THE ROLE OF GLUTATHIONE TRANSFERASES IN TNT DETOXIFICATION

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Abstract

Glutathione transferases (GSTs) are multi-functional enzymes with an important role in plant biochemical mechanisms of xenobiotic detoxification. The GSTs have been extensively studied due to their ability to detoxify herbicides; recently they have been implicated in numerous stress responses, including defence against pathogen attack, oxidative stress, heavy metal toxicity and the detoxification of a wide variety of xenobiotic compounds.

GSTs catalyse the conjugation of the tripeptide glutathione (GSH) (γ-Glutamyl-Cysteinyl-Glycine) to a variety of hydrophobic, electrophilic, and usually cytotoxic substrates to form a polar S-glutathionylated reaction product. Products of oxidative stress can also be a substrate as GSTs have a role in the protection of tissues against oxidative damage.

This project focused on the role of GSTs from *Arabidopsis thaliana* to detoxify 2,4,6-trinitrotoluene (TNT). Globally, manufacturing, use and storage of TNT has resulted in widespread soil contamination. Current strategies of TNT remediation (incineration, composting and landfilling) are expensive and damaging to the environment. Phytoremediation using the ability of plants to uptake, metabolise and detoxify xenobiotics *in situ* could present a cost-effective, non-invasive, environmentally friendly alternative.

In vitro studies focused on investigating the conjugation activity of purified recombinant GSTs with TNT. *In vivo* experiments aimed to determine the role of GSTs in TNT detoxification in Arabidopsis plants transformed with 35S-GST constructs in comparison with untransformed, wild type plants.

Purified recombinant GSTU24 and GSTU25 directly conjugated TNT to form a range of glutathionylated products. GSTU25 was found to produce 2-glutathionyl-4,6-dinitrotoluene, formed by nucleophilic substitution of a nitro group

by glutathione, previously identified in the conjugation reaction of mammalian GSTs with TNT. The data on two other compounds suggest the substances are C-glutathionylated 4-hydroxyaminodinitrotoluene and C-glutathionylated 2-hydroxyaminodinitrotoluene with the glutathione attached via the methyl group of TNT. The effect of pH and temperature on product formation and enzyme activity was also studied.

Experiments using transgenic lines overexpressing GSTU24 and GSTU25 showed better tolerance of the overexpressing lines compared to the wild type plants, as characterised by enhanced root growth in TNT containing medium and faster TNT removal from the growth medium.

In summary, this work presents biochemical data on the first identified glutathionylated TNT conjugates produced by plant GSTs *in vitro* and in the plants. It also shows that overexpressing detoxification enzymes in the plant results in increased transformation of TNT, improving the ability of plants to tolerate the pollutant, and could lead to the identification and breeding of native plant species with active detoxification systems suitable for the phytoremediation of contaminated sites.

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Author’s Declaration

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

Abbreviations

½ MS(A)	Murashige and Skoog medium half strength (with 8 g/l agar)
ABC transporters	ATP-binding cassette transporters
2-ADNT	2-amino 4,6-dinitrotoluene
4- ADNT	4- amino 2,6-dinitrotoluene
2-HADNT	2-hydroxylamino 4,6-dinitrotoluene
4- HADNT	4- hydroxylamino 2,6-dinitrotoluene
BITC	benzyl isothiocyanate
CDNB	1-chloro-2,4,-dinitrobenzene
DHAR	dehydroascorbate reductase
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency (USA)
GPOX	glutathione peroxidase
GST	glutahthione transferase
GSH	glutathione reduced
GSSG	glutathione oxidised (oxidised disulfide derivative)
HPLC	high-performance liquid chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
JA	jasmonic acid
k_{cat}	maximum turnover rate
LB	Luria-Bertani medium
LIC	ligation independent cloning
LC/MS	liquid chromatography–mass spectrometry
MS	mass spectrometry
NED	N-1-napthylethylenediamine dihydrochloride

NMR	nuclear magnetic resonance
NO-DNT	nitroso-dinitrotoluene
OCS	octopine synthase
OPDA	12-oxo-phytodienoic acid
OYE	Old Yellow Enzyme
PVPP	polyvinylpolypyrrolidone
ROS	reactive oxygen species
SA	salicylic acid
SAGE	serial analysis of gene expression
TCHQD	tetrachlorohydroquinone dehalogenase
TNT	2,4,6-trinitrotoluene
UDP	uridine diphosphate
UGT	UDP-glycosyltransferase

1. Introduction

1.1. Phytoremediation

1.1.1. TNT toxicity

2,4,6-Trinitrotoluene (TNT) is the most widely used military explosive and is associated with wide-scale soil contamination. It belongs to the nitroaromatics group of explosives that are characterized by an aromatic ring and three nitro groups. (Fig. 1.1). The nitro groups contribute to the stability of the chemical structure by withdrawing electrons from the aromatic ring. This makes TNT resistant to the enzymes involved in the microbial metabolism of aromatic compounds, making it recalcitrant to degradation in the environment (Hannink et al. 2002; Lima et al. 2011).

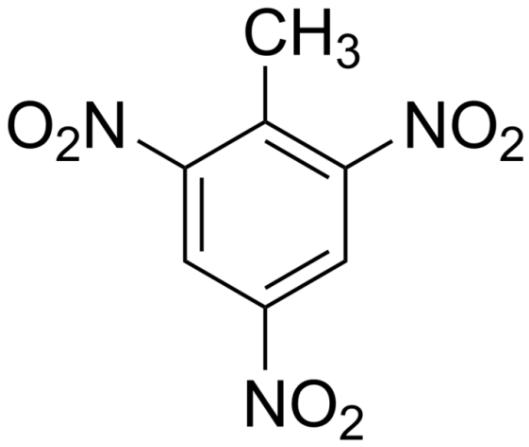


Fig. 1.1. The chemical structure of 2,4,6-Trinitrotoluene.

The nitro groups also have a strong oxidising power, making TNT highly toxic to living organisms (Rylott & Bruce 2009).

In humans, TNT is readily absorbed by the skin, respiratory tract and gastrointestinal tract (Lima et al. 2011). Exposure to TNT is known to cause rashes, skin haemorrhages, aplastic anemia and hepatitis (Hannink et al. 2002; Lachance et al. 1999).

Nitroreductases in the liver metabolize TNT into 2-aminodinitrotoluene (2-ADNT) and 4-aminodinitrotoluene (4-ADNT) as the major metabolites. The nitro and hydroxylamine groups responsible for the toxicity of TNT react with biological molecules causing carcinogenic and mutagenic effects (Lima et al. 2011). TNT is considered a possible human carcinogen by the EPA and its metabolites exhibited variable levels of mutagenicity and cytotoxicity in different assays using bacterial and mammalian cell systems (Honeycutt et al. 1996; Lachance et al. 1999; Tan et al. 1992; Tchounwou et al. 2001; Won et al. 1976).

In plants TNT causes chlorosis, stunting of roots and inhibition of lateral root growth (Pavlostathis et al. 1998) and affects seed germination and fresh seedling biomass (P. Gong et al. 1999).

1.1.2. TNT pollution

Large areas previously used as firing ranges and open detonation sites together with places of production, burning, detonation and dismantlement of munitions are heavily contaminated with TNT. Even though the United States ceased TNT production in the mid-1980s, the EPA reports more than 30 munitions sites covering several thousand square kilometres across the United States contaminated with explosives (www.epa.gov). The United States Department of Defense has identified more than 1000 sites with explosives contamination, of which more than 95% were contaminated with TNT (Rodgers & Bunce 2001). Similarly, many former military sites in Germany, often from World War I and II, have been contaminated

with TNT and other contaminants (Schoenmuth & Pestemer 2004). Explosives contamination levels are heterogeneous, with very high levels of pollution (50 - 200 g TNT /kg soil) found on relatively small hot spot areas and lower (<1 g TNT/kg soil) and diffuse levels present at battlefields or training and testing ranges (Stenuit & Agathos 2010).

1.1.3. Phytoremediation

Current strategies of TNT remediation (incineration, composting and landfilling) are expensive, insufficient to address the scale of the problem and damaging to the environment. Figures from 2005 showed that $6–8 billion per year was spent on environmental cleanup in the United States, and $25–50 billion per year worldwide (Pilon-Smits 2005). Phytoremediation using the ability of plants to uptake, metabolise and detoxify xenobiotics *in situ* could present a cost-effective, non-invasive, environmentally friendly alternative (Hannink et al. 2002; McCutcheon & Schnoor 2003; K. C. Makris et al. 2007a; K. C. Makris et al. 2007b). Plant biotechnology usually tolerates high concentrations of contaminants better than microorganisms (Rodgers & Bunce 2001). The EPA considers phytoremediation cheaper, requiring less equipment and labour than other methods. Trees and other plants can also make a site more attractive. The site can be cleaned up without removing and disposing of polluted soil (www.epa.gov). The U.S. phytoremediation market has grown two to threefold during 2000 - 2005. In Europe there is no significant commercial use of phytoremediation, but this may change in future because of increased interest and research, and the need for remediation in many polluted sites across the European Union countries. Phytoremediation may also become a suitable technology for remediation projects in developing countries because it is cost-efficient and easy to implement (Pilon-Smits 2005).

Phytoremediation involves several different processes (Fig. 1.2). Phytoextraction enables concentrating contaminants in the plant biomass without degradation; phytostabilisation reduces the bioavailability of contaminants by binding them in plant tissues. In phytodegradation, the plant enzyme systems metabolise the toxic compounds to a less toxic or less bioavailable compound, while phytovolatilisation uses plant transpiration to release pollutants in a modified form into the atmosphere. Additionally, rhizodegradation is the breakdown of contaminants in the soil through microbial activity, which is enhanced by root exudates (phytostimulation) (Hannink et al. 2002; Pilon-Smits 2005; Rodgers & Bunce 2001).

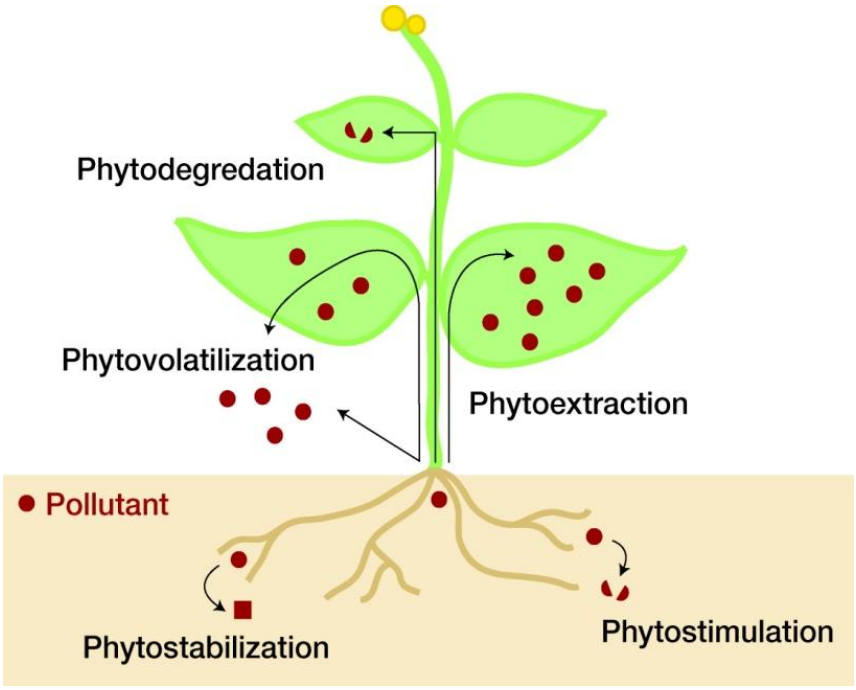


Fig. 1.2. Different processes of phytoremediation: the pollutant (represented by red circles) can be stabilized or degraded in the rhizosphere, sequestered or degraded inside the plant tissue, or volatilized. (Figure from Pilon-Smits 2005).

The limitations of phytoremediation lie in the use of a suitable plant capable of growing in given soil properties, toxicity level and climate. Phytoremediation is

also limited by root depth because the plants have to be able to reach the pollutant, and by availability of the contaminant.

Grasses can be particularly useful in cleaning up soils contaminated with TNT due to their fast growth and adaptability to various types of soil and climate. Vetiver grass (*Vetiveria zizanioides*) demonstrated high affinity for TNT without any visible toxic effects in hydroponic systems (40 mg TNT/l for 8 days) (K. C. Makris et al. 2007b) as well as in soil experiments (80 mg TNT/kg), especially due to the catalytic effect of urea (0.1 %) on TNT absorption by the plant (Das et al. 2010; K. C. Makris et al. 2007a). Several tree species have also been studied for their high phytoremediation potential (*Salix* sp. and *Populus* sp.) due to their longevity, low nutrient and soil quality requirements and high tolerance to many soil pollutants (Brentner et al. 2008; Schoenmuth & Pestemer 2004; B. Van Aken et al. 2004).

To increase the efficiency of phytoremediation technologies, it is important to study the biological processes involved. These include xenobiotic uptake, translocation, tolerance mechanisms (compartmentation, degradation) and transport and storage in the plant (Pilon-Smits 2005).

1.1.4. Xenobiotic metabolism in plants

Plants have evolved numerous and complex detoxification pathways to deal with the stress factors, including toxic chemicals, they are exposed to in their environment. Metabolism of xenobiotics has been classified into three phases. In phase I the compound is activated by adding functional groups (most often hydroxyl, amino or sulphuryl groups). These reactions create active sites in the substance prior to the Phase II transformation and although there is a possibility of transforming the substance to a more toxic metabolite, generally products of these reactions are less toxic than the original compound. This transformation involves one or more enzymes, and oxidation or reduction is the most

common type of reaction depending on the plant species and type of xenobiotic (Bhadra et al. 1999; Hannink et al. 2002).

During phase II the activated substance undergoes conjugation with a hydrophilic molecule (saccharide, malonate or glutathione) catalyzed by glucosyl-, malonyl-, or glutathione transferases, respectively, to produce a water soluble conjugate that is usually nontoxic or less toxic than the parent compound (Hannink et al. 2002; Rylott & Bruce 2009).

In phase III the conjugated xenobiotic is sequestered by the cell usually to the vacuole or cell wall, depending on its solubility (Hannink et al. 2002; Rylott & Bruce 2009).

1.1.5. Metabolism of TNT in plants

Although TNT is phytotoxic, toxicity is species dependent. The phytotoxicity of TNT to all plant species tested is demonstrated by chlorosis (change in colour from green to yellow, brown in severe toxicity) and by growth suppression. The lowest concentration of TNT to cause observable adverse effects was determined to be 50 mg/kg soil and several studied plant species were able to tolerate levels of the compound between 50 - 100 mg/kg soil (Hannink et al. 2002). Oat (*Acena sativa*) was found to be the most TNT resistant plant capable of tolerating 1600 mg TNT/kg soil. Uptake is also affected by the type of soil and TNT availability; TNT forms covalent bonds with soil humic acid, which effectively lowers its bioavailability and toxicity (Gong et al. 1999; Rylott & Bruce 2009; Hannink et al. 2002; Thorn & Kennedy 2002).

TNT concentrations higher than 30 mg/l (in agar plates) were found to affect germination in a range of species. With increasing TNT concentration, germination decreases linearly. In studies with bromegrass (*Bromus inermis*) TNT did not affect germination but reduced the root growth as the emerging radicle absorbed TNT (Peterson et al. 1998).

In an effort to determine how plant tolerance could be further improved, the biochemistry and enzymology underlying the ability of plants to detoxify TNT was studied. Gene expression studies have found genes that were upregulated in response to TNT exposure that correspond to the transformation, conjugation and sequestration steps of xenobiotic detoxification (Fig. 1.3) (Rylott et al. 2011).

Phase I of TNT detoxification in plants primarily follows the reductive pathway well known from microorganisms, in which nitroreductases reduce TNT to hydroxylaminodinitrotoluenes (HADNTs) and aminodinitrotoluenes (ADNTs). Data from microarray experiments have found that members of the small gene family of oxophytodienoate reductases (OPRs) in Arabidopsis were up-regulated following exposure to TNT. The OPRs share similarity with the Old Yellow Enzyme family; bacterial OYEs have been shown to transform explosives. Arabidopsis OPRs have the conserved amino acids in their active site necessary for this transformation and play a physiological role in xenobiotic detoxification (Beynon et al. 2009). Plants also contain nitroreductases and cytochromes P450 and other oxidases involved in oxidative transformation of TNT (methyl oxidation and/or aromatic hydroxylation), which introduce chemical substituents to the molecule susceptible to conjugation (Bhadra et al. 1999; Ekman et al. 2003; Pavlostathis et al. 1998).

Phase II of xenobiotic detoxification involves the conjugation of transformed intermediates to sugars or glutathione. Polar, water-soluble TNT conjugates were first observed in *Phaseolus vulgaris* (Harvey et al. 1990), Madagascar Periwinkle (*Catharanthus roseus*) (Bhadra et al. 1998) and *Myriophyllum aquaticum* (Wayment et al. 1999). UDP-glucosyltransferases (UGTs) from Arabidopsis were shown to have an important role in the detoxification process, conjugating HADNTs and, to a lesser extent, ADNTs, forming both *O*- and *C*-glucosidic bonds. Overexpressing some of these UGTs resulted in increased conjugate production in the plant and enhanced root growth of the transformed seedlings (Gandia-Herrero et al. 2008). The expression of glutathione transferases (GSTs) was also increased in response to TNT treatment in Arabidopsis and poplar (*Populus trichocarpa*) (Brentner et al. 2008; Ekman et al. 2003). Although commercially available GST was shown to

conjugate TNT to form 2-S-glutathionyl-4,6-dinitrotoluene, the identity of the glutathione-conjugated product in plants is not yet known (Brentner et al. 2008; Rylott & Bruce 2009).

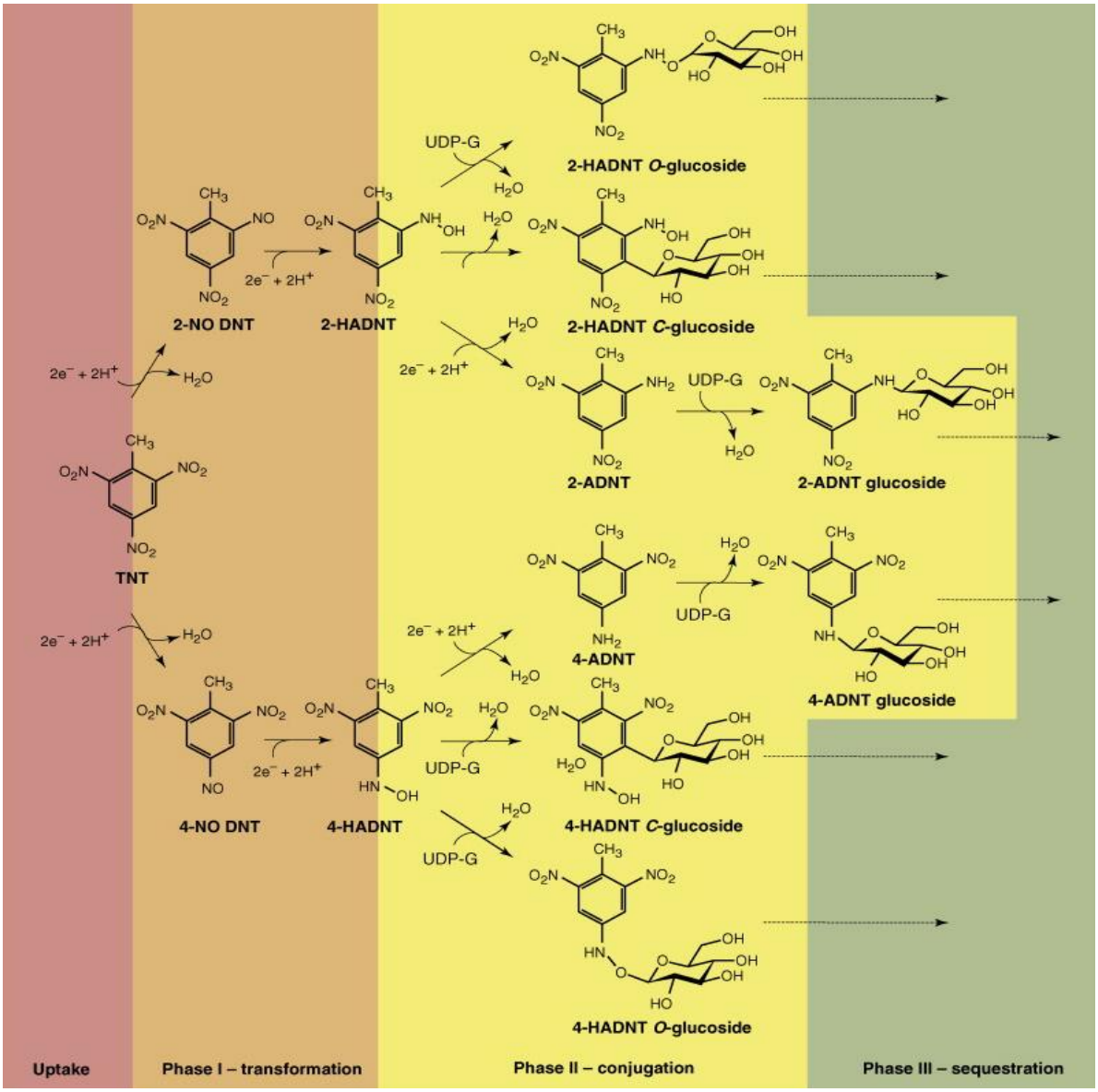


Fig. 1.3. Proposed metabolic pathway of TNT in plants. After uptake, TNT is reduced in Phase I of TNT detoxification via nitroso-dinitrotoluene (NO-DNT) to 2- and 4-hydroxylaminodinitrotoluene (HADNT) isomers. Phase II involves the conjugation of the transformed intermediates to endogenous hydrophilic plant compounds, e.g. sugars. During Phase III, the conjugates are sequestered into the plant biomass, probably into plant cell walls or vacuoles (Rylott & Bruce 2009, Figure from Gandia-Herrero et al. 2008).

Experiments using radiolabelled TNT in *Phaseolus vulgaris* and hybrid aspen (*Populus tremula x tremuloides* var. Etropole) showed that the majority (~95 %) of TNT intermediates and conjugates were found predominantly in the root, where label was distributed evenly between cytosolic and cell wall fractions, particularly in the lignin fraction (Harvey et al. 1990; Rylott & Bruce 2009; Sens et al. 1998; van Dillewijn et al. 2008). Several transporters have been found to be upregulated in plants, including ABC transporters responsible for ATP-dependent transport of GSH conjugates across membranes (Ekman et al. 2003, Lorenz, 2007), as well as cell wall modification enzymes abundantly upregulated especially in roots (Landa et al. 2010).

1.1.6. Engineering plants for the phytoremediation of explosives

Explosives are phytotoxic and detoxification rates of explosives pollutants are low in plants when compared with bacterial cultures. Although bacteria isolated from contaminated soil can rapidly detoxify explosives in laboratory conditions, the explosives persist in the environment suggesting that bacteria do not have enough biomass or metabolic activity to decontaminate these areas. Methods of genetic engineering could be used to transfer the TNT-detoxifying bacterial genes into plants, enhancing the ability of plants to both withstand and detoxify explosives. A nitroreductase gene *nsfI* from *Enterobacter cloacae* was constitutively expressed in *Nicotiana tabacum*, resulting in enhanced transformation of TNT by transgenic plants, which were able to tolerate levels of TNT contamination that would be toxic to untransformed plants (Hannink et al. 2007). Similarly, transgenic hybrid aspen (*Populus tremula* x *tremuloides* var. Etropole), which expressed the bacterial nitroreductase gene *pnrA*, were able to tolerate and take-up greater amounts of TNT from contaminated hydroponic media and soil than untransformed aspen plants (Van Dillewijn et al. 2008).

Overexpressing Arabidopsis detoxification enzymes in the plant also results in increased transformation of TNT and improved ability of plants to tolerate the pollutant, as was shown with seedlings overexpressing UGTs (Gandia-Herrero et al. 2008). Studying the biochemical mechanisms of TNT detoxification in plants could lead to identification and breeding of native plant species with active detoxification systems suitable for the phytoremediation of contaminated sites (Gandia-Herrero et al. 2008).

1.2. Glutathione transferases

Glutathione transferases (GSTs) are multifunctional enzymes found in all aerobic organisms from bacteria to humans (Frova 2003). GSTs catalyse the conjugation of the tripeptide glutathione (GSH) (γ-Glutamyl-Cysteinyl-Glycine) to a variety of hydrophobic, electrophilic, and usually cytotoxic substrates to form a polar S-glutathionylated reaction product. Products of oxidative stress can also be substrates, for example hydroperoxides, formed by the action of active oxygen species that are generated both as normal by-products of aerobic metabolism and as the result of pathogen infection or during abiotic stress (Axarli et al. 2009).

1.2.1. Nomenclature

The soluble GSTs have an ancient, monophyletic origin and a high degree of structural conservation in prokaryotes and eukaryotes revealed by the crystallography and x-ray diffraction analysis data (Dixon & Edwards 2010, Frova 2003). They are encoded by large gene families - in different plant species the family contains 25 - 60 members (Frova 2003). The proteins can be grouped into eight classes on the basis of nucleotide sequence identity, gene organization and active site residues in the proteins. The Theta and Zeta classes of GSTs are also present in animals, while the Phi and Tau classes are plant specific (Dixon, A. Lapthorn, et al. 2002). All these classes have an active site with a serine residue involved in the formation of the reactive thiolate anion of GSH, which is used in addition or substitution reactions with the hydrophobic substrate. Further plant-specific classes of GST-like proteins identified in Arabidopsis include the Lambda and dehydroascorbate reductase (DHAR) classes with dehydroascorbate reductase activity described by Dixon et al. 2002. These proteins have a cysteine residue in the active site which alters the catalytic properties of the enzymes. They cannot directly

catalyse the S-glutathionylation of substrates, but instead are more likely to catalyse redox reactions (Dixon et al. 2010). The tetrachlorohydroquinone dehalogenase-like (TCHQD) class contains a membrane associated protein with an, as yet, unknown function (Dixon et al. 2010).

GSTs are related to other GSH- and cysteine-binding proteins, which also contain a thioredoxin-like protein fold; and also to stress-related proteins from a range of organisms. Based on the conservation of introns, active site residue and function in all eukaryotes, Theta and Zeta GSTs are considered the predecessors of the family, preceding the separation of animal - plant structures (Frova 2003). Phylogenetic studies indicate that plant GSTs have mainly evolved after the divergence of plants; the two prevalent Phi and Tau classes being the result of recent multiple duplication events (Frova 2003).

1.2.2. Gene organisation

The Arabidopsis genome contains 54 GST genes, with the Tau (GSTU) and Phi (GSTF) classes being the most numerous, represented by 28 and 13 genes respectively, whereas there are only three Theta GSTs (GSTT), two Zeta GSTs (GSTZ), three Lambda GSTs (GSTL), four DHAR and one TCHQD (Fig. 1.4). Out of these genes 52 are subscribed and 41 of the proteins were shown to have GSH-dependent catalytic activity (Dixon, A. Lapthorn, et al. 2002; Dixon, Davis, et al. 2002; Dixon et al. 2010). The GST genes are predominantly organized in clusters non-randomly distributed in the genome (Fig. 1.5) Progress has been made in defining the function of some of the smaller groups of plant GSTs and the cellular localisation of the members of the family (Dixon et al. 2009).

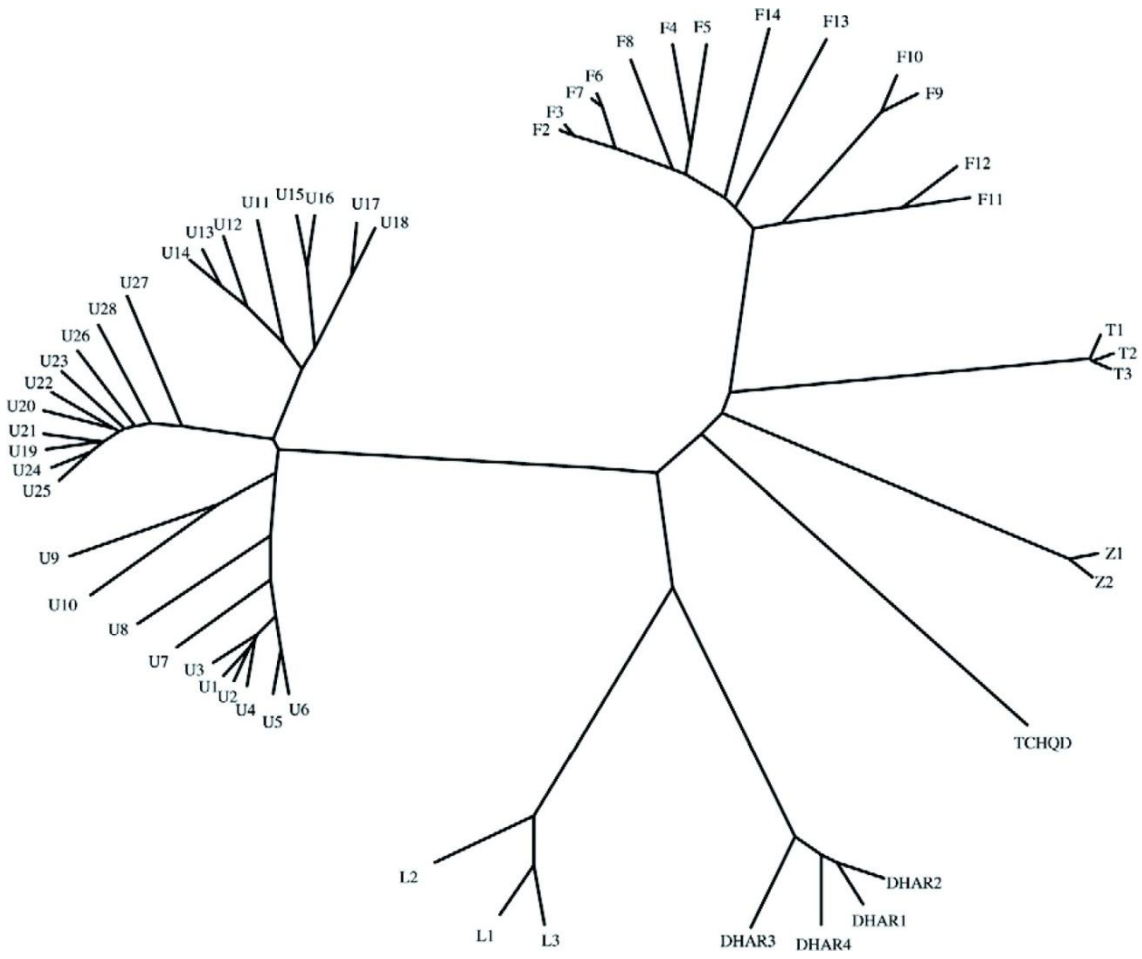


Fig. 1.4. Phylogenetic tree showing the diversity of Arabidopsis GSTs and the relationships between classes. Branch lengths correspond to the estimated evolutionary distance between protein sequences. GST class nomenclature: U, Tau class; F, Phi class; T, Theta class; Z, Zeta class; L, Lambda class; DHAR, dehydroascorbate reductase class, TCHQD, tetrachlorohydroquinone dehalogenase-like class; (Figure from Dixon & Edwards 2010, the tree was calculated and drawn using the PHYLIP package, using Protdist [JTT method], Neighbor [UPGMA method] and Drawtree).

