The Role of Glutathione Transferases in the Detoxification of TNT

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Abstract

Manufacture, use, storage and improper disposal of the explosive 2,4,6-trinitrotoluene (TNT) have led to widespread, global contamination of soil and groundwater. TNT is highly toxic and recalcitrant to degradation resulting in environmental build-up with far reaching ecological and health implications. It is therefore a priority to remove contaminating TNT from the environment. Phytoremediation is a promising solution; suitable plants possess some natural ability to transform TNT, high biomass, deep root systems, requirement for minimal nutrient input and ability to reduce contamination spread by wind or water erosion; making them an attractive remediation system. Key genes involved in the detoxification of TNT by plants have been recently identified by expression studies and the encoded enzymes characterised. This has lead to the thorough investigation of the enzymes in the pathway of TNT detoxification; Phase I transformation includes oxophytodienoate reductases, with uridine diphosphate (UDP) glycosyltransferases (UGTs) playing a role in the Phase II conjugation step. The expression studies identified additional enzymes also likely to be involved in these phases including glutathione transferases (GSTs). GSTs are known to detoxify compounds by conjugation to glutathione (GSH), and like UGTs are Phase II detoxification enzymes.

This thesis presents an investigation into whether plant GSTs play a role in the detoxification of TNT.

In vitro analysis of recombinant GSTs was performed to elucidate the activity of GSTs towards TNT. Seven GSTs were cloned, expressed and purified from *Escherichia coli*. TNT assays performed with pure enzyme indicated that at least two of the GSTs were able to transform TNT. Analysis of the reaction product by mass spectrometry showed that TNT was conjugated to glutathione through substitution of a nitro-group, a highly desirable reaction as the removal of a nitro group from TNT is likely to increase the likelihood of subsequent mineralisation of the pollutant. This is the first identification of enzymes capable of this transformation.

Abstract

The two GSTs which exhibited activity towards TNT were overexpressed in Arabidopsis to clarify if the conjugating activity observed *in vitro* was able to confer increased tolerance to TNT to the transformed plants. Transgenic lines showed no enhanced growth compared to wild type plants on TNT amended media, root lengths appeared slightly shorter while TNT uptake and biomass were reduced. The role of GSTs in the detoxification of TNT remains unresolved however it is likely that GSTs do not play an integral role in TNT detoxification in plants. Nonetheless, the two GSTs characterised in the project are the first examples of plant enzymes which are able to catalyse the removal of nitro groups from TNT. Engineering these GSTs to improve their ability to transform TNT could offer an opportunity for effective environmental remediation.

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Abbreviations

½ MS(S)(A)	Murashige and Skoog medium half strength (with 20 mM sucrose) (with 8 g/L agar)
2-ADNT	2- Amino-4,6-dinitrotoluene
2-HADNT	2- Hydroxylamino-4,6-dinitrotoluene
4-ADNT	4- Amino-2,6-dinitrotoluene
4-HADNT	4- Hydroxylamino-2,6-dinitrotoluene
A	Adenine
ADNT	Amino-dinitrotoluene
AI	Autoinduction
AOS	Active oxygen species
At	Arabidopsis, *Arabidopsis thaliana*
BITC	Benzylisothiocyanate
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
CaMV 35S	Cauliflower mosaic virus 35S promoter
cDNA	Complementary DNA
CDNB	1-Chloro-2,4-dinitrobenzene
Ch	Chromosome
cm	Centimetre
Col0	Columbia 0
DCNB	Dichloronitrobenzene
DHAR	Dehydroascorbate reductase
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EqGST	Crude extract of equine liver GST
EtOH	Ethanol
EV	Empty vector
g	Grams
Gm	*Glycine max,* Soya bean

GPOX	Glutathione peroxidase
GS^-	Thiolate anion of glutathione
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione transferase
HADNT	Hydroxylaminodinitrotoluene
His	Histidine
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
kb	Kilo base pairs
kg	Kilogram
L	Litre
LIC	Ligation independent cloning
m	Metre
M	Molar
m^3	Cubic meter
MAPEG	Membrane associated proteins in eicosanoid and glutathione metabolism
min	Minute(s)
mRNA	Messenger RNA
MS	Mass spectrometry
μL	Microlitre
μm	Micrometre
μM	Micromolar
mL	Millilitre
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
NR	Nitroreductase
ocs	Octopine synthase
Os	*Oryza sativa*, rice
OPDA	Oxophytodienoic acid
OPR	12-Oxophytodienoate reductase

OYE	Old yellow enzyme
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	Picomole
PETN(r)	Pentaerythritol tetranitrate (reductase)
RDX	Hexahydro-1,3,5-trinitro -1,3,5-triazine, Royal Demolition Explosive
RNA	Ribonucleic acid
RTPCR	Real time PCR
SAGE	Serial analysis of gene expression
s	Second(s)
SE	Standard error of the mean
Spec	Spectinomycin
STDEV	Standard deviation from the mean
T	Thymine
TCA	Trichloroacetic acid
TCQHD	Tetrachlorohydroquinone dehalogenase- like
TNT	2,4,6-trinitrotoluene
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
U	Units
UDP	Uridine diphosphate
UGTs	Uridine diphosphate glycosyltransferases
v/v	Volume to volume ratio
w/v	Weight to volume ratio
WT	Wild type (refers to *Arabidopsis thaliana* Ecotype Columbia0 unless otherwise stated)
Zm	*Zea mays*, maize

Author’s Declaration

I declare that I am the sole author of the work in this thesis and that it is original except where indicated by special reference in the text. No part of this degree has been submitted for any other degree to any other institution.

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Chapter 1: Introduction

1.1 Explosives

The first explosive compound was discovered by Chinese alchemists around 800 AD. In their search for an elixir they made an explosive combination of charcoal, sulphur and saltpetre (potassium nitrate), producing gunpowder (Ponting 2006). However, its explosive power could not be increased and it was very sensitive; any spark in production or storage would have catastrophic consequences. Despite this, it was not until 1847 when Ascanio Sobrero found a suitable alternative when he discovered nitroglycerine. This led the way for many more explosives to be created. From 1870 nitrocellulose became standard in military warheads and in 1863 2,4,6-trinitrotoluene (TNT) was first discovered by Joseph Wilbrand (Ponting 2006). TNT was more stable and easier to manufacture than previous explosives and was therefore heavily employed in combination with other explosives during World War I, when it became, and has since remained, the most common explosive for military use (Lewis *et al.* 2004). Between the two World Wars many more explosive compounds were developed including; hexahydro-1,3,5-trinitro-1,3,5-triazine (Royal demolition explosive, RDX), pentaerythritol tetranitrate (PETN) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Figure 1.1), which all came into wide use during World War II. The rise in petroleum production also increased availability of toluene for TNT manufacture. By 1945 global TNT production reached 150,000 tonnes per month (Snellinx *et al.* 2002). To date, formulations of TNT, RDX and, to a limited degree, HMX remain the most effective, safe, stable and economical explosives, and are therefore the explosives of choice for military applications (Rosenblatt 1980).

An explosive is a compound with rapid reaction rates, which create a high pressure shock wave resulting from a conversion of a substance into gaseous products with much greater volume and heat. For example, TNT detonation creates five gas molecules from each solid TNT molecule; within a sealed vessel this rapid expansion generates intense energy and high pressure causing the explosion (Kury *et al.* 1999).

Explosives can be classified as primary and secondary and these differ in their stability. Primary explosives are more sensitive and can be detonated by heat, friction or mechanical shock. Secondary explosives require a higher energy input and often a primary explosive is used for their detonation, this increased stability makes them commonly used for military applications. There are three main groups of secondary explosives, based on their structures. These include; nitrate esters (PETN, GTN), nitramines (RDX, HMX) and nitroaromatics (TNT, DNT) (Figure 1.1).

1.1.1 Nitroaromatics

Nitroaromatics are stable compounds, which contain an aromatic ring and one or more nitro groups. TNT is a nitroaromatic explosive with three nitro groups; produced by sequential nitration of toluene, producing mono-, then di- and finally tri-nitrotoluene (Figure 1.1) (Lewis *et al.* 2004). Waste produced during TNT manufacture contains the by-products 2,6-dinitrotoluene and 2,4-dinitrotoluene (2,6-DNT and 2,4-DNT).

The aromatic carbon ring of TNT is planar with equal angles of 120 ° (Carper *et al.* 1982). The π electrons of the ring are drawn away by the resonance of electron withdrawing nitro groups resulting in an electron deficient, highly stable ring structure (Qasim *et al.* 2007). The nitro groups also contribute a strong oxidising power, which makes TNT highly toxic to living organisms. This toxicity, combined with its widespread use and recalcitrance to degradation make TNT a priority target for remediation.

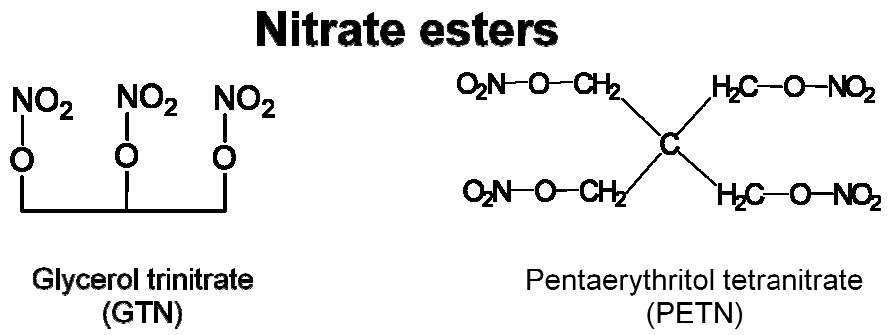


Figure 1.1: Major classes of secondary explosives. Examples of nitrate esters, nitramines and nitroaromatics.

1.2 TNT Pollution

The huge scales of production, use and demilitarisation of TNT over the past 100 years have contributed to its status as a major global environmental contaminant of soil and ground water (Honeycutt *et al.* 1996; Jarvis *et al.* 1998).

During the manufacture of TNT large amounts of water are required for purification; the aqueous wastes (red and pink water) are heavily contaminated with TNT, its precursors and its metabolites. A single manufacturing plant is capable of releasing 500,000 gallons of heavily contaminated red water each day and shell loading plants use large volumes of water to rinse out residual explosives (Yinon 1990). The release of this waste into rivers or its storage in unlined trenches has resulted in both soil and ground water contamination (Rodgers *et al.* 2001). The practices of open detonation and burning on military training sites have also caused significant pollution**:** Explosive fall-out from incomplete detonation of munitions causes repeated exposure of artillery ranges. In one case this pollution is so severe that a Massachusetts training range has heavily restricted live ammunition use, owing to its responsibility in causing the contamination of drinking water source for 700,000 residents of Cape Cod (EPA 2005). Large quantities of wastewater are also produced during decommissioning where munitions casings are 'rinsed' with high power water jets. Prior to this, explosives awaiting disposal are stored, often improperly, allowing leaching of TNT into the surrounding environment (Rodgers *et al.* 2001).

Between the US and Europe estimates for the cost of explosives remediation span 250 billion and 1 trillion US dollars, with reports of 1.2 million tonnes of TNT contaminated soil and 10 billion gallons of ground water in the US alone (Funk *et al.* 1993). The US Department of Defense has identified nearly 1000 sites contaminated with TNT, 87 % of which exceeded permissible levels of TNT is groundwater (Rodgers *et al.* 2001). Reported concentrations in soil range from 10 – 87, 000 mg/kg and in waste water can reach 300 μM (Talmage *et al.* 1999). Other countries with heavily contaminated sites include Argentina, Australia, Canada, Germany, Spain and the United Kingdom (references in Lewis 2004).

TNT has a high capacity to bind to the humic content of soil. The electron deficiency of the aromatic ring allows the compound to form complexes with clay in the soil reducing the mobility of TNT (Li *et al.* 2004; Qasim *et al.* 2005). This

low environmental migration has caused local build-up of TNT, resulting in points of discharge containing very high levels of the contaminant. Soil composition and maturity both play a role in the phytotoxicity of TNT; bioavailability of the explosive is reduced in highly organic or clay-containing soils, while the age of the soil is also important (Thompson *et al.* 1998). Hysteresis, or irreversible binding of TNT occurs gradually in soils over time indicating that younger soils may increase the toxicity of TNT due to increased bioavailability (Comfort *et al.* 1995).

1.3 Toxicity of TNT

1.3.1 Toxicity to Humans and Animals

Explosives are toxic and their presence in soil, groundwater and air can have potentially serious effects for human health and the environment. The first documented case relating to the toxicity of TNT was in 1917 when a worker at a large US TNT factory died of acute toxic jaundice, 17,000 further poisoning cases including 475 deaths occurred in the US and 96 deaths in the UK within the first 8 months of World War I (Yinon 1990). Following this, many fewer poisoning cases occurred due to a better understanding of the toxicity of explosives combined with improved prevention, diagnosis and treatment of poisoning. TNT causes jaundice, aplastic anaemia, liver atrophy and hepatitis in humans (Rosenblatt 1980). Other symptoms of TNT toxicity include; dermatitis, gastritis, cyanosis, reduced sperm count, dizziness, fatigue and nausea (Yinon 1990). As a Class C carcinogen, cell mutagenesis studies have implicated a link between TNT and cancer. Mutagenesis has occurred in three of four Ames assays, where the mutagenic potential of a compound is studied with *in vitro* tests; TNT and its reductive transformation product hydroxyl aminodinitrotoluene were both found to be carcinogenic (Whong *et al.* 1984). In mammalian cell lines both Chinese hamster ovary cells and mouse lymphoma cells experienced DNA-frameshift mutation as a result of 20 - 150 μM TNT treatment (Honeycutt *et al.* 1996), however Chinese hamster lung cells were exhibited effects of cytotoxicity but no mutagenicity with 25 - 500 μM TNT (Lachance *et al.* 1999). Erratic results from mutagenic tests have been proposed to indicate that TNT and its metabolites do not pose a serious genotoxic threat (Honeycutt *et al.* 1996).

1.3.2 Phytotoxicity of TNT

TNT is phytotoxic causing exposed plants to exhibit stunted roots, inhibition of lateral root growth and chlorosis (Pavlostathis *et al.* 1998). Germination and transpiration rates are affected (Hannink *et al.* 2001; Kim *et al.* 2004) and grass seedlings exposed to TNT also have reduced root hairs when grown on TNT (Peterson *et al.* 1998). The effect of TNT on plants is highly dependent on TNT dose, plant species and growth media.

Chlorosis is observed in cultures of *Myriophyllum spicatum* at TNT concentrations above 5.9 *μ*M, with a lethal dose of 23 *μ*M, however doses of 50 *μ*M are insufficient to adversely affect *Allium cepa* (onion) (Pavlostathis *et al.* 1998; Kim *et al.* 2004). Hybrid poplars present toxicity symptoms with 22 *μ*M when grown hydroponically including chlorosis, leaf loss and decreased transpiration, with complete inhibition of growth occurring over 440 *μ*M (Thompson *et al.* 1998). However, *Chrysopogon zizanioide* (vetiver grass) can withstand 180 *μ*M TNT before any morphological effects of TNT phytotoxicity can be observed (Makris *et al.* 2007). Arabidopsis and tobacco also exhibit chlorosis when grown in hydroponic media amended with TNT (Hannink *et al.* 2001; Rylott *et al.* 2006).

Reduced germination of cress and turnip occurs at 50 mg/kg soil, while oat can tolerate up to 1600 mg/kg (Gong *et al.* 1999). For some species, low doses of TNT ~25 mg/kg soil have even been found to stimulate seedling growth (Gong *et al.* 1999). *Phaseolus vulgaris* (bush bean) can grow on high TNT concentrations up to 500 mg/kg soil while *Festuca arundinacea (*tall fescue) suffers reduced root growth on just 1.9 mg/kg (Peterson *et al.* 1998; Scheidemann *et al.* 1998). Direct comparisons between different studies are limited by experimental variations of plant biomass and developmental stage, growth conditions, growth media and mode of TNT application. Despite this, it is clearly evident that TNT exhibits phytotoxic effects on all plant species tested but it is likely that some plants are more tolerant than others.

The mechanism of toxicity to plants is unclear. The reactivity of the nitro groups and the putative toxicity of reductive transformation products may contribute. Aminodinitrotoluenes (ADNTs) are known products of plant transformation of TNT, these and TNT are structural homologues of the dinitroaniline herbicides for

example; nitralin and butralin which disrupt cell division by binding to microtubules and preventing the formation of mitotic spindles, causing swelling of root tips without elongation (Anthony *et al.* 1999). TNT may also act in a similar manner to nitrobenzene which causes alterations in the nuclear membrane and leads to leaf ultrastructure damage (Farlane *et al.* 1990).

1.4 Current Strategies of Remediation

1.4.1 Incineration

Incineration was, for a long time, the only treatment process effective at TNT remediation and is still the mostly widely accepted method. It offers high levels of destruction and removal however the costs are high. Economically it is very expensive, with the requirement for construction of facilities and it is generally unviable for soil with low TNT concentrations. Soil must be excavated and transported to an incinerator, posing both high costs and safety hazards, and soil containing high concentrations of TNT (>12 %) is liable to detonate either by initiation by flame or by shock (EPA 2005). Estimations from 1992 calculated that incineration costs were 800-1000 US dollars per ton, depending on the size of the operation (Funk *et al.* 1993). Environmentally it is also damaging, owing to the high energy input required and the production of harmful airborne particulates, carbon monoxide and nitrous oxides (Yinon 1990; Rodgers *et al.* 2001; Lewis *et al.* 2004). Following incineration the soil is sterile, the physical structure of the soil has been destroyed and it has little application for cultivation or agriculture.

1.4.2 Composting

Composting was the first biological treatment technology to be approved for use on US military sites (EPA 2005). There are two main composting strategies in use for explosives remediation; static pile and windrow composting.

In both cases contaminated soil is mixed with organic material including woodchips and straw to increase microbial growth, bulking material to improve aeration and moisture. Aeration is required for both systems, static piles have

costly internal ventilation systems while windrow piles require regular turning. The moisture added can be contaminated TNT waste water which conveniently combines treatment of the two explosive wastes. Windrow composting costs approximately 200-800 US dollars per ton, which is much cheaper than incineration when used for low volumes of soil (Lewis *et al.* 2004). TNT toxicity is reduced due to it being bound to organic matter, however the possible release of bioavailable metabolites needs to be investigated further (Honeycutt *et al.* 1996). In addition to this, composting requires large land area and is slow.

1.4.3 Bioslurry

Bioslurry is performed by submerging soil in a bioreactor, where the addition of nutrients and optimised environmental conditions support microbial remediation. The results are similar to those of composting although this method provides a faster alternative. The costs involved are from soil excavation, sieving, reactor construction and maintenance, which are similar to those for composting, with small scale trials costing 200-600 US dollars per ton.

1.4.4 Phytoremediation

Owing to the sheer scale of pollution in the US alone; economic and environmental pressures are pushing for alternatives to current remediation technologies for the removal of both organic and inorganic pollutants. This has sparked significant interest in phytoremediation, where the ability of plants to uptake, concentrate or metabolise xenobiotics can be harnessed for low-cost environmental clean-up.

Plants can be used to perform a variety of different processes including phytoextraction, phytovolatilisation, phytostabilisation, phytostimulation and phytodetoxification (Pilon-Smits 2005). Generally plants for phytoremediation are required to be tolerant to pollution, fast growing, high biomass, high uptake and competitive.

The concept of phytoextraction, where a compound is accumulated with no degradation, was first discovered following the identification of the ability of certain plants to overcome the toxicity of heavy metals and accumulate them in

aerial tissues. Hyperaccumulators of nickel, zinc and copper can contain 10 to 500 times more metal that standard crop plants can tolerate. Although, in practicality hyperaccumulators are slow growing with low biomass and currently only the nickel hyperaccumulator *Alyssum bertolinii* is used commercially (Li *et al.* 2003). For phytoextraction high levels of translocation and accumulation in aerial tissue are important. Harvested xenobiotic-containing plants can be harvested and then either disposed by incineration or landfill, used for non-food purposes including cardboard production, or collected and concentrated, as for valuable metals in a process called phytomining.

Phytovolatilisation uses the plant's transpiration stream to release native or modified xenobiotics as gases into the atmosphere. The most successful example of this is for the element selenium, an agricultural toxin at widely varied concentrations depending on plant species; plants grown in selenium rich soil produce volatile dimethylselenide and dimethyldiselenide (Banuelos *et al.* 2005). *Oryza sativa*, *Brassica oleracea, Brassica juncea,* and *Brassica oleracea c.v Linne* have been shown to be especially efficient at this transformation. A phytovolatilising system has been developed for mercury remediation, Arabidopsis plants uptake mercury as Hg (II) from the soil and reduce it to Hg (0), which is released as a gas (Meagher *et al.* 2005). However, risks are associated with phytovolatilisation as it may result in release of highly toxic compounds into the air.

Phytostabilisation may imply the use of plants to maintain organic structure, reducing run-off and leaching of pollutants but it can also denote their use to reduce bioavailability by metabolism or incorporation into the structural biomass of plants (Pilon-Smits 2005). Poplar trees are a popular choice for phytostabilisation, owing to their deep root systems reducing downward flow and groundwater contamination, their fast transpiration stream is desirable for volatilisation and degradation.

Additionally plants can stimulate rhizosphere degradation by release of exudates and enzymes, which may themselves be active, or may improve activity of soil bacteria (Burken *et al.* 1996). This is called phytostimulation or rhizodegradation. Grasses such as *Festuca* and *Lolium* have dense roots, which allow good contact for phytostimulation of rhizosphere bacteria (Aprill *et al.* 1990).

Phytodegradation, harnessing the metabolic pathways of plants to degrade xenobiotics cannot be used for the remediation of inorganic compounds, which are undegradable. It can however be hugely successful for the remediation of organic compounds. Degradation is usually due to plant metabolism but may also be a result of plant endophytic bacterial (Barac *et al.* 2004). The main enzymes involved in phytodetoxification are; dehalogenases, mono and di-oxygenases, laccases, phosphatases and nitroreductases (Pilon-Smits 2005). If the degradation products are to be stored within the plants they are often conjugated by malonyl-, glucosyl- or glutathione- transferases and transported out of the cytosol (Coleman et al. 2007). The expression of foreign genes or the overexpression of native genes may also improve the ability of plants to detoxify specific compounds. The pathways of xenobiotic degradation in plants are outlined in Section 1.5. Plants with large dense roots and high levels of degradation enzymes are favoured for phytodegradation, for example grasses such as; *Festuca* and *Lolium*.

Phytoremediation is relatively inexpensive, with estimates that it is up to ten times cheaper than engineering-based strategies (Pilon-Smits 2005); it is usually performed *in situ*, reducing the hazards associated with transporting toxic waste. It is an attractive solution, economically, environmentally and aesthetically. In the US it is a fast growing market now comprising ~150 million US dollars, approximately 0.5 % of the remediation market. However it does have limitations. Phytoremediation sites need to have suitable climate for plant growth and transpiration; appropriate soil properties to allow xenobiotic uptake including pH, clay content and organic matter, in addition levels of the xenobiotic must not exceed phytotoxicity limits and the pollutant must be bioavailable to facilitate uptake. The contamination needs to be accessible by plant roots; however, deep reserves of contaminated water can be pumped to the surface for phytotreatment. Pollution may also be unevenly spread across a site; in the case of TNT on training ranges the contamination can be highly concentrated in small clusters within a site, posing accessibility and toxicity issues. Plants are also relatively slow remediators, often taking years to improve a site. Despite this, phytoremediation and non-biological remediation systems can be used in conjunction with one another, combining the benefits associated with each system (Pilon-Smits 2005) (EPA 2003).

1.5 Metabolism of Xenobiotics by Plants

As plants grow in our environment they are continuously exposed to toxic chemicals and therefore rely on detoxification pathways to secure their survival. These xenobiotics may be allelochemicals; toxins synthesised by other plants in competition situations, secondary metabolites or compounds produced by the plant itself as a result of general metabolic processes or in response to environmental stress (UV, temperature, animal predation, or microbial attack). Plants are also deliberately exposed to synthetic xenobiotics in the form of herbicides and pesticides or as a result of industrial pollution contaminating soil and groundwater (Coleman *et al.* 1997). Plants therefore employ various methods to respond to these xenobiotic compounds, and understanding this is valuable for agriculture, ecology and human health.

1.5.1 Four stages of Xenobiotic Detoxification by Plants

Plant metabolism of xenobiotics is similar to that observed in the livers of animals. The liver performs three main detoxification steps, activation, conjugation and excretion. Plants do not have an effective excretion pathway; instead xenobiotics are stored once detoxified. Due to the similarities of the enzymes and steps of plant metabolism to those of animals, the phrase 'Green Liver' has been applied to the plant detoxification. The steps or phases are outlined below and shown in Figure 1.2.

Phase I- Activation: Enzymes, for example cytochrome P450 or other monooxygenases, add functional groups including hydroxyl (-OH), sulfhydryl (–SH) or amino ($–NH_2$) to substrates. P450 enzymes play a major role in this Phase of detoxification, performing hydroxylation, sulfoxidation and N- or C-dealkylation. Reductive transformations as less common but have been observed by nitroreductase activity of oxophytodienoate reductases (OPRs) with TNT, sequentially producing hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) (Section 1.6.3.2), diaminonitrotoluenes have also been identified (Burken *et al.* 2000, Beynon *et al.* 2009). The products of these reactions are often less hydrophobic than the original xenobiotic but primarily these steps create 'reactive sites' which in some cases increase toxicity but allow

further transformation by the enzymes involved in Phase II (Sandermann 1994; Coleman *et al.* 1997).

Phase II- Conjugation: Functional groups already present on the xenobiotic or from Phase I are used as sites for covalent conjugation to an endogenous hydrophilic molecule. Xenobiotic substrates include the electrophilic herbicides as well as organic pollutants and some toxic secondary metabolites. The enzymes involved include malonyltransferases, glucosyltransferases and glutathione transferases, which catalyse conjugation producing a water-soluble conjugate of malonate, glucose or glutathione with reduced toxicity (Coleman *et al.* 1997). Malonate can be conjugated to hydroxyl and amino groups, glucose can bind to hydroxyl, sulfhydryl, amino and carboxyl and glutathione can bind to hydroxyl and sulfhydryl groups, often with the removal of a halogen or nitro group. As a result, the hydrophilic product is less mobile within the plant, with exposure time and accumulation within cells of the toxic xenobiotic is reduced (Sandermann 1992; Coleman *et al.* 1997).

Phase III- Compartmentation/Elimination: Any conjugates which remain in the cytosol have the potential to inhibit Phase II enzymes or be un-conjugated by cytosolic enzymes, restoring toxicity (Coleman *et al.* 1997). Therefore, conjugates are transported from the cytosol by ATP-dependent (GS-X) membrane pumps for sequestration or storage in the apoplast or vacuole, or cell wall incorporation (Marrs 1996; Coleman *et al.* 1997).

Phase IV- Transformation: In this step a conjugate may be metabolised, in the case of a glutathione conjugate; to a cysteine-conjugate by enzymatic removal of glutamate and glycine from GSH (Bartholomew *et al.* 2002; Ohkama-Ohtsu *et al.* 2007).

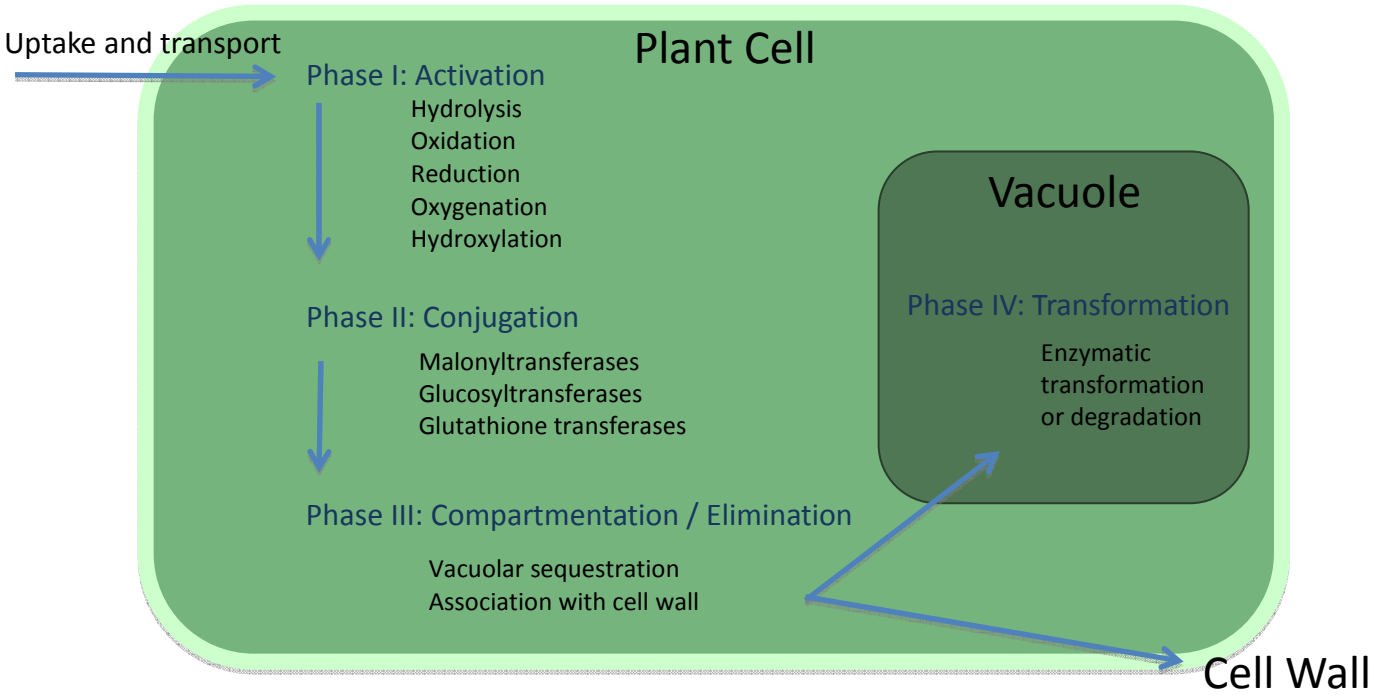


Figure 1.2: Phases of Xenobiotic Metabolism in Plants. Adapted from Van Aken, 2008.

1.6 Biotransformation of TNT

While TNT is a relatively recent, anthropogenic compound various organisms are able to transform TNT, including; bacteria, fungi and plants animals.

1.6.1 Bacterial Transformation of TNT

Both aerobic and anaerobic transformations of TNT have been observed in bacteria.

1.6.1.1 Aerobic Bacterial Transformation of TNT

Due to the difficulty of oxidative reactions, even when performed in the presence of oxygen, TNT metabolism is almost exclusively reductive. The most common pathway is nitro group reduction (Figure 1.3); bacteria reduce one or two of the nitro groups via a nitroso dinitrotoluene intermediate (NODNT) to hydroxylamino or amino groups producing hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) (Koder *et al.* 1998). *In vitro* experiments show these ADNTs usually undergo no further metabolism and hence accumulate in the culture media. The reduction of two nitro groups produces diaminonitrotoluene (DANT) (McCormick *et al.* 1976; Esteve-Nunez *et al.* 2001). Dimerisation of these compounds creates undesirable azo products; these dead-end structures have increased toxicity relative to TNT (Figure 1.3, F) (Honeycutt *et al.* 1996). Enzymes capable of reductive transformation of TNT have been isolated and characterised. These include; nitroreductases from *Enterobacter cloacae* (*nfsI*) and *Escherichia coli* (NfsA and B) and pentaerythritol tetranitrate reductase (PETNr) from *E. cloacae* (Bryant *et al.* 1991; French *et al.* 1999). PETNr was first identified by its ability to reductively liberate nitrite from PETN producing of pentaerythritol trinitrate and pentaerythritol dinitrate (Binks *et al.* 1996). It performs the same transformation with TNT but interestingly, it is also capable of catalysing hydride addition to the aromatic ring of TNT producing hydride and dihydride Meisenheimer products (Figure 1.4) (Symons *et al.* 2006). A condensation reaction occurs between the Meisenheimer dihydride complex and HADNT, from the nitro group reduction of TNT (Figure 1.3, B), to form stable secondary diarylamines and results in nitrite liberation (Figure 1.4) (van Dillewijn *et al.* 2008; Wittich *et al.* 2009). This reaction has also been observed for few other bacteria including species of *Pseudomonas*, *Mycobacterium* and *Rhodococcus* (Wittich *et al.* 2008).

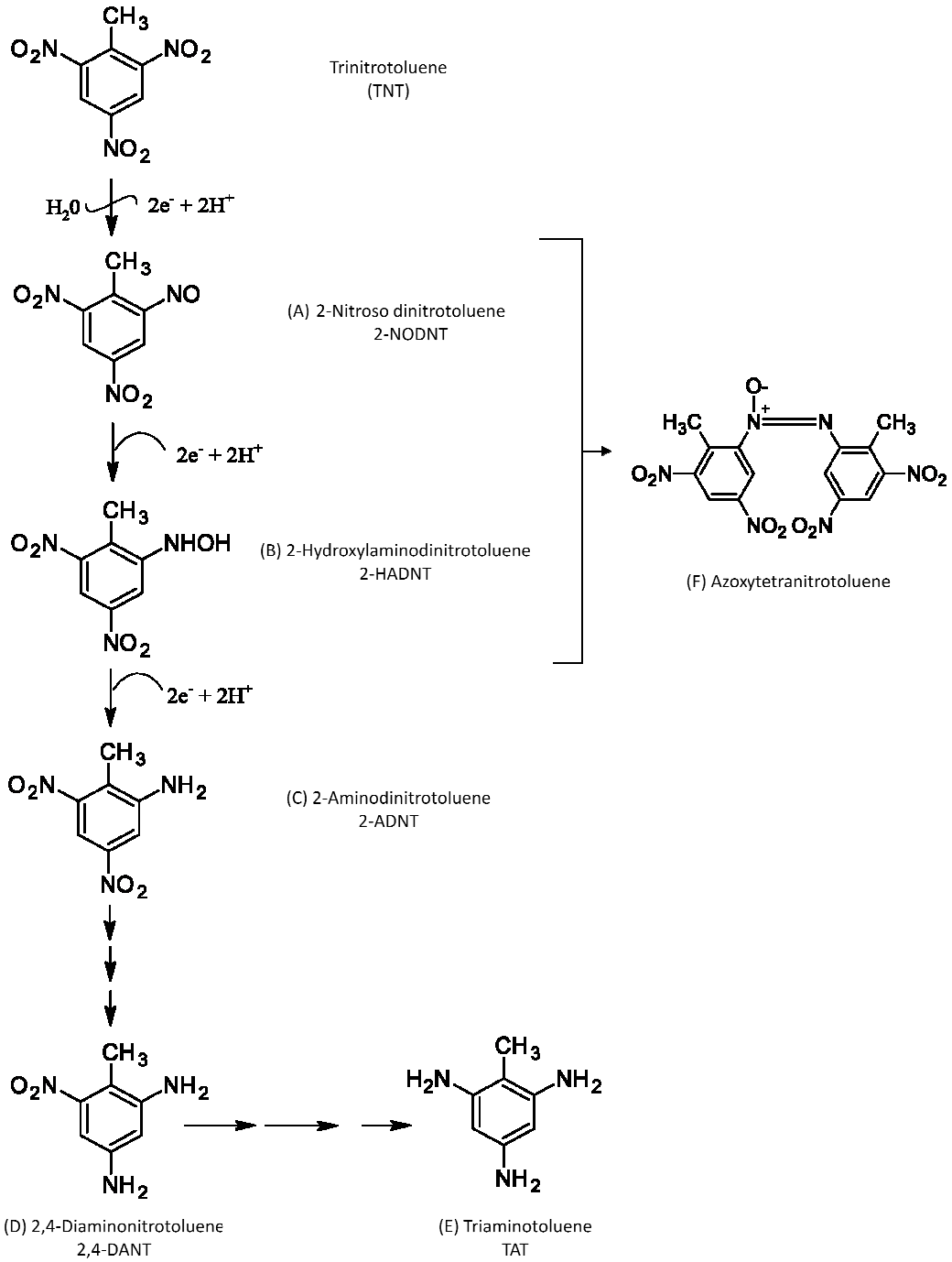


Figure 1.3: Aerobic transformation of TNT in bacteria and plants. Products A-F have been identified in bacteria and in plants A-C and F. Sequential reduction of via the putative intermediate (A) 2-nitroso-4,6-dinitrotoluene (2-NODNT) and (B) 2-hydroxylaminodinitrotoluene (2-HADNT), (C) 2-aminodinitrotoluene (2-ADNT) and (D) 2,4-diamino-nitrotoluene (2,4-DANT). Polymerisation of A and B can occur forming F; azoxytetranitrotoluenes. Product (E) triaminonitrotoluene (TAT) is a reductive product of (D) 2,4-diamino-nitrotoluene (2,4-DANT), only produced under anaerobic conditions. Figure adapted from Lorenz, 2007.

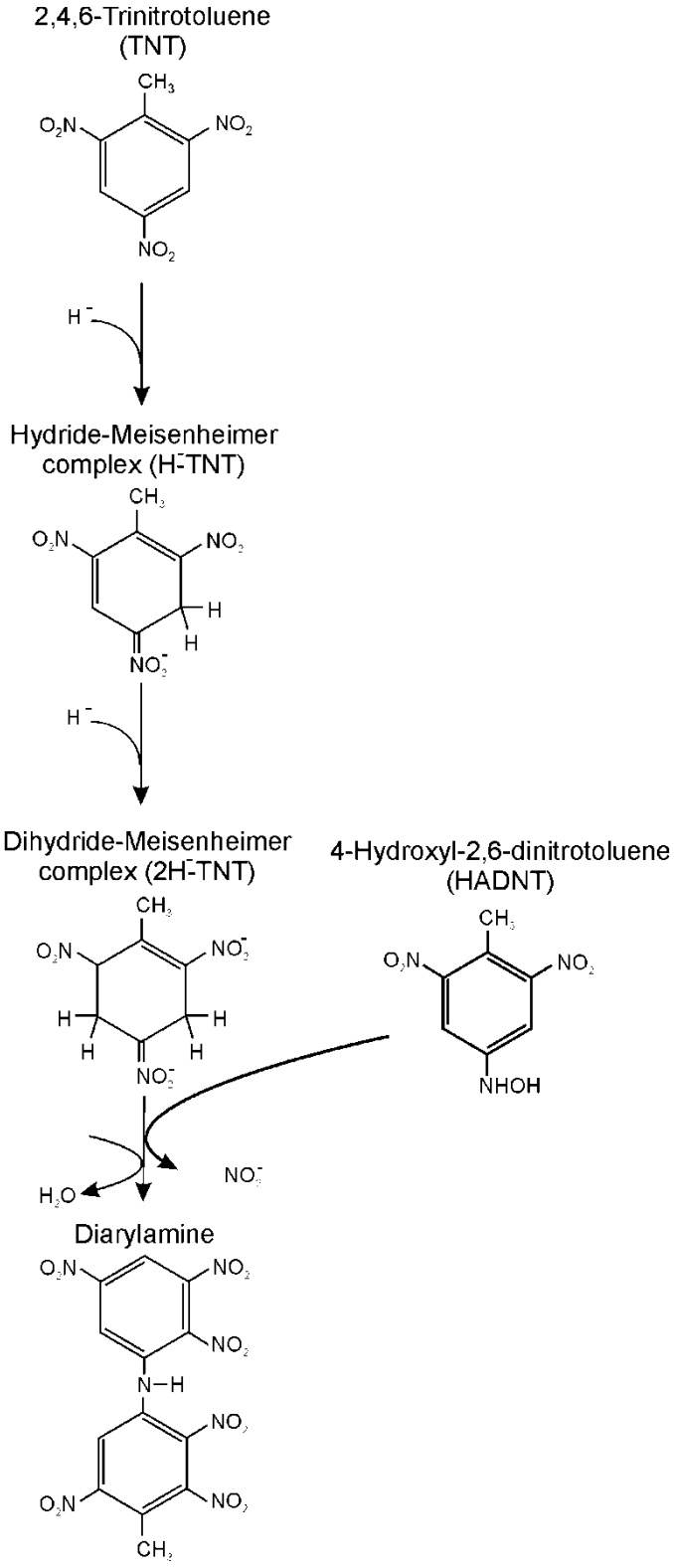


Figure 1.4: Hydride addition to the aromatic ring of TNT sequentially yields hydride and dihydride Meisenheimer complexes. The dihydride Meisenheimer complex forms a diarylamine with hydroxylaminodinitrotoluene from the nitro group reduction pathway with concominant nitrite release. Adapted from Rylott et al. (2011).

1.6.1.2 Anaerobic Transformation of TNT in Bacteria

Anaerobic transformation of TNT is more efficient than aerobic due to the low redox potential allowing for rapid reduction of substrates, additionally no toxic azo-oxy nitrotoluenes are produced (Figure 1.3). Despite this, few anaerobic bacteria have been found that express this ability. Methanogens (Boopathy *et al.* 1994) and fermentative bacteria such as *Clostridia* species are capable of producing triaminotoluene (TAT) (Figure 1.3F) (Ederer *et al.* 1997; Rosser *et al.* 2001). The production of TAT requires an electropotential below -200 mV and is therefore only possible in anaerobic environments. Sulfate reducing bacteria for example *Desulfovibrio* can use TNT as a sole nitrogen source, with the accumulation of toluene (Boopathy *et al.* 1994). It is assumed that this denitration reaction proceeds by a TAT intermediate, with reductive elimination of the amino groups; however no TAT has been detected in the culture media (Esteve-Nunez *et al.* 2001; Rosser *et al.* 2001). Esteve-Nunez and Ramos (1998) isolated a *Pseudomonas* strain also able to use TNT as a sole nitrogen source, 1 % mineralisation was observed with 45 % of the ^{14}C radiolabel from TNT found to be biomass associated. This suggests that anaerobic bacteria are capable of significant transformation of TNT, but current understanding of the mechanisms is limited.

1.6.2 Fungi

The transformation pathways of fungi are of significant interest due to their ability to completely mineralise TNT (Fernando *et al.* 1990). The best characterised example of this is wood white rot fungi *Phanerochaete chrysosporium*, however other white rot fungi are also capable of performing mineralisation, with >20 % efficiency (Fernando *et al.* 1990). Mineralisation requires lignolytic conditions and initial activity is likely to proceed by as for bacteria with sequential nitroreduction to HADNT, ADNT and polymerisation products azotetranitrotoluene. *Saccharomyces* species reduce TNT to HADNTs and ADNTs while *Candida* strains can transform TNT to HADNTs and H-TNT (Zaripov *et al.* 2002). Subsequent transformation varies depending on fungal species, environmental conditions and time. In many cases no further transformation occurs (Hawari *et al.* 2000). The mineralisation of TNT by *P. chrysosporium* is observed by recovery of 18 % of ^{14}C from labelled TNT as CO_2 after 90 days culture

(Fernando *et al.* 1990). The fungus *Irpex lacteus* does not reduce TNT initially but first denitrates it, producing DNT and H-TNT. This transformation produces >30% mineralisation due to the oxidation of denitrated metabolites (Kim *et al.* 2003; Smets *et al.* 2007). It is likely that efficient mineralisation by fungi is the result of activity by multiple enzymes including lignin and manganese peroxidases (Stahl *et al.* 1995). The mineralisation of HADNTs and ADNTs observed in *Phlebia radiaa* and *P. chrysosporium* likely results from activity of manganese peroxidases (Van Aken *et al.* 2004). The practical applicability of fungi as remediation solutions for TNT is hindered by TNT toxicity, low biomass of the organisms and the requirement for nutrient feeding (Spiker *et al.* 1992; Stahl *et al.* 1995).

1.6.3 Plant Metabolism of Nitroaromatics

1.6.3.1 Uptake and Translocation

Uptake and translocation of nitroaromatics is dependent on plant species, soil properties, climatic conditions and TNT bioavailability. Uptake is known to depend on solubility of the substrate in water, determined by the log 1-n-octanol/water partition coefficient (K_{ow}) (Sens *et al.* 1998). Log K_{ow} values of 3.5 or higher indicate low solubility and compounds bind to the root and soil surfaces, between 1 - 3.5 efficient uptake is observed, below this hydrophilic compounds are unable to pass through membranes. TNT has a log K_{ow} of 1.6 and is therefore readily absorbed. In liquid culture and soil studies, *Glycine max* (soya bean) *Triticum aestivum* (wheat), *Zea mays* (maize), *Spinacia oleracea* (spinach), *Populus sp*. (poplar) and Arabidopsis have all demonstrated significant levels of TNT uptake. The aquatic plants *Myriophyllum aquaticum* and *M. spicaticum* and tissue cultures of *Catharanthus roseus* uptake TNT rapidly, with 93 to 99 % recovery. Once taken up, radiolabelling studies show that much of the TNT remains in the roots. After 48 h *Panicum virgatum* transported just 2.5 % TNT to its foliage (Brentner *et al.* 2010), similarly studies of *T. Aestivum*, *P. Vulgaris, A. cepa* and poplar show less than 5 % of TNT is localised in aerial tissues (Sens *et al.* 1998; Sens *et al.* 1999; Kim *et al.* 2004). For *Z. mays*, *G. Max*, and *O. Sativa* more than 75 % of accumulated TNT and its metabolites are retained in the root (Thompson *et al.* 1998; Nepovim *et al.* 2005; Vila *et al.* 2005; Yoon *et al.* 2006).

1.6.3.2 Phase I: Nitroreduction

As for bacteria and fungi, TNT in plants is reduced to HADNTs and ADNTs via a nitroso intermediate (Figure 1.5). HADNTs are likely to be the major product; however their instability results in low detection levels. ADNTs are stable, but have been shown to only account for 15 % of initial TNT concentrations in *C. roseus* roots after 50 h (Wayment *et al.* 1999). Further evidence that HADNTs are the dominant product is that conjugates of HADNTs are more commonly detected than those of ADNTs (Vila *et al.* 2005; Subramanian *et al.* 2006; Gandia-Herrero *et al.* 2008). Nitroreductases, which perform this transformation of TNT are often upregulated in plants following TNT treatment (Ekman *et al.* 2003; Gandia-Herrero *et al.* 2008). Oxophytodienoate reductases (OPRs) have also been found to be upregulated; these enzymes are homologues of the bacterial Old Yellow Enzyme (OYE) family which are known to have nitroreductase activity with TNT (Symons *et al.* 2006; Beynon *et al.* 2009). Characterisation of the OPRs implicates them as having a role in TNT detoxification in plants. Arabidopsis overexpressing OPR1, 2 and 3 show faster uptake of TNT and increased production of ADNTs compared to unmodified plants. *In vitro* assays also show high rates of HADNT production, though HADNT stability limits their detection in plants (Beynon *et al.* 2009).

1.6.3.3 Phase I: Oxidation

TNT is a poor substrate for oxidation due to its electron deficient aromatic ring. One study using *M. aquaticum* suggests that oxidative transformation of the methyl group occurs, in addition to hydroxylation (Bhadra *et al.* 1999). It is however not clear whether these products solely result from plant processes as microbial or synergistic activities may have occurred in this system; the plants were not grown in a gnotobiotic environment. Endophytic bacteria have been shown to play a role in the TNT transformation observed in plants (Barac *et al.* 2004).

1.6.3.4 Phase II: Conjugation

Six-carbon conjugates of TNT and its Phase I metabolites have been identified in *P. vulgaris, M. aquaticum* and *C. roseus* (Hughes *et al.* 1997; Sens *et al.* 1998; Bhadra *et al.* 1999). SAGE and microarray analysis of TNT treated Arabidopsis and poplar show high upregulation of UDP-glycosyltransferases (UGTs) and GSTs compared to untreated plants (Ekman *et al.* 2003; Mezzari *et al.* 2005;

Gandia-Herrero *et al.* 2008). *In vitro* studies of UGTs yielded glycosyl conjugate production with ADNT and both *C*- and *O*- glycosides of HADNT (Figure 1.5). Characterisation of Arabidopsis plants overexpressing UGTs has shown increased production of 2- and 4-HADNT glucose conjugates, ADNT conjugates and increased levels of TNT uptake (Gandia-Herrero *et al.* 2008). This suggests that UGTs play a role in the Phase II metabolism of TNT by plants. Gene expression data have also implicated a role for glutathione transferases (GSTs) in TNT detoxification. However to date, no glutathione conjugates of TNT or TNT metabolites have been identified in plants.

1.6.3.5 Phase III Transport of Conjugates

Once conjugated, TNT might exit the cytoplasm through specific transporters such as ABC transporters (Mentewab *et al.* 2005). Various transporters have been found to be upregulated in plants by TNT including the Arabidopsis glutathione conjugate transporters (AtMRP1 and 2) (Lorenz 2007), P-glycoprotein transporters and phloem specific transporters (Landa *et al.* 2010). All of these may be involved in carrying TNT conjugates out of the cytosol.

1.6.3.6 Phase IV: Sequestration

Analysis of plant degradation pathways shows a possibility that the TNT ring structure can be broken and incorporated into new plant material, for example the cell wall. Cell wall modification enzymes are upregulated in response to TNT including; expansins, Touch4 and xyloglucan endotransglucosylase (Brazier-Hicks *et al.* 2007; Landa *et al.* 2010). These genes may be induced due to their role in associating TNT conjugates into the cell wall. This theory is strengthened by the repeated identification of TNT metabolites in cell wall fractions (Sens *et al.* 1998; Sens *et al.* 1999; Nepovim *et al.* 2005; Vila *et al.* 2005; Zhao *et al.* 2006).

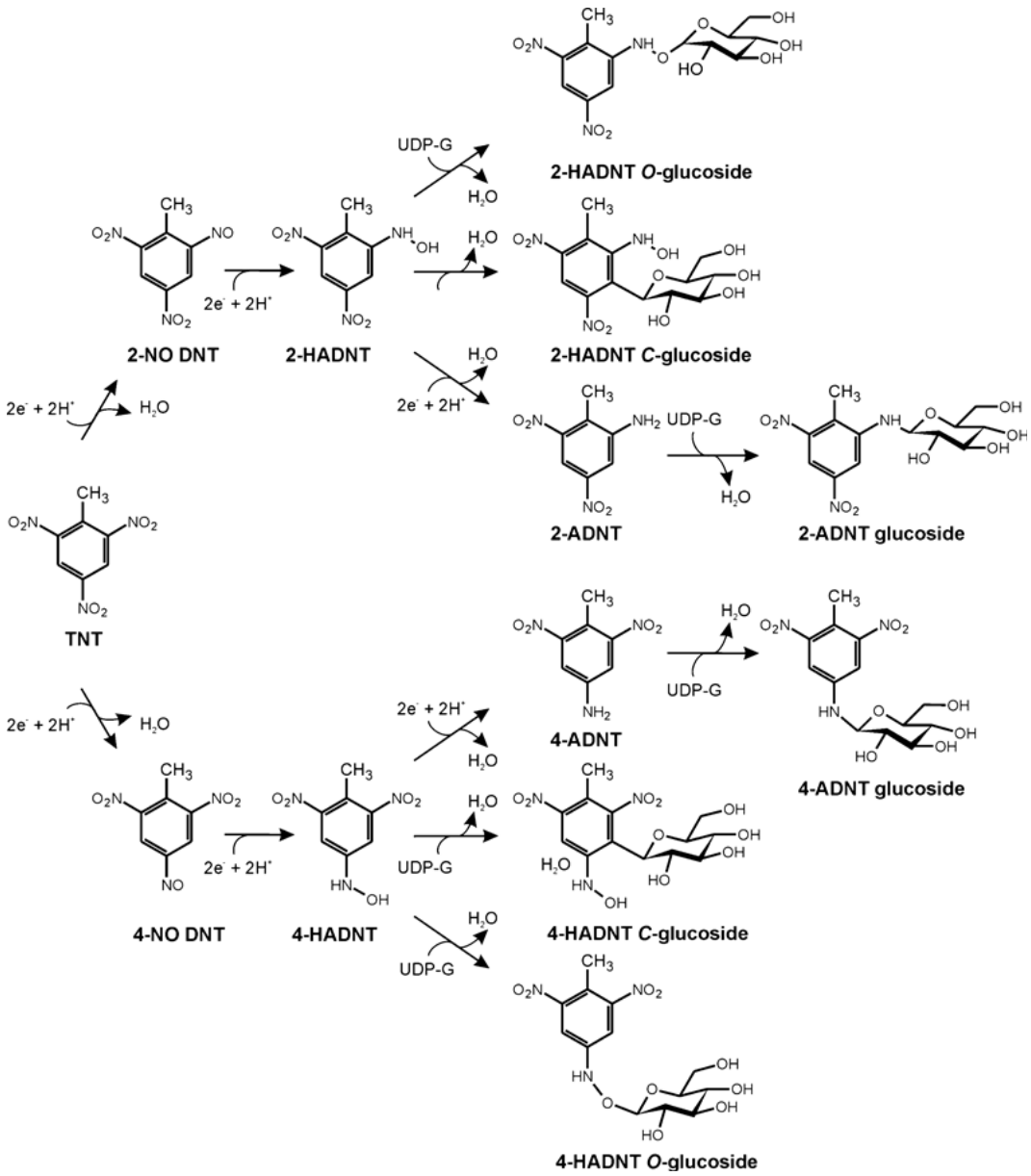


Figure 1.5: Metabolic pathway of TNT in plants. Reduction of TNT by nitroreductases and OPRs produces HADNT and ADNT which are then conjugated to sugars by UDP-glycosyltransferases. *O*- and *C*- linked HADNT conjugates have been isolated from extracts of TNT treated Arabidopsis. Figure taken from Beynon, 2008.

