IN NICOTIANA BENTHAMIANA USING TRANSIENT AGROBACTERIUM BASED EXPRESSION SYSTEM AND STABLE TRANSFORMATION

ABSTRACT

Atrazine is one of the most widely used herbicide in North America to control broadleaf weeds and it is frequently detected in water systems nearby agricultural areas. Since transgenic plants that can degrade atrazine may be useful for remediation of atrazine, an atrazine chlorohydrolase gene (TrzN) specific for metabolism of atrazine was transienly expressed in *Nicotiana benthamiana* using an agroinfiltraion approach. The plants that transiently expressed the atrazine chlorohydrolase TrzN along with P19 a suppressor of gene silencing tolerated atrazine. Based on these results, TrzN was stably integrated into *N. benthamiana* using *Agrobacterium*-mediated transformation. The result of RT-PCR in T$_1$ plants showed that atrazine chlorohydrolase TrzN gene was inserted into *N. benthamiana*. T$_2$ lines of transgenic *N. benthamiana* showed increasing tolerance to atrazine at concentrations of 0.1, 0.25, 0.5, and 1 mg/L in hydroponic culture when compared to wild-type plants. Moreover, transgenic plants expressing the atrazine chlorohydrolase TrzN (NBTrzN) tolerated atrazine at 250 g a.i./ha while wild-type plant died. This research indicates that protein expression using agroinfiltration in *N. benthamiana* and an herbicide tolerance bioassay can be used as a quick screening test prior to undertaking stable transformation. The transgenic plants expressing atrazine chlorohydrolase TrzN have a potential to remediate atrazine contaminated soil.
4.1 Introduction

Atrazine, a selective herbicide for broadleaf weed control is one of the common pesticides in North America (United States Environmental Protection Agency 2013). Approximate 33-35 million kilograms of atrazine are applied in agriculture areas mainly in Corn Belt (United States Environmental Protection Agency 2013). Atrazine has low $K_{OW}$, and is slightly adsorbed on soil (Giddings 2005). It is often detected in surface water and ground water when precipitation or irrigation occurs after the application of atrazine (Giddings 2005). Water systems adjacent to the treated area are most likely to be contaminated. More than 50% of water samples are contaminated with atrazine levels over the recommendation (Stone et al. 2014). Contaminated water is a concern for the public. To reduce the concentrations of atrazine found in the environment risk reduction strategies should be applied (United States Environmental Protection Agency 2003).

The use of plants to accumulate and degrade pesticides (phytoremediation) has been considered for decades (Maestri and Marmiroli 2011). Plants can be enhanced in their ability to remediate contaminants using genetic modification approach (Maestri and Marmiroli 2011). Genes of interest are usually incorporated by stable transformation so that the gene of interest can be expressed throughout the life of transgenic plants. To create a stable transgenic plant, the plant tissue has to go through selection and regeneration from callus, followed by further rounds of selection. To shorten the time required to determine whether a gene can be expressed and has the intended utility, transient agroinfiltration can be used as a quick turnaround method. In transient infiltration, a culture of *A. tumefaciens* harbouring the gene of interest is introduced into plant leaves directly via injection with a syringe or vacuum infiltration (Kapila et al.)
Once present in the plant the T-DNA from \textit{A. tumefaciens} is inserted into plant genome. As a result, the foreign protein is expressed over a number of days. Without regeneration into entire transgenic plants, the inserted gene can be detected within several days after infiltration (dpi) (Kapila et al. 1997). Because of the time saved, agroinfiltration has been used for many applications in protein expression. On the other hand, the stable genetic transformation approach ensures that the gene of interest is embedded in genetic material of transgenic plants and is transmitted to the subsequent generations (Gelvin 2003).

Based upon the results from Chapter 3 where the expression of atrazine chlorohydrolase TrzN could not be unequivocally shown and tolerance to atrazine was minimal when compared to the wild-type, it was decided to determine whether transient integration of atrazine chlorohydrolase TrzN could substantially increase tolerance of plants to atrazine. This was quickly determined using agroinfiltration of atrazine chlorohydrolase TrzN via \textit{A. tumefaciens} into \textit{N. benthamiana}. This strategy proved successful since transiently infected 3 to 4-leaf stage \textit{N. benthamiana} tolerated atrazine when compared to controls. Based on this success, TrzN was stably integrated in \textit{N. benthamiana} and the T$_2$ lines were studied for atrazine tolerance in hydroponic system and under field spray simulation.

