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# *The Activities Of Herbicide Safeners In Wheat (Triticum aestivum L)*

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## Abstract

Commercialised herbicide safeners (also known as protectants or antidotes) are synthetic chemicals used to enhance herbicide tolerance in cereal crops. They do this by causing an up-regulation in xenobiotic detoxifying enzymes such as glutathione transferases (GSTs). Seedlings of wheat (*Triticum aestivum* cv 'Einstein') were sprayed with the safeners cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl. All three compounds caused an identical up regulation of GSTs from the phi, tau and lambda classes, despite their differences in chemistry. Using cloquintocet mexyl as a classic wheat safener treatment, it was found that GST induction was both dose and time dependent. Safening was found to be associated with the rapid hydrolysis of the parent ester to cloquintocet acid. When the free acid was tested, the GST-induction response obtained was identical to that determined with the parent ester, suggesting that cloquintocet itself is the active safener. GST induction was found to be tissue specific within the wheat shoots, with the lambda GSTs being preferentially expressed in the meristematic tissue. Proteomic 2 D gel analysis revealed that the tau *TaGSTU3* was a major up-regulated GST. In addition, six GSTs that were previously shown in literature to be up-regulated by herbicide safeners in wheat were cloned, expressed and

characterized as the respective recombinant enzymes and renamed to bring them in line with existing nomenclature. The GSTs cloned included *TaGSTU3*, *TaGSTU6*, *TaGSTF4*, *TaGSTF10* and *TaGSTL1*. Metabolism studies showed that following the hydrolysis of cloquintocet mexyl, no further downstream metabolites could be identified and none of the up-regulated GSTs showed any activity toward the safener. However *TaGSTU3* was found to bind and be inhibited by cloquintocet free acid as determined by isothermal titration calorimetry. Safener treatment also led to a transient inhibition of GST activity in crude wheat extracts after spraying the seedlings. In addition to the induction of GSTs, safener treatments also resulted in an enhanced growth of wheat seedlings. The work presented in this thesis confirms that very different compounds can induce apparently identical downstream events at the level of GST enhancement and that these induction events underpin wider changes in plant physiology.

The Activities Of Herbicide  
Safeners In Wheat (*Triticum  
aestivum* L)

Victoria Louise Taylor

PhD Thesis

2012

Department Of Chemistry  
Durham University

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Publications Arising From Work Described In This Thesis

Taylor.VL, Cummins.I, Brazier-Hicks.M, Edwards.E  
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For mam and dad  
Thank-you and I love you

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## Abbreviations

ACCase:	acetyl coenzyme A carboxylase
ALS:	acetolactate synthase
AMP:	ampicillan
BCA:	bicinchronic acid
BCIP:	5-bromo-4-chloro-3-indolyl phosphate
BITC:	benzyl isothiocyanate
DMF:	<i>N,N,N',N'</i> -dimethylformamide
cDNA:	complementary deoxyribonucleic acid
CDNB:	1-chloro-2, 4-dinitrobenzene
CMTP:	4-chloro-6-(methylthio)-phenylpyrimidine
CYPs:	cytochrome P <sub>450</sub> – dependant mono_oxygenases
DCNB:	1,2-dichloro-4-nitrobenzene
DHAR:	dehydroascorbate reductases
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
EPTC:	S-ethyl dipropylcarbamoate
FAD:	flavin dinucleotide
FC:	S-(fenclozim)-cysteine conjugate
GAPDH:	glyceraldehydes-3-phosphate dehydrogenase
GPOX:	glutathione peroxidase
GSH:	glutathione
GSSG:	reduced glutathione
GST:	glutathione transferase
GSTF:	phi class glutathione transferase
GSTL:	lambda class glutathione transferase
GSTU:	tau class glutathione transferase
GSTT:	theta class glutathione transferase
GSTZ:	zeta class glutathione transferase
HEPES:	hydroxyethyl piperazineethanesulfonic acid

HPLC:	high pressure liquid chromatography
IAA:	indole-3-acetic acid
IC <sub>50</sub> :	half maximal inhibitory concentration
IPTG:	isopropyl-beta-D-thiogalactopyranoside
ITC:	isothermal calorimeter
IUPAC:	international union of pure and applied chemistry
LC:	liquid chromatography
MALDI :	matrix assisted laser desorption ionisation
mRNA:	messenger ribonucleic acid
MS:	mass spectroscopy
NADP:	nicotinamide adenine dinucleotide phosphate
NBC:	p-nitrobenzyl chloride
PCR:	polymerase chain reaction
PAGE:	polyacrylamide gel electrophoresis
RNA:	ribonucleic acid
RT-PCR:	real time polymerase chain reaction
RUBISCO:	Ribulose-1,5-bisphosphate carboxylase oxygenase
SDS:	sodium dodecyl sulphate
TAE:	tris-acetate
TBS:	tris buffered saline
TEMED:	tetramethylethylenediamine
TOF:	time of flight
UGTs:	glucosyltransferases

## Chapter One: Introduction

### 1.1 Overview

Grass weeds are the biggest threat to cereal crop yield in the U.K. An estimated 1.2 M hectares of land is infested with wild grasses, with estimated yield losses from black grass alone in wheat crops of 26 % if left untreated (Syngenta, 2010). Further more, losses in crop yield and herbicide resistance in weeds are increasing. This poses a problem for future crop sustainability. Land is a finite resource and therefore increasing productivity is one of the only viable options available to us at present. Intensive agricultural practises are needed at present to meet the demands of a growing population, and this requires the extensive use of agrochemicals.

Agrochemicals are continually subjected to media scrutinisation, especially since there is a growing trend in organic farming. There are fears about the safety and metabolic fate of agrochemicals, and this is understandable, but it also highlights a need for further research in the area. Without the use of agrochemicals crop yields and quality would plummet and we would be forced to change our predominantly urban lifestyles to one that revolves around rural agriculture.

After maize and rice, wheat is the third most produced cereal globally with estimated yields in the U.K of 15 M tonnes each year (UK agriculture, 2010). A total of 2 M hectares is cultivated with a value of 1.2 billion pounds, with 25 % of wheat grown in the U.K exported and 40 % used as animal feed. The remaining 35 % is used in various products and foods (UK agriculture, 2010).

Resistance of grass weeds to herbicides, with differing modes of action based on enhanced detoxification is compromising selective chemical weed control and poses a serious threat to this major cereal crop (Holt *et al.*, 1993). It is of vital importance to elucidate further the molecular mechanisms of xenobiotic detoxification within plants as this research can lead to the development of new weed control measures and can be used as a tool to develop new herbicides for multiple - herbicide resistant weed species.

One such chemical that can be used as a tool and the focus of this thesis is a class of agrochemicals called herbicide safeners. These are widely used to enhance herbicide selectivity in wheat when controlling wild grasses.

## 1.2 *Triticum aestivum* L.

Wheat is grown on more land area worldwide than any other crop and is the third largest crop behind rice and corn in total world production (UK Agriculture, 2010). Wheat constitutes 20% of the world food calories with the primary use being in bread manufacture. Other uses include in food stuffs such as cakes, biscuits and pasta, as a thickener in soups and sauces, and as livestock and poultry feed. Industrial uses include making starch, alcohol, oil and gluten.

*Triticum aestivum* L. is also known as modern bread wheat. It is an allohexaploid which is composed of twenty one pairs of chromosomes derived from three genomes termed AA, BB and DD (Sears, 1954). Modern hexaploid bread wheat is the product of two hybridisation events. Firstly the 'A' progenitor, identified as *Triticum uratu* L. (Kimber & Sears, 1987) hybridised with the 'B' progenitor to form a tetraploid wheat ( $2n = 28$ , AA BB). The identity of the 'B' progenitor remains unclear, proposals have been made for *Triticum longissimum* or *Triticum searsii* (Feldman & Kislev, 1977) and *Aegliops speltaides* (Sarkar & Stebbins, 1956). It now seems likely that the 'B' progenitor no longer survives in the wild but is a member of the *triticeae* closely related to *Aegliops speltaoides* (Feuillet *et al.*, 2007).

The second hybridisation event was with the 'D' progenitor, *Aegliops tauschii*, with the tetraploid wheat to form modern hexaploid bread wheat. During the domestication of wheat, farmers chose characteristics that benefitted them, this process eliminated the ability of the wheat to survive without farming intervention, such as favouring non shattering heads and huskless seeds (Eastham & Sweet, 2000).

Weed control or the losses from weed contamination can be the most significant loss in wheat production, and the presence of weeds can adversely affect crops in different ways. Losses are incurred through increased costs at harvest, storage and transportation costs resulting from the weed seed in the grain. The greatest negative impact is due to weeds competing with the crop for light, space and nutrients (WORC, 2002). It is due to the economic importance of *Triticum* spp. that this cereal crop was chosen as the focus of this thesis.

### 1.3 Overview of plant metabolism of xenobiotics

Plants are frequently exposed to synthetic foreign compounds (xenobiotics) which cannot be used for nutrition or as a source of energy. Since many of these compounds are toxic, plants mount specific and coordinated defence responses in order to survive (Zhang *et al.*, 2007). Plants are exposed to these xenobiotics as a result of industrial processes such as farming, in the form of agrochemicals such as herbicides. The complement of proteins involved in the biotransformation of xenobiotics has been collectively termed the xenome (Edwards *et al.*, 2005) and the biotransformation process can be sub – divided into four phases (Fig 1).

The first phase of metabolism can be mediated by esterases, amidases, peroxidases or cytochrome P<sub>450</sub> – dependant mono – oxygenases (CYPs) that are membrane bound five – liganded haem – containing proteins associated with the smooth endoplasmic reticulum. CYPs are present in multiple isoforms and this accounts for the wide range of substrates and reactions catalysed (Coleman *et al.*, 1997). Xenobiotics undergo reactions such as oxidations, hydroxylations and

alkylations in order to reveal or introduce a functional group (OH, NH<sub>2</sub>, COOH) (Davies & Caseley, 1999; Edwards *et al.*, 2005; Gaillard *et al.*, 1994; Zhang *et al.*, 2007). The most common CYP - mediated reactions are hydroxylations of aromatic rings or alkyl groups and heteroatom release (Kreuz *et al.*, 1996). Products from phase 1 do not always result in decreased phytotoxicity. Some xenobiotics already contain a functional group and bypass through to phase 2, where they are rendered non – toxic or less toxic (Fig 1).

The second phase of metabolism is catalysed by bioconjugating enzymes such as transferases, such as *O*- and *N*- glucosyltransferases (UGTs) and glutathione transferases (GSTs). In this phase the activated xenobiotic metabolite is conjugated to an endogenous hydrophilic substance such as glutathione (GSH) or glucose (Gaillard *et al.*, 1994) to form a water soluble conjugate. Glucose conjugates can then be further modified by reactions such as malonylation (Fig 1). Malonate can bind to hydroxyl and amino groups, glucose to hydroxyl, sulphhydryl, carboxyl and amino groups, and glutathione to electrophilic sites (Coleman *et al.*, 1997).

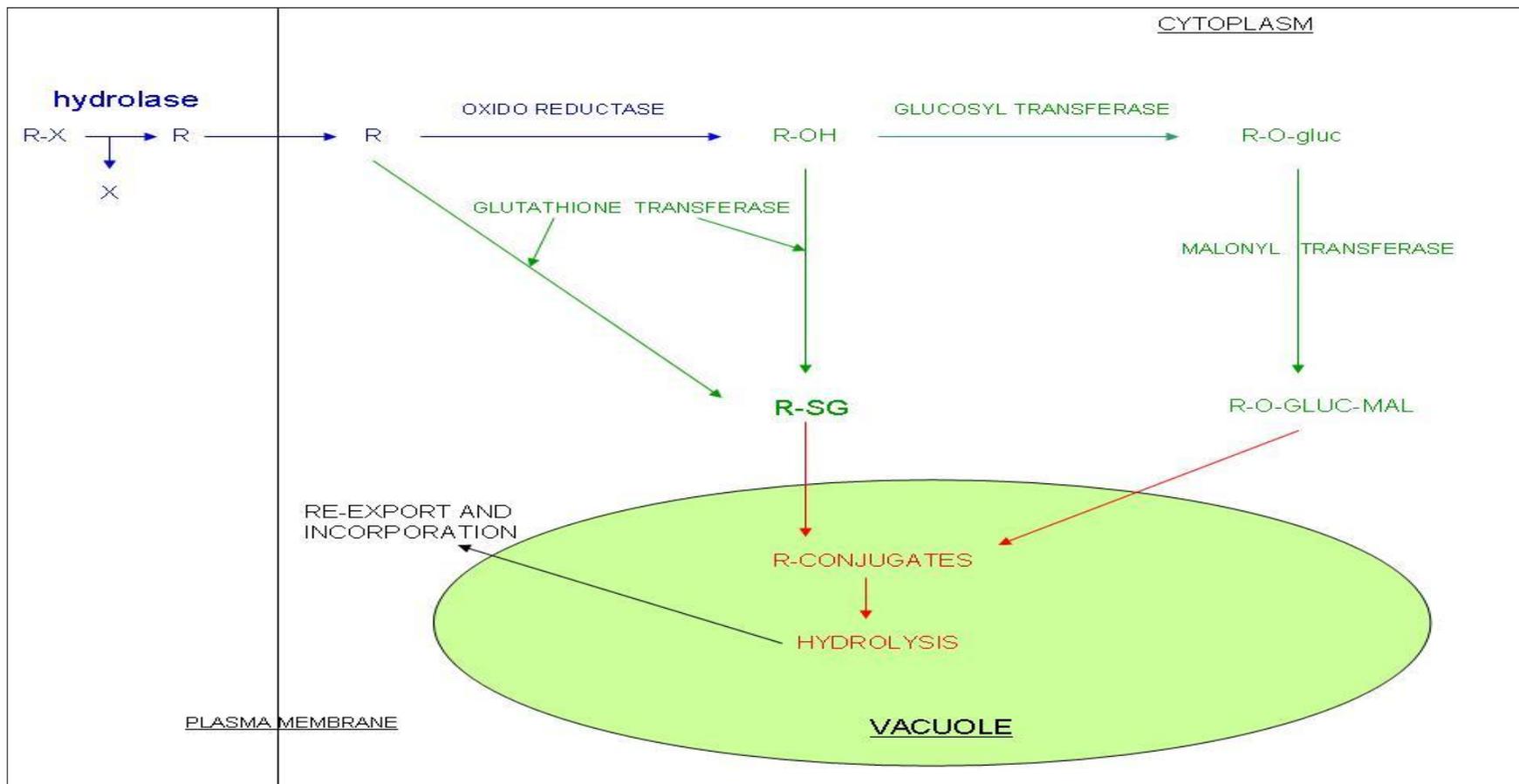


Figure 1 The four phases of xenobiotic metabolism

Diagram representing the four phases of xenobiotic metabolism. R is the xenobiotic. Phase 1: xenobiotic is metabolised in order to add or remove functional groups. Phase 2: xenobiotic conjugation. Phase 3: compartmentalisation of xenobiotic. Phase 4: Re – export and incorporation. Adapted from Edwards *et al.* (2005).

The third phase of metabolism (Fig 1), involves the xenobiotic conjugates being deposited in the large central vacuole (Edwards *et al.*, 2005), notably by the adenosine triphosphate binding cassette transporter proteins (ABC).

In phase four metabolism (Fig 1), conjugates that were imported into the vacuole are metabolised further, in the case of glutathionylated derivatives, by step – wise cleavage to a cysteine conjugate (Wolf *et al.*, 1996). Processed products may then be exported out into the cytoplasm and incorporated into cell wall components or other macromolecules (Gaillard *et al.*, 1994; Edwards *et al.*, 2005).

Xenobiotics containing electrophilic sites are particularly hazardous to plants and can be cytotoxic or genotoxic. Plants have systems in place to defend the plant from pathogenic and predatory attack and a lot of those systems are utilised in the detoxification of xenobiotics. One such system and the focus of this thesis is detoxification via conjugation to glutathione.

## 1.4 Herbicides

For a herbicide to be successful it must be highly selective in causing toxication in plants but not in other organisms, be efficiently delivered to the target site, be an effective inhibitor at the target site, be lethal at low dosages as well as act quickly, degrade rapidly in the environment and be economical to produce (Cobb & Kirkwood, 2000). To date, there have been only a limited number of target sites discovered which can cause problems with herbicide resistance occurring in weed due to repeated selection. Combinations of herbicide mixtures acting on different target sites and metabolised by different routes are needed to keep herbicide resistance under control, again reinforcing the fact that research into the agrochemicals is of economic importance.

### 1.4.1 Metabolism of herbicides in wheat

The enzymatic detoxification of herbicides in plants follows the stepwise process described in Fig (1), utilising the metabolic pathways pre-existing in plants for the metabolism of endogenous compounds.

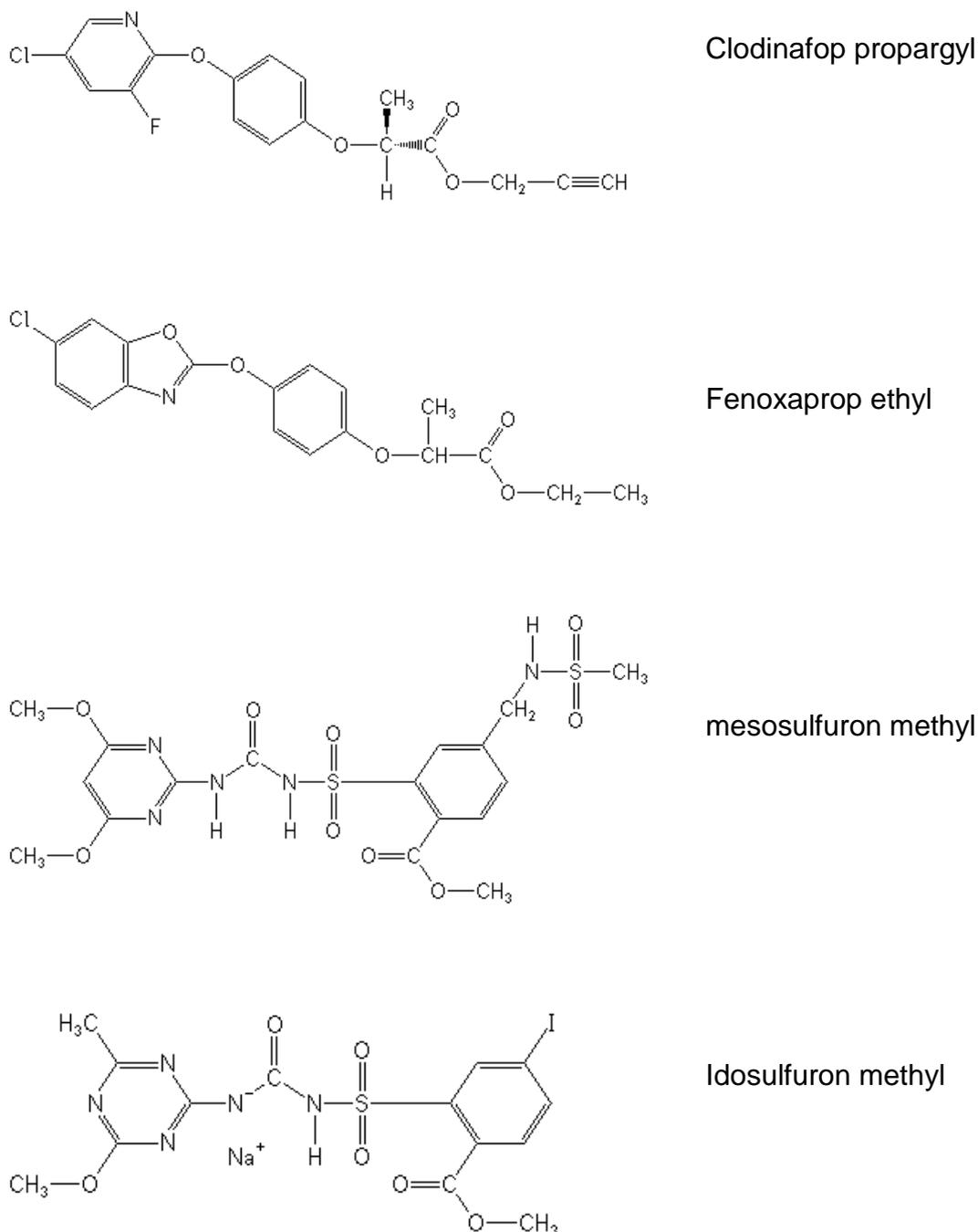
Herbicide metabolism reactions described in wheat include oxidations mediated by P450s including the *N*-dealkylation of phenylureas, aryl hydroxylation of sulfonylureas, phenylureas, and imidazolinones, and ring-methyl hydroxylation of metolachlor and chlortoluron (Hatzios, 2000). Currently the main herbicides used commercially in wheat are clodinafop propargyl, fenoxaprop ethyl and mesosulfuron methyl (Fig 2) and their metabolism is described in more detail in the following sections.

#### 1.4.2 Metabolism of selective herbicides used in *Triticum* spp

Clodinafop propargyl (2-propargyl-(*R*)-2-(4-(5-chloro-3-fluoro-2-pyridinyloxy)-phenoxy)propionate (IUPAC) is used as a post emergence herbicide on wheat, rye, triticale and durum wheat and is sprayed in combination with the safener cloquintocet mexyl. It is an aryl phenoxy-propionate herbicide and inhibits acetyl coenzyme A carboxylase (ACCase) which is part of the first step in lipid biosynthesis (Kreuz *et al.*, 1991; Medd *et al.*, 2000). In this way clodinafop propargyl acts by inhibiting fatty acid synthesis. It is taken up by the leaves and is thought to be translocated to meristematic tissue where it exerts its effect within 48 H (Medd *et al.*, 2000). Plant death occurs three to five weeks later.

Clodinafop propargyl is metabolised by hydrolysis of the parent ester to the acid, followed by arylhydroxylation at the six position of the pyridyl ring. This is followed by sugar conjugation and cleavage of the pyridinyloxy – phenoxy ether bridge forming the breakdown products (2-(4-hydroxyphenoxy)propanoic acid and the 2-hydroxy-3-fluoro-5-chloropyridine respectively (Kreuz *et al.*, 1991).

Fenoxaprop ethyl (2 ethyl-[4-[(6-chloro-1,3-benzoxazol-2-yl)oxy]phenoxy]propanoate) is a post emergence aryloxy phenoxyalkanoic acid herbicide, which is sprayed in combination with the herbicide safeners fenchlorazole ethyl or mefenpyr diethyl. The herbicide is taken up by the leaves and is translocated to the meristematic tissue. Fenoxaprop ethyl is also an ACCase inhibitor, being rapidly hydrolysed to the phytotoxic free acid fenoxaprop. The herbicide then undergoes nucleophilic displacement of the phenyl group by GSH (Tal *et al.*, 1993). The glutathione conjugate is further metabolised to the cysteine conjugate which can be further processed by conjugation to glucose forming an *N*-glucoside. The 4-hydroxyphenoxy propionic acid residue conjugates to glucose forming a  $\beta$ -*O*-glucoside conjugate (Tal *et al.*, 1993).



**Figure 2 Chemical structures of herbicides used in wheat**

Chemical structures of clodinafop propargyl, fenoxaprop ethyl, mesosulfuron methyl and idosulfuron methyl.

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Mesosulfuron methyl (2-methyl-[(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl]- $\alpha$ -(methanesulfonamido)-*p*-toluateis) (IUPAC) is a sulfonylurea herbicide sprayed post emergence with the safener mefenpyr diethyl. This herbicide interferes with the biosynthetic pathway leading to the production of branched chain amino acids leucine, valine and isoleucine via inhibition of acetolactate synthase (ALS). The action of the herbicide leads to a concomitant block of cell division in the meristematic tissue of the plant (Cobb & Kirkwood, 2000; Kocher, 2005). The metabolism of this herbicide has not been reported in detail but what is known is that the parent compound is metabolised to methyl 4-hydroxy-2-((((4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino)carbonyl)amino)sulfonyl)-benzoate and its carbohydrate conjugate (Anderson *et al.*, 1989).

A further metabolite methyl 2-((((4-(hydroxymethyl)-6-methoxy-1,3,5-triazin-2-yl)amino)carbonyl)amino)sulfonyl)benzoate was identified along with others that were attributed to the hydrolysis of the parent compound (Anderson *et al.*, 1989).

For all three herbicides the selectivity of the herbicides in wheat is based on the plants ability to metabolise the parent compound more rapidly than the competing weed species. The enhancement of herbicide metabolism in wheat is partly due to the herbicide safeners

that exert their protective effect in the crop without compromising weed control efficacy (Davies.J, 2001).

## 1.5 Herbicide Safeners

### 1.5.1 Overview

Commercialised herbicide safeners (also known as protectants or antidotes) are synthetic chemicals used to enhance herbicide tolerance in cereal crops (Davies, 2001; Rosinger & Kocher, 2007; Davies, 2001; Edwards *et al.*, 2005; Hatzios, 2003; Hatzios, 2004). Safeners have been sold commercially for over fifty years and are applied either pre – emergence as seed dressings (e.g naphthalic anhydride, oxabetrinil) or post – emergence, sprayed as a mixture with the herbicide (e.g cloquintocet mexyl, fenchlorazole ethyl) (Davies and Caseley, 1999).

The first safener was discovered accidentally in 1947 by Otto Hoffman, after he observed that tomato plants exposed to 2,4-D vapour drift showed no symptoms of injury when treated with trichlorophenoxyacetic acid (Hoffman, 1953; Davies & Caseley, 1999; Rosinger & Kocher, 2007). The potential for herbicide safeners was

recognised and so began research into safener discovery and development, with the first safener available for commercial use being 1,8-naphthalic anhydride (Davies and Caseley, 1999). A fundamental challenge in safener discovery is to find compounds that do not compromise weed control (Rosinger & Kocher, 2007). All safeners discovered so far are used exclusively to protect monocotyledonous crops.

Safeners range in their crop application and chemical specificity, with naphthalic anhydride being one of the most versatile safeners, protecting various crops such as maize (*Zea mays* L.), grain sorghum (*Sorghum bicolor*), oats (*Avena sativa* L.), wheat (*Triticum* spp.) and rice (*Oryza sativa* L.) against a range of herbicides (Miller *et al.*, 1978; Milhome & Batside, 1990; Chang *et al.*, 1978; Blair *et al.*, 1978; Hatzios, 1983).

### 1.5.2 Uses of herbicide safeners

Safeners improve the tolerance of cereals to newly developed herbicides showing limited selectivity, this means that they can extend the uses of existing herbicides; for example, helping to protect cereals from herbicide injury resulting from adverse weather conditions where

further crop damage can occur (Davies, 2001). Their use can also be extended to smaller crops which for economic reasons are not targeted for research for new product development.

One of the major uses for safeners at present is as a powerful research tool not only to identify and manipulate the biochemical mechanisms of herbicide selectivity but as a tool to explore associated biochemical and physiological pathways within plants.

### 1.5.3 Safener classification

Safeners are classed into a number of chemically diverse groups (Table 1 & 2) including the phenylpyrimidines (e.g fenclorim), dichloroacetamide derivatives (e.g dichlormid, benoxacor), oxime ether derivatives (e.g fluxofenim), thiocarbamates (e.g dimepiperate), methylbenzyl-tolylureas (e.g dymuron), and the naphthopyranones (e.g naphthalic anhydride) (Kömives and Hatzios 1991). In wheat these include the phenyl pyrazoles (e.g fenchlorazole ethyl and mefenpyr diethyl), and the quinolinoxycarboxylic acid esters (e.g cloquintocet mexyl).

<b>Safener</b>	<b>Class</b>	<b>Crop</b>	<b>Herbicide</b>	<b>Application Method</b>
Cloquintocet mexyl	quinolinoxycarboxylic acid esters	Wheat	Clodinafop propargyl	Spray as mixture with herbicide, post-emergence
Fenchlorazole ethyl	Phenyl pyrazole	Wheat	Fenoxaprop ethyl	Spray as mixture with herbicide, post-emergence
Mefenpyr diethyl	Phenyl pyrazole	Wheat, rye, triticale, barley	Fenoxaprop ethyl, Mesosulfuron methyl, idosulfuron methyl	Spray as mixture with herbicide, post-emergence
Benoxacor	Dichloroacetamide derivatives	Maize	Metolachlor	Spray as mixture with herbicide, post-emergence
Dichlormid	Dichloroacetamide derivatives	Maize	EPTC, butylate, vernolate	Seed treatment post-emergence
Naphthalic anhydride	Naphthopyranones	Maize	EPTC, butylate, vernolate	Seed treatment, post-emergence

**Table 1 Classification of herbicide safeners 1 of 2**

Table showing the classification of herbicide safeners in wheat and maize along with their partner herbicides and method of application.

Safener	Class	Crop	Herbicide	Application Method
Cyometrinil	Oxime ether	Sorghum	Metolachlor	Seed treatment, post-emergence
Flurazole	Thiazolecarboxylic acid	Sorghum	Alachlor	Seed treatment, post-emergence
Fluxofenim	Oxime ether	Sorghum	Metolachlor	Seed treatment, post-emergence
Oxabetrinil	Oxime ether	Sorghum	Metolachlor	Seed treatment, post-emergence
Fencloirim	Phenyl pyrimidine	Rice	Pretilachlor	Spray as mixture with herbicide, post-emergence
Furilazole	dichloroacetamide	Cereals	Halosulfuron methyl	Spray as mixture with herbicide, post-emergence

**Table 2 Classification of herbicide safeners 2 of 2**

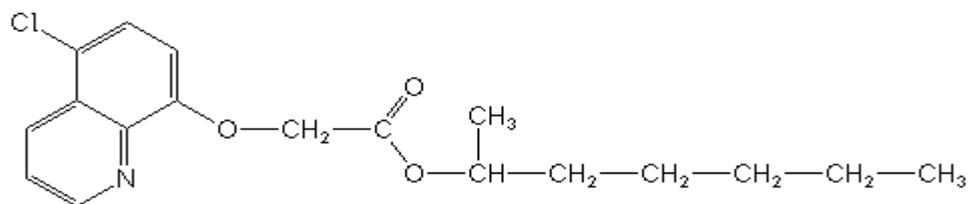
Table showing the classification of herbicide safeners in wheat and maize along with their partner herbicides and method of application.

#### 1.5.4 Safeners used in wheat (*Triticum* spp.)

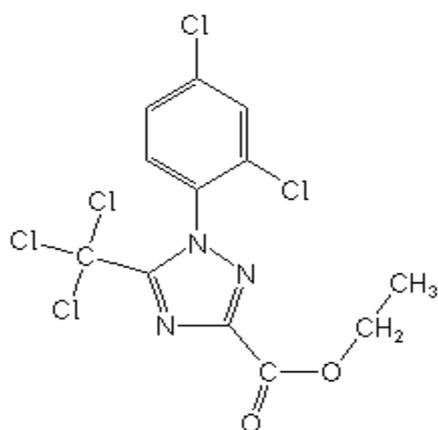
Three safeners are used commercially in wheat; cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl, and are the focus of this thesis.

Cloquintocet mexyl Fig (3) is a quinolinoxycarboxylic acid ester safener sprayed post-emergence with the herbicide clodinafop propargyl, in mixtures such as Topik®, Horizon®, and Discover®. This herbicide / safener combination can also be used in barley, rye and triticale but with reduced safening efficacy (Rosinger & Kocher, 2007; Syngenta, 2010).