1.7 Phytoremediation of TNT

Plants which have a potential for phytoremediation of TNT have been assessed; *Datura innoxia* and *Lycopersicon* grow well at 750 mg TNT/kg soil however at 1000mg/kg symptoms of phytotoxicity are observed. Despite this the plants can accumulate up to 30 times more TNT than the initial soil concentration with a 90-95 % removal rate after two weeks (Lucero *et al.* 1999). Reports of high uptake and transformation rates have also been published for maize, *P. vulgaris*, *T. aestivum* and *M. Aquaticum* (Sens *et al.* 1998; Bhadra *et al.* 1999; Sens *et al.* 1999). Despite this, the levels of accumulation and the rate of detoxification are not significant enough for efficient phytoremediation. For this reason genetic modifications of plants have been used to enhance the degradation abilities of plants. The shorter generation time of bacteria has allowed them to evolve effective degradation systems for recently developed xenobiotics. The combination of these genes with the benefits of phytoremediation has yielded successful results. Enzymes capable of reducing the nitro groups of TNT to produce HADNT have been isolated from bacteria including; PETNr (French *et al.* 1999) and *nfs*I (Hannink *et al.* 2001), *nfs*A and *pnr*A. When cloned into tobacco, both PETNr and *nfs*I conferred increased TNT uptake and higher levels of reductive metabolites, HADNT and ADNT (French *et al.* 1999; Hannink *et al.* 2001). Arabidopsis expressing *nfs*A had eight fold more uptake of TNT and twenty-times more nitroreductase activity (Kurumata *et al.* 2005). Of most interest is the transgenic poplar as these plants are well suited to phytoremediation, uptake and tolerance limits of TNT were greatly improved in these plants (van Dillewijn *et al.* 2008).

HADNTs and ADNTs are not dead-end products, they undergo further metabolism and investigation into this has identified a role for Phase II conjugating enzymes; UDP-glycosyltransferases (UGTs). Gandia-Herrero (2008) showed that overexpression of UGTs in Arabidopsis improves uptake and tolerance to TNT. Isolation of *O*- and *C*- glycosides of HADNTs confirmed the role of conjugation in TNT detoxification.

1.8 Glutathione Transferases

The glutathione transferases (GSTs) are an ancient superfamily found ubiquitously in aerobic organisms, from bacteria to humans (Frova 2003). They were first identified in the 1960s because of their importance for human metabolism and drug detoxification (Dixon *et al.* 2002b). The first plant GST was identified in 1970, due to its ability to provide herbicide resistance in maize (Dixon *et al.* 2002b). These Phase II enzymes catalyse the conjugation of glutathione (GSH) (γ-Glu-Cys-Gly) to an electrophilic, often hydrophobic and toxic substrate (R-X). This produces a non- or less-toxic peptide R-SG and H-X (Frova 2003). The reaction is thought to occur via a nucleophilic aromatic substitution reaction with a rate-determining step involving a Meisenheimer complex (delocalised carbanion) as a transient state (Van Der Aar *et al.* 1996; Patskovsky *et al.* 2006; Bowman *et al.* 2007). It is also emerging that plant GSTs also perform pivotal non-enzymatic functions involved in normal plant development and stress responses (Moons 2005).

Generally, GSTs are dimeric proteins, with two active ~26 kDa subunits, which may be identical (homodimers) or different (heterodimers). Heterodimers can only form with sub-units of the same class, as the linking mechanisms between dimers appear to be specific to each class (Dixon *et al.* 2002b). This results in a hydrophobic ~50 kDa protein with two independent catalytic sites. There are few exceptions, which are active in monomeric forms, which will be discussed later.

Though it is clear that prior to the introduction of xenobiotics, GSTs were maintained through evolution and therefore must play a crucial alternative role, the natural substrates of GSTs are not well characterised. They have been found to conjugate stress-induced metabolites, including 4-hydroxynonenal, a cytotoxic alkenal (Cummins *et al.* 1997). Plant specific GSTs can also conjugate phytotoxins produced by other plants, pathogens or herbivores, for example wheat and maize can detoxify isothiocyanates, produced by *Brassica* species (Cummins *et al.* 1997; Dixon *et al.* 1998; Frova 2003).

1.8.1 Classification

The abundance and variety of GSTs across the kingdoms has led to the requirement of a standardised classification system. Mammalian GSTs were the first to be investigated in detail and eight classes have been devised: Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta and Zeta (Dixon *et al.* 1998; Frova 2003). Plants GSTs were first divided into three groups based on sequence identity, all of which were thought to be closely linked to the mammalian Theta class (Droog *et al.* 1995). Soon after, the appreciation of the divergence within this class led to the creation of separate plant classes (Edwards *et al.* 2000). Plants GSTs have now been divided into eight GST-like classes; Phi and Tau, Theta and Zeta, Lambda, dehydroascorbate reductase (DHAR) tetrachlorohydroquinone dehalogenase- like (TCHQD) and membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Dixon *et al.* 2010).

1.8.2 Evolution of GSTs

Theta class GSTs are present in all aerobic bacteria and eukaryotes (Figure 1.6). It is therefore believed that they were present before eukaryotes and prokaryotes diverged and all other classes have originated from these. It is thought that GSTs arose originally in response to oxidative stress, from a thioredoxin-like ancestor, as they share structure and sequence similarities with these, and other protein families involved in the stress response (Armstrong 1998; Sheehan *et al.* 2001). The high number of classes, in addition to the high diversity within and among them, is believed to have originated though multiple gene duplications followed by exon shuffling of an ancestral GST (Figure 1.6) (Marrs 1996; Sheehan *et al.* 2001). Theta and Zeta GSTs are widely present in all eukaryotes, but there are few representative members in each organism, suggesting little duplication or loss of duplicated copies (Frova 2003). The plant specific GSTs, Phi and Tau on the other hand are well represented in all plant species, to varying degrees, suggesting extensive duplication and divergence (Figure 1.6) (Dixon *et al.* 2002a). The clustering of genes demonstrated later is common for these classes and indicates recent gene multiplication events (Frova 2003).

The differences of divergence within GST classes likely relates to varied selective pressures on the genes. Theta and Zeta classes have important roles in cell metabolism, so divergence is limited by the need to maintain cellular function. Phi and Tau respond to xenobiotic toxins, removing the threat by conjugating them to GSH. This rapidly changing environment allows for divergence presenting strong selective pressure to quickly adapt resistance to a wide range of compounds (Frova 2003).

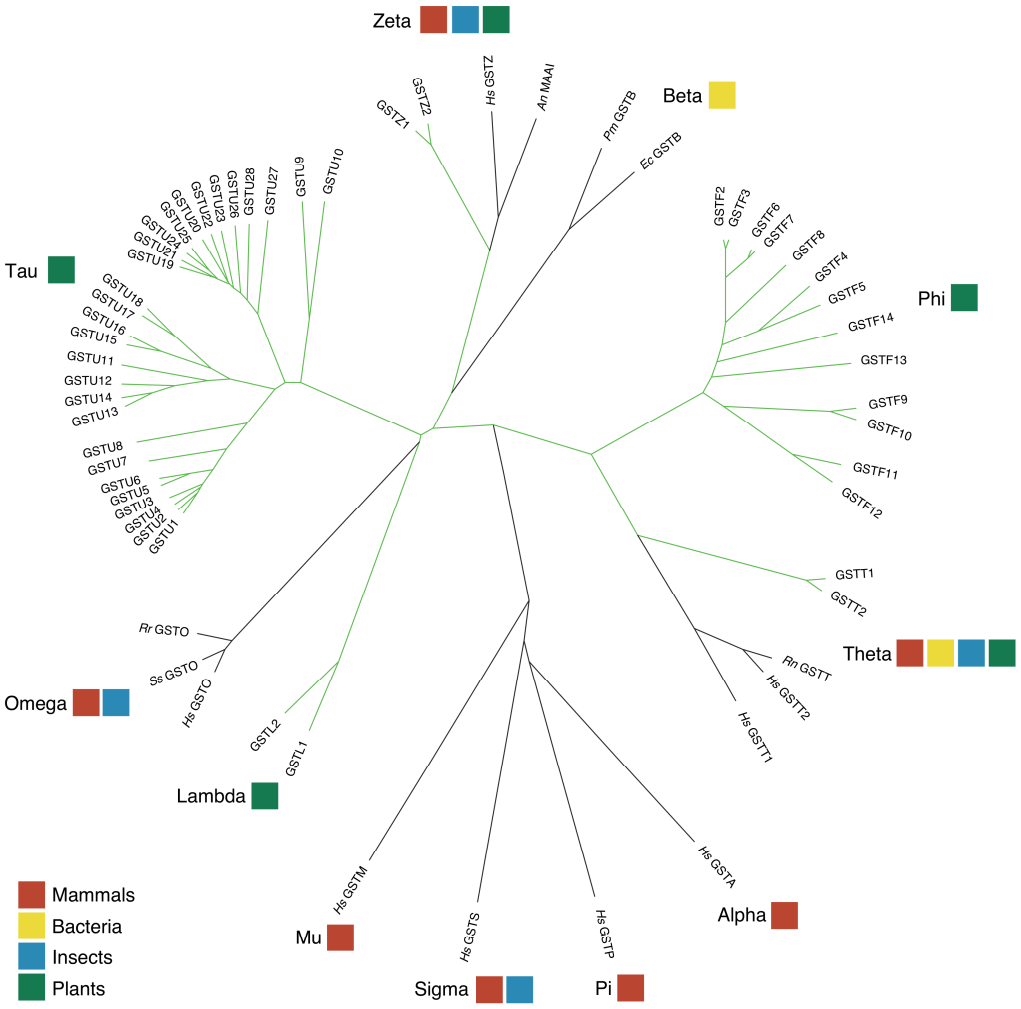


Figure 1.6: Phylogenetic tree of GSTs, illustrating the diversity of the different classes of GSTs and their evolutionary relationships. GSTs from Arabidopsis are shown in green. GSTs from other organisms are identified by black branches. The coloured dots indicate which GST classes can be found in which organisms Estimated evolutionary distances correspond to branch length. Figure adapted from Dixon *et al*. 2002.

1.8.3 Structural Features

Sequence identity within classes averages at >40 %, but can be as much as 98 % or as little as 17 % (Soranzo *et al.* 2004). Between plant classes, mean sequence identities are <20 % (Frova 2006), despite this, structures of the proteins show significant levels of conservation. The crystal structures of more than ten GSTs from the main mammal, plant and bacterial GST classes have been resolved, these all display dimeric structures with a two-fold symmetry and share a common tertiary organisation. Structural conservation is highest in the glutathione-binding site (G-site), which is adjacent to the hydrophobic-substrate binding site (H-site) (Dixon *et al.* 2002b). The G-site only serves to bind to GSH and closely related homologues, hence is conserved. The H-site on the other hand has broad specificity, binding to a diverse range of substrates and hence in these regions, sequence and structure identity between GSTs is low (Marrs 1996). The G- and H- sites form the two active components of the catalytic site, present in each sub-unit.

Figure 1.7 shows that each sub-unit is composed of two domains, amino- and carboxy-terminal, in green and blue respectively. The amino-terminal domain includes the G-site (yellow shading), with both alpha-helices and beta sheets, constituting one-third of the protein and is the most conserved region. The carboxy-terminal domain contains the H-site (blue shading) has only alpha-helices and is more variable in sequence and structure. Between the two domains is a 5-10 residue linker region (Figure 1.7, shown in red) (Dixon *et al.* 2002b).

The high conservation of the amino acids comprising the G-site indicates their importance for correct binding and orientation of glutathione. Within the G-site Glu66 and Ser67 form stabilising hydrogen-(H) bonds with the γ-glutamyl moiety of glutathione (Thom *et al.* 2002; Zeng *et al.* 2005). Two H-bonds are also formed between the cysteinyl group of GSH and the G-site, with Pro57 and Ile56; mutagenesis studies have shown Pro57 to be crucial for recognition and binding of GSH, while Ile56 is essential for protein folding. Lys40 also forms an H-bond with the glycyl of GSH. The thiolate anion of glutathione is stabilised through H-bonding to Ser13, essential for catalytic activity (Labrou *et al.* 2001).

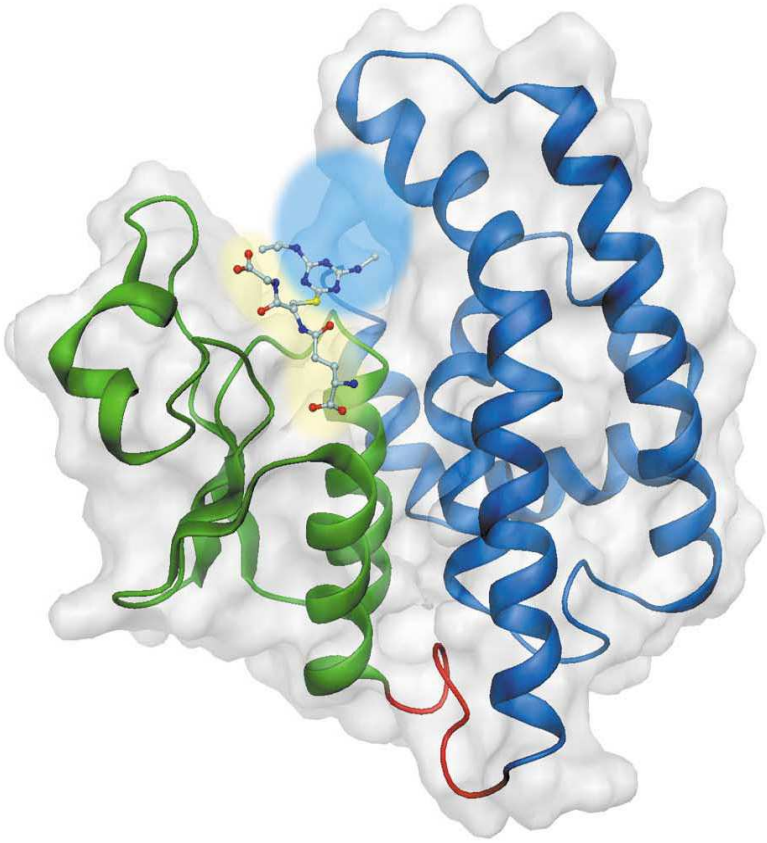


Figure 1.7: GST structure and substrate binding. GST monomer, in green is the amino-terminal, which contains the G-site, where glutathione binds (yellow). The carboxy-terminal is in blue and blue highlighting shows the H-site where the hydrophobic substrate binds. The amino- terminal is structurally conserved whilst the carboxy- terminal is diverse to allow the enzymes to conjugate a wide variety of molecules. The linker region between the two domains is in red. Figure adapted from Dixon, D.P. et al. 2002b.

GST catalysis relies upon the formation of the reaction thiolate anion of GSH (GS^-) which has a lower pKa than GSH, similar to physiological pH. Formation of the GS^- requires H-bonding with an adjacent hydroxyl group. For mammalian GSTs this is with tyrosine and for plants is a serine (Zeng *et al.* 2005; Bowman *et al.* 2007). The presence of cysteine residues in this position of Lambda GSTs and DHARs prevents GS^- stabilisation but instead allows the formation of mixed disulphides with GSH, accounting for their involvement in redox reactions but lack of conjugating activity (Sandermann 2004).

Heterodimeric GSTs have only been found with sub-units from the same class. This is likely due to specificities of the interface between the two sub-units; many of the strictly conserved residues lie on the dimer interface (Dixon *et al.* 2002b).

Two main types of interface have been recognised; ball and socket-type as seen with Alpha, Mu, Pi and Phi and hydrophilic-type for Theta, Sigma, Beta and Tau (Armstrong 1997). Each sub-unit is catalytically independent; therefore each could act as a monomer. It is not fully understood what is the significance of dimerisation, or in fact what functional roles heterodimers are. Erhardt and Dirr (1995) showed that the tertiary structure of a porcine Pi class GST was more stable as a dimer. This may be true for all GSTs but it is not yet known.

1.8.4 GST Function

The main function of GSTs is to inactivate toxic hydrophobic compounds which readily enter the cell and need to be eliminated. A schematic of the GST detoxification pathway is shown in Figure 1.8. Substrates of GSTs may first need to be functionalised by Phase I enzymes, including cytochrome P450s. GST catalyse the nucleophilic substitution or less often, addition, reaction of the substrate to the sulfyl group of glutathione. This reduces hydrophobicity and 'tags' the compound for transport. ATP-dependent pumps on the vacuolar membrane recognise and carry conjugates into the vacuole, The glutathione pump, or GS-X pump of plants is biochemically identical to that of mammals. It is comprised of three main units, a G-domain for glutathione recognition, a C-domain which binds the electrophilic moiety and a P-domain which is the site of phosphorylation (Landa *et al.* 2010). Within Arabidopsis these pumps are AtMRP1 and 2 (Lu *et al.* 1997; Lu *et al.* 1998). Once inside the vacuole, conjugates are targeted by transpeptidases which sequentially remove glutamate (GGT3) and glycine from the glutathione moiety, producing a cysteine-conjugate (Martin *et al.* 2007; Ohkama-Ohtsu *et al.* 2007) which is resistant to reverse-transport out of the vacuole. Further activity may involve a malonyl transferase which catalyses the formation of a malonyl-cysteine conjugate, the most abundant end-product in plants. It has been proposed that the next steps of detoxification involve sequestration or release. Conjugates have been found to be associated with pectin, lignin and hemicellulose in the cell wall or roots, cell shedding may therefore play a role in eventual the extracellular release of these xenobiotics (Sandermann 1994; Marrs 1996; Coleman *et al.* 1997).

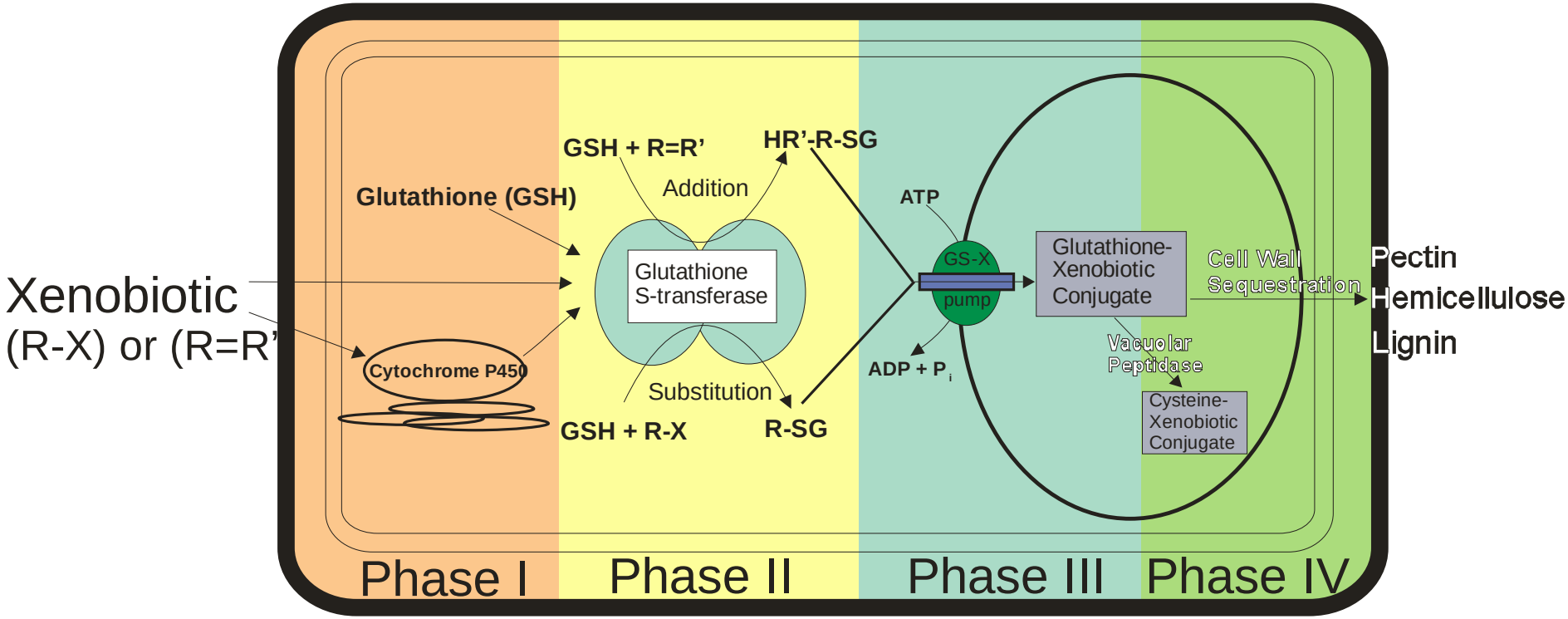


Figure 1.8: A detoxification process which involves glutathione transferases (GSTs). Xenobiotics enter the cell as they are lipophilic and in Phase I, the toxic compound undergoes activation, providing functional groups, which are required for Phase II. GSTs then conjugate the activated xenobiotic to glutathione (GSH), the product is then transported into the vacuole (or apoplast) through ATP-dependent membrane transporters. In the vacuole the conjugates can be degraded into Cysteine-conjugates (by enzymatic removal of Glutamate and Glycine) and may then be converted to malonylcysteine derivatives which are resistant to further metabolism or transport (Coleman *et al.* 1997; Bartholomew *et al.* 2002; Ohkama-Ohtsu *et al.* 2007). Conjugates may also be sequestered into the cell wall, bound to pectin, lignin or hemicelluloses (Marrs 1996; Coleman *et al.* 1997). Figure adapted from Yoon *et al.* 2006.

1.9 Aims

This project aims to expand current knowledge of the detoxification mechanisms of TNT employed by plants. Specifically, to elucidate the role of the detoxification enzymes; GSTs. GSTs have been selected for investigation as a microarray performed with TNT treated Arabidopsis tissue showed upregulation of GSTs, however it was unknown whether plants upregulated GSTs for conjugation of TNT. This project will use two approaches to tackle this question.

1.9.1 *In vitro* Studies

To understand if GSTs exhibit conjugation activity with TNT they will be recombinantly expressed in *E. coli*, purified and assayed with TNT. Further investigation of activity will reveal the identity of the product and mechanism of the reaction. This understanding will give an insight into a putative novel transformation reaction for TNT.

1.9.2 *In vivo* Characterisation

Plants are complex systems and while an *in vitro* reaction may occur, within the plant complex processes can be prohibitive. To determine whether GSTs play a role in detoxification *in planta*, the GSTs which show activity towards TNT will be assessed. To determine the TNT phytoremediation potential for GSTs homozygous *35SGST* Arabidopsis plants will be produced and studies will be performed comparing them to WT lines. This will include morphological, uptake and metabolite analysis of the plants.

Chapter 2: General Materials and Methods

2.1 Consumables and Reagents

Consumables and reagents used for this work were obtained from the following suppliers unless otherwise stated: Bioline Ltd (London, UK), Fisher Scientific UK Ltd. (Loughborough, UK), Invitrogen (Paisley, UK), Melford Laboratories Ltd. (Ipswich, UK), New England Biolabs (NEB) (Herts), Novagen (Merck Chemicals, Nottingham, UK), Oxoid Ltd. (Hampshire, UK), Promega (Southampton, UK), Qiagen (West Sussex, UK), Scotts (Ipswich, UK), Sigma-Aldrich Co. (Poole, UK), Starlab Ltd. (Milton Keynes, UK), Sterilin (Essex, UK), Stratagene (Agilent Technologies, Berks, UK) and VWR (Leicester, UK).

Primers were synthesised by Sigma-Genosys Ltd. (Haverhill, UK). DNA and protein gel markers were supplied by NEB UK and Promega, DNA Polymerases were purchased from NEB UK and Invitrogen UK, DNA restriction endonuclesases were bought from NEB UK and Promega and TNT was kindly donated by the Defence Science and Technology Laboratory (DSTL, Fort Halstead, UK). Water was purified with Elga Purelab Ultra water polisher (Elga Labwater, High Wycombe, UK).

2.2 Plasmids, Bacteria and Growth Conditions

2.2.1 Plasmids

The plasmids used for DNA manipulation and enzyme expression are shown in Table 2.1.

Table 2.1: Plasmids used for DNA cloning and enzyme expression

Plasmid	Selectable resistance	Antibiotic concentration μg/mL	Source
pET-YSBLIC3C	Kanamycin	30	Bonsor et al. 2006
pCR®2.1 TOPO	Kanamycin	30	Invitrogen (Paisley, UK)
pART7	Carbenicillin	100	Gleave 1992
pART27	Spectinomycin	100	Gleave 1992

2.2.2 Bacterial Methods

The bacterial strains used in this work are described in Table 2.2.

Table 2.2: Description of the bacteria used for cloning and expression of GSTs

Bacteria	Strain	Antibiotic	Purpose	Source
Escherichia coli	DH5α	None	Cloning, preparation of DNA for sequencing	Invitrogen (Paisley, UK)
Escherichia coli	BL21 (DE3)	None	General purpose expression host	Novagen, UK
Agrobacteria tumefaciens	GV3101	Gentamycin (50 μg/mL)	Transformation of Arabidopsis	Bruce Group Stocks

2.2.2.1 Preparation of Chemically Competent *Escherichia coli*

Five millilitre overnight cultures of *E. coli* were used to inoculate 100 mL Luria-Bertani media (LB) comprising 10 g/L tryptone, 10 g/L NaCl and 5 g/L yeast extract. Cultures were grown to an OD_{600} of 0.4-0.6 and centrifuged for 5 min at 5000 x g in a Swinging Bucket Rotor Centrifuge (Jouan CR312, SelectScience Ltd. Bath, UK). Pellets were resuspended in 10 mL of ice cold, sterile 80 mM $MgCl_2$/20 mM $CaCl_2$ solution and left on ice for 30 min. Cells were then centrifuged for 5 min at 5000 x *g* and resuspended in 1 mL ice cold, sterile 100 mM $CaCl_2$. Ten per cent v/v sterile glycerol (final concentration) was added and the cells were left on ice for a further 30 min. Fifty microlitre aliquots were snap-frozen in liquid nitrogen and stored at -80 ºC.

2.2.2.2 Transformation of Chemically Competent *E. coli*

Aliquots of 50 µL cells were defrosted at 37 ℃, 1 µL plasmid DNA was added and flicked to mix. The mixture was left on ice for 30 min, heat shocked for 90 s at 42 ℃ and returned to ice for a further 2 min. Five hundred microlitres of sterile LB was added and for recovery the cells were incubated at 37 ℃, 250 rpm for 1 h. The transformed cells were plated onto LB agar (LB with 15 g/L agar; LA) with appropriate antibiotics for selection (Table 2.1). Plates were incubated overnight at 37 ℃ or until colonies were visible.

2.2.2.3 Transformation of Chemically Competent *Agrobacterium tumefaciens*

Competent *A. tumefaciens* were provided by Dr E. Rylott. Fifty microlitre aliquots were defrosted at 37 ℃, 1 µL plasmid DNA was added and tubes were heat-shocked for 5 min at 37 ℃. One millilitre of LB was added to the mixture and incubated at 28 ℃ for 4 h at 100 rpm. Tubes were centrifuged at 4,000 rpm in a micro-centrifuge and supernatant was discarded. The cell pellet was resuspended in 100 µL LB and spread onto LA plates containing antibiotics selecting for both *A. tumefaciens* and the transformed plasmid (Table 2.1). Plates were incubated at 28 ℃ for 2-3 days.

2.2.3 Growth Conditions

Conditions of bacterial growth for protein expression are described in section 2.4.1.

2.2.3.1 Growth in Liquid Media

E. coli cultures were grown in sterile LB and appropriate antibiotic concentrations for plasmid selection. *A. tumefaciens* cultures were incubated in LB at 28 ℃ with 180 rpm shaking with required antibiotics (Table 2.1)

2.2.3.2 Growth on Solid Media

For the isolation of individual colonies, bacteria were grown on solid media (LA): LB containing 15 g/L agar. This was amended with the appropriate antibiotics for plasmid selection when required. *E. coli* colonies were grown overnight at 37 ℃ while *A. tumefaciens* colonies were grown at 28 ℃ for 2 to 3 days.

2.3 DNA Manipulation

2.3.1 Agarose Gel Electrophoresis

DNA fragments were separated by size using electrophoresis through an agarose gel. Gels were prepared with 1% agarose and 150 *μ*g/L ethidium bromide in 1x Tris-acetate-EDTA buffer (40 mM Tris-HCl, 18 mM glacial acetic acid and 1mM ethylenediaminetetraacetic acid (EDTA)). DNA was diluted 5:1 in loading dye (0.15 % w/v bromophenol blue, 0.5% w/v sodium dodecyl sulphate (SDS), 0.15 mM EDTA and 60 % w/v glycerol. Promega 1 kb DNA ladder was used as a molecular weight marker, with 0.5 *μ*g per well. DNA separation was achieved with a current of 80-120 V and UV light was used to visualise ethidium bromide stained DNA.

2.3.2 DNA Purification

2.3.2.1 Plasmid Purification

E. coli cultures were grown overnight at 37℃, 250 rpm in 5 mL LB containing the appropriate antibiotics. The cultures were harvested by centrifugation for 10 min

at 5000 x g in a Swinging Bucket Rotor Centrifuge (Jouan CR312, SelectScience Ltd. Bath, UK). The plasmids were extracted and purified using the QIAprep Spin Miniprep kit (Qiagen, Crawley, UK) as instructed by the manufacturer. DNA concentration of the plasmid preparation was determined using the Nanodrop (Thermo Fisher Scientific, USA).

2.3.2.2 DNA Fragment Purification

The Wizard SV Gel and PCR Clean-Up System (Promega) were used, as directed by the manufacturer's instructions, to purify DNA fragments resulting from PCR and restriction digest. Fragments produced by restriction digests were run on agarose gels, bands of the correct size were excised before using the Clean-Up System. PCR products did not require gel separation.

2.3.3 Sequencing

DNA sequencing was performed by the Genomics Lab, Technology Facility, University of York (York, UK). Sequencing from pCR2.1 TOPO used forward and reverse M13 primers (Table 2.3). For pETYSBLIC3C sequencing T7 and T7term primers were used (Table 2.3). Sequence analysis was performed with software packages including; Sequence Scanner (Applied Biosystems, USA), Clustalx 2.0.9 and BioEdit7 (Hall 1999; Larkin *et al.* 2007).

Table 2.3: Sequencing primers for Arabidopsis GSTs

Primer name	Sequence
M13 forward	GTA AAA CGA CGG CCA GTG
M13 reverse	GGA AAC AGC TAT GAC CAT G
T7	TTA TAC GAC TCA CTA TAG GG
T7term	TAT GCT AGT TAT TGC TCA GCG GT

2.3.4 PCR

Primers were synthesised by Sigma-Genosys and diluted in sterile dH_2O to make a master stock solution of 100 mM. This stock solution was further diluted to 20 mM to create a working stock. PCR amplifications were performed in 25 and 50 μL volumes. PCR reactions were performed in a Thermo Electron Corporation Px2 Thermal Cycler (Basingstoke, UK).

2.3.4.1 PCR for Cloning into *E. coli*

For recombinant expression of glutathione transferases (GSTs) in *E. coli*, PCR was performed using KOD Hot Start proofreading DNA polymerase (Merck, UK). Fifty ng/μL cDNA was used for each 50 μL reaction containing 5 μL 10x KOD buffer, 200 μM dNTPs, 0.4 μM forward and reverse LIC primers, 1 mM $MgSO_4$ and 1 unit of KOD Hot Start polymerase. Initial denaturing conditions were 95 ℃ for 2 min, followed by 30 cycles of 95 ℃ for 30 s, 45℃ for 30 sand 72 ℃ for 20 s. The final extension conditions were 72 ℃ for 3 min followed by a hold temperature of 4 ℃.

2.3.4.2 PCR for Cloning into Arabidopsis

Phusion High-Fidelity DNA polymerase (NEB) was used for amplifying *gst* genes for work in Arabidopsis. All *gst* genes in this study were amplified from Arabidopsis DNA isolated from plants exposed to TNT (see 2.5.4.2 and 2.5.4.3). 0.5 μL cDNA was used for each 25 μL reaction containing 5 μL of 5x HF buffer, 200 μM dNTPs, 0.4 μM forward and reverse primers and 0.5 units of Phusion High-Fidelity DNA polymerase. PCR followed a programme of; 98 ℃ for 30 s, 30 cycles of 98 ℃ for 10 s, 55 or 60 ℃ for 30 s and 72 ℃ for 30 s, followed by 72 ℃ for 5 min and samples were then held at 4 ℃ unt il use.

2.3.4.3 Diagnostic PCR

PCR to identify the presence of a gene in transformed bacteria was performed on single bacterial colonies. This was performed with *Taq* DNA polymerase in 25 μL reactions with 1x Thermo Pol buffer, 200 μM dNTPs, 0.4 μM forward and reverse primers, 0.5 units *Taq*. Colonies were dipped into this reaction then spread onto a stock plate. The PCR reactions were heated to 94 ℃ for 3 min, 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 1 min, a final extension of 10 min at 72 ℃ was followed by a 4 ℃ hold temperature. Analysi s was performed by agarose gel electrophoresis.

2.3.5 Restriction Endonuclease Digestion

Restriction endonuclease digestions contained 2 μg plasmid DNA or 200 ng PCR product in 20 or 10 μL reactions respectively, other components included 2 μg/mL bovine serum albumin (BSA), 10 or 5 units of the desired restriction endonuclease and corresponding buffer as specified by NEB.

2.4 Protein Expression and Purification

2.4.1 Protein Expression

Autoinduction (AI) protein expression media comprised; ZY broth 10 g/L tryptone and 5 g/L yeast extract with the addition of the solutions detailed in Table 2.4. The components of the AI stock solutions are shown in Table 2.5. For expression *E. coli* BL21(DE3) transformed with plasmids containing the gene of interest were grown on LBA overnight at 37 ℃ to produce individu al colonies. Starter cultures were grown from the colonies in LB and cells were grown at 37 ℃, 250 rpm. Per 1 L autoinduction expression culture, 1 mL starter culture was added and incubated at 30 ℃ with 120 rpm shaking for 12 h fo llowed by 48 h at 20 ℃ with 180 rpm shaking. Cells were pelleted by centrifugation at 5,000 x *g* for 10 min.

Table 2.4: Volumes of autoinduction solutions per 1 L culture.

Component	Volume (mL/L)
$MgSO_4$ (1 M)	1
1000 x metals	1
50 x 5052 solution	20
20 x NPS solution	50
ZY solution	928

Table 2.5: Quantities of ingredients for stock autoinduction solutions

AI solution	Ingredients	Quantity
1000 x metals (100 mL)	0.1 M $FeCl_3.6H_20$ (in 0.1 M HCl)	50 mL
	1.0 M $CaCl_2$	2 mL
	1.0 M $MnCl_2.4H_20$	1 mL
	1.0 M $ZnSO_4.7H_20$	1 mL
	0.2 M $CoCl_2.6H_20$	1 mL
	0.1 M $CuCl_2.2H_20$	2 mL
	0.2 M $NiCl_2.6H_20$	1 mL
	0.1 M $Na_2MoO_4.5H_2O$	2 mL
	0.1 M $Na_2SeO_3.5H_2O$	2 mL
	0.1 M H_3BO_3	2 mL
50 x 5052 solution 100 mL	Glycerol	25.0 g
	Glucose	2.5 g
	α-Lactose	10.0 g
20 x NPS solution 100 mL	Na_2SO_4	3.6 g
	NH_4Cl	13.4 g
	KH_2PO_4	17.0 g
	Na_2HPO_4	17.7 g
ZY solution 1L	Tryptone	10.0 g
	Yeast extract	5.0 g

2.4.2 Cell Lysis by Sonication

Cell pellets were resuspended to 0.5 g/ mL in PBS. Sonication was performed on ice Sonication was performed with an S-4000 Sonicator (Misonix) at 70 % amplitude for 4 min duration, with cycles of 3 s interrupted with 7 s cooling at 0 ℃. Cell lysates were centrifuged at 17,500 x *g*, 4 ℃ for 30 min to remove the cell debris. Supernatants were clarified by passage through 0.45 *μ*M syringe filters before being applied to the purification media.

2.4.3 Protein Purification

GSTs were purified with either HIS-Select Nickel Affinity Gel (Sigma-Aldrich USA) and Glutathione (GSH) Sepharose 4B (GE Healthcare, Little Chalfont, UK). For His-purification the wash buffer was 50 mM sodium phosphate pH 8, 0.3 M NaCl and elution was performed with 50 mM sodium phosphate pH 8, 0.3 M NaCl with

250 mM imidazole. Glutathione-purification used phosphate buffered saline (PBS) pH 7.4 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) for wash steps and 50 mM Tris-HCL, 10 mM reduced glutathione, pH 8.0 for elution. Centrifugation of His beads was performed at 5000 *x g*, while GSH beads were spun at 500 *x g*. For both methods 0.5 mL affinity resin was used per 1 L initial culture volume. Resin was washed three times in ten bed volumes of wash buffer before the clarified cell supernatant was added. After 20- 30 min incubation the supernatant was removed and the resin was washed three times in ten bed volumes of wash buffer. One bed volume of elution buffer was added and incubated for 10 min at room temperature, the supernatant was collected. The elution step was repeated for a total of three elutions.

2.4.4 Protein Visualisation by SDS-PAGE

A Mini-Protean system was used to separate proteins. Samples were solubilised in a 4 X SDS-PAGE loading buffer containing 2 mL water, 1.6 mL 10% SDS, 1 mL 0.5 M Tris-HCl, 1 mL glycerol, 0.4 mL β-mercaptoethanol and 20 mg bromophenol blue. A denaturing step was performed at 100 ℃ for 5 min. Gels comprised a 12 % w/v acrylamide separating gel and a 4 % acrylamide stacking gel. Samples were run through the stacking gel at 100 V and the separating gel at 200 V. Fifteen microlitres broad range protein marker were run on each gel for molecular weight determination.

2.5 Plant Methods

2.5.1 Seed Sterilisation

Seeds were dry sterilised by chlorine gas in an airtight container containing aliquots of seed and a beaker with 3 mL concentrated hydrochloric acid and 100 mL Chloros bleach. After 3 h the seeds were aired in a flow hood for 10 min to remove chlorine gas.

2.5.2 Stratification

Arabidopsis thaliana (ecotype Columbia 0) seeds to be grown in soil or on agar plates (see Section 2.5.3.2) were imbibed in the dark for 3 nights at 4 ℃ in sterile water. Seeds for soil growth were not sterilised unless germinated on plates.

For liquid culture; sterile Arabidopsis seeds were imbibed in the dark for 3 nights at 4 ℃ on ½ MS agar plates (0.215 % Murashige and Skoog Basal Salt mixture (MS), Sigma) with 20 mM sucrose (½ MSS).

2.5.3 Growth Conditions

2.5.3.1 Plant Growth in Soil

To bulk up seed stocks, gain tissue for nucleic acid extraction and for generation turnover, plants were grown in soil under non-sterile conditions in a greenhouse. For growth of Arabidopsis prior to floral dipping imbibed seeds were distributed evenly on top 3 inch pots, filled with F2 compost and covered in muslin. The plants were then propagated in the green house, and weeded to 12 plants per pot. The plants were left to flower, when floral dipping was performed (see Section 2.5.4.1).

2.5.3.2 Plant Growth on Solid Media

For selection of transformed lines imbibed seed were scattered onto ½ MSS agar plates supplemented with 30 μg/mL kanamycin. Resistant seedlings were transplanted to soil for seed bulking.

2.5.3.3 Hydroponic Conditions

For Real Time-PCR (RT-PCR) experiments ten sterile imbibed seeds were transferred to sterile 500 mL flasks each with 100 mL ½ MSS. After 13 days under constant light conditions (~15 μmol.m^{-2}.s^{-1}) and gentle shaking, 60 μM TNT or the equivalent volume of dimethyl formamide (DMF) was added (negative control). The cultures were incubated under the same conditions for a further 6 h when the media was removed and plants washed with pure water. Plants were then frozen at -80 ℃ until RNA extraction procedure.

For liquid culture assays to determine TNT uptake 8 imbibed seeds were transferred to sterile 100 mL flasks with 10 mL ml ½ MS and sucrose. After 13 days under constant light conditions (~15 μmol.m^{-2}.s^{-1}) at 120 rpm shaking, media was replaced with ½ MS with 200 μM TNT or ½ MS with 200 μL dimethyl sulfoxide (DMSO).

2.5.4 Manipulation of Arabidopsis

2.5.4.1 Plant Transformation

Agrobacterium tumefaciens GV3101 were transformed with pART27-GST constructs, grown on LA plates with gentamycin and spectinomycin, at 28 ℃ over 3 nights, transferred to 10 mL LB with antibiotics and grown overnight at 28 ℃, 130 rpm. Colony PCR was then performed to confirm presence of GST insert prior to inoculation of 1 L LA with antibiotics, for each construct. These cultures were centrifuged at 4,000 rpm in a Sorvall centrifuge using SLR-6000 rotor. Pellets were resuspended in 5 % sucrose and 0.05 % Silwet solution. The flowers of 12 pots per construct were dipped into this *A. tumefaciens* solution and shaken for 30 s. Plants were returned to the greenhouse and transformants were selected on kanamycin plates (Section 2.5.3.2).

2.5.4.2 RNA Extraction

Plant tissue from Arabidopsis grown on soil or in liquid culture was harvested. RNA was extracted using RNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol. Concentration of RNA was then measured on the Nanodrop (NanoDrop Technologies, USA) and samples were frozen at -80 ℃ until use.

2.5.4.3 Reverse Transcription from RNA

All steps used Invitrogen reagents, unless otherwise stated. RNAse-free water was added to 10 μg plant RNA, to make a volume of 24 μL. One microlitre oligo dT primer was added and the mixture was incubated for 2 min at 95 °C, then put on ice. Eight microlitres of 5x First strand buffer, 2 μL 2.5 mM dNTPs (Bioline), 2 μL of 0.1 M DTT, 2 μL RNAse out and 2 μL Superscript II were added to the 24 μL RNA dilution and incubated for 2 h at 42 ℃. All samples were stored at -80 ℃.

Chapter 3: The Cloning, Expression, Purification and Characterisation of Glutathione Transferases from Arabidopsis

3.1 Background

3.1.1 Glutathione Conjugation

Glutathione (GSH) conjugation is a major detoxification mechanism in plants. The substitution or addition reaction joining an electrophilic and often hydrophobic substrate (R-X) to glutathione, a γ-Glu-Cys-Gly tripeptide, increases its solubility enabling subsequent compartmentation and sequestration (Figure 3.1). The reaction is catalysed by glutathione transferases (GSTs), an ancient superfamily of enzymes present in all animals, plants fungi and aerobic bacteria investigated so far (Frova 2003).

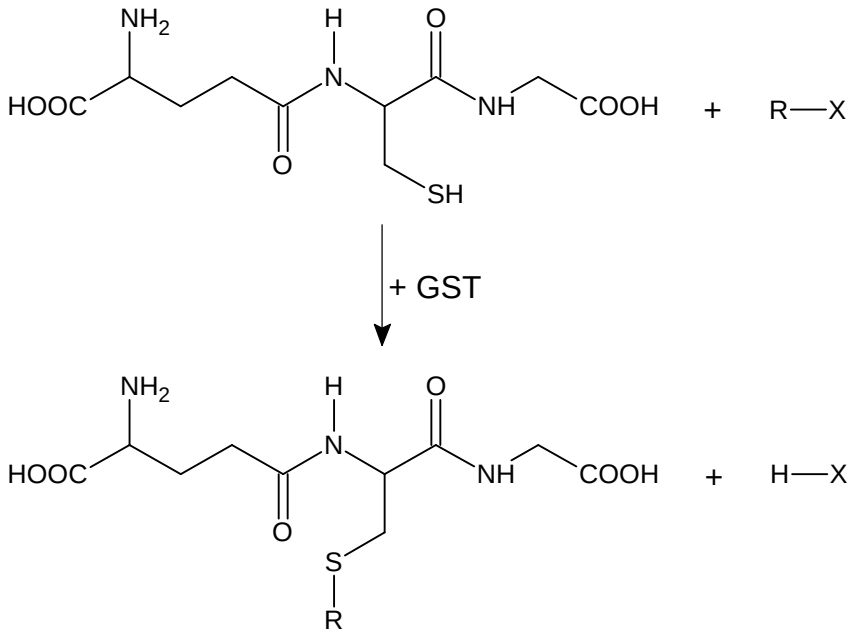


Figure 3.1: The nucleophilic substitution reaction between glutathione (GSH) and an electrophilic substrate, producing a hydrophilic conjugate with reduced toxicity. The reaction is catalysed by glutathione transferases (GSTs).

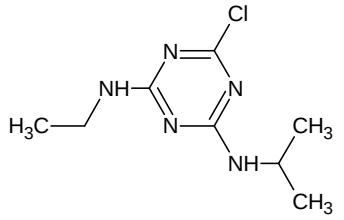
Glutathione conjugates of numerous xenobiotics in plants have been identified, most notably herbicides. GST activity with herbicides was first observed in the 1970s with the metabolism of atrazine by GSTs in maize (*Zm*GSTs) (Frear *et al.* 1970). Subsequently many other GSTs responsible for metabolism of herbicides

and other xenobiotics have been discovered in crops and weeds. The main classes of herbicides are shown in Figure 3.2. Domesticated crop species express much higher levels of GSTs than competing weed species, accounting for herbicide selectivity. In some crops GSTs can make up to 2 % total protein in cells (Scalla *et al.* 2002; Lederer *et al.* 2005).

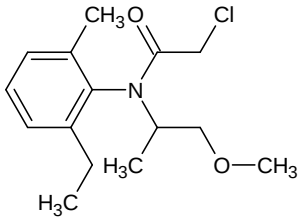
For the economic reasons associated with herbicide activities there has been much interest in plant GSTs especially those from *Zea mays* (maize, *Zm*) as well as other crop species, rice, soybean and wheat. Considerable amounts of research have also been performed with the model plant *Arabidopsis thaliana* (Arabidopsis, *At*), which has 58 GSTs (Dixon *et al.* 2010).

3.1.2 GST Classification

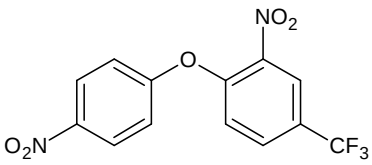
There are eight classes of GSTs or GST-like proteins in plants separated by sequence similarities and immunological cross-reactivity. These classes were named in line with the mammalian GST nomenclature, using Greek letters. The plant classes are Tau (GSTU), Phi (GSTF), theta (GSTT) and zeta (GSTZ). The more distantly related GST-like enzymes are called lambda (GSTL), dehydroascorbate reductase (DHAR), tetrachlorohydroquinone dehalogenase-like (TCHQD) and membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG). Most of these classes contain only 1 to 3 proteins each however of the 58 Arabidopsis GSTs; Phi and Tau contain the majority with 13 and 28 genes respectively (Figure 3.3) (Droog 1997; Edwards *et al.* 2000). These two classes are primarily responsible for herbicide detoxification: GSTFs are highly active against chloroacetanilide, chloro-*s*-triazine and thiocarbamate compounds; and GSTUs detoxify diphenyl ether and aryloxyphenoxypropionate herbicides (Figure 3.2) (Jepson *et al.* 1994; Thom *et al.* 2002; Frova 2003; Öztetik 2008).



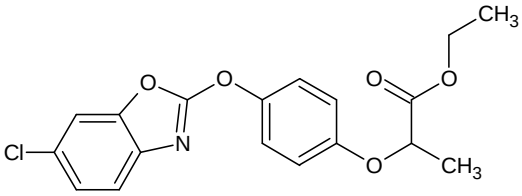
Atrazine - a chloro-*s*-triazine



Metolachlor - a chloroacetanilide



Fluorodifen - a diphenyl ether



Fenoxaprop ethyl - an aryloxyphenoxypropionate

Figure 3.2: Example herbicides the four main classes to which GSTs have conjugating activities.

GST nomenclature in plants uses an abbreviation of the first letter of genus and species as i.e. *Zm* for *Zea mays* or *At* for *Arabidopsis thaliana*, then GST, followed by the corresponding class letter (U, F, T, L, Z) and finally the GST gene number. In Arabidopsis these gene numbers roughly represent gene order on the chromosomes however for plants with unsequenced genomes; GSTs are numbered by order of identification. The most widely studied GST in Arabidopsis is identified as *At*GSTU19 and a homodimer of this GST is called AtGSTU19-19.

While there is at least one GST on each Arabidopsis chromosome (Figure 3.3), strikingly there is a high incidence of clustering; many of the GSTs are arranged as repeating units with the majority located on Chromosomes I and II. An example of clustering is seen on Ch II where GSTs U1-7 lie adjacent to one another, an indication that the high number of GSTs arose by a series of recent gene duplication events (Armstrong 1997; Edwards *et al.* 2000; McGonigle *et al.* 2000).

