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Glutathione Transferases in Soybean *Glycine max* (L.) Merr.

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Abstract

Glutathione transferases, also known as Glutathione *S*-transferases (GSTs), are a diverse group of enzymes that catalyse the conjugation of the tri-peptide glutathione to a wide range of electrophilic substrates. Their biological function in endogenous metabolism in plants is not well characterised, although their role in herbicide metabolism and herbicide selectivity is well documented. Many herbicides used in soybean, *Glycine max* (L.) Merr., are selective against weeds due to their rapid detoxification in the crop through conjugation with homoglutathione (γ-glu-cys-β-ala), the predominant free thiol in many legumes. However, an in depth characterisation of the GSTs which can potentially catalyse these reactions in soybean has never been performed. This work describes the biochemical and molecular characterisation of GSTs in soybean with emphasis on the identification of specific isoenzymes involved in herbicide metabolism.

GST activity toward the chloroacetanilide herbicides acetochlor and metolachlor, the diphenyl ethers acifluorfen and fomesafen and the sulphonyl urea chlorimuron-ethyl were all detected in crude protein extracts from five-day-old suspension cultured soybean cells. GST activity was also determined in five-day-old soybean seedlings, though this activity was significantly lower than that observed with the cell suspension cultures. Treatment of soybean plants with herbicides and herbicide safeners resulted in increased GST activity toward the model substrate 1-chloro-2,4-dinitrobenzene (CDNB), but no change in activity toward herbicide substrates. In both plant and cell cultures GST-catalysed conjugation of the diphenyl ethers acifluorfen and fomesafen was over five-fold greater in the presence of homoglutathione as compared with glutathione. The preferential detoxification of these herbicides in the presence of homoglutathione appeared to be an important determinant of their rapid detoxification in soybean and an important factor in herbicide selectivity.

GSTs were purified from five-day-old soybean cell cultures using *S*-hexylglutathione affinity chromatography and anion-exchange chromatography. A combination of reversed-phase HPLC, SDS-PAGE and MALDI-TOF mass spectrometry of the purified fractions indicated the presence of nine putative GST subunits, each with a molecular mass between 25 and 29 kDa.

Soybean GST cDNA clones were obtained using a combination of RT-PCR, utilising degenerate oligonucleotides designed to conserved regions within plant GSTs, and screening of cDNA libraries prepared from soybean plants and cell cultures. This process failed to identify any theta-type GSTs, the class associated with herbicide detoxification in maize. In contrast, seven distinct tau-type GSTs were isolated together with a number of clones showing minor variations in individual sequences. Expression of these cDNAs in *Escherichia coli* showed the purified recombinant GSTs were active toward a diverse range of substrates, and possessed additional glutathione peroxidase activity. GST activities for each recombinant enzyme varied with substrate and thiol type, with a marked preference for homoglutathione with selected substrates.

From the work reported in this study it would appear that the tau-type GSTs of soybean are at least as complex as those previously reported in cereals and have an important role in determining herbicide metabolism and selectivity in this major crop.

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Declaration

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Publications arising from work described in this thesis

Andrews C.J., Skipsey M., Townson J.K., Morris C., Jepson I. and Edwards R (1997) Glutathione transferase activities toward herbicides used selectively in soybean. *Pesticide Science* **51** 213-222.

Andrews C.J., Jepson I., Skipsey M., Townson J.K., Edwards R (1997) Nucleotide sequence of a glutathione transferase from soybean with activity toward herbicides. *Plant Physiology* **113** 1005.

Andrews C.J., Skipsey M., Edwards R., Hall G., Townson J.K., Jepson I (1997) Changes in glutathione transferase activities in soybean in response to treatment with herbicides and safeners. In: *The 1997 Brighton crop protection conference: Weeds*, vol. 1-3. British Crop Protection Council, UK.

Acknowledgements.

Abbreviations used in Text

ALS	acetolactate synthase
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSP	bromosulphophthalein
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
cv.	cultivar
Da	daltons (kDa = kiloDalton)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytodine triphosphate
dNTP	deoxynucleotide triphosphate
dH_2O	double-distilled water
DEAE	diethylaminoethyl
DIG	digoxigenin
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EA	ethacrynic acid
EDTA	ethylenediaminetetraacetic acid
EPTC	*S*-ethyl dipropylthiocarbamate
Fw	fresh weight
GSH	glutathione (reduced)
GST	glutathione transferase
h	hours(s)
HEPES	(*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid])
HIC	hydrophobic interaction chromatography
IAA	indole-3-acetic acid
IPTG	isopropyl-β-D-thiogalactoside
kat	katal (moles of product formed per second)

kb	kilobase
l	litre
MALDI-TOF	matrix-assisted laser desorption ionisation-time of flight
min	minutes
Mr	molecular mass
MS	mass spectrometry
mRNA	messenger RNA
NAA	α-Naphthaleneacetic acid
NBT	nitro-blue tetrazolium
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pfu	plaque-forming units
pI	isoelectric point
poly (A$^+$)RNA	polyadenylated RNA
PVPP	polyvinylpolypyrolidone
RNA	ribonucleic acid
RP-HPLC	reversed-phase high pressure liquid chromatography
RPM	revolutions per minute
sd	standard deviation
SDS	sodium dodecyl sulphate
Tris	tris (hydroxymethyl) aminomethane
TEMED	N, N, N', N'-tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Herbicides and Herbicide Safeners

Acetochlor	2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-methylphenyl) -acetamide
Acifluorfen	5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid
Alachlor	2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)-acetamide
Atrazine	6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine
BAS 145-138	1-dichloroacetyl-hexahydro-3,3,8a-trimethylpyrrolo(1,2a) pyrimidin-6-(2H)-one
Benoxacor	4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine
Chlorimuron-ethyl	ethyl 2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)-amino] carbonyl] amino]sulphony]benzoate
Dichlormid	2,2-dichloro-*N*,*N*-di-2-propenylacetamide
Ethacrynic acid	2,3-dichloro-4-(2-methylene-butyryl)phenoxyacetic acid
Fenchlorozole-ethyl	1-ethyl -(2,4-dichlorophenyl)-5-trichloromethyl-1*H*-1,2,4-triazole-3-carboxylate
Fenoxaprop-P	(*R*)-2-[4-[(6-chloro-2-benzoxazolyl)-oxy]phenoxy]propanoic acid
Fluorodifen	4-nitrophenyl α,α,α-trifluoro-2-nitro-*p*-tolyl ether
Fomesafen	5-[2-chloro-4-(trifluoromethyl)phenoxy]-*N*-methyl-sulphonyl-2-nitrobenzamide
Glyphosate	*N*-(phosphonomethyl)glycine
Metolachlor	2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide.
Naphthalic anhydride	1*H*,3*H*-naphtho(1,8-*cd*)-pyran-1,3-dione
Oxyfluorfen	2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoro-methyl) benzene.

1. Chapter One. Introduction

Soybean, *Glycine max* (L.) Merr., was first domesticated in China around the 11th century BC (Shoemaker *et al.*, 1996) and is one of man's oldest cultivated crops. Steady growth in consumption has resulted in soybean becoming a major agronomic crop, with an annual world-wide production in 1997 of 152.3 million tonnes (Wood Mackenzie, 1998). Soybean is the major oilseed crop, with soybean-derived products having a diverse range of industrial, food, pharmaceutical and agricultural applications (Verma and Shoemaker, 1996). They are particularly important in the production of edible oils, and the by-product of this process, soybean meal, is used as a source of high protein feed for livestock. As such, in terms of cash value, the soybean market in the USA, the world's major soybean producer, now ranks second only to maize (*Zea mays*) (Wood Mackenzie, 1998). Furthermore, legumes, such as soybean, have added agricultural benefit in that they are able to fix nitrogen as a result of their symbiosis with nodulating bacteria, which reduces the need for fertiliser application.

Pesticides, consisting of fungicides, herbicides and insecticides play an important role in protecting crops from damage, and thus allowing yields to be maximised. The total world soybean crop protection market in 1997 had an estimated value of $2,998 million, with herbicides representing the majority at $2,595 million (Wood Mackenzie, 1998). Therefore, the herbicide market associated with soybean is of significant importance to agrochemical companies.

Herbicides, by definition, are chemicals that have the ability to cause plant death and may be further classified as being either selective or non-selective. Herbicides which indiscriminately kill all plants are termed "non-selective", whereas compounds which preferentially kill weeds, with little or no adverse effect to the crop plant are termed "selective", implying that the crop plant is more tolerant of the herbicide than the weed controlled by the application. The discovery of novel selective herbicides is often difficult, due to a lack of knowledge regarding selectivity mechanisms. Studies with a range of crops and weeds have suggested that herbicides can be selective for the following reasons (Cole *et al.*, 1997):-

- reduced herbicide uptake or translocation by the crop plant relative to weeds, with crop tolerance due to the inability of the active ingredient to reach its target site.

- insensitivity to the herbicide due to an altered target site within the crop plant.

- rapid herbicide metabolism to inactive metabolites in the crop, which does not occur in the weed.

Recently, advances in biotechnology have allowed selectivity to be "engineered" into crops. For example, the Roundup Ready™ crops now commercialised contain a gene encoding a 5-enolpyruvylshikimic acid 3-phosphate synthase (EPSPS) that is tolerant of the normally non-selective herbicide glyphosate, a potent inhibitor of the native plant enzyme (Wells, 1995). However, in non-genetically modified crops selective herbicides owe their selectivity to naturally occurring tolerance mechanisms, such as glutathione conjugation, within the plant (Cole, 1994).

The available evidence (see later) suggests that metabolism, and particularly glutathione conjugation, are important in dictating the selectivity of many herbicides used commercially in soybean. Hence, the enzymes that are able to catalyse glutathione conjugation, the glutathione transferases (GSTs) are of potential interest in defining herbicide selectivity. Glutathione transferases are relatively well characterised in cereals, where their role in herbicide selectivity is well established (reviewed by Marrs, 1996). Detailed research has shown that the reason for the great diversity in the capacity of plants to conjugate electrophilic xenobiotics is derived from GSTs existing in multiple isoenzymic forms, with varying substrate specificity. Over the past two decades, plant GST research has focused on monocotyledonous crops with very little study on the GSTs in legumes such as soybean, or indeed dicotyledenous plants in general. A major objective of this project has been to further the understanding of GSTs involved in herbicide metabolism in soybean and competing weeds and to characterise these enzymes at both the biochemical and molecular level.

1.1 Herbicide Metabolism and Selectivity in Plants

All living organisms are now exposed to xenobiotic substances in their environment, many of which have the potential to cause detrimental, or even lethal effects if not efficiently metabolised. Xenobiotic metabolism in plants is analogous to that determined in animals and consists of three distinct phases: Phase I (conversion), Phase II (conjugation), and Phase III (deposition) as summarised in Figure 1.1. With respect to glutathione conjugation, many herbicides already contain an electrophilic centre susceptible to direct attack by glutathione. However, some herbicides require bio-activation by "Phase I metabolism", typically involving a cytochrome-P450, that introduces an electrophilic group. For example, the thiocarbamate herbicide EPTC and the triazinone herbicide metribuzin both undergo sulphoxidation, prior to conjugation with glutathione (Cole, 1994; Frear *et al.*, 1984). Phase II metabolism involves the conjugation of the xenobiotic substance with glutathione, glycosides and/or malonic acid. With respect to glutathione conjugation, this reaction is often found to be catalysed by a GST. Glutathione conjugates are often inhibitory to GST activity and their efficient removal from the cytosol is essential (Marrs, 1996). Removal occurs during Phase III metabolism, whereby the conjugate is imported into the vacuole by an ATP-binding cassette transporter located in the tonoplast membrane (Martinoia *et al.,* 1993; Lu *et al.,* 1998). Once in the vacuole, the glutathione conjugates can undergo further processing (Wolf *et al.,* 1996). Typically this involves degradation by a carboxypeptidase, to remove the glycine residue and a γ-glutamyl transpeptidase to release the *S*-cysteinyl derivative (Lamoureux and Bakke, 1984). Therefore, GSTs play an important role in plants as they catalyse the conversion of hydrophobic toxic electrophilic xenobiotics into water-soluble non-toxic glutathione conjugates, which can be selectively removed from the cytosol.
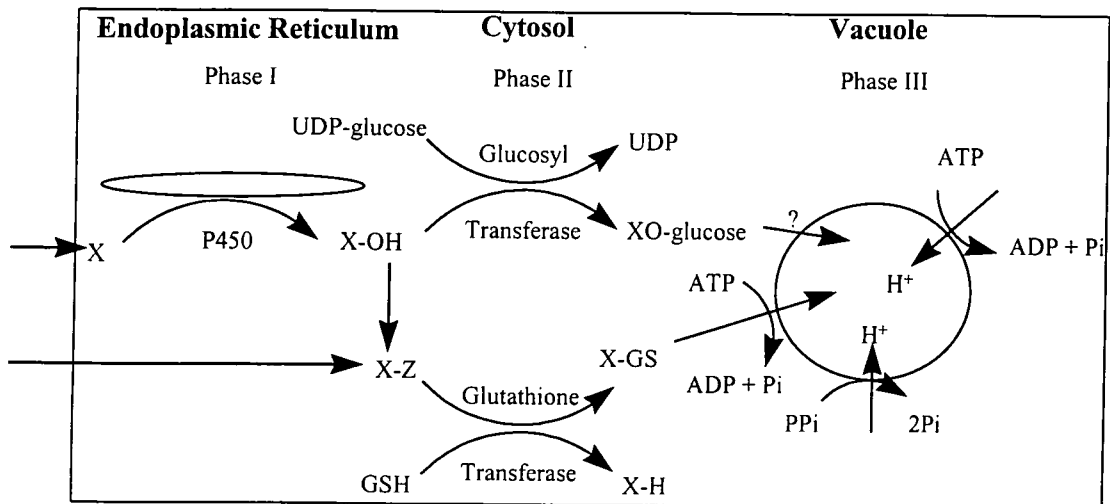
Figure 1.1. The three phases (I, II and III) of xenobiotic (X) metabolism in plants. (source: Kreuz *et al.*, 1996).

1.2 Glutathione Transferases

Glutathione transferases (GSTs, EC 2.5.1.18), also referred to as glutathione *S*-transferases, are a family of multi-functional enzymes that have evolved, together with glutathione, in most aerobic organisms including bacteria, fungi, yeast, insects, higher plants, fish and mammals (Fahey and Sunquist, 1991). They exist as homo- or heterodimers, with subunits typically between 24 kDa and 30 kDa. GSTs catalyse the conjugation of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) to a diverse number of electrophilic substances, including organic halides, esters, ethers, epoxides, lactones, quinones and activated alkenes (Wilce and Parker, 1994). 1-chloro-2,4-dinitrobenzene (CDNB) is often used as a model substrate with which to monitor GST activity, since the reaction involves a colour change that can be followed conveniently using a spectrophotometer. Examples of GST substrates are given in Figures 1.2 and 1.3. The catalytic activity of GSTs is due to the ability of the enzyme to promote the formation of the highly nucleophilic thiolate anion of glutathione, which then undergoes nucleophilic substitution or addition reactions with the electrophile (Mannervik, 1985). Although acting on diverse substrates, GSTs show specificity toward their substrates and the extensive range of detoxifying activities seen in most eukaryotes results from the presence of multiple GST isoenzymes.

CDNB   Glutathione (GSH)

Ether Substrate

GSH + R-O-R' → GS-R + R'-OH
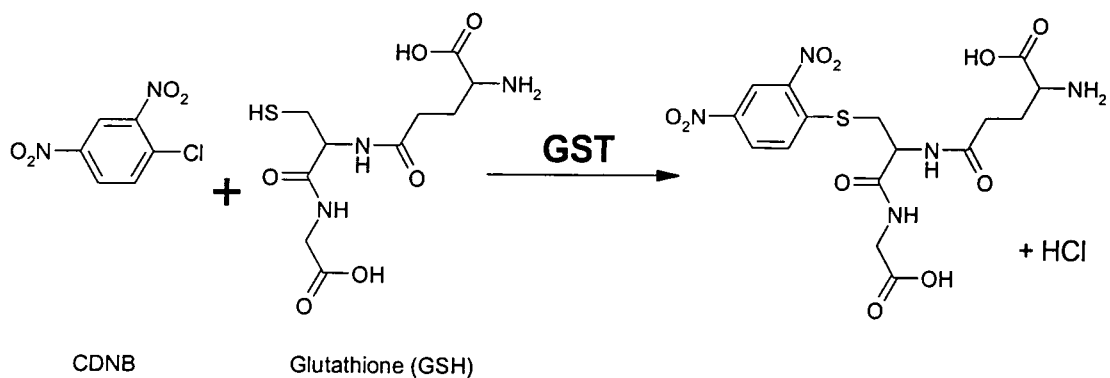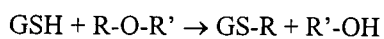
Alkene substrate addition reaction.

GSH + R-CH=CH-R' → R-CH(SG)-CH$_2$-R'
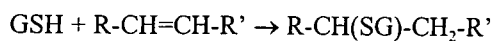
Figure 1.2. Examples of GST-catalysed reactions. CDNB is used as a model substrate with which to monitor GST activity, since the formation of the yellow conjugate can be conveniently measured at A$_{340nm}$ using a spectrophotometer.
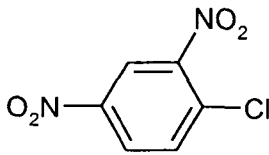
1-Chloro-2,4-dinitrobenzene (CDNB)
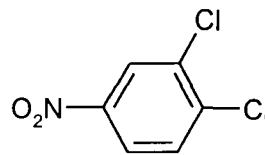
1,2-Dichloro-4-nitrobenzene (DCNB)
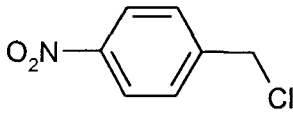
p-Nitrobenzyl chloride (NBC)
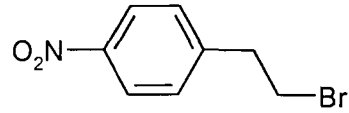
p-Nitrophenethyl bromide
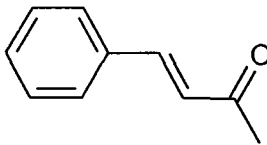
trans-4-Phenyl-3-buten-2-one
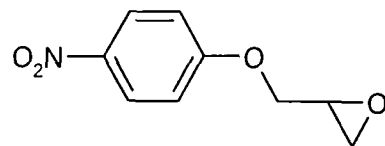
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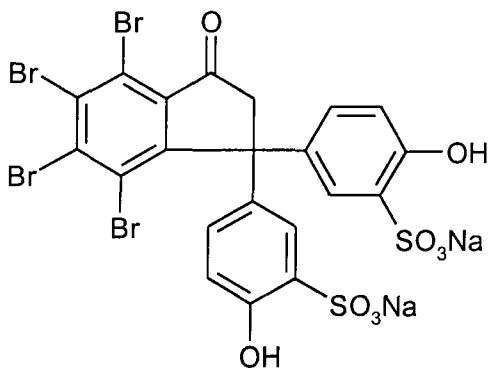
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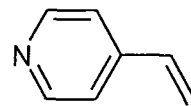
Vinylpyridine
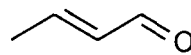
Crotonaldehyde
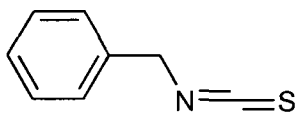
Benzyl isothiocyanate
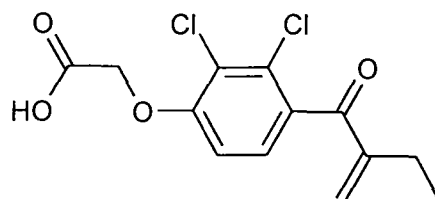
Ethacrynic acid

Figure 1.3 Examples of different chemistries susceptible to GST-catalysed glutathione conjugation.

1.2.1 Mammalian GSTs.

GSTs have been studied extensively in animals, due to their involvement in the detoxification of mutagens, carcinogens and other noxious chemicals (Mannervik and Danielson, 1988). They were first identified in rat liver (Booth *et al.*, 1961) and their involvement in the formation of mercapturic acid derivatives later characterised in this organ (Habig *et al.*, 1974). Since then they have been further implicated in the intracellular detoxification of both xenobiotic compounds and endogenously produced toxins (Wilce and Parker, 1994). Indeed, their importance in drug and antibiotic detoxification has led to GSTs becoming a target for rational drug design (Wilce and Parker, 1994). In addition to their detoxifying role, animal GSTs also exhibit activity in cellular metabolism, as they are involved in the isomerisation of 3-ketosteroids and in the synthesis of leukotrienes and prostaglandins (Mannervik and Danielson, 1988). Although GSTs comprise 5%-10% of the total cytosolic protein in human liver (Wilce and Parker, 1994), they have also been purified from other tissues including the placenta, erythrocytes, breast, lung and prostate (Wilce and Parker, 1994). All GSTs described to date are dimeric proteins, with each GST subunit containing a catalytically independent active site. Mammalian GSTs are only active following dimerisation, since a small part of the glutathione binding site from one subunit consists of residues from the other (Dirr *et al.*, 1994b). The specificity for glutathione at the binding site is high, with the γ-glutamic moiety and orientation of the sulphydryl group being critical (Danielson and Mannervik, 1985). However, the binding site for the electrophilic substrates is less conserved, which helps to explain the wide range of reactions the different isoenzymes can catalyse.

Five distinct classes of mammalian GSTs have been identified; alpha (α), mu (μ), pi (π), theta (θ) and zeta (ζ) (Mannervik and Danielson, 1988; Meyer *et al.*, 1991a; Board *et al.*, 1997). Classes are defined according to the substrate and inhibitor specificity of the enzyme, immuno-reactivity and similarity in amino acid sequence (Mannervik *et al.*, 1985). GSTs from the same class but different species show as much as 60-80% homology in primary protein sequence, whereas homology between classes drops to <30% (Mannervik and Danielson, 1988). In addition to the five classes described, a membrane bound microsomal form of GST has been identified (Tsuchida and Sato, 1992), and the *S*-crystallins of squid lenses appear to be related to

GSTs and have been assigned to the sigma (σ) class (Wilce and Parker, 1994). Although mammalian GSTs are known to exist as dimers, dimerisation between enzymes from different GST classes has never been observed (Armstrong *et al.*, 1995).

Crystallographic studies show that six residues appear to be conserved highly between mammalian GSTs notably: Tyr-7, Pro-53, Asp-57, Ile-68, Gly-145, Asp-152, with the numbering used based on human placental pi class GST (Wilce and Parker, 1994). The Tyr-7 residue has been shown to form a hydrogen bond with the sulphur atom of glutathione (Neuefeind *et al.*, 1997b), which lowers the pKa of the thiol group (Armstrong, 1993). It is this stabilisation of the thiolate anion of glutathione which allows it to react with the electrophilic centre of co-substrate, and gives rise to the catalytic activity of GST. Mutation of this tyrosine residue to alanine inhibited catalytic activity, confirming the importance of this residue (Board, 1995). The function of the other conserved residues is not known, although it is postulated that the last three may be involved in protein folding (Rushmore and Pickett, 1993). Overall, there does not appear to be any conservation of specific amino acids between all known GSTs. However, despite low overall sequence homology, crystallographic studies indicate that all GSTs studied to date possess a similar tertiary protein structure (Dirr *et al.*, 1994b). The first three-dimensional structure of a GST was elucidated in 1991 (Reinemer *et al.*, 1991) and since then the crystal structure of alpha (Cameron *et al.*, 1995), pi (Dirr *et al.*, 1994a), mu (Raghunathan *et al.*, 1994) and theta class (Wilce *et al.*, 1995) GSTs have all been elucidated. All the GSTs examined contain a highly specific glutathione binding site (G-site) and a less specific co-substrate binding site (hydrophobic site or H site). The typical GST folding pattern consists of a small N-terminal $\beta\alpha\beta\alpha\beta\beta$ domain (amino-acids 1-77) and a larger C-terminal α-domain (amino-acids 92-220) joined by a variable linker region (Dirr *et al.*, 1994b). The highly conserved N-terminal domain contains the residues that constitute the glutathione binding (G) site, whist the larger C-terminal domain is much less conserved and contains residues involved in the binding of the hydrophobic substrate (H) site.

In addition to GST activity some mammalian GSTs also possess secondary activity as selenium-independent glutathione peroxidases and steroid isomerases (Wilce and

Parker, 1994). Glutathione peroxidases (GPOX) catalyse the reduction of fatty acid hydroperoxides with the concomitant formation of oxidised glutathione (GSSG) (Bartling *et al.*, 1993). Such hydroperoxides are generated both as by-products of aerobic metabolism and as a result of exposure to oxidative stress. Organic hydroperoxides are potentially cytotoxic and their detoxification and removal is fundamental to an organism's survival. The peroxidase activity of GSTs is not inhibited by sodium azide, which differentiates these enzymes from selenium-dependent glutathione peroxidases (Dixon *et al.*, 1998b). It has also been suggested that GSTs are binding proteins and have been implicated in the transport of steroids, bilirubin and thyroid hormones (Ketley *et al.,* 1975) since binding of these ligands can markedly inhibit their glutathione conjugating activity (Wilce and Parker, 1994).

1.2.2 Plant GSTs

Just as GST research in animals focused initially on xenobiotic metabolism, it was soon discovered that GSTs in plants were responsible for detoxifying electrophilic herbicides (Frear and Swanson, 1970). GSTs were first reported in maize over 25 years ago (Frear & Swanson, 1970), where they were implicated in the metabolism of the chloro-*s*-triazine herbicide, atrazine. Today, GSTs have been reported in over 40 species of higher plants (Lamoureux & Rusness, 1989, Marrs, 1996, Dixon *et al.*, 1998b) and 20 species of fungi, algae and mosses (Pflugmacher and Schröder, 1995). GSTs have been successfully isolated from roots (Mozer *et al.*, 1983), seeds (Williamson and Berverley, 1988), green and etiolated foliage of monocotyledonous and dicotyledonous plants (Edwards, 1995), cell cultures (Edwards and Owen, 1986; Hatton *et al.*, 1998), and protoplasts (Takahashi and Nagata, 1992a). As such, GSTs are thought to be expressed at all stages of plant development and are often one of most abundant non-photosynthetic proteins in plants constituting, for example, 1-2% cytosolic protein in maize (Sari-Gorla *et al.*, 1993). Expression of GSTs in plants varies considerably, with both constitutive and inducible isoforms present. The majority of GSTs appear to be localised in the cytosol, even though the highest concentration of glutathione is reportedly in the chloroplast (Rennenberg and Lamoureux, 1990). Exceptions are the tobacco *par* A enzyme, which is located in the nucleus (Takahashi *et al.*, 1995), and more recently an enzyme from soybean which

was found in the apoplast (Flury *et al.*, 1996). Microsomal GSTs have also been reported in wheat (*Triticum aestivum*) (Schröder *et al.*, 1998).

1.2.3 Classification of plant GSTs

Plant GST sequences show little overall homology with GSTs in animals and insects, with all the enzymes described to date showing greatest identity to the mammalian theta-class. This may suggest that the theta-class is the most ancient class of GST, and the probable evolutionary forerunner of all GSTs (Marrs, 1996). Three classes of plant GSTs were originally proposed, based upon their intron / exon genetic structure, sequence conservation and primary sequence homology (Droog *et al.*, 1995). Type I GSTs, which include well characterised enzymes involved in herbicide detoxification possess two introns and include the maize *Zm*GST I, III and IV enzymes. Type-II GSTs contained nine introns, with the only reported sequence from plants being that from carnation (Meyer *et al.*, 1991b). Type III GSTs, first identified due to their regulation by auxins (Takahashi *et al.*, 1989; Droog, 1993) were all found to be encoded by genes containing a single intron. In an attempt to rationalise plant GSTs with the animal GST nomenclature, they have been further reclassified (Droog, 1997). Type I GSTs were considered members of the theta-class, due to the fact they showed greatest homology to the animal theta class. Type III GSTs were sufficiently divergent from the theta class to warrant their inclusion into a new family, assigned the tau (τ) class. And finally, the similarity of the type II GSTs to zeta-class identified in animals led to the inclusion of these enzymes into this class (Board *et al.*, 1997).

1.2.4 Plant GST structure.

Like mammalian GSTs, all plant GSTs characterised to date are dimeric enzymes with a native molecular mass of approximately 50 kDa and consist of subunits between 25 kDa and 30 kDa, with each type of subunit encoded by a distinct gene. Since plants contain GST multigene-families, and that subunits can associate to form both homo- and heterodimers, the permutations for dimer formation is potentially large. Recently, the crystal structure of three theta-type plant GSTs has been solved. A GST from *Arabidopsis thaliana* (Reinemer *et al.*, 1996) and the maize enzymes *Zm*GST I-I and

*Zm*GST III-III (Neuefeind *et al.*, 1997a/b). These studies indicated that plant GSTs have a similar overall structure to mammalian GSTs. However, minor differences were observed, the most pertinent being differences at the glutathione (G) binding site. In mammalian GSTs the G-site is composed of amino acid residues from both polypeptides of the dimer. However, in plant GSTs it was discovered that the glutathione binding site is composed completely of amino acids within the same polypeptide. Thus, unlike mammalian GSTs, each subunit is completely catalytically independent, each possessing its own binding site for glutathione and co-substrate, with the total activity the sum of the individual monomers (Neuefeind *et al.*, 1997a/b). In addition the conserved Tyr-7 residue present in mammalian GSTs, thought to play a crucial function in stabilising the thiolate anion, is not present in the crystallised plant GSTs. However, a Ser-11 residue which is absent in mammalian GSTs has been shown to function in a similar manner (Reinemer *et al.*, 1996). The hydrophobic cleft responsible for binding substrates was found to be larger in plant GSTs than mammalian GSTs, and may explain the varied spectrum of reactions catalysed by these enzymes in plants (Neuefeind *et al.*, 1997a). In addition, substrate binding appears to occur by an induced-fit mechanism, whereby a flexible loop closes around the substrate, possibly creating a more favourable hydrophobic environment following binding (Neuefeind *et al.*, 1997b).

1.2.5 Endogenous plant GST substrates.

Plant GSTs are unusually abundant enzymes, in some cases constituting between 1-2% of the total cytosolic protein, and they are subject to complex spatial and temporal regulation in response to plant development and numerous stress treatments. Therefore, perhaps the most intriguing question concerning plant GSTs is a consideration of their endogenous biological function. Evidence for their potential role in cellular metabolism was obtained following the discovery that the *Bronze-2* (*Bz-2*) gene in maize encoded a GST (Marrs *et al.*, 1995). The analysis of maize mutants has clearly shown that the *Bronze-2* gene is essential in ensuring the deposition of anthocyanin pigments in the vacuole, with inactivation of the enzyme leading to the accumulation of bronze pigments in the cytosol. Subsequent experiments demonstrated that *Bz-2* and reduced glutathione were required for the

import of anthocyanin precursors into isolated vacuoles, although evidence of actual glutathione conjugates of anthocyanins *in planta* has not been reported (Marrs *et al.*, 1995). Therefore, it appeared that *Bz-2* acts in conjunction with an ATP-dependant pump to transport cyanidin-3-glucoside and other anthocyanins across the tonoplast membrane and into the vacuole (Marrs *et al.*, 1995). Further work has shown that the *An9* gene in petunia encodes a theta-class GST that is also involved in vacuolar transport of anthocyanins. *An9* is able to complement *Bz-2* deficient mutants, whilst *Bz-2* complements *An9* deficient mutants of petunia (Alfentino *et al.*, 1998). The tau-type GST soybean *Gm*GST1 and theta-type maize GST-III have also been shown to complement the *Bz-2* mutation. However, other theta-type GSTs, including the *Arabidopsis* GST EST H36860, the most closely related plant GST to *An9*, *Zm*GST-I and *Zm*GST-IV failed to complement the mutation (Alfentino *et al.*, 1998). It was concluded that whilst GSTs closely related to *Bz-2* and *An9* were not necessarily active toward similar substrates, widely divergent GSTs were. There is little evidence of glutathione conjugation toward other natural products (Cheynier *et al.*, 1986), though conjugates of cinnamic acid (Edwards and Dixon, 1991), giberellins (Lamoureux and Rusness, 1993) and the isoflavonoid medicarpin (Li *et al.*, 1997) can be formed *in vitro*. In the case of cinnamic acid the conjugating activity described in extracts from French Bean cell cultures, termed glutathione *S*-cinnamoyl transferase, was increased upon exposure to fungal elicitors (Edwards and Dixon, 1991). However, it has now been discovered that the identification of plant GSTs with activity toward phenylpropanoids (Dean *et al.*, 1995) was incorrect and that this activity is in fact associated with an ascorbate peroxidase *in planta*, which possibly explains its apparent increase during fungal elicitation (Dean *et al.*, 1997).

In mammals, GSTs are known to protect against oxidative damage by reducing lipid hydroperoxides, and detoxifying the associated toxic aldehyde degradation products of such hydroperoxides (Mannervik and Danielson, 1988). It is therefore possible that they serve a similar function in plants, protecting the cell from reactive electrophiles formed during oxidative stress. Oxidative stress occurs when the production of active oxidative species (AOS) is greater than the cell's antioxidant scavenging ability. AOS are produced in all aerobic cells, and result in the formation of organic hydroperoxides, which are potentially cytotoxic (Marrs, 1996). Oxidative stress in

plants is caused by diverse biotic and abiotic stress treatments, including extremes in temperature and drought, air pollution (Alscher, 1989), pathogen infection (Dudler *et al.*, 1991), and exposure to heavy metals, ozone and ethylene (Marrs, 1996). Significantly, all these stress treatments have been reported to enhance selected GSTs, and it has been suggested that these inducible GSTs are functioning to counteract oxidative stress (Marrs, 1996). Thus, plant GSTs may be induced in response to oxidative stress in a similar manner to that seen in mammals (Daniel, 1993) and the most important antioxidant function for GSTs may be their secondary activity as glutathione peroxidases. Many plant GSTs have secondary activities as glutathione peroxidases, including enzymes isolated from pea (*Pisum sativum*) (Williamson *et al.*, 1987; Edwards, 1996), *Arabidopsis* (Bartling *et al.*, 1993) and wheat (Cummins *et al.*, 1997), and are able to detoxify products of lipid peroxidation in a similar manner to mammalian GSTs. Evidence for a "stress protection" function of GSTs has recently been demonstrated when transgenic tobacco seedlings, over-expressing a GST from tobacco, which possessed both GST and glutathione peroxidase activity, exhibited increased tolerance to stress conditions (Roxas *et al.*, 1997). However, GSTs are but one part of the antioxidant defence system in the plant and if this hypothesis is true, will function in concert with other classes of glutathione peroxidase (Mullineaux *et al.*, 1998) and other enzymes of antioxidant metabolism, such as glutathione reductase, superoxide dismutase and ascorbate peroxidase to counteract oxidative stress (Broadbent *et al.*, 1995).

A further biological function for GSTs is suggested by the number of auxin-inducible clones identified encoding these enzymes (Droog, 1993). Many GSTs are inhibited by the binding of auxins and auxin analogues, and it has been suggested that GSTs may be involved in the binding and transport of hydrophobic and amphiphatic compounds such as auxins (Dean *et al.*, 1995; Watahiki *et al.*, 1995), and as such provide modulation of auxin activity (Lamoureux and Rusness, 1989). Indole-3-acetic acid (IAA), α-Naphthaleneacetic acid (NAA), 2,4-Dichloroacetic acid (2,4-D), 2,3-Dichloroacetic acid (2,3-D) and 2,4,5-Trichloroactic acid (2,4,5-T) were all found to inhibit the GST activity toward CDNB of *GST1* from *Hyoscyamus muticus* (Bilang *et al.*, 1993; Bilang and Sturm, 1995). 2,4-D behaved as a competitive inhibitor, while IAA exhibited non-competitive inhibition. The GST activity of the tobacco GSTs

*Nt*103, *Nt*107 and potato GST1 was also inhibited by auxin (Droog, 1993). It is interesting to note that auxins regulate a number of cellular processes, most notably cell division and cell elongation, which lead to the production of oxidising products, suggesting a need for glutathione peroxidase protection. Inhibition of GST activity by porphyrins such as haemin and chlorophyllin suggest a further possible transport function, although this has not been proven (Singh and Shaw, 1988).

1.2.6 Role of GSTs in herbicide selectivity.

With respect to selectivity, the metabolism of a herbicide to a less toxic product is often the main factor in herbicide tolerance (Cole, 1994). Herbicide metabolism can occur via the hydrolysis, oxidation, dealkylation and conjugation of the active compound, with glutathione conjugation being well documented (Cole, 1994)
Herbicide chemistries susceptible to conjugation by glutathione include aryloxyphenoxy-propionates, chloroacetamides, chloroacetanilides, diphenyl ethers, thiocarbamates, sulphoxides, thiodiazolidines, triazines, triazinone sulphoxides, and sulphonyl ureas (Figure 1.4). Despite their characterisation as detoxification enzymes, it has been shown recently that GSTs can activate pro-herbicides. The isomerisation of thiadiazolidine herbicides to the more potent triazolidine herbicide was found to be catalysed by maize *Zm*GST II-II, acting as a thiadiazolidine isomerase (Nicolaus *et al.*, 1996). Fluthiacet-methyl also undergoes isomerisation by a GST to create a potent peroxidising urazole (Cole *et al.*, 1997).

Chloroacetanilides

Acetochlor*　　　　Alachlor*　　　　Metolachlor*

Diphenyl ethers

Acifluorfen*　　　　Fomesafen*

Fluorodifen
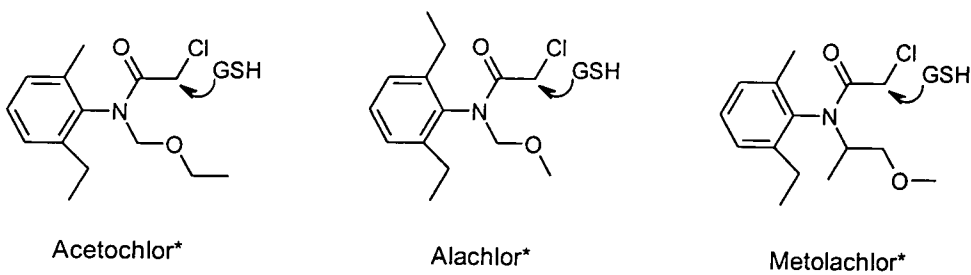
Aryloxyphenoxypropionate　　　　**Sulphonylurea**

Fenoxaprop

Chlorimuron Ethyl*
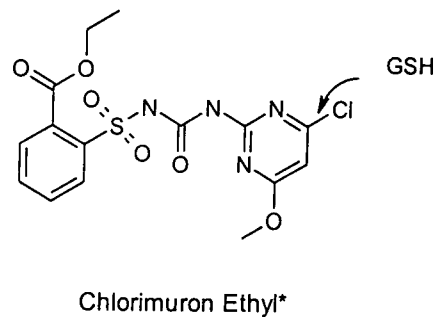
Triazine

Atrazine
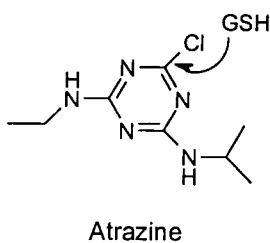
Figure 1.4 Examples of herbicides susceptible to glutathione conjugation. * Indicates herbicides known to owe their selectivity in soybean to rapid homoglutathione conjugation.

Plant GSTs are well known for their involvement in the detoxification of electrophilic xenobiotics, such as herbicides. Their role in herbicide selectivity was first discovered

when GST activity toward atrazine was found to be higher in resistant species such as maize and sorghum compared with susceptible species such as pea, wheat and barley (*Hordeum vulgare*) (Frear and Swanson, 1970). Further analysis showed that maize lines susceptible to atrazine possessed far lower GST activity than atrazine-resistant lines (Shimabukuro *et al.* 1971). Thus, the atrazine sensitive maize line GT112 contained <1% GST activity toward atrazine as compared with the resistant GT112 RfRf line (Shimabukuro *et al.*, 1971). Later studies, looking at alachlor metabolism in inbred maize lines revealed similar results with lines susceptible to alachlor, and lines showing intermediate sensitivity, exhibiting impaired function in their *Zm*GST IV and *Zm*GST I subunits, respectively (Rossini *et al.* 1996). The importance of the *Zm*GST IV subunit in chloroacetanilide tolerance has recently been confirmed in transgenic tobacco (Jepson *et al.*, 1997). Tobacco plants expressing the *Zm*GST IV showed markedly increased tolerance toward metolachlor as compared with controls. It is known that herbicides of the same class are not necessarily metabolised by the same GST isoenzyme. For example, the GST from pea catalysing glutathione conjugation with the diphenyl ether herbicide fluorodifen does not perform the same reaction with the structurally similar compound, acifluorfen as a substrate (Lamoureux & Rusness, 1989).

With respect to herbicides detoxified by glutathione conjugation, a study of several crops and weeds concluded that tolerant species were able to metabolise herbicides more readily than susceptible species (Breaux *et al.*, 1987). Studies with seedlings of maize and associated problematical weeds show higher levels of GST activity toward selective herbicides in the crop plant than in the weeds, and this could account for the observed selectivity (Hatton *et al.*, 1996). Recently, the acquired resistance to aryloxyphenoxypropionate herbicides seen in black-grass *(Alopercurus myosuroides)*, a problematical weed of wheat, was associated with higher levels of GST expression in resistant populations (Cummins *et al.*, 1997a).

Glutathione conjugation of herbicides in plants is not exclusively GST mediated and can occur spontaneously with reactive compounds. For example, the conjugation of fenoxaprop-ethyl with glutathione in many grass species is not thought to be enzyme-catalysed (Tal *et al.*, 1995). Additionally, it is thought that the chloroacetanilides are sufficiently electrophilic to react directly with glutathione *in planta* (Jablonkai and

Hatzios, 1993). Endogenous glutathione levels may indeed be important considerations in plant tolerance to specific herbicides, however it is thought that specific GST activities are the major determinants of herbicide tolerance at physiological pH (Hatton *et al.*, 1996). From considering the results of a number of studies it is clear that the efficiency of selective herbicides is dependent on the rate of detoxification of the active ingredient within the crop plant (Cole, 1994). In the case of glutathione conjugation this in turn is controlled by many factors, including the presence of specific GST isoenzymes, the relative abundance of these enzymes and possibly endogenous thiol levels.

1.2.6.1 Soybean herbicides and their metabolism

A major objective of the current work was to determine the role of soybean GSTs in herbicide selectivity. It was known that many herbicides used for selective weed control in soybean are detoxified by conjugation with homoglutathione (γ-glutamyl-cysteinyl-β-alanine), although the involvement of GSTs has never been determined. Fomesafen, commercially marketed as Reflex™ / Flexstar™ (Zeneca) is a selective diphenyl ether herbicide used in soybean to control broadleaf weeds and, at higher concentrations, some grasses (Evans *et al.*, 1987; Ridley, 1983). The diphenyl ether herbicides inhibit protoporphyrinogen oxidase (protox), a key enzyme involved in both the heam and chlorophyll synthetic pathways. Inhibition of protox causes the accumulation of protoporphyrin-IX, a photodynamic tetrapyrrole (Sherman *et al.*, 1991). In the presence of light and molecular oxygen protoporphyrin-IX generates singlet oxygen causing rapid membrane oxidation and chlorophyll bleaching. Metabolism studies with fomesafen in soybean showed that the major route of detoxification involves cleavage of the ether bond following homoglutathione conjugation (Evans *et al.*, 1987).

Acifluorfen, like fomesafen, is a substituted diphenyl ether herbicide. It is commercially sold as Blazer™ (BASF) and is used for selective post emergence control of annual broad leaf weeds, including *Abutilon, Amaranthus,* and *Ipomoea* species. Acifluorfen is also an inhibitor of protoporphyrinogen oxidase, although crop phytotoxicity, or "burn" associated with acifluorfen use in soybean is higher than that seen with fomesafen. Studies have shown that acifluorfen is rapidly metabolised in

soybean (Frear *et al.*, 1983). Within 24 hours 90-95% of the herbicide taken up by the plant was converted into soluble metabolites. Analysis of the products showed the diphenyl ether bond had been cleaved, and the presence of the homoglutathione conjugate was detected. The mechanism of selectivity of acifluorfen in soybean would therefore appear to be due to rapid metabolism of the herbicide in the crop.

Chlorimuron-ethyl, commercially sold as Classic™ (DuPont) is a highly effective selective, broad spectrum, sulphonyl-urea herbicide used for both pre- and post-emergence weed control in soybean at low application rates. The sulphonylurea herbicides act by inhibiting acetolactate synthase (ALS), an enzyme required for the synthesis of the essential branched chain amino acids valine, leucine and isoleucine. Inhibition of ALS leads to the rapid inhibition of cell division and plant growth (Brown and Neighbors, 1987). Studies have shown that chlorimuron-ethyl is rapidly taken up and translocated by both soybean and weeds, suggesting herbicide mobility is not the mechanism of selectivity. Instead, the literature indicates that the selectivity of chlorimuron-ethyl is due to its enhanced metabolism in soybean compared with weeds. It is reported that the half-life of chlorimuron in soybean was between one and three hours, whereas in susceptible weeds, such as *Xanthium* and *Amaranthus* species, the half-life was 30 hours. The major metabolite identified in soybean was the homoglutathione conjugate, formed by displacement of the chlorine from the pyrimidine ring (Brown and Neighbors, 1987).

Chloroacetanilide herbicides, such as metolachlor, sold as Dual™ (Ciba-Geigy) have an undefined mode of action, but appear to prevent cell division by inhibiting a number of processes, including protein synthesis, in susceptible germinating seedlings. They are applied pre-emergence to control grasses and some broad-leaf weeds in a variety of crops such as maize and soybean (Breaux, 1986 and Breaux *et al.*, 1987). In maize a good correlation has been determined between the glutathione content of seedlings and chloroacetanilide selectivity (Hatton *et al.*, 1996a). In soybean, it has been suggested that chloroacetanilide selectivity can be partially explained by the higher concentration of thiol available in the crop compared to the weeds (Breaux, 1986). Similarly acetochlor, sold as Surpass™ (Zeneca) and Harness™ (Monsanto) is another chloroacetanilide herbicide used in soybean. It is

mainly absorbed by the shoots of germinating plants and offers selective pre-emergence control of annual grasses and some broadleaf species.

1.2.7 Regulation of plant GSTs

The expression of GSTs in plants has been shown to be highly complex. Constitutive enzymes have been reported, such as the ZmGST I-I enzyme in maize (Holt *et al.*, 1995). However, many plant GST genes are induced by a wide variety of biotic and abiotic stimuli as well as being regulated by plant development. Known enhancers of GST expression include heat-shock and treatment with heavy metals (Czarnecka *et al.*, 1988; Marrs, 1996), ethylene (Zhou and Goldsbrough, 1993; Takahashi *et al.*, 1995) and auxins in a variety of plants including carnation (Meyer *et al.*, 1991b; Itzhaki *et al.*, 1994), *Arabidopsis* (Watahiki *et al.*, 1995) and tobacco (Droog *et al.*, 1993). GSTs are also induced following infection by fungi, viruses and bacteria (Marrs, 1996; Dudler *et al.*, 1991), wounding (Kim *et al.*, 1994) and a number of additional biotic stresses such as dehydration (Kiyosue *et al.*, 1993) and oxidative stress (Bartling *et al.*, 1993). Abiotic inducing agents of GSTs include ethanol, ethacrynic acid, herbicides (Mauch and Dudler, 1993) and herbicide safeners (Jepson *et al.*, 1994).

Due to their original classification as auxin-inducible genes, the tau-class of GST is perhaps the best studied group with respect to their gene regulation. *GST1* from *H. muticus* was found to be selectively induced by 2,4-D but not IAA or herbicides (Bilang *et al.*, 1993). Developmentally, plant GSTs appear to be strictly controlled. For example, the theta-type ZmGST II-II is only expressed constitutively in the roots (Holt *et al.*, 1995) and in carnation the zeta-class GST is only expressed during senescence (Meyer *et al.*, 1991b).

1.2.7.1 Herbicide safeners

In addition to their regulation by the wide range of endogenous and xenobiotic compounds, the GSTs in cereals are potently enhanced by herbicide safeners (Irzyk and Fuerst, 1997). Herbicide safeners, or antidotes, are chemicals which enhance the expression of GSTs and other herbicide detoxifying enzymes in maize, wheat and sorghum and often structurally resemble the herbicides they antagonise (Hatzios,

1997). They are used in agriculture to increase herbicide tolerance in the crop, but not in competing weeds, and this appears to be related to their ability to selectively enhance herbicide detoxification in cereals. They permit the selective control of grass weeds in cereals (e.g. wild oat in oats), expand the uses of older herbicides and improve the margin of herbicide selectivity with newer compounds (Hatzios, 1997). Safeners are chemically diverse and include dichloroacetamide derivatives (dichlormid, benoxacor, furilazole, R-29148), naphthopyranones (naphthalic anhydride), oxime ether derivatives (fluxofenim), 2,4-disubstituted thiazol-ecarboxylates (flurazole), phenyl pyrimidines (fenclorim), phenyl pyrazoles (fenchlorazole-ethyl) and quinolinoxycarboxylic acid esters (cloquintocet-methyl) (Hatzios, 1997). The mode of action of safeners is unclear but they are thought to exert their effect by either acting as antagonists at the target site in the plant, by preventing the herbicidal compound reaching the active site, or by means of reduced translocation or increased metabolism. Significantly, safeners are known to increase the levels of GSTs and mixed function oxidases involved in herbicide metabolism (Farago *et al.*, 1994). Therefore, increased metabolism would appear to be the most likely mechanism, with safeners enhancing the expression of specific detoxification enzymes in crop plants with activity toward specific herbicides (Hatzios, 1991). Examples of safeners and the herbicides they safen are shown in a number of species in Table 1.1. Safeners have also been shown to increase glutathione levels in the crop (Hatzios, 1997), although this mechanism is unlikely to increase herbicide selectivity since glutathione levels are rarely limiting during herbicide detoxification within the plant (Farago *et al.*, 1994).

Herbicide Class	Safener	Crop Usage
Chloroacetanilides	NA, Dichlormid Flurazole Fenclorim	Maize, wheat, rice Grain sorghum Rice
Sulphonylureas	NA, Dichlormid	Maize and sorghum
Aryloxyphenoxypropionates	Fenchlorazole-ethyl	Wheat, oats

Table 1.1 Herbicides and herbicide safeners used to protect specific crops from herbicide damage. Adapted from Hatzios, 1991.

Both theta-type (Jepson *et al.*, 1994) and tau-type (Dixon *et al.*, 1998a) GSTs have been shown to be safener-inducible in cereals. Thus, a number of GSTs are known to be responsive to enhancement by safeners in maize (Holt *et al.*, 1995, Wiegand *et al.*, 1986), wheat (Cummins *et al.*, 1997b), rice (Wu *et al.*, 1996) and sorghum (Gronwald *et al.*, 1987). The induction by dichlormid of *Zm*GST II-II in maize (Holt *et al.*, 1995), which has high activity toward the chloroacetanilides alachlor, acetochlor and metolachlor, can explain why dichlormid safens maize against these compounds. Dichlormid treatment results in a two to three-fold increase in GST activity in maize seedlings, with maximal induction achieved around 40 h post application (Jepson *et al.*, 1994). Safeners are not reported to increase herbicide tolerance in dicotyledons, although the herbicide oxadiazon increased GST activities in chickpea and broad bean (Hunaiti & Ali, 1991) and dichlormid enhanced GST activities in pea (Edwards, 1996).

1.2.8 Regulation of soybean GSTs

Like many other plant species, GSTs in soybean have been shown to be induced by exposure to auxins, non-auxin analogues, other plant hormones, heavy metals, hydrogen peroxide, DTT, GSH, salicylic acid and jasmonic acid (Ulmasov *et al.*, 1995). In nine-day-old soybean plants, treatment with 2,4-D and several auxin inhibitors, including 2,3,5-tri-iodobenzoic acid (TIBA), 2-(1-pyrenoyl)benzoic acid (PBA) all resulted in the increased accumulation of a 26 kDa GST subunit. This accumulation was maximal after 24 h incubation, and then decreased during the 48 h sampling period (Flury *et al.*, 1995). Furthermore, the addition of the *p*-nitrodiphenyl ether herbicide oxyfluorfen to soybean cell suspension cultures resulted in a six-fold increase in GST activity (Knörzer *et al.*, 1996).

1.2.9 Plant GST sequences.

Despite their importance in herbicide metabolism, GSTs have only been studied at the molecular level in a limited number of crops, although a large number of GST genes have been identified from a wide range of plants. Table 1.2 summarises all the plant GSTs identified to date. However, it is thought that this list will expand greatly over

forthcoming years. For example, an examination of the *Arabidopsis* expressed sequence tag (EST) database identified over 200 sequences showing significant homology to GSTs (Alfentino *et al.*, 1998).

Enzyme	Type	Molecular Mass	Regulation	Enzyme Activity	Accession No.	Reference
Arabidopsis						
BAC T7N9	Tau	nd	nd	nd	AC000348	Unpublished
ERD11	Theta	23.5 kDa	Dehydration	nd	P42760	Kiyosue et al., 1993
pm24	Theta	24 kDa	Ethylene	CDNB, GPOX, Auxin binding	p46422	Zhou & Goldsbrough 1993
ERD13	Theta	24.2 kDa	Dehydration	nd	P42761	Kiyosue et al., 1993
GST5	Tau	25.9 kDa	Wounding / HS	CDNB	P46421	Watahiki et al., 1995
AT GST TH	IV	nd	nd	nd	AB010072	Dixon et al., 1998a
pm239X14	Theta	25 kDa	nd	CDNB, GPOX	P42769	Bartling et al., 1993
*Maize**						
ZmGST I	Theta	29 kDa	Constitutive/ safener inducible	CDNB, alachlor, atrazine.	P12653	Mozer et al, 1983. Moore et al, 1986, Shah et al.,1986
ZmGST II	Theta	27 kDa	Safener inducible	Acetochlor, alachlor, metolachlor, GPOX.	P46420	Jepson et al., 1994; Holt, 1995.
ZmGST III	Theta	26 / 26 kDa Homodimer	Constitutive	CDNB, alachlor, metolachlor.	Pat. WO 96/23072 A1	Timmerman, 1989.
ZmGST V	Tau	28.5 kDa	Safener Inducible	CDNB, EA, metolachlor, fluorodifen, GPOX	Y12862	Dixon et al., 1998a
ZmGST VI	Tau	27.5 kDa	nd	nd	NA	Dixon et al. 1998a
Bronze-2	Tau	26 kDa	Cd, ABA	CDNB, cyanidin-3-glucoside	U14599	Marrs et al., 1995.
Soybean						
GmGST1	Tau	26 kDa	HS, Auxin, HM, SA etc.	CDNB, EA, NBC, VP, actifluorfen, fomesafen, fluorodifen, chlorimuron , metolachlor, GPOX	P32110	Ulmasov et al., 1995 Skipsey et al., 1997.
Tobacco						
parA/Nt114	Tau	25.7 kDa	2,4-D, Cd, SA	CDNB	P25317	Takahashi et al., 1989
parB	Theta	27 kDa	2,4-D	CDNB	P30109	Takahashi & Nagata, 1992b
parC/Nt107	Tau	nd	2,4-D	CDNB	P49332	Takahashi & Nagata, 1992a
C-7	Tau	nd	2,4-D	nd	X64399	Takahashi & Nagata, 1992a
Nt 103	Tau	25.7 kDa	2,4-D	CDNB	Q03664	Van der Zaal et al., 1991

Wheat*						
T. tauschii GST	Tau	26 kD	Safener inducible	nd	AF004358	Riechers et al., 1997
GST A1	Theta	29 kDa	Pathogen attack	CDNB	P30110	Dudler et al., 1991, 1993
TaGST T2	Zeta	nd	nd	nd	AF002211	Unpublished
TaGST 1	Tau	25 kDa	Safener inducible	CDNB, metolachlor, GPOX	N.A	Cummins et al.,1997b
TaGST 2	Tau	26 kDa	Safener inducible	CDNB, fenoxaprop-ethyl, atrazine, metolachlor, GPOX	N.A	Cummins et al.,1997b
TaGST 3	Tau	26 kDa	Safener inducible	CDNB, fenoxaprop-ethyl, atrazine, metolachlor, GPOX	N.A	Cummins et al.,1997b
TaGST 4	Tau	24 kDa	Safener inducible	CDNB, metolachlor, GPOX	N.A	Cummins et al.,1997b
Others						
Carica papaya	Tau	nd		nd	AJ000923	Unpublished.
Carnation						
DC SR8	Zeta	25 kDa	Ethylene	nd	P28342	Meyer et al. 1991b
Eucalyptus globulus	Tau		nd	nd	U80615	Unpublished
Hyoscyamus muticus	Theta	25 kDa	2,4-D, 2,4,5-T, 2,3-D	CDNB, auxin binding	P46423	Bilang et al., 1993
Mesembryanthemum crystallinum	Tau	nd	nd	nd	AF079511	Unpublished
Petunia						
PH GST/An9	Theta	nd	nd	CDNB	Y07721	Alfentino et al., 1998
Picea (Spruce)						
SB18	Tau	nd	nd	nd	AF051214	Unpublished
SB52	Tau	nd	nd	nd	AF051238	Unpublished
GST		23 kDa		Alachlor, fluorodifen	NA	Pflugmacher and Schröder
Potato PRP1	Tau	26 kDa	Fungal infection	CDNB, auxin binding	P32111	Taylor et al., 1990
Potato	Theta	nd	nd	nd	AF002692	Unpublished

Rice (Oryza sativa)						
ʔ						
Rgstl	Theta	nd	Safener	Pretilachlor	AJ002380	Wu et al., 1998a
Rgst II	Theta	nd	Safener	Pretilachlor	AJ002381	Wu et al., 1998b
AS1872	Tau	nd	nd	nd	AA753049	Unpublished
Silene cucubalus	Theta		Constitutive	nd	Q04522	Kutchan and Hochberger, 1992
Sorghum						
A1/A1		26 kDa	Constitutive	CDNB, metolachlor, alachlor, EA,GPOX, atrazine	NA	Gronwald and Plaisance,1998
B1/B2		26 / 28kDa	Inducible	CDNB, metolachlor, alachlor, EA, GPOX. atrazine	NA	Gronwald and Plaisance, 1998
Sugarcane	Theta	22.5 / 24 kDa heterodimer		DCNB, EA		Singhal et al., 1991
Vigna Radiata						
MII-4	Tau	nd	nd	nd	U20809	Chen et al., 1996

Table 1.2. Summary of all GSTs identified in plants to date. GenBank / SwissProt database accession numbers are given. NA = data not available. The maize GSTs have been classified according to the nomenclature provided by Dixon et al., 1998a. nd = not determined / not reported.

GST-like sequences have also been identified in broccoli (*Brassica oleracea* Acc. no, S43401,) (Lopez et al., 1994) and GST isoenzymes determined in chickpea (*Cicer arietinum*) (Hunaiti and Ali, 1990), French bean (*Phaseolus vulgaris*) (Edwards and Dixon, 1991), pea (Edwards and Dixon, 1991; Edwards, 1996), pumpkin (Fujita et al., 1995). GST activities have been characterised in many weed species including black-grass (*Alopercurus myosuroides*) (Cummins et al., 1997a) velvetleaf (*Abutilon theophrasti*) (Anderson & Gronwald, 1991) and giant foxtail (*Setaria faberi*) (Hatton et al., 1998).

1.2.9.1 GSTs in monocotyledonous plants.

Maize GSTs

GST activity in plants was first described in maize in 1970 (Frear and Swanson, 1970) and since then much progress regarding the isoenzyme profile in maize has been made. The identification of a GST activity in maize that catalysed the conjugation of glutathione to the herbicide atrazine first indicated the importance of GST enzymes in herbicide selectivity (Frear and Swanson, 1970). As such, that finding led the search for other herbicide active isoforms.

A number of constitutive GSTs have been characterised in maize with activity towards the chloroacetanilide herbicides metolachlor, alachlor and acetochlor (Table 1.2). In addition, the regulation of selected GSTs in cereals by herbicide safeners is a useful characteristic in their identification. Indeed, GSTs in maize have been shown to be enhanced following treatment with the safeners dichlormid (Holt *et al.*, 1995), benoxacor (Fuerst *et al.*, 1993; Irzyk and Fuerst, 1993; Dean *et al.*, 1991) and BAS 145-138 (Ekler *et al.*, 1993).

In total six GST subunits have been identified in maize. Historically, these subunits were numbered in their order of discovery and order of elution during anion-exchange separation. Thus, maize GST I eluted first following anion-exchange separation and was discovered before GST-III or GST-IV. Recently, with the subsequent discovery of new isoforms, this nomenclature has become confusing. This has now been addressed and the new nomenclature proposed by Dixon *et al.*, 1998b is used from now on in this thesis. The maize isoforms characterised to date are the theta-type enzymes *Zm*GST I (Mozer *et al.*, 1983; Wiegand *et al.*, 1986; Dixon *et al.*, 1997), *Zm*GST II (Jepson *et al.*, 1994; Holt *et al.*, 1995), *Zm*GST III (Moore *et al.*, 1986; Grove *et al.*, 1988; Dixon *et al.*, 1998c) and the tau-type enzymes *Zm*GST V (Dixon *et al.*, 1998a), *Zm*GST VI (Dixon *et al.*, 1998a) and Bronze-2 (Marrs *et al.*, 1995). These subunits are known to dimerise to give the native enzymes: *Zm*GST I-I (formerly GST-I), *Zm*GST I-II (formerly GST-II), GST II-II (formerly GST-IV), GST III-III (formerly GST III), GST V-V and GST V-VI which have overlapping but definable substrate specificity (Dixon *et al.*, 1997,1998b). It is now known that the 30 kDa monomeric protein formerly described as a GST which catalysed the glutathione

conjugation of phenyl-propanoids, such as cinnamic acid (Dean *et al.*, 1995) is an ascorbate peroxidase rather than a conventional GST (Dean *et al.*, 1997).

*Zm*GST I-I

Zm GST I-I was first purified from etiolated maize tissue by Mozer *et al.*, (1983). It was found to be a constitutively expressed homodimer consisting of 29 kDa subunits, which could be marginally induced following safener treatment (Holt *et al.*, 1995). *Zm*GST I-I possessed high activity toward CDNB but only modest activity toward chloroacetanilide herbicides. Later studies confirmed these findings and showed additional activity toward fluorodifen (Holt *et al.*, 1995; Dixon *et al.*, 1997), ethacrynic acid and atrazine (Dixon *et al.*, 1997). No activity was detected toward the glutathione peroxidase substrate cumene hydroperoxide. Both Wiegand *et al.* (1986) and Grove *et al.* (1988) published similar cDNA sequences corresponding to *Zm*GST I, and expressed the respective recombinant enzyme in *E. coli* (Grove *et al.*, 1988). In addition, Shah *et al.* (1986) have shown that the genomic DNA sequence of *Zm*GST I, contains two introns.

*Zm*GST I-II

Zm GST I-II was first purified by Mozer *et al.* (1983) and was only detected in etiolated maize seedlings following treatment with herbicide safeners. It eluted later than *Zm* GST I-I following anion exchange separation and was a heterodimer consisting of 29 kDa and 27 kDa subunits (Holt *et al.*, 1995). N-terminal protein sequencing determined that the 29 kDa subunit was identical to that in *Zm* GST I-I. No protein sequence information was obtained for the 27 kDa subunit. Reported GST activity shows that *Zm*GST I-II has lower activity toward CDNB but higher activity toward alachlor, metolachlor and fluorodifen herbicide substrates (Mozer *et al.*, 1983; Holt *et al.*, 1995; Dixon *et al.*, 1997). Antibodies raised to the 27kDa subunit were used to screen a maize cDNA library, which resulted in the isolation cDNAs corresponding to the 27 kDa subunit (Jepson *et al.*, 1994), now termed *Zm*GST II.

*Zm*GST III-III

*Zm*GST III-III was first purified using Orange A affinity chromatography (Timmerman and Tu, 1987), however it was initially incorrectly identified as a

heterodimer of *Zm*GST I and *Zm*GST III. Later studies showed it in fact to be a homodimer of *Zm* GST III-III, the misidentification probably having arisen due to contamination with *Zm*GST I-I. It is best characterised through molecular studies, whereby the cDNA encoding the enzyme is expressed in *E. coli* (Moore *et al.*, 1986). However, two different cDNA sequences reputed to encode *Zm*GST III have been reported (Grove *et al.*, 1988; Dixon *et al.*, 1998c). Subsequently, some ambiguity has existed as to the nucleotide sequence of the GST-III subunit. This has recently been resolved with the finding of two, near identical sequences (Dixon *et al.*, 1998c). The expression of *Zm*GST III-III has not been characterised in depth.

*Zm*GST II-II

*Zm*GST II-II, originally termed GST IV, was first purified by Irzyk and Fuerst (1993). It is a homodimer composed of 27 kDa subunits, which are identical to the 27 kDa subunit present in *Zm* GST I-II (Irzyk and Fuerst, 1993; Jepson *et al.*, 1994). *Zm*GST II-II is present at low levels in maize roots and is expressed at high levels in leaf tissue following safener treatment, confirming that *Zm*GST II is a safener-inducible subunit. In addition it has been shown that *Zm*GST II is enhanced following treatments with auxin, herbicides and glutathione (Dixon *et al.*, 1998a). Both Irzyk and Fuerst (1993) and Holt *et al.* (1995) showed that *Zm*GST II-II can be purified using either the affinity matrix *S*-hexyl glutathione or sulphobromophthalein-*S*-glutathione and that the enzyme eluted later than *Zm*GST I-I and *Zm*GST I-II during anion exchange separation. Purified *Zm* GST II-II has high activity toward the chloroacetanilide herbicide substrates acetochlor, alachlor and metolachlor but no activity toward CDNB (Irzyk and Fuerst, 1993). The *Zm*GST II subunit is also reported to possess glutathione peroxidase activity toward cumene hydroperoxide (Dixon *et al.*, 1997). cDNA clones encoding the *Zm* GST II subunit have been isolated by both Jepson *et al.* (1994) and Irzyk and Fuerst (1995).

Zm GST V-V

*Zm*GST V-V is a tau-type GST and was first characterised by Dixon *et al.* (1998a). It is a homodimer of 28.5 kDa subunits, and was purified from safener treated etiolated maize seedlings using *S*-hexyl glutathione chromatography. It has activity toward

CDNB, the diphenyl ether herbicide fluorodifen as well as detoxified toxic alkenal derivatives and also has glutathione peroxidase activity toward organic hydroperoxides (Dixon *et al.*, 1998). *Zm*GST V-V was shown to be enhanced following treatment with the herbicide safener dichlormid, therefore determining that both theta and tau type GSTs are responsive to safener treatment. Antibodies, raised to the *Zm* GST V-VI heterodimer, were used to screen a maize cDNA expression library, which resulted in the cDNA corresponding to *Zm*GST V being obtained.

Zm GST V-VI

Zm GST V-VI is a heterodimer of the 28.5 kDa *Zm* GST V subunit and a 27.5 kDa subunit, *Zm* GST VI (Dixon *et al.*, 1998a). *Zm* GST VI has not been characterised in detail.

Bronze-2

Bronze-2 was reported my Marrs *et al.* (1995). From sequence homology this enzyme belongs to the GST tau-class, but differs from the other maize enzymes described in that it has a defined endogenous function. As discussed previously, Bronze-2 has been implicated in the transportation of anthocyanin precursors into the vacuole.

Other Isoforms

Despite repeated attempts, the GST responsible for atrazine conjugation in maize is not well characterised, however there would appear to be greater atrazine conjugating activity in shoots than roots (Frear and Swanson, 1970; Shimabukuro *et al.*, 1970).

Wheat GSTs

Several herbicides used to control weeds in wheat (*Triticum aestivum* L.) owe their selectivity to rapid glutathione-mediated detoxification in the crop. Such compounds include the chloroacetanilides, dimethenamid and fenoxaprop-ethyl. As with maize, many herbicides used with wheat require the use of herbicide safeners to achieve selective weed control. However, the GSTs catalysing these conjugations have only recently been characterised in detail. Wheat appears to contain a similarly complex profile of tau- and theta-type GSTs to maize, with both constitutive and safener inducible isoforms present (Riechers *et al.*, 1996; Cummins *et al.*, 1997b).

Additionally, the GSTs in wheat seem to be immunologically related to those in maize (Cummins *et al.*, 1997b). GSTs were first purified from wheat flour by Williamson and Beverley (1988), with an enzyme composed of two 27.5 kDa subunits being identified. Further studies by Anderson *et al.* (1993) using DEAE anion exchange chromatography, showed the presence of seven distinct GST enzymes composed of subunits with molecular masses between 26.2 kDa and 35.2 kDa. A detailed purification of the different GSTs present showed that these enzymes in wheat could be separated into polar and hydrophobic types, which could then be further resolved based on their affinity for *S*-hexylglutathione agarose (Cummins *et al.*, 1997b). Four distinct subunits were determined, termed *Ta*GST1 (25 kDa), *Ta*GST2 (26 kDa), *Ta*GST 3 (26 kDa) and *Ta*GST 4 (25 kDa). *Ta*GST 1-1 consisted of two variants, *Ta*GST 1a and *Ta*GST 1b which showed immunological relatedness to the tau-type *Zm*GST V-VI and dimerised to give the most abundant enzyme in non-safener treated material. Accumulation of the enzymes *Ta*GST 1-2, *Ta*GST 1-3 and *Ta*GST 1-4 occurred following safener treatment. Pascal *et al.* (1998) also showed the presence of constitutive and safener-inducible isoforms and, using high performance reversed phase chromatography, suggested there may be as many as 20 GST subunits in wheat. Tal *et al.* (1993) suggested that the selectivity of fenoxaprop-ethyl in wheat may be due to the rapid non-enzymic conjugation with glutathione. However, findings by Edwards and Cole (1996) and Cummins *et al.* (1997b) show that GSTs with activity toward fenoxaprop-ethyl are present in wheat, suggesting catalysis of the reaction is likely to occur. Indeed GSTs have now been identified in safener-wheat treated that possess activity toward the herbicide substrates fenoxaprop-ethyl, atrazine, metolachlor, fluorodifen (Cummins *et al.*, 1997b) and dimethenamid (Riechers *et al.*, 1996) and a cDNA encoding a GST subunit with activity toward dimethanimid has been identified (Riechers *et al.*, 1997). In addition to their regulation by herbicide safeners, one theta-type GST was seen to be induced following infection of wheat with a fungal pathogen (Dudler *et al.*, 1991; Mauch and Dudler, 1993).

Sorghum GSTs

GSTs in sorghum (*Sorghum bicolor* L. Moench) were first identified by Gronwald *et al.* (1987). It was discovered that, like other monocotyledonous species, a number

safeners were able to enhance GST activity toward the herbicide metolachlor (Gronwald *et al.*, 1987). Anion-exchange chromatography showed that the number of isoforms present in sorghum with activity toward CDNB or metolachlor increased from two to seven following treatment with the safener oxabetrinil (Dean *et al.*, 1990). Two GST enzymes, GST A1/A1 and B1/B2 have been characterised in sorghum (Gronwald and Plaisance, 1998). The homodimer GST A1/A1 consists of 26 kDa subunits, and has high activity toward CDNB. The heterodimer GST B1/B2 consists of 26 and 28kDa subunits respectively, and possessed high activity toward the herbicide metolachlor. Both enzymes also exhibited glutathione peroxidase activity. N-terminal analysis of the A1, B1 and B2 subunits indicated a high degree of homology with the theta-type maize *Zm*GST I (Gronwald and Plaisance, 1998).