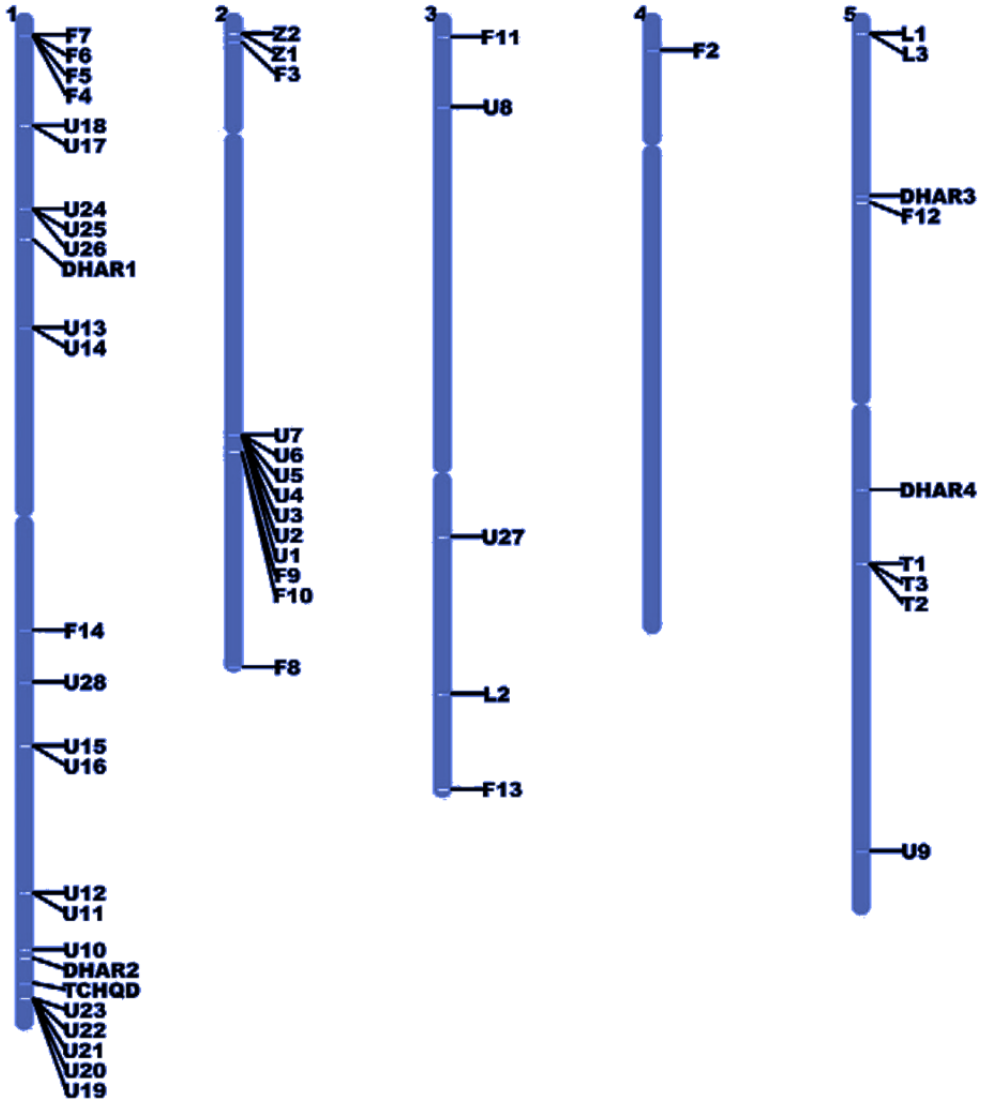


Fig. 1.5. Distribution of GST genes on the Arabidopsis chromosomes. GST class nomenclature: U, Tau class; F, Phi class; T, Theta class; Z, Zeta class; L, Lambda class; DHAR, dehydroascorbate reductase class, TCHQD, tetrachlorohydroquinone dehalogenase-like class (Figure from Dixon & Edwards 2010).

1.2.3. Structure

The GST sequences are divergent, even within a family. In plants, sequence identity within the larger Phi and Tau classes can be as low as 40 % and is less than 25 % between classes (Frova 2003). Structures of ten plant GSTs have been solved by x-ray crystallography, two of which were Arabidopsis GSTs: GSTF2 (Reinemer et al. 1996) and GSTZ1 (Thom et al. 2001). Despite differences in their primary

sequences the structures of these soluble GSTs are very similar, with comparable protein folds. All known GST structures consist of a conserved N-terminal, which is a GSH binding site (the G site), formed from a conserved group of amino-acid residues, which has evolved from the thioredoxin fold. The second component is a site that binds the hydrophobic substrate (the H site), which is much more structurally variable and is formed from residues in the C-terminal alpha-helical domain (Fig. 1.6) (Dixon & Edwards 2010; Frova 2003). The hydrophobic H-site is located next to the G-site. Catalytic activity of the protein is dependent on stabilisation of the reactive thiolate anion of GSH. GSTs facilitate proton removal from the thiol of GSH using serine residue Ser17 in the active centre (Dixon et al. 2010; Dixon & Edwards 2010).

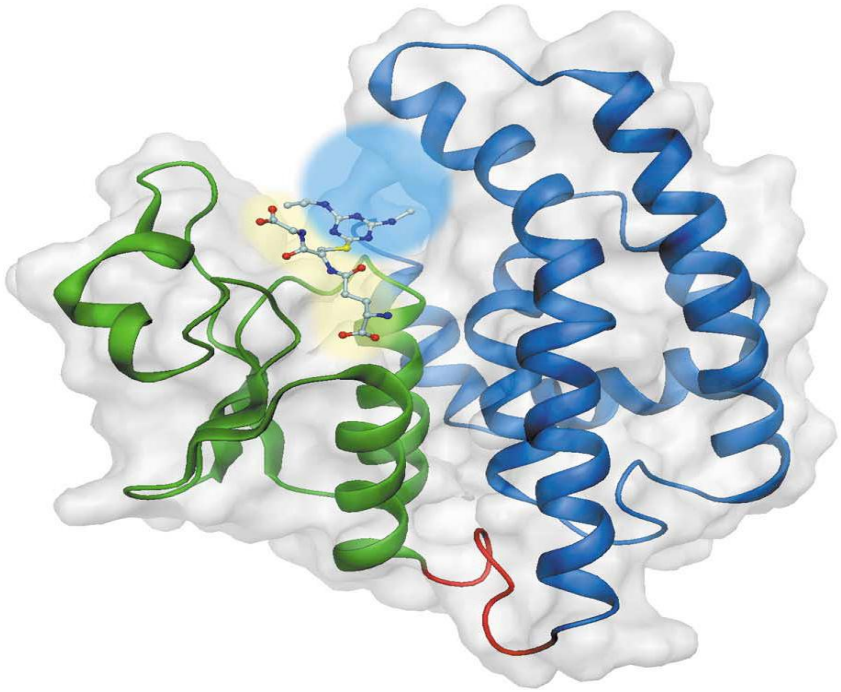


Fig. 1.6. GST structure and substrate binding. Representation of a typical GST subunit with the N-terminal domain in green, the linker region in red, the C-terminal domain in blue and the protein surface in gray. The GSH-binding site (G site) is highlighted in yellow and the hydrophobic site (H site) is highlighted in blue. (Figure from Dixon, A. Lapthorn, et al. 2002).

Eukaryotic GSTs are mostly cytosolic and in plants can constitute up to 2 % of soluble proteins (Dixon, A. Lapthorn, et al. 2002; Frova 2003). Recently, family members have also been shown to be selectively targeted to the nucleus and peroxisome (Dixon et al. 2009). As a general rule, GSTs are active as dimers of either identical (homodimers) or different (heterodimers) subunits, each encoded by independent genes (Frova 2003; Frova 2006), although the lambda and DHAR classes appear to be monomeric by gel filtration (Dixon, Davis, et al. 2002). The monomeric subunits associate to form the dimer with the central cleft containing an active site on each side and this dimerisation is essential for the function of soluble GSTs (Fig. 1.7) (Dixon & Edwards 2010). Molecular weights of the subunits range from 23 to 29 kDa, forming a hydrophobic 50 kDa protein with an isoelectric point in the pH range 4 - 5. Heterodimers form from subunits of the same class and further contribute to the diversity of GSTs (Dixon, A. Lapthorn, et al. 2002; Frova 2003).

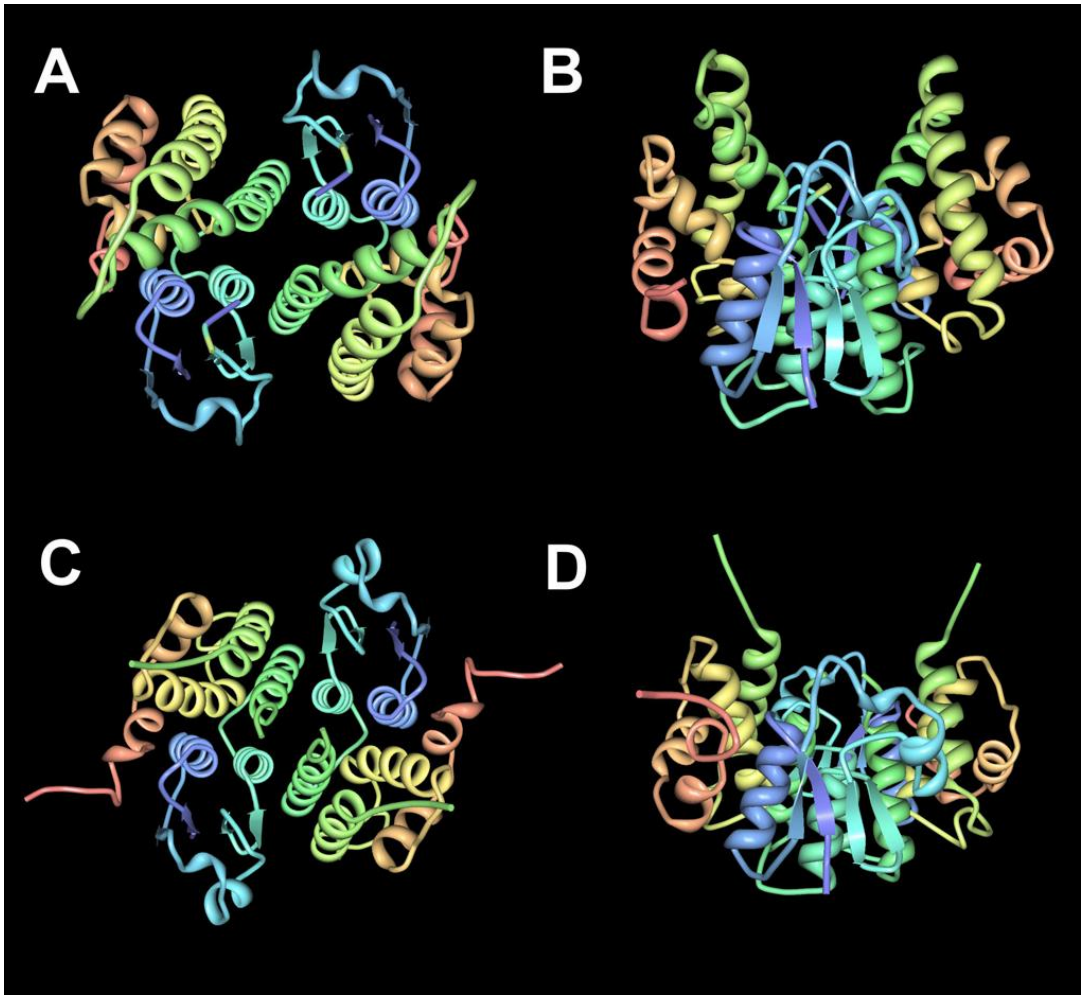


Fig. 1.7. The dimeric structure of Arabidopsis GST determined by x-ray crystallography: GSTF2 (**A** & **B**) and GSTZ1 (**C** & **D**). The structure is coloured from purple at the N-terminus to red at the C terminus for each polypeptide. **A** and **C** show a view looking into the active site showing the symmetry of the dimer, **B** and **D** show a side-on view of the dimer with a central cleft containing an active site on each side (Figure from Dixon & Edwards 2010).

1.2.4. Functions of GST classes

Zeta

The GSTZs are highly conserved in eukaryotes, which suggests they have an important function in the cell metabolism. The GSTZ genes contain eight or nine introns. Arabidopsis has two GSTZ genes (GSTZ1 and GSTZ2), although only GSTZ1 is transcribed at a significant level (Dixon & Edwards 2010). This enzyme

catalyses GSH-dependent isomerisation of maleylacetoacetate to fumarylacetoacetate, a reaction which is part of tyrosine degradation pathway functional in plants. Carnation GSTZs are induced during senescence, which is consistent with their function in amino acid catabolism (Dixon & Edwards 2010; Frova 2003; Sheehan et al. 2001).

DHAR

The DHARs are plant specific enzymes catalysing the reduction of dehydroascorbate to ascorbate, maintaining a reduced ascorbate pool. DHARs are monomers and have a cysteine residue in their active site which makes them unable to stabilise the thiolate anion of GSH, therefore they do not catalyse conjugating reactions using GSH. Instead, the cysteine forms a disulfide with GSH as a part of the catalytic mechanism of the enzyme. DHARs are most likely present in the organelles where the redox reactions are needed to maintain a pool of reductants. They have been reported in mitochondria, chloroplasts and peroxisomes and have been proposed to have a role during oxidative stress in plants (Dixon & Edwards 2010).

Lambda

The GSTLs are similar to the DHAR class, they are expressed as monomers, contain cysteine in their active centre forming disulfides with GSH, but their endogenous substrates are, as yet, unknown (Dixon & Edwards 2010).

Theta

Theta class of GSTs is conserved between plants and animals. Genes contain six introns; enzymes have an active site with a serine residue and high glutathione-dependent peroxidase (GPOX) activity. They are located in the peroxisomes, where the cellular metabolism generates quantities of hydrogen peroxide which could oxidatively damage endogenous fatty acids to form lipid hydroperoxides, which are

likely substrates of peroxisomal GSTs. Arabidopsis GSTT3 is transcribed as a fusion protein with the C-terminal domain resembling *myb*-like transcription factors. The fusion protein is localised exclusively to the nucleus. The significance of this accumulation is unclear and roles in regulating gene transcription under oxidative stress or detoxifying oxidatively damaged DNA have been suggested (Dixon et al. 2009; Dixon & Edwards 2010).

Phi

The GSTFs are a large, plant-specific class of enzymes, with apparent functional redundancy. This class includes some of the first GSTs identified due to their herbicide detoxifying activity. The Phi genes contain two introns at conserved positions (Frova 2003). A lot of the proteins have been studied in detail. GSTF2 is strongly inducible by oxidative stress and has a role in flavonoid metabolism (Smith et al. 2003). GSTF8 expression is induced by hydrogen peroxide, pathogens, salicylic acid and herbicides due to the presence of an *ocs* element in the promoter, mainly in the root tissue (Chen & Singh 1999). GSTF8 is also the most active of Arabidopsis GSTs in assays with the model substrate CDNB (Dixon et al. 2009). GSTF12 has a role in the transport of anthocyanins into the vacuole, its transcription being closely co-regulated with other anthocyanin synthesis genes (Brazier-Hicks et al. 2008; Dixon & Edwards 2010; Sun et al. 2012).

Tau

GSTUs are plant-specific enzymes and the most numerous GST class in Arabidopsis and other plants. The Tau genes each contain one intron in a conserved position. GSTU19 is the best studied of GSTs and is induced by drought and exposure to herbicide safeners as well as other stress reactions. GSTU24 transcription is induced by various xenobiotics including TNT; GSTU25 is characterised by high

activity towards CDNB and high GPOX activity (Brazier-Hicks et al. 2008; Dixon & Edwards 2010; Frova 2003).

TCHQD

Arabidopsis has a single TCHQD, showing sequence homology to the prokaryotic enzymes involved in metabolism of chlorinated xenobiotics. It is localised to the plasma membrane and contains a serine residue in its active site suggesting it could catalyse GST reactions (Dixon & Edwards 2010).

MAPEG

Plants also have members of phylogenetically unrelated GST family known as the membrane associated proteins in eicosanoid and glutathione metabolism. Arabidopsis has a single MAPEG-like protein with sequence similarity with mammalian proteins (Dixon & Edwards 2010).

1.2.5. Functions of GSTs

Initially GSTs were intensively studied because of their ability to detoxify herbicides; recently they have been implicated in numerous stress responses, including defence against pathogen attack, oxidative stress, and heavy-metal toxicity and the detoxification of a wide variety of xenobiotic compounds.

Following glutathionylation, the glutathione conjugates are transported to the vacuole by ABC transporters and processed by at least two distinct pathways. Based on molecular studies, these functions seem to be organ- and species-specific. Studies of herbicide metabolism in cereal crops suggests that glutathione conjugates are hydrolysed in the vacuole by carboxypeptidases to form γ-glutamylcysteine derivatives, which are processed to cysteine conjugates by γ-glutamyl

transpeptidases. In Arabidopsis, in the pathway, which based on enzyme expression studies predominates in roots, glutathionylated conjugates are sequentially processed in the vacuole to cysteinylglycine derivatives and then to the cysteine conjugates. In the pathway functioning in the leaves, conjugates are first metabolised to the γ-glutamylcysteine derivative in the cytosol through the carboxypeptidase activity of the enzyme phytochelatin synthase. Subsequent conversion to the cysteine conjugate is then catalysed by γ-glutamyltranspeptidase isoenzyme which is localized in the plasma membrane (Brazier-Hicks et al. 2008; Cummins et al. 2011; Dixon & Edwards 2010).

In the GSTs studies, gene expression is regulated at the transcription level, although evidence of alternative splicing was found for the Phi and Tau classes of Arabidopsis GSTs. Inducibility of GSTs by a wide range of signals suggests the presence of regulatory sequences in the promoters are activated by a number of different molecules. The plant GST promoters analysed so far do not contain responsive elements that are found in animal promoters. Octopine synthase (*ocs*) elements are the only regulatory motifs identified in a number of GST promoters from different plant species. *Ocs* elements were found to mediate induction of GST expression by auxin, salicylic acid, hydrogen peroxide, methyl jasmonate, wounding and cadmium (Frova 2003). A common effect of these factors is the generation of active oxygen species, causing membrane lipid peroxidation and cytotoxic product formation resulting from oxidative DNA injury. GSTs also have GPOX activity and reduce hydroperoxides to alcohols, metabolising the toxic products of lipid peroxidation and DNA injury and thus counteracting oxidative injury (Cummins et al. 2011; Frova 2003).

Research to gain a better understanding of GST functions may lead to the development of more tolerant plant and crop varieties. Overexpression of GSTs, or introduction of GST from different species, has been used to enhance the tolerance of plants to different stress factors, including toxic xenobiotics (Dixit,

et al. 2011b) and herbicides (Benekos et al. 2010). Improved tolerance to oxidative stresses induced in tobacco plants by heavy metals such as cadmium has also been demonstrated (Dixit, et al. 2011a), as well as improved drought and salt tolerance of transgenic tobacco (Ji et al. 2010).

In addition, plant GSTs participate in light signalling and can affect various aspects of Arabidopsis development. GSTs are also involved in the normal metabolism of plant secondary products like glucosinolates, anthocyanins and cinnamic acid (Dixon, Lapthorn, et al. 2002; Cummins et al. 2011; Jiang et al. 2010; Kitamura et al. 2012; Sun et al. 2012).

1.3. Aim of the current project

Experimental background

During previous research in Prof. Neil Bruce's group, a cDNA microarray assay using Arabidopsis seedlings treated with TNT was used to identify genes which could play a role in TNT detoxification. Many Phase I and II enzymes were found to be upregulated, including two Phi class GSTs and 12 Tau class GSTs, which were upregulated two fold or more (Gandia-Herrero et al. 2008).

During a previous PhD project (Sparrow, 2010), the seven Tau class GSTs, whose expression was most increased after TNT treatment, were further characterised (AtGSTU1, AtGSTU3, AtGSTU4, AtGSTU7, AtGSTU22, AtGSTU24 and AtGSTU25). The microarray data were confirmed using real-time PCR and the selected GSTs were cloned into the LIC vector system and conditions optimised for recombinant expression in *Escherichia coli*. These GSTs were also cloned into plant expression vectors under the control of the 35S promoter and a number of Arabidopsis over-expression lines were generated (Sparrow, 2010).

Based on the previous results of conjugation activity of purified GSTs with TNT (Sparrow, 2010), GSTU24 and GSTU25 were selected for further study because these GSTs gave the highest activity towards TNT. The aim of this MSc project was to characterise the biochemistry of these selected Arabidopsis GSTs.

***In vitro* experiments**

In vitro studies focused on investigating the conjugation activity of purified recombinant GSTs with TNT. The experiments investigated the mechanism and kinetics of the conjugation reaction and aimed to identify the reaction product.

In vivo experiments

In vivo experiments aimed to determine the role of GSTs in TNT detoxification in Arabidopsis plants transformed with 35S-GST constructs in comparison with untransformed, wild type plants. The characterisation of the transgenic lines included morphological analyses, rate of TNT uptake by plants and analyses of metabolites from transformed and wild-type plants.

2. Biochemical characterisation of AtGSTU24 and AtGSTU25

2.1. Materials

Consumables and reagents used for this work were obtained from the following suppliers: Sigma-Aldrich (Gillingham, UK), Thermo Fisher Scientific (Loughborough, UK), Invitrogen (Paisley, UK), Promega (Southampton, UK), Starlab Ltd. (Milton Keynes, UK), Waters Ltd. (Elstree, UK), GE Healthcare (Chalfont St. Giles, UK). The compost used in this work was Levington F2 compost sourced from Scotts (Suffolk, UK). Water used for all solutions and media was purified using an Elga Purelab Ultra water polisher (Elga Labwater, High Wycombe, UK) and sterilized by autoclave if necessary.

2.2. Methods

2.2.1. Cloning and expression of GSTs

Plasmids

Plasmids for transformation of *Escherichia coli* were created by Ligation Independent Cloning using the pET-YSBLIC3C vector by Sparrow, 2010. The vector contained the *gst* insert with 6 × His-Tag and a kanamycin resistance gene as well as a repressor gene (Lacl) for IPTG induction (Sparrow, 2010).

Expression of LIC-GSTs in *Escherichia coli*

The conditions for *in vitro* expression of GSTU24 and GSTU25 were optimised by Sparrow, 2010.

***Transformation of competent* E. coli**

Chemically competent *E. coli* cells of the BL21 (DE3) strain were transformed with pET-YSBLIC3C plasmid containing the respective *gst* gene. Aliquots of 50 µl of competent cells were defrosted on ice, incubated with 1 µl plasmid DNA for 30 min on ice, heat shocked for 90 s at 42 °C and returned to ice for another 2 - 3 min. For recovery, 500 µl of LB was added and the cells were incubated at 37 ºC with 250 rpm shaking for 1 h. The transformed cells were plated onto LB agar plates containing 50 µg/ml kanamycin. The plates were incubated overnight at 37 °C and a single colony was used to prepare a starter culture for protein expression.

Protein expression

The starter culture for expression of GSTU24 and GSTU25 was prepared from a single colony of transformed *E.coli* BL21 (DE3) cells grown on LBA with kanamycin. A single colony was transferred to 5 ml LB medium and incubated overnight at 37 °C with 250 rpm shaking. The starter culture was added to autoinduction (AI) medium (Table 1.1 & 1.2) and grown at 37 ºC with 180 rpm shaking until the optical density of the culture reached 0.8 - 1.0 at 600 nm. The culture was then incubated at 20 °C with 180 rpm shaking for an expression time of 60 h.

Table 2.1. The components of AI medium (per 1l):

ZY both (10 g/l tryptone, 5 g/l yeast extract)	928 ml
1 M $MgSO_4$	1 ml
1000 × metals	1 ml
50 × 5052 solution	20 ml
20 × NPS solution	50 ml

Table 2.2. Stock solutions for AI medium:

1000 x metals (100 ml)	0.1 M $FeCl_3.6H_2O$ (in 0.1 M HCl)	50 ml
	1 M $CaCl_2$	2 ml
	1 M $MnCl_2.4H_2O$	1 ml
	1 M $ZnSO_4.7H_2O$	1 ml
	0.2 M $CoCl_2.6H_2O$	1 ml
	0.1 M $CuCl_2.2H_2O$	2 ml
	0.2 M $NiCl_2.6H_2O$	1 ml
	0.1 M $Na_2MoO_4.2H_2O$	2 ml
	0.1 M $Na_2SeO_3.5H_2O$	2 ml
	0.1 M H_3BO_3	2 ml
	H_2O	36 ml
50 x 5052 solution (100 ml)	Glycerol	25 g
	Glucose	2.5 g
	α-Lactose	10 g
	H_2O	73 ml
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
	H_2O	90 ml

2.2.2. Cell lysis

After 60 h expression time the *E. coli* cells were centrifuged for 5 min at 5000 rpm and the cell pellets were re-suspended in PBS. Cell lysis was performed by sonication with S-4000 Sonicator (Misonix) at 70 % amplitude for 4 min, with cycles of 3 s interrupted by 7 s cooling at 0 ºC. Cell lysates were centrifuged at 17 500 g for 30 min to remove the cell debris. Prior to enzyme purification the supernatants were filtered through 0.45 μm syringe filters.

2.2.3. Purification of recombinant protein

GSTU24 and GSTU25 were purified by batch purification with Glutathione Sepharose 4B according to the manufacturer's instructions. Prior to use the resin was washed with PBS buffer (140 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), pH 7.4. The resin was added to protein extract, after incubation for 30 - 60 min at room temperature the mixture was centrifuged at 500 g for 5 min. The resin was washed 5 times with 10 bed volumes of PBS buffer. Protein was eluted with elution buffer (50 mM Tris-HCl, 10 mM GSH) after 10 min incubation at room temperature. The elution step was repeated for total of three to six elutions.

2.2.4. Protein visualisation by SDS-PAGE

The binding capacity of the Glutathione Sepharose 4B resin, the protein content of the wash steps and the purity of the enzyme was analysed by SDS-PAGE. The concentration of proteins in the analysed samples was quantified by Coomassie (Bradford) colorimetric protein assay at 595 nm, using Bovine Serum Albumin Standard to create a standard curve. Samples were solubilised in a 4 × 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 °C for 5 min. Samples containing 20 mg of protein extracts and 10 mg of purified proteins were run through an acrylamide gel (Biorad) at 200 V alongside 10 µl of broad range molecular weight marker and visualised using Coomassie brilliant blue stain.

2.2.5. CDNB activity assay

To assess the activity of the purified recombinant GSTs, an activity assay with CDNB was performed. GST catalyses the conjugation of GSH to CDNB through the thiol group of the glutathione. The reaction product, GS-DNB conjugate, absorbs at 340 nm, therefore the increase in absorption due to conjugate production can be measured spectrophotometrically following a protocol published by Habig et al. 1974 and adapted by Sparrow, 2010. The reaction mix contained 100 mM potassium phosphate buffer pH 6.5, 5 mM GHS, 1 mM CDNB and 5 μg of protein in the total reaction volume of 1 ml. The reaction was initiated by the addition of CDNB and followed over 1 min.

Kinetic analysis of GSTU24 with CDNB

The described spectrophotometric assay was performed using CDNB concentrations ranging from 0 to 3000 μM. The rates of conjugate production were measured in triplicate and Michaelis-Menten parameters were calculated using Sigma Plot 12.0.

2.2.6. TNT activity assay

An activity assay using TNT as the enzyme substrate was performed using similar conditions as in the experiment with CDNB in 100 mM potassium phosphate buffer at the temperature of 20 °C with 150 μg of the enzyme, 200 μM TNT and 5 mM GSH. Assays were performed in triplicate, aliquots of the samples were taken over a particular time course and the reaction was stopped with 1 % TCA to precipitate the protein. After centrifugation at 13 000 rpm for 10 min the products of the conjugation reaction were analysed by HPLC using a Waters Alliance 2695 separation module with a Waters 2996 photodiode array detector. The samples

(40 µl) were loaded onto a Waters X-Bridge C18 column (250 × 4.6 mm, 5 µm) heated to 25 °C and separated using gradient elution (Table 1.3). Integration was performed at 240 and 250 nm with Empower Pro software.

Table 2.3. HPLC conditions optimised for Waters X-Bridge C18 column:

Mobile phase A: acetonitrile			
Mobile phase B: water + 0.1 % formic acid			
HPLC gradient:	0 min	5 % A	95 % B
	5 min	5 % A	95 % B
	25 min	40 % A	60 % B
	30 min	100 % A	0 % B
	35 min	5 % A	95 % B

Equine liver GST was used as a positive control in the experiments, as it was previously shown to have activity towards TNT and LC-MS-MS analyses of the TNT conjugate confirmed the formation of 2-S-glutathionyl-4,6-dinitrotoluene (Brentner et al. 2008), therefore this reaction facilitated identification of the compound in the chromatogram.

The conjugation reactions were performed in triplicate using 10 U (173 mg) of enzyme (Sigma, 576 U/10g), 200 µM TNT and 5 mM GSH in 100 mM potassium phosphate buffer at 20 °C and 37 °C. Aliquots were taken from the reactions at the same time points as in Arabidopsis GST reactions. The reactions were stopped with 1 % (v/v) TCA and the samples were analysed by HPLC.

2.2.7. Griess assay

The Griess assay is a colorimetric assay used to measure the amount of free nitrite released in the reaction. The assay was used to obtain initial data on conjugation

of GSH by nucleophillic substitution of NO_2^- group of TNT. The reactions were performed in duplicates at 20 °C in 100 mM phosphate buffer pH 6.5 and 9.5 with 150 µg of the enzyme, 500 µM TNT and 5mM GSH in a total volume of 250 µl. After 1 h and 3 h the reaction was stopped by 1 % TCA. After centrifugation the samples were transformed to 1 ml cuvettes and water was added to bring the total volume to 760 ml. After adding 200 µl of acidified sulphanilamide solution, the reaction was incubated protected from light at room temperature for 10 min before adding 40 µl of N-1-napthylethylenediamine dihydrochloride (NED) solution. After incubation in the dark at room temperature for 10 min the samples were measured spectrophotometrically at 540 nm. The amount of free nitrite released in the reaction was quantified using a standard curve produced with 0 – 100 µM $NaNO_2$.