### 4.2 Material and Methods

#### 4.2.1 Construction of \textit{Nicotiana benthamiana} expression vector

Based on its coding sequence (Figure 4.1), the atrazine chlorohydrolase TrzN was codon optimized for expression in \textit{N. benthamiana}. It was incorporated to C-Myc tag and 6×Histidine tag for further detection purposes. The gene was synthesized and
inserted into binary vector p105T using SacI and BspEI restriction sites by GENEWIZ®. The expression of atrazine chlorohydrolase TrzN was under the control of CaMV35S promoter. A schematic diagram (Figure 4.2) of the atrazine chlorohydrolase TrzN fused to C-Myc tag and 6×Histidine tag, the construct for expression in *N. benthamiana*. TrzN was designed for expression in *N. benthamiana*. p105T binary plant expression vector containing atrazine chlorohydrolase (TrzN) construct was introduced into electrocompetent *A. tumefaciens* strain At542 cells by electroporation (Bio-Rad GenePulser Xcell™ Electroporation System) following the Gene Pulser Xcell Electroporation System Instruction Manual (Garabagi et al. 2012). The Agrobacterium clone containing the plasmid was propagated on LB-Miller agar containing 50 mg/L rifampicin and 50 mg/L carbenicillin for 48 h at 28°C. One isolated colony was selected and inoculated in 5 mL of liquid LB media containing 50 mg/L rifampicin and 50 mg/L carbenicillin for 24 h at 28°C, 220 rpm.
Figure 4.1 Sequence alignment of atrazine chlorohydrolase TrzN (EC 3.8.1.8) codon optimized for *Nicotiana benthamiana*. 
Figure 4.2 Schematic representation of atrazine chlorohydrolase TrzN (A), binary vector p105T (B), and p105T-TrzN (C) for expression of atrazine chlorohydrolase TrzN in *Nicotiana benthamiana*. LB and RB, T-DNA left and right borders, respectively; nptII, neomycin phosphotransferase gene (conferring kanamycin resistance); 35S, CaMV35S promoter; TrzN, atrazine chlorohydrolase TrzN; C, C-Myc tag; H, 6×Histidine tag.

### 4.2.2 Plant materials

Seedlings of *N. benthamiana* were transplanted two weeks after germination to 6 × 8-cm plastic pots, and grown in a greenhouse facility. Water containing fertilizer (20-8-20 (N-P-K)) was provided from the bottom two to three days until the plants were four weeks old.
4.2.3 Whole plant agroinfiltration in *Nicotiana benthamiana*

To express atrazine chlorohydrolase TrzN, plant cells were vacuum infiltrated with *Agrobacterium* containing TrzN gene with and without P19 gene plasmid. P19 is a protein of the tomato bushy stunt virus (TBSV) that suppresses post-transcriptional gene silencing (PTGS) (Garabagí et al. 2012). *A. tumefaciens* cultures were incubated overnight at 28°C and 220 rpm in LB media with 50 µg/mL rifampicin and 50 µg/mL carbenicillin or 50 µg/mL rifampicin for unmodified *A. tumefaciens* At542 to an OD$_{600}$ of approximately 1.8. Three of the *Agrobacterium* cultures containing atrazine chlorohydrolase TrzN, or P19, or untransformed *Agrobacterium* were mixed to prepare three *Agrobacterium* treatments. The first treatment was untransformed *Agrobacterium*, second treatment was the mixture of atrazine chlorohydrolase TrzN and untransformed *Agrobacterium*, third treatment was the mixture of atrazine chlorohydrolase TrzN and P19. Each mixture was centrifuged at 8,000 rpm for 5 min at room temperature. The pellet was resuspended in 2 L of agroinfiltration buffer (10 mM 1-(N-morpholino)ethanesulphonic acid – MES, pH 5.5, 10 mM MgSO$_4$ (Giritch et al. 2006), resulting in an OD$_{600}$ = 0.4 for each suspension. Four-week old, greenhouse-grown *N. benthamiana* plants were submerged in 2-L container containing 2 L of *Agrobacterium* bacterial suspension and placed in a vacuum chamber. A vacuum was applied for 2 min with pressure ranging from 0.5 to 0.9 bar and then slowly released (Marillonnet et al., 2005). Leaves of infiltrated *N. benthamiana* plants were glassy, and they are shown in Figure 4.3. Plants were returned to the greenhouse for seven days. The negative control was untransformed *Agrobacterium* At 542.
Figure 4.3 Appearance of *Nicotiana benthamiana* leaves after agroinfiltration. The plant on the left was infiltrated with *Agrobacterium* infiltration buffer and is translucent compared to the non-infiltrated plant (on the right).

4.2.4 Protein purification, SDS-PAGE and Western blot analysis

Seven days after infiltration, three plants of each group were harvested for protein purification and Western blot analysis. Plants were stored in -20°C until analyzed. Frozen plant samples were grounded using a chilled mortar and pestle. The samples were homogenously mixed with one volume of cold phosphate buffered saline (PBS). Total soluble protein (TSP) was clarified by centrifugation at 15,000 rpm for 30 min at 4°C. The supernatant was collected and applied to a His SpinTrap™ column containing Ni Sepharose medium (GE Healthcare, Canada) equilibrated with binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 20 mM imidazole, pH 7.4). The atrazine chlorohydrolase TrzN was eluted using elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 7.4). The eluates were stored at 4°C. Crude extract and purified TrzN protein was determined by SDS-PAGE and immunoblotting. Forty µL of crude plant extracts and purified protein were diluted with
an equal volume of 5 × reducing buffer, boiled, and separated on a 10.0% SDS–polyacrylamide gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA) for 1 h at 100 V, 4°C. The membrane was blocked with 4% w/v skim milk in water overnight at 4°C. The protein of interest atrazine chlorohydrolase TrzN tagged with hexahistidine was detected by probing with an anti-polyhistidine monoclonal antibody (mAb) (Penta-His™, Qiagen) followed by goat anti mouse antibody conjugated to alkaline phosphatase (Pierce Chemical Co., Rockford, IL) and developed in 1-Step™ NBT/BCIP Developing Solution (Pierce, Canada).