Rice GSTs

Unlike other cereals, the GSTs in rice (*Oryza sativa*) have not been studied in detail. Wu *et al.*, 1996 showed that the herbicide safener fenclorim can protect rice against injury caused by the chloroacetanilide herbicide pretilachlor by enhancing GST activity, and two partial GST cDNAs obtained from the safener treated roots of rice, have been reported (Wu *at al.*, 1998a/b).

1.2.9.2 GSTs in dicotyledonous plants.

Tobacco GSTs

Transcripts encoding GSTs were first identified in tobacco due to their up-regulation by auxins and were not initially identified as GSTs. It was later determined that one of the respective cDNAs, *Nt*103, encoded an enzyme that displayed GST activity toward CDNB (Droog *et al.*, 1993). Many *Nt*103 homologues have now been described in a number of species and this family has been classified as the type-III or tau-class (Droog, 1997). With the exception of the theta-type enzyme *par*B (Takahashi and Nagata, 1992b) all the tobacco GSTs characterised to date, *Nt*107, (Van der Zaal *et al.*, 1987), *par* C (Takahashi and Nagata, 1992a) and C-7 (Takahashi and Nagata, 1992a) show homology to the tau class.

GSTs in Legumes.

Prior to the work described in this thesis, GST activities had been identified in extracts of Chickpea (*Cicer arietinum*) (Hunaiti and Ali, 1990), French bean, alfalfa (Edwards and Dixon, 1991), mung bean (*Vigna radiata*) (Chen *et al.*, 1996), soybean (Flury *et al.*, 1995) and pea (Diesperger and Sandermann, 1979; Williamson and Beverley, 1988; Edwards, 1996). From the limited work carried out in legumes, the GSTs would appear significantly different to those in cereals. In particular, the diphenyl ether herbicides fluorodifen in pea, and fomesafen and acifluorfen in soybean, are selective in these legumes due to rapid (homo)glutathione conjugation (Frear and Swanson, 1983; Cole, 1996), but are not selective in maize. Similarly herbicides such as atrazine, which are readily detoxified by glutathione conjugation in maize, are not selective in legumes (Lamoureux & Rusness, 1993). Therefore, the GSTs involved in herbicide metabolism in soybean exhibit interesting specificity differences to the GSTs characterised in maize.

Legumes are of particular interest in that the predominant low molecular weight thiol present in some species is not glutathione but homoglutathione (Price, 1957: Breaux, 1986; Klapheck, 1988). The presence of homoglutathione in legumes is not universal. Members of the *Phaseoleae*, which includes soybean, mung bean, French bean and runner bean (*Phaseolus coccineus*) contain predominantly homoglutathione. Indeed, virtually all (>99%) of the available thiol in soybean is homoglutathione (Klapheck, 1988). In these species homoglutathione is utilised by the plant in the conjugation of herbicides (Frear *et al.*, 1983; Breaux, 1986; Brown and Neighbors, 1987; Evans *et al.*, 1987). However, species belonging to the *Trifolieae*, which include the clovers, contain a mixture of homoglutathione and glutathione, while *Vicieae*, such as pea and broad bean, contain glutathione only (Klapheck, 1988). Indeed, herbicide conjugates characterised from *Vicieae* species are all glutathione derivatives (Breaux, 1986).

The reasons for the selective accumulation of homoglutathione in certain legumes is not well understood. Experimental evidence suggests the formation of glutathione and homoglutathione is dependant on distinct (homo)glutathione synthetases, with different affinity for glycine and β-alanine, rather than one enzyme dependant on the bio-availability of either substrate (Macnicol, 1987). Thiol specificity of plant GSTs has never been assessed, although studies with mammalian species suggest

differences in affinity between enzymes does exist (Adang *et al.*, 1988). Evidence gained from the study of mammalian and yeast glutathione synthetases show that these enzymes are unable to utilise β-alanine as a substrate (Rathbun *et al.*, 1977; Mooz and Meister, 1967).

Although alternative glutathione derivatives have been reported in other species such as wheat (γ-glutamyl-cysteinyl-serine) (Klapheck *et al.*, 1991) and maize (γ-glutamyl-cysteinyl-glutamate) (Hell, 1997), the affinity of plant GSTs for these various thiols has not been reported.

GSTs in Soybean.

Like tobacco, the few reported soybean GST cDNA sequences were not initially identified as encoding GSTs. A 26 kDa heat shock inducible protein, termed *Gmhsp26-a* (Czarnecka *et al.*, 1988) or *GH2/4* (Ulmasov *et al.*, 1995) was later found to possess GST activity toward CDNB (Ulmasov *et al.*, 1995). The respective cDNA, now termed *Gm*GST1 (Skipsey *et al.*, 1997), was expressed in *E. coli* and the recombinant GST purified using *S*-hexylglutathione agarose. In addition to the GST activities reported by Ulmasov *et al.* (1995), the recombinant protein was found to be active toward the herbicide substrates acifluorfen, fomesafen, fluorodifen, chlorimuron-ethyl and metolachlor as well as natural stress metabolites and hydroperoxides. Studies using the *Gm*GST1 promoter fused to the reporter gene β-glucuronidase (GUS) showed that expression was upregulated in response to heat shock, treatment with auxins and other phytohormones, compounds resembling auxins but with no activity as phytohormones, heavy metals, H_2O_2, glutathione, salts and environmental stresses (Ulmasov *et al.*, 1995). Like many other auxin-inducible GSTs, *Gm*GST1 belongs to the tau-class.

In addition to *Gm*GST1, Flury *et al.* (1995) described the purification of a GST from soybean seedlings which accumulated following treatment with 2,4-D. The native 49 kDa enzyme consisted of two 26 kDa subunits, was highly active in catalysing glutathione conjugation to CDNB (4900 nmol min^{-1}mg protein^{-1}) and showed some activity toward the herbicide metolachlor (24 nmol min^{-1} mg^{-1}), but no activity toward the herbicides atrazine or fluorodifen. N-terminal amino acid sequence was determined for the enzyme, and this is discussed further in chapter five. Induction of

the enzyme was also observed following treatment with tri-iodobenzoic acid (TIBA), which inhibits auxin transport with consequential auxin accumulation. However, accumulation was not apparent following treatment with IAA or NAA, even though physiological auxin-effect was observed with these treatments, suggesting that the increase in GST activity was not related to auxin activity or to changes in auxin levels *per se*.

Whilst most GSTs are reported to be localised in the cytosol, GST activity in soybean has been reported in the apoplastic fluid (Flury *et al.*, 1996). Flury *et al.* (1996) described the purification of several GST isoenzymes from soybean, all of which had activity toward CDNB and had additional activities as glutathione peroxidases, reducing the hydroperoxides of linolenic acid and arachidonic acid to their respective monohydroxy-alcohols. Both GST and glutathione peroxidase activity was detected in the apoplastic fluid, although they were only apparent following treatment of the seedlings with TIBA. Inhibition of the golgi-based protein secretory pathway by monensin resulted in decreased apoplastic GST activity in TIBA treated hypocotyls, suggesting that the GST(s) were being actively secreted. Homoglutathione could be detected in the apoplast, and it was hypothesised that homoglutathione conjugates could be formed in the apoplast and then taken up by the cell via a plasma membrane transporter for further processing.

Besides the soybean GSTs reported, the nucleotide sequence of a cDNA described as encoding the glutathione dependant enzyme glyoxalase-I from soybean (database accession P46417) (Paulus *et al.*, 1993) shows high homology to tau-type GSTs. Glyoxalase-I catalyses the first step in the conversion of the cytotoxic compound methylglyoxal to D-lactate via the intermediate *S*-D-lactoylglutathione, an activity not previously associated with GSTs. GSTs are widely described as multi-functional enzymes (Marrs, 1996) and it was therefore of interest, to determine whether GSTs in soybean are able to function as glyoxalase I enzymes.

1.3 Aims of the Project.

From reviewing the literature at the beginning of this programme, it was clear that GSTs in cereals had well defined roles in herbicide metabolism and selectivity. However, in soybean, although herbicide detoxification by conjugation with homoglutathione had been shown to be a major determinant of the selectivity of many herbicides, the involvement of GSTs had not been established. The aims of this project were therefore:

1. To determine the range of GST activities in seedlings of soybean and a range of associated problem weeds and the role they play in selectivity, with GST activity towards the main selective herbicides used in soybean being of primary interest. These herbicides include chloroacetanilide, sulphonylurea and diphenyl ether compounds.

2. To determine whether GST activity can be induced in soybean plants by herbicide or safener treatment in a similar fashion to that seen in cereals.

3. To identify and purify GST isoenzymes from soybean and determine their substrate specificity (endogenous and xenobiotic).

4. To clone the GSTs of interest and express them in recombinant bacteria. Access to a large quantity of enzyme would assist thorough characterisation of the enzyme.

5. To determine the substrate and thiol specificity of pure recombinant soybean GSTs.

6. To relate the recombinant soybean GSTs to the respective enzymes found in soybean plants and cell cultures.

2. Chapter Two. Materials and Methods.

2.1 Biochemical Techniques.

2.1.1 Chemicals.

All general chemicals were obtained from the Sigma chemical company Ltd, Poole, UK or BDH Laboratory supplies Ltd, Poole, UK unless otherwise stated. HPLC grade solvents were obtained from Rathburn Chemicals Ltd, Walkerburn, UK. Analytical grade acifluorfen and chlorimuron-ethyl were obtained from British Greyhound Chromatography and Allied Chemicals, Merseyside, UK. Fomesafen, metolachlor and acetochlor were supplied by Zeneca Agrochemicals, Bracknell, UK.

[u-^{14}C-phenyl] fomesafen (1.8 Gbq mmol^{-1} > 99.0% pure) was obtained from Zeneca Agrochemicals. Homoglutathione was obtained in two batches. The first batch was prepared by the Physical Chemistry department, Zeneca Agrochemicals with a purity of ≈20% and the second batch by Bachem (UK) Ltd, Saffron Walden, UK with a purity of >95% as determined by HPLC.

2.1.2 Biological material.

General Plant Material

Soybean (*Glycine max* L. Merr.) variety D297 (formerly ICI297) was obtained from Zeneca Agrochemicals and was chosen for use throughout this project due to good germination rates, and previous non-published findings that suggested that this line possessed superior GST activity. Weed species *Abutilon theophrasti* Medic., *Amaranthus retroflexus* (L.), *Digitaria sanguinalis* (L.) Scop., *Echinochloa crus-galli* (L.) Beauv., *Ipomoea hederacea* (L.) Jacq., *Setaria faberi* Herrm, and *Sorghum halepense* (L.) Pers. were obtained from Herbiseed Ltd, Wokingham, UK. All plants used for GST characterisation were germinated and grown in a controlled environmental chamber in moist vermiculite or Levington's universal compost at 25°C under a 16h photo-period using white fluorescent lighting of intensity of 140 μmol m^{-2}s^{-1}. At harvest, whole soybean plants were separated into roots and shoots,

washed, blotted dry and then weighed and frozen in liquid nitrogen prior to storage at -80°C. With respect to the weed species, only the foliage was harvested.

Chemical Spray Trials

Chemical spray trials were carried out at Zeneca Agrochemicals, Bracknell. Two-week-old soybean plants were used to determine GST activity and for the purification experiments. Plants subjected to safener treatments were grown for three weeks (section 3.2.6). All plant material was grown in John Innes potting compost no.3 in a glass-house (16 h light (20°C) / 8 h dark (16°C) cycle). Both herbicides and safeners were prepared for spraying using the formulant JF5969 (Synperonic detergent NPE1800 33.3 g l^{-1}, Tween 85 16.7 g l^{-1} in cyclohexanone). Treatments were applied using a track sprayer fitted with a 8002E T-jet nozzle delivering an equivalent of 200 l ha^{-1}.

Plants were visually assessed for herbicide damage at 7 and 13 days post application using a % damage scoring method. For biochemical analysis the foliage of the plants was harvested 48 h following application and frozen in liquid nitrogen for storage at -80°C.

Soybean Cell Suspension Cultures.

Callus cultures of soybean (cv. Mandarin) were acquired from Zeneca Agrochemicals and maintained by sub-culturing every 30 days onto Gamborg's minimal growth medium (3.2 g l^{-1}) supplemented with 20.0 g l^{-1} sucrose, 1.0 mg ml^{-1} 2,4-D and 0.8% (w/v) agar (pH 5.5). Plates were incubated in darkness at 27°C. Cell suspension cultures were initiated from these calli in the same nutrient medium without agar present by adding lumps of friable callus to 10 ml of liquid medium in 100 ml flask. The cultures were grown at 25°C, on an orbital shaker at 130 rpm in the dark and were sub-cultured every 7 days. After establishing a vigorous suspension culture, a 10 ml inoculum was transferred into 50 ml medium in a 250 ml conical flask. These cultures were then sub-cultured every 10 days using a 10 ml inoculum. At harvest, cells were collected on a polyester filter by suction filtration through a Büchner funnel. Recovered cells were blotted dry between sheets of Whatman® 3MM paper, then frozen in liquid nitrogen and stored at -80°C.

2.1.3 Protein extraction.

All protein extractions were performed on ice or at 4°C. Tissue was ground to a fine powder under liquid nitrogen using a pestle and mortar and the powder re-suspended in 3x (v/w) 0.1 M Tris-HCl pH 7.5 containing 2 mM EDTA, 1 mM DTT and 5% (w/v) PVPP, and homogenised in a Waring blender for 30s. The resulting homogenate was filtered through two layers of muslin and centrifuged (17,000 g, 20 min, 4°C). Protein was precipitated from the supernatant by slowly adding ammonium sulphate to 80% saturation and the pellet recovered by centrifugation as before. The protein pellet was stored at -20°C and after resuspending in buffer, desalted using a PD-10 column as recommended by the manufacturers (Pharmacia, Milton Keynes, UK) prior to use. For further protein purification, the pellet was dissolved in appropriate column loading buffer and dialysed against the same buffer (100x sample volume) overnight.

Protein Concentration Determination

Protein determination on crude samples were performed using the Bio-Rad™ dye binding reagent (BioRad, Hemel-Hemstead, UK) as recommended by the manufacturers with γ-globulin as the standard (Bradford, 1976). The protein concentration of pure recombinant GSTs (chapter 6) were determined using calculated extinction coefficients at λ_{280mn}. Extinction coefficients (1 mg ml⁻¹ protein solution / 1 cm light path) were calculated from the content of amino acid residues using the following formula (Gill and Von Hipple, 1989):

Protein (mg ml⁻¹) = ((((No. of Cys x 120) + (No. of Tyr x 1280) + (No. of Trp x 5690))/MW)/A_{280nm})

2.1.4 Analytical methods.

Thin-layer chromatography

Thin-layer chromatography (TLC) analysis of herbicide conjugates was performed using aluminium-backed TLC plates (20 x 20 cm) pre-coated with 0.2 mm silica containing a fluorescent indicator (Merck, Darmstadt, Germany). After loading, samples were developed (butan-1-ol : water : acetic acid 4:1:1 v/v). UV-absorbing metabolites were visualised under UV light (254 nm) and amino-containing

metabolites identified by spraying with ninhydrin with (0.3% in acetone) and then heating the plates with a hairdryer. Herbicide conjugates were identified by the coincidence of their UV absorbance and positive reaction with ninhydrin. With radiolabelled herbicides, metabolites were located using autoradiography, whereby the developed plate was exposed to X-ray film.

High Performance Liquid Chromatography (HPLC).

Four different HPLC methods using either a Gilson HPLC model linked to an auto-injector (system A), or single manual injector Gilson HPLC model (system B) were used. Analysis of results on each system was performed using Gilson 715 software and Gilson 712 software respectively. Solvents were of HPLC grade unless stated and were filtered through a 0.22 μM nylon filter (Millipore, UK) prior to use.

- **Method 1.** Reversed-phase analysis of herbicide conjugates (system A).

A Sepherisorb ODS1 column (250 mm x 4.6 mm, particle size 5 μm) (Fisons chromatography, UK) was used to resolve herbicides and herbicide conjugates. The flow rate through the column was 0.8 ml min^{-1} for all experiments with solvent A consisting of 1% phosphoric acid : 99% water and solvent B being acetonitrile. The column was equilibrated with 5% solvent B : 95% solvent A prior to use, and the metabolites eluted with a two step gradient of increasing solvent B 5% to 10% over 10 min, followed by 10% to 75% over 30 min. The column was then washed in 100% acetonitrile (5 min) and equilibrated in 5% acetonitrile (5 min) prior to the next injection. Products eluted from the column were detected by their absorbance at 264 nm.

- **Method 2.** Preparative purification of herbicide conjugates by Reversed-phase (system A).

Preparative HPLC, using the same gradient conditions as method 1, was used to purify chemically synthesised reference herbicide-glutathione conjugates prior to further analysis. A Partisil ODS-3 column (150 mm x 10 mm, particle size 5 μm) (Alltech, UK) was used at a flow rate of 1 ml min^{-1} and fractions collected at 1 min intervals using a BioRad 2110 fraction collector.

- **Method 3.** Reversed-phase analysis of polypeptides (system B).

HPLC analysis of purified enzymes was performed using a VYDAC C-18 column with solvent A = 0.1% TFA and solvent B = Acetonitrile + 0.08% TFA. The column was equilibrated in 90% A at 0.5 ml min^{-1} prior to sample application. Samples were loaded in 90% solvent B over a period of 5 min. Polypeptides were eluted with a two-step linear gradient of increasing solvent B 10% to 80% over 50 min. The column was then washed in 100% B (4 min) and re-equilibrated in 10% B (4 min) prior to the next injection. Proteins eluted from the column were detected by their absorption at 280nm. Individual peaks were collected manually for N-terminal protein sequencing and MALDI-TOF MS analysis. Where necessary the solvent was removed from the fractions under a stream of nitrogen.

- **Method 4.** Reversed-phase analysis of derivatised thiols (system A).

Bromobimane derivatised thiols (section 2.1.7) were separated on a PhaseSep ODS2 column (250 mm x 4.6 mm, particle size 5 μm) using 15% (v/v) methanol : 85% 50 mM K$_2$HPO$_4$ adjusted to pH 6.0 with glacial acetic acid at a flow rate of 0.5 ml min^{-1} to separate adducts. Bromobimane conjugates were detected by there florescence using a Gilson 121 fluorometer. At the conclusion of each run the column was washed with 100% methanol.

2.1.5 Enzyme assays.

Unless otherwise stated all enzyme assays were determined in triplicate and expressed as mean activity +/- standard deviation (n=3) (calculated using Microsoft Excel™).

2.1.5.1 Spectrophotometric assays.

1-chloro-2,4-dinitrobenzene (CDNB)

GST activity toward CDNB was determined by monitoring the increase in absorbance at 340nm. 0.9 ml 0.1 M potassium phosphate buffer pH 6.8 were placed in a 1 ml cuvette and 33 μl 30 mM CDNB (in ethanol, final concentration 1mM) added. The cuvette was incubated at 30°C for 5 min prior to the addition of 33 μl 100 mM glutathione or homoglutathione pH 7.0 (final concentration 3.3 mM), followed by

33μl (0-1mg) protein. To determine the non-enzymic rate, 33 μl extraction buffer was added in place of enzyme. The change in absorbance at 340 nm was observed over a 30 s period and the activity toward CDNB in nkatal (nkat) (1 nkatal = 1nmol product produced per second) determined using the extinction coefficient for the reaction products $\Delta\varepsilon_{340nm}$= 9.6 mM^{-1}cm^{-1} (Habig *et al.*, 1974). As such, activity toward CDNB in a 1 ml assay was calculated as follows.

$$\Delta OD_{340nm} \ min^{-1} \ x \ 1.74 = nkat \ activity$$

Specific activity was quoted as nkat mg^{-1} protein.

Ethacrynic Acid

900 μl of 100 mM potassium phosphate buffer, pH 6.5, 25 μl 8 mM ethacrynic acid (in ethanol) and 25 μl enzyme solution (extraction buffer for chemical rate) were added to a 1 ml quartz cuvette and incubated at 30°C for 5 min. The reaction was started by the addition of 50 μl 100 mM thiol co-substrate and the change in absorbance at 270 nm monitored for 2 min. The amount of conjugate formed in the reaction was calculated using the extinction coefficient $\Delta\varepsilon_{270nm}$ = 5.0mM^{-1}cm^{-1} (Habig *et al.*, 1974).

Therefore: nmol of conjugate formed = (OD change (+enzyme) - OD change (-enzyme)) x 200.

Glutathione Peroxidase Assay.

The method used to measure glutathione peroxidase activity was based upon the procedure of Heath and Tappel (1976). 0.5 ml reaction buffer (0.25 M potassium phosphate pH 7.0, 2.5 mM EDTA, 2.5 mM sodium azide), 100 μl glutathione reductase (6 units ml^{-1}), 100 μl 10 mM glutathione pH 7.0 and 100 μl 2.5 mM NADPH (in 0.1% sodium hydrogen carbonate) were added to a 1 ml cuvette and incubated at 37°C for 10 min. The reaction was started by the addition of 100 μl 12 mM cumene hydroperoxide and 100 μl enzyme solution (or extraction buffer for control). The reaction was monitored by recording the decrease in absorbance at 366 nm over 2 min.

Enzyme activity was reported as OD change min^{-1}mg^{-1} protein and could be converted to nkat using the extinction co-efficient for NADPH (A$_{366nm}$ = 2.83 mM^{-1}cm^{-1}).

Glyoxalase I

100 μl 35 mM methylglyoxal, 100 μl 100 mM GSH/hGSH pH 7.0 and 850 μl assay buffer (0.1 M sodium phosphate pH 7.5 + 16 mM $MgSO_4$) were added to a 1 ml quartz cuvette and incubated at 30°C for 5 min. The reaction was started by the addition of 25 μl enzyme extract and the change in absorbance monitored at 240 nm over 30 s. Enzyme activity was calculated using the extinction coefficient $A_{240nm} =$ 3.37 mM^{-1} cm^{-1} (Vander Jagt *et al.*, 1972).

2.1.5.2 GST activity toward herbicide substrates.

Chemical synthesis of herbicide-glutathione conjugates.

Herbicide conjugates of fomesafen and chlorimuron-ethyl were synthesised by incubating 10 mM herbicide (1 ml) with 100 mM glutathione in 100 mM Tris-HCl pH 9.0 (9 ml) under aseptic conditions. The reaction was allowed to proceed for 5 days at room temperature. The conjugate was resolved from non-reacted glutathione and herbicide using HPLC method 2, by collecting 1 ml fractions. The purity of the conjugates was determined by thin layer chromatography and HPLC (method 1). The glutathione conjugates of fomesafen and chlorimuron-ethyl were analysed by Dr David O'Hagen, Dept. of Chemistry, University of Durham by Fast Atom Bombardment Mass Spectrometry (FAB-MS) to confirm their authenticity.

High Performance Liquid Chromatography (HPLC) Assays.

HPLC assays were established to monitor GST activities toward herbicides. The herbicide (10 μl of 10 mM, final concentration 0.5 mM) were added to an eppendorf containing 50 μl assay buffer (0.4 M Tris-HCl pH 9.5 for fomesafen, chlorimuron-ethyl, and acifluorfen or 0.1 M potassium phosphate pH 6.8 for metolachlor and acetochlor). 120 μl enzyme extract (0-2 mg protein) were added and the reaction started by the addition of 20 μl 100 mM thiol co-substrate (final concentration 10 mM). Controls consisted of i) omitting glutathione or homoglutathione to correct for any co-chromatographing material, and ii) omitting the enzyme to correct for the non-enzymic rate of conjugation. The sample tubes were incubated at 37°C for 1 h and the reaction stopped by the addition of 5 μl 6 N HCl. The samples were freeze/thawed

and centrifuged at 13,000 g for 2 min to remove precipitated protein, prior to product separation using HPLC method 1.

Authenticity of the herbicide glutathione conjugates produced was confirmed by comparing the retention time with the chemically synthesised standards.

Assay with Radiolabelled Fomesafen.

The assay was based on the principle of separation of the radiolabelled hydrophilic glutathione conjugate product from the [^{14}C]-fomesafen by partitioning with organic solvent (diethyl ether). The unconjugated herbicide partitioned into the ether, whereas the conjugate remained in the lower, aqueous phase. Therefore, the reaction rate was determined by observing the increase in radioactivity in the aqueous phase.

5 µl [^{14}C]-Fomesafen (0.045 µCi) were placed into a 1 ml eppendorf tube containing 25 µl 0.4 M Tris-HCl pH 9.5. 60 µl enzyme extract were added and the reaction initiated with 10 µl 100 mM thiol co-substrate. The reaction mixture was incubated at 37°C for 30 min. Controls containing no enzyme were included to detect any non-enzymic reaction rate. The reaction was stopped with 6 N HCl (5 µl), and diethyl ether (600 µl) added immediately. Each tube was vortexed for 10 s and centrifuged (13,000 g) for 2 min. 50 µl of the lower, aqueous phase were added to scintillation fluid (4 ml, Ecoscint, National Diagnostics, UK) and radioassayed using a Packard scintillation counter operating with an external standard for quench correction.

2.1.6 Free thiol determination.

Bromobimane Derivatisation.

Thiol labelling with monobromobimane was performed using a protocol modified from Cummins *et al.* (1997a). Tissue (1 g) was weighed accurately, frozen in liquid nitrogen, ground to a fine powder and transferred to a clean tube containing 3 ml 0.1 M HCl. After incubation on ice for 30 min with occasional mixing, the slurry was transferred to an eppendorf tube and centrifuged (13,000 g, 3 min). Two volumes of supernatant (100 µl) were each transferred to two clean eppendorfs, and 10 µl water added to one and the other spiked with 1 mM glutathione or homoglutathione (10 µl). Available thiols were reduced by adding 10 µl 1 M NaOH followed by 10 µl 1 M

NaOH containing 20 mg ml^{-1} NaBH$_4$ and the solution incubated for 10 min at room temperature. The reaction was stopped by the addition of 10 µl 3.6 M HCl and the samples centrifuged (13,000 g, 5 min). The supernatant (100 µl) was transferred to a fresh tube and 10 µl 5 mg ml^{-1} monobromobimane dissolved in acetonitrile added, followed by 5 µl of 35% v/v *N*-ethylmorpholine. The samples were placed in the dark for 20 min, and the reaction stopped by the addition of 880 µl 5% acetic acid (5 µl v/v).

A standard curve was prepared by derivatising glutathione or homoglutathione (0-20 mmol), and the *S*-bimane conjugates analysed using HPLC method 4.

2.1.7 GST purification using column chromatography.

All protein purification work was performed using a "Gradifrac" apparatus (Pharmacia). The elutant was monitored for UV absorbance at 280 nm and fractions collected using an automated fraction collector.

2.1.7.1 Synthesis of affinity columns.

S-hexylglutathione was synthesised from glutathione and iodohexane (Mannervik and Guthenberg, 1981). *S*-hexylglutathione, glutathione and homoglutathione were linked to epoxy-activated sepharose 4B using the following procedure.

Epoxy-activated Sepharose (1g) was placed into 50 ml water and allowed to swell. The Sepharose was recovered by filtration in a sintered glass funnel, and washed with 2 x 50 ml water. The gel was then resuspended in 5 ml water containing 100 mg of the relevant thiol which had been adjusted to pH 12.0 with NaOH. The slurry was mixed overnight on a shaker at room temperature and the Sepharose then recovered by filtration, and washed with 200 ml water. Residual activated groups were blocked by adding 10 ml of 2 M ethanolamine to the gel and incubating for 4 h, 30°C. The Sepharose was then recovered by filtration and sequentially washed with 2x 200 ml water, 3x 20 ml 100 mM sodium acetate pH 4.0, 3x 20 ml 0.5 M NaCl, and 3 x 20 ml 100 mM Tris-HCl pH 8.0, 0.5 M NaCl. The synthesised affinity matrix was packed into a column at 1.5x the flow rate used for normal chromatography. The column was then stored in 20% ethanol at 4°C prior to use.

2.1.7.2 Hydrophobic interaction chromatography (HIC).

Hydrophobic interaction chromatography was performed using phenyl-Sepharose packed in a 50 ml XK 26 column (Pharmacia) at a flow rate of 4 ml min^{-1}. The column was equilibrated with 500 ml loading buffer A (20 mM Tris-HCl, 1 M $(NH_4)_2SO_4$, 200 mM KCl, 1 mM DTT pH 7.4) prior to loading the sample which had been previously dialysed against loading buffer. The column was washed with loading buffer until the output from UV chart recorder had returned to its basal value, indicating that all the unbound protein had been eluted. Bound protein was then recovered with elution buffer B (20 mM Tris-HCl, 1 mM DTT, pH 7.4) followed by buffer C (20 mM Tris-HCl, 1 mM DTT, 50% ethylene glycol, pH 7.4). Fractions were collected (10 ml for buffer B, 5 ml for buffer C) and assayed for GST activity. Active fractions containing ethylene glycol were passed through a 5 ml Fastflow™ Q-sepharose (Pharmacia) ion exchange column at 2 ml min^{-1} in order to remove the ethylene glycol and allow further analysis. After washing the column with 50 ml 20 mM Tris-HCl, 1 mM DTT, pH 7.4., the GSTs present were eluted using 50% buffer B (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, pH 7.4). The proteins were dialysed overnight against affinity loading buffer prior to further purification.

2.1.7.3 Affinity chromatography.

Affinity chromatography, using either *S*-hexylglutathione, glutathione or homoglutathione coupled to Sepharose was performed as follows. The affinity column was equilibrated in 10 column volumes of buffer A (20 mM Tris-HCl, pH 7.4, 1 mM DTT). Crude samples, or those obtained from HIC, were dialysed against buffer A and applied onto the column at a flow rate of 1 ml min^{-1}. After washing with buffer A until the UV absorbance returned to basal level, loosely-bound protein was eluted from the column using 20 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM DTT and 2 ml fractions collected and assayed for GST activity. The column was then re-equilibrated in loading buffer and the remaining affinity bound protein eluted using 20 mM Tris-HCl pH 7.4 containing 5 mM of the counter affinity ligand (*S*-hexylglutathione, glutathione or homoglutathione) in the presence of 1 mM DTT. Fractions (1 ml) were collected and tested for GST activity. Purification using Orange A affinity chromatography was performed in a similar manner to the above, except loading

buffer was 10 mM potassium phosphate pH 6.0, 1 mM DTT. The wash buffer was 50 mM potassium phosphate pH 7.0, 1 mM DTT, and the affinity elution buffer was wash buffer containing 2 mM glutathione.

2.1.7.4 Anion-exchange chromatography.

Anion-exchange chromatography was used to remove the counter ligand from the affinity elution, and to facilitate the resolution of the various isoenzymes present. A 1 ml HiTrap-Q ion-exchange column (Pharmacia) was equilibrated in loading buffer C (20 mM Tris-HCl, pH 7.8, 1 mM DTT) at a flow rate 0.5 ml min^{-1}. The protein sample was then added and the column washed in loading buffer until the absorbance returned to near its basal level. Proteins were eluted from the column using a linear increasing salt gradient up to 0.25 M NaCl with a total gradient volume 25 ml. Fractions (1 ml) were collected which were tested for GST activity and active fractions subjected to further characterisation.

2.1.8 Production of antisera.

Antibodies toward purified soybean GSTs were raised by Jane Bird and Andrew Dinsmore, Alderley Park, Macclesfield using two young female New Zealand white rabbits per antigen. Immunisations were administered sub-cutaneously at four sites on each animal using the protocol outlined in Table 2.1. Blood samples were allowed to clot at room temperature and incubated at 4°C overnight. Serum was recovered by centrifugation 1500 g, 20 min, 4°C.

Time	Procedure	Comments
	500μl Pre-bleed	
0	Dose 1	200μg GST in 1ml Freund's complete adjuvant.
4 weeks	Dose 2	200μg GST in 1ml Freund's incomplete adjuvant
6 weeks	Test bleed	≈10ml sera
8 weeks	Dose 3	200μg GST in 1ml Freund's incomplete adjuvant
12 weeks	Dose 4	200μg GST in Freund's incomplete adjuvant
14 weeks	Term bleed	≈15ml sera

Table 2.1 Antibody production protocol, courtesy of Bird and Dinsmore.

2.1.9 Protein analysis.

2.1.9.1 SDS - polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis was performed using a BioRad Minigel apparatus. 10μl of protein sample or molecular mass markers (BioRad low range, 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 kDa respectively) were denatured by the addition of 2x loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% Bromophenol Blue, 20% Glycerol, 200 mM DTT) and boiled for 5 min. The samples were loaded onto a 12.5% or 17.0% polyacrylamide gel (0.75 mm thick) containing 0.33% or 0.17% N,N'-methylene-bisacrylamide respectively. The gels were run in glycine running buffer (15 g Tris, 72 g glycine and 5 g SDS per litre) until the dye front reached the base of the gel (approx. 90 min at 150 V) using protocols supplied by the manufacturer.

Gels were fixed for 30 min in water : methanol : acetic acid (5:4:1 v/v) then stained using B/T Blu (BT Scientific Technologies, USA), and de-stained using fixative until a clear background was obtained. Where protein concentrations were low, polypeptides were detected using the BioRad silver staining protocol as recommended by the manufacturer.

2.1.9.2 Western blotting of SDS-PAGE gels.

Polypeptides were separated by SDS-PAGE using visible low molecular weight markers of 46, 30, 21.5, 14.3, 6.5, 3.4, 2.35 kDa (Amersham) for calibration. The blotting membrane used was either nitro-cellulose (Hybond-C) or PVDF (Hybond-P, Pharmacia), the latter requiring pre-wetting with methanol prior to use. Both the SDS-

PAGE gel and blotting membrane were equilibrated in transfer buffer (19.3 g Tris, 90 g glycine per litre) for 5 min. Western blotting was performed using a tank electroblotter (mini Trans-Blot cell, BioRad) in transfer buffer at 100 V for 30 min. After blotting the membrane was blocked in Tris-buffered saline (TBS = 0.2 M NaCl, 50 mM Tris-HCl pH 7.4) containing 3% (w/v) milk powder for one hour.

The blocked membrane was incubated in fresh TBS containing 3% (w/v) milk powder and the primary antiserum of interest diluted 1:2000, for either one hour at room temperature, or overnight at 4°C. The membrane was washed twice in TBS + 0.1% Triton X-100 (20 min each) and once in TBS (20 min). The secondary antibody, anti-rabbit IgG (Sigma) coupled to either horseradish peroxidase (HRP) or alkaline-phosphatase was added at 1:10,000 dilution in TBS containing 3% (w/v) milk powder for one hour at room temperature. After this time, the membrane was washed as described following treatment with the primary antibody. For HRP-linked antibodies the ECL detection kit (Amersham) was used to identify immunoreactive proteins according to the manufacturer's guidelines. Membranes incubated with alkaline phosphate linked antibodies were developed in 10 ml BCIP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) containing 33µl 100 mg^{-1} nitro blue tetrazolium (NBT) in 70% dimethylformamide (DMF) and 33µl 50 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in DMF.

2.1.9.3 Protein sequencing.

Proteins were purified prior to N-terminal sequencing either by reversed-phase HPLC or by electroblotting from SDS-PAGE gels onto PVDF membrane. Once blotted, proteins were visualised using Ponceau-S and excised. Protein sequencing was performed using an Applied Biosystems 477A protein sequencer.

2.1.9.4 Matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS).

Proteins intended for Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) were prepared by HPLC and analysis performed by M-Scan Ltd, Berkshire, UK, using a Voyager Elite Biospectrometry Research Station laser-desorption mass spectrometer.

2.2 Molecular Biology Techniques.

All media compositions are listed in an appendix at the end of this chapter. Unless otherwise indicated all basic molecular biology procedures were performed as described by Sambrook *et al.* (1989)

Molecular biology grade chemicals were obtained from Sigma Chemical Company, Poole, Dorset. Enzymes were purchased from Promega, UK or New England Biolabs, UK with the exception of *Taq* DNA polymerase, which was purchased, with license for PCR, from Gibco-BRL, UK. Bacterial growth media was obtained from Lab M laboratories, UK. All solutions were autoclaved at 120 psi for 20 min where appropriate, or filter sterilised. Special precautions were observed when handling RNA including the baking of all glassware, the wearing of gloves, and the use of water treated with diethyl pyrocarbamate (DEPC). Radiolabelled substrates were acquired from Amersham (α-[^{32}P] dCTP with a specific activity of 3,000 Ci mmol^{-1} and a concentration of 10 mCi ml^{-1}; γ-[^{32}P] ATP with a specific activity of 6,000 Ci mmol^{-1} and a concentration of 10 mCi ml^{-1}; and α-[^{35}S] dATP with a specific activity of 1,250 Ci mmol^{-1} and a concentration of 12.5 mCi ml^{-1}).

2.2.1 General.

Bacterial Strains.

The following *Escherichia coli* strains were used in the work described in this thesis. XL1-Blue MRF'(Tet^r) (Stratagene Inc.), BL21 (DE3) (Novagene, UK), SolR (Stratagene Inc.) and INVαF' (Invitrogen, Netherlands). For plant transformation the host strain *Agrobacterium tumefaciens* LBA4404 (rif^r, str^r, T-DNA$^-$) was obtained from Zeneca Agrochemicals.

Vectors.

The bacterial cloning vectors pBluescript-II SK+ (Stratagene) and pCR 2.1 (Invitrogen) were used for cDNA cloning. Both pBluescript and pCR 2.1 contain a multiple cloning site (MCS) in the *lacZ* gene which allows blue-white colour selection for cDNA inserts. When no insert is present, the *lacZ* gene is expressed normally and is able to cleave 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) resulting in the formation of a blue colour. Insertion of a cDNA into the MCS disrupts the *lacZ*

gene and consequently no expression of the gene occurs, resulting in no X-gal cleavage and these colonies appear white in colour. The *lacI^q* strains used, such as XL1-Blue, required the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to induce *lacZ* expression. The INVαF' strain is not *lacI^q* and therefore does not require IPTG addition. For bacterial recombinant expression work the vector pET-11d (Novagen) was chosen. This vector allows the insertion of the desired cDNA as an *Nco*1 / *Bam*H1 fragment, since the translational "ATG" start signal is contained within the *Nco*1 site. The expression host used was *E. coli* BL21 (DE3) which contains the gene encoding the T7 RNA polymerase (λDE3 lysogen) within the bacterial genome under the control of the *lacUV5* promoter. Thus transcription of the desired cDNA is regulated by the addition of IPTG.

For plant transformation work the intermediate cloning vector pMJB1 (Zeneca Agrochemicals, Figure 2.1) was used. pMJB1 is a PUC based intermediate cloning plasmid containing the double enhanced cauliflower mosaic virus (CaMV35S) promoter, tobacco mosaic virus (TMV) omega (Ω) leader sequence and nopaline synthase (*nos*) transcriptional terminator.
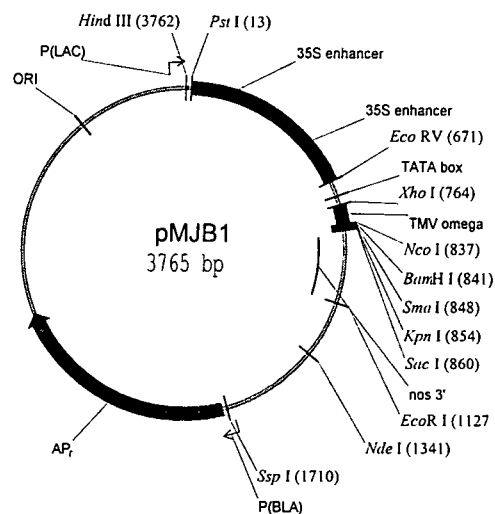
Figure 2.1 PUC based vector pMJB1 containing the double enhanced CaMV35S promoter, TMV Ω leader sequence and *nos* terminator.

Plant Transformation Binary Vectors.

Plant transformation was carried out using the Bin19 (Bevan, 1984) derived binary vector pJR1i obtained from Zeneca Agrochemicals (Figure 2.2).
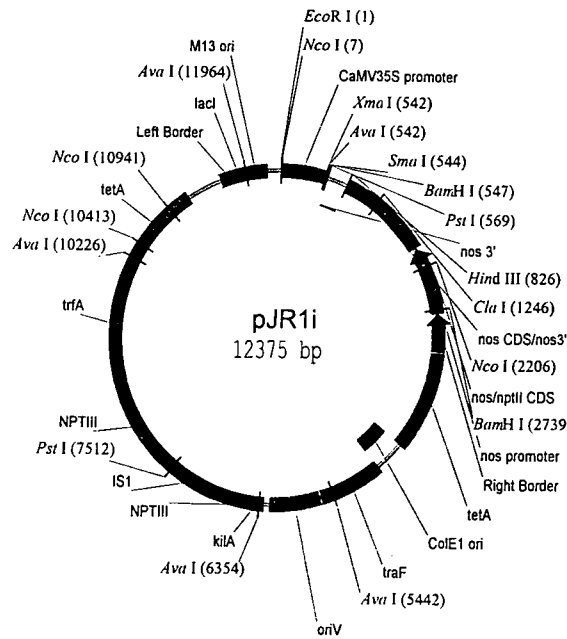
Figure 2.2 Binary vector pJR1i used for plant transformation.

Growth of Bacterial cultures

Liquid medium was inoculated using a sterile loop with a single bacterial colony obtained either direct from a transformation or from a bacterial streak from a glycerol. Selective antibiotics were included where necessary (ampicillin 100 μg ml^{-1}, kanamycin 50 μg ml^{-1}, carbinicillin 100 μg ml^{-1}, rifampicin (in methanol) 100 μg ml^{-1}, streptomycin 500 μg ml^{-1}, tetracycline 12.5 μg ml^{-1}). Cultures were grown on an orbital shaker with *E. coli* grown at 37°C and *A. tumefaciens* at 28°C. Bacterial growth was monitored by determining the optical density (OD) at 600 nm. For agar plates, 20 g l^{-1} of agar was added to the liquid media prior to autoclaving and the required antibiotics were added when the molten media was <50°C.

Storage of Bacterial Cultures

Glycerol stocks of bacterial were made by the addition of 0.15 ml autoclaved glycerol to 0.85ml overnight culture in a 1.5ml Cryotube™ (Nalgene). The tube was frozen in liquid nitrogen and stored at -80°C.

Plasmid Recovery.

Plasmid recovery from 5-10 ml overnight cultures was performed using a Wizard SV mini-preparation kit (Promega) or the Qiaprep™ Spin Miniprep kit (Qiagen) according to the manufacturers guidelines. In all cases plasmid was eluted from the spin-columns in distilled water (dH_2O) and stored at -20°C.

Nucleic Acid Quantification

Nucleic acids were quantified by their absorbance at 260nm using the equation:

Concentration (mg ml^{-1}) = A_{260nm} x ε x dilution factor.

Where ε = 33 (single stranded DNA), 50 (double stranded DNA) and 40 (RNA), A = absorbance. Quality of RNA was determined from the ratio of A_{260nm}:A_{280nm}, with a ratio in ≥ 1.8 deemed acceptable.

Restriction Enzyme Analysis.

Restriction enzyme digests were performed using the supplier's recommended guidelines. For simultaneous double restriction digests, Promega buffers were selected using compatibility tables provide by Promega.

DNA Separation using Agarose Gels.

Agarose (0.8 - 2.0% w/v, Gibco-BRL) was prepared in 1x TAE / TBE buffer (10x TAE: 49.5g Tris-base, 11.2 ml glacial acetic acid, 20 ml 0.5 M EDTA per litre pH 8.0. 10x TBE: 108 g Tris Base, 55 g Boric acid, 5.8 g EDTA per litre pH 8.3). TAE gels were used if DNA fragments were intended for gel purification. Ethidium bromide was added to the molten agarose to a final concentration of 0.05 μg ml^{-1} from a 10 mg ml^{-1} stock. A tenth volume of 6x DNA loading buffer (0.25% Xylene Cyanol FF, 0.25% Bromophenol Blue, 15% Ficoll (Type 400)) was added to each sample prior to loading. Gels were run in appropriate 1x running buffer containing 0.05 μg ml^{-1} at a constant 100 V until desired resolution fragments had been achieved. DNA was visualised on a UV trans-illuminator and recorded using a BioRad GelDoc system. A 1 kb DNA ladder (Gibco-BRL) was used to size DNA fragments.

Purification of DNA from TAE Agarose Gels.

The QIAquick Gel Extraction Kit (Qiagen) was used to purify DNA from TAE agarose gels according to the manufacturer's protocol.

2.2.2 Isolation of total RNA from soybean seedlings & cell cultures.

For cDNA Library Construction.

RNA from 5-day-old seedlings and cell cultures was isolated using the method described by Jepson *et al.* (1991).

Tissue (10 g) was ground using a baked pestle and mortar under liquid nitrogen and the fine powder transferred to a clean 250 ml beaker and 50 ml RNA homogenising buffer (50 mM Tris HCl, 0.5 mM EDTA, 400 mM NaCl, 20 U/ml heparin, 1 mM ATT, 10 mM DTT, 1% SDS) and 25 ml phenol/cresol (22.5 ml phenol + 2.5 ml m-cresol) added. This solution was transferred to a DEPC-treated Oakridge™ tube and centrifuged (9,000 g, 10 min, 4°C) in swing-out rotor. The upper phase was removed, 10 ml phenol / chloroform (25:24:1 v/v/v phenol (buffered in Tris-HCl pH 8.0) : chloroform : isoamylalcohol) added to it and mixed for five minutes. The solution was then centrifuged (9,000 g, 10 min, 4°C and the supernatant transferred to a 30 ml Corex™ tube and the phenol/chloroform extraction repeated. The supernatant was then removed and adjusted to a final concentration of 2 M LiCl using 12M LiCl, sealed with Nescofilm™ and left overnight at 4°C. Precipitated RNA was recovered by centrifugation (6,500 g, 7 min, 4°C), re-suspended in 5 mM Tris-HCl pH 7.5 (5 ml) and transferred to a 15 ml Corex tube for re-precipitation of the RNA as before. The resulting pellet was washed twice in 70% ethanol, dried under a vacuum and re-suspended in 0.5 ml of DEPC-treated water.

For RT-PCR and other Applications.

For RT-PCR, RNA was isolated using from 50-100 mg of plant material by homogenising in 1 ml Tri-Reagent® (Sigma). The homogenate was centrifuged (13,000 g, 10 min, 4°C) and the supernatant transferred to a clean eppendorf tube and allowed to stand at room temperature for 5 min. Chloroform (200 µl) was added and the sample vortexed for 15 s and allowed to stand for 2-15 min at room temperature.

Following centrifugation (13,000 g, 15 min, 4°C) the colourless, upper aqueous phase was removed carefully and 0.5 ml isopropanol added. The sample was allowed to stand for 5-10 min at room temperature and the precipitated RNA recovered by centrifugation (13,000 g, 10 min, 4°C). The RNA pellet was washed in 75% (v/v) ethanol (1 ml), dried under vacuum, re-suspended in 50 µl RNase free water and stored at -20°C.

2.2.3 cDNA library construction.

cDNA libraries were constructed using the λ ZAP-II system provided by Stratagene Inc.). Poly-A$^+$ mRNA was isolated from total RNA using the Promega PolyATtract™ system and cDNA synthesised using the kit provided. *Eco*R1 linkers were ligated onto the 5' end of the synthesised cDNA, prior to size-fractionation of the cDNA. The cDNA was then digested with *Eco*R1:*Xho*1 and ligated into the supplied UNI-ZAP XR vector. The recombinant vector was packaged into phage using the Gigapack II packaging extract. The proportion of inserts was tested by titering the packaged extract onto NZY plates containing IPTG / X-Gal and the average insert size determined by PCR of random plaques using M13 primers.

2.2.4 DNA screening of phage cDNA libraries.

cDNA library screening of 160,000 plaque forming units (pfu) was carried out according to guidelines supplied by Stratagene using 243 x 243 mm bio-assay plates (Nalgene, UK). Phage competent XL1-Blue *E. coli* were produced by inoculating 50 ml of LB medium, supplemented with 0.2% maltose and 10 mM $MgSO_4$ with a 500 µl overnight culture. The cells were harvested by centrifugation when OD_{600nm} = 0.5 and re-suspended in ice cold 10 mM $MgSO_4$ to an equal OD. The required volume of phage stock, diluted in SM buffer (5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml 1 M Tris-HCl, 5 ml 2% (w/v) gelatin per litre pH 7.5) was added to 2 ml of phage competent *E. coli* and incubated for 15 min at 37°C prior to addition of 25 ml of melted NZY-Top agar (48°C). After a brief mix, the top-agar was poured onto pre-warmed (37°C) NZY-plates and the plates incubated at 37°C. Plaques were usually visible 4 to 6

hours after plating. The plates were cooled to 4°C before lifts were made to prevent removal of the top-agar.

Lifts were made using Hybond-N$^+$ nitro-cellulose membranes (Amersham). Bound DNA was denatured by placing the filters onto 3MM Whatman paper saturated with 1.5 M NaCl + 0.5 M NaOH for 10 min. The filters were then neutralised by washing with 1.5 M NaCl + 1 M Tris-HCl (pH 7.5) for 10 min and equilibrated in 2x SSC (SSC = 150 mM NaCl, 15 mM sodium citrate per litre adjusted to pH 7.0 with NaOH) for 5 min. Finally, the filters were air dried and the DNA bound to the filter using a UV Crosslinker delivering 150 MJ (Stratagene).

2.2.4.1 [γ-^{32}P]-labelling of oligonucleotide DNA probes.

Oligonucleotide probes were end labelled using the following procedure. 50 ng desired oligonucleotide (2.0 µl) were added to 10x Kinase Buffer (0.5 M Tris pH 7.6, 0.1 M MgCl$_2$, 1.5 M NaCl, 50 mM DTT and 10 mM Spermidine, 5.0 µl) and the volume made up to 40 µl with dH$_2$O. [γ-^{32}P] ATP (6.0 µl) and T-4 polynucleotide kinase (4.0 µl) were then added and the reaction allowed to proceed 37°C for 30 min. The labelled oligonucleotides were used without the need for denaturation or clean-up.

2.2.4.2 [α-^{32}P] labelling of double-stranded DNA probes.

Labelling of double stranded DNA probes was achieved using the Ready to go™ labelling kit (Pharmacia). Denatured DNA (25 ng) was added to the supplied tube and 50 µCi [α-^{32}P] dCTP added in a total volume of 50 µl. The labelling reaction was allowed to proceed for 60 min.

2.2.4.3 Non-radioactive labelling of double-stranded DNA by PCR.

DNA probes were labelled with digoxigenin-dUTP by PCR using the PCR DIG Probe Synthesis Kit (Böehringer Mannheim, Germany). Briefly, PCR was performed using Primer 1 (100 ng µl^{-1}) (3 µl), Primer 2 (100 ng µl^{-1}) (3 µl), 10x PCR DIG Mix (2 mM dNTP containing 1:20 dig-dUTP) (5 µl), Expand™ Taq polymerase (0.75 µl), template DNA (100 ng µl^{-1}) (100 ng) and dH$_2$O to a total volume of 50 µl. Successful

incorporation of the DIG-nucleotide was confirmed by the retardation of the DIG-labelled PCR products during electrophoresis.

2.2.4.4 Hybridisation of labelled probes.

All hybridisations were performed using hybridisation ovens and roller bottles from Techne, UK.

Hybridisation Conditions for Radio-labelled Probes.

Filters were incubated in pre-hybridisation solution (5x SSC, 0.5% SDS, 1% milk powder and 4 μg ml^{-1} pre-boiled salmon sperm DNA) at 55°C (oligonucleotide probe) or 65°C (DNA probe) for at least three hours. The labelled probe was added to the pre-hybridisation solution and allowed to hybridise overnight. The filters were washed using 6x SSC, 0.1% (w/v) SDS (2 x 20 min, 55°C) for oligos or 0.1x SSC; 0.1% (w/v) SDS (2 x 20 min, 1 x 10 min, 65°C) for homologous DNA probes; 1x SSC, 0.1% (w/v) SDS (2 x 20 min, 1 x 10 min, 65°C) for heterologous DNA probes. After washing the filters were wrapped in Saran™ wrap and exposed to X-ray film using intensifying screens at -70°C overnight.

Hybridisation conditions for Digoxigenin-labelled DNA probes.

Plaque lifts and filter processing were performed as described in section 2.2.4. Filters were pre-hybridised in 5x SSC, 0.1% *N*-laurylsacrosine, 0.02% SDS, 1x Blocking solution (Böehringer Mannheim) at 65°C for 1 hour. The DIG-labelled probe was boiled for 5 min and 5 μl added to the filters in 12.5 ml fresh pre-hybridisation solution prior to hybridisation overnight at 65°. The filters were then washed twice in 2x SSC, 0.1% SDS, 15 min, 65°C and cooled to room temperature, equilibrated in DIG washing buffer (100 mM maleic acid buffer, 0.3% (w/v) Tween 20) and treated for one hour with blocking buffer (maleic acid buffer (100 mM maleic acid, 150 mM NaCl adjusted to pH 7.0 with NaOH) + 1x blocking solution). DIG-positive clones were then identified by adding 6 μl of the secondary DIG-antibody in 30 ml blocking buffer and incubating for 1 h at room temperature. After washing twice in DIG washing buffer for 15 min each, the binding of the peroxidase coupled antibody was

visualised by incubating the membrane in BCIP detection buffer using BCIP/NBT as described in section 2.1.9.2.

2.2.4.5 Antibody screening of cDNA expression library.

For immunoscreening of cDNA expression libraries, 160,000 pfu were added to XL1-blue MRF' plating cells (2 ml) and incubated with gentle shaking at 37°C, 15 min. The cells were mixed with 20 ml NZY-top agar (48°C) and plated onto Nalgene Bio-assay dishes (160,000 pfu/plate) and incubated at 37°C until plaques were just visible (approx. 3-4 h). At this time a Hybond-C membrane (pre-soaked in 10 mM IPTG and dried between Whatmann 3MM paper) was overlaid and the plate incubated for a further 3 h. The plate was then placed at 4°C for 10 min and the membrane carefully removed, rinsed in TBS, blocked in TBS containing 3% milk powder and processed as for a Western blot (section 2.1.9.2).

2.2.4.6 Plaque storage and titering.

Plaques of interest were cored from the plates using a pipette tip and placed into an eppendorf tube containing 500 µl SM buffer and 20 µl chloroform and stored at 4°C. Secondary screening was performed in a similar manner primary screening. Phage stocks were titred and *c.* 1000 pfu's plated using 200 µl competent cells in 3 ml NZY-Top agar per petri dish (82 mm diameter).

2.2.4.7 *In vivo* excision.

Plasmid was recovered from phagemids using the *in vivo* excision protocol detailed by Stratagene. 200 µl of XL1-Blue MRF' cells (OD_{600nm}=1.0) were added to 200 µl desired phage stock (>1x10^5 pfu) and 1 µl ExAssist™ helper phage and incubated at 37°C for 15 min. LB broth (3 ml) was then added and the tube incubated overnight with shaking prior to heating at 65-70°C for 20 min. After centrifuging (1,000g, 15 min), the supernatant was transferred to a fresh tube and 10µl of this phagemid stock added to 200 µl SOLR cells (OD_{600nm}=1.0) after incubating at 37°C for 15 min. 200 µl of this mixture was plated onto LB-ampicillin plates which were incubated at 37°C overnight. Plasmid was then recovered from overnight cultures from antibiotic resistant colonies as described previously.

2.2.5 Polymerase chain reaction (PCR).

The polymerase chain reaction (PCR) (Mullis *et al.*, 1986) was used to amplify small quantities of DNA. PCR reactions were performed in a Techne Progene™ thermal cycler. Oligonucleotide primers were synthesised by Applied Biosystems, UK or MWG Biotech, Germany. The following codes are used to describe nucleotides throughout this thesis.

```
G = Guanosine      R = G or A          B = C, G or T not A
A = Adenosine      Y = C or T          D = A, G or T not C
T = Thymidine      W = A or T          H = A, C or T not G
C = Cytidine       S = C or G          V = A, C or G not T
I = Inosine        K = G or T          N = A, C, G or T
                   M = A or C
```

A typical PCR consisted of primer A (100 ng μl^{-1}) (1.5 μl), Primer B (100 ng μl^{-1}) (1.5 μl), 11x PCR buffer (167 μl 2 M Tris-HCl pH 8.8, 83 μl 1 M $(NH_4)_2SO_4$, 33.5 μl 1 M $MgCl_2$, 3.6 μl β-mercaptoethanol, 3.4 μl 10 mM EDTA pH 8.0, 75 μl of each 100 mM dNTP stock and 85 μl 10 mg ml^{-1} BSA) (2.27 μl), Taq DNA polymerase (5 U μl^{-1}) (1 μl), DNA template (X μl) and dH_2O to 25 μl total volume. PCR conditions were calculated for specific applications but typical conditions were:

1 cycle 94°C, 5 min, followed by

X cycles 94°C, 45 s; 51°C, 30 s; 72°C°, 60 s and a final extension period of

1 cycle 72°C, 7 min, 4°C hold.

Annealing temperatures were determined for individual oligonucleotides using Tm calculation's where $Tm = 81.5 + 16.6 (\log_{10}[Na^+] + 0.41(\%G + C) - (600/N)$, with N = chain length. For oligonucleotides 24 bases or less the approximation of $Tm = 2 \times (A+T) + 4 \times (G+C)$ was used.

2.2.5.1 Reverse-transcriptase polymerase chain reaction (RT-PCR).

SuperScript™ Reverse Transcriptase (Gibco-BRL) was used for all reactions. First strand cDNA synthesis was prepared from Primer OG2 (5' GAG AGA GGA TCC TCG AGT TTT TTT TTT TTT TTT T 3') (100 ng μl^{-1}) (5 μl), Total RNA (1 μg μl^{-1}) (5 μl) and dH_2O to a total volume of 2 μl. The mixture was heated to 70°C for 10 min, rapidly chilled on ice, and the contents recovered by brief centrifugation. 5x first

strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 0.1 M DTT) (4 µl), 0.1 M DTT (2 µl), 10 mM dNTPs (1 µl) were then added and the tube contents gently mixed and incubated 42°C, 2 min. 1 µl reverse transcriptase was added and the tube incubated at 42°C, 50 min. The reverse transcriptase was inactivated by heating to 70°C, 15 min.

PCR cDNA amplification

Specific cDNA was amplified from the RT-PCR as described above.

General PCR conditions used were 1 cycle 94°C, 5 min; 40 cycles 94°C, 45 s; 51°C, 30 s; 72°C, 1 min; 1 cycle 72°C, 7 min; 4°C hold. Slight modifications to the annealing temperature and primer concentration were made depending on degeneracy and characteristics of primers used. 10x PCR Buffer (10 µl), 50mM $MgCl_2$ (3 µl), 10mM dNTPs (2 µl), primer OG9 (CGC ACT GAG AGA GGA TCC TCG AG) (100 ng $µl^{-1}$) (5 µl), specific primer (100 ng $µl^{-1}$) (5 µl), Taq-DNA polymerase (5 U $µl^{-1}$) (1 µl), first strand cDNA from RT reaction (2 µl) and dH_2O (72 µl).

2.2.5.2 Cloning of PCR products.

Taq-polymerase derived PCR products were cloned using the original TA cloning® Kit (Invitrogen). Direct aliquots of the PCR reaction, or gel purified products, were ligated into the pCR 2.1 vector according to the manufacturer's guidelines. To improve efficiency overnight ligations were performed at 4°C. Ligated products were transformed into *E. coli* INVαF' (>$1x10^8$ (amp') $µg^{-1}$ pUC 18) supplied using the protocol provided by the manufacturers and transformed colonies selected on LB agar plates containing 100 µg ml^{-1} and 40 µg ml^{-1} X-gal (IPTG not required since INVαF' is not *lacIq*). White colonies were analysed for the presence of the desired insert by PCR and restriction digest.

Ligation of DNA fragments.

Purified DNA was mixed at a 3:1 molar ratio of insert : vector before the addition of 10x ligase buffer and T_4 DNA ligase. The typical total ligation volume was 10 µl, and this was incubated overnight at 14°C.

2.2.6 Bacterial transformation.

2.2.6.1 Preparation of electro-competent *E. coli.*

One litre of pre-warmed 2xYT growth medium, containing relevant antibiotics, was inoculated with 1/100th volume of overnight culture containing the desired *E. coli* strain. This culture was grown at 37°C until OD_{600nm} = 0.5 to 0.7, after which the culture was chilled on ice for 30 min. Cells were recovered by centrifugation (4000 g, 20 min) in a pre-chilled rotor and carefully re-suspended in an original volume of ice cold 1 mM HEPES, pH 7.0. The cells were then re-centrifuged and the cells re-suspended in half the original volume using 1 mM HEPES, pH 7.0. The cells were once again recovered and washed in 20 ml 10% glycerol (w/v), 1 mM HEPES, pH 7.0. Finally the cells were centrifuged again and, resuspended in 2.5 ml 10% (w/v) glycerol prior to dispensing into 50 µl aliquots, freezing in liquid nitrogen and storage at -80°C.

2.2.6.2 Transformation of *E. coli.*.

One 50µl vial of electro-competent *E. coli* was thawed on ice and transferred to a pre-chilled 0.2 cm electroporation cuvette, containing 1µl of ligation mixture. The cuvette contents were shaken gently, and placed on ice for 1 min. A Gene Pulser™ (BioRad) electroporator was set to 25 µF, 2.5 kV, 200 Ω and the dried cuvette placed into the electroporation chamber. After applying the pulse the cells were quickly re-suspended in 1 ml pre-warmed 2x YT medium. Pulsed cells were allowed to recover for 1 h at 37°C with shaking before plating onto selective LB plates.

2.2.6.3 Transformation of *Agrobacterium tumefaciens.*

Transformation of *Agrobacterium tumefaciens* was achieved using the method first described by Holsters *et al.*, 1978.

LB media (100 ml), supplemented with 100 µg ml^{-1} rifampicin and 500 µg ml^{-1} streptomycin, were inoculated with *A. tumefaciens* strain LBA 4404 and grown at 28°C, 200 rpm until an OD_{600nm} 0.5-1.0 was reached. The culture was chilled on ice, centrifuged (3000 g, 6 min, 4°C) and the pellet re-suspended in 500 µl chilled 20 mM $CaCl_2$. Aliquots (100 µl) were dispensed into chilled microfuge tubes and recombinant binary vector (100 ng) was added. The microfuge tube was frozen in liquid nitrogen

and the cells thawed by incubation at 37°C for 5 min. LB medium (1 ml) was added and the cells allowed to recover for 3-4 h at 28°C on an orbital shaker (100 rpm). After this time the cells were centrifuged (13,000 g, 30 s), re-suspended in 100 μl LB and spread onto LB plates containing rifampicin (100 μg ml⁻¹), streptomycin (500 μg ml⁻¹) and kanamycin (50 μg ml⁻¹). The plate was incubated at 28°C and transformed colonies appeared within 2-3 days. The recombinant nature of the *Agrobacterium* was checked by plasmid restriction digest and PCR analysis.

2.2.6.4 Identification of bacterial transformation events.

Identification of transformed bacterial was achieved, where applicable using the blue/white colour selection described in section 2.2.1. Following plasmid recovery, the identity of the inserts was determined by PCR, restriction analysis and DNA sequencing.

2.2.7 DNA sequencing.

DNA sequencing was performed both manually and automatically. To ensure sequence integrity, both top and bottom DNA strands were sequenced. The DNA sequence of cDNA clones inserted into vector flanked by M13 forward and reverse primer sites (pBluescript, pCR 2.1) or T7 promoter (pET) was obtained using the following sequencing primers .

T7 Sequencing: CGA AAT TAA TAC GAC TCA CTA TAG G

SEQ M13 REVERSE: CAC ACA GGA AAC AGC TAT GAC

SEQ M13 FORWARD: TTG TAA AAC GAC GGC CAG T

2.2.7.1 Manual DNA sequencing.

12 μl plasmid DNA (1-2 μg) were denatured by the addition of 8 μl 1 M NaOH and the DNA precipitated adding 40 μl ethanol and 2 μl 3 M sodium acetate by incubating at -20°C for 20 min. The DNA was recovered by centrifugation (13,000 g, 10 min), and the pellet washed with 1.5 ml 80% ethanol, before air drying. The DNA pellet was re-suspended in 10 μl annealing mixture (7 μl H₂O, 2 μl 5x Sequenase® reaction

buffer, 1 µl primer (0.5 pmol)) and vortexed carefully. The tube was placed in a 65°C water bath for 2 min, and cooled to 30°C over a period of 30 min. The tube contents were spun down and placed on ice. DNA labelling was performed using a T7 DNA polymerase sequencing kit (USB) and chain terminating ddNTPs. 3.5 µl labelling solution (1 µl DTT, 2 µl labelling mix (diluted 1:5), 0.5 µl [$\alpha^{35}S$] dATP (5 µCi)) were added to 2 µl diluted Sequenase® (1 µl enzyme: 8 µl enzyme dilution buffer)). The resulting 5.5 µl reaction solution was added to the annealed DNA/primer mix (10 µl) and the tube contents mixed, spun down and incubated at room temperature for 5 min. Each dideoxy termination mix (2.5 µl) (A, T, G, C) were placed into separate wells in a microtiter plate pre-equilibrated at 45°C and 3.5 µl of the labelling reaction mix were added to each of the wells for exactly 5 min. The reaction was stopped by the addition of 4 µl stop solution and the reaction cooled on ice.

Gel electrophoresis of labelled products.

Labelled DNA fragments were separated using a 6% TBE polyacrylamide resolving gel. The gel was poured, avoiding the introduction of air bubbles, with a comb inserted upside-down and allowed to polymerise overnight. The tape from the lower edge was removed and the gel placed into the gel tank. The upper and lower reservoirs were filled with 500 ml 1x TBE and the comb removed from the gel and replaced the correct way around. The wells were washed and the gel pre-warmed by running at 60W for 1 h. The reaction samples were heated at 80°C for 20 min, cooled on ice, and 2 µl loaded into appropriately marked lanes on the gel. The gel was run at 60W for 90 min (for a short run) or 5 h (for a long run). Once run, the small plate was carefully removed and the gel, on the large plate, placed into fixative (5% ethanol / 5% acetic acid) for 20 min. The gel was then washed in distilled water, dried onto Whatman 3MM paper and exposed to X-ray film at room temperature overnight.

2.2.7.2 Automated Taq DyeDeoxy™ terminator sequencing.

Automated DNA sequencing was carried out using an Applied Biosystems 373A or 377 DNA sequencer. DNA was fluorescently labelled using an Applied Biosystems PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit using 3.2 pmol primers according to the manufacturer's guidelines.

2.2.8 Data handling.

DNA sequence analysis was performed using the Sequencher® and DNAStrider® programmes on an Apple Macintosh computer. CLUSTAL V (Higgins *et al.*, 1996) was used to create multiple sequence alignments and PHYLIP (Joseph Felsenstein) used to construct sequence dendograms. DNA and protein similarity searches were performed using BLAST-P and BLAST-N programmes respectively at Internet site *http://www.ncbi.nih.nlm.gov*. Database sequences were obtained using sequence retrieval software (SRS) at the European Bioinformatics Institute (EBI) at *http://www.ebi.ac.uk*.

2.2.9 Heterologus GST expression.

2.2.9.1 Bacterial pET protein expression.

Cloning of cDNA into recombinant host.

Bacterial expression of recombinant soybean GSTs was achieved using the pET heterologous cDNA expression system (Novagen) in *E. coli* strain BL21(DE3). cDNAs of interest were introduced into the expression vector pET 11-d as an *Nco*1 and *Bam*H1 restriction fragment. cDNAs were engineered with the relevant restriction sites by PCR, using oligonucleotide primers described in chapter five. The PCR product was purified as described previously and digested using *Nco*1 and *Bam*H1 in Promega Multicore™ buffer. The digested PCR product was re-purified and ligated into pET-11d, which had also been pre-digested with *Nco*1 and *Bam*H1. Ligated plasmid (1μl) was then used transform *E. coli* BL21 DE3 as described in (2.2.6). Plasmid was recovered from putative transformed colonies and checked for authenticity by restriction enzyme analysis and by DNA sequencing.

Expression of soybean GST in *E. coli*.

LB media (100 ml), containing 100 μg ml^{-1} carbinicillin, which had been pre-warmed at 37°C was inoculated with 1 ml of a 5 ml overnight culture of BL21 DE3 *E. coli* containing the desired pET-GST plasmid. The culture was incubated on an orbital shaker at 37°C, 200 rpm until an OD$_{600}$ 0.5 was reached. For induction, 1 ml 1 M IPTG was added and the culture incubated for a further 3 h. After this time cells were

recovered by centrifugation, re-suspended in 10 ml extraction buffer (20mM Tris-HCl pH 7.4, 1 mM DTT) and sonicated for 30 s using an ultrasonic probe set at $\lambda=15$ μm. Cell debris was removed by centrifugation (4000 g, 5 min) and the recombinant GST purified from the bacterial lysate by affinity chromatography and anion exchange chromatography as described previously (2.1.7).

2.2.9.2 Tobacco transformation.

Tobacco plants were transformed using the leaf disk transformation method modified from Bevan (1984), with all work performed under aseptic conditions in a laminar flow cabinet. For transformation work plants were grown 30 cm below two neon lights (Osram, 125 W) at 25°C under a 16 h light / 8 h dark regime.

Wild type tobacco (*Nicotiana tabacum* var. Samsun) were sub-cultured on Murashige-Skoog medium (1x MS1S) and grown sterile tissue culture for approximately four weeks. LB media (100 ml), supplemented with 50 μg ml⁻¹ kanamycin, were inoculated with *A. tumefaciens* LBA 4404 containing the desired plasmid and grown at 28°C, 200 rpm until an OD_{600} of 0.6 was reached. The cells were recovered by centrifugation (3,000 g, 10 min) and re-suspended in 100 ml 1x MS1S liquid medium.

Leaves were removed from 20 tobacco plants and their outer edge excised and discarded. Leaves were immediately submerged in the *A. tumefaciens* suspension, dissected into five pieces, and left in the inoculating medium for 30 min. After this time, the leaf discs were transferred to NBM plates, which were sealed with parafilm and incubated for 2 days. When *A. tumefaciens* growth was visualised on the plates, the leaf discs were transferred into NBM tubs supplemented with 500 μg ml⁻¹ carbenicillin and 100 μg ml⁻¹ kanamycin. Tubs were incubated until kanamycin resistant shoots arose from the calli around the leaf tissue (5-6 weeks). These shoots were removed and transferred to 1x MS1S tubs supplemented with 200 μg ml⁻¹ carbenicillin and 100 μg ml⁻¹ kanamycin. Once these shoots had rooted (3-4 weeks) two cuttings were taken and re-rooted on the same media. Plantlets were analysed by PCR and western blotting and transferred to soil in the glass-house, where they were kept covered and in a humid condition for the first week. Duplicate plants were kept in tissue culture.

2.2.9.3 Isolation of plant DNA for PCR.

The following method, reported by Edwards *et al.,*(1991), was used to isolated DNA from putative transgenic plants for PCR.

Two discs were cut from a leaf using eppendorf lids and homogenised in 1 ml DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), vortexed for 5 s and centrifuged (13,000 g, 10 min). The supernatant (200 μl) was transferred to a fresh eppendorf and the DNA precipitated by the addition of 400 μl 100% ethanol and 20 μl 3 M sodium acetate. The tube contents were then inverted and left for a minimum 20 min at -20°C. Precipitated DNA was recovered by centrifugation (13,000 g, 10 min), washed in 70% ethanol, air dried and re-suspended in 100 μl dH$_2$O.