2.2.8. LC-MS analysis of conjugation products

Mass spectrometry of the conjugated TNT products was performed using Finnigan Surveyor Autosampler 1.4 (Thermo Electron Corporation), Finnigan Surveyor LC pump 1.4 SP1, Finnigan Surveyor PDA detector 1.0 and LCQ detector Finnigan MAT 2.0 with a Waters X-Bridge C18 column (250 × 4.6 mm, 5 µm) and the same HPLC conditions as used for analytical LC with 10 µl injection (section 2.2.6). The LCQ detector was tuned using TNT standard. Data were collected for peaks from 5.11 to 32.77 minutes. ESI was used to produce ions in negative mode with mass range 100 - 1000. Data were analysed with Excalibur 2.0 SUR 1 software.

2.2.9. Purification of conjugation products

The conjugation products were formed in the reaction of GSTU25 with 2 mM TNT, as GSTU25 forms all of the conjugates possible. The reaction was performed in 100 mM potassium phosphate buffer pH 9.5 at 20 °C for 6 h using 1.5 mg of

the purified enzyme with 2 mM TNT and 25 mM GSH in a total volume of 500 µl. To increase the solubility of TNT in the aqueous solution, 50 µl of DMSO was added to the reaction. The TNT conjugates were purified from the reaction by means of analytical HPLC (conditions in section 2.2.6). Acetonitrile from the HPLC mobile phase was evaporated in a fume hood and water was removed by freeze-drying. The dried purified compounds were re-suspended in water after determining weight and stored at -80 °C for use as HPLC standards. For NMR analyses, the purified substances were dissolved in 0.7 ml D_2O and transferred into NMR tubes.

2.2.10. NMR analysis of conjugation products

Bruker AVII 700 MHz spectrometer was used to obtain $_{13}C$, COSY, HSQC, HMBC and NOESY NMR spectra to identify the products of the TNT conjugation reactions.

2.2.11. Kinetic assay of GSTU24 and GSTU25 with TNT

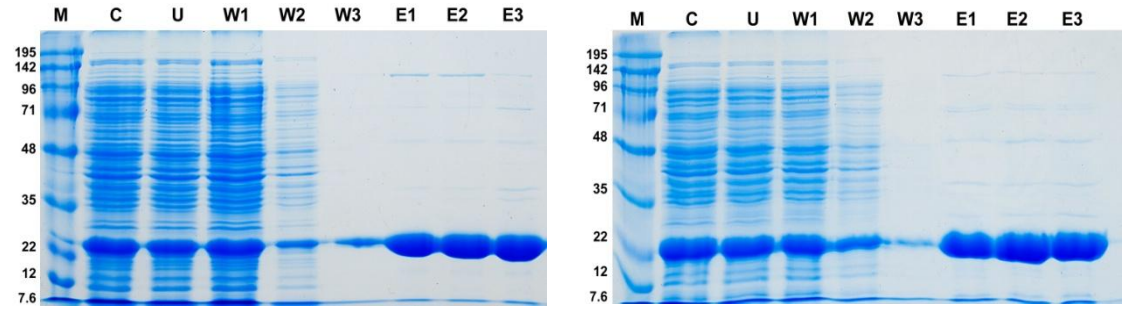
Kinetic assays of GSTs with TNT were performed at conditions optimal for the enzyme activity: 100 mM phosphate buffer pH 9.5 at 42 °C with 150 µg of the enzyme. The concentration of TNT ranged from 25 - 5000 µM and GSH concentration ranged between 5 to 45 mM. To ensure TNT was solubilised in the solution, stock solutions of different TNT concentrations were prepared in DMSO and 5 % DMSO (10 % DMSO to solubilise 3, 4 and 5 mM TNT) was added to the total volume of the reaction, as this amount of DMSO was found to have no effect on GST protein activity and at the same time solubilised TNT in the solution (Sparrow, 2010). Experiments were performed in triplicate under the optimised conditions, aliquots of the samples were taken at 0, 5, 10, 15, 20 and 25 min and the enzymatic reaction was stopped with 1 % TCA. Following centrifugation, the samples were analysed by HPLC (section 2.2.6). Michaelis-Menten parameters were calculated using Sigma Plot 12.0.

2.3. Results

2.3.1. Cloning and expression of GSTU24 and GSTU25

Chemically competent *E. coli* cells, BL21 (DE3) strain, were transformed with pET-YSBLIC3C containing the *gst* of interest (created by Sparrow, 2010), or the empty vector as a negative control. The expression conditions using autoinduction medium and expression time of 60 h at 20 °C were also optimized by Sparrow, 2010.

GSTU24 and GSTU25 were purified with Glutathione Sepharose 4B according to the manufacturer's instructions, as these enzymes were found to bind effectively to the Sepharose beads (Sparrow, 2010). The binding capacity of the Glutathione Sepharose 4B resin, the protein content of the wash steps and the purity of the enzyme were analysed by SDS-PAGE. Samples containing 20 mg of protein were run through an acrylamide gel alongside a broad range molecular weight marker and visualised using Coomassie brilliant blue stain (Fig. 2.1 & 2.2).



A **B**

Fig. 2.1. Coomassie Brilliant Blue-stained SDS–PAGE gel showing purification profile of GSTU24 (**A**) and GSTU25 (**B**). Lanes show: **M**, molecular weight marker (kDa); **C**, crude protein extract from *E. coli* after 60 h expression time; **U**, unbound fraction of the purification process; **W1 - 3**, wash steps, **E1 - 3**, purified fractions.

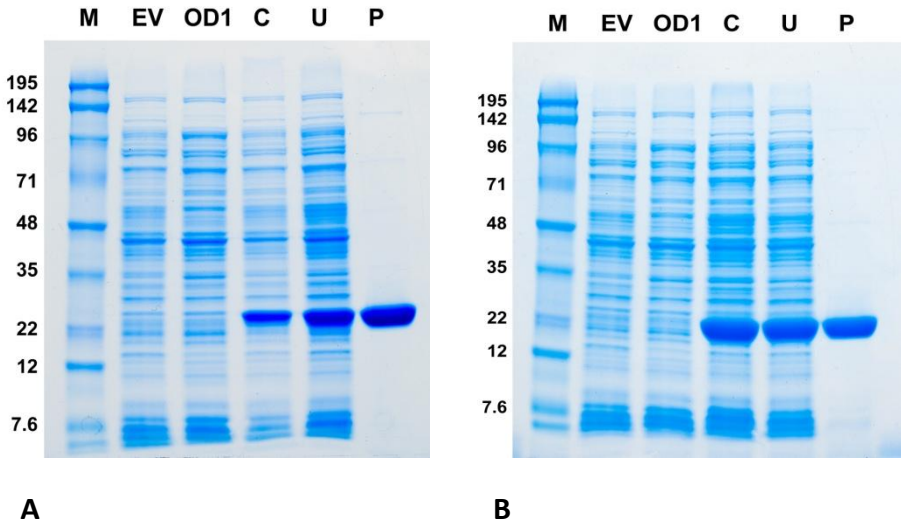


Fig. 2.2. SDS-PAGE gel of expression profile and purification of GSTU24 (**A**) and GSTU25 (**B**). **M**, molecular weight marker (kDa); **EV**, protein extract from *E. coli* transformed with empty vector; **OD1**, protein extract from *E. coli* culture with optical density 0.8-1 at 600 nm before the induction of the protein expression; **C**, crude protein extract from *E. coli* after 60 h expression time; **U**, unbound fraction of the purification process; **P**, purified protein.

2.3.2. Activity of GSTU24 and GSTU25 with CDNB

GSH-conjugating activity of the purified proteins was assessed spectrophotometrically using the generic substrate CDNB. The CDNB assay was originally developed as a simple colorimetric assay to measure mammalian GST activity (Habig et al. 1974). Although many GSTs are not active with this exogenous substrate, GSTU24 and GSTU25 were previously found to be capable of catalysing the conjugation of several model substrates, particularly CDNB and benzyl isothiocyanate (BITC), which is a potential natural GST substrate in cruciferous plants derived from the degradation of glucosinolates (Dixon et al. 2009; Dixon & Edwards 2010). GSTs catalyse the removal of a proton from GSH to generate the thiolate anion GS^-, which is more reactive than GSH. In the case of CDNB, the conjugation of the thiolate anion occurs at carbon 1 where the chloride is bound, producing a Meisenheimer complex. The complex is unstable and the chloride dissociates leaving glutathionyl-dinitrobenzene conjugate (Fig. 2.3).

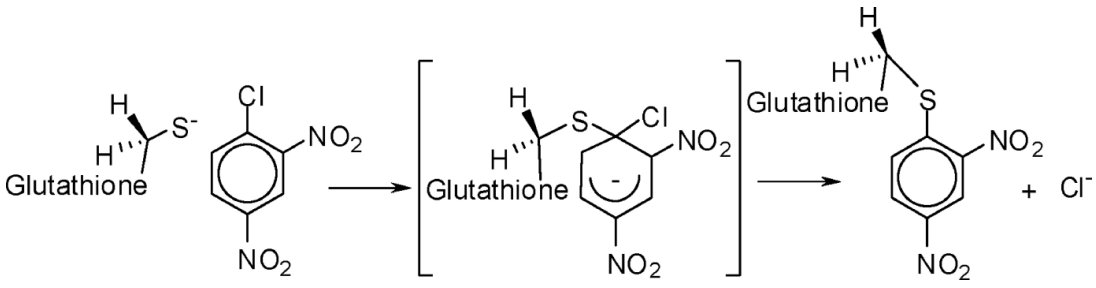


Fig. 2.3. The conjugation of glutathione anion with CDNB via the Meisenheimer complex (centre). Figure from Bowman et al. 2007.

A conjugation assay with CDNB was performed to test activity and functionality of the purified enzymes (Fig. 2.4 **A**). The K_m value of GSTU24 of 954.9 µM CDNB (648 nkat.mg^{-1}) and V_{max} of 38.9 µM/min were calculated based on the Michaelis-Menten plot presented in Fig. 2.4 **B**.

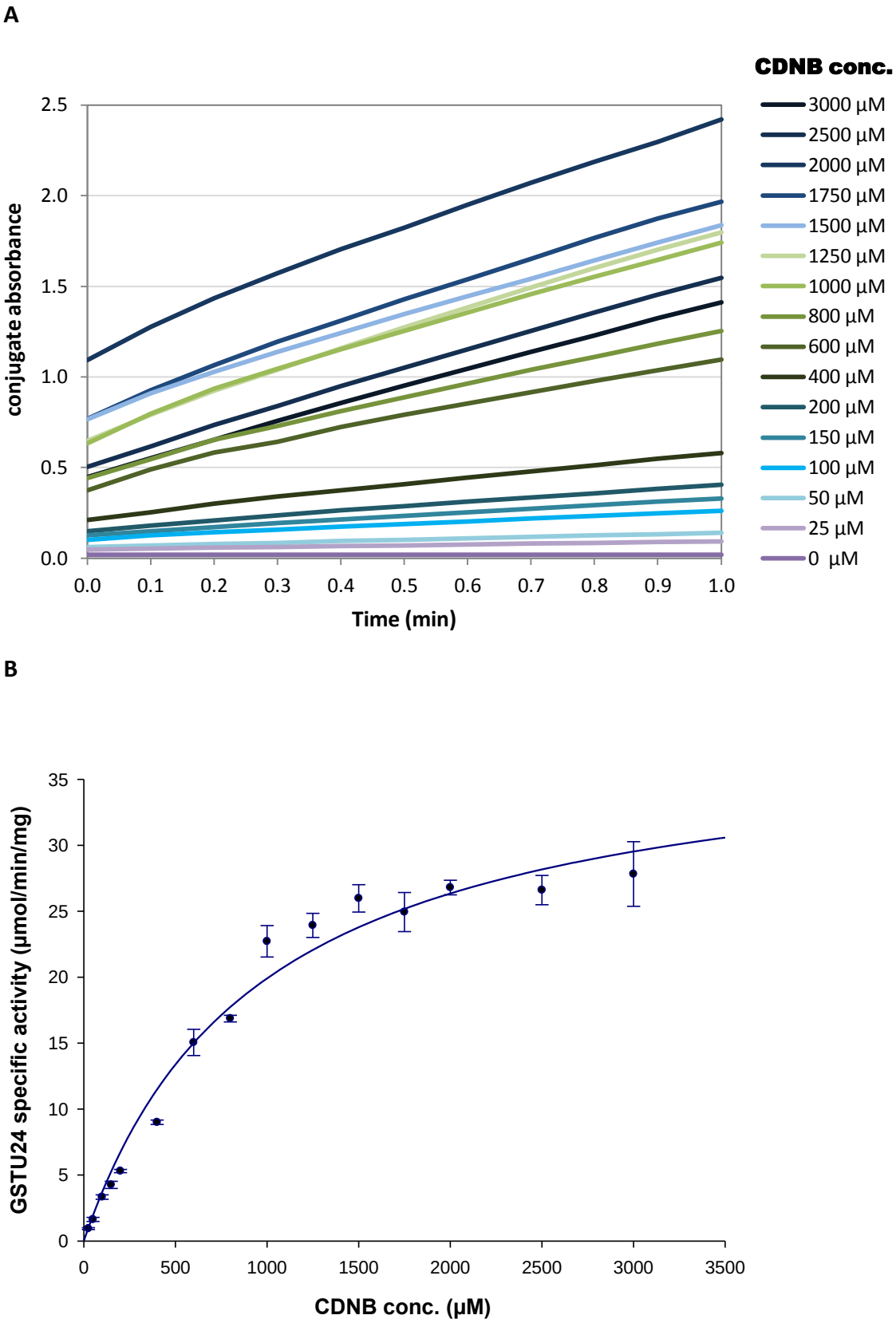


Fig. 2.4. Kinetic assays of GSTU24 with CDNB. **A**, rate of conjugate production determined spectrophotometrically over 1 min at 340 nm. Reactions contained 0 - 3000 µM CDNB, 5 µg of purified recombinant protein and 5 mM GSH in 100 mM

phosphate buffer pH 6.5. Absorbance values are the means of the reactions performed in triplicate. **B,** Michaelis-Menten plot showing the rate of conjugate production in the set of samples described in **A**. Points are the mean and error bars represent ± 1 standard deviation of the experiments performed in triplicate.

The kinetic analysis of GSTU24 with CDNB provided a K_m value of 648 nkat.mg^{-1}, which is in accordance with the value established by Dixon et al., 2009, of 635 nkat.mg^{-1}. Of the 41 Arabidopsis GSTs tested by Dixon et al., 2009, GSTU25 was found to be one of the most active with CDNB. The kinetic analyses of GSTU25 were performed by Sparrow, 2010, who calculated a K_m of 1560 nkat.mg^{-1}, in agreement with the value of Dixon et al., 2009, of 1240 nkat.mg^{-1}.

2.3.3. Activity of GSTU24 and GSTU25 with TNT

The rate of conjugation of TNT in an enzymatic reaction is affected by both enzyme quantity and substrate concentration. Preliminary assays to establish a suitable amount of enzyme in the reaction were performed by Sparrow, 2010. Concentrations of GSU25 from 50 - 800 μg produced reliably detectable levels of a conjugation product (Sparrow, 2010). For consistency, in the current project the amount of enzyme used in all reactions was 150 μg. The solubility limit of TNT in aqueous solutions is 512 μM at 20 °C, to ensure complete solubilisation of TNT the concentration used in all pH and temperature assays was 200 μM.

Analyses of the conjugation reaction products of GSTU24 and GSTU25

The conjugation activities of GSTU24 and GSTU25 were studied in assays containing 100 mM potassium phosphate buffer with the pH ranging from 5.5 - 9.5, increasing by 0.5 units, at a temperature of 20 °C over a time course of 6 h.

The HPLC analyses show formation of different products by the respective enzymes. GSTU24 produces a single major product with a molecular weight of 518, as determined by the results of LC-MS analyses. The spectrum of conjugates formed by GSTU25 varies across the pH range. At pH below 7, the main conjugate produced has a molecular mass of 487 corresponding to 2-glutathionyl-4,6-dinitrotoluene (conjugate 3), previously identified by LC-MS in the conjugation reaction of commercially available GSTs from equine liver (Sigma) with TNT (Brentner et al. 2008). At pH above 7.5 two major compounds are formed, both with masses of 518 (Fig. 2.5), one of which is identical to the compound produced by GSTU24 (conjugate 2) and the other substance (conjugate 1) is present in GSTU24 reactions in trace amounts. All compounds have characteristic UV absorption spectra (Fig 2.6).

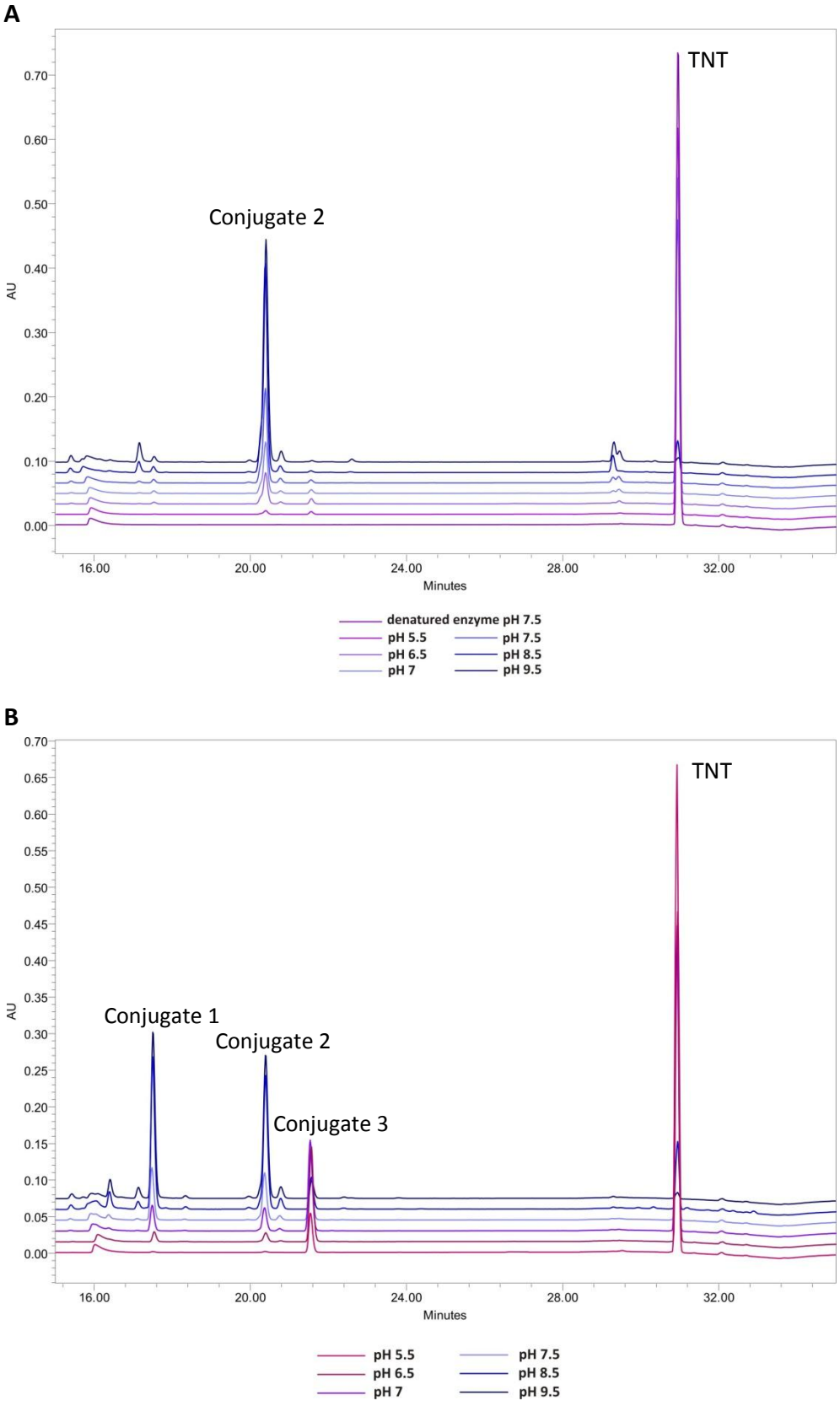


Fig. 2.5. HPLC chromatogram (analysed at 250 nm) of conjugation products of TNT with GSH formed by GSTU24 (**A**) and GSTU25 (**B**) across the pH range of the reaction (5.5 - 9.5). The reactions were performed in 100 mM potassium

phosphate buffer at the temperature of 20 °C with 150 µg of the enzyme, 200 µM TNT and 5 mM GSH.

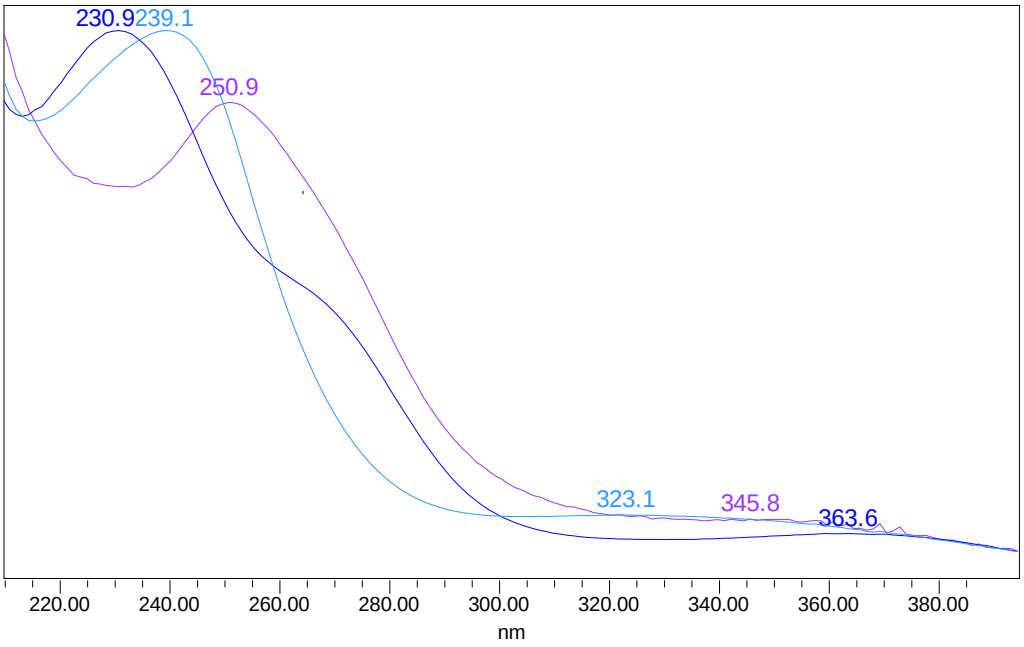


Fig. 2.6. UV absorption spectra and absorption maxima of TNT conjugates: conjugate 1 (blue), conjugate 2 (dark blue), conjugate 3 (purple).

To confirm the conjugated products are formed by the enzymatic activity, and not in a chemical reaction between GSH and TNT, control reactions were performed under the same conditions as the enzyme reactions but using the enzyme denatured by heating to 95 °C for 5 min. No compounds were found to be formed in the reactions with the heat deactivated enzyme (Fig. 2.5 **A**).

Griess assay

Apart from MS analysis, Griess assays were performed to obtain data on the conjugation of GSH by nucleophillic substitution of the NO_2^- group of TNT. The Griess assay is a colorimetric assay used to measure the amount of free nitrite released in the reaction. This assay relies on a diazotization reaction that was originally described by Griess in 1879 and has been modified over the years.

The chemical reaction uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic conditions (Fig. 2.7).

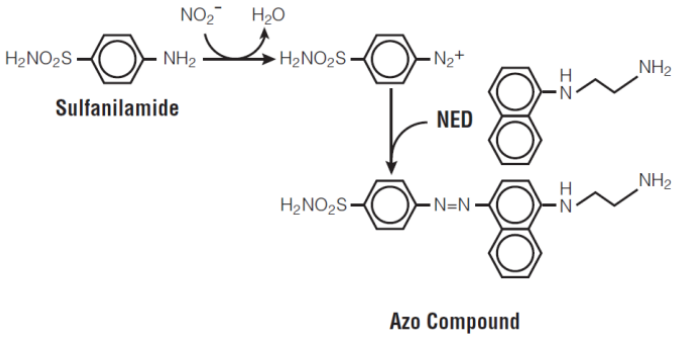
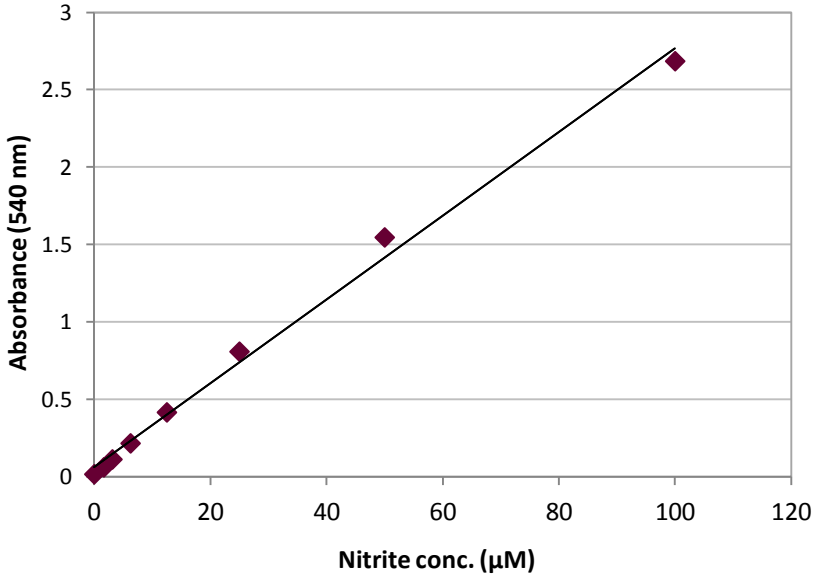


Fig. 2.7. Chemical reactions involved in the measurement of NO_2^- using the Griess reaction (Figure from Promega Griess Reagent System protocol).

Results of the Griess assay performed on the products of the conjugation reaction of GSTU24 and GSTU25 indicated that only conjugate 3 (expected 2-glutathionyl-4,6-dinitrotoluene), produced by GSTU24 in small amount and as the major product by GSTU25 at the pH of 6.5 and lower, is formed in a substitution reaction via the removal of a nitro group (Fig. 2.8).

A



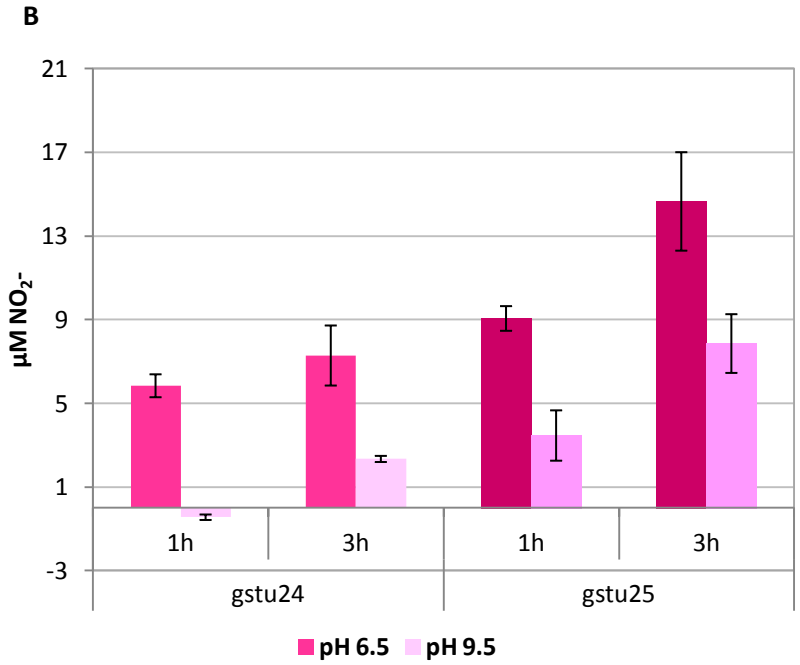


Fig. 2.8. Standard curve for the Griess assay (**A**) and the Griess assay of TNT conjugation reaction (**B**). TNT conjugation reactions were performed for 1 and 3 h at 20 °C in 100 mM phosphate buffer pH 6.5 and 9.5 with 150 µg of the enzyme, 500 µM TNT and 5mM GSH in total volume of 250 µl. After the reaction was stopped the concentration of NO_2^- produced by substitution of GSH for NO_2^- group of TNT was measured spectrophotometrically at 540 nm due to pink coloration indicating the presence of free nitrite. Quantification was performed based on standard curve produced with 0 – 100 µM $NaNO_2$.

Chemical structure of TNT conjugates

To elucidate the complete chemical structure, all three TNT conjugates were purified and their NMR spectra measured. The NMR data confirmed the structure of 2-glutathionyl-4,6-dinitrotoluene (conjugate 3, *m/z* 486 $[M-H]^-$) formed by nucleophilic substitution of NO_2^- group by GSH. The data on the remaining compounds (*m/z* 517 $[M-H]^-$) suggest the substances are C-glutathionylated 4-HADNT (conjugate 1) and C-glutathionylated 2-HADNT (conjugate 2) with GSH attached via CH_3 group of TNT (Fig. 2.9 and 2.10).