4.2.5 Atrazine tolerance bioassay

Tolerance of N. benthamiana transiently expressing atrazine chlorohydrolase TrzN to atrazine was assessed seven days after infiltration. The soil for plants in 2-L pots was covered with aluminium foil to prevent atrazine contamination of the soil (Figure 4.4). The plants were sprayed with suspension of 0, 25, 100, 250, 500, and 1,000 g of a.i./ha of atrazine in 0.5% Turbocharge (surfactant kindly provided by Peter Smith) using an automatic hood sprayer (RC-5000-100EP, Mandel Scientific) equipped with a flat fan nozzle (SS80015E Spraying Systems) equipped to deliver 200 L/ha of various atrazine concentrations.

Seven days after application of atrazine, height of the plants and number of leaves were measured. Only the shoots above soil were weighed. Fresh biomass was recorded. They were oven dried at 60°C for 72 h prior to the determination of dry weight. Each endpoint were used to calculate percent of control (% of control).

Measuring PSII quantum yield was measured using a MINI-PAM fluorometer (Heinz Walz GmbH, Eiffeltrich, Germany) following the manufacturer’s instructions.
Samples were analyzed in the light and were considered to be light adapted. Quantum yield was determined as effective photosynthetic efficiency of PSII based on the effective quantum yield at 665 nm from a single saturation pulse. The probe of MINI-PAM was set to zero in the air prior to measuring the samples. In each plant sample, quantum yield of the last three measurements (out of five) were selected for the calculation of the mean quantum yield. Leaf samples were measured with a probe placed in the middle of leaf. The probe was rinsed with DI-water and dried with Kimwipe®. Effective fluorescence after saturating pulse ($F'_{M}$) and the steady-state fluorescence ($F$) of the samples were measured using the PAM fluorometer. Effective quantum yield ($\Phi_{PSII}$) was calculated using $F'_{M}$ and $F$.

$$\Phi_{PSII} = \frac{F'_{M} - F}{F'_{M}}$$

The effective quantum yield of each sample ($\Phi_S$) at each exposure concentration and the effective quantum yield of the control ($\Phi_C$) were used to calculate percent of control (% control).

$$\% \text{ control} = \frac{\Phi_S}{\Phi_C} \times 100$$

Data of five endpoints from *N. benthamiana* were analyzed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc. CA, USA). All endpoints were presented as percent of control using a log atrazine concentration versus response curve with standard error of mean (n = 3).
4.2.6 Stable transformation in *Nicotiana benthamiana* using *Agrobacterium*-mediated transformation

The transgenic lines of *N. benthamiana* were produced by *Agrobacterium*-mediated transformation. The cointegrated plasmid contained nptII gene (conferring resistance to kanamycin). The binary vector containing the atrazine chlorohydrolase TrzN gene as well as the nptII gene, which confers kanamycin resistance for selection, was transferred into *A. tumefaciens* At542 by electroporation. *Agrobacterium* containing the atrazine chlorohydrolase TrzN gene was propagated on LB agar containing 50 mg/L rifampicin and 50 mg/L kanamycin. An isolated colony on the selective medium was then incubated in 10 mL of LB containing 50 mg/L rifampicin and 50 mg/L kanamycin at 220 rpm, 28°C until the OD$_{600}$ was around 1.0. Fully expanded *N. benthamiana* leaves were
were surface-sterilized by immersing in 70% ethanol for 1 min, followed by 20% (v/v) bleach for 10 min and three rinses in sterile distilled water. Approximately 1 × 1 cm cut pieces of *N. benthamiana* explants were co-cultivated with *Agrobacterium* suspension for 30 min. The explants were then plated onto Murashige and Skoog (MS) medium, containing 0.1 mg/L naphthaleneacetic acid and 1 mg/L benzyladenine supplemented with 0.8% agar. Plates were kept in dark for four days for co-cultivation. The explants were transferred to a new selective medium; MS medium containing 0.1 mg/L naphthaleneacetic acid, 1 mg/L benzyladenine, 300 mg/L timentin, and 200 mg/L kanamycin to produce callus. Each of the spatially distinct callus formed were kept separately to ensure that different transgenic events were selected. The newly formed shoots were moved to MS medium containing 300 mg/L timentin and 200 mg/L kanamycin without NAA and BA in Magenta boxes for root induction. Only 6 lines of transgenic plants were produced. After roots were formed, plants were transplanted to soil for the production of seeds. An agar-solidified (0.8%) medium was used for axenic growth of plants. It consisted of 1× Murashige and Skoog salts and vitamins (Gibco Laboratories, Grand Island, NY) and 30% (w/v) sucrose. Kanamycin (200 mg/L) was added to the medium after autoclaving. Seeds were surface-sterilized by immersing in 70% ethanol for 1 min, and then 20% (v/v) bleach for 10 min followed by three rinses in sterile distilled water. After the seeds dried they were placed in a dish containing MS agar medium (in the presence or absence of kanamycin). The seedlings were grown to maturity. Mature plants, which were grown in kanamycin were transplanted to soil and were self-pollinated. These seeds were T₂ seeds.
4.2.7 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to confirm the transcription of atrazine chlorohydrolase TrzN gene inserted in T₁ plants. T₀ plants were grown to produce T₁ seeds by self-pollination. T₁ plants were grown on MSO medium containing 200 g/L kanamycin. Only T₁ plants of lines 1 and 3 were analyzed for gene expression using total RNA from whole kanamycin-resistant plants using an RNA easy Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instruction. To synthesize cDNA, 2 µg of RNA was added with 1 µL oligo dT primer and water to bring the volume to 11 µL. A master mix containing 4 µL 5× First strand Buffer, 2 µL 0.1 M DTT, 1 µL RNase OUT™, and 1 µL 10 mM dNTP was prepared. Reactions were run in a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) at 65°C for 5 min, samples were placed on ice for 2 min, after which the master mix was added. The reaction was run at 42°C for 2 min, then 1 µL SuperScript™ II Reverse Transcriptase (Invitrogen, Canada) was added. The reaction was run at 42°C for 60 min followed by 70°C for 15 min. cDNA from reverse transcription product was amplified using PCR with primers 5’-AGAGTTTGCTGGGAGGGATT-3’ and 5’-TTCCTCTCTTGGAGGAACGA-3’. All primers were synthesized by the Laboratory Services Division, University of Guelph. A master mix consisted of 2.5 µL 10× Buffer, 2.5 µL 2 mM dNTPs, 1 µL 50 mM MgCl₂, and 0.13 µL 5U/µL Platinum® Taq Polymerase, was added to the reactions containing 1 µL of sample cDNA, 0.5 µL of 20 pmol/µL forward and reverse primers. Total volume was adjusted to 25 µL with water. The reaction was run in the PTC-100 thermal cycler. The cycles were 1 cycle of 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 58°C, and
2 min at 72°C, and 1 cycle of 5 min at 72°C. The reaction product was run on a 1% agarose gel, using EZ-Vision® Two 6 × dye to visualize DNA.