2.2.10 Appendix 1. Growth media.

Bacterial Growth Media

Luria Bertani (LB) Agar (per litre)

10 g NaCl
10 g tryptone (bacto-peptone)
5 g yeast extract
20 g agar
Adjusted to pH 7.0 with 5 M NaOH

LB Broth (per litre)

10 g NaCl
10 g tryptone (bacto-peptone)
5 g yeast extract
Adjusted to pH 7.0 with 5 M NaOH

NZY Agar (per litre)

5 g NaCl
2 g $MgSO_4.7H_2O$
5 g yeast extract
10 g NZ amine casein (hydrolysate)
15 g agar
Adjusted to pH 7.5 with NaOH

NZY Top Agar (per litre)

5 g NaCl
2 g $MgSO_4.7H_2O$
5 g yeast extract
10 g NZ amine (casein hydrolysate)
0.7% (w/v) agarose
Adjusted to pH 7.5 with NaOH

2x YT liquid media (per litre)

10 g NaCl
10 g yeast extract
16 g bactopeptone
Adjust to pH 7.5 NaOH

Plant Culture Media

1x MS1 Liquid

0.47% (w/v) MS salts; pH 5.9.

1x MS1

1x MS1 Liquid medium supplemented
with 0.8% (w/v) Bacto®-agar

1x MS1S Liquid

1x MS1 Liquid medium supplemented
with 3% (w/v) sucrose.

1x MS1S

1x MS1S Liquid medium supplemented
with 0.8% (w/v) Bacto®-agar.

NBM

1x MS1S supplemented with
1 μg ml^{-1} 6-BAP and 100 ng ml^{-1} NAA.

3. Chapter Three. GST Activities in Soybean and Associated Weeds.

3.1 Introduction.

GSTs with activity toward specific herbicide substrates have been characterised in a number of crops (Lamoureux and Rusness, 1993), and these have been implicated in both herbicide tolerance and selectivity. Recently, investigations into the role of GSTs in herbicide selectivity between crops and weeds have confirmed that the relative rate of glutathione conjugation of herbicides is a major determinant of selectivity (Hatton *et al.*, 1996a). The herbicides acetochlor (Breaux, 1986), acifluorfen (Frear *et al.*, 1983), chlorimuron-ethyl (Brown and Neighbors, 1987), fomesafen (Evans *et al.*, 1987) and metolachlor (Breaux *et al.*, 1987) are all used to give selective weed control in soybean, since they are rapidly detoxified by conjugation with homoglutathione in the crop plant. The role of GSTs in catalysing the conjugation of homoglutathione to these herbicides in soybean has not been reported, although it has been suggested that the selectivity of the chloroacetanilide herbicides metolachlor and acetochlor may be partially attributed to higher homoglutathione concentrations in soybean compared with the weed species studied (Breaux *et al.*, 1987). However, studies into the metabolism of selective herbicides in maize seedlings and associated weeds showed that GSTs were the major determinant of herbicide tolerance, with the bio-availability of glutathione being less important (Hatton *et al.*, 1996). In this chapter the GST activities present in soybean plants, along with the activities in associated problematical weed species will be examined to determine the role of GSTs in herbicide selectivity in soybean. The major problematical weeds associated with soybean (Dr J Townson, Personal communication) investigated are listed below, along with their abbreviated nomenclature which is used throughout this thesis.

Grass Weeds:

1. Foxtails (e.g. Giant Foxtail *Setaria faberi*, SETFA).

2. Barnyard Grass (*Echinochloa crus-galli*, ECHCG).

3. Crabgrass (*Digitaria sanguinalis*, DIGSA).

4. Johnson Grass (*Sorghum halepense*, SORHA).

Broadleaf Weeds:

1. Velvetleaf (*Abutilon theophrasti*, ABUTH).

2. Morningglory (*Ipomoea hederacea*, IPOHE).

3. Pigweed (Redroot pigweed: *Amaranthus retroflexus*, AMARE).

In addition to examining the GST activities in whole plants, the GST activities in soybean cell suspension cultures was of interest. GSTs have been identified in suspension cultures of maize (Edwards and Owen 1986, 1988; Miller *et al*, 1994), pumpkin (Fujita *et al*., 1995), tobacco (Droog *et al*., 1995) and *S. faberi* (Hatton *et al*., 1998). Comparative metabolism studies indicate that qualitatively there would appear to be little difference in pesticide metabolism between plant cell suspension cultures and whole plants (Swisher, 1987). In many cases, cell cultures contain greater GST activity than the corresponding whole plant, and as such are a good source for purification of these enzymes (Hatton *et al*., 1998). However, it is reported that some activities apparent in native plants become absent during de-differentiation in cell suspension cultures. For instance, comparative studies of GSTs in maize seedlings and cell cultures (Black Mexican Sweetcorn) showed the latter contained significantly less activity toward the herbicide atrazine, even though activity toward CDNB and metolachlor was increased (Edwards and Owen, 1986). It is important, therefore, to determine activity toward a wide range of GST substrates when comparing GST enzymes in plants and cell cultures to ensure that the complement of isoenzymes is similar in the two systems.

As discussed previously, GSTs in plants, animals, insects and micro-organisms are known to be induced by a number of chemical and environmental factors (Lamoureux and Rusness, 1993), a good example being the induction of GSTs by herbicide safeners in cereal crops (section 1.5.1). Many safeners are known to increase herbicide tolerance in cereals by enhancing the level of GSTs involved in herbicide metabolism (Hatzios, 1991). There is also evidence that GSTs may be induced following sub-toxic herbicide treatment (Mauch and Dudler, 1993). Although GST induction by herbicide safeners is well documented in maize, wheat and sorghum (Hatzios, 1997), little is known regarding safener induction of GSTs in dicotyledonous species. Treatment of chickpea with sub-toxic levels of the herbicide oxadiazon enhanced GST activity

(Hunaiti and Ali, 1990), suggesting that these enzymes are xenobiotic-inducible. Therefore, it was of interest to determine whether or not GST activities in soybean could be enhanced by treating soybean plants with herbicides, or compounds that were active inducers of GSTs in monocotyledons as herbicide safeners.

3.2 Results.

3.2.1 Development of assays to determine GST activity toward herbicides.

HPLC-based assays were developed to monitor *in vitro* GST activity toward the herbicides acetochlor, acifluorfen, chlorimuron-ethyl, fomesafen and metolachlor. Optimal conditions for biosynthesising conjugates were determined using crude protein extracts from cell cultures with both glutathione and homoglutathione. Most conjugates were formed between either glutathione or homoglutathione and the parent herbicides. However conjugations involving the diphenyl ether herbicides acifluorfen and fomesafen resulted in the cleavage of the diphenyl ether bond, and the formation of the (homo)glutathione conjugates of *o*-nitrobenzoic acid (GS-NBA) and 3-*N*-methane-sulphonyl-carbamoyl-4-nitrophenol (GS-MSCNP) respectively (Figure 3.1).
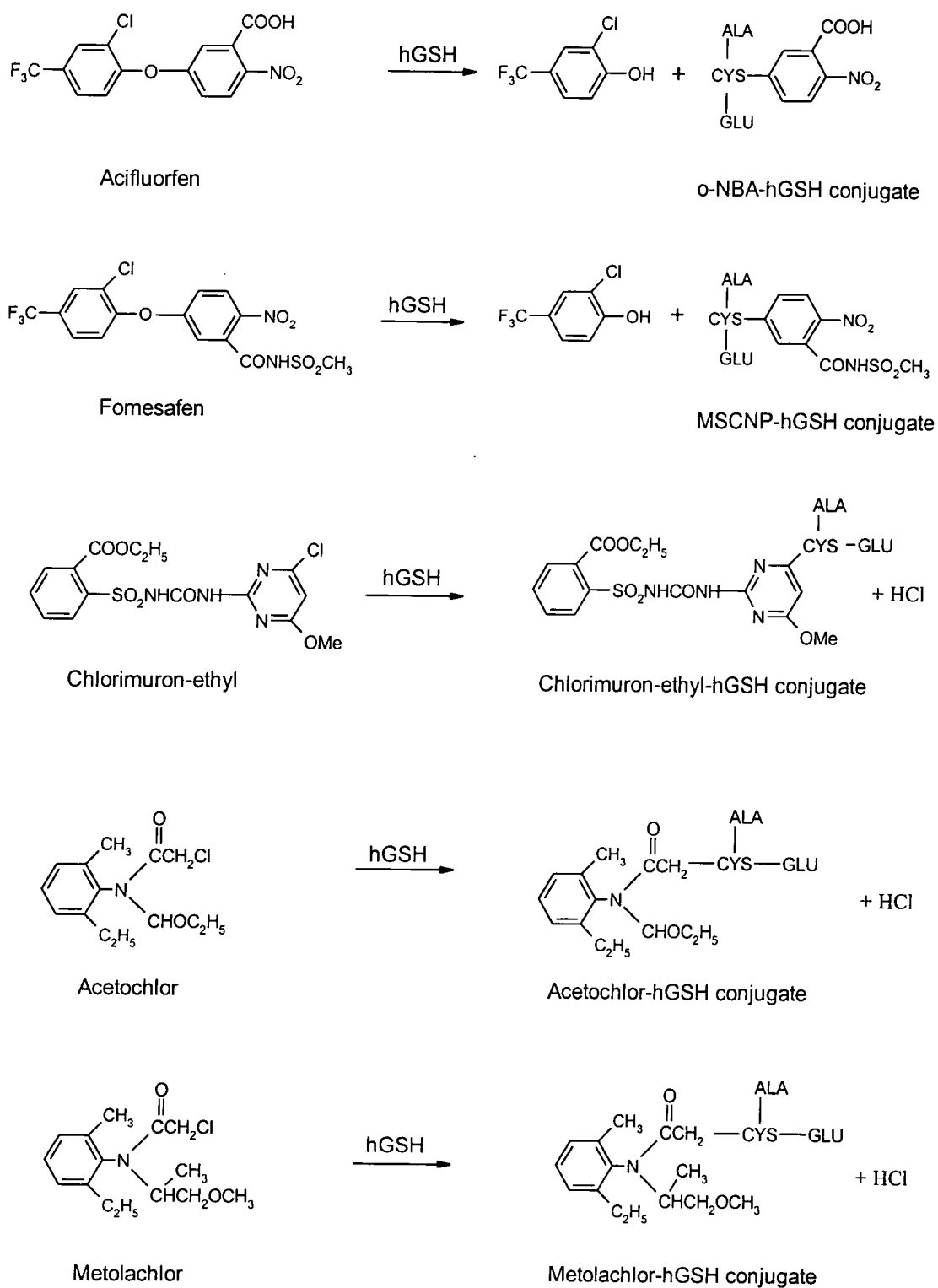
Figure 3.1 Homoglutathione (hGSH) conjugation of herbicides used selectively in soybean. NBA-hGSH = homoglutathione conjugate of *o*-nitrobenzoic acid, resulting from the cleavage of acifluorfen and MSCNP-hGSH = 3-*N*-methane-sulphonyl-carbamoyl-4-nitrophenol, resulting from the cleavage of fomesafen.

Herbicide conjugates were identified by co-chromatography using HPLC and TLC in conjunction with chemically synthesised standards (section 2.1.5.2). The identity of these reference conjugates was in turn confirmed by fast-atom-bombardment-mass-

spectrometry (FAB-MS) as required. The FAB-MS spectrum of the glutathione conjugate of chlorimuron-ethyl is shown for example in Figure 3.2.
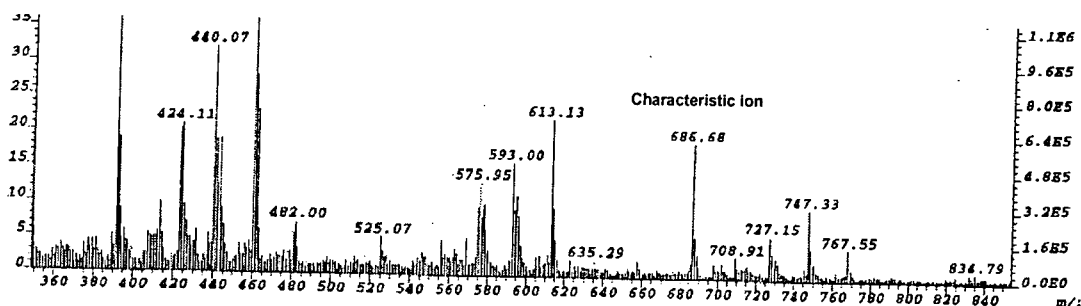
Figure 3.2 FAB-MS spectrum obtained for the glutathione conjugate of chlorimuron-ethyl.

The signal at m/z 686 corresponds to the expected mass of the chlorimuron-ethyl glutathione conjugate. The additional mass ions are consistent with the fragmentation pattern of the conjugate.

Figures 3.3 to 3.7 show representative HPLC chromatograms, obtained using HPLC method 1 (section 2.1.4.2), for each of the herbicides studied, with the parent herbicide and the respective homoglutathione conjugate indicated. The various peaks are quantified according to their absorbance at 264 nm using the analogue signal obtained from the UV detection device. Information regarding non-enzymic rates of conjugation for the herbicides and the conjugation retention times of the respective glutathione and homoglutathione conjugates are given in Table 3.1. Previous studies have indicated that the production of the herbicide conjugate formed over time in similar *in vitro* assays was linear within the time period of the assay (Hatton, 1997; Dixon, 1998).
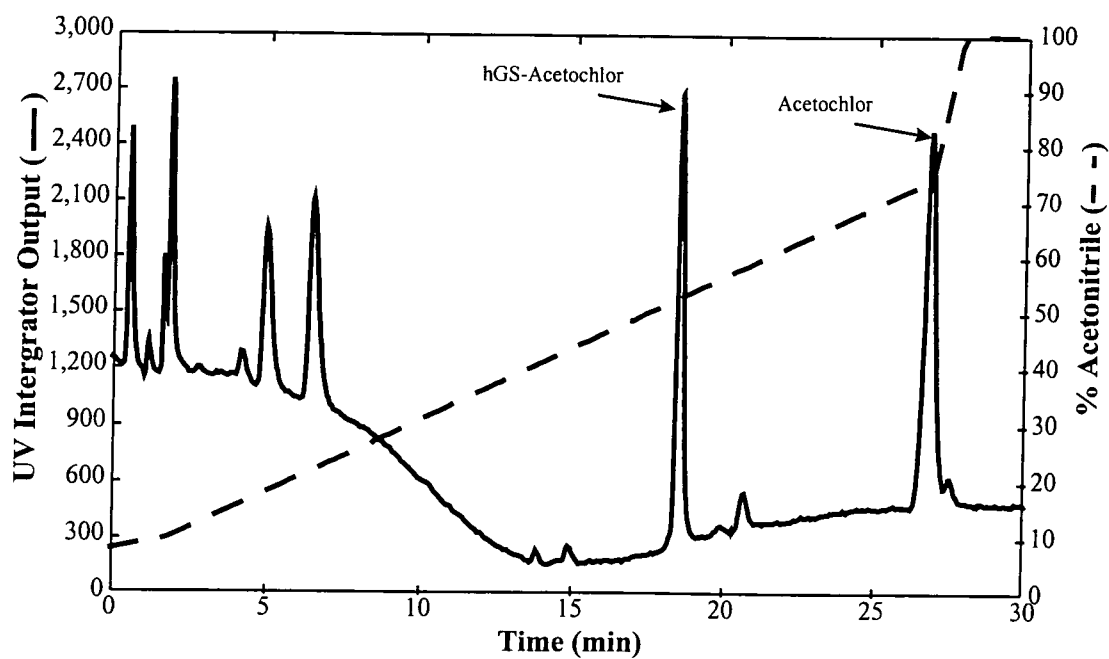
Figure 3.3 Separation of acetochlor assay products by RP-HPLC.
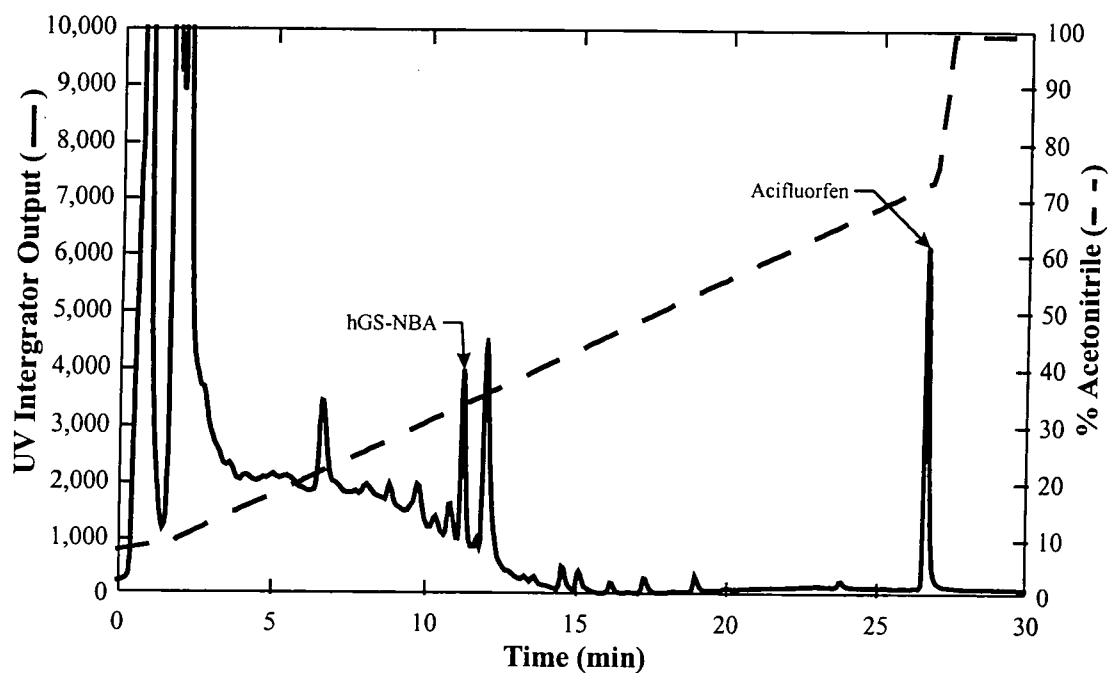
Figure 3.4 Separation of acifluorfen assay products by RP-HPLC.
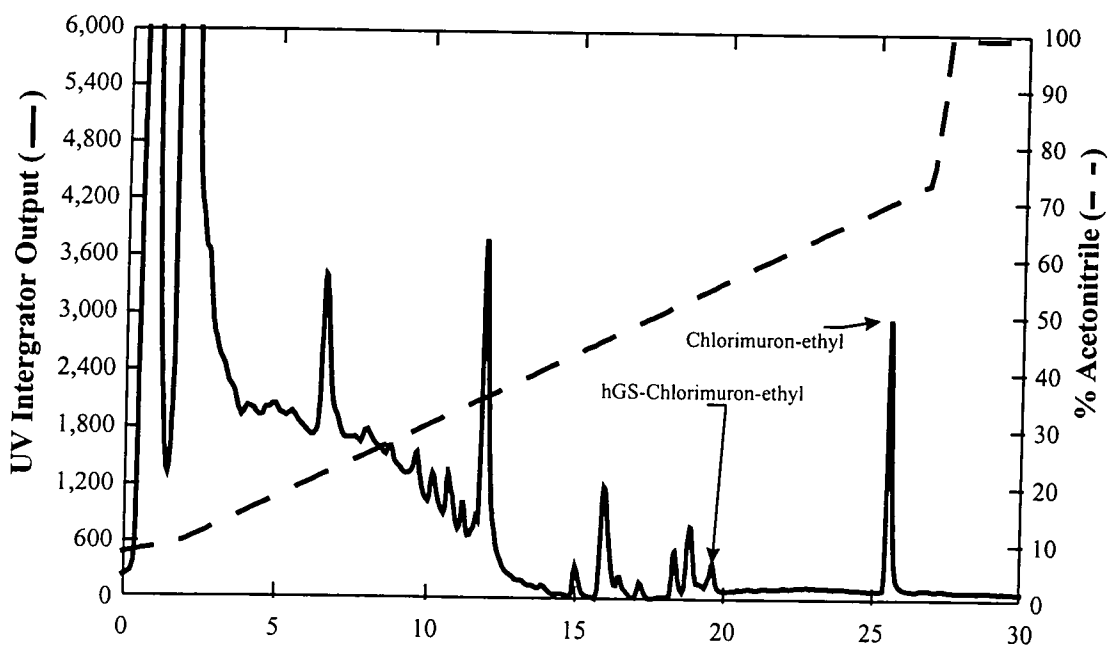
Figure 3.5 Separation of chlorimuron-ethyl assay products by RP-HPLC.
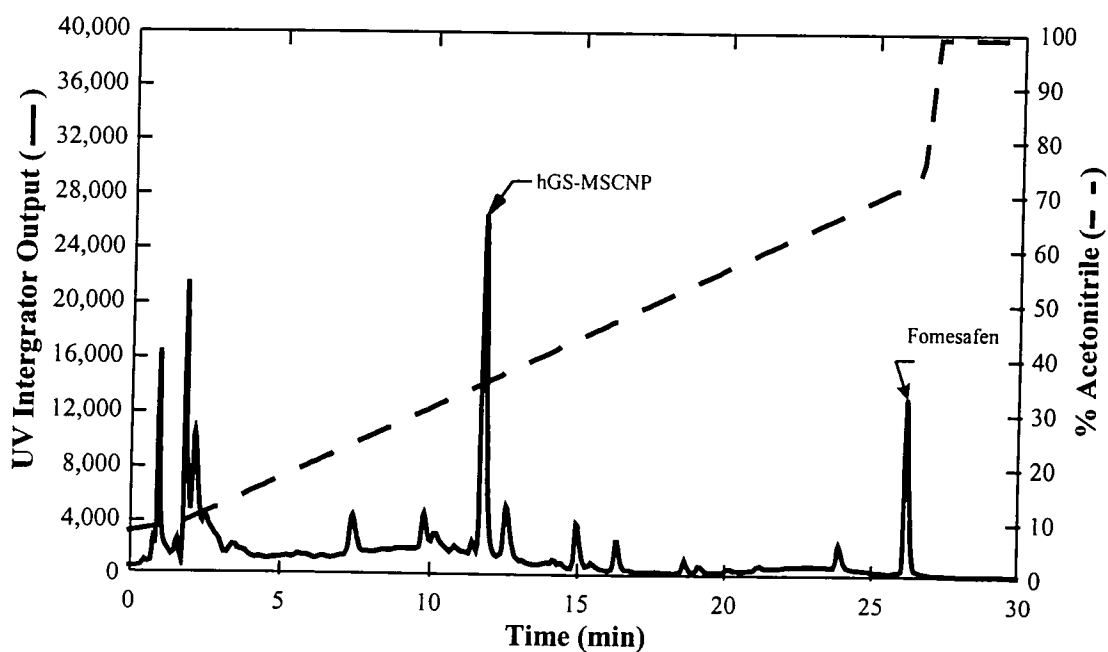
Figure 3.6 Separation of fomesafen assay products by RP-HPLC.
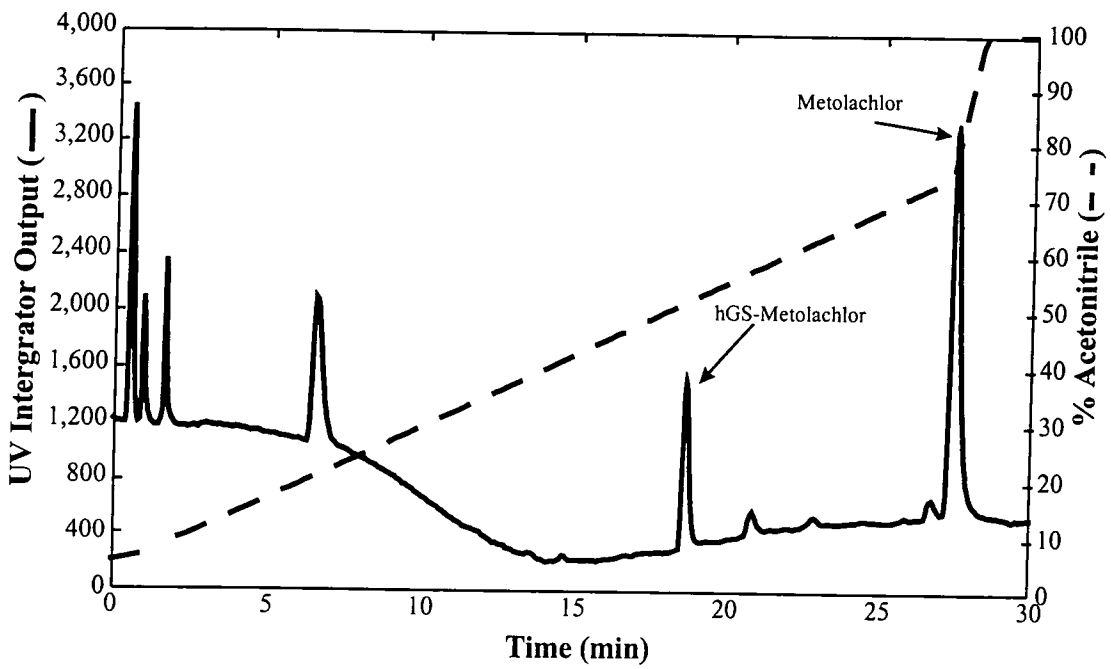
Figure 3.7 Separation of metolachlor assay products by RP-HPLC.

Herbicide/ Conjugate	pH	Retention Time (Min)	Peak Area Units (PAU)/nmol	Limit of Detection (nmol)	Chemical Rate (pkat)
Acetochlor		26.85	62,000	0.323	
GS-Acetochlor	6.8	18.34	62,000	0.323	1.49
hGS-Acetochlor	6.8	18.56	62,000	0.323	1.14
Acifluorfen		26.65	506,785	0.039	
GS-NBA	9.5	10.72	253,390	0.079	ND
hGS-NBA	9.5	11.21	253,390	0.079	ND
Chlorimuron		25.64	310,680*	0.064	
GS-Chlorimuron	9.5	19.46	310,680*	0.064	ND
hGS-Chlorimuron	9.5	19.61	310,680*	0.064	ND
Fomesafen		26.16	463,750	0.043	
GS-MSCNP	9.5	10.70	231,870	0.086	ND
hGS-MSCNP	9.5	11.11	231,870	0.086	ND
Metolachlor		27.54	39,000*	0.513	
GS-Metolachlor	6.8	18.47	39,000*	0.513	0.45
hGS-Metolachlor	6.8	18.70	39,000*	0.513	0.21

Table 3.1 pH values refer to the optimal pH for *in vitro* monitoring of conjugate formation. Quantification of the conjugates used calibration values obtained for the parent herbicides with acetochlor, chlorimuron-ethyl and metolachlor and for MSCNP and NBA conjugates the absorbance coefficient was assumed to be half that of the native herbicide, following its cleavage. "*" indicates data obtained from previous studies (Hatton *et al.*, 1996). The limit of detection indicates the smallest quantity of conjugate that could be detected reliably and enzyme rates described as "ND" (non-detectable) are therefore below this level.

3.2.2 Identification of thiols in soybean.

Prior to characterising the GST activity it was considered important to confirm the identity of the free thiol available for conjugation within the soybean tissues of interest. The free thiols in cell cultures, roots and shoots of soybean were identified and quantified using fluorescence-HPLC following monobromobimane derivatisation of thiols as described in section 2.1.6. Figures 3.8-3.13 show the fluorescent *S*-bromobimane adducts identified in extracts of 5-day-old soybean roots, shoots and cell cultures, together with reference glutathione and homoglutathione conjugates. Fluorescence was detected using a Gilson 121 fluorometer.

No Thiol
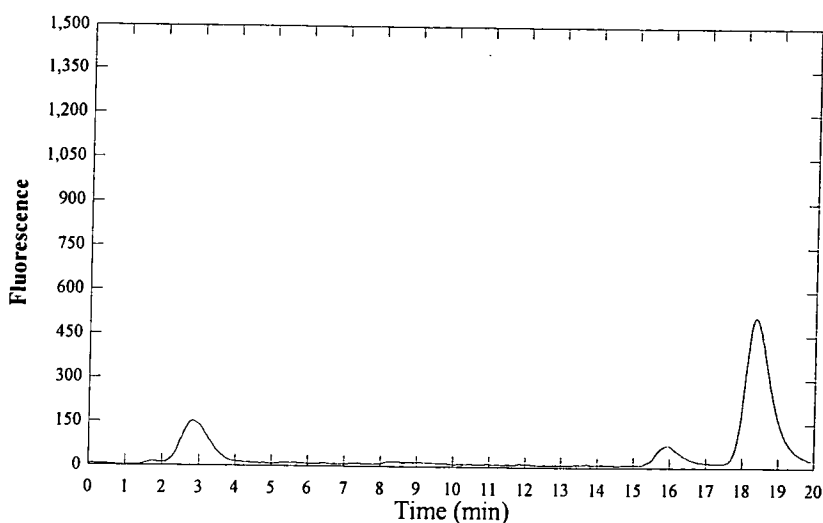
Figure 3.8 Elution profile obtained with bromobimane only (no thiol).

Glutathione Standard
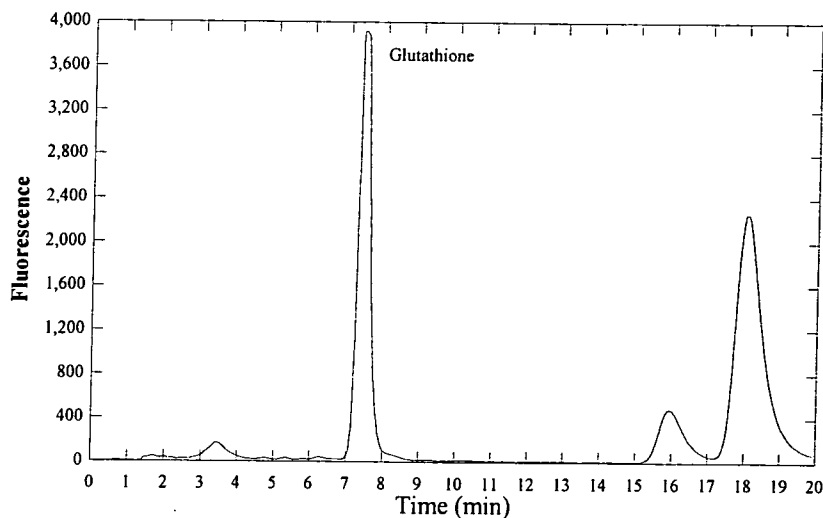
Figure 3.9 10 nmol glutathione standard derivatised with monobromobimane.

Homoglutathione Standard
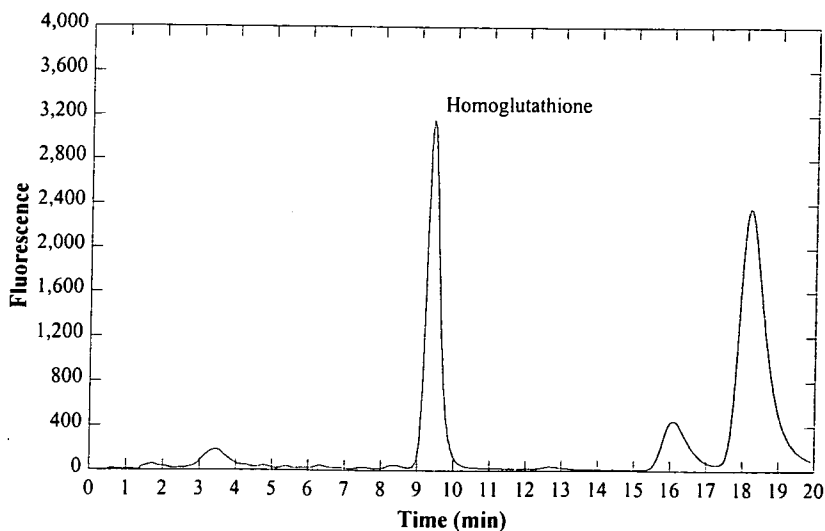
Figure 3.10 10 nmol homoglutathione standard derivatised with monobromobimane.

Cell Culture
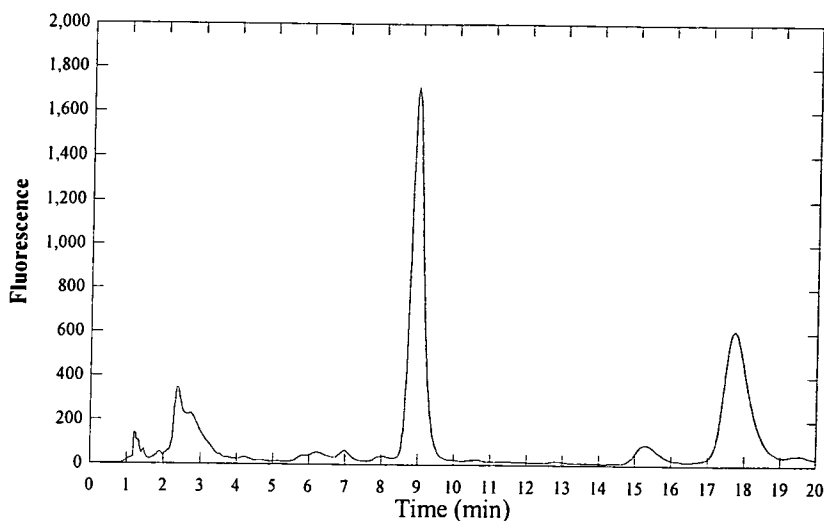
Figure 3.11 Monobromobimane derivatised free thiol in soybean cell cultures.

Soybean Roots
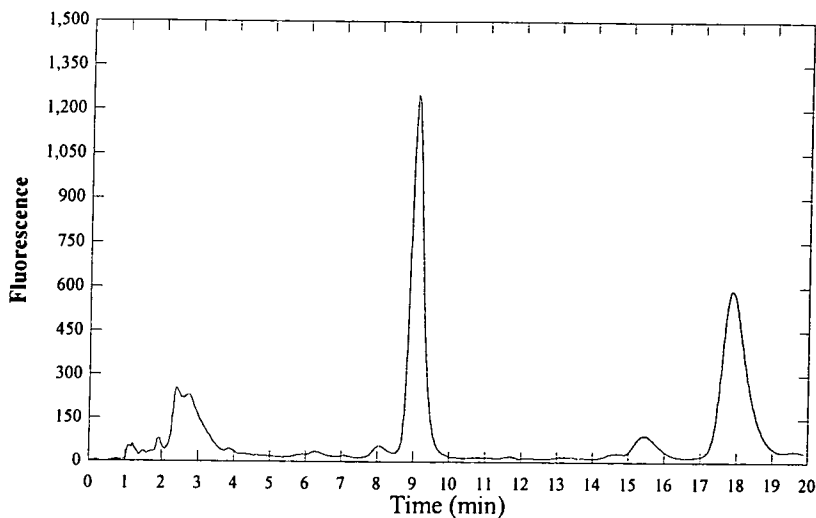
Figure 3.12 Monobromobimane derivatised free thiol in soybean roots.

Soybean Shoots
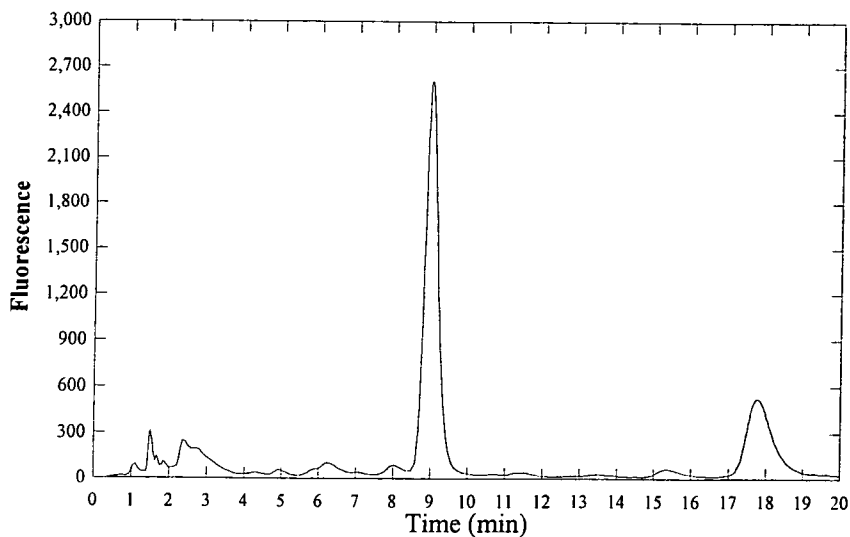
Figure 3.13 Monobromobimane derivatised free thiol in soybean shoots.

Tissue	hGSH concentration μmol g^{-1} FW	GSH concentration μmol g^{-1} FW
Root	1.45	ND
Shoot	3.12	ND
Cell culture	1.97	ND

Table 3.2 Free thiol concentrations in soybean tissues. Values represent single measurements, ND = not detectable.

The results confirmed that homoglutathione was the major (>99%) free thiol available in all tissues, as reported previously (Klapheck *et al.*, 1988). The concentration of homoglutathione was greatest in shoots, with the levels in cell cultures and roots of a similar value (Table 3.2). The figure obtained for cell cultures compared well with the value of 1.84 μmol g^{-1} Fw reported in previous studies (Knörzer *et al.*, 1996). However, the levels of homoglutathione in the whole plant tissues were significantly higher than the 0.41 μmol g^{-1} Fw reported by Breaux *et al.*, 1987. No other significant fluorescent thiol-derivatives were observed in crude soybean extracts. The fluorescent products detected in the no thiol control (Figure 3.8) are due to the presence of unreacted bromobimane and its degradation products.

These initial findings revealed the importance of using homoglutathione as co-substrate whenever possible when defining the detoxifying activity of soybean GSTs in soybean herbicides.

3.2.3 GST in soybean organs and cell cultures.

GST activity toward CDNB and the herbicides acetochlor, acifluorfen, chlorimuron-ethyl, fomesafen and metolachlor was determined in soybean seedlings and in suspension cultured cells (cv. Mandarin). In order to optimise experimental conditions, the GST activity in soybean cell cultures was determined at various time points after sub-culturing (Figure 3.14).
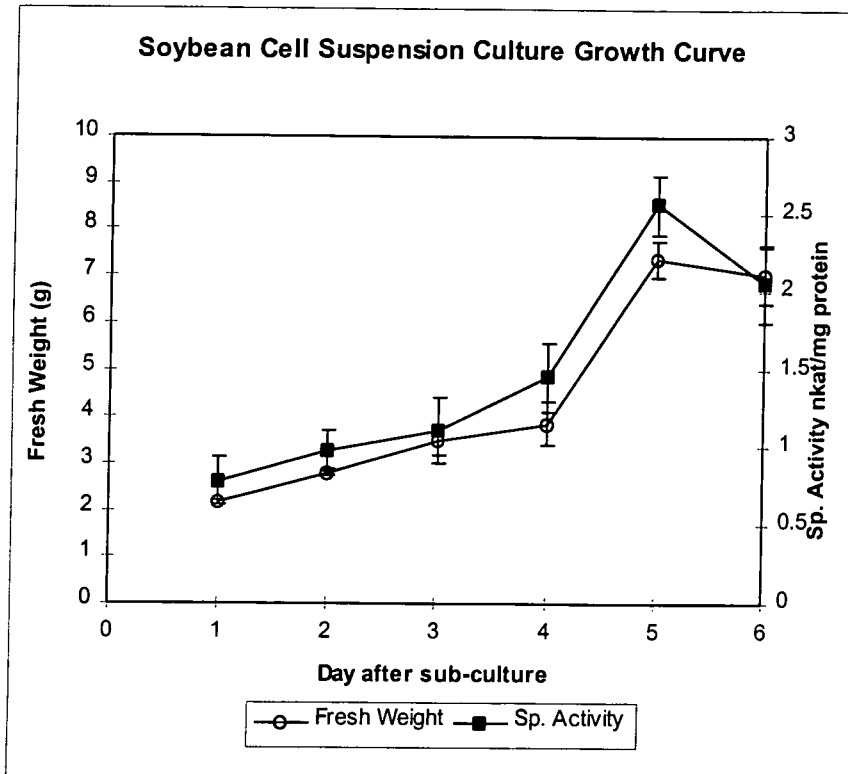
Figure 3.14. Increase in fresh weight and GST activity toward CDNB following sub-culturing of suspension cultured soybean cells. Value represent mean of duplicate results, with variations from the mean indicated by error bars.

Maximal GST activity toward CDNB coincided with maximal cell growth at 5-days post sub-culturing. This was in contrast to suspension-cultured cells of *Setaria faberi*, which showed maximal activity toward CDNB during early logarithmic growth, three days after sub-culture (Hatton *et al.*, 1998). Unless otherwise stated, 5-day-old cell cultures were used as the source for all subsequent characterisation and purification studies with GSTs from soybean cultures.

For whole plant studies, soybean variety D297 was chosen due to good germination efficiency and previous non-published studies finding that this cultivar possessed higher GST activity toward CDNB than a range of other cultivars tested (Dr R Edwards, personal communication). GST activity toward CDNB was determined in soybean seeds and the roots, stems and cotyledons of seedlings and the results compared with those obtained with extracts from 5-day-old suspension cultures (Table 3.3 /ᵃFigure 3.15). In these initial characterisation studies homoglutathione was unavailable and so glutathione was used as co-substrate.

Tissue	Specific Activity nkat mg^{-1} protein
Cell Culture	1.66 ± 0.15
Seed	0.45 ± 0.1
Root	0.21 ± 0.12
Stem	0.20 ± 0.11
Cotyledon	0.26 ± 0.07

Table 3.3 GST activity toward CDNB in extracts from soybean seeds, five-day-old seedlings and five-day-old cell cultures. Specific activity is reported along with the standard deviation (n=3).
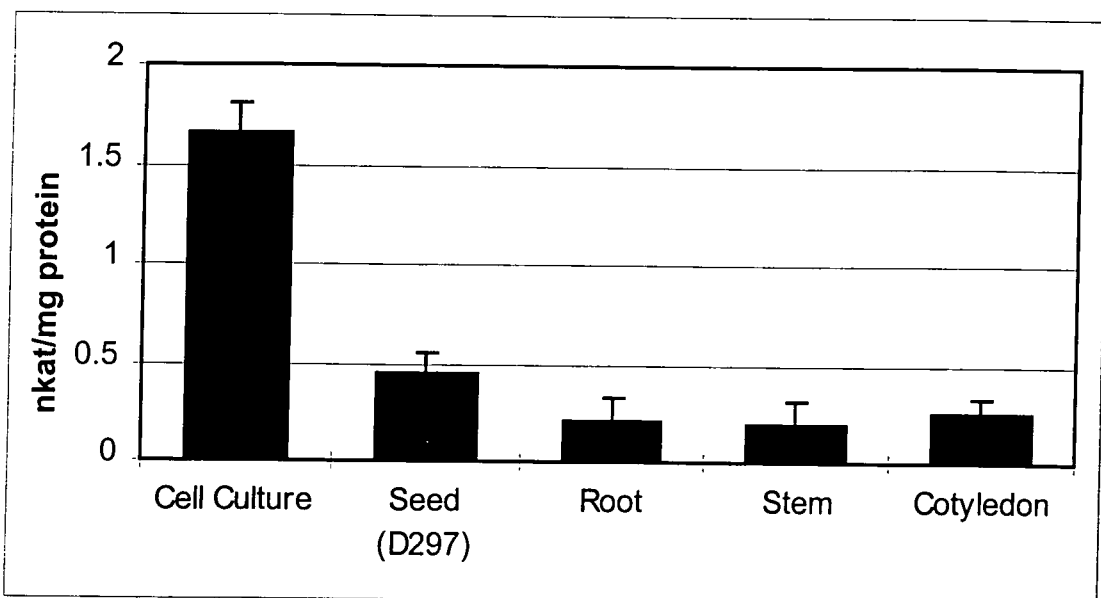
Figure 3.15 Histogram showing results from Table 3.3. GST activity toward CDNB in extracts from soybean seeds, seedlings and cell cultures. Mean values are indicated along with the standard deviation (n=3).

These results showed that cell cultures contain greater than 4-fold GST activity toward CDNB than whole soybean plants. Although GST activities in different plant features have not been reported previously in soybean, they have been determined in other legumes such as pea (Williamson and Beverly, 1988; Edwards, 1996) and chickpea (Hunaiti and Ali, 1990). In pea seedlings GST activity toward CDNB was considerably higher in epicotyls than roots (Edwards, 1996). In chickpea, the order of specific activity (nmol product formed min^{-1} mg^{-1} protein) was roots<leaves<stems (Hunaiti and Ali, 1991).

3.2.4 GST activity toward CDNB in crude extracts from soybean plants and cell cultures using glutathione and homoglutathione as co-substrates.

To determine whether GST activity in soybean differed when assayed in the presence of homoglutathione rather than glutathione, enzyme activity with CDNB as substrate was determined in the presence of both thiols using crude extracts from 5-day-old cell cultures and the foliage of 14-day-old plants (Table 3.4 / Figure 3.16).

Tissue	Thiol	Specific activity nkat mg^{-1} protein
5-day-old cell culture	GSH	2.16 ± 0.20
	hGSH	2.16 ± 0.21
14-day-old soybean	GSH	0.31 ± 0.01
	hGSH	0.36 ± 0.12

Table 3.4 GST activity toward CDNB in 14-day-old soybean plants vs. 5-day-old cell cultures. Results show the means of triplicate assays with error bars showing standard deviation (n=3).
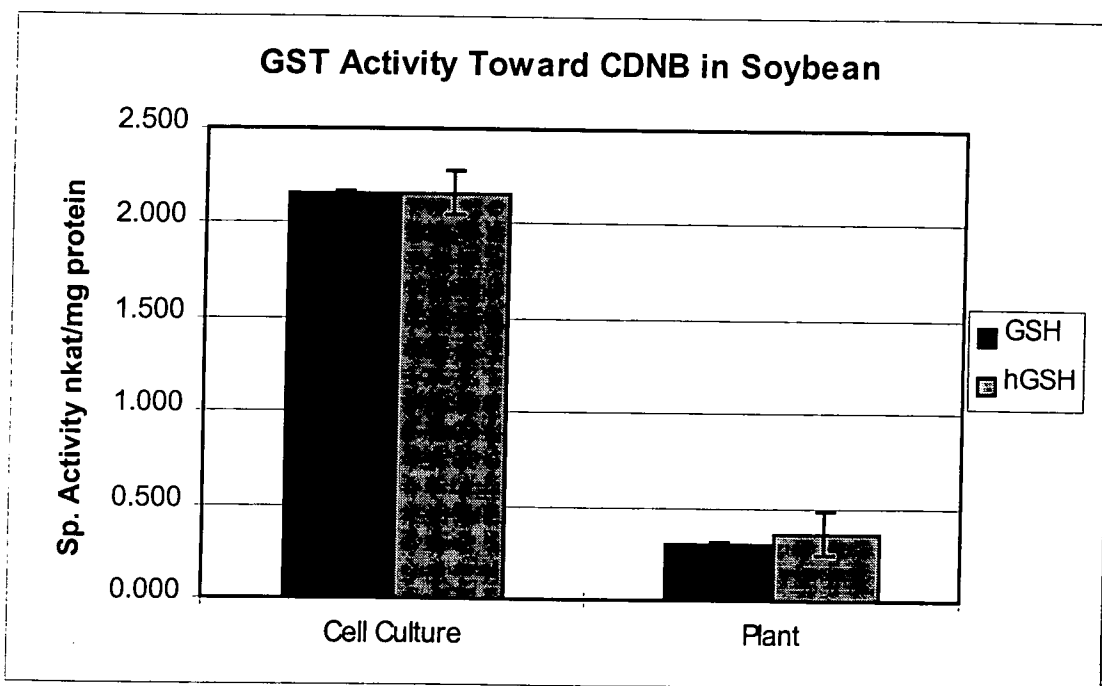
Figure 3.16 Histogram showing results from Table 3.4. GST activity toward CDNB in 14-day-old soybean plants vs. five-day-old cell cultures. Results show the means of triplicate assays with error bars showing standard deviation (n=3).

Using crude protein preparations, it appeared that soybean GST activity toward CDNB showed no preference overall toward either thiol. In the absence of enzyme,

conjugation to CDNB was identical for both glutathione and homoglutathione (0.07 nkat). However, during these studies it was noted that the stability of homoglutathione was significantly lower than glutathione at pH 7.0, and as a result homoglutathione preparations were always prepared fresh for each experiment.

3.2.5 GST activity toward herbicides in 2-week-old soybean plants and 5-day-old cell cultures.

Since soybean GSTs involved in herbicide selectivity were of particular interest it was considered important to determine activity toward specific herbicides, rather than CDNB, as some GSTs important in herbicide detoxification, show little, if any activity toward CDNB (Holt *et al.,*1995). GST activity was determined in 2-week-old soybean plants (Table 3.5; Figure 3.17) and 5-day-old cell cultures (Table 3.5 / Figure 3.18) using both glutathione and homoglutathione.

Herbicide	Thiol	Specific activity pkat mg⁻¹ protein	
		Plants	Cell Culture
Acetochlor	GSH	1.80	12.14
	hGSH	2.01	7.34
Acifluorfen	GSH	ND	0.22
	hGSH	ND	2.53
Chlorimuron-ethyl	GSH	ND	0.28
	hGSH	ND	0.31
Fomesafen	GSH	ND	2.35
	hGSH	1.00	15.32
Metolachlor	GSH	1.23	5.42
	hGSH	1.11	3.26

Table 3.5. GST activity toward herbicide substrates in 14-day-old whole soybean plants and 5-day-old soybean cell cultures. Activity was determined with both glutathione and homoglutathione. Values given represent the average of duplicate measurements.

GST Activity Toward Herbicides in Soybean Plants
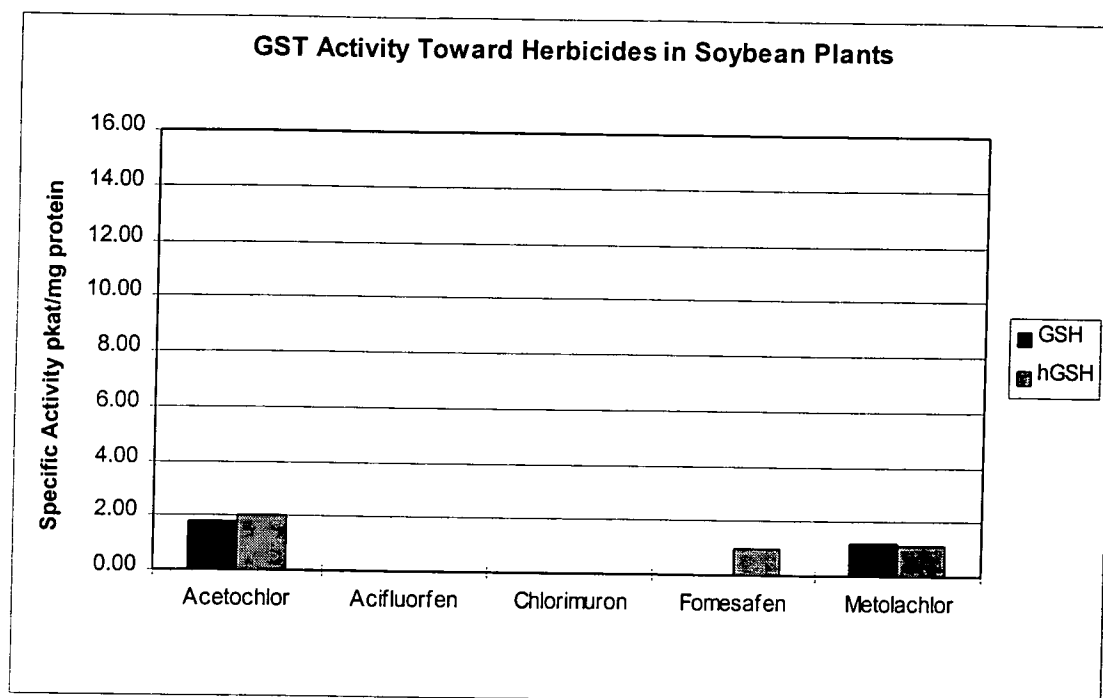
Figure 3.17. Histogram showing results from Table 3.5. GST activity toward herbicide substrates in 14-day-old soybean plants. Values represent the average of duplicate measurements.

GST Activity Toward Herbicides in Soybean Cell Cultures
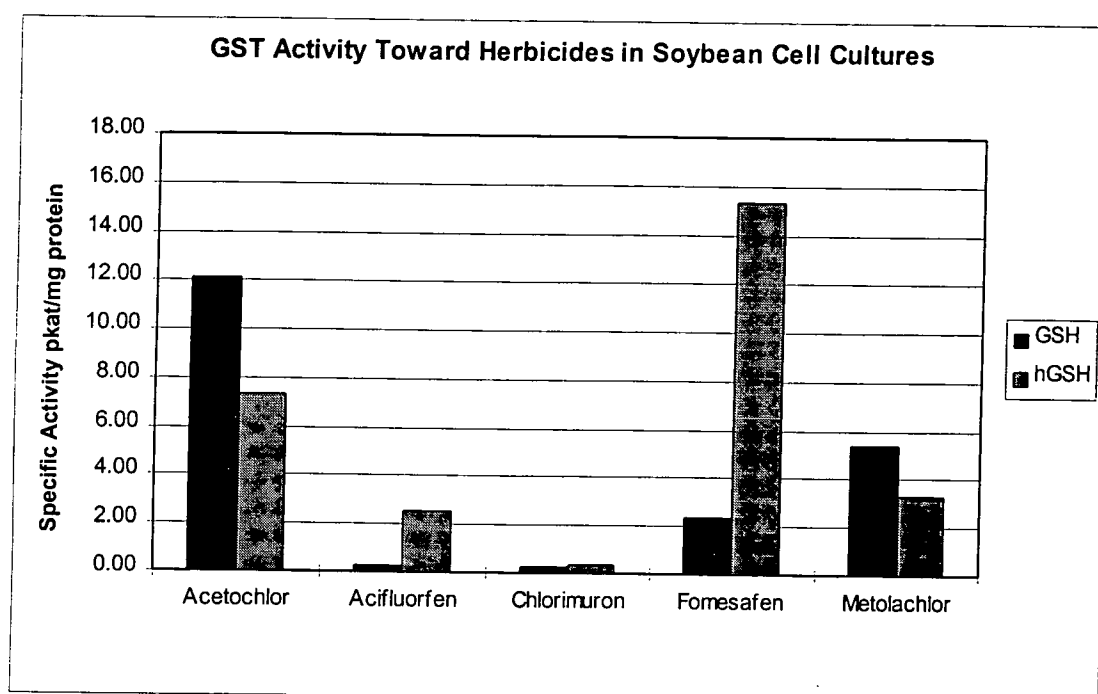
Figure 3.18. Histogram showing results from Table 3.5. GST activity toward herbicide substrates in 5-day-old soybean cell cultures. Values represent the average of duplicate measurements.

Figures 3.17 and 3.18 show that crude protein extracts from soybean cell cultures contain superior activity toward all the herbicide substrates tested, compared with the plant extracts, reflecting the results obtained with CDNB (Figure 3.16). However, interesting differences in thiol preference were observed with the herbicide substrates, which were not apparent with CDNB. In the extracts from whole plants, no GST

activity could be determined toward acifluorfen or chlorimuron-ethyl with either glutathione or homoglutathione. GST activity toward fomesafen in plants was only determined in the presence of homoglutathione, while there was no obvious thiol preference with the chloroacetanilides metolachlor and acetochlor. This result gave the first indication that soybean GSTs responsible for fomesafen conjugation show a preference for homoglutathione. The results obtained from cell culture analysis confirmed this finding. Although activity toward fomesafen could be detected with both glutathione and homoglutathione, the rate of conjugation was greater than 5-fold more rapid in the presence of the latter. A similar observation was apparent with the other diphenyl ether acifluorfen, but not with the chloroacetanilides.

3.2.6 Development of a GST assay with ^{14}C-fomesafen.

A major disadvantage of using an HPLC system to determine GST activity toward herbicides was the time taken to process each sample. In order to monitor GST activity toward fomesafen in a large number of samples, an assay using radiolabelled fomesafen was developed (section 2.1.5.2). In Figure 3.19, the biosynthesis of radioactive polar metabolites by a crude extract from cell cultures (5-day) is shown at two pH values in the presence of either glutathione or homoglutathione. Briefly, the assay consisted of incubating [^{14}C]-fomesafen with the enzyme extract and glutathione or homoglutathione. Control incubations consisted of a) omitting thiol and b) omitting enzyme extract. At end of the reaction period the unconjugated herbicide was removed by phase-partitioning with diethyl ether, leaving the polar conjugates in the aqueous fraction. The quantity of radioactivity in the aqueous phase was therefore an indication of enzyme activity. The linearity of the assay was determined by sampling the assay mixture at 15, 30, 45 and 60 min.
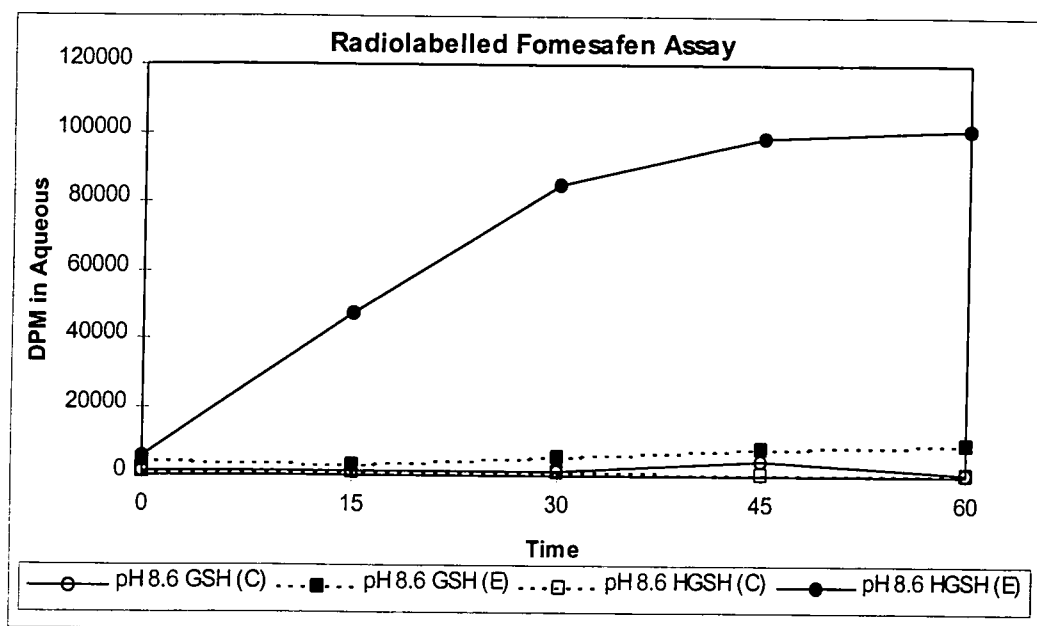
Figure 3.19 Conjugation of radiolabelled fomesafen by extracts from 5-day-old cell cultures.

Unlike the HPLC based method, the GST-catalysed reaction was only linear up until 30 min, probably because of the limited concentrations of fomesafen present as compared with the HPLC assay. As determined with the HPLC-based assay, the assay with radioactive fomesafen confirmed the finding in the previous section that fomesafen metabolism shows a strong preference for conjugation with homo-glutathione.

3.2.7 GST activity in 14-day-old seedlings of soybean and associated weeds.

To determine whether GST activity and herbicide tolerance could be correlated in soybean, GST activity toward a range of substrates was assessed in 14-day-old soybean plants and problematical weeds of the same age. As mentioned previously, these weeds were the dicotyledons *A. theophrasti*, *A. retroflexus* and *I. hederacea* and the monocotyledons *D. sanguinalis*, *E. crus-galli*, *S. faberi* and *S. halepense* (Figure 3.20). Due to differing rates of growth and growth habit it was difficult to compare plants at equivalent stages of development and so plants of similar age (14-day post sowing) were used in each study. Ammonium sulphate precipitated preparations from crude soybean extracts were assayed for GST activity with glutathione and homoglutathione, whereas the weeds examined were only assayed using glutathione, since all species studied were non-leguminous and contained glutathione as the major thiol (Klapheck, 1988; Skipsey *et al.*, 1997).

Figure 3.20 14-day-old soybean and weed species used in the study (shown glass-house grown).

GST activity toward CDNB.

Although CDNB is not a herbicide, it is frequently used to assay GSTs in plants and it was therefore of interest to determine activity toward this model substrate in soybean and weed species. Table 3.6 and Figure 3.21 show the results obtained.

Species	Thiol	Specific Activity nkat mg^{-1} protein
Glycine max (var. D297)	GSH	0.26 ± 0.07
	hGSH	0.07 ± 0.02
A. theophrasti	GSH	1.13 ± 0.16
A. retroflexus	GSH	0.58 ± 0.12
D. sanguinalis	GSH	0.58 ± 0.04
E. crus-galli	GSH	1.25 ± 0.14
I. hederacea	GSH	0.52 ± 0.16
S. faberi	GSH	0.13 ± 0.02
S. halepense	GSH	0.14 ± 0.03

Table 3.6 GST activity toward CDNB in 14-day-old soybean and 14-day-old associated weeds ($\pm$ SD, n=3).
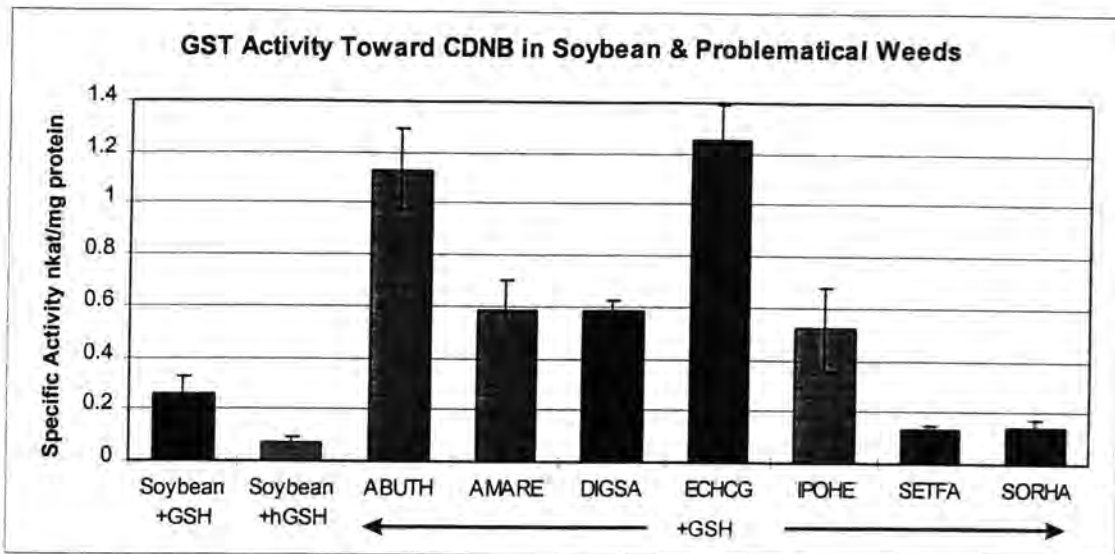
Figure 3.21. Histogram showing results from Table 3.6. GST activities toward CDNB in 14-day-old soybean (D297) and associated weeds ($\pm$ SD, n=3).

In the 14-day-old plants, GST activity toward CDNB appeared to be lowest in soybean and the grasses *S. faberi* and *S. halepense*. The highest specific activity for GST mediated conjugation of CDNB was determined in the broadleaf species *A. theophrasti*, *A. retroflexus*, and *I. hederacea*, and the grasses *E. crus-galli* and *D. sanguinalis*. GST activity has been previously reported in *A. theophrasti* (0.110 ± 0.0 nkat mg^{-1} protein), *D. sanguinalis* (0.040 ± 0.0 nkat mg^{-1} protein), *E. crus-galli* (0.060 ± 0.0 nkat mg^{-1} protein) and *S. faberi* (0.061 ± 0.0 nkat mg^{-1} protein) (Hatton *et al.*, 1996). Whilst there is some difference in the degree of activity between this study and that of Hatton *et al*, (1996) the trend in activity between the species studied, with the exception of *E. crus-galli*, was similar.

3.2.9 GST activity toward herbicide substrates in soybean and problematical competing weeds.

Of the herbicides tested, GST activity toward acetochlor, metolachlor and fomesafen, but not acifluorfen or chlorimuron-ethyl, was detected in crude extracts from soybean seedlings (Figure 3.17). Therefore, GST activities toward fomesafen (Table 3.7; Figure 3.22) and metolachlor (Table 3.7; Figure 3.23) were chosen to further assess detoxifying activity toward herbicide substrates in soybean and problematical weeds.

Species	Thiol	Specific Activity pkat mg^{-1} protein	
		Fomesafen	Metolachlor
Glycine max (D297)	GSH	0.16 ± 0.02	6.26 ± 0.11
	hGSH	0.10 ± 0.04	6.17 ± 0.05
A. theophrasti	GSH	ND	4.88 ± 0.09
A. retroflexus	GSH	ND	0.08 ± 0.03
D. sanguinalis	GSH	0.50 ± 0.02	ND
E. crus-galli	GSH	0.58 ± 0.02	ND
I. hederacea	GSH	ND	0.04 ± 0.01
S. faberi	GSH	0.44 ± 0.02	ND
S. halepense	GSH	0.41 ± 0.02	0.312 ± 0.12

Table 3.7 GST activity toward the herbicides fomesafen and metolachlor in 14-day-old soybean (D297) and associated weeds. Error is given as SD from the mean (n=3). ND = activity below the limit of detection.
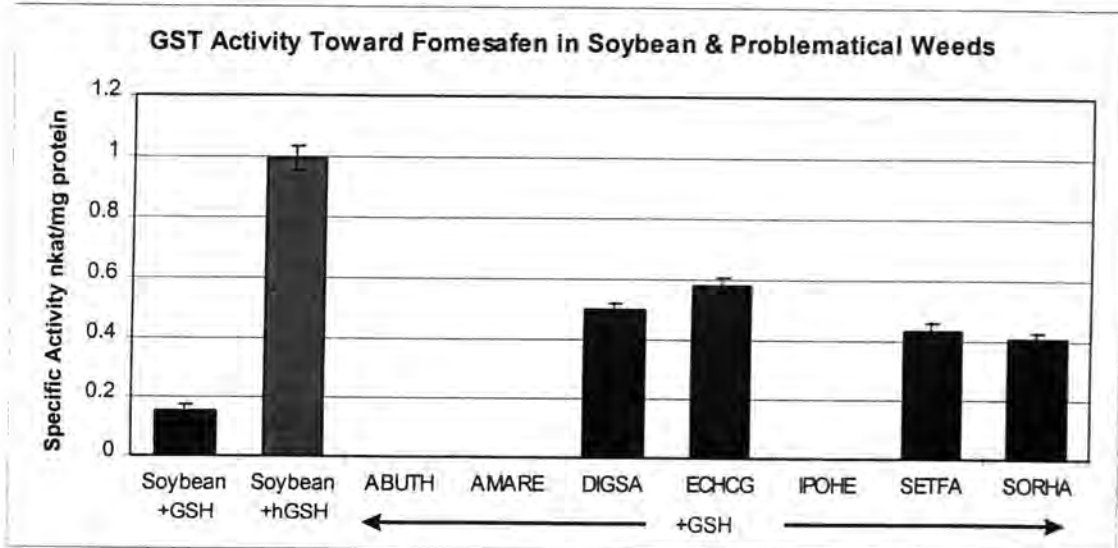
Figure 3.22 Histogram showing data presented in Table 3.7. GST activity toward fomesafen in soybean and associated weeds. Error bars represent SD from the mean (n=3).

GST activity toward fomesafen was greatest in soybean with the endogenous thiol homoglutathione as co-substrate (Figure 3.22). Activities toward fomesafen were non-detectable in the other broadleaf plants, although in *A. theophrasti* it was possible that fomesafen was undergoing conjugation at a slow rate, but this was difficult to quantify due to the co-chromatography of a natural product with the GS-MSCNP conjugate following HPLC separation. In contrast to the dicotyledon weeds, all the

grass weeds were able to conjugate fomesafen *in vitro*, though all with approximately half the specific activity of soybean.
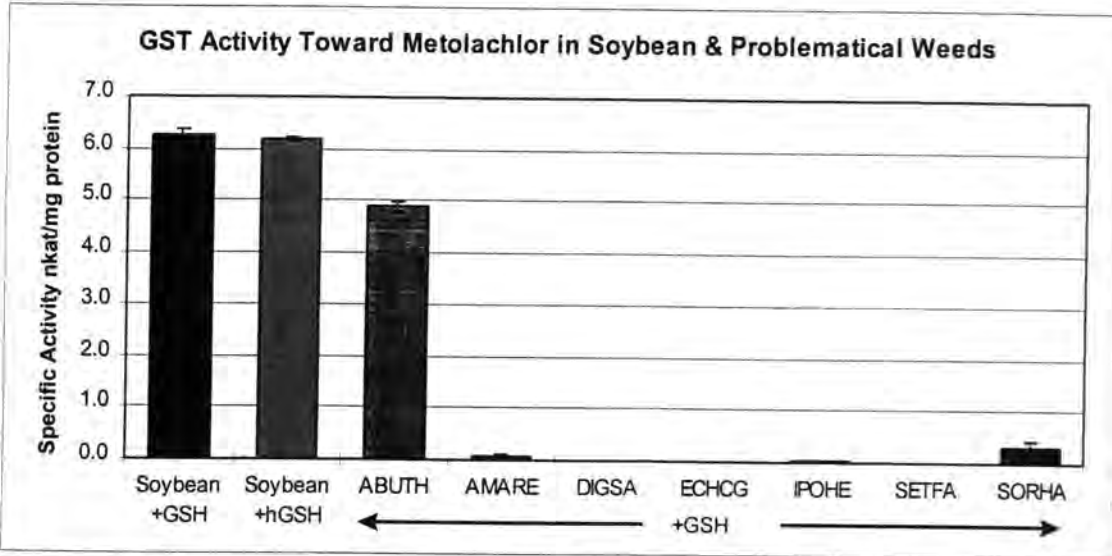
Figure 3.23 Histogram showing results presented in Table 3.7. GST activity towards metolachlor in soybean and associated weeds. Error bars represent SD from the mean (n=3).

With the exception of *A. theophrasti*, there was negligible GST activity toward metolachlor in the grass weeds analysed (Figure 3.23). Previous studies have reported low GST activity toward *D. sanguinalis, E. crus-galli* and *S. faberi* but high activity toward *A. theophrasti* (Hatton *et al.*, 1996). GST activity toward metolachlor in soybean were higher than previously determined in 14-day-old plants (Figure 3.17). The reasons for these differences appear to be due to differences in growth stage of the plants used in each experiment.

3.2.10 Herbicide selectivity in soybean.

Soybean plants were treated with various herbicides to determine whether the GST activities observed could be correlated to physical herbicide tolerance. The experiment was carried out, according to the method described in section 2.1.2, with the assistance of Carol Morris, Zeneca Agrochemicals. Physiological damage caused by each herbicide at various application rates was assessed at 7 and 13 days post-application. When studying metabolism as a basis of herbicide selectivity, damage at day 7 was considered more relevant, as recovery from non-lethal damage due to other factors, for example new growth, could conceivably occur after this time. For this reason only data obtained at day 7 is reported in Figures 3.24-3.27. Due to the various

modes of action of the herbicides used, damage assessment was made on the basis of plant necrosis with acifluorfen and fomesafen, which are both photo-bleaching herbicides, and recorded as twisting and stunting in chlorimuron-ethyl treated plants. Metolachlor, which is a growth inhibitor, is traditionally used as a pre-emergence herbicide, therefore it was necessary to apply a more general assessment of damage when applied as a post-emergence herbicide. Damage was assessed on an arbitrary scale from 0% (no effect) to 100% (plant death). Table 3.8 shows the growth stage at which the herbicide was applied.