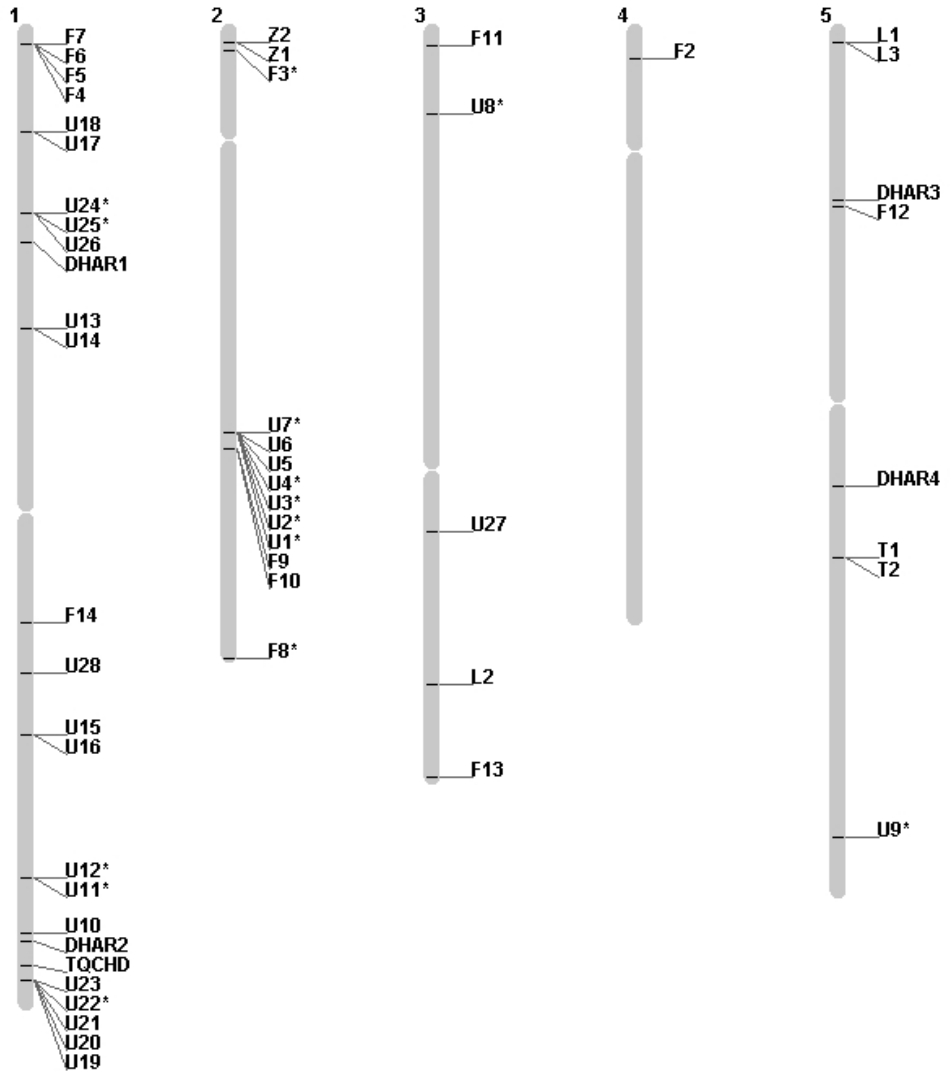


Figure 3.3: Chromosome map of Arabidopsis GSTs. All of the upregulated GSTs selected for study (*) are members of Tau class. Arabidopsis has 58 GSTs or GST-like proteins, 28 of which are Tau, making this by far the most dominant class, followed by the other plants specific class, Phi, with 13 members (Edwards *et al.* 2005). Figure from www.arabidopsis.org. GST class nomenclature: U= Tau, F= Phi, L= Lambda, Z= Zeta, T= Theta, D= DHAR (dehydroascorbate reductase), involved in ascorbate recycling (Dixon 2002a) and T= TCHQD (tetrachlorohydroquinone dehalogenase-like), a relative of the GSTs with unknown function.

3.1.3 Induction of GST Expression

EST analysis of Arabidopsis has indicated that 85 % of the GST genes are expressed (Dixon *et al.* 2002). Within the EST libraries it is evident that the GST classes are not expressed equally; maize contains 54 % Phi, 41 % Tau and 4 % Zeta GSTs while of the expressed GSTs in soya bean 92 % are Tau, 6 % are Phi and 2 % are Zeta (McGonigle *et al.* 2000). This does not correlate with the class abundance of these species. It has also been observed that some GSTs are tissue specific or are only expressed in response to specific stresses. Sari- Gorla *et al.* (1993) found that maize pollen contains a single GST, while the scutellum expresses five different GSTs. This tissue specific expression can be overridden by stress treatments, inducing GST expression more widely throughout the plant (Dixon *et al.* 2002).

At least 34 of the 58 Arabidopsis GSTs or GST-like proteins are expressed in the proteome, although specific expression levels were not accurately determined (Dixon *et al.* 2010). Many GSTs have only been identified by their high expression profiles following a wide variety of stress treatments. These include; heavy metals, temperature, dehydration, plant hormones, pathogen attack, glutathione and hydrogen peroxide (Marrs 1996; Droog 1997; Öztetik 2008; Sappl *et al.* 2009).

Chemicals called safeners or antidotes can be used to protect monocotyledonous crops from damage caused by herbicides which can be damaging to the grass crops. Pre-treatment with the non-toxic safeners differentially induces expression of detoxifying enzymes including GSTs in monocot crop species and to a lesser extent in dicot weeds (Hatzios 1983; DeRidder *et al.* 2002). The application of specific safeners therefore enhances tolerance of the crops to particular herbicides by accelerating the rate of their detoxification by GSH conjugation (Hatzios 1983; Kriton 1984; Dean *et al.* 1990; Cummins *et al.* 1997).

3.1.4 Non Glutathione -Conjugating Roles

Aside from the relatively well characterised herbicide-conjugating activities of GSTs, they play pivotal roles in plant development and stress response (Mueller *et al.* 2000). It is even likely that their conjugating ability is not their primary function, as there is a striking lack of evidence for glutathionylated endogenous compounds (Dixon 2010). Across the classes of plant GSTs a range of alternative functions have been proposed. The known catalytic roles include; GSH-dependent peroxidase reactions (GPOX), GSH-dependent isomerisation, GSH-dependent thioltransferase activity and dehydroascorbate reductase reactions by dehydroascorbate reductases (DHAR). The non-catalytic roles include ligandin or carrier protein functions which facilitate the distribution of phytohormones or anthrocyanins (Bartling *et al.* 1993; Marrs *et al.* 1995). Non-conjugating activities may be derived from their thioredoxin-like ancestry (Mueller *et al.* 2000; Sheehan *et al.* 2001).

3.1.5 Experimental Background

In our laboratory a microarray of cDNA from 14 day-old Arabidopsis seedlings treated with 60 μM TNT for 6 h to provide a library of genes which may play a role in the detoxification of TNT (Gandia-Herrero *et al.* 2008). Many genes were upregulated in response to TNT including a wide range of Phase I and II detoxification enzymes one of which was the Phase II GST family. This project is therefore interested in discovering the role GSTs may play in the detoxification of TNT. Figure 3.4 shows the GSTs which were upregulated two fold or more in the microarray. Arabidopsis has 13 Phi class GSTs, two of which, GSTF3 and GSTF8 are seen to be upregulated, 2.84 and 2.5 fold respectively. The Phi class is a plant specific class, like Tau and the two classes are the best studied as they exhibit broad ranging conjugation activity towards xenobiotics, primarily herbicides (Edwards *et al.* 2005). Twelve of a total 28 Arabidopsis Tau class GSTs are upregulated more than two fold in response to TNT treatment. The red line is drawn at eight fold upregulation and the seven GSTs on or above that line have been selected for further investigation (Figure 3.4).

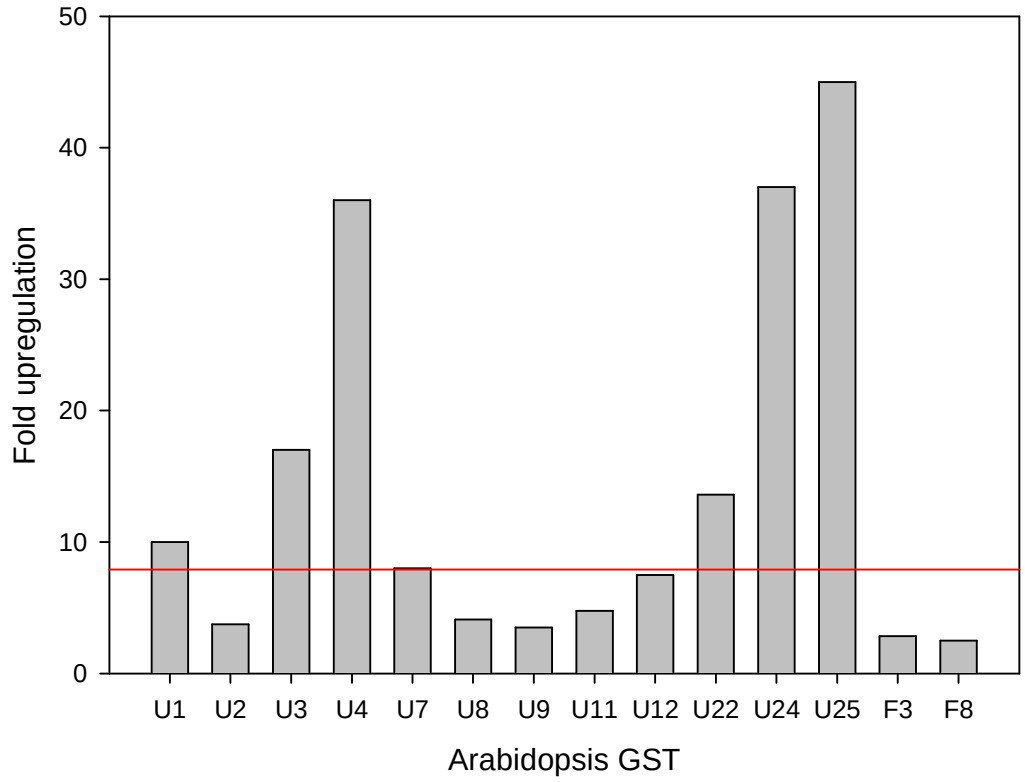


Figure 3.4: Microarray data showing Arabidopsis GSTs upregulated more than 2 fold following TNT treatment. All data has P values <0.05. Those on or above the line were selected for study. Data kindly provided by Dr Astrid Lorenz, 2007.

Of the upregulated GSTs, there is a good representation from the major clusters of the Tau genes, from U1-U7 all but U5 and U6 are upregulated, U8 and U9 are also clustered together as are U11 and U12, and U24 and U25 (Figure 3.3). The upregulation of closely related GSTs may be due to functional overlap of genes arising from a recent gene duplication or the fact that microarray targets for Arabidopsis genes have been shown to not differentiate between certain Tau GSTs including GSTU3 and U4 (Dixon *et al.* 2010).

Chlorodinitrobenzene (CDNB) is regularly used as a generic substrate and standard activity assay for the GST superfamily (Habig *et al.* 1974; Bowman *et al.* 2007; Dixon *et al.* 2010). It is a xenobiotic benzene derivative used mainly for the production of azo-dyes though it is also an intermediate product in the preparation of explosives including picric acid. Its ability to act as a broad substrate for GSTs was first described in 1974 (Habig *et al.* 1974). CDNB is not a universal substrate as GSTs exhibit a high variation in levels of conjugating

activity against this substrate, with many GSTs showing no activity at all (Dixon *et al.* 2009).

The nucleophilic aromatic substitution between GSH and CDNB reaction produces 1-(S-glutathionyl)-2,4-dinitrobenzene, where the chlorine of CDNB is removed and the sulphyl group of GSH binds to the aromatic ring instead (Figure 3.5). The reaction is proposed to begin with the ionisation of GSH to yield GS- which attacks the C1 of CDNB, conjugation then proceeds via π–complex intermediates and rate limiting Meisenheimer complex intermediates (Van Der Aar *et al.* 1996; Bowman *et al.* 2007). The non-enzymatic conjugation of CDNB and GSH is also likely to follow the same mechanism, though at a slower rate.

Many of the known substrates for GSTUs are nitrobenzene derivatives e.g. CDNB, dichloronitrobenzene (DCNB) or aromatic compounds with nitro- groups, for example the herbicide fluorodifen. These substrates have structural similarities to TNT, however in these cases no nitro group removal is observed and the enzyme prefers to substitute GSH elsewhere on the molecule (Pflugmacher *et al.* 2000). There is however some evidence of TNT conjugation with GSH; commercially available equine liver GST extract can catalyse the production of 2-*S*-glutathionyl-4,6-dinitrotoluene, a conjugate of TNT and GSH, a substitution reaction with nitro-group removal (Brentner *et al.* 2008).

Previous research has proposed that GSTs play a role in the detoxification of TNT by plants. Ekman et al. (2003) found GST transcripts to be upregulated following TNT treatment, although long term treatment (Mentewab *et al.* 2005) does not significantly upregulate GST mRNAs. It is possible that this difference arises due to GST upregulation induced as a generalised stress response resulting from TNT treatment rather than transformation activity of GSTs with TNT. However, the conjugation of TNT to GSH observed *in vitro* by equine liver GST does suggest that some detoxification may be performed by GSTs (Brentner *et al.* 2008).

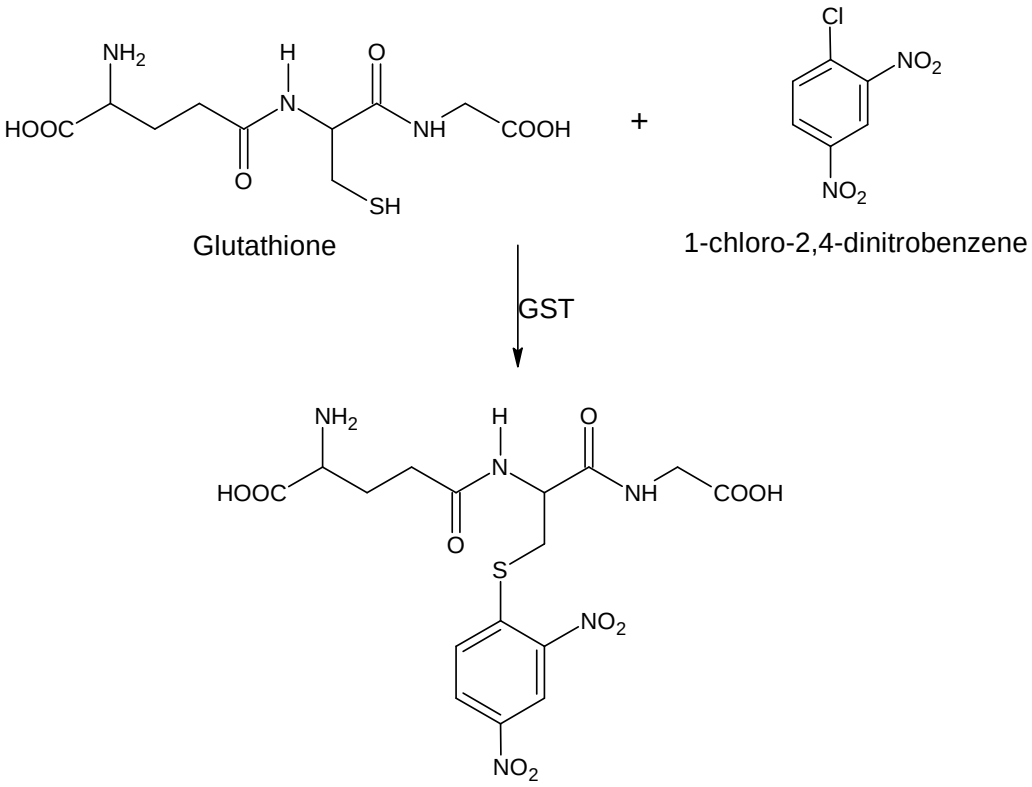


Figure 3.5: Conjugation of Glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) producing 1-(S-glutathionyl) 2,4-dinitrobenzene (GS-DNB). The reaction proceeds via *π*- and Meisenheimer complexes (not shown).

GSTs are active as dimers, forming homo-dimers or hetero-dimers with closely related GSTs, usually within the same class (Dixon *et al.* 2002b). This, coupled with the high similarities between related GSTs arising from recent gene duplication makes it hard to study individual enzymes from plant extracts. Specific enzymes are therefore preferentially expressed in recombinant *E. coli* for characterisation. Purification of many GSTFs and GSTUs can usually be achieved by affinity chromatography with glutathione agarose, due to their GSH binding sites (Edwards *et al.* 2005).

This chapter investigates whether the GSTs found previously to be upregulated by exposure to TNT exhibit any conjugative activity to this compound. The GSTs are cloned, expressed, purified and assayed with CDNB and TNT.

3.2 Methods

3.2.1 RNA Extraction, cDNA Production

WT Col0 *Arabidopsis thaliana* seeds were sterilised as described in Section 2.5.1 and imbibed on ½ MSSA plates for three nights in the dark at 4 °C. After 18 h in 80 μmol.m^{-2}.s^{-1} light, ten seedlings were transferred from the plate to 250 mL flasks containing 100 mL ½ MSS. Flasks were incubated at 20 °C for 13 days with 120 rpm shaking, under low light conditions (~15 μmol.m^{-2}.s^{-1}) to minimise stress. After 14 days half the flasks were dosed with 60 μM TNT diluted in the solvent N,N-dimethylformamide (DMF) or with the same volume of DMF only. After 6 hours plants were rinsed in dH_2O and snap frozen at -80 °C until RNA extraction which was performed with the Qiagen kit as detailed in Section 2.5.4.2. The cDNA was reverse transcribed from the RNA as described in Methods (2.5.4.3).

3.2.2 RT-PCR of GSTs from TNT treated Arabidopsis

Primers for each GST were designed based on published GST sequences using Primer Express v3.0 (Table 3.1) (Applied Biosystems). Once cDNA concentrations had been determined, primer efficiency tests were performed to ensure the designed primers were suitable. Real-time (RT) PCR reactions were performed in 96 well plates with 1, 10 or 100 ng cDNA. 5 μL DNA dilutions were added to 12.5 μL Power SYBR Green Mix (Applied Biosystems), 0.4 μM of forward and reverse primer and made up to 25 μL with nuclease free water. Plates were then centrifuged for 2 min at 5000 x *g* before being placed in a 7000 sequence detection system RT-PCR machine (Applied Biosystems). Cycle conditions were 2 min at 50 ℃, 10 min at 95 ℃, 40 cycles of 15 s at 95 ℃ and then 1 min at 60 ℃.

Table 3.1: Primers used for RT-PCR amplification of AtGSTUs

Primer name	Sequence
RTU1F	CGTGCCATACGAATACTTGGAA
RTU1R	TTCTTGTGAAGCGGGTTTAGC
RTU3F	ACCAAACATGGACAAACAATCCT
RTU3R	CGACAAATTTGGCCCAGAA
RTU4F	AAGCCCTTTTACTCGTAGAGTTGAGA
RTU4R	TTTGTAGACAAGAACCGGAACCTT
RTU7F	TCCGGTTCTTGTTCATAATGGTA
RTU7R	TCATCGACGAATTTAGACCAGAAT
RTU22F	TCGAAGCATCAGAGAAACTAGCTAAC
RTU22R	CCTCTTAGCCGAAGCCATCA
RTU24F	TCCCTCCGATCCTTACAAGAGA
RTU24R	TCGCCGTAACATTCACCTTTT
RTU25F	TGTCAAATTCGATTACAGAGAACAAG
RTU25R	GGTATTTTCTTATGAACCGGATTCA

3.2.3 Ligation Independent Cloning System

The ligation independent cloning (LIC) system was adopted for rapid, semi-automated cloning of the GSTs (Bonsor *et al.* 2006). Complementary long overhangs on the insert and plasmid allow for simple annealing prior to transformation into *Escherichia coli.*

The long overhangs are produced by the addition of a single dNTP and a DNA polymerase which removes nucleotides from a single strand up to a specific nucleotide. The pET-YSBLIC 3C has been designed as a LIC vector. It is based on pET-28a with a cleavable N-terminal hexahistidine tag (Figure 3.6). *Gst* inserts were derived by PCR from Arabidopsis cDNA.

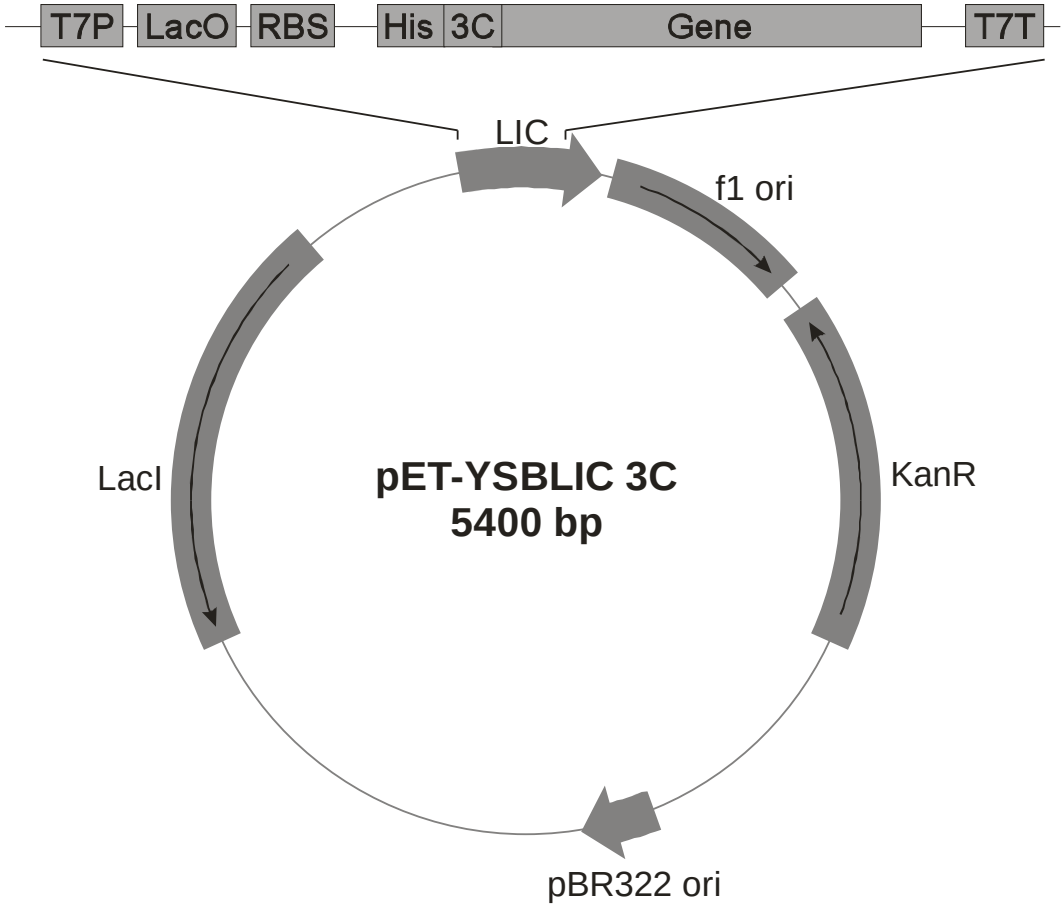


Figure 3.6: LIC vector containing gene of interest. This modified pET-28a (+) plasmid has a cloning site comprising; a T7 promoter (T7P), Lac operator (LacO), ribosome binding site (RBS), a 6x His-tag (His) which can be cleaved at the HRV 3C protease site (3C). The plasmid also contains two origins of replication (f1 and pBR322), a kanamycin resistance gene and a repressor gene (LacI) for IPTG induction.

3.2.3.1 Vector Preparation

The pET-YSBLIC3C plasmid was transformed into *Escherichia coli* DH5α and grown in LB at 37 ℃, 250 rpm overnight with 30 *μ*g/ *μ*L kanamycin. Plasmid DNA was extracted using a miniprep kit according to the manufacturer's instructions (Qiagen), see Chapter 2.3.2.1. The vector was then linearised with the restriction endonuclease BseRI (NEB). The digest reaction contained; 5 *μ*g vector DNA, 5 *μ*L *Bse*RI, 10 *μ*l 10x NEB buffer 2 and water to 100 *μ*L, and was incubated at 37 ℃ for 2 h. Digested plasmid was separated from undigested plasmid by gel electrophoresis. The linearised vector was then extracted from the gel using the Wizard extraction kit (Promega) as detailed in Chapter 2.3.2.2 and quantified on a nanodrop (Thermo Fisher Scientific, USA).

3.2.3.2 PCR Amplification

Oligonucleotide primers for the seven selected glutathione transferase genes were designed specifically for Ligation Independent Cloning (LIC) with the pET-YSBLIC3C vector (http://bioltfws1.york.ac.uk/cgi-bin/primers.cgi) (Table 3.2). cDNA template from Section 3.2.1 was used for PCR amplification with the LIC primers to produce inserts for each *gst* gene. 50 ng/μl cDNA was used for each 50 μl reaction containing 5 μl 10x KOD buffer, 200 μM dNTPs and 0.4 μM forward and reverse LIC primers, 1mM $MgSO_4$ and 1 U of KOD Hot Start polymerase (Novagen). PCR was performed with a Px2 Thermal Cycler (Thermo Scientific). Initial denaturing conditions were 95 ℃ for 2 min, followed by 30 cycles of; melting at 95 ℃ for 30 s, annealing at 45℃ for 30 s and elongation at 72 ℃ for 20 s. The final extension conditions were 72 ℃ for 180 s, followed by a hold step at 4 ℃ until use or storage at -20 ℃.

Table 3.2: Primers used for PCR amplification of *gsts* for recombinant expression in *E. coli*. Red text indicates the sequence for LIC specific overhangs.

Primer name	Sequence
GSTU1LIC F	CCAGGGACCAGCAATGGCGGAGAAAGAAGAGAGTGTGAAG
GSTU1LIC R	GAGGAGAAGGCGCGTTAGGCAGACTTAATTGTCTCTGCAATTTTGGT
GSTU3LIC F	CCAGGGACCAGCAATGGCCGAGAAAGAAGAGGGTGTGAA
GSTU3LIC R	GAGGAGAAGGCGCGTTAGACCGCTTTGATTCGTCCTACAATTTTCAT
GSTU4LIC F	CCAGGGACCAGCAATGGCGGAGAAAGAAGAGGATGTGAAG
GSTU4LIC R	GAGGAGAAGGCGCGTTAGGCTGATTTGATTCTTTCTACAACTTTCTTC
GSTU7LIC F	CCAGGGACCAGCAATGGCGGAGAGATCAAATTCAGAGGAAG
GSTU7LIC R	GAGGAGAAGGCGCGTTATCAAGCAGATTTGATATTGAGTTTCTCCATACG
GSTU12LIC F	CCAGGGACCAGCAATGGCTCAAAATGGTTCGAATACTACTGTG
GSTU12LIC R	GAGGAGAAGGCGCGTTACTAAACACTGAATTTCTTTTTGGCAAACTCGAT
GSTU22LIC F	CCAGGGACCAGCAATGGCGGATGAAGTGATACTTTTGGATTTTTG
GSTU22LIC R	GAGGAGAAGGCGCGTTAGACACAGTATATCTTCCTAATCTTATAGGC
GSTU24LIC F	CCAGGGACCAGCAATGGCAGATGAGGTGATTCTTCTGGATTTC
GSTU24LIC R	GAGGAGAAGGCGCGTTACTCCAACCCAAGTTTCTTCCTACGTTC
GSTU25LIC F	CCAGGGACCAGCAATGGCAGACGAGGTGATTCTTCTTGATTTC
GSTU25LIC R	GAGGAGAAGGCGCGTTACTATTCGATTTCGATCCCAAGTTTTTTCCTTAG

3.2.3.3 T4 Polymerase Treatment

Digested vector and PCR products were treated with T4 polymerase and complimentary bases (A and T) to produce sticky ends and remove the requirement for ligation.

Vector T4 polymerase reactions were performed in a 40 μL volume containing; 0.5 pmol vector DNA, 4 μL 10x T4 pol buffer, 6.25 mM dTTP, 12.5 mM DTT and 2.5 units T4 DNA polymerase (Merck). T4 polymerase reactions with the insert contained 0.2 pmol PCR product, 2 μL 10x buffer, 2.5 mM dATP, 5 mM DTT and 1 unit T4 DNA polymerase (Merck). Both reactions were incubated at 22 ℃ for 30 min and then inactivated by heating at 75 ℃ for 20 min. The vector reaction was then purified with the Wizard extraction kit (Promega) and its DNA concentration quantified on a nanodrop (Thermo Fisher Scientific, USA).

3.2.3.4 Annealing and Transformation

The LIC annealing was performed by the addition of 2 μL insert to 1 μL vector DNA, incubation for 10 min at room temperature, addition of 1 μL 25mM EDTA and further incubation for 10 min at room temperature. The product was then transformed into *E. coli* expression hosts as described in Chapter 2 and positive clones were identified by colony PCR and agarose gel electrophoresis (Section 2.3.4.3 and 2.3.1).

3.2.4 Expression of LIC-GSTs in *Escherichia coli* BL21-DE3

The optimum conditions for expression of the LIC-GST constructs were; *E. coli* BL21-DE3 freshly transformed with pET-YSBLIC3C vector containing the *gst* insert. Cultures were grown in 500 mL flasks containing 200 mL ZY media, autoinduction additives (see Section 2.4.1) and 30 μg/mL kanamycin, at 37 ℃ with 180 rpm shaking until an optical density of 0.8 – 1.0 at 600 nm was reached. Cultures were then transferred to a 20 ℃ incubator with 180 rpm shaking and expression time varied from 24 h to 72 h. Cultures were harvested by centrifugation at 8500 x *g* in a Sorvall Evolution RC centrifuge. The pellets were weighed and resuspended to 0.5 g/mL in cold PBS, pH 7.4. Cells were then lysed by sonication prior to purification. Sonication was performed as described in Section 2.4.2. Cell lysates were centrifuged at 17,500 x *g*, 4 ℃ for 30 min to

remove the cell debris. Supernatants were passed through 0.45 μM syringe filters before being applied to the purification media.

3.2.5 Purification of GSTs by Affinity Chromatography

Purification by both GSH and nickel affinity chromatography was performed as described in Section 2.4.3. SDS-PAGE was performed to assess the purity and yields of the purified enzymes. The purified protein concentration was determined with Pierce Coomassie Dye Binding Reagent, with 1 mL reagent and 1-10 μL purified protein. The increase in absorbance at 595 nm was measured with reference BSA standards of known protein levels to calculate the purified protein concentration, see Figure 3.7.

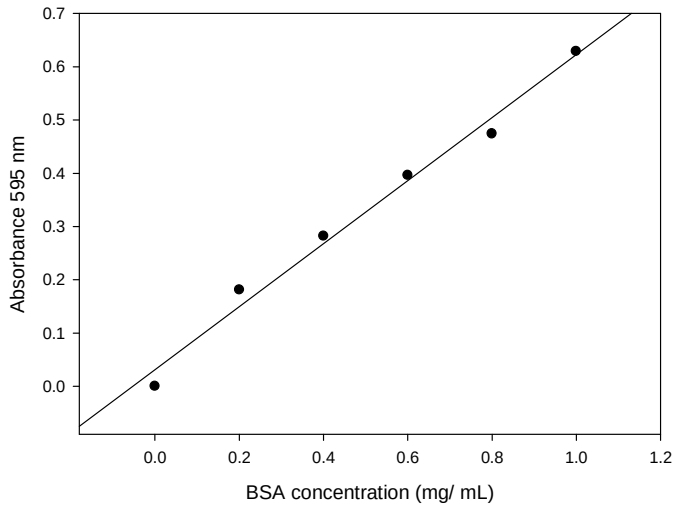


Figure 3.7: Concentration curve of bovine serum albumin standards and Pierce Coomassie Dye Binding Reagent. Change in absorbance was measured at 595 nm.

3.2.6 GST Activity Assays

To establish assay conditions and determine activity, each GST was assessed for activity with CDNB using adaptations of published protocols (Habig *et al.* 1974). Conditions were then repeated with TNT as a substrate, with the addition of higher enzyme concentrations and over a longer time course. TNT assays were analysed by both HPLC and Griess assay.

3.2.6.1 CDNB Assay

Supernatant or pure protein was added to reaction mix which contained; 1 mM CDNB, 100 mM phosphate buffer pH 6.5, 5 mM GSH. Total reaction volume was 1 mL, The reaction was initiated by the addition of CDNB. Increase in absorption due to conjugate production was followed over 1 min by UV-spectrophotometry at 340 nm. Protein concentrations were determined in triplicate by Bradford Assay, with BSA standards (Section 3.2.5).

3.2.6.2 TNT Assay

The TNT assays were performed in 100 mM phosphate buffer pH 6.5, 5 mM GSH, 200 μM TNT and 100 μg pure protein. Samples were taken over a time course and analysed by HPLC or Griess, see below.

The samples analysed by HPLC had been stopped by the addition of 1% TCA. A Waters Alliance 2695 separations module and a Waters 2996 Photodiode Array was used to follow TNT transformation. Fifty microlitres of each sample were loaded onto a Sunfire C18 5 μm column (Waters, Wexford, Ireland) and run at 1 mL/ min with the solvent conditions outlined in Table 3.3 where; Buffer A is methanol and buffer B is 2 mM ammonium acetate in 5 % methanol.

For the Griess reactions, to measure nitrite release from TNT conjugation to GSH, 180 μl of TNT assay samples were transferred to 96-well plates. 50 μL of 10 mg/mL sulphanilamide (acidified in 0.68 M HCl) were added and mixed by pipetting. Following at 10 min incubation at room temperature 20 μL of 5 mg/mL N-(1-naphthyl)-ethylenediaminedihydrochloride was added. The assay was incubated for 10 min at room temperature before change in absorbance at 595 nm was determined, using a Sunrise plate reader (Tecan, Austria). A standard nitrite curve was also produced to quantify results.

Mass spectrometry of the TNT assay samples from Equine GST, GSTU24 and GSTU25 was performed using a Q-STAR Pulsar i LC-MS system (Applied Biosystems) with a Phenomenex Columbus column (5 μm C18 150 x 3.2 mm) and run with HPLC method B (Table 3.3).

Table 3.3: HPLC run conditions. Method A is optimised for the Sunfire column, and method B for the Phenomenex Columbus column. Buffer A is methanol and buffer B is 2 mM ammonium acetate in 5 % methanol

Method A:

Time (min)	Buffer A	Buffer B
0	60	40
3	60	40
4	20	80
7	60	40
15	60	40

Method B:

Time (min)	Buffer A	Buffer B
0	20	80
4	20	80
7	60	40
12	60	40
15	20	80
25	20	80

3.3 Results

3.3.1 GST Gene Structure and Amino Acid Alignment

The seven GST DNA sequences selected from the microarray data (Lorenz 2007) were obtained from Arabidopsis information resource website (www.tair.org). All the GSTs have complete gene lengths between 950 – 1100 bp, with a single intron reducing cDNA lengths to 650-680 bp (Figure 3.8). Their calculated amino acid lengths range from 218 – 227 and have predicted pI values between 4.9 and 6.9. They all belong to the Tau class (GSTU), a plant specific class and the largest in Arabidopsis. Many members of the GSTUs have well characterised detoxifying activities (Moons 2005). Although no crystal structure has been elucidated for an Arabidopsis Tau class GST the structures of *Ta*GSTU4 (bread wheat, *Triticum tauschii*), *Gm*GSTU4 (soya bean, *Glycine max*) and a *Zea mays* (maize) Tau Gst have been determined, and structural comparisons have been inferred for other GSTs from these (Neuefeind *et al.* 1997; Thom *et al.* 2002; Zeng *et al.* 2005; Axarli *et al.* 2009).

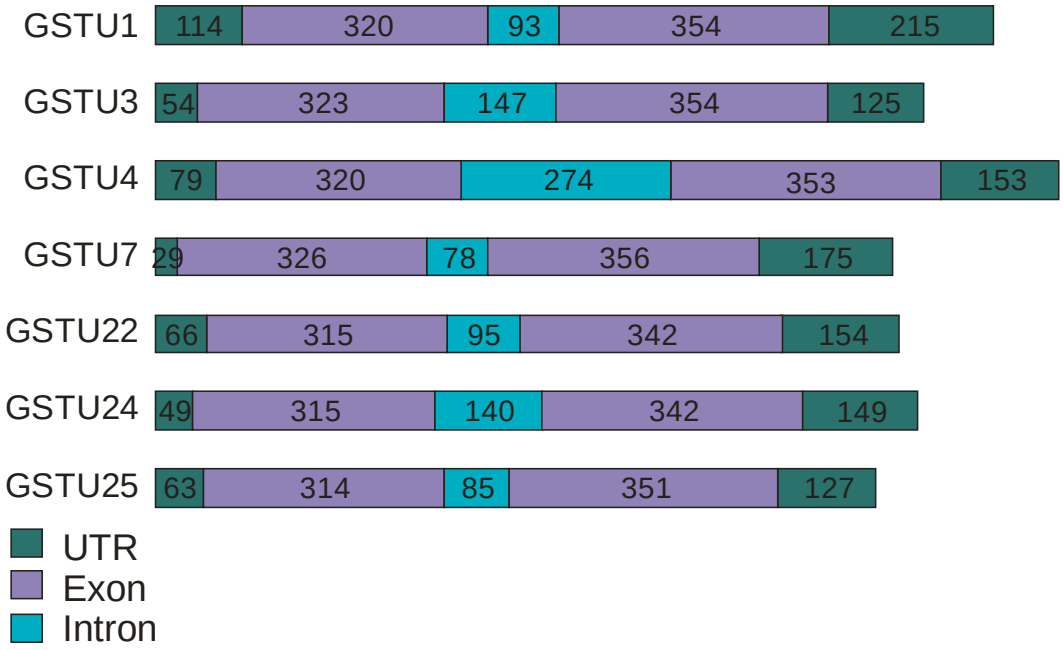


Figure 3.8: Gene structure of seven Tau GSTs. All GSTs have a single intron with two ~300-350 bp coding regions either side.

The protein sequences of the seven selected GSTUs were aligned using Clustalx 2.0.9 and BioEdit7 (Figure 3.9) (Hall 1999; Larkin *et al.* 2007).There are 35 amino acids conserved amongst all these GSTs, four of which are known to be involved in glutathione binding (Thom *et al.* 2002). The amino terminal domain is the site of glutathione binding (G-site), while the carboxy-terminal domain binds the hydrophobic substrate (H-site). Much of the sequence conservation is maintained at the G-site, which only binds to GSH or other very similar compounds including gamma-glutamyl linked peptides. Important residues at the amino terminal are Ser13, Lys40, Glu66 and Ser67, which are critical G-site residues for glutathione binding, boxed in blue in Figure 3.9 (Thom *et al.* 2002; Zeng *et al.* 2005).

The H-site lies adjacent to the G-site in the active site cleft; its open nature and hydrophobicity allow it to accept a wide range of ligands and substrates. Across the GSTs the H-sites demonstrate much higher sequence diversity than the G-sites, allowing conjugation of GSH to a diverse range of hydrophobic substrates (Figure 3.9) (Edwards *et al.* 2000; Dixon *et al.* 2002b). The residues thought to be important for substrate binding are boxed in green.

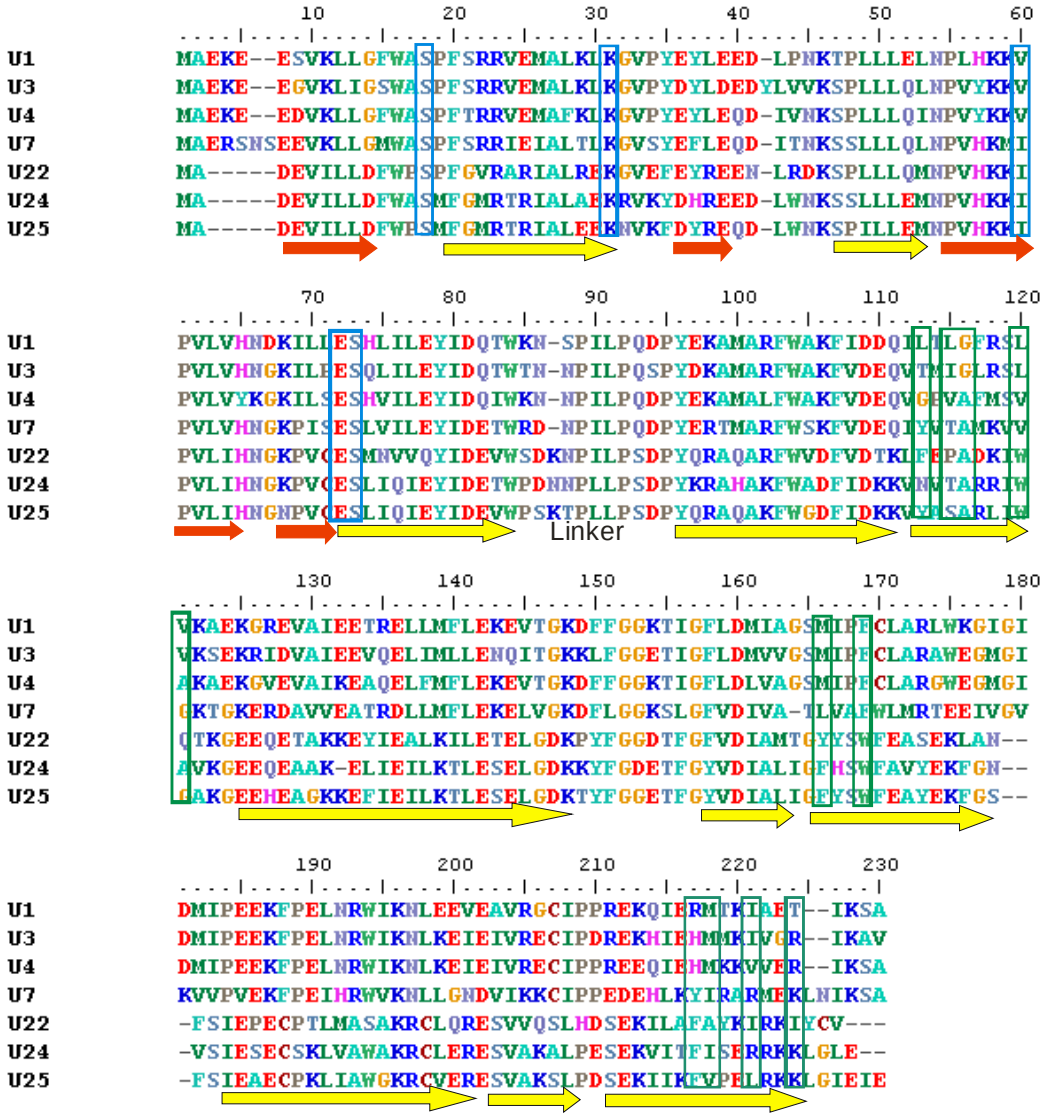


Figure 3.9: Sequence alignment of seven Tau GSTs with major structural features demonstrated by arrows. Red arrows are beta-sheets and yellow arrows are alpha-helices, the 5-10 residue linker region between the two domains is also marked. The blue boxes highlight residues involved in GSH binding (G-site), the green boxes indicate the residues involved in substrate binding (H-site). Produced using Clustalx 2.0.9 and BioEdit7, adapted from Thom et al., 2002.

3.3.2 Expression of GSTs *in planta*

To confirm the microarray data, real-time PCR was carried out with plant material grown under the same conditions using cDNA from 14-day old Arabidopsis seedlings grown hydroponically, treated with 60 μM TNT or DMF-only for 6 h.

The transcription levels observed by RT-PCR generally mimicked those from microarray, but with much more accuracy due to the higher sensitivity and wider linear dynamic range achieved by RT-PCR (Figure 3.10).

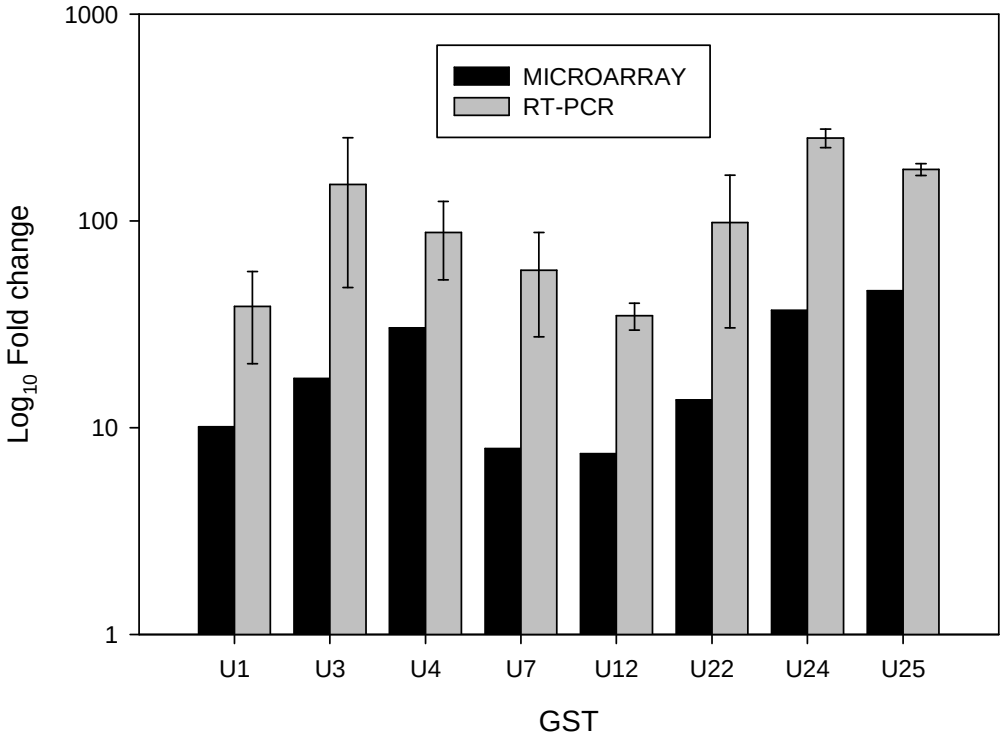


Figure 3.10: Comparison of microarray and RT-PCR results from cDNA of 14-day old Arabidopsis treated with 60 μM TNT for 6 h. Microarray data (black bars) was taken from Lorenz, 2007, All values are significant with P values < 0.05. RT-PCR (grey bars) was performed as a comparison between TNT and DMF only with actin primers used to calibrate to an endogenous control gene. Errors are ± Standard Error of the mean.

Table 3.4: Expression levels of GSTs in different tissues of Arabidopsis plants ± SD. Expression levels are absolute values from microarray data of 22,000 genes from Arabidopsis as represented on the ATH1 GeneChip from Affymetrix. Arbitrary cut offs of 10 or less for low expression are shown in blue and for 250 or higher for high expression are show in in red. Data taken from the Arabidopsis eFP browser (www.http://bar.utoronto.ca/efp).

	GSTU1	GSTU3	GSTU4	GSTU7	GSTU22	GSTU24	GSTU25
Dry Seed	22.49 ± 2.73	7.77 ± 0.23	416.49 ± 45.0	382.84 ± 41.7	6.82 ± 0.18	2.15 ± 8.02	0.31 ± 0.13
Imbibed Seed	22.67 ± 12.28	3.53 ± 3.15	33.73 ± 1.52	210.29 ± 73.08	14.46 ± 10.05	94.02 ± 14.89	19.36 ± 11.45
Cotyledons	3.73 ± 1.77	3.05 ± 3.43	8.61 ± 1.45	70.73 ± 7.46	9.56 ± 3.21	9.29 ± 2.72	2.16 ± 1.49
Hypocotl	33.56 ± 1.24	2.35 ± 2.08	8.56 ± 1.45	234.8 ± 12.48	16.36 ± 1.51	27.13 ± 1.63	8.41 ± 0.41
Root (young)	84.18 ± 5.84	4.6 ± 1.76	8.35 ± 1.3	208.81 ± 1.72	116.25 ± 5.35	119.88 ± 16.7	77.95 ± 14.41
Vegetative rosette	10.53 ± 2.77	1.65 ± 1.41	11.51 ± 2.7	65.81 ± 6.94	9.95 ± 2.34	11.28 ± 2.64	7.96 ± 7.16
Root (mature)	131.36 ± 12.68	5.33 ± 2.77	10.3 ± 1.06	218.45 ± 10.82	105.51 ± 5.37	131.71 ± 9.93	58.2 ± 3.23
Carpel	3.18 ± 1.19	26.1 ± 6.65	17.75 ± 3.79	147.33 ± 19.11	7.1 ± 3.32	16.94 ± 1.46	2.53 ± 1.45
Petal	8.71 ± 1.67	76.34 ± 3.31	126.08 ± 13.71	179.06 ± 15.39	7.88 ± 2.4	51.91 ± 4.43	2.65 ± 2.51
Stamen	16.65 ± 4.73	273.2 ± 14.67	341.5 ± 28.61	691.33 ± 12.36	11.8 ± 3.82	70.46 ± 10.02	6.3 ± 4.35
Sepal	135.65 ± 6.36	256.31 ± 14.04	413.76 ± 28.47	505.68 ± 22.56	13.81 ± 4.49	410.86 ± 34.22	87.25 ± 14.33
Mature pollen	3.0 ± 1.75	7.66 ± 7.8	5.38 ± 3.31	13.15 ± 8.73	19.28 ± 1.49	14.76 ± 5.38	6.8 ± 1.78

Expression levels of the GSTs studied in this work under non-stress conditions, are shown in Table 3.1. It is clear that the GSTs are variably expressed throughout the different tissues of Arabidopsis. Interestingly, GSTU25, which is expressed most highly of these GSTs in response to TNT, shows the lowest levels of expression in these tissues under non-stress conditions. U22 also expresses at relatively low levels while U4 and U7 show high levels of expression, especially in dry seed, stamen and sepal. It is therefore possible that the expression levels of GSTs in TNT treated plants contains more similar levels of GSTU7 as GSTU25 because the microarray data of these plants was normalised, to show upregulation of the GSTs, not total expression values.

3.3.3 Creation of GST Expression Constructs

To create recombinant enzymes, the genes of seven GSTs most upregulated in response to TNT were cloned into the LIC vector system described in Section 3.2.3.

Inserts containing the GSTs were amplified from Arabidopsis cDNA by PCR as described previously. The products are shown in Figure 3.11 A. The products were then inserted into the pETYSBLIC3C plasmid by an annealing step. To verify the correct insertion of each GST gene into the vector, colony PCR and restriction digests were performed after transformation into cloning host *E. coli* DH5α (Figure 3.11 B and C). The sizes of the PCR products observed on the gel correspond with the lengths of each *gst*. To confirm correct insertion and lack of mutations, genes were sequenced and aligned with those from the Arabidopsis information resource website (www.tair.org). The double restriction digests produced a band the same size as the inserts for each gene (~700 bp) and one the same size as the empty vector (5369 bp) while the single digests linearised the vector (not shown). The empty vector control is smaller due to lack of insert and double digest does not liberate a *gst*.

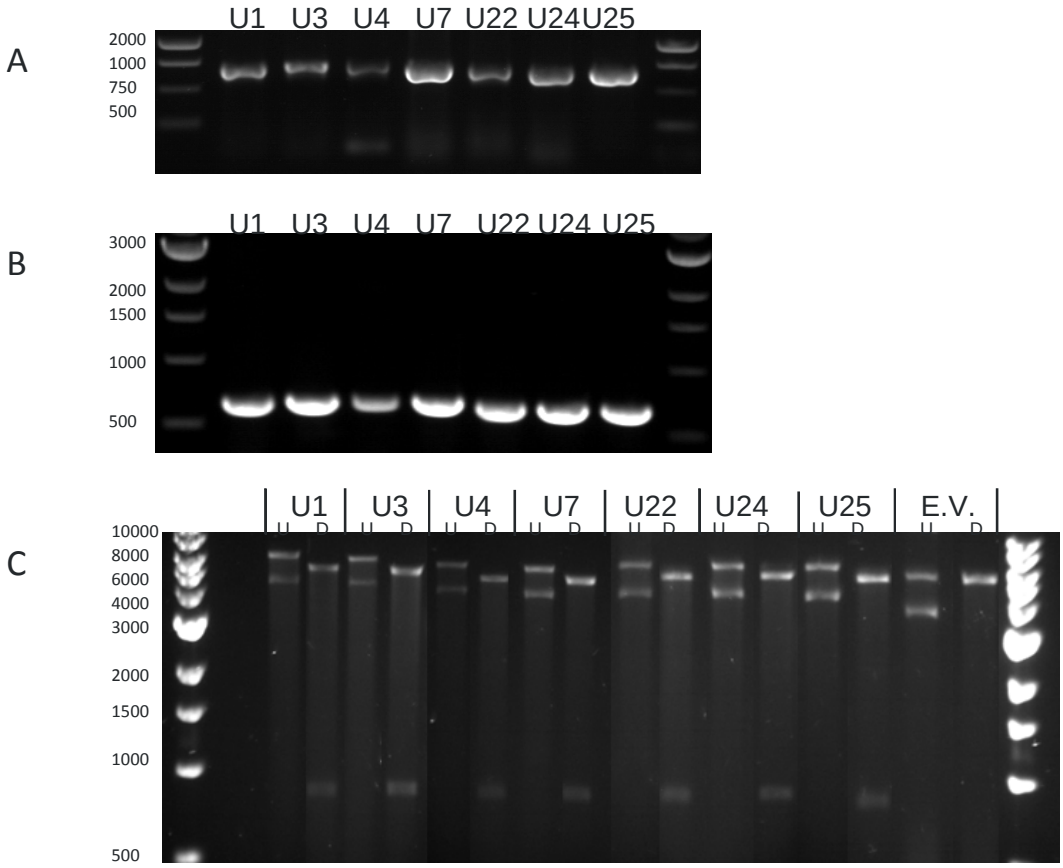


Figure 3.11: Cloning of seven Arabidopsis Tau GSTs. **A:** The amplification of GSTs 1, 3, 4, 7, 22, 24 and 25 from Arabidopsis cDNA, to be annealed into the pETYSBLIC 3C vector. **B:** Colony PCR of LIC-GSTs. *E. coli* DH5α were transformed with each GST-YSBLIC construct; selected colonies clearly show they contain the respective GST gene. Negative controls were performed and no bands were present, data not shown. **C:** Restriction digest of LIC-GST constructs. Uncut plasmids (U) and double digests with *Xba*I and *EcoR*I (D) are shown for each *gst*-LIC construct including an empty vector control.