4.2.8 Atrazine tolerance bioassay using hydroponic medium

T2 seeds from self-pollinated T1 plants (line # 1-5) were grown on MS agar containing 200 mg/L kanamycin. Wild-type N. benthamiana seeds were plated on MS agar with and without 200 mg/L kanamycin, and were used as positive and negative control, respectively. After two weeks, transgenic plants surviving on media containing kanamycin were collected. This ensured that plants had the atrazine chlorohydrolase TrzN gene. Wild-type N. benthamiana plants from regular MS agar were also used in the experiment. To acclimatize plants before treatment, wild-type and transgenic N. benthamiana were acclimatized in sterile liquid modified Hoagland’s E+ medium containing 15 µM ZnSO4·H2O without sucrose and EDTA for five days. MS agar remaining on the roots was removed completely from plant samples. At day 0, each of the plants were treated in 500 µL of liquid modified Hoagland’s E+ medium without sucrose and EDTA plus 15 µM ZnSO4·H2O containing atrazine. Atrazine concentrations were 0.1, 0.25, 0.5, 0.75 and 1.0 mg/L plus two untreated controls (medium and 0.1% acetone in medium). Experimental treatments were performed in triplicate. Sterile conical bottom glass tubes were used to contain test samples. Tubes were capped with aluminium foil and wrapped with surgical tape to maintain sterile conditions. Plant samples were placed in a rack vertically and gently shaken using an orbital shaker. Plants were visually inspected for chlorosis. Growth reduction was monitored as compared to controls. After seven d of treatment, plant samples were harvested and gently blotted on
Kimwipe™ to remove excess medium. Fresh weight was immediately determined followed by lyophilisation prior to being weighed again (dry weight).

The data were analyzed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, Inc. CA, USA). Fresh and dry weights were presented as percent of control in log (inhibitor) versus response curve with standard error of mean (n = 3).

4.2.9 SDS-PAGE and Western blot analysis

T₂ seeds from self-pollinated T₁ plants (line # 1-5) were grown for two weeks on MS agar containing 200 mg/L kanamycin, while wild-type N. benthamiana seeds were grown on the same medium without kanamycin. Plants were moved to soil and grown in a greenhouse facility. Seven-week-old plants were harvested for Western blot analysis. Plants were stored in -20°C until analyzed. Frozen plant samples were ground using Mixer Mill MM 300. The samples were homogenously mixed with one volume of cold phosphate buffered saline (PBS). Total soluble protein (TSP) was clarified by centrifugation at 15,000 rpm for 30 min at 4°C. The supernatant and pellet were collected. Thirty-two µL of crude plant extract were diluted with an equal volume of 5× non-reducing buffer. Pellets were diluted with 2× non-reducing buffer. Samples were boiled and separated on a 10.0% SDS–polyacrylamide gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA) for 1 h at 100 V, 4°C. Membranes were blocked with 4% w/v skim milk in water for 3 h at room temperature. atrazine chlorohydrolase TrzN protein tagged with hexahistidine was detected by probing with an anti-polyhistidine monoclonal antibody (mAb) (Penta-His™, Qiagen) overnight at 4°C, followed by goat anti mouse antibody conjugated to alkaline phosphatase (Pierce
Chemical Co., Rockford, IL) and developed in 1-Step™ NBT/BCIP Developing Solution (Pierce, Canada)

### 4.2.10 Atrazine tolerance bioassay in field spray simulation

Seven-week-old plants were sprayed with suspension of 0, 25, 100, 250, 500, 1,000, and 2,000 g a.i./ha of atrazine in 0.5% Turbocharge (surfactant kindly provided by Peter Smith) using an automatic hood sprayer (RC-5000-100EP, Mandel Scientific) equipped with a flat fan nozzle (SS80015E Spraying Systems) equipped to deliver 200 L/ha of spray suspension. Seven days after application of atrazine, the endpoints were measured and statistical analysis was performed as described in section 4.2.5.