Species	Leaf no.	Height (cm)
Soybean (D297)	2 trifoliate leaves	31-33
DIGSA	5 leaves	7
ECHCG	4 leaves	7.5
SETFA	3 leaves	4-7
SORHA	4 leaves	4.5-7
ABUTH	2.5-3 leaves	2.5-7
AMARE	4-5 leaves	3.5
IPOHE	2 leaves	4-8

Table 3.8 Growth stage of plants used in herbicide selectivity trials.

The plants were of similar age to those used to assess GST activity, allowing direct comparisons to be made, though the plants for selectivity trials were grown in glass-house facilities rather than in environmentally controlled growth conditions. The broken line indicates broad leaf weeds, the thin solid line indicates grass weeds. Application rates are given in g active ingredient / hectare (g ai ha^{-1}).

Fomesafen Selectivity
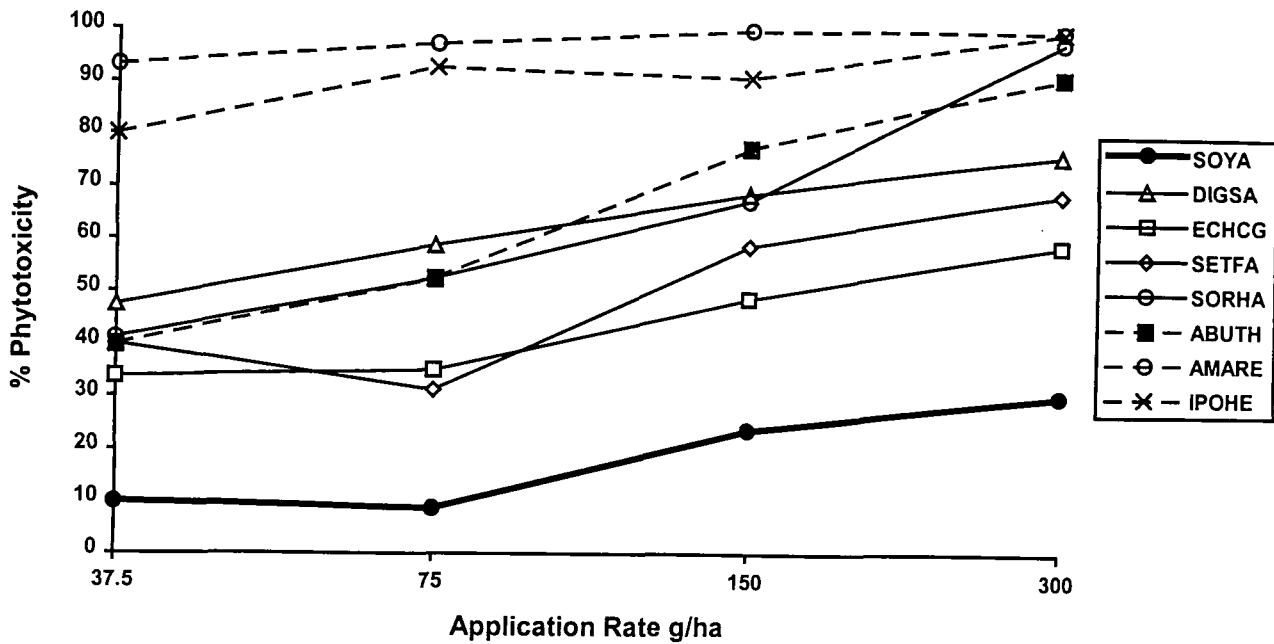
Figure 3.24. Fomesafen phytotoxicity in soybean and associated weeds seven days after spraying.

Acifluorfen Selectivity
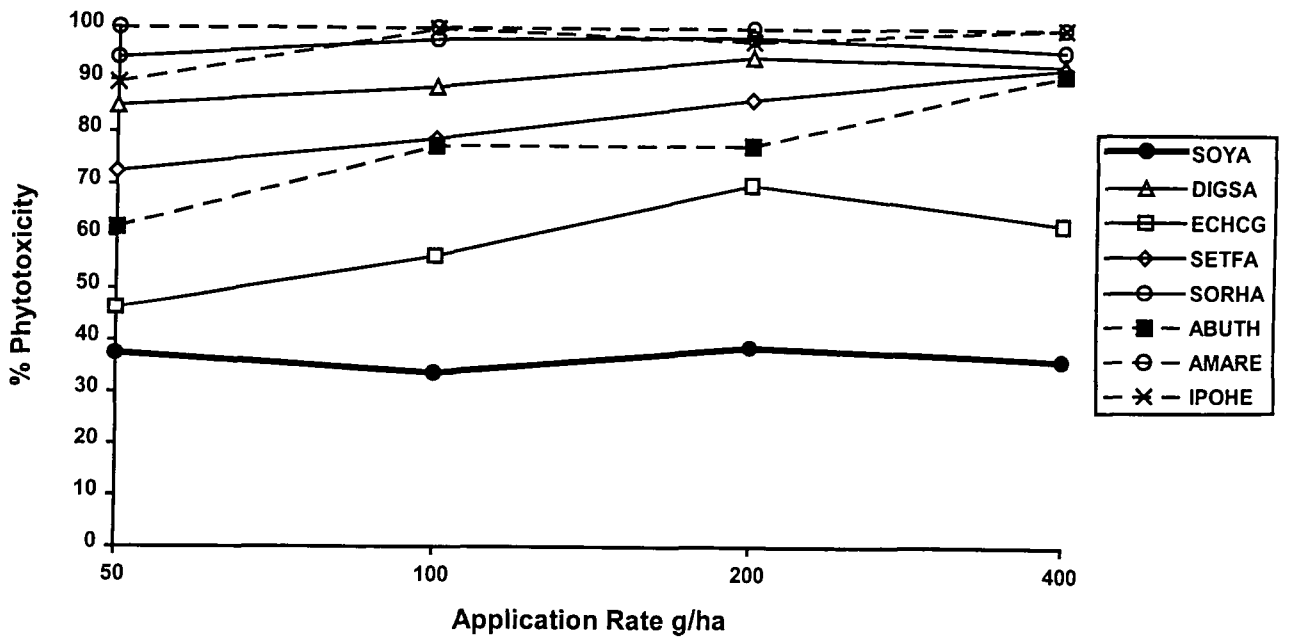
Figure 3.25 Acifluorfen phytotoxicity in soybean and associated weeds seven days after spraying.

Chlorimuron-ethyl Selectivity
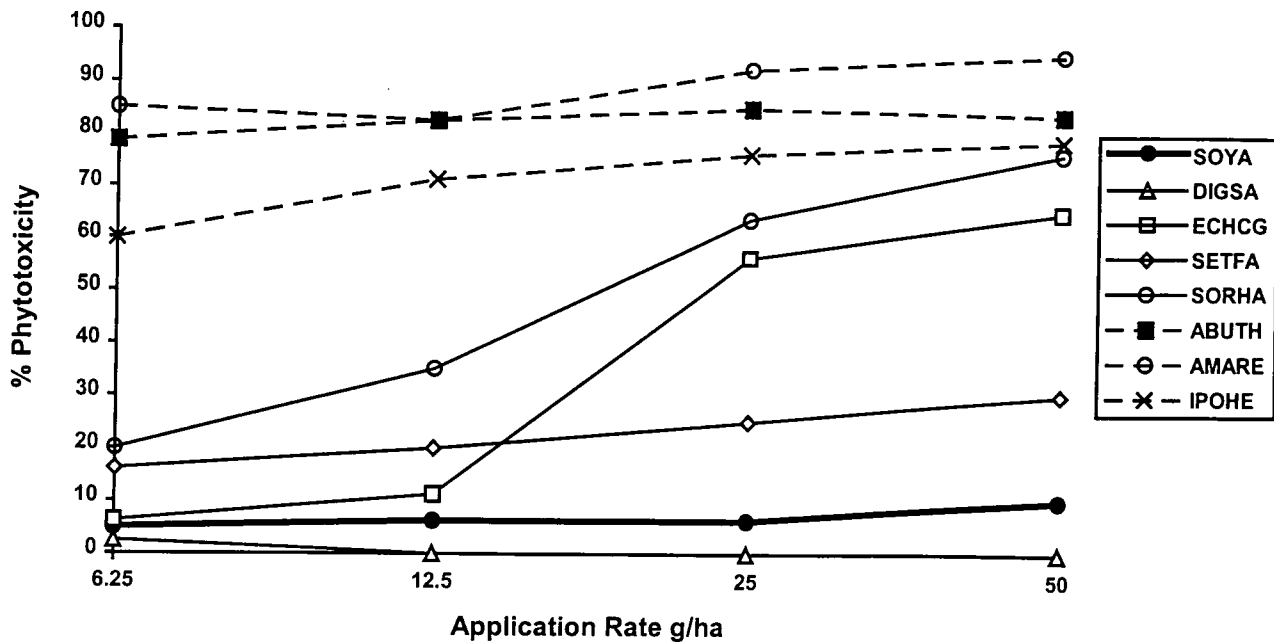
Figure 3.26 Chlorimuron-ethyl phytotoxicity in soybean and associated weeds seven days after spraying.

Metolachlor Selectivity
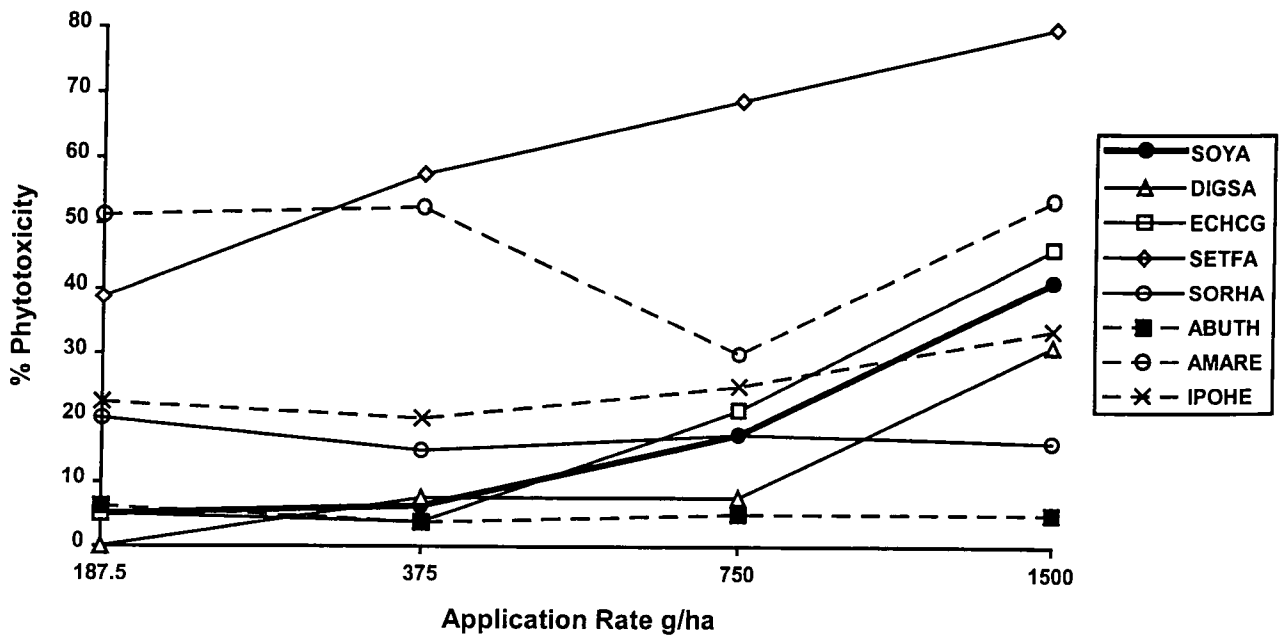
Figure 3.27. Metolachlor phytotoxicity in soybean and associated weeds seven days after spraying.

At the application rates used, both acifluorfen and fomesafen gave good selective control of the broadleaf weeds *A. theophrasti, A. retroflexus* and *I. hederacea*. In

addition, some control of the grasses was observed at the higher application rates used, especially with acifluorfen. These findings are consistent with the use of these compounds as post-emergence herbicides effective against broad-leaf weeds but also showing some activity toward grasses. However, the phytotoxicity associated with acifluorfen was significantly higher in soybean than observed with fomesafen at similar application rates.

Chlorimuron-ethyl was effective toward all the broad-leaf species tested, and showed little detrimental effect toward soybean. At application rates above 25 g ai ha^{-1} some control of the grasses *E. crus-galli* and *S. halepense* was observed. However little control of *D. sanguinalis* or *S. faberi* was apparent. This is in accordance with chlorimuron-ethyl's commercial use as an effective post-emergence broadleaf herbicide.

Metolachlor, commercially used as a selective pre-emergent herbicide, was not effective as a selective post-emergent treatment in this study. Phytotoxicity observed in soybean was similar to that in many of the weeds examined, with the notable exception on *S. faberi* which showed relatively high levels of damage, especially at higher application rates.

3.2.11 GST activities in 21-day-old soybean plants following treatment with herbicides and herbicide safeners.

Three safeners, known to be active in increasing GST expression in maize (Hatzios, 1991) were selected to determine whether these compounds were able to exert a similar enhancing effect on GST activity in soybean. The chosen compounds were BAS 145-138, dichlormid and naphthalic anhydride (NA) (Figure 3.28).
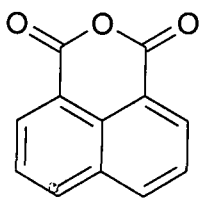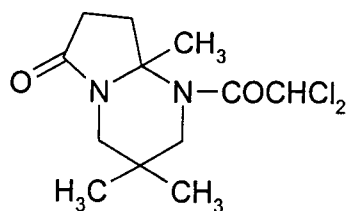
Naphthalic anhydride   BAS 145-138   Dichlormid
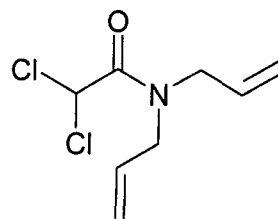
Figure 3.28 Safeners used in trial.

Significantly, both BAS 145-138 and naphthalic anhydride safen against acifluorfen and chlorimuron-ethyl damage in maize (Böger and Miller, 1994, Lamoureux & Rusness, 1991), and both these herbicides are used for selective broadleaf weed control in soybean. Similarly, dichlormid has been shown to protect maize against damage caused by the chloroacetanilide herbicides, such as metolachlor (Fuerst & Lamoureux, 1992), again used for weed control in soybean.

The effect of sub-toxic herbicide application on soybean GST activity was determined using the diphenyl ether herbicides fomesafen and oxyfluorfen. Oxyfluorfen was included in this study as although it has a common mode of action to fomesafen it is not detoxified by homoglutathione conjugation in soybean (Knörzer et al., 1996). Following application of herbicides or herbicide safeners at differing treatment rates, plants were analysed 48 h later for signs of visible phytotoxicity and changes in GST activity toward CDNB and the herbicides acetochlor and fomesafen (Table 3.9).

Treatment	App. Rate g ai ha^{-1}	VP	Specific Activity nkat* / pkat** mg^{-1} protein		
			CDNB*	Fomesafen**	Acetochlor**
Oxyfluorfen	2,500	+++	0.61 ± 0.11	1.27 ± 0.05	4.27 ± 0.57
	312	+++	0.55 ± 0.06		
	39	+++	0.52 ± 0.04		
Fomesafen	1,000	++	0.60 ± 0.02	0.99 ± 0.05	3.48 ± 0.42
	125	+	0.41 ± 0.02		
BAS 145-138	125	Nil	0.34 ± 0.03	1.55 ± 0.05	3.67 ± 1.17
Dichlormid	4,000	Nil	0.46 ± 0.05	1.11 ± 0.06	4.61 ± 0.08
	1,000	Nil	0.33 ± 0.03		
	125	Nil	0.24 ± 0.03		
Naphthalic Anhydride	1,000	Nil	0.42 ± 0.05	1.29 ± 0.09	3.96 ± 0.44
	125	Nil	0.30 ± 0.01		
WC	-	Nil	0.26 ± 0.01	1.21 ± 0.05	3.34 ± 0.70
FC	-	Nil	0.27 ± 0.02	1.03 ± 0.04	3.93 ± 0.66

Table 3.9 Foliar treatment application rates of herbicides and herbicide safeners. Herbicide and safener treatments are shown, along with WC = water only application control and FC = formulation only control. App. Rate = application rate in g active ingredient per hectare (g ai ha^{-1}). VP = Visible phytotoxicity associated with treatment: + = minor phytotoxicity, ++ = intermediate phytotoxicity, +++ = severe phytotoxicity, Nil = No phytotoxicity observed. Standard deviation is shown (n=3).

Figure 3.29 Soybean plants 48-h after treatment with the herbicides fomesafen, oxyfluorfen and typical safener treatment. Leaf necrosis on the herbicide treated plants is highlighted.
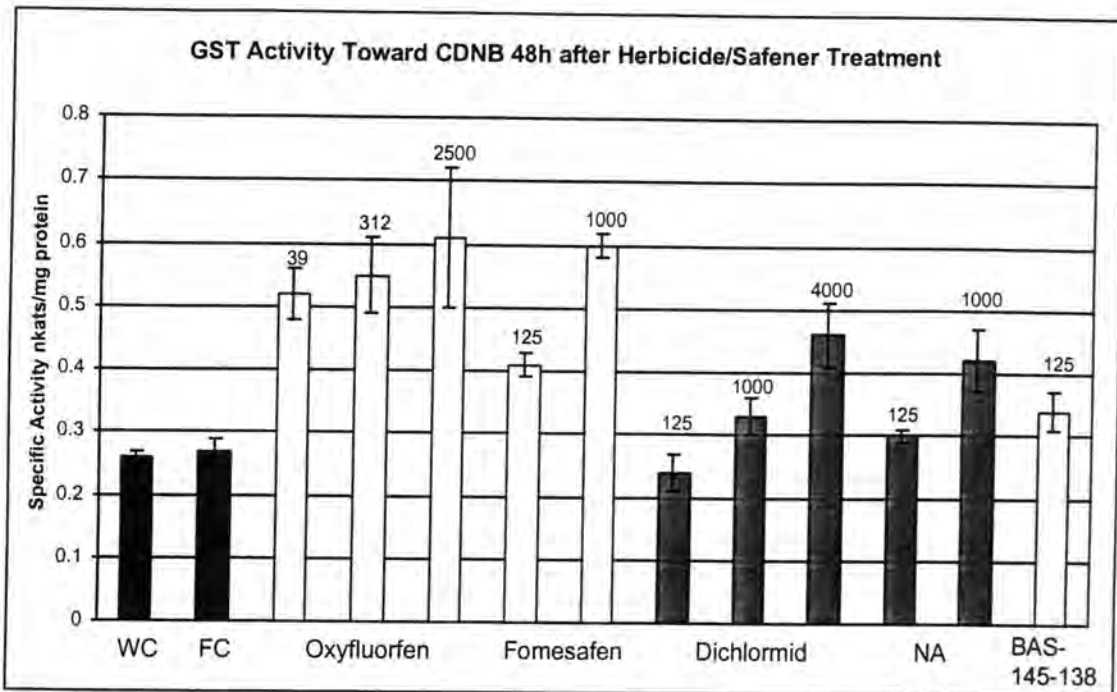
Figure 3.30. Histogram showing results presented in Table 3.9. GST activity toward CDNB in soybean 48 h following treatment with herbicides and herbicide safeners at the application rates indicated in g ai ha^{-1}. Results represent the mean of triplicate determinations with error bars indicating the standard deviation. WC= water only application control. FC = formulation application only control. Standard deviation is shown (n=3).
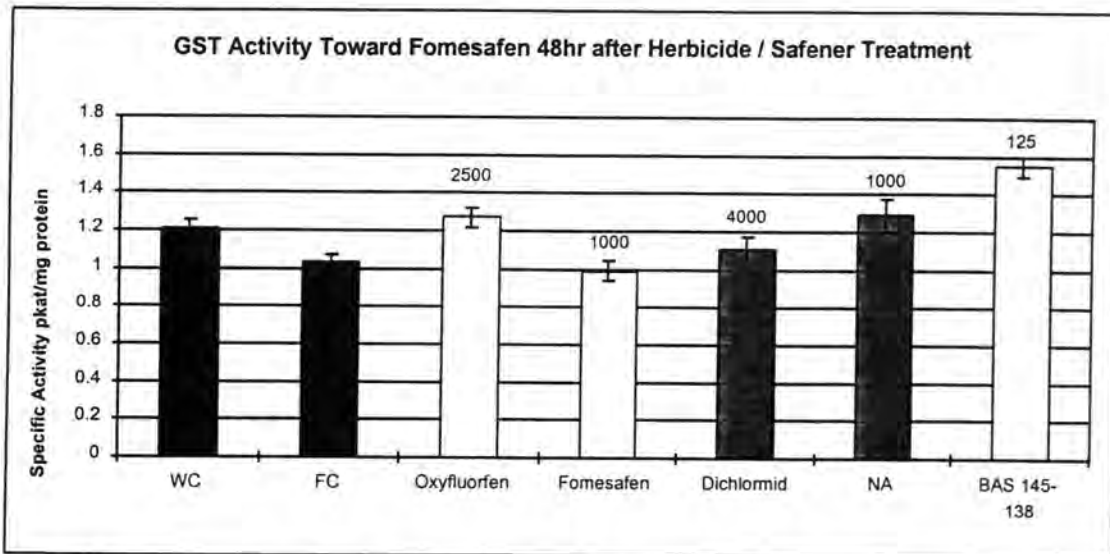
Figure 3.31. Histogram showing results presented in Table 3.9. GST activity toward fomesafen in soybean 48 h following treatment with herbicides and herbicide safeners at the application rates indicated in g ai ha^{-1}. Results represent the mean of duplicate measurements with error bars indicating the variation from the mean. WC= water only application control. FC = formulation application only control. Error bars represent SD from the mean (n=3).
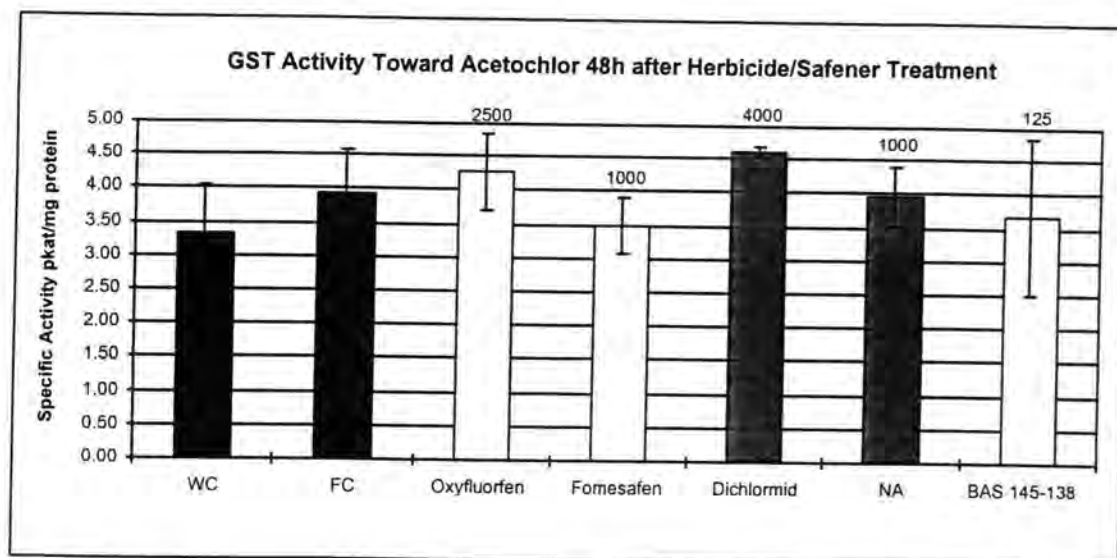
Figure 3.32. Histogram showing results presented in Table 3.9. GST activity toward acetochlor in soybean 48 h following treatment with herbicides and herbicide safeners at the application rates indicated. Results represent the mean of duplicate determinations with error bars indicating the variation from the mean. WC= water only application control. FC = formulation application only control. Error bars represent SD from the mean (n=3).

Figure 3.30 shows that treatment of 21-day-old soybean with the diphenyl ether herbicides oxyfluorfen and fomesafen caused a significant increase in GST activity toward CDNB in the foliage of treated plants. Severe necrosis was observed in oxyfluorfen treated plants (Figure 3.29), whilst phytotoxicity was only apparent at higher application rates of fomesafen. Safener treatments resulted in small increases in GST activity toward CDNB, but this was not associated with any visible damage (Figure 3.29). The safeners dichlormid and naphthalic anhydride enhanced GST activity toward CDNB in a dose dependent fashion, whilst the safener BAS 145-138 could only be applied at one application rate due to formulation characteristics, but also enhanced GST activity toward CDNB.

Changes in activity toward the herbicides fomesafen and acetochlor were assessed using treatments that gave optimum enhancement of activity toward CDNB. Neither treatment with herbicides nor with safeners caused any major change in activity toward either substrate, however the BAS 145-138 safener appeared to give a minor enhancement of GST activity toward fomesafen.

3.3 Discussion.

3.3.1 GST activity in soybean.

GST activity toward CDNB was detectable in all soybean tissues tested (Figure 3.15), with soybean cell cultures containing between 4 and 8-fold greater specific activity than whole plants. Furthermore, using HPLC based assays, activity toward diphenyl ether, sulphonylurea and chloroacetanilide herbicides (Figures 3.17 and 3.18) was also detected, and again shown to be significantly higher in cell suspension cultures than seedlings, with no activity toward the substrates acifluorfen or chlorimuron-ethyl determined in plants. GST metabolism toward specific herbicides has been shown to be both higher (Hatton *et al.*, 1998) and lower (Edwards and Owen, 1986) in plant cell cultures than in the respective whole plants, while GSTs with activity toward CDNB are generally higher in cell cultures (Edwards and Owen, 1986; Fujita *et al.*, 1995; Hatton *et al.*, 1998). Increased GST activity in soybean cell cultures may be due to the inclusion of 2,4-D in the culture media since it is a known inducer of tau-type GSTs (Droog, 1997). Therefore it is likely that the inclusion of 2-4-D in the media causes the enhancement of isoenzymes which are either expressed at low levels, or not at all in normal whole healthy plants. Indeed, it has been reported that the removal of 2-4-D from the culture medium of pumpkin cells causes a decrease in specific GST activity toward CDNB (Fujita *et al.*, 1995). Alternatively, it is known that plants contain natural inhibitors of GSTs (Lamoureux and Rusness, 1993) which if present in soybean plants, may inhibit GST activity in crude plant extracts and thus exaggerate the differences in GST activity in plants and cell cultures. This latter explanation seems unlikely, as the crude protein extraction process used in preparing the plant extracts for assay includes protein precipitation with ammonium sulphate and subsequent desalting to remove low molecular weight inhibitors.

With the exception of the chloroacetanilides metolachlor and acetochlor, conjugation rates in the absence of the enzyme suggest none of the herbicides are sufficiently electrophilic to undergo rapid conjugation with glutathione or homoglutathione *in planta* (Table 3.1). In metabolism studies with soybean plants, the conjugation of homoglutathione to acifluorfen (Frear *et al.*, 1983), chlorimuron-ethyl (Brown and Neighbors, 1987) and fomesafen (Evans *et al.*, 1987) is reported as being rapid, and essentially complete within 24 h. In all these previous studies the relative rate of

herbicide conjugation with glutathione or homoglutathione in soybean and competing weeds was cited as the major determinant of selectivity, as herbicide uptake was comparable in both tolerant and susceptible species. Furthermore, acetolactate synthetase (ALS) in soybean and weeds such as common cocklebur was equally sensitive to inhibition by chlorimuron-ethyl, inferring that target site insensitivity in soybean was not responsible for the selectivity (Brown and Neighbors, 1987). It was concluded that the conjugation of homoglutathione to acifluorfen, fomesafen and chlorimuron-ethyl was rapid in soybean and considerably slower in susceptible weeds (Frear et *al.*, 1983; Evans *et al.*, 1987; Molsey *et al.*, 1992), which led to the selectivity of these herbicides. Because conjugation of these herbicides with homoglutathione has been determined *in planta*, but does not occur *in vitro* at physiological pH in the absence of enzyme then it is probable that GSTs are involved in the metabolism and thus selectivity of these herbicides.

It was discovered that the GST activity toward fomesafen is significantly lower in plants than in cell cultures (Figures 3.17 / 3.18). For this reason it is probable that the GST activity toward acifluorfen and chlorimuron-ethyl in crude plant extracts falls below the limit of detection of the HPLC assay. In the case of chlorimuron-ethyl, soybean may not require appreciable GST activity to confer tolerance since the field application rate of chlorimuron-ethyl is very low and can be between 100 and 200-fold less than that of the chloroacetanilide herbicides (McGonigle and O'Keefe, 1997). Therefore, the rate of conjugation of chlorimuron-ethyl required for tolerance *in planta* could be between 100 and 200-fold less than that determined with metolachlor or acetochlor. Given the data in Figure 3.16, this would correlate to a GST activity below the detection limit of the HPLC-based assay. Soybean may therefore only possess minor GST activity toward chlorimuron-ethyl, but this activity may be sufficient to confer selectivity.

It is interesting to note the differences in GST activity toward the similar diphenyl ether herbicides acifluorfen and fomesafen. It is possible that the significantly lower GST activity toward acifluorfen than fomesafen in soybean may explain the increased phytotoxicity or "burn" associated with acifluorfen use in the field. This indicates that the GST isoform(s) responsible for acifluorfen detoxification are less abundant than those involved in fomesafen metabolism, or that an enzyme able to detoxify both herbicides exhibits a lower activity toward acifluorfen.

The detection of GST activity toward the chloroacetanilides acetochlor and metolachlor in soybean plants may be important in determining selectivity. Unlike the other herbicides studied these compounds are sufficiently electrophilic to undergo direct conjugation with glutathione / homoglutathione (as indicated in Table 3.1) and it has been reported that endogenous thiol levels may indeed be important in selectivity (Breaux *et al.*, 1987). However, the discovery of GST activity toward these substrates in soybean compares with findings in maize (Hatton *et al.*, 1996), suggesting that both specific GST isoenzymes and thiol concentration are important in chloroacetanilide selectivity.

Of significant importance was the finding of substrate-dependent variation in thiol preference for glutathione and homoglutathione by soybean GSTs. The GST activities toward the diphenyl ether herbicides fomesafen and acifluorfen showed a strong preference for the use of homoglutathione rather than glutathione as co-substrate (Figures 3.17 and 3.18). Activity toward the chloroacetanilide herbicides showed a preference for glutathione in cell culture. However, no thiol preference was seen in the conjugation of this herbicide in soybean plants. GST activity toward chlorimuron-ethyl and CDNB also exhibited little apparent thiol preference. This thiol preference suggests that either a) there are unique fomesafen and acifluorfen conjugating GSTs present in soybean which exhibit increase activity in the presence of homoglutathione or b) that soybean GSTs preferentially conjugate fomesafen with homoglutathione as co-substrate. This was the first time that plant GSTs have been reported to exhibit different specificity for thiol co-substrate, and demonstrates the importance of using the endogenously occurring thiol when monitoring GST activity. CDNB has been shown to be a useful model substrate with which to monitor GST activity. However this result reveals the limitations of using CDNB to study GST activity in plants, since thiol selectivity was less pronounced with this substrate. Similar observations concerning the limitations of using CDNB as a substrate for GSTs have been reported in maize, where only two isoenzymes could be determined in extracts from safener treated plants when the activity was assessed using CDNB. Further analysis with the herbicide substrates atrazine and metolachlor indicated the presence of further, safener inducible GSTs not detected using CDNB previously (Dean *et al.*, 1991).

These initial studies have identified GST activities in soybean toward a number of selective herbicides used in soybean. The spectrum of activities observed gave an

indication that the complexity of enzymes present is likely to be as high as determined in other well studied species, most notably maize.

3.3.2 GST activity and herbicide selectivity in soybean and associated weeds.

GST activity toward CDNB in soybean was much lower than in the majority of weed species examined. Initially, the expectation was that if GSTs were involved in herbicide selectivity, then the level of GST activity would be higher in soybean than the weeds. GST activity toward specific herbicide substrates was indeed shown to be generally higher in soybean than the weeds. Comparison of the GST activity (section 3.2.4) with herbicide selectivity data (section 3.2.5) shows interesting correlation with fomesafen. Soybean, the tolerant crop, contained the highest GST activity toward the herbicide of all the plants tested. In contrast the two susceptible broadleaf weeds *A. retroflexus and I. hederacea* possessed no detectable GST activity toward fomesafen. The grass weeds *D. sanguinalis, S. halepense, S. faberi and E. crus-galli* showed some tolerance, with activity toward fomesafen detectable in all species, albeit at least 50% lower than in soybean. *A. theophrasti* was the only exception to the relationship, as it showed some tolerance to fomesafen but no GST activity. The most likely explanation was that *A. theophrasti* did show some GST activity toward fomesafen but using the HPLC assay, the fomesafen conjugate could not be quantified due to co-chromatographing plant products. Alternatively, factors other than GSTs could account for partial herbicide tolerance in this species.

Correlation between GST activity toward metolachlor and tolerance to the herbicide was less obvious. Soybean and the broadleaf weed *A. theophrasti*, both metolachlor tolerant, contained the highest GST activity toward metolachlor of all the species tested. *A. theophrasti* is a well known weed associated with maize, showing high tolerance toward chloroacetanilide herbicides and high GST activities toward metolachlor and alachlor (Hatton *et al.,* 1996). Therefore, tolerance to chloroacetanilides at least in part, is due to the weed possessing it's own GSTs that are able to detoxify the herbicide. In contrast, GST activity toward metolachlor in *A. retroflexus* and *I. hederacea,* which were both sensitive to this herbicide, could not be detected. Therefore, a correlation would appear to exist between the GST activity and tolerance in soybean and the broadleaf weeds. Both the grass weeds *D. sanguinalis*

and *E .crus-galli* showed comparable tolerance to metolachlor to soybean. However, no GST activity toward metolachlor could be detected in either species. Conversely, both *S. faberi* and *S. halepense* contain measurable GST activity toward the herbicide but were more susceptible to physiological application than *D. sanguinalis* or *E. crus-galli*. Previous studies report that *D. sanguinalis, E. crus-galli* and *S. faberi* do contain GST activity toward metolachlor (Hatton *et* al., 1996). The authors note that considerable variation was apparent in GST activities in *S. faberi* between different seed batches, possibly due to the genetic diversity within *Setaria* species. The significance of GST activity toward metolachlor in post-emergent soybeans in determining tolerance may also be misleading. Chloroacetanilides are growth inhibitors, applied pre-emergence, and it is understood that the GST(s) with activity toward chloroacetanilide herbicides in maize vary at different stages of development (Sari-Gorla, 1993; Hatton *et al.*, 1996). It would therefore be more accurate to determine biochemical characteristics in germinating seedlings rather than post-emergent material.

GST activities towards acifluorfen and chlorimuron-ethyl could not be detected using HPLC in crude extracts of any of the plants. It was therefore impossible to make conclusions regarding the correlation of *in vitro* GST activity with the selectivity of these herbicides. Previous studies suggested that chlorimuron-ethyl was rapidly conjugated to homoglutathione in tolerant soybean, whereas the rate of conjugation in susceptible *Amaranthus* and *Xanthium* species was slow (Brown and Neighbors, 1987).

3.3.3 Enhancement of GST activities by safeners.

While GST enhancement by safeners was considerably lower than that seen with the herbicide treatments, it is important to note that the increased activity was achieved without any signs of phytotoxicity. As discussed previously, the diphenyl ether herbicides act by inhibiting protoporphyrinogen oxidase, with the subsequent formation of reactive oxygen species that cause membrane destruction and chlorophyll bleaching. Therefore the GST induction seen following diphenyl ether herbicide treatment may in fact be due to an oxidative stress response, rather than a true "safening effect", which is observed in the absence of obvious oxidative injury.

This would explain why the induction following oxyfluorfen treatment was highest, since this herbicide exhibited the highest degree of photo-bleaching and phytotoxicity. Oxyfluorfen is known to induce GST activity in soybean cell cultures (Knörzer *et al.*, 1996), although the herbicide is not detoxified by GST catalysed conjugation. Again, the authors suggest that the enhancement is part of the antioxidant protective system of plants during the formation of active oxygen species. The differential enhancement of GST activity toward CDNB and herbicides suggested that these substrates are detoxified by distinct enzymes. In other studies, dichlormid has been shown to selectively induce CDNB activity three-fold in pea roots, but had no effect on the activity toward other substrates, including metolachlor and fluorodifen (Edwards, 1996). Therefore, it is unknown whether diphenyl ether herbicides and safeners enhance GST activity in soybean using similar, or distinct, signalling mechanisms.

These results suggest that safener induction of GSTs is not unique to cereals, but can also be determined in dicotyledons such as soybean. However, safener treatment of maize (Fuerst *et al.*, 1993; Holt *et al.*, 1995), wheat (Cummins *et al.*, 1997b) and sorghum (Gronwald and Plaisance, 1998) all resulted in the induction of GSTs active toward herbicide substrates, whilst in soybean this was not apparent. The failure to enhance herbicide-detoxifying activities in soybean probably accounts for the inability of herbicide safeners to protect soybean from herbicide injury. It should also be noted that many safeners are applied as seed treatments and it will be of interest to determine whether the enhancement of GST activity is more effective if the safeners are applied as seed dressings.

4. Chapter Four: Purification of GSTs from Soybean.

4.1 Introduction.

GSTs have been purified from a number of plant species, most notably maize where much is known about the protein structure, spatial regulation and substrate specificity of the individual isoenzymes present (Marrs, 1996). Results presented in the previous chapter showed that soybean contains GST activity toward several xenobiotic substrates, including herbicides used for selective weed control in soybean. From the diversity of conjugations catalysed, and the observation of substrate-dependent thiol specificity, it would appear that soybean contains multiple GSTs active in herbicide metabolism. In this chapter the purification and characterisation of GSTs from soybean with activity toward CDNB and the herbicides acetochlor, acifluorfen, chlorimuron-ethyl, fomesafen and metolachlor is reported.

Results presented in chapter three indicated that cell suspension cultures were an optimal source for GST purification (Figure 3.16), with activity detected toward a number of different substrates. Indeed activity toward the herbicides acifluorfen and chlorimuron-ethyl were only detectable in cell cultures. Therefore, cell cultures were chosen for all purification studies detailed in this chapter. Since differences in the GST compliment of whole plants and cell cultures are likely to occur, the profile of purified GSTs determined in the cultured cells was compared with that in plant tissue to highlight any differences.

Review of purification strategy

A number of different methodologies have been reported in the successful purification of plant GSTs. GSTs are generally hydrophobic enzymes (Mannervik and Danielson, 1988) and as such hydrophobic interaction chromatography (HIC), typically using phenyl-Sepharose (Williamson and Beverley, 1988), can often be a useful initial purification step from crude protein preparations. A number of affinity matrices have been used to specifically purify GSTs from heterogeneous mixtures (O'Connel *et al.*, 1988), most notably *S*-hexylglutathione and glutathione agarose (Mannervik and Guthenberg, 1981). Orange-A-agarose (Dixon *et al.*, 1997) and *S*-bromo-sulphophthalein glutathione-agarose (Mozer *et al.*, 1983; Holt *et al.*, 1995) have been

used as effective affinity matrices for the purification of specific GST isoforms, and as such these ligands have the potential for resolving different types of plant GSTs. For example, Orange A agarose has been shown to specifically bind the theta class *Zm*GST-I enzyme from maize, probably due to its structural similarity to the chloro-*s*-triazine herbicides known to be metabolised by this enzyme (Dixon *et al.*, 1997). In view of the complexity of GSTs in plants, it is frequently desirable to resolve individual isoenzymes. Separation of native, dimeric GSTs has traditionally been achieved using anion-exchange chromatography such as DEAE-Sepharose (Mozer *et al.*, 1983) or Mono-Q FPLC (Dean *et al.*, 1991), since GST enzymes typically have isoelectric points between pH 5.0 and pH 7.0. Additionally, individual GST subunits have been resolved on the basis of their hydrophobicity using reversed-phase HPLC (Cummins *et al.*, 1997b; Pascal *et al.*, 1998). In this chapter a number of these methodologies were utilised in order to study GSTs isoenzymes in soybean.

4.2 Results.

4.2.1 Purification of GSTs from cell cultures with activity toward CDNB.

Purification of GSTs from soybean cell cultures was initially performed using phenyl-Sepharose CL-4B hydrophobic interaction chromatography, followed by affinity chromatography using S-hexyl-glutathione-Sepharose. Total crude protein was extracted, precipitated with 80% ammonium sulphate and loaded onto the phenyl-Sepharose column in the presence of salt as described in section 2.1.7. Proteins were eluted from the column by decreasing the salt concentration and GST activity monitored in individual fractions using CDNB as substrate. Hydrophobic proteins, including the majority of the GST activity, remained tightly bound to the phenyl-sepharose column and could only be eluted with 50% (v/v) ethylene glycol, which was subsequently removed by anion-exchange chromatography prior to further purification.

Crude Extract

HIC

Low Salt   50% Ethylene Glycol

S-hexylglutathione affinity   Q-Sepharose

S-hexylglutathione affinity
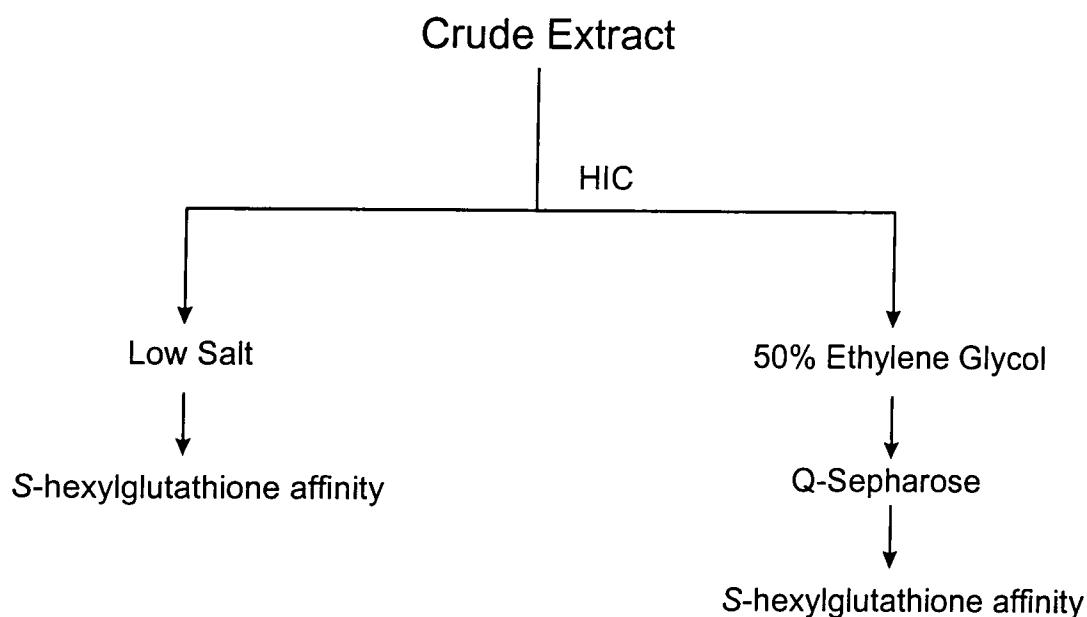
Figure 4.1 Schematic representation of the initial purification of GSTs from soybean with activity toward CDNB using Phenyl-sepharose hydrophobic interaction chromatography (HIC) and S-hexyl-glutathione affinity chromatography.

Table 4.1 summarises the purification of GST activity toward CDNB using the strategy outlined in Figure 4.1.

Fraction	Total Activity nkat	Total Protein mg	Specific Activity nkat mg^{-1} protein	Yield %
Crude Extract	700	421	1.66	100
HIC-Phenyl Sepharose				
No Salt	104	89	1.17	15
Ethylene Glycol	178	34	5.21	25
Hexyl-GSH Affinity				
HIC No Salt	15	0.63	23.8	2.1
HIC Ethylene Glycol	100	1.08	92.9	14.3

Table 4.1 Purification of GST activity toward CDNB from 5-day-old cell suspension cultures.

These results showed that GSTs with activity toward CDNB in soybean can be subdivided into two separate classes, based on the differences in hydrophobicity of the enzymes. Approximately 15% of the total activity loaded onto phenyl-sepharose column eluted with the no salt buffer, suggesting the presence of polar isoforms, whereas 25% of the applied activity eluted in 50% ethylene glycol, suggesting these GSTs are more hydrophobic in nature. The resolution of GSTs based on differences in hydrophobicity either suggests that certain GSTs may aggregate with hydrophobic proteins or other moieties which in turn bind to the column, or may indeed reflect true differences in enzyme hydrophobicity. The reason for the relatively poor final recovery (40%) of GST activity from the total loaded was not understood. Extensive washing of the column failed to release further active GSTs. In general GSTs are considered stable enzymes, however some variation in the stability of isoforms has been observed (Timmerman, 1989). Affinity purification of GSTs from both the polar and hydrophobic fractions, using *S*-hexyl-glutathione chromatography, indicated that 2.1% and 14.3% respectively (Table 4.1) of the total GST activity toward CDNB which was in the original extract was recovered. With respect to the hydrophobic fraction, this represented an overall 92 fold purification of GST activity from the initial crude extract. However, it should be noted that in this study, fractions eluted from the *S*-hexylglutathione affinity column were assayed in the presence of 5 mM *S*-hexylglutathione, a known inhibitor of GST activity (Dixon *et al.*, 1997). Therefore, the true recovery of GST activity toward CDNB and herbicide substrates may be considerably higher in these fractions than suggested in Table 4.1.

In order to improve the effectiveness of the purification process it was decided to use additional affinity matrices to attempt the selective purification of specific isoforms. The hydrophobic GSTs eluting from the phenyl-Sepharose column were dialysed and initially applied onto Orange-A agarose, with GST activity eluting from the column monitored using CDNB as substrate. Proteins not retained on the Orange-A column, and the polar proteins eluted from the phenyl-Sepharose column without requiring ethylene glycol were applied independently to a 5 ml *S*-hexylglutathione affinity column and GSTs eluted as described in section 2.1.7. The typical elution of protein and GST activity toward CDNB during the purification of an extract from soybean cell cultures is shown following hydrophobic interaction chromatography (Figure 4.2), affinity chromatography using Orange-A agarose (Figure 4.3) and affinity chromatography on *S*-hexylglutathione agarose (Figure 4.4). A summary of the different fractions obtained from this purification strategy is given in Figure 4.5, with the activities in the fractions A-H given in Table 4.2.
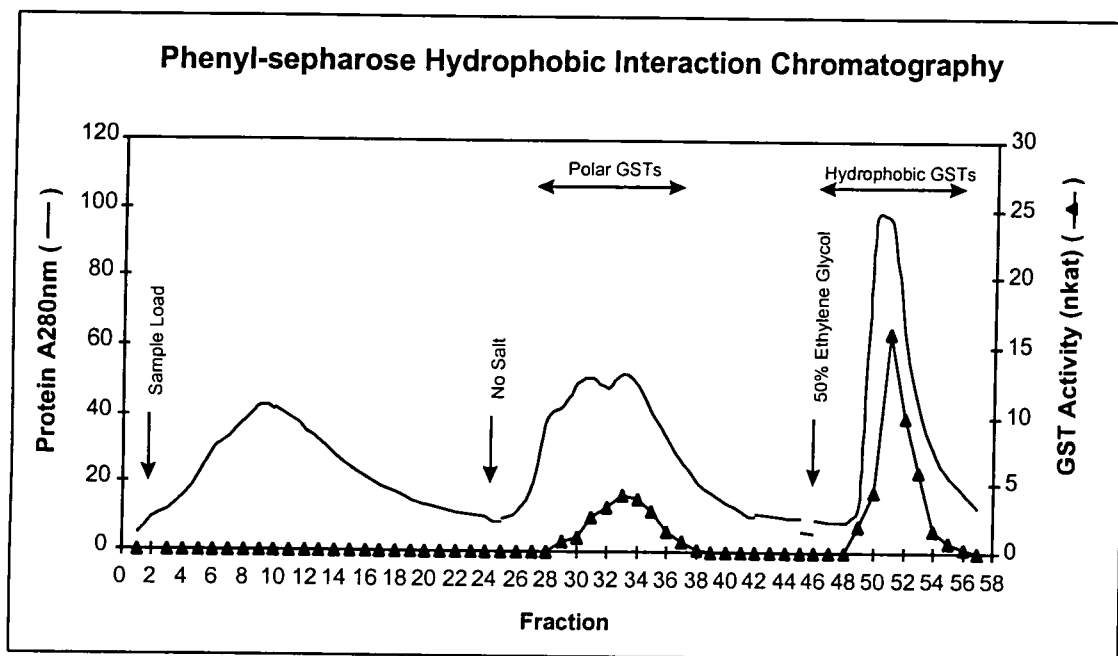
Figure 4.2 Separation of GSTs from crude protein extracts from 5-day-old soybean cell cultures using phenyl-Sepharose hydrophobic interaction chromatography. The crude protein extract was loaded in 20 mM Tris-HCl pH 7.4, 1 M $(NH_4)_2SO_4$, 200 mM KCl. Polar proteins were eluted in 20 mM Tris-HCl pH 7.4, 1 mM DTT and hydrophobic proteins eluted using 20 mM Tris-HCl pH 7.4, 1 mM DTT, ethylene glycol (50% v/v).
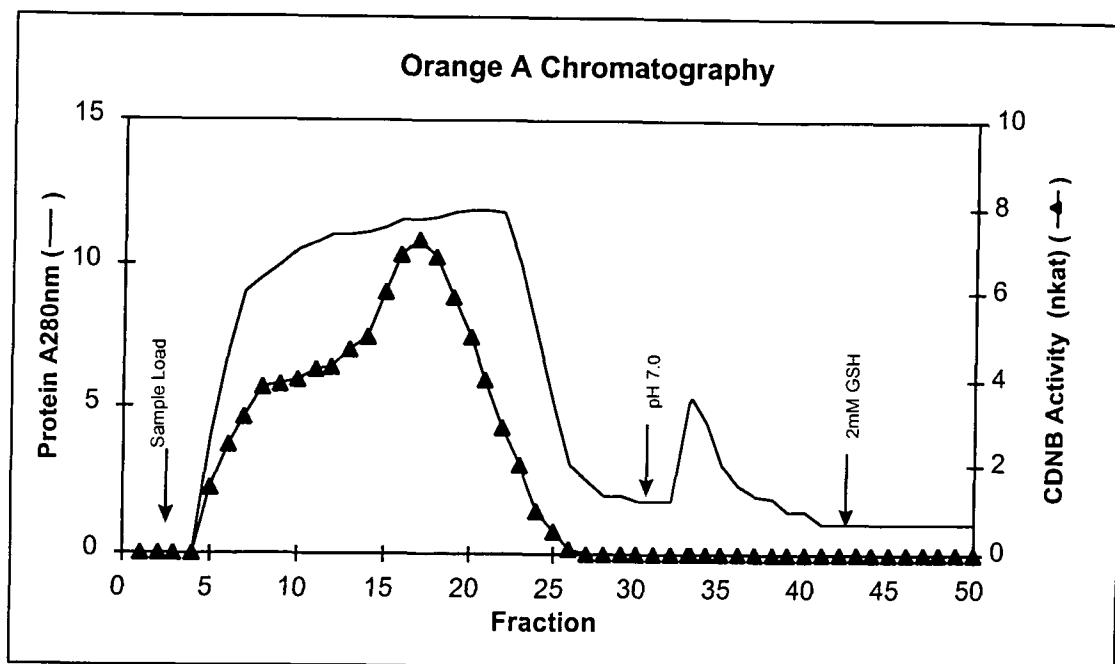
Figure 4.3 Purification of GSTs with activity toward CDNB using Orange-A-agarose. Hydrophobic proteins eluted from the phenyl-Sepharose column were loaded onto the Orange-A-agarose affinity column in 10mM phosphate buffer pH 6.0, 1mM DTT. Proteins were eluted from the column using 50mM phosphate buffer pH 7.0, 1mM DTT. Affinity bound protein was eluted in 50 mM phosphate buffer pH 7.0, 2mM GSH, 1mM DTT.

No GST activity toward CDNB or any of the herbicides was retained on Orange-A-agarose (Figure 4.3). Thus, Orange-A affinity chromatography was not considered a suitable matrix for purification of soybean GSTs and was excluded from all further experiments. However, selective purification of soybean GSTs was achieved using the affinity ligand *S*-hexylglutathione.

S-hexylglutatione Affinity Chromatography
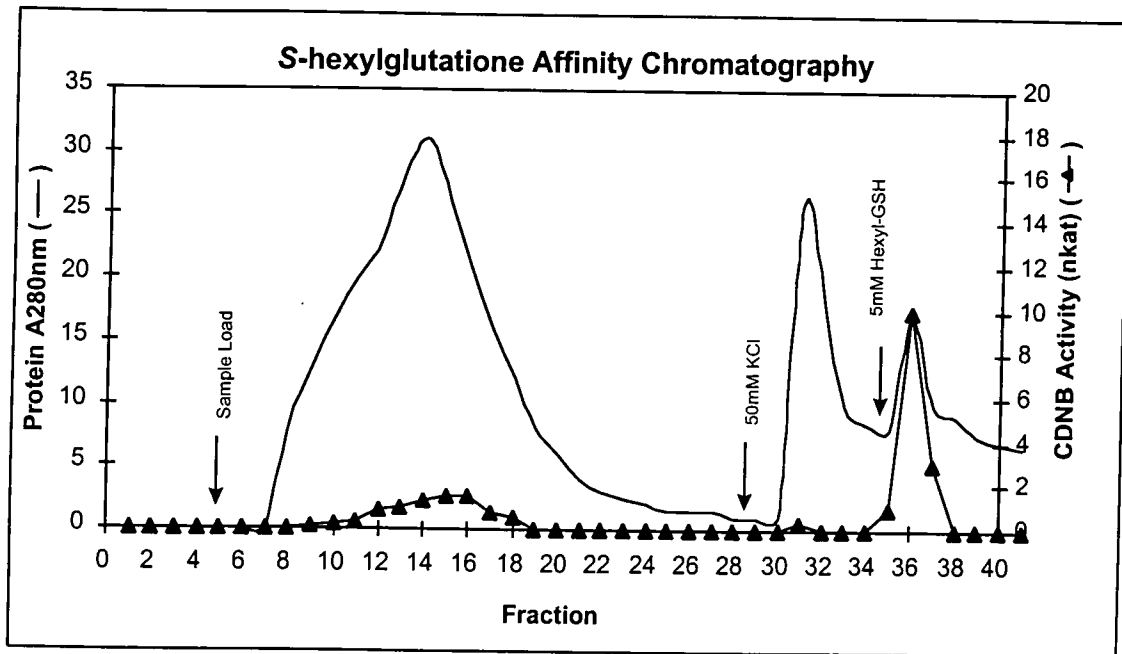
Figure 4.4 Purification of hydrophobic GSTs eluted from phenyl-Sepharose using *S*-hexylglutathione agarose. Affinity bound proteins were eluted using 5mM *S*-hexylglutathione.

Figure 4.4 indicates that *S*-hexylglutathione agarose, unlike Orange-A, is a good affinity matrix for the purification of soybean GSTs. Since the purification of soybean GSTs was conveniently monitored using CDNB as substrate it was possible that GSTs with activities toward herbicides, but little activity toward CDNB, were not being identified. To test this possibility fractions A-H, indicated in Figure 4.5, were assayed for GST activity toward CDNB, and the herbicides acifluorfen, chlorimuron-ethyl, fomesafen and metolachlor. The activity results obtained are presented in Table 4.2.

Crude Extract

Dialysed Crude Extract (Fraction A)

Phenyl-Sepharose Column

50% Ethylene Glycol   Low Salt Wash

Non-Bound (Fraction C)

pH 7.0   2mM GSH

Orange-A Affinity Column

ND   Non-Bound   ND

50mM KCl   5mM S-hexyl-GSH

50mM KCl / 5mM S-hexyl-GSH

Non-Bound

Hexyl-GSH Affinity Column

Fraction G   Non-Bound (Fraction F)   Fraction H   Fraction D   Fraction E

Anion Exchange Chromatography and RP-HPLC

Figure 4.5 Schematic representation of modified purification strategy for soybean GSTs. Each fraction derived from the chromatographic steps showing GST activity was labelled A to F (see Table 4.2 for activities) and was assayed for activity toward CDNB and herbicide substrates. ND = No GST activity detected and these fractions were not processed further.

Fraction	A	%	C	%	D	%	E	%	F	%	G	%	H	%
CDNB	462	100	0.5	0	5	1	25	5	44	10	3.5	1	170	37
Acifluorfen*	3,000	100	0	0	174	6	170	6	170	6	70	2	179	6
Chlorimuron	961	100	0	0	ND	?	145	15	76	8	27	3	317	33
Fomesafen*	10,628	100	0	0	632	6	600	6	1,923	18	866	8	748	7
Metolachlor	7,443	100	0	0	0	0	560	8	510	7	43	1	3,180	43

Table 4.2 Purification of GST with activity toward herbicides from 5-day-old cell cultures. Fractions A to H are described in Figure 4.5. Values refer to the average of duplicate enzyme activities determined with hGSH and for CDNB are given as nkat mg^{-1} protein, and for herbicide substrates pkat mg^{-1} protein. % values indicate the proportion of the total activity present in each fraction relative to fraction A (100%).

When considering the results obtained with CDNB as substrate a greater than 2-fold increase in activity recovered from the *S*-hexylglutathione column as compared with the previous purification attempt (Table 4.1). However, the relative proportion of GST activity in the polar and hydrophobic fractions was comparable (1:7) between the experiments. This suggested that the stability of the GSTs varied during different purification runs. The affinity purified GST preparations showed activity toward herbicides, demonstrating that these fractions were likely to contain isoenzymes involved in herbicide metabolism. The majority of GST activity (58%) toward chlorimuron-ethyl was present in the polar *S*-hexylglutathione bound fraction E and

hydrophobic S-hexylglutathione bound fraction H, indicating that S-hexylglutathione is a relatively good matrix for purifying GSTs active toward this substrate. The fractionation of the activity into polar and hydrophobic types suggested the presence of multiple isoenzymes. Similarly, with respect to metolachlor, 51% was present in fractions E and H, with the majority (43%) of activity in fraction H, suggesting that the GST(s) responsible for conjugation of metolachlor were retained by S-hexylglutathione but were hydrophobic enzymes. However, S-hexylglutathione agarose was not such an effective matrix for purification of the GSTs with activity toward the diphenyl ether compounds acifluorfen and fomesafen, with only 12% and 13% respectively of the applied activity retained in fractions E and H.

4.2.2 SDS-PAGE analysis of purified fractions

The polypeptides present in the fractions derived from purification by HIC and S-hexylglutathione affinity chromatography (Figure 4.5 / Table 4.2) were separated using SDS-PAGE and visualised by silver staining.
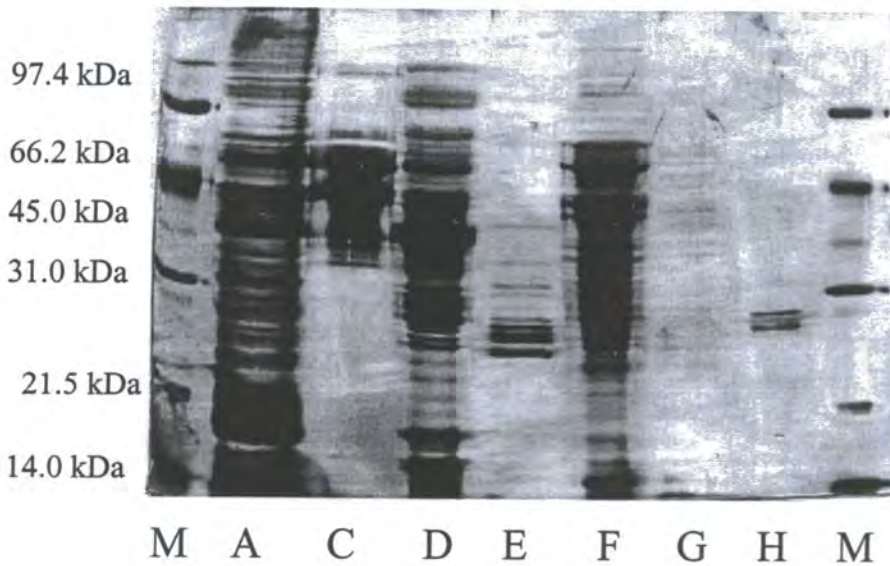
Figure 4.6 Fractions obtained from purification of GST from soybean cell cultures. Letters refer to fractions described in Figure 4.5. M = molecular mass marker, A = crude protein, C = unbound protein from phenyl-Sepharose column, D= polar proteins recovered from HIC which were not bound to S-hexylglutathione, E = Polar proteins recovered from HIC which eluted from S-hexylglutathione column with 50 mM KCl and 5 mM S-hexylglutathione, F = hydrophobic proteins recovered from HIC which did not bind to S-hexylglutathione, G = hydrophobic proteins from HIC recovered from S-hexylglutathione column with 50 mM KCl, H = hydrophobic proteins from HIC recovered from S-hexylglutathione column with 5 mM S-hexylglutathione. The protein concentration shown is not representative of the relative protein abundance in each fraction.

This analysis clearly showed the presence of polypeptides of molecular mass 25-30 kDa in the *S*-hexylglutathione purified fractions of polar GST activity (Fraction E) and hydrophobic GST activity (Fraction H). Fraction E consisted of a combination of both the loosely bound protein recovered from *S*-hexylglutathione in 50 mM KCl and tightly bound protein eluted with 5mM *S*-hexylglutathione. However, fraction H represents only the affinity bound fraction of the hydrophobic protein eluted in 5 mM *S*-hexylglutathione. Fractions E and H each contain multiple polypeptides with molecular mass between 26-29 kDa, consistent with the molecular mass of known GSTs. The polypeptides present in fraction H were used to raise polyclonal antibodies (termed ABT) which were described in chapter seven of this thesis.

4.2.3 Separation of *S*-hexyl-glutathione purified hydrophobic soybean GST isoenzymes using anion-exchange chromatography.

Figure 4.7 shows the GST activity toward CDNB and herbicides of the hydrophobic GSTs bound to *S*-hexylglutathione, (fraction H), following their separation by Q-sepharose anion-exchange chromatography. The active fractions were analysed by SDS-PAGE (Figure 4.8). For comparative purposes, recombinant *Gm*GST1, supplied by Dr Mark Skipsey, is shown on the SDS-PAGE gel, as this GST is known to be expressed in soybean (Ulmasov *et al.*, 1995).
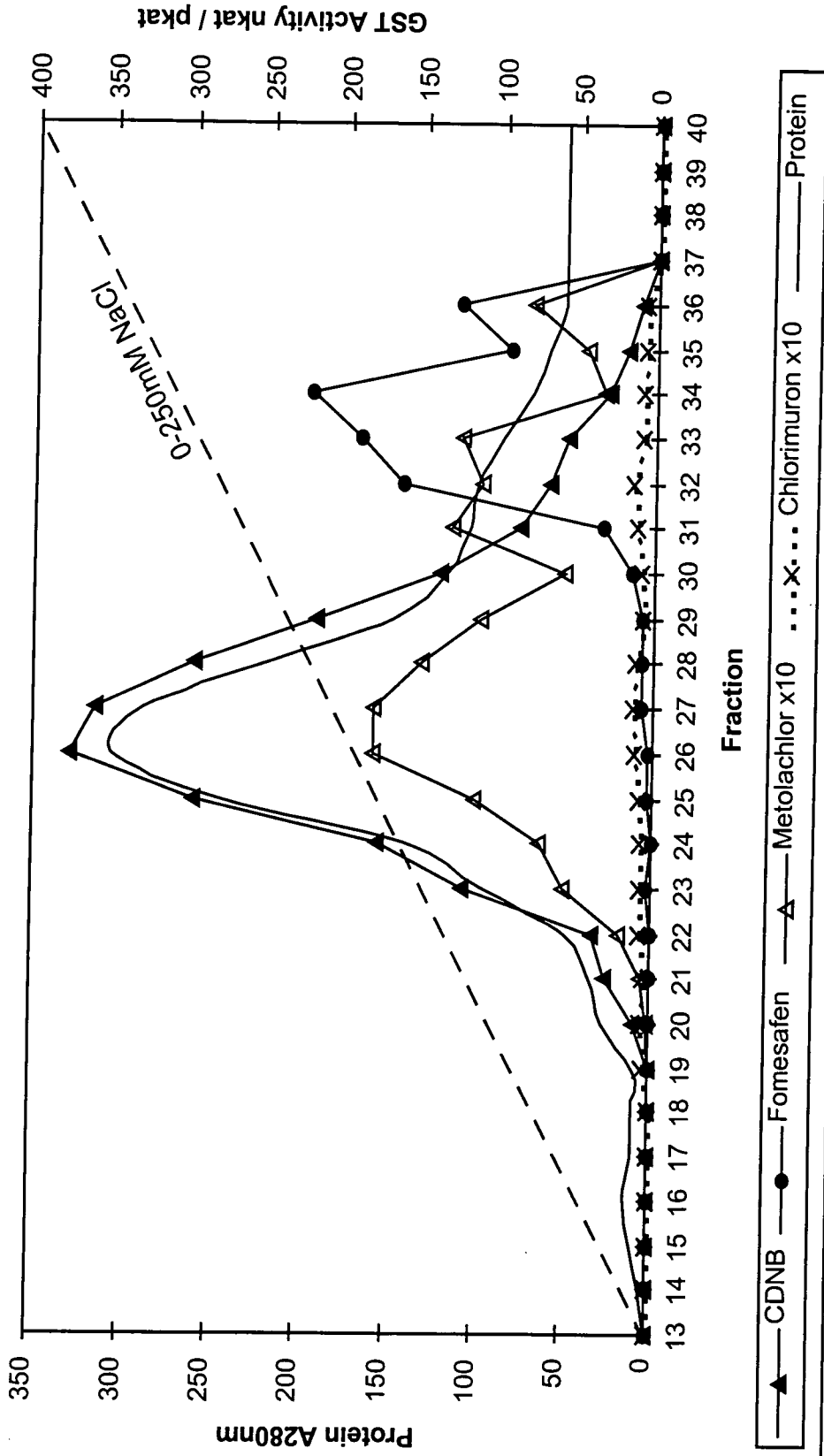
Figure 4.7 GST activity in fractions eluting from a Q-sepharose anion-exchange column following application of the affinity purified hydrophobic GSTs from soybean cell cultures. Activity toward CDNB are shown in nkat, while for the herbicides they are expressed in pkat. For the herbicides metolachlor and chlorimuron-ethyl, activities are shown at 10x observed rate for illustrative purposes. All assays were performed with homoglutathione.

The affinity purified hydrophobic GSTs (Fraction H) contained at least four distinct polypeptides when analysed by SDS-PAGE (Figure 4.6). However, the number of isoenzymes assembled as dimers from such polypeptides was unknown. From the UV absorbance shown in Figure 4.7, one broad protein peak was observed, though it was clear from its poor definition and inflexions that multiple proteins were present. This was confirmed when individual fractions were monitored for GST activity, where the activity profile showed clearly that multiple GSTs were present. Activity toward CDNB and metolachlor appeared to mirror the protein eluted from the column, suggesting the isoforms present in the fraction are responsible for the activity toward these substrates. Activity toward chlorimuron-ethyl was very low, and also appeared to correlate with the elution of total protein. Of most interest was the observation that the GST(s) responsible for fomesafen conjugation, eluted much later from the column than the predominant isoforms present. The specific activity of this fomesafen-conjugating enzyme is high, since the protein present in the active fractions was relatively low. Interestingly, little fomesafen conjugating activity was detected when glutathione was used to assay fractions 31-36, suggesting homoglutathione is an essential requirement for the activity of the enzyme(s) present. Additionally, little activity toward CDNB was detected in these fractions with either glutathione or homoglutathione, suggesting the substrate specificity of the enzyme is high.

SDS-PAGE analysis of anion-exchange fractions (Figure 4.7).

31kDa →
21.5 kDa→

M   19   20   21   22   23   24   25   26   27

31kDa →
21.5kDa →

M   28   29   30   31-36   *Gm*GST1
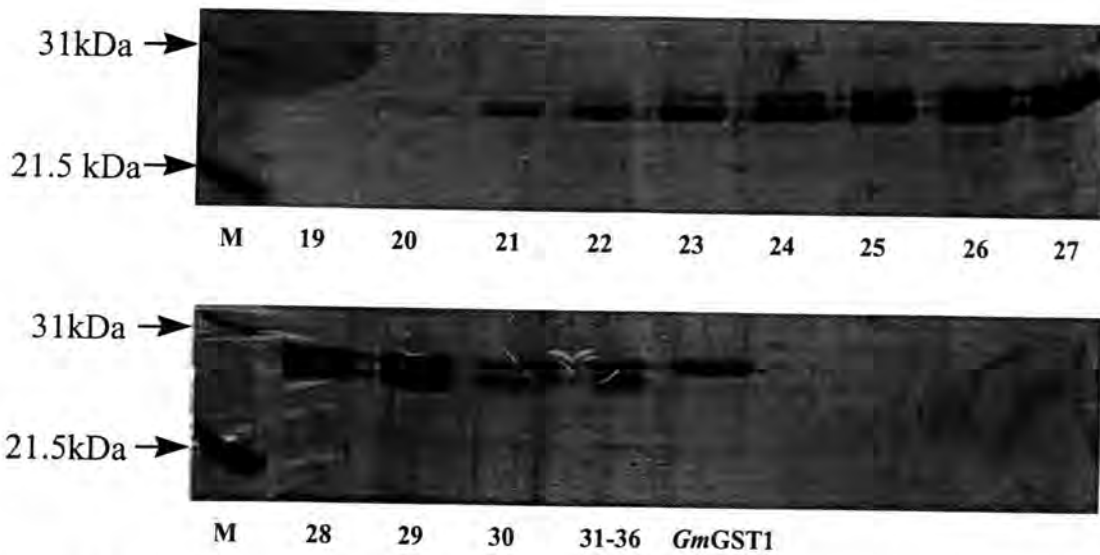
Figure 4.8 SDS-PAGE analysis of polypeptides present in fractions separated by anion-exchange chromatography (Figure 4.7) with the molecular mass markers (M) indicated. For fractions 31-36 protein contents were low and these fractions were combined and concentrated prior to analysis. *Gm*GST1 refers to the recombinant *Gm*GST1 polypeptide (Skipsey *et al.*, 1997).

SDS-PAGE analysis indicated the presence of three distinct polypeptides associated with GST activity, two polypeptides of approximately 27 kDa and one larger 29 kDa polypeptide. The two 27 kDa sub-units eluted early from the column, with the 29 kDa subunit appearing in fraction 22 onwards. The recombinant *Gm*GST1 migrated with the upper 29 kDa sub-unit, although it was clear from subsequent analysis that the 29 kDa polypeptide from the anion-exchange analysis constituted multiple polypeptides (discussed later). It was interesting to note that *Gm*GST1 had a predicted molecular mass of 26 kDa, but appeared as a 29 kDa protein on SDS-PAGE. This is in agreement with previous reports that GSTs migrate as larger proteins than they really are following SDS-PAGE (Dixon *et al.*, 1998).