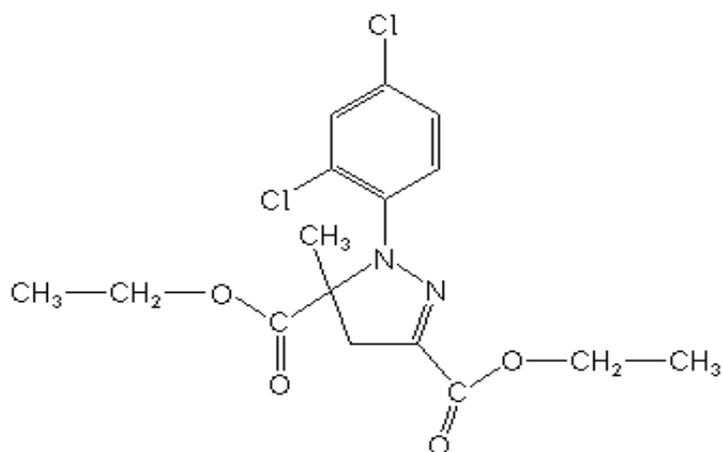
Fenchlorazole ethyl (Fig 3) is a phenyl pyrazole safener sprayed post emergence with the herbicide fenoxaprop ethyl. Mefenpyr diethyl has now replaced fenchlorazole ethyl as it is a more versatile safener. Mefenpyr diethyl can be used not only in wheat but in rye and barley, and can also be used in conjunction with a wider variety of herbicides such as fenoxaprop ethyl (Puma®), idosulfuron-methyl-sodium (Hussar®) and mesosulfuron methyl (Atlantis®).



Cloquintocet mexyl



Fenchlorazole ethyl



Mefenpyr diethyl

**Figure 3 Chemical structures of wheat herbicide safeners**

Chemical structures of cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl.

### 1.5.5 Safener mode of action

Safeners work by reducing the availability of the herbicide to act at its target site (Davies, 2001). There are three main theories on how this might happen:

The first is the competitive antagonist theory. Several safener / herbicide combinations are similar in structure leading to the theory that safeners compete with herbicides at their target site or in biochemical processes targeted by herbicide action. Proposals for this theory led from the structural similarities of the herbicide EPTC and the safener dichlormid (Stephenson & Chang, 1978; Komives & Hatzios, 1991), and diclofop-methyl and 2,4-D which has been shown to exert some protective activity (Taylor & Loader, 1984). A study by Walton & Cassida (1995) showed competitive binding of a dichloroacetamide safener R-29148 to a maize protein (SafBP) which also bound the herbicides EPTC and metolachlor. In contrast Kocher *et al.* (1989) examined this theory by treating wheat chloroplast suspensions with fenoxaprop ethyl and its partner safener fenchlorazole ethyl, as well as the safeners free acid fenchlorazole. Results showed that neither the parent safener or its free acid moiety altered the IC<sub>50</sub> of fenoxaprop ethyl for its target enzyme ACCase. A similar study was carried out by

Polge *et al.* (1987) with chlorsulfuron and the safener 1,8-naphthalic anhydride. Again no competition for the target site was observed between the herbicide and the safener.

Although this theory is a possibility for specific herbicide / safener combinations it is not the main reason for safening in crops. For example some safeners are used with a broad spectrum of herbicides with different target sites e.g mefenpyr diethyl is used with herbicides that are both ALS and ACCase inhibitors.

The second theory of safener mode of action is that safeners influence the uptake and translocation of herbicides. The majority of studies in this area have concluded that herbicide uptake is unaffected by safener treatment. Kocher *et al* (2005) found that mefenpyr diethyl had no effect on the uptake of mesosulfuron methyl or idosulfuron methyl. In cases where uptake was apparently affected, this has been attributed to the interference of other processes by the safener in the plant (Davies & Caseley, 2001; Rosinger & Kocher, 2007). For example the reduction in the uptake of metolachlor by cyometrinil was attributed to a decrease in transpiration rate after application of the herbicide safener (Ketschersid *et al.*, 1982).

Herbicides acting as ACCase or ALS inhibitors exert their effect in the meristematic tissue in the plant. After foliar application, the herbicide must be transported within the plant. It is therefore possible that safeners can effect the translocation of the herbicides by exerting their effect before the herbicide reaches its site of action. For example it is well documented that safeners increase the metabolism of herbicides by inducing enzymes responsible for their detoxification (Cummins *et al.*, 1997; DeRidder *et al.*, 2002), thereby reducing the amount of herbicide available for translocation.

The third theory, and the one that is widely accepted as the main mode of action of safeners, is that safening is due to the induction of enzymes involved in xenobiotic metabolism, which in turn increases the metabolism of the herbicide, this will be discussed in more detail in the following section.

## 1.6 Induction of enzymes involved in herbicide detoxification by herbicide safeners

There is a large body of research done on how safeners increase the activity of detoxification enzymes but knowledge of the molecular mechanisms underpinning the related signalling pathways involved is scarce. Several studies have demonstrated that safeners induce enzymes involved in the metabolism of herbicides during phase one reactions (Fig 1), thereby increasing the degradation of the herbicide.

### 1.6.1 Cytochrome P450 monooxygenases (P450)

P450s are heme containing mixed function oxidases and range in molecular mass from 45 – 62kDa (Katagi *et al.*, 2000; Feldmann, 2001). P450s are associated with the endoplasmic reticulum and are critical in plant metabolic pathways such as phenylpropanoid, terpenoid, and alkaloid pathways. Major reactions catalysed include alkyl and ring hydroxylation, heteroatom dealkylation, and heteroatom oxygenation (Feldmann, 2001; Cojocaru *et al.*, 2007). For the catalysis of a reaction the substrate and molecular oxygen are required to

bind in the active site with a membrane bound flavoprotein NADPH-P450 reductase acting as the electron donor (Katagi, 2000; Cojocaru *et al.*, 2007). After the reaction, the products must leave the protein. NADPH-P450 reductase is an electron transfer partner protein thought to bind on the side of the protein proximal to the heme. Reactions are based on two, one electron transfer steps. Electrons derived from NADPH are shuttled through the FAD and FMN domains of the protein into the heme containing iron centre (Jensen *et al.*, 2010). Protons have been proposed to reach the active site via hydrogen-bond networks involving water molecules (Cojocaru *et al.*, 2007). P450s constitute a large enzyme family divided into nine classes, classification is based on the electron transfer components involved in the catalytic reaction (Jensen *et al.*, 2010).

Several studies have shown P450s to be induced by safeners. In a study by Persans *et al* (1995), P450s (CYP71C1, CYP71C3, CYP92A1 and CYP72A5) involved in the DIMBOA biosynthetic pathway were up-regulated in response to the safener naphthalic anhydride. In a study by Fonne-Pfister *et al* (1990) hydroxylation of primisulfuron was increased by an inducible P450 by the safener CGA154281 and naphthalic anhydride was also found to increase the rate of P450 mediated

O-demethylation of the sulfonylurea herbicide pyrazosulfuron methyl (Yun *et al.*, 2001).

Many herbicides and safeners are formulated as esters to facilitate their diffusion across the waxy cuticle of the plant, as such hydrolytic reactions play a vital role in activating the herbicides. A study by Tal *et al.* (1995) showed that the safener fenchlorazole ethyl enhanced the de-esterification of fenoxaprop ethyl in wheat.

### 1.6.2 Glycosyl transferases (UGTs)

Glycosylation is the most commonly reported fate of agrochemicals in plants with functional groups such as –OH, –NH, –SH, or –COOH (Edwards *et al.*, 2000). UGTs attach a sugar molecule to a specific acceptor (Keegstra & Raikhel, 2001). UGTs are also involved in the conjugation of hormones such as auxins, abscisic acid, cytokinins, brassinosteroids and salicylic acid. Conjugation to a sugar results in increased stability and water solubility of the products and is a key step in the inactivation and detoxification of xenobiotics (Gachon *et al.*, 2005). UGTs are classified according to the activated molecule that donates the sugar, the kind of sugar transferred and whether the

enzyme forms an  $\alpha$  or a  $\beta$  glycoside linkage (Keegstra & Raikhel, 2001; Gachon *et al.*, 2005).

The ability of safeners to enhance the detoxification of herbicides by glucose conjugation was first recognised by Kreuz *et al* (1991) where cloquintocet mexyl was shown to enhance the metabolism of clodinafop propargyl by glucosylation. More recent studies have found that cloquintocet mexyl increases OGT activity toward xenobiotics (4-nitrophenol, 2,4,5-trichlorophenol) and flavonoids (quercetin, luteolin, genistein) (Brazier *at al.*, 2002). Cummins *et al* (2006) also found that cloquintocet mexyl induced the accumulation of ferulic acid and triclin, associated with enhanced expression of O-methyltransferase activity toward caffeic acid and luteolin. In support of this a study by Zhang & Riechers (2004) found a selective induction of 3-O-methyltransferase after treatment with fluxofenim.

Further more several studies have found that safeners also induce MRP transporters (Multi-drug resistance associated protein) which are a member of the ATP binding cassette (ABC) transporters. These proteins transport molecules across cellular membranes. MRP transporters have been found to be responsible for the transport of

glucose conjugates into the vacuole (Gaillard *et al.*, 1994; Theodoulou *et al.*, 2003; Zhang *et al.*, 2007).

### 1.6.3 Glutathione transferases (GSTs)

GSTs were first discovered in animals in the 1960s due to their importance in drug metabolism. This was soon followed by their discovery in plants in the 1970s, when a GST in maize was identified that conjugated atrazine (Edwards *et al.*, 2000; Dixon *et al.*, 2002). Further GSTs have since been identified in plants, animals and fungi. Classically, GSTs catalyse the conjugation of the tri – peptide glutathione ( $\gamma$  – glutamyl – cysteinyl - glycine) to an electrophilic centre.

GSH is the major plant cellular thiol typically exceeding 1 mM in the cytoplasm (Noctor & Foyer, 1998). Conjugation to GSH can occur spontaneously or be mediated by GSTs. The reaction happens by attack of the GSH negatively charged thiolate anion to a suitable electrophilic centre of a xenobiotic (Kreuz *et al.*, 1996). GSH is a nucleophilic scavenger and is an important metabolite that protects the cell from oxidative stress, by acting as a reducing agent (Edwards *et al.*, 2005, Coleman *et al.*, 1997). GSH undergoes disulphide formation with itself (GSSG) when acting as a reductant, this oxidised form can

then be reduced back to GSH by the action of GR (Noctor & Foyer, 1998). GSH is in turn used to maintain the ascorbic acid pool through the reduction of dehydroascorbate (Edwards *et al.*, 2005). Plants contain a number of GSH – dependant enzymes including the GSTs, which helps GSH deliver a central protective role within the plant.

GSTs can constitute > 1 % of the soluble protein in plants (Marrs *et al.*, 1996), and are mainly cytosolic. GSTs are composed of two subunits and are either homodimers or heterodimers, with molecular masses in the range of 25 – 27 kDa (Edwards *et al.*, 2000), with an isoelectric point in the pH range 4 - 5. With the phi and tau GSTs only subunits from the same class will dimerize (Dixon *et al.*, 1999). The ability to form heterodimers may contribute to the broad range of substrate specificities and diversity of the plant GSTs (Dixon *et al.*, 1999).

The plant GSTs can be grouped into six classes (Dixon *et al.*, 2002), namely the phi (GSTF), tau (GSTU), theta (GSTT), zeta (GSTZ) and lambda (GSTL) GSTs, and the dehydroascorbate reductases (DHAR). The phi, tau, lambda and DHAR are plant specific. Using a system suggested by Edwards *et al* (2000) GSTs can be identified by their origin species, class, and polypeptide composition, for example the

lambda class GST first identified in winter wheat '*Triticum aestivum* L.' would be *TaGSTL1*.

Each subunit has an active site with two components, a conserved GSH – binding site (G site) located in the *N* – terminal domain and a *C* – terminal cosubstrate – binding domain (H site) (Edwards *et al.*, 2000, Edwards *et al.*, 2005). The G – site is GSH specific and is responsible for the formation of the catalytically active thiolate anion of GSH. Between the two domains is a short variable linker region of 5 – 10 residues (Dixon *et al.*, 2002). The subunits are related by two – fold symmetry. GSTs have one of two types of subunit interface, a hydrophilic interface or a hydrophobic one, an incompatibility of interfacial residues prevent subunits from different classes dimerizing (Dixon *et al.*, 2002).

#### 1.6.3.1 Endogenous roles of GSTs

GST induction is specific to the particular stress. Despite studies showing that GSTs have roles in herbicide detoxification, their endogenous role within the plant remains speculative. GSTs have not evolved as a response to synthetic xenobiotics and it can therefore be assumed that they have an endogenous role *in planta* (Table 3).

The ability to detoxify toxic compounds is crucial to the survival of a plant as most will encounter them in their environment either from pathogen attack, agrochemicals or pollutants in the environment.

Glutathione conjugation 'tags' xenobiotics and endogenous substrates for sequestration into the vacuole. In addition GSTs and GSH are involved in transporting anthocyanin pigments in the vacuole. Without this anthocyanins would accumulate and be toxic to the plant preventing their further synthesis (Mars *et al.*, 1995).

The products of oxidative damage (hydroxyl radicals, membrane lipid peroxides etc) are highly toxic to plants. In addition to their roles in glutathione conjugation GSTs have also been shown to have activity as GSH – dependant peroxidases (Fig 4), protecting the plant against organic hydroperoxides produced during oxidative stress, by reducing them to the corresponding monohydroxy – alcohols. (Cummins *et al.*, 1999, Roxas *et al.*, 1997).

<b>Class</b>	<b>Abbreviation</b>	<b>Localisation</b>	<b>Endogenous Function</b>
Phi	GSTF	Cytosol, chloroplast	Flavonoid transport (Smith <i>et al.</i> , 2003; Cummins <i>et al.</i> , 2003)
Tau	GSTU	Cytosol, nucleus	Unknown (Mueller <i>et al.</i> , 2000; Cummins <i>et al.</i> , 2003)
Lambda	GSTL	Cytosol, chloroplast, peroxisome	(Dixon and Edwards, 2010)
Theta	GSTT	Peroxisome , nucleus	Hydroperoxide reduction (Dixon <i>et al.</i> , 1999)
Zeta	GSTZ	Cytosol	Tyrosine catabolism (Thom <i>et al.</i> , 2001)
DHAR	DHAT	Cytosol, chloroplast, peroxisome	Ascorbate recycling (Foyer & Mullineaux, 1998)

**Table 2 Classes of plant GSTs and known function within the plant**

Table showing the abbreviation, localisation and known function of plant GSTs. (Adapted from Dixon *et al.*, 2010)

GSTs can also serve as nonenzymatic carrier proteins (ligandins) and have been implicated in the transport of compounds such as steroids and indole-3-acetic acid (IAA) (Bilang *et al.*, 1995). Another group of potential GST ligands are the electrophilic oxylipins such as OPDA (Dixon *et al.*, 2010; Riechers *et al.*, 2010). OPDA is an intermediate in jasmonate synthesis, and must pass through the cytosol to the peroxisome without modification (Dixon *et al.*, 2010). Studies by Dixon *et al.* (2009) and Mueller *et al.* (2008) demonstrated that the GSTs *AtGSTF* and *AtGSTU* can bind the glutathione conjugate of OPDA suggesting that these enzymes may play a role in the transport of these compounds in the cell. There have also been observation that mutations in two GSTs, a maize tau GST (Bronze2) and a phi GST from *Petunia hybrida* (An9) resulted in the inability of the plant to deposit flavonoid derived pigments in the vacuole (Marrs *et al.*, 1997; Mueller *et al.*, 2000).

One of the most studied functions of GSTs in plants and the focus of this thesis is the role GSTs play in detoxifying synthetic xenobiotics they encounter in the environment, and specifically how they are induced by herbicide safeners.

### 1.6.5 Induction of GSTs by herbicide safeners

The first studies examining the induction of GSTs by herbicide safeners were in maize and associated with the enhanced metabolism of chloroacetanilide herbicides by benoxacor and dichlormid (Edwards *et al.*, 2005; Davies and Caseley, 1999). The major safener inducible GST was identified as *ZmGSTF2*. Phi GSTs have also been shown to be inducible in sorghum (Gronwald & plaisance, 1998), and barley (Scalla & Roulet, 2002). Tau GSTs have also been shown to be induced by safeners in rice (Deng & Hatzios 2002), wheat (Cummins *et al.*, 1997) and maize (Dixon *et al.*, 1998) as have the lambda GSTs in wheat (Theodoulou *et al.*, 2003; Dixon and Edwards, 2010).

### 1.7 Safener metabolism

It has been hypothesised that safeners are metabolised in the same way as herbicides, and by the same enzymic pathways, with some safeners forming glutathione conjugates within plants. The thiazolecarboxylate safener flurazole is applied to grain sorghum and was used in a study by Breaux *et al* (1989) where it was absorbed and metabolised rapidly by etiolated shoots of corn and grain sorghum. The major metabolite detected was a GSH conjugate.

Similarly Miller *et al* (1996) identified a di (GSH) conjugate of the dichloroacetamide safener benoxacor as the most abundant metabolite. In this study suspension cultures of *Zea mays* (cv Black Mexican sweet) were used. Other metabolites identified were mono ( $\gamma$ -Glu - Cys) and di ( $\gamma$  - glu - cys) conjugates of benoxacor resulting from peroxidase activity on the mono and di GSH conjugates, a disaccharide S-O-(diglycoside) conjugate formed from the mono GSH conjugate by the action of glucosyl transferases, and an S-(S'-cys) GSH conjugate formed by a peptidase and a dipeptidase activity on the di GSH conjugate (Miller *et al.*, 1996).

The phenylpyrimidine safener fenclorim used to increase tolerance to chloroacetanilide herbicides in rice was also shown to be glutathionylated in rice and *Arabidopsis* (Brazier-Hicks *et al.*, 2008). The fenclorim conjugate was processed to two further metabolites, an S-(fenclorim)- $\gamma$ -glutamyl - cysteine and an S-(fenclorim)-cysteine conjugate (FC). The latter was then further metabolised by *N*-acetylation to malonic acid or catabolised to 4-chloro-6-(methylthio)-phenylpyrimidine (CMTP by a cysteine conjugate  $\beta$  - lyase and an S-methyltransferase. A malonyl - CoA - dependant *N*-malonyltransferase then acting on FC forms a fenclorim-*N*-malonylcysteine conjugate which was further processed to make an S-fenclorim-*N*-acetylcysteine

Intermediate which underwent a second round of glutathione conjugation mediated by the GSTs. CMPT was found to induce GSTs and herbicide safening in rice (Brazier *et al.*, 1998) against the herbicide pretilachlor. This was an unusual example of a metabolic reactivation rather than an essential bioactivation step.

Conjugation to glutathione has not been reported for any other safeners, and to date these studies shed no light on any associated role of GSTs in the signalling pathways involved in safening.

### 1.8 Aims and objectives of Thesis

As discussed previously it is well documented that herbicide safeners induce GSTs from all classes. This thesis focuses on the induction of GSTs in the modern hexaploid bread wheat *Triticum aestivum*, chosen because of its economic importance and widespread use. It is also easily cultivated and analysed. The safeners that are the focus of these studies are fenchlorazole ethyl, cloquintocet mexyl and mefenpyr diethyl and are all used in *Triticum aestivum*.

These safeners have different chemistries (Fig 3) and it was of interest to determine if safeners from different chemistries induce the same classes of GSTs in a single cereal crop. If this was the case this would indicate a similar mode of action for the safeners despite their chemical class. Because GSTs are known to be induced upon safener treatment, it will be used as an indicator of safening. In chapter three wheat was treated with each safener and assayed for GST activity toward CDNB and for GPOX activity. Western blots were also be used to determine induction of particular classes of GSTs (tau, phi and lambda).

The induction of GSTs has not been fully characterised in wheat and further studies aim to elucidate the pattern of induction. Some questions that this chapter aims to provide answers for are: 1) What is the time course for induction of GSTs? 2) Is the induction of GSTs dependant on dose? 3) Is there any localisation of GST activity within the wheat shoot? 4) Is the effect of safening in wheat with regards to GST induction additive or saturative?

Cloquintocet mexyl is unique in its chemistry as it has a large ester moiety. This work aimed to determine if the ester moiety of safeners was important in their ability to safen or if the free acid moiety was the active part of the compound. GST induction studies were carried out for cloquintocet mexyl and its free acid cloquintocet using enzyme assays and western blots.

Chapter four uses a proteomic approach to try and determine which particular GSTs are induced by cloquintocet mexyl using hydrophobic chromatography (phenyl sepharose), and affinity chromatography (glutathione agarose, S-hexyl-glutathione) coupled with 2-D gel electrophoresis. Putative GST polypeptides were sent for MALDI-TOF analysis.

Chapter five takes the GSTs identified by proteomics in chapter four, through to cloning, expression and characterisation of the respective proteins. A range of known GST assays were used to help determine the specific activities of the inducible GSTs including CDNB, DCNB, BITC, NBC, thiol transferase and GPOX assays. The safener inducible GSTs were also assayed by HPLC and analysed by MS for activity toward cloquintocet mexyl, fenchlorazole ethyl, mefenpyr diethyl, benoxacor, clodinafop propargyl, fenoxaprop ethyl and alachlor.

In chapter six RT-PCR was used to identify the induction of transcripts of the safener inducible GSTs over a time course of 24 H in response to cloquintocet mexyl. The metabolism of cloquintocet mexyl has not been reported and a focus of this chapter was to further elucidate any further downstream metabolites, as well as to look at the cleavage of cloquintocet mexyl *in vivo* and *in vitro*. One further theory of safener mode of action explored in this chapter is the inhibition of GSTs by herbicide safeners, as inhibition could cause a possible induction of further GSTs through a feedback response. This theory was explored using crude protein extracts coupled with CDNB and GPOX assays, and using isothermal calorimetry with the safener inducible GSTs and the herbicide safeners. The final part of this chapter looks at safener induced changes in flavonoids. Perturbations in the enzymes in

secondary metabolism have been known to effect flavonoid levels in plants. This section further builds on work done by Cummins *et al* (1997) by conducting a study of changes in flavonoid levels 24 H after safener treatment.

This thesis will further clarify the induction of GSTs in *Triticum aestivum* by herbicide safeners, by analysing their expression, function and role in safener induction and metabolism.

## Chapter Two: Materials and Methods

### 2.1 Plant growth and spray treatment

Winter wheat seed cv 'Einstein' (*Triticum aestivum* L.) were obtained from Nickerson-Advanta LTD (Lincolnshire). Seeds were imbibed for 1 H in water prior to sowing in compost (John Innes loam based compost N<sup>o</sup> 2) to promote germination. Wheat was grown in an environmental chamber (Sanyo MLR-350H) at 25 °C and 60 % humidity for a photoperiod of 16 H light (150  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) and 8 H dark until treatment and harvest.

Cloquintocet mexyl, cloquintocet, fenclorazole ethyl and mefenpyr diethyl were obtained from Sigma-Aldrich and were prepared as 1 mg ml<sup>-1</sup> stock solutions in acetone and diluted according to the treatment required.

Seven day old wheat shoots were sprayed with the field rate of cloquintocet mexyl / cloquintocet (15 g hec in 200 L) in 0.1 % v/v Biopower, (obtained from Bayer Crop Science), using a fine mist spray.

For spray treatments involving all three safeners seeds were imbibed overnight in a 10 mg L<sup>-1</sup> solution of the safeners cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl prior to sowing. Wheat was sprayed daily with 25 ml of a 0.1 % v/v acetone (control) or a 10 mg L<sup>-1</sup> solution of the safeners cloquintocet mexyl, fenchlorazole ethyl, and mefenpyr diethyl until harvest. Plant growth was determined by measuring fifty wheat shoots from two replicates at each harvest. At each harvest wheat was dissected into three parts, meristem, mid section and tip, to determine any localisation of GST activity.

## 2.2 Extraction of plant GSTs and enzyme assays

### 2.2.1 Extraction of plant GSTs

The extraction procedure described by Edwards & Dixon (2005) was followed. All steps were carried out at 4 °C unless otherwise stated. After treatment plant tissue was weighed, frozen in liquid nitrogen and stored at - 80 °C. Frozen plant tissue was ground to a fine powder using a pestle and mortar and then extracted in 3 v/w 0.1 M Tris-HCl, pH 7.5, containing 2 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 5 % w/v polyvinylpolypyrrolidone. After straining through miracloth (Calbiochem, Nottingham, UK) followed by

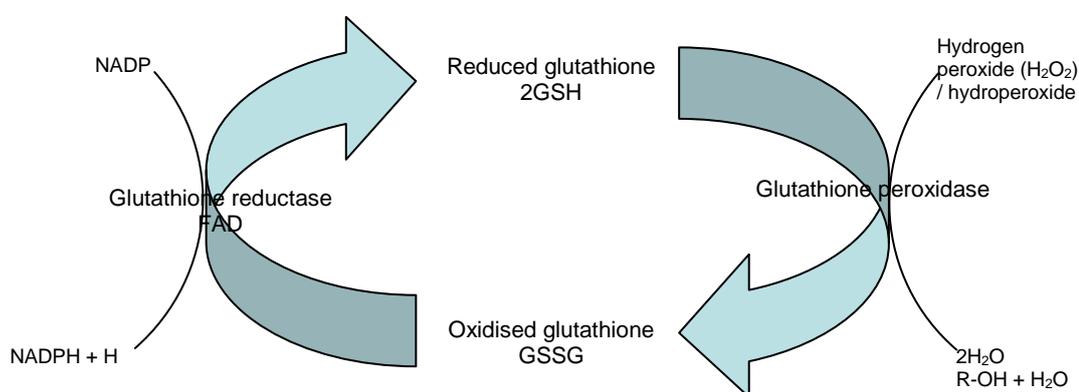
centrifugation (Beckman Coulter™ Allegra™ X22-R) (10,000 g, 30 min), the supernatant was adjusted to 80 % saturation with  $(\text{NH}_4)_2\text{SO}_4$  and the protein pellet recovered after re-centrifuging (4500 g, 20 min). Protein pellets were stored at  $-20\text{ }^\circ\text{C}$  until needed, and desalted prior to use on a sephadex spin column (4500 g, 2 min), pre-equilibrated with 3 v/w 0.1 M Tris-HCl, pH 7.5, containing 2 mM EDTA, 1 mM DTT.

### 2.2.2 Protein determination

A BCA™ protein assay kit (Pierce) was used for protein determination. This is based on the reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$  by protein with the selective colorimetric detection of the cuprous cation by bicinchronic acid. A set of protein standards were prepared using the provided albumin standard ( $2\text{ mg ml}^{-1}$  bovine serum albumin), and used to produce a standard curve. Prior to incubation for 30 min ( $37\text{ }^\circ\text{C}$ )  $40\text{ }\mu\text{l}$  of buffer (3 v/w 0.1 M Tris-HCl, pH 7.5, containing 2 mM EDTA, 1 mM DTT), and  $10\text{ }\mu\text{l}$  of extracted enzyme were added to an eppendorf along with 1 ml of working reagent from the assay kit. The control contained  $10\text{ }\mu\text{l}$  of buffer instead of extracted enzyme. The contents of each eppendorf were transferred to a cuvette and left to cool at room temperature for 4 min. Cuvettes were then transferred to a

spectrophotometer (Beckman Coulter™ DU®530) and absorbance read at 562nm.

### 2.2.3 Glutathione peroxidase assay (GPOX)

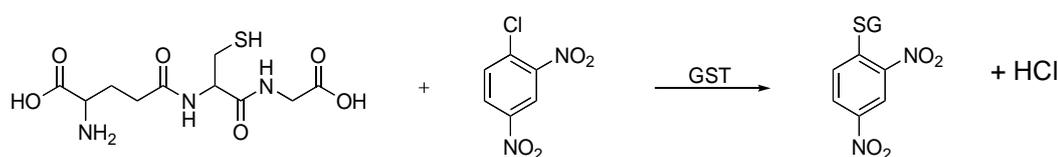


**Figure 4 Schematic representation of the glutathione oxidation / reduction (redox) cycle**

The GPOX assay is a coupled assay measuring NADPH oxidation during the reduction of GS-SG, formed by the enzymatic reduction of hydroperoxides (Flohe & Günzler, 1984). 500 µl of a 0.25 M potassium phosphate buffer, pH 7.0, containing 2.5 mM EDTA was added to a 1 ml cuvette. To this 100 µl of glutathione reductase in 0.25 M potassium phosphate buffer, pH 7.0 (6 units ml<sup>-1</sup>) was added along with 100 µl of 10 mM reduced glutathione, pH 7.0, and 100 µl of 2.5 mM NADPH prepared in 0.1% w/v aqueous NaHCO<sub>3</sub>. Each cuvette was incubated in a water bath at 37 °C for 10 min. A cuvette was placed in the

spectrophotometer and 100  $\mu\text{l}$  of an aqueous 12 mM solution of cumene hydroperoxide and 100  $\mu\text{l}$  of enzyme extract was added. The change in absorbance was determined over 2 min at 360 nm. As a control the change in absorbance was determined by replacing the extracted enzyme with 100  $\mu\text{l}$  of potassium phosphate buffer.  $E_{360} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ .

#### 2.2.4 1-chloro-2, 4-dinitrobenzene (CDNB) assay



**Figure 5 Schematic representation of the CDNB assay**

This assay involves the detection of the product of enzymatic conjugation of glutathione to CDNB (Habig *et al.*, 1974). 875  $\mu\text{l}$  of a 0.1 M potassium phosphate buffer, pH 6.5, was added to a cuvette along with 25  $\mu\text{l}$  of 40 mM CDNB. After incubation at 30  $^{\circ}\text{C}$  for 10 min in a water bath, 50  $\mu\text{l}$  of extracted enzyme and 50  $\mu\text{l}$  of 100 mM glutathione were added, mixed by inversion and transferred to a spectrophotometer. The increase in absorbance was monitored by

replacing the enzyme extract with potassium phosphate buffer.  $E_{340} = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$

### 2.2.5 Benzyl isothiocyanate assay (BITC)

BITC is a GST substrate converting isothiocyanate ( $\text{R-N=C=S}$ ) into the corresponding dithiocarbamate ( $\text{R-NH-C(=S)-SG}$ ) with a corresponding increase in U.V. Using a quartz cuvette 950  $\mu\text{l}$  of 10 mM phosphate buffer pH 6.5 buffer was incubated at 30  $^{\circ}\text{C}$  for 5 min, then 25  $\mu\text{l}$  of enzyme added along with 10  $\mu\text{l}$  of 100 mM GSH. The reaction was started with 10  $\mu\text{l}$  of 16 mM BITC. The increase in absorbance was monitored at 274 nm over 30 sec. The BITC was added last as it irreversibly inhibits GSTs.  $E_{274} = 9.25 \text{ mM}^{-1} \text{ cm}^{-1}$

### 2.2.6 Crotonaldehyde assay ( $\alpha$ , $\beta$ unsaturated aldehyde)

In a cuvette 10  $\mu\text{l}$  of 10 mM crotonaldehyde and 900  $\mu\text{l}$  of 10 mM phosphate buffer pH 6.5 were incubated for 5 min at 37  $^{\circ}\text{C}$ . Prior to the addition of 50  $\mu\text{l}$  of enzyme and 10  $\mu\text{l}$  at 100 mM GSH. The decrease in absorbance over 1 min was monitored at 230 nm.  $E_{230} = 10.7 \text{ mM}^{-1} \text{ cm}^{-1}$

### 2.2.7 *p*-nitrobenzyl chloride assay (NBC)

940  $\mu$ l of 10 mM phosphate buffer pH 6.5 was added to a cuvette along with 40 mM NBC and incubated for 5 min at 30  $^{\circ}$ C. Prior to the addition of 50  $\mu$ l of 100 mM GSH and 10  $\mu$ l of enzyme were added. The increase in absorbance over 30 sec was monitored at 310 nm.

$$E_{310} = 1.9 \text{ mM}^{-1} \text{ cm}^{-1}$$

### 2.2.8 1,2-dichloro-4-nitrobenzene (DCNB)

940  $\mu$ l of 10 mM phosphate buffer pH 6.5 was added to a cuvette along with 40mM DCNB and incubated for 5 min at 30  $^{\circ}$ C. Following incubation 50  $\mu$ l 100 mM GSH and 10  $\mu$ l enzyme were added and the increase in absorbance monitored over 30 sec at 345 nm.  $E_{345} = 8.5 \text{ mM}^{-1} \text{ cm}^{-1}$

## 2.3 Gel analysis and western blotting

### 2.3.1 SDS-PAGE

The method of Laemmli (1970) was used to prepare SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels

using the mini-PROTEAN III kit from Biorad. Resolving gels were polymerised from 12.5% *bis*-acrylamide in 375 mM Tris.HCl, pH 9.0, 0.1% (w/v) ammonium persulphate, 0.1 % (v/v) *N,N,N',N'* – tetramethylethylenediamine (TEMED), and 0.1 % (w/v) sodium dodecyl sulphate. The stacking gel was polymerised from 4 % acrylamide/*bis*-acrylamide, 126 mM Tris/HCl, pH 6.8, 0.1 % (v/v) TEMED, 0.05 % (w/v) ammonium persulphate and 0.1 % (w/v) sodium dodecyl sulphate. Protein concentration was determined using the BCA™ protein assay kit. 50 µg of enzyme extract and 20 µl of 2x SDS loading buffer (100 mM Tris-HCl, pH 6.7, 20 % glycerol, 200 mM DTT, 4 % w/v SDS, 0.2 % w/v bromophenol blue) were incubated at 95 °C for 5 min prior to gel loading. Samples were loaded into the wells and electrophoresed in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) at 150 V until the dye reached the bottom of the gel. Gels were washed thoroughly with water for 2 x 5 min to remove any residual SDS.