3.3.4 Optimisation of GST Expression and Purification

The expression of GSTs in *E. coli* was optimised by varying certain conditions including; expression host, time and temperature of induction, and induction method. Two methods of expression induction were compared, autoinduction media (Section 2.4.1) or IPTG addition. A comparison of the two methods is shown for GSTU24 in Figures 3.12 and 3.13. Both induction methods successfully stimulated expression and the resultant protein was similarly soluble and active, however autoinduction media was favoured for subsequent studies as the cell density of cultures grown in this media is much higher, hence more protein is produced in total (Studier 2005).

All seven GSTs were successfully cloned, expressed and at least a fraction of the expressed protein was soluble (Figure 3.14). SDS-PAGE gels show GSTU1 and U4 express little soluble GST, but the other GST construct appear to express more soluble protein with U3 and U25 expressing the most protein of the right molecular weight (~25 kDa). Purification was initially performed with GSH sepharose as previous literature states that the majority of Tau GSTs can be purified by this method (Dixon *et al.* 2002b). However, only GSTU24 and 25 effectively bound to the beads and were purified, with lesser yields of GSTU7 and U22 (data not shown). Successful purification for all GSTs was only achieved with a nickel affinity column, via the His-Tag of the LIC-vector (Figure 3.14). An SDS-PAGE of the purification profile of GSTU25 is shown as a representation of purification of all the GSTs (Figure 3.15). Three wash steps appear adequate to yield pure protein. Where expression levels are especially high, as seen for GSTU25 the purification appears to be less effective, with contaminating bands visible in the first elution fraction (Figure 3.15). For this reason further purifications of GSTU25 were performed on a packed GSH column, and the first elution fraction was discarded.

The GSTs U1 and U4 yielded very little soluble protein despite ample expression levels as determined by SDS-PAGE. This low solubility may have hindered purification, allowing contaminants to bind the column. This would explain the impurities observed in the pure protein shown by SDS-PAGE (Figure 3.14). Explanations for this low solubility could be mis-folding or inclusion bodies, as previous work with GSTs has identified the expression of poplar GSTs in *E. coli* as inclusion bodies (Lan *et al.* 2009).

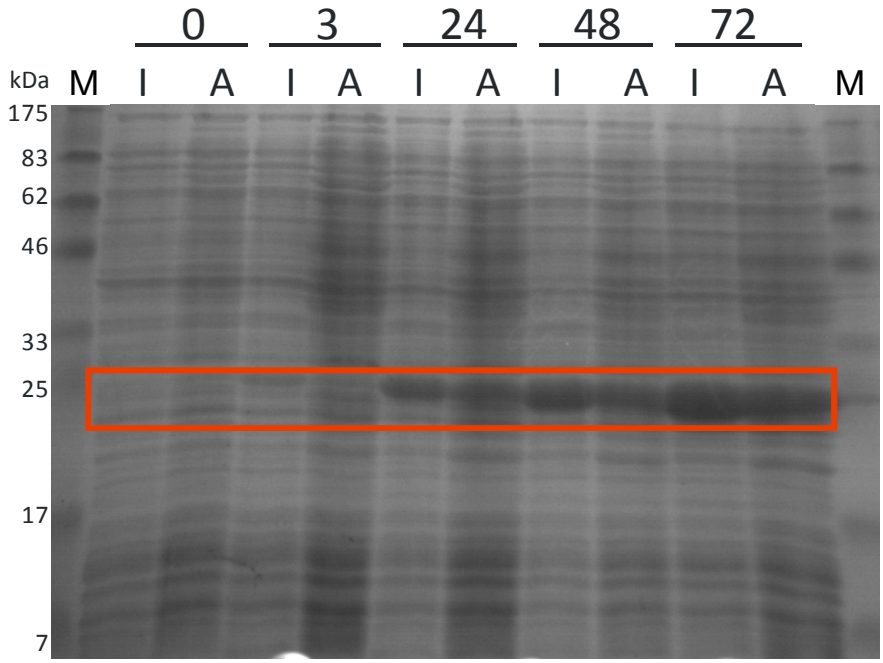


Figure 3.12: SDS-PAGE comparison of expression by IPTG induction (I) and autoinduction (AI) methods. SDS-PAGE of soluble fraction of *E. coli* BL21 expression GSTU24 Cell pellets were normalised for weight prior to sonication. GSTU25 has a molecular weight of ~25kDa and its expression is therefore observed within the red box. M denotes a molecular weight marker.

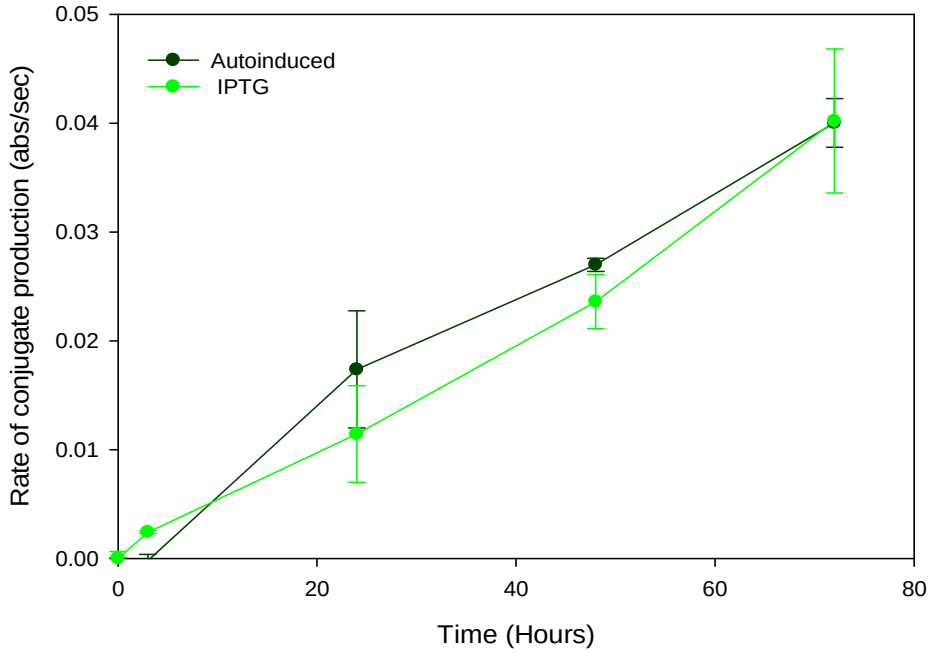


Figure 3.13: Activity of GSTU24 with CDNB, comparison of autoinduction and IPTG. Assays were performed with 50 μL of crude lysate from the two different expression conditions; IPTG or auto- induction produced. No difference in activity with CDNB can be seen. Samples contained the same cell pellet weight prior to sonication and centrifugation.

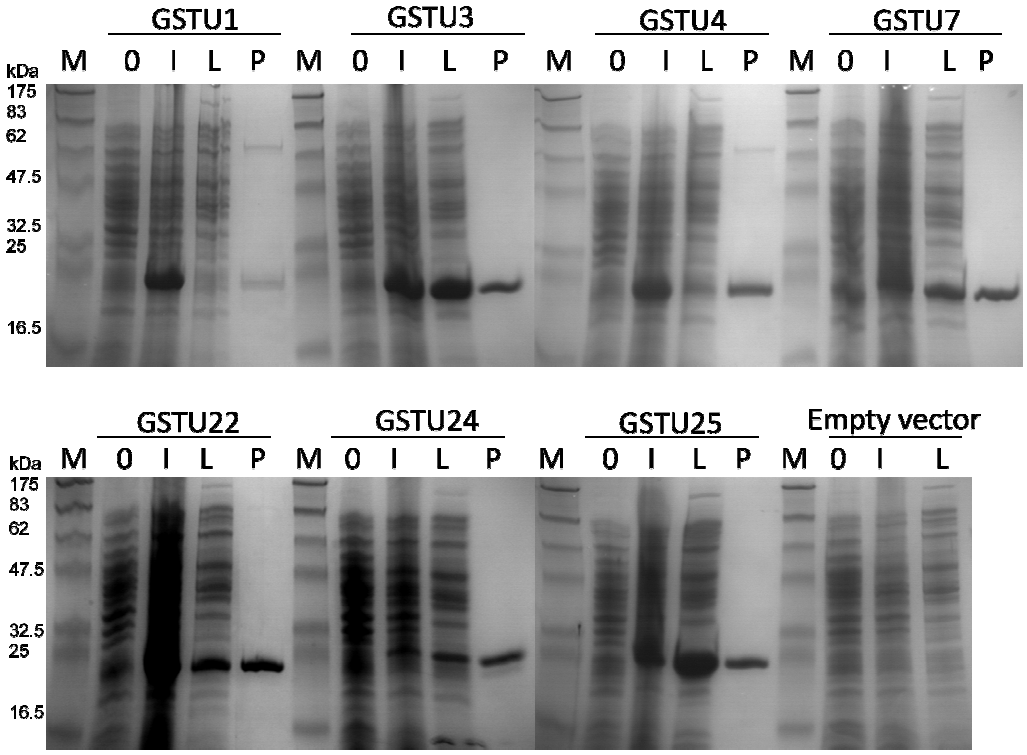


Figure 3.14: Expression profile and purification of all GSTs including empty vector control. Lanes show; cells grown in LB (0), induced cell culture (I), lysed cell supernatant (L) and purified protein. Expected GST size is ~26 kDa and the large bands at this weight in the induced samples demonstrate that all GSTs express well and the GSTs are soluble. The empty vector control cells do not produce any additional observable bands at ~25 kDa following induction. M denotes a molecular weight marker.

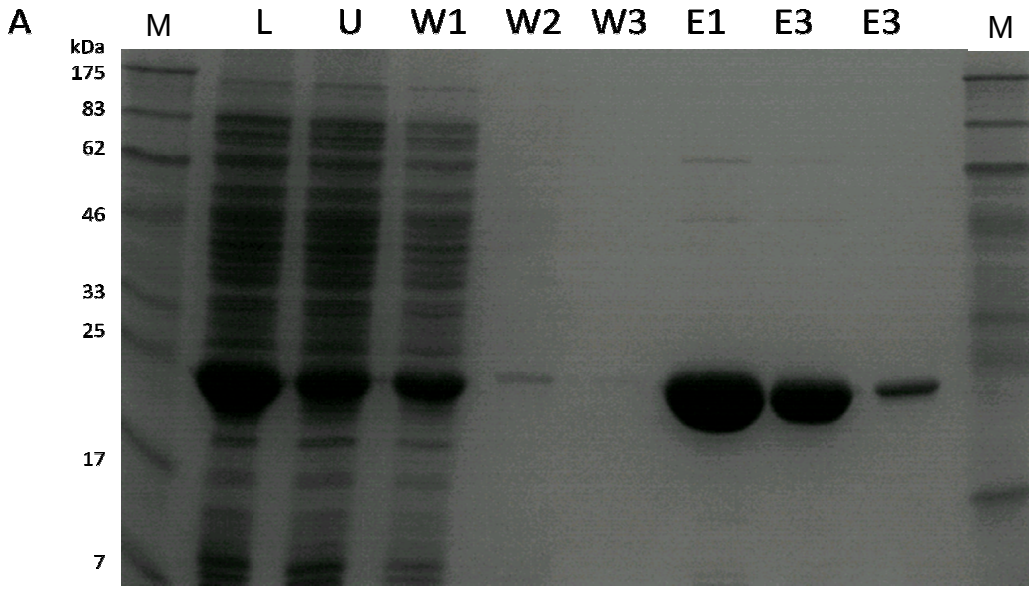


Figure 3.15: Purification of GSTU25. A purification profile by SDS-PAGE showing cell lysate (L) unbound fraction (U), wash steps (W), elutions (E) and purified protein. M denotes a molecular weight marker.

3.3.5 Activity of GSTs with CDNB

Activity of the recombinant GSTs towards CDNB was tested spectrophotometrically both in lysed cell supernatant (Figure 3.16) and with purified protein (Figure 3.17). The GSTs showed variability in activity towards CDNB, both lysed cell and purified protein. This is in accord with the findings of Dixon 2009, where activities of GSTs range from 1.8 – 1240 nkat.mg^{-1}. Five of the seven GSTs exhibited some level of activity with GSTU25 proving to be the most active in both cases. GSTs U1 and U4 were the only two enzymes not to exhibit significantly more activity the empty vector (E.V) control, this may be due to their low solubility levels; however previous work has shown that these two GSTs have very low activities with CDNB (U1 =17.8 and U4 =10.4 nkat.mg^{-1}), so perhaps the activity is simply too low to observe under these assay conditions (Dixon *et al.* 2009). This suggests that CDNB is not suitable as a universal substrate for assessment of activity; however, its structural similarities to TNT may make it a good indicator of activity towards this explosive. Assays of activity with purified enzyme and TNT were performed to determine this.

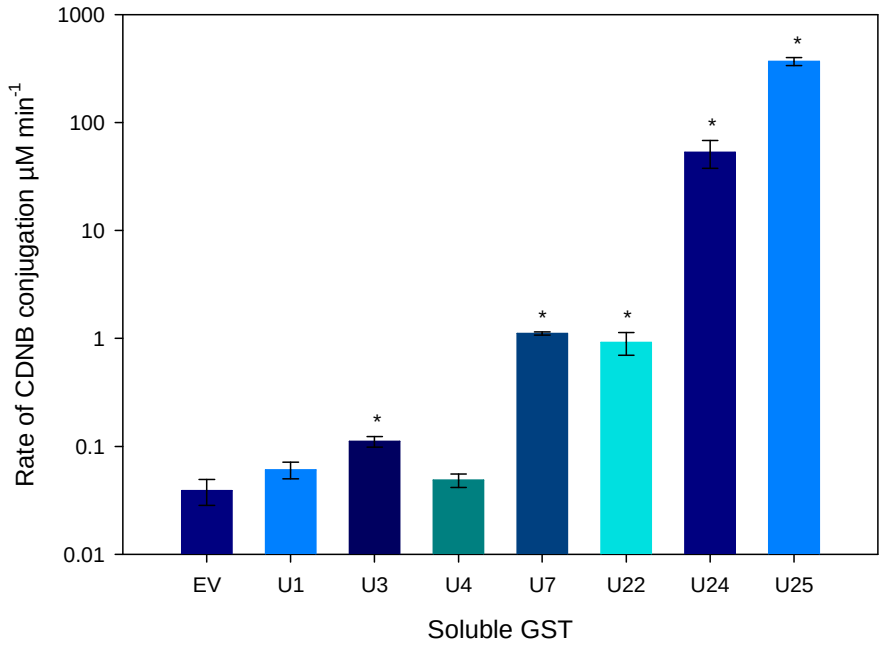


Figure 3.16: Rate of conjugation of CDNB by 200 µg crude cell lysate for each of the cloned GSTs and an empty vector control. Lysates were normalised for whole protein concentration by Bradford assay. Asterisks show significant differences to EV control where P values > 0.05 as determined by Student's t-Test.

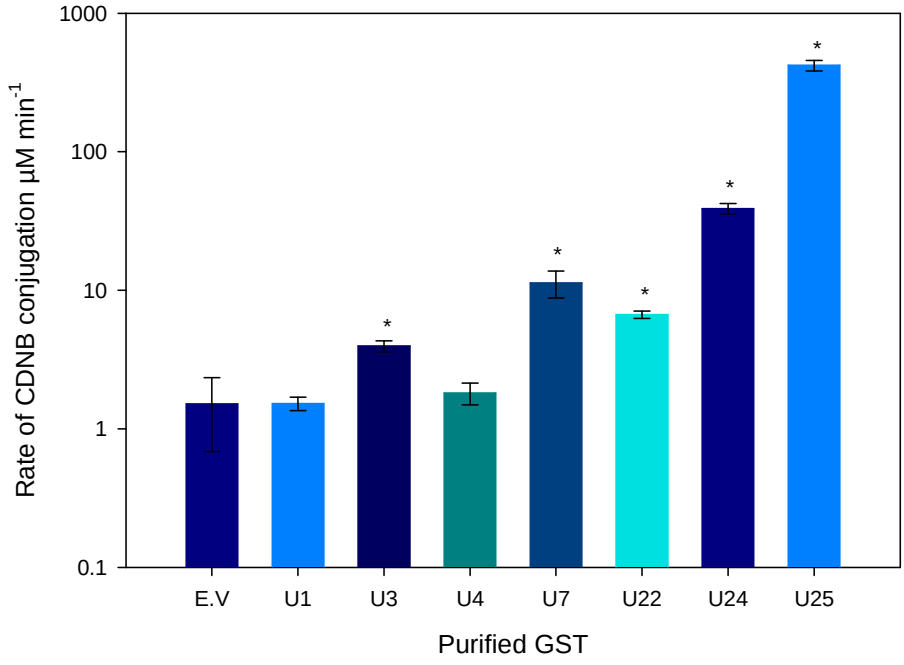


Figure 3.17: Rate of conjugation of CDNB by 10 µg purified enzyme, for each of the cloned GSTs and equivalent volume of purified empty vector lysate (E.V) for the negative control. Protein concentration was determined by Bradford assay. Asterisks show significant differences to EV control where P values > 0.05 as determined by Student's t-Test.

3.3.6 Activity of GSTs with TNT

A preliminary assay was performed with EqGST and TNT to determine a suitable HPLC method (Figure 3.18). The assay conditions employed were a variation on those used for CDNB, replacing CDNB with TNT, over 18 h rather than the five day incubation employed by Brentner et al. 2008. The TNT concentration used was 200 μM, as used previously for pure enzyme assays with TNT derivatives (Lorenz 2007). Numerous HPLC methods were trialled with both isocratic and gradient flows of many solvent compositions to gain sufficient separation of the product and substrate peaks, within an acceptable timeframe. The optimised method is described in Table 3.3 A. Figure 3.18 shows a chromatogram of the separation of peaks as well as the absorption spectra for the substrate and product. The same traces are observed for, GSTU24, U25 and equine GST. The product and TNT spectra differ indicating different extinction coefficients of the compounds therefore calculations made from TNT standards for TNT assay peaks cannot be performed on product peaks. To stop reactions 1 % TCA was used as the TCA peak eluted between 2-4 min and was shown not to interfere with peaks of interest at 6.5 and 11.6 min.

HPLC results were generated by UV/Vis absorption detection and peaks generated on the traces are assumed to be proportional to concentration (Figure 3.18). However, to determine the concentration of TNT a standard curve was performed. A range of known concentrations of TNT were run on the HPLC in triplicate for each HPLC assay, using the same run conditions. A standard curve of peak areas was then used to calculate the TNT concentrations in assay samples (Figure 3.19). Unfortunately no standards were commercially available for the product so concentration values could not be determined.

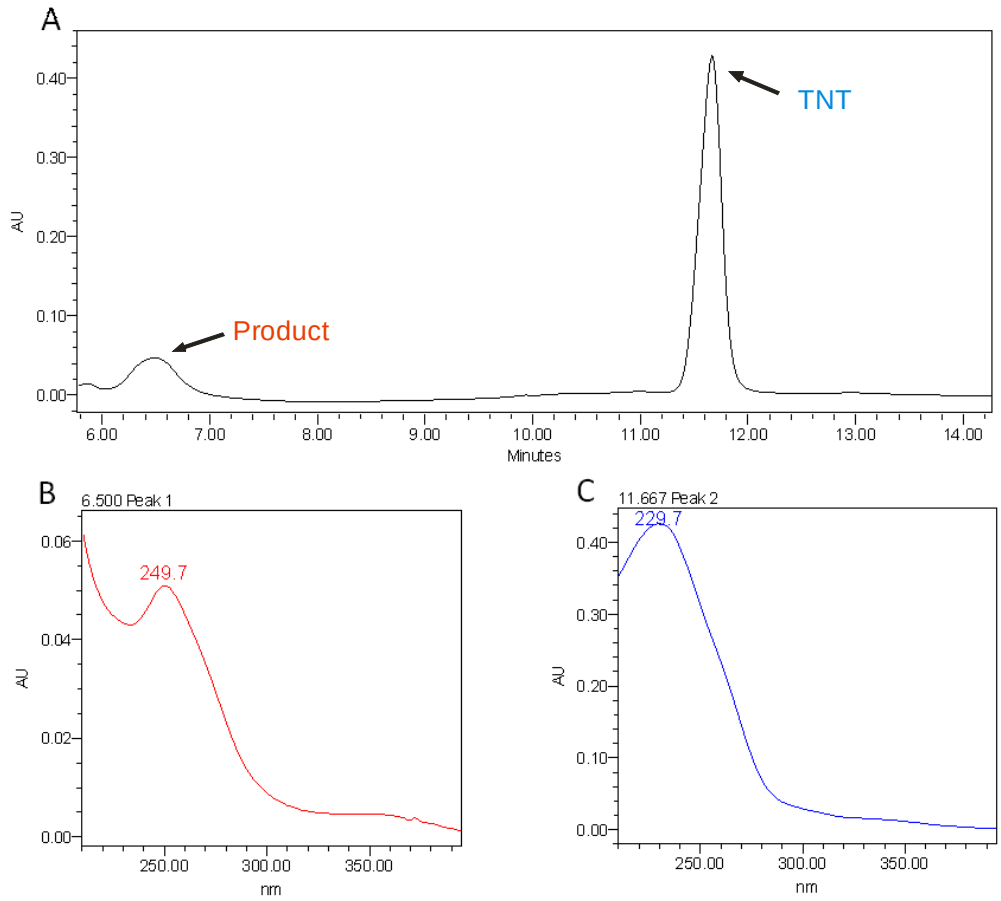


Figure 3.18: HPLC trace of TNT assay with GST. A: Chromatogram at 230 nm of the reaction between GSH and TNT as catalysed by GSTs (GSTU24, U25 and Equine GST). Assay conditions were 200 μM TNT, 5 mM GSH and 10U/ 100 μg enzyme in 100 mM phosphate buffer pH 6.5. Samples were incubated at 20 ℃ for 18 h; reactions were stopped with 1% TCA. The HPLC method is described in section 3.2.. The product has a retention time of 6.5 min while TNT has a retention time of 11.67 min. **3.18 B: Absorption Spectra of product. 3.18 C: Absorption spectra of TNT.**

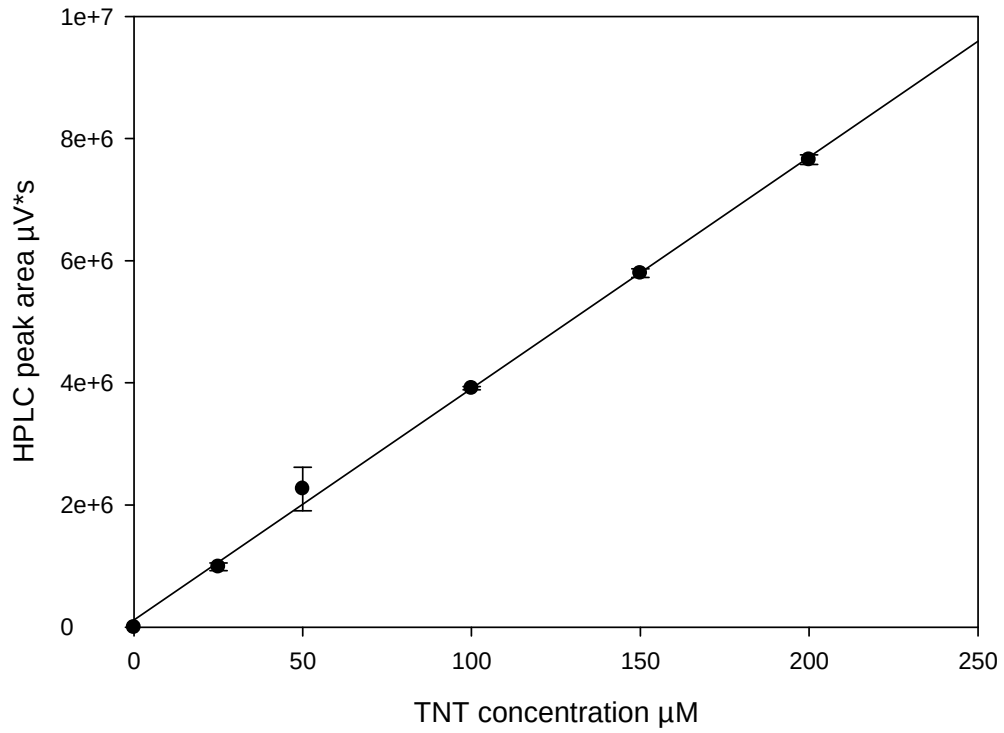


Figure 3.19: TNT standard curve: Trend line equation y = 38736*x* can be used in TNT assay results from the HPLC to calculate TNT concentration from peak area. A new standard curve was produced for every HPLC run.

Investigations to determine if these plant GSTs are able to conjugate GSH to TNT were performed using both HPLC and Griess assays. HPLC analysis measuring TNT removal from assay samples provides a quantitative determination of any activity with TNT (Fig. 3.20). As previous reports with equine GST indicate that TNT conjugates to GSH by nitrite removal (Brentner *et al.* 2008) the Griess assay was employed. The Griess assay is a colorimetric assay, where free nitrite binds to sulphanilamide, which in the presence of N-(1-naphthyl) ethylenediamine produces an azo product with pink colouration (λ_{max} = 540 nm). This indicates whether activity with TNT releases free nitrite.

Figure 3.20 shows the TNT concentrations in assays for each of the GSTs over 18 h. Depletion in TNT concentration indicates conjugation to GSH as the boiled enzyme control shows no TNT removal. This TNT removal is evident for at least three of the AtGSTs (U22, U24 and U25) demonstrating detectable levels of activity against TNT.

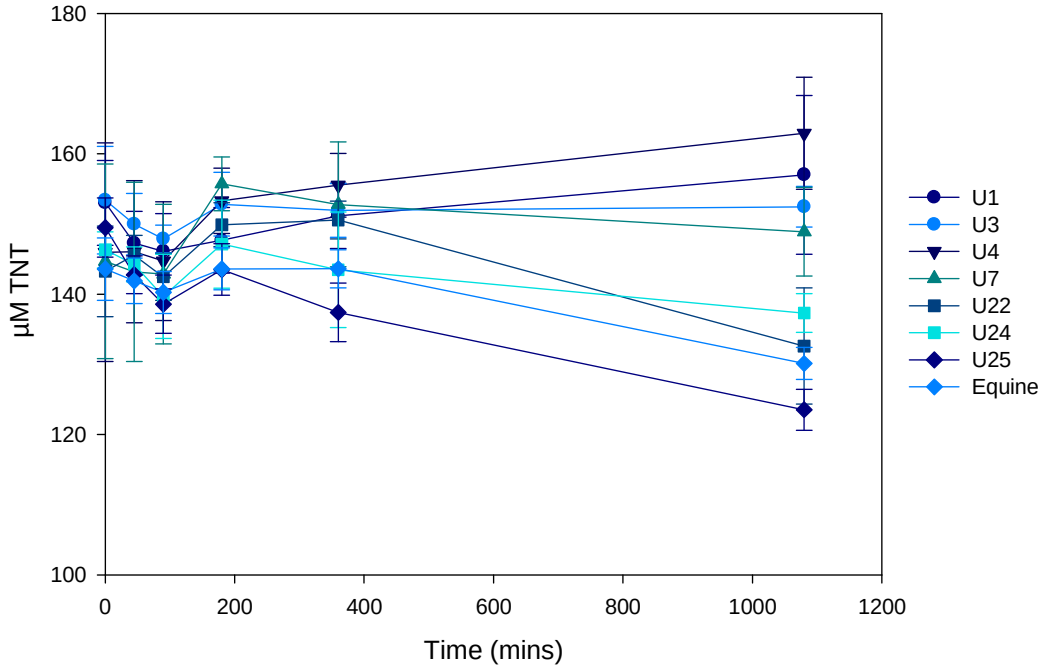


Figure 3.20: TNT assay of purified GSTs with 100 μg / mL protein and 10U for Equine GST. Assay conditions were 200 μM TNT, 5 mM GSH and 100 ug / mL protein in 100 mM phosphate buffer pH 6.5. Samples were incubated at 20 ℃ for 24 h reactions were stopped with 1% TCA. The HPLC method is described in Section 3.2.6

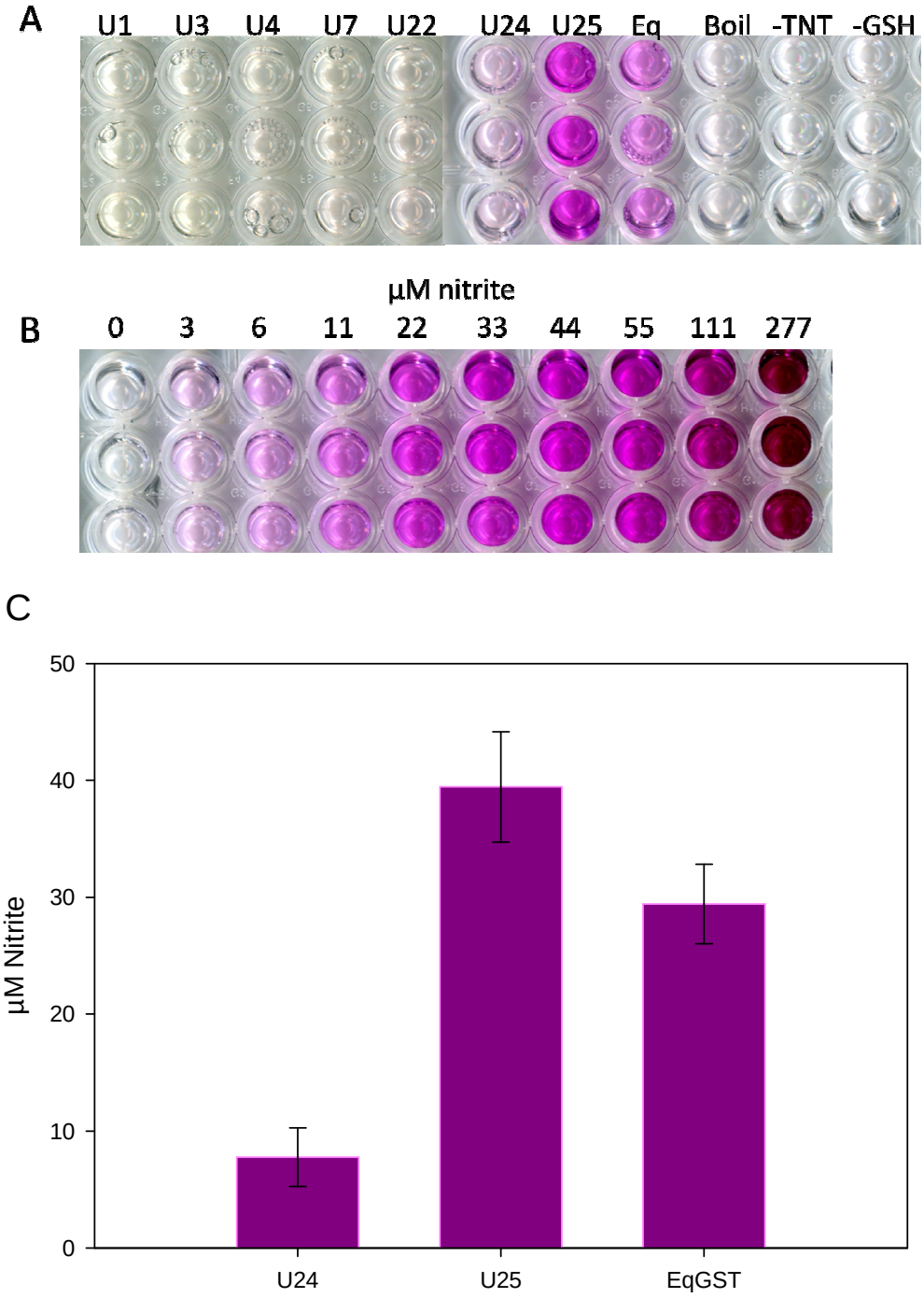


Figure 3.21: Griess assay of a 24 h TNT assay. Pink coloration indicates the presence of free nitrite and likely suggests conjugation of TNT to GSH through substitution of a nitro-group **3.21 B:** Standard curve of nitrite **3.21 C:** Nitrite produced by GSTs from TNT conjugation, concentration determined by A_{540} of standard curve in B.

The Griess assays (Figure 3.21) show pink coloration for GSTs U24, U25 and equine. A standard curve for nitrite was produced to quantify these results and this showed that GSTU25 yields 40 μM nitrite which equates to 20 % of the 200 μM starting TNT concentration. Equine GST released ~30 μM nitrite and GSTU24 released ~10 μM after a 24 h reaction.

Interestingly, analysis of TNT assays by both HPLC and Griess shows that GSTU24 and GSTU25, the enzymes with the highest CDNB activity and most upregulated in Arabidopsis in response to TNT are also active against TNT. These positive results from the Griess assay and the presence of the product observed by HPLC analysis suggest that conjugation reaction between TNT and GSH, via nitrite removal, observed by Brentner et al. 2008 for equine liver extract may also be performed by these Arabidopsis GSTs.

3.3.7 Identification of the TNT Conjugation Product

Mass spectrometry (MS) was performed on TNT enzyme assays of GSTU24, U25 and Equine GST; Figure 3.22A shows the LC traces of GSTU25, representative for each sample. Equine, GSTU25 and GSTU24 assays all contained a TNT peak at ~11.4 min with a $[M-H]^-$ of 225.98 and a product peak with a retention time of 8.98 min and a $[M-H]^-$ of 486.04. Further fragmentation of the product by MS/MS analysis was performed; the conjugate (486) fragments into compounds with sizes representative of; GSH(272) and TNT (213). The masses of the two compounds correlate to a conjugation via the sulphur group of glutathione with the removal of a nitro group of TNT (Figure 3.23). Interestingly a further (dominant) product was identified from the GSTU24 assay only, eluting at 5.64 min with a total $[M-H]^-$ of 517. This fragmented to 272 (glutathione) and further peaks (179, 209, 254 and 306), but none of these, when combined with the 272 from GSH, add up to the 517 of the complete product. No further investigation was performed on this product as it is likely to be a conjugated contaminant rather than an additional product as the assay contained only pure enzyme and substrates.

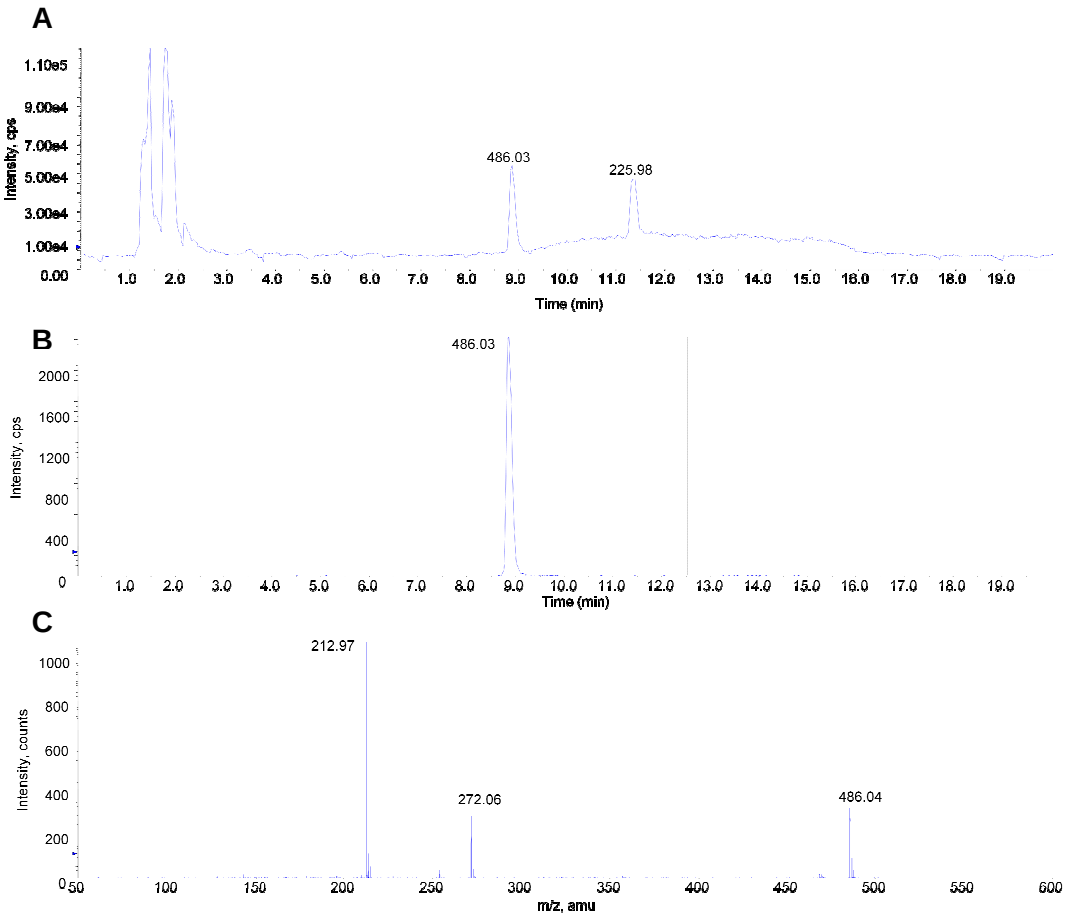


Figure 3.22: Mass Spectrometry of GST assay with TNT. A: Total ion chromatogram (TIC) of GSTU25. Peaks were also evident for Equine and U24. **3.22 B** Mass spectrum of GSTU25 (-TOF MS). Peak at 11.4 min (TNT) has a m/z ratio of 225.96, Peak at 9.0 min (Conjugate) has a peak of 486.04 **3.22 C:** MS/MS spectrum GSTU25 product peak (-TOF product) at 9.0 min. Product of 486.04 further fragments to 212.97 and 272.06.

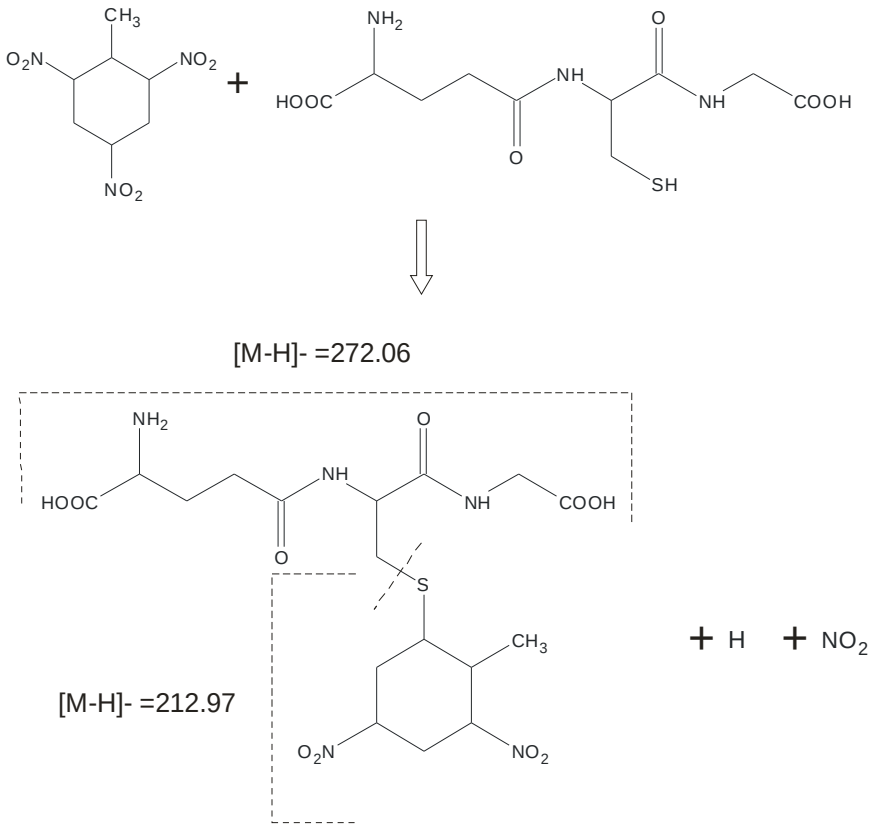


Figure 3.23: Structure of GST, TNT and their conjugation product. TNT is bound with the sulphyl group from glutathione releasing a nitro group, producing a glutathionyl-dinitrotoluene.

3.4 Conclusions

To elucidate the mechanisms behind TNT transformation in plants, the detoxification enzymes found to be upregulated in Arabidopsis were investigated. Microarray data identified seven Tau class GSTs to be upregulated greater than eight fold following TNT treatment. Microarrays are valuable for identification of transcript expression changes from an entire genome however their accuracy is low so verification of GST upregulation by RT-PCR was performed. The SYBR green dye used for RT-PCR has reliable detection limits from as little as two copies of the target, is more sensitive and specific than microarray. It therefore produces a more precise representation of transcript expression levels for individual genes than a microarray. The results for the RT-PCR followed the microarray results showing that all seven GSTs were indeed upregulated but with ~ 40 to 300 fold upregulation compared to 8 to 46 fold as seen from the microarray.

In contrast to the results from the microarray data, GSTU24 appears to be the most upregulated by TNT when measured by RT-PCR analysis, not GSTU25, however this is in accordance with the results observed by SAGE analysis, where this was the only GST shown to be upregulated following TNT treatment (Ekman *et al.* 2003).

The ATP-binding cassettes associated with transporting glutathione conjugates out of the cytosol, into the vacuole are AtMRP1 and AtMRP2. The transcripts for these transporters are also upregulated in the microarray data, 2 and 4.8 fold respectively (Lorenz 2007), suggesting that Arabidopsis may conjugate TNT to GSH and transport the resultant hydrophilic molecule into the vacuole.

This upregulation suggests that the GSTs may be active against TNT by catalysing the conjugation of TNT to GSH. However the induction and regulation of GSTs is complex and GSTs perform a variety of roles besides conjugation. Plant GST promoters contain *ocs* elements and other motifs which are activated by a variety of electrophilic agents including both active hormones, auxin, salicylic acid and inactive hormone analogues, as well as other oxidative stress causing agents like heavy metals and hydrogen peroxide (Ulmasov *et al.* 1995; Frova 2003). It is therefore possible that the upregulation of these seven GSTs is caused by a general stress response resulting from the other stresses induced by

TNT treatment, not a specific detoxification response (Zhang *et al.* 1994; Chen *et al.* 1999). The GSTs are commonly upregulated in response to a variety of stresses (Section 3.1.3), many of which induce a predictable, coordinate GST response although no increase in conjugation products are seen, suggesting another role for GSTs (DeRidder *et al.* 2002; Wagner *et al.* 2002; Sappl *et al.* 2009). Also, many GSTs appear to exhibit close co-regulation in microarray data, for example GSTs U3 and U4; but this may also be due to the inability of microarray probes to distinguish between these two genes, or perhaps that they share promoter sequence (Dixon *et al.* 2010). This suggests that upregulation of GSTs in response to TNT stress is not due to a role in TNT detoxification but instead is the result of a stress responsiveness pattern. However, the observed activity of the upregulated GSTs with TNT does infer that there may be some *in vivo* conjugating activity toward the explosive, and not just a coordinated stress response. This will be investigated in the remaining chapters of this thesis.

To investigate the roles that these enzymes might play in the response of plants to TNT, the GSTs were successfully cloned, expressed and purified.

Purification difficulties were observed when using GSH sepharose for all the recombinant GSTs except GSTU24 and U25. It has been observed that binding affinities to GSH vary depending on the electrophilic co-substrate used (Axarli 2008). For example, the K_m^{GSH} for ZmGSTF1 varies between 3.6 μM and 937 μM for cumene hydroperoxide and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) respectively (Axarli *et al.* 2008). It has been proposed that this results from intra-subunit modulation between the G-site and the H-site (Labrou *et al.* 2001). Subsequently, it is therefore possible that the binding affinities for these tau GSTs are not sufficient for GSH affinity purification in the absence of an electrophilic co-substrate. These difficulties were not observed when purification was achieved with a nickel affinity column, via the His-Tag of the LIC-vector.

Previous characterisation of the Arabidopsis Tau GSTs has shown GSTU1, U3, U4, U7 and U22 to have very little CDNB activity while GSTU24 and U25 are highly active (Dixon *et al.* 2009).

Many GSTs have little or no activity with CDNB which shows it is not a universal assay for catalytic activity, but in addition it is also possible that not all GSTs

exhibit any conjugation ability, let alone with CDNB but instead act as ligandins, or GSH-dependent peroxidases (GPOX). The lack of activity observed for GSTs U1 and U4 does not therefore prove inactivity as they may still be active against a different substrate. Previous work with the GSTs used in this study has shown that only GSTU24 and U25 show significant GPOX activity (determined by activity with cumene hydroperoxide) and these are also the most active CDNB conjugating enzymes (Dixon *et al.* 2009). Glutathionylation by GSTs is their best studied activity, however little evidence suggests this to be a major role of GSTs with endogenous substrates. Perhaps this is due to instability or rapid metabolism of conjugates by the corresponding processing pathway. It is likely that some natural conjugation occurs, otherwise the pressure to maintain this co-regulation would be lost. However there are other characterised roles of GSTs; non conjugative as well as non-enzymatic for example transport and stabilisation of flavonoid pigments. They likely play important roles in the metabolism of endogenous compounds for example fatty acid derivatives and porphyrinogens, though further characterisation of putative natural substrates is required (Dixon *et al.* 2008; Dixon *et al.* 2009).

Purified enzymes were assayed for activity with TNT; samples were analysed by Griess and HPLC. The GSTs which are most active with CDNB (GSTU25 and U24) are similarly the most active with TNT. This could either be an indication of overall activity of the enzymes, or it could be a reflection of the structural similarities of the two xenobiotics. Characterisation of CDNB conjugation has shown that a glutathionyl-dinitrobenzene conjugate is produced by removal of the chloride ion (Van Der Aar *et al.* 1996; Bowman *et al.* 2007). Although TNT lacks any chloride atoms, active site residues may still play a similar role for the two substrates. Previous work has also found Equine GSTs to have activity towards TNT (Brentner *et al.* 2008), conjugating it to GSH via the removal of a nitro- group producing glutathionyl-dinitrotoluene. There is an absence of colour for GSTU22 which had also caused a decrease in TNT concentrations when measured by HPLC. This could be due to its activity with TNT by other means than nitro-group removal. However it is more likely that the Griess assay is less sensitive as the product spectra observed on the HPLC traces for GSTU22 was identical to U24 and U25, which do cause nitrite liberation.

Mass spectrometry of the conjugates produced by GSTU24 and U25 show the conjugation of GSH and TNT via liberation of nitrite, as also observed by Brentner et al. 2008 with equine GST. U25 was the more active of those tested, releasing more than four times as much nitrite and GSTU24. Therefore U25 was selected for more detailed biochemical characterisation, presented in subsequent chapters within this thesis. The *in vitro* conjugation activity of GSTs U24 and U25 suggests that they have an *in vivo* role in the transformation of TNT, this will also be investigated.

Chapter 4: The Biochemical Characterisation of AtGSTU25

4.1 Introduction

Hundreds of plant GSTs have been characterised due to their importance in herbicide detoxification or to elucidate their endogenous roles. This includes whole families of GSTs from Arabidopsis (Dixon *et al.* 2009) and poplar (Lan *et al.* 2009) and many individuals from maize, rice and wheat. Studies have used mixed GSTs purified directly from a crude plant extract using GSH columns; however, this approach has only proven effective for a limited number of GSTs, mostly within the Tau and Phi classes. Because of the limitations, this method has only been used to give an idea of the broad activity present in the GST family rather than that of individual enzymes. To characterise individual GST enzymes, the genes have been cloned, expressed in bacterial systems and purified using affinity tags or GSH binding (Thom *et al.* 2002; Dixon *et al.* 2009; Lan *et al.* 2009)

The activities of purified GSTs towards a range of substrates have been tested. These include herbicides and endogenous substrates such as porphyrinogens and oxylipins, in addition to chlorodinitrobenzene (CDNB) and benzylisothiocyanate (BITC), a natural plant compound with antibiotic properties (Brusewitz *et al.* 1977), which are considered to be a generic substrates, as many GSTs have been found to have activity towards them (Figure 4.1). For Arabidopsis, 41 of the total 54 GSTs have now been purified, recombinantly expressed and assayed for activity (Dixon *et al.* 2009). However, while many of the GSTs have activity towards the generic substrate CDNB and/ or BITC, others have no known substrates including AtGSTU11 and AtGSTF5 and 14 (Dixon *et al.* 2009). It has been proposed that some GSTs have no conjugating activities at all but instead may be involved in intracellular signalling, responses to cytokinin and auxin hormones and transport of secondary metabolites (Lan *et al.* 2009; Dixon *et al.* 2010). This is discussed in more detail in Chapter 5.

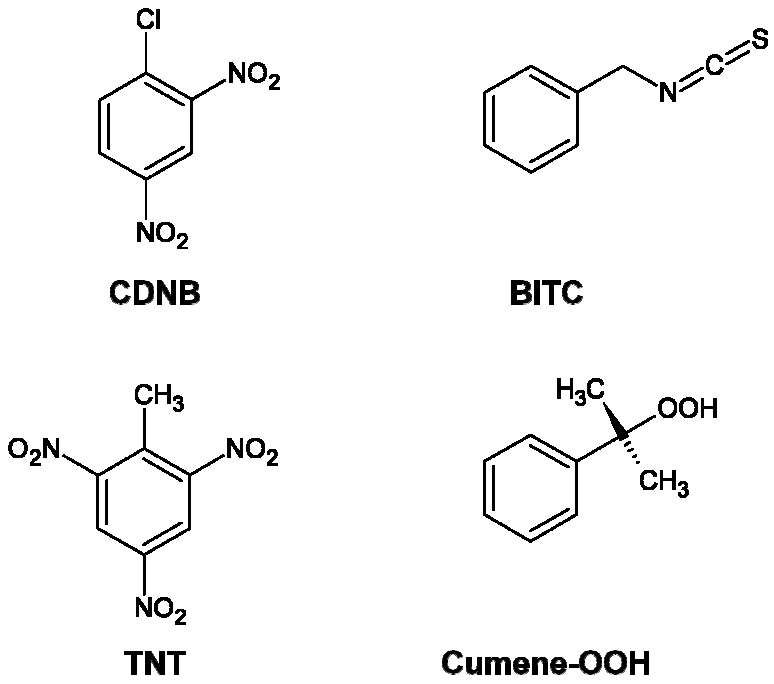


Figure 4.1: Structures of known GSTU25 substrates: chlorodinitrobenzene (CDNB), benzylisothiocyanate (BITC), 2,4,6-trinitrotoluene (TNT) and cumene hydroperoxide (Cumene-OOH).