### 4.3 Results

#### 4.3.1 Expression of atrazine chlorohydrolase TrzN in transiently infiltrated *Nicotiana benthamiana*

To compare the transient expression of atrazine chlorohydrolase TrzN from whole plant infiltration, *N. benthamiana* plants were infiltrated with non-transformed *Agrobacterium*, TrzN, or TrzN with the addition of P19. Plants were harvested at day 7 and atrazine chlorohydrolase TrzN was purified using Ni sepharose column from crude extract. SDS-PAGE and western blots were performed. Under reducing condition, the expression of atrazine chlorohydrolase TrzN was not detectable directly by Western blot. However, after being purified, the expression of atrazine chlorohydrolase TrzN was detectable. The infiltration along with P19 shows a greater concentration of atrazine chlorohydrolase TrzN produced as compared to the treatment without P19 (Figure 4.5).
Figure 4.5  Expression of atrazine chlorohydrolase TrzN in transiently infiltrated *Nicotiana benthamiana*. The immunoblot was probed with anti-histidine antibody and goat anti-mouse alkaline phosphatase. Lane 1) standard 6×histidine ladder, lane 2) blank, lane 3) crude extract of P19 + TrzN, lane 4) crude extract of At 542 + TrzN, lane 5) crude extract of At 542, lane 6) blank, lane 7) purified protein of P19 + TrzN, lane 8) purified protein of At 542 + TrzN, and lane 9) purified protein of At 542.
4.3.2 Induction of atrazine tolerance in *Nicotiana benthamiana* by agroinfiltration with atrazine chlorohydrolase TrzN

A dose-response bioassay was conducted by spraying atrazine formulation to determine protective effect of transiently expressed atrazine chlorohydrolase TrzN in *N. benthamiana*. Tests of difference were analyzed using endpoints measured. Figure 4.6 – 4.10 present the response of each endpoint as a percent of control versus concentrations. Overall, *N. benthamiana* plants which were infiltrated with the mixture of atrazine chlorohydrolase TrzN with P19 showed more tolerance to atrazine. Fresh weight and dry weight were sensitive endpoints. In effective quantum yield, *N. benthamiana* plants appeared to be protected from atrazine by atrazine chlorohydrolase TrzN either with or without co-expression of P19 at all concentrations applied. Especially, at higher concentrations; 250, 500, and 1,000 g a.i./ha, more protection was found. Similarly, the same trend was seen in data representing the number of leaves/plant.
Figure 4.6  Effect of atrazine on effective quantum yield in *Nicotiana benthamiana*. Endpoint shows response as a percentage of the untreated control. Error bars represent the standard error of the mean (n=3).
Figure 4.7  Effect of atrazine on number of leaves in *Nicotiana benthamiana*. Endpoint shows response as a percentage of the untreated control. Error bars represent the standard error of the mean (n=3).
Figure 4.8  Effect of atrazine on height in *Nicotiana benthamiana*. Endpoint shows response as a percentage of the untreated control. Error bars represent the standard error of the mean (n=3).
Figure 4.9   Effect of atrazine on fresh weight in *Nicotiana benthamiana*. Endpoint shows response as a percentage of the untreated control. Error bars represent the standard error of the mean (n=3).
Figure 4.10  Effect of atrazine on dry weight in *Nicotiana benthamiana*. Endpoint shows response as a percentage of the untreated control. Error bars represent the standard error of the mean (n=3).
### 4.3.3 Development of stable TrzN T₂ plants

Six T₀ plants were produced and selected for self-pollination to produce T₁ seeds based on the kanamycin resistant screening. T₁ seeds were screened using MSo containing 200 mg/L kanamycin. Segregation on kanamycin of T₁ seedlings is presented in Table 4.1. Only line # 1 and 3 were transferred into MSo containing 200 mg/L kanamycin in Magenta boxes. The gene of interest in T₁ line # 1-1 to -11 and # 3-1 plants was confirmed with RT-PCR. Figure 4.11 displays RT-PCR product of plants tested.

With confirmation of RT-PCR products, two of T₁ plants were selected and moved to soil and self-pollinated to produce T₂ seeds. T₂ seeds were tested for segregation (Table 4.2). T₂ plants of line # 1-5 used for atrazine tolerance bioassays.

**Table 4.1 Phenotypic characterization of transgenic *Nicotiana benthamiana*.** The T₁ (self-pollinated generation 1) progeny were screened using 200 mg/L kanamycin. Two weeks after seeding, T₁ plants in each line were scored as tolerant or susceptible. Plants that were living were scored as tolerant (R) and plants that were dying or were dead were scored as susceptible (S).

<table>
<thead>
<tr>
<th>Lines</th>
<th>Observed T₁ phenotype</th>
<th>R</th>
<th>S</th>
<th>Total</th>
</tr>
</thead>
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<td>28</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td># 2</td>
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<td># 6</td>
<td></td>
<td>21</td>
<td>7</td>
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Figure 4.11 Agarose gel electrophoresis of RT-PCR with mRNA isolated from transgenic *Nicotiana benthamiana* containing TrzN. Lane 1) wild-type *N. benthamiana* (negative control), lane 2) to lane 12) T1 line # 1 of transgenic *N. benthamiana* 13) T1 line # 3 of transgenic *N. benthamiana*, and lane 14) cDNA from *Agrobacterium tumefaciens* (positive control) containing TrzN.