4.2.4 One-step *S*-hexylglutathione affinity chromatography.

In order to shorten the time taken to purify GSTs from soybean cell cultures the strategy was modified. A one-step affinity purification procedure was adopted, using *S*-hexylglutathione as the affinity matrix, since of all the matrices tested it appeared to be a relatively good affinity ligand for the GSTs with activity toward CDNB, the chloroacetanilides and chlorimuron-ethyl. It also had the advantage of binding some,

albeit minor, activity toward fomesafen. Thus, the HIC purification step was omitted and crude cell culture protein extracts were applied directly to a 5ml S-hexylglutathione column and retained proteins eluted as described previously. Table 4.3 shows the binding of GST activity toward CDNB and herbicide substrates on the column. This one-step procedure had the advantage over the previous method in that it speeded up the purification process and reduced losses in activity due to the possible degradation of the enzymes. In addition, this method did not resolve polar and non-polar GSTs, resulting in one comprehensive pool of GSTs for further analysis.

Substrate	Crude	%	Non-Bound	%	50mM KCl	%	Hex-GSH	%
CDNB (GSH)	106.6nkat	100	30.3 nkat	28	0	0	14.6 nkat	14
CDNB (hGSH)	106.6nkat	100	23.5 nkat	22	6.3 nkat	6	13.9 nkat	13
Acetochlor	292.4pkat	100	0	0	3.1 pkat	1	61.8 pkat	21
Acifluorfen	86.2pkat	100	48.1 pkat	56	0	0	3.1 pkat	4
Chlorimuron	10.5pkat	100	4.3 pkat	41	2.3 pkat	22	0.77 pkat	7
Fomesafen	605.8pkat	100	302.5 pkat	50	15.7 pkat	3	7.5 pkat	1
Metolachlor	111.1pkat	100	6.7 pkat	6	5.5 pkat	5	19.9 pkat	18

Table 4.3 Purification of GSTs from crude soybean cell culture extracts using S-hexylglutathione. affinity chromatography. All assays with herbicides were performed using homo-glutathione and values refer to the mean of duplicate enzyme assays which are given as nkat mg^{-1} protein for CDNB and pkat mg^{-1} protein for all herbicide substrates. % refers to percentage of total activity in each fraction relative to that in the crude extract applied.

The one-step S-hexylglutathione strategy resulted in the selective purification of an appreciable proportion of GSTs active toward CDNB, and the herbicide substrates. The % recovery of some of these activities in the fractions recovered using S-hexylglutathione may have underestimated true recoveries due to the presence of S-hexylglutathione inhibiting GST activity. The data obtained confirmed that S-hexylglutathione was a useful affinity ligand for the purification of GSTs with activity toward CDNB and the chloroacetanilides acetochlor and metolachlor, but a poor matrix for the purification of the diphenyl ethers acifluorfen and fomesafen. The purification of activity toward chlorimuron-ethyl using a one-step purification procedure was less effective than obtained using an initial HIC-purified protein (Table 4.2). The GST isoenzymes eluted from the affinity column using 5 mM S-hexylglutathione were separated as before using Q-sepharose anion-exchange chromatography. Each resulting fraction was assayed for GST activity using homoglutathione (Figure 4.9) and fractions analysed by SDS-PAGE (Figure 4.10).
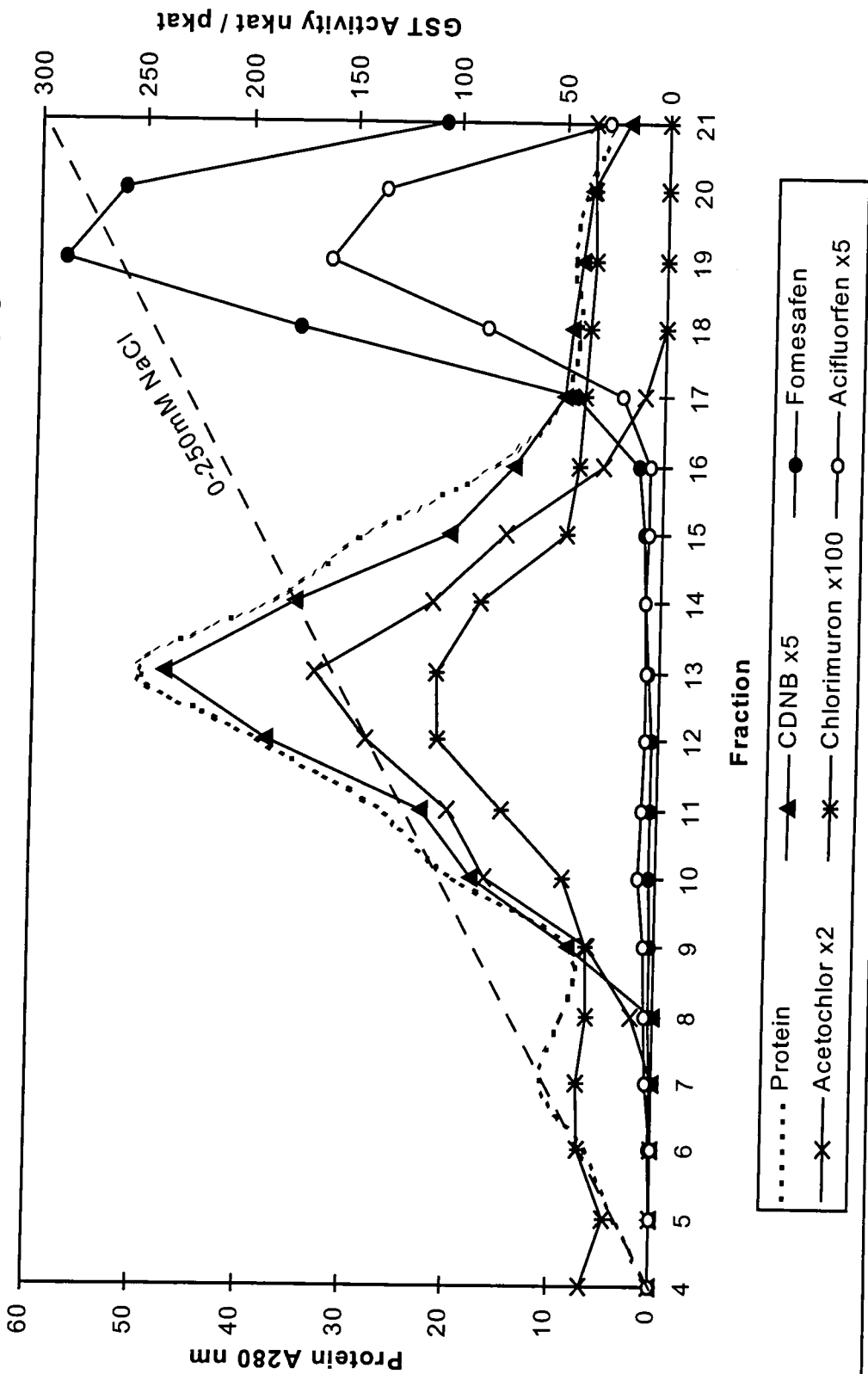
Figure 4.9 GST activity in fractions eluting from a Q-sepharose anion-exchange column following application of the affinity purified hydrophobic GSTs from soybean cell cultures. Activity toward CDNB are shown in nkat, while for the herbicides they are expressed in pkat. For the herbicides metolachlor and chlorimuron-ethyl activities are shown 10x observed rate for illustrative purposes. All assays were performed with homoglutathione.

The anion-exchange protein/activity profile obtained is similar to that seen with the separation of *S*-hexylglutathione purified hydrophobic GSTs following HIC as described in section 4.2.2. Activity toward CDNB, acetochlor and chlorimuron-ethyl co-eluted with the major peak of UV-absorbing protein, whereas activity toward fomesafen eluted from the column in the later fractions. Interestingly, GST activity toward the diphenyl ether acifluorfen displayed a similar elution profile to fomesafen, suggesting these two substrates are metabolised by the same enzyme(s). Again, no activity toward either diphenyl ether was apparent when any of the fractions were assayed with glutathione.

SDS-PAGE analysis of the fractions also showed a similar pattern of polypeptides associated with GST activity as before (Figure 4.8), with the early elution of 27 kDa polypeptides, followed by the subsequent appearance of a larger, 29 kDa polypeptide. However, an additional 32 kDa protein was seen in fractions 12 and 13 that was not observed previously. No GST activity toward the substrates tested was apparent in this fraction, and therefore this protein did not appear to be a GST.

Figure 4.10 SDS-PAGE analysis of polypeptides present in fractions 11-17 from the anion-exchange separation of GSTs from crude extracts of soybean cell cultures affinity purified using *S*-hexyl-glutathione. The 32 kDa protein (arrowed) was not detected previously and is not thought to be a GST. M = marker, numbers refer to fractions described in Figure 4.9.

Other affinity matrices.

In an attempt to selectively purify the fomesafen-conjugating GSTs different affinity matrices were tested. It was reported in chapter 3, that the GSTs active toward both acifluorfen and fomesafen showed a thiol preference for homoglutathione over glutathione. It was therefore suspected that the poor binding of the GSTs active toward fomesafen and acifluorfen could be due to the use of affinity columns containing glutathione rather than homoglutathione. To test this hypothesis

homoglutathione was coupled to epoxy-activated sepharose and was tested as an affinity ligand in an attempt to selectively purify GSTs with activity toward fomesafen. Proteins were loaded onto the column using similar conditions for chromatography as used for the S-hexylglutathione method. However, no GST activity toward these herbicides was seen to bind to this column. As an alternative ligand homoglutathione was reacted with fomesafen, and the hGS-MSCNP coupled to epoxy-activated sepharose to serve as a highly specific affinity ligand. However, the limited amounts of conjugate which could be prepared limited the development of such an affinity matrix. Finally, fomesafen was directly coupled to epoxy activated sepharose, but this affinity column acted as a non-specific hydrophobic interaction column rather than as an affinity column.

4.2.5 Reversed-phase HPLC separation of individual GST subunits.

RP-HPLC resolves polypeptides based on their hydrophobicity, and as such polypeptides of similar molecular mass and net charges can be separated. Reversed-phase HPLC was used to resolve individual protein subunits eluted from the S-hexylglutathione affinity column following the application of crude cell culture protein extract directly to the column. It was also considered important to determine any differences between GST subunits in whole plants and cell cultures, the model system adopted in these purification studies. Of particular concern was the possibility that GSTs present in cell suspension cultures used for enzyme purification, however interesting, may not reflect accurately the profile present in the native plant. Figure 4.11 shows the RP-HPLC resolved individual polypeptides (labelled 1-11) identified in the S-hexylglutathione affinity bound fraction obtained from 5-day-old cell cultures compared to those purified in an identical manner from 3-week-old soybean plants.

Reversed-Phase HPLC Analysis of *S*-Hexylglutathione Bound Polypeptides
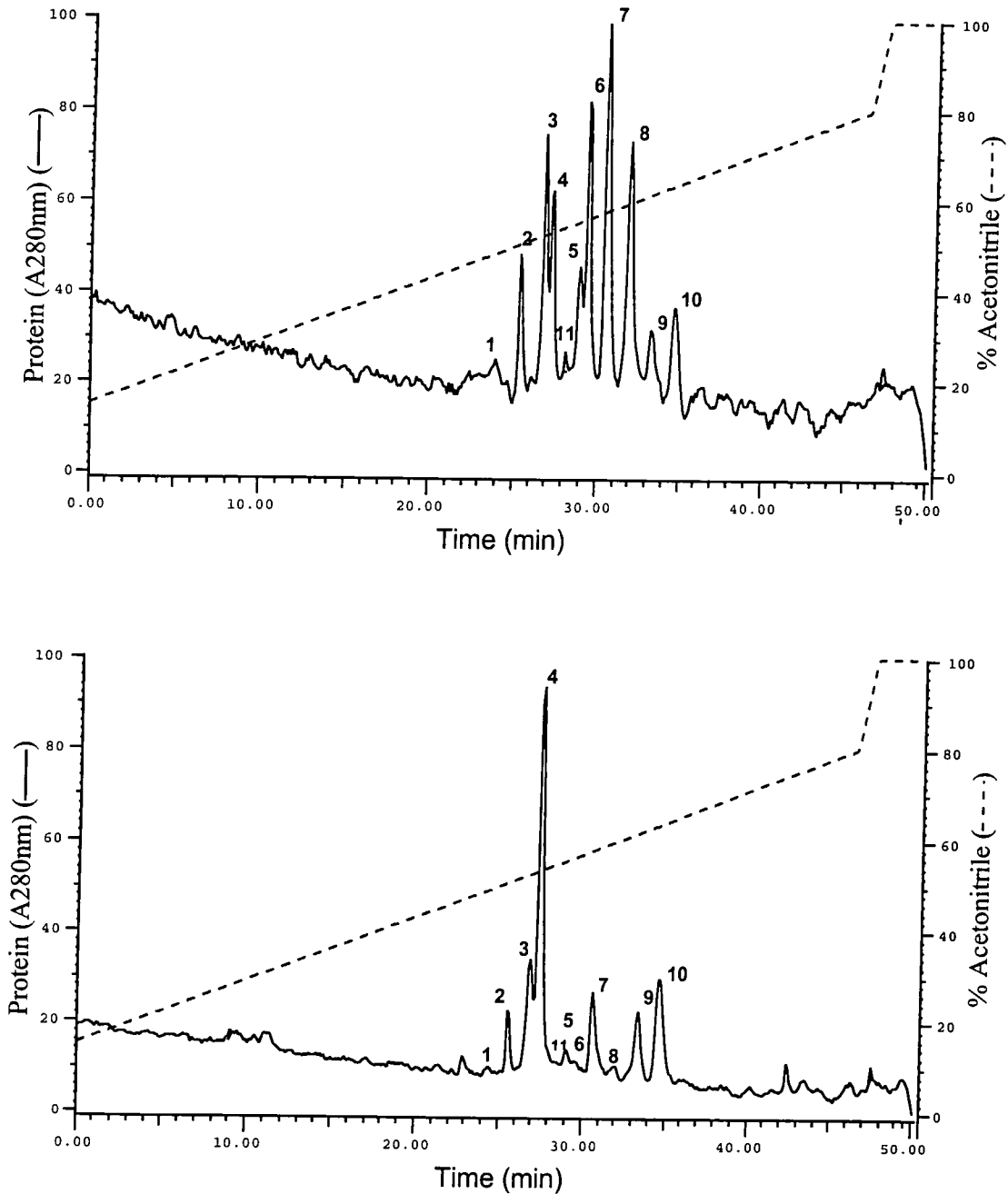
Figure 4.11 Reversed-phase HPLC separation of *S*-hexyl glutathione affinity bound proteins from 5-day-old cell cultures (top) and three-week-old soybean plants (bottom). Protein was detected by absorbance at 280 nm, and individual UV-absorbing peaks indicated 1-11.

Reversed-phase analysis of *S*-hexylglutathione purified enzymes indicated that there were differences in polypeptide content between whole plants and cell cultures. However, in both samples the same 11 UV-absorbing peaks were identified and only differed in their relative abundance. Therefore, cell cultures were considered a valid and useful model system with which to study soybean GSTs. In soybean plants, peak

4 constituted the major polypeptide, with peaks 5, 6 and 8 only present at very low levels. Conversely, peaks 6 and 8 are major polypeptides in cell cultures, with peak 7 the most abundant. Table 4.4 summarises the relative abundance of each polypeptide in plant and cell culture. Such inferences assume that each polypeptide has a similar extinction co-efficient, since abundance is correlated to A_{280nm}.

Peak	Cell Culture	Plants
1	+	0/+
2	++	++
3	+++	++
4	+++	++++
5	++	+
6	+++	0/+
7	++++	++
8	+++	0/+
9	++	++
10	++	++
11	+	0/+

Table 4.4 Relative abundance of putative GST sub-units in plants and cell culture from not present (0) to highly abundant (++++). Protein abundance shown in Figure 4.11 is assumed to be proportional to the UV absorbance at 280nm of the eluting peak.

This strategy demonstrates that whilst there are disadvantages associated with separation of polypeptides by reversed-phase HPLC, the resolution achieved is considerably greater than that obtained with anion-exchange or SDS-PAGE. Although it was not possible to identify these polypeptides as GST subunits at this stage, it seemed likely from demonstrating that the fraction from which the isoenzymes were purified contained high levels of GST activity, that a good proportion of these UV absorbing peaks do indeed represent GST subunits.

As no isoforms were present in the plant which were absent in the cell cultures it was decided not to pursue a characterisation of soybean GST enzymes at various developmental stages in whole plants, but to concentrate on the isoenzymes in cell cultures. RP-HPLC analysis indicates that the superior GST activity of crude cell

culture extracts as compared with extracts from plants observed in chapter three is probably due to the high levels of expression of GSTs in cell cultures.

4.2.6 Analysis of purified polypeptides using SDS-PAGE and MALDI-TOF MS.

4.2.6.1 SDS-PAGE analysis of RP-HPLC purified polypeptides.

Each of the polypeptides identified in section 4.2.4 were collected manually from the reversed-phase HPLC column, the acetonitrile removed under a stream of nitrogen, and the samples subjected to SDS-PAGE analysis (Figure 4.12).
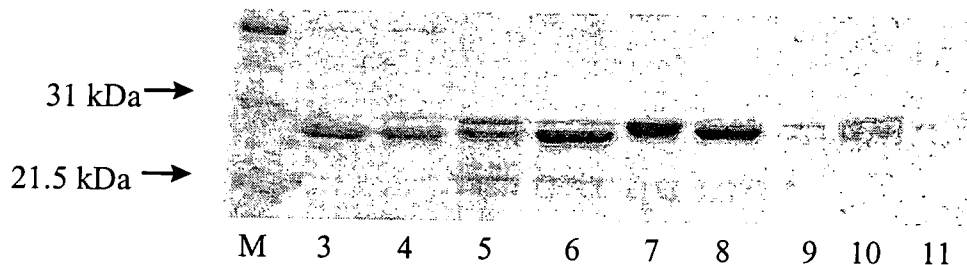
Figure 4.12 SDS-PAGE analysis of *S*-hexylglutathione purified polypeptides separated by reversed-phase HPLC.

No polypeptides appeared to be associated with peak 1, which is probably caused by the presence of a UV absorbing contaminant. Peak 2 corresponded to the ≈32 kDa polypeptide first described in Figure 4.10, and was not associated with any GST activity toward the substrates tested. Therefore, neither peaks 1 or 2 were subjected to further analysis. Additionally, due to the poor resolution of peaks 3 and 4, and 5 and 6 by HPLC it was not possible to resolve these polypeptides as single entities following RP-HPLC (Figure 4.11).

4.2.6.2 Molecular mass determination of proteins by MALDI-TOFMS.

Polypeptides 3 to 11 were purified using RP-HPLC, analysed for purity by SDS-PAGE (Figure 4.12) and sent for MALDI-TOF MS analysis to determine the molecular mass of the polypeptides. Poor resolution of peaks 3 and 4, and 5 and 6 dictated that these polypeptides could not separated from one another, and cross contamination of the samples was likely. Samples were lyophilised and sent to M-

Scan Ltd, Berkshire for MALDI-TOF MS analysis. The results obtained for each subunit are summarised in Table 4.5.

In all cases where a mass was observed, a mass ion of half the molecular mass was observed which is consistent with the parent ion in a doubly-charged state. These signals are not highlighted in the following analyses.

Peak	Mass Ions Present
3	No signal
4	25,459
5	25,844 (20,469)
6	No Signal
7	25,733 (20362)
8	26,535
9	25,953
10	24,928 and 25,909
11	No Signal

Table 4.5 Molecular masses of putative GST polypeptides as determined by MALDI-TOF MS. Major mass ions are indicated, with minor signals shown in brackets.

With the exception of peaks 3, 6 and 11, molecular mass peaks information was obtained for all the polypeptides analysed.

4.2.7 Summary of SDS-PAGE and MALDI-TOF analysis.

Peak 3

Peaks 3 and 4 could not be completely resolved by RP-HPLC, although only one major ≈27 kDa polypeptide was observed in the purified sample of peak three following SDS-PAGE analysis. Therefore, it is likely that this 27 kDa polypeptide corresponds to peak 3. No MALDI-TOF signal was obtained from this sample, despite the large quantity of lyophilised polypeptide supplied for analysis.

Peak 4

SDS-PAGE analysis of peak indicates the presence of two components, a major 27 kDa polypeptide and a minor 29 kDa polypeptide, which is consistent with the poor resolution of peaks 3 and 4. Since peak three is thought to correspond to a 27 kDa polypeptide (see above), the assumption is made that peak 4 corresponds to the larger 29 kDa polypeptide. MALDI-TOF analysis showed only one strong signal, with an average mass at m/z 25,459 Da.

Peak 5

Peaks 5 and 6, like peaks 3 and 4, were not completely resolved by reversed-phase HPLC. SDS-PAGE analysis of peak 5 showed that two polypeptides, of molecular mass 27 kDa and 29 kDa were present. Since peak 6 is thought to correspond to the 27 kDa polypeptide (see below), it is inferred that peak 5 probably corresponds to the larger 29 kDa polypeptide. MALDI-TOF analysis showed one m/z signal at 25,844 Da. A minor signal was observed at m/z 20,469 Da, which may represent a degradation product of the mature protein. This smaller polypeptide was apparent following SDS-PAGE analysis (Figure 4.12).

Peak 6

Only one 27 kDa polypeptide was apparent in purified peak 6 following SDS-PAGE analysis. Despite the large amount of peak 6 provided for MADI-TOF analysis no mass ion signals were observed.

Peak 7

Peak 7, a major polypeptide in soybean cell cultures, corresponded to a 29 kDa polypeptide following SDS-PAGE. A strong MALDI-TOF signal was detected with an average mass at m/z 25,733 Da. A minor peak was observed at m/z 20,362 Da, which, as with peak 5, may indicate a degradation product of the native enzyme. This smaller polypeptide was also detected by SDS-PAGE (Figure 4.12).

Peak 8

SDS-PAGE analysis showed that peak 8 contained a 28 kDa polypeptide. MALDI-TOF analysis indicated this polypeptide possessed a mass at m/z 26,535 Da.

Peak 9

Peak 9 was a low abundant polypeptide in soybean cell cultures, and in fact 2 polypeptides of 28 kDa and 29 kDa were determined in this fraction using SDS-PAGE. However, only one major polypeptide was detected using MALDI-TOF, with an average mass of 25,953 Da.

Peak 10

SDS-PAGE analysis on peak 10 indicated two polypeptides of 28 kDa and 29 kDa were present. MALDI-TOF analysis confirmed the presence of two polypeptides, with masses at m/z 25,909 Da and 24, 928 Da.

Peak 11

Peak 11 is an extremely low abundant polypeptide in soybean cell cultures, and as such it was difficult to visualise the polypeptide by SDS-PAGE. No MALDI-TOF signal was detected, again probably due to the very low concentration of protein in the sample provided.

4.2.8 N-terminal protein sequencing.

N-terminal protein sequence analysis was performed using an Applied Biosystems 477A protein sequencer as described in 2.1.9.3. All HPLC purified polypeptides were sent for analysis, however sequence information was only obtained for 3 of the polypeptides (Table 4.6).

Polypeptide	Protein Sequence
5	SNPVHKKIPV
7	KNPVHKKVP (F/V)
11	VRPVLPKCLT (S/I) I

Table 4.6 Protein sequence obtained from RP-HPLC polypeptides 5, 7 and 11.

Sequence obtained from polypeptides 5 and 7 corresponded to known internal sequences of plant GSTs centred around a highly conserved motif between residues 50 to 60 (Figure 5.24). This suggested that these proteins had undergone proteolysis

during purification and significantly, both of these preparations contained a ≈ 21 kDa polypeptide when analysed by SDS-PGE and MALDI-TOF MS. The 21 kDa polypeptide is the correct size to correspond to a GST subunit minus the first 50-60 amino acids, which suggested these two GSTs undergo cleavage at a specific amino-acid, exposing an internal N-terminus susceptible to sequencing. Interpretation of the sequence obtained from polypeptide 11 was less obvious. The presence of the proline and valine residues at positions 3 and 4 was consistent with the internal sequence of a GST, being similar to the internal sequence obtained for peaks 5 and 7, since the proline and to a lesser degree the valine are conserved residues. However, the remaining sequence does not show any of the other highly conserved residues which would be expected within this domain. Amino-acid residues within this conserved region are thought to be involved with the interaction of glutathione at the binding site (Neuefeind, 1997a). As the GST composed of subunit 11 polypeptide(s) show a specificity for homoglutathione in the conjugation of fomesafen and acifluorfen, it is possible that the "glutathione" binding domain of polypeptide may be fundamentally different to that seen in other GST subunits. Alternatively, the sequence obtained could have been derived from the N-terminal of the native protein after the removal of the methionine or some other internal sequence.

N-terminal sequence has previously been obtained from a soybean GST (Flury *et al.*, 1995) and it is surprising that so few of the polypeptides purified in this study were amenable to sequencing. N-terminal modifications resulting in N-terminal blocking have been seen before in a number of GSTs in maize. Thus, attempts to sequence *Zm*GST IV-IV (Holt *et al.*, 1995), and *Zm*GSTs V and VI (Dixon *et al.*, 1997) were unsuccessful even though the 29 kDa *Zm*GST I subunit sequenced successfully (Holt *et al.*, 1995). The sequences obtained from these polypeptides 5, 7 and 11 were used to design degenerate oligonucleotides which were used in RT-PCR. These experiments are described in chapter 5.

4.2.9 Identification of RP-HPLC-subunits as GSTs.

Due to the denaturing conditions of reversed-phase HPLC, individual polypeptide subunits rather than the native enzymes were isolated. This resulted in the loss of biological activity, making it more difficult to assign activity to a particular isoform.

An attempt was made to collect eluted polypeptides directly into buffer (100 mM Tris-HCl pH 7.4, 1 mM DTT) to reconstitute activity, since previous reports suggested this was viable with some type III GSTs (Cummins *et al.*, 1997b). However, in the case of soybean GSTs, this approach proved unsuccessful. To try to identify these subunits as catalytically active GSTs, *S*-hexylglutathione bound polypeptides from soybean cell cultures were separated by anion-exchange chromatography as before (Figure 4.9). Aliquots from resolved fractions were then subjected to reversed-phase HPLC chromatography in order to visualise the individual polypeptides present (Figures 4.14 - 4.21). Predictions were made as to catalytic function and the identity of specific peaks by comparing changes in polypeptide composition and activity detected in each fraction. Figure 4.13 shows the actual UV trace obtained following resolution of the affinity-purified GSTs by Hi-Trap Q-sepharose anion-exchange chromatography. The GST activity observed in each of the fractions was identical to that profiled in Figure 4.9.
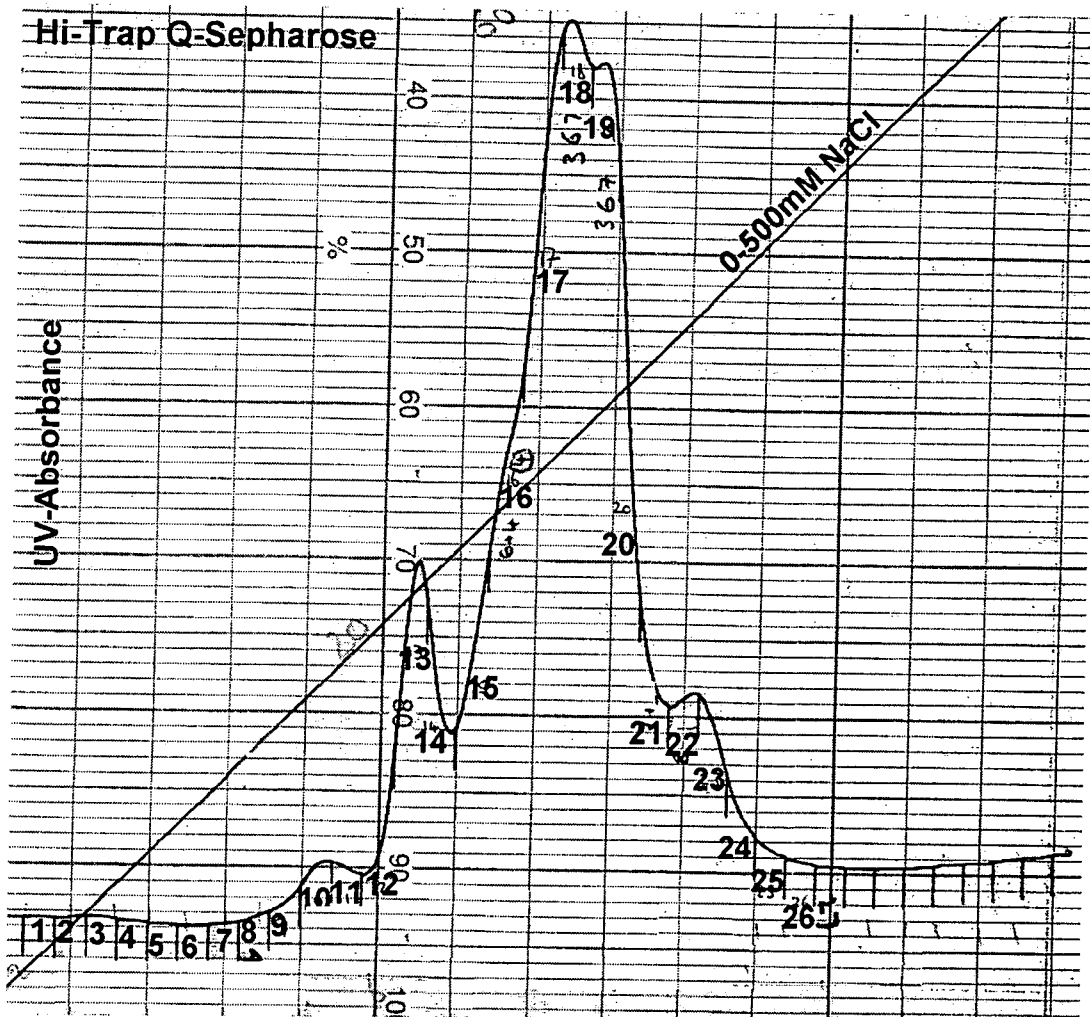
Figure 4.13 Q-sepharose anion-exchange separation of proteins purified from soybean cell cultures using *S*-hexylglutathione affinity chromatography. Each fraction was independently analysed by reversed-phase HPLC (Figures 4.14 to 4.21).

Figures 4.14 to 4.21 show RP-HPLC of fractions taken following anion-exchange chromatography of *S*-hexylglutathione affinity purified GSTs, shown in Figure 4.13.
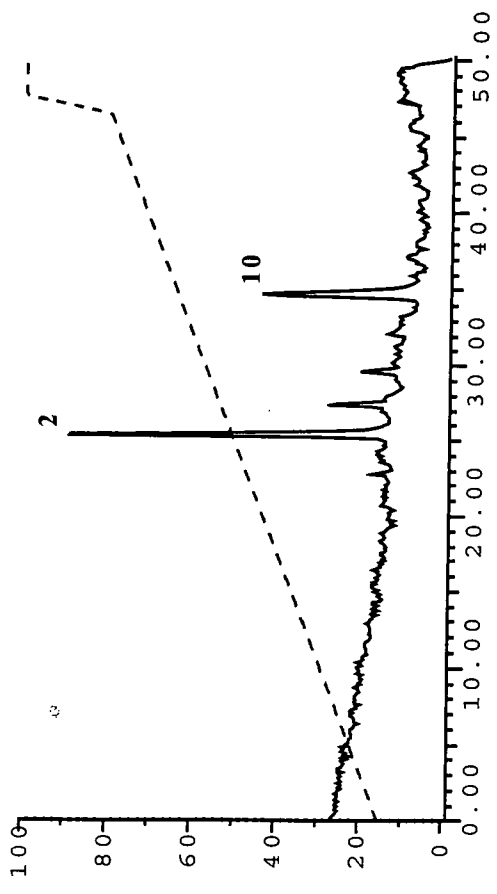
Figure 4.14 Polypeptide subunits in fraction 13
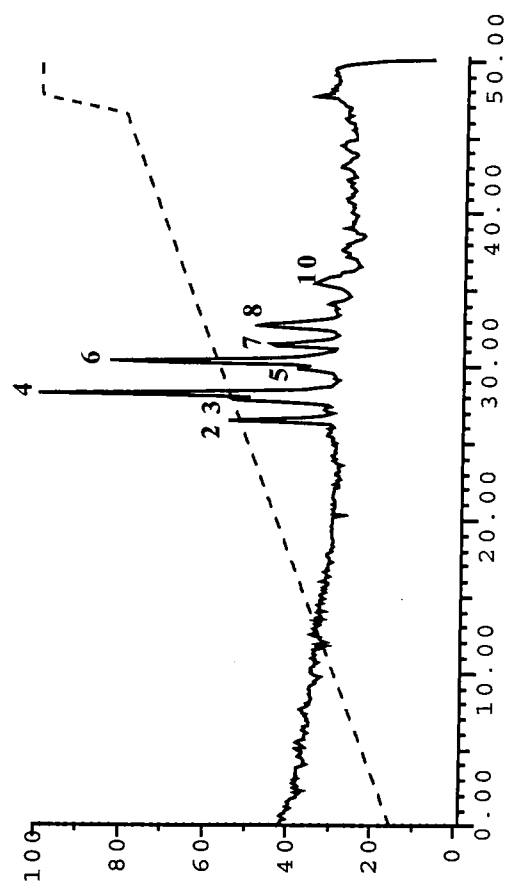
Figure 4.15 Polypeptide subunits in fraction 16
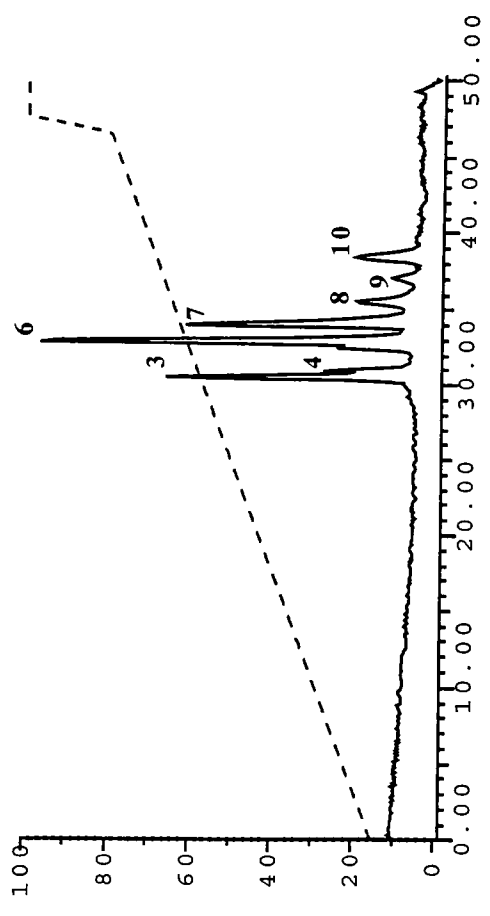
Figure 4.16 Polypeptide subunits in fraction 18
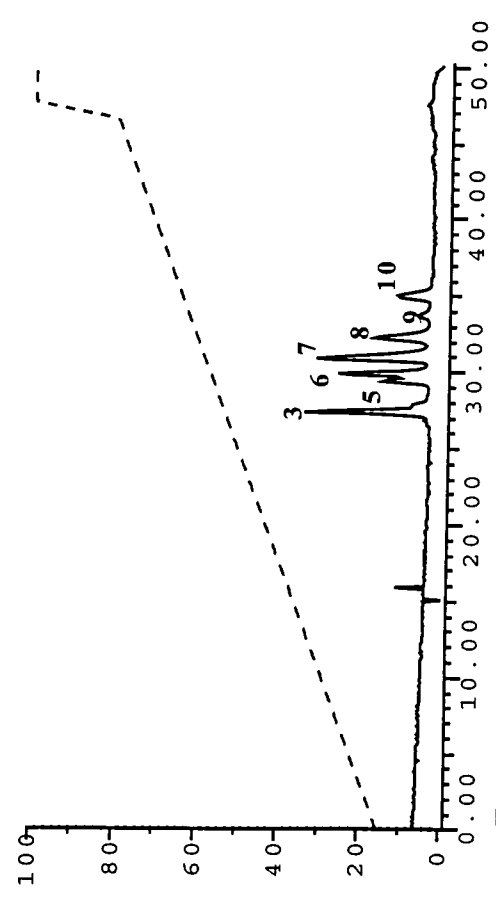
Figure 4.17 Polypeptide subunits in fraction 19
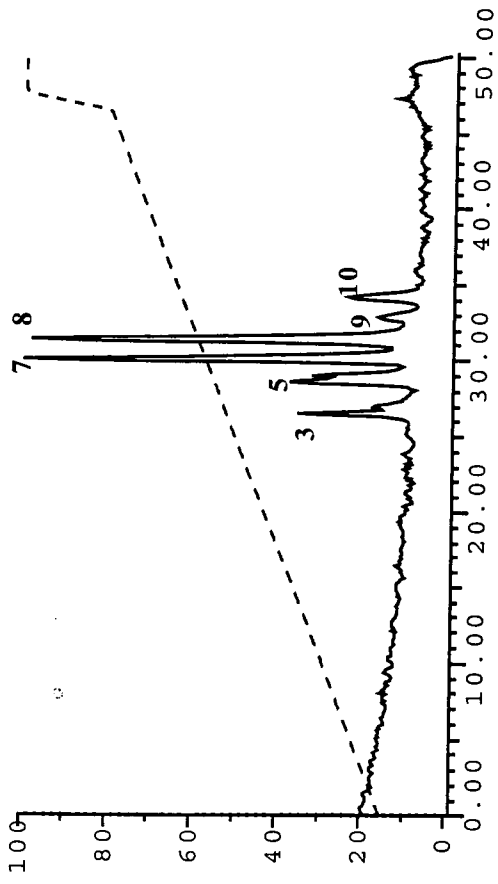
Figure 4.18 Polypeptide subunits in fraction 21
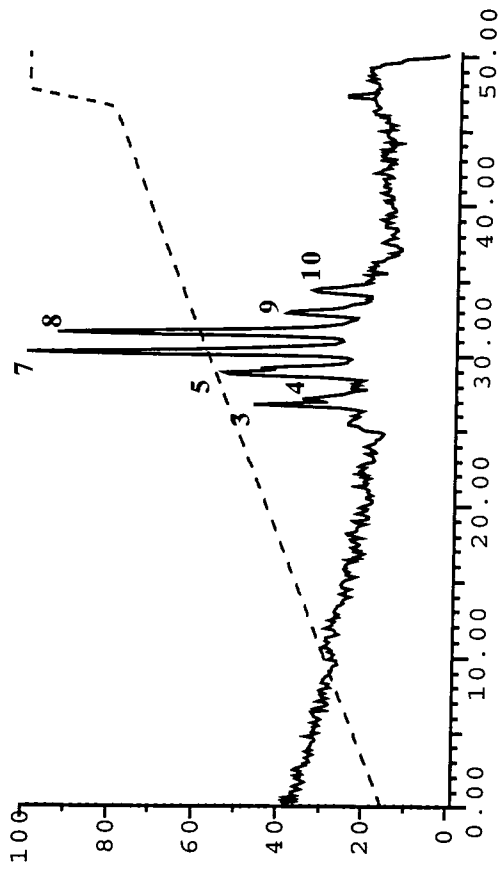
Figure 4.19 Polypeptide subunits in fraction 22
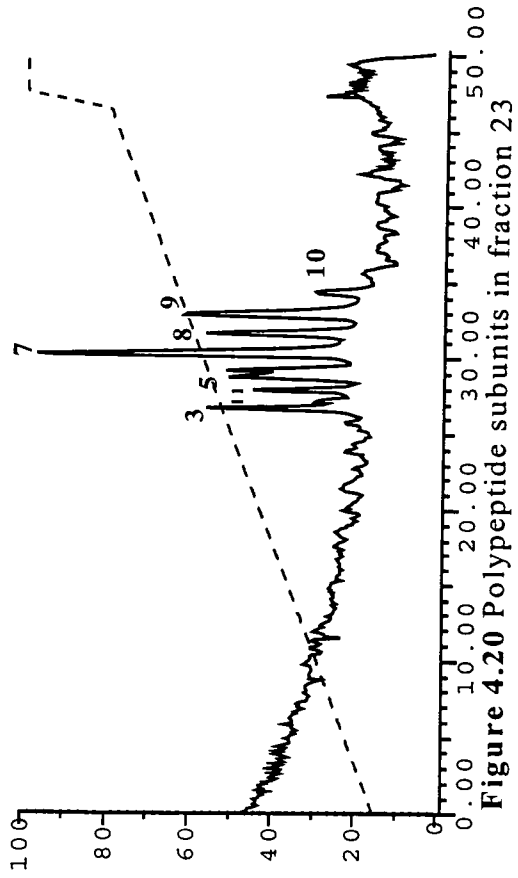
Figure 4.20 Polypeptide subunits in fraction 23
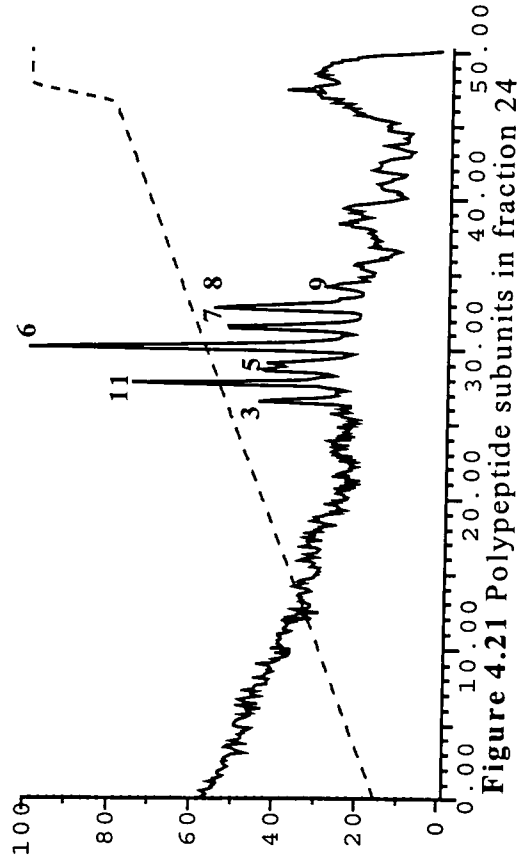
Figure 4.21 Polypeptide subunits in fraction 24

Marked variation was apparent in the constituent polypeptides present in each of the fractions eluting from the anion-exchange column. Figure 4.14 indicates that polypeptide 2 was the predominant polypeptide present in the early-eluting fraction 13, and was not associated with any GST activity. The presence of polypeptide 10 in fraction 13 appears to be due to a contaminant on the reversed-phase column during this analysis, since it was not detected in subsequent analysis. It was concluded that polypeptide 2 was a glutathione binding protein with no measurable GST activity toward CDNB or the herbicides tested, as hypothesised previously.

Although most polypeptides were detected in fraction 16, polypeptides 4 and 6 were relatively enriched, suggesting fraction 16 may contain homodimers or a heterodimer of these polypeptides. The predominant polypeptides in fraction 18 were 3, 6 and 7. This suggested that a number of native GST isoforms may actually be present in fraction 18 composed of these polypeptides, but that these were not effectively resolved by anion-exchange chromatography. Again, a number of polypeptides were detected in fraction 19, however a significant increase in peak 7 was apparent in this fraction, suggesting that like fraction 18, fraction 19 probably contains a number of different homo- and hetero-dimeric GST enzymes. In fraction 21 polypeptide 8 was apparent, whilst fraction 22 contained predominantly polypeptides 6 and 7, with an increase also observed in polypeptide 9. These were still the major polypeptides present in fraction 23, however the appearance of polypeptide 11 was noted. This is of particular interest as activity toward fomesafen and acifluorfen is first detected in this fraction and was maximal in fraction 24, where polypeptide 11 is seen to be the most abundant. Therefore, it is probable that polypeptide 11 is responsible for the GST activity toward the diphenyl ether herbicides observed eluting late from the anion-exchange column (Figures 4.7 and 4.9).

4.2.10 Summary of results obtained.

Table 4.7 summarises the data obtained for each putative GST subunit.

Subunit	Abundance	MW by SDS-PAGE (kDa)	MW by MALDI-TOF (Da)
3	+++	27	ND
4	+++	29	25,459
5	++	29	25,844
6	+++	27	ND
7	++++	29	25,790
8	+++	28	26,535
9	++	28/29	25,953
10	++	28/29	24,928 / 25,909
11	+	ND	ND

Table 4.7 Summary of abundance and molecular weight of putative GST polypeptides isolated from five-day-old soybean cell cultures. Relative abundance is indicated from (+) low abundance to (++++) high abundance. ND = not determined.

4.3 Discussion.

The results presented in this chapter suggest that soybean contains multiple hydrophobic and polar GST isoforms differing in substrate specificity, composed of subunits with molecular masses of 25-29 kDa. CDNB was generally a good substrate to monitor GSTs purification, however the GST isoform active in fomesafen and acifluorfen conjugation did not exhibit activity toward this substrate. Such differences in specificity have been observed in other plants. For example, in maize, *Zm*GST IV-IV shows high activity toward chloroacetanilide herbicides but little activity toward CDNB (Holt *et al.*, 1995). It has also been suggested that the elusiveness of the atrazine conjugating GST in maize is due to the enzyme's low specific activity toward CDNB (Timmerman, 1989). The findings in maize and soybean highlight the limitation of using CDNB as a marker for GST activity in plant GST purification.

Purification of GSTs from crude soybean cell culture protein preparations using *S*-hexylglutathione affinity and subsequent anion-exchange separation of the purified protein revealed that differing GST activities toward herbicides could be partially

resolved. GST activity toward fomesafen and acifluorfen eluted late from the anion-exchange column, suggesting that the isoenzymes responsible for this conjugation were distinct from other isoforms. Similarly, GST purification from pea has shown the activity toward the diphenyl ether herbicide fluorodifen also eluted late during ion-exchange chromatography (Edwards, 1996). However, the relatedness of the fluorodifen active enzyme to those active toward acifluorfen and fomesafen in soybean is not known, since activity toward fluorodifen was only determined using glutathione. It will be of interest to determine whether or not other homoglutathione containing legumes resistant to acifluorfen and fomesafen, such as French beans, contain a similar specific detoxification isoenzyme.

The initial purification characteristics of soybean GSTs showed similarities to the reported purification of GSTs from cereals. Purification using hydrophobic interaction chromatography, showed the presence of both polar and hydrophobic isoforms. Similarly GSTs in wheat (Cummins et al., 1997b) and to a lesser degree in maize (Dixon et al., 1997), can be resolved into both polar and non-polar pools. The presence of similar polypeptides in both polar and non-polar fractions could indicate GST interaction with other hydrophobic entities, or post-translational modifications resulting in changes in hydrophobicity. A detailed study of the hydrophobic and polar GSTs in soybean in this study was not performed. However, differences in the binding characteristics of soybean GSTs toward various affinity matrices indicates that soybean contains marked differences in the GST isoenzymes present as compared with cereals. Affinity purification of GSTs from maize, using Orange A agarose (Dixon et al., 1998) and sulphobromophthalein-S-glutathione agarose (Holt et al., 1995), resulted in the selective retention of theta-type GSTs. Thus, Orange A selectively bound the theta class ZmGST I, whilst sulphobromophthalein-S-glutathione bound both ZmGST I and ZmGST II. In cereals, tau-type GSTs are selectively retained on S-hexylglutathione agarose in both maize (Dixon et al., 1998a) and wheat (Cummins et al., 1997b). Conversely, in wheat the use of S-hexylglutathione agarose has shown that theta-type enzymes, identified by reactivity with antibody raised to the theta-GST ZmGST I-II, were eluted from the column in 200 mM KCl, suggesting they were loosely bound with low affinity for S-hexylglutathione (Cummins et al., 1997b). In this study with soybean, no GST

activity was retained by the Orange A affinity matrix. Additionally, only a small percentage of the activity toward CDNB was eluted from the S-hexylglutathione in low salt buffer. The different pattern of activity binding in soybean may suggest that theta-class GSTs may not be as abundant in soybean as cereals. Although the majority of GSTs described in dicotyledons belong to the tau-class, theta-type enzymes have been identified in *Arabidopsis* (Bartling *et al.*, 1993; Kiyosue *et al.*, 1993; Zhou and Goldsbrough, 1993) and tobacco (Takahashi and Nagata, 1992). Indeed, a DNA sequence encoding a soybean GST showing high homology toward the theta-type ZmGST-I has been reported in the literature, however no details of the purification or characterisation of this enzyme have been published (McGonigle and O'Keefe, 1997). The theta-type ZmGST I-I enzyme of maize, which can be selectively purified using Orange A agarose, has been shown to possess activity toward atrazine (Dixon *et al.*, 1997). Unlike maize, soybean is sensitive to atrazine and other chloro-*s*-triazine herbicides (Lamoureux and Rusness, 1993) and the apparent lack of theta-type GSTs in soybean may partially explain soybean's sensitivity to atrazine. By inference from what is known about GSTs binding to affinity matrices it seems most likely that the GST subunits from soybean, purified using S-hexylglutathione, belong to the tau-class as they are tightly bound to this ligand.

Reversed-phase HPLC analysis of S-hexylglutathione bound GSTs in soybean detected the presence of 11 UV absorbing peaks, of which nine were considered to be putative GSTs subunits. The reason for the difference in polypeptide profile of the affinity-purified proteins from plants and cell cultures is not known. Assuming that most of the UV absorbing peaks are GST subunits then the enrichment of peaks 5,6,7 and 8 in the cell culture preparations may reflect their enhancement due to the presence of 2,4-D in the cell culture medium, which is known to cause the induction of tau-type GST isoforms (Droog *et al.*, 1993). Indeed, such isoforms have been reported in soybean seedlings treated with auxin (Flury *et al.*, 1995). Additionally, the age of the plant material studied may be relevant. The GST compliment of maize is known to vary at different stage of development (Sari-Gorla *et al.*, 1993). Therefore, it is possible that if the soybean plants had been assayed at alternative stages of plant development other subunits may have been enhanced.

SDS-PAGE and MALDI-TOF MS analysis provided further information regarding the physical properties of the polypeptides identified by RP-HPLC. Most importantly, all the polypeptides identified possessed a molecular mass between 25 kDa and 27 kDa, which is consistent with all other plant GSTs described to date (Marrs, 1996). It is also in agreement with previous reports of two distinct classes of soybean GST subunits with masses between 26 kDa and 28 kDa (Flury *et al.*, 1996). Large differences were apparent between the predicted molecular mass of the polypeptide by SDS-PAGE compared with the more accurate results obtained by MALDI-TOF analysis. This was unsurprising, since SDS-PAGE has been reported previously as overestimating the molecular mass of proteins (Skipsey *et al.*, 1997). No MALDI-TOF signal was observed for polypeptides 3 and 6, which were relatively abundant polypeptides in HPLC purified fractions. This may suggest that these polypeptides did not redissolve following lyophilisation and subsequent injection into the MALDI-TOF MS. SDS-PAGE and MALDI-TOF analysis of fraction 10 indicated that two distinct polypeptides were present in this sample. This suggested there may be an additional putative GST subunit present in the *S*-hexylglutathione affinity bound fraction from soybean cell cultures.

It is plausible that the use of *S*-hexylglutathione affinity chromatography will lead to the purification of other glutathione related proteins in addition to GSTs. Indeed, it is reported that glyoxalase I has been purified from soybean cell cultures using *S*-hexylglutathione affinity chromatography (Paulus *et al.*, 1993). Paulus *et al* (1993) did not report GST activity in the *S*-hexylglutathione purified pool, but a single hetero-dimeric protein which was assigned as glyoxalase I. The glyoxalase I protein could not be resolved into individual subunits by RP-HPLC and it is surprising that the researchers did not identify the multitude of polypeptides discovered in the current study, given the similar source material and purification strategy. The identity of soybean GSTs and the glyoxalase I enzyme are discussed further in chapters 5 and 6.

Assigning GST activity to the individual subunits identified was difficult, due to the inability to clearly resolve the isoenzymes using anion-exchange chromatography. However, the major polypeptides present in cell cultures (3,6,7,8) and the minor polypeptides (4,5,9 and 10) all appeared to be associated with activity toward CDNB, the chloroacetanilide herbicides and chlorimuron-ethyl. Significantly, polypeptide 11

was associated with the highly active conjugation of the diphenyl ether herbicides acifluorfen and fomesafen when utilising homoglutathione as co-substrate. The native enzyme containing polypeptide 11 appears to have little activity toward other substrates and exhibited only minor activity toward acifluorfen and fomesafen in the presence of glutathione. The discovery of a specific GST responsible for conjugating fomesafen and acifluorfen correlates well with metabolism data in the literature. Metabolism of fomesafen to the homoglutathione conjugate was more rapid in tolerant soybean than susceptible maize or spiny cocklebur (*Xanthium spinosum*) (Evans *et al.*, 1987). Studies with acifluorfen suggest the rate of metabolism was also rapid, with 85 to 95% of the absorbed herbicide to be conjugated within 24 h (Frear *et al.*, 1983). Unfortunately, the attempted use of S-hexylglutathione to purify the fomesafen / acifluorfen detoxifying GST was not successful, with much of the activity not binding to the affinity matrix. Purification methods using other matrices were attempted but were similarly unsuccessful.

Due to the poor separation of native enzymes on anion-exchange chromatography it was difficult to identify the types and numbers of native GST isoenzymes present. The cross contamination of these isoenzymes also prevented an accurate kinetic assessment of individual GSTs, which would have indicated the relative importance of individual enzymes in herbicide metabolism *in planta*. For example, whilst polypeptide 11 would appear to be responsible for a great proportion of fomesafen / acifluorfen conjugation *in vitro*, Km and Vmax analysis may prove that other isoforms present, although possessing a much lower specific activity *in vitro*, may play a more prominent role at physiological substrate concentration. This could certainly be the case with chlorimuron-ethyl. Whilst *in vitro* analysis suggests activity toward this herbicide is very low, and seemingly associated with the major isoforms present, kinetic analysis may indicate that this activity alone may be sufficient to confer selectivity in soybean without the need for a specific metabolising enzyme due to the very low application rates of this herbicide.

The purification of GSTs from soybean seedlings has recently been described (Flury *et al.*, 1996). The enzymes were purified from active crude protein preparations using ion-exchange chromatography and S-hexylglutathione affinity chromatography, and eluted protein monitored for GST activity toward CDNB. Resolution of individual

isoforms bound to the *S*-hexylglutathione matrix was achieved using mono-Q FPLC with 5 partially resolved peaks were obtained, which, when subjected to SDS-PAGE showed the presence of 2 groups of distinct 26 kDa and 28 kDa polypeptides. All sub-units had distinct pI between 6.4 & 5.8 and cross reacted with antibodies raised to GSTs in maize between 27 kDa-29 kDa, now thought to be tau-type GSTs (Dixon *et al.*, 1998). These results agree with findings in the current study in that the GSTs had similar acidic isoelectric points and were composed of similar subunits.

5. Chapter Five. Molecular Characterisation of Soybean GSTs.

5.1 Introduction.

Over 30 cDNAs encoding GSTs have been identified from a number of plant species, including the cereals maize (Wiegand *et al.*, 1986; Grove *et al.*, 1988; Jepson *et al.*, 1994; Dixon *et al.*, 1998), wheat (Mauch and Dudler, 1993; Riechers *et al.*, 1997) rice (Wu *et al*, 1998a/b) and the dicotyledons *Arabidopsis* (Kiyosue *et al.*, 1993) and tobacco (Droog *et al.*, 1993) (Table 1.2). Unlike animal GSTs, it is difficult to classify plant GSTs based on their activity toward various substrates, and the cDNAs encoding plant GSTs have instead been classified based on nucleotide similarities. In addition, the cloning and heterologous expression of plant GST cDNAs in both bacteria (Dixon *et al.*, 1998) and transgenic plants (Roxas *et al.*, 1997; Jepson *et al.*, 1997) has provided further information as to the substrate specificity and possible biological function of specific enzymes.

Prior to the work described in this thesis only 1 soybean GST DNA sequence, *Gmhsp26a*, was listed on the EMBL database. This gene encoded a heat-shock protein, which was first identified following due to its up-regulation by a range of physiological stresses (Czarnecka *et al*, 1988). In later studies *Gmhsp-26a* was found to be identical to the soybean *GH 2/4* gene and expression of this gene in *E. coli* showed the recombinant enzyme possessed GST toward CDNB (Ulmasov *et al.*, 1995). Subsequently it has been demonstrated that GH 2/4 is active in conjugating (homo)glutathione to a number of herbicides and homologues of natural stress metabolites (Skipsey *et al.*, 1997). To avoid further confusion this *Gmhsp26a / GH 2/4* was termed *Glycine max* GST1 (*Gm*GST1), in recognition that it was the first GST described in soybean.

Results presented in chapter 4 showed that soybean contains a number of GST isoenzymes, each with different substrate specificity. The results presented in this chapter describe the molecular characterisation of soybean GSTs, including the cloning of the corresponding cDNAs. Isolation of soybean GST cDNAs would enable their accurate classification, with heterologous expression permitting a thorough assessment of their detoxifying activity (chapter 6).

5.2 Results.

Throughout this section percentage identity and percentage similarity figures are quoted for aligned predicted amino acid sequences. Identity (%) refers to the percentage of amino-acids perfectly conserved between the sequences. Similarity (%) refers to the percentage of identical and conservative amino-acid substitutions between the sequences.

5.2.1 Soybean cDNA library construction.

Soybean cDNA libraries were constructed using 5-day-old cell cultures and 5-day-old soybean seedlings (cv. D297). Total RNA was extracted using the method described by Jepson *et al* (1991) and poly A$^+$ mRNA recovered. cDNA was synthesised, size fractionated, and the cDNA library constructed in the λZAP-II vector according to protocols supplied by Stratagene. The primary libraries were titred, and blue-white colour selection utilised to determine the ratio of plaque forming units (pfu) with insert to those without. The results obtained are shown in Table 5.1. PCR was performed on randomly selected plaques to determine the average cDNA size within the library, using M13RSPL (5' GCG GGA TCC GGA AAC AGC TAT GAC CAT GAT TAC GCG 3') and M13FL (5' GCG GGA TCC CAC GAC GTT GTA AAA CGA CGG CCA GTG 3') primers (Figure 5.1).
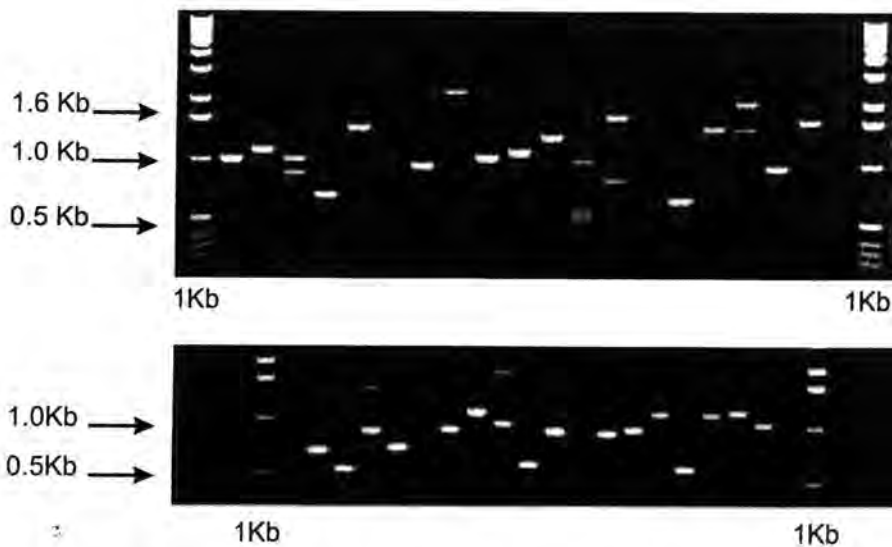
Figure 5.1 PCR of randomly selected library plaques, using M13RSPL and M13FL primers, showing cDNA sizes in the 5-day-old seedling library (top) and the 5-day-old cell culture library (bottom).

Library	Total pfu	cDNA Range (Kb)	Average cDNA Size	No. White	No. Blue	Ratio
Cell Culture	2×10^6	0.6-1.3	1.0Kb	350	3	116:1
Seedling	1×10^7	0.6-2.5	1.4Kb	928	15	62:1

Table 5.1 Characteristics of 5-day-old soybean cell culture and seedling cDNA libraries constructed. Total pfu refers to the total number of pfu obtained, prior to amplification. cDNA range refers the smallest and largest cDNAs present in the library, as sampled by PCR, with the average size indicated. Ratio white colonies : blue colonies indicates that proportion of pfu in the library containing a cDNA insert.

These results suggest both cDNA libraries were of good quality, and they were amplified ($>10^{10}$ pfu ml^{-1}) and stored using protocols provided by Stratagene.

5.2.2 cDNA library screening using degenerate oligonucleotides.

Initial experiments involved the screening of the above cDNA libraries with γ-[^{32}P]-dATP-labelled degenerate oligonucleotide primer, termed SGST1. SGST1 was designed to the N-terminal protein sequence of an auxin-inducible soybean GST described by Flury *et al.* (1995), which is shown below.

?-Ser-Asp-Glu-Val-Val-Leu-Leu-Asp-Phe-Trp-Pro-Ser-Pro-Phe-Gly-Met

?=not determined

The highlighted residues were used to design the 27mer degenerate oligonucleotide SGST1 (GAY TTY TGG CCI WSI CCI TTY GGI ATG). This oligonucleotide had the following characteristics: degeneracy =32, max Tm = 69°C, min Tm = 63°C.

SGST1 was end-labelled with γ-[^{32}P]-dATP and used to screen 300,000 pfu's from the primary (non-amplified) seedling cDNA library. Ten putative positives were picked and subjected to a second round of screening. Six of these isolates appeared positive and were subjected to third round of screening, with reduced titre to enable identification of individual plaques. *In vivo* excision was performed on plaque-pure phage stocks, and plasmid recovered, and sequenced, from overnight cultures initiated from ampicillin resistant bacterial colonies. Identical DNA sequence was obtained

from all 6 cloned cDNAs. However, it was apparent that no cDNAs with a full length open reading frame were obtained from this initial round of screening. In order to isolate a full length cDNA the longest partial cDNA obtained was labelled with [^{32}P]-dCTP and used to re-screen the library. Automated sequencing using M13 forward and M13 reverse sequencing primers identified a 920bp cDNA, with a 651bp ORF, encoding a 216 amino-acid protein. Analysis of the sequence using the BLAST alignment programme confirmed that the ORF encoded a protein showing homology to tau-type GSTs, and this cDNA was termed *GmGST2* (Figure 5.2). The DNA sequence of *GmGST2* was submitted to the EMBL database and assigned the accession number Y10820. Table 5.1 shows the result of a GenBank database search showing most closely related protein sequences to *Gm*GST2 (Table 5.2).

*Gm*GST2

```
1/1                                          31/11
CTT GAA TCT TCG TTA TCC TTC TTT TTC TCT CCT TGA ACT CGA ATA TTC ACT ATG GCA GAT
                                                                     M   A   D
61/21                                        91/31
GAG GTG GTT CTG CTA GAT TTC TGG CCA AGT CCA TTT GGG ATG AGG GTC AGG ATT GCA CTT
E   V   V   L   L   D   F   W   P   S   P   F   G   M   R   V   R   I   A   L
121/41                                       151/51
GCT GAA AAG GGT ATC AAA TAT GAG TCC AAA GAA GAG GAC TTG CAG AAC AAG AGC CCT TTG
A   E   K   G   I   K   Y   E   S   K   E   E   D   L   Q   N   K   S   P   L
181/61                                       211/71
CTC CTC AAA ATG AAC CCG GTT CAC AAG AAA ATC CCG GTT CTC ATC CAC AAT GGC AAA CCC
L   L   K   M   N   P   V   H   K   K   I   P   V   L   I   H   N   G   K   P
241/81                                       271/91
ATT TGT GAA TCT CTC GTT GCT GTT CAG TAC ATT GAG GAG GTC TGG AAT GAC AGA AAT CCC
I   C   E   S   L   V   A   V   Q   Y   I   E   E   V   W   N   D   R   N   P
301/101                                      331/111
TTG TTG CCT TCT GAC CCT TAC CAG AGA GCT CAG GCT AGA TTC TGG GCT GAC TTT GTT GAC
L   L   P   S   D   P   Y   Q   R   A   Q   A   R   F   W   A   D   F   V   D
361/121                                      391/131
AAT AAG ATA TTT GAT CTT GGA AGA AAG ATT TGG ACA TCA AAG GGA GAA GAA AAA GAA GCT
N   K   I   F   D   L   G   R   K   I   W   T   S   K   G   E   E   K   E   A
421/141                                      451/151
GCC AAA AAG GAG TTC ATA GAG GCC CTT AAA TTA TTG GAG GAA CAG CTG GGA GAC AAG ACT
A   K   K   E   F   I   E   A   L   K   L   L   E   E   Q   L   G   D   K   T
481/161                                      511/171
TAT TTT GGA GGA GAC GAT CTA GGT TTT GTG GAT ATA GCA CTT ATT CCA TTC GAC ACT TGG
Y   F   G   G   D   D   L   G   F   V   D   I   A   L   I   P   F   D   T   W
541/181                                      571/191
TTC AAG ACT TTT GGC AGC CTC AAC ATA GAG AGT GAG TGC CCC AAG TTT GTT GCT TGG GCC
F   K   T   F   G   S   L   N   I   E   S   E   C   P   K   F   V   A   W   A
601/201                                      631/211
AAG AGG TGC ATG CAG AAA GAC AGT GTT GCC AGG TCT CTT CCT GAT CAA CAC AAG GTC TAT
K   R   C   M   Q   K   D   S   V   A   R   S   L   P   D   Q   H   K   V   Y
661/221                                      691/231
GAG TTC ATT ATG GGC ATA AGA AAG AAG TTC GAC ATT GAG TAG GTT CAT GTT GGA TCT TAA
E   F   I   M   G   I   R   K   K   F   D   I   E
721/241                                      751/251
TAG CCA CAG TGA CGT ATT GAT CAT TCT TGG CCT TTC AAC TAA ATA GTA TTT GTG TAG AAA
781/261                                      811/271
TTA AAG GCA CTT GGA TGT ACC AAA CTT CAT GCT TTT TGT AGG AGT GCG TAG GTT TTA AAA
841/281                                      871/291
ATT TTC TGA TGT ATC TTT CAT GTG TTT GTT GGT TTT GCA ATA GAA TAT TTC CTA TAT TAT
901/301
ACA AAA AAA AAA AAA AAA AA
```

Figure 5.2 DNA sequence of *GmGST2* showing deduced ORF. The sequence corresponding to the degenerate oligonucleotide used to isolate the cDNA is highlighted.

No.	Species	cDNA Identity	Accession	Similarity (%)	Identity (%)
1	Papaya	pPGST1	AJ000923	88	87
2	Tobacco	Nt103	Q03666	87	72
4	Tobacco	par C	P49332	86	71
5	Tobacco	C-7	S19182	84	71
6	Tobacco	par A	P25317	83	64
7	Eucalyptus	GST	U80615	79	64
8	*Mesembryanthemum crystallinum*	GST	AF079511	83	64

Table 5.2 GSTs present in the GenBank database showing similarity in predicted protein sequence to *GmGST2*.

The N-terminal protein sequence of *Gm*GST2 differs to that reported for the GST purified by Flury *et al.* (1995) in that the amino acid at position two in *Gm*GST2 is alanine and not serine, suggesting that the two proteins may not be identical. *GmGST2* was sub-cloned into the pET vector and the recombinant protein expressed in *E. coli*. Thus further comparisons of the two proteins, based on activity similarity, is described chapter six.

5.2.3 Screening of cDNA libraries using herbicide selection.

Several of the herbicides detoxified by GSTs in soybean have the potential to be toxic to bacteria. It was therefore of interest to establish whether bacteria expressing recombinant soybean GSTs could be selected for on media containing the ALS inhibitor chlorimuron-ethyl and the protoporphyrinogen oxidase inhibitor fomesafen. A mass excision of the cDNA library was performed, and the resulting *E. coli* plated onto media containing IPTG. Unfortunately, neither fomesafen or chlorimuron-ethyl tested proved toxic to *E. coli* within their solubility range in aqueous media (10μM to 100μM) and this approach could not be developed further.

5.2.4 Reverse-transcription polymerase chain reaction (RT-PCR).

RT-PCR was used to obtain soybean GST cDNA sequences using i) degenerate oligonucleotide primers designed to protein sequences obtained from GSTs purified

from soybean (chapter 4) and ii) degenerate primers designed to consensus sequences present in the different plant GSTs classes.

i) RT-PCR using degenerate oligonucleotides designed to protein sequence.

In chapter four the amino-acid sequence obtained from purified GST polypeptides 5 and 7 was described, a sequence corresponding to highly conserved region within plant GSTs. Since the sequence obtained from both proteins was near identical a single degenerate oligonucleotide CAD1 (Table 5.3) was designed. Since CAD1 is designed to such a conserved region it is likely that the primer will be useful in amplifying a range of soybean GST sequences rather than being specific for cDNAs encoding polypeptides 5 or 7.

	1	2	3	4	5	6	7	8	9	10
GST5	S	N	P	V	H	K	K	I	P	V
GST7	K	N	P	V	H	K	K	V	P	F/V
CAD1	AAR	AAY	CCI	GTI	CAY	AAR	AAR	GTI	CC	

Table 5.3 Protein sequence obtained from purified soybean GSTs five and seven showing the design of degenerate oligonucleotide CAD1. CAD1 characteristics: max Tm = 70°C, min Tm = 60°C, degeneracy = 32.

In addition to CAD1, a further degenerate oligonucleotide primer, termed CAD11, was designed to the protein sequence obtained from polypeptide 11 (Table 5.4).

		1	2	3	4	5	6	7	8	9	10
GST11	M	V	R	P	V	L	P	K	C	L	T
CAD11	ATG	GTI	MGI	CCI	GTI	YTI	CCI	AAR			

Table 5.4 Construction of degenerate oligonucleotide CAD11, designed toward the amino-acid sequence obtained for the fomesafen active GST subunit 11. CAD11 characteristics: max Tm = 58°C, min Tm = 52°C, degeneracy 16.

Total RNA was isolated from 5-day-old plant and cell culture tissue and first strand cDNA synthesised using the poly-T primer OG2. PCR amplification was achieved using either CAD1 or CAD11 together with the primer OG9 (OG9 CGC ACT GAG AGA GGA TCC TCG AG), which anneals to OG2 primed first-strand cDNA. The

PCR conditions used were (94°C 2 min, 1 cycle) (94°C, 45 s; 51°C, 30 s; 72°C, 1 min, 35 cycles) (72°C, 7 min; 4°C hold).

Figure 5.3 shows the products obtained with the CAD1 primer. Several attempts to amplify products with the CAD11 primer proved unsuccessful.
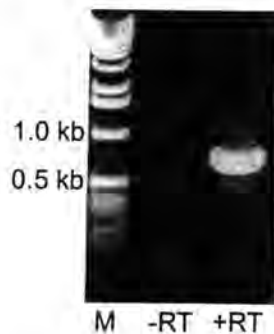
Figure 5.3 RT-PCR products obtained using the degenerate CAD1 primer. M=1kb marker, - RT = PCR excluding first-strand cDNA, + RT = PCR including first-strand cDNA.