The conditions for assaying GSTs are mostly variations of those first determined by Habig et. al (1974) to measure the conjugation of CDNB and GSH by crude rat liver GST extracts. The spectrophotometric assay contains 100 mM potassium phosphate pH 6.5, 1 mM CDNB, 5 mM GSH (in excess) at 25 ℃ (Habig *et al.* 1974) and monitors the increase in absorbance at 340 nm as CDNB is conjugated (see Fig 3.5). Activity assays of other substrates are often based on the same method, though as many conjugation reactions do not cause large changes in spectral characteristics, analysis by HPLC is often preferred. Generally no cofactors or reducing factors are necessary for activity, though the addition of BSA as a carrier protein may aid activity in reactions with low levels of purified GST. All plant GSTs characterised with CDNB have pH optima between 6.5 – 8.5 and are assayed between 25 ℃ and 37 ° C, with protein inactivation at temperatures over 55 ℃ (Scalla *et al.* 2002; Edwards *et al.* 2005; Nutricati *et al.* 2006; Cho *et al.* 2007) .

Members of the GST family exhibit a wide range of substrate specificities, not only within the distinct classes, a reflection of the low sequence identity in the hydrophobic binding site but single enzymes can be catalytically active towards a variety of structurally dissimilar substrates. The H-site is a large hydrophobic cleft with numerous putative binding residues, lying adjacent to the glutathione binding site (see Figure 1.8) (Thom *et al.* 2002). The most studied Arabidopsis GST, AtGSTU19 (previously known as AtGST8), is a good example of a GST with a wide substrate range. It is active against both commonly used GST generic substrates CDNB, BITC and has high activity towards a range of herbicides including chloroacetanilides and diphenyl ethers in addition to binding many fatty acids, oxophytodienoic acid and 2-*S*-glutathionylchlorogenic acid (DeRidder *et al.* 2006; Dixon *et al.* 2009).

Recently, GSTU25 has been identified as having high glutathione peroxidase (GPOX) activity towards the synthetic substrate cumene hydroperoxide (Figure 4.1), however it has surprisingly little activity with the putative *in planta* substrates; fatty acid hydroperoxides (Dixon *et al.* 2009). GSTU25 has the ability to bind short chain length fatty acids adducts, and when expressed in *E. coli* hydroxylated fatty acids bind specifically to the enzyme, while the closely related GSTU28 only accepts non-hydroxylated fatty acids (Dixon *et al.* 2009). When the Arabidopsis GSTs are expressed in tobacco, U25 and U28 both accept a wide range of fatty acid derivates including oxophytodienoic acid (OPDA), which is thought to be conjugated through Michael addition to the α,β- unsaturated carbonyl group (Dixon *et al.* 2009). This is an example of the substrate diversity of closely related GSTs. Additionally; GSTU25 may conjugate endogenous substrates to GSH, or act as a carrier for spontaneously formed conjugates which, once de-glutathionylated, may regain activity. BITC is a compound commonly used as a generic substrate, although unlike CDNB, BITC is mainly specific to the Tau class GSTs (Figure 4.1). Despite this, the Tau GST; GSTU25 has low activity with BITC, only 62 nkat.mg^{-1}, compared to 124 nkat.mg^{-1} of GSTU24 (which has 78 % protein identity with GSTU25), though this low activity is not unusual within the phylogenetic clade to which GSTU25 belongs (Dixon *et al.* 2009).

In Chapter 3 the activity of GSTU25 with TNT, producing a conjugate with the removal of a nitro-group, was shown. As GSTs are well known to have roles in detoxification through conjugation and other means (Marrs 1996), then the conjugation of TNT to GSH could be an important pathway in the detoxification of TNT by plants. In this Chapter the activity of AtGSTU25 with TNT has been further characterised. Optimal assay conditions including; substrate concentration, pH, temperature and time have been identified. Substrate specificity was assessed with compounds related to TNT, including explosives and TNT transformation products. Kinetic values of the reaction for both CDNB and TNT are also presented.

4.2 Methods

4.2.1 Cloning, Expression and Purification

Cloning and protein expression were performed as described in Chapter 3. Pure protein was produced by affinity chromatography through a 5 mL glutathione (GSH) sepharose column; GSTrap 4B (GE Healthcare, Little Chalfont, UK). The column was equilibrated with five column volumes of PBS pH 7.4. Filtered lysate (see Section 2.4.2) was applied at a flow rate of 0.5 mL/ min. Five column volumes of PBS was used to wash the column before elution with one column volume of 50 mM Tris-HCl with 10 mM GSH pH 8.0. The first 1 mL of eluate was discarded due to impurities and low yield. Protein concentration was measured by Bradford assay and purity determined by SDS-PAGE analysis (Section 2.4.4). Aliquots of pooled eluate were frozen at -80 ℃ until use.

4.2.2 TNT Enzyme Assay

Assays with TNT contained GST, TNT, GSH and buffer, as determined by optimisation assays. Reaction times were three to 24 h at 30 ℃ with 100 mM potassium phosphate buffer pH 6.5, 200 μM TNT, 5 mM GSH and enzyme was added to start the reaction. Reactions were stopped with 10% (v/v) TCA (240 mg / mL) and stored at -80 ℃ until analysis. Samples were centrifuged at 16 000 x *g* for 10 min after defrosting to remove precipitate prior to HPLC analysis. HPLC analyses were performed on a Waters Alliance 2695 separations module and a Waters 2996 Photodiode Array, with a C18 Techsphere ODS 80 A 5 μ column (250 mm x 4.6 mm) (Chromacol). Data analysis was performed with Empower Pro Analysis software. Fifty microlitres of sample was injected onto the column and run conditions are outlined in Table 4.1

Table 4.1: HPLC run conditions, Buffer A is methanol and Buffer B is 2 mM ammonium acetate with 5 % methanol. Sample and column chamber were set at 25 ℃.

Time (min)	Buffer A (%)	Buffer B (%)
0	20	80
4	20	80
7	60	40
12	60	40
15	20	80
25	20	80

4.2.3 ADNT RDX, PETN, DNT

The TNT, hexahydrotrinitrotriazine (RDX), and pentaerythritoltetranitrate (PETN) were supplied by the Defence Science and Technology Laboratory (DSTL, Fort Halstead, UK). Dinitrotoluene (DNT) was purchased from Sigma Aldrich (Poole, UK). Stock solutions were prepared to a 1 M concentration in dimethylsulphoxide (DMSO). RDX and DNT were assayed at 180 μM, the solubility limit of RDX at 20 ℃. PETN was assayed at 6.6 μM as it is highly insoluble. Aminodinitrotoluenes (ADNTs) were obtained from Accustandard (New Haven, US), as a 1 mg/ml stock, 40 μM ADNT were used for reactions. Assays were performed in triplicate and contained appropriate substrate, 1 mg/mL enzyme and 5 mM GSH in potassium phosphate pH 6.5. A positive TNT control was also run in parallel. Experiments were carried out at 30 ℃ over 24 h and stopped with TCA. Samples were analysed at various intervals and reactions were stopped with 10 % (v/v) TCA (240 mg / mL) and centrifuged at 16 000 x *g* for 10 min prior to loading on the HPLC and run according to the conditions outlined in Table 4.2, with a C18 Techsphere ODS 80 A 5 μ column (250 mm x 4.6 mm) (Chromacol) for PETN and a Sunfire C18 5 μm column (Waters, Wexford, Ireland) for all other substrates. For PETN an isocratic run of 50 % Methanol and 50 % water for 25 min was used. TNT, RDX, DNT PETN and ADNT peaks were analysed at a wavelength of 230 nm (Figure 4.2).

Table 4.2: HPLC run conditions, Buffer A is methanol and Buffer B is 2 mM ammonium acetate with 5 % methanol. Sample and column chamber were set at 25 ℃.

Time (min)	Buffer A	Buffer B
0	60	40
3	60	40
4	20	80
7	60	40
15	60	40

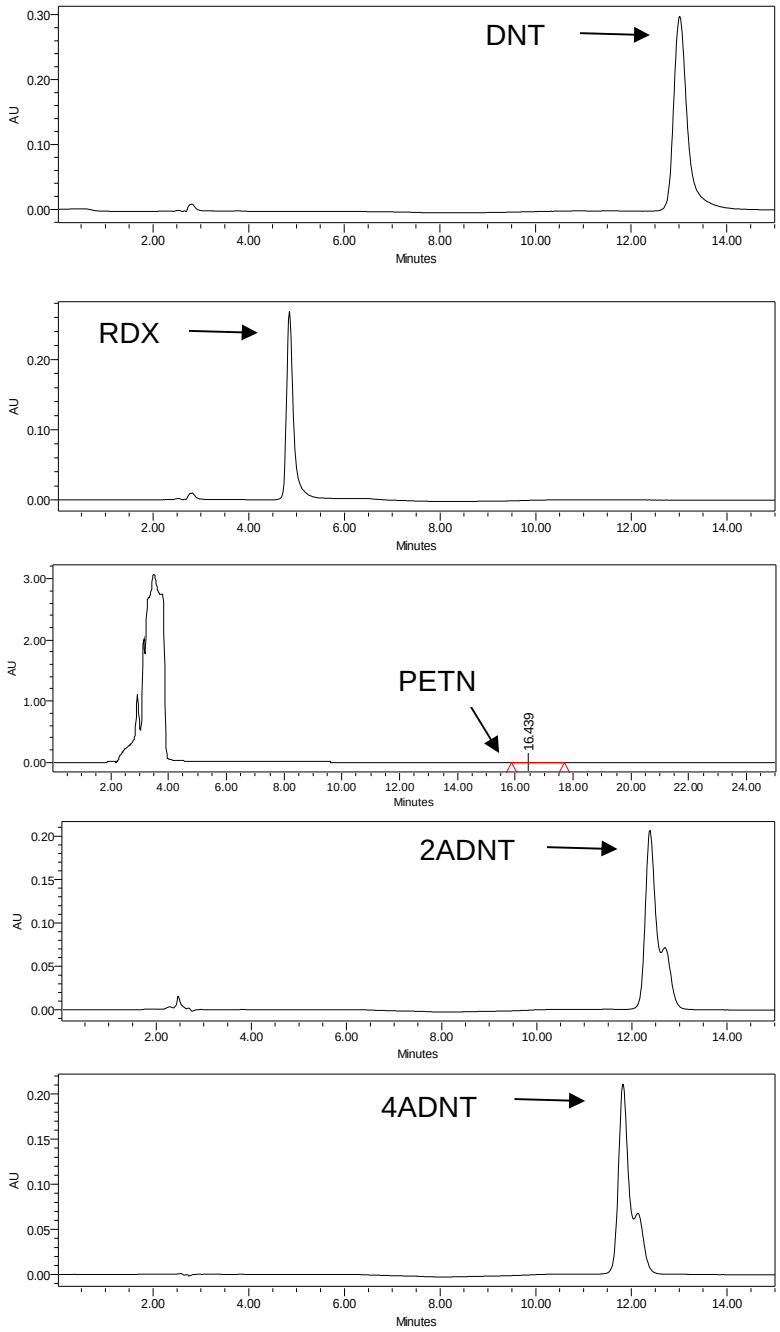


Figure 4.2: HPLC traces of the substrates assayed for GST activity.

4.2.4 CDNB Activity Assay

A spectrophotometric assay was performed with GSTU25 and CDNB as described in Chapter 3.2.6.1. A range of CDNB concentrations was used, from 25 to 1000 μM, the rates of CDNB conjugate production were measured in triplicate over 1 min and Michaelis-Menten kinetic parameters were calculated.

A non-linear regression was applied to the rate plotted against CDNB concentration, using the equation;

$$y = \frac{ax}{b + x}$$

where a =V_{max} and b = K_m.

4.2.5 Kinetic Analysis with TNT

The TNT solubility in DMSO was measured by adding various concentrations (25 to 1000 μM) of TNT to solutions of 1, 5 and 10 % DMSO. These were then analysed by HPLC analysis for apparent TNT concentration. Effects of DMSO on enzyme activity were analysed by measuring conjugate production in a standard TNT assay with the addition of 0 to 20 % DMSO. Kinetic experiments were performed with 0 to 1000 μM TNT, 5 mM GSH, 2.27 mg / mL enzyme in 100 mM potassium phosphate buffer pH 6.5, with samples removed every 5 min over a 27.5 min time course. Aliquots were stopped with 1 % TCA (v/v) and analysed by HPLC. To calculate kinetic values the rate of the points over 25 min were plotted and a non linear regression was applied by sigma plot;

$$y = \frac{ax}{b + x}$$

where a =V_{max} and b = K_m.

4.3 Results

In Chapter 3 it was shown that GSTU25 exhibits the highest activity against TNT when compared to all the other GSTs upregulated in Arabidopsis plants after 6 h TNT exposure. It is also one of only two Arabidopsis GSTs found to release nitrite from TNT. For these reasons it was selected for further characterisation. Due to the lack of previous analysis of recombinant GSTs with TNT, optimisation of assay conditions was undertaken and kinetic parameters were established.

4.3.1 Establishing Assay Conditions: GST Concentration

To assess a suitable quantity of enzyme for use in assays a time course was performed over 24 h with various concentrations of enzyme (determined by Bradford assay, see Chapter 3.2.5). Concentration of the product could not be determined due to lack of commercially available standards and it was not possible to purify significant quantities to establish the extinction coefficient. A non-enzymatic conjugation reaction with GSH and TNT was attempted at pH 9 and pH 10 to produce conjugate standards; however, this was unsuccessful and no decrease in TNT was observed by HPLC analysis of these samples. This may have been due to rapid oxidation of GSH in the system. Conjugate concentrations are therefore represented by HPLC peak area measurements. All enzyme concentrations tested produced conjugate at reliably detectable levels. Equine GST was assayed at 11.5 U /mL. Figure 4.3 shows the decrease in TNT concentration appears to be inversely related to the increase in product. Over 24 h only half the TNT had been transformed, therefore subsequent assays, which were run over shorter time courses, sometimes contain higher concentrations of enzyme. There is also a striking levelling off of activity between 6 h and 24 h, likely due to oxidation of GSH as TNT concentration remains high, another reason could be product inhibition. Further assays were run over a shorter time course to minimise the consequences of this.

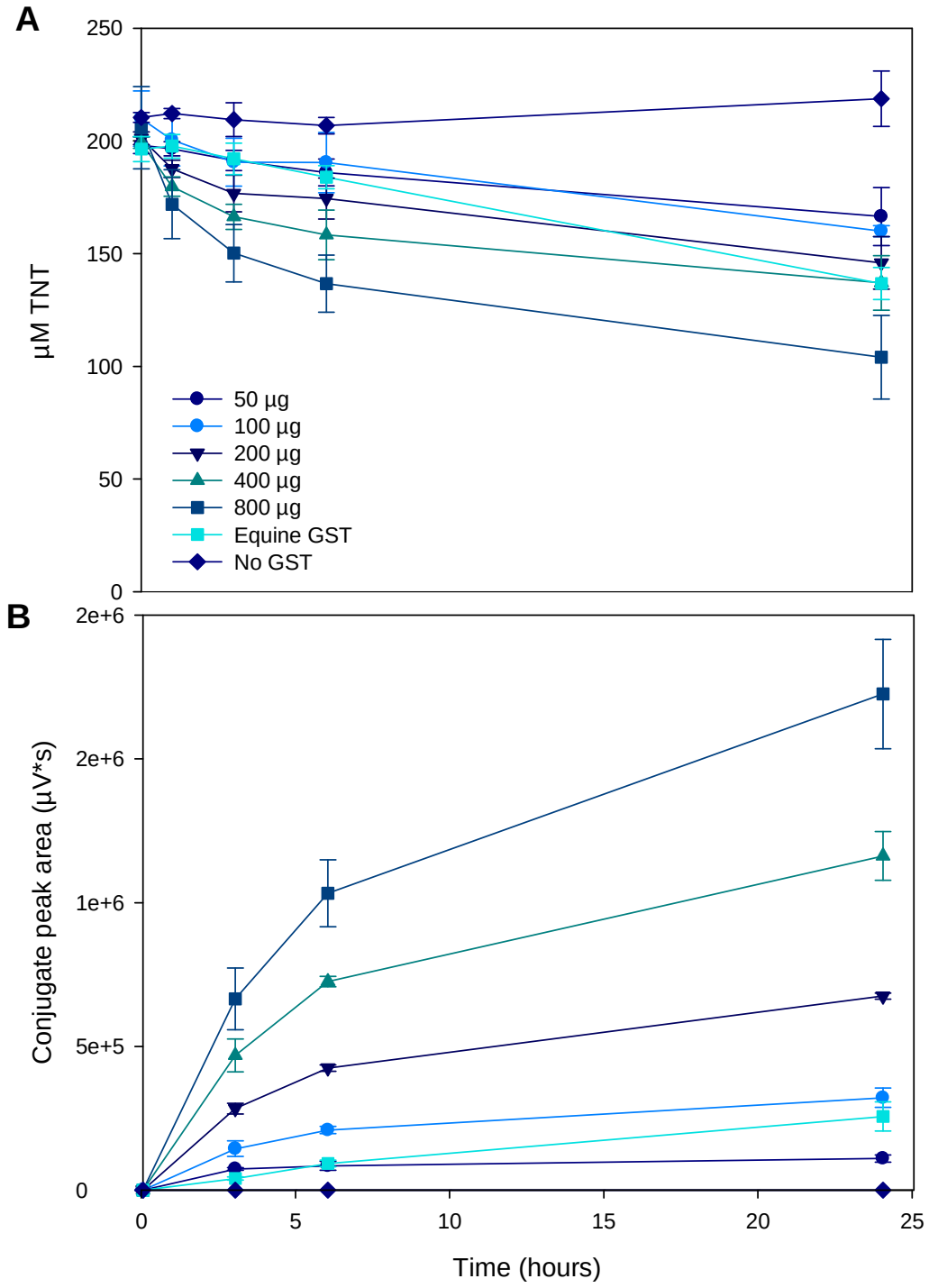


Figure 4.3: Effect of enzyme concentration on GST activity towards TNT:TNT depletion and conjugate production. A 24 h time course assay with 200 μM TNT, 5 mM GSH and 50 – 800 μg GSTU25 in 100 mM phosphate buffer pH 6.5. **4.3 A: TNT concentration and 4.3 B: Conjugate peak area.**

4.3.2 Establishing Assay Conditions: TNT Concentration

To ensure that TNT and product levels were detectable and that their respective decline and production is linear, a TNT concentration assay was performed. The enzyme was incubated in potassium phosphate buffer pH 6.5 with 10 to 200 μM TNT, 5 mM GSH for 120 min. Samples were removed at 0, 15, 30, 60, 120 and 180 min and stopped with 1 % TCA (v/v) to precipitate the protein. TNT has an aqueous solubility limit of 512 μM at 20 ℃ therefore to ensure all the TNT was solubilised; the highest TNT concentration used was just 200 μM. As anticipated, Figure 4.4 shows the reaction rates were fastest with the highest concentrations used; 100 and 200 μM TNT. Subsequent assays with TNT were performed with 200 μM TNT, to maximise conjugate production.

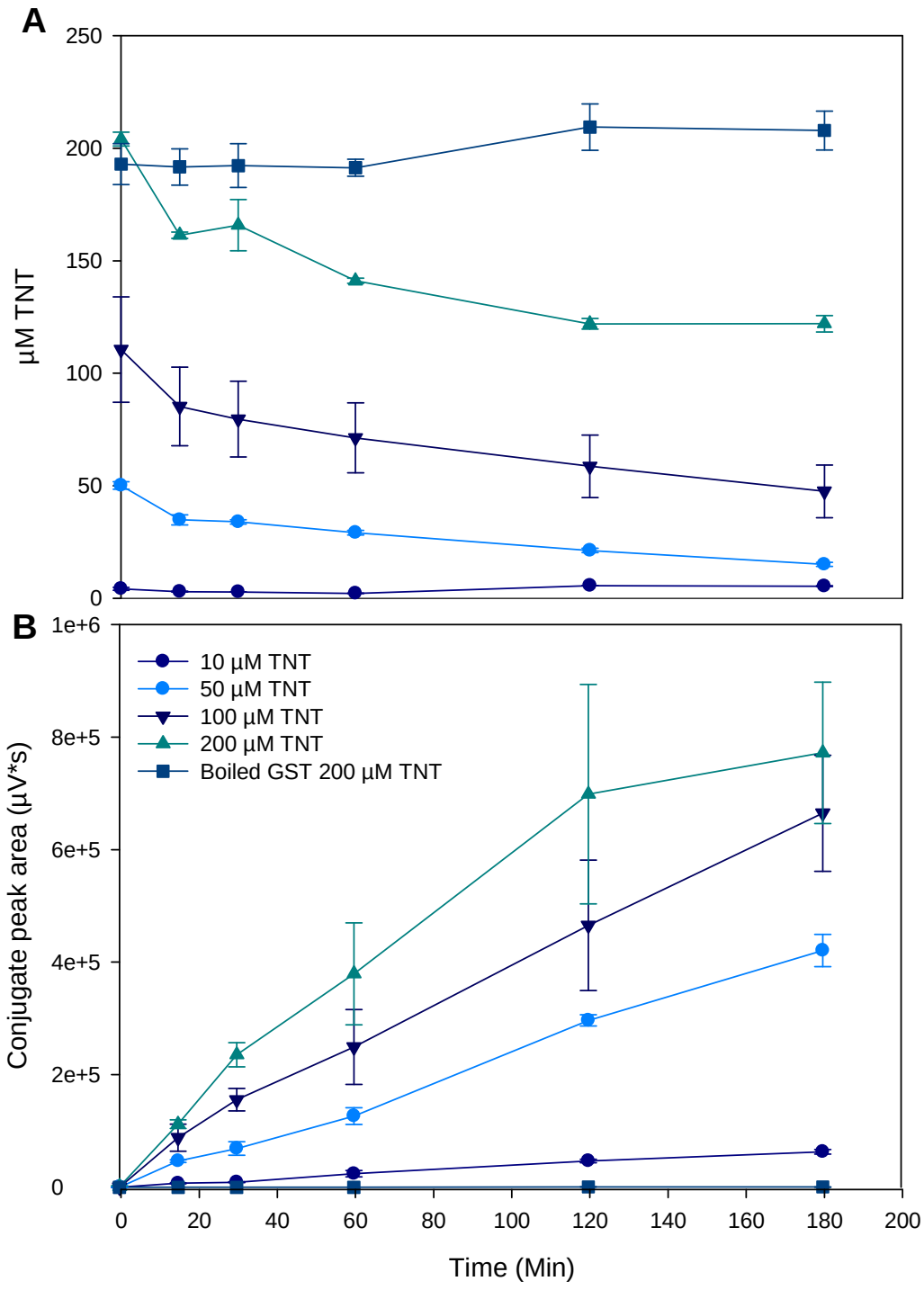


Figure 4.4: Effect of TNT concentration on GST activity towards TNT:TNT depletion and conjugate production. A 3 h time course assay with variable concentrations of, 5 mM GSH and 800 μg GSTU25 in 100 mM potassium phosphate buffer pH 6.5. A: shows TNT concentration and B: conjugate peak area.

4.3.3 Effect of pH on GST Activity

To determine the optimum pH for the reaction of GST with TNT, three different buffers were used spanning a range in pH from 5.5 to 8.5. The amount of TNT conjugate produced by 800 μg/mL GSTU25 in 400 μL after 3 h was determined by HPLC analysis (Figure 4.5). TNT depletion was also measured but as pH increases the observed TNT concentration decreases, likely due to alkaline hydrolysis of TNT (Emmrich 1999). Figure 4.5 shows the optimum pH for activity as observed by maximal GS-DNT production is pH 6.5. At pH 5.5, conjugate production by GSTU25 with TNT is reduced four-fold and at pH 8.5, a five-fold reduction was observed, though in both cases some enzymatic activity remained. No significant differences were observed between the two buffers (potassium phosphate and MOPS) at the optimum pH and further experiments were performed with potassium phosphate buffer, as its buffering range spans the optimum, where MOPS is at the limits of its capacity. The error bars are also smaller for potassium phosphate than for MOPS, this again may be due to the buffering range, but corroborates the decision to select potassium phosphate.

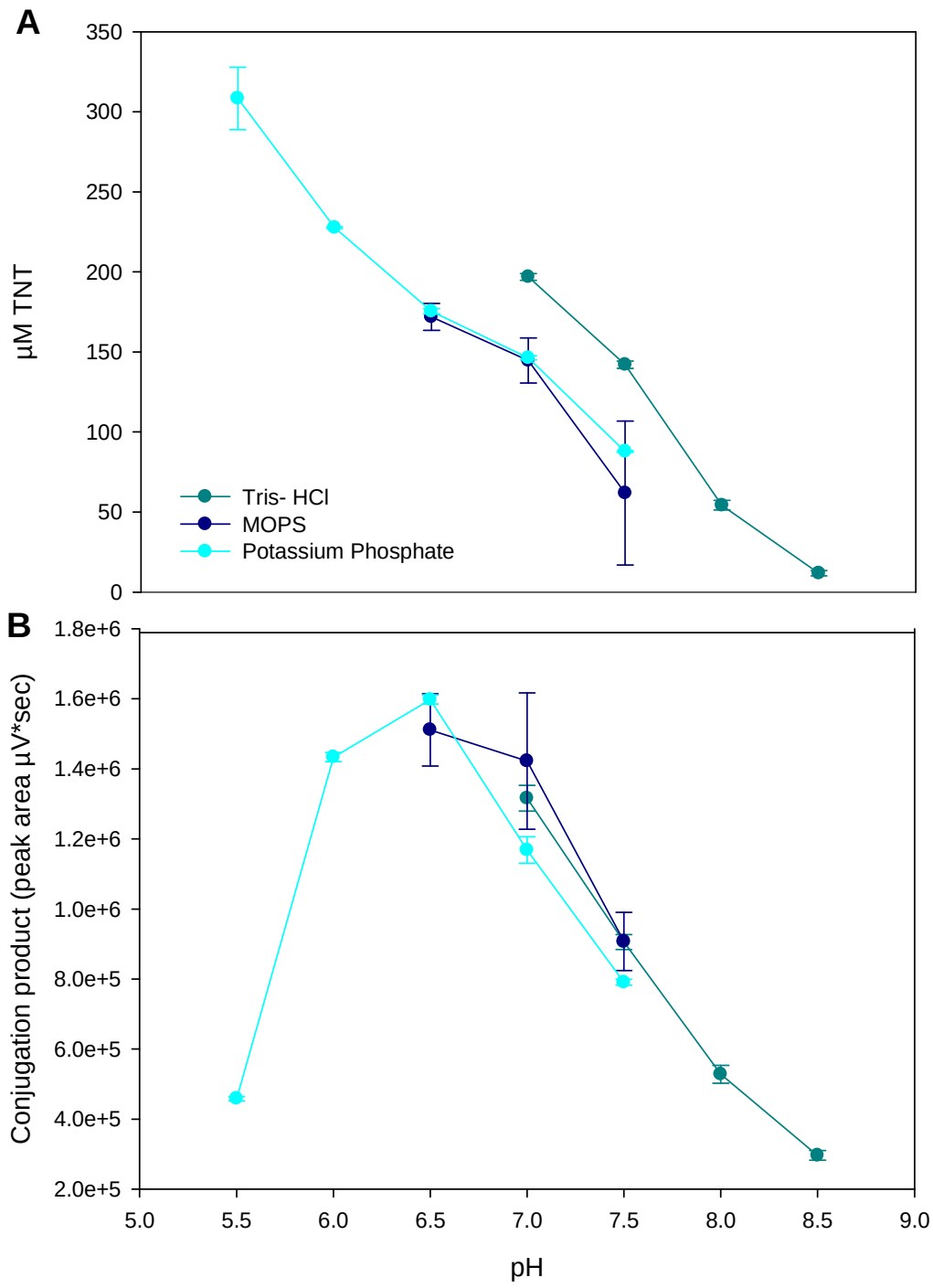


Figure 4.5: Effect of pH on GST activity towards TNT. HPLC analysis of assays performed with GSTU25, 200 μM TNT, 5 mM GSH and various buffers. The reactions were incubated at 30 ℃ over 3 h. All experiments were performed in triplicate, points are the mean and error bars represent ±1 standard deviation from the mean. 4.5 A: TNT concentration and 4.5 B: Conjugate peak area.

4.3.4 Effect of Temperature on GST Activity and Stability

It is established that enzyme activity increases with temperature, however so does enzyme denaturation. The optimum temperature for assaying an enzyme is deemed the highest temperature at which the enzyme remains stable over the length of the reaction. To discover at which temperature the reaction proceeds fastest without denaturation, GSTU25 was incubated at temperatures ranging from 4 ℃ to 42 ℃ in potassium phosphate buffer pH 6.5 with 200 μM TNT, 5 mM GSH for 120 min. Samples were removed at 15, 30, 60 and 120 min and stopped with 1 % TCA (v/v). Figure 4.6 shows the highest activity was observed at 37 ℃, the lowest activity was at 4 ℃. The rate at 42 ℃ was almost half that of 37 ℃, likely due to denaturation. The thermal stability of the enzyme was therefore measured (Figure 4.7) by incubating GSTU25 at the various temperatures for 1 h prior to addition of substrates and assaying over 3 h at 30℃. HPLC analysis of the TNT concentration was used to determine remaining activity. It is assumed that this decrease in activity is due to denaturation as at the higher temperatures, 42 ℃ and 55 ℃ precipitate was visible prior to ad dition of assay components to the enzyme. Temperature is also known to affect rates of GSH oxidation; however a large excess of GSH (5 mM) was used such that over the time course of the reaction enough GSH should remain in excess and assayed at 30 ℃ even for stability assay (Habig *et al.* 1974). The first GST assay method with CDNB used 5 mM GSH and other researchers now use between 1 to 10 mM GSH (Habig *et al.* 1974; Edwards *et al.* 2005; Dixon *et al.* 2008), all of which contain GSH in excess. A temperature of 30 ℃ was selected as the optimal temperature for further assays as GSTU25 has the highest activity at this temperature (Figure 4.6) without any loss of stability over the time course tested (Figure 4.7).

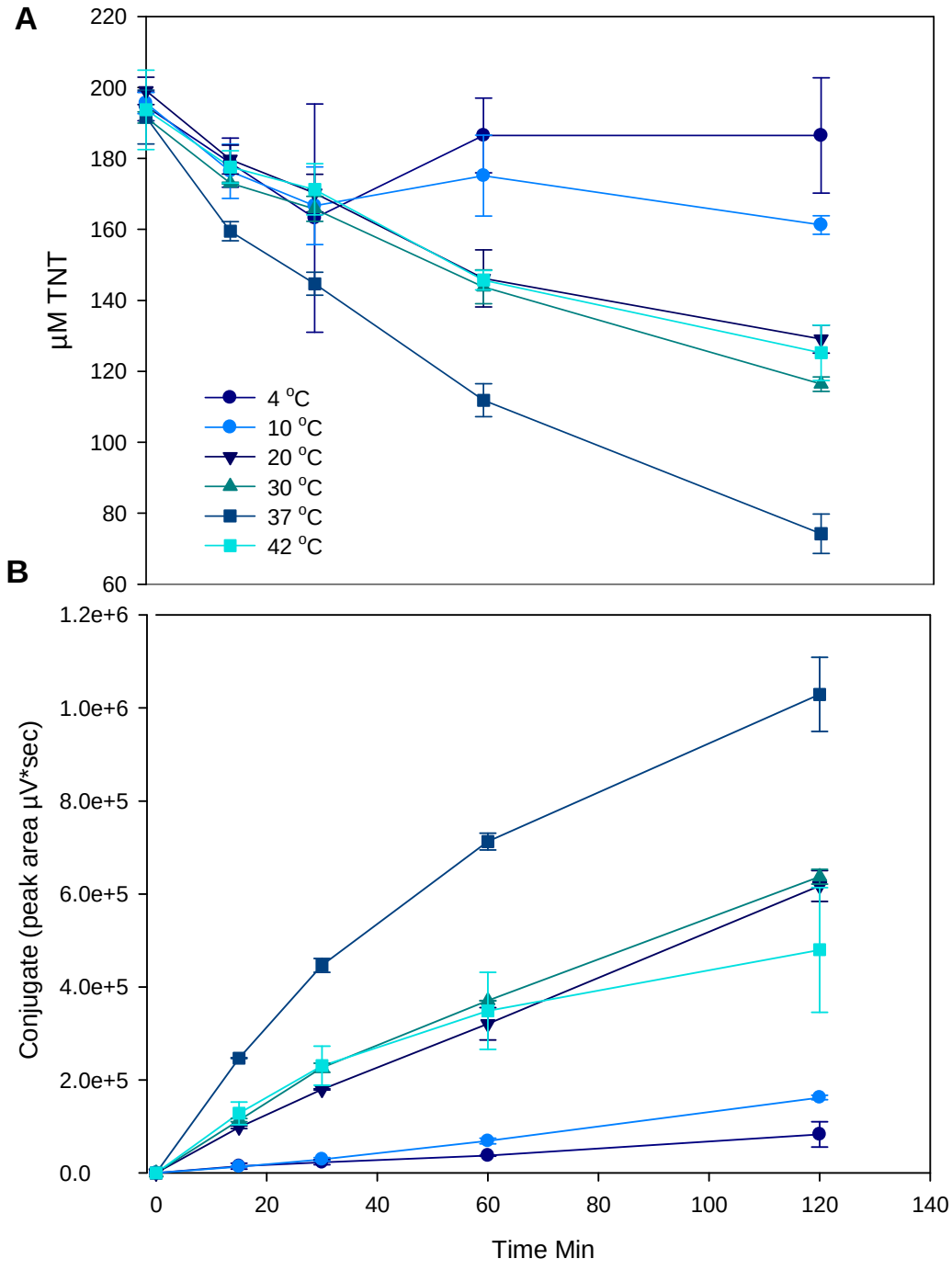


Figure 4.6: Effect of temperature on GST activity towards TNT. HPLC analysis of assays performed with GSTU25, 200 μM TNT, 5 mM GSH and potassium phosphate pH 6.5. The reactions were incubated at various temperatures from 4 – 42 ℃ over 3 h. All experiments were performed in triplicate, points are the mean and error bars represent ±1 standard deviation from the mean. A: TNT concentration and B: conjugate peak area.

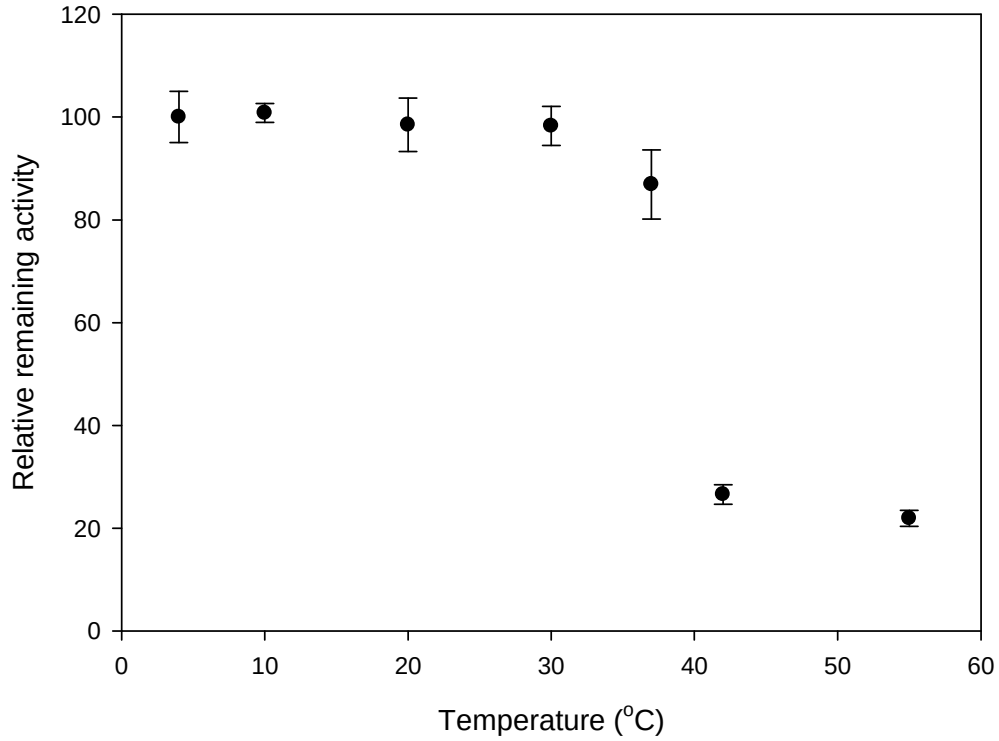


Figure 4.7: Temperature stability of GSTU25. Purified GSTU25 was preincubated for 1 h in buffer at various temperatures from 4 – 55 ℃. Solutions were cooled/ warmed to 30 ℃ before GSH and TNT were added to begin the reaction. After 3 h the samples were stopped with 1 % TCA before HPLC analysis. All experiments were performed in triplicate, points show the means and error bars represent ± standard deviation.

4.3.5 Time Course of GSTU25 and TNT

A time course reaction of GSTU25 with TNT was performed to identify the linear phase of the reaction under the optimised conditions (Figure 4.8). The decrease in TNT and the production of conjugate inversely correlate with one another, indicating that this is a simple 1st order reaction. For the first 30 min the reaction is linear, and starts to tail off by 6 h (360 min), by which time more than 60 % of the TNT is transformed, with conjugate production following the same curve. The levelling off could be due to enzyme instability or more likely, the rapid oxidation of GSH. Oxidised glutathione (GSSG) cannot act as a substrate for GSTs so the excess of GSH added at the beginning of the reaction could become limiting to the reaction. For this reason it was decided that experiments to determine the enzyme kinetics with TNT would be performed over 25 min, to ensure initial rate measurements (Section 4.5.5).

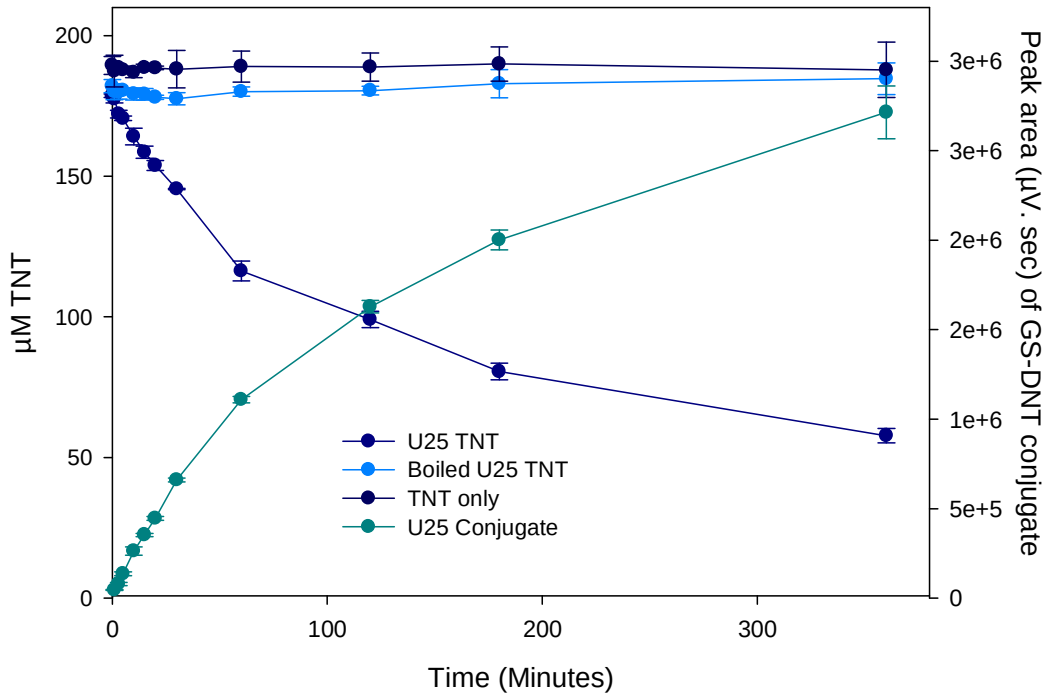


Figure 4.8 Time course of GSTU25 with TNT and GSH. Optimised conditions have been employed to produce a time course, phosphate buffer pH 6.5, 200 μM TNT, 5 mM GSH and 2mg/ml enzyme with 30 ℃ incubation. All experiments were performed in triplicate and error bars represent ± 1 standard deviation of the mean.

4.3.6 Other Substrates

In the environment, TNT pollution is often found with other organic pollutants, particularly hexahydrotrinitrotriazine (RDX) and dinitrotoluene (DNT). It is therefore of interest to test activity of GSTU25 with these substrates, as activity may reduce the requirement for alternative solutions to the other explosives. Activity towards another explosive, PETN, was also assessed, as were the reductive transformation products of TNT; ADNTs, which have been found in plants following TNT treatment (Figure 1.5) (Gandia-Herrero *et al.* 2008). Samples were analysed by HPLC and substrate consumption compared to boiled enzyme negative controls. No detectable activity was observed for these substrates (Figures 4.9 and 4.10).

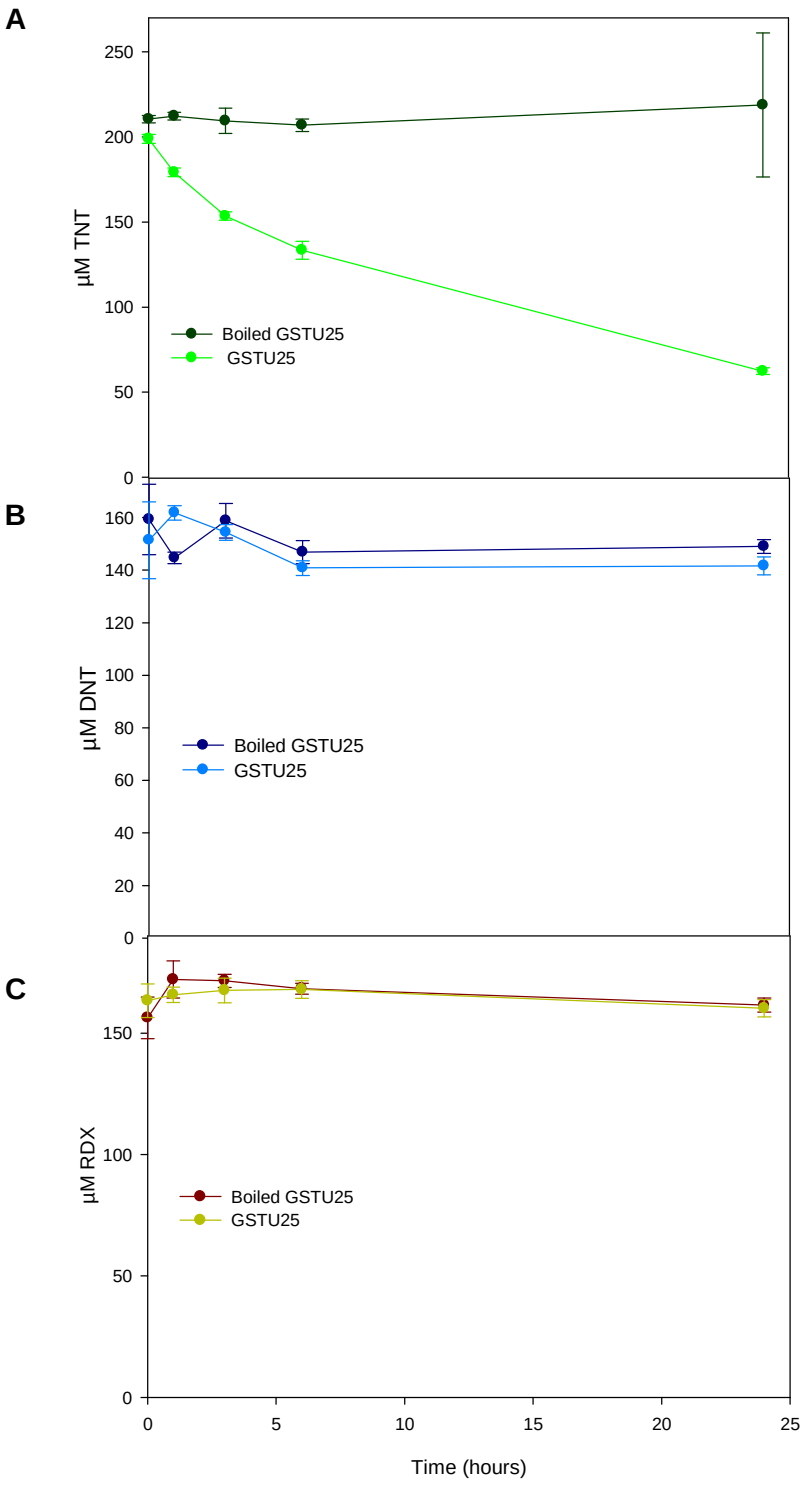


Figure 4.9: Time course of GSTU25 with alternative substrates. A: TNT, B: DNT, C: RDX. All experiments were performed in triplicate under the conditions optimised for TNT activity; 1 μg enzyme with 5 mM GSH, in phosphate buffer pH 6.5, samples were incubated at 30 ℃ over 24 h. Aliquots were removed and stopped with TCA over the time course. Reactions were analysed by HPLC.

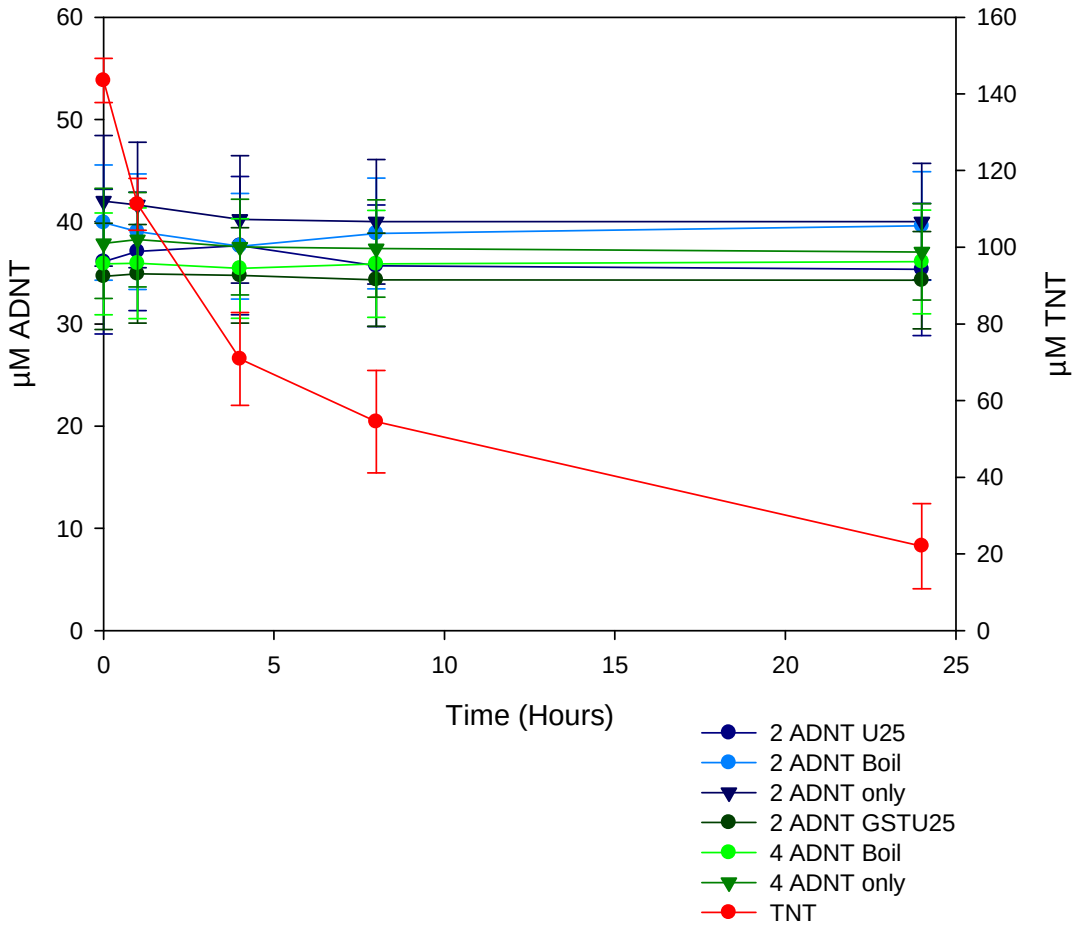


Figure 4.10: Activity of GSTU25 with ADNTs: 2-ADNT and 4-ADNT were tested as substrates for GSTU25. All experiments were performed in triplicate with 200 μM TNT or 40 μM ADNT, under the conditions optimised for TNT activity; 1 μg enzyme/ boiled enzyme with 5 mM GSH, in 100 mM phosphate buffer pH 6.5. Samples were incubated at 30 ℃ over 24 h. Aliquots were removed at 1 h, 4 h, 8h and 24 h and stopped with 1 %TCA. Reactions were analysed by HPLC.

4.3.7 Activity of GSTU25 with CDNB

A previously reported activity value for of GSTU25 and CDNB is 1240 nkat.mg^{-1} (Dixon *et al.* 2009). Assays of GSTU25 with CDNB give an average activity of 1560 nkat.mg^{-1}. Kinetic analysis of this reaction performed with 0 to 1000 μM of CDNB gives a K_m of 30.55 µM CDNB and a V_{max} of 28.06 μM.sec^{-1}. The Michaelis-Menten plot used to calculate these values, by non-linear regression is shown in Figure 4.11.

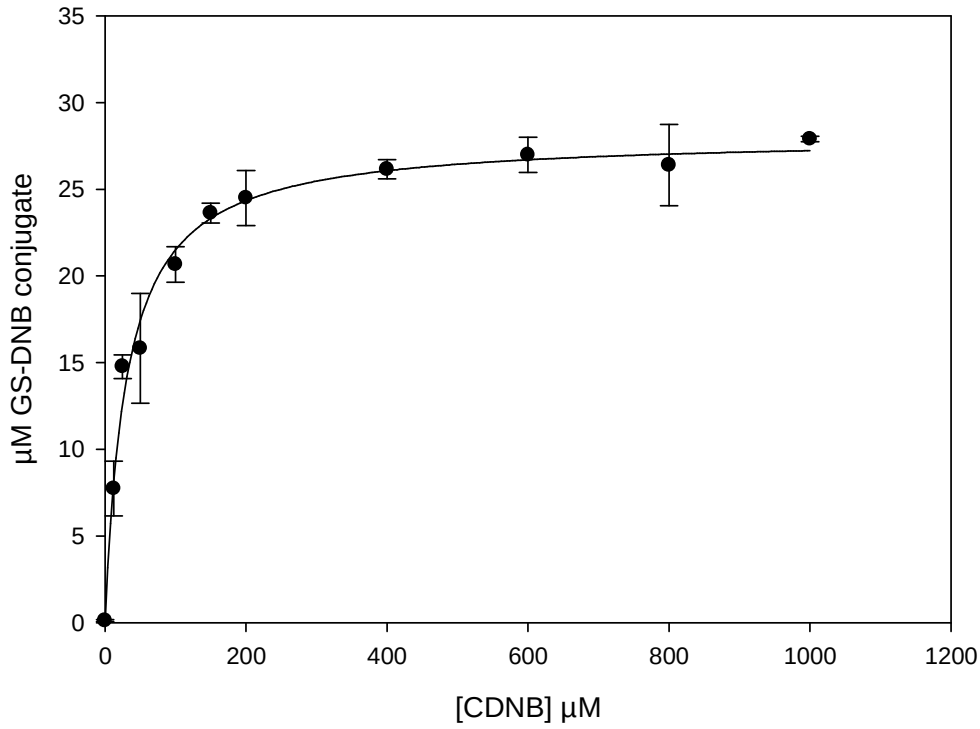


Figure 4.11: Michaelis-Menten plot of GSTU25 with CDNB. Rates were calculated from triplicate experiments with various CDNB concentrations, measured by 1 min spectrophotometric assays at 340 nm. 1 mL reactions contained 5 mM GSH, 0 – 1000 μM CDNB and 9.6 μg of GSTU25 in potassium phosphate buffer pH 6.5.