Table 4.2 Phenotypic characterization of transgenic *Nicotiana benthamiana line # 1*. The T₂ (self-pollinated generation 2) progeny were screened using 200 mg/L kanamycin. Two weeks after seeding, T₁ plants in each line were scored as tolerant or susceptible. Plants that were living were scored as tolerant (R) and plants that were dead or dying were scored as susceptible (S).

<table>
<thead>
<tr>
<th>Lines</th>
<th>Observed T₂ phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
</tr>
<tr>
<td># 1 - 2</td>
<td>80</td>
</tr>
<tr>
<td># 1 - 5</td>
<td>100</td>
</tr>
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</table>
4.3.4 Atrazine tolerance bioassay using hydroponic system

Tolerance to atrazine of $T_2$ seedlings from self-pollinated $T_1$ plants was determined using bioassay in liquid hydroponic medium. Two week-old transgenic plants, which survived from 200 mg/L kanamycin were picked for the assay. Seven days after exposure, transgenic NBTrzN1-5 was tolerant to atrazine as shown in Figure 4.12 and 4.13. In contrast, wild-type (control) were adversely affected by atrazine. The growth reduction and chlorosis were found at concentration 0.1 mg/L and higher. Based on fresh and dry weight data presented as percent of control (Figure 4.14 and 4.15), it was found that with exposure to 0.1, 0.25, 0.5, 0.75, and 1 mg/L atrazine transgenic plants had increased tolerance to atrazine in the range of 13% - 71% for fresh weight. Dry weights were 40% (0.1 mg/L), 64% (0.25 mg/L), 31% (0.5 mg/L), 38% (0.75 mg/L) and 21% (1.0 mg/L) greater than those for wild-type plants.
Figure 4.12 Comparison of wild-type and transgenic *Nicotiana benthamiana*. Samples are shown after seven days of treatment. Five treatments of atrazine contained in liquid modified Hoagland’s E+ medium without sucrose. (A) Transgenic plants, (B) wild-type plants. Transgenic plants were more tolerant to atrazine treatment than wild-type plantlets which show chlorosis and reduction of growth.
Figure 4.13 Appearance of transgenic (A) and wild-type (B) *Nicotiana benthamiana* seven days after treatment.
Figure 4.14  Dose-response curves of *Nicotiana benthamiana* Wild-type vs. NBTrzN1-5 after seven days of treatment with atrazine. Standard error of means are presented as bars (n = 3).
Figure 4.15  Dose-response curves of *Nicotiana benthamiana* Wild-type vs. NBTrzN1-5 after seven days of treatment with atrazine. Standard error of means are presented as bars (n = 3).
4.3.5 Expression of atrazine chlorohydrolase TrzN in transgenic *Nicotiana benthamiana*

Total soluble protein was extracted using phosphate buffered saline. SDS-PAGE and western blots were performed. Under non-reducing condition, the expression of atrazine chlorohydrolase TrzN was not detectable. However, atrazine chlorohydrolase TrzN was detected in the pellet Figure 4.16.

![Expression of atrazine chlorohydrolase TrzN in transgenic *Nicotiana benthamiana*.](image)

Figure 4.16 Expression of atrazine chlorohydrolase TrzN in transgenic *Nicotiana benthamiana*. The immunoblot was probed with anti-histidine antibody and goat anti-mouse alkaline phosphatase. Lane 1) standard dual protein ladder, lane 2) standard 6×Histidine ladder, land 3) blank, lane 4) crude extract of wild-type *N. benthamiana*, lane 5) crude extract of NBTrzN1-5, lane 6) blank, lane 7) pellet of wild-type *N. benthamiana*, and lane 8) pellet of NBTrzN1-5
4.3.6 Atrazine tolerance bioassay: atrazine field spray simulation

Tolerance to atrazine of T\textsubscript{2} plants from self-pollinated T\textsubscript{1} plants was determined using an atrazine tolerance bioassay using field spray simulation. Seven weeks old transgenic plants, which survived from kanamycin were picked and were sprayed with six different atrazine concentrations. Seven days after application of atrazine, transgenic line # 1-5 was tolerant to atrazine as shown in Figure 4.19 – 4.22. In contrast, wild-type plants were affected by atrazine. With exposure to 25, 100, 250, 500, 1000, and 2000 g a.i./ha of atrazine, transgenic plants had increased tolerance to atrazine in the range of 22% - 27% for height. It was found that NBTrzN1-5 were 26% (25 g a.i./ha), 33% (100 g a.i./ha), 26% (250 g a.i./ha), 19% (500 g a.i./ha), 21% (1000 g a.i./ha) and 23% (2000 g a.i./ha) greater than wild-type plants for fresh weight. Based on the dry weight, growth reduction of transgenic plants was ranged from 42 to 50%, when growth reduction of wild-type plants was ranged from 71 to 84%.
Figure 4.17  Comparison in size of transgenic NBTrzN1-5 (A) versus wild-type (B) Nicotiana benthamiana. Six concentrations of atrazine were used to determine atrazine tolerance. Atrazine concentrations were shown at the bottom of each picture.
Figure 4.18  Representative plants of each concentration of atrazine.
Figure 4.19  Concentration-response curves of *Nicotiana benthamiana* Wild-type vs. transgenic line NBTrzN1-5 after seven days of treatment with atrazine. Standard error of the means are presented as bars (n = 4).
Figure 4.20 Concentration-response curves of *Nicotiana benthamiana* Wild-type vs. transgenic line NBTrzN1-5 after seven days of treatment with atrazine. Standard error of the means are presented as bars (n = 4).
Concentration-response curves of *Nicotiana benthamiana* Wild-type vs. transgenic line NBTrzN1-5 after seven days of treatment with atrazine. Standard error of the means are presented as bars ($n = 4$).
4.4 Discussion