The PCR products obtained with the CAD1 primer were gel purified, cloned into the pCR2.1 vector, and sequenced using M13 forward and reverse sequencing primers.

The cDNA was termed *CAD1PART* and was DIG-labelled in order to probe a soybean cell culture cDNA library and isolate a full length cDNA. Following three rounds of screening 20 positive clones were identified and the recovered plasmid analysed by PCR (using CAD1 and OG9) and restriction analysis using *Apa* I : *Sac* I (to size the cDNA), and *Hind* III and *Ssp* I for diagnostic purposes. Ten cDNAs of the correct predicted size, but differing in restriction pattern were sent for DNA sequencing.

The DNA sequence analysis showed that three distinct, but highly related cDNAs, termed *CADGST2* (Figure 5.4), *CADGST5* (Figure 5.5) and *CADGST30* (Figure 5.6), were obtained, all of which closely resembled the previously characterised soybean GST, *GmGST1* (Skipsey *et al.*, 1997).

CADGST2

```
1/1                                      31/11
TTT TTT GAG TGA AGG TTA GAA GTG CTA CAA ACA ATG GCA ACT AAT CAG GAA GAG GTG TCT
                                             M   A   T   N   Q   E   E   V   S
61/21                                    91/31
CTT TTG GGA GTT GTT GGA AGC CCA TTT GTG TGC AGG GTG CAG ATT GCC CTC AAG TTG AAG
L   L   G   V   V   G   S   P   F   V   C   R   V   Q   I   A   L   K   L   K
121/41                                   151/51
GGA ATT CAA TAC AAG TTT TTT GAA GAA AAT TTG GTC AAC AAG AGT GAA CTG CTT CTC AAA
G   I   Q   Y   K   F   F   E   E   N   L   V   N   K   S   E   L   L   L   K
181/61                                   211/71
TAC AAC CCT GTT CAC AAG AAG GTT CCG GTG TTT GTT CAC AAT GAG AAG CCC ATA GCA GAG
Y   N   P   V   H   K   K   V   P   V   F   V   H   N   E   K   P   I   A   E
241/81                                   271/91
TCT CTT GTG ATT GTT GAA TAC ATT GAT GAG ACA TGG AAG AAC AAC CCC ATC TTG CCT TCT
S   L   V   I   V   E   Y   I   D   E   T   W   K   N   N   P   I   L   P   S
301/101                                  331/111
GAT CCT TAC CAA AGA GCC TTG GCT CGT TTC TGG TCC AAA TTC ATT GAT GAC AAG ATT GGG
D   P   Y   Q   R   A   L   A   R   F   W   S   K   F   I   D   D   K   I   G
361/121                                  391/131
GGT GCT GTA TGG AAA TCT GTT TTC ACG GTT GAT GAG AAA GAG CGT GAG AAG AAT GTT GAA
G   A   V   W   K   S   V   F   T   V   D   E   K   E   R   E   K   N   V   E
421/141                                  451/151
GAA TCG TTG GAG GCT CTG CAG TTT CTT GAG AGT GAG ATC AAG GGC AAG AAG TTC TTT GGA
E   S   L   E   A   L   Q   F   L   E   S   E   I   K   G   K   K   F   F   G
481/161                                  511/171
GGA GAG GAG TTT GGG ATG GTA GAT ATT GCT GCT ATC TTC ATA GCA ATT TGG GTC CCT ATG
G   E   E   F   G   M   V   D   I   A   A   I   F   I   A   I   W   V   P   M
541/181                                  571/191
GTT CAA GAA ATT GCA GGG TTG GAA TTA TTC ACA AGT GAG GAA TTT CCT AAG CTC TAC ATT
V   Q   E   I   A   G   L   E   L   F   T   S   E   E   F   P   K   L   Y   I
601/201                                  631/211
GGG AGC CCA GAG TTC ATG AAC CAC CCT GTT GTG AAA GAA GTT CTT CCT CCT AGA GAC CCA
G   S   P   E   F   M   N   H   P   V   V   K   E   V   L   P   P   R   D   P
661/221                                  691/231
CTT TTT GCC TTT TTC AAA GCC CGG TAC GAA AGC CTG CTC GCT GAT TCA AAA TAG ATT TAT
L   F   A   F   F   K   A   R   Y   E   S   L   L   A   D   S   K
721/241                                  751/251
TTA AGG ATA CTT GTG TGA ACA ACT TGT CTC TTC GTT GAG TTA TTG ATG TTT GAA TTT CAT

781/261                                  811/271
GTC AAT TTG ATA CTA TAT GTA ATG TAA CTT AGG ATC TTA TTC CCA AAA AAA AAA AAA AAA

841/281
AAA
```

Figure 5.4 cDNA sequence of *CADGST2*, showing deduced open reading frame.

CADGST5

```
1/1                                      31/11
TCG AAT AGA TTA TCC TAA GTC TTT GGG AAT AGT GCT GCA ATG GCT TCA AGT CAG GAG GAG
                                                          M   A   S   S   Q   E   E
61/21                                    91/31
GTG ACC CTT TTG GGA GCT ACT GGA AGC CCA TTT GTG TGC AGG GTT CAT ATT GCC CTC AAG
V   T   L   L   G   A   T   G   S   P   F   V   C   R   V   H   I   A   L   K
121/41                                   151/51
TTG AAG GGA GTT CAA TAC AAA TAT GTC GAA GAA AAT TTG AGG AAC AAG AGT GAA CTG CTT
L   K   G   V   Q   Y   K   Y   V   E   E   N   L   R   N   K   S   E   L   L
181/61                                   211/71
CTC AAA TCC AAC CCA GTT CAC AAG AAG ATT CCA GTG TTT ATT CAC AAT GAG AAG TCC ATA
L   K   S   N   P   V   H   K   K   I   P   V   F   I   H   N   E   K   S   I
241/81                                   271/91
GCA GAG TCT CTT GTG ATT GTT GAA TAC ATT GAT GAG ACA TGG AAG AAC AAT CCC ATC TTG
A   E   S   L   V   I   V   E   Y   I   D   E   T   W   K   N   N   P   I   L
301/101                                  331/111
CCT TCT GAT CCT TAC CAA AGA GCC TTG GCT CGT TTC TGG TCC AAA TTC ATT GAT GAC AAG
P   S   D   P   Y   Q   R   A   L   A   R   F   W   S   K   F   I   D   D   K
361/121                                  391/131
GTT TTT GGT GCT TCA TGG AAA GCT GTT TTC ACA GCT GAT GAG AAA GAG CGT GAG AAG AAT
V   F   G   A   S   W   K   A   V   F   T   A   D   E   K   E   R   E   K   N
421/141                                  451/151
GTT GAG GAA GCA ATT GAT GCT CTG CAG TTT CTT GAG AAT GAG ATA AAG GAC AAG AAG TTC
V   E   E   A   I   D   A   L   Q   F   L   E   N   E   I   K   D   K   K   F
481/161                                  511/171
TTT GGA GGT GAG GAG ATT GGG TTG GTA GAT ATT GCT GCT GTC TAC ATA GCA TTT TGG GTC
F   G   G   E   E   I   G   L   V   D   I   A   A   V   Y   I   A   F   W   V
541/181                                  571/191
CCT ATG GTT CAA GAA ATT GCA GGG TTG GAG TTA TTC ACA AGT GAC AAA TTT CCT AAG CTC
P   M   V   Q   E   I   A   G   L   E   L   F   T   S   D   K   F   P   K   L
601/201                                  631/211
CAC AAT TGG AGC CAA GAA TTT TTG AAC CAT CCA ATT GTC AAA GAA AGT CTG CCC CCT AGA
H   N   W   S   Q   E   F   L   N   H   P   I   V   K   E   S   L   P   P   R
661/221                                  691/231
GAT CCT GTT TTT GCC TTT TTC AAG GGT CGC TAT GAA ATC CTT TTT ACT TCA AAA TAG ATT
D   P   V   F   A   F   F   K   G   R   Y   E   I   L   F   T   S   K
721/241                                  751/251
TGA TGA TGT GGT GTG AGA CGT AGA ATT TCT AAG AAT TAT GTG TTT GTT ATT GAG TTT TTG
781/261                                  811/271
CTG TTA AAA TGA TGC TTG TAA GTT GTA ATC TAG AAT TTC TCC ATG TCA AAG AAA TAT TCC
841/281                                  871/291
AAG GTT GTT ATT GAC ATT TTG CTA TTT CAA TGA ATA AAT TAT ATT GCA TCT ATC TAA AAA
901/301
AAA AAA AAA AAA AAA AAA AAA
```

Figure 5.5 DNA sequence cDNA *CADGST5*, showing deduced open reading frame.

CADGST30

```
1/1                                   31/11
TAC CAG CTA TAC TTG TTC CTT TTG AAG GTT AGA AGT GCT ACA ATA CAA ACA ATG GCA GCT
                                                                      M   A   A
61/21                                 91/31
ACT CAG GAA GAT GTG AAG CTT TTG GGT ATT GTG GGA AGC CCA TTT GTG TGC AGG GTC CAG
T   Q   E   D   V   K   L   L   G   I   V   G   S   P   F   V   C   R   V   Q
121/41                                151/51
ATT GCC CTT AAG TTG AAG GGA GTT GAA TAC AAA TTT TTG GAA GAA AAT TTG GGC AAC AAG
I   A   L   K   L   K   G   V   E   Y   K   F   L   E   E   N   L   G   N   K
181/61                                211/71
AGT GAT TTG CTT CTC AAA TAC AAC CCT GTT CAC AAG AAG GTT CCA GTG TTT GTT CAC AAT
S   D   L   L   L   K   Y   N   P   V   H   K   K   V   P   V   F   V   H   N
241/81                                271/91
GAG AAG CCC ATA GCA GAG TCT CTT GTG ATT GTT GAA TAC ATT GAT GAG ACA TGG AAG AAC
E   K   P   I   A   E   S   L   V   I   V   E   Y   I   D   E   T   W .  K   N
301/101                               331/111
AAC CCC ATC TTA CCT TCT GAT CCT TAC CAA AGA GCC TTG GCT CGT TTC TGG TCC AAA TTC
N   P   I   L   P   S   D   P   Y   Q   R   A   L   A   R   F   W   S   K   F
361/121                               391/131
ATT GAT GAT AAG ATT GTG GGT GCT GTA TGG AAA TCT GTT TTC ACG GTT GAT GAG AAA GAG
I   D   D   K   I   V   G   A   V   W   K   S   V   F   T   V   D   E   K   E
421/141                               451/151
CGT GAG AAG AAT GTT GAA GAA ACA TAT GAG GCT CTT CAG TTT CTT GAG AAT GAG CTG AAG
R   E   K   N   V   E   E   T   Y   E   A   L   Q   F   L   E   N   E   L   K
481/161                               511/171
GAC AAG AAG TTT TTT GGA GGA GAG GAA TTT GGG TTG GTA GAT ATT GCT GCT GTC TTC ATA
D   K   K   F   F   G   G   E   E   F   G   L   V   D   I   A   A   V   F   I
541/181                               571/191
GCA TTT TGG ATC CCA ATT TTT CAG GAA ATA GCA GGG TTG CAG TTA TTC ACC AGT GAG AAA
A   F   W   I   P   I   F   Q   E   I   A   G   L   Q   L   F   T   S   E   K
601/201                               631/211
TTT CCT ATA CTC TAC AAA TGG AGC CAA GAA TCC CTT AAC CAC CCT TTT GTG CAA GAA GTC
F   P   I   L   Y   K   W   S   Q   E   S   L   N   H   P   F   V   Q   E   V
661/221                               691/231
CTT CCT CCT AGA GAC CCA CTT TTT ACC TTT TTC AAA GCC CGC TAT GAA AGT TTT TTT GCT
L   P   P   R   D   P   L   F   T   F   F   K   A   R   Y   E   S   F   F   A
721/241                               751/251
TCA AAA TAG AAT TAT TTA AGG ATA TTT GTT GAA CAA CTT GTG TCT TGT TGA GTT ATT GAT
S   K
781/261                               811/271
GTT TGA ATT TCA TGT CAA ATG ATA CTA GCT ATA TGT AAA TCC CAA AAA AAA AAA AAA AAA
```

Figure 5.6 DNA sequence of cDNA *CADGST30* showing deduced open reading frame.

Thus, the use of the CAD1 degenerate primer in RT-PCR resulted in the isolation of a group of related cDNAs encoding *Gm*GST1-type enzymes. DNA and protein sequence alignments showing differences between the three cDNAs obtained are shown in an alignment of all new soybean sequences identified in Figures 5.12 and 5.13. Percentage identities and similarities of these proteins to *Gm*GST1 are given in Table 5.5.

cDNA	Identity (%)	Similarity (%)
CADGST2	84	91
CADGST5	79	88
CADGST30	96	96

Table 5.5 Identity and similarity of expressed protein sequence of cDNAs isolated using RT-PCR with the degenerate primer CAD1 to *GmGST1* (Skipsey *et al*, 1997).

5.2.5 RT-PCR using degenerate oligos designed to conserved Plant GST motifs.

The majority of all plant GST sequences discovered to date belong to either the tau- or theta- GST classes. Alignment of specific regions of the tau- and theta-type GST sequences (Figure 5.23) indicates that distinct regions of homology exist within each class of GST. Furthermore, it would appear that within the tau class, there are two distinct groups of closely related sequences (indicated by phylogenetic analysis in Figure 5.24). It was hypothesised that the conserved regions could be exploited using RT-PCR in conjunction with degenerate oligonucleotides in order to identify novel soybean GST. The degenerate primers designed were named CJACON1, CJACON2 and CJACON3, and their design based on nucleotide homology is detailed in Figures 5.6-5.8. The corresponding regions of protein homology used to design the primers are indicated in Figure 5.23, which is presented in the discussion section of this chapter.

CJACON1

AtPM24	(D)	GATGGAGACCTCAAGCTCTTCGAATCA
HmGST	(D)	GATGGAGATCTCAAGCTTTTGAGTCA
AtERD11	(D)	GATGGAGACTTCAAGATTTTCGAATCA
NtparB	(D)	GATGGGGACTTGAAGCTTTTGAATCA
ScGST	(D)	GATGGAGAAATCAAGCTTTTTGAGTCA
ZmGSTI	(M)	GATGGTGACTTGTACCTCTTCGAATCA
		***** ** * * * ** ** ***
CJACON1		GATGGIGAYITSWASCTYTTYGARTCA

Table 5.6 Region of homology in theta class GSTs used to design PCR primer CJACON1. (D) indicates dicotyledonous species, whereas (M) indicates monocotyledonous species. At = *Arabidopsis thaliana*, Hm = *Hyoscyamus muticus*, Nt = *Nicotiana tabacum* Sc = *Silene cucubalus*, Zm = *Zea mays*. Oligo characteristics: min Tm = 68°C, max Tm = 76°C, degeneracy = 128.

CJACON2

EgGST	(D)	TTCTGGGCCGACTACGTGGACAAGAAG
GmGST2	(D)	TTCTGGGCTGACTTTGTTGACAATAAG
GmGST3	(D)	TTCTGGGTGGACTACATTGACAAAAAG
Nt_C-7	(D)	TTTTGGGCTGATTATGTTGACAAAAAG
NtparA	(D)	TTCTGGGCCGACTATATTGACAAGAAG
NtparC	(D)	TTCTGGGCTGACTACATTGACAAGAAG
ZmGSTV	(M)	TTCTGGGCGGACTACGTCGACAAGAAG
		** **** ** * ***** ***
CJACON2		TTCTGGGYKRASTWCITYGACRAIAAG

Table 5.7 Region of homology in tau class GSTs used to design PCR primer CJACON2. (D) indicates dicotyledonous species, whereas (M) indicates monocotyledonous species. Eg = *Eucalyptus globulus*, Gm = *Glycine max*, Nt = *Nicotiana tabacum*, Zm = *Zea mays*. Oligo characteristics: min Tm = 70°C, max Tm = 80°C, degeneracy = 128.

CJACON3

AtGST5	(D)	GAGTCTCATGTGATTCTTGAATACATCGATGAG
Nt103	(D)	GAGTCTATGGTCATTCTTGAATACATTGATGAG
StPRP1	(D)	GAGTCTATGGTCATTCTTGAATACATTGATGAG
Vr MII-4	(D)	GAGTCCCTTGTGATTGTTGAGTACATCGATGAG
GmGST1	(D)	GAGTCTCTTGTGATTGTTGAATACATTGATGAG
		***** ** *** **** ***** ******
CJACON3		GAGTCYMWKGTSATTSTTGARTACATYGATGAG

Table 5.8 Region of homology in tau class GSTs used to design PCR primer CJACON3. (D) indicates dicotyledonous species, whereas (M) indicates monocotyledonous species. At = *Arabidopsis thaliana*, Nt = *Nicotiana tabacum*, St = *Solanum tuberosum*, Vr = *Vigna Radiata*, Gm = *Glycine max*. Oligo characteristics: min Tm = 88°C, max Tm = 98°C, degeneracy = 256.

First strand cDNA was prepared from total RNA by RT-PCR using OG2 as described previously. Amplification of specific products was achieved by PCR (94°C, 45 s; 51°C 30 s and 72°C, 60 s: 35 cycles) using primers CJACON1, CJACON2 and

CJACON3 each used independently in conjunction with OG9. The reaction products obtained with each primer are shown in Figure 5.7.
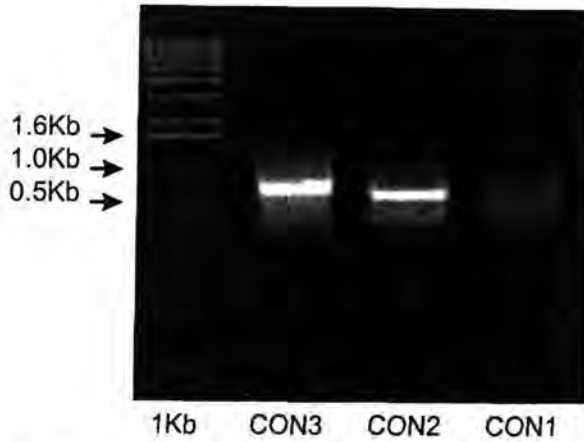
Figure 5.7 Gel showing RT-PCR products obtained using CJACON primers. Products of the correct predicted size can be visualised for CJACON2 and CJACON3. Several attempts to obtain specific products with CJACON1 were unsuccessful.

RT-PCR products were obtained using both CJACON2 and CJACON3 primers, both of which were designed to tau-type GSTs. No RT products were obtained using CJACON1, designed to the theta-class, under any of the various conditions tested. However, when used in conjunction with OG2 the CJACON1 primer did amplify a product of the correct size product from a plasmid containing the theta-type maize ZmGSTI cDNA (supplied by Dr Ian Jepson) (Figure 5.8), which suggested the primer was functional in the amplifying of theta-type GST sequences.
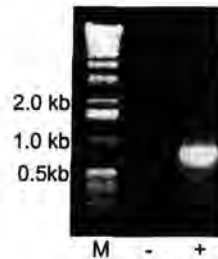
Figure 5.8 Positive RT-PCR control using CJACON1. Gel shows PCR products obtained after 25 cycles using CJACON1 and OG2 to amplify ≈700bp fragment from the plasmid pIJ21 which contained the cDNA encoding the theta-type ZmGST1. M=1kb marker, - = without pIJ21, + = with pIJ21.

The amplified products obtained using the CJACON2 and CJACON3 primers were ligated into the pCR2.1 vector and transformed into *E. coli* INVαF' competent cells (Invitrogen). Transformed colonies were selected on LB media containing 100 μg ml⁻¹

ampicillin and 40 µg ml⁻¹ X-GAL. Plasmid was recovered from 5 ml overnight cultures, initiated from each of 90 individual white colonies (45 x CJACON2, 45 x CJACON3) as described previously. The various plasmids were subjected to restriction analysis (*Eco*R1 to size cDNA, *Ssp*1, *Ssp*1:*Sph*1, and *Rsa*1). These enzymes were chosen since they allow the grouping of the cDNAs into different classes and specifically identify the cDNAs already known, based on characteristic digest patterns. In addition PCR was performed using either CJACON2 or CJACON3 and OG9 primers to confirm identity (Figure 5.9). Representative cDNAs were sent for automated sequencing.
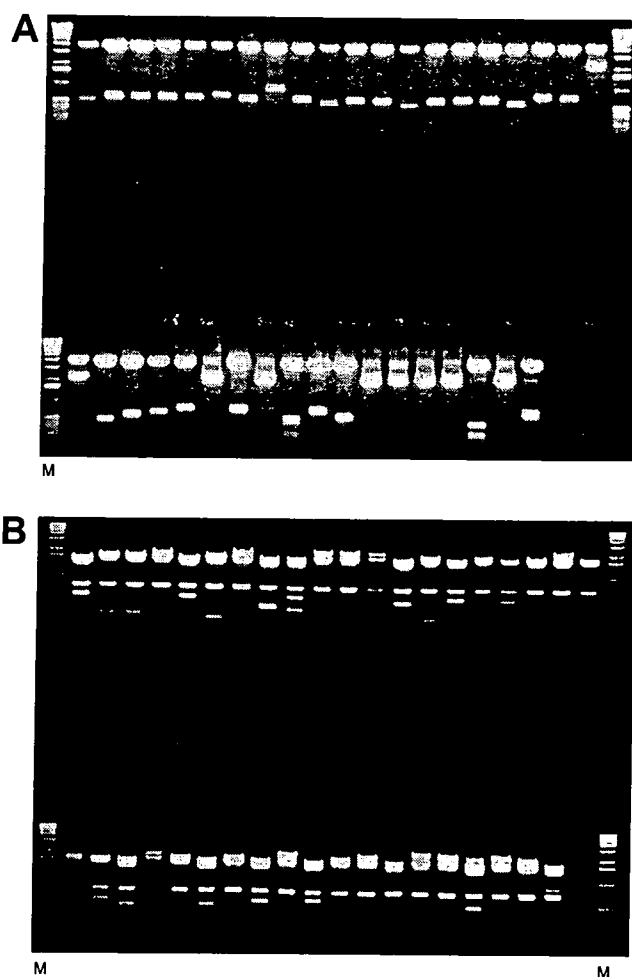
Figure 5.9 Examples of restriction enzyme analysis of cDNAs cloned into the pCR 2.1 vector after RT-PCR using the CJACON2 / 3 and OG9 primers. Gel A shows *Eco*R1 digest, which releases the cDNA from the pCR 2.1 vector. Gel B shows restriction analysis with *Rsa* I.

The DNA sequences obtained showed that a number of cDNAs showing similarity to both *Gm*GST1 and *Gm*GST2 had been obtained. This was expected, since both the CJACON2 and CJACON3 primers were designed to conserved regions that are

present within these previously characterised sequences. In addition, a number of partial cDNAs with identity to a to a cDNA sequence described as encoding soybean glyoxalase I (P46417) were isolated (Paulus *et al*, 1993). The glyoxalase I cDNA shows little homology toward other plant glyoxalases in the database (Espartero *et al.*, 1995), and, as will be discussed later this cDNA does in fact encode a GST which has been re-termed *GmGST3*.

In addition to the *GmGST1*, *GmGST2* and *GmGST3*-like sequences obtained, four completely novel tau-type cDNAs were identified. These clones, termed *GSTCON26a*, *GSTCON31a*, *GSTCON32* and *GSTCON33a*, were DIG-labelled and used to screen the cDNA library for full length GSTs.

5.2.6 Comparison of soybean GST cDNAs.

All the soybean GST cDNAs obtained by the different screening methods were compared.

5.2.7 *GmGST1*-type sequences.

RT-PCR using primers CJACON2 and CJACON3 resulted in the isolation of multiple *GmGST1* variants, with partial cDNAs, identical to the *CADGST2*, *CADGST5* and *CADGST30* already described being identified. In addition, two further partial cDNAs, termed *GSTCA311* (Figure 5.10) and *GSTCA39* (Figure 5.11) were obtained. Relatedness of these genes to *Gm*GST1 is given in Table 5.9.

GSTCA311

```
1/1                                       31/11
GAG TCT ATT GTC ATT GTT GAA TAC ATT GAT GAG ACA TGG AAG AAC AAC CCC ATC TTG CCT
E   S   I   V   I   V   E   Y   I   D   E   T   W   K   N   N   P   I   L . P
61/21                                     91/31
TCT GAC CCT TAC CAA AGA GCC TTG GCT CGT TTC TGG TCC AAA TTC ATT GAT GAT AAG GTT
S   D   P   Y   Q   R   A   L   A   R   F   W   S   K   F   I   D   D   K   V
121/41                                    151/51
TTT GGT GCT GCA TGG AAA TCC GTT TTC ACA GCT GAT GAG AAA GAG CGT GAG AAG AAT GTT
F   G   A   A   W   K   S   V   F   T   A   D   E   K   E   R   E   K   N   V
181/61                                    211/71
GAG GAA GCA ATT GAG GCT CTG CAG TTT CTT GAG AAT GAG ATA AAG GAC AAG AAG TTC TTT
E   E   A   I   E   A   L   Q   F   L   E   N   E   I   K   D   K   K   F   F
241/81                                    271/91
GGA GGA GAG GAG ATT GGG TTG GTA GAT ATT GCT GCT GTC TAC ATA GCA TTT TGG GTC CCT
G   G   E   E   I   G   L   V   D   I   A   A   V   Y   I   A   F   W   V   P
301/101                                   331/111
ATG GTT CAA GAA ATT GCA GGG TTG GAG TTA TTC ACA AGT GAG AAA TTT CCT AAG CTC CAC
M   V   Q   E   I   A   G   L   E   L   F   T   S   E   K   F   P   K   L   H
361/121                                   391/131
AAT TGG AGC CAA GAA TTT TTG AAC CAT CCA ATT GTC AAA GAA AGT CTG CCC CCT AGA GAT
N   W   S   Q   E   F   L   N   H   P   I   V   K   E   S   L   P   P   R   D
421/141                                   451/151
CCT GTT TTC TCC TTT TTC AAG GGT CTC TAT GAA AGC CTT TTT GGT TCA AAA TAG ATT TGA
P   V   F   S   F   F   K   G   L   Y   E   S   L   F   G   S   K
481/161                                   511/171
TGA TGT GGT GTG AGA CTT AGT ATT TCT AAG AAT TAT GTG TTT GTT AAA GGC TTC TAT GAA

541/181                                   571/191
AGC CTC ACT GCT TCA AAA TAG ATT CAT GTA TGT GAG ACT CAG AAT CTC TGG GGA AAA TTG

601/201                                   631/211
TGT GTG GTG TGG ACT ACT TGT TTT GTT TGT CAT TGA GCT ATA TCG CTG TTA ATT AGG ATT

661/221                                   691/231
TTG TTT CAA AAT GAT GCT TAT AAG TTG TAA TCT AGG ATT TCT CCC TTT GAA ATC CTA GGT

721/241                                   751/251
TGT TCT TGA CAT TTG CTA TTT CAA AGA ATA AAT ATA TAG CAT CTT TCT ATT TAA AAA AAA

781/261
AAA AAA AAA AA
```

Figure 5.10 DNA sequence of partial cDNA *GSTCA311* with deduced ORF.

GSTCA39

```
1/1                                      31/11
GAG TCT CAT GTC ATT GTT GAA TAC ATC GAT GAG ACA TGG AAG AAC AAC CCC ATC TTA CCT
E   S   H   V   I   V   E   Y   I   D   E   T   W   K   N   N   P   I   L   P
61/21                                    91/31
TCT GAT CCT TAC CAA AGA GCC TTG GCT CGT TTC TGG TCC AAA TTC ATT GAT GAT AAG ATT
S   D   P   Y   Q   R   A   L   A   R   F   W   S   K   F   I   D   D   K   I
121/41                                   151/51
GTG GGT GCT GTA TCG AAA TCT GTT TTC ACG GTT GAT GAG AAA GAG CGT GAG AAG AAT GTT
V   G   A   V   S   K   S   V   F   T   V   D   E   K   E   R   E   K   N   V
181/61                                   211/71
GAA GAA ACA TAT GAG GCT CTT CAG TTT CTT GAG AAT GAG CTG AAG GAC AAG AAG TTT TTT
E   E   T   Y   E   A   L   Q   F   L   E   N   E   L   K   D   K   K   F   F
241/81                                   271/91
GGA GGA GAG GAA TTT GGG TTG GTA GAT ATT GCT GCT GTC TTC ATA GCA TTT TGG ATC CCA
G   G   E   E   F   G   L   V   D   I   A   A   V   F   I   A   F   W   I   P
301/101                                  331/111
ATT TTT CAG GAA ATA GCA GGG TTG CAG TTA TTC ACC AGT GAG AAA TTT CCT ATA CTC TAC
I   F   Q   E   I   A   G   L   Q   L   F   T   S   E   K   F   P   I   L   Y
361/121                                  391/131
AAA TGG AGC CAA GAA TCC CTT AAC CAC CCT TTT GTG CAA GAA GTC CTT CCT CCT AGA GAC
K   W   S   Q   E   S   L   N   H   P   F   V   Q   E   V   L   P   P   R   D
421/141                                  451/151
CCA CTT TTT ACC TTC CTC AAA GCC CGC TAT GAA AGT CTT TCT GCT TCA AAA TAG ACT TAT
P   L   F   T   F   L   K   A   R   Y   E   S   L   S   A   S   K
481/161                                  511/171
TTA AGG ATA TTT GTT GAA CAA CTT GTG TCT TGT TGA GTT ATT GAT GTT TGA ATT TCA TGT

541/181                                  571/191
CAA ATG ATA CTA GCT ATA TGT AAA TCC AAA AAA AAA AAA AAA AAA
```

Figure 5.11 DNA sequence of cDNA *GSTCA39* with deduced open reading frame.

cDNA	Identity (%)	Similarity (%)
GSTCA39	96	96
GSTCA311	82	89

Table 5.9 Protein identity and similarity of partial length cDNAs GSTCA39 and GSTCA311 compared with the corresponding region in *Gm*GST1.

Screening of soybean libraries for full length cDNAs for *GSTCA39* and *GSTCA311* was not attempted, since the high homology between these clones would have undoubtedly resulted in the re-isolation of *GmGST1* clones, as observed previously using the sequences derived from RT-PCR using the CAD1 primer.

Alignment of all *GmGST1*-type cDNAs

All the variant *GmGST1* type cDNA sequences (full length and partial) obtained from soybean using the CAD1, CJACON2 and CJACON3 degenerate primers were compared using the Clustal W sequence alignment programme. Both DNA (Figure 5.13) and protein alignments (Figure 5.12) are shown, since differences in the 3' and 5' untranslated DNA regions (UTR's) may be indicative of variations between the clones and suggest the presence of distinct genes.

```
GmGST1       MAATQEDVKLLGIVGSPFVCRVQIALKLKGVEYKFLEENLGNKSDLLLKYNPVHKKVPVF
GSTCA30      MAATQEDVKLLGIVGSPFVCRVQIALKLKGVEYKFLEENLGNKSDLLLKYNPVHKKVPVF
GSTCA2       MATNQEEVSLLGVVGSPFVCRVQIALKLKGIQYKFFEENLVNKSELLLKYNPVHKKVPVF
GSTCA5       MASSQEEVTLLGATGSPFVCRVHIALKLKGVQYKYVEENLRNKSELLLKSNPVHKKIPVF
GSTCA311     ------------------------------------------------------------
GSTCA39      ------------------------------------------------------------


GmGST1       VHNEQPIAESLVIVEYIDETWKNNPILPSDPYQRALARFWSKFIDDKIVGAVSKSVFTVD
GSTCA30      VHNEKPIAESLVIVEYIDETWKNNPILPSDPYQRALARFWSKFIDDKIVGAVWKSVFTVD
GSTCA2       VHNEKPIAESLVIVEYIDETWKNNPILPSDPYQRALARFWSKFIDDKIGGAVWKSVFTVD
GSTCA5       IHNEKSIAESLVIVEYIDETWKNNPILPSDPYQRALARFWSKFIDDKVFGASWKAVFTAD
GSTCA311     --------ESIVIVEYIDETWKNNPILPSDPYQRALARFWSKFIDDKVFGAAWKSVFTAD
GSTCA39      --------ESHVIVEYIDETWKNNPILPSDPYQRALARFWSKFIDDKIVGAVSKSVFTVD
                      ** ************************************  **   * .*** *


GmGST1       EKEREKNVEETYEALQFLENELKDKKFFGGEEFGLVDIAAVFIAFWIPIFQEIAGLQLFT
GSTCA30      EKEREKNVEETYEALQFLENELKDKKFFGGEEFGLVDIAAVFIAFWIPIFQEIAGLQLFT
GSTCA2       EKEREKNVEESLEALQFLESEIKGKKFFGGEEFGMVDIAAIFIAIWVPMVQEIAGLELFT
GSTCA5       EKEREKNVEEAIDALQFLENEIKDKKFFGGEEIGLVDIAAVYIAFWVPMVQEIAGLELFT
GSTCA311     EKEREKNVEEAIEALQFLENEIKDKKFFGGEEIGLVDIAAVYIAFWVPMVQEIAGLELFT
GSTCA39      EKEREKNVEETYEALQFLENELKDKKFFGGEEFGLVDIAAVFIAFWIPIFQEIAGLQLFT
             **********. .****** *.*  ******** *.*****..** *.*. ******.***


GmGST1       SEKFPILYKWSQEFLNHPFVHEVLPPRDPLFAYFKARYESLSASK-
GSTCA30      SEKFPILYKWSQESLNHPFVQEVLPPRDPLFTFFKARYESFFASK-
GSTCA2       SEEFPKLYIGSPEFMNHPVVKEVLPPRDPLFAFFKARYESLLADSK
GSTCA5       SDKFPKLHNWSQEFLNHPIVKESLPPRDPVFAFFKGRYEILFTSK-
GSTCA311     SEKFPKLHNWSQEFLNHPIVKESLPPRDPVFSFFKGLYESLFGSK-
GSTCA39      SEKFPILYKWSQESLNHPFVQEVLPPRDPLFTFLKARYESLSASK-
             *. ** *    * * .*** *.* ******.*.. *   **
```

Figure 5.12 Clustal W (1.7) protein sequence alignment of *Gm*GST1 type enzymes. * indicates totally conserved amino-acids, . indicates conserved amino-acids.

```
GmGST1     --------------------------------------------------------ATGGCAGCT
GSTCA30    TACCAGCTATACTTGTTCCTTTTGAAGGTTAGAAGTGCTACAATACAAACAATGGCAGCT
GSTCA2     ----------------ATTTTTTGAGTGAAGGTTAGAAGTGCTACAAACAATGGCAACT
GSTCA5     ----------TTCGAATAGATTATCCTAAGTCTTTGGGAATAGTGCTGCAATGGCTTCA
GSTCA311   ------------------------------------------------------------
GSTCA39    ------------------------------------------------------------


GmGST1     ACTCAGGAAGATGTGAAGCTTTTGGGTATTGTGGGAAGCCCATTTGTGTGCAGGGTCCAG
GSTCA30    ACTCAGGAAGATGTGAAGCTTTTGGGTATTGTGGGAAGCCCATTTGTGTGCAGGGTCCAG
GSTCA2     AATCAGGAAGAGGTGTCTCTTTTGGGAGTTGTTGGAAGCCCATTTGTGTGCAGGGTCCAG
GSTCA5     AGTCAGGAGGAGGTGACCCTTTTGGGAGCTACTGGAAGCCCATTTGTGTGCAGGGTGCAG
GSTCA311   ------------------------------------------------------------
GSTCA39    ------------------------------------------------------------


GmGST1     ATTGCCCTTAAGTTGAAGGGAGTTGAATACAAATTTTTGGAAGAAAATTTGGGCAACAAG
GSTCA30    ATTGCCCTTAAGTTGAAGGGAGTTGAATACAAATTTTTGGAAGAAAATTTGGGCAACAAG
GSTCA2     ATTGCCCTCAAGTTGAAGGGAATTCAATACAAGTTTTTTGAAGAAAATTTGGTCAACAAG
GSTCA5     ATTGCCCTCAAGTTGAAGGGAGTTCAATACAAATATGTCGAAGAAAATTTGAGGAACAAG
GSTCA311   ------------------------------------------------------------
GSTCA39    ------------------------------------------------------------


GmGST1     AGTGATTTGCTTCTCAAATACAACCCTGTTCACAAGAAGGTTCCAGTGTTTGTTCACAAT
GSTCA30    AGTGATTTGCTTCTCAAATACAACCCTGTTCACAAGAAGGTTCCAGTGTTTGTTCACAAT
GSTCA2     AGTGAACTGCTTCTCAAATACAACCCTGTTCACAAGAAGGTTCCGGTGTTTGTTCACAAT
GSTCA5     AGTGAACTGCTTCTCAAATCCAACCCAGTTCACAAGAAGATTCCAGTGTTTATTCACAAT
CSTCA311   ------------------------------------------------------------
GSTCA39    ------------------------------------------------------------


GmGST1     GAGCAGCCCATAGCGGAGTCTCTTGTGATTGTTGAATACATTGATGAGACATGGAAGAAC
GSTCA30    GAGAAGCCCATAGCAGAGTCTCTTGTGATTGTTGAATACATTGATGAGACATGGAAGAAC
GSTCA2     GAGAAGCCCATAGCAGAGTCTCTTGTGATTGTTGAATACATTGATGAGACATGGAAGAAC
GSTCA5     GAGAAGTCCATAGCAGAGTCTCTTGTGATTGTTGAATACATTGATGAGACATGGAAGAAC
GSTCA311   --------------TGAGTCTATTGTCATTGTTGAATACATTGATGAGACATGGAAGAAC
GSTCA39    --------------GAGTCTCATGTCATTGTTGAATACATCGATGAGACATGGAAGAAC
                       * * * * * *     * * *   * * * * * * * * * * * * *   * * * * * * * * * * * * * * * * *


GmGST1     AACCCCATCTTACCTTCTGATCCTTACCAAAGAGCCTTGGCTCGTTTCTGGTCCAAATTC
GSTCA30    AACCCCATCTTACCTTCTGATCCTTACCAAAGAGCCTTGGCTCGTTTCTGGTCCAAATTC
GSTCA2     AACCCCATCTTGCCTTCTGATCCTTACCAAAGAGCCTTGGCTCGTTTCTGGTCCAAATTC
GSTCA5     AATCCCATCTTGCCTTCTGATCCTTACCAAAGAGCCTTGGCTCGTTTCTGGTCCAAATTC
GSTCA311   AACCCCATCTTGCCTTCTGACCCTTACCAAAGAGCCTTGGCTCGTTTCTGGTCCAAATTC
GSTCA39    AACCCCATCTTACCTTCTGATCCTTACCAAAGAGCCTTGGCTCGTTTCTGGTCCAAATTC
            * *   * * * * * * *   * * * * * * *   * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *


GmGST1     ATTGATGATAAGATTGTGGGTGCTGTATCGAAATCTGTTTTTCACGGTTGATGAGAAAGAG
GSTCA30    ATTGATGATAAGATTGTGGGTGCTGTATGGAAATCTGTTTTTCACGGTTGATGAGAAAGAG
GSTCA2     ATTGATGACAAGATTGGGGGTGCTGTATGGAAATCTGTTTTTCACGGTTGATGAGAAAGAG
GSTCA5     ATTGATGACAAGGTTTTTGGTGCTTCATGGAAAGCTGTTTTTCACAGCTGATGAGAAAGAG
GSTCA311   ATTGATGATAAGGTTTTTGGTGCTGCATGGAAATCCGTTTTTCACAGCTGATGAGAAAGAG
GSTCA39    ATTGATGATAAGATTGTGGGTGCTGTATCGAAATCTGTTTTTCACGGTTGATGAGAAAGAG
            * * * * * * * *   * * *   * *       * * * * * *     * *   * * * *   *   * * * * * * * *   *   * * * * * * * * * * * * *


GmGST1     CGTGAGAAGAATGTTGAAGAAACATATGAGGCTCTTCAGTTTCTTGAGAATGAGCTGAAG
GSTCA30    CGTGAGAAGAATGTTGAAGAAACATATGAGGCTCTTCAGTTTCTTGAGAATGAGCTGAAG
GSTCA2     CGTGAGAAGAATGTTGAAGAATCGTTGGAGGCTCTGCAGTTTCTTGAGAGTGAGATCAAG
GSTCA5     CGTGAGAAGAATGTTGAGGAAGCAATTGATGCTCTGCAGTTTCTTGAGAATGAGATAAAG
GSTCA311   CGTGAGAAGAATGTTGAGGAAGCAATTGATGCTCTGCAGTTTCTTGAGAATGAGATAAAG
GSTCA39    CGTGAGAAGAATGTTGAAGAAACATATGAGGCTCTTCAGTTTCTTGAGAATGAGCTGAAG
            * * * * * * * * * * * * * * * * *   * * *   *       * *   * * * * *   * * * * * * * * * * *   * * * *   *   * * *


GmGST1     GACAAGAAGTTTTTTGGAGGAGAGGAATTTGGGTTGGTAGATATTGCTGCTGTCTTCATA
GSTCA30    GACAAGAAGTTTTTTGGAGGAGAGGAATTTGGGTTGGTAGATATTGCTGCTGTCTTCATA
GSTCA2     GGCAAGAAGTTCTTTGGAGGAGAGGAGTTTGGGATGGTAGATATTGCTGCTATCTTCATA
GSTCA5     GACAAGAAGTTCTTTGGAGGTGAGGAGATTGGGTTGGTAGATATTGCTGCTGTCTACATA
GSTCA311   GACAAGAAGTTCTTTGGAGGAGAGGAGATTGGGTTGGTAGATATTGCTGCTGTCTACATA
GSTCA39    GACAAGAAGTTTTTTGGAGGAGAGGAATTTGGGTTGGTAGATATTGCTGCTGTCTTCATA
            *   * * * * * * * * *   * * * * * * *   * * * * *     * * * * * * * * * * * * * * * * * * *   * * *   * * * *
```

```
GmGST1     GCATTTTGGATCCCAATTTTTCAGGAAATAGCAGGGTTGCAGTTATTCACCAGTGAGAAA
GSTCA30    GCATTTTGGATCCCAATTTTTCAGGAAATAGCAGGGTTGCAGTTATTCACCAGTGAGAAA
GSTCA2     GCAATTTGGGTCCCTATGGTTCAAGAAATTGCAGGGTTGGAATTATTCACAAGTGAGGAA
GSTCA5     GCATTTTGGGTCCCTATGGTTCAAGAAATTGCAGGGTTGGAGTTATTCACAAGTGACAAA
GSTCA311   GCATTTTGGGTCCCTATGGTTCAAGAAATTGCAGGGTTGGAGTTATTCACAAGTGAGAAA
GSTCA39    GCATTTTGGATCCCAATTTTTCAGGAAATAGCAGGGTTGCAGTTATTCACCAGTGAGAAA
           *** ***** **** **  **** ***** ******** * ******* ***** **

GmGST1     TTTCCTATACTCTACAAATGGAGCCAAGAATTTCTTAACCACCCTTTTGTGCACGAAGTC
GSTCA30    TTTCCTATACTCTACAAATGGAGCCAAGAATCCCTTAACCACCCTTTTGTGCAAGAAGTC
GSTCA2     TTTCCTAAGCTCTACATTGGGAGCCCAGAGTTCATGAACCACCCTGTTGTGAAAGAAGTT
GSTCA5     TTTCCTAAGCTCCACAATTGGAGCCAAGAATTTTTGAACCATCCAATTGTCAAAGAAAGT
GATCA311   TTTCCTAAGCTCCACAATTGGAGCCAAGAATTTTTGAACCATCCAATTGTCAAAGAAAGT
GSTCA39    TTTCCTATACTCTACAAATGGAGCCAAGAATCCCTTAACCACCCTTTTGTGCAAGAAGTC
           *******  *** ***   ****** *** *   * ***** **  **** * ***

GmGST1     CTTCCTCCTAGAGACCCACTTTTTGCCTACTTCAAAGCCCGCTATGAAAGTCTTTCTGCT
GSTCA30    CTTCCTCCTAGAGACCCACTTTTTACCTTTTTTCAAAGCCCGCTATGAAAGTTTTTTTGCT
GSTCA2     CTTCCTCCTAGAGACCCACTTTTTGCCTTTTTTCAAAGCCCGGTACGAAAGCCTGCTCGCT
GSTCA5     CTGCCCCCTAGAGATCCTGTTTTTGCCTTTTTTCAAGGGTCGCTATGAAATCCTTTTTACT
GSTCA311   CTGCCCCCTAGAGATCCTGTTTTTCTCCTTTTTTCAAGGGTCTCTATGAAAGCCTTTTTGGT
GSTCA39    CTTCCTCCTAGAGACCCACTTTTTACCTTCCTCAAAGCCCGCTATGAAAGTCTTTCTGCT
           ** **  ******** **  **** ***  ***  ****  *  *  ** ****   *     *

GmGST1     ---TCAAAATAGACTTATTTAAG-GATATTTGT-TGAACAACTTGTGTCTT-GTTGAGTT
GSTCA30    ---TCAAAATAGAATTATTTAAG-GATATTTGT-TGAACAACTTGTGTCTT-GTTGAGTT
GSTCA2     GATTCAAAATAGATTTATTTAAG-GATACTTGTGTGAACAACTTGTCTCTTCGTTGAGTT
GSTCA5     ---TCAAAATAGATTTGATGATGTGGTGTGAGACGTAGAATTTCTAAGAATTATGTGTTT
GSTCA311   ---TCAAAATAGATTTGATGATGTGGTGTGAGACTTAGTATTTCTAAGAATTATGTGTTT
GSTCA39    ---TCAAAATAGACTTATTTAAG-GATATTTGT-TGAACAACTTGTGTCTT-GTTGAGTT
           ********* **  *   *   *     *    *   *     *   *    **

GmGST1     ATTGCTGTTTGAATTTCATGTAAAAT-GATACTAGCTATA----TGTAA-----------
GSTCA30    ATTGATGTTTGAATTTCATGTCAAAT-GATACTAGCTATA----TGTAA-----------
GSTCA2     ATTGATGTTTGAATTTCATGTCAATTTGATACTATATGTAA---TGTAACTTAGGATCTT
GSTCA5     GTTATTGAGT--TTTTGCTGTTAAAATGATGCTTGTAAGT----TGTAATCTAGAATTTC
GSTCA311   GTTAAAGGCTTCTATGAAAGCCTCACTGCTTCAAAATAGATTCATGTATGTGAGACTCAG
GSTCA39    ATTGATGTTTGAATTTCATGTCAAAT-GATACTAGCTATA----TGTAA-----------
           **    *   *      *     *          * * *          ****

GmGST1     ATCCCAGAAAAAAAAAAAAAAAA-------------------------------------
GSTCA30    ATCCCAAAAAAAAAAAAAAAAAA-------------------------------------
GSTCA2     ATTCCCAAAAAAAAAAAAAAAAAAAA---------------------------------
GSTCA5     TCCATGTCAAAGAAATATTCCAAGGTTGTTATTGACATTTTGCTATTTCAATGAATAAAT
GSTCA311   AATCTCTGGGGAAAATTGTGTGTGGTGTGGACTACTTGTTTTGTTTGTCATTGAGCTATA
GSTCA39    ATCCAAAAAAAAAAAAAAAAAAA-------------------------------------
                ***

GmGST1     ------------------------------------------------------------
GSTCA30    ------------------------------------------------------------
GSTCA2     ------------------------------------------------------------
GSTCA5     TATATTGCATCTATCTAAAAAAAAAAAAAAAAAAAAAAAAAAA-----------------
GSTCA311   TCGCTGTTAATTAGGATTTTGTTTCAAAATGATGCTTATAAGTTGTAATCTAGGATTTCT
GSTCA39    ------------------------------------------------------------

GmGST1     ------------------------------------------------------------
GSTCA30    ------------------------------------------------------------
GSTCA2     ------------------------------------------------------------
GSTCA5     ------------------------------------------------------------
GSTCA311   CCCTTTGAAATCCTAGGTTGTTCTTGACATTTGCTATTTCAAAGAATAAATATATAGCAT
GSTCA39    ------------------------------------------------------------

GmGST1     ----------------------------
GSTCA30    ----------------------------
GSTCA2     ----------------------------
GSTCA5     ----------------------------
GSTCA311   CTTTCTATTTAAAAAAAAAAAAAAAAAA
GSTCA39    ----------------------------
```

Figure 5.13 Clustal W (1.7) alignment of all *GmGST1* type cDNA sequences. ATG translational start codon and TAG stop codon are shown underlined. * indicates conserved nucleotide.

Differences in 3' and 5' UTR sequences were apparent between the clones suggesting *GmGST1* is probably encoded by multiple genes in soybean. The variation in the length of the 3' UTR regions observed in cDNAs *GSTCA5* and *GSTCA311* also suggested the presence of alternative polyadenylation signal sites within the genetic sequence.

5.2.8 *GmGST2*-type sequences.

Partial cDNA sequence identical to the similar region in *GmGST2* was obtained by RT-PCR using the CJACON2 and CJACON3 primers. In addition, another *GmGST2*-type GST, *GSTCA23* (Figure 5.14) was also obtained.

GSTCA23

```
1/1                                          31/11
TTC TGG GTT GAC TTC GTT GAC AAG AAG ATA TTT GAT CTT GGA AGA AAG ATT TGG ACA TCA
F   W   V   D   F   V   D   K   K   I   F   D   L   G   R   K   I   W   T   S
61/21                                        91/31
AAG GGA GAA GAA AAA GAA GCT GCC AAA AAG GAG TTC ATA GAG GCC CTT AAA TTA TTG GAG
K   G   E   E   K   E   A   A   K   K   E   F   I   E   A   L   K   L   L   E
121/41                                       151/51
GAA CAG CTG GGA GAC AAG ACT TAT TTT GGA GGA GAC GAT CTA GGT TTT GTG GAT ATA GCA
E   Q   L   G   D   K   T   Y   F   G   G   D   D   L   G   F   V   D   I   A
181/61                                       211/71
CTT ATT CCA TTC GAC ACT TGG TTC AAG ACT TTT GGC AGC CTC AAC ATA GAG AGT GAG TGC
L   I   P   F   D   T   W   F   K   T   F   G   S   L   N   I   E   S   E   C
241/81                                       271/91
CCC AAG TTT GTT GCT TGG GCC AAG AGG TGC CTG CAG AAA GAC AGT GTT GCC AAG TCT CTT
P   K   F   V   A   W   A   K   R   C   L   Q   K   D   S   V   A   K   S   L
301/101                                      331/111
CCT GAT CAA CAC AAG GTC TAT GAG TTC ATT ATG GAC ATA AGA AAG AAG TTC GAC ATT GAG
P   D   Q   H   K   V   Y   E   F   I   M   D   I   R   K   K   F   D   I   E
361/121                                      391/131
TAG GTT CAT GTT GGA TTT TAA TAG CCA TAG TGA CGT ATT GAT CAT TCT TGG CCT TTC AAC

421/141                                      451/151
TAA ATA GTA TTT GTG TAG TAA ATT AAA GGC ACT TGG ATG TAC CAA ACT TCA TGC TTT TTG

481/161                                      511/171
TAG GAG TGC GTA GGT TTT AAA AAT TTT CTG ATG TAT CTT TCA TGT GTT TGT TGG TTT TGT

541/181                                      571/191
AAC AGA ATA TTT CCT ATA TTA TAC ATA TAT TTG AGT CAC TAG TAA AAA AAA AAA AAA AAA

601/201
AAA AA
```

Figure 5.14 DNA sequence of partial cDNA *GSTCA23*. Predicted open reading frame is shown. The identity of the initial nine amino acids may be inaccurate due to the use of the degenerate primer CJACON2 to obtain the cDNA.

5.2.9 Alignment of *GmGST2*-type cDNA sequences.

Figure 5.15 shows a Clustal W alignment of all *Gm*GST2-type enzymes known. In addition to *Gm*GST2 and GSTCA23, two further sequences are shown. GSTa is the predicted protein sequence of *GSTa*, a *Gm*GST2 type clone recently reported in the literature (McGonigle and O'Keefe, 1998a). *Gm*GST2b is the corresponding protein sequence of a cDNA obtained by Dr Mark Skipsey, isolated by RT-PCR using the GLY5 primer (described in chapter 6). Both GSTa and *Gm*GST2b are included here for comparative purposes, to show that like *Gm*GST1 large amount sequence variation is observed in the *Gm*GST2 family of soybean GSTs. Relatedness of these *Gm*GST2-like enzymes is given in Table 5.10.

```
GmGST2     MADEVVLLDFWPSPFGMRVRIALAEKGIKYESKEEDLQNKSPLLLKMNPVHKKIPVLIHN
GSTa       MSDEVVLLDFWPSPFGMRVRIALAEKGIKYEYKEEDLRNKSPLLLQMNPVHKKIPVLIHN
GmGST2b    MTDEVVLLDFWPSPFGMRVRIALAEKGIEYEYKEEDLRNKSPLLLQMNPVHKKIPVLIHN
GSTCA23    ------------------------------------------------------------


                                                            I
GmGST2     GKPICESLVAVQYIEEVWNDRNPLLPSDPYQRAQARFWADFVDNKIFDLGRKIWTSKGEE
GSTa       GKPICESLIAVQYIEEVWNDRNPLLPSDPYQRAQTRFWADYVDKKIYDLGRKIWTSKGEE
GmGST2b    GKPISESLIAVQYIEEVWNDRNPLLPSDPYQRAQARFWADYVDIKIHDLGKKIWTSKGEE
GSTCA23    ---------------------------------FWVDFVDKKIFDLGRKIWTSKGEE
                                                ** *.** ** ***.********


GmGST2     KEAAKKEFIEALKLLEEQLGDKTYFGGDDLGFVDIALIPFDTWFK---TFGSLNIESECP
GSTa       KEAAKKEFIEALKLLEEQLGDKTYFGGDNLGFVDIALVPFYTWFKAYETFGTLNIESECP
GmGST2b    KEAAKKEFIEALKLLEEQLGDKTYFGGDNIGFVDIALVPFYTWFKVYETFGSLNIENECP
GSTCA23    KEAAKKEFIEALKLLEEQLGDKTYFGGDDLGFVDIALIPFDTWFK---TFGSLNIESECP
           ************************** .*******.** ****   ***.**** ***


GmGST2     KFVAWAKRCMQKDSVARSLPDQHKVYEFIMGIRKKFDIE
GSTa       KFIAWAKRCLQKESVAKSLPDQQKVYEFIMDLRKKLGIE
GmGST2b    RFVAWAKRCLQKESVAKSLPDQHKVYEFVVEIRKKLVIE
GSTCA23    KFVAWAKRCLQKDSVAKSLPDQHKVYEFIMDIRKKFDIE
           .*.******.**.***.*****.*****.. .*** **
```

Figure 5.15 Clustal W (1.7) alignment of predicted amino acid sequences of *Gm*GST2-type cDNAs. * indicates identical amino-acids, . indicates conserved amino-acids.

cDNA	Identity (%)	Similarity (%)
GSTa	88	94
GmGST2b	87	94
GSTCA23	95	96

Table 5.10 Identity and similarity of *Gm*GST2 type cDNAs to *Gm*GST2 at the protein level.

Protein sequence alignments indicate that *Gm*GST2-type enzymes are very closely related. The most notable difference is the absence of three amino acids in enzymes

*Gm*GST2 and GSTCA23. An alignment of the cDNA sequences was performed to see whether further differences were apparent (Figure 5.16).

```
GmGST2     ---------------CTTGAATCTTCGTTATCCTTCTTTTTCTCTCCTTGAACTCGAATA
GSTa       ------------------------------------------------------------
GmGSt2b    GTTTTTTACTTGAATTTTCTTCATCCTTCTCTGTTCTCCTAGAACTTGATTACTTGAACA
GSTCA23    ------------------------------------------------------------

GmGST2     TTCACTATGGCAGATGAGGTGGTTCTGCTAGATTTCTGGCCAAGTCCATTTGGGATGAGG
GSTa       ------ATGAGTGATGAGGTAGTGTTATTAGATTTCTGGCCAAGTCCATTTGGGATGAGG
GmGST2b    TTCCCTATGACAGATGAGGTGGTTCTTCTGGATTTCTGGCCAAGTCCATTTGGGATGAGG
GSTCA23    ------------------------------------------------------------

GmGST2     GTCAGGATTGCACTTGCTGAAAAGGGTATCAAATATGAGTCCAAAGAAGAGGACTTGCAG
GSTa       GTCAGGATTGCACTTGCTGAAAAGGGTATCAAATATGAGTACAAAGAAGAGGACTTGAGG
GmGST2b    GTCAGGATTGCACTTGCTGAAAAGGGTATCGAATATGAGTACAAAGAAGAGGACTTGAGG
GSTCA23    ------------------------------------------------------------

GmGST2     AACAAGAGCCCTTTGCTCCTCAAAATGAACCCGGTTCACAAGAAAATCCCGGTTCTCATC
GSTa       AACAAGAGTCCTCTTCTCCTCCAAATGAACCCGGTTCACAAGAAGATTCCGGTTCTCATC
GmGST2b    AACAAGAGTCCTCTTCTCTTACAAATGAACCCGGTTCACAAGAAGATTCCGGTTCTCATC
GSTCA23    ------------------------------------------------------------

GmGST2     CACAATGGCAAACCCATTTGTGAATCTCTCGTTGCTGTTCAGTACATTGAGGAGGTCTGG
GSTa       CACAATGGCAAACCCATTTGTGAATCCCTCATTGCTGTTCAGTACATTGAGGAGGTTTGG
GmGST2b    CACAATGGCAAACCCATTTCCGAATCCCTCATTGCTGTTCAGTACATTGAGGAGGTTTGG
GSTCA23    ------------------------------------------------------------

GmGST2     AATGACAGAAATCCCTTGTTGCCTTCTGACCCTTACCAGAGAGCTCAGGCTAGATTCTGG
GSTa       AATGACAGAAATCCCTTGTTGCCTTCTGACCCTTACCAGAGAGCTCAGACTAGATTCTGG
GmGST2b    AATGACAGAAATCCCTTGTTGCCTTCAGACCCTTACCAGAGAGCTCAGGCTAGATTCTGG
GSTCA23    ----------------------------------------------------TTCTGG
                                                               ******
GmGST2     GCTGACTTTGTTGACAATAAGATATTTGATCTTGGAAGAAAGATTTGGACATCAAAGGGA
GSTa       GCTGATTATGTTGATAAGAAGATATATGATCTTGGAAGGAAGATTTGGACATCAAAAGGA
GmGST2b    GCTGATTATGTTGACATTAAGATACATGATCTTGGAAAGAAGATTTGGACATCAAAGGGA
GSTCA23    GTTGACTTCGTTGACAAGAAGATATTTGATCTTGGAAGAAAGATTTGGACATCAAAGGGA
           *  *** *    ***** *   ****** ********** ***************** ***
GmGST2     GAAGAAAAGAAGCTGCCAAAAAGGAGTTCATAGAGGCCCTTAAATTATTGGAGGAACAG
GSTa       GAAGAAAAGAAGCTGCCAAGAAGGAGTTCATAGAAGCCCTTAAATTGTTGGAGGAACAG
GmGST2b    GAAGAAAAGAAGCTGCCAAGAAGGAGTTCATAGAGGCCCTTAAATTGTTGGAGGAACAG
GSTCA23    GAAGAAAAGAAGCTGCCAAAAAGGAGTTCATAGAGGCCCTTAAATTATTGGAGGAACAG
           ******************* ************** ********** ***********
GmGST2     CTGGGAGACAAGACTTATTTTGGAGGAGACGATCTAGGTTTTGTGGATATAGCACTTATT
GSTa       CTGGGAGACAAGACTTATTTTGGAGGAGACAATCTAGGTTTTGTGGATATAGCGCTTGTT
GmGST2b    CTGGGAGATAAGACTTATTTTGGAGGAGACAATATTGGTTTTGTGGATATAGCACTTGTT
GSTCA23    CTGGGAGACAAGACTTATTTTGGAGGAGACGATCTAGGTTTTGTGGATATAGCACTTATT
           ******* ******************** ** * ***************** *** **
GmGST2     CCATTCGACACTTGGTTCAA---------GACTTTTGGCAGCCTCAACATAGAGAGTGAG
GSTa       CCATTCTACACTTGGTTCAAAGCCTATGAGACTTTTGGCACCCTCAACATAGAGAGTGAG
GmGST2b    CCATTCTACACTTGGTTCAAAGTCTATGAGACTTTTGGCAGCCTCAACATTGAGAATGAG
GSTCA23    CCATTCGACACTTGGTTCAA---------GACTTTTGGCAGCCTCAACATAGAGAGTGAG
           ****** ************          ********** ******* **** ****
GmGST2     TGCCCCAAGTTTGTTGCTTGGGCCAAGAGGTGCATGCAGAAAGACAGTGTTGCCAGGTCT
GSTa       TGCCCCAAGTTTATTGCTTGGGCCAAGAGGTGCCTTCAGAAAGAAAGCGTTGCCAAGTCT
GmGST2b    TGCCCCAGGTTTGTTGCTTGGGCCAAGAGGTGCCTACAGAAAGAGAGTGTTGCAAAGTCT
GSTCA23    TGCCCCAAGTTTGTTGCTTGGGCCAAGAGGTGCCTGCAGAAAGACAGTGTTGCCAAGTCT
           ******* **** ********************* *  ******** ** ***** * ****
GmGST2     CTTCCTGATCAACACAAGGTCTATGAGTTCATTATGGGCATAAGAAAGAAGTTCGACATT
GSTa       CTTCCTGATCAGCAAAAGGTTTATGAGTTCATTATGGATCTAAGAAAGAAGTTAGGCATT
GmGSt2b    CTTCCTGATCAGCACAAGGTCTATGAGTTCGTTGTGGAGATAAGAAAGAAGTTAGTCATC
GSTCA23    CTTCCTGATCAACACAAGGTCTATGAGTTCATTATGGACATAAGAAAGAAGTTCGACATT
           ********** ** ***** ******** ** ***    ************ * ***
```

```
GmGST2     GAGTAGGTT----CATGTTGGATCTTA-ATAGCCACAGTGACGTATTGATCATTCTTGGC
GSTa       GAGTAGGTTGGAGCTTAATGGCCATTGTGAAGTAGTGGTTTTCCATTGGTCGTTCTTAGC
GmGST2b    GAGTAGGTTT---CATGTTGGATCTTA-ATAGCCATAGTGAAGTATTGGTCGTTCTTGAC
GSTCA23    GAGTAGGTT----CATGTTGGATTTTA-ATAGCCATAGTGACGTATTGATCATTCTTGGC
           *********    *  *  ***   **     **      **   **** ** *****  *

GmGST2     CTTTCAACTAAATAGTATTTGTGTAGAAATT-AAAGGCACTTGGATGTACCAAACTTCAT
GSTa       CTTTCAAATAAGTAATATTTGTGTAATAAA----AGGCACTTAGATGTGCCAAACTTCGT
GmGST2b    CTTTCAACTAAATAATATTTGTGTAATAAA---AAGGCATTTGGATGTGCCAAACTTCAT
GSTCA23    CTTTCAACTAAATAGTATTTGTGTAGTAAATTAAAGGCACTTGGATGTACCAAACTTCAT
           ******* *** ** ********** **     ***** ** ***** ********* *

GmGST2     GCTTTTTGTAGGAGTGCGTAGGTTTTAAAAATTTTCTGATGTATCTTTCATGTGTTTGTT
GSTa       GCTTTCTGTAGGAATGTGTGGGTTTTGGAAAATCTCTGATGTATCTTTCATGTGTTTGTT
GmGST2b    GCTTTCTGTTGGATTGTGTGTAGGTTTTAAAATTTTTCTGATGTATCTTTCATGTGTTTGTT
GSTCA23    GCTTTTTGTAGGAGTGCGTAGGTTTTAAAAATTTTCTGATGTATCTTTCATGTGTTTGTT
           *****  *** *** ** ** ******   **   * ************************

GmGST2     GGTTTTGCAATAGAATATTTCCTATATTAT----------------ACAAAAAAAAAA
GSTa       GGTTTTGTAATTTTTTTTTGGTATTGTCTTATACTTGAATAATTTGAGACTAAAAAAAAA
GmGST2b    GGTTTTGCAATAGAGTATTTTCCGTATTAT-------------CATAAAAAAAAAAAAA
GSTCA23    GGTTTTGTAACAGAATATTTCCTATATTATACATATATTTGAGTCACTAGTAAAAAAAA
           *******  **     *  **   *  *  *              *   *********

GmGST2     AAAAAAAA-----
GSTa       AAAAAAAAAAAAA
GmGST2b    AAAAAAAAAAAA-
GSTCA23    AAAAAAAAAAAAA
           *******
```

Figure 5.16 Clustal W (1.7) DNA sequence alignment of *GmGST2*-type cDNAs. ATG start and TAG stop codons are indicated. * indicates conserved nucleotides.

When compared with *GSTa* and *GmGST2b* the absence of the nine nucleotides in sequences *GSTCA23* and *GmGST2* could be due to a mRNA splicing error. Tau-class GSTs are known to contain one intron (Droog, 1997), but since genomic DNA clones were not obtained for any of the soybean GSTs, the intron position was not determined. However, the intron present within the genomic clone of *Gm*GST1 (Czarnecka *et al.*, 1988) corresponds to a region between the lysine and isoleucine residues marked by 'I' in Figure 5.15. This is well away from the deleted amino acids and, since the position of introns is generally conserved within gene families, it is unlikely that the differences are due to mRNA splicing errors.

5.2.10 *GmGST3*-type sequences.

A number of cDNAs were obtained using CJACON2 and CJACON3 that showed similarity to *Gm*GST3 (formerly glyoxalase 1). All the sequences obtained were identical, except for changes at the extreme 5' end which were due to primer differences associated with the use of degenerate primers. The representative sequence GSTCA23a (Figure 5.17) is shown aligned to the amino-acid sequence of *Gm*GST3 (Figure 5.18).

GSTCA23a

```
1/1                                          31/11
TGG GCG GAG TTC GTC GAC GAG AAG ATT TAT GAT ACT TGG AAG AAA ATG TGG CTT TCT AAA
W   A   E   F   V   D   E   K   I   Y   D   T   W   K   K   M   W   L   S   K
61/21                                        91/31
GGA GAA GAG CAT GAA GAA GGG AAG AAG GAG TTG ATC TCT ATC TTT AAG CAA TTA GAA GAG
G   E   E   H   E   E   G   K   K   E   L   I   S   I   F   K   Q   L   E   E
121/41                                       151/51
ACA CTA ACT GAC AAA CCC TTT TAT GGG GAT GAC ACG TTT GGC TTT GTT GAT CTT TGT TTG
T   L   T   D   K   P   F   Y   G   D   D   T   F   G   F   V   D   L   C   L
181/61                                       211/71
ATC ACT TTC TCT AGT TGG TTT TAT ACT TAT GAG ACA TAT GGG AAC TTC AAA ATG GAA GAA
I   T   F   S   S   W   F   Y   T   Y   E   T   Y   G   N   F   K   M   E   E
241/81                                       271/91
GAG TGT CCT AAA CTC ATG GCT TGG GTC AAG AGA TGC ATG GAG AGA GAG ACT GTG TCC AAT
E   C   P   K   L   M   A   W   V   K   R   C   M   E   R   E   T   V   S   N
301/101                                      331/111
ACT CTT CCT GAT GCT AAG AAG GTG TAT GGT CTT ATT GTG GAA CTG CAG AAG ACA CTT GAA
T   L   P   D   A   K   K   V   Y   G   L   I   V   E   L   Q   K   T   L   E
361/121                                      391/131
TCG AAA TAG AAG ATT TCA ATA AAT CAA CCC ATT AAA TAA TAT TTT CAT GTT AAA TAT GTT
S   K
421/141                                      451/151
GTT GTA AGG TCT TGT GTA CTT TTC CTC TAT GGT TGT GTG GGT TGG GTC AGT CAT TTA TGT

481/161                                      511/171
GGT TTA CTA GAC ACT AAT ACA TCT TCT CTA TTG AAG ATT AAG GTA TCT AAT TAT TTT TCC

541/181                                      571/191
CAT TAC TAT ATA TTG AAT ATT ATC GTC TTT GAC ATT GAA AAG AAA GAA AAC GTA GAA AAA

601/201
GCA AAA AAA AAA AAA AAA A
```

Figure 5.17 DNA sequence of GSTCA23a with deduced ORF indicated.

*Gm*GST3	MSDEVVLLDTWASMYGMRARIALAEKGVRYEYKEENLMNRSPLLLQMNPIHKKIPVLIHN
GSTCA23A	--

*Gm*GST3	GKPICESAIIVQYIDEVWNDKSPLMPSDPYKRSQARFWVDYIDKKIYDTWKKMWLSKGEE
GSTCA23A	-----------------------------------WAEFVDEKIYDTWKKMWLSKGEE
	* ...* **************

*Gm*GST3	HEEGKKELISIFKQLEETLTDKPFYGDDTFGFVDLCLITFSSWFYTYETYGNFKMEEECP
GSTCA23A	HEEGKKELISIFKQLEETLTDKPFYGDDTFGFVDLCLITFSSWFYTYETYGNFKMEEECP
	**

*Gm*GST3	KLMAWVKRCMERETVSNTLPDAKKVYGLIVELQKTLESK
GSTCA23A	KLMAWVKRCMERETVSNTLPDAKKVYGLIVELQKTLESK
	***************************************

Figure 5.18 Clustal W (1.7) protein sequence alignment of GSTCA23a and *Gm*GST3. N-terminal changes observed in GSTCA23a are due to errors caused by the use of degenerate primer CJACON2. * indicates identical amino-acids, . indicates conserved amino-acids.

None of the variation in sequence identity observed with *Gm*GST1 and *Gm*GST2 sequences was apparent within the *Gm*GST3-type sequences described. A DNA alignment of *GSTCA23a* and *GmGST3* is not shown, but such alignment shows that the two clones differ by only four base pairs over the 375 base pair 3' UTR, and as such are probably PCR-introduced errors. This finding may suggest *GmGST3* is present as a single copy gene within the soybean genome. However, further Southern-blot analysis would be required to confirm this. Additionally, *Gm*GST3 would appear to be conserved between soybean cultivars, since the *GmGST3* and *GSTCA23a* cDNAs were obtained from cultivars Orsay and Mandarin respectively.

5.2.11 Novel tau-type soybean GST cDNA sequences.

Four completely novel soybean GST cDNA sequences were obtained using the CJACON2 and CJACON3 primers, termed G*STCON26a* (Figure 5.19), *GSTCON31a* (Figure 5.20), *GSTCON32* (Figure 5.21) and *GSTCON33a* (Figure 5.22) The partial length cDNAs cloned were DIG-labelled and used to screen the soybean cell culture library for full length cDNAs. 160,000 pfu's were screened with each probe, resulting in the following number of positives shown in brackets: *GSTCON26a* (8), *GSTCON31a* (33), *GSTCON32* (7) and *GSTCON33a* (16). cDNAs containing full length open reading frames were obtained for *GSTCON26a* and *GSTCON33a*. However, of all the library clones examined only 5' truncated cDNAs were obtained for *GSTCON31a* and *GSTCON32*, despite extensive analysis and re-screening. The alignment shown in Figure 5.23 suggested GSTCON31a and GSTCON32 were

truncated by approximately 33 and 8 amino acids respectively. Existing GSTs showing greatest homology to these novel sequences are given in Tables 5.11-5.14. Relatedness to known soybean GSTs is shown in given in Table 5.15.