4.3.8 Kinetics of GSTU25 with TNT

To increase the concentrations of TNT in reactions for kinetic analysis, stock solutions of TNT were made by dissolving TNT in DMSO, a common solvent for stock solutions as it has high dissolving abilities, low chemical reactivity and low vapour pressure. To determine the concentration of DMSO required for increasing solubility to 1 mM, various TNT concentrations were dissolved in 0, 5 and 10 % DMSO (Figure 4.12). These samples were analysed by HPLC to determine which concentrations give an accurate representation of TNT concentration. Although both 10 and 5 % DMSO concentrations solubilise TNT to 1 mM, 5 % was selected as high DMSO concentrations (> 10 %) have documented effects on analytical instruments (Tjernberg *et al.* 2006).

DMSO has varied effects on proteins and enzyme activity, at high concentrations has been found to have stabilising or denaturing effects on different proteins. In activity assays it can behave as an inhibitor or can increase activity (Perlman *et al.* 1968). Even at low concentrations it has been shown to lead to protein aggregation or degradation and alter binding properties (Tjernberg *et al.* 2006). It was therefore decided that if the addition of DMSO was necessary for increasing TNT solubility, its effects on GSTU25 should be assessed. Figure 4.13 shows that no effect on TNT conjugate formation by GSTU25 was seen, even up to 20 % DMSO.

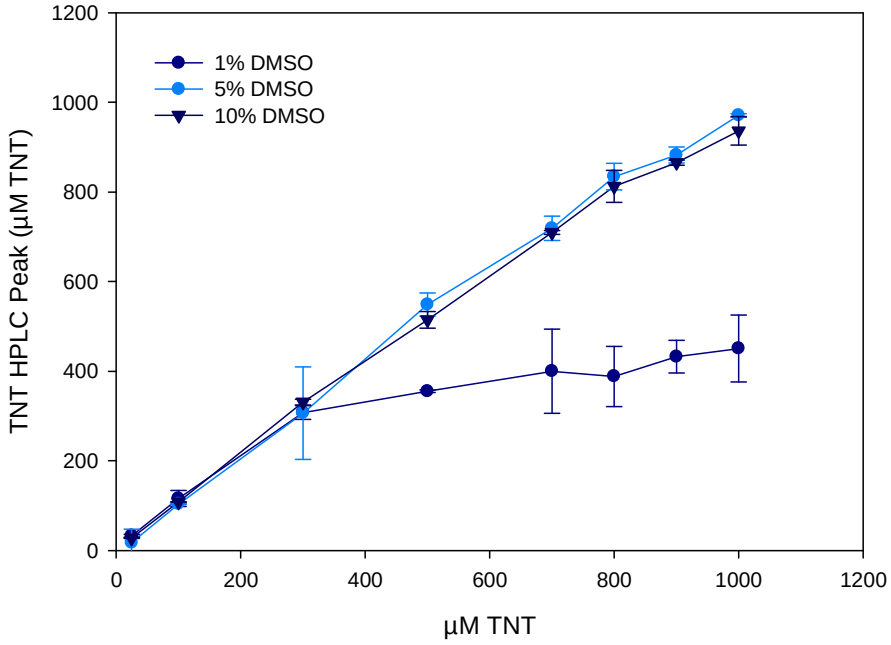


Figure 4.12: Effect of DMSO concentration on TNT solubility. Various concentrations of TNT were dissolved in 1, 5 and 10 % DMSO before HPLC analysis.

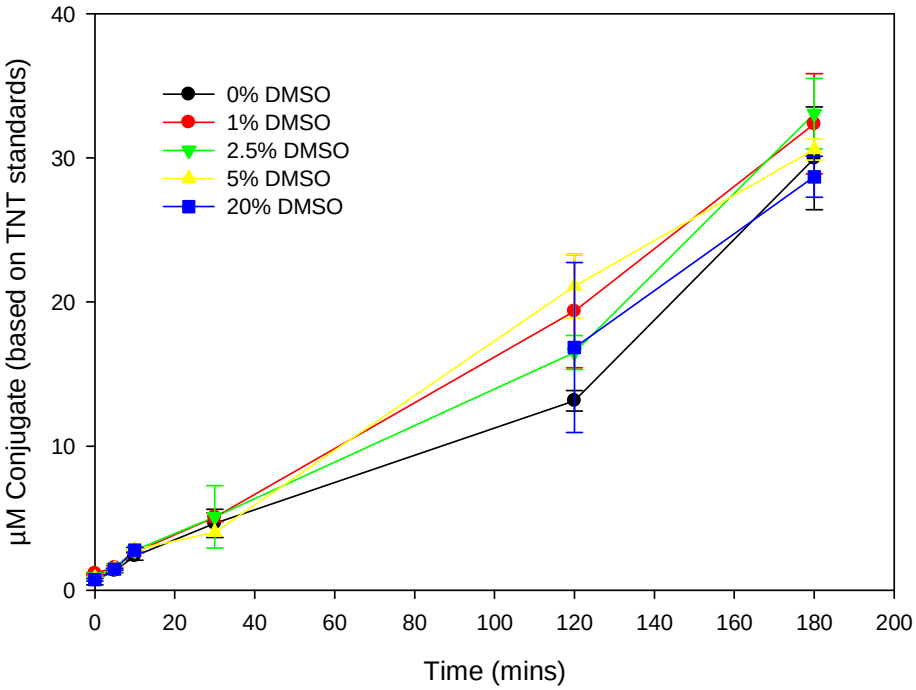


Figure 4.13: Effect of DMSO on GSTU25 activity. Activity assays were performed with five different DMSO concentration in experiments containing 1 mg GSTU25 with 200 μM TNT, 5 mM GSH in 100 mM potassium phosphate buffer pH 6.5. Experiments were performed in triplicate with error bars representing standard deviations.

A saturation curve was performed to measure K_m and V_{max} of GSTU25 with TNT. Assays were performed under the optimised conditions, 5 % DMSO, 5 mM GSH and 2.86 mg GSTU25 in 100 mM phosphate buffer pH 6.5 with various TNT concentrations of 25 to 1000 μM. Assays were performed over 27.5 min to ensure measurement of the linear reaction phase, samples were taken every 5 min, stopped with 1 % TCA (v/v) and frozen at -80 °C until run on the HPLC. Figure 4.14 shows the time course reactions at each TNT concentration, with values for both TNT depletion and conjugate production.

Using the gradients of the lines from Figure 4.14, the reaction rates were calculated for TNT depletion, these were then plotted against TNT concentration to produce a Michaelis-Menten plot (Figure 4.15). The kinetic parameters V_{max} and K_m were calculated from experimental steady state reactions by non linear regression analysis using SigmaPlot. For TNT, a linear relationship between rate and concentration was observed even up to 1000 μM TNT and the V_{max} was not reached so values were extrapolated. Clearly for TNT the linearity causes problems of accuracy of extrapolation but the values calculated for TNT depletion are K_m 8219 ± 232.4 μM and V_{max} 210.2 ± 5.4 μM min^{-1}. For the conjugate production a Michaelis-Menten plot was also produced and the rate does demonstrate some levelling off at higher substrate concentrations, however again, a V_{max} was not reached. Due to the lack of standards for product concentration no kinetic values could be determined.

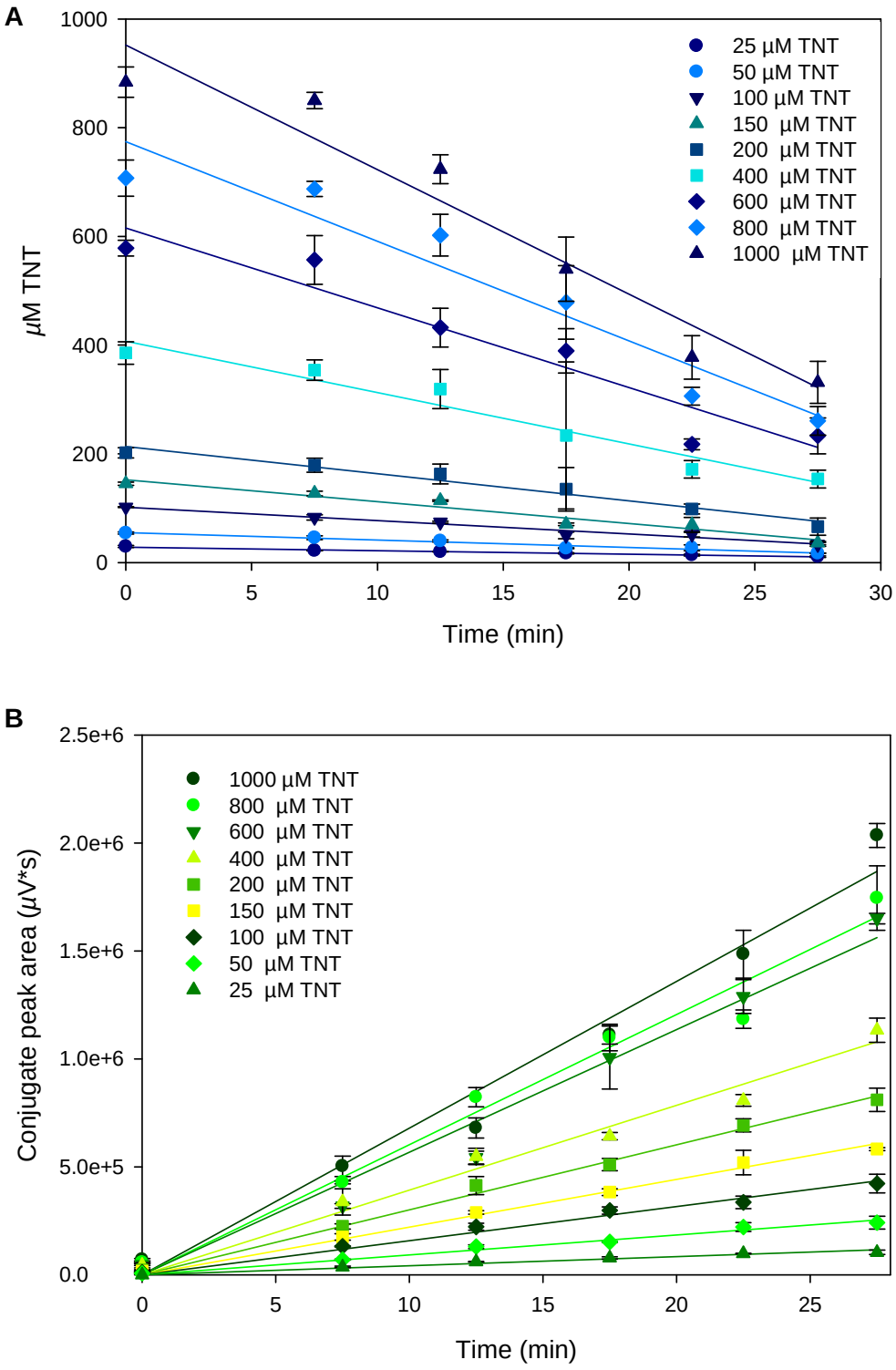


Figure 4.14: TNT depletion and conjugate production over 27.5 min. Assays contained 0 – 1000 µM TNT, 5 % DMSO, 5 mM GSH and 2.86 mg GSTU25 in 100 mM phosphate buffer pH 6.5. Experiments were performed in triplicate with error bars indicating standard deviation. Lines show regression of points for each initial TNT concentration, used to calculate the reaction rates.

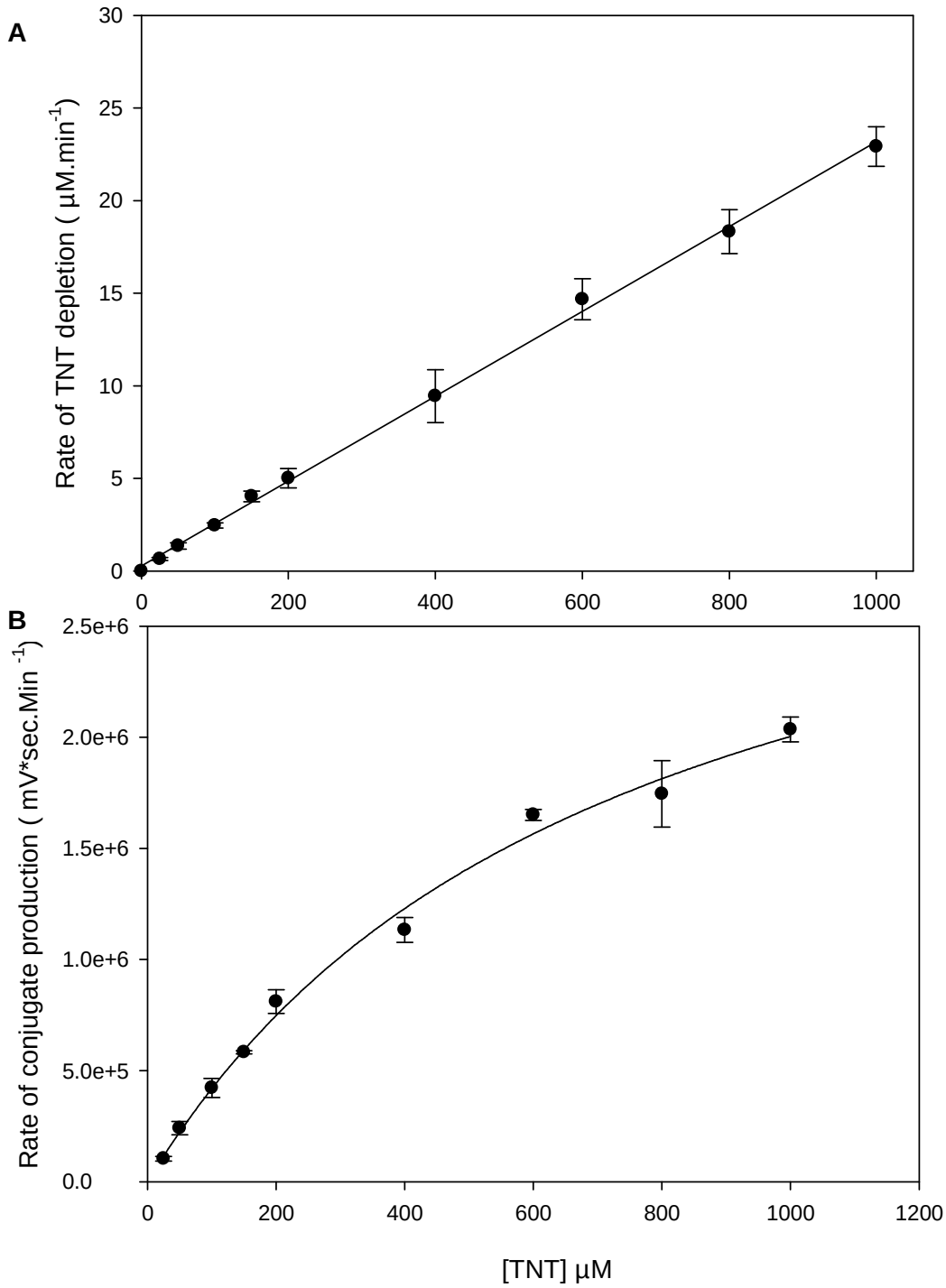


Figure 4.15: Michaelis-Menten plots showing**: A:** Rate of TNT depletion at different initial TNT concentrations and **B**: Rate of conjugate production in the same samples as A. Experiments were performed with 0 – 1000 μM TNT, 5 mM GSH, 2.27 mg / mL enzyme in potassium phosphate buffer pH 6.5, with samples removed every 5 min over a 25 min time course. Aliquots were stopped with 1 % TCA (v/v) and analysed by HPLC.

4.4 Conclusions

As established in Chapter 3 purified recombinant Arabidopsis GSTs U24 and U25 are active against TNT; conjugating it to glutathione by removal of a nitro group. This activity is of special interest as it could facilitate mineralisation of TNT in the environment; GSTU24 and 25 are the first plant enzymes identified which can perform nitrite removal from TNT. It was therefore crucial to investigate this reaction further. This Chapter presented a biochemical characterisation of the conjugation of TNT to GSH by GSTU25 as this enzyme demonstrated higher activity than GSTU24.

Previous characterisations of plant GSTs have revealed a broad range in pH optima for GST activity. Analysis of *At*GSTU25 with TNT in this Chapter yields an optimum activity at pH 6.5, this appears to be at the lower end of the superfamily, which has average optima of pH 9 for *Os*GSTF5, pH 8.3 for barley GSTs (both with CDNB) and pH 7.5- 8 for metolachlor and a *Zm*GST (Habig *et al.* 1974; Irzyk *et al.* 1993; Kunieda *et al.* 2005; Nutricati *et al.* 2006; Cho *et al.* 2007; Dixon *et al.* 2009). Another interesting observation in the literature is the use of various pH conditions for the same enzyme depending on substrate stability, for example; pH 7.5 for CDNB assays and pH 6.5 for OPDA assays with *At*GSTUs, pH 6.8 for chloro-s-triazine and chloroacetanilide herbicides and pH 8.5 for the diphenylethers, aryloxyphenoxyprioionates and sulfonylureas as recommended for all Tau or Phi GSTs (Edwards *et al.* 2005; Dixon *et al.* 2009). Investigation into the catalytic mechanism of GSTs has also shown that for activation of glutathione to a thiolate anion, which can attack the electrophilic substrate, the optimum pH is 6.2 (Labrou *et al.* 2001). It is therefore important to note that the pH optimum depends on enzyme biochemistry, glutathione anion production and to a large degree by the stability of the electrophilic substrate (Habig *et al.* 1974).

Temperature affects not only reaction rate but also protein inactivation and glutathione oxidation. To reduce oxidation, glutathione was added after the enzyme pre-incubation for temperature inhibition studies, to ensure that

glutathione availability was not limited in the assay. Previous plant GST temperature optima have documented fastest rates of activity at 40 ℃ (Park *et al.* 2005; Cho *et al.* 2007) however, the stability of these enzymes also drops off at temperatures over 30 ℃. These studies also meas ured temperature inhibition in the presence of DTT and EDTA to prevent glutathione oxidation (Park *et al.* 2005; Cho *et al.* 2007). The published inactivation rates align with those in this work suggesting that GSH oxidation does not appear to be the major cause of reduced activity at higher temperatures and that DTT and EDTA addition are unnecessary. Agreeing with the results found here, the majority of GST conjugating assays are performed at 25 or 30 ℃, to reduce enzyme denaturation which is likely to be the main cause of the reduced activity observed at higher temperatures (Edwards *et al.* 2005; Nutricati *et al.* 2006; Farkas *et al.* 2007; Dixon *et al.* 2009).

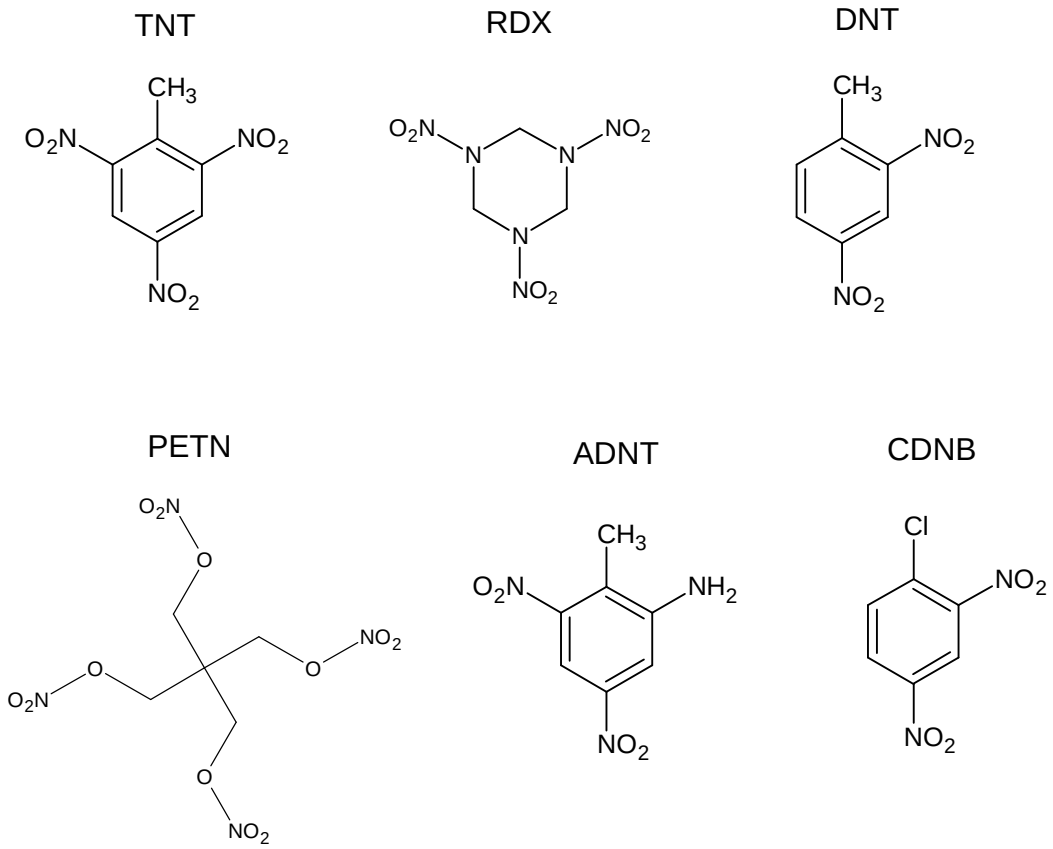


Figure 4.16: Compounds tested for substrate activity with GSTU25.

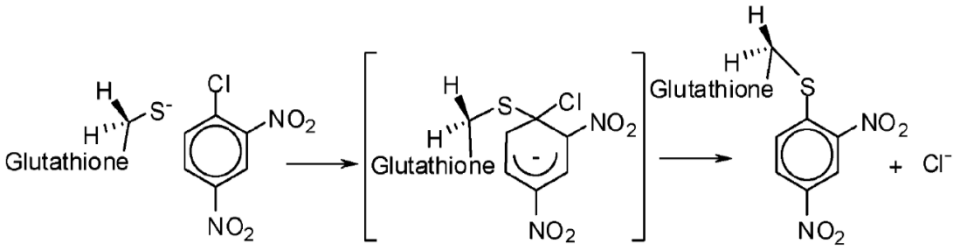


Figure 4.17: Reaction mechanism for CDNB conjugation proceeds via a Meisenheimer complex of CDNB and GSH. Glutathione is deprotonated within the active site of GSTs, it is then able to attack the C1 of CDNB through nucleophilic addition. This results in an unstable Meisenheimer intermediate which readily eliminates chloride to produce the stable glutathionyl-dinitrobenzene conjugate. Taken from Bowman et al. 2007.

Various substrates shown in Figure 4.16 have been tested to determine the specificity of GSTU25. Activity was only observed for TNT and CDNB. The conjugation of GSH to electrophiles occurs through anion addition, GSTs catalyse the removal of a proton from GSH to generate the thiolate anion; GS^- (Armstrong 1991). All active GSTs contain an essential active site serine, which in GSTU25 is Ser^{13}, this amino acid enhances the nucleophilicity of the bound thiolate anion through hydrogen bond donation from the hydroxyl group of Ser^{13} (Labrou *et al.* 2001). The GS^- anion is much more reactive than GSH and attacks the ring carbon of the electronegative leaving group by nucleophilic addition, producing a Meisenheimer complex (Figure 4.17) (Bowman *et al.* 2007). In the case of CDNB, the conjugation of the thiolate anion occurs at carbon1, where the chloride is bound. This Meisenheimer complex is very unstable and the dissociation of the chloride is highly favourable, yielding glutathionyl-dinitrobenzene conjugates (Figure 4.17). The presence of more-electronegative leaving groups, for example NO_2, F and COOH, is likely to facilitate Meisenheimer-intermediate formation, but prevent its degradation; therefore in the case of 1,3,5-trinitrobenzene (TNB), which has three highly electronegative nitro groups, the stable Meisenheimer complex is a reversible dead end product (Graminski *et al.* 1989; Armstrong 1991). The nitro groups of CDNB are not the leaving groups as they have resonance-induced stability which the chloride ion does not and the less electronegative chloride ion increases the reactivity of the Meisenheimer complex allowing conjugate production instead of the dead end product observed for TNB. In the case of TNT, which is structurally very similar to TNB, it is proposed that the methyl group, which has a slight inductive effect, provides electrons to the aromatic ring, making the neighbouring nitro groups more labile, allowing for degradation of the Meisenheimer complex and nitrite release. The inductive effect of the methyl- group is local, exerting its electron donating effects only over a short distance, therefore it is possible that for TNT only 2- and 6- nitro groups are putative leaving groups as the 4- nitro group will remain too electron withdrawing to allow conjugation, although no experimental evidence is available to confirm this.

Figure 4.16 shows the structure of 2-amino-4,6-dinitrotoluene (2-ADNT), which with 4-amino-2,6-dinitrotoluene (4-ADNT), is the product of reductive attack on a nitro-group of TNT. The amino group is electron donating, which prevents the nucleophilic addition of the thiolate anion to the aromatic ring and therefore glutathione conjugation is unfavourable. The compound 2,4- DNT has one less nitro-group than TNT and therefore the aromatic ring has reduced stability provided by electron resonance in addition to reduced hydrophobicity compared to TNT (Johnson *et al.* 2001). The cyclic hexanitramine RDX contains carbon and nitrogen atoms in alternating positions (Figure 4.17). Three nitro groups are bound to the three ring nitrogen atoms. RDX lacks the resonance stability of the π system as is in non-planar and non-aromatic. Therefore the RDX nitro groups do not act as an electron withdrawing sink and are therefore unlikely to be sites of thiolate anion addition.

It is well known that for nucleophilic attack by the thiolate anion of glutathione, the lipophilic substrate requires an electrophilic centre (Keen *et al.* 1976). Based on these results it can therefore be concluded that of the substrates tested with GSTU25, only CDNB and TNT have the required electrophilic centre to facilitate GS- binding through nucleophilic attack. This suggests that specificity of the enzymes depends strictly on the hydrophobicity of the substrate. The electron resonance by electron withdrawing groups may also be required for activity. This may also explain the relatively low activity of GSTU25 with benzyl isothiocyanate (BITC) (Figure 4.1) which is a natural plant compound with antibiotic properties (Brusewitz *et al.* 1977). It possesses single benzene ring with an isothiocyanate functional group - C-N=C=S, conjugation occurs through an addition reaction, following nucleophilic attack of the carbon, a different mechanism to that employed for TNT and CDNB.

Optimisation of reaction conditions is essential before kinetic analysis is performed, however conditions must also be optimized to minimize non-enzymatic conjugation. For CDNB kinetic analysis, a non-enzymatic control was assayed and the rate was deducted from the enzymatic rate. In the case of TNT, boiled enzyme controls were assayed for all optimisation experiments but no non-

enzymatic rate was detected, therefore the kinetic data gathered with the optimised conditions is expected to be through enzymatic activity only.

Kinetic values for GSTU25 have been calculated for both CDNB and TNT. For CDNB the K_m is 30.55 µM CDNB and V_{max} is 1683.6 μM min^{-1}. For TNT, results were extrapolated as the V_{max} was not reached, the derived values are; K_m 8219 ± 232.4 μM and V_{max} 210.2 ± 5.4 μM min^{-1}. It is evident that GSTU25 is much more active towards CDNB than TNT, this indicates that either the H-site, or hydrophobic substrate binding site has better specificity towards CDNB than TNT or that the thiolate anion (GS-) has better reactivity with CDNB. It is likely that the latter is true as the non-enzymatic rate of CDNB glutathionylation is high, suggesting a high affinity for nucleophilic attack of the C1 carbon of CDNB. It may also be true that the H-site is better suited to accept CDNB as a ligand than TNT, active site mutagenesis of GSTU25 could determine this.

The disparities between the two Michaelis-Menten plots for conjugate production and TNT depletion may suggest the presence of a diconjugate, although mass spectrometry in Chapter 3 did not identify and additional peaks which indicated glutathione conjugation. A literature search yielded no instance of a diconjugate to GSH, however cases of a di-glutathione conjugate have been observed (Takahashi *et al.* 1987). But, in this case the diconjugate of 1,4-napthoquinone was produced non-enzymatically, it is therefore possible that the production of a diconjugate by GSTs is unfavourable due to steric effects. Glutathione conjugation occurs through thiol binding to the substrate, each glutathione only has a single thiol group so the conjugation of two substrates to a single glutathione must proceed through another conjugation mechanism. In addition to this if the diconjugate was a glutathione diconjugate then the rate of TNT depletion should decrease at the higher concentrations as diconjugate production would not affect the TNT pool as it would use the conjugate pool as a substrate source. Further explanations for the levelling off of conjugate production could be; product insolubility, or non-enzymatic breakdown of the conjugate, however the conjugate is likely to be soluble and stable as glutathione conjugation is known to reduce hydrophobicity and glutamyl- transferases are required for conjugate

breakdown (Marrs 1996; Dixon *et al.* 1998). Although no stability characterisation of the conjugate itself has yet been performed. To fully understand the biochemistry, the kinetics should be repeated and mass spectrometry performed to identify the presence of a diconjugate or fragments, which may indicate conjugate breakdown.

This *in vitro* characterisation has optimised the conditions for TNT conjugation and determined the kinetic values of the reaction. Given this reaction is achieved *in vitro* it is likely that this pathway also occurs *in planta,* the upregulation of conjugate transporters in Arabidopsis following TNT treatment supports this hypothesis. To further investigate if GSTs do have a role in TNT detoxification *in planta* it was necessary to perform analysis of plants with altered levels of GST to determine if any effects are observed in the presence of TNT compared to wild type plants. Results of this are shown in Chapter 5.

Chapter 5: Characterising the *in vivo* role of Glutathione Transferases in Trinitrotoluene Transformation

5.1 Introduction

Plants have developed numerous detoxification pathways allowing them to cope with environmental pressures. The glutathione transferases (GSTs) are well-known Phase II conjugation enzymes and, as discussed previously in this thesis, their upregulation in Arabidopsis in response to 2,4,6-trinitrotoluene (TNT) treatment makes them candidates for TNT detoxification. Chapters 3 and 4 showed that two Arabidopsis GSTs have activity against TNT when assayed *in vitro,* indicating an involvement in TNT turnover in plants. This chapter investigated the role GSTs play in plant TNT detoxification, by characterising GST-overexpressing plants for altered responses to TNT treatment.

5.1.1 *In vivo* Detoxification of TNT

Phase I detoxification of TNT has been shown to be reductive transformation of one or more of the nitro groups, via a nitroso intermediate, resulting initially in an unstable hydroxylaminodinitrotoluene (HADNT) which is further reduced to aminodinitrotoluene (ADNT) (Figure 5.1). In Arabidopsis transformation is likely performed by the oxophytodienoate reductases (OPRs) and nitroreductases, some of which are upregulated in the roots in response to TNT (Biesgen *et al.* 1999; Ekman *et al.* 2003; Beynon *et al.* 2009). The OPRs are a small gene family which show homology to members of the Old Yellow Enzyme (OYE) family of flavoenzymes, including pentaerythritol tetranitrate reductase (PETNr) from *Enterobacter cloacae* which has been characterised to perform nitroreduction of TNT, to produce hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) (Figure 5.1), in addition to aromatic ring reduction to produce hydride and dihydride Meisenheimer products (French *et al.* 1999). To investigate if the

sequence homology of the OPRs to PETNr confers activity homology the role of OPRs in Arabidopsis was investigated (Beynon *et al.* 2009). *In vitro* analysis of the three main OPRs in plants, OPR1, OPR2 and OPR3 showed that all can transform TNT to HADNT and ADNT, with OPR1 also exhibiting aromatic ring reduction of TNT. Overexpression of OPR1 in Arabidopsis increased TNT uptake from liquid media and yielded higher levels of transformation products than unmodified plants while knockdown lines of OPR1 and OPR2 took up less TNT and produced lower levels of transformation products than unmodified plants (Beynon *et al.* 2009). This strongly suggests a role of OPRs in the Phase I transformation of TNT in plants.

Phase II detoxification, conjugation, of TNT in plants is known to occur as six-carbon conjugates of ADNTs, likely glycosyl-conjugates, have been identified in plant extracts following TNT treatment. Transferases (conjugating enzymes) have also been found to be upregulated in plants in response to TNT treatment (Ekman *et al.* 2003; Mezzari *et al.* 2005; Gandia-Herrero *et al.* 2008). In microarray studies of 6 h TNT treated Arabidopsis, seven uridine diphosphate (UDP) glycosyltransferases (UGTs) were found to be upregulated 14 fold or higher (Lorenz 2007). The UGTs are a large family of Phase II detoxification enzymes, with 107 putative members in Arabidopsis. To determine if they are active in TNT detoxification in plants, the upregulated UGTs have been investigated. *In vitro* analysis of the UGTs showed that six of the seven had conjugating activity towards both isomers of HADNT and ADNT, with preferences for HADNTs and the 4-isomers of HADNTs and ADNTs over 2-HDNT and 2-ADNT (Gandia-Herrero *et al.* 2008). Arabidopsis lines overexpressing the enzymes with highest activity towards 2-HADNT and 4-HADNT produced 28 to 41 % more conjugates than untransformed plants; however they showed no enhanced removal of TNT. Extraction of metabolites from these plants also identified that 4-HADNT conjugates are produced by either an *O*- or *C*-glucosidic bond (Figure 5.1) (Lorenz 2007; Gandia-Herrero *et al.* 2008). This indicates that UGTs upregulated in plants in response to TNT do have a role in the Phase II detoxification pathway of TNT.

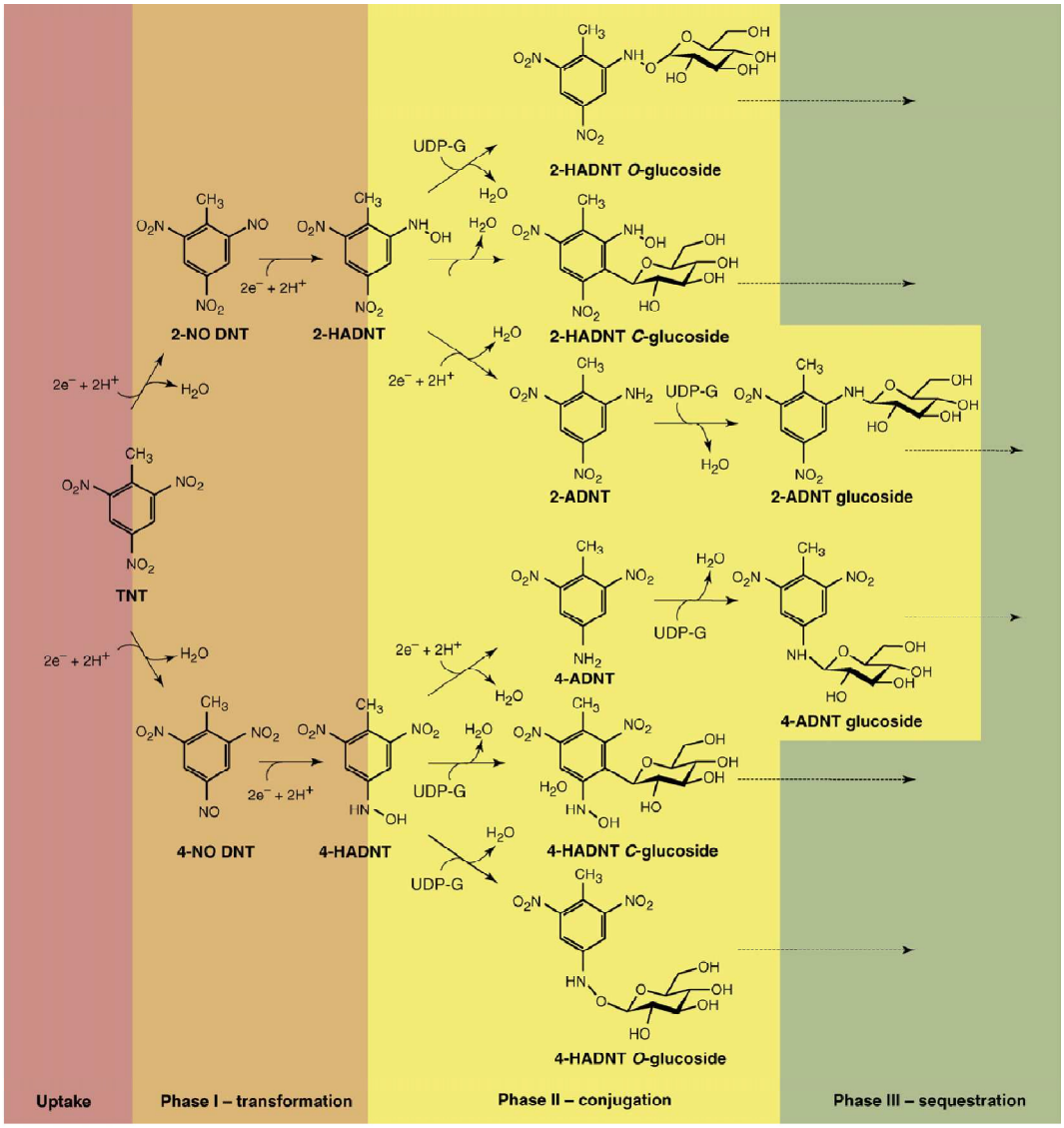


Figure 5.1: Metabolism of TNT in Arabidopsis with Phase I transformation shown to occur by oxophytodienoate reductases (OPRs) and Phase II conjugation of TNT to sugar molecules by uridine diphosphate (UDP) glycosyltransferases (UGTs).

5.1.2 GPOX Activity of GSTs

Glutathione peroxidases (GPOXs) reduce organic hydroperoxides of fatty acids and nucleic acids by using GSH as an electron donor. This produces monohydroxyl alcohols, which have lower cytotoxicity, reducing oxidative injury and the oxidation of GSH to GSSG triggers signals for further stress responses (Marrs 1996; Dixon *et al.* 1998; Cummins *et al.* 1999).

$$R{-}O{-}OH + 2GSH \longrightarrow R{-}OH + GS{-}SG + H_2O$$

The GSTs were first identified due to their conjugation activities, later, GSTs with peroxidase activity (GPOX-GSTs) were identified (Bartling *et al.* 1993) and shown to play an important role in protecting cells from oxidative damage, a vital component of the oxidative stress tolerance of plants. This function of GSTs could also account for the upregulation of GSTs in response to stresses which produce active oxygen species (AOS) including; pathogen attack, wounding, auxins, ethylene, heat-shock, hydrogen peroxide and heavy metals (Marrs 1996). GSTs with GPOX activities have been widely identified in plants including both crop and weed species (Edwards 1996; Benekos *et al.* 2010; Dixon *et al.* 2010).

5.1.3 Overexpression of GSTs in Plants

Overexpression of GSTs to improving stress tolerance has been widely studied, especially for resistance to herbicides. There are numerous examples of genetic modifications of GSTs including their expression in another species, as well as the overexpression of a native enzyme, both of these approaches can increase the tolerance of plants to stress factors.

Transgenic plants containing a GST from another species has resulted in transfer of resistance to numerous stresses. This has been observed for GSTs taken from *Suaeda salsa* to Arabidopsis, conferring increased salt tolerance (Qi *et al.* 2010); expression of soya bean GST, GmGSTU4 in tobacco conferred enhanced GST conjugating activity towards the herbicides fluorodifen and alachlor, as well as GPOX towards cumene hydroperoxide (Benekos *et al.* 2010). Another soya bean GST, GmGSTU21, was also expressed in tobacco, providing resistance to diphenyl ether herbicides (Skipsey *et al.* 2005). ZmGSTF2 has been cloned into

both wheat and tobacco hosts, conferring increased tolerance to chloroacetanilide and thiocarbamate herbicides (Skipsey *et al.* 1997; Milligan *et al.* 2001). Karavangeli et al. (2005) increased tobacco resistance to alachlor by the incorporation of another GST from maize (Karavangeli *et al.* 2005). Interestingly this GST had no detectable peroxidase activity. When a cotton GST was expressed in tobacco plants, the transformed plants were more resistant to oxidative damage arising from treatment with the herbicide Paraquat (Yu *et al.* 2003). No conjugation to this herbicide was observed, and it was concluded that the GST activity in this case towards products arising from the oxidative stress, putatively by conjugation to membrane lipid peroxidases or products of oxidative DNA damage (Pickett *et al.* 1989; Dudler *et al.* 1991; Bartling *et al.* 1993; Yu *et al.* 2003).

There are also many examples of overexpression of a native GST conferring increased tolerance to stresses: OsGSTL1, a rice GST, when constitutively overexpressed using the 35*S* promoter gave enhanced tolerance to chlorsulfron and glyphosphate, these *35SOsGSTL1* plants also performed better under other stress conditions, displaying lower superoxide levels than wild type plants (Hu *et al.* 2009). Tobacco lines overexpressing a Tau GST with high GPOX activity were more tolerant to abiotic stresses than unmodified plants. When these lines were treated with a variety of stress conditions, including chilling and salt treatment, they exhibited a reduced oxidative stress response compared to wild type plants and contained higher levels of monodehydroascorbate reductase (MDAR) activity and the glutathione pool was more oxidised (Roxas *et al.* 1997; Roxas *et al.* 2000).

All of the examples of GST expression or overexpression in plants have shown improved resistance against stress treatments. In some of these cases it is not the herbicide to which GSH is conjugated, but products of the resulting oxidative stress responses (Yu *et al.* 2003). The GPOX activity of GSTs is also likely to have an important role in minimising the oxidative damage following stress treatment. This confirms a role for GSTs in countering the oxidative stress response in addition to direct detoxification of xenobiotics.

5.1.4 GST Knockdown in Plants

To gain functional information for a specific enzyme it is common to perform a knock-down study where the expression of the protein of interest is reduced. RNAi of GSTs has previously been performed in Arabidopsis for phi class GSTs (Sappl *et al.* 2009). RNAi co-reduced the expression of ATGSTF6, F7, F9, and F10, however a high degree of functional redundancy in GSTs was identified. A larger decrease in protein levels would be required to determine whether there is an effect on oxidative stress tolerance. A SALK T-DNA insertion line exists for AtGSTU24, one of the genes selected for study, however previous characterisation of this plant line with TNT showed no altered tolerance to TNT by root length studies (Yoon *et al.* 2007). The high functional redundancy in GSTs could therefore require a multiple knockdown of not just the seven GSTs selected for study, but perhaps also many of the remaining 21 Arabidopsis Tau GSTs. For these reasons no knock-down studies were performed in this investigation.

5.1.5 Safeners

Safeners are chemicals which, when applied with specific herbicides to certain plant species, enhance tolerance of monocot crop species to the herbicide without impairing its efficacy towards target weeds. The mode of action of safeners is not fully understood, though it is likely that they act in a variety of ways including; competing with herbicides for binding sites of receptor proteins, enhancing synthesis of detoxification enzymes and reducing the susceptibility to herbicide inhibition by induction of less sensitive isoenzymes (Davies *et al.* 1999). Much research has been performed on the effect of safeners on the detoxification route by GSTs. Many herbicides are known to be conjugated to GSH and the addition of safeners enhances the rate of this conjugation step thus improving tolerance to the herbicide. For example dichloroacetamide safeners increase the levels of conjugation of the herbicides metolachlor, metazachlor and acetolachor (Fuerst *et al.* 1991; Rowe *et al.* 1991; Fuerst *et al.* 1992; Ekler *et al.* 1993). Fenchlorim and fenchlorazole ethyl increase conjugation of acetochlor and fenoxaprop-ethyl respectively (Ekler *et al.* 1993; Tal *et al.* 1993). Another route of detoxification could be by increasing the levels of reduced glutathione, by upregulating glutathione reductase which converts oxidised glutathione (GSSG) to the active, reduced form, GSH (Farago *et al.* 1994). The safeners dichlormid,

benoxacor, flurazole and fenchlorim increase GSH levels in maize, sorghum and tobacco (Gronwald *et al.* 1987; Cottingham *et al.* 1991; Kunkel *et al.* 1991). In the plant stress response GSH functions as a free radical scavenger, protecting cells from oxidative damage. Oxidative stress induces the production of GSH which oxidises to GSSG, this reaction can be catalysed by GSTs with GPOX activity and therefore GSTs could provide a dual protective mechanism.

Safeners have also been shown to induce GSTs in dicots including pea and Arabidopsis (Edwards 1996; DeRidder *et al.* 2002). The indication that these plants can perceive and respond to safeners, yet remain sensitive to herbicides suggests that an additional factor is involved in the selection between monocots and dicots. DeRidder et al. (2002) found that the localisation of GST expression following safeners treatment is important. In Arabidopsis the levels of GST in appropriate tissues was too low to confer herbicide tolerance. The same was observed for pea, where GST expression was mainly root localised following safener treatment (Edwards 1996).

5.1.6 Experimental Outline

In this chapter, Arabidopsis GSTs have been overexpressed to determine if GSTs can improve the tolerance to and transformation of TNT in plants as was observed for the UGTs (Gandia-Herrero *et al.* 2008). Arabid*o*psis lines overexpressing GSTs U24 and U25 under the control of the CaMV 35*S* promoter were created and independent, homozygous lines with T3 segregation ratios indicative of single T-DNA insertion sites have been characterised. Preliminary experiments were performed to identify altered tolerance to TNT compared to untransformed wild type (WT) plants. Expression levels were determined by real-time (RT) PCR and protein activity assays before further analysis of TNT tolerance of selected independent lines.

5.2 Methods

Arabidopsis over-expressing GSTs U1, U3, U4, U7, U22, and U25 were produced and analysed for altered resistance to TNT treatment. T2 *35SGSTU24* lines were donated by Dr E. Rylott.

5.2.1 Cloning

The pART7 and 27 binary vector system (Gleave 1992) was employed for CaMV-35*S* constitutive expression of GST U1, U3, U4, U7, U22, U24 and 25. Primer design was facilitated using Primer3 design (Table 5.1) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Arabidopsis cDNA from TNT treated plants was used as a template from which the *gsts* were amplified by PCR was performed with Phusion High-Fidelity DNA polymerase (NEB), as described in Section 2.3.4.2. Primers are listed in Table 5.1 and PCR programme is shown in Table 5.2.

Table 5.1: Primers for amplification of *gsts* from Arabidopsis thaliana cDNA were designed with Primer3 design: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. GSTU24 primers were designed by Dr Liz Rylott.

Primer	Restriction site	Sequence
35S GSTU1F	*EcoR* I	GAA TTC ATG GCG GAG AAA GAA GAG AG
35S GSTU1R	*BamH* I	GGA TCC TTA GGC AGA CTT AAT TGT C
35S GSTU3F	*EcoR* I	GAA TTC ATG GCC GAG AAA GAA GAG G
35S GSTU3R	*BamH* I	GGA TCC TTA GAC CGC TTT GAT TC
35S GSTU4F	*EcoR* I	GAA TTC ATG GCG GAG AAA GAA GAG G
35S GSTU4R	*BamH* I	GGA TCC TTA GGC TGA TTT GAT TC
35S GSTU7F	*EcoR* I	GAA TTC ATG GCG GAG AGA TCA A
35S GSTU7R	*BamH* I	GGA TCC TCA AGC AGA TTT GAT ATT G
35S GSTU22F	*EcoR* I	GAA TTC ATG GCG GAT GAA GTG
35S GSTU22R	*BamH* I	GGA TCC TTA GAC ACA GTA TAT CTT CC
35S GSTU25F	*EcoR* I	GAA TTC ATG GCA GAC GAG GTGA
35S GSTU25R	*BamH* I	GGA TCC CTA TTC GAT TTC GAT CC
35S GSTU24F	*Kpn* I	GGT ACC ATG GCA GAT GAG GTG ATT CTT
35S GSTU24R	*Xba* I	TCT AGA TTA CTC CAA CCC AAG TTT GTT

Table 5.2: PCR cycle conditions for the amplification of *gsts* from Arabidopsis cDNA and colony PCR for verification of subsequent cloning steps.

Step	Temperature	Time	
Initial denaturing	98 ℃	30 s	
Denaturing	98 ℃	10 s	30 cycles
Annealing	60 ℃	30 s	
Extension	72 ℃	30 s	
Final Extension	72 ℃	5 min	

TOPO cloning protocol was employed to clone the of PCR products of the *gsts* into the pCR2.1-TOPO vector. GST inserts were excised from TOPO with the restriction sites shown in Table 5.1. They were cloned into pART 7 which houses a cassette containing a CaMV-35*S* promoter, multiple cloning site and ocs terminator, flanked by *Not*I restriction sites. This cassette containing the gene was cut from pART 7 with *Not*I and ligated into pART 27. This secondary vector houses T-DNA borders which surround the incoming the cassette, which allow transformation into Arabidopsis, in combination with the Ti plasmid from *Agrobacterium tumefaciens*. Constructs were confirmed by colony PCR and restriction digests (Sections 2.3.4.2 and 2.3.5).

5.2.2 Arabidopsis Transformation

Flowering Arabidopsis plants were transformed by floral dipping with *A. tumefaciens* GV3101 expressing the pART27GST plasmids (Section 2.5.4.1).

T1 seed was collected from dipped plants and were grown on kanamycin plates. Resistant seedlings were collected and transferred to soil where the plants were allowed to self fertilise. T2 seed with segregation ratios indicative of T-DNA insertion at a single locus were selected by their 3:1 kanamycin resistant: sensitive segregation. One hundred percent kanamycin resistant T3 seeds of *35SGSTU24* and *35SGSTU25* were propagated in soil to produce T4 seed which was used for analysis.

5.2.3 Analysis of Expression Levels

5.2.3.1 Western Blot of *35SGSTU* Plant Tissue

SDS PAGE gels were prepared and loaded as described in Section 2.4.4. Following electrophoresis, gels were equilibrated in chilled Towbin buffer for 15 min. Filter paper and nitrocellulose membrane were cut to the size of the gel and soaked in Towbin buffer. Three pieces of filter paper were placed on the anode of the transfer unit (Bio-Rad, UK) a single sheet of nitrocellulose membrane was put on top of the filter paper. The gel was laid on the pile and three more soaked sheets of filter paper were placed on top. The cathode was then applied and a 10 V current was applied for 40 min. The membrane was rinsed in buffer A (PBS) and blocked in PBS with 3 % BSA and 2 % milk powder (Marvel) for 60 min. The blot was then incubated with primary antibody at various concentrations for 60 min at room temperature or at 4 ℃ overnight. The blot was then washed for 5 min twice in buffer B (PBS with 0.1 % tween 20), then twice in buffer C (PBS with 0.1 % tween 20, 1 M NaCl) and briefly rinsed in buffer B followed by buffer A. A five min incubation with 10 mM Tris pH 9.6 was performed prior to development with a NBT and BCIP tablet (Sigma). Development was stopped with two 5 min washes in buffer B followed by rinse steps in buffer A followed by water.

5.2.3.2 RT-PCR of *35SGSTU* Arabidopsis Lines

The cDNA was extracted from leaf tissue of Arabidopsis plants grown on soil for six weeks, with three lines for each GST and five replicates of each line. RNA was extracted and reverse transcribed to cDNA prior to analysis of GST expression levels by RT-PCR as described previously (Chapter 3.2.2). The primers used are shown in Table 5.3.

Table 5.3: RT-PCR primers for GSTU24, GSTU25 and GSTU22

RT-PCR Primer	Sequence
RT GSTU25F	TGTCAAATTCGATTACAGAGAACAAG
RT GSTU25R	GGTATTTTCTTATGAACCGGATTCA
RT GSTU24F	TCCCTCCGATCCTTACAAGAGA
RT GSTU24R	TCGCCGTAACATTCACCTTTT
RT GSTU22F	TCGAAGCATCAGAGAAACTAGCTAAC
RT GSTU22R	CCTCTTAGCCGAAGCCATCA

5.2.3.3 Protein Extraction from Plants

Leaf tissue from Arabidopsis grown in soil for 6 weeks was harvested and ground to a powder under liquid nitrogen. At 4 ℃, 400 μL extraction buffer (0.1 M Tris-HCl pH 8, 2 mM EDTA, 1mM DTT and 50 g/kg PVPP) were added to 200 μg of frozen plant tissue, samples were then passed through a 0.45 μM filter and centrifuged at 10,000 x *g* for 30 min. Protein concentration of the supernatant was then assessed by Bradford assay (Chapter 3.2.5) and GST activity was determined by spectrophotometric CDNB assays measured over 1 min at 340 nm with 0.2 mg/mL protein, 1 mM CDNB, 5 mM GSH in potassium phosphate buffer pH 6.5.