### 2.3.2 Gel staining

Gels were stained with Coomassie blue reagent (0.01% w/v Coomassie brilliant blue, 5% v/v 95% ethanol: water (95:5v/v) and 10% v/v phosphoric acid: water (85:15 v/v).

### 2.3.4 Western blotting and immunodetection

Proteins were separated by SDS-PAGE prior to electroblotting onto a polyvinylidene fluoride membrane (Hybond-P, Amersham Biotech) using a mini Trans-Blot cell (Biorad) at 100 v for 45 min. After blotting the non specific binding sites were blocked with 3 % skimmed milk powder in Tris-buffered saline (1.93 % w/v Tris, 9 % w/v glycine) for 1 H at room temperature. Antisera (Rabbit) raised to specific GSTs were added at a 1:1000 – 1:5000 dilution and incubated overnight at 4 °C. The membrane was washed twice for 5 min with TBST (TBS with 0.1 % Tween-20), and then washed once in TBS. The secondary antibody (monoclonal anti Rabbit IgG ( $\gamma$ -chain specific) alkaline phosphatase, antibody produced in mouse from Sigma) was incubated at a 1:10 000 dilution in 3 % milk powder in TBS for 1 H at room temperature. The membrane was washed again twice for 5 min in TBST and once for 5 min in TBS. The membrane was then left for 5 min in 100 mM Tris/HCl, pH 9.5 to equilibrate. and developed in 0.3 % (v/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP 50 mg ml<sup>-1</sup>) dissolved in *N,N,N',N'*-dimethylformamide (DMF) and 0.3 % (v/v) nitro blue tetrazolium (NBT 100 mg ml<sup>-1</sup>) dissolved in 70 % DMF. The reaction was stopped with large amounts of water after visible bands appeared.

### 2.4 Metabolism studies

Samples were analysed by reversed-phase HPLC/MS using a Waters AQUITY HPLC system with diode array detection linked to a Q-TOF PREMIER electrospray Time-Of-Flight mass spectrometer. 1 g of seven day old wheat shoots were left to incubate overnight at 18 °C, in MS media containing 50 mM cloquintocet mexyl, and 30 g L<sup>-1</sup> sucrose. Using a pestle and mortar, wheat was homogenized in 4 ml methanol before centrifuging (4500 g, 10 min), and the supernatant partitioned with hexane. The aqueous phase was retained for analysis and injected onto an Acuity UPLC™ BEH C18 (1.7 µM, 2.1 x 100 mm) column at a flow rate of 0.2 ml min<sup>-1</sup> and eluted using a gradient starting at 5 % B rising to 100 % B over 9 min. The eluent was analyzed using a Waters Q-TOF Premier Mass Spectrometer after ESI (capillary 2.55 kV, sample cone 41 kV, extraction cone 5.0 kV, source 100 °C with desolvation at 180 °C). Samples were analysed in positive ion mode.

## 2.5 Gene cloning and expression of GSTs

Using Polymerase Chain Reaction (PCR) The following primers were used in the following sections:

Oligo	Sequence	T <sub>m</sub> (°C)
TaGSTF4	F: GCG CGC CAT ATG GCG CCG GTG AAG GTG	74.1
	R: CGC GCG CTC GAG TCA CGG CTT CTT GGG AAC	75
TaGSTF10	F: GCG CGC CAT ATG GCG CCG GTG AAG GTC TTC	71.8
	R: CGC GCG CTC GAG TCA CGG CTT CTT GGG AAC CAT	71
TaGSTU3	F: GCG CGC CAT ATG GCG GGC GAG AAG GGC	75
	R: CGC GCG CTC GAG TCA CTC GAT GCC GTA CTT	73.6
TaGSTU6	F: GCG CGC CAT ATG GCC GGA GGA GAT GAC	72.6
	R: CGC GCG CTC GAG TCA CTT AGA CGC TGC AGC	75
TaGSTL1	F: GCG CGC CAT ATG GCC GCA GCT GCA GCA ATA	73.6
	R: GCG CGC CTC GAG TCA AGC AAT CTT GAG ATG CCT	73.2

**Table 3 Primers used for the cloning and expression of GSTs**

The reaction mix contained 29 µl H<sub>2</sub>O, 10 µl 5 x Phusion™ buffer, 10 µl, 2 mM dNTP, 10 mM reverse primer, 10 mM forward primer, 0.5 µl Phusion™ (DNA polymerase), 1 µl template. The reaction mixture was placed into a thermocycler (Eppendorf Mastercycler Gradient PCR machine) using the programs in Table 5.

<b>Step</b>	<b>Temperature °C</b>	<b>Duration (min)</b>
<b>1</b>	98	01:00
Add Phusion™		
<b>Repeat steps 2 - 4 for 25 cycles then proceed to step 5</b>		
<b>2</b>	98	00:15
<b>3</b>	60 (TaGSTF4, TaGSTF10, TaGSTU3, TaGSTU6, TaGSTL1)	00:30
<b>4</b>	72	00:30
<b>5</b>	72	10:00
<u>END</u>		

**Table 4 PCR program used**

PCR products were analysed on a 0.8 % agarose gel, containing 1 µl ethidium bromide. Bands were visualised using a Bio-Rad Gel Doc™ and Quantity One® 4.5.0 1 D analysis software (Bio-Rad Laboratories, UK), and cut out prior to purifying using a Promega Wizard® SV gel and PCR clean up kit (Promega UK, Southampton).

### 2.5.1 Addition of single 'A' ends

Single 'A' ends were added to the purified PCR product. 7 µl of the purified PCR product was added to 1 µl Taq Polymerase, 1 µl dATP,

and 1  $\mu$ l of 10 x buffer (containing 0.5 M Tris/HCl, pH 8.8, 50 mM MgCl<sub>2</sub>, 125 mM ammonium sulphate, 50  $\mu$ M EDTA, 2.2  $\mu$ M each of dATP, 12.5 % (w/v) bovine serum albumin, 12.5 mM DTT) prior to being incubated at 70 °C for 30 min.

### 2.5.2 Ligation into pGEM® -T Easy

The 'A' tailed PCR product from section (2.5.1) was ligated into pGEM®-T using the pGEM®-T Easy Vector System (Promega) following manufacturers instructions. The following was added to a small PCR tube prior to being incubated for 1 H at room temperature, 1  $\mu$ l pGEM®-T Easy (Promega), 5  $\mu$ l 2 x buffer (as provided), 1  $\mu$ l T4 ligase (as provided), and 3  $\mu$ l of 'A' tailed PCR product.

### 2.5.3 Transformation and blue / white selection

Following ligation, the vector containing the insert was transformed into chemically competent cells ( $\alpha$  – gold chemically competent cells, Bioline) following the manufacturers instructions. The  $\alpha$  – golds are chemically competent cells with bacteriophage T1 resistance, containing a *lacZ* marker that provides blue/white colour screening (Bioline, 2008). 25  $\mu$ l of competent cells in an eppendorf were thawed on ice prior to use. 5  $\mu$ l of ligation reaction was pipetted directly into the

vial of competent cells and mixed by tapping gently. After incubating on ice for 30 min, the cells were heat shocked for 30 sec in a 42 °C water bath then placed in ice. 250 µl of pre-warmed MS medium was added and the cells left to recover at 37 °C whilst being agitated gently on a shaker for 1 H at 225 rpm. Once recovered the cells were plated on to LB agar (10 g L<sup>-1</sup> NaCl, 10 g L<sup>-1</sup> bacteriological peptone, 5 g L<sup>-1</sup> yeast extract, 15 g L<sup>-1</sup> agar, with AMP selection) and left to incubate overnight at 37 °C.

#### 2.5.4 Isolating plasmid DNA

A single transformed *E.Coli* colony was used to inoculate a 10ml LB starter culture (10 g L<sup>-1</sup> NaCl, 10 g L<sup>-1</sup> bacteriological peptone, 5 g L<sup>-1</sup> yeast extract, with AMP selection). The 10 ml cultures were grown overnight at 37 °C then pelleted at 4500 rpm. The supernatant was discarded and the plasmid DNA purified from the cells using the Promega Wizard<sup>®</sup> Plus Minipreps DNA purification system.

#### 2.5.5 Digestion

The following was added to a PCR tube prior to incubating for 1 H at

37 °C. 20 µl water, 1 µl plasmid DNA, 3 µl buffer D (Promega), 1 µl Nde1, 1 µl Xho1. Digested plasmids were run on an agarose gel (400 mg agarose, 50 ml TAE (40 mM Tris-acetate, 1 mM EDTA), 1 µl ethidium bromide) using a Bio-Rad Sub-Cell<sup>®</sup> GT agarose gel electrophoresis system. Inserts were visualised using a Bio-Rad Gel Doc<sup>™</sup> EQ and Quantity One<sup>®</sup> 1-D analysis software, and digested inserts cut out. Plasmid DNA was purified using Bio-Rad Prep-a-gene<sup>™</sup>.

#### 2.5.6 Ligation into pET- STRP3

Purified plasmid DNA was ligated into pET – STRP3 (N-terminal T7 promoter). The following was added to a PCR tube prior to incubation for 1 H at room temperature; 5 µl 2 x buffer (Promega), 1 µl T4 ligase (Promega), 2 µl pET-STRP3, 2 µl purified insert.

#### 2.5.7 Transformation

Following ligation, the vector containing the insert was transformed into chemically competent cells (α – gold chemically competent cells, Bioline) prior to the plasmid DNA being purified using the Promega

Wizard<sup>®</sup> Plus Minipreps DNA purification system. 25 µl of competent cells in an eppendorf were thawed on ice prior to use. 5 µl of ligation reaction was pipetted directly into the vial of competent cells and mixed by tapping gently. After incubating on ice for 30 min, the cells were heat shocked for precisely 30 sec in a 42 °C water bath, then placed in ice. 250 µl of pre-warmed MS medium was added and the cells left to recover at 37 °C whilst being agitated gently on a shaker for 1 H at 225 rpm. Once recovered the cells were plated on to LB agar (10 g L<sup>-1</sup> NaCl, 10 g L<sup>-1</sup> bacteriological peptone, 5 g L<sup>-1</sup> yeast extract, 15 g L<sup>-1</sup> agar, with chloramphenicol and kanamycin selection) and left to incubate overnight at 37 °C.

### 2.5.8 Transformation into Tunetta cells

The pET-STRP3 constructs were transformed into *E.coli* strain Tuner (DE3) (Novagen) containing the pRARE plasmid from the strain Rosetta (Novagen). 1 µl of purified DNA was incubated with the Tunetta cells on ice for 5 min, prior to being heat shocked at 42 °C for 30 seconds. Cells were left to recover for 2 min on ice. 250 µl of pre-warmed MS medium was added and the cells left to recover at 37 °C whilst being agitated gently on a shaker for 1 H at 225 rpm. Once recovered the cells were plated on to LB agar (10 g L<sup>-1</sup> NaCl, 10 g L<sup>-1</sup>

bacteriological peptone, 5 g L<sup>-1</sup> yeast extract, 15 g L<sup>-1</sup> agar, with chloramphenicol and kanamycin selection) and left to incubate overnight at 37 °C.

### 2.5.9 Expression

A single colony of transformed *E.Coli* was used to inoculate a 10 ml starter culture containing 100 µg ml<sup>-1</sup> kanamycin and 35 µg ml<sup>-1</sup> chloramphenicol and incubated overnight at 37 °C. A 1 L culture was inoculated with the overnight culture and grown under the same conditions until an optical density of 0.5 at 600 nm. The cells were then induced with 1 mM IPTG and incubated overnight at 37 °C. Cultures were then centrifuged for 10 min at 8, 000rpm. Pellets were stored until needed at -80 °C.

### 2.5.10 Purification

The pelleted bacteria were resuspended in buffer A containing 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.6, and sonicated. After adding DTT (1 mM) and avidin (8 µg ml<sup>-1</sup>) to remove endogenous biotin and biotinylated proteins, the bacterial lysate was sonicated three times for thirty seconds with twenty seconds between each burst. The

bacterial lysate was then centrifuged for 15 min at 4,500 rpm and the pellet discarded. A Strep-Tactin macroprep column (Stratech Scientific Ltd, Soham, UK) was pre-equilibrated with buffer A and the supernatant loaded onto the column using an FPLC.

Recombinant protein was eluted with buffer A containing 2.5 mM desthiobiotin. The column was regenerated with buffer A containing 1mM 2-(4-hydroxy-benzeneazo)-benzoic acid. Eluted recombinant protein was flash frozen in liquid nitrogen in 10% glycerol and stored at - 80 °C until needed.

## 2.6 Proteomics 2 D gel electrophoresis

### 2.6.1 Treatment

Wheat was sprayed at 7 D with the field rate of cloquintocet mexyl or a control formulation then harvested 24 H after treatment. The GST extraction procedure from Edwards *et al.*, 2005 was followed with a slight adjustment of a 40 – 80 % saturation with ammonium sulphate with 10 g fresh weight tissue.

### 2.6.2 Phenyl sepharose column

Pellets were resuspended in 20 mM Tris – HCl pH 7.5 containing 500 mM  $(\text{NH}_4)_2\text{SO}_4$ . A phenyl sepharose column was pre – equilibrated with the same buffer. The supernatant was loaded onto the column using an FPLC system and eluted with 20 mM Tris – HCl pH 7.5.

### 2.6.3 S-Hexyl glutathione affinity chromatography

Pellets were resuspended in 20 mM Tris HCl pH 7.5. An S-Hexyl glutathione column was pre – equilibrated with the same buffer. The supernatant was loaded onto the column using a FPLC system and eluted with 5 mM hexyl-glutathione in 20 mM Tris – HCl pH 7.5.

### 2.6.4 Glutathione agarose affinity chromatography

Protein pellets were resuspended in 20 mM Tris HCl pH 7.5 and a glutathione agarose column pre – equilibrated with the same buffer. The supernatant was then loaded onto the column using an FPLC system and after washing eluted with 5 mM glutathione in 20 mM Tris – HCl pH 7.5.

### 2.6.5 1<sup>st</sup> dimension (Isoelectric focussing)

Samples were concentrated using a Sartorius Vivaspin column (4500 g) prior to acetone precipitation overnight at - 20 °C in 4 v/v ice cold acetone. Precipitates were centrifuged at 14, 000 g for 10 min and the supernatant decanted off. Pellets were resuspended in Destreak ® rehydration solution (GE Healthcare). Immobiline™ DryStrip (GE Healthcare) 7cm pH4 – pH7 strips were used (Strips were left overnight in a re-swelling tray covered in mineral oil.) Strips were focussed using an IPGphor3 isoelectric focussing unit (GE Healthcare). Phase 1: 200 V, 0.05mA SW 0.01 H 1vh, Phase 2: 3500 V, 0.05mA, SW 1.30 H , 2800 vh, Phase 3: 3500 V, 0.05mA, SW 1.30 H, 3700 vh.

After focussing, strips were rinsed in water and equilibrated for 10 min in 10 ml of buffer A containing 50 mM Tris pH8.8, 6M urea, 30 % glycerol, 2 % SDS, and 2 % bromophenol blue, and 100 mg DTT. Strips were rinsed in water and equilibrated secondly in buffer B containing 250 mg idoacetamide.

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### 2.6.6 2<sup>nd</sup> dimension ( SDS – PAGE)

The method of Laemmli (1970) was used to prepare SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels using the mini-PROTEAN III kit from Biorad. Resolving gels were polymerised from 12.5% *bis*-acrylamide in 375 mM Tris.HCl, pH 9.0, 0.1% (w/v) ammonium persulphate, 0.1 % (v/v) *N,N,N',N'* – tetramethylethylenediamine (TEMED), and 0.1 % (w/v) sodium dodecyl sulphate. The IEF strips were placed ontop of the gel along with a marker and secured in place with warm 1 % agarose containing bromophenol blue, prior to electrophoresis in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) at 200 V until the dye has reached the bottom of the gel. Gels were then washed thoroughly with water for 2 x 5 min to remove any residual SDS.

### 2.6.7 Gel staining

Gels were stained with Coomassie blue reagent (0.01% w/v Coomassie brilliant blue, 5% v/v 95% ethanol: water (95:5 v/v) and 10% v/v phosphoric acid: water (85:15 v/v). Polypeptides appearing in the GST molecular mass range were cut out and sent for MALDI – TOF analysis for identification.

## 2.7 HPLC based assays

### 2.7.1 Synthesis of glutathione conjugates

Conjugates were synthesised following the protocol in Edwards *et al* (2005). Herbicides and Safeners were obtained from Sigma-Aldrich (Gillingham, Dorset, UK). The herbicide or safener were dissolved in 4 ml of ethanol / acetonitrile (1:1 v/v). 1 ml of 100  $\mu$ M glutathione was added prior to adjusting the mixture to pH 9.5 with triethylamine and made up to 6 ml with distilled water and incubating for 35 H at RT. 14 ml of ice cold acetone was then added and the mixture stored at - 20 °C for 24 H. The precipitate was collected on filter paper and the conjugate further purified using an HPLC C18 reverse-phase column (Dionex) and eluted using a gradient of 10 - 80% acetonitrile and 0.5% v/v trifluoroacetic acid. Identity of the conjugates was confirmed by MS.

### 2.7.2 HPLC assays

The method described by Edwards *et al* 2005 was used. Crude enzyme preparations were re-suspended in 20 mM Tris-HCl, pH7.5 and desalted using Sephadex G25 columns. Protein content was

determined using a BCA™ protein assay kit (Pierce). 120 µl of 0.1 M phosphate buffer (pH 6.8), 20 µl glutathione, 50 µl of buffer (phosphate buffer for assays withalachlor, 0.1M Tris-HCl for assays with fenoxaprop or fenoxaprop ethyl) were incubated for 60 min at 37 °C along with 10 µl of enzyme. Assays were terminated with 10 µl of 3 M HCl every ten minutes prior to being incubated on ice for 30 min followed by centrifugation at 12 000 rpm for 5 min. 50 µl of supernatant was run on a reverse phase HPLC. Boiled enzyme controls were used to correct for non-enzymic rate of conjugation and controls lacking GSH were used to identify compounds that were not reaction products. Standard curves were used to quantify any conjugate made during the assay using the synthesised glutathione conjugates described previously. Samples were loaded onto a C18 reversed-phase HPLC column and eluted using 1% v/v acetonitrile and phosphoric acid.

### 2.8 Real time polymerase chain reaction (RT-PCR)

A suitable control for amplification was identified which is highly expressed in equal amounts in all tissues. Four housekeeping genes were subjected to RT-PCR in wheat treated with the field rate of cloquintocet mexyl and a 0.1 % acetone control and harvested after 30

min, 1 H, 2 H, 3 H, 4 H, 5 H, 6 H and 24 H post treatment. House-keeping genes selected were GAPDH (glyceraldehyde-3-phosphate dehydrogenase),  $\alpha$ -tubulin,  $\beta$ -tubulin, ubiquitin and actin.

Oligo	Sequence	T <sub>m</sub> (°C)
GAPDH	F: GGA GGA GTC TGA GGG AAA CC	61.4
	R: GCT GTA TCC CCA CTC GTT GT	59.4
$\alpha$ -Tubulin	F: GTC CTG TCC ACC CAC TCA CT	61.4
	R: TGA AGT GGA TCC TCG GGT AG	59.4
$\beta$ -Tubulin	F: ATC CCG AAC AAC GTC AAG TC	57.3
	R: CTC TGC GCC TCA GTG AAC TC	61.4
Actin	F: GTC GGT GAA GGG GAC TTA CA	59.4
	R: TTC ATA CAG CAG GCA AGC AC	57.3
Ubiquitin	F: AAG GAG TCC ACC CTT CAC CT	59.4
	R: AAC CAC AGG ACT CGA TGG TC	59.4

**Table 5 RT - PCR housekeeping gene primers**

Primers and melting temperatures (T<sub>m</sub>) for housekeeping genes tested for amplification.

The GAPDH primer was used as a control for amplification due to being highly expressed at equal levels in both control and treated tissue at all time courses. RT-PCR primers were designed for

*TaGSTF4*, *TaGSTF10*, *TaGSTU6*, *TaGSTU3* and *TaGSTL1*, flanking an intron where possible to improve accuracy.

Oligo	Sequence	T <sub>m</sub> (°C)
TaGSTF4	F: CCC GAT CTC TCA CTC TCT CG	61.4
	R: GAG ATG CTC AGG GCT CTT GT	59.4
TaGSTF10	F: CAG TCA CAC ACA GCA ACA CAC C	62.1
	R: GAA ATC GAT GTC GAC CAC CT	57.3
TaGSTU3	F: CAA CGA GTC CCT CAT CAT CC	59.4
	R: GAG GGT CTT GAG GAT GTC CA	59.4
TaGSTU6	F: AGA TAC CCG TGC TCA TCC A	59.4
	R: GCT TCT TTC CCT CGG ATT TC	57.3
TaGSTL1	F: GCA CTG CTT CCT CAA GAT CC	59.4
	R: GTC ACG TAC GCA ATG TCC AC	59.4

**Table 6 RT - PCR primers for GSTs**

Primers and melting temperatures (T<sub>m</sub>) for the GSTs up-regulated by cloquintocet mexyl in wheat.

### 2.8.1 RNA extraction and quantification

Tissue samples from each time course were homogenised using a pestle and mortar in TRI Reagent (Sigma-aldrich) (1 ml per 100 mg

tissue) and left to stand for 5 min at RT. 0.2 ml of chloroform was added per 1 ml of TRI Reagent used and mixed for 15 sec and left to stand at RT for 15 min. The resulting mixture was centrifuged for 15 min at 12 000 rpm, 4 °C to separate the mixture into three phases, a red organic phase containing protein, an interphase containing DNA and an upper aqueous phase containing RNA. The upper aqueous phases was transferred to a fresh eppendorf and 0.5 ml of isopropanol added per ml of TRI Reagent prior to being left to stand for 10 min at RT. The mixture was then further centrifuged at 12 000 rpm for 10 min at 4 °C. The pellet formed by the RNA precipitate was washed in 75 % ethanol and centrifuged at 7 500 rpm for 5 min at 4 °C. The pellet was then air dried for 10 min and resuspended in 25 µl of water.

The quantification of RNA was determined by making a 1/100 dilution in 10 mM Tris-HCl, pH 7.5 and measuring the absorbance at 260 nm ( $A_{260}$ ). To estimate purity, an absorbance value was obtained using the ratio of readings at 260 nm and 280 nm ( $A_{260}/A_{280}$ ), readings should be greater the 0.15 to ensure significance. an absorbance of 1 unit at 260 nm is equal to 40 µg of RNA per ml. ( $A_{260} = 1 = > 40\mu\text{g} / \text{ml}$ ).

### 2.8.2 Synthesis of cDNA

After quantification 5 µg of RNA was added to an eppendorf containing 6.5 µl of water, and 3.5 µl of OG2 primer prior to heating for 10 min at 65 °C, then cooling on ice for 5 min. To make a final volume of 20 µl, 4 µl of 5 x MMLV (Moloney murine leukaemia virus) buffer, 2 µl of 10 mM dNTP, 0.5 µl RNasin, 1 µl MMLV (Promega) and 2.5 µl of water were added and heated at 37 °C for 90 min, samples were stored at -80 °C until needed.

### 2.8.3 RT-PCR

RT-PCR was carried out using a Rotorgene 3000 (Qiagen) using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma). Expression of the GSTs was determined by comparative quantification, with expression being normalised against GAPDH. The RT-PCR reaction mix was 8 µl of a 1/500 dilution of cDNA made from 5 µg of RNA, 10 µl of 2 x SYBR® Green reaction mix, and 1 µl of 20 µM of primer stock. Optimisation was done in triplicate with varying primer concentrations (10 µM, 15 µM and 20 µM) and cDNA concentrations (1/100, 1/250, 1/500, 1/700) prior to the study.

Cycle	Cycle point
Hold at 930C, 2 min	
Cycling (40 repeats)	Step 1 at 930C, hold 10 sec
	Step 2 at 600C, hold 15 sec
	Step 3 at 720C, hold 20sec, acquiring to cycling A(FAM)
Melt (57-950C) hold 45 sec on the 1 <sup>st</sup> step, hold 5 sec on next step, Melt A(FAM)	

**Table 7 Cycling programme used for RT - PCR**

## 2.9 Inhibition of GSTs

### 2.9.1 Crude assays

7 D old wheat shoots were sprayed with the field rate of cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl as described in section 2. Wheat was harvested at a time course of 30 min, 1 H, 2 H, 3 H, 4 H and 24 H after spraying. Crude extracts were assayed for enzyme activity toward CDNB and for GPOX activity.

### 2.9.2 Isothermal calorimetry

*Ta*GSTU3, *Ta*GSTU6, *Ta*GSTF4, *Ta*GSTF10 and *Ta*GSTL1 were left to dialyse in Strep-buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA) pH 7.6, overnight. 100 µM of either cloquintocet mexyl, fenchlorazole

ethyl, mefenpyr diethyl, cloquintocet, or a fenclorim-GSH conjugate were dissolved in 2  $\mu$ l of DMSO and 998  $\mu$ l of strep-buffer and degassed. Using a VP-ITC Isothermal Titration Calorimeter (MicroCal Inc, Northampton). The ITC was programmed to inject the substrate solution into the enzyme solution in the sample cell. 0.01 mM of substrate (cloquintocet mexyl, cloquintocet, mefenpyr diethyl, fenclorazole ethyl, fenclorim-GSH) was added to the sample cell containing 0.1 mM of enzyme at 25  $^{\circ}$ C at 10  $\mu$ l injections. Origin  $^{\circ}$  7.0 was used to determine the binding constant ( $K_B$ ).

### 2.10 Flavonoid study

Wheat was sprayed with the field rate of cloquintocet mexyl prior to harvesting at a time course of 30 min, 1 H, 4 H and 24 H. Wheat was ground in 4 x ice cold methanol prior to centrifugation at 10 000 rpm for 15 min. Extracts were subjected to LC – MS analysis using a Waters Q-TOF Premier Mass Spectrometer after ESI (capillary 2.55 kV, sample cone 41 kV, extraction cone 5.0 kV, source 100  $^{\circ}$ C with desolvation at 180  $^{\circ}$ C). Metabolites were identified using Cummins (2006) as a guide reference, and quantified using an apigenin standard curve as described by Cummins *et al* (2006).

## 2.11 Metabolite analysis

### 2.11.1 Identification of cloquintocet mexyl metabolites

Wheat was sprayed with the field rate of cloquintocet mexyl prior to harvesting at 24 H. Wheat was ground in 4 x ice cold methanol prior to centrifugation at 10 000 rpm for 15 min. Extracts were subjected to LC – MS analysis using a Waters Q-TOF Premier Mass Spectrometer after ESI (capillary 2.55 kV, sample cone 41 kV, extraction cone 5.0 kV, source 100 °C with desolvation at 180 °C).

### 2.11.2 Quantification of cloquintocet mexyl metabolites

7-day-old wheat shoots were harvested and cut into 1 cm strips prior to being floated on MS sucrose media containing 50 mM cloquintocet mexyl. Flasks were gently agitated at 18 °C and harvested at 30 min, 1 H, 2 H, 3 H, 4 H, 5 H, 6 H and 24 H. Flasks containing no cloquintocet mexyl were used as controls. Tissue was rinsed in water thoroughly to remove any excess media and safener from the surface of the wheat. Wheat was extracted in 4 x methanol prior to centrifugation for 15 min at 10 000rpm and subjected to LC-MS analysis. A standard

curve using cloquintocet mexyl and the free acid cloquintocet were used to quantify the respective metabolites.

## Chapter 3: Induction Of GST Polypeptides in *Triticum aestivum* L.

### 3.1 Treatment of wheat seedlings

To compare the effect of different safeners on the induction of GST polypeptides a uniform treatment was required. Field rates for safener application vary according to the tank mix, crop, safener type and the herbicide. A treatment regime was developed whereby winter wheat was soaked overnight in 0.1% v/v acetone (control), or an identical solution containing 10 mg L<sup>-1</sup> of the safeners cloquintocet mexyl, fenchlorazole ethyl or mefenpyr diethyl. The seeds were then planted in soil and grown in an environmental growth chamber. Shoots were sprayed daily with 25 ml of either the 0.1% v/v acetone (control), or the 10 mg L<sup>-1</sup> solution of the respective safeners. Shoots were harvested at 7 D, 8 D and 9 D, weighed, frozen in liquid nitrogen and stored at - 80 °C until needed. CDNB and GPOX assays were used to determine if the safeners had induced GSTs. Western blot analysis was then used to determine the type of GSTs induced based on using antisera raised to GSTs from the phi, tau and lambda classes.