Agroinfiltration of *N. benthamiana* using *A. tumefaciens* containing atrazine chlorohydrolase TrzN gene showed the capability of expressing the recombinant protein within seven days after infiltration. It also indicated that, with the transient expression of atrazine chlorohydrolase TrzN in *N. benthamiana*, there was tolerance to atrazine, when compared to the wild type. However, the expression of atrazine chlorohydrolase TrzN was increased by co-infiltration of P19 and, as a result plants were more tolerant to atrazine. These results prove that agroinfiltration can be useful as rapid screening step to confirm the construct, expression, and enzyme activity in plants prior to proceeding with stable transformation. However, there are some limitations in this agroinfiltration. Expression of protein of interest occurs within the a few days after infiltration and usually
peaks with a week and then decreased thereafter, which has also been shown previously (Faizal and Geelen 2012). During the current study, the highest quantity of the protein expression in *N. benthamiana* was detected at six to eight days post infiltration, thus seven days was selected to determine expression of atrazine chlorohydrolase TrzN and *in vivo* tolerance assay. An investigation to determine the optimum time (i.e., day) that provides the highest amount of the protein may be required. Another factor affecting expression is the OD of the *Agrobacterium* culture. In this study, the research protocol was a standardized practice by the research group. It might be useful to adjust the OD for this particular construct (Faizal and Geelen 2012) to optimize *Agrobacterium* infiltration and hence expression rates.

In the study of stable plant transformation, the expression of TrzN improved atrazine tolerance in transgenic *N. benthamiana*. With a whole plant atrazine application using simulated standard field spraying technique, tolerance to atrazine was enhanced. The results of the studies suggested that transgenic *N. benthamiana* expressing atrazine chlorohydrolase TrzN may have potential use for atrazine remediation of contaminated sites. Using *N. benthamiana* as a model plant for genetic transformation for herbicide remediation is beneficial since this plant is not a cash crop, it can be made sterile, and is an annual that will not overwinter thus proving less of a hazard to escaping into the general environment.

Phytoremediation, the use of plants to ameliorate contaminated soils, and aquatic sites, is a sustainable approach (Kawahigashi 2009, Maestri and Marmiroli 2011). Wild-type, terrestrial plants, such as poplar trees (Burken and Schnoor 1997), switch grasses (Lin et al. 2011), and *Lolium multiflorum* (Merini et al. 2009), have been used in atrazine
remediation of contaminated soil. Although plants have the capability to remove atrazine from the environment, it has been shown here that their capability can be enhanced (Maestri and Marmiroli 2011, Abhilash et al. 2009). Several successful studies using genetic modification to mitigation atrazine have been investigated. Transgenic potato (*Solanum tuberosum cv.*) expressing a rat cytochrome P450 (CYP1A1) shows that atrazine residue is less in transgenic plants comparing to non-transgenic plants (Yamada et al. 2002). The researchers suggested that this could be the result of atrazine metabolic reaction in transgenic plants. The use of transgenic rice in phytoremediation has also been reported. (Kawahigashi et al. 2008) reveal that the expression of three human cytochrome P450s; CYP1A1 CYP2B6, and CYP2C19 could increase atrazine metabolism in rice (*Oryza sativa*). The present study also similarly demonstrated another potential genetic transformation for environmental management purpose.
5. CONCLUSIONS AND FUTURE DIRECTIONS

The ability of plants to remediate contaminants in the environment is the primary focus in the present study, and this technique has also been widely applied, especially in environmental management. With the addition of recombinant protein expression, phytoremediation can be enhanced. In this dissertation, the production of an antibody fragment and an enzyme in plants has been shown. It can also be considered as proof-of-concept for remedial tool development. In this investigation, genetic modification of \( L. \) \( \text{minor} \) to express an anti-atrazine scFv fragment as well as evaluation of the efficacy of this transgenic aquatic \( L. \) \( \text{minor} \) as a tool for remediation of atrazine in water systems were performed. Experiments were carried out by determining biological response of transgenic \( L. \) \( \text{minor} \) produced by \( Agrobacterium \)-mediated transformation to atrazine. One of the transgenic \( L. \) \( \text{minor} \) lines expressing anti-atrazine scFv exhibited tolerance to atrazine. According to quantification of the protein using immunoblot, the scFv was produced at approximately 3.7 mg/kg. Upon challenging the transgenic and wild-type plants with atrazine, results showed no statistical significant difference in uptake of atrazine. It should be noted that employing \( L. \) \( \text{minor} \) as experimental plant species presents challenges as the plant is small, leading to stress and low expression of anti-atrazine scFv protein.