5.2.4 Analysis of Growth on TNT

5.2.4.1 Root Length Studies

Root lengths were measured on 9 cm petri-dishes containing 30 mL ½ MSSA amended with 0, 2 and 7 μM TNT. Sterile, stratified seeds (Section 2.5.1 and 2.5.2) were placed in a single row across the plates, which were then placed vertically under 80 μmol.m^{-2}.s^{-1} light, with 16 h light, 8 h dark cycles at 20 ℃. Root lengths of the seedlings were measured after 7 days.

5.2.4.2 Liquid Culture Assays

Eight, sterile stratified seedlings (Section 2.5.1 and 2.5.2) were transferred to 100 mL flasks containing 20 mL ½ MSS. Flasks were incubated at 20 ℃ for 14 days with 120 rpm shaking, under low light conditions (~15 μmol.m^{-2}.s^{-1}) to minimise stress. After 14 days media was replaced 20 mL ½ MSS containing 200 μM TNT diluted in the solvent N,N-dimethylformamide (DMSO). Samples of the media were removed for HPLC analysis over 9 days.

5.2.4.3 Mass Spectrometry Analysis of TNT-Transformation Products

Eight Arabidopsis seedlings were grown in 20 mL ½ MSS for 14 days and treated with 200 μM TNT in DMSO for 7 days. After incubation 300 μL of the media was removed and stored at -80 C until analysis. Plant tissue was washed in distilled water then weighed and freeze dried overnight. The dried plant tissue was ground to a powder by shaking vertically with a ball bearing inside the polypropylene tube. Methanol extraction of the plant compounds was performed three times per sample with 3 mL methanol over 24 h under vertical shaking.

Methanol was collected, centrifuged to remove debris and evaporated to dryness in a fume hood. Residues were re-dissolved in methanol according to the initial plant fresh weight. Media and plant extract were then analysed by LC-MS (see Section 3.2.6.2).

5.3 Results

To identify the role of GSTs in Arabidopsis in the detoxification of TNT, 35*S*-overexpression lines were produced. No knockdown lines were produced as previous attempts to study the effects of Arabidopsis GSTs, even with multiple knockout lines has shown that the functional overlap of GSTs is too high to observe any effects of GST knockdown (Sappl *et al.* 2009).

Homozygous lines constitutively overexpressing GSTs were created for GSTU24 and U25 (*35SGSTU24* and *35SGSTU25*) and following preliminary experiments with TNT, their various levels of expression were assessed, by both RT-PCR and CDNB assay. Further characterisation with TNT was then performed to elucidate the roles of GSTs in TNT detoxification.

5.3.1 Cloning and Transformation of 35*SGSTU* Lines

All *gst* genes were amplified by PCR from Arabidopsis cDNA (Figure 5.2A). TOPO cloning was successful and fragments of the correct lengths were cut from pCRTOPO2.1 (Figure 5.2B) and ligated first into the pART7 plasmid, excised with the restriction endonuclease *Not*I (Figure 5.2C) and then ligated into pART27 (Figure 5.3A). All constructs were transformed into electrocompetent *Agrobacterium tumefaciens* (Figure 5.3B) and floral dip transformation of Arabidopsis Col0 plants was performed with positive clones of *A. tumefaciens*. Cloning of GSTU24 is not shown as this was performed by Dr E Rylott. Heterozygous T2 lines of Arabidopsis were created for all GSTs, however following this the *in vitro* analysis of the GSTs had identified the proteins GSTsU24 and U25 as active towards TNT and therefore subsequent generations were only produced for lines of these two *gsts*.

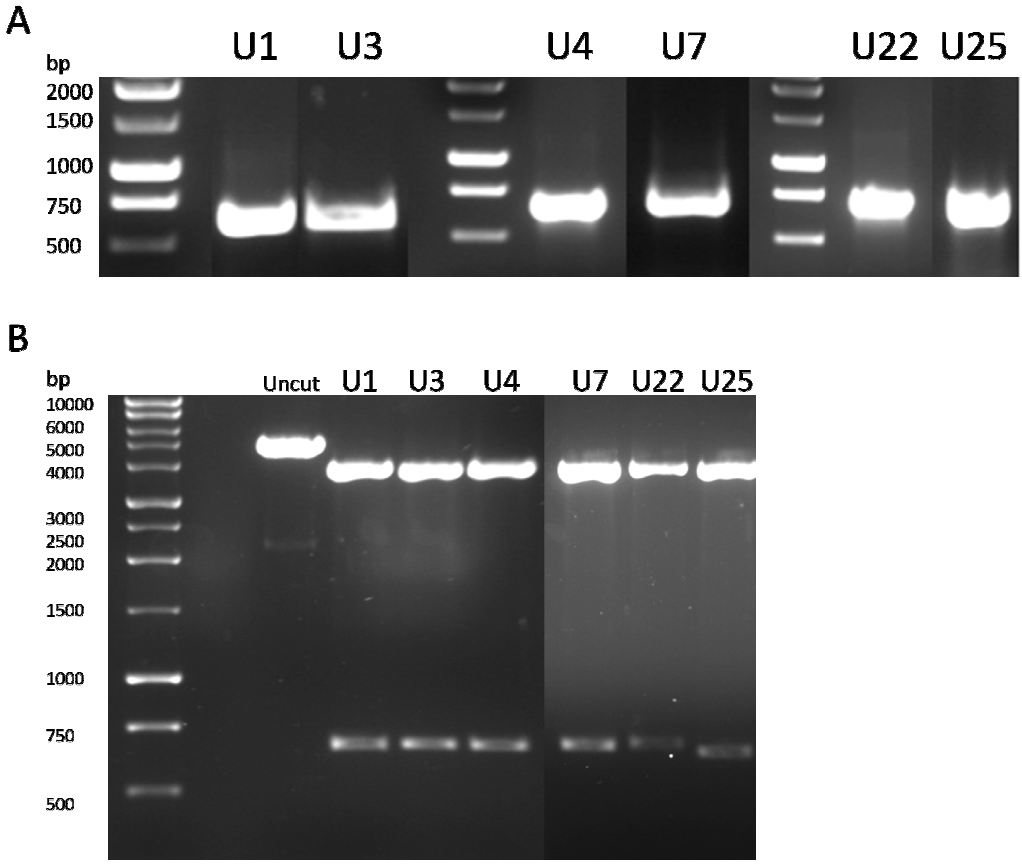


Figure 5.2: TOPO cloning of *gsts.* **A:** PCR amplification of *gsts* from Arabidopsis cDNA using gene specific primers. Subsequent sequencing confirmed correct genes sequences. **B:** *EcoR*I and *BamH*I double digest of pCRTOPO 2.1 containing *gsts.* The larger bands in each lane correspond with that of pCRTOPO 2.1. The smaller fragments of approximately 700 bp are the excised *gst* genes.

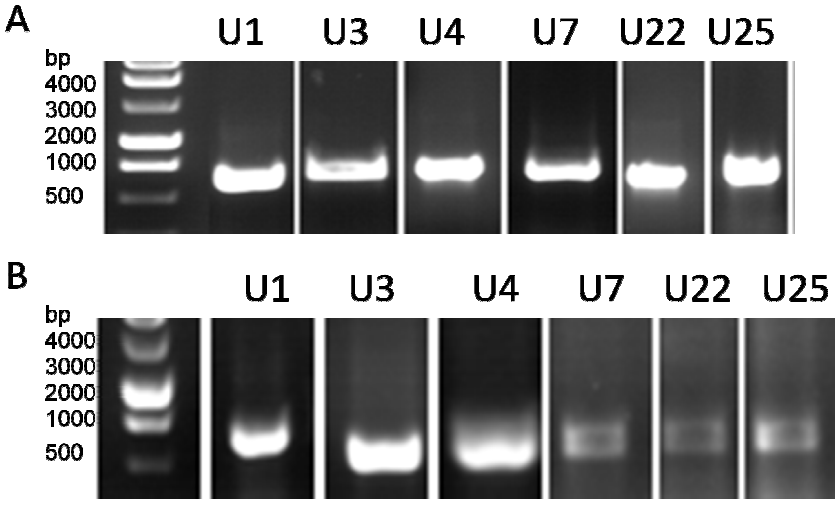


Figure 5.3: Diagnostic colony PCR of **A**: *E. coli* DH5 α with pART27 plasmid containing *gst*. **B:** *Agrobacterium tumefaciens* GV3101 with pART27 plasmid containing *gst*. Presence of a band of ~700 bp indicates insertion of the *gst* in the plasmid. Scales are approximate.

5.3.2 Preliminary Analysis of GST Overexpression Lines

5.3.2.1 Root Growth of 35S-GST Lines on TNT

Preliminary screening of homozygous T4 seed overexpressing GSTU24 and U25 was performed with the aim of identifying, relatively quickly, key lines with altered tolerance to TNT prior to subsequent gene and protein expression analysis. Root length has previously been shown to be a good indicator of TNT tolerance (Yoon *et al.* 2007; Gandia-Herrero *et al.* 2008; Beynon *et al.* 2009; Rao *et al.* 2009). Wild type lines have severely stunted roots when grown on concentrations of 2 *μ*M TNT and higher. Root lengths of all independent lines grown on ½ MSSA amended with TNT were measured after seven days. Seven *μ*M TNT was primarily assessed as this has been shown to significantly arrest root growth of WT (Lorenz 2007; Gandia-Herrero *et al.* 2008). Seeds for ten and nine independent lines of *35SGSTU24* and *U25* respectively were germinated and grown on ½ MSSA plates with 7 *μ*M TNT, as well as on control plates with no TNT. Root lengths were measured after nine days. Figure 5.4A shows the results for *35SGSTU24* lines and 5.4B shows the root lengths of *35SGSTU25* lines. When grown on the negative control plates without TNT six of the ten *35SGSTU24* lines had root lengths significantly longer than WT, as determined by a Dunnett's test where significant values are $P < 0.05$. Figure 5.5 shows the plant lines root length relative to WT and on the negative control plates with no TNT (½ MSSA only), there is a clear trend for enhanced root growth of the transgenic plants compared to WT, especially for *35SGSTU24* lines (Figure 5.5A and B). The seedlings grown on TNT amended media suffered severe root length stunting and no effects of TNT on root branching were observed. In comparison to WT seedlings, only two *35SGSTU24* lines shows any significant difference, line 4.2 had shorter roots while line 12.3 had longer roots. For *35SGSTU25* lines only one line showed a significant difference to WT without TNT, line E had roots which were significantly longer. But when plotted relative to WT root lengths (Figure 5.5B), there is a general trend for *35SGSTU25* lines when grown on 7 *μ*M TNT to be shorter than WT, this was not observed for *35SGSTU24* (Figure 5.5A).

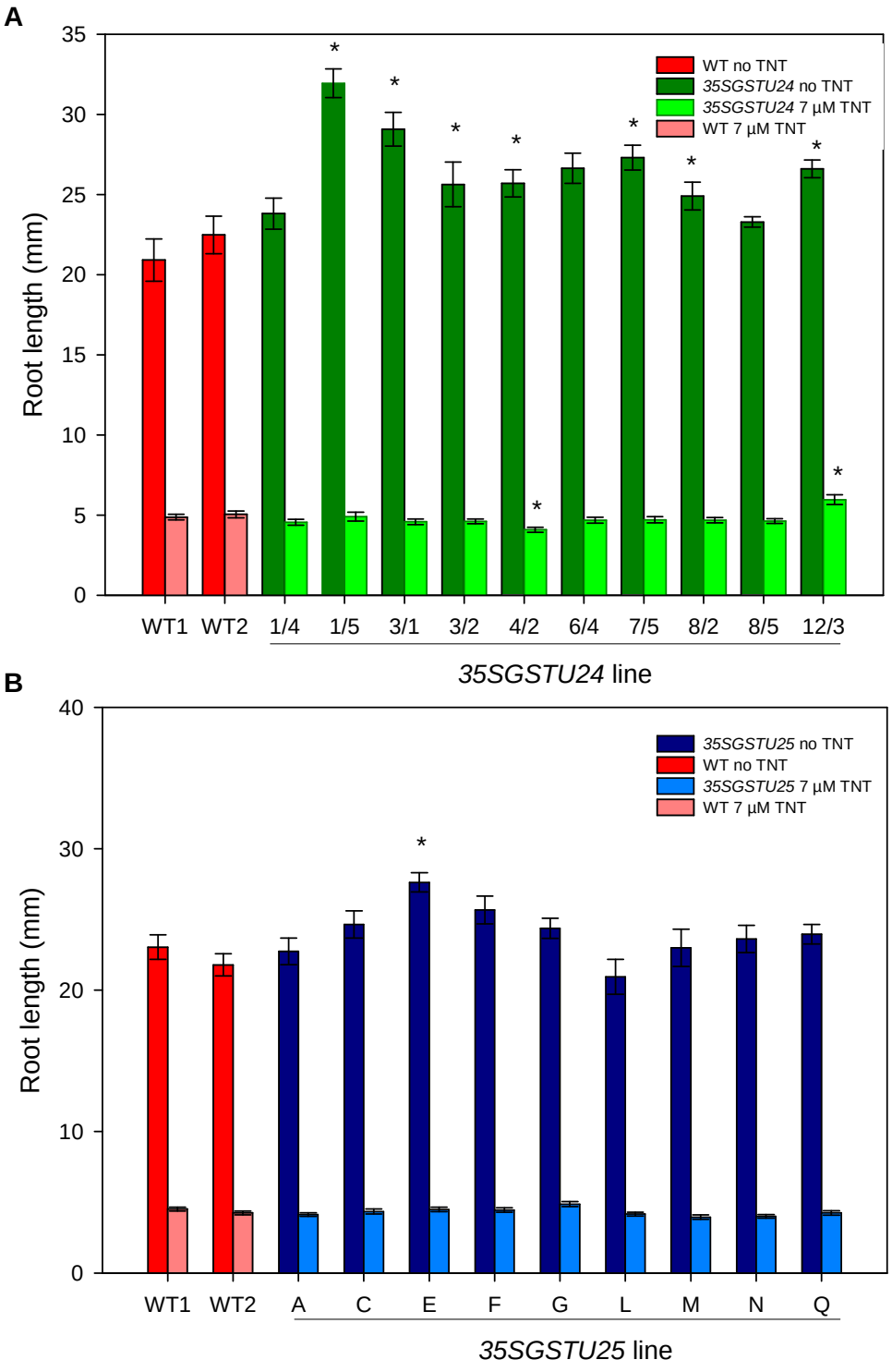


Figure 5.4: Effect of 7 *µ*M TNT on root growth of Arabidopsis *35SGSTU* lines A: *35SGSTU24*, B: *35SGSTU25* and WT lines. Seedlings were grown vertically on ½ MSSA plates amended with 7 *µ*M TNT. Root lengths were measured after 9 days, dark bars show root length on ½ MSSA with TNT and light bars shown root length on or ½ MSSA only. Results are the means of 20 seedlings ± standard error of the mean (SE). A one-way ANOVA followed by a Dunnett's test was performed to compare the results for each of the transgenic lines against the all the WT root lengths; mean differences are significant at the 0.05 level.

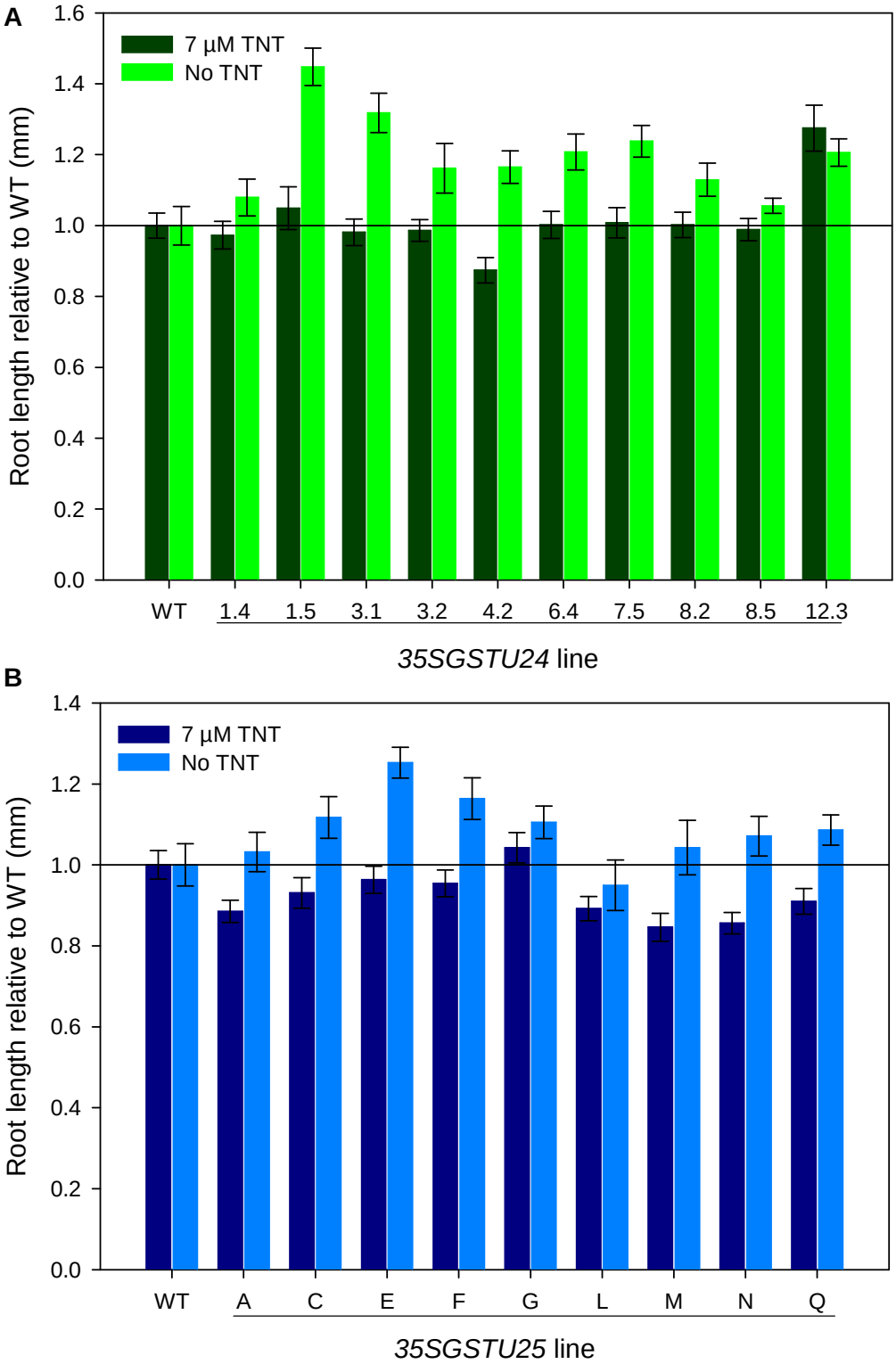


Figure 5.5: Root lengths of *35SGSTU* lines on 7 *µ*M TNT relative to WT. A: *35SGSTU24*, B: *35SGSTU25* and WT lines. Seedlings were grown vertically on ½ MSSA plates amended with 7 *µ*M TNT. Root lengths were measured after 9 days, dark bars show root length on ½ MSSA with TNT and light bars shown root length on or ½ MSSA only. Results are the means of 20 seedlings ± standard error of the mean (SE).

The addition of 7 *μ*M TNT to the media resulted in severe root stunting. It is possible that any minor morphological differences indicating tolerance to TNT would be masked by the high toxicity of TNT at this concentration. The experiment was therefore repeated with a lower concentration of TNT; 2 *μ*M was selected as previous analysis has shown this to significantly affect WT root length (Beynon *et al.* 2009) and have a lower toxic effect on the plant. When the *35SGSTU24* lines were grown without TNT for seven days one line; 6.4 had root lengths significantly longer than WT (Figure 5.6). This is in contrast to the previous experiment where six of the ten *35SGSTU24* lines had longer roots than WT in the absence of TNT and 6.4 was not among them. Figure 5.7 shows that the general trend for longer roots on ½ MSSA only is clear, for both *35SGSTU24* and *35SGSTU25* lines. The addition of 2 *μ*M TNT caused no significant differences between WT roots and those of the *35SGSTU24* lines. For *35SGSTU25* lines, no lines showed longer roots than WT when grown without TNT, whereas the previous experiment showed the roots of line E to be significantly longer (Figures 5.4 and 5.6). In the presence of 2 *μ*M TNT lines G, L and Q showed significant differences to WT, line Q is longer and the other two are shorter. General trends were again observed when the root lengths were plotted relative to WT (Figure 5.7), many of the lines appear shorter than WT when grown on TNT and this is more evident for *35SGSTU25* lines.

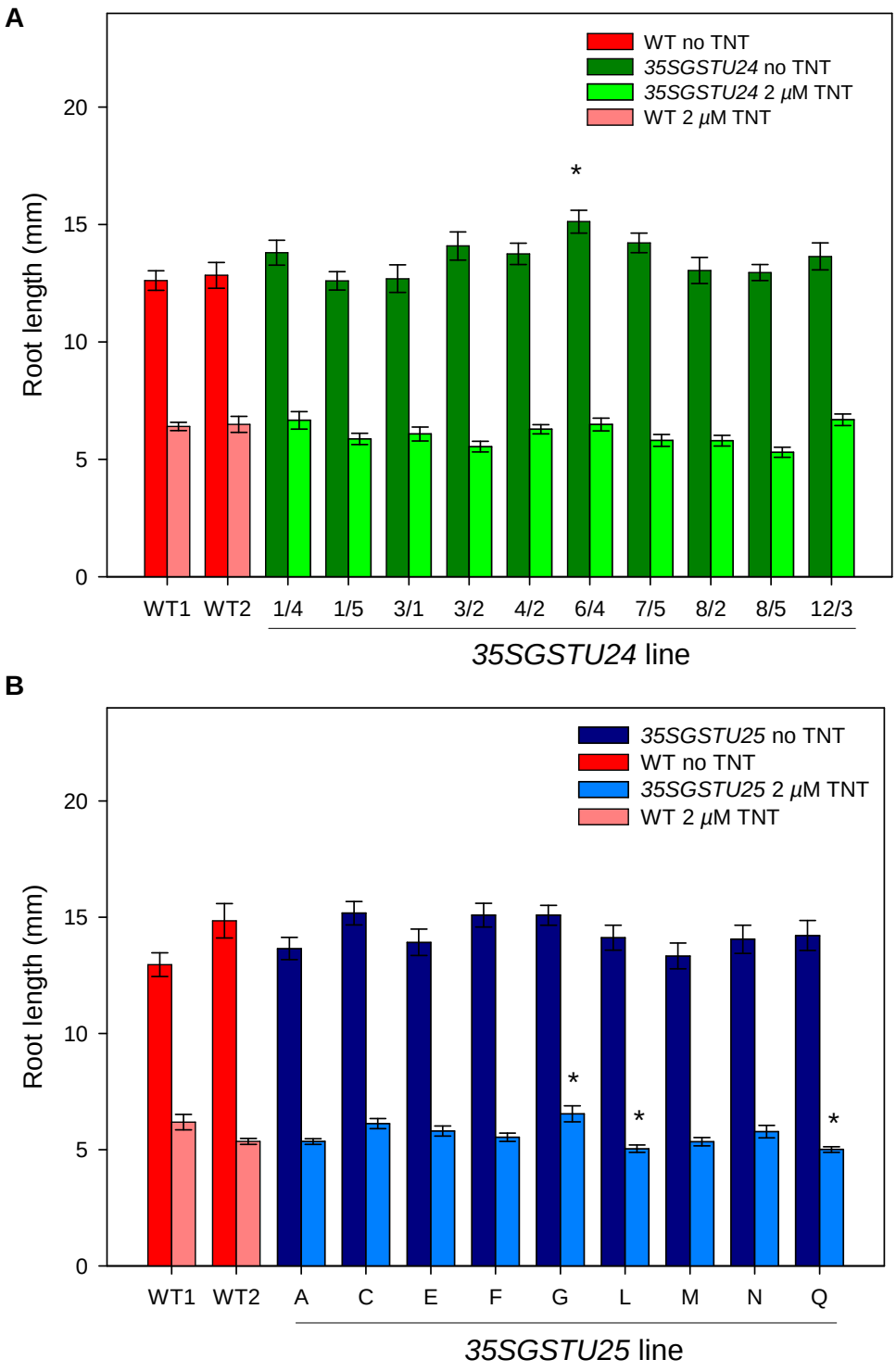


Figure 5.6: Effect of 2 µM TNT on root growth of Arabidopsis *35SGSTU* lines. A: *35SGSTU24*, B: *35SGSTU25* and WT lines. Seedlings were grown vertically on ½ MSSA plates amended with 2 µM TNT. Root lengths were measured after 7 days, dark bars show root length on ½ MSSA with TNT and light bars shown root length on or ½ MSSA only. Results are the means of 20 seedlings ± standard error of the mean (SE). A one-way ANOVA followed by a Dunnett's test were performed to compare the results for each of the transgenic lines against the all the wild type root lengths; mean differences are significant at the 0.05 level.

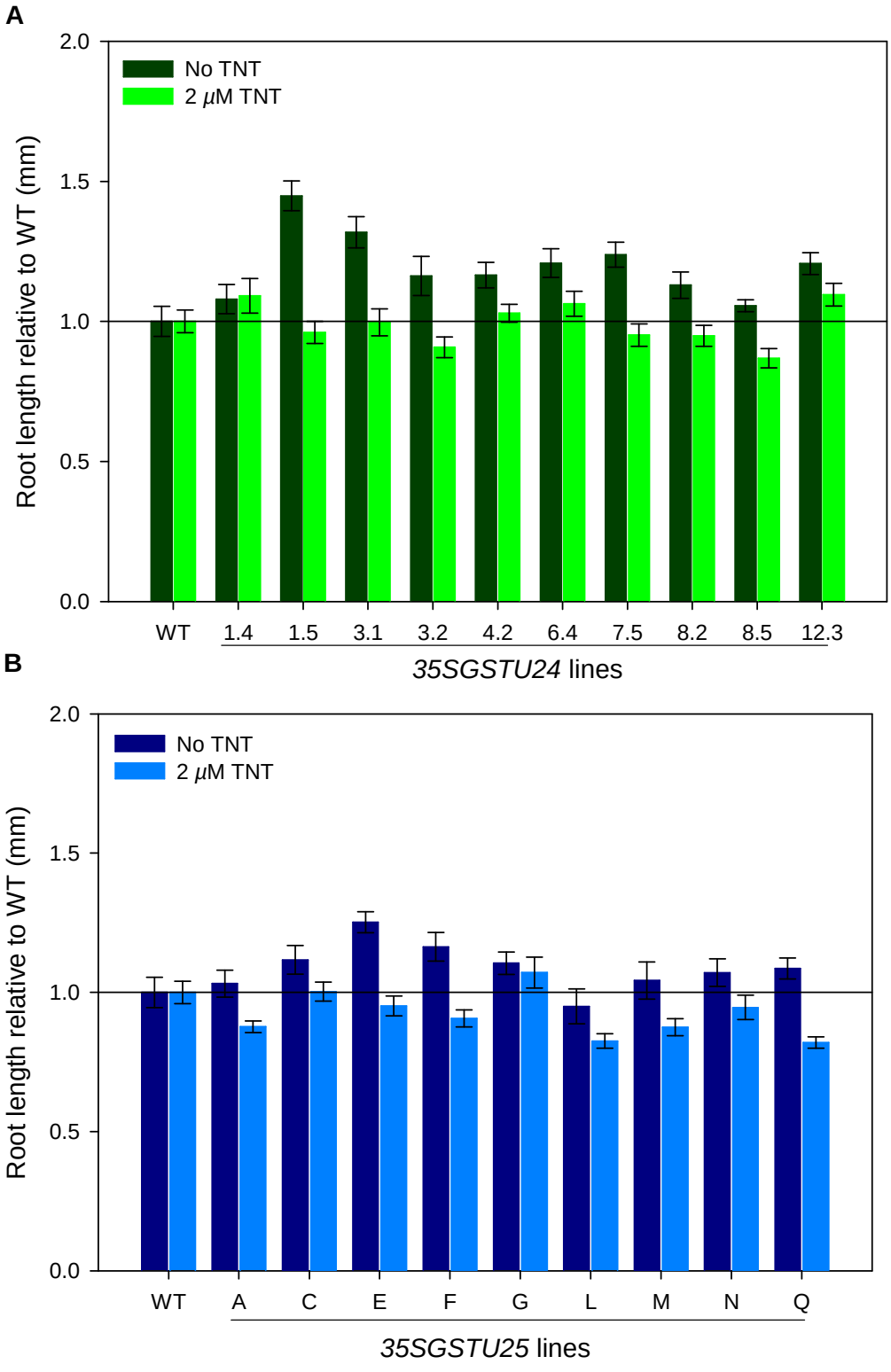


Figure 5.7: Root lengths of *35SGSTU* lines on 2 μM TNT relative to WT. A: *35SGSTU24*, B: *35SGSTU25* and WT lines. Seedlings were grown vertically on ½ MSSA plates amended with 2 μM TNT. Root lengths were measured after 7 days, dark bars show root length on ½ MSSA with TNT and light bars shown root length on or ½ MSSA only. Results are the means of 20 seedlings ± standard error of the mean (SE).

5.3.2.2 Preliminary Liquid Culture Studies of 35S-GST Lines

It has been observed that silencing of bacterial nitroreductase overexpressed in *NAtI* lines of tobacco occurs over four generations. This silencing causes a dramatic reduction in the enhanced root length phenotype observed, however TNT uptake remains significantly higher than WT even in the 'silenced' lines. This suggests that TNT uptake is a more sensitive indicator of TNT tolerance than root length. For this reason it was decided that before any lines were selected for analysis of expression and further characterisation a preliminary liquid culture assay was performed for all T4 homozygous *35SGSTU* lines to identify key lines for subsequent expression analysis. Axenic liquid culture experiments are commonly used to study uptake of compounds by plants and is a commonly used method for studying TNT detoxification (Gandia-Herrero *et al.* 2008; Beynon *et al.* 2009). Seedlings were grown in liquid media for 14 days before they were dosed with TNT. Samples of the media taken over a period of seven days were analysed for TNT concentration to determine uptake by the plants. It is also possible to measure further morphological and physiological effects of TNT treatment by this experimental system, including biomass and chlorosis.

Preliminary screening was performed on T4 homozygous independent lines. Nine independent lines of *35SGSTU25* were tested and ten independent lines of *35SGSTU24*. Wild type plants and flasks containing no plant material (NPC) were employed as controls.

Figure 5.8 shows the TNT concentration of the media in which the seedlings were grown over 96 h. For *35SGSTU24*, the lines removed between 68 and 94 % however WT lines removed TNT at a faster rate than all the GST overexpression lines with only 97.4 % ± 1.6 % of the initial TNT concentration remaining after 96 h (Figure 5.8A). The *35SGSTU25* lines also consistently removed less TNT than WT with between 79 to 95 % TNT removal by the lines. Further analysis of these data is shown in Figure 5.9 where the values from 48 h have been statistically analysed by a one-way ANOVA followed by a Dunnett's test and plotted in a bar chart. For *35SGSTU24* lines there is a general trend of slower TNT removal than WT. However, only line 8.2 shows any significant difference to the WT control. *35SGSTU25* lines have removed more TNT from the media than *35SGSTU24* lines and when compared to WT four lines show a significant difference in TNT

uptake; F, G, M and Q. Lines G and Q were previously identified by the root length experiments as G showed longer roots than WT and Q had shorter roots when grown on plates containing TNT.

Three lines for each construct were selected for further characterisation. These are *35SGSTU24* lines 6.4, 7.5 and 8.2 and *35SGSTU25* lines F, M and Q. These lines are shown in Figure 5.9, indicated by their lighter fill colours. They were selected because they represent a range of phenotypes within the lines over the different experiments.

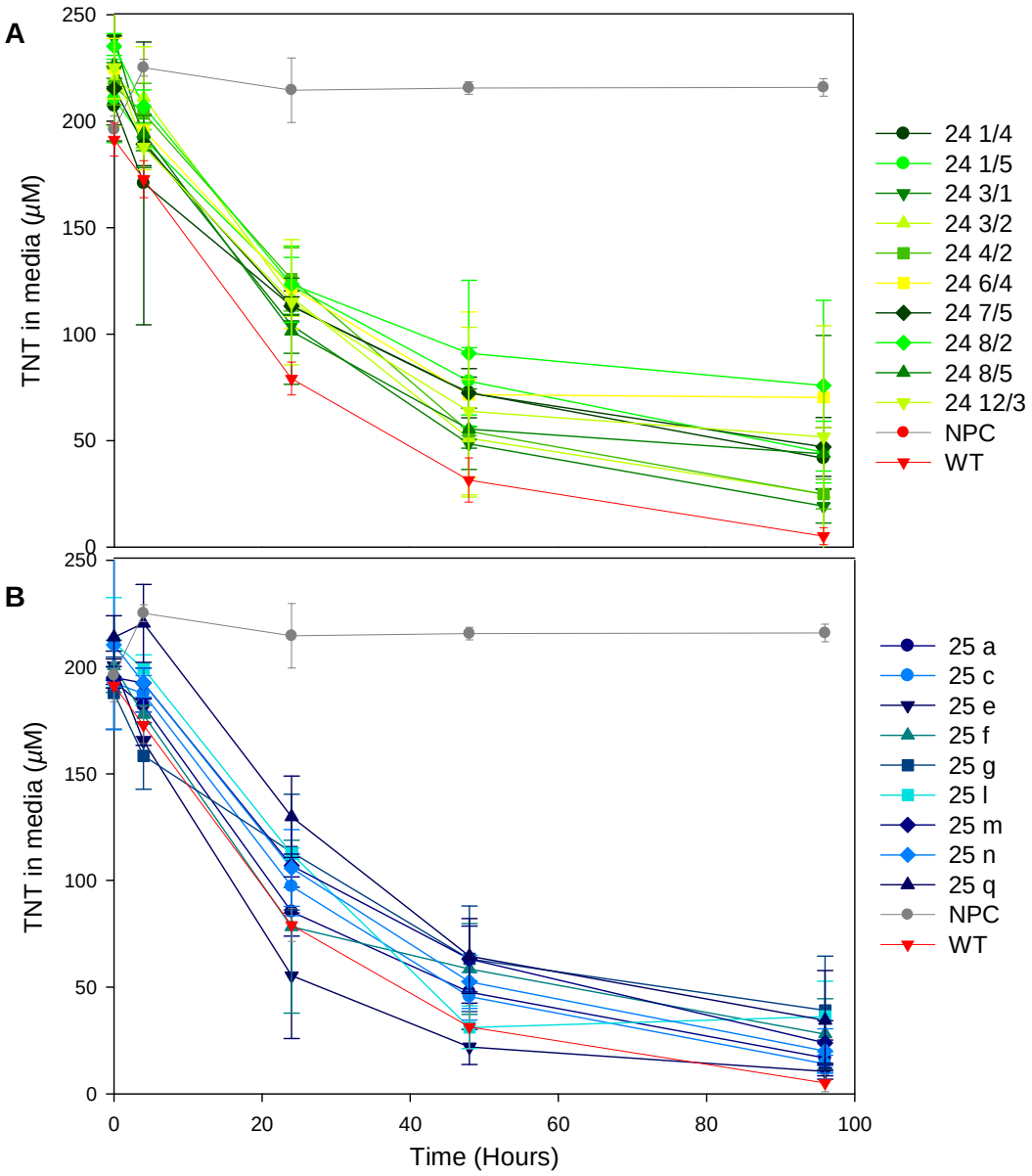


Figure 5.8: TNT removal from liquid culture by WT, *35SGSTU24* and *35SGSTU25* lines. Eight T4 homozygous seedlings for each independent line were grown in 20 mL ½ MSS for 13 days before the media was replaced with; ½ MS containing 20 mM sucrose and 200 μM TNT (in DMSO). Aliquots of the media were then removed at 0 h, 4 h, 24 h, 48 h and 96 h and analysed for TNT concentration by HPLC. Results for *35SGSTU24* lines are shown in green in the top panel, while *35SGSTU25* lines are shown in blue underneath. Red denotes the wild type (WT) and grey is the no plant control (NPC). Results are the means of three biological replicates ± one standard deviation of the mean (SD).

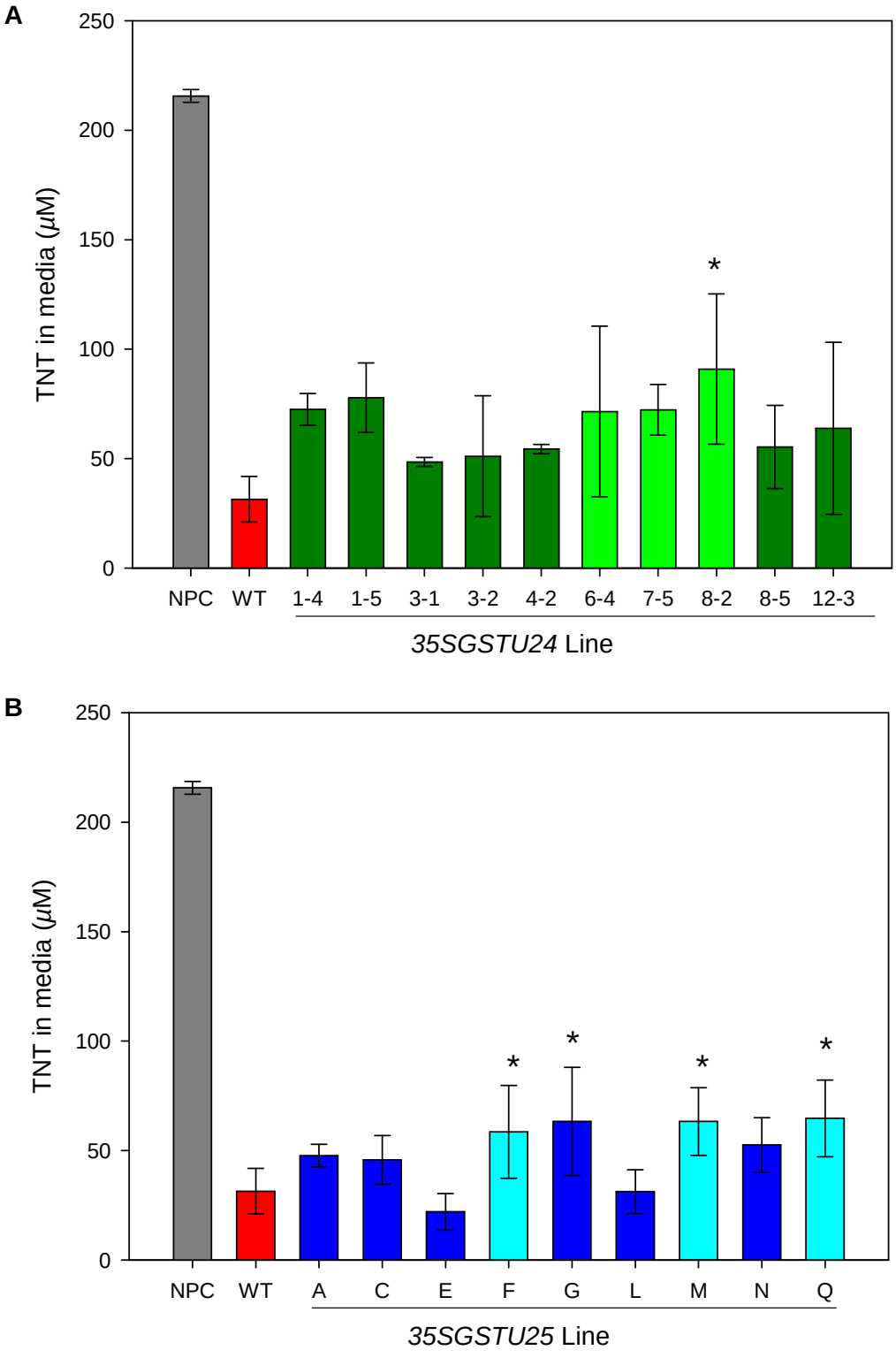


Figure 5.9: TNT concentrations of media from liquid culture of *35SGSTU* lines at 48 h following TNT dosing. Lines selected for further analysis are indicated by lighter fill colour. A one-way ANOVA followed by a Dunnett's test was performed to compare the results for each of the transgenic lines against the all the wild type values; mean differences are significant at the 0.05 level.

5.3.3 Characterisation of Arabidopsis 35S GST Lines

5.3.3.1 Analysis of Overexpression Levels

Expression of GSTs was assessed by RT-PCR and western blot analysis to identify transcript levels and protein levels of the *35SGSTU* lines.

To assess the transcript expression levels RT-PCR was performed on cDNA transcribed from RNA extracted from three lines of each gene (*35SGSTU24* 6.4, 7.5 and 8.2 and *35SGSTU25* F, M and Q) and a wild type (WT) line (Figure 5.10). The RT-PCR was performed using the same primers (GSTU22, GSTU24 and GSTU25) and methods as described in Section 3.2.1 and 3.2.2.

To determine levels of expressed protein western blots were performed with two different antibodies, A *Zm*GSTU1-2 antibody supplied by Dr D. Dixon (University of Durham, UK) and an *At*GSTU19 antibody from Prof. P. Goldsburgh (Purdue University, USA). These were used at a range of concentrations to determine optimal conditions however very little signal was seen for the pure enzyme positive control and none was observed for any of the plant lines.

To measure the levels of transcript produced by the *35SGSTU* lines, an RT-PCR experiment was performed. The six selected lines were grown in soil for six weeks then RNA was extracted from leaf tissue. TNT has been shown to accumulate in root tissue (Brentner *et al.* 2010) however the 35S promoter produces constitutive expression throughout the plant. This allows the simpler and cleaner extraction of RNA from leaves to be employed. Transcript levels of the respective *gsts* were assessed for each line, also those of the alternate GST, i.e. *35SGSTU24* lines were tested for levels of *gstu24* and *gstu25*. Although the primers used for cloning and those used for RT-PCR have been shown to clearly differentiate between the two closely related genes, it is possible that some co-regulation may occur. For this reason, both sets of lines were also measured for expression of *gstu22*. GSTU22 is also induced by TNT (see Chapter 3) and of those upregulated by the treatment; it is the most similar to GSTU24 and GSTU25, with 64 % and 68 % identity to them, respectively.

Figure 5.10 shows the transcript levels of each gene, normalised by the internal standard actin, then compared to WT plants. Graph 5.10A shows the levels of GSTU22 transcript expressed by the *35SGSTU* lines. While *35SGSTU24* lines show very little variation to WT, two of the *35SGSTU25* lines; F and M show a two-fold upregulation of this gene. The levels of GSTU24 transcript are shown in Figure 5.10B: The three *35SGSTU24* lines all show high amount of overexpression, ranging from the 350 fold increase seen for 8.2 to 1000 fold increase of line 7.5. Line 6.4 has 750 times more transcript of U24 than WT. The *35SGSTU25* lines show no altered expression of GSTU24 compared to WT. GSTU25 transcript levels are shown in Figure 5.10C. *35SGSTU24* lines do not have increased levels of GSTU25, except for line 8.2 which has a 3.4 fold increase of this related gene. The *35SGSTU25* lines all overexpress GSTU25, but line M is only producing four times the amount of transcript of WT. The other two lines, F and Q and expressing 1500 and 2400 fold more transcripts for GSTU25 than WT plants.

It is unlikely that these transcript levels directly correspond to the amount of protein produced, so to quantify the levels of protein expression antibodies were acquired from Dr David Dixon (University of Durham, UK) and Prof. Peter Goldsborough (Purdue University, USA) for a western blot. These antibodies were raised against ZmGSTU1-2 and AtGSTU19 respectively and were expected to work with the Tau GSTs used in this study. A range of concentrations of the antibodies were used to optimise conditions, however very little signal was produced by the positive control of pure protein at high concentrations and no signal was observed for the *35SGSTU* lines. Due to time constraints it was not possible to raise an antibody specific for GSTU24 or GSTU25 so a CDNB assay for GST activity was performed instead.

Extracts of GST-overexpressing plants have previously been shown to have increased activity than WT in spectrophotometric CDNB assays (Takesawa *et al.* 2002). A protein extraction from leaf tissue of each of the *35SGSTU* lines was analysed for activity towards CDNB. Figure 5.11 shows the results of this assay, the bars represent the amount of conjugate produced by samples of equal total

protein concentration. Interestingly, *35SGSTU25* line M, which had the lowest GST expression, just four fold increase, but CDNB assays show has less activity than WT. Aside from this there is a general trend of increased activity towards CDNB compared to WT, though only *35SGSTU25* line F has a statistically significant increase, with three times more conjugate being produced by this line than for WT. This line (F) is also the line expressing the most GST transcript, with a 2400 fold increase.

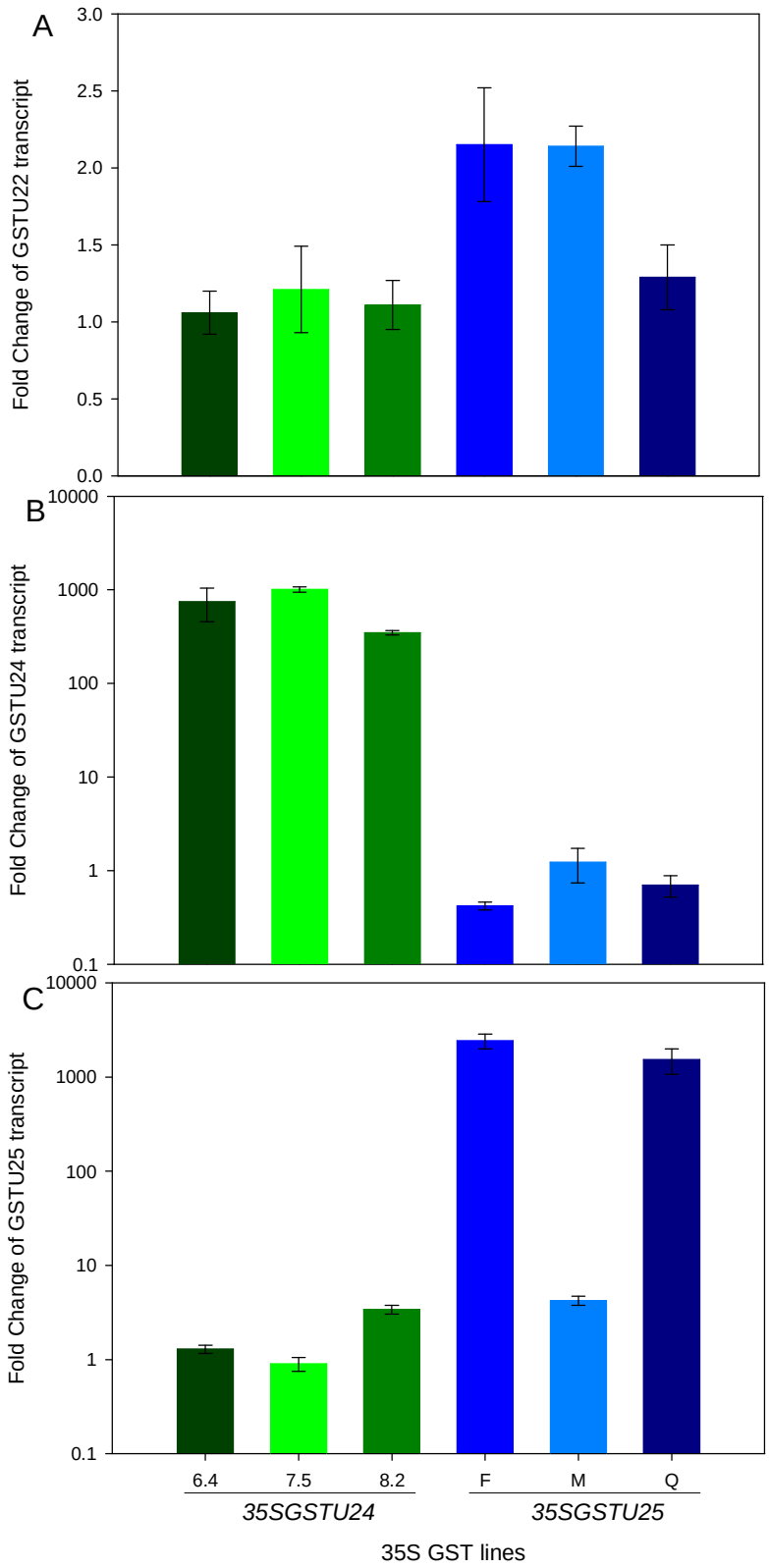


Figure 5.10: RT-PCR of *35SGSTU24* and *35SGSTU25* plants with GSTU22, U24 and U25 primers. A: transcript levels of GSTU22, a related GST. B: Transcript levels of GSTU24. C: Transcript levels of GSTU25.

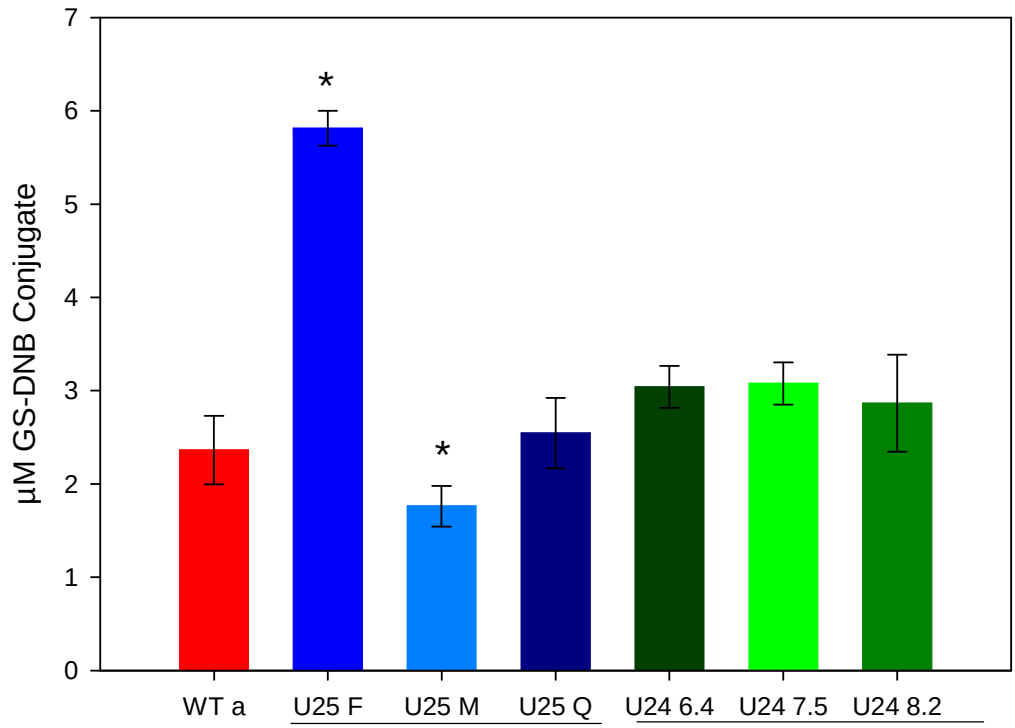


Figure 5.11: CDNB assay of protein extracts from *35SGSTU* lines. Assays contained 10 μg protein, 1 mM GSH and 1 mM CDNB in 100 mM potassium phosphate pH 6.5. Change in absorbance at 340 nm was measured over 1 min. Significant difference compared to WT is shown by an asterisk and determined by Dunnett's test $P<0.05$. Results are the means of three technical replicates and error bars represent standard deviation.