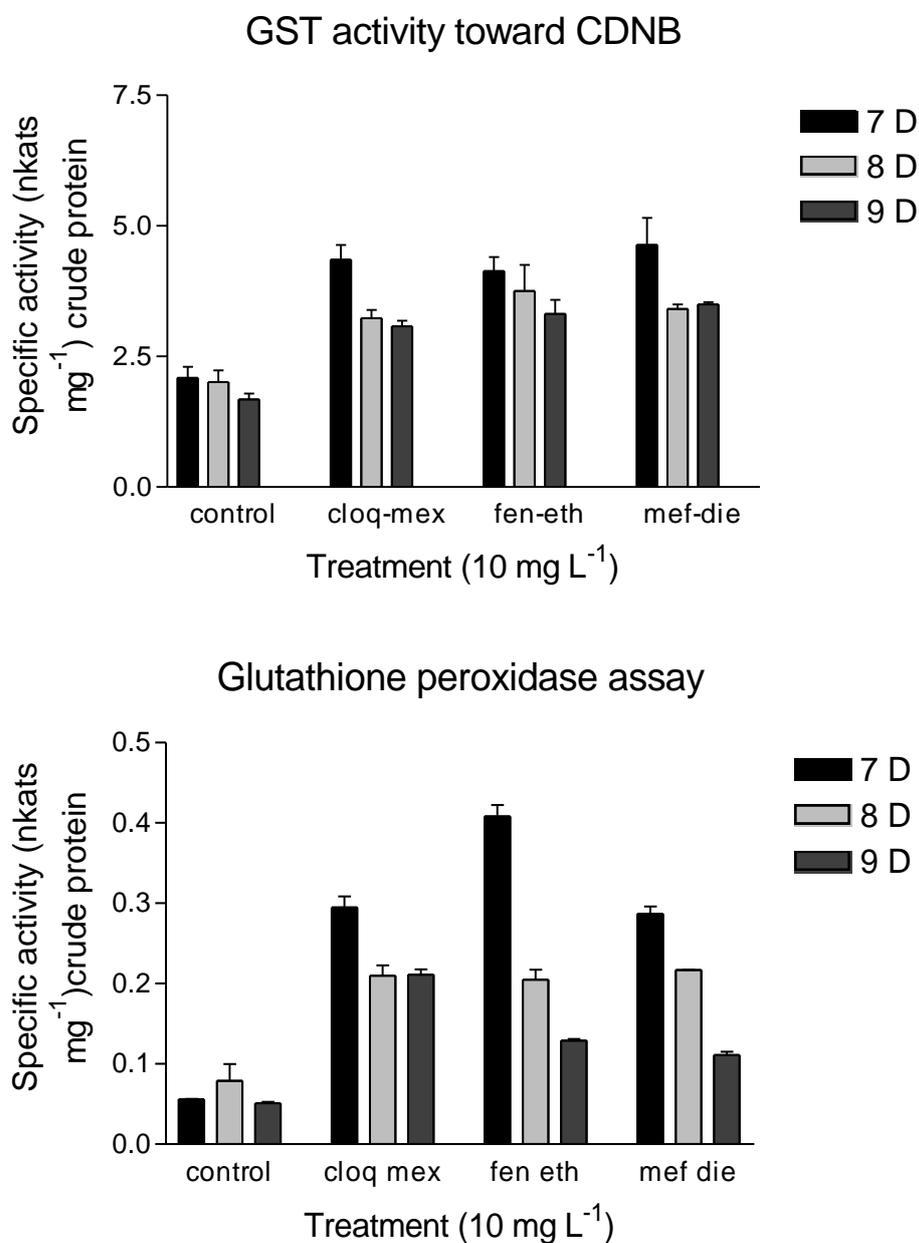
### 3.1.1 Results

Shoots treated with the herbicide safeners cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl showed an overall two fold increase in GST activity toward CDNB over three days relative to the control (Fig 6) with the activity being highest at 7 D and declining over the three days. No significant difference (95 % confidence interval) in the induction of GST activity toward CDNB was found between the safeners at 7 D, 8 D and 9 D.

Over three days, an overall increase in GPOX activity was observed with cloquintocet mexyl and mefenpyr diethyl inducing a six fold increase and fenchlorazole ethyl inducing an eight fold increase at 7 D relative to the control (Fig 6). As with the GST activity toward CDNB, the GPOX activity declined over three days.

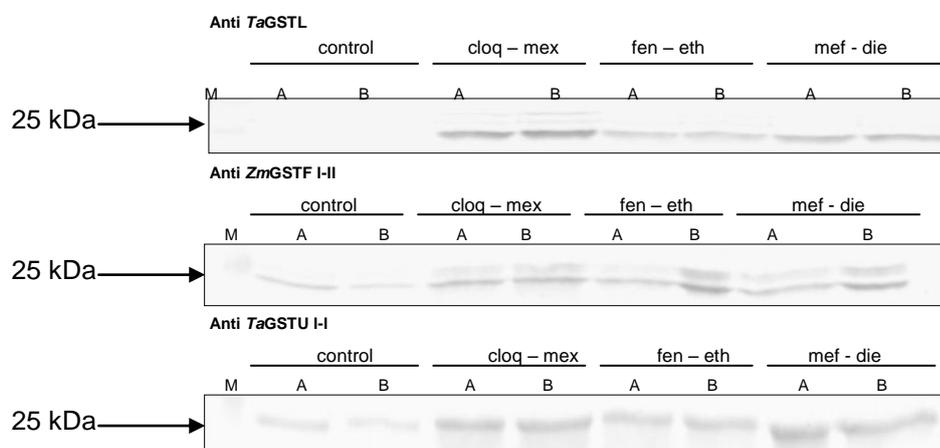
Western blots using antisera raised to specific GSTs (*ZmGSTFI-II*, *TaGSTL* and *TaGSTUI-I*) were used to determine if the safeners were inducing the same classes of GSTs. It can be seen in (Fig 7) that the lambda GST is highly induced by all three safeners, with no detectable lambda GST in the controls. GSTs from the tau and phi classes were

also induced by all three safeners, with little tau and phi GSTs detected in the control.



**Figure 6 GST activity toward CDNB and GPOX activity**

GST activity toward CDNB, and glutathione peroxidase activity in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed for 24 H in 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safeners cloquintocet mexyl, fenclorazole ethyl, and mepfenpyr diethyl, then sprayed daily with 25 ml of either 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safeners (n = 4 ± SD).



**Figure 7 Western blots using antisera raised to specific tau, phi and lambda GSTs**

Induction of glutathione transferases by herbicide safeners in *Triticum aestivum* L. at 9 D  
Seeds were imbibed for 24 H in 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safeners cloquintocet mexyl, fenchlorazole ethyl, and mefenpyr diethyl, then sprayed daily with 25 ml of either 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safeners. Protein extracts were normalised and resolved by SDS-PAGE before probing with antisera raised to the tau class wheat GSTU I-I, phi class maize GST I-II, or the lambda class wheat GSTL. The molecular mass of the immunodetected GST subunits are shown.

### 3.2 Effect of safener treatment on the growth of *Triticum aestivum* L.

#### 3.2.1 Aims and objectives

It has been observed during conducting studies in this thesis that safeners have a growth promoting effect on wheat shoots. This has also been observed in independent studies (Theodoulou *et al.*, 2003). To further quantify this effect, winter wheat was soaked overnight in 0.1% v/v acetone (control) or an identical solution containing 10 mg L<sup>-1</sup> of the safeners cloquintocet mexyl, fenchlorazole ethyl or mefenpyr diethyl respectively, then planted in soil and grown in an environmental growth chamber. Shoots were sprayed daily with 25 ml of 0.1% v/v acetone (control) or the 10 mg L<sup>-1</sup> solution of the safeners. Fifty wheat shoots from four replicates for each treatment were measured (mm) from seed to tip, and the dry and fresh weights recorded from harvests at 7 D, 10 D, 12 D and 14 D.

### 3.2.2 Results

There was a significant increase in height observed in the safener treated wheat shoots after 14 D of treatment (95 % confidence interval), relative to the controls (Table 9). The dry weight of the safener treated wheat was determined using 1 g of fresh weight of wheat to determine whether increased height was due to increase assimilation or due to cell expansion alone, over 14 D (Table 10).

There was no increase in dry weight after 7 D in the safener treated wheat compared to the control. After 10 D, both the fenchlorazole ethyl and mefenpyr diethyl treated wheat showed a 10 % increase in dry weight compared to the controls. In contrast, cloquintocet mexyl-treated wheat showed no increase in dry weight after 10 D (Table 10). After 12 D, the safener treated wheat all showed an increase in dry weight between 10 % - 20 % as compared to the controls (Table 10). After 14 D, cloquintocet mexyl treated wheat showed no increase in dry weight whereas the fenchlorazole ethyl-treated wheat showed a 20 % increase and the mefenpyr diethyl-treated wheat showed a 10 % increase in dry weight compared to the controls (Table 10).

Mean heights of safener – treated and untreated  
wheat shoots (mm)

Treatment	Day of harvest			
	7	10	12	14
Control	96.08 (± 23.1)	156.34 (± 13.1)	195.32 (± 9.7)	235.32 (± 6.5)
Cloquintocet mexyl	96.46 (± 14)	159.3 (± 14.6)	195.18 (± 8.4)	264.12 (± 8.7)*
Fenchlorazole ethyl	105.56 (± 10.3)*	181.32 (± 15.3)*	229.6 (± 11)*	264.1 (± 14.7)*
Mefenpyr diethyl	101.26 (± 9.9)	172.72 (± 13.6)*	196.9 (± 9.2)	245 (± 10.5)*

**Table 8 Safener induced increase in height**

Safener induced increase in height (mm) in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed for 24 H in 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safeners cloquintocet mexyl, fenchlorazole ethyl, and mefenpyr diethyl, then sprayed daily with 25 ml of either 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safeners. Values represent the means of fifty measured shoots at each time course and for each treatment. Values marked with \* are significantly different from the control at a 95% confidence interval.

Mean dry weight (mg) from 1 g fresh weight of safener  
treated and  
untreated wheat shoots (n = 4 ± S.D)

Treatment	Day of harvest			
	7	10	12	14
Control	90 (± 4.8)	100 (± 2.2)	100 (± 1.9)	110 (± 1.5)
Cloquintocet mexyl	90 (± 3.5)	100 (± 5.0)	110 (± 3.3)	110 (± 3.6)
Fenclorazole ethyl	90 (± 3.2)	110 (± 3.1)	120 (± 3.1)	130 (± 3.4)
Mefenpyr diethyl	90 (± 6.1)	110 (± 2.4)	110 (± 2.6)	120 (± 3.0)

**Table 9 Dry weights of wheat shoots treated with safeners**

Increase in dry weight (mg) from 1g fresh weight in wheat shoots (*Triticum aestivum* L.).

Seeds were imbibed for 24 H in 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> of the safeners cloquintocet mexyl, fenclorazole ethyl and mefenpyr diethyl then sprayed daily with 25 ml of either 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safeners. Values represent the mean of four replicates ± S.D.

### 3.3 Studies with cloquintocet mexyl in *Triticum aestivum* L.

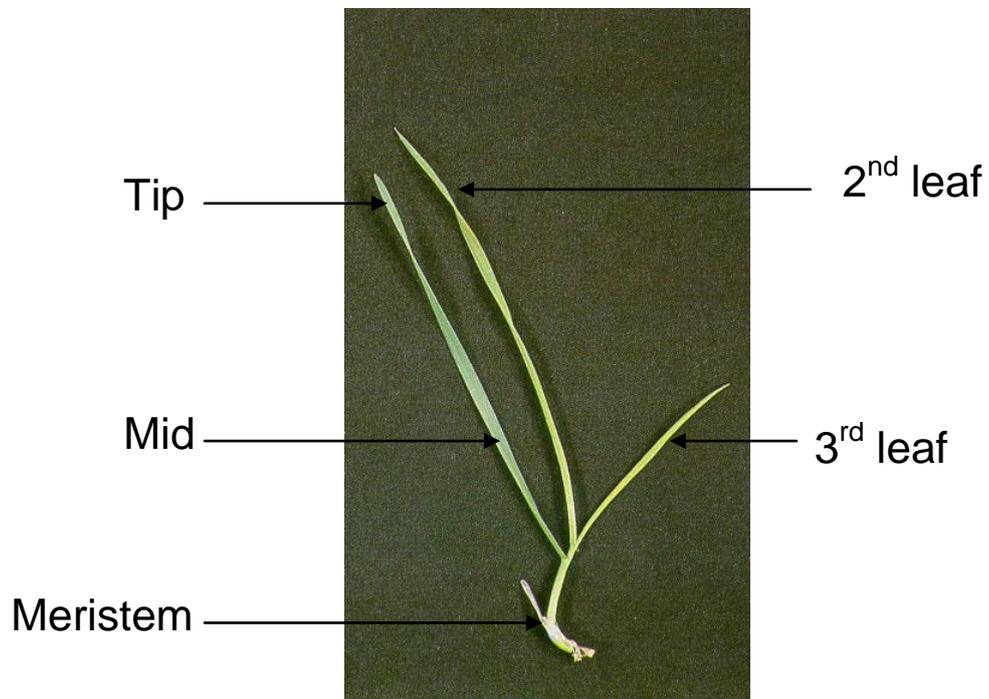
#### 3.3.1 Aims and objectives

After showing that treatment with cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl effectively gave an induction of GSTs from the phi, tau and lambda classes, studies focused on the safener cloquintocet mexyl. The aim of these studies was to determine the localisation of induction of these proteins within the wheat shoot, the time dependence of safening, and the dose responsiveness. In addition it was of interest to determine whether or not safening has an additive or saturative effect on GST induction. In each case the increase in GST activity was determined by assaying with CDNB. Western blots were used to monitor the safening of different GST classes.

### 3.3.2 Localisation of GST activity

#### 3.3.2.1 Treatment

Winter wheat was soaked overnight in a 0.1% v/v acetone (control) or a 10 mg L<sup>-1</sup> solution of the safener cloquintocet mexyl then planted in soil and grown in an environmental growth chamber. Shoots were sprayed daily with 25 ml of a 0.1% v/v acetone (control) or a 10 mg L<sup>-1</sup> solution of the safener cloquintocet mexyl. Shoots were harvested at 7 D and dissected into tip, mid and meristem. Each section was weighed and assayed separately for GST activity toward CDNB and western blots using antisera raised to specific GSTs were used to determine the localisation of GST induction.



**Figure 8 Wheat shoot**

Wheat shoot showing the dissections made of the tip, mid, meristem, 2<sup>nd</sup> and 3<sup>rd</sup> leaves.

Image can be found at <http://www.summitfertz.com.au/calcium & magnesium.htm> visited 10/06/08

### 3.3.2.2 Results

As with study 3.1 (Fig 6), an overall increase in GST activity toward CDNB was observed in the safener treated wheat shoots (Table 11). The induction of GST activity within the wheat shoots was seen in all plant tissue, with the meristematic tissue showing the greatest increase in GST activity in both the control and treated plants.

Western blots using antisera raised to the tau, phi and lambda class GSTs showed a strong induction of all classes in the meristematic tissue of the wheat shoots (Fig 9). As with study 3.1 (Fig 7), the lambda class GST was very safener inducible with no lambda detected in the control sections. One strong band dominated the western blot and can be seen in the meristematic tissue with little detected in the mid and tip sections of the safener treated wheat (Fig 9).

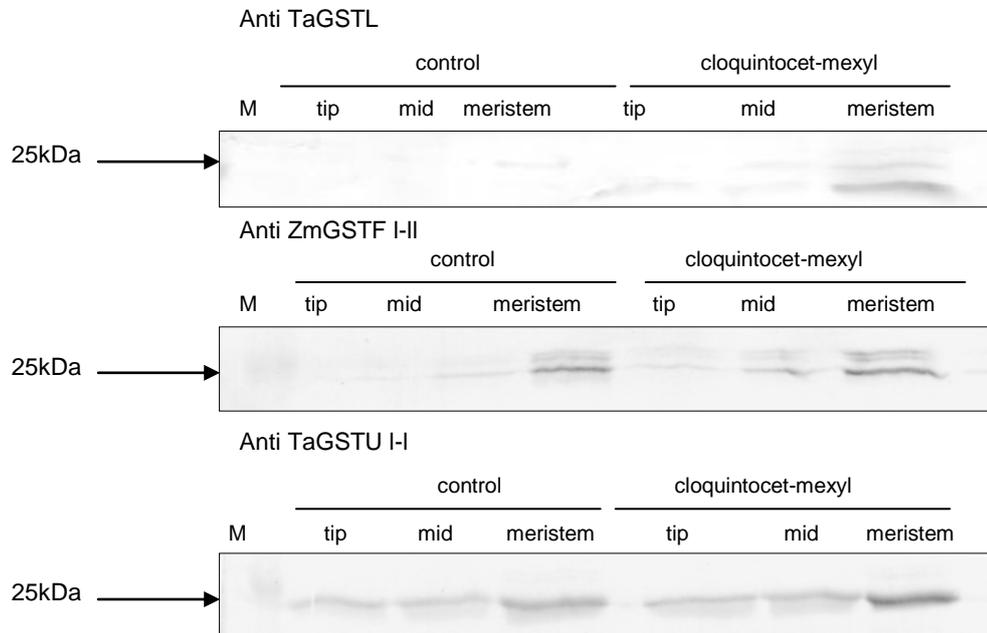
Compared with the safener treated wheat where phi class GST can be seen to be induced in the mid and tip sections, little can be detected in the mid and tip sections of the control and instead the immunoreactive polypeptides are localised to the meristematic tissue (Fig 9). Tau class GSTs were detected in all sections of both the control and the

safener treated wheat though relative abundance was greatest in the meristem.

Treatment	Specific activity (nkats mg <sup>-1</sup> ) crude protein
	Day harvested
	7 D
Control tip	0.46 (± 0.005)
Control mid	0.55 (± 0.021)
Control meristem	1.98 (± 0.009)
Cloquintocet mexyl tip	0.99 (± 0.021)*
Cloquintocet mexyl mid	1.01 (± 0.012)*
Cloquintocet mexyl meristem	3.05 (± 0.021)*

**Table 10 Localisation of GST activity**

GST activity toward CDNB in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed for 24 H in 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the cloquintocet mexyl, then sprayed daily with 25 ml of either 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safener. Values represent the means of triplicate determination with the standard deviation showing the extent of variation between replicates. Values marked with \* are significantly different from the control at a 95% confidence interval.



**Figure 9 Western blots showing localised induction of GSTs in the wheat shoot**

Induction of glutathione transferases by herbicide safeners in wheat shoots (*Triticum aestivum* L.) at 7 D. Seeds were imbibed for 24 H in 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the cloquintocet mexyl, then sprayed daily with 25 ml of either 0.1 % v/v acetone (control), or 10 mg L<sup>-1</sup> solution of the safener. Protein extracts were normalised and resolved by SDS- PAGE before probing with antisera raised to the tau class wheat GSTU I-I, phi class maize GST I-II, or the lambda class wheat GSTL. The molecular mass of the immunodetected GST subunits are shown.

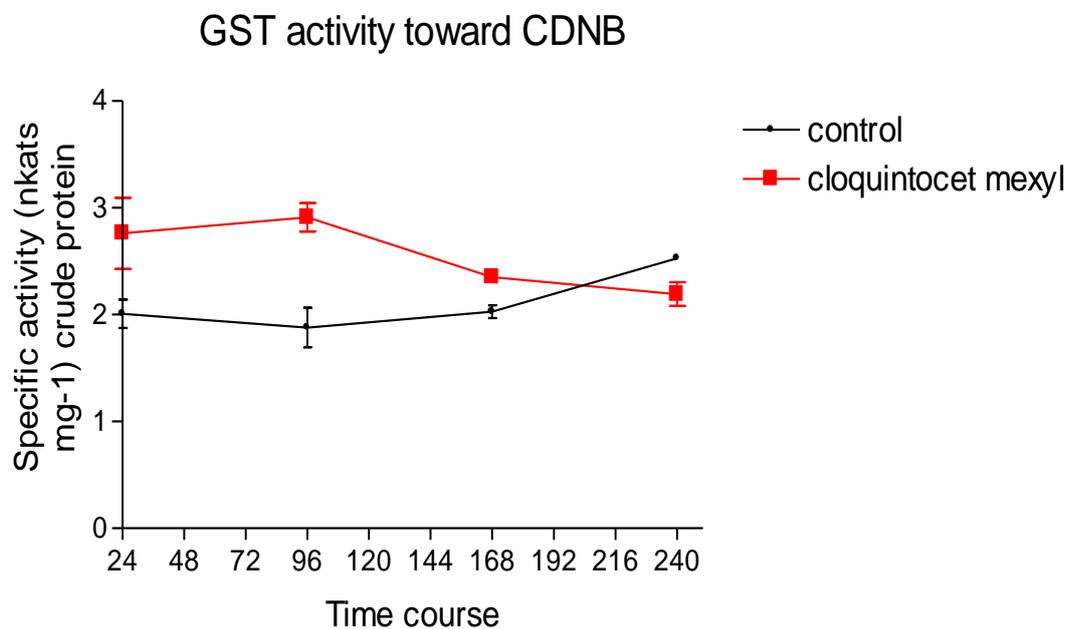
### 3.3.3 Longevity of GST Induction

#### 3.3.3.1 Treatment

7-day-old wheat shoots were sprayed once with the field rate of cloquintocet mexyl (15 g hec in 200 L), or a 0.1 % v/v acetone control. Wheat was harvested at 24 H, 96 H, 168 H and 240 H after treatment, weighed, frozen in liquid nitrogen, and stored at - 80 ° C until needed. In each case GST activity toward CDNB was used as an indicator of GST induction.

#### 3.3.3.2 Results

The time dependence study shows that the induction of GST activity is limited to 10 D after treatment as no enhancement in activity toward CDNB was detected after that time. Cloquintocet mexyl treated wheat showed an increase in GST activity toward CDNB 24 H after treatment of 48 %, then increased to 58 % after 96 H. At 168 H GST activity toward CDNB decreased to 17.5 % with no activity seen after 240 H (Fig 10).



**Figure 10 Longevity of GST induction**

GST activity toward CDNB in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed in water for 1 H to promote germination before sowing on soil. Wheat shoots were sprayed with the field rate of cloquintocet mexyl and a control at 7 D and harvested after 24 H, 96 H, 168 H and 240 H to determine the time dependence of safening ( $n = 4 \pm \text{S.D.}$ ).

### 3.3.4 Dose responses to safening

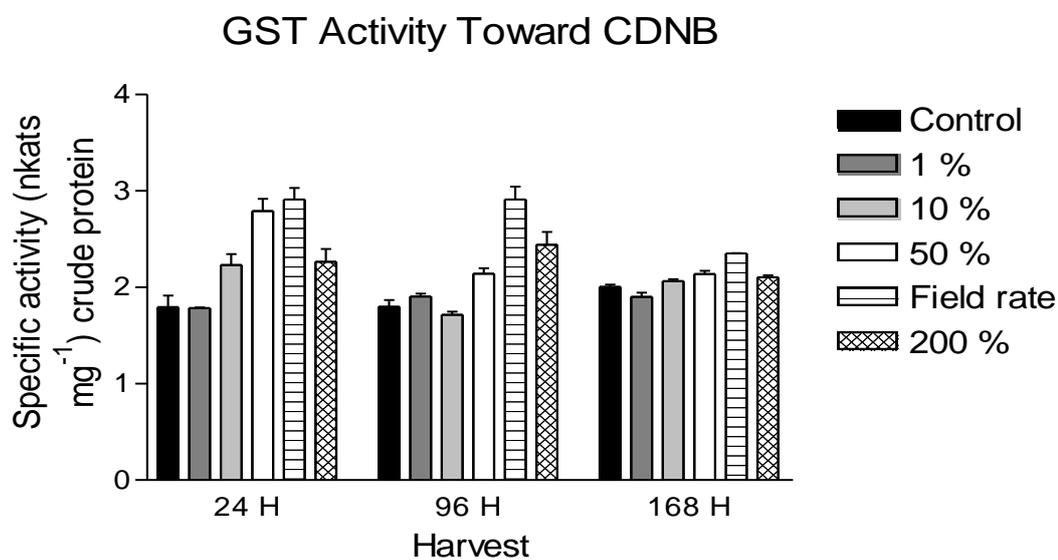
#### 3.3.4.1 Treatment

7-day-old wheat shoots were sprayed once with 1 %, 10 %, 50 %, and 200 % of the field rate of cloquintocet mexyl (15 g hec in 200 L), or a 0.1 % v/v acetone control to determine the dose dependence of safening. Wheat was harvested 24 H, 96 H, and 168 H after treatment, weighed, frozen in liquid nitrogen, and stored at - 80 ° C until needed. GST activity toward CDNB was used as an indicator of GST induction.

#### 3.3.4.2 Results

The response to the safeners can be observed in Fig 11, with 1 %, and 10 % of the field rate showing no significant enhancement in GST activity toward CDNB relative to the control, at any time point. There was a significant and similar increase in GST activity 24 H after treatment with both the field rate and with the 50 % dose relative to the control ( 95 % confidence interval), with an average increase in GST activity of 58 % (Fig 11)

There was an increase in GST activity after 96 H in the wheat shoots treated with 50 %, field rate and 200 % dose, with the field rate dose having the highest increase in activity of 62 % relative to the control (Fig. 0). After 168 H only the 50 % dose and the field rate showed any increase in activity, with the field rate showing the greatest increase at 17 % relative to the control (Fig 11).



**Figure 11 Dose responsiveness of GST induction**

GST activity toward CDNB in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed in water for 1 H to promote germination before sowing on soil. Wheat shoots were sprayed with the field rate of cloquintocet mexyl and a control at 7 D and harvested after 24 H, 96 H, and 168 H to determine if there is any dose response ( $n = 4 \pm SD$ ).

### 3.3.5 Additive / saturative effect of safeners on GST induction

#### 3.3.5.1 Treatment

Following the results of the dose dependence study, a further study was set up to determine if the increase in GST activity caused by the safeners is additive and saturatable. 7-day-old wheat shoots were sprayed once with the field rate of cloquintocet mexyl (15 g hec in 200 L) or a 0.1 % v/v acetone control. After 48 H, half of the wheat was harvested, weighed, frozen in liquid nitrogen, and stored at - 80 °C until needed. The remaining wheat was sprayed for a second time with the field rate of cloquintocet mexyl and harvested a further 48 H later. GST activity toward CDNB was used as an indicator of safening and western blots using antisera raised to specific lambda and tau class GSTs were used to identify any difference in GST induction between samples.

### 3.3.5.2 Results

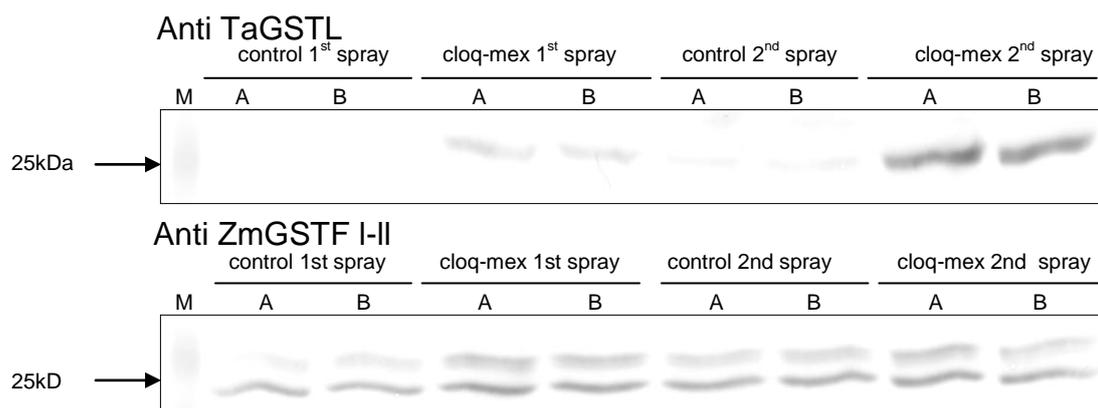
There was a significant increase in GST activity toward CDNB between the safener treated wheat and the controls (Table 12) (95% confidence interval) in the first spray. No further induction was determined between the 1<sup>st</sup> and 2<sup>nd</sup> sprays of cloquintocet mexyl-treated wheat. This saturation of GST activity was also seen in study 3.3.4 (Fig 11) where an increase in dose was shown to have no additive effect on GST activity, showing that once the GSTs with activity toward CDNB have been induced, this effect cannot be increased further.

Although there was no increase in GST activity toward CDNB after the second spray (Table 12), there was an increase in induction of lambda class GST (Fig 12) after the second spray. Lambda is induced after the first spray with safener treatment, with no lambda detected in the control (as seen in Figs. 7, 9 and 12), but unlike the phi GSTs this induction was also observed after the second spray. Lambda induction is unlikely to correlate with CDNB activity due to them having a different catalytic function.

Treatment	Specific activity (nkats mg <sup>-1</sup> crude protein)	
	Day harvested	
	1 <sup>st</sup> spray	2 <sup>nd</sup> spray
Control	1.68 (± 0.03)	2.00 (± 0.08)
Cloquintocet mexyl	2.48 (± 0.03)*	2.46 (± 0.10)*

**Table 11 Additive / saturable effect of safening on GST induction**

Induction of glutathione transferases by herbicide safeners in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed in water for 1 h to promote germination before sowing on soil. Wheat shoots were sprayed with the field rate of cloquintocet mexyl and a control at 7 D and harvested after 48 H, when wheat was sprayed for a second time with the field rate of cloquintocet mexyl. Wheat was harvested a further 48 H after the second spray. Values marked with \* are significantly different from the control at a 95% confidence interval (n = 4 ± SD).



**Figure 12 Western blots showing the additive or saturable effect of safeners on GST induction**

Induction of glutathione transferases by herbicide safeners in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed in water for 1 H to promote germination before sowing on soil. Wheat shoots were sprayed with the field rate of cloquintocet mexyl and a control at 7 D and harvested after 48 H, when wheat was sprayed for a second time with the field rate of cloquintocet mexyl. Wheat was harvested a further 48 H after the second spray. Protein extracts were normalised and separated by SDS-PAGE before probing with antisera raised to the phi class maize GST I-II, or the lambda class maize GSTL. The molecular mass of the immunodetected subunits are shown

### 3.3.6 Cloquintocet / cloquintocet mexyl studies

#### 3.3.6.1 Treatment

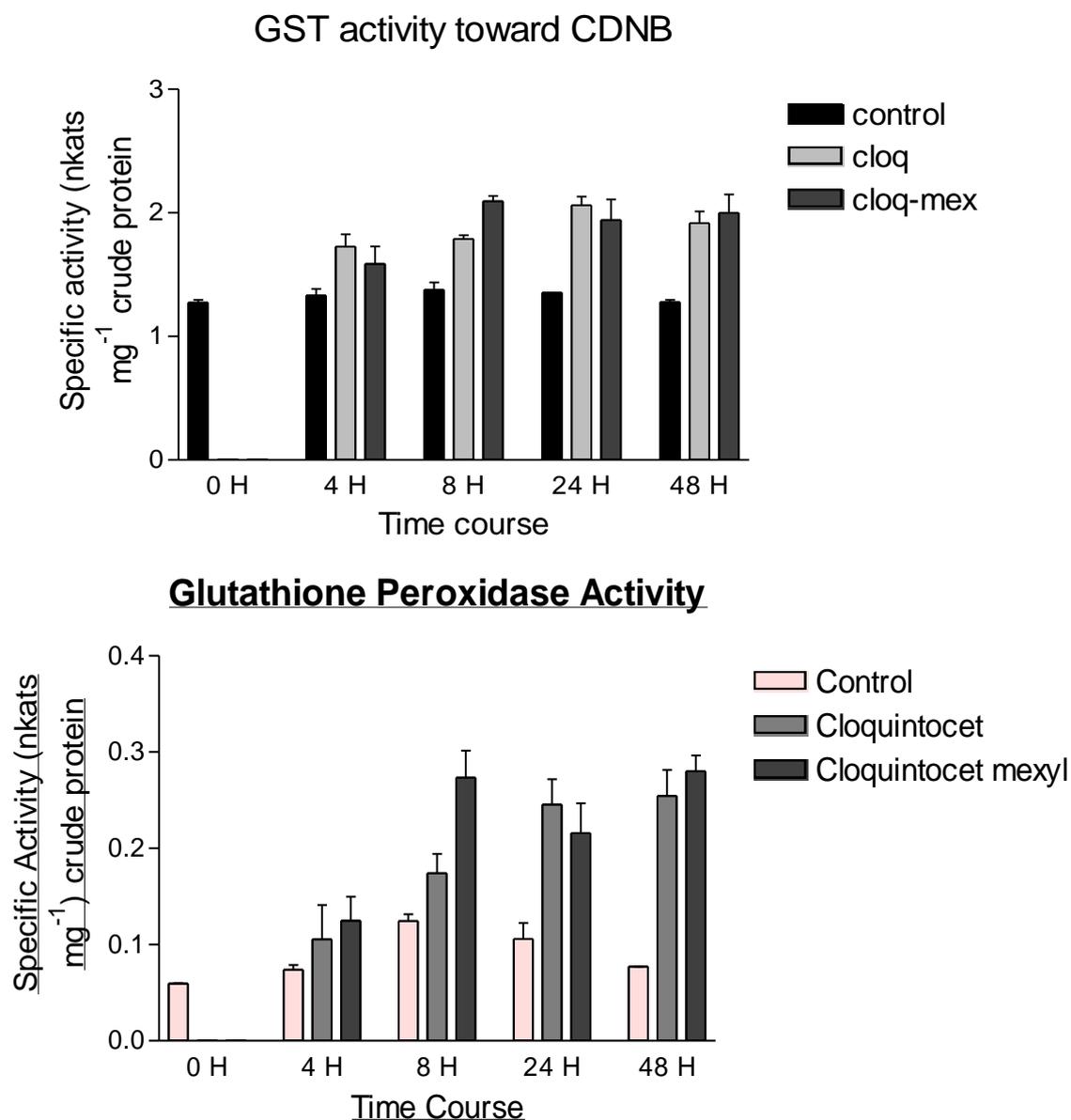
7-day-old wheat shoots were sprayed with the field rate (15 g hec in 200 L) of cloquintocet mexyl and the free acid cloquintocet to determine whether the ester group on cloquintocet mexyl has any function in its ability to safen wheat. Wheat was harvested 4 H, 8 H, 24 H, and 48 H after treatment in order to determine how quickly cloquintocet mexyl and cloquintocet exert safening. After harvest wheat was weighed, frozen in liquid nitrogen and stored at - 80 °C until needed. GST activity toward CDNB was used as an indicator of safening, together with a GPOX assay. Western blots using antisera raised to specific GSTs were also used to identify specific classes of GST induced.