In attempting to express atrazine chlorohydrolase TrzN \( L. \) \( \text{minor} \) was genetically modified to express atrazine chlorohydrolase TrzN gene using \( Agrobacterium \)-mediated transformation. The atrazine chlorohydrolase TrzN gene was successfully inserted into \( L. \) \( \text{minor} \). Results from atrazine tolerance bioassay confirmed that transgenic \( L. \) \( \text{minor} \) expressing atrazine chlorohydrolase TrzN showed increased tolerance to atrazine at low
concentrations. However, the concentration of hydroxyatrazine and other atrazine metabolites were not significantly different between transgenic and wild-type *L. minor*. This finding might be due to the low expression of the enzyme, which was not detectable using Western blot analysis.

Upon investigating the construct of atrazine chlorohydrolase TrzN in the model plant *Nicotiana benthamiana*, the atrazine chlorohydrolase TrzN sequence was optimized for *N. benthamiana*. The agroinfiltration approach confirmed the expression of atrazine chlorohydrolase TrzN, which was co-expressed with P19 to increase protein expression. Furthermore, the result of an atrazine tolerance bioassay indicated that the agroinfiltration could be used as a rapid bioassay for herbicide-tolerant transgenes in plants. In terms of remediation of atrazine, transgenic *N. benthamiana* expressing atrazine chlorohydrolase TrzN showed a potential to resist the effects of atrazine. The result of RT-PCR in T₁ plants showed that atrazine chlorohydrolase TrzN gene was inserted into *N. benthamiana*. T₂ lines of transgenic *N. benthamiana* showed increasing tolerance to atrazine at different concentrations as compared to wild-type plants. Moreover, transgenic plants expressing the atrazine chlorohydrolase TrzN tolerated atrazine of 250 g a.i./ha while wild-type plant died. These findings indicated that protein expression using agroinfiltration in *N. benthamiana* and an herbicide tolerance bioassay can be used as a quick screening test prior to further stable transformation proceeded. The transgenic plants expressing atrazine chlorohydrolase TrzN have a potential to remediate atrazine-contaminated soil.

**Future studies**

Based on this work, future investigation should explore and verify antigen-antibody interaction in plants. The goal is to further identify where atrazine is
sequestered by the antibody. Optimization of organelle-specific expression of antibody could also improve the protection mechanism conferred by the antibody, and should be explored. The central concept behind immunomodulation is binding of antibody to antigen at one-to-one ratio. By increasing the amount of antibody in plants, improved tolerance in plants is likely. Based on expression of atrazine chlorohydrolase TrzN in *L. minor*, identifying ways to increase protein expression is also of interest. One method to increase protein expression is to employ another promoter that is perhaps more efficient for monocotyledons. In future studies, it is also recommended to improve the study system by employing a different experimental unit (i.e., employing a different plant species), which may increase protein expression.

Based on the ability of atrazine chlorohydrolase TrzN, the enzyme has the efficacy to metabolize both atrazine and ametryn, which contains s-triazine ring (Shapir et al. 2005, Seffernick et al. 2010). Study of metabolic pathway to ametryn may also be of interest for developing remedial tool for these types of pesticide. This possibly brings the genetic modified plants to a broader application of phytoremediation of herbicides.

To continue the preliminary work with atrazine chlorohydrolase TrzN in planta, the requirements for complete phytoremediation of atrazine may involve insertion of combination of different genes into single plant. The gene stacking approach may be employed to further express atzB, and atzC genes in the plants expressing TrzN. The atzB and atzC genes from *Pseudomonas sp.* strain ADP have been shown to metabolize hydroxyatrazine, N-isopropylammelide, cyanuric acid, consecutively. In the best management practice, switchgrass (*Panicum virgatum* L.) has been shown the effectiveness to be used in vegetative filter strip for remediation of atrazine (Mersie et al.
2006). Therefore, a recommendation for future study would be to insert TrzN, atzB, and atzC genes in promising terrestrial plant species such as switchgrass in order to enhance bioremediation property.

With the efforts to produce and analyze transgenic *L. minor* tolerant to atrazine in the laboratory such as in this study, the larger scale experiment such as microcosm or mesocosm, which can simulate how atrazine runoff to water systems, is also important to investigate the efficacy of transgenic *L. minor* under the simulated but relevant contaminated site. Investigation of efficacy of the two transgenic *L. minor* expressing anti-atrazine scFv and atrazine chlorohydrolase TrzN in constructed wetlands would be useful.

Although genetically modified plants are a potentially useful tool for environmental remediation, releasing the plant into the environment remains an issue of concern because it may have potential risks to the environment. The risks include toxic effects in grazers of the modified plants, invasiveness of the GM plants, and gene flow to related species. Since *L. minor* is a source of food for fish and ducks in nature, a study of toxicological response to the consumption of transgenic *L. minor* that may hyper-absorbed and accumulate atrazine or atrazine’s metabolites, in fish or duck should be conducted. Genetic modifications can potentially enhance the ability of a plant to become an invasive species. It is therefore important to study the invasiveness of the transgenic plants. Tests with other herbicides for the control of transgenic *L. minor* could also provide an answer to the question of controlling the invasiveness of a GM plant. Moreover, whether wild-type *L. minor*, which has vegetative reproduction, could interbreed with transgenic *L. minor* and decreased genetic variability of native plants due
to interbreeding is also a research topic of interest for future study. Last but not least, the study of gene flow between microorganisms and this transgenic plant is also of research interest, as information obtained would be considered as environmental risk. Assessment of these risks would, no doubt, be beneficial for the decision-making process, and thus, are of interest for future study.
6. LITERATURE CITED


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