5.3.3.2 Effect of TNT in Hydroponic Studies of 35S GST Lines

A liquid culture study was performed for the six selected *35SGSTU* lines, with wild type (WT) and no plant controls (NPCs). Eight seedlings were grown in liquid culture for 14 days, before media was replaced with ½ MS amended with 200 μM TNT. The removal of TNT was measured over 168 h. Ten flasks were set up for each line, although for some lines up to three flasks were lost to contamination. WT and NPC controls were also analysed. Figure 5.12 shows that the lines follow the same trend as observed in preliminary experiments (Figure 5.8). All *35SGSTU* lines are capable of TNT uptake from the liquid culture media, however in all cases this appears to occur at a slower rate than for WT plants. *35SGSTU25* lines have removed all TNT by 96 h, where as it takes the *35SGSTU24* lines up to 168 h to completely deplete the TNT. This disparity between the lines of the two different GSTs was also observed in the preliminary experiment. The data from the 48 h time point were plotted and statistical analyses by Dunnett's test were performed (Figure 5.13). A clear trend of decreased TNT depletion is observed for the *35SGSTU* lines compared to WT. All of the *35SGSTU24* lines have removed significantly less TNT by 48 h, with line 6.4 having removed only 66 % of the starting TNT concentration, and 7.5 and 8.2 showing 73 and 77 % uptake. In comparison WT has taken up 92 %. TNT concentrations of the media of *35SGSTU25* lines look higher than for WT with mean uptakes of 78 %, 85 % and 81 %, though these values are not significant compared to WT.

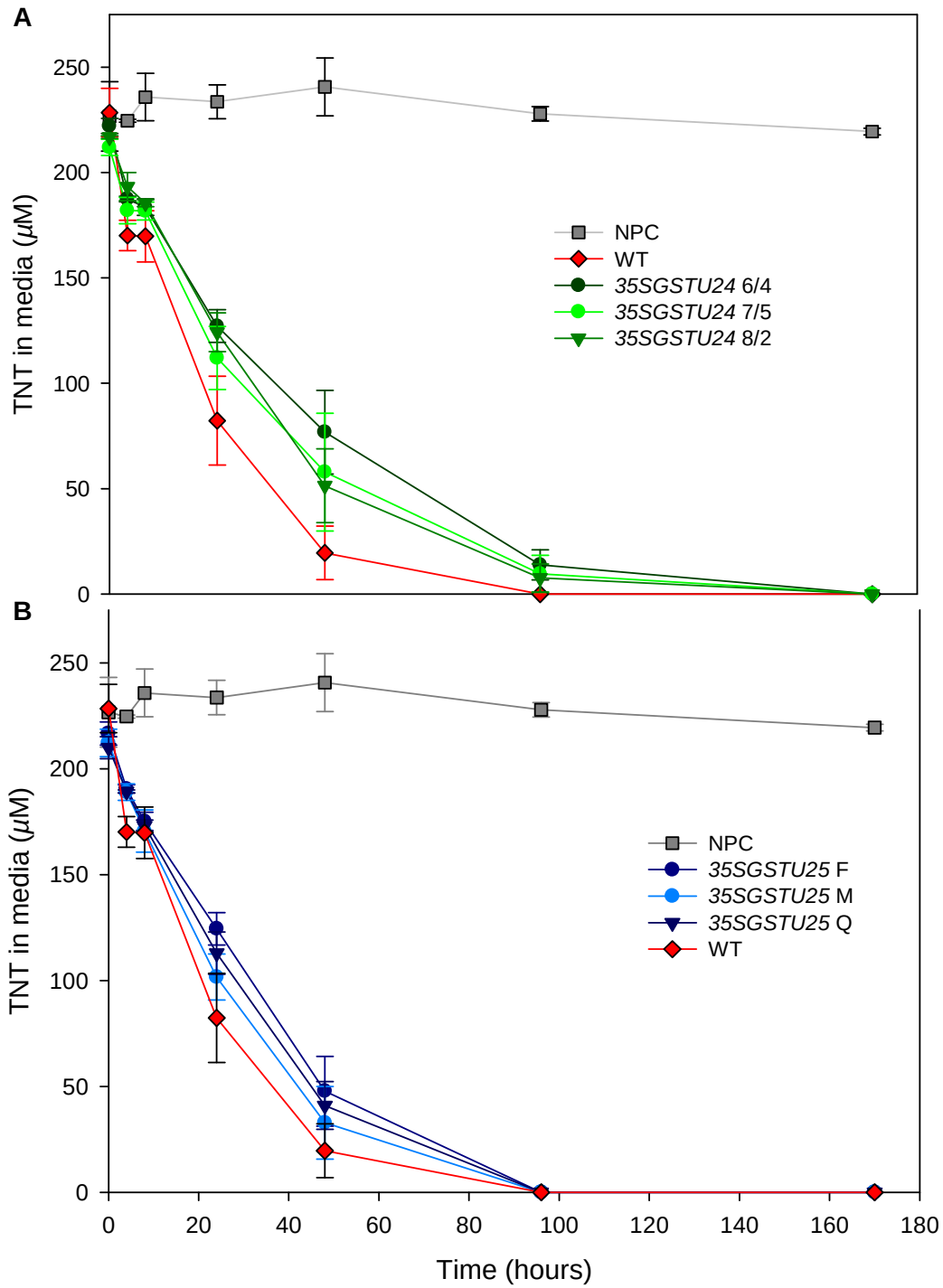


Figure 5.12: TNT removal from liquid culture by WT, *35SGSTU24* and *35SGSTU25* lines. Eight T4 homozygous seedlings for each independent line were grown in 20 mL ½ MSS for 13 days before the media was replaced with; ½ MS containing 20 mM sucrose and 200 μM TNT (in DMSO). Aliquots of the media were then removed at 0 h, 4 h, 24 h, 48 h and 96 h and analysed for TNT concentration by HPLC. Results for *35SGSTU24* lines are shown in green, the top panel, while *35SGSTU25* lines are shown in blue underneath. Red denotes the wild type (WT) and no plant control (NPC). Results are the means of seven biological replicates ± one standard deviation of the mean (SD).

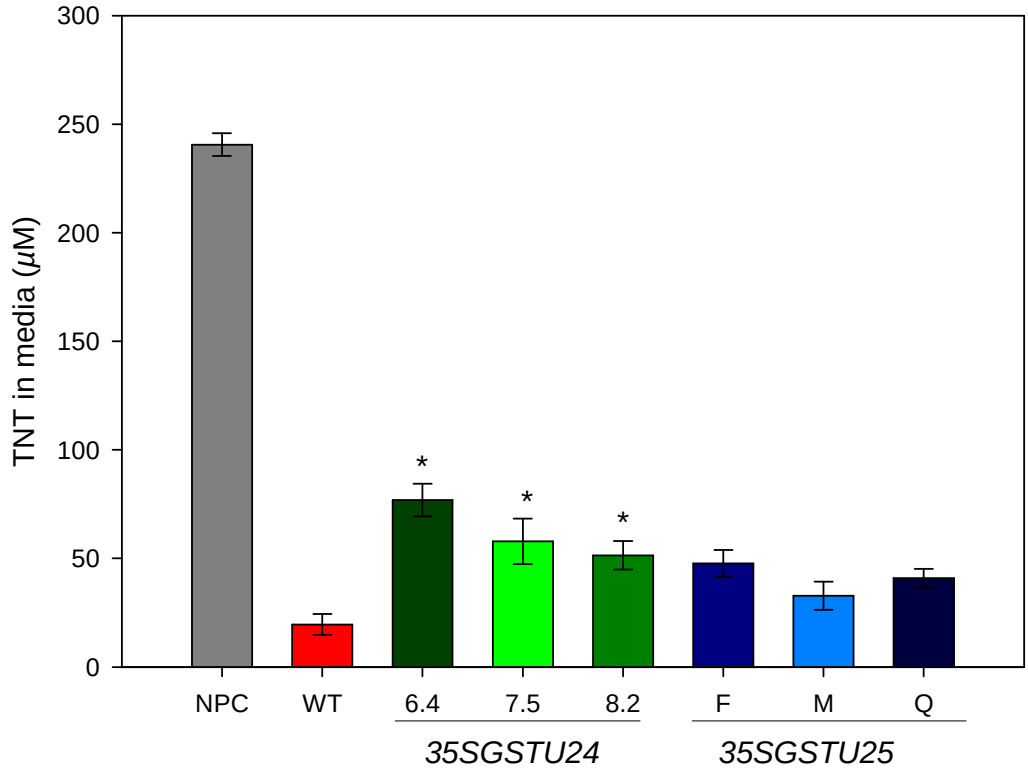


Figure 5.13: TNT depletion by *35SGSTU* lines at T= 48 h. Significant difference compared to WT is shown by an asterisk and determined by Dunnett's test, P<0.05. Results are the means of seven technical replicates and error bars represent standard error (SE) of the mean.

5.3.3.3 Morphology of *35SGSTU* Lines in Liquid Culture

When grown in liquid culture some differences were apparent between the 35SGST lines and WT. Without TNT treatment all the lines are comparable to WT, with no clear chlorosis or changes in biomass. However, in the presence of TNT many of the 35SGSTU lines, most notably *35SGSTU24* lines 6.4 and 8.2 and *35SGSTU25* lines F and Q appear to be smaller than WT (Figure 5.14). This correlates with the diminished uptake of TNT.

To investigate the observed difference in biomass, at 168h of the liquid culture experiment, the *35SGSTU24* lines were harvested and their fresh weights were compared to WT. Figure 5.15 shows the fresh weights of seven plants per line, all of the *35SGSTU24* lines have significantly less biomass than WT following TNT treatment over 168 h. Line 6.4 has a mean weight of 0.38 g, less than one third of the 1.17 g mean of the WT lines. The other two lines 7.5 and 8.2 have means of 0.6 g and 0.42 g respectively.

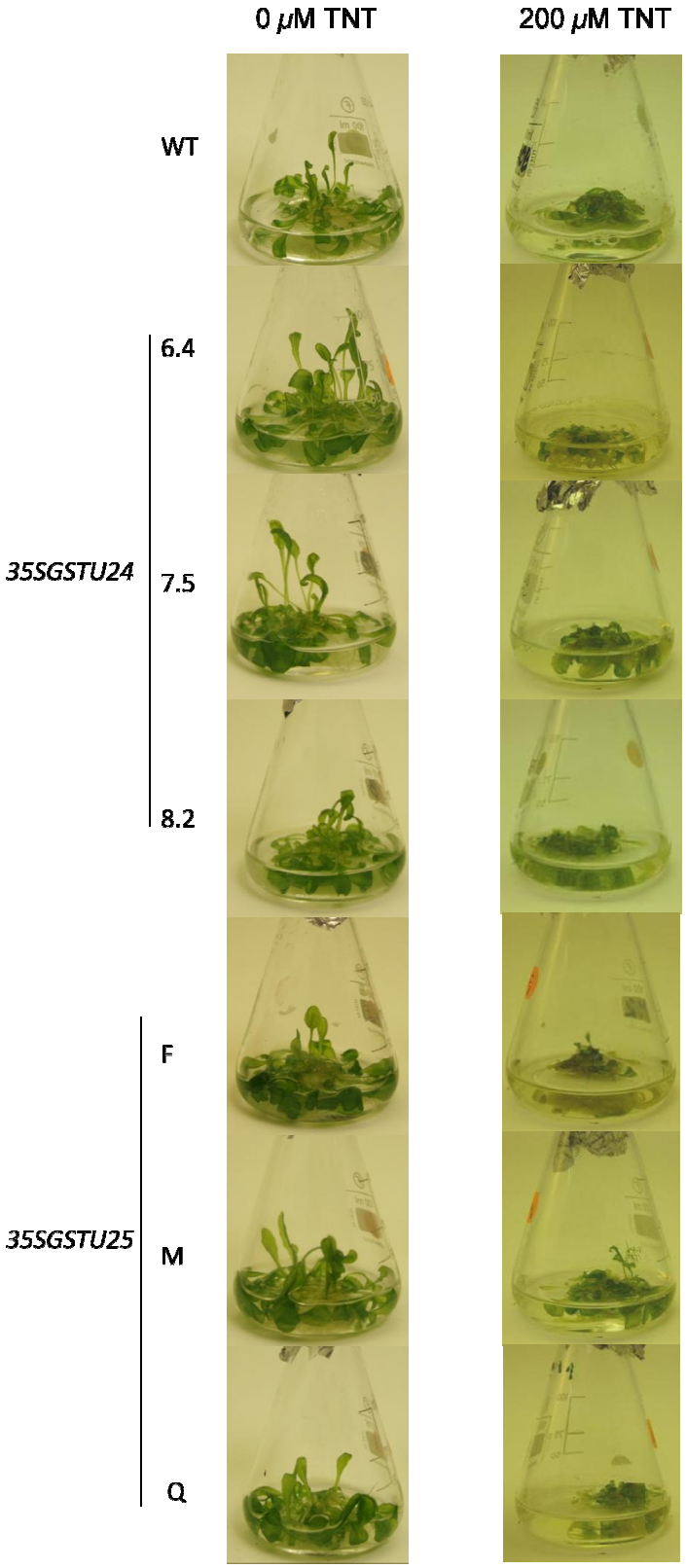


Figure 5.14: Response of *35SGSTU* grown in liquid culture with and without TNT. Eight T4 homozygous seedlings for each independent line were grown in 20 mL ½ MSS for 13 days before the media was replaced with; ½ MS containing 20 mM sucrose and 200 μM TNT (in DMSO) or DMSO only. Images show the flasks following 168 h treatment.

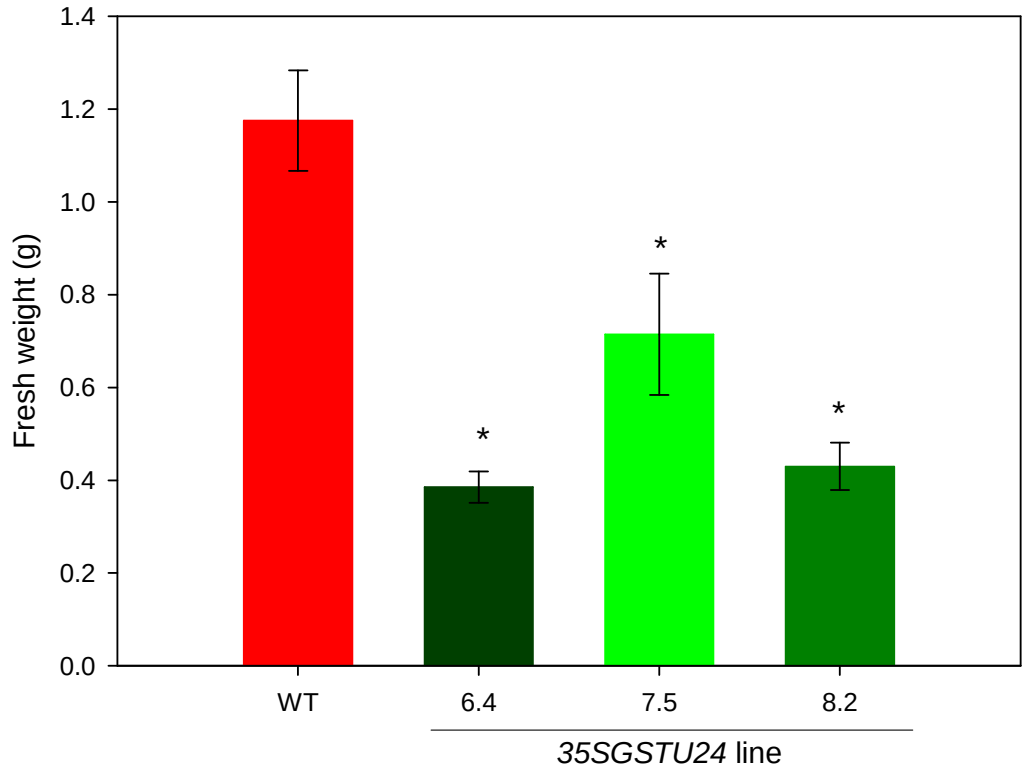


Figure 5.15: Fresh weight of *35SGSTU24* lines following 168 h TNT treatment. Eight T4 homozygous seedlings for each independent line were grown in 20 mL ½ MSS for 13 days before the media was replaced with; ½ MSS containing 200 μM TNT (in DMSO) or DMSO only. Results are the means of seven biological replicates ± one standard error of the mean (SE). Significant difference compared to WT is shown by an asterisk and determined by Dunnett's t-test, $P<0.05$.

5.3.3.4 Identification of Conjugates

Mass spectrometry was performed on the liquid culture samples shown in Figure 5.16 (t= 168 h), to identify any conjugates which may be in the media. The standard was an enzyme assay, which showed products at 7 min of 468 which fragmented to 212 and 247 (See Section 3.3.9). None of the samples showed this peak. This does not verify the lack of conjugate, but could be due to sequestration of the conjugate, limiting its release into the media (Ekman *et al.* 2003; Yoon *et al.* 2006). To investigate if a sequestered conjugated could be identified; methanol extraction of plant tissue was performed following a liquid culture assay. Mass spectrometry of these samples revealed no conjugate.

5.3.3.5 Toxicity of Conjugates

Growth curves of recombinant *E. coli* BL21-DE3 expressing GSTU24 and GSTU24 were produced to determine the toxicity of the s-glutathionyl dinitrotoluene product of the reaction between GSTs and TNT (Figure 5.16). Expression of the GSTs was induced by 1 mM IPTG. Bacterial cultures were grown overnight before dilution in LB to an OD_{600} of 0.1. The cultures were added to 96 well plates with the IPTG and TNT, with various controls. The OD_{600} was measured at 30 min intervals over 6 h. The LB only control cultures grew the fastest, with the empty vector (EV) control performing better than either GST culture. This could be because of leaky expression of the protein causing an increase in metabolic load to the recombinant cells. The presence of TNT only shows the GST cultures respond much better than the EV, again likely due to the leaky expression of GSTs, and resulting detoxification of TNT by conjugation. The EV cultures have reduced capability to detoxify TNT compared to the GST lines and are therefore more susceptible to its toxicity. The difference in growth of the GST cultures with and without TNT is small indicating that the detoxification by conjugation is not detrimental to *E. coli* growth and the conjugate produced is unlikely to be toxic. The addition of 1 mM IPTG severely limits the growth of all cultures; this was not unexpected as IPTG is toxic to bacteria, especially at such high concentrations (Baneyx 1999). This combined with the increased metabolic load arising from protein production causes the GST cultures to grow more slowly than EV.

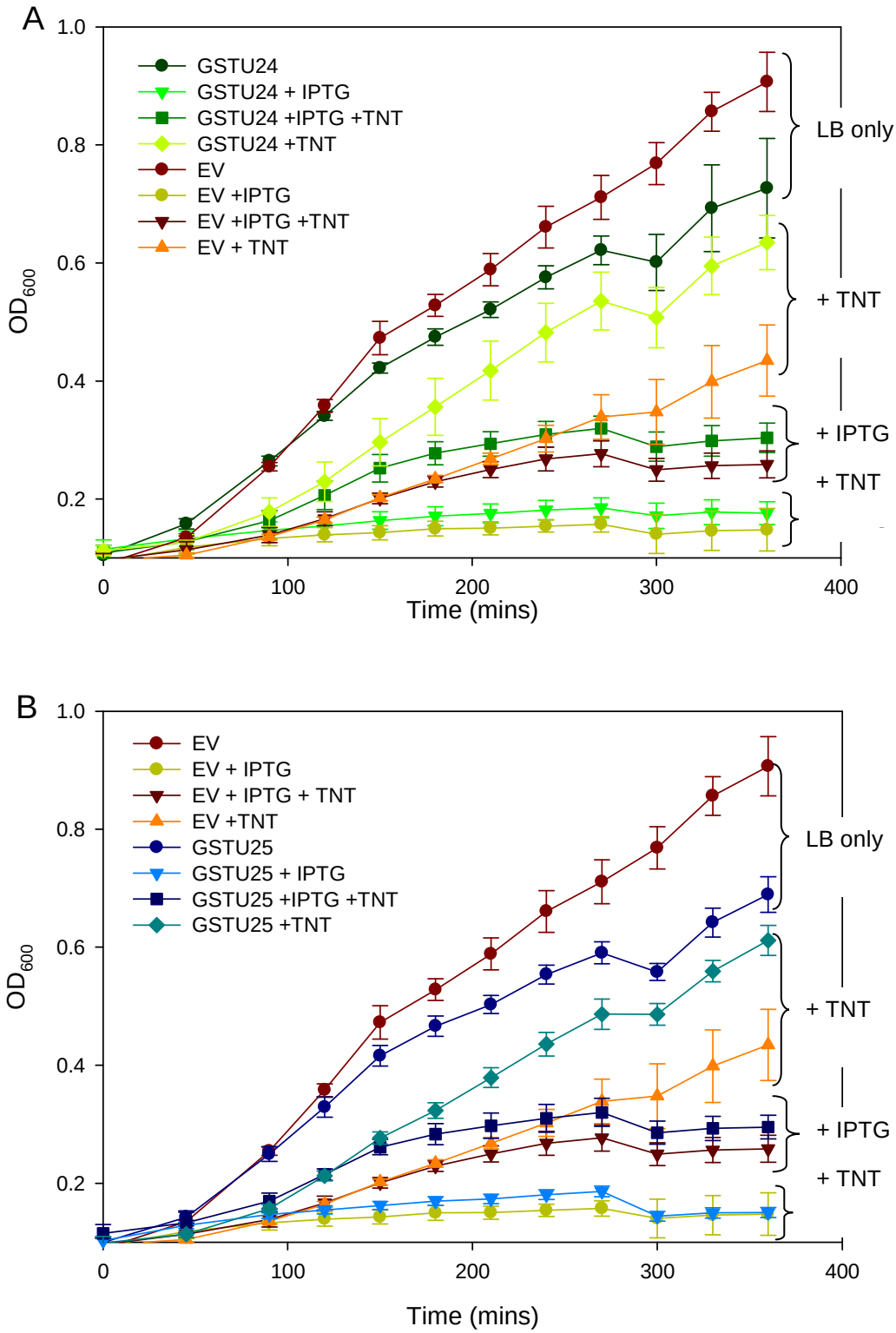


Figure 5.16: Growth curve of *E. coli* expressing GSTU24 and GSTU25 over 6 h. Cultures were grown at 37 ℃ for 6 h with shaking. Additives IPTG and TNT were added at t=0; IPTG to induce expression and TNT as a substrate for the enzymes expressed. **A**: *E. coli* expressing GSTU24 and an empty vector control. **B:** *E. coli* expressing GSTU25 and an empty vector control. Culture OD_{600} was determined using a plate reader at 30 min intervals.

5.4 Conclusions

Experiments were performed to determine the roles GSTs play in TNT detoxification. GSTs are hypothesized to have a role in TNT detoxification as microarray studies of TNT treated Arabidopsis have shown upregulation of GSTs and purified enzymes are shown to conjugate TNT to glutathione *in vitro*. GST overexpression lines were produced to identify altered tolerance compared to wild type lines; to determine if they are potential targets for phytoremediation of TNT.

5.4.1 Overexpression of GSTs

All the GSTs were successfully cloned into Arabidopsis, however only *35SGSTU24* and *35SGSTU25* overexpression lines were analysed further as *in vitro* characterisation indicated these GSTs had activity towards TNT. Ten, independent homozygous lines were produced for GSTU24 and nine were produced for GSTU25. Lines with reduced expression were not produced as previous attempts to study GSTs in this way have shown that high functional similarities exist. Both RNAi studies and SALK T-DNA insertion lines indicate that knockdown of individual GSTs or closely related groups is not sufficient to yield observable differences to stress tolerance, including TNT treatment (Yoon *et al.* 2007; Sappl *et al.* 2009). RT-PCR was performed to measure transcript levels of the GSTs in the overexpressor lines. Low increases of transcript of a closely related GST, GSTU22 which is also upregulated in response to TNT. One to two fold increases of this GST were found in all GST lines tested, for both *35SGSTU24* and *35SGSTU25* lines. Such low values indicate that co-upregulation of GSTs is not occurring to a significant degree in the 35*S*-lines. For *35SGSTU24* lines, all over expressed U24 transcript between 350 and 1000 fold more than WT. In all lines this is substantially more than the 250 fold increase in transcript for this *gst* following TNT treatment of WT seedlings. Two of the *35SGSTU25* lines, F and Q are also producing 8 and 12 fold more transcript than WT does following TNT treatment, with 1500 and 2500 fold increases in transcript levels compared to untreated WT plants respectively. However, one of the GSTU25 lines, M, is only producing 4 fold more transcript than WT, which is 50

fold less than when WT plants are TNT treated. With the exception of this line, these expression levels indicate that, as long as protein levels are also higher, any effect GSTs might have on TNT uptake or transformation should be increased in these plants. In the case of *35SGSTU25* lines, F and Q should perform better than the low overexpressor M, just as they should against WT. However, there are examples of other stress related detoxification genes being upregulated with no increase in protein levels, however further knowledge of these post-transcriptional modifications is lacking, and has never previously been observed for *35SGST* lines (Xu *et al.* 1994; Moons 2005). The western blots performed to quantify protein levels of the GSTs in the overexpressor lines were unsuccessful, likely due to inactive antibody. The use of a positive control for the antibodies would have confirmed this, i.e. a plant line overexpressing the same GST that the antibody was raised against.

A CDNB assay was performed on protein extracts of the plant tissue, to determine if the overexpressing lines were more active towards CDNB as a result of increased GST levels. Only line F was significantly more active than WT, with three times more conjugate produced than WT, although a general trend of increased conjugation by the different lines was apparent. This increase could be due to the fact that the majority of CDNB activity in plant extracts is known to result from the activity of AtGSTF8 and AtGSTU19, so even large increases in the levels of GSTU24 and GSTU25 could have a minimal overall effect on the CDNB activity (Dixon *et al.* 2009; Dixon *et al.* 2010). It is also possible that the GSTs are post-translationally modified. There are only two reports of plant GSTs being phosphorylated or glycosylated but in neither case does this reduce activity of the enzymes (Gronwald *et al.* 1998; Zhang *et al.* 2004); it is thought to assist protein folding, protect from proteases and increase solubility.

5.4.3 The Effect of GSTU24 and GSTU25 on TNT Tolerance

Preliminary root length studies compared these lines to WT when grown on media containing TNT. Both 2 and 7 μM TNT yielded slight differences compared to WT lines and when grown without TNT many of the *35SGSTU24* and *35SGSTU25* lines were longer than WT, yet in the presence of TNT they were generally shorter then WT, especially *35SGSTU25* lines. Hydroponic studies of all the *35SGSTU* lines showed that all the lines had lower TNT uptake levels than

WT, with *35SGSTU24*s performing worse than *35SGSTU25*s. Moreover, the biomass of *35SGSTU24* lines was significantly less than WT following seven days TNT treatment. Together these results indicate that the tolerance of the *35S* overexpressing lines is reduced compared to WT when grown on solid and liquid media. It must be noted that these general trends are subtle, this was also observed for overexpressing lines of OPRs and UGTs when tested on TNT. These transgenic lines clearly showed improved tolerance to TNT, however only slight differences were observed compared to untransformed lines, when tested under specific conditions optimised to visualise the variations (Gandia-Herrero *et al.* 2008; Beynon *et al.* 2009).

Glutathione conjugates are formed in the cytosol and transported into the vacuole (Coleman *et al.* 1997) and may be subsequently sequestered in the cell wall (Fricker *et al.* 2000) this restricts their release into the media and might explain why no conjugates were observed in media samples. In the vacuole, glutathione conjugates are degraded, first to *γ*-glutamylcysteinyl-*S*-conjugate, then to a cysteine-conjugate by vacuolar carboxypeptidases, final transformation by a malonyltransfrease may occur, producing a malonylcysteine conjugate, all of which will have different masses than those searched for by mass spectrometry (Marrs 1996; Wolf *et al.* 1996). However, it is possible that TNT-conjugates are not transported to the vacuole, due to inhibition of the ABC-transporters (Mezzari *et al.* 2005). Microarray data does show increased expression of AtMRP1 and 2 which are the transporters involved in conjugate vacuolar transport, suggesting that they could be involved in transporting TNT conjugates into the vacuole (Lu *et al.* 1997; Lu *et al.* 1997; Lu *et al.* 1998; Lorenz 2007).

5.4.4 Hypotheses of the Role GSTs Play in TNT Detoxification

5.4.4.1 GSTs Have a Role in Detoxification and Conjugate TNT

The most studied function of GSTs is their conjugation activity between hydrophobic compounds and glutathione. This mechanism is used by plants to detoxify xenobiotics, notably herbicides and safeners, including many with nitro-containing phenol groups, similar to TNT. Upregulation of GSTs in crop plants

and Arabidopsis have been observed following treatment by numerous herbicides and subsequent *in vitro* characterisation of these enzymes has shown high conjugating activity. Furthermore, overexpression of GSTs known to be active towards herbicides often resulted in an increase in tolerance of plants to these herbicides (Karavangeli *et al.* 2005; Hu *et al.* 2009; Benekos *et al.* 2010). It is therefore possible that the upregulation of GSTs observed following TNT treatment is an indication of their conjugation activity towards it. Moreover, *in vitro* studies with the most upregulated GSTs have shown that conjugates are produced (Chapter 3). In light of this evidence it is hypothesised that GSTs have a role in TNT transformation *in planta*, producing a GSH-conjugate with reduced toxicity (Rylott *et al.* 2009). The overexpression of these GSTs would then be expected to improve the tolerance of plants to TNT, however, results in this chapter suggest otherwise. Root lengths of overexpressing lines were not significantly different to WT when grown on either 7 or 2 μM TNT, in liquid culture experiments TNT uptake was reduced compared to WT and biomass was also reduced. While maintaining the conjugation hypothesis, a possible explanation for these results could be from increased toxicity of the conjugate. This is unlikely as glutathione conjugates identified thus far have increased solubility, elevated transport ability and decreased toxicity. Additionally the *E. coli* growth curve presented here indicated that conjugation by these GSTs does not inhibit cell growth. Despite this result in bacteria, it is possible that TNT affected conjugate transport into the vacuole *in planta*. Following exposure of Arabidopsis to TNT, a fluorescent conjugate of monochlorobimane has been shown to accumulate in the cytosol, suggesting dysfunctional vacuolar ABC-transporters blocking the Phase III and IV stages of the GST detoxification pathway (Sandermann 1994; Marrs 1996; Mezzari *et al.* 2005). This effect was also observed for plants treated with NaN_3 which inhibits ATP synthesis and conjugates remained in the cytosol (Mezzari *et al.* 2005).

Arabidopsis is a dicot, GST conjugation activity has been found to occur primarily in monocot species, providing the selectivity of herbicides, it is therefore possible that, despite the activity of the GSTs with TNT, within plant cells there are further unfavourable conditions limiting this detoxification pathway, one such factor could be glutathione levels. Arabidopsis is a monocot with relatively low levels of reduced glutathione, which are postulated to be responsible for the plants susceptibility to herbicides (DeRidder *et al.* 2002; Mezzari *et al.* 2005). However

the levels have been shown to be adequate for conjugation of xenobiotics and additionally TNT has been shown to induce glutathione reductase, which increases the pool of reduced GSH (DeRidder *et al.* 2002; Ekman *et al.* 2003; Mezzari *et al.* 2005). Consequently, it is possible that conjugation of TNT to GSH occurs, however *in vivo* results from overexpressing lines indicate that this is not a primary detoxification route as no improved tolerance to TNT was observed. GSTs may play a minor role but as no GSH-TNT conjugates have yet been identified from plant extracts, it is likely that where TNT conjugates have been identified from plants; they are likely to be glycosyl-conjugates (Gandia-Herrero *et al.* 2008; Rylott *et al.* 2009; Landa *et al.* 2010). Where any slight different has been observed between the 35SGSTU lines and WT, the overexpression of GSTs showed a negative impact on the plant. One possible explanation for this could be that GSH-conjugates of TNT are being produced but they are inhibitory to the proposed primary detoxification pathway of OPR-UGT metabolism, accounting for the reduced tolerance and uptake of 35SGSTU lines to TNT. It is also possible that GSTs do not conjugate TNT to GSH in plants but instead play a role in general oxidative stress response rather than direct conjugation.

5.4.4.2 GSTs are Involved in the Oxidative Stress Response

In addition to upregulation of GSTs, TNT treatment causes an oxidative stress response in Arabidopsis (Ekman *et al.* 2003). Enzymes involved in reactive oxygen species (ROS) scavenging are often upregulated during environmental stress including; oxidative stress, pathogen attack, herbicide treatment and exposure to abiotic treatments (Zhu *et al.* 1994; Moons 2005). The reactive oxygen species produced during oxidative stress cause membrane lipid peroxidation and DNA damage, producing cytotoxic products for example H_2O_2, which needs to be eliminated. GSTs can conjugate GSH to these molecules however this has not been observed in plants and the major role of GSTs in oxidative stress it likely to be through their glutathione peroxidase (GPOX) activity (Pickett *et al.* 1989; Dudler *et al.* 1991; Bartling *et al.* 1993). Cytotoxic hydroperoxides are produced as a result of oxidative stress and their reduction to alcohols is performed by selenium-dependent GPOX, non-selenium dependent phospholipid hydroperoxide glutathione peroxidases (PHGPX) and GSTs with GPOX activity (GST-GPOX), with the oxidation of two molecules of reduced

glutathione as electron donors (Eshdat *et al.* 1997; Dixon *et al.* 1998). However, it has been reported that the GST-GPOXs have limited activity, reducing only organic hydroperoxides of fatty acids produced by oxidative membrane damage and showing no activity towards phospholipid hydroperoxides or hydrogen peroxide. Not only that but each GST with GPOX activity has distinct substrate specificities, suggesting individual roles for the GSTs in stress tolerance (Moons 2005). Both GSTU24 and U25 have been demonstrated to have GPOX activities, with U25 displaying very high activity towards cumene hydroperoxide, but as yet no lipid hydroperoxide substrates have been established (Dixon *et al.* 2009). It is therefore possible that GSTs play a role as ROS-scavenging enzymes, and their upregulation is a result of the oxidative stress resulting from TNT treatment. The *in vitro* conjugation of TNT could be simply a side-reaction resulting from the structural similarities between TNT and CDNB and no significant conjugation occurs *in planta* following TNT treatment. However, if GSTs with GPOX activity are involved in countering the stress response, it seems likely that plants over expressing these enzymes would show improved tolerance to TNT. Interestingly, the opposite is seen and the overexpression of GSTs U24 and U25 hinders TNT uptake and reduces biomass following TNT application. However, 0.1 and 1.0 mM TNT treatment has been shown to inhibit peroxidase activity in horseradish plants, despite the stimulation of expression by the explosive (Nepovim *et al.* 2004). It is also possible that the upregulation of GSTs by TNT occurs by the same mechanism as safener- induced expression and these GSTs offer no stress response or TNT transformation capability to the plant.

5.4.4.3 TNT is a Safener, Inducing GSTs

The structural similarity of TNT to other known substrates of GSTs may be adequate to induce expression and allow for low levels of *in vitro* activity but this is not sufficient to cause a physiological effect on TNT treated plants (Marrs 1996). This gene induction would be a result of a safeners-effect, enhancing synthesis of detoxification enzymes. Safeners are non-phytotoxic compounds which confer protection to cereal crops from herbicide injury (Riechers *et al.* 2010). They reduce the toxicity in a number of ways; direct interaction with biochemical targets or receptor proteins of herbicides, increasing rates of degradation or reducing uptake and translocation of herbicides (Davies *et al.*

1999). The most studied mechanism is promotion of herbicide metabolism in plants (Marrs 1996). It is assumed that safeners modulate the activity of transcription factors which interact with specific regulatory elements of the promoters of metabolic enzymes (Davies *et al.* 1999). Safener structures often resemble those of their respective herbicide and act by inducing detoxification genes GSTs, UGTs and P450s, stimulating vacuolar transport of conjugates and increasing the levels of cofactors for example GSH (Hatzios 1983). The induction of GSTs by TNT could be a result of the same induction pathway as observed for safeners. Regulatory elements on the promoters of *Gsts* react to both specific and broad signals. Octopine synthase (*ocs*) elements, present in the promoter regions of many GSTs are stress inducible, stimulated by hormones, heavy metals, oxidative stress and pathogen attack (Marrs 1996; Riechers *et al.* 2010). Whether TNT itself is a signal or if GST expression is a response to the resultant oxidative stress is not yet known. Additionally, although safeners stimulate GST expression in dicots, they are not effective at protecting them from herbicide injury. This suggests that unknown additional factors must be integral for the protection of monocot cereal crops from herbicide injury (DeRidder *et al.* 2002; DeRidder *et al.* 2006; Riechers *et al.* 2010). The work presented here is with the dicot Arabidopsis, hence the upregulation of GSTs by TNT may not be protective (DeRidder *et al.* 2006). This is observed for safeners which do induce GSTs in dicots, yet herbicides remain active towards them. This implies that GSTs could play a detoxification role in monocots.

5.4.5 Concluding Remarks

The overexpression of GSTs in Arabidopsis reduces TNT tolerance and uptake rates. No glutathionyl-TNT conjugates have been observed from plant samples, this may be due to their absence or their metabolism in the vacuole, though previous reports suggest that vacuolar transport is arrested by TNT. Three hypotheses have been proposed to explain the data presented in this chapter however none are fully justified by the results.

Chapter 6: Discussion

The detoxification of 2,4,6-trinitrotoluene (TNT) in plants is thought to follow the 'green liver model', with Phase I transformation, Phase II conjugation and Phase III transport and sequestration (Sandermann 1994). However for an effective phytoremediation approach it is imperative to gain a better understanding of the specific mechanisms of TNT detoxification used by plants. Previous investigations within this group have stemmed from a microarray of TNT treated Arabidopsis, which highlighted the upregulation of numerous detoxification gene families which could indicate their involvement in TNT transformation, including reductases, P450s and transferases (Lorenz 2007). Thus far, TNT detoxification studies of upregulated genes have been performed for oxophytodienoate reductases (OPRs), which can catalyse Phase I detoxification of TNT catalysing nitro group reduction producing; hydroxylaminodinitrotoluenes (HADNTs) and aminodinitrotoluenes (ADNTs) in addition to aromatic ring reduction yielding hydride and dihydride Meisenheimer products (Beynon *et al.* 2009). Investigation into the glycosyl- transferases (UGTs) has also been performed, these Phase II enzymes catalyse the conjugation of glycosyl molecules to both isomers of HADNT and ADNT, likely reducing the toxicity of TNT and leading to sequestration (Gandia-Herrero *et al.* 2008). Also upregulated in the microarray were a number of the glutathione transferase (GST) family (Lorenz 2007). GSTs are Phase II detoxification enzymes which function by conjugating the tripeptide, glutathione (GSH) to an electrophilic substrate (Dixon *et al.* 2010). Their activity in plants has been widely studied owing to their role in herbicide detoxification (Tal *et al.* 1993; Cummins *et al.* 1997; Dixon *et al.* 2003; Mezzari *et al.* 2005). It has been proposed on numerous occasions that GSTs play a role in TNT detoxification in plants, although no direct evidence has been observed (Mezzari *et al.* 2005; Rylott *et al.* 2009; Landa *et al.* 2010).

The aims of this project were to identify if GSTs are active towards TNT and whether they are involved in TNT detoxification by Arabidopsis. To investigate this, both *in vitro* and *in vivo* characterisations of Arabidopsis GSTs were

performed. Seven GSTs were selected due to their high levels (8 to 47 fold) of upregulation following TNT treatment of Arabidopsis as detected by microarray analysis (Lorenz 2007).

Real-time PCR (RT-PCR) of Arabidopsis treated with TNT verified the upregulation of the seven selected GSTs observed by the microarray experiments (Lorenz 2007), however the increased sensitivity of RT-PCR revealed expression levels to be 40 to 300 fold upregulated. The seven GSTs were cloned and expressed in *Escherichia coli* and purified by both GSH- and His- affinity chromatography. His-purification was more successful than GSH-purification as many of the GSTs (U1, U3, U4 and U7) did not bind well to the GSH- sepharose. This was unexpected as Tau class GSTs are commonly successfully purified from plant extracts by GSH- affinity chromatography (Edwards *et al.* 2005). Activity assays of the GSTs were performed with 1-chloro,2,4-dinitrobenzene (CDNB) considered to be a generic substrate for GST studies. The observed activities were similar to rates previously published for these GSTs (Dixon *et al.* 2009); five of the seven GSTs were able to conjugate this substrate to GSH significantly more than lysate of *E. coli* containing empty vector, with GSTU24 and U25 exhibiting the highest rates of activity whilst GSTU1 and U4 activities were not significantly more than the negative control. The failure of GSH-purification and the lack of activity towards CDNB suggest that GSH has a reduced affinity to GSTU1 and U4; however sequence alignment of the GSTs showed that the catalytically important residues of the GSH-binding site are the same for all GSTs in this investigation. An assay was performed to determine if any of the GSTs had conjugation activity towards TNT. Previous reports of GSTs with conjugating activity towards TNT are limited to crude extract of equine liver GST (Brentner *et al.* 2008), which was therefore used as a positive control. TNT assays confirmed that the equine GST was able to reduce TNT concentration and produce a conjugate, and plant GSTs U24 and U25 were also active, with GSTU25 showing the most activity of the two. However, the assays with TNT and purified GSTs U1, U3, U4, U7 and U22 did not yield a decrease in TNT concentration or production of conjugate. It is therefore interesting that GSTU24 and U25 were the GSTs upregulated the most in the microarray and RT-PCR data, and the most active of the seven enzymes towards both the

generic GST substrate CDNB and TNT. This strengthens the likelihood that these enzymes play a role in Phase II conjugation of TNT in plants. To identify the structure of the conjugate mass spectrometry of TNT assays with purified GSTU24 and U25 were performed. This showed conjugation between TNT and GSH occurs through substitution of one of the nitro groups of TNT, also previously identified for equine liver GST (Brentner *et al.* 2008). However, it was not possible to further fragment the conjugate ion sufficiently to allow identification of the specific nitro group removed. The removal of a nitro group from TNT is a highly sought after reaction, it is the presence of three electron withdrawing nitro groups on TNT which provide stability to the aromatic ring through resonance (Qasim *et al.* 2007). This reduced stability could subsequently allow degradation and complete mineralisation.

In Chapter 4 the activities of GSTU25 towards TNT and other substrates were characterised. The assay conditions were optimised, with pH 6.5 phosphate buffer producing the highest results for conjugate production and TNT stability, a relatively low pH compared to other characterised plant GSTs (Habig *et al.* 1974; Irzyk *et al.* 1993; Edwards *et al.* 2005; Kunieda *et al.* 2005). Optimal temperature was 30 ℃, which produced the fastest reaction rate with minimal protein inactivation and GSH oxidation occurring over the reaction length. This concurs with optimal temperatures for other plant GSTs (Dixon *et al.* 2005; Nutricati *et al.* 2006; Farkas *et al.* 2007). The optimised conditions were employed for kinetic analysis of the reaction between GSTU25, GSH and TNT. Due to the low solubility of TNT, the substrate concentration used was not high enough to identify a V_{max} and determine an accurate K_m of the reaction. Interestingly, the Michaelis-Menten plots for TNT depletion and conjugate production were different. The rate of conjugate production appeared to level off, while TNT depletion remained linear (Figure 4.15). Despite careful analysis of previous mass spectrometric data, the presence of a diconjugate was not identified and breakdown of GSH- conjugates due to stability is not expected to occur. Extrapolated values from the TNT Michaelis-Menten plots compared to values from CDNB kinetics indicate that CDNB is a significantly better substrate than TNT for GSTU25.

The activity of GSTU25 towards structurally similar compounds; hexahydrotrinitrotriazine (RDX), dinitrotoluene (DNT) and aminodinitrotoluenes (ADNTs) was assayed to determine the substrate specificity of the enzyme. GSTU25 showed no activity to any of these tested substrates except TNT and CDNB. This suggests that the electrophilic strength of the aromatic centre is important for substrate binding in the active site. It also provided evidence for the reaction mechanism. The nitro groups of CDNB and the GST inhibitor trinitrobenzene (TNB) are resistant to substitution by the glutathione anion (Armstrong 1991; Bowman *et al.* 2007), however for TNT a nitro group is the leaving group. It is therefore proposed that the presence of the methyl group in TNT has an important role; it has an inductive effect supplying electrons to the neighbouring 2- and 4- nitro groups, reducing their resonance stability. This makes nucleophilic substitution by the thiolate anion favourable; making GSTU24 and U25 the first enzymes identified which are capable of catalysing nitro group removal from TNT. This reaction is desirable for remediation purposes as the stability of the dinitrotoluene (DNT)- moiety of the conjugate is reduced compared to TNT and could allow complete mineralisation of TNT, as observed for 2,4-DNT which in bacteria can be further denitrated by dioxygenases, to produce 2,4,5-trihydroxytoluene and eventual ring cleavage (Nishino *et al.* 2000).

In Chapter 5 *35SGSTU* overexpression lines were produced in Arabidopsis to assess the role of GSTs in TNT transformation *in planta*. No lines with reduced expression were created as previous studies have found the functional overlap of GSTs too high to observe effects of individual genes (Yoon *et al.* 2007; Sappl *et al.* 2009). Overexpressing lines were produced for GSTU24 and U25, the two enzymes which exhibited activity towards TNT *in vitro*. RT-PCR analysis of transcript levels show that of the lines selected for study all had hundred or thousand fold- higher transcript levels of the appropriate GST than WT, except for *35SGSTU25* line M, which was only four-fold overexpressed. There was also little overlap in overexpression of the GSTs, despite their high sequence identity. Analysis of protein levels by western blot was unsuccessful, possibly the supplied antibodies were no longer functional. A CDNB assay of protein extracts from *35SGSTU24* and *25* overexpression lines was performed to investigate if increased protein levels reflected the increased transcript levels shown by RT-

PCR. The overexpressing lines generally had some increase in activity towards this generic substrate, though not at a significant level for most lines, this is probably due to the activity of GSTs U19 and F8, which account for the majority of CDNB activity in plants and are likely to have overwhelmed the response of GSTU24 and U25 overexpression (Dixon *et al.* 2010).

The lack of reliable data for protein overexpression levels limits the conclusions that can be drawn from the experiments performed with the *35SGSTU* lines. If this work were to be repeated, the GSTs would be cloned into plants with the addition of an affinity tag, an extraction and purification method would have allowed easy determination of the levels of overexpressed protein in each line.

Analysis of TNT tolerance of the overexpressing lines by root length and liquid culture studies was performed. In general these results showed that the *35SGSTU* lines showed reduced TNT tolerance and uptake compared to untransformed, wild type (WT) plants on TNT media, although root lengths of the plant lines when on media containing no TNT appeared enhanced or the same as for WT. This interesting observation, along with the reduced tolerance on TNT amended media suggests that GSTs do have a role in the plant's response to TNT, but it does not significantly improve tolerance to the xenobiotic.

The combination of *in vitro* and *in vivo* results from this thesis has provided three putative explanations for the role of GSTs in the TNT response of plants. The first is that GSTs are able to detoxify TNT by catalysing its conjugation to GSH. The *in vitro* results from recombinant GSTs in Chapter 3 indicated that GSTU24 and U25 are capable of conjugating TNT to GSH, and this product (GS-DNT) was shown to have reduced toxicity by a growth curve of TNT and recombinant *E. coli* expressing Arabidopsis GSTs. However, if conjugation were to occur within the plant then the overexpressing lines would be expected to exhibit improved tolerance to TNT than untransformed lines. Experimental evidence shows that the opposite is true and that the overexpression of GSTs in Arabidopsis leads to slightly reduced tolerance to TNT. In addition to this, the presence of conjugates in plant extracts has not been observed. It is possible that the GS-DNT conjugates are exerting a toxic effect on the OPR-UGT pathway, which might account for the decreased tolerance of the overexpressing lines. To test for this,

an OPR assay with TNT could be spiked with the conjugate to determine if any inhibition occurs. It is also possible that TNT is inhibiting the transport of conjugates into the vacuole and their accumulation within the cytosol is detrimental to the plant. Results suggesting the inhibition of ABC transporters have been shown by TNT treatment, which inhibits the transport of fluorescent conjugates (Mezzari *et al.* 2005). If this were to occur in the *35SGSTU* overexpressing lines, then the build up of conjugates could account for the reduced tolerance to TNT of those lines compared to WT. This could be assessed with a similar method to Mezzari et al. (2005) by investigating the localisation of conjugates of other compounds, for example monochlorobimane, in the presence of TNT with the 35SGSTU lines. The identification of TNT conjugates within the *35SGSTU* lines by mass spectrometry should also be optimised, with a positive control of CDNB treated plants.

To assess the fate of TNT in the *35SGSTU* lines, radiolabelled TNT could be used. This would allow visualisation of the localisation of TNT within the plant (Brentner *et al.* 2010) and fractionation of plant samples would show where the TNT was sequestered, for example in the vacuole or cell wall. However, the difficulties in obtaining such a chemical were prohibitive in this project.

GSTs have an emerging role in the oxidative stress response of plants (Marrs 1996). GSTU25 and to a lesser extent, GSTU24 have glutathione peroxidase (GPOX) activity (Dixon *et al.* 2009; Dixon *et al.* 2009). It is therefore possible that, following TNT treatment, GSTs act as reactive oxygen species (ROS) scavenging enzymes, removing cytotoxic products resulting from oxidative stress. However, if this were a protective role of GSTs, their overexpression in Arabidopsis would be expected to confer increased tolerance to TNT treatment.

Reduced toxicity resulting from GPOX expression has been previously observed when a cotton GST with GPOX activity was expressed in tobacco; the transformed plants had lower levels of expression of the antioxidant enzymes peroxidase and superoxide dismutase (Yu *et al.* 2003). To determine if these two GSTs are protecting from oxidative stress, the *35SGSTU* lines from this work should be tested for improved resistance to oxidative stress, it would also be interesting to assess tolerance on other abiotic stresses such as chilling and salt treatments (Pickett *et al.* 1989; Bartling *et al.* 1993; Yu *et al.* 2003), which have been shown to be improved by GST overexpression. GSTU25 is known to have

high activity towards cumene hydroperoxide, it would also be interesting to determine if the overexpressing plants show enhanced GPOX activity towards this substrate (Dixon *et al.* 2009). The levels of glutathione in the *35SGSTU* lines should also be determined as they might also indicate differential oxidative stress responses to WT.

It is possible that, despite the activity of GSTs towards TNT *in vitro*, their upregulation does not confer any activity towards TNT or products of oxidative stress in plants. Safeners are compounds which prepare crops for herbicide application, priming their detoxification pathways (Davies *et al.* 1999). Interestingly, whilst safeners induce gene expression, only in monocots are active proteins produced. It is this distinction which is exploited when safeners are applied to monocot crops. Safeners, like TNT share structural similarities with herbicides and function by inducing gene expression of GSTs and other detoxifying genes (Marrs 1996; DeRidder *et al.* 2002). The upregulation of GSTs in Arabidopsis could, as for safeners, simply induce expression without providing any further activity towards the compound. It is important to note that the induction of GSTs by safeners in Arabidopsis does not result in protection from herbicides as it does for monocot crop species (DeRidder *et al.* 2006). This indicates that detoxification by glutathionylation is less effective in dicotyledonous plants and it is possible that if this work were to be repeated in a monocot crop species, glutathione conjugation of TNT may be observed. If the plants show no increased conjugating activity to other substrates or no improved tolerance to stress treatments then the hypothesis for induction of expression but no further activity would be favoured.

This work has investigated the role of GSTs in TNT detoxification. The activity of two Arabidopsis GSTs towards TNT has been identified, with *in vitro* assays and mass spectrometry showing that conjugation of TNT to GSH occurs through nitrite release. Despite this, overexpression of GSTs in Arabidopsis does not improve tolerance to TNT indicating that GSTs are unlikely to play a major role in TNT transformation in plants. However further work outlined above must be performed to confirm this.

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