### 3.3.6.2 Results

There was a significant increase in GST activity toward CDNB 4 H, 8 H, 24 H and 48 H after treatment, relative to the control (95 % confidence interval), with both treatments, there being no significant difference between the ability of cloquintocet acid and cloquintocet mexyl to induce GSTs with activity toward CDNB at any time course except 8 H where the ester increases GST activity by 22 % more than cloquintocet (Fig 13).

Glutathione peroxidase activity did not increase until 8 H after treatment and increased at each consecutive time course after, relative to the control (Fig 13). There was no significant difference between the ability of the cloquintocet acid and cloquintocet mexyl to induce GSTs that also function as glutathione peroxidase at any time point except 8 H, as observed with the GST activity toward CDNB (Fig 13).

As with previous studies (Figs 7, 9 and 12) GSTs from the tau, phi and lambda classes were induced by cloquintocet mexyl. It can be seen (Fig. 14) that there is no difference in the relative activity of cloquintocet or cloquintocet mexyl to induce GSTs from the tau, phi and lambda classes.



**Figure 13 Induction of GSTs by cloquintocet mexyl and cloquintocet**

GST activity toward CDNB, and glutathione peroxidase activity in wheat shoots (*Triticum aestivum* L.). Seeds were imbibed in water for 1 H to promote germination before sowing on soil. Wheat shoots were sprayed with the field rate of cloquintocet mexyl, the free acid cloquintocet and a control at 7 D. Wheat was harvested at 0 H, 4 H, 8 H, 24 H and 48 H ( $n = 4 \pm SD$ ).



**Figure 14 Western blots showing induction of GSTs by cloquintocet methyl and cloquintocet**

Induction of glutathione transferases by herbicide safeners in wheat shoots (*Triticum aestivum* L.) 24 H after treatment. Seeds were imbibed for 1 H in water to promote germination before sowing in soil. Wheat was sprayed at 7 D with the field rate of cloquintocet methyl and the free acid cloquintocet, then harvested 4H, 8H, 24H, and 48H after treatment. A, B and C represent replicates. Protein extracts were normalised and separated by SDS-PAGE before probing with antisera raised to the tau class wheat GSTU I-I, phi class maize GST I-II, or the lambda class maize GSTL. The molecular mass of the immunodetected GST subunits are shown.

### 3.4 Discussion

Three wheat safeners, cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl, were each tested for their ability to induce GSTs in wheat. The over all question to be addressed is whether or not these safeners all have the same effect on the induction of GST polypeptides. Safeners protect the crop plant from herbicide damage without effecting weed control efficacy and in every case reported this is associated with an increase in the induction of GSTs (Edwards *et al.*, 2000; Davies and Caseley, 1999). Therefore an increase in GST induction was used as an indicator of safening by means of assaying GST activity toward CDNB, GPOX (Fig 6) and immunoblotting using antisera raised to specific GSTs (Fig 7).

Antisera used was raised to *Ta*GSTUI-I, *Zm*GSTFI-II and *Ta*GSTL have been shown previously to recognise wheat GSTs in a class specific manner (Edwards *et al.*, 2000; Cummins *et al.*, 1997; Cummins *et al.*, 2003). The anti – GSTL- serum has also been shown to recognise lambda class specific GSTs in previous unpublished studies (Chapman.H, 2006).

Study (3.2) Fig (6) demonstrates that all three safeners induce GST activity toward CDNB relative to the control. This was also found by Scarponi *et al.* (2006), Cummins *et al.* (2002) in *Triticum aestivum*, and by Riechers *et al.* (1996) in *Triticum tauschii*. GPOX activity (Fig 6) increased significantly on exposure of the wheat to all three safeners. This suggested that plants respond to oxidative injury caused by the safener by enhancing GST mediated GPOX activity. The GSTs protect the plant from oxidative injury by functioning as GPOXs.

GSTs from the phi and tau class have been shown to have GPOX activity (Cummins *et al.*, 1999) with the GSTs using glutathione to reduce organic hydroperoxides of fatty acids and nucleic acids to the corresponding monohydroxyalcohols (Dixon *et al.*, 2002). The Western blotting studies (Fig 7) demonstrated an enhancement of GST expression after exposure to all three safeners. Previous studies by Cummins *et al.* (2002, 2003), and Brazier *et al.* (2002) also showed this effect with GSTs from the phi and tau classes, however the lambda class of GST was not tested in these two studies. It can be seen from Fig (7) that the lambda GSTs are very safener inducible with a negligible amount seen in the control sample, this is in contrast to the phi and tau GSTs which can be seen in small quantities in the control samples. A study by Edwards *et al.* (2000) using the same antisera

demonstrated that treatment over 48 H with cloquintocet mexyl gave a negligible enhancement of tau GSTs, and weak enhancement of phi.

Treatment with fenchlorazole ethyl gave enhancement of tau GSTs but not phi.

The varying factor in these studies has been the treatment of the wheat. In study (3.2) the wheat was soaked in a 10 mg L<sup>-1</sup> solution of the safeners then sprayed with 25 ml of a 10 mg L<sup>-1</sup> solution of the safeners daily. Showing that the induction of GSTs may be affected by the length of exposure to the safeners and time of harvest, treatment regimes, and treatment. With this in mind studies (3.3) investigated this further.

Tables (9 & 10) show that all three safeners significantly enhance growth in wheat shoots. This has been observed in previous unpublished studies but never fully quantified. By 14 D, all safened wheat showed a significant increase in height with only fenchlorazole ethyl treated wheat demonstrating an increase over all four harvests.

Studies (3.3) focussed on the safener cloquintocet mexyl after demonstrating that all three safeners effectively promoted an enhancement of GST induction in *Triticum aestivum*. The first

question posed was is there any localisation of GST activity within the wheat shoot (3.3.2), a question that has been previously largely unanswered in the literature. Wheat shoots were dissected into tip, mid and meristematic tissues (Fig 8) and each section assayed separately. The results show that there was a localisation of GST induction within wheat shoots following safener treatment and that this induction is stronger in the meristematic tissue. Induction of lambda class GSTs was localised to the meristem of safener treated wheat, whereas the phi and tau classes were induced in all three sections. Again as in study (3.2) the lambda GSTs have been shown to be highly safener inducible.

Study (3.3.3) aimed to determine the time dependence of GST induction. From Fig (10) it can be seen that GST activity towards CDNB is no longer present after ten days, despite further treatment with the cloquintocet mexyl. This shows that there is possibly some kind of feedback mechanism or inhibition that prevents the safeners from having an additive effect on the induction of GSTs after their initial increase. This was also seen in study (3.2) (Fig 6) where GST activity toward CDNB and GPOX activity declined after seven days of treatment.

The dose response study (3.3.4) (Fig 11) also showed this decline in GST activity even with a different treatment. From Fig (11) It can be seen that GST activity is dependent on the dose of safener applied, with the greatest increase in activity seen in wheat sprayed with the field rate.

This shows that the manufacturers field rate is the best rate to achieve optimum safening in wheat, as well as demonstrating that GST activity is only increased up to a certain level before it begins to decrease. This effect was also observed in study when wheat shoots were dosed up with  $10 \text{ mg L}^{-1}$  of the safeners from seed. Although the treatment differed it can be seen also from study (3.3.3) that after 7 D (Fig 11) GST activity decreases despite further safener treatment. The increases in GST activity toward CDNB observed with the field rate were also similar to those observed in the time dependence study (Fig 10) showing that the increases in GST activity and induction is replicable. With the previous studies demonstrating that GST induction is time dependent.

Study (3.3.5) aimed to show whether GST activity and induction was a saturated or additive effect by means of western blots and CDNB assays. Wheat was sprayed once at 7 D with the field rate of

cloquintocet mexyl and a sample harvested 48 H later, with the remaining plants sprayed a second time then harvested a further 48 H later. The induction of GST activity toward CDNB was shown to be saturable as there was no significant difference between spray treatments (Table 12). However upon analysis with western blotting using Anti GSTL and Anti *ZmGSTF* I-II antisera it can be seen that there is an additive effect with the lambda (Fig 12) GST. It is possible that the lambda GSTs are slower to be induced compared to the tau and phi GSTs, or that the GSTs are induced via different signalling pathways.

Cloquintocet mexyl has a unique chemistry from other safeners in that it has a large ester moiety (Fig 3). This poses the question of whether the ester group is important in the safeners ability to induce safening.

Wheat was sprayed with the free acid cloquintocet and cloquintocet mexyl in order to determine this. It can be seen from Fig (13) that the free acid does have the ability to induce GST activity, suggesting that the ester is present to aid passage into the plant through the waxy cuticle (Roberts, 1998).

Cloquintocet and cloquintocet mexyl both induce GSTs from the same classes as can be seen from Fig (13) again showing that the ester

group is not important in the chain of signalling events leading to induction of GSTs. For the purpose of this study equal molar amounts of each chemical were not necessary as the aim was solely to determine if the free acid could safen. Both the free acid and the safener were applied at the recommended field rate. In order to quantify the ability of cloquintocet mexyl and cloquintocet relative to each other this study must also be repeated using equal molar amounts of each chemical in future work.

It can also be seen from this study that GST induction appears as early as 4 H after spray treatment (Fig 13 ). Fig (13) shows that safening can be seen between 4 H and 10 D after spray treatment.

This chapter has demonstrated that although the wheat safeners vary in their structure (Fig 3), they all induce a similar subset of GSTs from the phi, tau and lambda classes. This activity has been shown to be dose responsive and saturable. In addition to this, cloquintocet mexyl was found to induce GSTs from the lambda class and this was found to be localised in the meristematic tissue of the wheat shoot. It is interesting to note that this is also the target site for clodinafop propargyl (ACCCase inhibitor). It has also been demonstrated that safeners have a growth promoting effect on wheat shoots and that the

free acid moiety of cloquintocet mexyl is responsible for the induction of GSTs.

## Chapter 4: Identification of Safener Inducible GSTs using a Proteomics Approach

### 4.1 Introduction

After demonstrating that herbicide safeners induce GSTs from the lambda, phi and tau classes in chapter 3, 2 D gel analysis was used to determine exactly which GST polypeptides were being enhanced as a prelude to cloning the respective cDNA and characterising the respective enzymes.

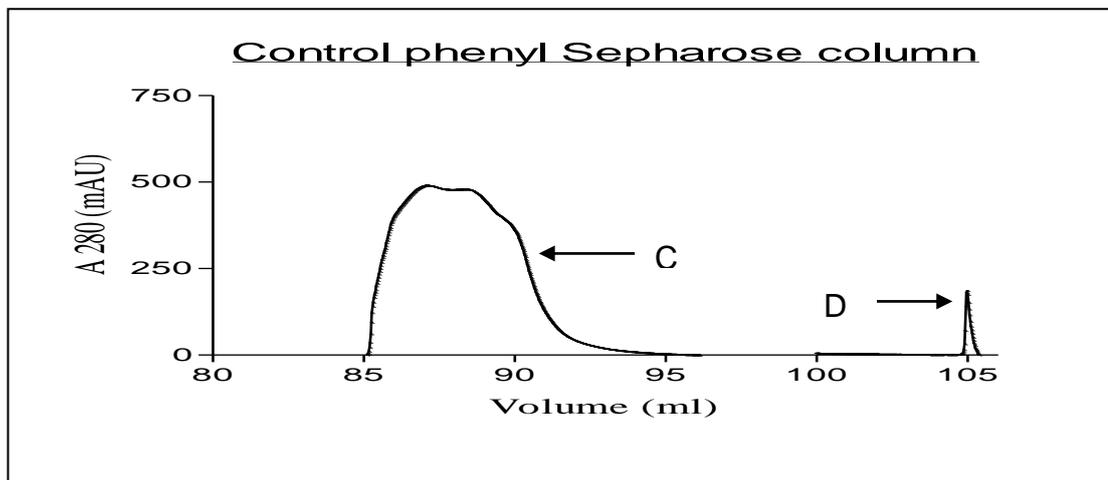
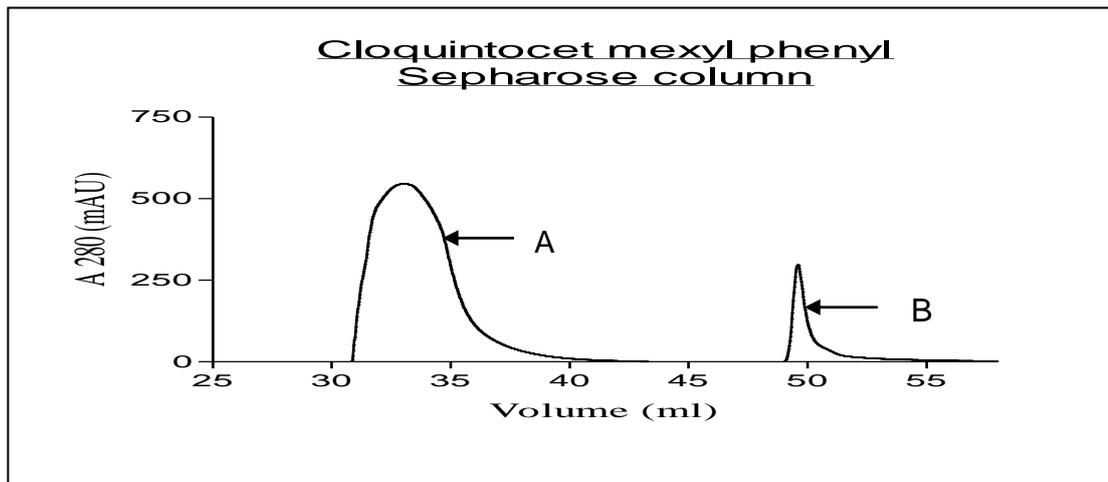
Other studies in the literature describe a variety of techniques to identify safener induced GSTs. For example, subtractive suppression hybridisation was used to identify genes in wheat which were upregulated by cloquintocet mexyl (Theodoulou *et al.* 2003). A study by De Ridder *et al.* (2002) used GSH – affinity chromatography coupled with 2 D gel electrophoresis to identify one of the major safener inducible GSTs in *Arabidopsis thaliana* AtGSTU19. Based on the success of this affinity method in *Arabidopsis*, it was of interest to try a similar approach in *Triticum aestivum* L. Three types of GST affinity column were tested to, identify further GSTs, namely glutathione agarose, hexyl glutathione and phenyl sepharose.

Following previous studies in chapter three focusing on cloquintocet mexyl, wheat was treated at 7 D with cloquintocet mexyl (15 g.a.i ha<sup>-1</sup>), or a 0.1 % acetone control prior to being harvested in duplicate after 24 H.

Wheat extracts were then precipitated between 40 - 80 % saturation using (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, prior to desalting on a PD-10 column. Crude protein extracts were assayed for CDNB conjugating activity to ensure GSTs had been induced. Crude extracts were then purified using two different affinity columns (glutathione agarose and hexyl glutathione), or by hydrophobic interaction using a phenyl sepharose column. Crude protein from 10 g fresh weight of plant tissue was loaded onto each column. Fractions containing the affinity bound protein were then subjected to 2 D gel analysis to determine which GSTs were safener inducible. Protein content was normalised prior to 2 D gel analysis to ensure even protein loading.

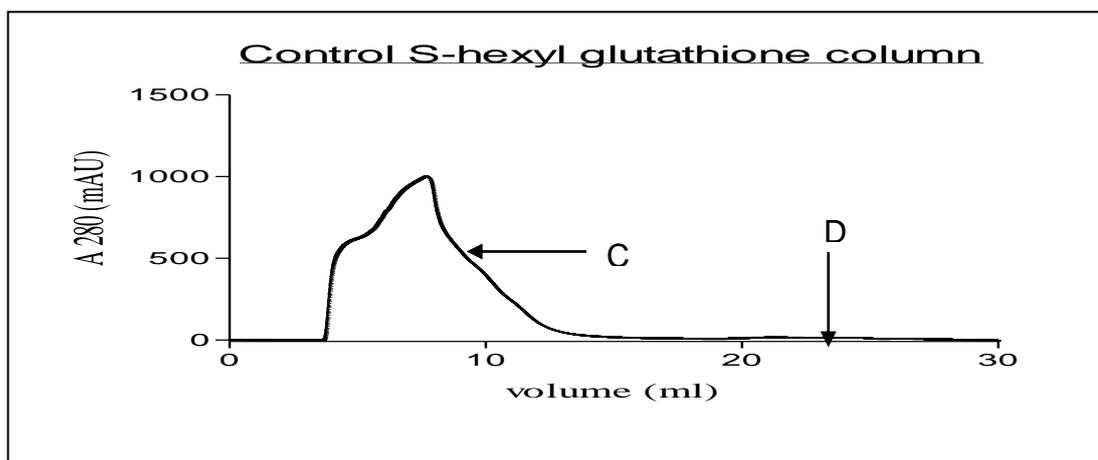
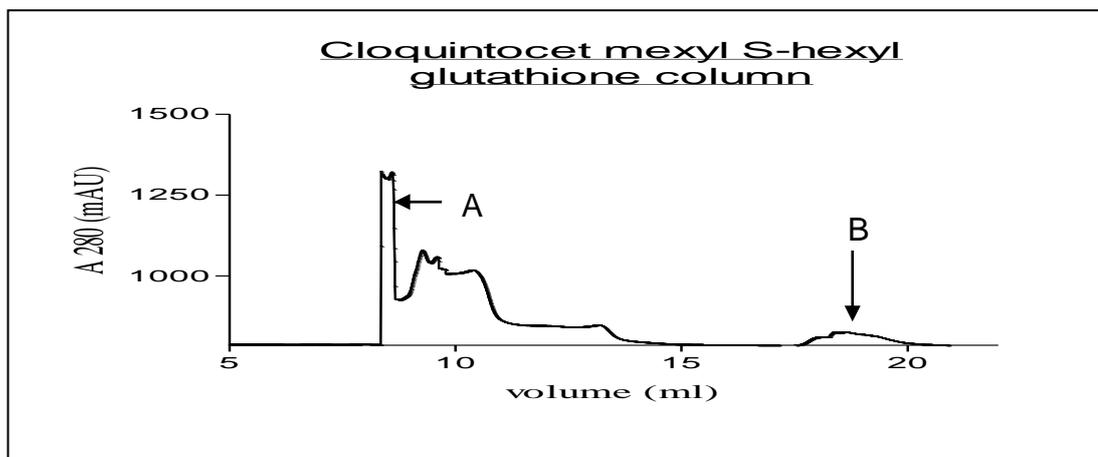
## 4.2 Results

Crude extracts were purified by affinity chromatography using glutathione agarose (Fig 17), S-hexyl-glutathione (Fig 16) and hydrophobic interaction (Fig 15). Two peaks were observed in both the control and the treated extracts on the chromatograms (Figs 15, 16, 17). The first peak was always the largest and represented the unbound protein, which was discarded. The second smaller peak was the elution of the bound protein. This eluent was then subjected to 2 D gel analysis. The first dimension separates proteins by their isoelectric point (the pH at which the protein has no net charge). The eluent was concentrated and added to a buffer containing ampholytes and applied to a gel strip. The pH gradient is established along the gel strip by the ampholytes. During electrolysis the ampholytes migrate along the strip until they reach a region where their pH is equal to their pI (thereby separating the strip into zones of a defined pH).



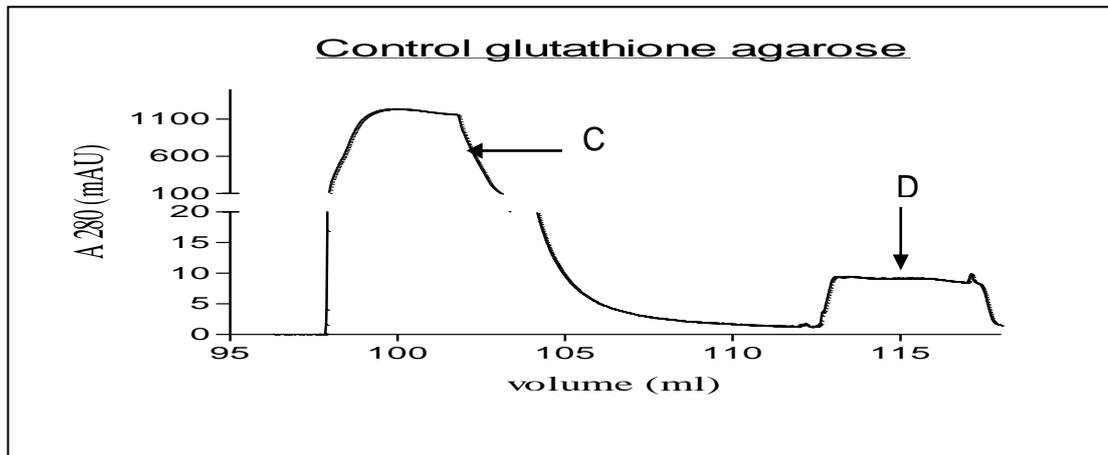
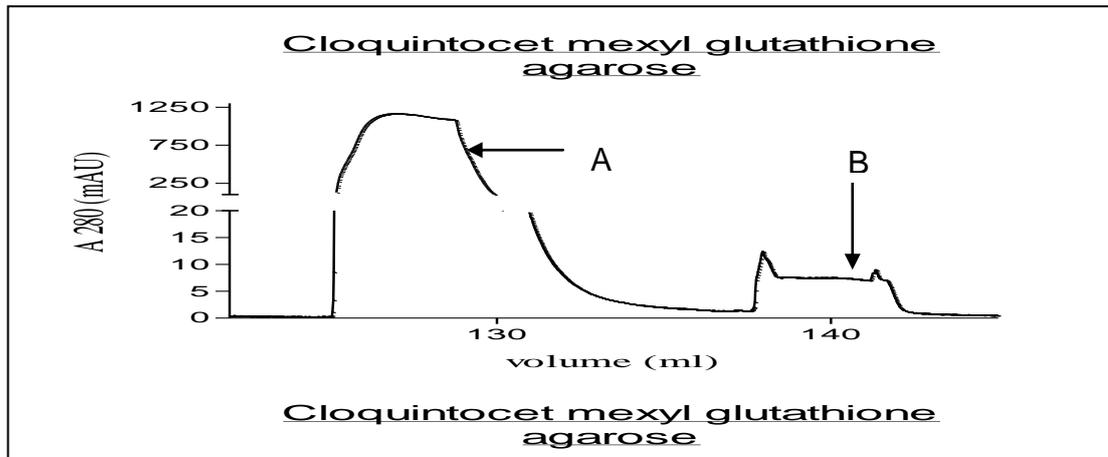
**Figure 15 Chromatographs showing elution of bound protein from the phenyl sephacrose column**

A and C are unbound protein peaks. B is the bound protein from the extracts treated with cloquintocet mexyl. D is the bound protein from the control.



**Figure 16 Chromatographs showing elution of bound protein from the S-hexyl glutathione column**

A and C are unbound protein peaks. B is the bound protein from the extracts treated with cloquintocet mexyl. D is the bound protein from the control.

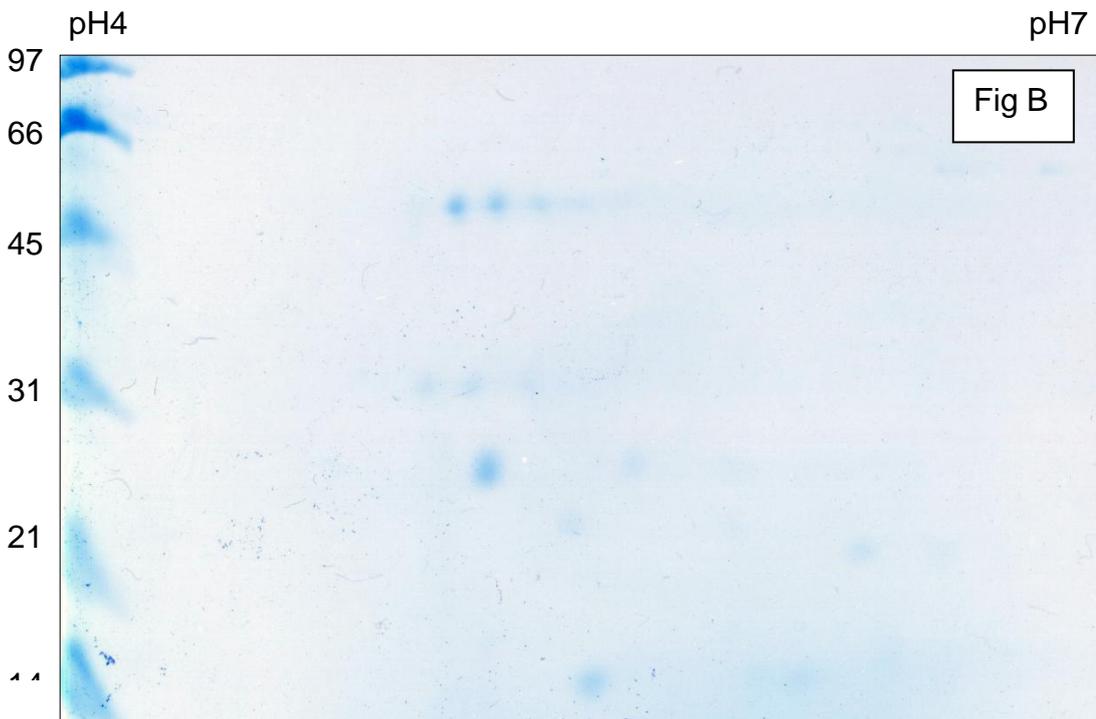
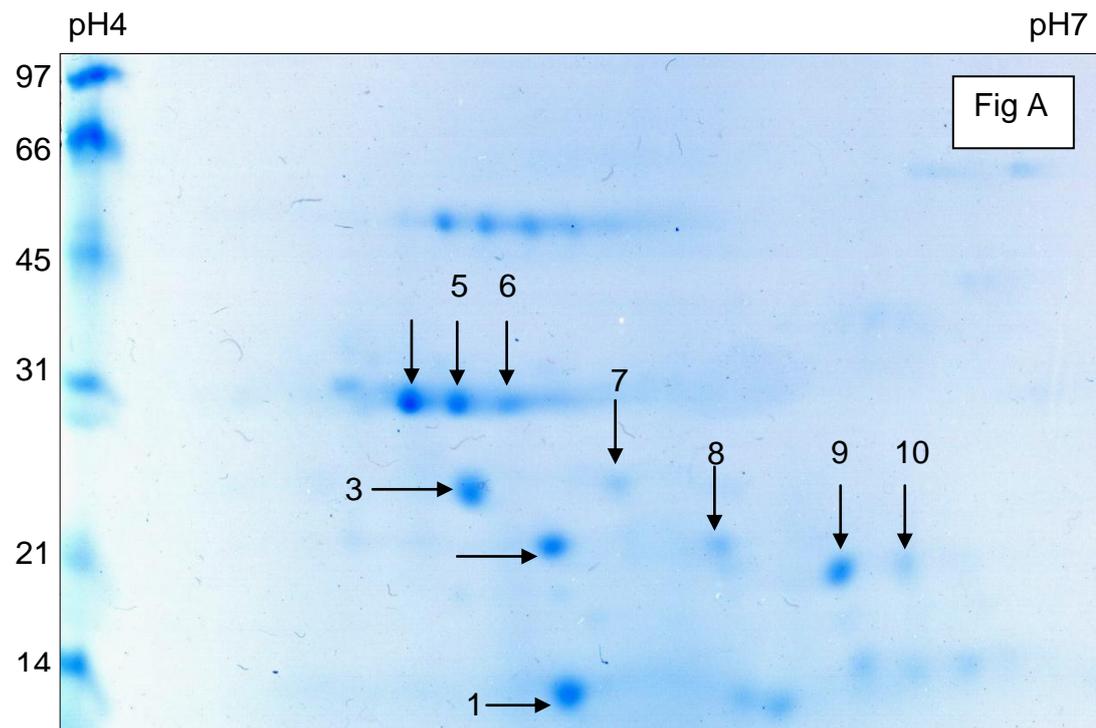


**Figure 17 Chromatographs showing elution of bound protein from the glutathione agarose column**

A and C are unbound protein peaks. B is the bound protein from the extracts treated with cloquintocet mexyl. D is the bound protein from the control.