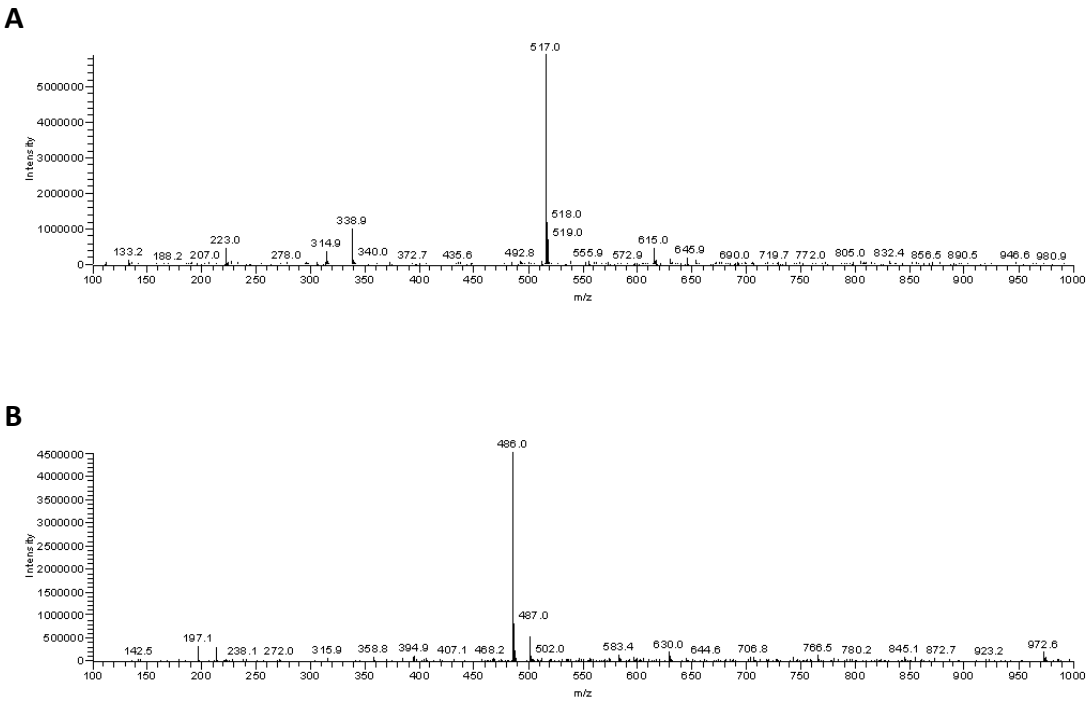
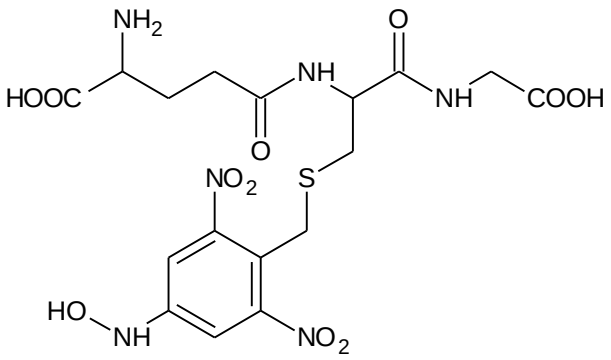
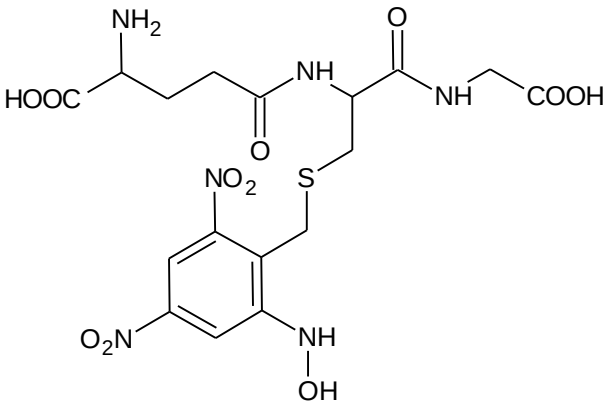


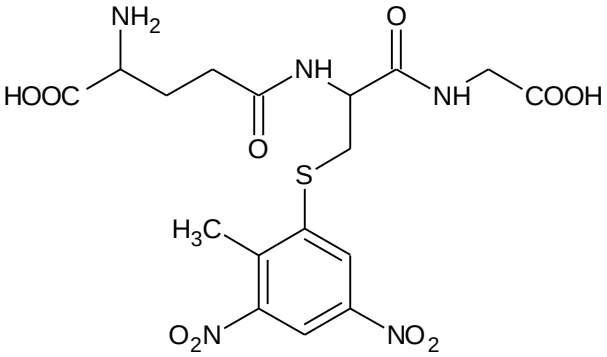
Fig 2.9. Mass spectrometry data from LC/MS analyses of products of GSTU24 and GSTU25 conjugation reactions. **A**, mass spectrum of conjugate 1 and 2 in negative mode with $[M-H]^-$ ion of 517 and molecular mass of 518; **B**, mass spectrum of the conjugate 3 with $[M-H]^-$ ion of 486 and mass of 487.



Conjugate 1



Conjugate 2



Conjugate 3

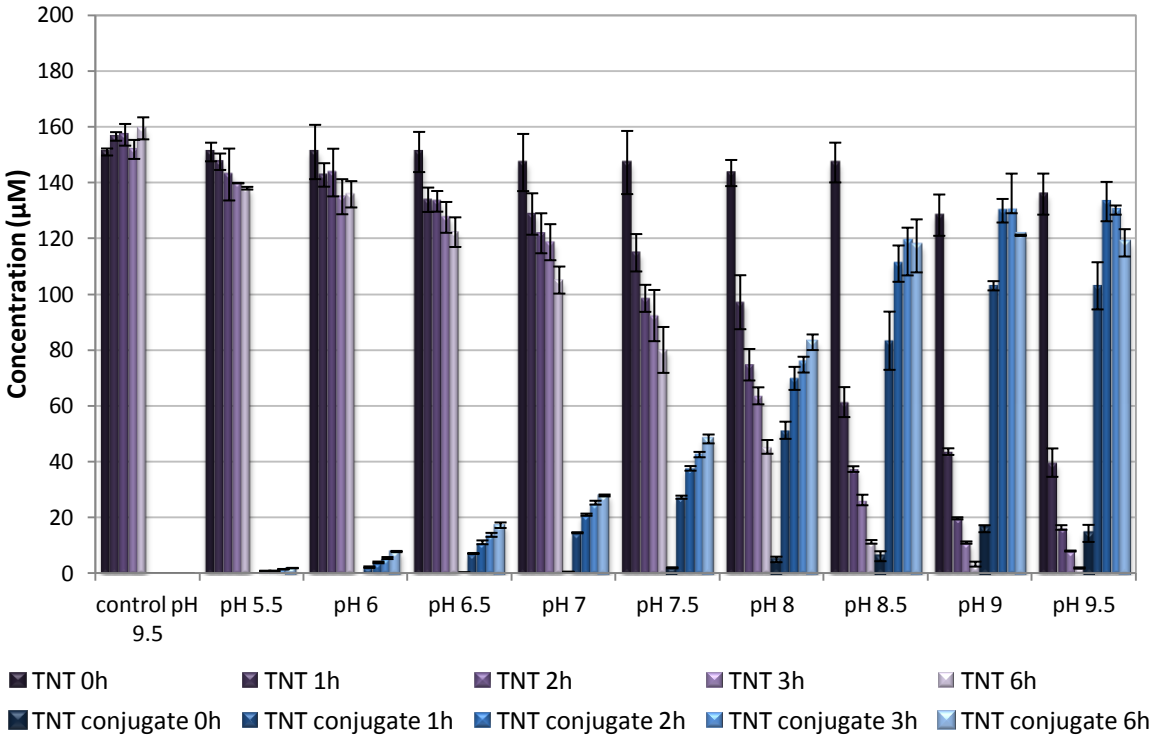
Fig. 2.10. Chemical structure of TNT conjugation products elucidated by NMR.

2.3.4. Effect of pH on GST activity

To determine the effect of pH on the conjugation activity of GSTU24 and GSTU25, the conjugation reaction was performed over 6 h in 100 mM potassium phosphate buffer with the pH ranging from 5.5 - 9.5, increasing by 0.5 units, at the temperature of 20 °C. To establish the rate of non-enzymatic chemical transformation of TNT under the assay conditions, control reactions were performed across the pH range with enzymes denatured by heating to 95 °C for 5 min.

Results of the HPLC analyses show that both enzymes were most active towards TNT at the higher pH values tested, transforming 96 - 98 % of TNT into the respective conjugates at pH 9 - 9.5 within 6 h, while no quantitative or qualitative changes of TNT were observed in the reactions without the active enzyme (Fig. 2.11 & 2.12).

A



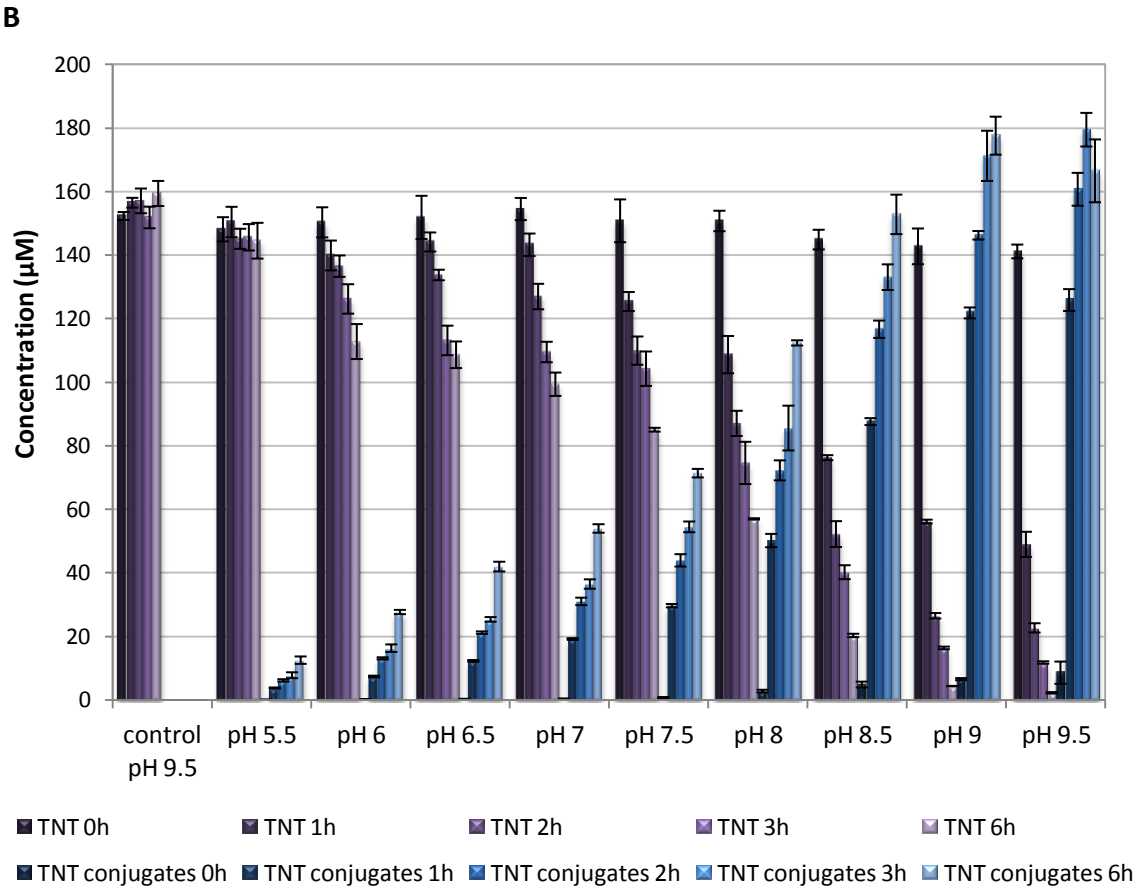


Fig. 2.11. Effect of pH on conjugation activity of GSTU24 (**A**) and GSTU25 (**B**). Concentrations of TNT and TNT conjugates were analysed by HPLC for 6 h at 20 °C at pH ranging from 5.5 - 9.5. The results show mean of experiments performed in triplicate with 150 µg of the enzyme, 200 µM TNT and 5 mM GSH; error bars represent standard deviation from the mean.

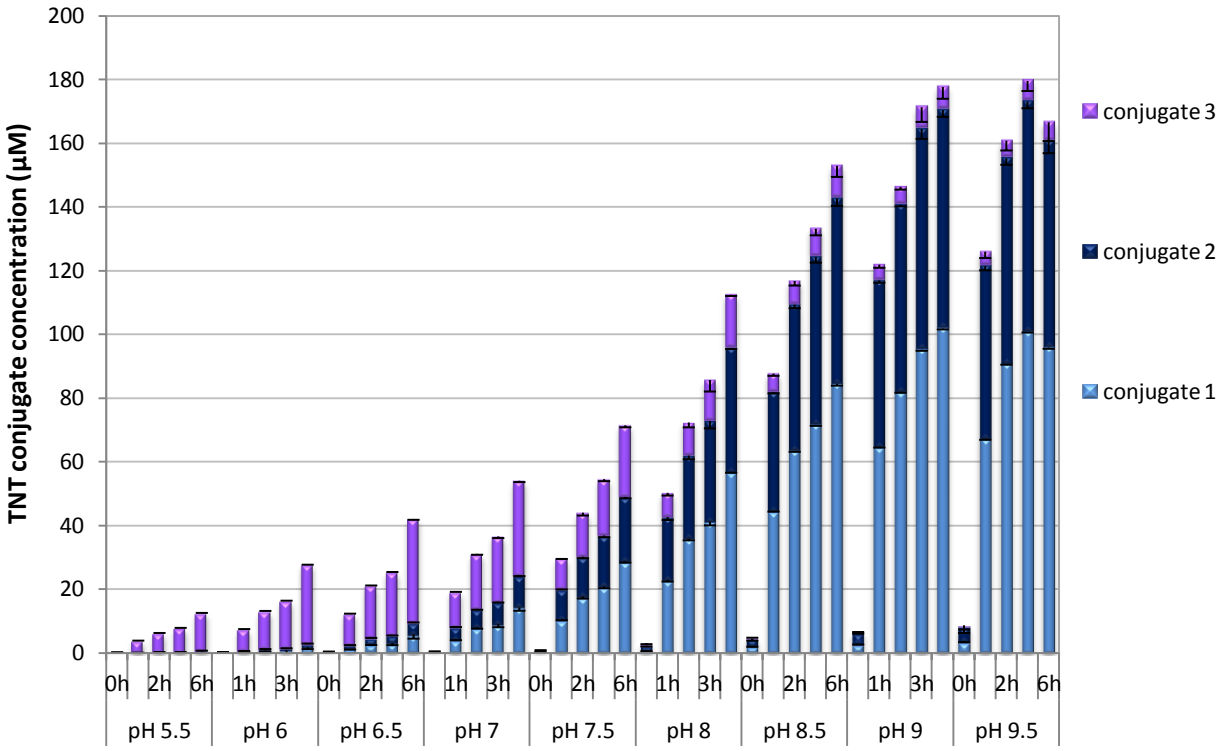


Fig. 2.12. Effect of pH on the quantity and spectrum of conjugates formed by GSTU25. Concentrations of TNT conjugates were analysed by HPLC. The results show mean of experiments performed in triplicate; error bars represent standard deviation from the mean.

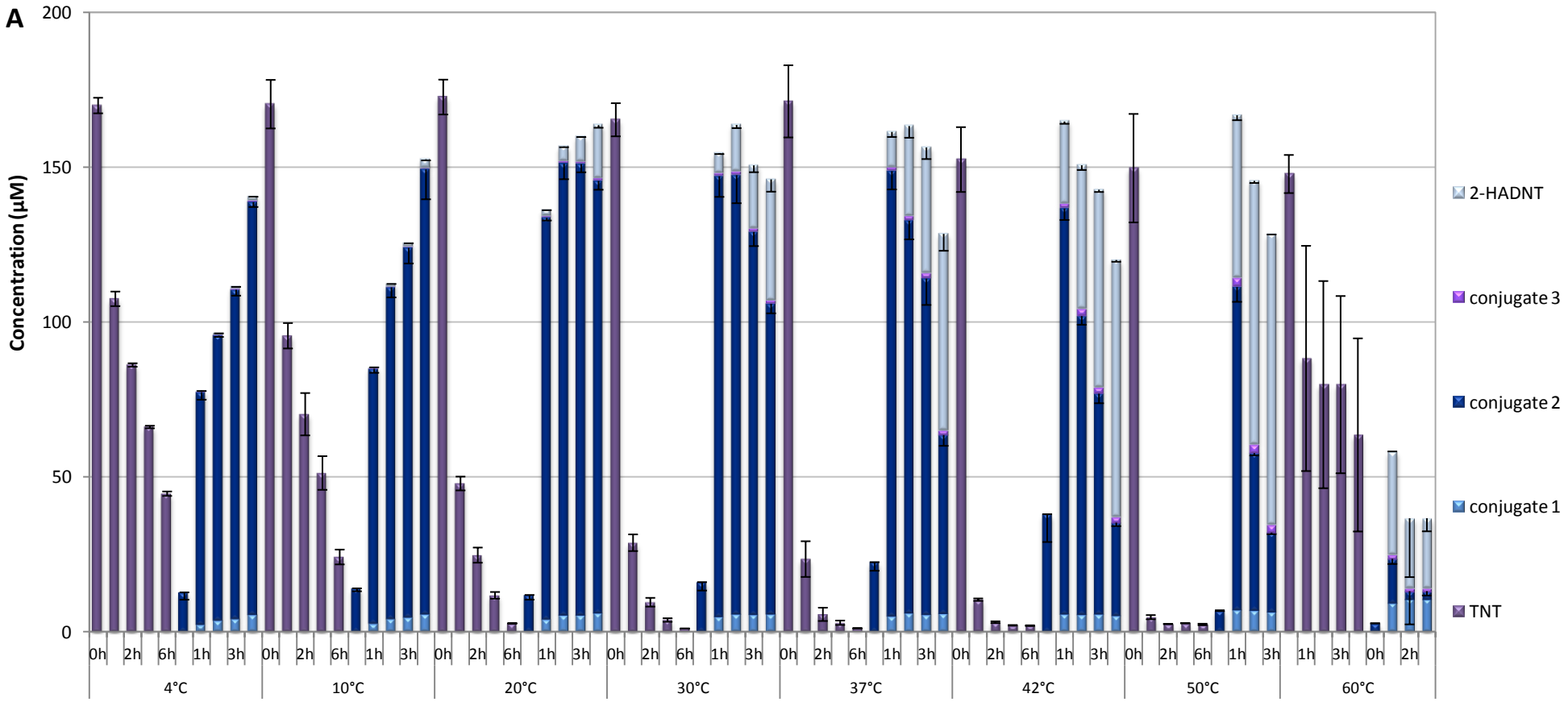
2.3.5. Effect of temperature on the enzyme activity

Enzyme activity increases with temperature, but so does denaturation. To study the effect of temperature on the activity of GSTU24 and GSTU25, enzymatic reactions were performed in triplicate over 6 h with 150 µg of enzyme, 200 µM TNT and 5 mM GSH in 100 mM potassium phosphate buffer pH 9 at temperatures ranging from 4 - 60 °C.

The enzymes are active in a wide range of temperatures; at 4 °C GSTU24 can transform 75 % of initial TNT concentration in the reaction within 6 h and GSTU25 conjugates 65 % of TNT into the respective conjugates. The highest rate of TNT transformation by GSTU24 and GSTU25 was observed at 42 °C when 93 % and 90 % of TNT, respectively, were conjugated within 1 h (Fig. 2.13).

The temperature of 50 °C causes visible aggregation of the enzymes, and although does not affect the tertiary structure of the protein significantly to reduce activity with the substrate concentration studied, it affects together with high pH the stability of the products causing chemical degradation of the unstable HADNT. At 60 °C the activity significantly decreased, probably due to changes in the enzyme structure.

Temperatures from 30 °C had a negative effect on the accumulation of reaction product (conjugate 2) in the GSTU24 reactions after 1 h, when nearly all available TNT was used, and an increase in 2-HADNT concentration, a possible degradation product of the conjugate, was observed. The degradation might be of enzymatic origin, as the isolated, purified conjugates were found to be stable when subjected to temperatures of 50 °C for 3 h in water solution, therefore it was further investigated.


A
Concentration (µM)
200
150
100
50
0
0h 2h 6h 1h 3h 0h 2h 6h 1h 3h 0h 2h 6h 1h 3h 0h 2h 6h 1h 3h 0h 2h 6h 1h 3h 0h 2h 6h 1h 3h 0h 2h 6h 1h 3h 1h 3h 0h 2h
4°C
10°C
20°C
30°C
37°C
42°C
50°C
60°C
2-HADNT
conjugate 3
conjugate 2
conjugate 1
TNT

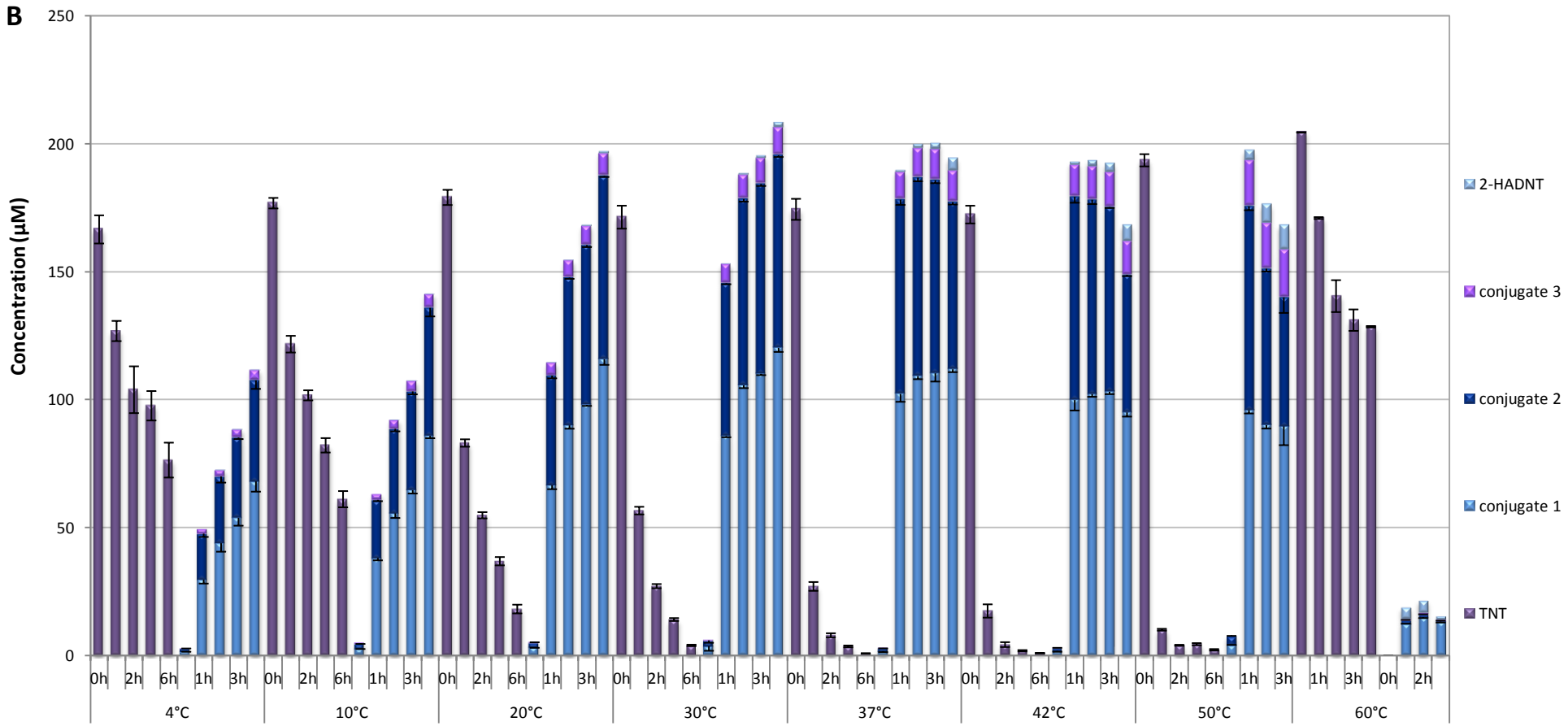


Fig. 2.13. Effect of temperature on conjugation activity of GSTU24 (**A**) and GSTU25 (**B**). Concentrations of TNT and TNT conjugates were analysed by HPLC for 6 h at pH 9 and temperature range 4 - 42 °C. The results show mean of experiments performed in triplicate with 150 µg of the enzyme, 200 µM TNT and 5 mM GSH; error bars represent standard deviation from the mean.

2.3.6. Substrate specificity of GSTU24

To study the accumulation of 2-HADNT (identified based on the comparison of retention time and UV spectra of 2-HADNT standard) in the reaction, parallel experiments were conducted using the same conditions at the temperature of 42 °C (150 μg of enzyme, 200 μM TNT and 5 mM GSH in 100 mM potassium phosphate buffer pH 9). In the first experiment the enzyme was active for the duration of the experiment (6 h), in the second experiment the reaction was stopped with TCA after 1 h, the time at which all available TNT had been converted to conjugate 2, and then incubated at 42 °C for 5 h. The results of the HPLC analyses showed an increase in 2-HADNT concentration in reactions with GSTU24 active for 6 h, while there were no changes in the amount of conjugate 2 and no accumulation of 2-HADNT in the second experiment (Fig. 2.14). This suggests that conjugate 2 could be a substrate for GSTU24 when no TNT is present in the reaction mixture. This hypothesis was tested using purified conjugate 2 (200 μM) as the substrate for GSTU24 using the same conditions as the conjugation reaction with TNT (150 μg of enzyme in 100 mM potassium phosphate buffer pH 9, 20 °C and 42 °C for 6 h). The HPLC results showed that GSTU24 was capable of using conjugate 2 as a substrate, forming two products: 2-HADNT and a novel substance with a shorter retention time and the same molecular weight of 518 (Fig. 2.15). The reaction rate of GSTU24 with conjugate 2 was slower than with TNT and only ~23 % of the compound was metabolised after 6 h at 20 °C compared with 98.5 % of TNT conjugated in the same conditions, although the reaction rate was significantly higher at the increased temperature (87 % of the conjugate 2 metabolised at 42 °C after 6 h). 2-HADNT, 4-HADNT, 2-ADNT and 4-ADNT were also tested as possible substrates for the GSTs, but no product of conjugational activity was observed for GSTU24 and GSTU25 using a range of different conditions with low and high pH (6.5 and 9.5) and a range of temperatures (20 – 42 °C) (Fig. 2.16). It is therefore possible to conclude that only TNT is the substrate of GSTU24 and GSTU25, but not the products of TNT activation reactions during Phase I of xenobiotic detoxification, although GSTU24 is capable of unique de-glutathionylation reaction using conjugate 2 as a substrate. Only traces of 2-HADNT and 4-HADNT were found in the GSTU25 reactions, which suggests this enzyme is capable of the de-glutathionylation reaction in a very small extent.

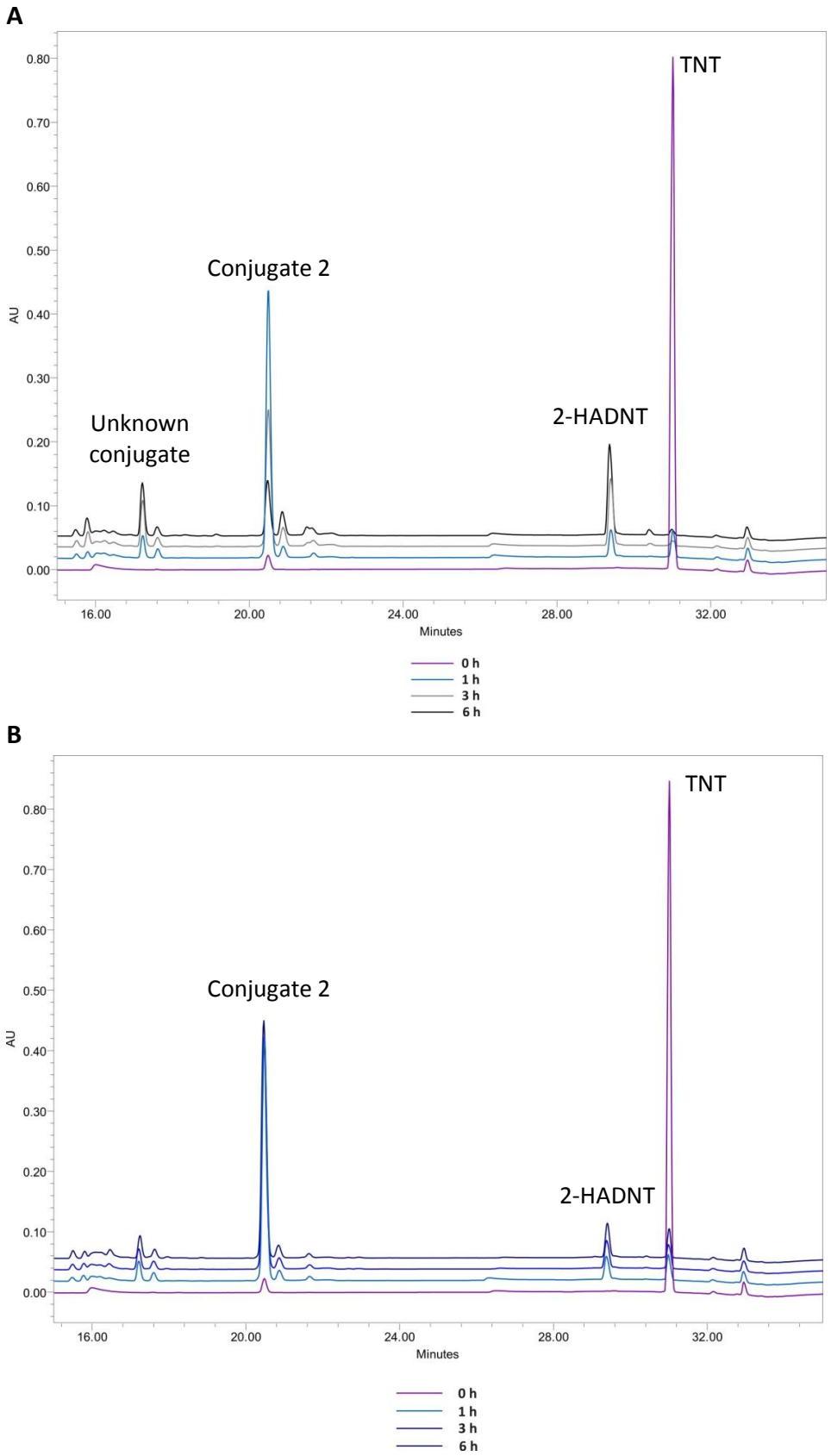


Fig. 2.14. HPLC analysis of GSTU24 enzymatic activity analysed at 250 nm with TNT as a substrate. Reactions were performed with 150 µg of enzyme, 200 µM TNT,

5 mM GSH in phosphate buffer pH 9 and temperature of 42 °C for 6 h. **A,** TNT was conjugated into conjugate 2 in 1 h, after 6 h 2-HADNT and an unidentified conjugate with molecular mass of 518 was accumulated in the reaction mixture; **B,** GSTU24 enzymatic activity was stopped by precipitating the enzyme with 1 % TCA after 1 h when TNT was conjugated into the respective conjugate, no further chemical changes of conjugate 2 were observed in the reaction for following 5 h.