GSTCON26a

```
1/1                                           31/11
GCA ATG TCT TCA AGT CAG GAA GAG GTG ACC CTT TTG GGA GTT GTG GGA AGC CCA TTT CTA
    M   S   S   S   Q   E   E   V   T   L   L   G   V   V   G   S   P   F   L
61/21                                         91/31
CAC AGG GTT CAG ATT GCT CTC AAG TTG AAG GGA GTT GAA TAC AAA TAT TTG GAA GAC GAT
H   R   V   Q   I   A   L   K   L   K   G   V   E   Y   K   Y   L   E   D   D
121/41                                        151/51
TTG AAC AAC AAG AGT GAT TTG CTC CTC AAG TAT AAC CCA GTT TAC AAA ATG ATT CCA GTG
L   N   N   K   S   D   L   L   L   K   Y   N   P   V   Y   K   M   I   P   V
181/61                                        211/71
CTT GTT CAC AAT GAG AAG CCC ATT TCA GAG TCC CTT GTG ATT GTT GAG TAC ATT GAT GAC
L   V   H   N   E   K   P   I   S   E   S   L   V   I   V   E   Y   I   D   D
241/81                                        271/91
ACA TGG AAA AAC AAT CCC ATC TTG CCT TCT GAT CCC TAC CAA AGA GCC TTG GCT CGT TTC
T   W   K   N   N   P   I   L   P   S   D   P   Y   Q   R   A   L   A   R   F
301/101                                       331/111
TGG GCT AAG TTC ATT GAT GAC AAG TGT GTG GTT CCA GCA TGG AAA TCT GCT TTT ATG ACT
W   A   K   F   I   D   D   K   C   V   V   P   A   W   K   S   A   F   M   T
361/121                                       391/131
GAT GAG AAA GAG AAA GAG AAG GCT AAA GAA GAG TTA TTT GAG GCT CTG AGT TTT CTT GAG
D   E   K   E   K   E   K   A   K   E   E   L   F   E   A   L   S   F   L   E
421/141                                       451/151
AAT GAG TTG AAG GGC AAG TTT TTT GGT GGA GAG GAG TTT GGC TTT GTG GAT ATT GCT GCT
N   E   L   K   G   K   F   F   G   G   E   E   F   G   F   V   D   I   A   A
481/161                                       511/171
GTG TTA ATA CCT ATA ATT CAA GAG ATA GCA GGG TTG CAA TTG TTC ACA AGT GAG AAA TTC
V   L   I   P   I   I   Q   E   I   A   G   L   Q   L   F   T   S   E   K   F
541/181                                       571/191
CCA AAG CTC TCT AAA TGG AGC CAA GAC TTT CAC AAC CAT CCA GTT GTC AAC GAA GTT ATG
P   K   L   S   K   W   S   Q   D   F   H   N   H   P   V   V   N   E   V   M
601/201                                       631/211
CCT CCT AAG GAT CAA CTT TTT GCC TAT TTC AAG GCT CGG GCT CAA AGC TTC GTT GCT AAA
P   P   K   D   Q   L   F   A   Y   F   K   A   R   A   Q   S   F   V   A   K
661/221                                       691/231
AGA AAG AAT TAA TAT AGT GAG ACT CAG AAT TTC CAT CGA GGT TTC AGT ATT GTA TGA AAT
R   K   N
721/241                                       751/251
GAA AGC TAC TTG TCT ATG TTT CGT TAT TGC GGT TGT ATT TTC ATT TTT CAA TGA ATT ATG

781/261                                       811/271
TGA TAT AGG ATT TCT CCA TGT CAA AAG ATA GTT CAA TTC AAT CAA TAA AAT AAA CGA ATG

841/281                                       871/291
AGT CGT GTT AGA GCA AAA AAA AAA AAA AAA AAA AAA AAA AAA A
```

Figure 5.19 DNA sequence of *GSTCON26a* showing ORF.

No.	Species	cDNA Identity	Accession	Similarity (%)	Identity (%)
1	Soybean	*Gm*GST1	P32110	84	74
2	Mung Bean	VR MII-4	U20809	79	64
3	Tobacco	Nt103	Q03663	71	54
4	Potato	PRP-1	P32111	66	49
5	Arabidopsis	PM24	P46421	67	47

Table 5.11 GSTCON26a protein identity and similarity to other plant GSTs.

GSTCON31a

```
1/1                                 31/11
GTT GAA GAA GAC TTG AGA AAT AAG AGT GAT TTG CTT CTA AAG TAC AAC CCT GTT CAC AAG
 V   E   E   D   L   R   N   K   S   D   L   L   L   K   Y   N   P   V   H   K
61/21                               91/31
AAG GTT CCT GTA CTT GTT CAT AAT GGA AAG GCC ATT GCT GAA TCC ATG GTG ATC CTT GAG
 K   V   P   V   L   V   H   N   G   K   A   I   A   E   S   M   V   I   L   E
121/41                              151/51
TAT ATT GAT GAA ACA TGG AAA GAT GGT CCT AAA CTG CTT CCA AGT GAT TCT TAC AAA CGA
 Y   I   D   E   T   W   K   D   G   P   K   L   L   P   S   D   S   Y   K   R
181/61                              211/71
GCC CAA GCT CGA TTC TGG TGT CAT TTC ATC CAG GAT CAG TTA ATG GAG AGC ACT TTT CTA
 A   Q   A   R   F   W   C   H   F   I   Q   D   Q   L   M   E   S   T   F   L
241/81                              271/91
GTA GTC AAA ACT GAT GGA GAA GCA CAA CAA AAG GCC ATT GAC CAC GTG TAT GAG AAA CTG
 V   V   K   T   D   G   E   A   Q   Q   K   A   I   D   H   V   Y   E   K   L
301/101                             331/111
AAA GTG CTA GAA GAT GGA ATG AAG ACC TAT CTG GGA GAA GGC AAT GCT ATT ATC TCT GGT
 K   V   L   E   D   G   M   K   T   Y   L   G   E   G   N   A   I   I   S   G
361/121                             391/131
GTT GAA AAC AAC TTT GGA ATC CTT GAC ATT GTG TTT TGT GCT TTA TAT GGT GCC TAC AAG
 V   E   N   N   F   G   I   L   D   I   V   F   C   A   L   Y   G   A   Y   K
421/141                             451/151
GCT CAT GAA GAA GTT ATT GGC CTC AAG TTC ATA GTG CCA GAA AAG TTT CCT GTG TTG TTT
 A   H   E   E   V   I   G   L   K   F   I   V   P   E   K   F   P   V   L   F
481/161                             511/171
TCT TGG TTG ATG GCT ATT GCT GAG GTT GAA GCT GTG AAA ATT GCA ACT CCT CCA CAT GAA
 S   W   L   M   A   I   A   E   V   E   A   V   K   I   A   T   P   P   H   E
541/181                             571/191
AAA ACA GTG GGA ATT CTT CAG TTG TTC AGG CTG TCT GCA CTG AAA TCT TCT TCT GCC ACA
 K   T   V   G   I   L   Q   L   F   R   L   S   A   L   K   S   S   S   A   T
601/201                             631/211
GAA TGA TAT ATA CTT CAA CAC TTT AAT AGA CTG TCC ATC GTT TGC TTC TTC TGC GAG TCT
 E
661/221                             691/231
TTA ATG TAT GTA TCT TTC AAT AAC AGG ATG AGT AAC ACC TGA GTA TGT AAA GCG TGA TGA

721/241                             751/251
TAT AGA GAT ATA CCT CTA TAT ATC AAA TAC TCT TCT ATA AAC ACT TCT TTC TTT CCT TAA

781/261
AAA AAA AAA AAA AAA AA
```

Figure 5.20 DNA sequence of partial cDNA *GSTCON31a* showing ORF.

No.	Species	cDNA Identity	Accession	Similarity (%)	Identity (%)
1	Soybean	*Gm*GST1	P32110	60	44
2	Papaya	GST	AJ000923	50	42
3	Spruce	GST	AF051214	56	40
4	Mung Bean	VR MII-4	U20809	59	43
5	Tobacco	Nt110	Q03662	54	40

Table 5.12 GSTCON31 identity and protein similarity to other plant GST sequences.

GSTCON32

```
1/1                                        31/11
CTT CAT GGA TTT TGG TAT AGT CCC TAC ACT    TTG AGG GTG GTA TGG ACC TTA AAG TTA AAG
 L   H   G   F   W   Y   S   P   Y   T      L   R   V   V   W   T   L   K   L   K
61/21                                      91/31
GAT ATA CCA TAT CAA AAC ATA GAA GAA GAC    CGC TAC AAT AAG AGT CTT CAA CTT CTT GAA
 D   I   P   Y   Q   N   I   E   E   D      R   Y   N   K   S   L   Q   L   L   E
121/41                                     151/51
TAC AAC CCA GTA TAC AAG AAA ACT CCA GTG    CTT GTC CAT AAT GGA AAA CCC TTA TGT GAG
 Y   N   P   V   Y   K   K   T   P   V      L   V   H   N   G   K   P   L   C   E
181/61                                     211/71
TCC ATG CTT ATT GTT GAA TAC ATT GAT GAG    ATT TGG TCA CAT AAT TCA TTA CTT CCT GCT
 S   M   L   I   V   E   Y   I   D   E      I   W   S   H   N   S   L   L   P   A
241/81                                     271/91
GAT CCC TAC GAG AGA GCT CTG GCA AGG TTT    TGG GTT AAA TAT GCT GAT GAT GAC ATG TTT
 D   P   Y   E   R   A   L   A   R   F      W   V   K   Y   A   D   D   D   M   F
301/101                                    331/111
TCT GCA GTT ATT GCA TTC TTC CTT AGC AAT    AAT GAT GAA GAG CGA GAA AAG AGC ATA GAG
 S   A   V   I   A   F   F   L   S   N      N   D   E   E   R   E   K   S   I   E
361/121                                    391/131
AAG ATA TGG GAG CAT CTC AGG GTT GTT GAG    AAT CAG TGT TTT GGT GAT CAG AAG AAA TTT
 K   I   W   E   H   L   R   V   V   E      N   Q   C   F   G   D   Q   K   K   F
421/141                                    451/151
TTT GGG GGA GAC ATT ATT AAC ATT ATG GAC    ATA GCT TTT GGG TCC ATA TTC AAA ATT CTT
 F   G   G   D   I   I   N   I   M   D      I   A   F   G   S   I   F   K   I   L
481/161                                    511/171
GTG GTT GCA GAA GAT ATT CTT GAC GCG AAG    GTC CTG GAA GAT GAG AAA TTC CCT CAC TTG
 V   V   A   E   D   I   L   D   A   K      V   L   E   D   E   K   F   P   H   L
541/181                                    571/191
CAT TCA TGG TAT AAT AAT TTC AAG GAT GTT    GCA GTT ATT AAA GAA AAC CTC CCA GAC CAT
 H   S   W   Y   N   N   F   K   D   V      A   V   I   K   E   N   L   P   D   H
601/201                                    631/211
GAG AAA ATG GTG GCT TTT GCT AAG TTT ATT    AGA GAA AAA CGT TTG GCA TGT ACC TAA GAA
 E   K   M   V   A   F   A   K   F   I      R   E   K   R   L   A   C   T
661/221                                    691/231
AGT AAT CTT ATA TGA GAT CAA GTA TGA ATC    ACT TTG TAT CTG TCT GAA TCG TTT TGT TAT

721/241                                    751/251
GCG TGT TTC TTT AGT TTC CAC TCC ATT ATT    AGG ATG TCT TGA CAT ATC TGT GAA AGC AAT

781/261                                    811/271
AAA AGT TTA ATG GGA TGT ACT GGA TTA AAA    AAA AAA AAA AAA AAA
```

Figure 5.21 DNA sequence of partial cDNA *GSTCON32* showing ORF.

No.	Species	cDNA Identity	Accession	Similarity (%)	Identity (%)
1	Tobacco	nt111	Q03663	66	47
2	Mung Bean	VRII-4	U20809	66	44
3	Soybean	*Gm*GST1	P32110	63	43
4	Wheat	GST TSI-1	Af004358	60	41
5	Spruce	GST	AF051214	59	41

Table 5.13 GSTCON32 protein identity and similarity to other plant GSTs.

GSTCON33a

```
1/1                             31/11
CAT AAA ACT CCA CAT TTC CTG CTG AGT AAC CTA ACA AAA CAA ACA CAA TAT TGC TCC GTG

61/21                           91/31
TTT GAC CTG TTA TAG TAA ACA GTG ATG GCT GAA AGG GAC TTG AGG CTT TTG GGT GCT TGG
                            M   A   E   R   D   L   R   L   L   G   A   W
121/41                          151/51
TTC AGT CCA TTT GCC CTG AGG GTG CAG ATT GCC CTT AAC CTC AAG GGT TTG GAT TAT GAG
F   S   P   F   A   L   R   V   Q   I   A   L   N   L   K   G   L   D   Y   E
181/61                          211/71
GTT GTT GAA GAG ACT TTG AAT CCC AAA AGT GAA TTG CTT CTT AAG TCC AAC CCT GTG CAC
V   V   E   E   T   L   N   P   K   S   E   L   L   L   K   S   N   P   V   H
241/81                          271/91
AAG AAA ATC CCA GTT TTC TTC CAT GGA GAT AAA GTC ATA TGT GAA TCT GCA ATC ATA GTT
K   K   I   P   V   F   F   H   G   D   K   V   I   C   E   S   A   I   I   V
301/101                         331/111
GAG TAC ATA GAT GAG GTT TGG TCC AAC AAT GCT CTC TCC ATC CTT CCA CAA AAT GCA TAT
E   Y   I   D   E   V   W   S   N   N   A   L   S   I   L   P   Q   N   A   Y
361/121                         391/131
GAT CGA GCT AAT GCC CGA TTT TGG GTT TCT TAC ATC GAT GAC AAG TGG CTT ACG TCC TTG
D   R   A   N   A   R   F   W   V   S   Y   I   D   D   K   W   L   T   S   L
421/141                         451/151
AAA AGT GTT CTA GCG ACT GAA GAT GAT GAG GCA AAG AAG CTA CAC TTT GAG CAA GCG GAA
K   S   V   L   A   T   E   D   D   E   A   K   K   L   H   F   E   Q   A   E
481/161                         511/171
GAA GTG CTT GAG AAG GTG GAA GAA GTG TTC AAC AAG TGC AGT GAA GGG AAG GCC TAT TTC
E   V   L   E   K   V   E   E   V   F   N   K   C   S   E   G   K   A   Y   F
541/181                         571/191
GGA GGA GAT ACG ATT GGA TTT GTT GAC ATT GGT TTT GGA AGC TTT TTG AGT TTC ATT AGA
G   G   D   T   I   G   F   V   D   I   G   F   G   S   F   L   S   F   I   R
601/201                         631/211
GTC TCA GAG AAT ATG AAT GAA AGA AAA TTG CTT GAT GAA ACG AAG TAC CCT GGT TTG ACC
V   S   E   N   M   N   E   R   K   L   L   D   E   T   K   Y   P   G   L   T
661/221                         691/231
CTA TGG GCT GAA ACT TTT GCT GCT GAT CCT GCT GTG AAG GGC CTT CTG CCA GAG ACT GAA
L   W   A   E   T   F   A   A   D   P   A   V   K   G   L   L   P   E   T   E
721/241                         751/251
AAG CTT GTT GAG TTT GCA AAG ATT CTT CAG CTA AAA TGG GCT GCT GCA GCT GCT GCA AAG
K   L   V   E   F   A   K   I   L   Q   L   K   W   A   A   A   A   A   A   K
781/261                         811/271
TAA ATG GAA TCA AAT TAA TTG CTG GAT GAA TTT CAA AAA TTG TTG TGC AAG TTA TTT ATA

841/281                         871/291
TCT GAG GCT ATG TTT GTT GCA ACT TTA TAT ATT TAA AAG TCA AAA TAA ATG TTA TGA TAA

901/301
TAT AGT AAA AAA AAA AAA
```

Figure 5.22 DNA sequence of cDNA *GSTCON33a* showing ORF.

No.	Species	cDNA Identity	Accession	Similarity (%)	Identity (%)
1	Wheat	GST TSI-1	AF004358	64	48
2	Arabidopsis	BAC Clone	AC000348	64	47
3	Spruce	GST1	AF051214	60	43
4	Spruce	GST2	AF051238	61	41
5	Soybean	*Gm*GST1	P32110	59	41

Table 5.14 GSTCON33a protein identity and similarity to other plant GSTs.

Unlike *Gm*GST1 and *Gm*GST2, and similar to *Gm*GST3 no variability was found within each of these groups of cDNAs obtained for each GST.

cDNA	GmGST1		GmGST2		GmGST3	
	Identity %	Similarity %	Identity %	Similarity %	Identity %	Similarity %
GSTCON26a	74	84	44	61	39	57
GSTCON31a	44	60	53	69	35	54
GSTCON32	43	63	39	57	35	53
GSTCON33a	41	59	44	61	38	55

Table 5.15 Protein identity and similarity of novel soybean GSTs compared with *Gm*GST1, *Gm*GST2 and *Gm*GST3.

5.2.12 Alignment of all known soybean GST sequences.

In order to assess the overall similarities of the soybean GSTs obtained in these experiments they were aligned using Clustal W with the known soybean GST, *Gm*GST1 (Figure 5.23). For reasons of clarity the multiple minor variations of *Gm*GST1 and *Gm*GST2 are not shown on this alignment.
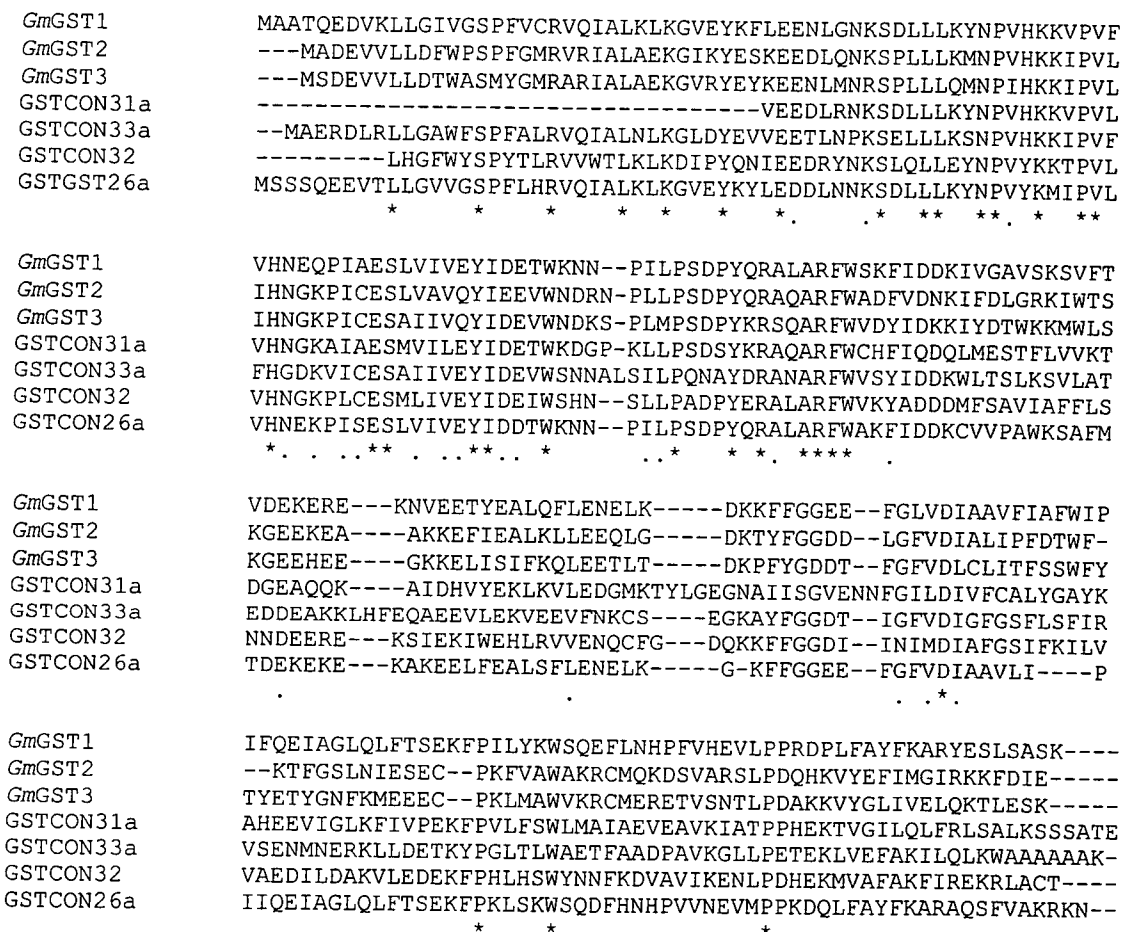
```
GmGST1     MAATQEDVKLLGIVGSPFVCRVQIALKLKGVEYKFLEENLGNKSDLLLKYNPVHKKVPVF
GmGST2     ---MADEVVLLDFWPSPFGMRVRIALAEKGIKYESKEEDLQNKSPLLLKMNPVHKKIPVL
GmGST3     ---MSDEVVLLDTWASMYGMRARIALAEKGVRYEYKEENLMNRSPLLLQMNPIHKKIPVL
GSTCON31a  -----------------------------VEEDLRNKSDLLLKYNPVHKKVPVL
GSTCON33a  --MAERDLRLLGAWFSPFALRVQIALNLKGLDYEVVEETLNPKSELLLKSNPVHKKIPVF
GSTCON32   ---------LHGFWYSPYTLRVVWTLKLKDIPYQNIEEDRYNKSLQLLEYNPVYKKTPVL
GSTGST26a  MSSSQEEVTLLGVVGSPFLHRVQIALKLKGVEYKYLEDDLNNKSDLLLKYNPVYKMIPVL
              *     *     *     *  *   *    *.    .*  **  **. *  **

GmGST1     VHNEQPIAESLVIVEYIDETWKNN--PILPSDPYQRALARFWSKFIDDKIVGAVSKSVFT
GmGST2     IHNGKPICESLVAVQYIEEVWNDRN-PLLPSDPYQRAQARFWADFVDNKIFDLGRKIWTS
GmGST3     IHNGKPICESAIIVQYIDEVWNDKS-PLMPSDPYKRSQARFWVDYIDKKIYDTWKKMWLS
GSTCON31a  VHNGKAIAESMVILEYIDETWKDGP-KLLPSDSYKRAQARFWCHFIQDQLMESTFLVVKT
GSTCON33a  FHGDKVICESAIIVEYIDEVWSNNALSILPQNAYDRANARFWVSYIDDKWLTSLKSVLAT
GSTCON32   VHNGKPLCESMLIVEYIDEIWSHN--SLLPADPYERALARFWVKYADDDMFSAVIAFFLS
GSTCON26a  VHNEKPISESLVIVEYIDDTWKNN--PILPSDPYQRALARFWAKFIDDKCVVPAWKSAFM
             *.  . ..**  . ...**.. *      ..*   * *. ****  .

GmGST1     VDEKERE---KNVEETYEALQFLENELK-----DKKFFGGEE--FGLVDIAAVFIAFWIP
GmGST2     KGEEKEA----AKKEFIEALKLLEEQLG-----DKTYFGGDD--LGFVDIALIPFDTWF-
GmGST3     KGEEHEE----GKKELISIFKQLEETLT-----DKPFYGDDT--FGFVDLCLITFSSWFY
GSTCON31a  DGEAQQK----AIDHVYEKLKVLEDGMKTYLGEGNAIISGVENNFGILDIVFCALYGAYK
GSTCON33a  EDDEAKKLHFEQAEEVLEKVEEVFNKCS----EGKAYFGGDT--IGFVDIGFGSFLSFIR
GSTCON32   NNDEERE---KSIEKIWEHLRVVENQCFG---DQKKFFGGDI--INIMDIAFGSIFKILV
GSTCON26a  TDEKEKE---KAKEELFEALSFLENELK-----G-KFFGGEE--FGFVDIAAVLI----P
               .               .              .  .*.

GmGST1     IFQEIAGLQLFTSEKFPILYKWSQEFLNHPFVHEVLPPRDPLFAYFKARYESLSASK----
GmGST2     --KTFGSLNIESEC--PKFVAWAKRCMQKDSVARSLPDQHKVYEFIMGIRKKFDIE-----
GmGST3     TYETYGNFKMEEEC--PKLMAWVKRCMERETVSNTLPDAKKVYGLIVELQKTLESK-----
GSTCON31a  AHEEVIGLKFIVPEKFPVLFSWLMAIAEVEAVKIATPPHEKTVGILQLFRLSALKSSSATE
GSTCON33a  VSENMNERKLLDETKYPGLTLWAETFAADPAVKGLLPETEKLVEFAKILQLKWAAAAAAK-
GSTCON32   VAEDILDAKVLEDEKFPHLHSWYNNFKDVAVIKENLPDHEKMVAFAKFIREKRLACT----
GSTCON26a  IIQEIAGLQLFTSEKFPKLSKWSQDFHNHPVVNEVMPPKDQLFAYFKARAQSFVAKRKN--
                *      *        .      *
```

Figure 5.23 Clustal W multiple alignment of all known soybean GST protein sequences (*Gm*GST1 and *Gm*GST2 variants not shown). * indicates identical amino-acids, . indicates conserved amino-acids.

The overall homology of the soybean GSTs identified is low, despite the fact that they all belong to the same GST tau-class. However, of the amino acids that are conserved

between the enzymes there are some interesting observations. Of particular note is the conserved serine-16 (numbering relative to *Gm*GST1). This serine is thought to function in a similar manner to a conserved tyrosine in animal GSTs, in that it stabilises the thiolate anion of GSH. Mutation of this serine to alanine results in loss of catalytic activity of the GST (Board *et al.*, 1995). In addition, conserved residues arginine-21, glutamic acid-69 and serine-70 are all thought to constitute part of the conserved GSH binding site (Neuefeind *et al.*, 1997b). Indeed, it is thought that during substrate binding the GSH binds first, with the GSH-γ-glutamate attracted to the negative charge on glutamic acid-69 (Neuefeind *et al.*, 1997b). The conserved proline-58 is apparent in all known GST crystal structures and is responsible for backbone hydrogen bonding to the GSH substrate (Neuefeind *et al.*, 1997a). Of the remaining conserved amino acids some are likely to be involved in hydrophobic interactions, whilst others function in the structural formation of the GST.

Figure 5.24 shows a Clustal W protein alignment of all known plant GSTs. All soybean GST cDNA sequences detailed in Figure 5.23 have been included in the line-up for comparative purposes. Again, the conserved residues discussed above are noted.

Figure 5.24 Clustal W (1.5) alignment of all known plant GST sequences. Class is indicated and regions used to design degenerate primers shown. Totally conserved residues are indicated in black. New nomenclature has been used for maize GSTs (Dixon *et al*, 1998a)
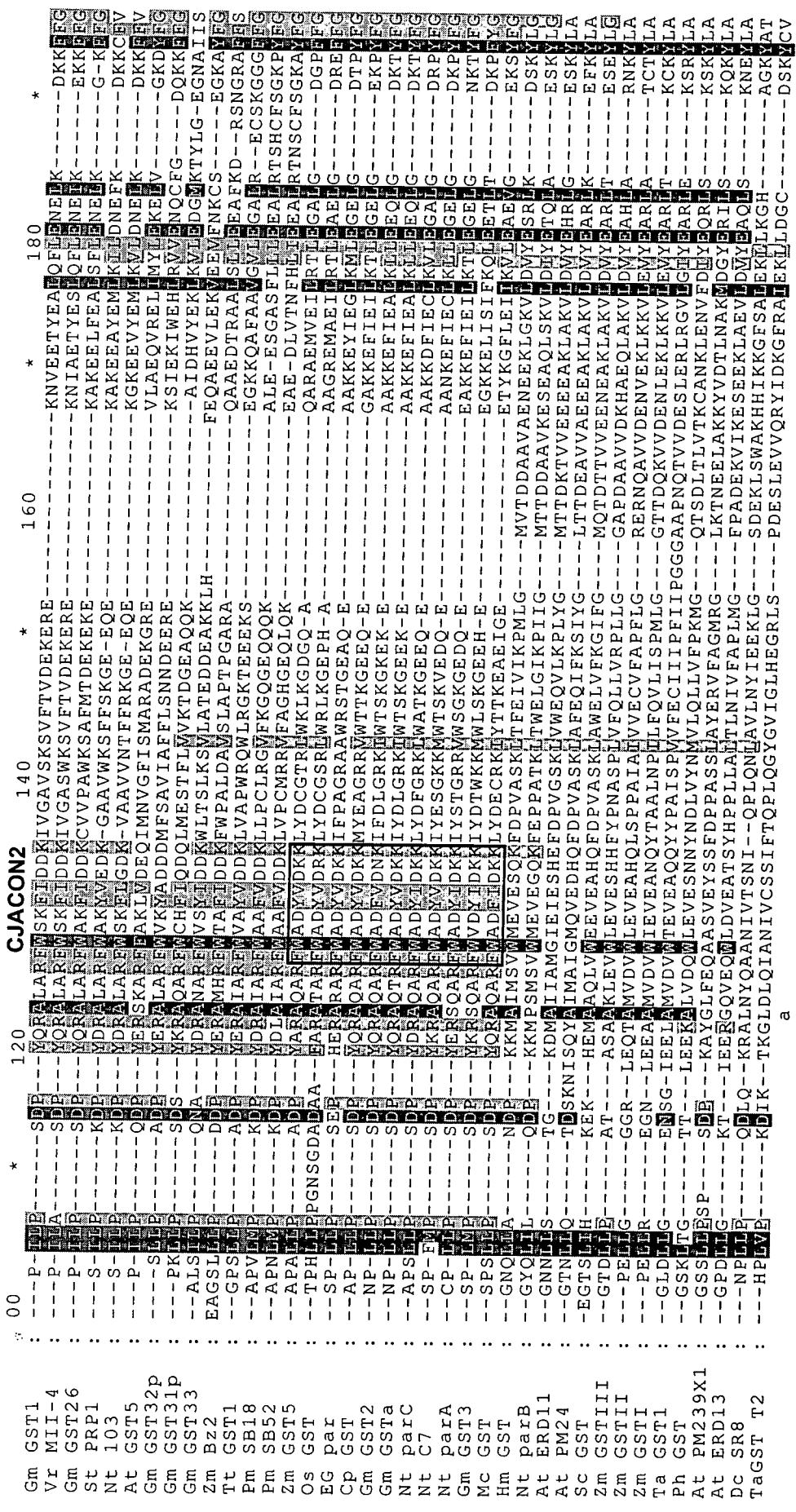
Figure 5.24 Clustal W (1.5) alignment of all known plant GSTs *cont.*
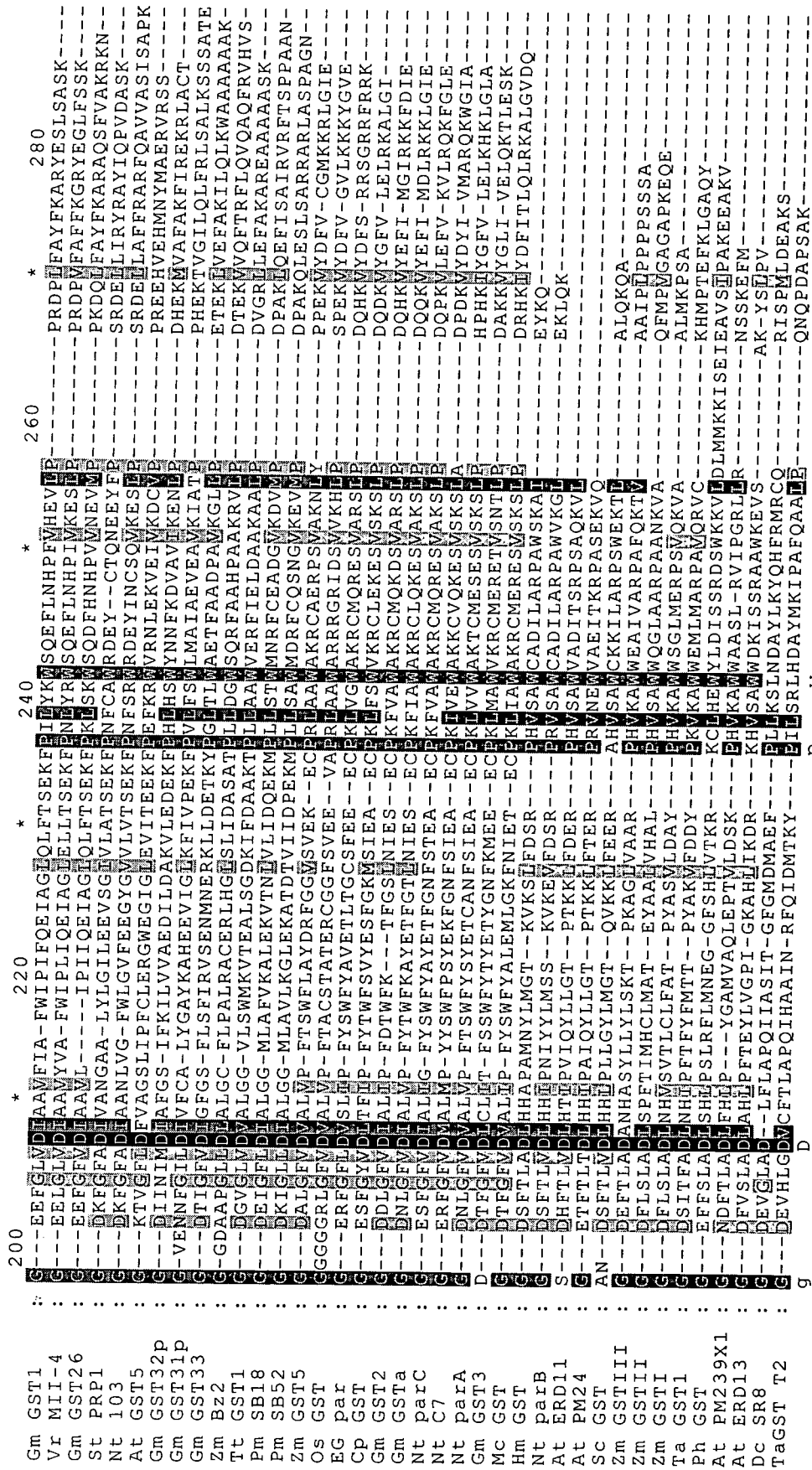
**Figure 5.24** Clustal W (1.5) alignment of all known plant GST sequences *cont.*

5.3 Discussion.

Various molecular cloning strategies were used to identify six distinct soybean GST cDNAs, as well as the previously characterised *Gm*GST1. Previous studies, reporting the isolation of plant GST cDNAs have described the use of degenerate primers designed to N-terminal protein sequence (Jepson *et al.*, 1994) or cDNA expression library screening using GST antisera (Grove *et al.*, 1988). Library screening with a degenerate oligonucleotide resulted in the isolation of the *GmGST2* cDNA. The predicted protein sequence of *Gm*GST2 has a near identical N-terminal identity to the GST purified from soybean seedlings described by Flury *et al*, (1995). This was as expected, since the N-terminal sequence reported was used to design the degenerate oligonucleotide used to identify *GmGST2*. However the predicted protein sequence of *Gm*GST2 and the isoenzyme reported by Flury *et al.* (1995) differed by one amino acid residue in the N-terminal region, and as such it is possible that sequences are derived from distinct isoenzymes. N-terminal amino-acid homology is a characteristic of plant GSTs where, for example, there is only one amino-acid difference in the first eight between maize *Zm*GST-I and *Zm*GST-III (Figure 5.24). Further evidence, based on the activity properties, suggests that *Gm*GST2 is a distinct enzyme from that previously described by Flury *et al* (1995), and these results are presented in chapter six.

An RT-PCR strategy to isolate GST-type sequences, was successfully developed to assist the isolation of soybean GST cDNAs. Multiple alignment of the many plant GSTs now present in the GenBank database indicated that regions of homology exist between both the tau- and theta-type GSTs. This finding was exploited in the design of degenerate oligonucleotides to these regions, which were used in RT-PCR to amplify GST sequences in soybean. This experiment identified the soybean GST-like cDNAs *GmGST1* (characterised previously) and the novel sequences *GmGST2*, *GmGST3*, *GSTCON26a*, *GSTCON31a*, *GSTCON32* and *GSTCON33a*. In addition, a number of variant sequences of *Gm*GST1 and *Gm*GST2 were obtained, but the other soybean GSTs described appeared to be highly conserved, with no variant sequences observed. The reason for the variation in *GmGST1* and *GmGST2* sequences is not fully understood. The most plausible explanation is that *GmGST1* and *GmGST2* exist in multiple copies throughout the soybean genome, and the variations reflect distinct

genes arising from soybean being a stable tetraploid composed of diploidised genomes, which behaves genetically as a diploid (Shoemaker *et al.*, 1996). Many examples of duplicated loci have been observed in soybean and it is thought that the vast majority of the genome exists in multiple copies (Palmer *et al.*, 1996). Southern blot analysis was not performed in this study, but such analysis should determine whether *GmGST1* and *GmGST2* are present in multiple copies within the soybean genome and thus explain the variants in these two GSTs. The differences between the reported soybean GST cDNA, *GSTa* (McGonigle and O'Keefe, 1998a) and *GmGST2* may be due to the different soybean cultivars used to obtain the cDNAs. More than 100,000 individual accessions exist for the cultivated soybean *Glycine max* (L.) Merr., indicating a wide diversity must exist within the germplasm (Shoemaker *et al.*, 1996). However, variation is not seen in all soybean GSTs obtained from different cultivars. There were only two nucleotide differences between *GmGST3*, obtained from cv. Mandarin and the sequence described as encoding "glyoxalase I", obtained from cv. Orsay, and these differences are most likely due to PCR-introduced errors. Variations in the sequences of individual plant GST isoenzymes have been reported in other species. Three cDNA sequences, all described as encoding *Zm*GST-III in maize, have been reported (Moore *et al.*, 1986, Grove *et al.*, 1988 and Dixon *et al.*, 1998c). Whilst some of these differences are undoubtedly due to shifts in the deduced translated reading frame, true differences are undoubtedly present, and at least two true isoenzymes can be predicted (Dixon *et al.*, 1998c). Certainly further detailed genetic analysis is required to determine the reason for the variation in similar DNA encoding GST sequences in soybean.

Tau-type GSTs from soybean, with relatively little homology to tau-type GSTs in other species were identified by RT-PCR using the CJACON2 and CJACON3 degenerate primers. Figure 5.25 shows the phylogenetic relationship between all known plant GSTs and the new GST cDNA sequences identified in soybean.
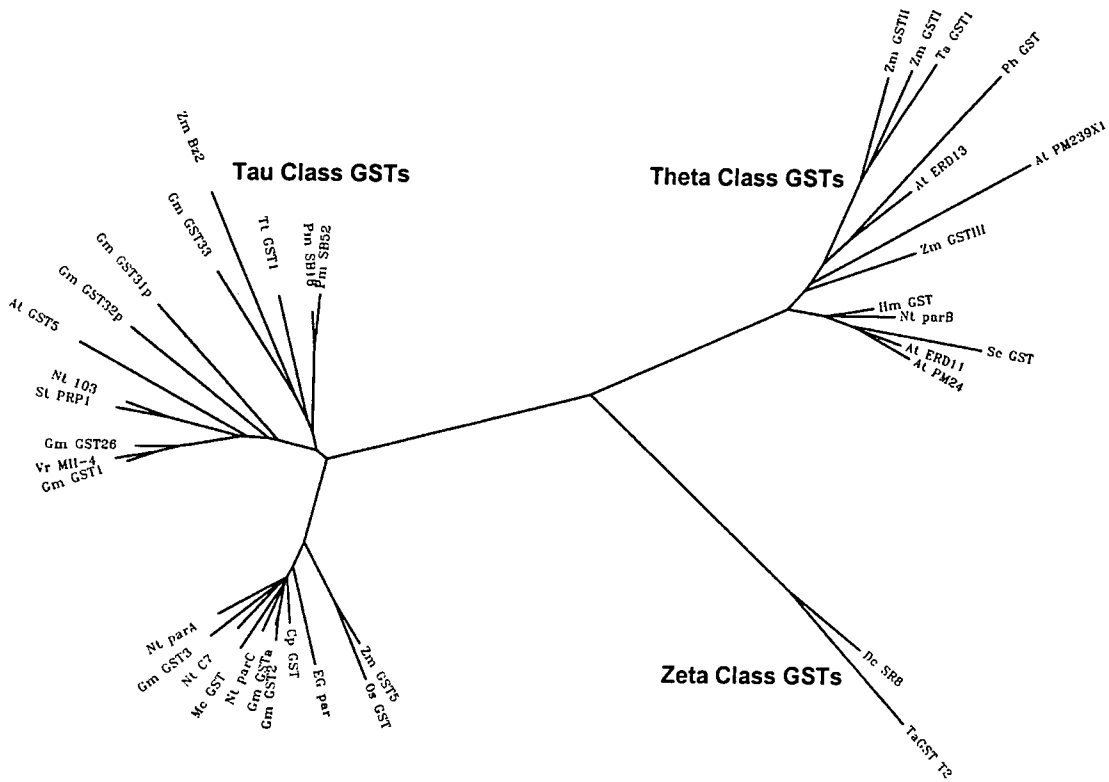
Figure 5.25 Phylogenetic alignment of all known higher plant GSTs based on protein sequence alignments. All soybean GST cDNA sequences described in this thesis are indicated. The partial protein sequences from GSTCON31a (Gm GST 31p) and GSTCON32 (Gm GST 32p) are included for comparative purposes. The length of the line between sequences is proportional to evolutionary relatedness.

The phylogenetic analysis shown in Figure 5.25 is similar to that published by Droog (1997) with Dixon *et al.* (1998b) showing the relationship of the plant GSTs with the human alpha, mu, pi, theta and zeta classes. As expected the plant theta class of GSTs shows greatest similarity with the human theta class. However, the discovery of an *Arabidopsis* expressed sequence tag (EST) sequence (GenBank Z35742 and T46668) with even greater homology to the human theta class suggests the classification of theta-type plant GSTs may be more complex than previously thought (Dixon *et al.*, 1998b).

No theta-type GST sequences were obtained from soybean by RT-PCR using the CJACON1 primer, designed to a highly conserved region within plant theta-class GSTs, although the primer did amplify the maize theta-type *Zm*GST-1 used as a positive control. Whilst not confirming that soybean does not contain theta type GSTs, it would appear from the molecular work presented here, the purification data presented in chapter four, and the differences in herbicides used selectively in maize

and soybean, that theta-class GSTs are much less abundant than tau-class GSTs in soybean. Interestingly, few theta-type GSTs have been reported in dicotyledonous plants (Kiyosue *et al.*, 1993) and few tau-type GSTs have been reported in grasses (Dixon *et al.*, 1998b). The success in isolating novel GST isoforms in soybean using RT-PCR suggests that the CJACON1, CJACON2 and CJACON3 oligonucleotide primers could also be useful tools for identifying GSTs in other species.

6. Chapter Six. Heterologous Expression of Soybean GSTs.

6.1 Introduction.

The purification of soybean GSTs described in chapter 4 revealed that cell suspension cultures contained several GST isoenzymes with activity toward diverse substrates. The attempted resolution of the native dimeric GST enzymes, utilising a range of biochemical purification strategies was not successful, even though multiple putative GST subunits were resolved using RP-HPLC. For the purposes of studying the GST activity of specific enzymes, RP-HPLC purification was undesirable, since the denaturing conditions associated with the process result in the loss of enzyme activity. Additionally, detailed biochemical analysis of purified GSTs from cell cultures required the utilisation of large quantities of biological starting material. Therefore, many technical difficulties existed which limited the use soybean GSTs purified from soybean when studying the substrate specificity and kinetic properties of specific GSTs.

In chapter 5, the cDNA sequences of a number of full length soybean GSTs were reported. To assist the biochemical characterisation of soybean GSTs it was anticipated that expression of these cDNAs in *E. coli* could provide a source of material suitable for detailed enzyme analysis. Bacterial expression as a tool to study plant GSTs is desirable, as the technique often facilitates the production of relatively large quantities of recombinant protein, since *E. coli* itself possesses only very low levels of endogenous GSTs (Sheehan and Casey, 1993). Successful expression of recombinant maize GSTs in *E. coli* has been described previously (Grove *et al.*, 1988 Moore *et al.*, 1996, Dixon *et al.*, 1998a). However, there can be problems associated with recombinant protein expression in bacteria. Not all recombinant proteins are folded properly or expressed efficiently, and often they are incorporated into insoluble inclusion bodies, significantly hindering the purification of active enzymes. Additionally, the post-translational processing of recombinant proteins differs in prokaryotes compared with eucaryotes. With respect to plant GSTs there is little evidence to suggest that these enzymes undergo post-translational modification in plants, though it has recently been reported that GSTs from sorghum are glycosolated (Gronwald and Plaisance, 1998). However, these post-translational modifications are

not reported to alter GST activities and overall there is little doubt that expression in *E. coli*, where successful, has assisted the characterisation of GSTs and provided access to large quantities of protein suitable for further studies including structural analysis following protein crystallisation.

In this chapter the expression of the soybean GSTs, *Gm*GST2 and *Gm*GST3 in *E. coli* is described. The purified recombinant proteins were characterised with respect to their enzyme activity and subjected to RP-HPLC and MALDI-TOF analysis in order to identify the corresponding GSTs purified from soybean cell cultures. The pET system (Novagen) was chosen for expression work, due to favourable heterologous expression results obtained previously with plant GSTs (Dixon *et al.*, 1998a).

6.2 Results.

6.2.1 Sub-cloning of soybean GST cDNAs into *E. coli* expression vectors.

*Gm*GST2.

*Nco*1 and *Bam* H1 sites were introduced into *GmGST2* by PCR (94°C, 45 s; 51°C, 30 s; 72°C, 60 s) using the oligonucleotide primers CJA215 (5' GCG CCA TGG CAG ATG AGG TG 3') (to introduce 5' *Nco* 1 restriction site) and OG2 (to introduce 3' *Bam* H1 restriction site). For the template, 50 ng pBluescript™ SK+ containing the *GmGST2* cDNA was used with the number of PCR cycles limited to 15 to reduce the likelihood of PCR-introduced errors. The amplified product was ligated into the *Taq*-PCR cloning vector pGEM-T (Promega), and transformed into *E. coli* XL1-Blue MRF'. Transformed bacteria were selected on LB media containing 100 µg ml⁻¹ ampicillin, X-Gal and IPTG. White colonies were analysed by PCR and plasmid recovered from 5 ml overnight cultures initiated from the positive bacteria. *GmGST2* was excised from the pGEM-T vector using *Nco* 1 and *Bam* H1 (Figure 6.1) and the fragment ligated into pET-11d (pre-digested with *Nco* 1 and *Bam* H1) prior to transformation into *E-coli* BL21 (DE3), with selection on LB plates containing 100 µg ml⁻¹ ampicillin. Plasmid was recovered from transformed colonies and the DNA sequenced, to confirm that the sub-cloned DNA sequence was identical to that of the original cDNA. For reasons unknown, the *Bam* H1 site used to clone the cDNA into the pET-11d vector was altered during the cloning process. As such, it was not possible to excise the cDNA from pET-11d using *Nco* 1 and *Bam* H1.
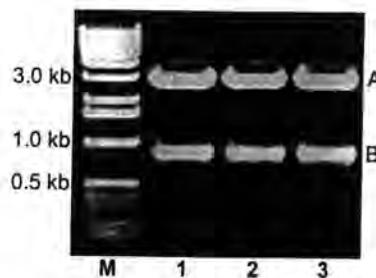
Figure 6.1 Introduction of *Nco*1:*Bam*H1 sites into *Gm*GST2 cDNA to allow sub-cloning into the expression vector pET-11d. Gel shows *Nco* I / *Bam* H1 digest of three independent pGEM-T plasmids (1,2,3) all containing the cloned PCR product. Linearised pGEM-T vector (A) and *Nco* 1:*GmGST2*:*Bam* H1 fragment (B) are visible.

*Gm*GST3

The full length soybean cDNA *GmGST3* (previously described as glyoxalase I, database accession P46417) was kindly provided in the vector pSB73 (Promega) by Prof. H-J Jacobsen, Department of Molecular Genetics, University of Hannover, Germany. The sequence was checked, using the T7 promoter primer, and found to be one base pair different to the clone described in the database (T to a G at position 370). This resulted in a change in the predicted amino acid sequence from histidine to glutamine at position 121. However, the sequences of the partial cDNAs homologous to this clone described in chapter 5 suggest the true residue at this position is probably histidine. The cDNA was excised from the pSB73 vector using *Xho*1 and *Eco* R1, and ligated into pBluescript™ KS⁺ (pre-digested with *Xho* 1:*Eco* R1). PCR (15 cycles) was performed with this plasmid, using the oligonucleotide primers GLY5 (5' GCG CCA TGG CGG ACG AGG TAG 3') and M13RSPL in order to engineer the required *Nco* 1 and *Bam* H1 sites, with the *Bam* H1 site obtained from the pBluescript polylinker using the M13RSPL primer. The PCR product was gel purified and digested directly with *Nco* 1 and *Bam* H1 overnight. This digested cDNA was gel-purified, ligated into pET-11d digested with *Nco* 1:*Bam* H1 and transformed into *E. coli* BL21 (DE3) cells (Figure 6.2). Plasmid was recovered from transformed bacteria and the DNA sequence checked. The introduction of the 5' *Nco* 1 site resulted in a change in the fourth nucleotide in the coding sequence, causing a consequent change in the second amino acid from serine to alanine. Therefore, two amino acid differences existed within the recombinant protein compared with the predicted sequence of the "glyoxalase I" cDNA previously described.
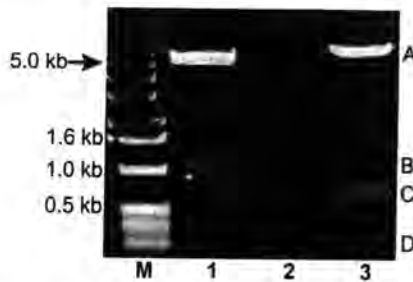
Figure 6.2 Cloning of *GmGST3* into pET-11d. Lane one shows *Nco*1 and *Bam*H1 digest of *GmGST3* in pET-11d, with linearised pET-11d (A) and cDNA insert of the correct size (B). Lane three shows characteristic *Ssp*1 digest pattern (A,C,D) *GmGST3* in pET-11d.

## 6.2.2 Expression of recombinant protein in *E. coli.*

Overnight cultures were initiated from a single colony containing either the pET:*GmGST2* or pET:*GmGST3* plasmid as desired. Expression of the heterologous protein was initiated by the addition of 1 mM IPTG to the culture medium.

6.2.3 Purification of recombinant enzymes.

*Gm*GST2 and *Gm*GST3 were purified from the recombinant bacteria as described in section 2.2.9. The quantity of recombinant *Gm*GST2 and *Gm*GST3 protein obtained from 100 ml starting culture was approximately 8.0 mg and 3.0 mg respectively. Figure 6.3 shows SDS-PAGE analysis of the time scale of induction of recombinant soybean GSTs *Gm*GST2 and *Gm*GST3 in *E. coli.* Figure 6.4 shows SDS-PAGE analysis of purified *Gm*GST2 and *Gm*GST3. In addition, recombinant *Gm*GST1 and *Gm*GST2b, supplied by Dr Mark Skipsey, are shown for comparative purposes. This analysis showed that pure recombinant *Gm*GST2 and *Gm*GST3 migrated as 28 kDa polypeptides, rather than as the predicted 25 kDa polypeptides.
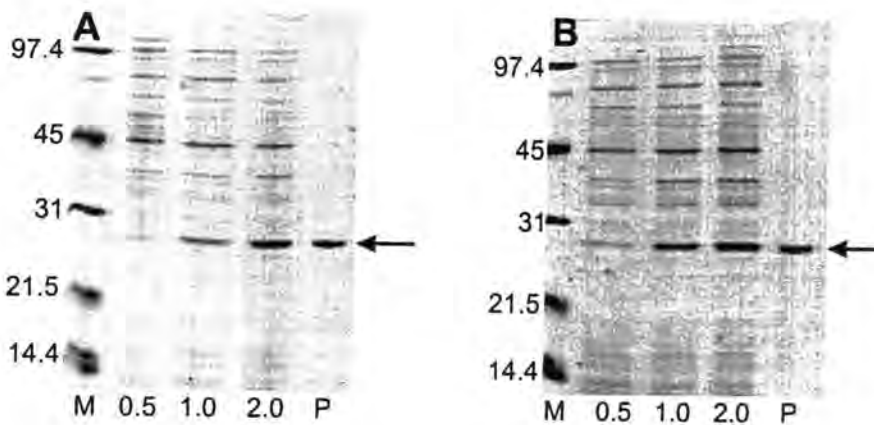
Figure 6.3 SDS-PAGE analysis showing recombinant expression of recombinant soybean GSTs (arrowed) *Gm*GST2 (A) and *Gm*GST3 (B). Sonicated crude extracts are shown 0.5, 1.0 and 2.0 hours following the addition of IPTG to the culture medium. P indicates the *S*-hexylglutathione purified recombinant protein. The molecular masses of reference proteins are given in kDa.
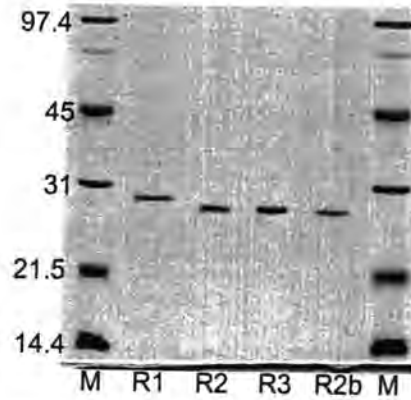
Figure 6.4 SDS-PAGE analysis of purified recombinant soybean GSTs. M= protein standard markers, with molecular masses given in kDa. R1=*Gm*GST1 (50 ng), R2=*Gm*GST2 (50 ng), R3=*Gm*GST3 (50 ng). R2b = *Gm*GST2b (50 ng).

Antibodies were subsequently raised to purified recombinant *Gm*GST1 (supplied by Dr Mark Skipsey), *Gm*GST2 and *Gm*GST3, termed AB1, AB2 and AB3 respectively. The detailed use of these antibodies is described in chapter 7.

6.2.4 Characterisation of recombinant soybean GSTs.

6.2.4.1 Activity of recombinant enzymes.

The catalytic activity of *Gm*GST2 and *Gm*GST3 was determined toward a number of substrates including herbicides, known to be conjugated with homoglutathione in soybean. All *in vitro* assays were performed with both glutathione and homoglutathione to determine the thiol preference exhibited by these enzymes. The results from these analyses are given in Table 6.1.

Substrate	*Gm*GST1*		*Gm*GST2		*Gm*GST3	
	Activity	Error	Activity	Error	Activity	Error
GST						
CDNB (GSH)	121.8	2.4	107.9	2.0	215.5	1.1
CDNB (hGSH)	65.4	3.0	384.6	9.2	107.4	1.1
Ethacrynic Acid (GSH)	3.7	1.4	1.7	0.3	2.5	0.1
Ethacrynic Acid (hGSH)	1.0	0.3	ND		2.7	0.7
GST - Herbicides						
Acetochlor (GSH)	NA		202.1	17.1	251.3	4.8
Acetochlor (hGSH)	NA		224.3	3.9	200.0	14.4
Acifluorfen (GSH)	12.9	0.3	9.7	0.1	ND	
Acifluorfen (hGSH)	42.0	2.0	18.4	0.6	ND	
Chlorimuron-ethyl (GSH)	9.8	1.6	3.7	0.7	ND	
Chlorimuron-ethyl (hGSH)	16.3	0.9	5.6	0.5	ND	
Fomesafen (GSH)	27.9	1.9	23.5	1.5	ND	
Fomesafen (hGSH)	138.5	6.9	47.4	3.1	ND	
Metolachlor (GSH)	228.3	18.9	417.6	2.9	410.7	19.9
Metolachlor (hGSH)	91.9	16.1	45.6	1.7	51.1	4.7
Glutathione Peroxidase						
Cumene Hydroperoxide (GSH)	0.44	0.13	16.7	0.5	4.0	0.2
Cumene Hydroperoxide (hGSH)	0.50	0.15	9.4	0.1	2.8	0.2
Glyoxalase						
Glyoxalase I	ND		ND		ND	

Table 6.1 Enzyme activity of recombinant *Gm*GST1, *Gm*GST2 and *Gm*GST3. *Data for *Gm*GST1 is obtained from Skipsey *et al.*, 1997 and is included for comparative purposes. CDNB data is expressed as nkat mg^{-1} protein with cumene hydroperoxide activity quoted as OD change at 366nm min^{-1} mg^{-1} protein and with herbicides the activity is given as pkat mg^{-1} protein. Error is given as SD from the mean (n=3). ND = Not detectable, NA = Not available.

Table 6.1 shows that both *Gm*GST2 and *Gm*GST3, like *Gm*GST1 characterised previously (Skipsey *et al.*, 1997), exhibit catalytic activity toward a diverse range of substrates. No glyoxalase I activity was detected with either enzyme, confirming the mis-identity of *Gm*GST3 as a glyoxalase I enzyme. Lactoylglutathione, the product of glyoxalase I, has been shown to tightly bind at the active site of maize *Zm*GST I (Neuefeind *et al.*, 1997a), although to date no glyoxalase I activity has ever been associated with a GST enzyme. Furthermore, it has now been shown that a distinct glyoxalase I enzyme exists in soybean, which shows a high degree of sequence homology to glyoxalase I enzymes identified in other species (Skipsey *et al.*, 1998). The original mis-identification of *Gm*GST3 as a glyoxalase probably arose due to the co-purification of GST and glyoxalase I by *S*-hexylglutathione affinity chromatography (Allen *et al.*, 1993). GSTs in soybean are far more abundant than glyoxalase I and antisera raised to proteins purified by *S*-hexylglutathione affinity chromatography are likely to contain GST antibodies. Consequently, it is probable that immuno-screening of a cDNA library using such antisera would result in the identification of GST clones.

Increasingly, plant GSTs are reported to have additional activities as glutathione peroxidases (Bartling *et al.*, 1993). Both recombinant soybean GSTs characterised in this study displayed glutathione peroxidase activity toward cumene hydroperoxide, with *Gm*GST2 showing an appreciably higher degree of activity than *Gm*GST3. The lower glutathione peroxidase activity value obtained in both cases with homoglutathione may reflect affinity differences for the thiol. Alternatively, this may result from a difference in affinity of the glutathione reductase enzyme used in the linked assay, though the thiol preference of the yeast glutathione reductase was not investigated further. Both enzymes showed activity toward ethacrynic acid, a phenylacetic acid derivative that contains an electrophilic group structurally analogous to α-β-alkenals generated in mammals under oxidative stress (Edwards, 1996). However, no activity was observed with this substrate when *Gm*GST2 was assayed with hGSH. These results suggest that in addition to catalysing xenobiotic (h)GSH-conjugation, *Gm*GST2 and *Gm*GST3 may additionally serve to detoxify both naturally-occurring propenals and fatty acid hydroperoxides which accumulate as a

result of oxidative stress imposed by infection, chemical injury and heat-shock (Marrs, 1996).

Intriguingly, under the assay conditions used, differences in both thiol and substrate specificity of GmGST2 and GmGST3 were observed. With respect to CDNB, GmGST2 showed a preference for conjugation with homoglutathione, whereas GmGST3 showed a preference for glutathione. Activity toward the chloroacetanilide acetochlor was comparable with both thiols for both enzymes. However, both enzymes showed an approximately 10-fold greater activity toward metolachlor when using glutathione as co-substrate. The major difference in GST activity of GmGST2 and GmGST3 was determined with the herbicide substrates acifluorfen, chlorimuron-ethyl and fomesafen. GmGST2 exhibited activity toward all three substrates, with homoglutathione the preferred thiol for conjugation in all cases. However, GmGST3 was not active toward any of these substrates. These findings provide further evidence that tau-type GSTs are likely to play an important role in herbicide selectivity in addition to the theta-type GSTs already known to function in this manner (Marrs, 1996). The activities of GmGST2 and GmGST3 were consistent with the observed tolerance of soybeans toward these herbicides, although the precise role of these GSTs in herbicides *in planta* was not determined. These GST isoenzymes are unlikely to be the major catalysts of fomesafen / acifluorfen detoxification which was associated with a single isoenzyme (chapter 4). The relatively low activity toward fomesafen observed with GmGST2 and GmGST3 may explain the low level of activity seen toward this substrate observed during the anion-exchange purification from cell cultures which eluted prior to the highly active isoform 11 described in chapter 4.

These results suggested that GmGST2 exhibited a broader spectrum of activity than GmGST3. With respect to the substrates tested there is little difference in activity between GmGST1 and GmGST2, except that GmGST2 shows considerably greater glutathione peroxidase activity.

6.2.4.2 Enzyme kinetics of *Gm*GST2 and *Gm*GST3.

Km and Vmax

The use of *in vitro* assays at a single defined concentration of substrates provides a useful insight into the characteristics of GST activities. However, due to their nature, such assays can often mis-represent probable activities *in planta*, due to the non-physiological substrate concentrations used to monitor activity. Kinetic enzyme analysis provides information as to the affinity of an enzyme for it's substrate, and also, in the case of two-substrate enzymes, provides information as to whether the binding of the first substrate can affect the enzyme's affinity for the second substrate, and define the catalytic independence of each subunit in the dimer (Gronwald and Plaisance, 1998). These more complex two-substrate models have been used extensively to study mammalian GSTs, and have revealed that the order of substrate binding is specific to the isoenzyme (Gronwald and Plaisance, 1998). The use of two-substrate models when studying GSTs can be problematical for two reasons, a) many xenobiotic substrates become insoluble at sub-saturating concentrations, b) the significant rate of non-enzymic (homo)glutathione conjugation at high substrate concentrations. For these reasons, complex kinetic analysis was not performed. In this basic study the kinetic parameters Km and Vmax were determined using initial velocity data as analysed by the standard Michaelis-Menten equation for a single substrate reaction. In order to perform analysis in this way it is important to assay one substrate whilst the other is held at saturating concentration. Vmax is a theoretical measure of the maximum catalytic velocity of an enzyme, and thus occurs at saturating substrate concentrations. Km is a measure of the affinity of the enzyme for a given substrate, and is defined as the substrate concentration required to achieve ½ Vmax. Therefore, if an enzyme has a small Km value, it possesses high affinity toward its substrate and achieves maximal catalytic efficiency at low substrate concentrations. Whilst the determination of Vmax and Km will not provide information regarding the specific catalytic mechanism of the enzymes, the data will provide an insight as to the substrate and thiol preference of soybean GSTs. In this study analysis was performed on *Gm*GST2 and *Gm*GST3 using CDNB, glutathione and homoglutathione as substrates. Data analysis was performed using Hyperbolic Regression Analysis of Enzyme Kinetic Data (HYPER, J.S Easterby).
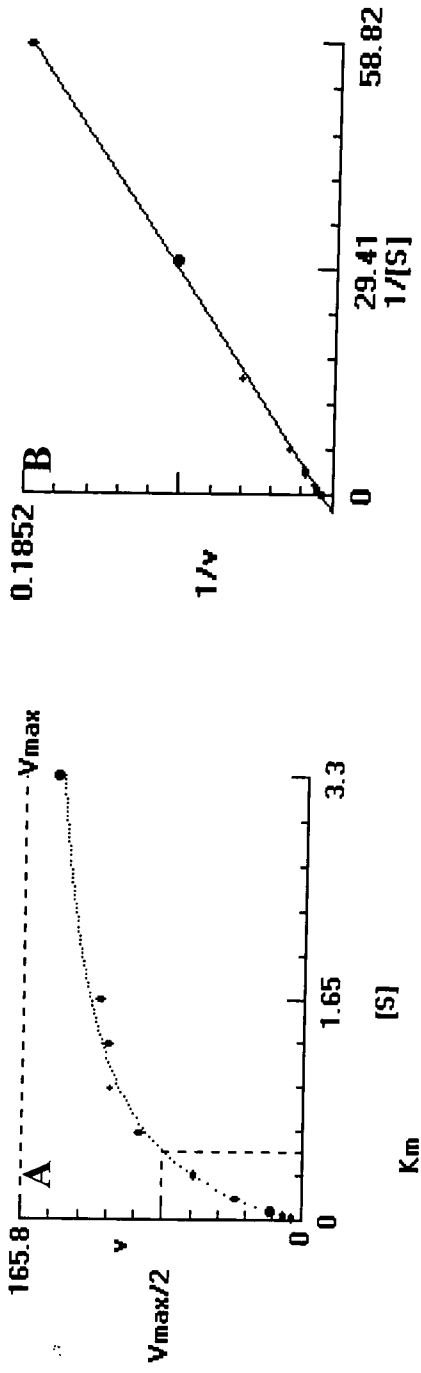
Figure 6.5 Determination of Vmax and Km for CDNB with *Gm*GST2 as determined by: A, a plot of V/S and B, a Lineweaver-Burke plot.
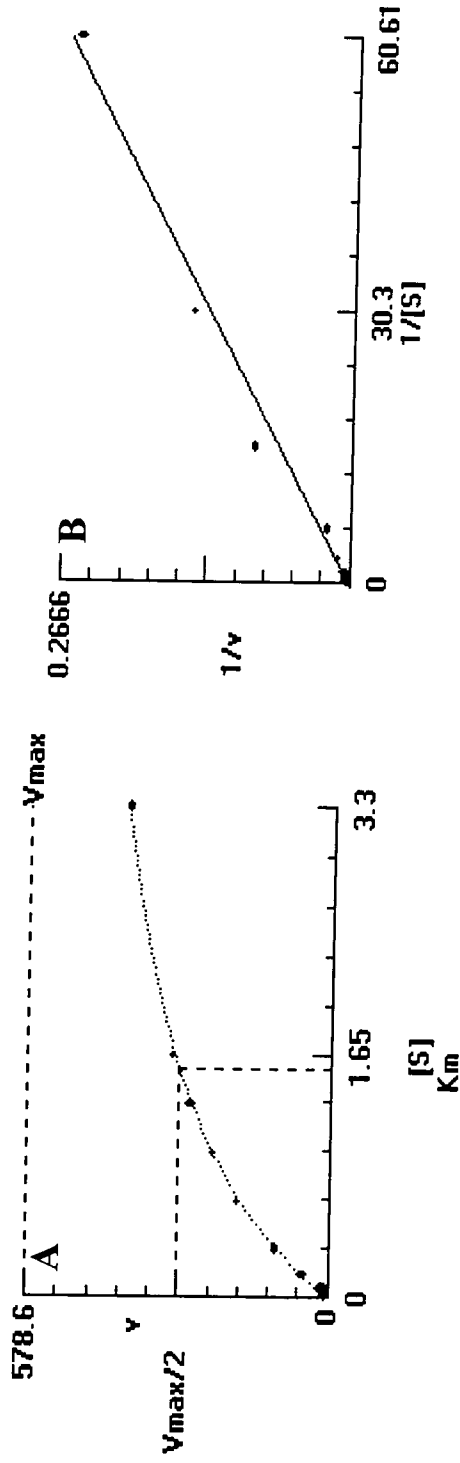

Figure 6.6 Determination of Vmax and Km for CDNB with *Gm*GST3 as determined by: A, a plot of V/S and B, a Lineweaver-Burke plot.
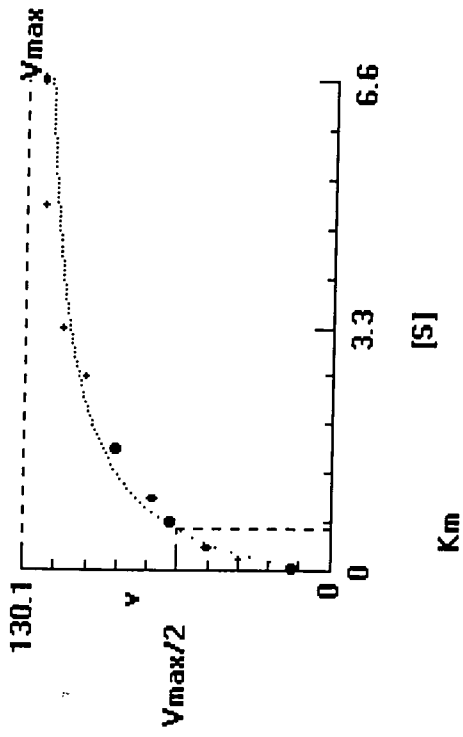
Figure 6.7 Determination of Vmax and Km for glutathione with *Gm*GST2 as determined by: A, a plot of V/S and B, a Lineweaver-Burke plot.
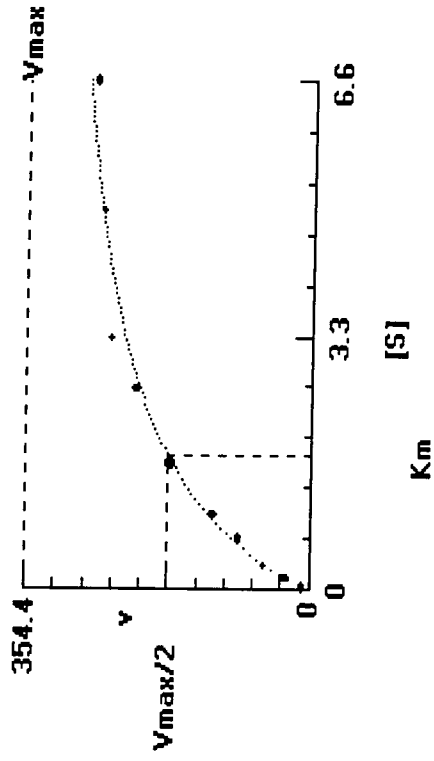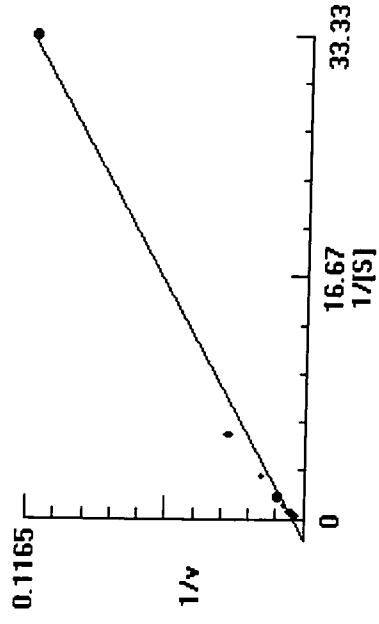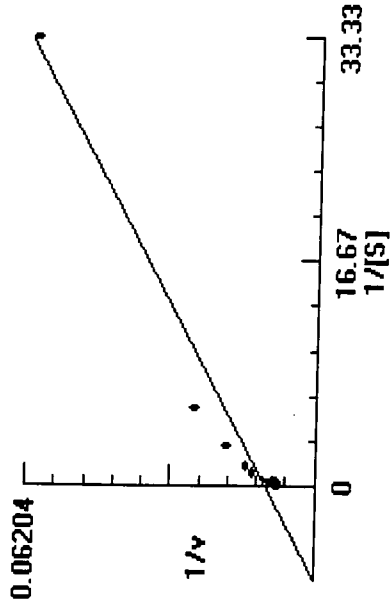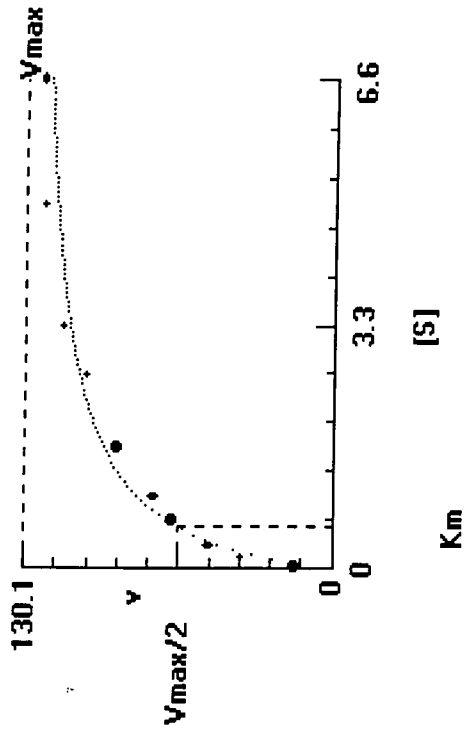

Figure 6.8 Determination of Vmax and Km for homoglutathione with *Gm*GST2 as determined by: A, a plot of V/S and B, a Lineweaver-Burke plot.