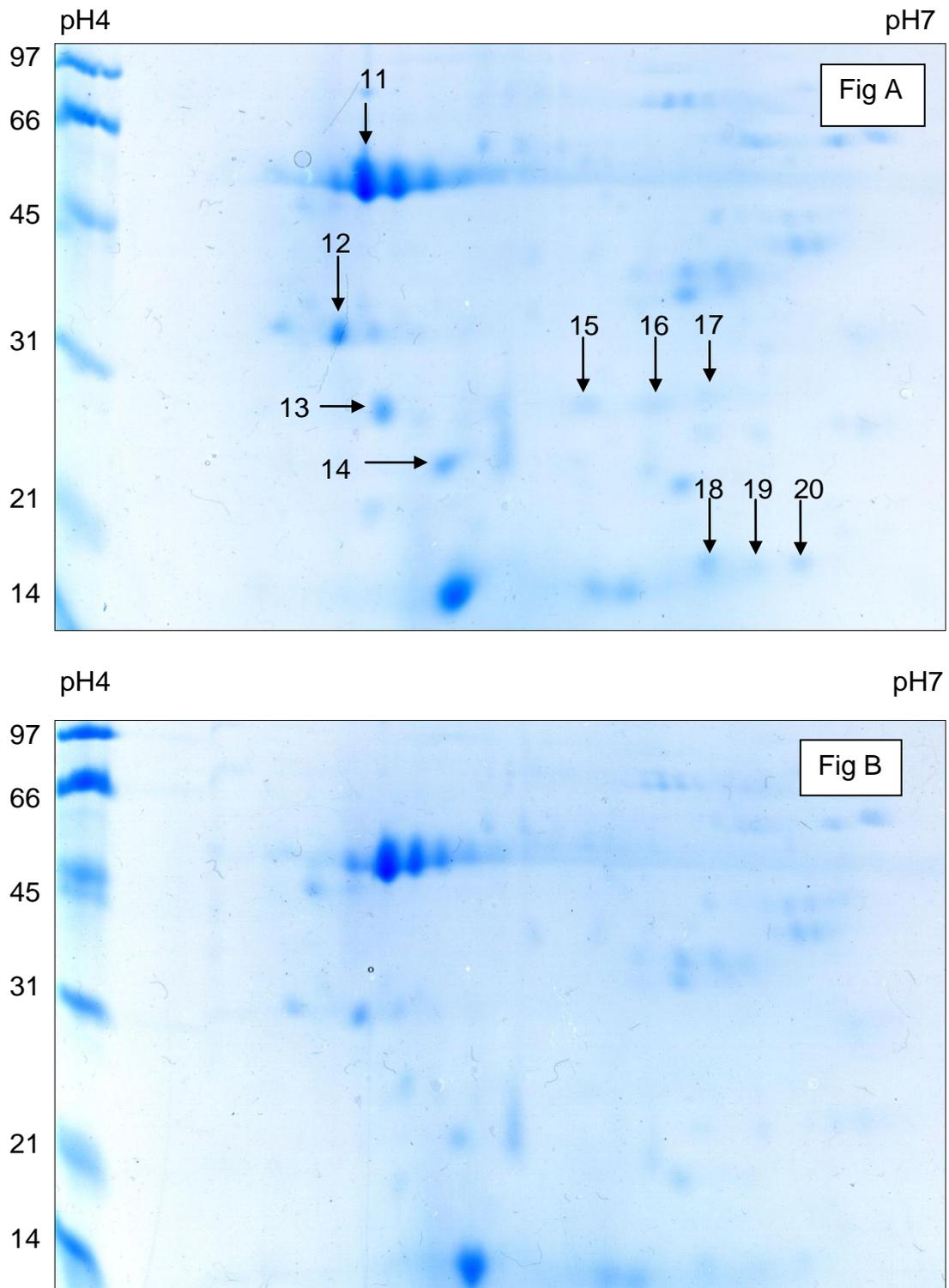
The second dimension involved taking the gel strip from the first dimension and running it on an SDS PAGE gel to further separate the proteins by their mass – to - charge ratio. The SDS gives the proteins a negative charge, allowing migration toward the anode. The resulting gel was stained with coomassie to enable visualisation of the protein spots (Figs 18, 19, 20).

Upon visual inspection no spots were up-regulated by safener treatment in the gels resulting from the eluent of the phenyl sepharose column (Figs 18, 19, 20). The glutathione and hexyl-glutathione affinity chromatography columns were far more successful in isolating up-regulated GSTs than the hydrophobic interaction column (Figs 18, 19, 20). However there was still contamination with Ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO) in all of the affinity purified preparations. The glutathione agarose column was successful in isolating two GST polypeptides which were up-regulated following treatment with cloquintocet mexyl (spots 3 and 7; Fig 18). Spots within the GST size range were subjected to MALDI-TOF analysis for identification. Multiple variants were found to be proteins derived from homologous genes in wheat. MASCOT (a search engine using mass spectrometry data) was used to identify the proteins from a primary database sequence.



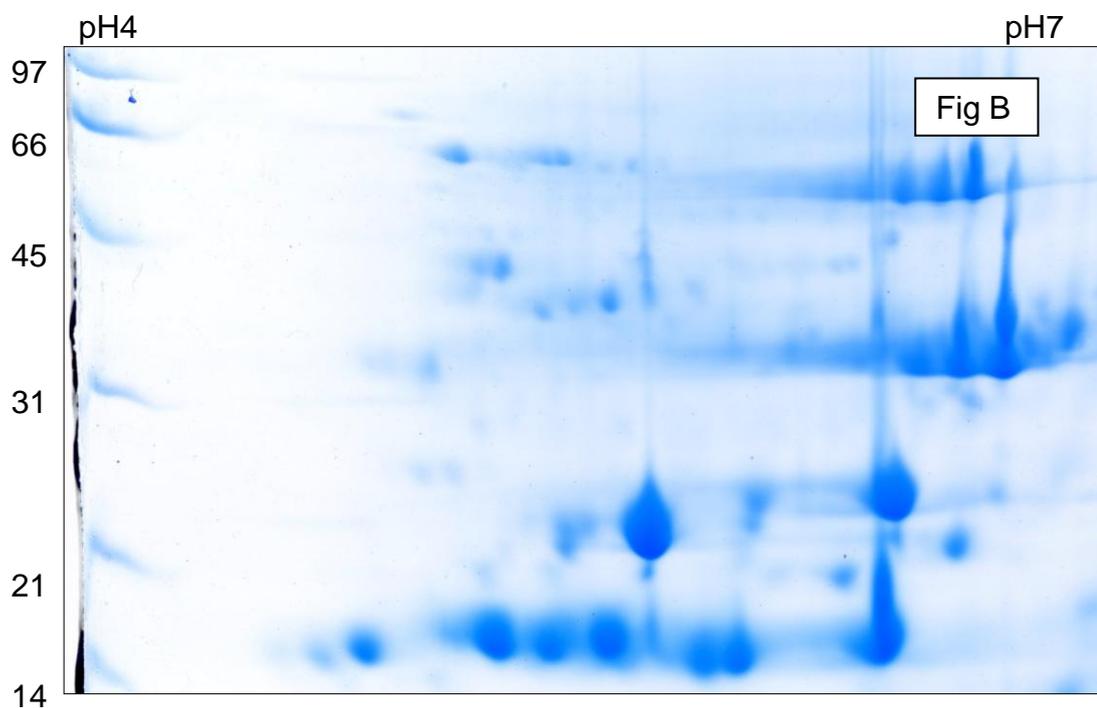
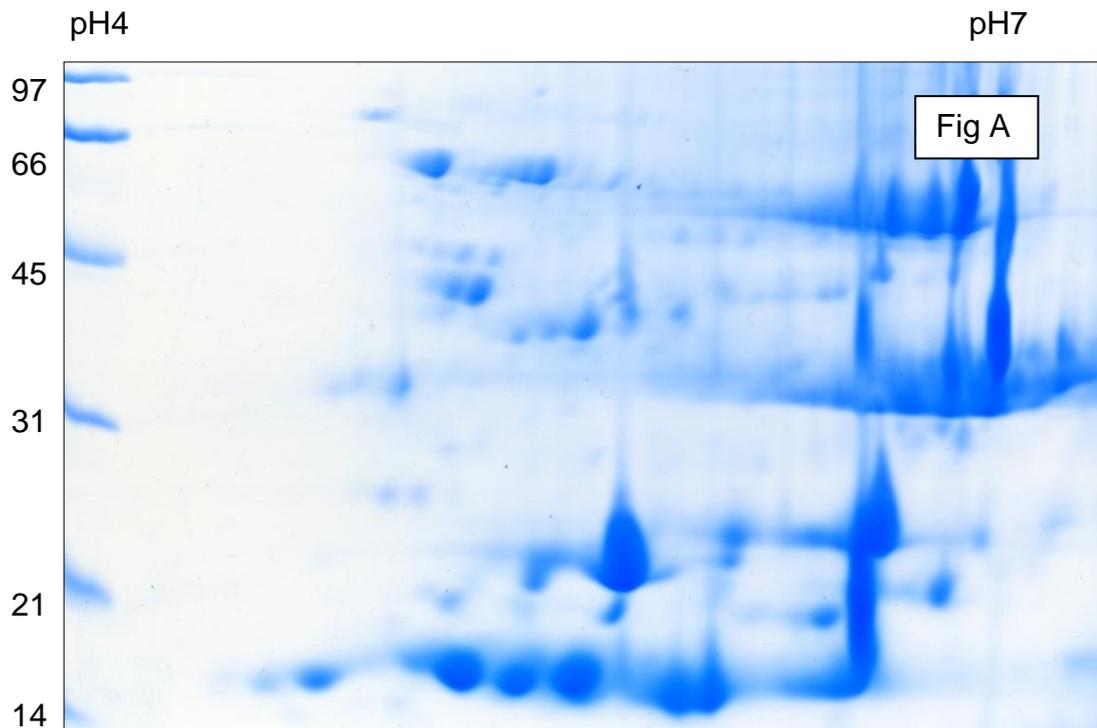
**Figure 18 Proteome of *Triticum aestivum* using a glutathione agarose column**

Proteome of *Triticum aestivum* L shoots. Wheat was treated with the field rate of cloquintocet mexyl (A) ( $15 \text{ g.a.i ha}^{-1}$ ) or a 0.1% acetone control (B) at 7 D then harvested 24 H later. Affinity bound proteins were eluted from a glutathione agarose column. Arrows show safener upregulated spots that were analysed by MALDI-TOF.



**Figure 19 Proteome of *Triticum aestivum* using a S-hexyl glutathione column**

Proteome of *Triticum aestivum* L shoots. Wheat was treated with the field rate of cloquintocet mexyl (A) ( $15 \text{ g.a.i ha}^{-1}$ ) or a 0.1% acetone control (B) at 7 D then harvested 24 H later. Affinity bound proteins were eluted from a glutathione agarose column. Arrows show safener upregulated spots that were analysed by MALDI-TOF



**Figure 20 Proteome of *Triticum aestivum* using a phenyl sepharose column**

Proteome of *Triticum aestivum* L shoots. Wheat was treated with the field rate of cloquintocet mexyl (A) ( $15 \text{ g.a.i ha}^{-1}$ ) or a 0.1% acetone control (B) at 7 D then harvested 24 H later. Affinity bound proteins were eluted from a glutathione agarose column. Arrows show safener upregulated spots that were analysed by MALDI-TOF.

Spot Number	Identity
1 (Fig 18, A)	Ribulose 1,5-bisphosphate carboxylase/oxygenase, large chain ( <i>Triticum aestivum</i> )
2 (Fig 18, A)	rbcL ( <i>Triticum aestivum</i> )
3 (Fig 18, A)	glutathione transferase ( <i>Triticum aestivum</i> ), GSTU1c
4 (Fig 18, A)	Ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit ( <i>Hordeum bulbosum</i> )
5 (Fig 18, A)	Ribulose 1,5-bisphosphate carboxylase/oxygenase, large chain ( <i>Triticum aestivum</i> )
6 (Fig 18, A)	rbcL ( <i>Triticum aestivum</i> )
7 (Fig 18, A)	Ribulose 1,5-bisphosphate carboxylase/oxygenase, large chain ( <i>Triticum aestivum</i> )
8 (Fig 18, A)	glutathione transferase ( <i>Triticum aestivum</i> ), GSTU1c
9 (Fig 18, A)	Ribulosebiphosphate carboxylase ( <i>Hordeum lechleri</i> )
10 (Fig 18, A)	Ribulose-1,5-bisphosphate carboxylase/oxygenase, small subunit ( <i>Triticum aestivum</i> )
11 (Fig 19, A)	Ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit ( <i>Halosarcia indica</i> )
12 (Fig 19, A)	glutathione transferase ( <i>Triticum aestivum</i> ), GSTU1c
13 (Fig 19, A)	glutathione transferase ( <i>Triticum aestivum</i> ), GSTU1c
14 (Fig 19, A)	glutathione transferase ( <i>Triticum aestivum</i> ), GSTU1c
15 (Fig 19, A)	Ribulose-1,5-bisphosphate carboxylase/oxygenase, small subunit ( <i>Triticum aestivum</i> )
16 (Fig 19, A)	Ribulose-1,5-bisphosphate carboxylase/oxygenase, small subunit ( <i>Triticum aestivum</i> )
17 (Fig 19, A)	Ribulose 1,5-bisphosphate carboxylase/oxygenase, large chain ( <i>Triticum aestivum</i> )
18 (Fig 19, A)	Ribulose 1,5-bisphosphate carboxylase, large subunit ( <i>Atherosperma moschatum</i> )
19 (Fig 19, A)	glutathione transferase ( <i>Triticum aestivum</i> ), GSTU1c
20 (Fig 19, A)	Ribulose-1,5-bisphosphate carboxylase, large subunit ( <i>Euclinia longiflora</i> )

**Table 12 Results of MALDI-TOF analysis of selected spots**

Crude protein extracts were purified using a glutathione agarose, S-hexyl glutathione and phenyl sepharose column, eluents were subjected to 2 D gel analysis. Selected spots were cut out and sent for MALDI TOF analysis. MASCOT (a search engine using mass spectrometry data) was used to identify the proteins from a primary database sequence.

The *S*-hexyl glutathione column was successful in isolating four spots (spots 13, 15, 16, 17; Fig 19). All GSTs isolated were identified by MALDI TOF analysis as corresponding to the tau class *TaGSTU1c* (Genbank accession number : AJ414699.1).

### 4.3 Discussion

All spots picked and sent for MALDI - TOF analysis were identified as being GSTU1c. Because *Triticum aestivum L* has a hexaploid genome the spots probably corresponded to the multiple variants of *TaGSTU1c* notably *TaGSTU1a* (Genbank acc: AJ414697.1) and *TaGSTU1b* (Genbank acc: AJ414698.1). From previous literature and from assays and western blots in chapter one it has been shown that GSTs from the phi and lambda classes were also induced by safeners. Therefore, it can be assumed that the lambda and phi class GSTs induced either do not recognise the affinity ligands or are present in such small quantities that they are not visible on the 2 D gels. De Ridder *et al.* (2002) also found that the tau class *AtGSTU19* was prominent on the 2 D gels, being enhanced with plant treatment with benoxacor. It is interesting to note that this study also only highlighted GSTU1c, and that GSTU1c and *AtGSTU19* are homologues of each other, suggesting that safeners may induce a very similar subset of GSTs even in different species of plant.

There is a significant level of RUBISCO contamination on the affinity – purified gels. RUBISCO is very abundant in green tissue and has overloaded the column despite a prior clean up by treating with 40 – 80

% saturation with ammonium sulphate. The RUBISCO contamination could have been overcome by using etiolated wheat. However for the purpose of these studies treatments were kept as close to field treatment as possible, and light grown seedlings were used.

Based on this study and previously published observations (Theodoulou *et al.*, 2003; Cummins *et al.*, 2003) several GSTs were chosen as representative of each class that are known to be safener inducible for cloning, expression and characterisation, namely, Cla 30 (lambda) (Theodoulou *et al.*, 2003), 28e45 (tau) (Theodoulou *et al.*, 2003), U1c (tau) (Cummins *et al.*, 2003), F6b (phi) (Cummins *et al.*, 2003) and 19e50 (phi) (Theodoulou *et al.*, 2003). These proteins were selected for cloning because Cla 30, 28e45 and 19e50 were shown to be very strongly up-regulated by the safener cloquintocet mexyl in wheat cv. Darius (Theodoulou *et al.* 2003). Similarly F6b was shown by Cummins *et al.* (2003) to be upregulated upon safener treatment with fenchlorazole ethyl, while U1c was shown to be upregulated in this study and in a previous study (Cummins *et al.* 1997), in response to safener treatment with fenchlorazole ethyl. These representative GSTs were then cloned and the respective recombinant protein purified (chapter 5).

## CHAPTER 5 : Cloning, Expression and Characterisation of Safener Inducible GSTs from *Triticum aestivum*

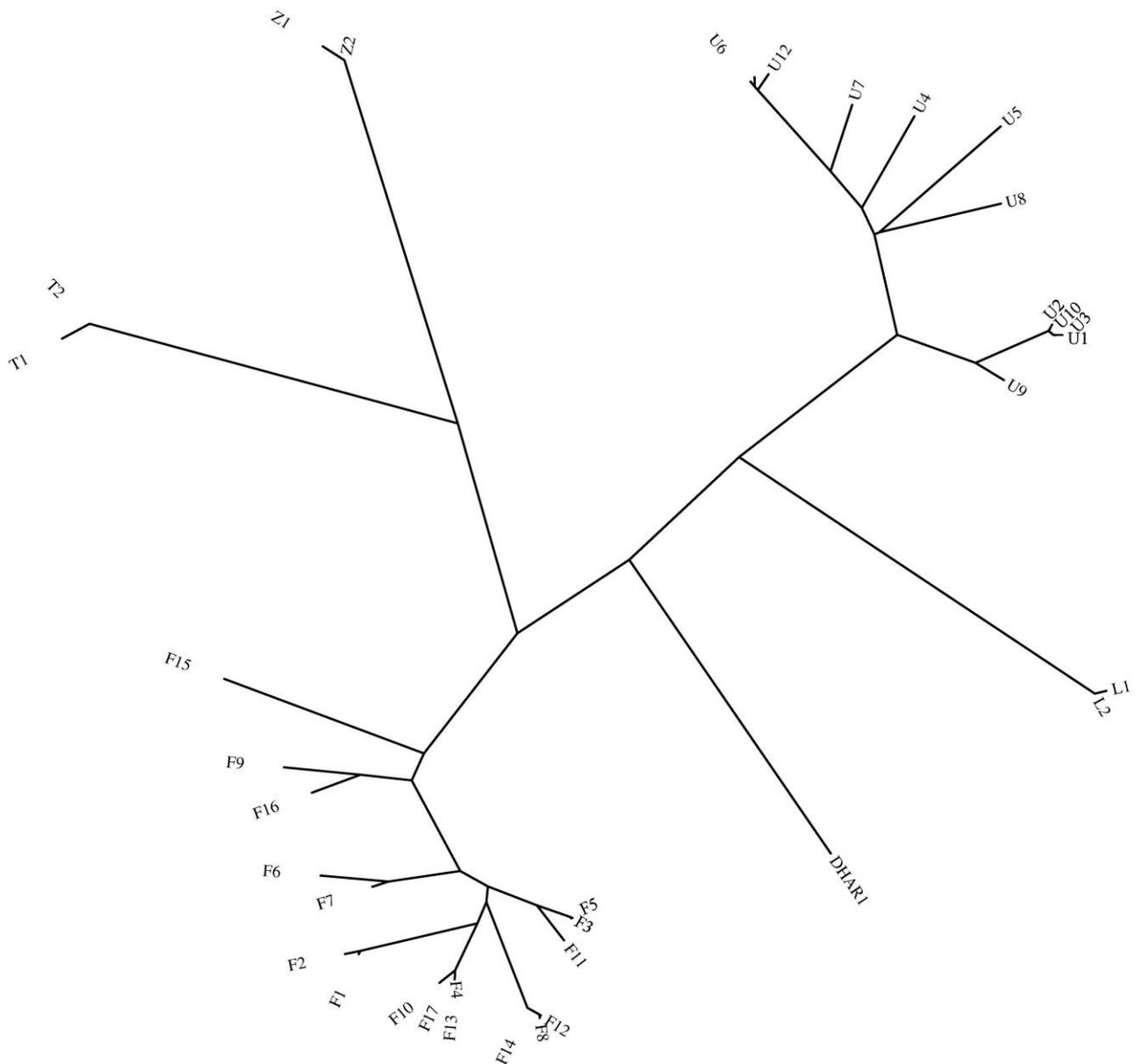
### 5.1 Introduction

This chapter describes the cloning, expression, purification and biochemical characterisation of six wheat GSTs from the phi, tau and lambda classes, following the identification of the major safener inducible GSTs from safener treated seedlings in chapter four.

### 5.2 *Triticum aestivum* GST phylogeny

The current naming of GSTs in *Triticum aestivum* is confusing and needs to be brought in line with current nomenclature to simplify future assignments. Using the system suggested by Edwards *et al.* (2000) for gene and enzyme nomenclature the sequences available from a Genbank search for GSTs in *Triticum aestivum* were compiled and renamed. Naming is split into three parts, the first describes the species, the second the enzyme, and the third the class of GST (tau – U, F – phi, L – lambda, Z – zeta, T – theta) e.g. a lambda GST from *Triticum aestivum* would be named *TaGSTL*. Alignment was by

sequence similarity using the Clustal W (Larkin *et al.*, 2007) and Phylip (Felsenstein.J, 2003) programs to create an unrooted phylogenetic tree (Fig 21). Identified GSTs were then grouped according to sequence similarity and renamed using a species designation, a gene class identifier and a number within that class (the number given was based on the date of submission to Genbank) (Table 14) e.g the first lambda class GST identified from *Triticum aestivum* would be *TaGSTL1*.



**Figure 21 Unrooted phylogenetic tree of GSTs from *Triticum aestivum***

Phylogenetic relationships of available TaGSTs based on multiple sequence alignments of the full length amino acid sequences available in Genbank for *Triticum aestivum*. (Clustal X, Larkin *et al.*, 2007; Phylip, Felsenstein.J, 2003).

<b>GST Class</b>	<b>Accession</b>	<b>Date Sub</b>	<b>Old name</b>	<b>New name</b>	<b>Pub/unpub</b>
<b>Theta</b>	BT009196	20.06.03	N/A	TaGSTT1	U
	BT009476	20.06.03	N/A	TaGSTT2	U
<b>Zeta</b>	AAQ83840	02.09.03	N/A	TaGSTZ2	U
	AF109714	10.11.99	N/A	TaGSTZ1	P (ref 1)
<b>DHAR</b>	AAL71854	22.01.02	N/A	TaDHAR1	P (ref 2)
<b>Lambda</b>	Y17386	01.06.98	Cla30	TaGSTL1	P (ref 3)
			N/A	TaGSTL2	
<b>Phi</b>	AJ441055	05.04.02	F6b	TaGSTF10	U* (ref 8)
	X56012	17.08.90	GSTA1	TaGSTF1	P (ref 4)
	X56004	17.08.90	GSTA2	TaGSTF2	P (ref 5)
	AJ440792	03.04.02	F3	TaGSTF7	U
	AJ440791	03.04.02	F2	TaGSTF6	U
	BT009210	20.06.03	N/A	TaGSTF15	U
	AF440793	03.04.02	F4	TaGSTF8	U
	AY064481	30.11.01	19 e 50	TaGSTF4	P (ref 6)
	AJ440796	03.04.02	F1	TaGSTF5	U
	AF184059	05.09.99	GST1	TaGSTF3	P (ref 7)
	BT009155	20.06.03	N/A	TaGSTF11	U
	BT009505	20.06.03	N/A	TaGSTF12	U
	BT009600	20.06.03	N/A	TaGSTF13	U
	BT009443	20.06.03	N/A	TaGSTF14	U
	CAD29478	03.04.02	F5	TaGSTF9	U
	BT009137	20.06.03	N/A	TaGSTU16	U
<b>Tau</b>	AF479764	04.02.02	28 e 45	TaGSTU6	P (ref 6)
	AJ414700	02.10.01	GSTU2	TaGSTU4	U
	AJ414701	02.10.01	GSTU3	TaGSTU5	U
	AJ414698	02.10.01	GSTU1b	TaGSTU2	U
	AJ414699	02.10.01	GSTU1c	TaGSTU3	U
	AJ414697	02.10.01	GSTU1a	TaGSTU1	U
	BT009438	20.06.03	N/A	TaGSTU7	U
	BT009217	20.06.03	N/A	TaGSTU8	U
	BT009150	20.06.03	N/A	TaGSTU9	U
	BT009437	20.06.03	N/A	TaGSTU10	U

**Table 13 Available TaGSTs**

Sequences from Genbank. Accession numbers, date submitted, old name, new name based on nomenclature suggested by Edwards *et al.* (2000), and journal articles published.

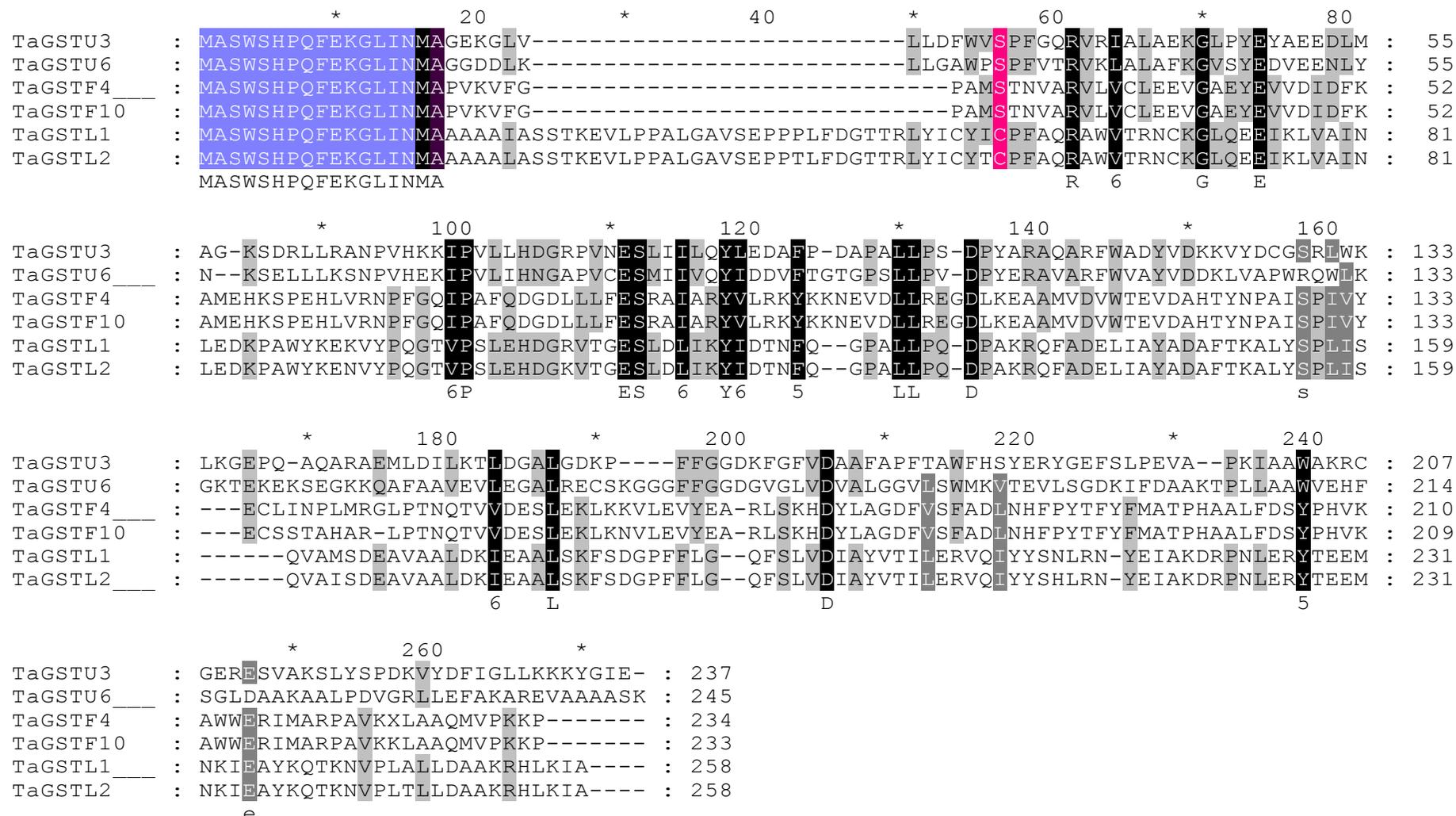
- 1 Subramaniam.K *et al.* (1998) *Biochim.Biophys.Acta* 1447(2-3): 348-356
- 2 Chen.Z *et al.*(2003) *Proc.Natl.Acad.Sci.U.S.A* 100(6):3525-3530
- 3 Theodoulou.F.L *et al.* *The Electronic Plant Gene Register Plant Physiol* 119(4):1567-1568
- 4 Dudler.R *et al.* (1991) *Mol.Plant.Microbe.Interact* 4(1):14-18
- 5 Mauch.F *et al.* (1991) *Plant.Mol.Biol* 16(6): 1089-1091
- 6 Theodoulou.F.L *et al.* (2003) *Pest.Manag.Sci* 59:202-214
- 7 Goetzberger.C *et al.* (2000) *Plant.Physiol* 122(1):292
- 8 Cummins *et al.*, (2003) *Plant.Mol.Biol* 52:591-603

### 5.3 Cloning and expression of GSTs

A cDNA library was available from previous studies which had been prepared from green shoots of winter wheat c.v. 'Hunter' (Cummins *et al.*, 2003). The library was mass excised and PCR performed. The PCR products were obtained using sequence specific primers designed to the tau class *TaGSTU3* identified from 2D gel analysis in chapter four. Four further GSTs shown to be safener inducible were also cloned and expressed using PCR and selective primers. These were the phi class GSTs *TaGSTF10* (Cummins *et al.*, 2003) and *TaGSTF4* (Theodoulou *et al.*, 2003), the tau class GST *TaGSTU6* (Theodoulou *et al.*, 2003), and the lambda class GST *TaGSTL1* (Theodoulou *et al.*, 2003). During the cloning process one further GST was amplified during PCR due to its sequence similarity to *TaGSTL1*. This GST was also cloned and expressed and named according to the existing nomenclature described by Edwards *et al* (2000) as *TaGSTL2*.

In each case the PCR products were ligated into pGEM<sup>®</sup>-T Easy following excision from an agarose gel and purification. The recombinant plasmid was used to transform chemically competent cells

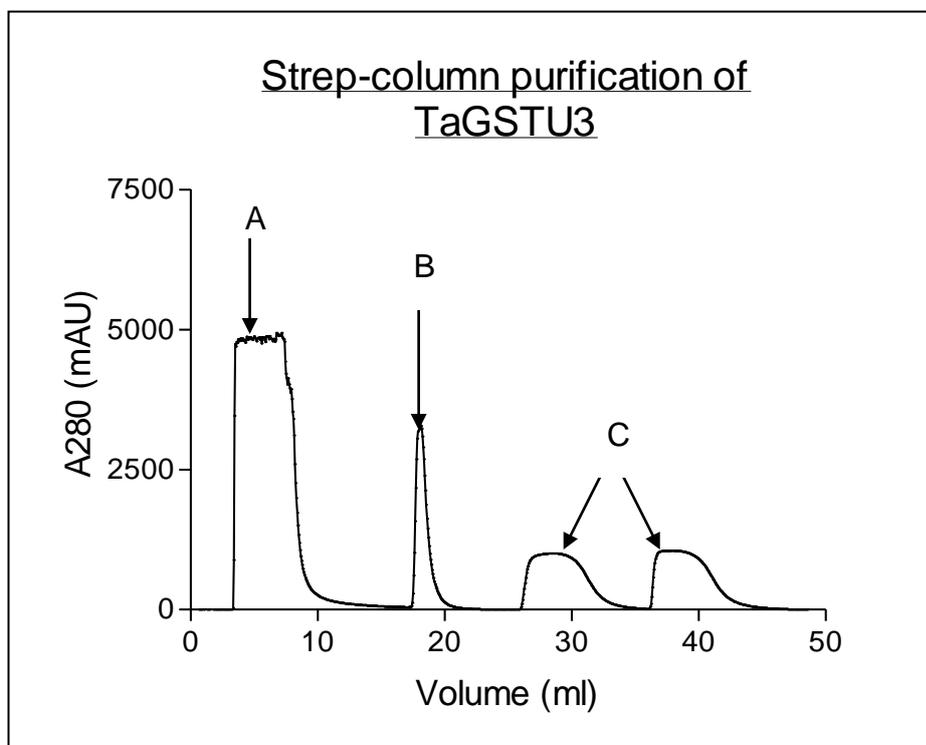
( $\alpha$  – select gold efficiency, Bioline) using blue / white selection, resulting in numerous white colonies. Several colonies were selected and mini-preps performed prior to analysis by restriction digestion. Colonies with inserts were sequenced and the cDNA compared with the expected products by searching the Genbank database at NCBI.