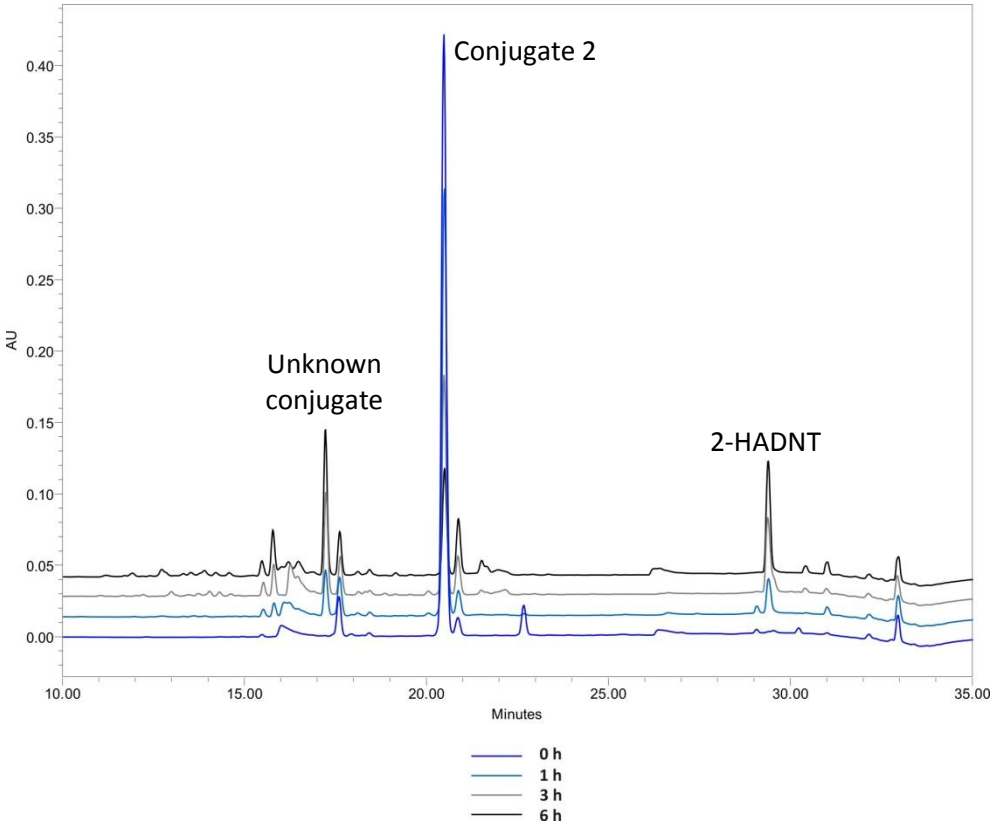


Fig. 2.15. HPLC analyses of GSTU24 enzymatic activity with conjugate 2 as a substrate analysed at 250 nm. Reactions were performed with 150 µg of enzyme, 200 µM conjugate 2, 5 mM GSH in phosphate buffer pH 9 and temperature of 42 °C for 6 h.

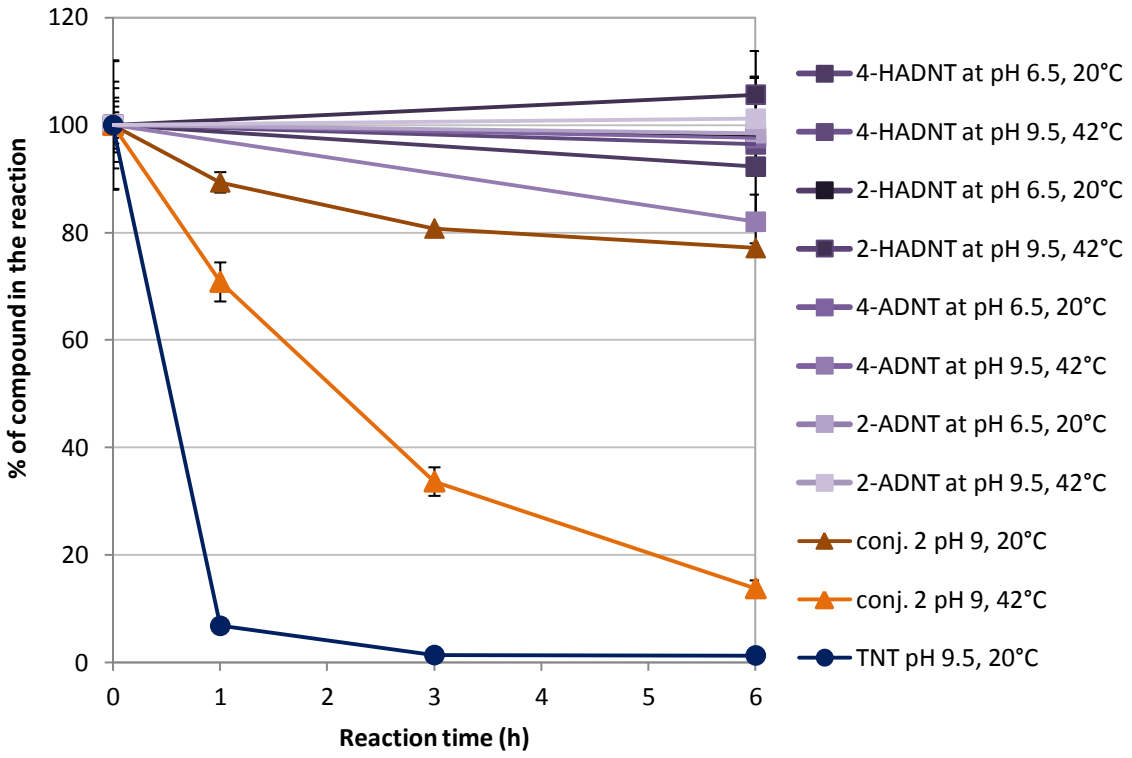
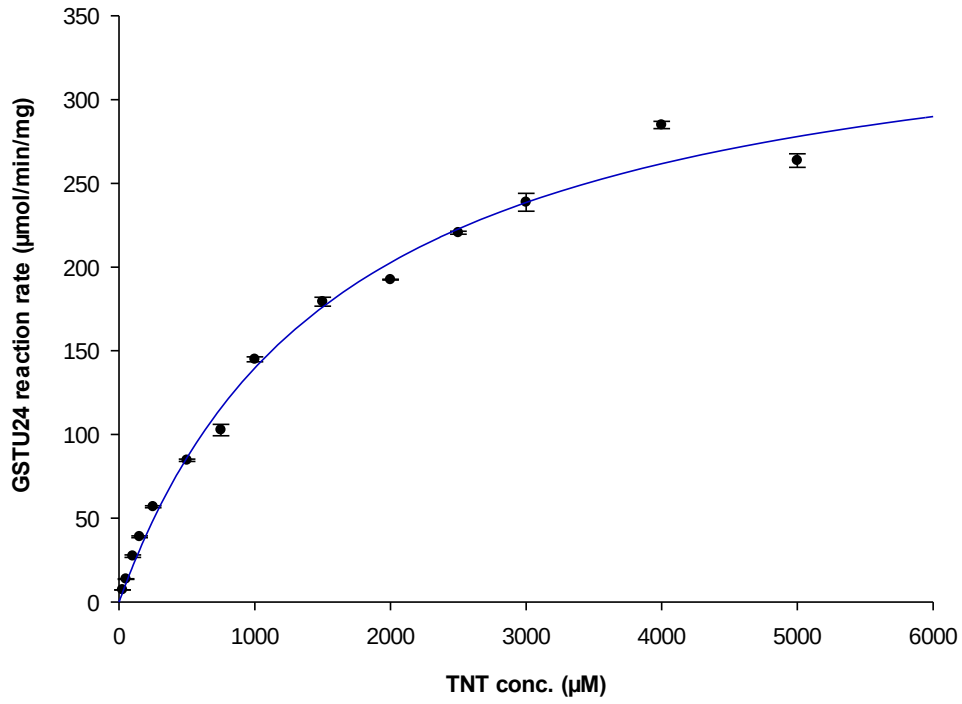


Fig. 2.16. Substrate specificity of GSTU24 was tested using different substances and different conditions. The reactions were performed with 150 µg of enzyme, 200 µM substrate and 5 mM GSH at 20 °C and 42 °C and at pH 6.5 and 9.5. The results show mean of experiments performed in duplicate; error bars represent standard deviation from the mean.

2.3.7. Kinetic analyses of GSTU24 and GSTU25

Kinetic assays of GSTs with TNT were performed at the conditions optimal for the enzyme activity and product stability- in 100 mM phosphate buffer pH 9.5 at 42 °C with 150 µg of enzyme. The concentration of TNT ranged from 25 - 5000 µM and GSH concentration ranged between 5 - 45 mM. To ensure TNT was solubilised in the solution, stock TNT concentrations were prepared in DMSO and 5 % DMSO added to the total volume of the reaction, as this amount of DMSO was found to have no effect on GST protein activity and at the same time solubilise TNT (Sparrow, 2010). The reaction rates of conjugate production were calculated and plotted against TNT concentration to produce Michaelis-menten plots for GSTU24 and GSTU25 (Fig. 2.17).

A



B

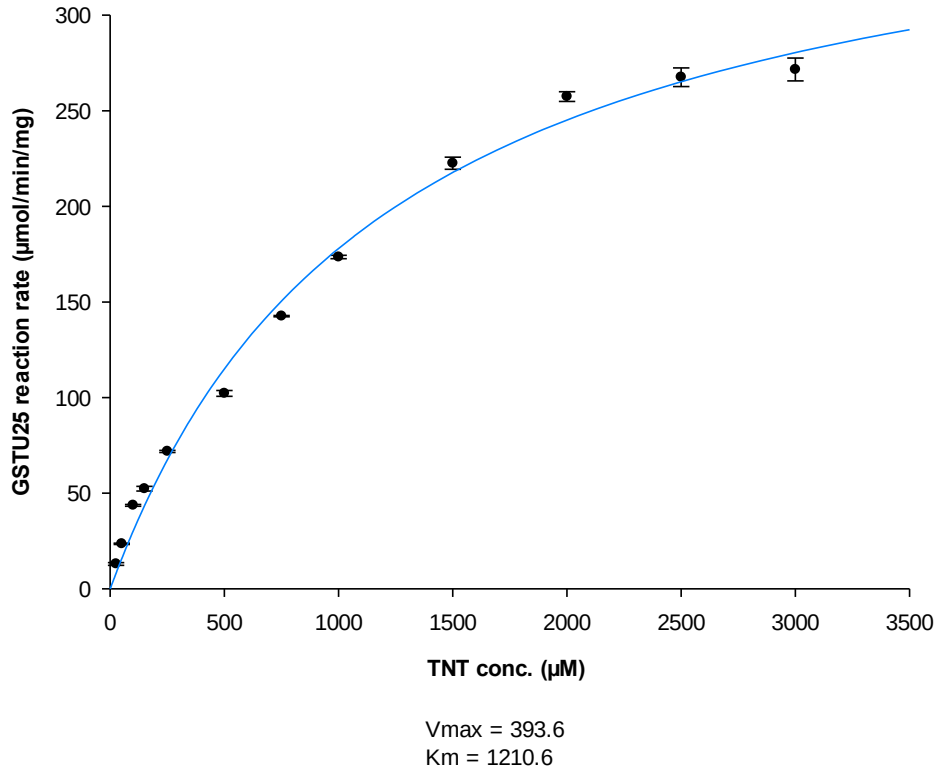


Fig. 2.17. Michaelis-Menten plot of GSTU24 (**A**) and GSTU25 (**B**) rate of conjugate production with different initial TNT concentrations. The reactions were performed in triplicate with 150 µg of the enzyme, 0 – 5 mM TNT and 5 - 45 mM GSH in phosphate buffer pH 9.5 at 42 °C, samples were analysed during 25 min time course by HPLC; error bars represent standard deviation from the mean.

The kinetic analyses of GSTU24 with TNT provided a K_m value of 1644.2 µM TNT and V_{max} of 369.3 µmol.min^{-1} (6155 nkat.mg^{-1}). The K_m value of GSTU25 calculated from the kinetic analyses (based on the sum value of the concentrations of three reaction products) was 1210.6 µM TNT and V_{max} was 393.6 µmol.min^{-1} (6560 nkat.mg^{-1}). The turnover rate values k_{cat} calculated based on the V_{max} values were 1029 ± 26 s^{-1} and 1088 ± 40 s^{-1} for GSTU24 and GSTU25, respectively. The results of the kinetic analyses show that in the optimal conditions both enzymes have a similar activity towards TNT with GSTU25 being the more active of the two enzymes studied.

2.3.8. Equine liver GST

Commercially available purified GSTs (Sigma) from equine liver are characterised by their activity towards CDNB (1 unit conjugates 1 μmol CDNB/min). Equine liver GST has been used as a positive control in the experiments helping to identify the reaction product, as it was previously shown to have activity towards TNT and LC–MS–MS analyses of the TNT conjugate confirmed the formation of 2-S-glutathionyl-4,6-dinitrotoluene (Brentner et al. 2008) (Fig. 2.18).

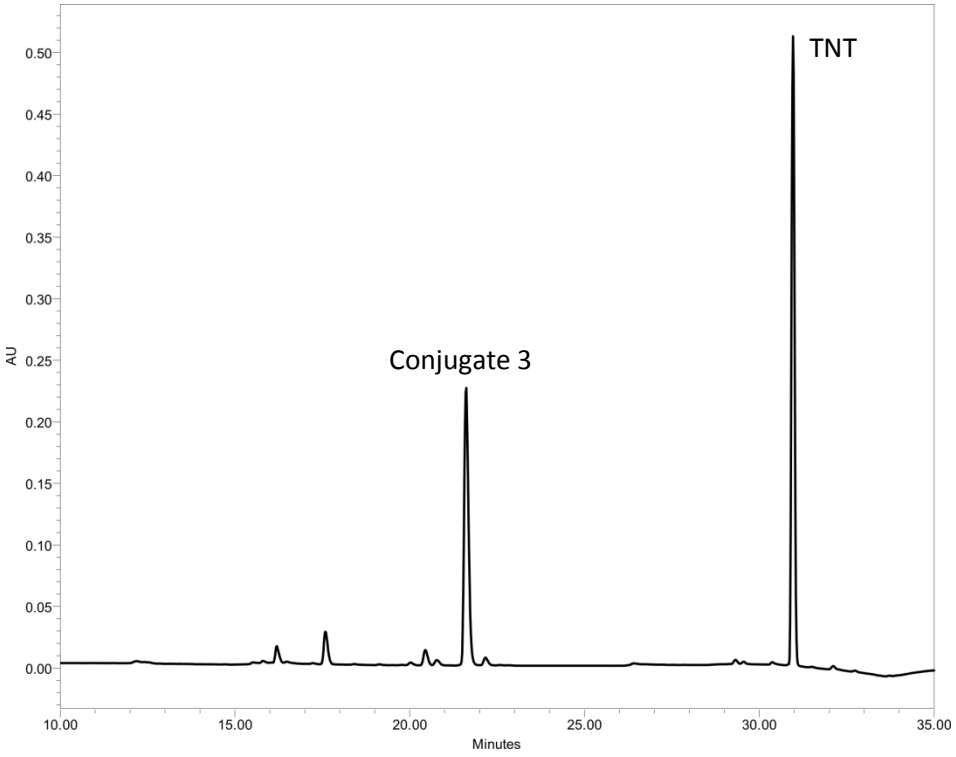


Fig. 2.18. HPLC chromatogram of conjugation product of TNT with GSH formed by equine liver GST at pH 7.5 and temperature 37 °C after 3 h reaction time (analysed at 250 nm).

In the HPLC analyses equine liver GST produced a product with a molecular weight of 487 corresponding to the 2-*S*-glutathionyl-4,6-dinitrotoluene, matching in retention time and UV spectrum the conjugate 3 from GSTU24 and GSTU25

reactions. The enzyme produced only trace amounts of the product at 20 °C. At 37 °C, the enzyme showed TNT conjugating activity increasing with pH, similar to that seen by GSTU24 and GSTU25 (Fig. 2.19)

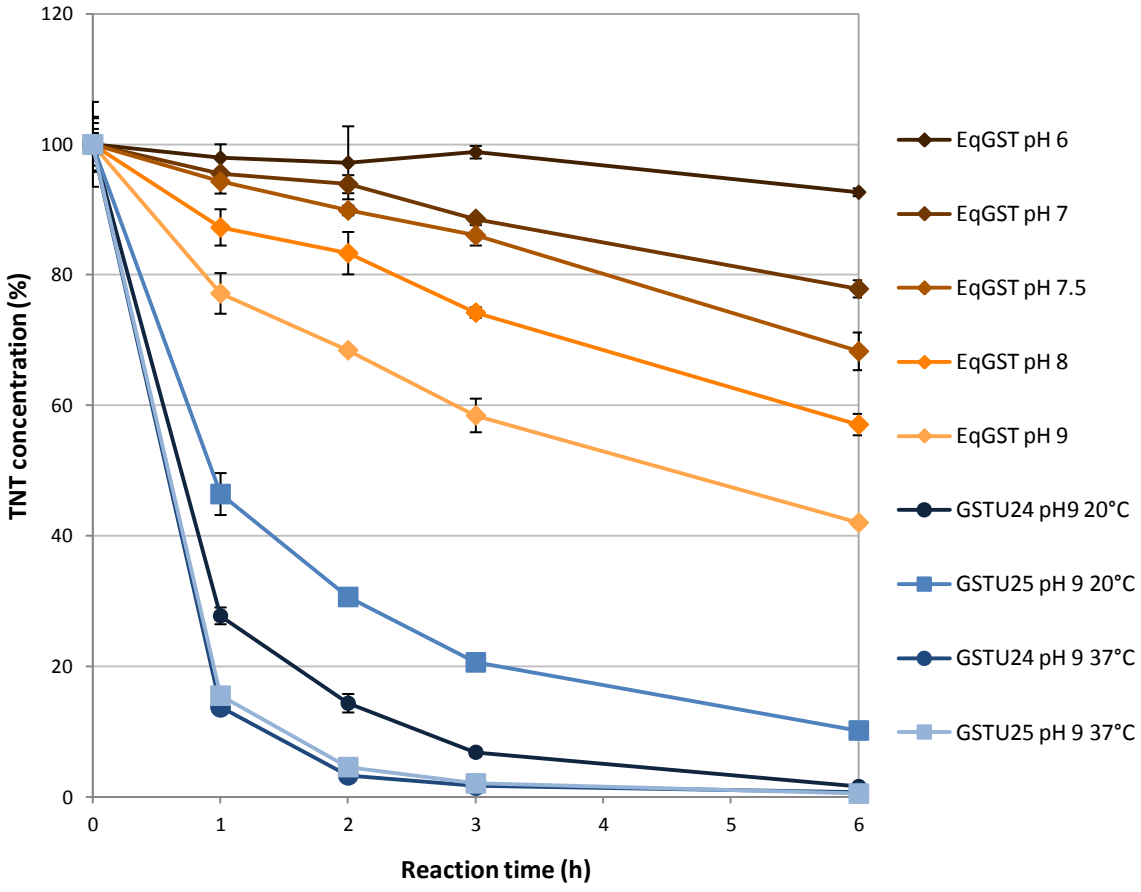


Fig. 2.19. Effect of pH on TNT conjugating activity of equine liver GST (Sigma) compared to the activity of GSTU24 and GSTU25. Concentrations of TNT in equine liver GST reactions (10 U of enzyme, 200 µM TNT and 5 mM GSH) were analysed by HPLC for 6 h at 37 °C at pH ranging from 5 - 9 and compared to the activity of GSTU24 and GSTU25 with 150 µg of enzyme, 200 µM TNT and 5 mM GSH at 20°C and 37 °C at pH 9. The results show mean of experiments performed in triplicate; error bars represent standard deviation from the mean.

2.4. Discussion and Conclusions

GSTU24 and GSTU25 are phylogenetically the closest of Arabidopsis GSTs and share 77 % sequence identity. There are, however, differences in some amino acids present in the active centres of GSTU24 and GSTU25 (Fig. 2.20), which could explain the differences in TNT product formation.

```
GSTU24    MADEVILLDFWASMFGMRTRIALAEKRVKYDHREEDLWNKSSLLLEMNPVHKKIPVLIHN 60
GSTU25    MADEVILLDFWPSMFGMRTRIALEEKNVKFDYREQDLWNKSPILLEMNPVHKKIPVLIHN 60
          ***********.*********** **.**:*:**:****▲*.:**********▲****** 

GSTU24    GKPVCESLIQIEYIDETWPDNNPLLPSDPYKRAHAKFWADFIDKKVNVTARRIWAVKGEE 120
GSTU25    GNPVCESLIQIEYIDEVWPSKTPLLPSDPYQRAQAKFWGDFIDKKVYASARLIWGAKGEE 120
          *:***▲▲*********.**.:.********:**:****.******* .:** **..****

GSTU24    QEAAK-ELIEILKTLESELGDKKYFGDETFGYVDIALIGFHSWFAVYEKFGNVSIESECS 179
GSTU25    HEAGKKEFIEILKTLESELGDKTYFGGETFGYVDIALIGFYSWFEAYEKFGSFSIEAECP 180
          :**.* *:***************.***.*************:*** .*****..***:**.

GSTU24    KLVAWAKRCLERESVAKALPESEKVITFISERRKKLGLE-- 218
GSTU25    KLIAWGKRCVERESVAKSLPDSEKIIKFVPELRKKLGIEIE 221
          **:**.***:*******:**:***:*.*:.* ******:*
```

Fig. 2.20. CLUSTAL 2.1 Multiple Sequence Alignments of AtGSTU24 and AtGSTU25. Ser17 is marked by *, residues involved in GSH binding are marked with red ▲. Amino acid residues forming the H-site are in blue □, those considered important to substrate specificity are highlighted in blue. (Amino acid identification according to Thom et al. 2002).

The increased activity towards TNT at high pH conditions seems to be a common feature of the GSTs studied. The pH of the solution in which the enzymes perform their catalytic reactions has a significant effect on enzyme activity. Amino acid residues play important roles in the binding of proteins to other molecules and in enzyme mechanisms. They also have a large influence on protein structure, stability and solubility. The types of interactions these side chains will have with their environment depend on their protonation state (Di Russo et al. 2012). Acidic amino acids have carboxyl functional groups in their side chains, while basic amino acids have amino functional groups in their side chains. If the state of ionization of amino acids in a protein is altered, the ionic bonds that help to

determine the tertiary structure of the protein can be altered. This can lead to change in protein recognition of the substrate or an enzyme might become inactive (http://academic.brooklyn.cuny.edu/biology/bio4fv/page/lect_o.htm).

The amino acids forming the active centres of GSTU24 and GSTU25 are generally non-polar, containing a number of amino groups in their side chains, which will be protonised at lower pH values, as the amino group accepts a proton at pH 7 and carries a positive charge at pHs below 7 (http://academic.brooklyn.cuny.edu/biology/bio4fv) affecting the activity and product identity of the enzymes.

Glutathione might also contribute to the higher activity of the enzymes at pH 9 - 9.5 due to the increased reactivity at high pH, as the sulfhydryl group has a pKa value of 9.4 and therefore the reactive thiolate anion is more stable at these conditions (Dixon & Edwards 2010).

Kinetic assays with recombinant proteins at different conditions were most often performed using CDNB as a substrate. Most assays using CDNB to characterise GSTs were performed at pH 6.5 to minimize non-enzymatic reaction (Habig et al. 1974). Results of a detailed kinetic study of PtGSTU1 from *Pinus tabulaeformis* showed the enzyme had the highest activity towards CDNB in the pH range 8.5 - 9.0. The enzyme still had 56 % and 49% of its maximum activity at pH 7.5 and 9.5, respectively, at pH 5.5 and 10.5 enzyme activity was barely detectable (Zeng et al. 2005). The pH optima for GSTs from animal species using CDNB are pH 6.5 - 7.5 (Akkemik et al. in press, Samra et al. 2012).

GSTs from different plant and animal species had a broad temperature optimum, with higher activity at 40 - 45 °C than at 24 °C. PtGSTU1 from *Pinus tabulaeformis* also retains 60 % of its enzymatic activity at 15 °C, probably contributing to the ability of the plant to cope with the temperature fluctuations throughout its life span as well as

with variations across its extensive distribution range (Akkemik et al. in press, Samra et al. 2012; Zeng et al. 2005).

Biotransformation of TNT was studied in several plant species and cell cultures without analyzing contributions of different enzymes to the production of metabolites (Bhadra et al. 1998; Duringer et al. 2010; Hawari et al. 1999; Martin et al. 1997; Pavlostathis et al. 1998; B. Singh et al. 2011; Hawari et al. 1998). The pH optimum of Arabidopsis UGTs towards TNT was determined to be 7.0 (Gandia-Herrero et al. 2008), but there are no literature available on optimum activity conditions of recombinant GSTs with TNT.

To conclude, Arabidopsis GSTs conjugate TNT into different transformation products when compared to previously characterised mammalian GSTs. The mammalian GSTs from the liver fraction are active only at body temperature, whereas plant GSTs are active across wider temperature range from 4 - 42 °C, exhibiting higher conjugation reaction rates. Increase in activity with increasing pH value seems to be a common characteristic of all GSTs studied.

3. Role of GSTs in TNT detoxification in Arabidopsis plants

3.1. Methods

3.1.1. Plant material

The experiments were performed using wild type Arabidopsis plants and GST overexpressing lines, wild type and all lines were Col 0 ecotype. The overexpressing lines were created by transforming Arabidopsis plants with 35SGSTU24 and 35SGSTU25 constructs by Dr E. Rylott and Dr H. Sparrow using *Agrobacterium tumefaciens* (Sparrow, 2010). T2 seeds of GSTU25 transformed plants were sown on ½ MS agar with kanamycin as a selective marker; lines with T-DNA insertion in a single locus were selected based on a 3:1 ratio of kanamycin resistant and sensitive seedlings. Homozygous T3 seeds originating from kanamycin resistant plants grown in soil were used in all experiments. T3 lines of GSTU24 overexpressing plants were screened for segregation in kanamycin resistance and homozygous plants were grown in the soil to produce T4 seeds used for analyses.

3.1.2. Plant methods

Seed sterilisation

Seeds intended for growing in sterile conditions were sterilised by chlorine gas produced by mixing 100 ml Chloros bleach with 3 ml concentrated HCl in an airtight container. After 4 h the seeds were aired in the flow hood to remove chlorine.

Stratification of seeds

Before planting, Arabidopsis seeds were stratified in the dark for 3 nights at 4 °C on ½ MS agar plates or in 500 µl of sterile water to ensure even germination.

Growth conditions

Plant growth on solid medium

Transformed lines were selected by scattering the sterile seeds on ½ MS agar (0.215 % MS Basalt Salt mixture, 20 mM sucrose, 8 % agar) plates, supplemented with 50 µg/ml kanamycin. The seeds were imbibed in the dark for 3 nights at 4 °C before transferring to a growth room with 20 ºC/18 ºC day/night temperatures with a 16 hour light (80 $\mu mol.m^{-2}.s^{-1}$)/8 hour dark cycle.

Sterile seedlings were produced by sowing the seeds on ½ MS agar plates without kanamycin and 7- day old seedlings were transferred to liquid ½ MS medium.

Root length studies were carried out by imbibing sterile seeds in 500 µl of sterile water in the dark for 3 nights at 4 °C. Stratified seeds were placed in a single line on a ½ MS agar plate containing TNT dissolved in DMSO and transferred to the growth room.

Plant growth in soil

For seed and plant tissue production the plants were grown in non-sterile conditions in the greenhouse. Kanamycin resistant seedlings were transferred from ½ MS agar plate to the trays filled with F2 compost and propagated in the greenhouse until the seeds were collected.

Plant growth in liquid medium

The uptake of TNT by plants in liquid cultures was studied by growing seedlings initially on ½ MS agar plates without kanamycin. After seed stratification, the plates

were placed in the growth room and 7- day-old seedlings were transferred to sterile 100 ml flasks containing 20 ml ½ MS medium (8 seedlings per flask). After growing for 14 days under reduced light conditions (~15 μmol $m^{-2}.s^{-1}$) with 120 rpm shaking, the medium was replaced with 20 mM sucrose solution with 200 μM TNT in DMSO.

Hydroponic growth conditions

Arabidopsis was grown according to the method described by Taylor (2011). Rafts were made from circular lightweight plastic, 70 mm in diameter and 6 mm thick, with approximately 100 holes (3 - 4 mm diameter) drilled into each disk. The rafts were sterilised by autoclaving and the holes were plugged with ½ MS agar. Sterile Arabidopsis seeds stratified in the sterile water for three nights in the dark at 4 ºC were pipetted onto the plugged holes (8 seeds per raft). Rafts were then placed on liquid ½ MS medium in the sterile jars. Plants were grown in the sealed jars for 21 days at 20 ºC/18 ºC day/night temperatures with 16 h photoperiod (80 μmol.$m^{-2}.s^{-1}$). After three weeks the medium was removed and replaced with sterile ½ MS medium containing 50 μM and 100 μM TNT in DMSO.

3.1.3. Extraction of proteins from plant material

Rosette leaves of 3-week-old plants were used for protein extraction. Approximately equal weights of fresh leaves (60 - 80 mg) were homogenised in 400 μl of protein extraction buffer (0.1 M Tris-HCl pH 8, 2 mM EDTA, 1 mM DTT, 50 g/kg PVPP). Samples were centrifuged 15 min at 13 000 rpm; the supernatant was kept on ice and used in GST activity measurements.

3.1.4. GST activity measurements

The general activity of all GSTs was measured in the whole cell protein extracts from fresh rosette leaves spectrophotometrically using a CDNB assay (section 2.2.5) Solutions of 825 µl of 100 mM potassium phosphate buffer pH 6.5 and 25 µl of 100 mM CDNB were pre-incubated at 30 °C for 5 min. After this time, 50 µl of 100 mM GSH and 100 µl of the plant protein extract were added to the buffer and the increase in the reaction product concentration was measured spectrophotometrically at 340 nm.

3.1.5. Root length image analysis

To measure the effect of TNT on Arabidopsis root length, stratified seeds of Col 0 (wild type) and overexpressing lines were placed in a single row on ½ MS agar plate containing 0, 2, 7, 15 and 50 µM TNT dissolved in DMSO. The plates were placed vertically in the growth room. Photographs of the seedlings were taken after 9, 14 and 20 days. Photographs were subsequently analysed by image processing software. To measure the root area the photographs were processed by Image Pro 6.2 (Media Cybernetics Inc. USA). Image spatial calibrations were provided by ruler included in each image (calibrated over 65 mm of the ruler). Pre-analysis processing (to remove reflections and enhance root contrast):

1× 50 pixel flatten filter (dark)

1 × Sobel edge enhancement

1 × dilate (5 × 5 pixel circle)

1 × erode (5 × 5 pixel circle)

Inage J software was used to measure the root lengths where seedlings were too small for Image Pro software to process correctly.

Statistical differences were calculated using ANOVA and LSD multiple comparisons performed by IBM SPSS Statistics 19.