Figure 6.7 Determination of Vmax and Km for glutathione with *Gm*GST2 as determined by: A, a plot of V/S and B, a Lineweaver-Burke plot.
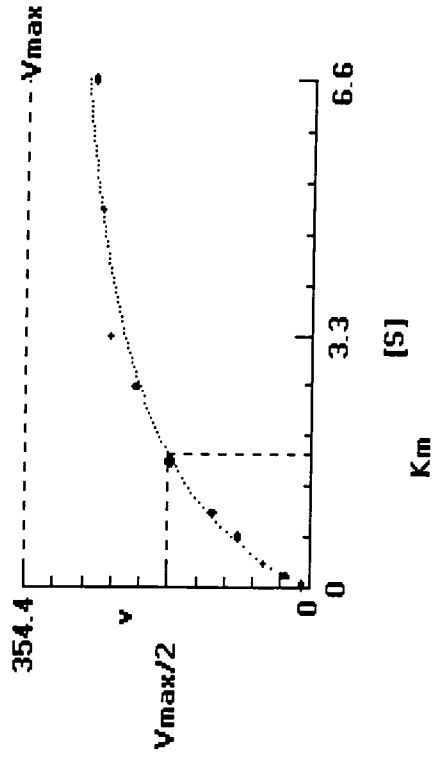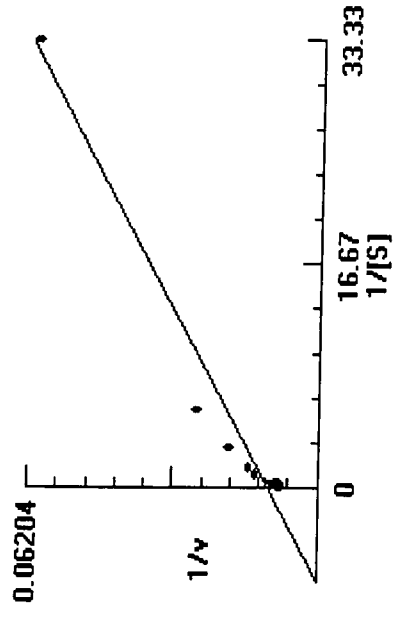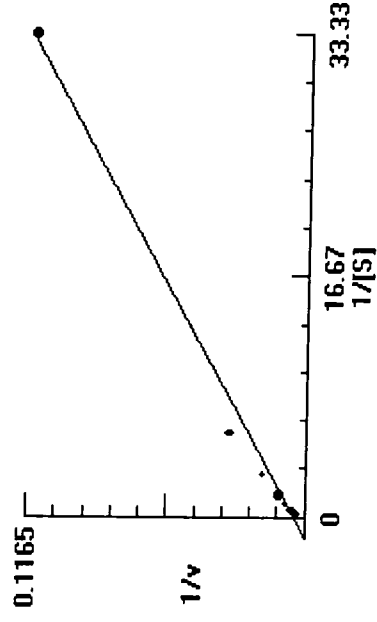

Figure 6.8 Determination of Vmax and Km for homoglutathione with *Gm*GST2 as determined by: A, a plot of V/S and B, a Lineweaver-Burke plot.

Tables 6.2 to 6.4 summarises the kinetic data obtained. Km (mM) and Vmax (nkat mg^{-1} pure protein) values are given along with the standard error (SE).

Enzyme	Km (mM)	SE	Vmax	SE
*Gm*GST2	0.51	0.04	165.8	4.57
*Gm*GST3	1.56	0.10	578.6	17.72

Table 6.2 Km and Vmax determinations for *Gm*GST2 and *Gm*GST3 using CDNB. In each case the concentration of glutathione in the assay was fixed at 3.3 mM.

Enzyme	Km	SE	Vmax	SE
*Gm*GST2	0.55	0.08	130.1	5.02
*Gm*GST3	1.73	0.18	354.4	13.70

Table 6.3 Km and Vmax determinations for *Gm*GST2 and *Gm*GST3 using glutathione. In each case the concentration of CDNB in the assay was fixed at 1 mM.

| Enzyme	Km	SE	Vmax	SE
*Gm*GST2	0.68	0.07	401.5	11.17
*Gm*GST3	1.75	0.30	142.5	9.30

Table 6.4 Km and Vmax determinations for *Gm*GST2 and *Gm*GST3 using homoglutathione. For both enzymes the concentration of CDNB in the assay was fixed at 1 mM.

The kinetic data obtained indicates that *Gm*GST2 has a lower Km for CDNB than *Gm*GST3, which suggests that *Gm*GST2 shows a higher affinity for CDNB than *Gm*GST3. However, Vmax data indicates that *Gm*GST3 has greater catalytic activity toward CDNB at saturating substrate concentration than does *Gm*GST2. Previously reported Km values for GSTs toward CDNB are rat = 0.06-0.1 mM (Habig *et al.*, 1974), tobacco Nt107 = 0.2 mM and tobacco Nt103 = 0.86 mM (Droog *et al.*, 1993). Flury *et al.* (1996) reported Km values toward CDNB of three partially purified GSTs from soybean as 0.434 ± 0.103 mM, 0.560 mM ± 0.053 mM, 0.319 mM ± 0.037 mM. These are similar to the values obtained for *Gm*GST2, and indicate that soybean may contain a number of GSTs with similar Km toward CDNB. The Km of *Zm*GST V-V from maize, another tau-type GST similar to *Gm*GST1 and *Gm*GST2, is reported as 2.82 mM ± 0.28 mM (Dixon *et al.*, 1998). Therefore, it would appear that plant GSTs of the same class exhibit appreciable variation in affinity toward similar substrates.

The kinetic data obtained for glutathione and homoglutathione revealed further differences between the two recombinant enzymes. The Km values toward glutathione and homoglutathione were similar. However, *Gm*GST3 had a higher Vmax in the presence of glutathione rather than homoglutathione, while *Gm*GST2 shows a significantly greater Vmax in the presence of homoglutathione. This finding that plant GSTs exhibit different maximal activities with different thiols is an important observation, since there is a lack of information in the literature regarding the differential use of thiol substrates by plant GSTs. This study indicates that in soybean there are important differences in the rates of detoxification between GST isoenzymes in the presence of different thiols when catalysing conjugation to specific xenobiotic substrates.

6.2.4.3 MALDI-TOF MS analysis.

MALDI-TOF MS analysis was performed on the purified recombinant GSTs in order to determine the difference between the theoretically and experimentally determined protein molecular weights of known samples. This information was also used to indicate the accuracy of the MALDI-TOF information obtained regarding the GST subunits purified from soybean cell cultures described in chapter four. A representative MALDI-TOF chromatogram obtained from recombinant *Gm*GST2 is shown in Figure 6.17. Table 6.5 summarises the results obtained.
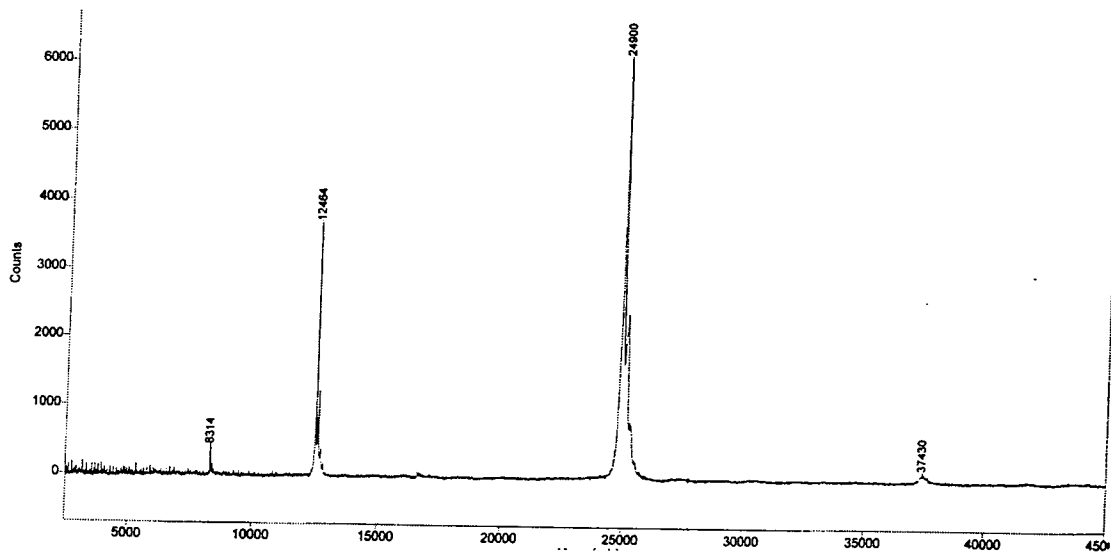
Figure 6.17 MALDI-TOF MS spectrum of recombinant *Gm*GST2. A signal was observed with an average peak top mass at m/z 24,900. The signal at m/z 12,464 is consistent with the major component in a doubly-charged state.

It was apparent from these spectra obtained that all the recombinant samples analysed contained two mass ion peaks, 130 Da apart. The reason for this phenomenon is not clearly understood. It may be that the presence of the two mass-ions indicates the cleavage of the N-terminal methionine in *E. coli*. Both prokaryotes and eucaryotes are known to contain an aminopeptidase, which can catalyse the cleavage of the N-terminal methionine (Freifelder, 1987). The difference in molecular weight observed is consistent with such N-terminal methionine cleavage.

| Protein	MALDI	Theoretical	Theoretical (-M)	Theoretical pI
*Gm*GST1	25,872	26,013	25,881	5.38
*Gm*GST2	24,900	25,047	24,916	5.85
*Gm*GST3(exp)	25,748	25,878	25,747	5.42
*Gm*GST3	ND	25,903	25,772	5.60
*Gm*GST2b	25,439	25,580	25,449	5.81

Table 6.5 Characterisation of recombinant soybean GSTs. Data obtained from MALFI-TOF analysis is shown, along with theoretically determined MW (from cDNA ORF translation). Theoretical (-M) indicates theoretical mass of protein with cleavage of the N-terminal methionine. pI indicates theoretically determined isoelectric point. *Gm*GST3 (exp) refers to the recombinant *Gm*GST3 protein actually expressed (with the two amino-acid changes described in section). Theoretical data for *Gm*GST3 refers the translated *Gm*GST3 provided in the GenBank database, which indicates differences between *Gm*GST3 (exp) and *Gm*GST3. Data generated using *EditSeq* (DNASTAR, Inc.) All molecular weight data is given to the nearest 1 Da.

If the explanation regarding methionine cleavage is correct then the comparison of the experimental data obtained with the theoretically determined molecular masses shown in Table 6.5 suggest that the N-terminal methionine is cleaved in all the recombinant proteins analysed. Thus, the real difference between the experimentally determined and theoretical masses is between 1 and 16 Da, which is within the 0.1% error limits of the MALDI-TOF technique. Therefore, it may be concluded that the molecular weight data obtained in chapter four regarding the GSTs purified from soybean should be accurate.

It is interesting to note that the theoretical calculation of isoelectric points (pI) reveals that all the recombinant soybean GSTs examined possesses very similar values between pH 5.42 and pH 5.83. The similarity in pI values may explain the poor anion-exchange resolution of S-hexylglutathione purified GSTs from soybean cell cultures detailed in chapter four. These findings support those of Flury *et al.* (1996) who identified a number of GSTs in soybean plants with pI between pH 5.8 and pH 6.4.

6.2.4.4 RP-HPLC analysis of recombinant proteins.

In chapter 4, reversed phase HPLC analysis of GSTs purified from soybean cell cultures proved a powerful tool to assist in the identification of individual GST subunits purified from soybean cell cultures. Since two independent strategies had been adopted to study soybean GSTs, namely purification of the cell culture enzymes and cloning and expression of the recombinant GSTs, it was deemed important to determine which, if any, of the recombinant GSTs expressed corresponded to the purified polypeptides described in chapter 4. In order to achieve this each of the purified recombinant proteins, *Gm*GST1, *Gm*GST2 and *Gm*GST3, was analysed by RP-HPLC (Figure 6.18).
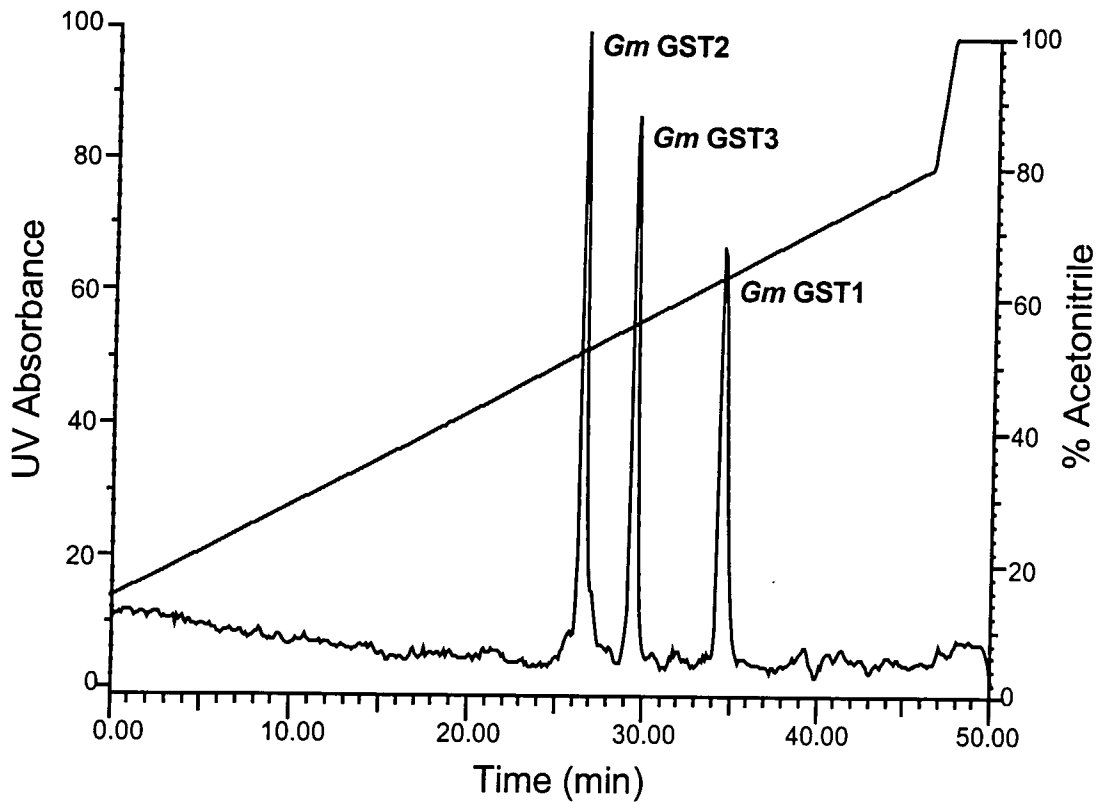
Figure 6.18 Reversed-phase HPLC separation of recombinant *Gm*GST1, *Gm*GST2 and *Gm*GST3 purified by *S*-hexylglutathione affinity chromatography and then pooled for comparative purposes.

Figure 6.18 shows that the three recombinant GSTs were clearly resolved from one another. To determine whether any of these recombinant GSTs corresponded with any of the polypeptides identified in plants the three recombinant GSTs were used to spike a sample of *S*-hexylglutathione affinity-purified GSTs from soybean cell cultures (Figure 6.19).
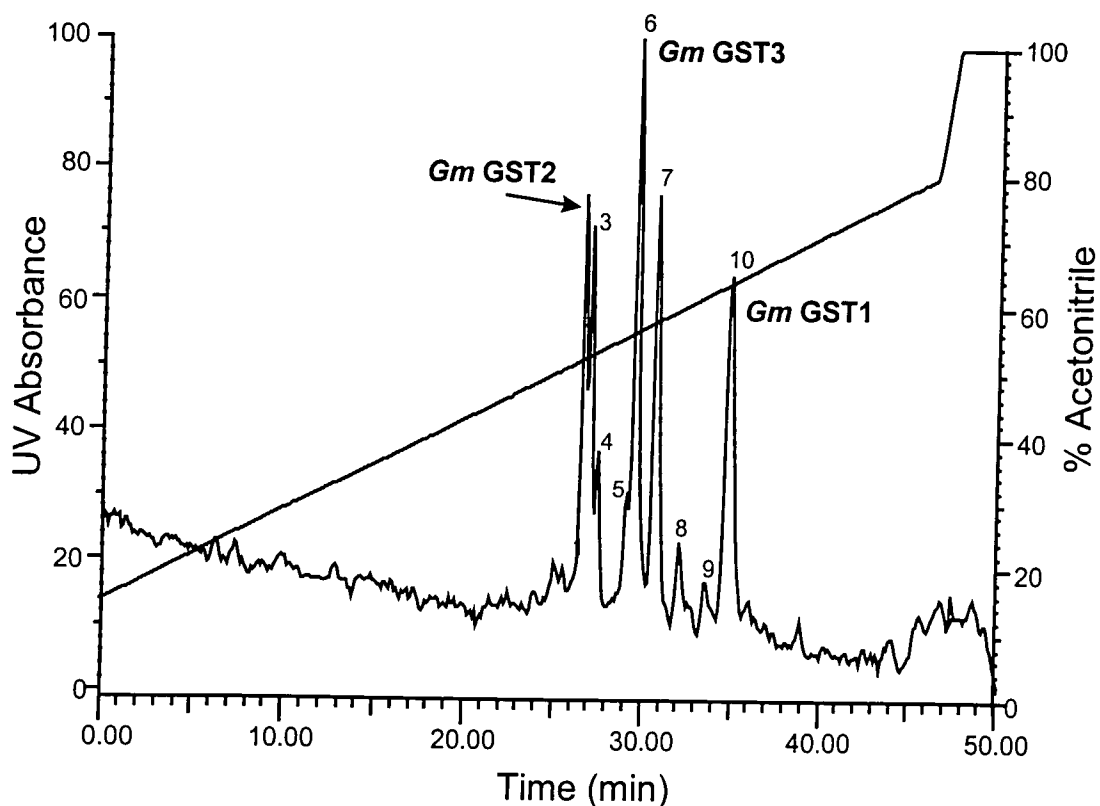
Figure 6.19 *S*-hexylglutathione purified GSTs from 5-day-old cell cultures (Figure 4.18) spiked with purified recombinant *Gm*GST1, *Gm*GST2 and *Gm*GST3.

These results indicate that during RP-HPLC, *Gm*GST1 migrates with polypeptide 10 and *Gm*GST3 with polypeptide six, the latter being a major polypeptide present in soybean cell cultures. *Gm*GST2 did not migrate with any of the polypeptides identified, and was observed as an additional peak. It is somewhat surprising that, given the transcript abundance of *Gm*GST2 in the soybean cell culture cDNA library, that *Gm*GST2 did not correspond to any of the GST polypeptides in soybean. This may suggest that either *Gm*GST2 undergoes post-translational modification, altering its mobility during HPLC or that it is in fact a low abundant protein in soybean. It is possible that one of the other *Gm*GST2-type proteins identified in chapter 5 is more abundant *in planta*. Minor changes in the protein sequence are likely to result in changes in the hydrophobic characteristics of the enzyme. Indeed, evidence does exist that GSTs with similar protein sequences do show very different RP-HPLC retention times (Pascal *et al.*, 1998). Either scenario may explain the different migration following RP-HPLC separation. To test the hypothesis that the minor variations in the sequence of *Gm*GST2 result in different RP-HPLC retention characteristics, another *Gm*GST2 type cDNA, *Gm*GST2b (described in chapter 5), was expressed in *E. coli*.

However, despite differences in protein sequence, including the three additional amino acids in *Gm*GST2b, the recombinant protein migrated with *Gm*GST2 during reversed phase HPLC (personal communication, Dr Mark Skipsey). Further speculation as to the identity of *Gm*GST2 in soybean is provided in chapter 7. This data provides further evidence that two of the polypeptides purified from soybean cell cultures in chapter 4 are indeed GST subunits.

6.3 Discussion.

In this chapter, bacterial expression has been used to characterise the specific properties of two recombinant soybean GSTs, *Gm*GST2 and *Gm*GST3. The discovery in chapter three that soybean GSTs exhibited differences in thiol specificity was of particular interest, and the utilisation of recombinant DNA technology has allowed this observation to be investigated further. No common thiol preference was apparent for either *Gm*GST2 or *Gm*GST3, although differences in activity between enzymes toward individual substrates were shown to exist. In some cases, such as the conjugation of the metolachlor, catalytic activity of both enzymes was significantly enhanced in the presence of glutathione, a thiol not endogenously present in soybean.

It is not clear whether or not *Gm*GST2 was identical to the auxin-inducible GST sequenced by Flury *et al.* (1995). This sequence was used to design a degenerate oligonucleotide DNA probe to isolate *GmGST2* from a soybean seedling cDNA library. The N-terminal sequence of this clone differed by one amino acid (serine to alanine at position two) in the series of 17 amino acids reported by Flury *et al.* (1995). GST activity of the enzyme described by Flury *et al.* (1995) is reported at 81.7 ± 3.8 nkat mg^{-1} toward CDNB and 400 ± 50 pkat mg^{-1} toward metolachlor. No activity was detected with the herbicide substrates fluorodifen or atrazine. Conversely, the recombinant *Gm*GST2 did exhibit detectable activity toward fluorodifen when glutathione was used to assay the enzyme (data not shown). Therefore, given this variation in activity and the amino-acid difference in the N-terminal region, it is likely that *Gm*GST2 is different to that reported by Flury *et al.* (1995). Multi-variant *Gm*GST2-type genes exist in soybean, as discussed in chapter five. It may be possible that these minor differences in protein sequence do results in different catalytic properties of the enzyme. Indeed preliminary experiments have suggested that minor differences in the activity of *Gm*GST2 and *Gm*GST2b enzymes do exist (data not shown). In chapter 5 it was hypothesised that the multiplicity of *Gm*GST2 sequences may be due to the complex genome of soybean and that the variants were due to multiple copies of the same gene. Interestingly, the GST described by Flury *et al.* (1995) was auxin inducible and auxin treatment of soybean results in H$_2$O$_2$ and O$_2^-$ formation (Flury *et al.*, 1998). This may explain why GSTs such as *Gm*GST2, which has high glutathione peroxidase activity, are induced following auxin treatment.

Reversed phase HPLC analysis of the recombinant enzymes provided evidence that *Gm*GST1 corresponded to peak 10 and *Gm*GST3 to peak 6 purified from soybean cell cultures (described in chapter 4). MALDI-TOF analysis of recombinant *Gm*GST1 gave an apparent molecular mass of 25,881 Da, which resulted from N-terminal methionine cleavage of the parent polypeptide. MALDI-TOF MS analysis of peak 10 purified from soybean, showed the presence of two polypeptides, of molecular mass 24,928 Da and 25,909 Da. Since the MALDI-TOF data was derived from a single analysis, it is not possible to determine accurately the degree of error. It may be that the 25,909 Da polypeptide in peak 10 corresponded to *Gm* GST1, since the observed 28 kDa difference in mass is within the 0.1% error limit quoted for the analytical equipment used. Incomplete MALDI-TOF data for recombinant *Gm*GST3 meant a comparative analysis of recombinant *Gm*GST3 and peak six could not be performed. *Gm*GST2 did not correspond to any polypeptide isolated from the cell cultures and, as discussed, it is likely that all the other variants of *Gm*GST2 would co-migrate with recombinant *Gm*GST2 and fail to correspond exactly to any of the GST subunits purified from cell cultures or whole plants. It is possible that *Gm*GST2 undergoes post-translational modification in soybean. Post-translational modifications, including phosphorylation, methylation, glycosylation and acetylation have been reported for mammalian GSTs (Lopez *et al.*, 1994), with the glycosylation of plant GSTs recently reported (Gronwald and Plaisance, 1998). The definitive reason for such modifications is not known, but the process may be involved in proper folding, protection from protease degradation, improved solubility or recognition for cellular targeting (Gronwald and Plaisance, 1998). GSTs that undergo post-translational modification are likely to exhibit different hydrophobic properties, and thus their migration during RP-HPLC is likely to be altered.

7. Chapter Seven. Immunological Studies using Antisera Raised to Soybean GSTs.

7.1 Introduction.

The discovery of multiple GST polypeptides in chapter 4, and cDNA sequences in chapter 5 suggests that soybean probably contains a number of distinct GST isoenzymes. In this chapter the use of antibodies raised to soybean GSTs is detailed, allowing further characterisation of the polypeptides described in chapter 4 based on their immunological properties. Antiserum, termed Antibody T (ABT) was raised to the total *S*-hexylglutathione affinity bound GSTs from soybean cell cultures. In addition, individual antibodies were raised to the purified recombinant enzymes *Gm*GST1, *Gm*GST2 and *Gm*GST3, described in chapter 6, and these were termed AB1, AB2 and AB3 respectively. Antibodies were raised according to the procedure described in section 2.1.8, with two rabbits immunised per antigen. Western analysis performed using bleeds taken prior to immunisation showed that the rabbit serum did not contain antibodies which recognised soybean GSTs prior to immunisation. The sensitivity of the ABT antibody was assessed by Western blotting using *S*-hexyl glutathione purified GSTs from soybean cell cultures, the protein to which the antibody was raised. The sensitivity of antibodies AB1, AB2 and AB3 were kindly assessed by Dr Jane Bird, Zeneca Pharmaceuticals, using enzyme-linked immunosorbent assays (ELISA). The optimal working concentration of each primary antibody for Western blot analysis was determined as being a 1:2000 dilution of the crude antisera.

7.2 Results

7.2.1 Characterisation of soybean GST antibodies.

Each of the antibodies raised was assessed for cross-reactivity with the recombinant enzymes *Gm*GST1, *Gm*GST2, *Gm*GST2b and *Gm*GST3. In addition, each antibody was used to probe western blots of the total crude protein from 5-day-old soybean cell cultures and 5-day-old soybean roots and soybean shoots (Figure 7.1).
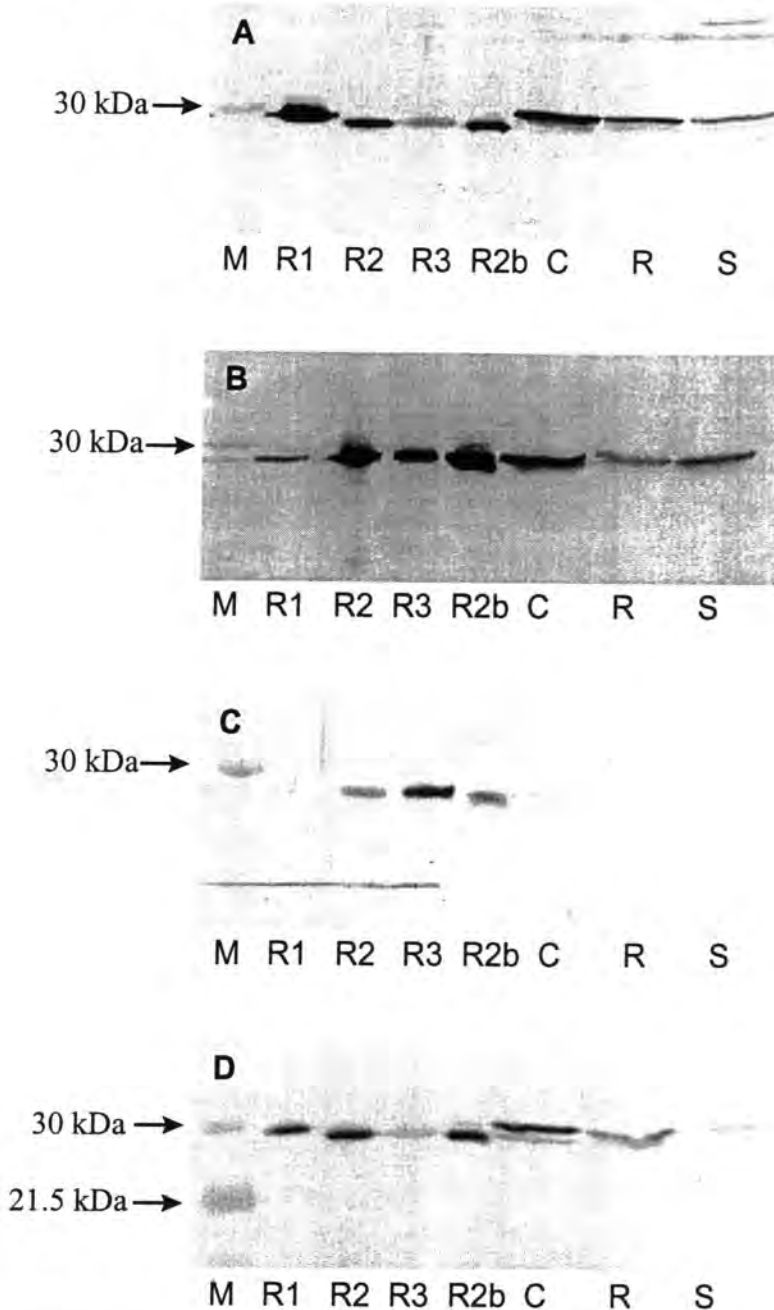
Figure 7.1 Western blot analysis using AB1 (A), AB2 (B) AB3 (C) ABT (D). M = molecular weight markers, R1 = recombinant *Gm*GST1, R2 = recombinant *Gm*GST2, R3 = recombinant *Gm*GST3 and R2b = recombinant *Gm*GST2b. Total protein extracts from soybean are shown as c) 5-day-old cell cultures, R) roots and S) shoots. Protein loadings were 50 ng of each recombinant protein and 10μg of total crude protein extracts.

As expected AB1(A) cross-reacted strongly with *Gm*GST1, the recombinant protein to which it was raised. In addition, it reacted with the *Gm*GST2-type GSTs, though detection of *Gm*GST3 was poor. Immuno-reactive polypeptides of $\approx$ 29 kDa and 28 kDa were clearly detected in cell cultures, and to a lesser degree in extracts from soybean shoots and roots. AB2 (B) reacted strongly with *Gm*GST2 to which it was raised and the close variant *Gm*GST2b. *Gm*GST1 was poorly recognised, however reactivity toward *Gm*GST3 was relatively good. Again, strong recognition of polypeptides of $\approx$ 29 kDa was apparent in cell cultures, and to a lesser extent in both shoots and roots. AB3 (C) showed relatively poor affinity toward any of the proteins tested. *Gm*GST3, the protein used to raise the antibody, was recognised strongest, with recognition of the *Gm*GST2 type proteins. This antibody reacted weakly with a polypeptide of similar mass to *Gm*GST3 present in the cell cultures but gave no reaction with any polypeptides in roots or foliage. ABT (D), raised to *S*-hexylglutathione purified GSTs from cell culture (section 4.2.1) reacted with *Gm*GST1 and *Gm*GST2. Immunologically reactive bands of 28 - 29kDa were detected in cell cultures, as expected, and to a lesser degree in roots and shoots. These results are summarised in Table 7.1.

Protein	ABT	AB1	AB2	AB3
*Gm*GST 1	+++	++++	+	-
*Gm*GST 2	+++	+++	++++	+
*Gm*GST 3		+	+++	++
*Gm*GST 2b	+++	+++	++++	+
Cell Culture	+++	+++	+++	+
Roots	++	++	+	-
Shoots	+	+	+	-

Table 7.1 Summary of cross-reactivity of AB1, AB2, AB3 and ABT with recombinant protein and crude soybean protein extracts. Relative cross-reactivity is given from no observed cross-reactivity (-) to strong cross-reactivity (++++).

7.2.2 Immunological characterisation of GSTs purified from soybean cell cultures.

Reversed-phase HPLC analysis of S-hexylglutathione purified GSTs from soybean cell cultures (Figure 4.11) identified the presence of 9 distinct putative GST subunits. Results presented in chapter 6 indicated that two recombinant enzymes, GmGST1 and GmGST3 co-chromatographed with two of these subunits. Western blots were performed using antibodies ABT, AB1, AB2 and AB3 in order to further characterise polypeptides resolved from soybean cell cultures.
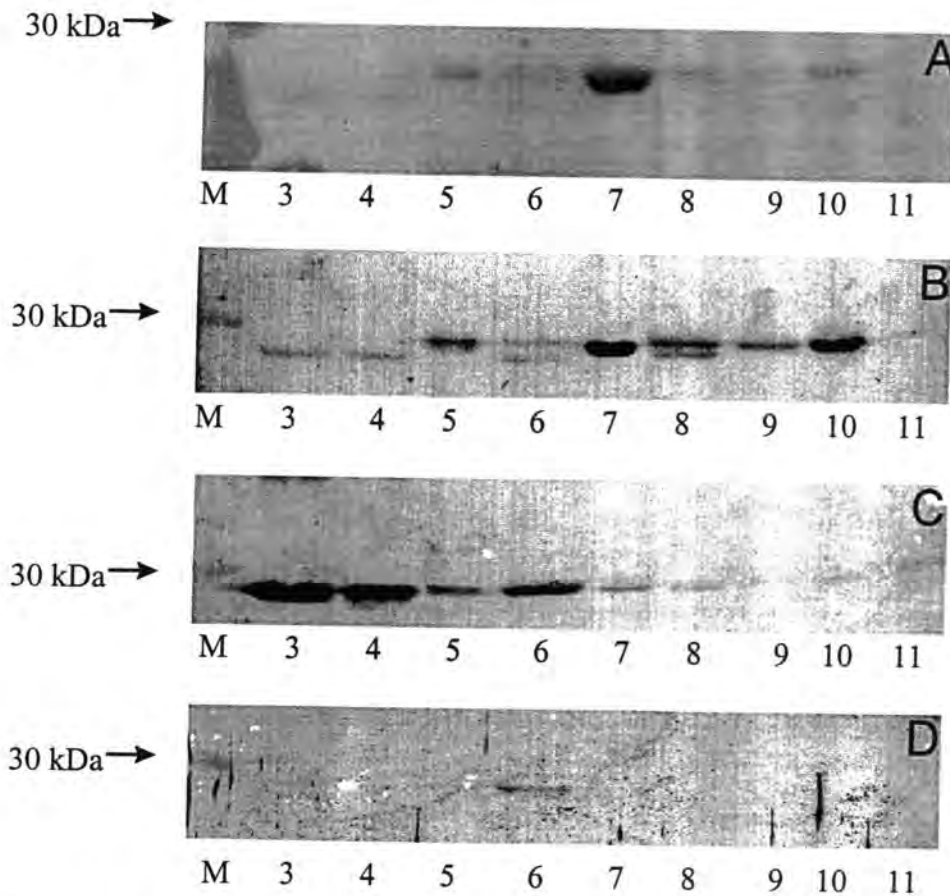
Figure 7.2 Western blot analysis of S-hexylglutathione purified GSTs from soybean cell suspension cultures resolved into the individual subunits indicated using antibodies ABT (A), AB1 (B), AB2 (C) and AB3 (D). Protein loading as shown in Figure 4.12. All primary antibodies were used at 1:2000 dilution and detected using a donkey anti-rabbit secondary conjugated to alkaline phosphatase.

Figure 7.2 clearly shows that all the RP-HPLC purified putative GSTs from soybean cell cultures have different immunological properties. Table 7.2 summarises the information obtained.

Peak	ABT	AB1	AB2	AB3
3	+	+	++++	-
4	+	+	++++	-
5	++	++	++	-
6	++	+	+++	+
7	++++	++++	++	-
8	++	++	++	-
9	+	++	-	-
10	++	++++	-	-
11	?	?	?	?

Table 7.2 Summary of the immunological properties of *S*-hexylglutathione purified GST polypeptides from 5-day-old cell soybean suspension cultures. Cross-reactivity is given from no observed cross-reactivity (-) to strong cross-reactivity (++++). (?) indicates insufficient protein available to perform analysis.

Eight of the nine polypeptides associated with these peaks could be identified using the different anti-GST sera, providing further evidence that they all indeed correspond to GST subunits. Results obtained using the antibody ABT suggest it is highly active toward peak 7, with some affinity toward polypeptides 3, 4, 5, 6, 8, 9 and 10. Since this antibody was raised to the total *S*-hexylglutathione purified population it is somewhat expected that this antibody recognises such a multitude of isoforms. The observation that peak seven reacts the strongest probably reflects the fact that this is the most abundant form in the population, as indicated in Figure 4.11. Polypeptide 11, the fomesafen active enzyme, was the only polypeptide not detected using the antisera. This probably reflects the very low loading of this protein on the gel, due to its low abundance, rather than the definitive non-reactivity of the polypeptide.

AB1 (B) showed strong affinity toward polypeptides 5, 7 and 10, with lower affinity toward polypeptide 3, 4, 6, 8 and 9. Thus, the immunoreactivity of AB1 resembled that of ABT. AB1 was raised toward recombinant *Gm*GST1, which has been shown to co-chromatograph with polypeptide 10 in chapter 6. This result therefore provides further evidence that one of the two polypeptides observed in peak 10 corresponds to *Gm*GST1. It also indicates immunological similarity between polypeptides 5, 7 and *Gm*GST1.

AB2 (C) showed strong affinity toward polypeptides 3, 4 and 6 and lower affinity toward polypeptides 5, 7 and 8, providing further evidence that polypeptides 3 and 4 are represent *Gm*GST2 type subunits. However, it has been shown in chapter six that neither polypeptides 3 or 4 correspond exactly to *Gm*GST2 when analysed by reversed-phase HPLC.

AB3 (D) exhibited weak affinity toward polypeptide 6, with none of the other proteins detected. Poor detection with this antibody was expected, since AB3 exhibited poor affinity toward recombinant *Gm*GST3, the protein to which it was raised (Figure 7.1). The finding that AB3 shows affinity toward polypeptide 6 provides further evidence that this is a *Gm*GST3 subunit, which was first indicated by their co-chromatography during reversed-phase HPLC reported in chapter 6. It is interesting to note that although peak 5 closely migrates with peak 6 during RP-HPLC, the two enzymes display very different immunological characteristics. Specifically, polypeptide 6 was identified using AB2 whereas polypeptide 5 was not. Conversely, polypeptide 5 was detected using AB1 whereas 6 was not. This finding suggests the amino-acid sequence of peak 5 is more closely related to *Gm*GST1.

7.2.3 Library screening using GST antisera.

Results shown in Figure 7.2 indicated that, with the exception of peak 11, all the polypeptides affinity purified from soybean cell cultures could be detected by using one or more of the soybean GST antibodies. Immuno-screening of cDNA expression libraries enables the isolation of specific cDNAs, based on the immunological characteristics of the expressed protein. cDNA expression in the plated phage was initiated by the placement of an IPTG-impregnated nitro-cellulose filter onto the plated phage during growth (section 2.4.2), which causes the induction of the T7 promoter in the pBluescript vector, and thus the expression of the cloned cDNA as a β-galactosidase fusion protein. Since the Stratagene λ-ZapII library is uni-directional, there is statistically a one in three chance that a desired cDNA will be in the correct reading frame, and will thus be expressed. This expressed protein adheres to the nitro-cellulose membrane, and can be detected using antibodies raised toward proteins of interest. Therefore expression library screening was performed using the different

soybean anti-GST-sera. All antibodies were used at 1:2000 dilution and primary, secondary and tertiary screens performed to isolate individual positive plaques.

Following screens with all the individual antisera, only two positive plaques were identified with the ABT antibody, which were termed ABT2 and ABT5. *In vivo* excisions were performed, plasmid recovered and the cDNAs sequenced as described previously. The sequence data showed that neither cDNA contained a GST like open reading frame, in fact ABT2 did not appear to contain any significant ORF, whereas clone ABT5 showed low homology to a pyrophosphate-dependant phosphofructo-1-kinase (accession U93272). A number of attempts to screen with AB1 and AB2 were unsuccessful, which was surprising given the strong cross-reactivity of these antibodies with soybean GST polypeptides (Figure 7.2).

7.3 Discussion.

The use of the various soybean GST antibodies in this chapter has assisted the characterisation of the putative GST subunits isolated from soybean cell cultures. Immuno-reactive proteins were detected in cell cultures, roots and shoots of soybean using antibodies raised to *Gm*GST1, *Gm*GST2 and *S*-hexylglutathione purified GSTs from cell cultures. Significantly, the expression of immunoreactive GST subunits was greatest in cell suspension cultures, which is in agreement with the biochemical data discussed in chapters 3 and 4. In addition, antibodies raised to *Gm*GST3 detected two 28-29 kDa polypeptides in cell cultures. The overall immunological response of *Gm*GST3 appears to have been rather poor, suggesting this polypeptide may exhibit poor antigenic properties.

The use of antibodies to characterise the purified putative soybean GST polypeptides described in chapter 4 suggests all the polypeptides identified are indeed GSTs, and has provided useful information as to the possible identity of the nine distinct putative GST subunits identified. Eight of the nine purified polypeptides were recognised using the various antibodies, with AB1 and AB2 showing different immunological characteristics. AB1 showed higher affinity toward polypeptides 5, 7, 8, 9 and 10, whereas AB2 showed affinity toward polypeptides 3, 4 and 6. Therefore, it is possible to partially classify these purified subunits based on their immunological reactivity profile with *Gm*GST1 and *Gm*GST2 antibodies. Such classification suggests that polypeptides 5, 7, 8 and 9 are related to *Gm*GST1, which is now thought to correspond to polypeptide 10, whereas polypeptides 3, 4 and 6 (polypeptide six = *Gm*GST3) show homology toward *Gm*GST2. It is interesting to note that the phylogenetic tree of all known plant GST cDNA sequences, shown in Figure 5.24, indicates that *Gm*GST2 and *Gm*GST3 are closely related and belong to the same phylogenetic group, suggesting they may also be immunologically related. *Gm*GST1 however is seen to belong to another branch of the tree, therefore providing a possible explanation as to why antisera raised to this protein show distinct characteristics to *Gm*GST2 and *Gm*GST3.

Much evidence now exists to suggest that polypeptide 6 and 10 correspond to recombinant enzymes *Gm*GST3 and *Gm*GST1 respectively. However, the identity of *Gm*GST2 in soybean remains elusive, which is surprising given the apparent

abundance of the gene transcript in the library. RP-HPLC data presented in chapter 6 showed that this recombinant enzyme does not exactly correspond to any of the polypeptides purified from soybean cell cultures. However polypeptides 3 and 4, which migrated most similarly to *Gm*GST2, are clearly immunologically related to the recombinant protein. This may suggest that in plants *Gm*GST2 undergoes post-translational modification to yield polypeptides 3 and 4. Such modification is likely to result in proteins with similar immunological characteristics, but with altered RP-HPLC retention properties, due to changes in the hydrophobicity of the enzyme. It is unlikely that polypeptides 3 and 4 correspond to the *Gm*GST2 genetic variants described in chapter 5, since the corresponding recombinant enzymes to these variants have been shown to migrate identically following RP-HPLC. Alternatively, it may be that polypeptides 3 and 4 correspond to as yet unidentified GSTs, which probably show protein sequence homology to *Gm*GST2.

The reason for the poor results obtained with immunoscreening of the cDNA expression library is not understood, given the number of GST subunits immunologically detected in the plant organs used to construct library. However, the cDNA sequences of soybean GSTs presented in chapter 5 shows that many of the cDNAs described (*GmGST2*, *GSTCA2*, *GSTCA5* and *GSTCON33a*) contained at least one in-frame translational stop codon in the 5' untranslated region (UTR). This will result in the termination of the translation of the β-galactosidase fusion protein in the 5' UTR of the cDNA, and consequently no translation of the GST cDNA itself. If characteristic of other soybean GSTs, this observation may indicate why library screening using the antibodies raised to GSTs was unsuccessful.

8. Chapter Eight. Additional and Future Work.

8.1 Transgenic expression of Soybean GSTs in tobacco.

The role of GSTs in herbicide selectivity is well understood, and has been discussed at length in this thesis. It is generally regarded that the increasing pressure on world food production, as a result of a growing world population, is likely to dictate that pesticides, and biotechnology continue to play an important role in agriculture well into the next century. Thus, the requirement for effective weed control will dictate the development of effective selective herbicides. Modern herbicides, such as glyphosate (RoundUp™) and glufosinate (Liberty™) are high efficacy compounds that possess a good registration portfolio. However, the major agricultural disadvantage of these compounds is that they are non-selective, indiscriminately killing both weeds and crop species. This problem has been addressed and overcome with the use of biotechnology, by creating genetically modified plants containing an appropriate herbicide resistance gene. Consequently, products such as Roundup Ready™ and Liberty Link™ crops are now available that are resistant to glyphosate and glufosinate respectively.

With conventional selective herbicides, such as the chloroacetanilides, selectivity may require the use of a herbicide safeners to enhance selectivity by increasing the levels of specific GST isoforms within the crop which show activity toward that particular herbicide (Jepson *et al.*, 1994). An alternative to the use of herbicide safeners would be to genetically modify the crop plant to over-express the specific safener-inducible GST isoforms. Engineering of GSTs with activity toward herbicides into normally susceptible species would also be achievable. Indeed it has been shown that the over-expression of the maize GST *Zm*GST-IV in tobacco confers herbicide resistance to chloroacetanilide in this normally susceptible species (Jepson *et al.*, 1997). In addition to herbicide detoxification, the glutathione peroxidase activity exhibited by many plant GSTs may be of added agronomic value. Roxas *et al.* (1997) over-expressed the tobacco GST *Nt*-107, which also possesses glutathione peroxidase activity, in transgenic tobacco. The transgenic seedlings were found to exhibit higher levels of tolerance toward both salt and cold stresses. Crop resistance to such stresses is an important consideration in agriculture since the low soil temperatures early in the

growing season and heavily irrigated soils, which often contain high levels of salt, can severely affect seed germination and thus crop productivity (Roxas *et al.*, 1997).

In addition to their agronomic value, herbicide resistance genes are increasingly being used as selectable markers in transgenic plants. Selectable markers are co-introduced into a transgenic plant with the gene of interest to allow specific selection of transgenic plants. Traditional selectable markers include antibiotic resistance genes, which are undesirable in commercial products, due mainly to public concern regarding the fate of such genes in the environment. Thus, herbicide resistance genes are able to operate as selectable markers by rendering the transgenic material resistance to herbicide treatment. For this reason it is hypothesised that GSTs may utilised as novel selectable markers. Therefore, it was of interest to explore the benefits of introducing soybean GSTs into transgenic plants. The aim of the work initiated here was to establish whether a tau-type GST obtained from soybean could provide useful herbicide and stress tolerance characteristics when heterologously expressed in transgenic plants.

8.2 Results.

8.2.1 Heterologous expression of *Gm*GST2b in transgenic tobacco.

The soybean GST *Gm*GST2, described in chapters 5 and 6, possessed GST activity toward a spectrum of herbicide substrates and additionally showed relatively high glutathione peroxidase activity. In this study, the cDNA chosen for expression in transgenic tobacco was the variant *GmGST2b* (shown in Figure 5.15), due to non-published findings that indicated this isoenzyme may possess a slightly enhanced activity spectrum (Personal communication, Dr Mark Skipsey). *Gm*GST2b contains significant amount of both GST and glutathione peroxidase activity, exhibits GST activity toward a number of herbicides owning their selectivity to GSTs and will be expressed in transgenic tobacco using an optimised plant expression vector. This last point is of particular importance. Due to the different assay procedures used by different groups to characterise GST and glutathione peroxidase activity it is difficult to make direct comparisons between published data. For example, Roxas *et al.* (1997) only reported the GPOX activity of *Nt* 107 in crude bacterial lysates, with no

indication as to the level of expression, making comparison of its activities with purified *Gm*GST2b difficult. In the study with *Gm*GST2b, the double enhanced CaMV35S promoter with the Tobacco Mosaic Virus (TMV) omega leader sequence was used for expression studies. This vector has been shown to increase expression of transgenes by up to 5 times compared with expression using the single CaMV35S promoter in tobacco (Gallie *et al.*, 1987). Therefore, low activity levels of *Gm*GST2b toward certain substrates may be compensated for by increased expression levels. Tobacco was chosen as the model system due to its susceptibility to herbicides, coupled with a relatively easy transformation procedure.

8.2.2 Plant binary vector design.

Oligonucleotide primers MS27*Nco*1 (5' GCG CCA TGG CAG ATG AGG TGG TTC 3') and M13FL were used in PCR experiments to engineer the *Nco* I and *Kpn* I restriction enzyme sites required for the subsequent cloning of the *GmGST2b* cDNA. A 50μl PCR reaction was performed according to section 2.7. 15 cycles were used to reduce the risk of PCR error and an annealing temperature of 51°C used.

The resulting PCR product was purified on a 1% TAE agarose gel and recovered according to the protocol described in section 2.1.10. The DNA was digested overnight using *Nco* I and *Kpn* I in Multicore™ reaction buffer (Promega) and re-purified before ligating into vector pMJB1 (section 2.2.1), and transformed into XL1-Blue MRF' electrocompetent cells. Transformed colonies were selected on LB-Agar plate containing 100 μg ml⁻¹ ampicillin and colonies obtained were analysed by PCR (MS27*Nco*1, OG2, 35 cycles). PCR positive colonies were used to inoculate a 5 ml overnight from which plasmid was recovered and re-sequenced to check for any PCR errors introduced. This plasmid was termed pMJB-*GmGST2b*.

Construction of Binary Transformation Vector.

The CaMV35S:Ω:*GmGST2b*:*nos* cassette was sub-cloned from pMJB-*GmGST2b* into the plant binary transformation vector pJR1i (section 2.2.1) as a *Hind* III / *Eco* R1 fragment using methods described previously. The plasmid was transformed into electrocompetent *E. coli* XL1-Blue MRF' cells and transformed colonies selected on LB agar plates containing 50 μg ml⁻¹ kanamycin. Plasmid recovered from transformed

colonies were checked for authenticity by PCR and automated sequencing. This plasmid was termed pB*GmGST2b*.

Agrobacterium Transformation

pB*Gm*GST2b was transformed into *Agrobacterium tumefaciens* strain LBA4404 using the freeze thaw process and transformed bacteria selected on LB media containing 100 μg ml^{-1} rifampicin, 100 μg ml^{-1} kanamycin and 500 μg ml^{-1} streptomycin. Plasmid was recovered from transformed colonies and digested using *Eco*R1 / *Hind* III to check the authenticity of the binary vector (Figure 8.1).
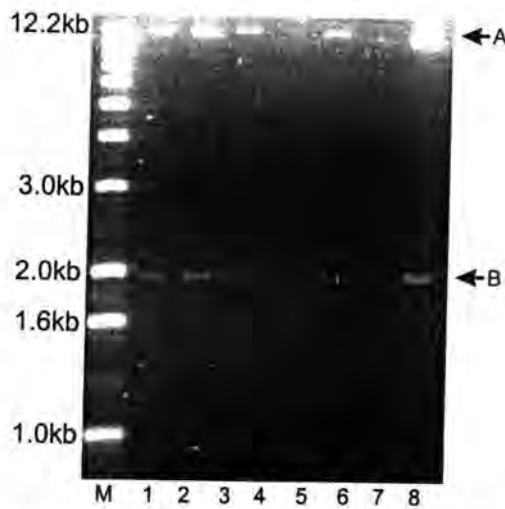
Figure 8.1 *Hind* III / *Eco*R1 digest of plasmid pB*Gm*GST2b showing ≈2.0kb 2xCaMV35S:Ω: *Gm*GST2b:*nos* fragment (B) cut from the pJR1i vector (A).

8.2.3 Tobacco transformation.

A single *Agrobacterium* colony containing the checked insert was used to initiate an overnight culture that was to be used for the tobacco transformation. Following selection on media containing kanamycin, 15 plantlets rooted and were transferred to soil in the glasshouse.

8.2.4 Analysis of transgenic plants.

Leaf samples were taken form the each plantlet and protein extractions made using the method described in section 2.1.9.2. Protein concentrations were standardised to 1 mg

ml^{-1} western blot analysis performed on the extracts using the antibody AB2 (Figure 8.2).
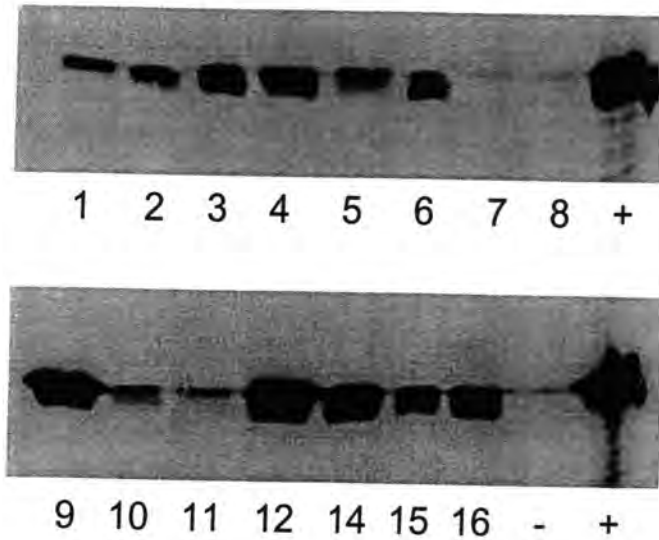
Figure 8.2 Western blot of crude protein extracts obtained from transgenic plants containing the pB*Gm*GST2a gene. 10µg crude protein and 50ng recombinant *Gm*GST2 (+) were loaded. (-) indicated wild-type tobacco extract.

The transgenic plants created will now be further studied and their tolerance to both herbicide and stress treatment determined. The effectiveness of soybean GSTs in tobacco may be limited by the lack of homoglutathione, which may be especially apparent in tolerance to the diphenyl ether herbicides fomesafen and acifluorfen.

8.3 Future Work

Whilst this thesis described much novel information regarding the GSTs in soybean, the sheer scope of the project dictated that it simply was not possible to undertake a complete and thorough analysis of every aspect of soybean GSTs. The work described below outlines follow up work from this thesis that may be of interest.

1. Full evaluation of transgenic tobacco lines generated including *in vitro* GST / GPOX assays to identify high expressing lines. These studies would include herbicide spray trials and stress experiments to determine the effect of transgene expression on tolerance to biotic and abiotic stress.

2. Further characterisation of GSTs purified from soybean cell cultures. Protein sequencing of the N-terminal region of these proteins was not possible since they were blocked. Therefore peptide digest/cleavage may facilitate the sequencing of internal regions which could then be used to design degenerate oligonucleotide primers suitable for library screening. This approach has proved successful in the study of maize GSTs (Holt *et al.*, 1995). Additionally, it would be desirable to purify soybean GSTs directly involved in herbicide detoxification, such as the fomesafen active enzyme containing subunit 11. This may be achieved by using higher performance purification chromatography, such as FPLC, or the use of further affinity matrices.

3. Clone and express the cDNAs identified in chapter 5 to determine to which, if any, of the polypeptides purified form soybean cell cultures in chapter 4 they correspond. Antibody data suggests that polypeptides 3 and 4 are immunologically related to *Gm*GST2 and that subunits 5, 7, 8 and 9 are related to *Gm*GST1. Like *Gm*GST1 and *Gm*GST2, all the new soybean GST cDNA sequences described in chapter 5 are tau-type GSTs. Therefore, there is some evidence that some of the purified soybean GSTs which remain unidentified will be encoded by the new cDNAs described in chapter 5.

4. Characterise the GSTs responsible for herbicide detoxification which do not bind to the hydrophobic interaction column described in chapter 4.

5. Make further use of the recombinant GSTs to characterise fully the differing activities of soybean GSTs toward different substrates and thiols using more complex kinetic models.

6. Determine, with the use of antibodies and reversed-phase HPLC, which soybean GSTs are upregulated following the herbicide or safener treatments described in chapter 3.

7. Determine the regulation of specific GST isoforms in plants. Due to the limitations of time it has not been possible to study expression of specific GSTs at the molecular and biochemical level. GSTs have been shown to be differentially expressed at various points in plant development and in response to diverse stimuli (hormones, chemical stress, infection). Therefore it will be of interest to determine expression in different tissues at different developmental stages or exposed to biotic and abiotic stress with the use of northern blots and / or antibodies. It will also be of interest to determine the sub-cellular localisation of the various GST isoforms described, especially since GST activity in soybean has been recently identified in the apoplastic space (Flury *et al.*, 1996). It will also been of interest to see whether changes in GST activity occur during legume specific processes, such as nodulation.

9. Chapter Nine: Concluding Remarks.

Detailed information regarding the biochemical, molecular and physiological characteristics of GSTs in soybean has been presented in this thesis. Inferences drawn from these results suggest that the GST biology in soybean is amongst the most complex of any plant species studied to date. Soybean GSTs have been implicated in the selectivity of a number of herbicides used in soybean, including diphenyl ether, sulphonylurea and chloroacetanilide compounds. Purification and biochemical analysis of the individual GSTs present active toward these herbicide substrates identified a number of GST subunits, of similar molecular mass and isoelectric point. However, successful resolution of the native enzymes by anion-exchange chromatography proved impossible. Identification of individual polypeptides was only achieved using RP-HPLC, with subsequent MALDI-TOF MS and SDS-PAGE analysis identifying 9 putative GST subunits. Individual GST activities could not be assigned to these enzymes, due to their denaturation during the purification process. This also prevented an assessment of dimerisation characteristics of the enzymes. However, further molecular and immunological data obtained provided evidence that all were in fact GSTs.

The purification results presented in chapter 4 suggested that soybean almost certainly contains additional GST subunits to the 9 detailed in this thesis. It was apparent that *S*-hexylglutathione was a useful purification matrix with which to purify soybean GSTs, but it was noted that GSTs active toward certain substrates remained unbound. Alternative purification strategies, utilising additional matrices showed no improvement in performance. It is possible that despite the wide range of GST activities monitored, additional activities may have remained undetected. Furthermore, Flury *et al.* (1996) describe the presence of extracellular GST activity in soybean following auxin treatment. The authors hypothesise that these extracellular GSTs may be acting as glutathione peroxidases, limiting oxidative stress following the oxidative burst responses invoked by pathogen infection (Alvarez *et al.*, 1998). However, the extracellular fluid in the cell suspension cultures was not examined for GST activity in this study.

In addition to the biochemical characterisation, 6 soybean cDNAs, which encoded distinct GSTs were obtained and 2 of these GSTs, *Gm*GST1 and *Gm*GST3 were

shown to correspond to GST subunits expressed in soybean cell cultures. Immunological and chromatographic characteristics of the unidentified polypeptides 3, 4, 5, 7, 8 and 9 suggest they all belong to the tau-class of plant GSTs. It is not unreasonable to expect that the gene products from the other four tau-type cDNAs identified, *GSTCON26a*, *GSTCON31a*, *GSTCON32* and *GSTCON33a* may correspond to some of these unidentified subunits. A number of cDNA variants encoding *Gm*GST1 and *Gm*GST2 were observed, which showed high overall homology but minor, though significant variation. It is not known whether these variants may result in enzymes with subtle differences in catalytic activity, or simply result from the complex genome of soybean.

It is interesting that all the cDNAs obtained in this study belonged to the GST tau-class, which to date have only been identified in plants. Is it possible that the tau class, with its broad multi-functional activity is characteristic of, and unique to plants? Species specific GSTs are known, such as the sigma class which is only found in the lenses of squid. Undoubtedly, further investigation in a number of species is required before firm conclusions can be made. However, it was surprising that theta-type GSTs were not identified in soybean. Both molecular and biochemical strategies, designed specifically to identify theta-type enzymes, were unsuccessful. It may be that the lack of theta type GSTs may explain soybean's sensitivity to atrazine, which is known to be detoxified in part by this class of enzyme in maize (Dixon *et al.*, 1997). However, knowledge obtained from other plant species suggest that it is unreasonable to assume that soybean does not contain theta-, and even zeta-type GSTs.

Table 9.1 summarises all the results obtained concerning soybean GSTs described in this thesis.

Table 9.1. Summary of all information regarding attempts to summarise all the information gained from purifying GST subunits from soybean cell cultures described within this thesis. ND = Not-determined. MALDI-TOF data represents the most abundant peak observed in each purified fraction. [1] Maldi-Tof and SDS-PAGE analysis of Sub-unit 10 showed the presence of two polypeptides. [2]Inferences drawn from cross-reactivity with antibodies. Relative abundance / cross-reactivity are given from zero (-) to high (++++).

Subunit	MW	Relative Abundance		Antibody Characteristics						Notes
	MALDI-TOF	Whole Plants	Cell Cultures	AB1	AB2	AB3	ABT	ID		
3	ND	++	+++	+	++++	-	+	ND		Related to *Gm*GST2[2]
4	25,459	+++	+++	+	++++	-	+	ND		Related to *Gm*GST2[2]
5	25,844	+	++	++	++	-	++	ND		Related to *Gm*GST1[2]
6	ND	+	+++	+	+++	+	++	*Gm*GST3		
7	25,733	++	++++	++++	++	-	++++	ND		Related to *Gm*GST1[2]
8	26,535	+	+++	++	++	-	++	ND		
9	25,953	++	++	++	-	-	+	ND		
10a[1]	24,928	++	++					ND		Related to *Gm*GST1[2]
10b[1]	25,909	++	++	++++	-	-	++	*Gm*GST1		Multi-variant sequences observed
11	ND	+	-/+	ND	ND	ND	ND	ND		Active toward fomesafen/ acifluorfen

Heterologus expression of *Gm*GST2 and *Gm*GST3 in *E. coli* showed both recombinant enzymes possessed additional glutathione peroxidase activity. This finding adds to mounting experimental evidence that an important endogenous function of plant GSTs may be to protect cells from damage imposed by oxidative stress. It is known that many inducers of GSTs in plants have the ability to cause oxidative stress, for example wounding, exposure to ozone, ethylene, heavy metals, or pathogen attack. Many plant GSTs are characterised by their induction by auxin and it is of particular note that the treatment of soybean with 2,3,5-triiodobenzoic acid (TIBA), a compound which causes auxin accumulation in plants, results in the production of H_2O_2 and O^{2-}, with a subsequent increase in GST activity (Flury *et al.*, 1998). Additionally, exogenously applied H_2O_2 to soybean cell cultures was seen to induce the accumulation of GST transcripts (Tenhaken *et al.*, 1995). Treatment of the plants with antioxidants ascorbate and glutathione prior to TIBA treatment reduced the GST induction, suggesting that it was in fact the oxidative stress was responsible for the induction. The time-scale of reactive oxygen species production was similar to the oxidative burst observed during pathogen response (Levine *et al.*, 1994) and it was postulated that both auxin treatment and pathogen attack impose oxidative stress in a similar manner. This observation suggested that oxidative stress may be a common signalling mechanism for the variety of biotic and abiotic treatments able to enhance GSTs in plants, many of which show additional glutathione peroxidase activity. In physiological terms such induction could be involved in the spatial limitation of hypersensitive cell death, imposed by the oxidative burst which follows pathogen infection or fungal elicitation (Flury *et al.*, 1998). Ulmasov *et al* (1995) propose that an "*ocs*" element, located in the promoter region of many auxin-inducible genes, is responsive to oxidative stress and that it is this, rather than auxin *per se* that is responsible for the induction. This "*ocs*" site is thought analogous to the "*AP-1*" site that is thought to be responsive to oxidative stress located in many mammalian GSTs (Daniel, 1993). Indeed a common oxidative stress signalling pathway is thought to be responsible for GST induction by structurally diverse compounds in mammals (Daniel, 1993). However, evidence also exists to suggest oxidative stress may not in fact be a common signalling pathway in plants. Differential GST induction has been shown in wheat, where xenobiotic treatments such as cadmium and paraquat, both of

which are known to impose oxidative stress in plants, induced different isoforms to those induced by pathogen attack, suggesting that induction mechanism is more complex (Mauch and Dudler, 1993). Also, a GST in carnation has been identified that is upregulated during senescence. At first this may suggest that oxidative stress during senescence is responsible for the induction. However, it is somewhat perplexing that a plant is mounting a defence response to prevent this process of programmed cell death, unless it is in some way regulatory. The discovery of ethylene responsive elements within the carnation GST promoter suggests that this may indeed be the case (Itzhaki *et al.*, 1994).

All plant GSTs described to date show greatest homology to the archaic theta-class. This class is thought to be the evolutionary fore runner of all GST classes, and it has been proposed that the theta-GST class may have originally evolved in prokaryotes to protect against oxidative stress (Pemble and Taylor, 1992). Therefore it is possible that a primary function of plant GSTs, like mammalian GSTs, is the detoxification of lipid hydroperoxides generated by reactive oxygen species during oxidative stress (Levine *et al.*, 1994). In fact, close similarities appear to exist between many plant GSTs and the alpha-class GSTs in mammals, for which endogenous substrates are not well defined but seem to protect against oxidative stress by detoxifying reactive products generated by lipid peroxidation (Daniel, 1993). Experimental evidence suggests that GST regulation in plants may be linked to oxidative stress, and there is much evidence from studies in soybean to support this hypothesis. It is known that herbicide safeners, able to enhance specific herbicide detoxifying GSTs, also cause stress, indicated by growth inhibition of developing seedlings following safener treatment (Fuerst and Gronwald, 1986). The primary function of the induced GST may therefore be to protect against lipid peroxidation products generated by such stress. It may be purely coincidental that the safener-induced GSTs are able to catalyse herbicide detoxification. In order to confirm this hypothesis it will be of interest to determine whether other abiotic stresses are able to protect plants from herbicide treatment. It will also be interesting to extend the herbicide and herbicide safener study described in this thesis. It was shown that increased activity toward CDNB could be observed in soybean plants following treatment with certain herbicides or safeners. However, induction of activity toward herbicide substrates was

not observed. This differential regulation is intriguing and it will be of interest to determine whether the isoforms induced which have activity toward CDNB also show glutathione peroxidase activity.

The discovery of the secondary function of many GSTs as glutathione peroxidases is perhaps the most intriguing observation of these enzymes. Much debate has centred on the biological activity of plant GSTs, with the only report of specific function being their involvement in anthocyanin sequestration in the vacuole (Marrs *et al.*, 1995). However, glutathione conjugates of these natural products have never been reported. Indeed, other biological molecules, such as many auxin and flavonoid derivatives which have been shown to be inhibitors of GSTs, have not been reported to undergo glutathione conjugation, nor do they contain an electrophilic group suitable for such reaction. It may be that the Bronze-2 GST does not in fact catalyse the formation of glutathione *S*-linked conjugates, but acts as a carrier which binds, and presents natural products to the vacuole transporter where they are transported in the presence of glutathione. Could it be that the initial identification of GSTs as enzymes able to catalyse the formation of *S*-linked glutathione conjugates may in fact be confusing the primary endogenous function of these enzymes *in planta*? The quiescent nature of plants dictates that they must be capable of adapting to a diverse range of environmental stresses and toxins in order to survive. GSTs, and in particular the tau-class, may have evolved in plants to act as general detoxification or stress reducing enzymes which may explain their abundance in the cytosol. This hypothesis may also explain why the active "H-site" in many plants GSTs is generally larger than that in mammalian enzymes (Neuefeind *et al.*, 1997a), indicating the more diverse substrates acceptable by plant enzymes. Plants GSTs are traditionally assessed using *in vitro* assays toward xenobiotic substrates. However the possibility that their primary biological function may in fact be related to their glutathione peroxidase activity or as a binding or carrier protein should not be overlooked.

An important finding of this research is that for the first time it has been shown that plant GSTs exhibit thiol specificity in addition to substrate specificity. With respect to soybean, this finding suggests that the presence of homoglutathione may be fundamentally important in the metabolism, and thus selectivity of the diphenyl ether herbicides fomesafen and acifluorfen. Certainly detailed kinetic analysis is required

on the isoforms reported in this thesis to further explore this hypothesis. The reason for the apparent affinity of certain isoforms for homoglutathione is not understood. However, the report of an induced fit mechanism of substrate binding reported for maize GSTs (Neuefeind *et al.*, 1997a) may explain the observations. It may be that, with respect to fomesafen and acifluorfen, the binding of homoglutathione results in a structural reconfiguration that allows the preferential binding of these diphenyl ether compounds. Conversely, the binding of glutathione may constrain the binding of the co-substrate, and thus decrease catalytic activity. A study of *Gm*GST1 showed that ternary complex formation is part of the reaction mechanism, and the binding of substrates takes place in random order (McGonigle and O'Keefe, 1998b). In the case of chlorimuron-ethyl conjugation rates with homoglutathione exceeded those with glutathione, since a high dissociation constant made the formation of a glutathione containing ternary complex unfavourable. The opposite was observed with alachlor, whereby conjugation rates were higher in the presence of glutathione. It will be of interest to see whether the strong preference of the diphenyl ether herbicides for homoglutathione conjugation is due to a more favourable ternary complex with homoglutathione.

The discovery of this specificity may be of fundamental importance when considering herbicide selectivity in other major crop species, since other plant species, including maize and wheat have both been shown to contain glutathione variants (Hell, 1997; Klapheck *et al.*, 1992). Many herbicides used to control weeds in cereals owe their selectivity to GSTs, and it will be of interest to re-visit herbicide metabolism in these species to determine whether similar thiol preferences are apparent. Crude extracts of maize have been shown to exhibit GST activity toward fomesafen when assayed with homoglutathione but not glutathione (Skipsey *et al.*, 1997). Fomesafen activity is not normally associated with maize, since the herbicide is not selective in this species. This data suggests that maize GSTs are able to conjugate fomesafen in the presence of a thiol not normally encountered. This raises an interesting possibility that the presence of particular thiols in plants may be just as important in herbicide selectivity as specific GST isoforms. Little work has been reported regarding the selectivity of plant GSTs for different glutathione derivatives. Differences in selectivity of different thiols, including homoglutathione, have been studied in mammals where significant

differences in affinity were seen (Adang *et al.*, 1988). However, in this study none of the activities tested were increased with homoglutathione, suggesting it was in fact a much poorer co-substrate than glutathione. The biological reason for these the variation in thiols between species is not understood. Since no homoglutathione conjugated natural products have been identified *in vivo*, it is difficult to hypothesise the evolutionary pressure on soybean to synthesise homoglutathione instead of glutathione. Of course, it may be that the presence of homoglutathione is not linked to GST affinity, and is more dependant on substrate availability in soybean, or its requirement in some other biological function.

Despite the increasing numbers of plant GSTs reported their conclusive endogenous biological function remains elusive. The finding that the total GST complement of some plant species may constitute as much as 2% of the total soluble protein (Sari Gorla *et al.*, 1993) suggests they play a fundamental role in plant biochemistry. The data in this thesis provides further evidence that soybean, like many other plant species, contains multiple GST isoenzymes, indicating they probably play a fundamental importance in the well being of plants. The exploration of thiol specificity, and determination of endogenous function and regulation remain the major goals of plant GST research and hopefully, with the increasing numbers of enzymes being reported these functions will at last be elucidated.

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