**Figure 22 GST clones isolated from a cDNA library prepared from safener treated wheat**

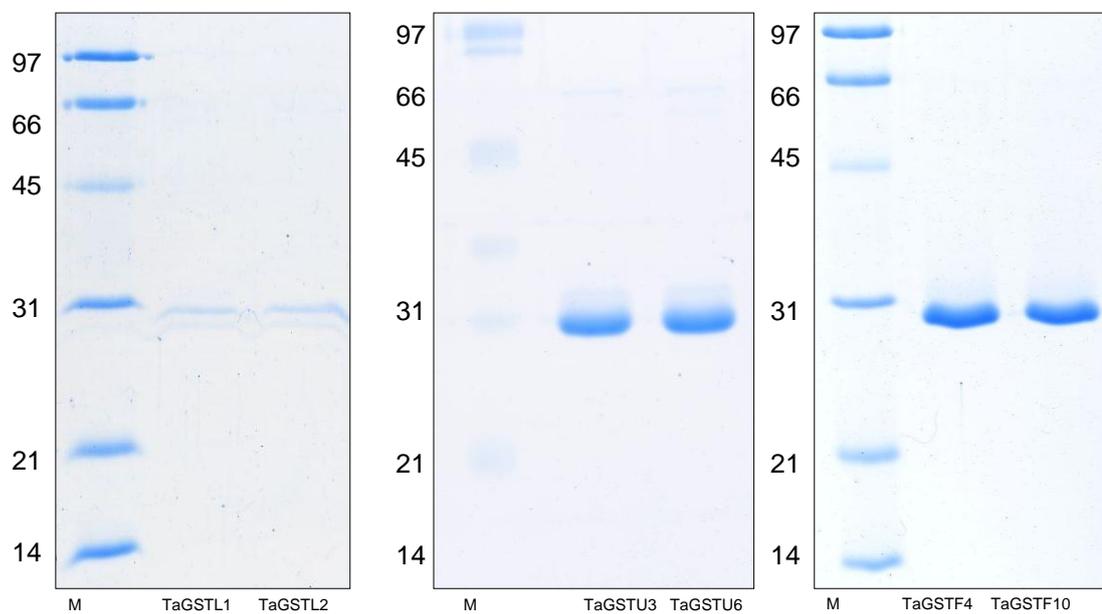
Multiple sequence alignment produced using Clustal W (Thompson *et al.*, 1994) and visualised using Genedoc Version 2.6.001 (Nicholas *et al.*, 1997). Genbank accession numbers are listed in Table ( 13), Chapter 4. Purple shading shows the strep-tag used for purification. Pink shading shows the active site serine and cystine residues. Black shading shows 100 % conservation of amino acids between sequences, dark grey shading shows 80 % or greater conservation and light grey shading shows 60 % or greater conservation.

Digested inserts were ligated into pET-STRP3 (Dixon *et al.*, 2008), the plasmid was used to transform chemically competent cells ( $\alpha$  – select gold efficiency, Bioline) and mini-preps performed from colonies grown on LB cultures using appropriate antibiotic selection. The resulting recombinant plasmid was used to transform *E.coli* strain Tuner (DE3), which contains the pRARE plasmid from strain Rosetta (Novagen). The resulting LB cultures were then used to inoculate larger 1 L LB cultures until a density of 0.5 O.D had been reached. IPTG was added to induce expression. GSTs were purified by affinity chromatography, loading the lysate onto a 1ml Strep-Tactin macroprep column (Stratech Scientific Ltd, Soham, UK) and eluted with desthiobiotin. Purified GSTs were run on an SDS - PAGE gel and stained with coomassie.



**Figure 23 Strep column purification of TaGSTU3**

Lysate was loaded onto a strep-tactin macroprep column. Peak A is the unbound protein. Peak B is the bound protein peak eluted with DTB. Peak C is the column recharging with HABA.



**Figure 24 SDS-PAGE gels showing purified recombinant protein from the lambda, phi and tau classes**

GSTs were purified using a 1ml Strep-Tactin macroprep column (Stratech Scientific Ltd, Soham, UK) and eluted with desthiobiotin. SDS - PAGE gels were stained with coomassie.

## 5.4 Characterisation of safener induced recombinant GSTs

### 5.4.1 Spectrophotometric assays

Detoxification of a broad range of chemical binding, electrophilic groups by GSTs is important as many electrophiles are cytotoxic as well as genotoxic (Berhane., *et al* 1994).

A range of assays were used in order to determine if any of the safener inducible GSTs had the capacity to inactivate electrophilic model substrates. GSTs were assayed toward crotonaldehyde (Berhane *et al.*, 1994), benzyl isothiocyanate (Kolm *et al.*, 1995), p-nitrobenzyl chloride, 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene, ethacrynic acid (Habig *et al.*, 1974), and cumene hydroperoxide (flohe and gunzler, 1984). GSTs were also assayed for disulphide exchange activity using 2 - hydroxyethyl disulfide (Vlami-Gardikas *et al.*, 1997).

Glutathione conjugates of  $\alpha$ ,  $\beta$ -unsaturated aldehydes are produced during lipid peroxidation, and may serve as signaling molecules in plants such compounds also frequently occur as a result of pollution

from industrial processes and exhaust fumes. A study in *Cucurbita maxima* by Masayuki & Mohammed (2003) indicated that plant GSTs may be involved in the detoxification of physiologically and environmentally hazardous aldehydes, with crotonaldehyde causing a nine fold induction of the tau class GSTs *CmGSTU3* and *CmGSTF1*.

Ethacrynic acid has been shown to both induce and inhibit GSTs (Shen *et al.*, 1995). Benzyl isothiocyanate has been shown to be a good substrate for human glutathione transferases converting the isothiocyanate to the corresponding dithiocarbamate (Kolm *et al.*, 1995). Cumene hydroperoxide was used as a substrate for GSTs functioning as glutathione peroxidases (Cummins *et al.*, Chapter 3). The substrates CDNB, DCNB and NBC represent a range of electrophilic compounds that have been shown to be substrates for GSTs (Habig *et al.*, 1974). Until the true substrates of lambda GSTs can be found their disulphide exchange activity using HED as a substrate was used to monitor their activity (Edwards *et al.*, 2000, Vlammi-Gardikas *et al.*, 1997).

### 5.4.2 Results

None of the recombinant GSTs had activity toward crotonaldehyde, ethacrynic acid, or BITC, and only the lambda GSTs acted as thiol transferases (Table 15). Both the phi and tau class GSTs show activity toward CDNB with the tau class GSTs having a higher activity toward this substrate. *TaGSTU3* shows the highest activity toward CDNB and was also the only GST to have activity toward DCNB. The tau GSTs show the highest activity toward NBC, with *TaGSTU6* being the highest. GPOX activity was shown by both tau and phi class GSTs, though the activity was over three fold higher with the phi GSTs compared with the tau (Table 15).

Specific activity (nkat mg <sup>-1</sup> recombinant protein)								
Substrate								
TaGST	CDNB	DCNB	NBC	GPOX	Thiol Transferase	Crotonaldehyde	Ethacrynic acid	BITC
U3	294.6 ± 12.5	1.5 ± 0.03	1.9 ± 0.2	6.4 ± 0.1	n.d	n.d	n.d	n.d
U6	206.0 ± 9.6	n.d	3.4 ± 0.2	8.2 ± 0.05	n.d	n.d	n.d	n.d
F4	113.5 ± 4.8	n.d	1.5 ± 0.2	29.3 ± 0.2	n.d	n.d	n.d	n.d
F10	143.0 ± 1.8	n.d	0.5 ± 0.1	29.8 ± 1.4	n.d	n.d	n.d	n.d
L1	n.d	n.d	n.d	n.d	126.8 ± 3.2	n.d	n.d	n.d

**Table 14 Results of the recombinant protein colourmetric assays**

*TaGSTU3*, *TaGSTU6*, *TaGSTF4*, *TaGSTF10*, *TaGSTL1* activity toward 1-chloro 2,4-dinitrobenzene (CDNB), 1,2-dichloro 4-nitrobenzene (DCNB), p-nitrobenzyl chloride (NBC), crotonaldehyde, ethacrynic acid, and benzyl isothiocyanate (BITC). Glutathione peroxidase activity (GPOX) was determined with cumene hydroperoxide, while thiol transferase activity was determined using HED. n.d represents no activity determined. Values represent the means ± S.D (n = 4).

## 5.5 HPLC based assays

### 5.5.1 Introduction

GSTs have been shown to conjugate a range of herbicides with glutathione during phase two metabolism to form polar, non toxic peptide conjugates. To determine if the safener induced GSTs identified in chapter four had activity toward any of the herbicide safeners and their associated herbicides, HPLC based assays described in Edwards *et al.* (2005) were carried out. The safeners tested were cloquintocet mexyl, cloquintocet free acid, fenchlorazole ethyl, mefenpyr diethyl and benoxacor. Herbicides used were alachlor, clodinafop propargyl, and fenoxaprop ethyl. Boiled enzyme was used in the controls, with assays carried out without glutathione. Prior to the HPLC based assays, reference glutathione conjugates were synthesized to create standard curves in order to quantify the reaction products.

### 5.5.2 Results

Assays were run with all substrates (Table 16), with the recombinant GSTs *TaGSTU3*, *TaGSTU6*, *TaGSTF4*, *TaGSTF10* and *TaGSTL1*. Chromatograms showing the basis of the assay are shown in Figs (25, 26 and 27). Of the herbicides, alachlor and fenoxaprop ethyl underwent a chemical conjugation to glutathione and showed an enzyme mediated increase in conjugate formation. The phi GSTs *TaGSTF4* and *TaGSTF10* showed activity toward alachlor, while the tau GST *TaGSTU6* conjugated with fenoxaprop ethyl. Clodinafop propargyl did not undergo any conjugation with glutathione.

Of the safeners only benoxacor underwent a chemical conjugation with glutathione, with *TaGSTF10* increasing the amount of conjugate formed. No other safener including the free acid of cloquintocet mexyl was conjugated, either chemically or through the action of the GSTs.

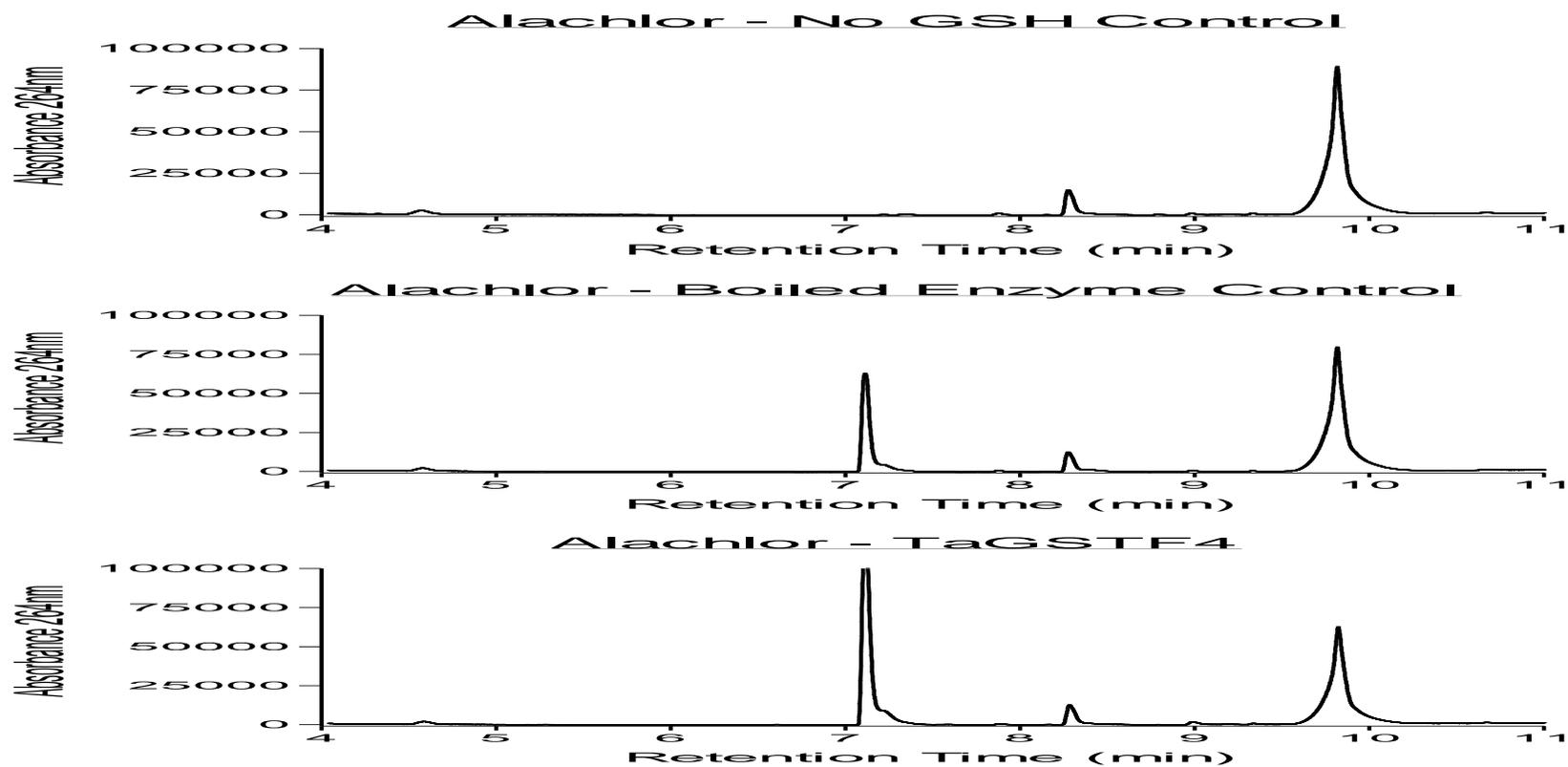
Over the forty minute time course, the amount of conjugate formed per assay showed a linear increase. After forty minutes the reaction is not linear presumably due to substrate depletion and product inhibition.

		nmol <sup>-1</sup> GSH - conjugate formed Time Course (min)		
Substrate	Recombinant GST	0	20	40
Alachlor	TaGSTF4	n.d	10.9 ± 1.1	19.2 ± 2.1
	TaGSTF10	n.d	12.1 ± 0.15	23.5 ± 0.3
Fenoxaprop ethyl	TaGSTU6	n.d	5.5 ± 1.64	9.3 ± 1.5
Benoxacor	TaGSTF10	n.d	23.0 ± 4.3	63.4 ± 8.8

	nmol <sup>-1</sup> GSH - conjugate formed BSA control Time Course (min)		
Substrate	0	20	40
Alachlor	n.d	4.5 ± 1.2	6.8 ± 0.3
Fenoxaprop ethyl	n.d	4.8 ± 2.7	9.6 ± 3.1
Benoxacor	n.d	3.1 ± 0.2	5.3 ± 0.7

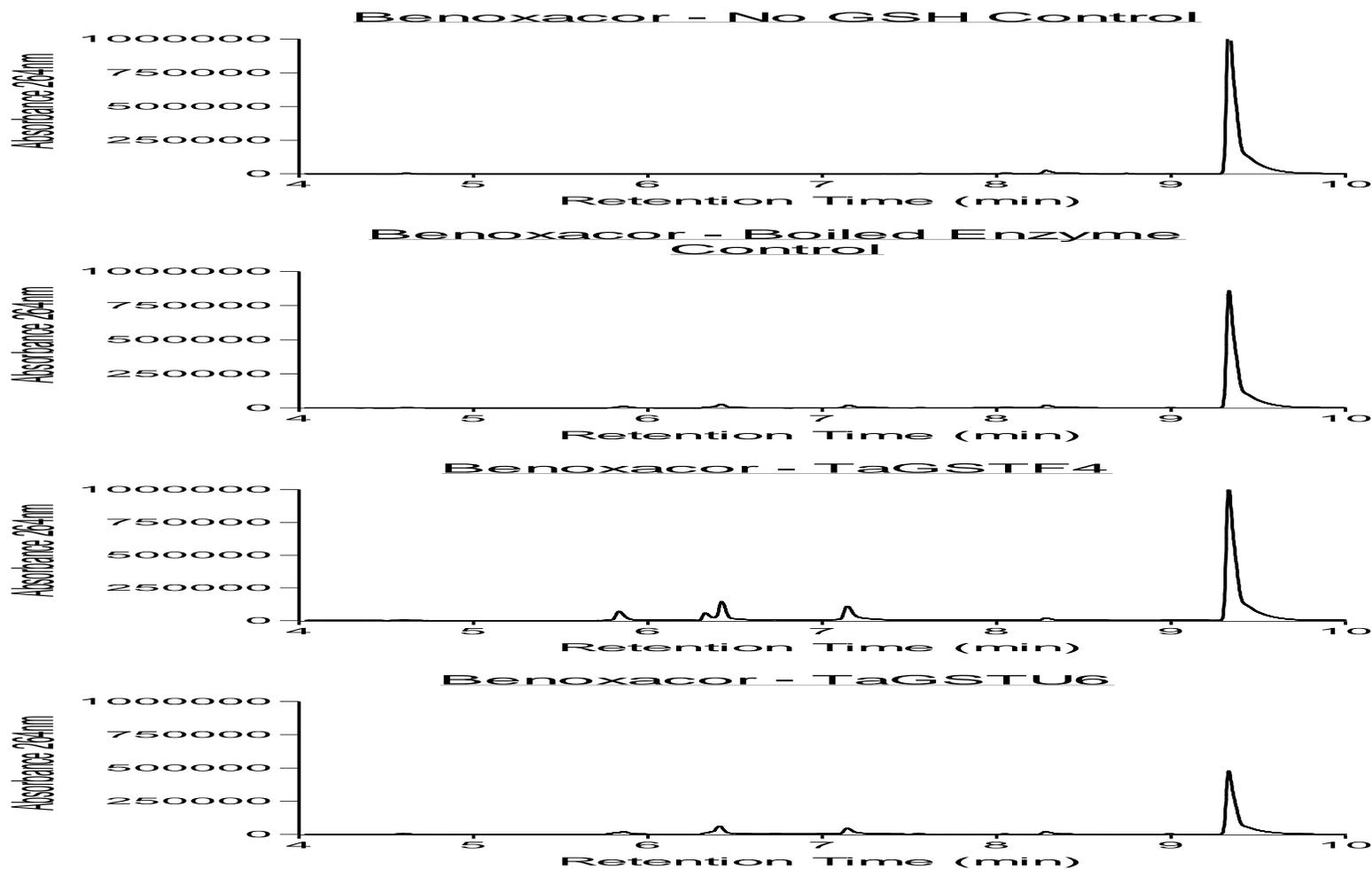
**Table 15 Results of the HPLC assays**

Table showing nmol<sup>-1</sup> of conjugate formed per 200 µl assay containing 20 µg<sup>-1</sup> of recombinant protein or boiled enzyme control, over a time course of 0, 20 min and 40 min. Assays were stopped with 3 M hydrochloric acid and run on an LC – MS.



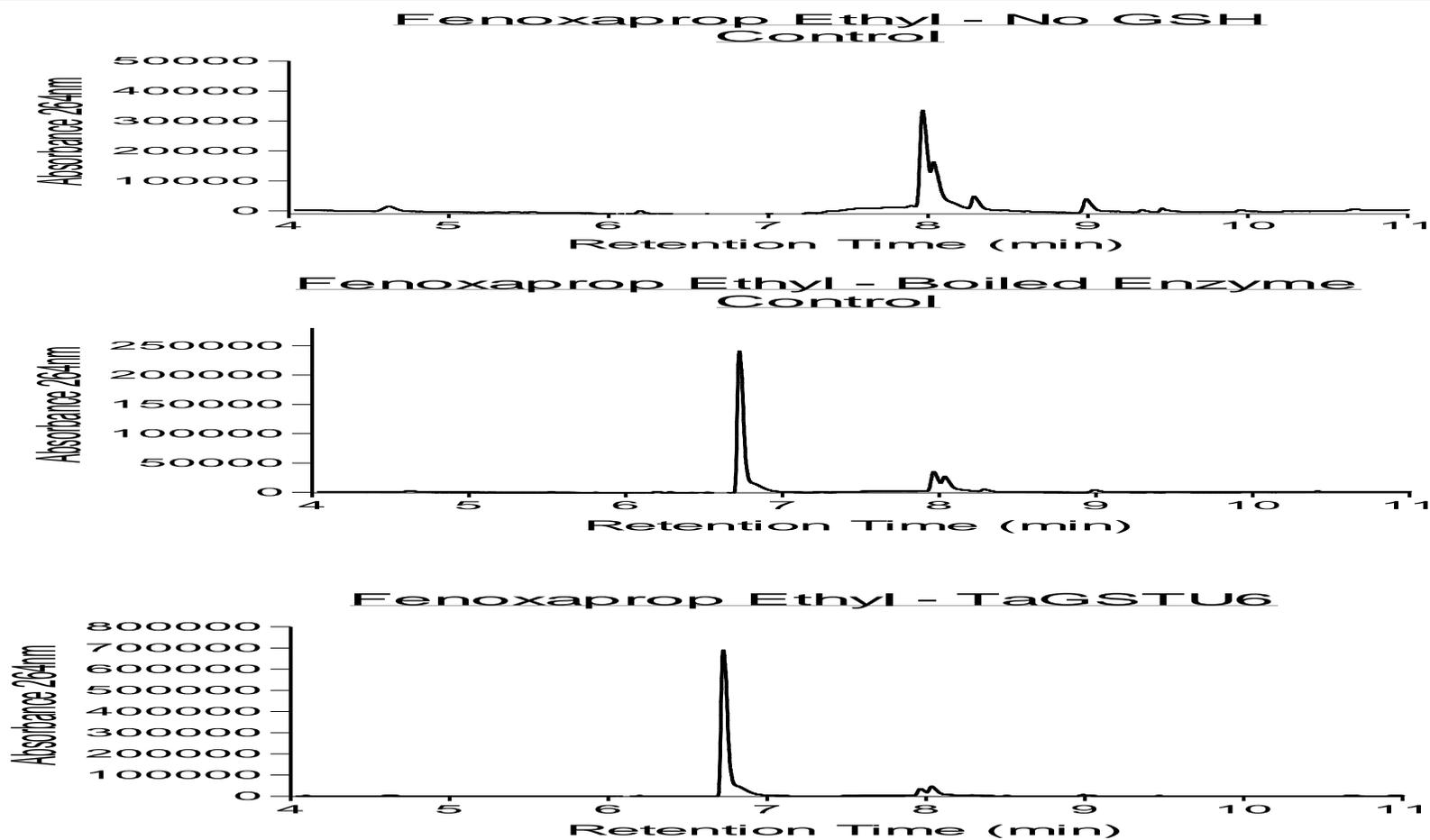
**Figure 25 Alachlor chromatograms**

Chromatograms showing the chemical conjugation of alachlor to glutathione and the *TaGSTF4* mediated conjugation of alachlor to glutathione as well as a no GSH control. Absorbance was measured at 264nm.



**Figure 26 Benoxacor chromatograms**

Chromatograms showing the chemical conjugation of benoxacor to glutathione, and the *TaGSTU6*, *TaGSTF4* mediated conjugation of glutathione to benoxacor as well as a no GSH control. Absorbance was measured at 264nm.



**Figure 27 Fenoxaprop ethyl chromatograms**

Chromatograms showing the chemical conjugation of fenoxaprop ethyl to glutathione, the *TaGSTU6* conjugation of fenoxaprop ethyl to glutathione as well as a no glutathione control. Absorbance measured at 264nm.

The safener induced *TaGSTU3*, *TaGSTU6*, *TaGSTF4*, *TaGSTF10*, and *TaGSTL1* did not conjugate any of the wheat safeners, including the free acid of cloquintocet mexyl. The maize safener benoxacor was conjugated by *TaGSTU6* and *TaGSTF4* (Table 16) whereas the herbicide clodinafop propargyl did not undergo conjugation. Fenoxaprop ethyl formed a glutathione conjugate to glutathione both with boiled enzyme and as mediated by catalysis with *TaGSTU6*. Alachlor also showed a chemical conjugation to glutathione with boiled enzyme which was enhanced in the presence of *TaGSTF4*.

## 5.6 Discussion

The GSTs induced by cloquintocet mexyl in chapter four were cloned, expressed and subjected to a range of spectrophotometric assays as well as HPLC based assays using herbicides and safeners as substrates. Despite the GSTs being induced by the same safener, cloquintocet mexyl, they demonstrated differing but overlapping substrate specificities. The GSTs induced by cloquintocet mexyl showed no activity toward the safener or toward its partner herbicide clodinafop propargyl, suggesting that cloquintocet mexyl is not conjugated to glutathione as part of its metabolism within the plant. Roberts (1998) also found that in animals cloquintocet mexyl was not conjugated to glutathione but excreted in the bile as the acid.

The lambda GSTs *TaGSTL1* showed no detectable activity toward any substrates except HED Table (16). The function of the lambda GSTs is largely unknown but it is likely that they catalyse a glutathione dependent oxidoreductase reaction (Edwards *et al.*, 2005) due to the presence of a cysteine residue in the active site. The lambda GSTs showed no activity toward any of the safeners or herbicides, this suggests that the lambda GSTs are unlikely to be involved in the direct metabolism of the parent compounds, but may be induced due to

chemical stress, or that they are induced by a metabolite of the parent compound.

The safener inducible phi class GSTs F4 and F10 showed activity toward GPOX, CDNB, and NBC. The tau class GSTs TaU6 and TaU3 showed activity toward CDNB, DCNB, NBC, and GPOX. TaF10 also had activity toward NBC and to a lesser extent TaF4 showing that these GSTs have a broad substrate range.

## Chapter Six: Mode Of Action Of Cloquintocet Mexyl In *Triticum aestivum*

### 6.1 Introduction

This chapter is aimed at further elucidating the mode of action of the herbicide safener cloquintocet mexyl. Section one is concerned with quantifying the induction of the safener inducible GSTs cloned in chapter five, upon treatment with cloquintocet mexyl using RT – PCR.

The second section is concerned with further elucidating the metabolism of cloquintocet mexyl by identifying down stream metabolites *in vivo*. The first part is aimed at confirming that cloquintocet mexyl is firstly hydrolysed to its free acid. Roberts (1998) identified tha cloquintocet acid was the major metabolite found (4.4% of the extractable residue). Continuing on from this the next section is therefore aimed at determining if the levels of cloquintocet increase *in vivo* following treatment with cloquintocet mexyl, and whether any increase in the free acid is correlated to an increase in GST induction.

The third section explores the possibility of a chemical or enzymically mediated conjugation of glutathione to the herbicide safeners with an

aim to rule out or further clarify the role of the safener induced GSTs in the initial metabolism of the safeners.

The fourth section is involved in exploring the possibility that the safeners may be inhibiting GSTs and that this is then causing them to be induced through a negative feedback mechanism.

Many of the proteins involved in xenobiotic detoxification are derived from secondary metabolism and perturbations in their expression can affect the levels of phenylpropanoids and flavonoids *in vivo* (Cummins *et al.*, 1997). The study was conducted 24 H post safener treatment and it was of interest in this fifth section to further clarify whether there are changes in flavonoid content upon safening can be correlated with the increase in cloquintocet and the induction of the GSTs at an earlier time course of 0 – 24 H.

## 6.2 Quantitative real time PCR of safener inducible GSTs

### 6.2.1 Aims and objectives

GST activity toward CDNB can be detected four hours post safener treatment (Chapter three), but this does not tell us which GST is induced and in what quantities. A time course of induction of the safener inducible GSTs was carried out using RT-PCR to determine this. 7-day-old wheat was sprayed with the field rate of cloquintocet mexyl and a time course of 30 min, 1 H, 2 H, 3 H, 4 H, 5 H, 6 H, and 24 H harvested. Each harvest was in triplicate. RNA was extracted using TRI<sup>®</sup> Reagent and 5 µg used to make cDNA. Primers were designed to the tau class GSTs *TaGSTU3*, and *TaGSTU6*, the phi class GSTs *TaGSTF4* and *TaGSTF10*, and the lambda class GST *TaGSTL1*. Differential expression of house keeping genes has been observed (McCurdy *et al.*, 2008) in other studies and with this in mind primers were designed for five housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin, ubiquitin  $\beta$ -tubulin and  $\alpha$ -tubulin. SYBR<sup>®</sup> Green binds to increasing amounts of DNA and was used as a fluorescent dye for quantification.

### 6.2.2 Results

For each biological replicate at each time course a technical duplicate was also carried out to correct for any pipetting errors. The Comparative Quantification (CQ) supplied as part of the Rotorgene software (Corbett Research) was used to analyse the results. This method provides comparable data to the comparative threshold cycle method. The CQ method does not require extra PCR reactions to calculate PCR efficiencies, is cheaper, less time consuming and uses fewer reagents (Rasmussen, 2001, McCurdy *et al.*, 2008). A study by McCurdy *et al.* (2008) found that house keeping genes used to normalise expression were differentially expressed in healthy control samples, this was also observed in this study with the actin and  $\alpha$ -tubulin house keeping genes. Therefore GAPDH was used as the reference gene to normalise expression as this did not vary. There was no amplification found for the genes of interest at the 30 min, 1 H, 2 H and 3 H time points (Table 17). After 4 H post treatment all of the genes of interest were slightly amplified with a few GSTs having a notable increase in amplification above the rest. At the 4 H time course a 2.4 fold induction of *TaGSTU3* was observed relative to the GAPDH control. This two fold induction was also observed at the 5 H time point

with the addition of a two fold increase in *TaGSTF10*. At the 6 H time point *TaGSTL1* increased 10.2 fold relative to the control. It is interesting to note that at the 24 H time point only the tau GST *TaGSTU3* showed a fold increase in transcript levels (Table 17).