3.1.6. TNT analyses in the liquid medium

Samples of plant growth medium containing TNT were taken from the flasks or sterile jars at 0, 8, 24, 48, 72, 96 and 168 h. Samples were analysed by HPLC using a Waters Alliance 2695 separation module with a Waters 2996 photodiode array detector. The samples (50 µl and 100 µl) were loaded onto a Techsphere C18 column (250 × 4.6 mm, 5 µm) heated to 35 °C and separated using isocratic elution with methanol:water mixture (60:40). Integration was performed at 230 nm with Empower Pro software.

3.1.7. Extraction of TNT metabolites from plant tissues

Plants for the metabolite extraction were harvested at 0, 4, 6, 8, and 24 h after transferring the plants in the TNT containing medium in the liquid cultures. Plants growing hydroponically were harvested 0, 24, 48, 72, 96 and 168 h after adding TNT to the medium.

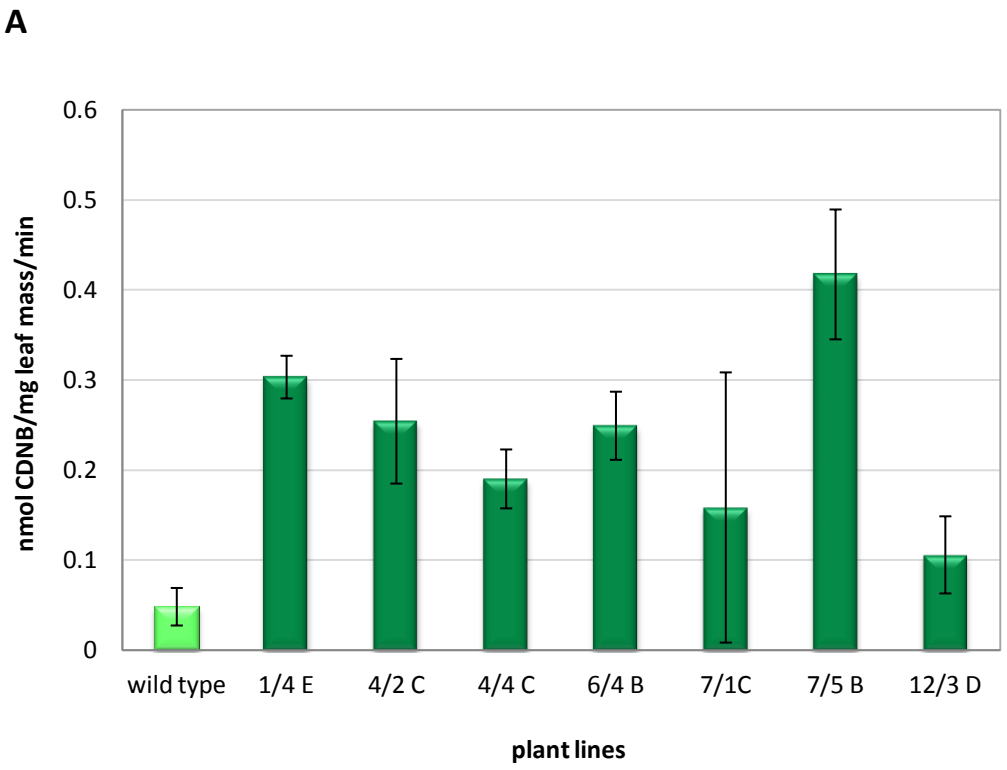
Fresh plant tissues were weighed and frozen by liquid nitrogen and ground to powder. The metabolites were extracted from the tissues with methanol:water mixture (60:40). After centrifugation the supernatant was removed and evaporated using Genevac centrifugal evaporator (SP Industries). Residues were re-dissolved in methanol:water mixture (60:40) according to the initial plant fresh weight and analysed by HPLC (conditions in section 2.2.6) and LC/MS (condition in section 2.2.8)

3.2. Results

3.2.1. Activity of GSTs in fresh leaves

The physiological importance of the TNT conjugates formation in plants was studied using transgenic Arabidopsis lines overexpressing GSTU24 and GSTU25 under the control of CaMV 35S promoter, compared to the wild type Columbia 0 plants. The total activity of all GSTs in the plant lines was measured in protein extracts from fresh rosette leaves using CDNB spectrophotometric assay (section 2.2.5). The increase in absorbance due to reaction product accumulation was measured over 1 min, data were normalised against no-enzyme control.

The transgenic lines with the highest GST activity were selected for future studies of the role of the GSTs in TNT detoxification (Fig. 3.1).



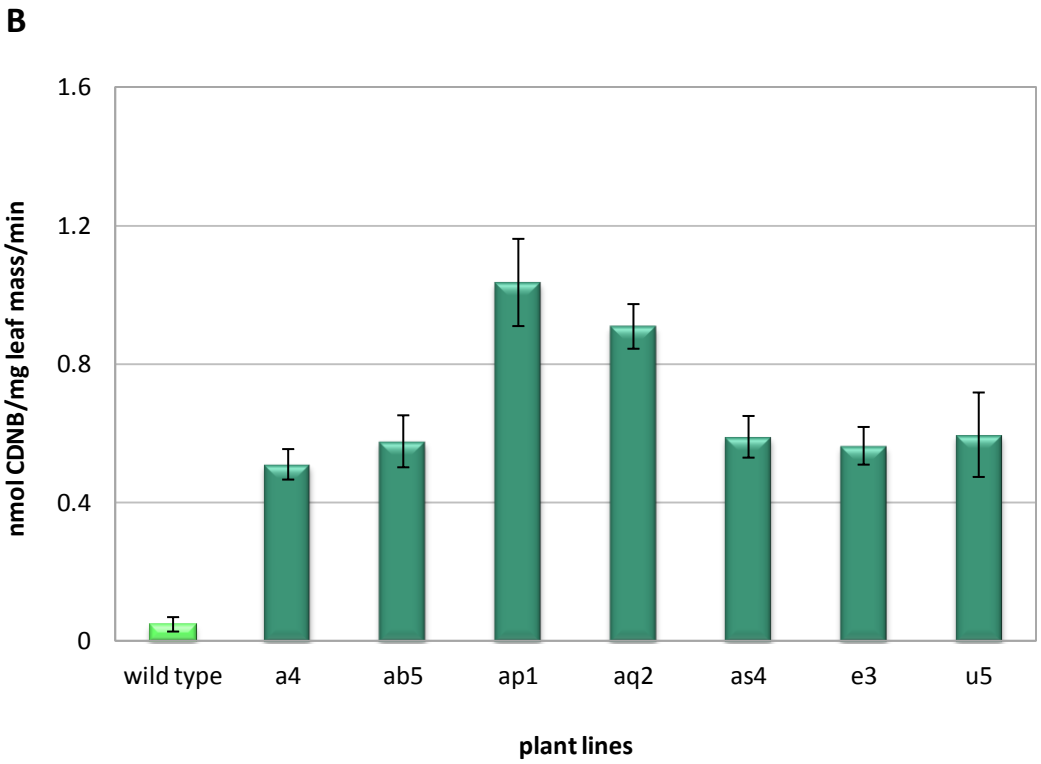


Fig. 3.1. Activity of GSTU24 (**A**) and GSTU25 (**B**) was measured using CDNB conjugation assay in protein extracts from fresh rosette leaves. Results are mean of 3 - 5 biological replicates for each plant line, error bars represent standard deviation from the mean.

GST activity in selected Arabidopsis lines overexpressing GSTU24 and GSTU25 was 2.2 - 8.7 fold and 10.6 - 21.5 fold higher, respectively, compared to the wild type plants; these lines were used in following experiments to characterise the role of GSTU24 and GSTU25 in TNT detoxification in plants.

Western blot analysis was not performed as the levels of GSTU24 and GSTU25 protein expression underlying the increased GST activity in the transgenic levels compared to the wild type plants could not be analysed due to the unavailability of specific antibodies.

3.2.2. Root length studies of 35S-GST lines

Root lengths of Arabidopsis seedlings grown in medium containing TNT were found to be an indicator of the tolerance of plants towards TNT (Beynon et al. 2009; Gandia-Herrero et al. 2008), as one of the symptoms of TNT phytotoxicity is characterised by severe root stunting.

Sterile imbibed seeds were placed in a single row on ½ MSA plates containing 0, 2, 7, 15 and 50 µM TNT in DMSO. Three plates each containing 20 seeds of each plant line were analysed. Photographs of 9-, 14- and 20-day-old seedlings were taken and root lengths and root areas of the plants under control conditions and in TNT containing medium were measured using Image J and Image Pro software (section 3.1.5).

TNT present in the medium had a severe effect on root growth affecting both root length and branching (Table 3.1 & 3.2). The results of root measurements also revealed that particularly GSTU25 overexpressing lines have 7 - 28 % (on average 17 %) smaller root biomass compared to the wild type plants in control conditions.

A	wild type	12-3D	7-1C	6-4B	4-4C	7-5B	1-4E	4-2B
Control (mm^2)	268.5	271.2	280.6	315.7	278.0	302.4	284.9	295.6
2 µM TNT (mm^2)	198.6	235.1	256.4	243.3	237.9	310.4	218.5	354.0
7 µM TNT (mm^2)	100.9	120.1	138.1	127.6	151.7	144.3	126.8	126.0
15 µM TNT (mm)	0.367	0.428	0.444	0.475	0.438	0.390	0.482	0.433
50 µM TNT (mm)	0.078	0.071	0.062	0.101	0.058	0.104	0.054	0.070

B	wild type	12-3D	7-1C	6-4B	4-4C	7-5B	1-4E	4-2B
Control (mm^2)	765.3	680.2	690.1	965.6	925.6	815.9	797.8	918.6
2 µM TNT (mm^2)	622.6	797.7	835.9	939.7	885.5	1130.9	647.8	1045.8
7 µM TNT (mm^2)	452.0	820.1	825.8	628.6	950.2	1140.2	835.2	756.9
15 µM TNT (mm^2)	207.5	399.8	466.3	652.1	332.6	289.3	639.1	500.0
50 µM TNT (mm)	0.119	0.108	0.112	0.169	0.097	0.159	0.093	0.138

C	wild type	12-3D	7-1C	6-4B	4-4C	7-5B	1-4E	4-2B
Control (mm^2)	1983.1	1642.1	1771.7	1707.0	1762.4	1854.5	1779.3	1902.5
2 µM TNT (mm^2)	1766.3	1857.6	1702.9	2040.8	1975.6	2020.0	1447.2	2089.6
7 µM TNT (mm^2)	1169.0	1579.6	1509.2	1446.6	1464.4	1763.8	1518.8	1362.9
15 µM TNT (mm^2)	621.7	994.0	1082.9	1285.6	1069.0	732.4	1321.1	1217.5
50 µM TNT (mm)	0.165	0.261	0.298	0.477	0.111	0.320	0.127	0.349

Table 3.1. Root area (mm^2)/length (mm) of 9-day-old (**A**), 14-day-old (**B**) and 20-day-old (**C**) Col 0 (wild type) and GSTU24 overexpressing lines grown vertically on ½ MSA plates containing 0 - 50 µM TNT. Results are mean of 3 replicates of each plant line (root areas of the plates) or ~60 replicates of each plant line (root lengths of the seedlings).

A	wild type	a4	ab5	ap1	aq2	as4	e3	u5
Control (mm^2)	280.1	258.9	217.5	233.1	203.5	251.5	232.3	229.7
2 µM TNT (mm^2)	224.9	131.2	138.5	157.4	206.0	180.2	210.1	202.2
7 µM TNT (mm^2)	105.5	81.7	91.0	73.7	114.0	118.9	88.9	111.3
15 µM TNT (mm)	0.363	0.376	0.419	0.373	0.399	0.390	0.362	0.401
50 µM TNT (mm)	0.104	0.166	0.094	0.088	0.088	0.113	0.096	0.102

B	wild type	a4	ab5	ap1	aq2	as4	e3	u5
Control (mm^2)	878.0	969.7	575.1	769.3	547.7	663.7	710.8	749.1
2 µM TNT (mm^2)	806.7	543.0	554.4	742.9	774.0	676.2	811.9	773.7
7 µM TNT (mm^2)	499.4	256.3	379.4	371.3	543.4	618.4	355.4	554.4
15 µM TNT (mm^2)	267.7	205.4	180.7	277.8	230.3	237.6	131.0	283.1
50 µM TNT (mm)	0.500	0.407	0.237	0.440	0.724	0.821	0.547	0.244

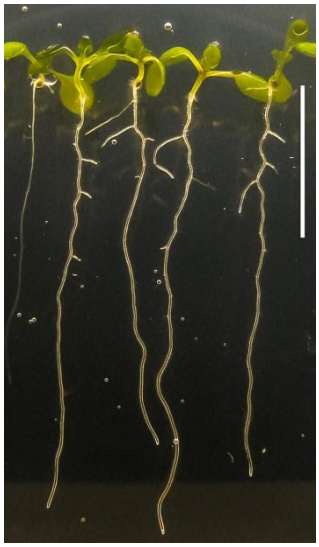
C	wild type	a4	ab5	ap1	aq2	as4	e3	u5
Control (mm^2)	1576.8	1357.0	1463.5	1184.4	1445.5	1450.0	1308.0	1512.8
2 µM TNT (mm^2)	1057.0	1406.3	1579.1	1729.1	1499.8	1423.0	1576.8	1557.4
7 µM TNT (mm^2)	778.9	1457.3	1073.2	810.4	1232.7	1104.8	1427.7	1374.0
15 µM TNT (mm^2)	354.7	562.4	751.2	889.1	988.1	710.8	592.2	906.0
50 µM TNT (mm)	0.721	0.771	0.512	0.622	0.282	0.422	0.353	0.329

Table 3.2. Root area (mm^2)/length (mm) of 9-day-old (**A**), 14-day-old (**B**) and 20-day-old (**C**) Col 0 (wild type) and GSTU25 overexpressing lines grown vertically on ½ MSA plates containing 0 - 50 µM TNT. Results are mean of 3 replicates of each plant line (root areas of the plates) or ~60 replicates of each plant line (root lengths of the seedlings).

A **DMSO control**

wild type

GSTU24

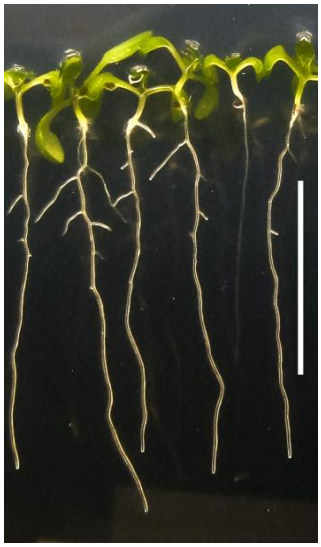


GSTU25

2 µM TNT

wild type

GSTU24



GSTU25

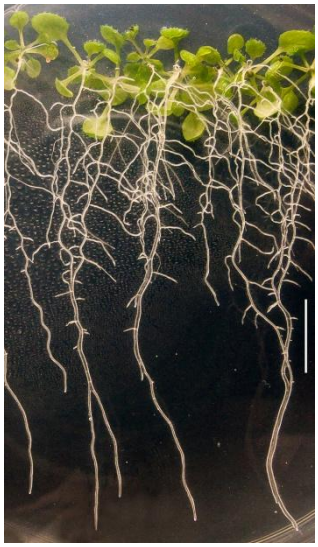
7 µM TNT

wild type

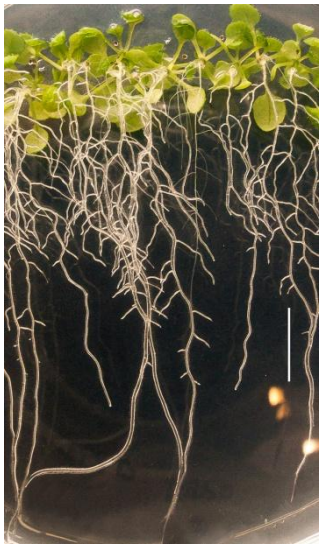
GSTU24

GSTU25

B **DMSO control**



wild type



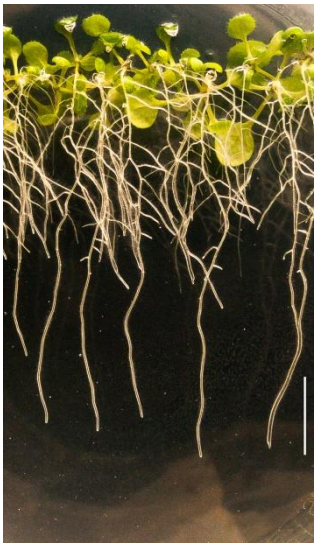
GSTU24

GSTU25

2 µM TNT

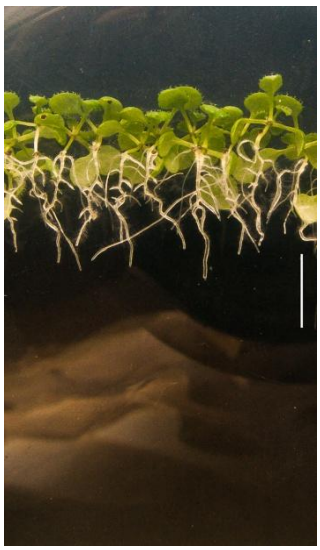
wild type

GSTU24



GSTU25

7 µM TNT



wild type

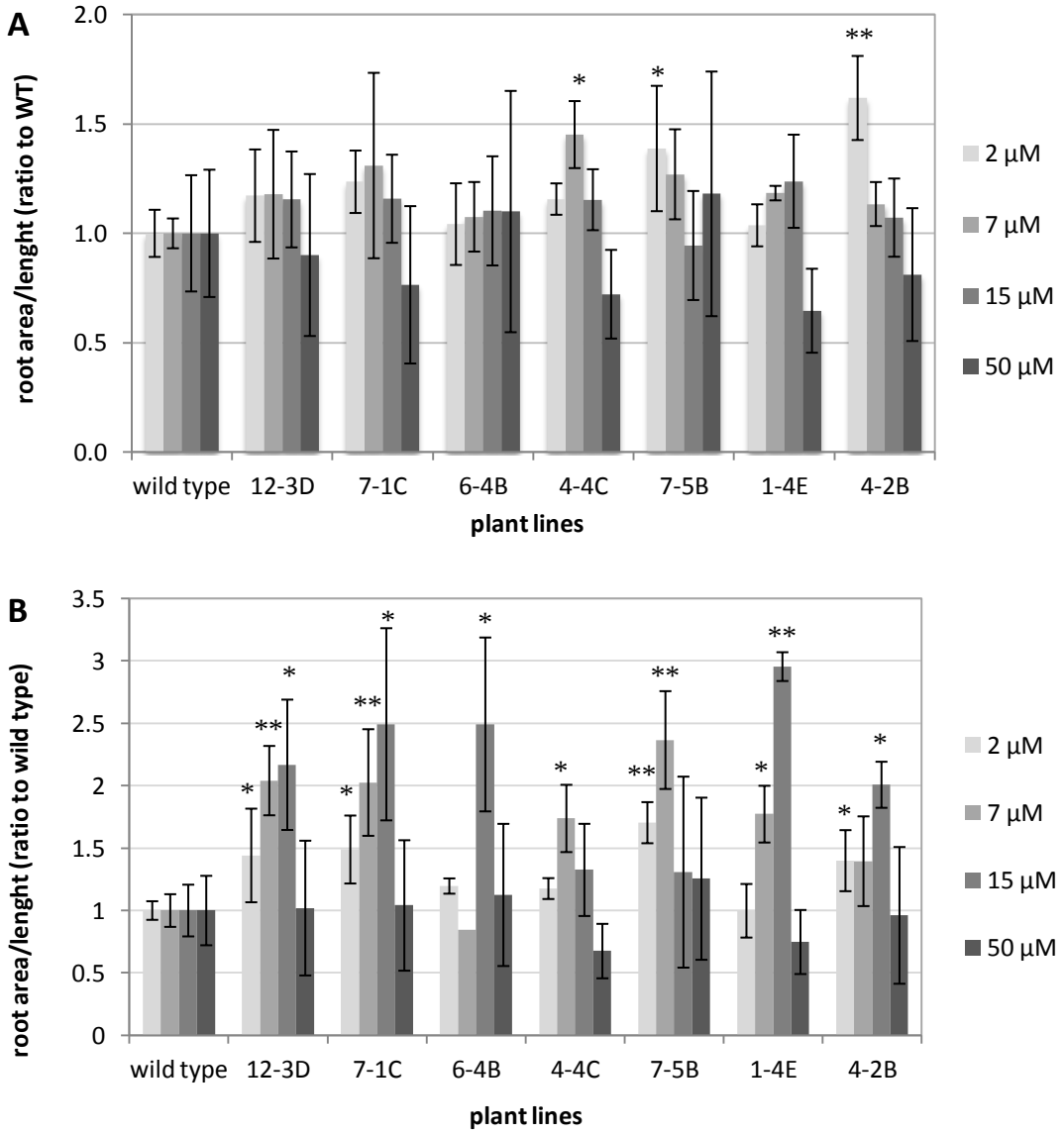
GSTU24

GSTU25

Fig. 3.2. Effect of TNT on root growth of Arabidopsis seedlings. Photographs of 9-day-old (**A**), 14-day-old (**B**) and 20-day- old (**C**) seedlings. Plants of Col 0 (wild type), GSTU24 and GSTU25 overexpressing lines were grown vertically on ½ MSA plates containing 0 - 50 µM TNT. Scale bar is 10 mm in each photograph.

The differences in the root growth between the wild type and the transgenic plants are not significant in 9-day-old seedlings, with root areas on average 1.2 and 1.12 fold larger in GSTU24 and GSTU25 transgenic lines, respectively, and reaching 1.62 and 1.51 fold increase in root area of the best performing lines (Fig. 3.2, 3.3 A & 3.4 A).

Most of the analysed 14- and 20-day-old plants of the transgenic lines, however, had significantly (1.6 fold on average) larger root area/length compared to the wild type, with 3.21 and 3.34 increases in the most successful GSTU24 and GSTU25 plant lines, respectively (Fig. 3.2 & 3.3), suggesting that overexpression of these particular detoxifying enzymes in plants can result in better tolerance of higher concentrations of TNT in plant growth medium.



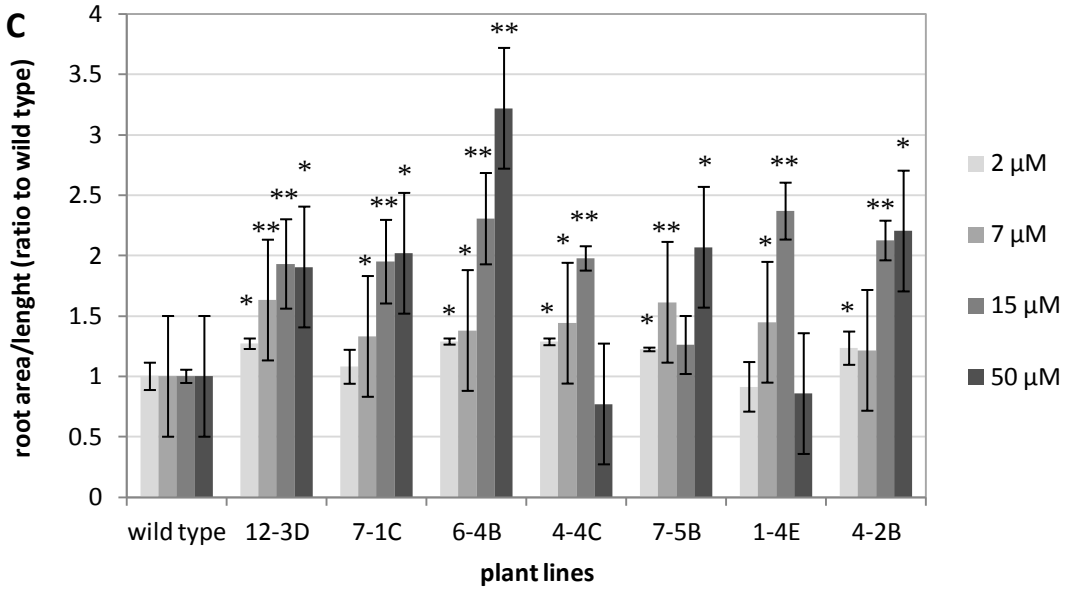
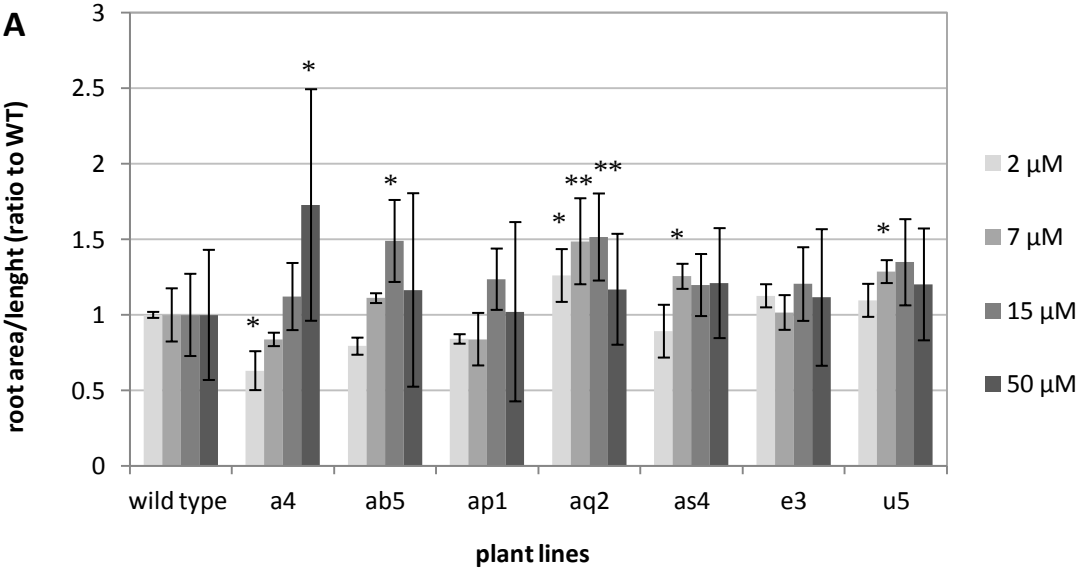


Fig. 3.3. Root area (mm^2)/length (mm) of 9-day-old (**A**), 14-day-old (**B**) and 20-day-old (**C**) Col 0 (wild type) and GSTU24 overexpressing lines grown on vertically on ½ MSA plates containing 0 - 50 µM TNT relative to wild type. Results are mean of 3 replicates (plates) of each plant line or ~60 root length replicates of each plant line, error bars represent standard deviation from the mean; * denotes statistical difference compared to wild type ($p<0.05$); ** denotes statistical difference compared to wild type ($p<0.001$).



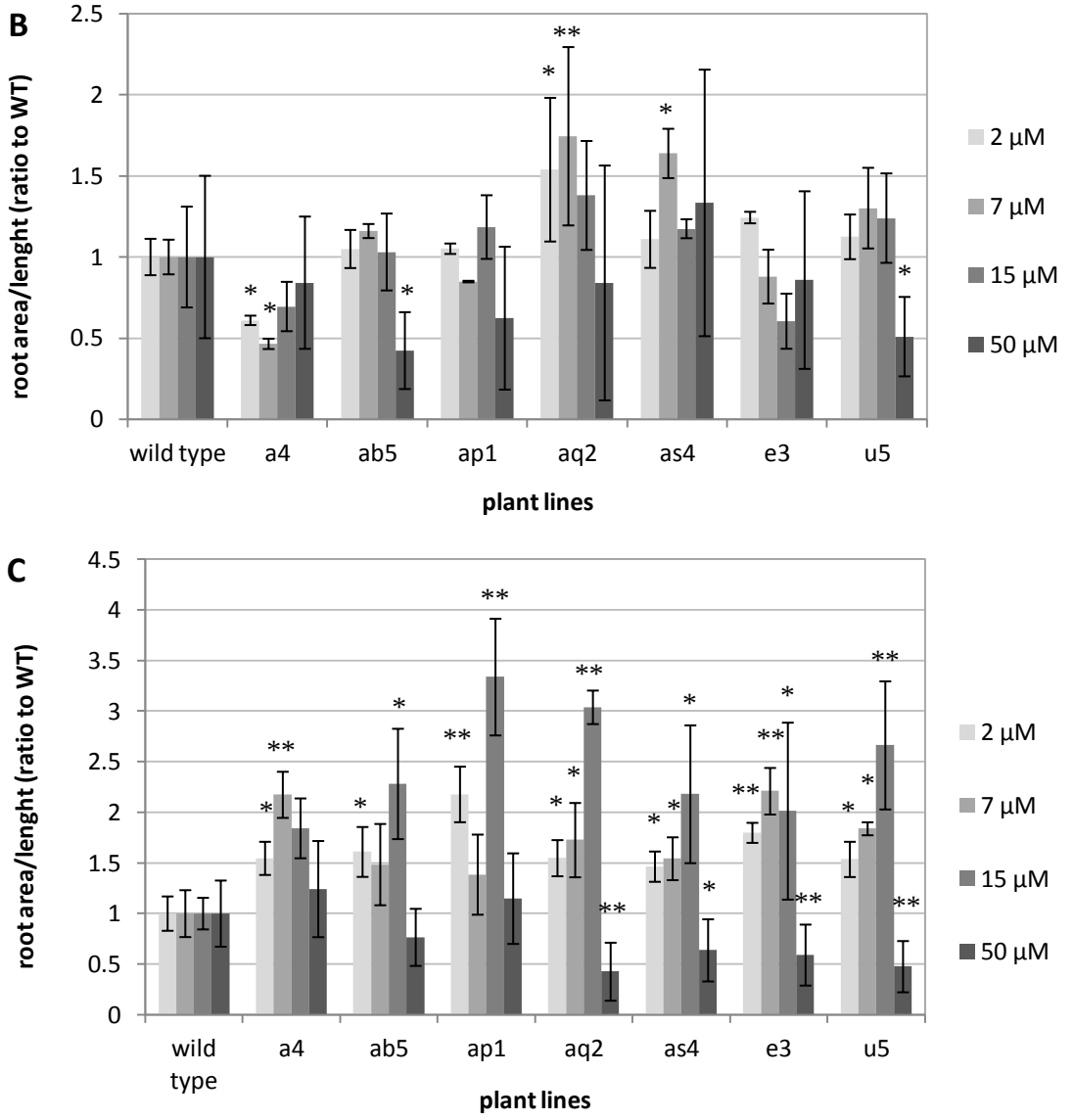


Fig. 3.4. Root area (mm^2)/length (mm) of 9-day-old (**A**), 14-day-old (**B**) and 20-day-old (**C**) Col 0 (wild type) and GSTU25 overexpressing lines grown on vertically on ½ MSA plates containing 0 - 50 µM TNT relative to wild type. Results are mean of 3 replicates (plates) of each plant line or ~60 root length replicates of each plant line, error bars represent standard deviation from the mean; * denotes statistical difference compared to wild type ($p<0.05$); ** denotes statistical difference compared to wild type ($p<0.001$).

3.2.3. Liquid culture studies of 35S-GST lines

Growing Arabidopsis plants in liquid cultures enabled the uptake of TNT from the medium by wild type and GSTU24 and GSTU25 transformed lines to be followed. It was also possible to extract and analyse the TNT conjugates formed in the plants.

In this experiment imbibed seeds were germinated on ½ MSA plates and 7-day-old seedlings were transformed to 100 ml flasks containing 20 ml of ½ MS. After 14 days the medium was replaced by 20 mM sucrose solution containing 200 µM TNT. Samples of the medium were taken at 0, 8, 24, 48, 72, 96 and 168 and analysed by HPLC (section 3.1.6) (Fig. 3.5).

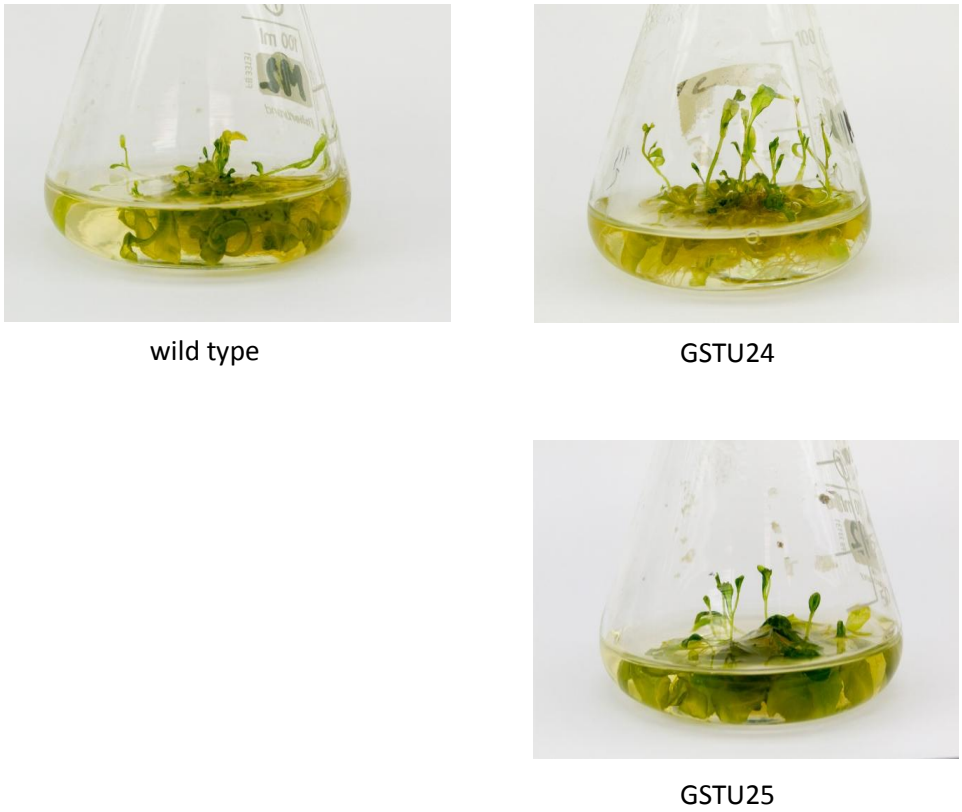
A

wild type

GSTU24

GSTU25

B



wild type

GSTU24

GSTU25

Fig. 3.5. Appearance of Col 0 (wild type) and GSTU24 and GSTU25 overexpressing lines growing in ½ MS medium for three weeks (**A**) and after growing in 20 mM sucrose solution containing 200 µM TNT for 7 days (**B**).

The results of the analyses show that there is a significant difference in the amount of TNT removed from the medium between the transgenic lines and wild type plants only at the 24 h time point (Fig. 3.6). The extracts of the plants growing in the liquid medium containing TNT were therefore analysed to study and compare the formation of different TNT conjugates in the plants.

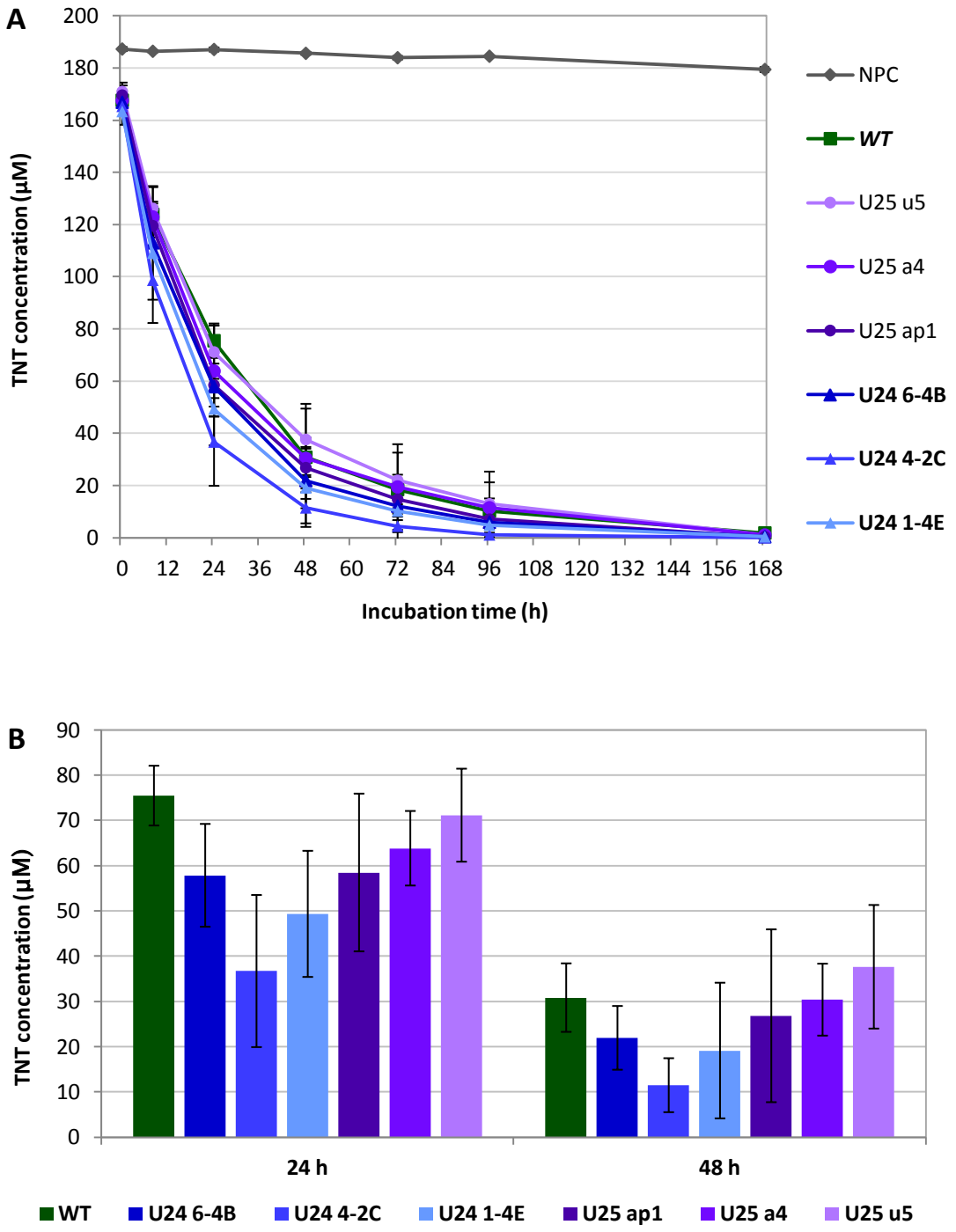


Fig. 3.6. Uptake of TNT from the medium of Col 0 (wild type) and GSTU24 and GSTU25 overexpressing lines. **A**, Eight 21-day-old seedlings were grown for 7 days in flasks containing 20 ml of 20 mM sucrose solution with 200 µM TNT. Samples of the medium were taken from the flasks at regular time points and analysed by HPLC; **B**, uptake of TNT from the liquid medium 24 h and 28 h after the seedlings were transferred to the TNT containing medium. **NPC**, no plant control. Results are

mean of 5 replicates of each plant line, error bars represent standard deviation from the mean.

For the conjugate analysis the plants were grown in the same conditions but the samples were taken at 0, 4, 6, 8 and 24 h. Metabolites were extracted from the whole plants by methanol:water mixture (60:40) and analysed by HPLC and LC/MS. The results of the analyses show presence of free TNT in the plant tissues 4 h after the plants were transferred to the TNT containing media. Within 24 h most of the TNT in the tissues was conjugated (Fig. 3.7). The metabolite analyses show that the prevalent detoxification pathway is the conjugation of TNT by UDP-glycosyltransferases (UGTs), and the major TNT conjugation product had a molecular weight of 375 corresponding to *O*-glucosylated HADNT identified previously during UGT conjugation reaction analyses (Fig. 3.8) (Gandia-Herrero et al. 2008). The LC/MS analyses also confirmed formation of glutathionylated products in the transgenic plants, GSTU24 overexpressing plants contained conjugate 2 (Fig. 3.9), analyses GSTU25 overexpressing plants showed formation of conjugate 3 but not conjugate 1 and 2 in the physiological conditions in the plant cells (Fig. 3.10). The concentration of the conjugates increased with time during the analysis, reaching maximum at the 24 h time point characterised by the biggest differences between the wild type and the transgenic lines.

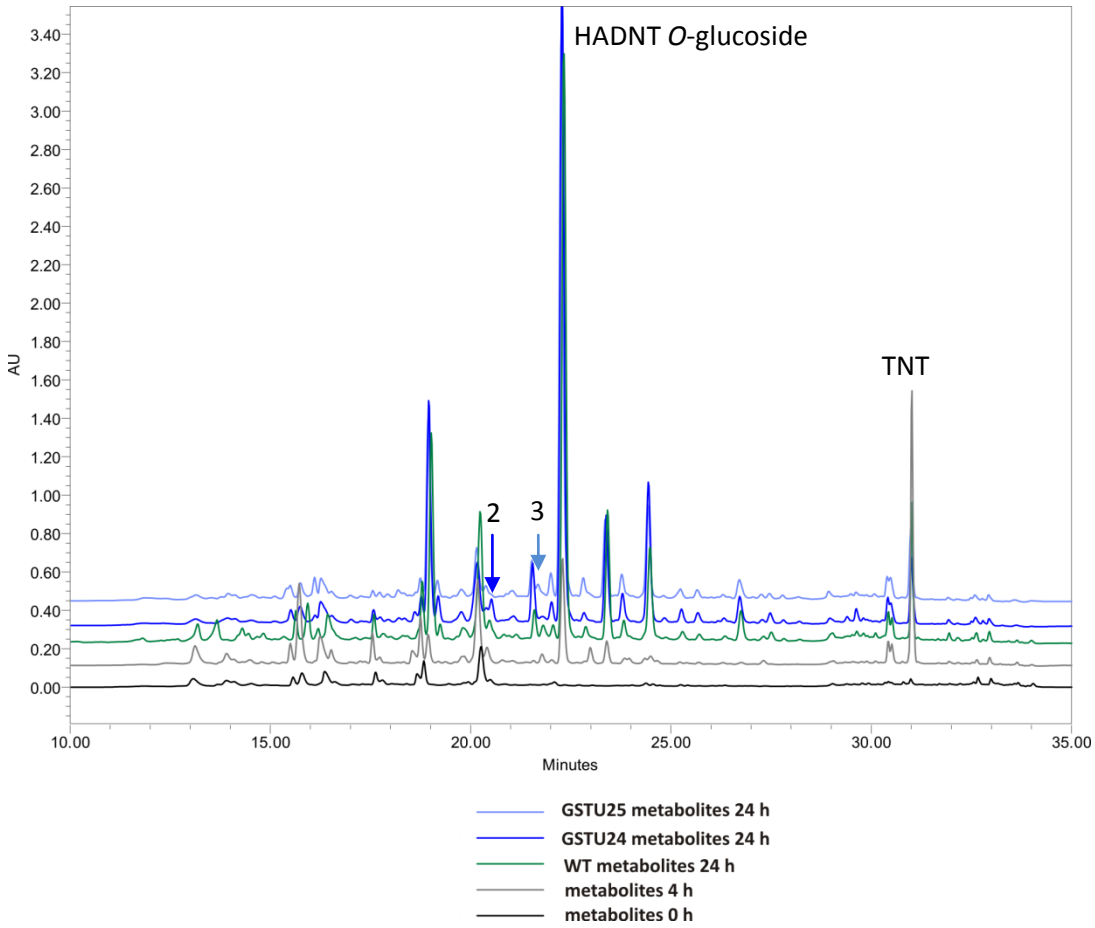


Fig. 3.7. HPLC chromatogram of metabolite analyses in the extracts of Col 0 (wild type) and transgenic plants grown in the liquid cultures (analysed at 250 nm). Metabolites were extracted from the whole plants by methanol:water mixture (60:40) at different time points after transferring the plants into the TNT containing ½ MS medium. **2**, conjugate 2; **3**, conjugate 3.

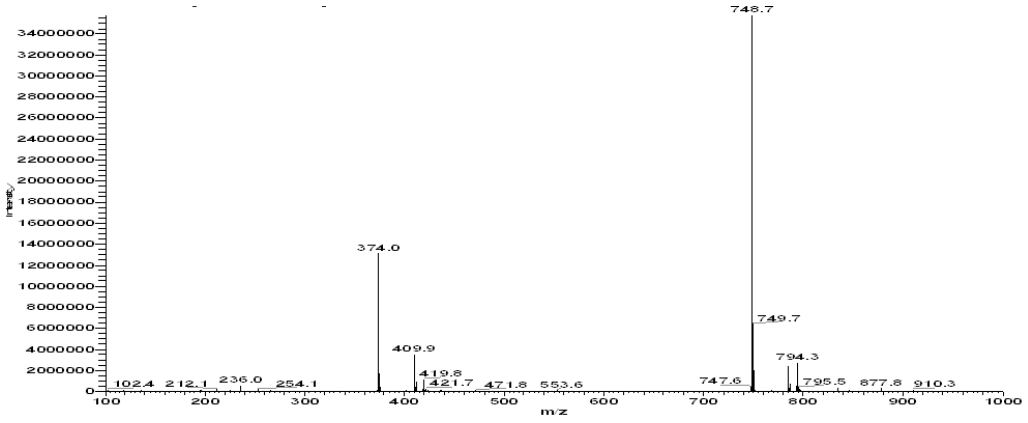
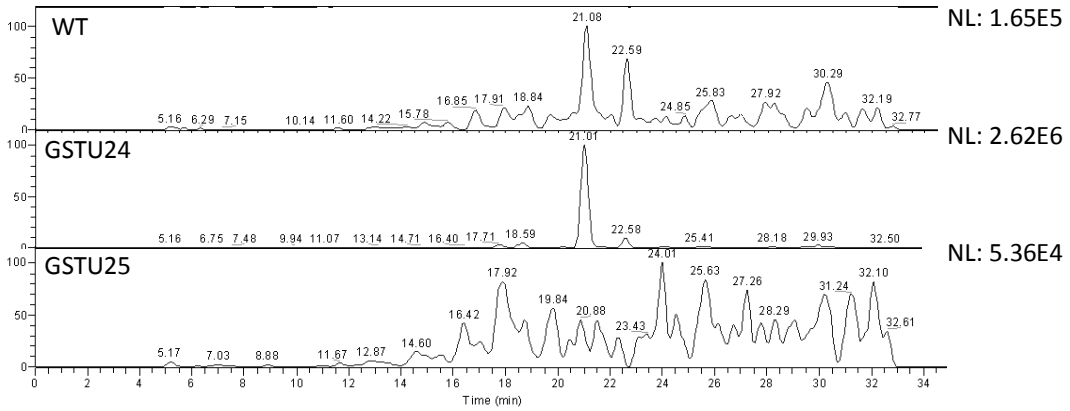
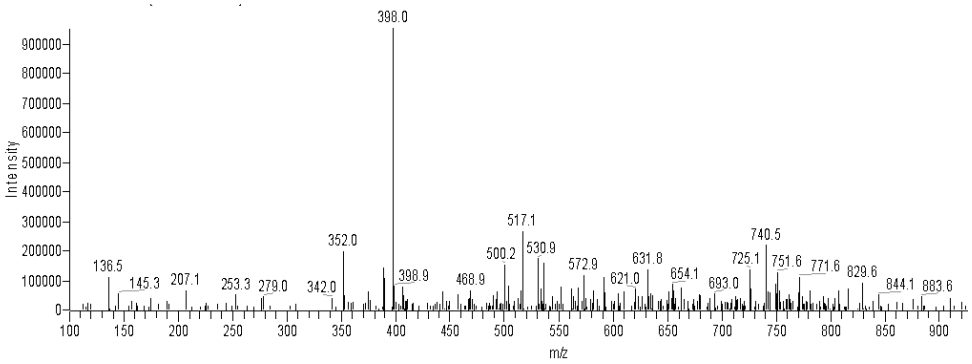


Fig. 3.8. MS spectrum of *O*-glucosylated HADNT with *m/z* 374 [M-H] corresponding to the molecular mass of 375, the main TNT conjugate formed in plant during TNT metabolism.

A



B



C

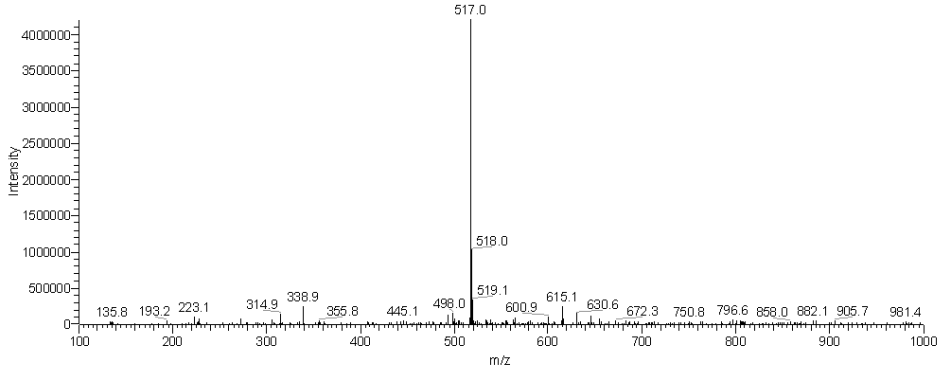
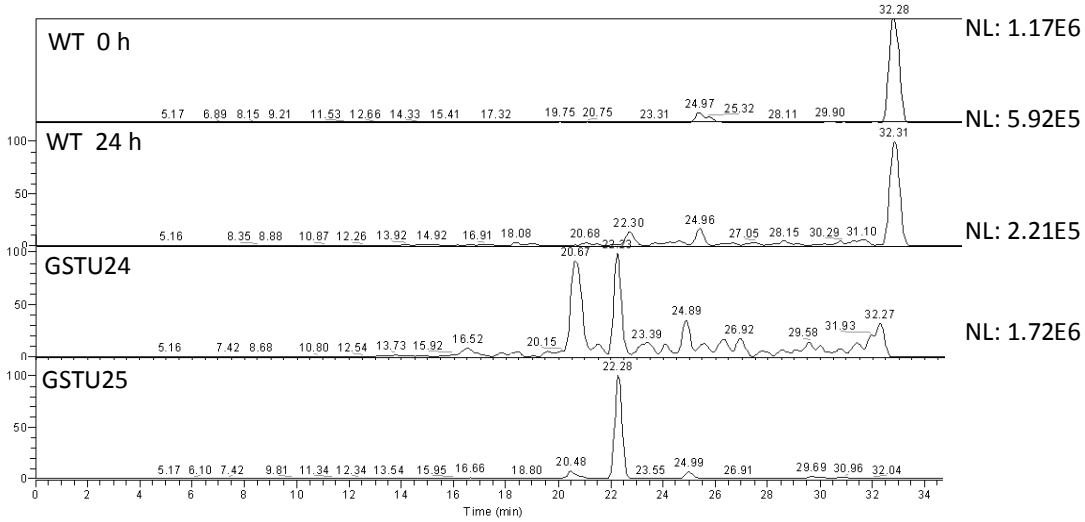
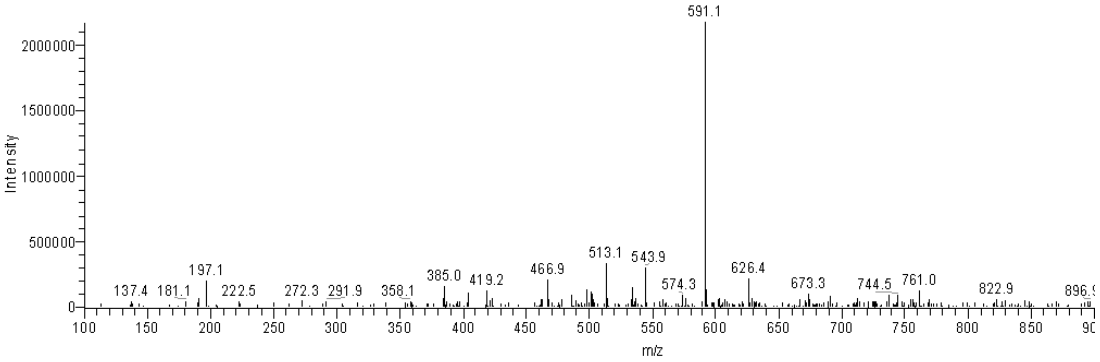


Fig. 3.9. LC/MS scan of TNT metabolite analyses analysed at *m/z* = 517-519 (**A**). No peak corresponding to the conjugate 2 was registered in the GSTU25 transgenic plant extracts. **B,** MS spectrum of a peak with the retention time of 21.01 min (WT); extract of Col 0 (wild type) plants contains a smaller peak with a correct retention time but MS spectra did not correspond to the conjugate 2; **C,** MS spectra of conjugate 2; GSTU24 overexpressing plants contain a compound with the right retention time and mass of 517 characteristic of conjugate 2.

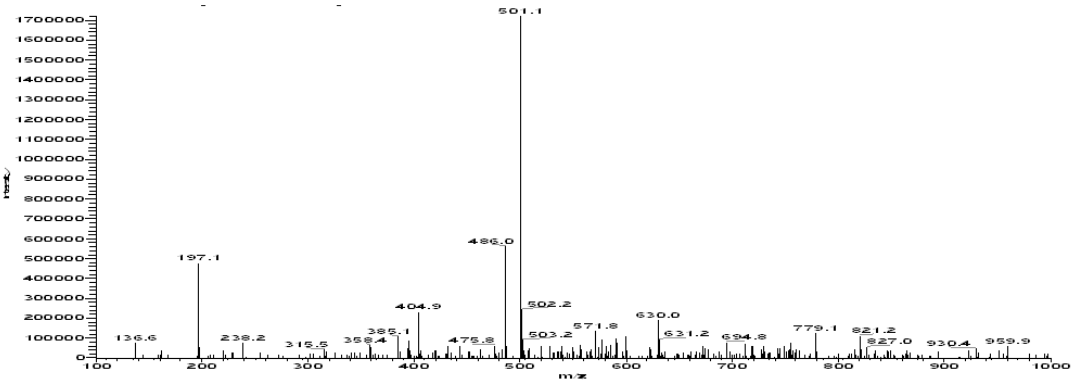
A


WT 0 h
NL: 1.17E6
NL: 5.92E5
WT 24 h
NL: 2.21E5
GSTU24
NL: 1.72E6
GSTU25
Time (min)

B


Intensity
m/z

C


m/z

D

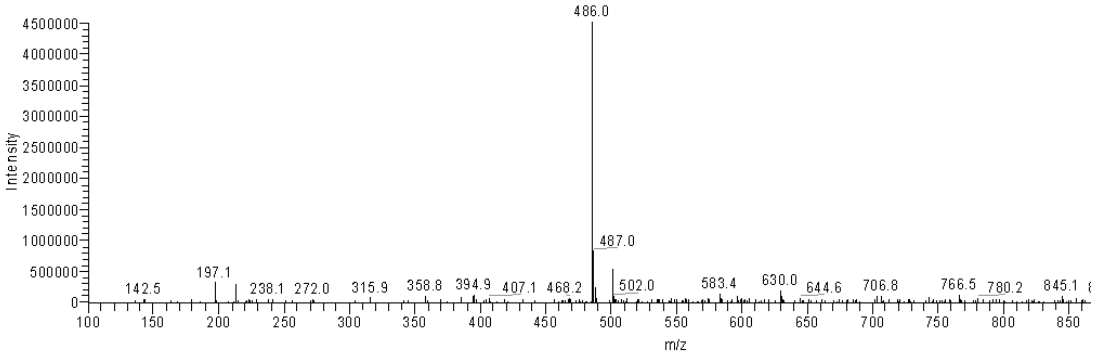


Fig. 3.10. LC/MS scan of TNT metabolite analyses analysed at *m/z* = 485-487 (**A**). **B**, Extracts of Col 0 (wild type) plants do not contain a peak with the correct retention time and MS spectra to correspond to the conjugate 3; **C**, GSTU24 overexpressing plants contain a compound with the right retention time of conjugate 3 and but different mass; **D,** MS of conjugate 3; GSTU25 overexpressing plants contain a compound with the right retention time and mass of 487 characteristic of conjugate 3.

3.2.4. Hydroponic experiment

When Arabdiopsis plants are grown in the liquid culture flasks (as described in section 3.1.2 *Plant growth in liquid medium*), the physiology of the plant is altered. The waxy cuticle layer is reduced and much of the plant is submerged. These factors are likely to enable TNT to penetrate all the plant organs equally. These conditions are far removed from the in-field conditions of a plant growing on a military training range, where only the roots are in contact with TNT. To isolate exposure to the roots, and thus more closely resemble the natural environment, plants were grown hydroponically on top of plastic rafts with holes drilled through them to allow roots grow into the liquid medium under the rafts inside the sterile jars, as shown in Fig. 3.11 and described in section 3.1.2 *Hydroponic growth conditions*.

Fig. 3.11. Hydroponic growing of Arabidopsis seedlings for TNT uptake experiments. Twenty-day-old plants growing hydroponically in liquid ½ MS medium in the sterile jars before placing the plants in the TNT containing medium. Rafts were made from circular lightweight plastic, 70 mm on diameter and 6 mm thick, with approximately 100 holes (3-4 mm diameter) drilled into each disk. Sterile stratified Arabidopsis seeds (8 seeds per raft) were pipetted onto the hole filled with ½ MSA.

After 21 days ½ MS medium was replaced with 30 ml of liquid ½ MS medium containing 50 and 100 µM TNT. Samples of the medium were taken every 24 h during 7 days and analysed by HPLC (conditions in section 3.1.6) (Fig. 3.12).

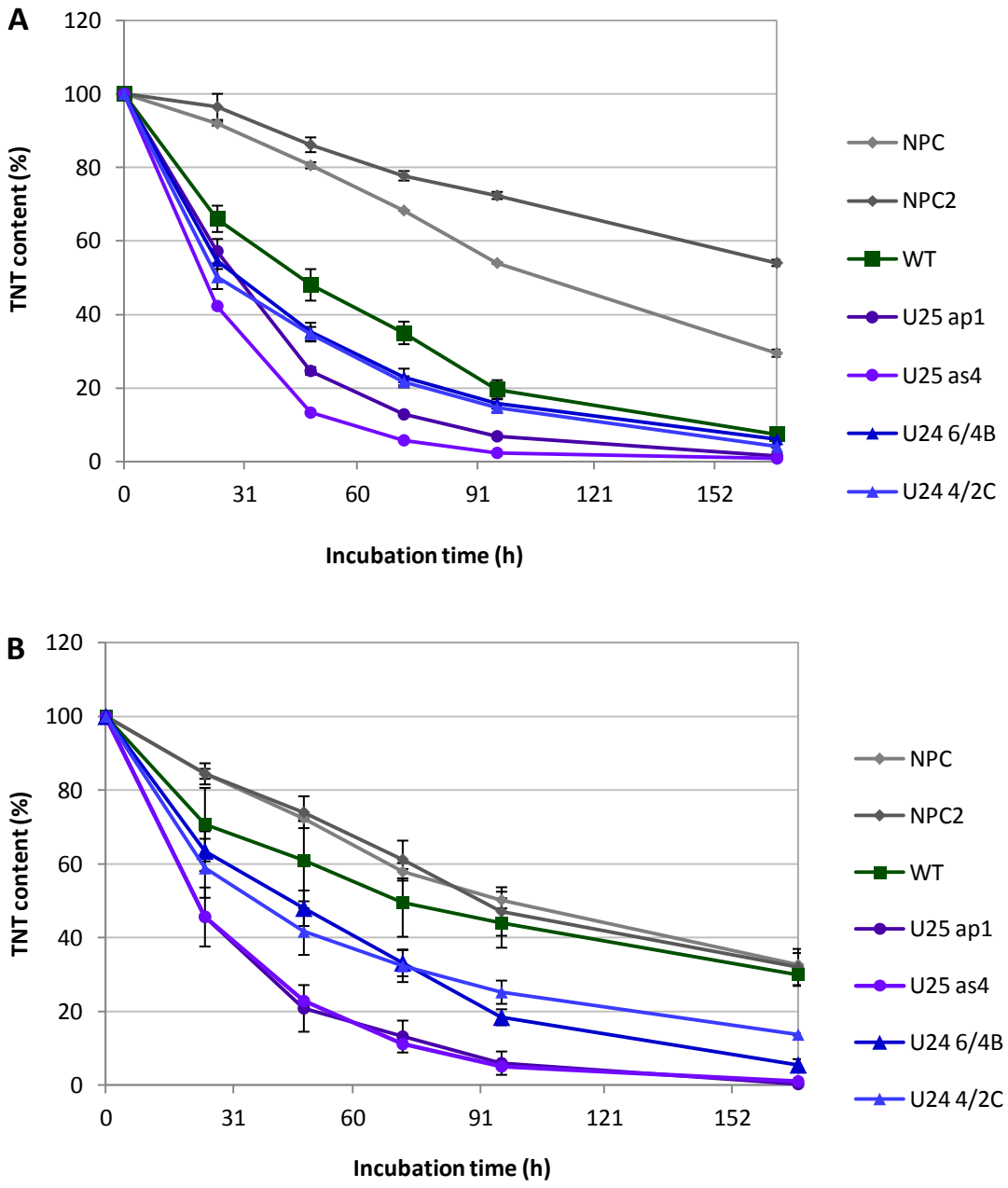


Fig. 3.12. Uptake of TNT from the medium of Col 0 (wild type) and GSTU24 and GSTU25 overexpressing lines. Eight 21-day-old seedlings were grown hydroponically for 7 days in jars containing 30 ml of ½ MS with 50 µM TNT (**A**) and 100 µM TNT (**B**). Samples of the medium were taken from the flasks at regular time points and analysed by HPLC. **NPC**, no plant control without raft; **NPC2**, no plant control with raft to check the absorption of TNT by the plastic rafts. Results are mean of 5 - 6

replicates of each plant line, error bars represent standard deviation from the mean.