Time Course (H)	Gene of interest	Comp. quantification	Amplification $\pm$ SD	Fold Increase
4	GAPDH	0.503	$1.8 \pm 0.12$	/
	<i>TaGSTU3</i>	1.2	$1.8 \pm 0.04$	2.4
	<i>TaGSTU6</i>	0.970	$1.8 \pm 0.04$	1.9
	<i>TaGSTF4</i>	0.651	$1.8 \pm 0.04$	1.3
	<i>TaGSTF10</i>	0.921	$1.8 \pm 0.03$	1.8
	<i>TaGSTL1</i>	0.786	$1.79 \pm 0.03$	1.6
5	GAPDH	0.537	$1.8 \pm 0.07$	/
	<i>TaGSTU3</i>	1.1	$1.8 \pm 0.05$	2.04
	<i>TaGSTU6</i>	0.868	$1.8 \pm 0.03$	1.6
	<i>TaGSTF4</i>	0.540	$1.8 \pm 0.04$	1.0
	<i>TaGSTF10</i>	1.11	$1.8 \pm 0.01$	2.04
	<i>TaGSTL1</i>	0.824	$1.79 \pm 0.02$	1.5
6	GAPDH	0.572	$1.8 \pm 0.08$	/
	<i>TaGSTU3</i>	0.897	$1.8 \pm 0.02$	1.6
	<i>TaGSTU6</i>	0.719	$1.8 \pm 0.02$	1.3
	<i>TaGSTF4</i>	0.856	$1.79 \pm 0.04$	1.5
	<i>TaGSTF10</i>	0.562	$1.8 \pm 0.06$	0.9
	<i>TaGSTL1</i>	5.73	$1.8 \pm 0.1$	10.02
24	GAPDH	0.411	$1.8 \pm 0.02$	/
	<i>TaGSTU3</i>	0.827	$1.8 \pm 0.02$	2.01
	<i>TaGSTU6</i>	0.376	$1.8 \pm 0.01$	0.9
	<i>TaGSTF4</i>	0.241	$1.75 \pm 0.02$	0.6
	<i>TaGSTF10</i>	0.527	$1.8 \pm 0.03$	1.3
	<i>TaGSTL1</i>	0.322	$1.75 \pm 0.02$	0.8

**Table 16 Results of the RT-PCR comparative quantification analysis**

The CQ is expressed as a ratio between control and treated samples, GAPDH is used to determine a fold increase in the gene of interest. The amplification is used as a quality control.

### 6.3 The metabolism of cloquintocet mexyl

#### 6.3.1 Aims and objectives

To identify any downstream metabolites of cloquintocet mexyl, 7 D old wheat shoots *Triticum aestivum* were cut into 1 cm strips and floated in MS - sucrose media containing 50  $\mu\text{M}$  cloquintocet mexyl or an acetone control. The purpose of this was to determine if there was an accumulation of cloquintocet mexyl within the plant. As a further control, 50  $\mu\text{M}$  cloquintocet mexyl in MS – media was incubated at the same time to determine if there was any chemical changes in cloquintocet mexyl caused by the media. Shoots were left to shake at 100 rpm, 18  $^{\circ}\text{C}$  and harvested 24 H after treatment. Tissue was thoroughly rinsed prior to extraction to eliminate any residue on the leaf surface. Methanol extracts were subjected to LC-MS to identify any change in the free acid content in the plants.

#### 6.3.2 Results

Upon analysing the results of the methanol extracts using masslynx software. The parent compound cloquintocet mexyl (336  $\text{M}^+\text{H}^+$ , retention time: 10.50 min) was identified, along with its free acid cloquintocet (238  $\text{M}^+\text{H}^+$ ; retention time: 6.50 min), confirming that

cloquintocet mexyl is cleaved to its free acid after safener treatment. It has been shown in chapter 3 section 3.3.6, that the free acid moiety safens wheat, it can therefore be hypothesised that it is the cleavage of the parent compound that activates the GST induction within the plant. No further downstream metabolites of the safener could be identified at 24 H.

## 6.4 Cleavage of cloquintocet mexyl In Vivo

### 6.4.1 Aims and objectives

As discussed in section 3.3, the study was repeated over a shorter time course using the same experimental conditions in order to further identify any metabolites. An induction of GST activity is observed after 4 H (chapter 3, section 3.3) and a further aim of this study is to further elucidate the rate of cleavage of the safener, to correlate any increase in the free acid to an induction of GSTs. 7 D old wheat plants *Triticum aestivum* were cut into 1 cm strips and floated in MS - sucrose media containing 50  $\mu$ M cloquintocet mexyl. Shoots were left to shake at 100 rpm, 18 °C and harvested at a time course of 0 H, 30 min, 1H, 2 H, 3H, 4 H, 5 H, 6 H, and 24 H. Tissue was thoroughly rinsed prior to extraction to eliminate any residue on the leaf surface. Methanol extracts were subjected to LC - MS to identify any change in the free acid content in the plants.

### 6.3.2 Results

Methanol extracts were analysed using mass lynx software and at the 0 H and 30 min time points no cloquintocet mexyl or cloquintocet could be detected within the extracts (Table 17). At the 1 H time point the

parent compound can be detected as can the free acid moiety. There is a two fold increase in the levels of cloquintocet (Table 17) compared with cloquintocet mexyl at 1 H. This two fold increase in the free acid relative to the parent compound continues throughout the time course with  $\text{nmol}^{-1}$  of the free acid staying at a constant level relative to the parent compound. Analysis of the MS – sucrose media containing the parent compound showed negligible levels of the free acid.

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Time Course (H)	nmol g <sup>-1</sup> fresh weight wheat shoots $\pm$ s.d n = 3	
	Cloquintocet free acid	Cloquintocet mexyl
0.5	None Detected	None Detected
1	8.05 $\pm$ 0.08	4.24 $\pm$ 0.8
2	8.14 $\pm$ 0.08	4.27 $\pm$ 0.3
3	8.20 $\pm$ 0.3	4.49 $\pm$ 0.3
4	8.25 $\pm$ 0.8	4.43 $\pm$ 0.3
5	8.32 $\pm$ 0.1	4.51 $\pm$ 1
6	8.36 $\pm$ 0.2	4.86 $\pm$ 0.1
24	9.83 $\pm$ 0.08	5.39 $\pm$ 0.2

**Table 17 The cleavage of cloquintocet mexyl to its free acid moiety cloquintocet in 7-day-old wheat shoots.**

The cleavage of cloquintocet mexyl to its free acid moiety cloquintocet in 7-day-old wheat shoots (nmol g<sup>-1</sup>). Wheat shoots were cut into 1cm strips and floated in MS - sucrose media containing 50  $\mu$ M cloquintocet mexyl and an acetone control. Plants were harvested at a time course of 30 min, 1 H, 2 H, 3 H, 4 H, 5 H, 6 H, and 24 H.

## 6.5 Cleavage of cloquintocet mexyl *in vitro*

### 6.5.1 Aims and objectives

The aim of this section is to determine if any of the GSTs that are induced by the safeners are also responsible for their cleavage into the free acid, and whether the safeners can be conjugated to glutathione either chemically or enzymically.

It was of interest to determine if any of the safener inducible GSTs cloned in chapter five were responsible for the cleavage of the parent compound, as it has been demonstrated in the literature that GSTs can function as esterases (Hall *et al.*, 1995).

It had been demonstrated in chapter five that selected GSTs have activity toward alachlor, benoxacor and fenoxaprop ethyl conjugating them to glutathione. These compounds can also be chemically conjugated to glutathione. Fenchlorazole ethyl, mefenpyr diethyl, cloquintocet mexyl and cloquintocet were incubated with glutathione to try and chemically conjugate them to glutathione following a protocol for synthesising glutathione conjugates in Edwards *et al* (2005). The safeners were also incubated with each of the five cloned GSTs to determine if there is any GST mediated conjugation.

### 6.5.2 Results

Following analysis of the samples by LC – MS, it was found that fenchlorazole ethyl, mefenpyr diethyl, cloquintocet mexyl or cloquintocet do not undergo a chemical conjugation to glutathione. A number of experimental conditions were tried including a range of buffers, incubation times and temperatures. There was also no enzyme mediated conjugation of glutathione to any of the safeners including the cloquintocet free acid by any of the safener inducible GSTs.

## 6.6 Inhibition of GSTs by herbicide safeners

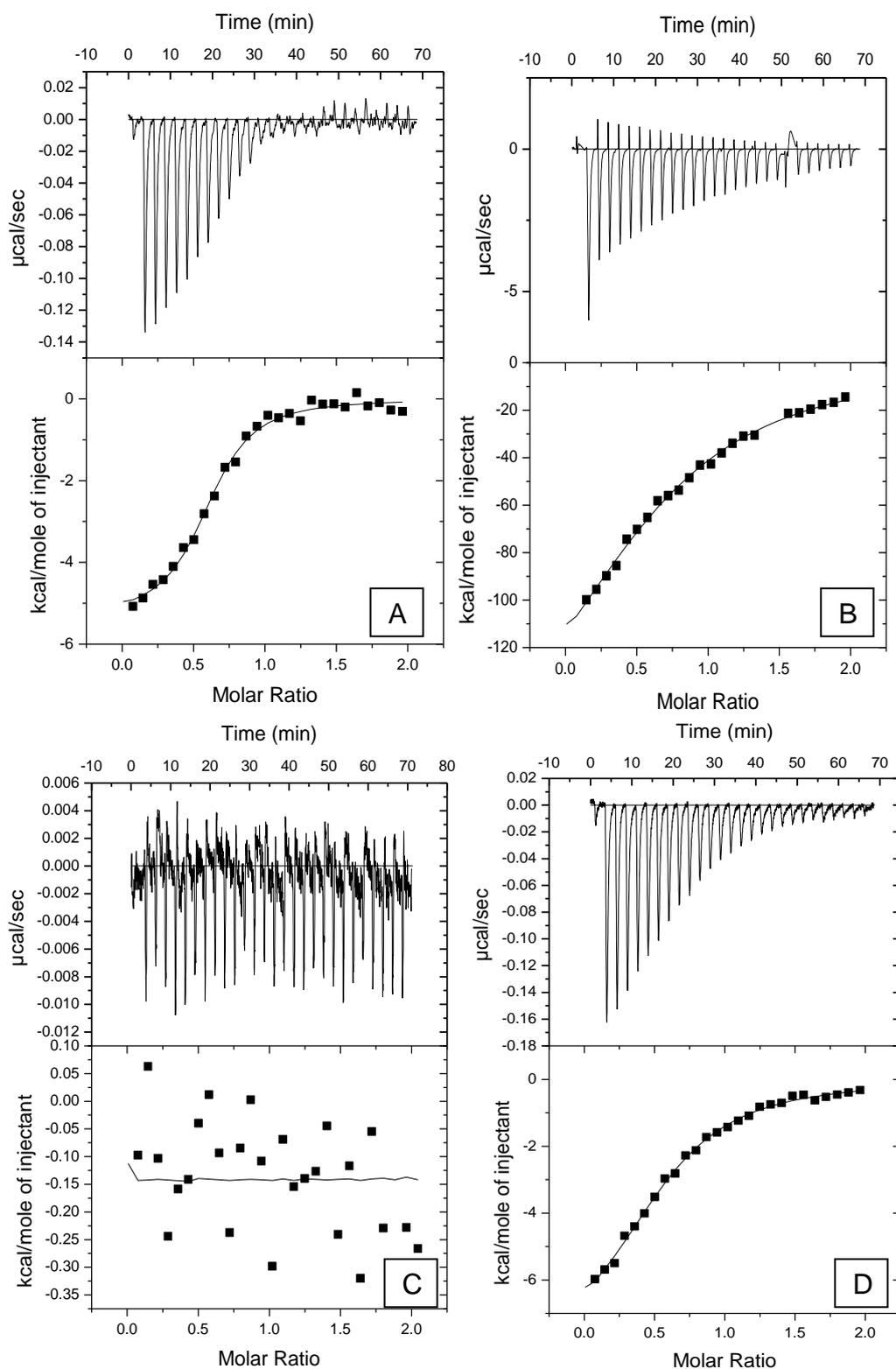
### 6.6.1 Aims and objectives

A number of human GSTs are inhibited by xenobiotics. Examples are ethacrynic acid and its glutathione conjugate which can be conjugated chemically or enzymically and are both inhibitors of human GSTs (Awasthi *et al.*, 1993) as are ellagic acid and curcumin (Hayeshi *et al.*, 2007). It was of interest to determine whether the herbicide safeners used in wheat cause a modulation of signal transduction by inhibiting GSTs. This was done in two ways. The first was using crude protein from 7-day-old wheat plants sprayed with the field rate of cloquintocet mexyl, mefenpyr diethyl and fenchlorazole ethyl and harvested at a time course of 30 min, 1 H, 2 H, 3 H, 4 H, and 24 H post treatment. GST inhibition was determined by CDNB and GPOX assays. The second method was to use Isothermal calorimetry to determine if any of the safener inducible GSTs cloned in chapter five were bound to any of the safeners, notably cloquintocet mexyl, cloquintocet acid, fenchlorazole ethyl and mefenpyr diethyl. It was also of interest to determine if any GSH - conjugates inhibited these GSTs, and since none of the wheat safeners could be conjugated to glutathione, a fenclorim – GSH conjugate was synthesised and used instead. A boiled enzyme buffer control (Fig 28, C) was used along with a positive

control using *AtGSTU19* was utilised as this GST is known to be inhibited by the fenclorim – GSH conjugate.

### 6.6.2 Results

In the plant study there was an inhibition of GST activity toward CDNB 3 H after treatment with all three safeners (Fig 19), with cloquintocet mexyl exhibiting the highest rate of inhibition. There is also an inhibition of GPOX activity 3H after treatment again with cloquintocet mexyl exhibiting the highest rate of inhibition. When tested using the ITC binding assay there was no inhibition seen with the lambda GSTs or the phi GSTs with any of the safeners or the fenclorim GSH conjugate. *AtGSTU19* also bound the fenclorim GSH conjugate as predicted but not with any of the wheat safeners. *TaGSTU3* also bound the fenclorim-GSH conjugate. This was also the GST that was found to be up regulated in chapter four. None of the other recombinant GSTs showed binding toward the fenclorim – GSH conjugate, nor did they show any signal toward cloquintocet mexyl, fenchlorazole ethyl and mefenpyr diethyl. Interestingly *TaGSTU6* did bind the free acid moiety of cloquintocet mexyl.



**Figure 28 ITC analysis**

Selected results from the ITC analysis. (A) *At*GSTU19 with the fenclorim – GSH conjugate, (B) *Ta*GSTU6 with the fenclorim – GSH conjugate, (C) buffer control, (D) *Ta*GSTU3 with cloquintocet free acid.

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GST and Ligand	Binding Affinity $K_d \text{ M}^{-1} \pm \text{s.d}$
<i>At</i> GSTU19 + fenclorim - GSH	$2.57 \times 10^{-6} \pm 2.8 \times 10^{-5}$
<i>Ta</i> GSTU3 + fenclorim - GSH	$4.69 \times 10^{-7} \pm 3.4 \times 10^{-6}$
<i>Ta</i> GSTU6 + cloquintocet F.A	$5.26 \times 10^{-6} \pm 5.4 \times 10^{-5}$

**Table 18 Binding affinities for the GSTs inhibited**

Binding affinity for *At*GSTU19 and *Ta*GSTU3 for the fenclorim- glutathione conjugate, and *Ta*GSTU6 for cloquintocet F.A ( $K_d \text{ M}^{-1}$ ) with s.d showing the extent of variation between replicates. n = 2.

GST activity toward CDNB (nkat mg<sup>-1</sup> crude protein  $\pm$  s.d n = 4)

Time Course (H)

Treatment	0.30	1	2	3	4	24
Acetone control	1.71 $\pm$ 0.04	1.65 $\pm$ 0.12	1.22 $\pm$ 0.01	1.67 $\pm$ 0.02	1.71 $\pm$ 0.02	2.08 $\pm$ 0.2
Cloquintocet mexyl	1.68 $\pm$ 0.03	1.78 $\pm$ 0.12	1.51 $\pm$ 0.01	0.84 $\pm$ 0.06	1.97 $\pm$ 0.02	4.35 $\pm$ 0.2
Fenchlorazole ethyl	1.97 $\pm$ 0.09	2.07 $\pm$ 0.03	1.84 $\pm$ 0.15	1.23 $\pm$ 0.08	2.30 $\pm$ 0.10	4.13 $\pm$ 0.2
Mefenpyr diethyl	1.78 $\pm$ 0.14	1.62 $\pm$ 0.06	1.69 $\pm$ 0.05	1.26 $\pm$ 0.15	1.83 $\pm$ 0.02	4.63 $\pm$ 0.5

**Table 19 Inhibition of GST activity toward CDNB in crude extracts**

Inhibition of GST activity toward CDNB in wheat shoots (*Triticum aestivum* L.). Wheat was sprayed with the field rate of each respective safener. Values represent the means of triplicate determination with the standard deviation showing the extent of variation between replicates.

	<u>GPOX activity (nkat mg<sup>-1</sup> crude protein ± s.d n = 4)</u>					
	<u>Time Course (H)</u>					
Treatment	0.30	1	2	3	4	24
Acetone control	0.08±0.001	0.09 ± 0.01	0.08 ± 0.04	0.10 ± 0.01	0.10 ± 0.004	0.14 ± 0.01
Cloquintocet mexyl	0.06 ± 0.001	0.11 ± 0.005	0.22 ± 0.001	0.021±0.001	0.09 ± 0.001	0.24 ± 0.01
Fenchlorazole ethyl	0.08 ± 0.009	0.18 ± 0.006	0.14 ± 0.013	0.05 ± 0.004	0.20 ± 0.01	0.34 ± 0.01
Mefenpyr diethyl	0.14 ± 0.01	0.32 ± 0.05	0.24 ± 0.01	0.08 ± 0.003	0.18 ± 0.009	0.22 ± 0.007

**Table 20 Inhibition of GPOX activity in crude extracts**

Inhibition of GPOX activity in wheat shoots (*Triticum aestivum* L.). Wheat was sprayed with the field rate of each respective safener. Values represent the means of triplicate determination with the standard deviation showing the extent of variation between replicates.

## 6.7 Analysis of flavonoid content in safened wheat.

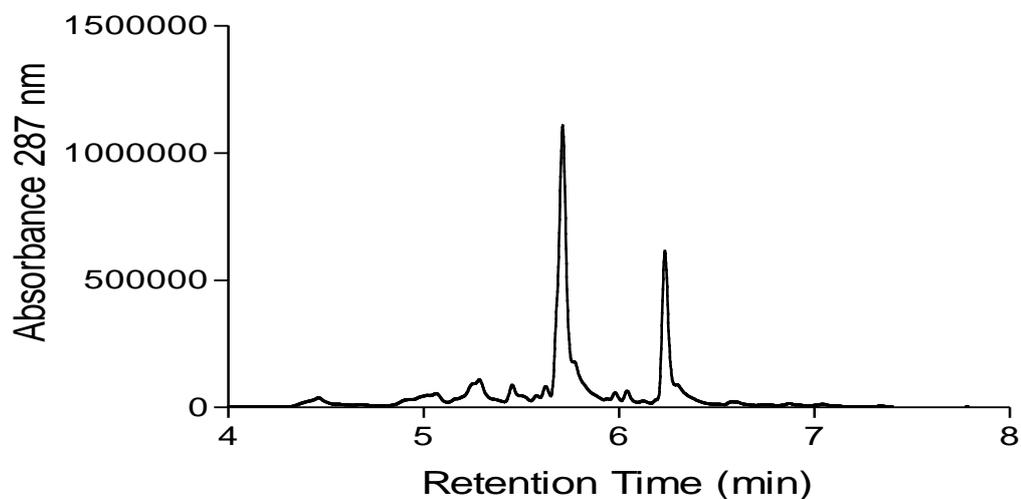
### 6.7.1 Aims and objectives

It has been suggested that GSTs protect flavonoids from oxidation and or guide them to the central vacuole (Mueller *et al.*, 2000). A study by Cummins *et al* (2006) identified an accumulation of ferulic acid and triclin in wheat shoots after treatment with cloquintocet mexyl at a time course of 2-7 days. This thesis has demonstrated that GST induction can be observed four hours post safener treatment (Table 16). It was therefore of interest to repeat this study over a 48 H time course and observe any perturbations in flavonoid levels. Wheat shoots were grown for 7 D in an environmental chamber prior to being sprayed with the field rate of cloquintocet mexyl (15 g / hec) and a 0.1% acetone control. Wheat was harvested at a time course of 0 H, 30 min, 1 H, 4 H, and 24 H in biological triplicate and samples subjected to LC – MS.

### 6.7.2 Results

No perturbations in flavonoid content were detected over a 24 H time course after treatment with cloquintocet mexyl. This study identified no change in flavonoid content that would correlate to the induction of the

GSTs. Cummins *et al* (2006) found that cloquintocet mexyl caused a depletion of the flavone C - glycosides with an accumulation of ferulic acid and triclin in the original study. The concentration of luteolin was reduced by 50 % with apigenin and 3'-O-methyl luteolin undergoing a more modest decline with the study being conducted over a time course of 2 – 7 D post safener treatment. It is possible that a downstream metabolite of cloquintocet mexyl may cause a depletion or accumulation of the flavonoids at a later time course.



Metabolite	Retention time (min)	Identity	[M + H] <sup>+</sup>
1	5.36	Luteolin C-glucoside C-xyloside	581.2
2	5.48	Luteolin C-glucoside C-xyloside	581.2
3	5.78	Apigenin C-glucoside C-xyloside	565.2
4	6.25	Luteolin 6-C-glucoside	449
5	6.30	3'-O-methyluteolin6-C-glucosylglucoside	625

**Table 21 Identified flavonoid metabolites**

**Figure 29 Chromatogram showing identified flavonoid metabolites**

Identification of flavonoid metabolites (retention time (min), and mass (M + H)<sup>+</sup>). Wheat was sprayed with the field rate of cloquintocet mexyl.

This study suggests that the GSTs suspected of being involved in the transport and or protection of the flavonoids are not the same GSTs that are being induced a few hours after safener treatment, otherwise a perturbation in flavonoid content would be expected. Cummins *et al.*, (2006) attributed the depletion of the respective C -glycosides of luteolin, apigenin and 3' - O - methyluteolin to the up regulation of the O - methyltransferases and the C - glucosyltransferases and possibly to the GSTs. It has been shown in previous studies that the lambda GSTs are expressed later then the phi and tau GSTs (Chapter 3 and Chapter 6, table 16) and may play a role in flavonoid metabolism.

Metabolite	0 Time	Untreated metabolite concentration (nmol g <sup>-1</sup> fresh weight ± s.d n-3)				Safener treated metabolite concentration (nmol g <sup>-1</sup> fresh weight ± s.d n-3)			
		30 min	1 H	4 H	24 H	30 min	1 H	4 H	24 H
Luteolin C-glucoside C-xyloside	6.9 ± 0.4	6.3 ± 0.7	6.4 ± 0.5	6.4 ± 0.5	5.98 ± 0.5	7.2 ± 0.2	7.1 ± 0.1	5.6 ± 1.2	6.65 ± 0.2
Apigenin C-glucoside C-xyloside	50.1 ± 1.5	49.6 ± 5.1	55.8 ± 0.6	47.3 ± 2.6	52.6 ± 1.3	49.7 ± 4.4	50 ± 8.8	48.3 ± 7.9	42.6 ± 0.6
3'- O-methyluteolin 6-C-(2" O-rhamnosyl) – glucoside	3.37 ± 0.9	3.9 ± 1.5	3.5 ± 0.1	2.9 ± 0.2	3.1 ± 0.9	3.0 ± 0.2	3.0 ± 0.2	2.7 ± 0.4	3.0 ± 0.2
Luteolin 6-C-glucoside	18.7 ± 1.7	18.2 ± 4.6	20.2 ± 2.5	14.7 ± 1.6	22.9 ± 3.1	21.5 ± 3.9	20.6 ± 1.5	19.0 ± 2.4	18.3 ± 1.6

**Table 22 Quantification of flavonoid metabolites in wheat**

Wheat was sprayed with the field rate of cloquintocet mexyl or a 0.1% acetone control.

### 6.8 Discussion

The aim of this chapter was to elucidate further the mode of action of safeners in wheat. Little is known about the metabolism of the wheat safeners. We determined that cloquintocet mexyl is hydrolysed to its free acid, but no further metabolites were identified. This chapter has shown that none of the GSTs that are up-regulated by cloquintocet mexyl have activity toward it, or its free acid. It was plausible that the chlorine group would provide a site for nucleophilic attack. However, no glutathione conjugates were detected in crude extracts, and cloquintocet or its free acid could not be conjugated enzymically or chemically. Although this does not rule out a different GST being able to do this, the fact that no glutathione conjugates were identified in crude extracts means this is unlikely. GST activity toward CDNB is at its highest after 24 H, no glutathione conjugates were detected at 24 H making it unlikely that cloquintocet mexyl is metabolised this way. The free acid however is still detected after 24 H, and it was shown in chapter three that the free acid moiety safens as well as the parent compound. The results of the inhibition experiments suggest that it is the free acid that safens, Intriguingly the safener treatments caused an inhibition in GST activity in plants. It is interesting to speculate that this inhibition in GST activity could cause the further induction of GSTs.

It is possible that the metabolism of the safener is quite rapid and a shorter time course is required to identify any further metabolites. This initial delay in the uptake of the safener may indicate that the safener is slow to diffuse across the waxy cuticle of the plant but once absorbed cloquintocet mexyl is rapidly cleaved to its free acid.

This shows that there is a constant uptake of the safener into the plant and that the plant is further metabolising the free acid. If this were not the case then there would be an accumulation of the free acid within the plant. This also suggests that the initial delay of safening for four hours can in part be accounted for by the time taken for the safener to cross the waxy cuticle. It can also be hypothesised that the ester group that is cleaved is there solely to facilitate the passage of the safener across the cuticle, again demonstrating that cleavage is caused by the plant and does not happen chemically or is caused by the experimental conditions. Although the safeners induce these GSTs, they are not directly involved in the conjugation of the free acid but possibly a further down stream metabolite. It is also possible that another GST conjugates glutathione to cloquintocet mexyl. All other GSH – conjugates studied (chapter 5) had a chemical conjugation to glutathione which the GSTs increased, no such chemical conjugation could be found in this study.

This does not however rule out any conjugation to glutathione by other GSTs, but it does show that none of the GSTs that are induced by the safener are involved in the early stage metabolism of cloquintocet mexyl. It is therefore possible that after the rapid hydrolysis of cloquintocet mexyl to its free acid, and the inhibition of *Ta*GSTU6 by cloquintocet, that this then causes a further induction of GSTs which can be measured as an increase in CDNB activity in crude extracts after four hours. It can also be hypothesised that the same is also true for any glutathione conjugates of safeners in other plants. No further metabolites were found after the hydrolysis of the parent compound, this does not however mean that they do not exist. If cloquintocet mexyl were being deposited in the vacuole of plants an increase in levels of the free acid would be expected (section 6.3), instead levels of the free acid stay constant, indicating that it is being metabolised further to as yet an unidentified product.

## Chapter Seven: Discussion

This thesis set out to elucidate further the way in which GSTs respond to safener application in wheat (*Triticum* spp).

The third chapter was aimed at answering some of the basic unanswered questions about the safening response in wheat with regards to the induction of GSTs. This chapter identified that although the wheat safeners differ in their chemistries, they all induced a similar set of GSTs from the phi, lambda, and tau classes, this similarity suggested that all the safeners tested appeared to elicit an identical signalling pathway which led to the co – induction of family members. These results led to further studies investigating the factors affecting the induction of GSTs by safeners in *Triticum aestivum* L. Using a variety of treatment regimes. It was observed throughout the studies, that fenclorazole ethyl and mefenpyr diethyl in particular significantly enhanced the growth of wheat seedlings. In recent studies (Dixon and Edwards, 2009) phi and tau GSTs have been shown to bind glutathione to the electrophilic oxophytodienoic acid, which is an intermediate in Jasmonate synthesis. Jasmonic acid is a hormone that plays a role in plant growth and regulation. It is therefore possible that if

GSTs have an endogenous role involved in the shuttling of the metabolite between cellular compartments, that when they are up-regulated by herbicide safeners, this also causes a perturbation in levels of Jasmonic acid and thereby affecting the growth of the wheat shoots. Studies using the free acid moiety of cloquintocet mexyl showed that it exerted the same safening effect as the parent ester, this finding suggests that it is the free acid that is the active agent. The esters function is primarily to aid diffusion across the waxy cuticle (Roberts, 1998).

Studies then focussed in on cloquintocet mexyl. With respect to tissue responsiveness, it was demonstrated that the induction of GSTs by cloquintocet mexyl was not uniform in wheat seedlings. In addition the types of GSTs induced by the safener in different plant parts was class specific, with the lambda GSTs up-regulated in the meristematic tissue and the tau and phi GSTs up-regulated throughout the shoot. Again, it is worth while noting that the herbicide sprayed in combination with cloquintocet mexyl is clodinafop propargyl, and that its target site of action as an ACCase is in the meristematic tissues of plants.

It was then found that GST induction is time and dose dependent, and that while repeated applications did not give an additive effect on the induction of tau and phi GSTs, a cumulative effect

was seen with the lambda GSTs. If the lambda GSTs accumulate at the site of action of the herbicide it is possible that the lambda GSTs may play an important role in protecting the wheat from injury caused by the herbicide. Using the lambda GSTs as a biomarker of induction, it was found that the induction starts after four hours and continues for several days after treatment.

Chapter four focussed in on the studies in chapter three by further clarifying that it was *TaGSTU3* that appeared to be one of the main GST polypeptides up-regulated by safener treatment. These proteomic studies did not identify other classes of GSTs such as the phi and lambda classes, which are also known to be up-regulated, and it can therefore be assumed that they did not recognise the affinity ligands used to enrich for GSTs, or that they were present in much smaller quantities. The lambda GSTs were unlikely to correlate to CDNB activity or the affinity ligands due to their catalytic function.

GST polypeptides identified in chapter four were then cloned, expressed and assayed for activity toward a range of substrates. The GSTs induced by cloquintocet mexyl were found to have no activity toward the safener or its partner herbicide clodinafop propargyl as determined using the spectrophotometric and HPLC assays.

Chapter six was aimed at further elucidating the role of metabolism in the mode of action of cloquintocet mexyl in wheat. It was determined that cloquintocet mexyl is rapidly hydrolysed to its free acid once absorbed into shoot tissue Roberts (1998) also found this. However no further down stream metabolites were identified such as glutathione conjugates, confirming that it is unlikely to be metabolised by GSTs, despite causing their up-regulation. In animals the free acid of cloquintocet mexyl is excreted (Roberts, 1998), this can not occur in plants and an accumulation of the free acid or metabolites was expected. While the levels of parent ester rapidly declined, there was no corresponding accumulation of the free acid suggesting cloquintocet is being metabolised further within the plant to as yet undetermined metabolites.

Although it has been determined that cloquintocet mexyl causes an up-regulation of GST polypeptides from the tau, phi and lambda classes, it is still unclear why these specific GSTs are up-regulated as they appear to play no part in the conjugation of the parent compound, its free acid or its partner herbicide to glutathione. Due to time constraints

it was not possible to study the down stream metabolites of cloquintocet mexyl. This could be a possible focus of future study,

using radiolabelled safener to allow the detection of related metabolites, as it is possible that it is a downstream metabolite that causes the up-regulation of the GSTs. If this was found to be true it could inform future herbicide safener design.

In terms of future work, the next obvious challenge is the characterisation of the safener receptor system in wheat and indeed in other plants. The work presented in this thesis confirms that very different compounds can induce apparently identical downstream events at the level of GST enhancement. This would argue against a single protein – safener binding event as being the primary means of recognition. Work in the Edwards lab is continuing on the mode of action of safeners, with the recent work on a safener chemical series in *Arabidopsis* suggesting a close link to the response of plants to oxylipin stress signalling agents. It is therefore possible that safeners may act by disrupting endogenous stress pathways linked to oxylipin generation and turnover.

The work in this thesis focused on the induction of GSTs by the herbicide safener. It would be of interest in future work to conduct a comparative study using the techniques used in this thesis with the parent herbicide sprayed in combination with its safener. Especially

looking at tissue specific localisation of GST activity with a focus on the lambda GST induction.

An account of some of the work contained in this thesis has been accepted for publication in Environmental and Experimental Botany as part of the Special Issue on Plants and Global Change.

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