Analyses of TNT metabolites extracted from the plants by LC/MS revealed similar composition and quantity of conjugates, with *O*-glucosylated HADNT being the dominant compound, and with the concentration of conjugate 2 and 3, respectively, increasing in GSTU24 and GSTU25 plant extracts during the duration of the experiment.

3.3. Conclusions and discussion

Glutathionylated conjugates undergo a sequence of chemical modification in the plant, which were well studied using herbicide safeners in Arabidopsis suspension cell cultures. In these experiments, glutathionylated herbicide safener fenclorim was found present in the highest concentration in the cells 4 h – 8 h after treatment. After 24 h the concentration of glutathionylated fenclorim in the cell cultures declined and a number of new compounds appeared, resulting from the sequential processing of the conjugates in the vacuoles to the cysteine conjugates (Brazier-Hicks et al. 2008). Uptake of TNT by the whole plant in current research is a slower process compared to the suspension cultures; the highest concentration of free unconjugated TNT was found in plants 4 h after adding TNT into the medium. The very rapid uptake of the safener in plant cultures compared with the foliage was also observed in the previous metabolism studies (Skipsey et al. 2011). The free TNT was rapidly conjugated by UDP-glucosyltransferases and glutathione transferases and the concentration of the conjugates increased during the experiment duration until TNT was removed from the medium. In the present study, LC/MS did not reveal presence of metabolites resulting from the processing of glutathionylated TNT (cysteinglycine, γ-glutamylcysteine or cysteine derivatives).

Overexpressing Arabidopsis detoxifying enzymes, which were found to play role in the TNT detoxification, was successfully used to prove the function of these genes in the xenobiotic metabolism and also to produce plants tolerating higher levels of TNT than untransformed wild type plants. The oxophytodienoate reductases, which are upregulated in response to the TNT treatment, were shown to produce TNT derivatives with reduced nitro group (HADNTs and ADNTs). Overexpression of OPR1 and OPR2 resulted in longer roots of seedlings growing on solid medium containing TNT compared to the wild type plants. Transgenic plants also had faster TNT uptake form liquid medium and produced more TNT transformation products

than wild type (Beynon et al. 2009). Similarly, overexpression of Arabidopsis UGTs resulted in improved root growth and TNT uptake from the medium (Gandia-Herrero et al. 2008). The results of experiments presented here suggest that glucosylation of reduced TNT derivatives is the main detoxification pathway of TNT metabolism in Arabidopsis.

Based on microarray data of GST expression in Arabidopsis tissues, *GSTU24* and *GSTU25* expression is localised predominantly in roots (GSTU24 is localised mainly in the root endodermis, GSTU25 is expressed primarily in the root epidermal atrichoblasts) (Dixon & Edwards 2010). This localisation could be important for the function of the GSTs in TNT detoxification, as TNT is not transported to the aerial parts of the plant (Sens et al. 1998; van Dillewijn et al. 2008). Based on results of SAGE studies in Arabidopsis, GSTs were considered to be primarily responsible for TNT conjugation reactions (Ekman et al. 2003a). In another study of the activity of detoxifying enzymes towards several different xenobiotics in Arabidopsis GSTU24 was the highest induced GST by all chemical treatments (TNT, RDX, herbicides metolachlor and acetochlor). The induction of GSTU24 in 2-week-old Arabidopsis plants exposed to 0.6 mM TNT showed an average 40-fold increase in gene expression 6 h after treatment. In the same experiment, *in vivo* analysis of GSH conjugation activity showed no conjugation of GSH to TNT or RDX in contrast to strong activity towards CDNB and herbicides (Mezzari et al. 2005).

Overexpressing GSTU24 and GSTU25 in the current study had a significant effect on the root length of transformed seedlings growing on TNT containing medium and also improved TNT uptake from liquid medium. The glutathionylated conjugates, which the enzymes produce in *in vitro* conjugation assays, were also found to be formed *in vivo* in plants exposed to TNT. These conjugates are, however, only found in small amounts in plants compared to the glucosylated metabolites, which suggests

a possible different mechanism of GST action in addition to the direct TNT conjugation, resulting from the GPOX activity of both enzymes.

4. Discussion

Glutathione transferases play critical roles in the detoxification of xenobiotics and the protection of tissues against oxidative damage. GSTs are important enzymes in plant responses to a number of environmental stresses including herbicides and pathogen attack, heavy metal toxicity and reactive oxygen species (Chen & Singh 1999).

Plant GSTs have been intensively studied for their ability to detoxify a range of herbicides. They catalyze the conjugation of GSH to herbicide molecules to form glutathione-S-conjugates, which are then imported to vacuoles, thus protecting the plants from herbicide damage. Pre-treatment with herbicide safeners greatly elevates the capability of herbicide detoxification via GSH conjugation by activation of detoxification systems, including upregulating GST activity (H. Gong, et al. 2005a; Riechers et al. 2010). A number of mechanisms have been proposed for safener activity. Herbicide safeners protect monocotyledoneous crops from herbicide injury but have little effect on dicotyledonous species (target weed species). Safeners are also able to induce the expression of herbicide detoxifying enzymes in dicots, such as Arabidopsis (DeRidder et al. 2002). In experiments studying the effect of various safeners on gene expression in Arabidopsis, induction of AtGSTU19 and AtGSTF2 expression was observed predominantly in roots. Transgenic plants overexpressing AtGSTU19 had an increased level of AtGSTU19 protein in roots, but this had no effect on tolerance to chloroacetamide herbicides (DeRidder & Goldsbrough 2006). It has since been suggested that the localisation of expression of GST proteins expression in the outer cell layers of the coleoptiles in monocotyledonous crops is important to prevent the herbicide from reaching the sensitive new leaves of etiolated shoots as they emerge from the soil (Riechers et al. 2003; Riechers et al. 2010). In addition to upregulating GST expression, safeners increase the activity of enzymes involved in GSH biosynthesis (Riechers et al. 2010).

The glutathione-mediated metabolism of fenclorim suggested that the changes in GSH metabolism could play an active role in the safener response. Changes in GSH content, and the ratio to its oxidized form GSSG, have been found to be involved in redox stress signalling in a number of plants (Foyer & Noctor 2009). Both CDNB and fenclorim are known to be rapidly glutathionylated in Arabidopsis cell cultures and, therefore, have the potential to affect gene expression by disturbing thiol homeostasis. CDNB and fenclorim treatments of Arabidopsis root cultures caused depletion of the thiol pool in the cells and lead to increases in GSTU19 and GSTU24 transcription (Skipsey et al. 2011).

New research suggests that safeners might be using oxidised lipid (oxylipin)-mediated pathway, which subsequently leads to the increase in expression of detoxification enzymes, including GSTs. Oxylipins are structurally diverse metabolites derived from fatty acid oxidation and can be formed non-enzymatically or in enzymatic reactions. Non-enzymatically generated oxylipins are formed via free radical-catalyzed reactions in or near cell membranes, e.g. during wounding, where membrane polyunsaturated fatty acids are precursors for oxylipin synthesis. These unstable, reactive oxylipins have been used as biochemical markers for monitoring oxidative stress and membrane damage in plant and animal systems. Enzymatically produced oxylipins include jasmonic acid (JA) and 12-oxo-phytodienoic acid (OPDA), which are part of the jasmonate biosynthetic pathway in plants. JA-conjugates and biosynthetic precursors have a hormonal activity in defence gene activation in response to stress. Highly reactive oxylipins, for example A1-type phytoprostanes and OPDA, have a similar effect to safeners on strong, coordinated induction of genes and enzymes involved in detoxification and stress responses (Riechers et al. 2010; Skipsey et al. 2011). To examine the link between safener- and oxylipin-mediated signalling and metabolism, GSTs were tested for detoxifying activities toward oxylipins. Studies have shown that OPDA can be conjugated by the Arabidopsis enzymes GSTU6, GSTU10, GSTU17, GSTU19 and

GSTU25 (Dixon & Edwards 2009). However, no activity towards OPDA was detected for GSTU24 (Skipsey et al. 2011).

Many other factors apart from herbicides (pathogen attack, wounding, plant hormones, heavy metal toxicity, and hydrogen peroxide) were found to induce GST expression in plants. The promoters of several GSTs were found to contain a well characterised plant enhancer sequence, the *ocs* element. The *ocs* element was found to have an important role in the transcriptional response of GST genes to H_2O_2 and salicylic acid (SA) (Chen et al. 1996; Chen & Singh 1999). One mechanism by which a number of diverse compounds could induce plant GST expression via the *ocs* element is by inducing conditions of oxidative stress at the cellular level (Gong et al. 2005a). GST upregulation in response to SA may also be attributed to H_2O_2 accumulation. It is known that SA inhibits catalase (Gong et al. 2005b), an enzyme involved in H_2O_2 degradation, and exogenous application of SA has been shown to cause H_2O_2 accumulation (Chen et al. 1993).

Hydrogen peroxide and superoxide (O_2^-), reactive oxygen species (ROS), are produced in a number of cellular reactions. The main cellular components susceptible to damage by ROS are lipids (unsaturated fatty acids in membranes), proteins and nucleic acids (Blokhina et al. 2003). Oxidative stress is often manifested as leaf chlorosis (and subsequent necrosis) in a wide variety of both abiotic and biotic stresses (Mullineaux et al. 2000). To counteract the production of ROS, plants have evolved an antioxidant system consisting of low molecular weight antioxidants (ascorbic acid, glutathione, tocopherols), enzymes regenerating the reduced forms of antioxidants, and ROS detoxifying enzymes (peroxidases and catalases) (Blokhina et al. 2003).

In experiments with animal cell systems and tissues, the toxic effect of TNT exposure was associated with generation of ROS (Sun et al. 2006). Morphological studies showed damaged mitochondria, dilatated Golgi apparatus, vacuolization and

accumulation of lipid material. The production of H_2O_2 by mitochondria was more pronounced in the liver than in other organs, but its production by microsomes was more pronounced in the brain tissue (Kong et al. 1989). The results suggested that ROS formed during nitroreduction of TNT may cause the observed adverse effects (Zitting et al., 1982; Sun et al. 2006). In plants, the effect of TNT at the cellular level has not been studied in detail; results of electron microscopy radioautography showed the presence of radio-labelled TNT in vacuoles, plastids, mitochondria, endoplasmic reticulum, and cytoplasm and substantially damaged ultrastructure of differentiated cells (Ghoghoberidze et al. 2009).

Many GSTs also act as GSH-dependent peroxidases by catalyzing the reduction of organic hydroperoxide to the less toxic monohydroxy alcohols (GPOX activity). GSTU24 and GSTU25 both have GPOX activity, with GSTU25 being the most active of the Arabidopsis GSTs (Dixon et al. 2009). In the current research GSTU24 and GSU25 were shown to be able directly metabolise TNT in the plants. The analyses of TNT conjugation products from Arabidopsis plants showed, however, that the dominant pathway of TNT detoxification in Arabidopsis is the glucosidation of reduced TNT derivatives. This result suggests that, in addition to their GSH conjugating activity, GSTs could exhibit their detoxification activity in plants (resulting from the GPOX activity of both enzymes) by protecting the cellular components from ROX damage generated by exposure of the cells to TNT.

Arabidopsis, the object of the current research, is an annual plant with a relatively small root system, unsuitable for phytoremediation application. Perennial grass species, such as switchgrass species (*Panicum virgatum*), which are native to military training ranges in temperate regions and produce dense root systems extending over a metre below the soil surface would be more suitable (Rylott et al. 2006). The results of current study show that overexpressing plant detoxification systems results in plants that are significantly more resistant to TNT and that GSTs play an important role in the detoxification mechanism of many xenobiotics,

including TNT. Overexpression of plant endogenous detoxification enzymes might lead to a breeding of native species with effective detoxification systems for phytoremediation of contaminated soil.

5. References

Van Aken, B., Yoon, J.M. & Schnoor, J.L., 2004. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic Methylobacterium sp. associated with poplar tissues (*Populus deltoides* x *nigra* DN34). *Applied and Environmental Microbiology*, 70(1), pp.508–517.

Akkemik, E. et al., Purification and characterization of glutathione S-transferase from turkey liver and inhibition effects of some metal ions on enzyme activity. *Environmental Toxicology and Pharmacology*, (0). Available at: http://www.sciencedirect.com/science/article/pii/S1382668912001263 [Accessed November 21, 2012].

Axarli, I. et al., 2009. Crystal structure of *Glycine max* glutathione transferase in complex with glutathione: investigation of the mechanism operating by the Tau class glutathione transferases. *Biochemical Journal*, 422, pp.247–256.

Benekos, K. et al., 2010. Overexpression of a specific soybean GmGSTU4 isoenzyme improves diphenyl ether and chloroacetanilide herbicide tolerance of transgenic tobacco plants. *Journal of biotechnology*, 150(1), pp.195–201.

Beynon, E.R. et al., 2009. The Role of Oxophytodienoate Reductases in the Detoxification of the Explosive 2,4,6-Trinitrotoluene by Arabidopsis. *Plant Physiology*, 151(1), pp.253 –261.

Bhadra, R. et al., 1999. Characterization of Oxidation Products of TNT Metabolism in Aquatic Phytoremediation Systems of *Myriophyllum aquaticum*. *Environ. Sci. Technol.*, 33(19), pp.3354–3361.

Bhadra, R. et al., 1998. Confirmation of Conjugation Processes during TNT Metabolism by Axenic Plant Roots. *Environ. Sci. Technol.*, 33(3), pp.446–452.

Blokhina, O., Virolainen, E. & Fagerstedt, K.V., 2003. Antioxidants, Oxidative Damage and Oxygen Deprivation Stress: a Review. *Annals of Botany*, 91(2), pp.179 –194.

Bowman, A.L. et al., 2007. Molecular Determinants of Xenobiotic Metabolism: QM/MM Simulation of the Conversion of 1-Chloro-2,4-dinitrobenzene Catalyzed by M1-1 Glutathione S-Transferase†. *Biochemistry*, 46(21), pp.6353–6363.

Brazier-Hicks, M. et al., 2008. Catabolism of Glutathione Conjugates in *Arabidopsis thaliana*. *Journal of Biological Chemistry*, 283(30), pp.21102 –21112.

Brentner, L.B. et al., 2008. Expression of glutathione S-transferases in poplar trees (*Populus trichocarpa*) exposed to 2,4,6-trinitrotoluene (TNT). *Chemosphere*, 73(5), pp.657–662.

Chen, W, Chao, G. & Singh, K B, 1996. The promoter of a H2O2-inducible, Arabidopsis glutathione S-transferase gene contains closely linked OBF- and OBP1-binding sites. *The Plant Journal: For Cell and Molecular Biology*, 10(6), pp.955–966.

Chen, W & Singh, K B, 1999. The auxin, hydrogen peroxide and salicylic acid induced expression of the Arabidopsis GST6 promoter is mediated in part by an *ocs* element. *The Plant journal: for cell and molecular biology*, 19(6), pp.667–677.

Chen, Wenqiong & Singh, Karam B, 1999. The auxin, hydrogen peroxide and salicylic acid induced expression of the Arabidopsis GST6 promoter is mediated in part by an *ocs* element. *The Plant Journal*, 19(6), pp.667–677.

Chen, Z., Silva, H. & Klessig, D.F., 1993. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science (New York, N.Y.)*, 262(5141), pp.1883–1886.

Cummins, I. et al., 2011. Multiple roles for plant glutathione transferases in xenobiotic detoxification. *Drug Metabolism Reviews*, 43, pp.266–280.

Das, P. et al., 2010. Vetiver grass is capable of removing TNT from soil in the presence of urea. *Environmental Pollution (Barking, Essex: 1987)*, 158(5), pp.1980–1983.

DeRidder, B.P. et al., 2002. Induction of Glutathione S-Transferases in Arabidopsis by Herbicide Safeners. *Plant Physiology*, 130(3), pp.1497 –1505.

DeRidder, B.P. & Goldsbrough, P.B., 2006. Organ-Specific Expression of Glutathione S-Transferases and the Efficacy of Herbicide Safeners in Arabidopsis. *Plant Physiology*, 140(1), pp.167 –175.

Van Dillewijn, P. et al., 2008. Bioremediation of 2,4,6-Trinitrotoluene by Bacterial Nitroreductase Expressing Transgenic Aspen. *Environ. Sci. Technol.*, 42(19), pp.7405–7410.

Dixit, P., Mukherjee, P.K., Sherkhane, P.D., et al., 2011. Enhanced tolerance and remediation of anthracene by transgenic tobacco plants expressing a fungal glutathione transferase gene. *Journal of Hazardous Materials*, 192(1), pp.270–276.

Dixit, P., Mukherjee, P.K., Ramachandran, V., et al., 2011. Glutathione transferase from *Trichoderma virens* enhances cadmium tolerance without enhancing its accumulation in transgenic Nicotiana tabacum. *PloS one*, 6(1), p.e16360.

Dixon, D.P. et al., 2009. Enzyme activities and subcellular localization of members of the Arabidopsis glutathione transferase superfamily. *Journal of Experimental Botany*, 60(4), pp.1207 –1218.

Dixon, D.P., Davis, B.G. & Edwards, R., 2002. Functional Divergence in the Glutathione Transferase Superfamily in Plants. *Journal of Biological Chemistry*, 277(34), pp.30859 –30869.

Dixon, D.P. & Edwards, R., 2010. Glutathione Transferases. *The Arabidopsis Book*, p.e0131.

Dixon, D.P. & Edwards, R., 2009. Selective binding of glutathione conjugates of fatty acid derivatives by plant glutathione transferases. *The Journal of Biological Chemistry*, 284(32), pp.21249–21256.

Dixon, D.P., Lapthorn, A. & Edwards, R., 2002. Plant glutathione transferases. *Genome Biology*, 3(3), p.REVIEWS3004.

Dixon, D.P., Skipsey, M. & Edwards, R., 2010. Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry*, 71(4), pp.338–350.

Duringer, J.M. et al., 2010. Uptake and transformation of soil [$_{14}$C]-trinitrotoluene by cool-season grasses. *Environmental Science & Technology*, 44(16), pp.6325–6330.

Ekman, D.R. et al., 2003a. SAGE analysis of transcriptome responses in Arabidopsis roots exposed to 2,4,6-trinitrotoluene. *Plant physiology*, 133(3), pp.1397–1406.

Ekman, D.R. et al., 2003b. SAGE Analysis of Transcriptome Responses in Arabidopsis Roots Exposed to 2,4,6-Trinitrotoluene. *Plant Physiology*, 133(3), pp.1397 –1406.

Foyer, C.H. & Noctor, G., 2009. Redox Regulation in Photosynthetic Organisms: Signaling, Acclimation, and Practical Implications. *Antioxidants & Redox Signaling*, 11(4), pp.861–905.

Frova, C., 2006. Glutathione transferases in the genomics era: new insights and perspectives. *Biomolecular Engineering*, 23(4), pp.149–169.

Frova, C., 2003. The plant glutathione transferase gene family: genomic structure, functions, expression and evolution. *Physiologia Plantarum*, 119(4), pp.469–479.

Gandia-Herrero, F. et al., 2008b. Detoxification of the explosive 2,4,6-trinitrotoluene in Arabidopsis: discovery of bifunctional O- and C-glucosyltransferases. *The Plant Journal*, 56(6), pp.963–974.

Gong, H., Jiao, Y., et al., 2005a. Expression of glutathione-S-transferase and its role in plant growth and development in vivo and shoot morphogenesis in vitro. *Plant Molecular Biology*, 57(1), pp.53–66.

Gong, H., Hu, W.-W., et al., 2005b. Molecular characterization of a Phi-class mustard (*Brassica juncea)* glutathione S-transferase gene in *Arabidopsis thaliana* by 5′-deletion analysis of its promoter. *Plant Cell Reports*, 24, pp.439–447.

Gong, P., Wilke, B.-M. & Fleischmann, S., 1999. Soil-Based Phytotoxicity of 2,4,6-Trinitrotoluene (TNT) to Terrestrial Higher Plants. *Archives of Environmental Contamination and Toxicology*, 36(2), pp.152–157.

Ghoghoberidze, M., Zaalishvili, G., Ramishvili, M., Gogava, M., Chelidze, N., 2009. Ultrastructural study of the effect of TNT on callus cells and cells of intact plants of *Yucca gloriosa* L. Cytology and Genetics 43, 18–21.

Habig, W.H., Pabst, M.J. & Jakoby, W.B., 1974. Glutathione S-Transferases. *Journal of Biological Chemistry*, 249(22), pp.7130 –7139.

Hannink, N.K. et al., 2007. Enhanced transformation of tnt by tobacco plants expressing a bacterial nitroreductase. *International journal of phytoremediation*, 9(5), pp.385–401.

Hannink, N.K., Rosser, S.J. & Bruce, N.C., 2002. Phytoremediation of Explosives. *Critical Reviews in Plant Sciences*, 21(5), pp.511–538.

Harvey, S.D. et al., 1990. Analysis of 2,4,6-trinitrotoluene and its transformation products in soils and plant tissues by high-performance liquid chromatography. *Journal of Chromatography A*, 518(0), pp.361–374.

Hawari, J. et al., 1999. Biotransformation of 2,4,6-Trinitrotoluene with *Phanerochaete chrysosporium* in Agitated Cultures at pH 4.5. *Applied and Environmental Microbiology*, 65(7), pp.2977–2986.

Hawari, J. et al., 1998. Characterization of Metabolites in the Biotransformation of 2,4,6-Trinitrotoluene with Anaerobic Sludge: Role of Triaminotoluene. *Applied and Environmental Microbiology*, 64(6), pp.2200–2206.

Honeycutt, M.E., Jarvis, A.S. & McFarland, V.A., 1996. Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites. *Ecotoxicology and Environmental Safety*, 35(3), pp.282–287.

Ji, W. et al., 2010. Over-expression of a glutathione S-transferase gene, GsGST, from wild soybean (*Glycine soja*) enhances drought and salt tolerance in transgenic tobacco. *Biotechnology letters*, 32(8), pp.1173–1179.

Jiang, H.-W. et al., 2010. A glutathione S-transferase regulated by light and hormones participates in the modulation of Arabidopsis seedling development. *Plant physiology*, 154(4), pp.1646–1658.

Kitamura, S. et al., 2012. Molecular characterization of an anthocyanin-related glutathione S-transferase gene in cyclamen. *Journal of plant physiology*, 169(6), pp.636–642.

Lachance, B. et al., 1999. Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 444(1), pp.25–39.

Landa, P. et al., 2010. Transferases and transporters mediate the detoxification and capacity to tolerate trinitrotoluene in Arabidopsis. *Functional & Integrative Genomics*, 10(4), pp.547–559.

Lima, D.R.S. et al., 2011. Impact of ammunition and military explosives on human health and the environment. *Reviews on Environmental Health*, 26(2), pp.101–110.

Makris, K.C. et al., 2007a. Chemically catalyzed uptake of 2,4,6-trinitrotoluene by *Vetiveria zizanioides*. *Environmental Pollution*, 148(1), pp.101–106.

Makris, K.C. et al., 2007b. High uptake of 2,4,6-trinitrotoluene by vetiver grass – Potential for phytoremediation? *Environmental Pollution*, 146(1), pp.1–4.

Martin, J.L. et al., 1997. Denitration of 2,4,6-trinitrotoluene by *Pseudomonas savastanoi*. *Canadian journal of microbiology*, 43(5), pp.447–455.

McCutcheon, S.C. & Schnoor, J.L., 2003. *Phytoremediation: transformation and control of contaminants*, John Wiley & Sons.

Mezzari, M.P. et al., 2005. Gene Expression and Microscopic Analysis of Arabidopsis Exposed to Chloroacetanilide Herbicides and Explosive Compounds. A Phytoremediation Approach. *Plant Physiology*, 138(2), pp.858 –869.

Mullineaux, P. et al., 2000. Are diverse signalling pathways integrated in the regulation of arabidopsis antioxidant defence gene expression in response to excess excitation energy? *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 355(1402), pp.1531–1540.

Pavlostathis, S.G. et al., 1998. Transformaton of 2,4,6-trinitrotoluene by the aquatic plant *Myriophyllum spicatum*. *Environmental Toxicology and Chemistry*, 17(11), pp.2266–2273.

Peterson, M.M. et al., 1998. Germination and seedling development of switchgrass and smooth bromegrass exposed to 2,4,6-trinitrotoluene. *Environmental pollution (Barking, Essex: 1987)*, 99(1), pp.53–59.

Pilon-Smits, E., 2005. Phytoremediation. *Annual Review of Plant Biology*, 56(1), pp.15–39.

Reinemer, P. et al., 1996. Three-dimensional structure of glutathione S-transferase from Arabidopsis thaliana at 2.2 Å resolution: Structural characterization of herbicide-conjugating plant glutathione S-transferases and a novel active site architecture. *Journal of Molecular Biology*, 255(2), pp.289–309.

Riechers, D.E. et al., 2003. Tissue-specific expression and localization of safener-induced glutathione S-transferase proteins in *Triticum tauschii*. *Planta*, 217(5), pp.831–840.

Riechers, D.E., Kreuz, K. & Zhang, Q., 2010. Detoxification without Intoxication: Herbicide Safeners Activate Plant Defense Gene Expression. *Plant Physiology*, 153(1), pp.3 – 13.

Rodgers, J.D. & Bunce, N.J., 2001. Treatment methods for the remediation of nitroaromatic explosives. *Water Research*, 35(9), pp.2101–2111.

Di Russo, N.V. et al., 2012. pH-Dependent Conformational Changes in Proteins and Their Effect on Experimental pKas: The Case of Nitrophorin 4. *PLoS Comput Biol*, 8(11), p.e1002761.

Rylott, E.L. et al., 2006. An explosive-degrading cytochrome P450 activity and its targeted application for the phytoremediation of RDX. *Nature Biotechnology*, 24(2), pp.216–219.

Rylott, E.L. & Bruce, N.C., 2009. Plants disarm soil: engineering plants for the phytoremediation of explosives. *Trends in Biotechnology*, 27(2), pp.73–81.

Rylott, E.L., Lorenz, A. & Bruce, N.C., 2011. Biodegradation and biotransformation of explosives. *Current Opinion in Biotechnology*, 22(3), pp.434–440.

Samra, A.I. et al., 2012. Cloning and characterization of two glutathione S-transferases from pyrethroid-resistant *Culex pipiens*. *Pest Management Science*, 68(5), pp.764–772.

Schoenmuth, B.W. & Pestemer, W., 2004. Dendroremediation of trinitrotoluene (TNT). Part 1: Literature overview and research concept. *Environmental science and pollution research international*, 11(4), pp.273–278.

Sens, C. et al., 1998. Distribution of14C-TNT and derivatives in different biochemical compartments ofPhaseolus vulgaris. *Environmental Science and Pollution Research*, 5(4), pp.202–208.

Sheehan, D. et al., 2001. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochemical Journal*, 360(Pt 1), pp.1–16.

Singh, B., Kaur, J. & Singh, K., 2011. 2,4,6-Trinitrophenol degradation by *Bacillus cereus* isolated from a firing range. *Biotechnology letters*, 33(12), pp.2411–2415.

Skipsey, M. et al., 2011. Xenobiotic Responsiveness of *Arabidopsis thaliana* to a Chemical Series Derived from a Herbicide Safener. *Journal of Biological Chemistry*, 286(37), pp.32268 –32276.

Smith, A.P. et al., 2003. Arabidopsis AtGSTF2 is regulated by ethylene and auxin, and encodes a glutathione S-transferase that interacts with flavonoids. *The Plant journal: for cell and molecular biology*, 36(4), pp.433–442.

Sparrow, H., 2010. The Role of Glutathione Transferases in the detoxification of TNT. *PhD Thesis*. The University of York, UK.

Stenuit, B.A. & Agathos, S.N., 2010. Microbial 2,4,6-trinitrotoluene degradation: could we learn from (bio)chemistry for bioremediation and vice versa? *Applied Microbiology and Biotechnology*, 88, pp.1043–1064.

Sun, Yang, Sumi, D. & Kumagai, Y., 2006. Serine 1179 phosphorylation of endothelial nitric oxide synthase caused by 2,4,6-trinitrotoluene through PI3K/Akt signaling in endothelial cells. *Toxicology and applied pharmacology*, 214(1), pp.55–60.

Sun, Yi, Li, H. & Huang, J.-R., 2012. Arabidopsis TT19 functions as a carrier to transport anthocyanin from the cytosol to tonoplasts. *Molecular plant*, 5(2), pp.387–400.

Tan, E.L. et al., 1992. Mutagenicity of trinitrotoluene and its metabolites formed during composting. *Journal of toxicology and environmental health*, 36(3), pp.165–175.

Taylor,A. 2011. Gold uptake and tolerance in Arabidopsis. *PhD Thesis*. The University of York, UK.

Tchounwou, P.B. et al., 2001. Transcriptional activation of stress genes and cytotoxicity in human liver carcinoma cells (HepG2) exposed to 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. *Environmental toxicology*, 16(3), pp.209–216.

Thom, R. et al., 2002. Structure of a Tau Class Glutathione S-Transferase from Wheat Active in Herbicide Detoxification†,‡. *Biochemistry*, 41(22), pp.7008–7020.

Thom, R. et al., 2001. The structure of a zeta class glutathione S-transferase from *Arabidopsis thaliana*: characterisation of a GST with novel active-site architecture and a putative role in tyrosine catabolism. *Journal of Molecular Biology*, 308(5), pp.949–962.

Thorn, K.A. & Kennedy, K.R., 2002. 15N NMR Investigation of the Covalent Binding of Reduced TNT Amines to Soil Humic Acid, Model Compounds, and Lignocellulose. *Environ. Sci. Technol.*, 36(17), pp.3787–3796.

Wayment, D.G. et al., 1999. A Transient Study of Formation of Conjugates during TNT Metabolism by Plant Tissues. *International Journal of Phytoremediation*, 1(3), pp.227–239.

Won, W.D., DiSalvo, L.H. & Ng, J., 1976. Toxicity and mutagenicity of 2,4,-6-trinitrotoluene and its microbial metabolites. *Applied and environmental microbiology*, 31(4), pp.576–580.

Zeng, Q.-Y., Lu, H. & Wang, X.-R., 2005. Molecular characterization of a glutathione transferase from *Pinus tabulaeformis* (Pinaceae). *Biochimie*, 87(5), pp.445–455.

Zitting, A., Szumanska, G., Nickels, J. & Savolainen, H. 1982. Acute toxic effects of trinitrotoluene on rat brain, liver and kidney: role of radical production. *Archives of Toxicology*, 51**,** 53-64.