Glutathione transferases in maize (zea mays)

Dixon, David Peter

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PhD Thesis, submitted 1998

Abstract

The glutathione transferases (GSTs) of maize have been the most studied GSTs in plants, however much is still not known about these enzymes. In the course of the current study six GST subunits (*Zm* GSTs I, II and III, which have been reported previously, and *Zm* GSTs V, VI and VII, which have not been previously reported) have been identified in the dimers *Zm* GST I-I, I-II, I-III, V-V, V-VI and V-VII. Maize GSTs are known to be important in herbicide detoxification and the purified maize enzymes were each found to have differing activities toward a number of herbicides, and also a range of other potential GST substrates. Additionally, *Zm* GST I-II and *Zm* GST V-V possessed glutathione peroxidase activity. The developmental regulation and chemical inducibility of maize GSTs were studied in maize seedlings using western blotting, with different subunits showing markedly different responses. *Zm* GST I was constitutively present in all plant parts and unaffected by chemical treatment, *Zm* GST II was only detected in young roots but was induced in roots and shoots by many different chemical treatments, and *Zm* GST V was present at low levels throughout maize plants, with levels enhanced greatly by treatment with the safener dichlormid but not by other chemicals tested. cDNA clones corresponding to *Zm* GST subunits I, III, V, VI and VII were isolated by library screening using antibody or DNA probes. The cDNA sequences for *Zm* GST subunits V, VI and VII were different from those of previously cloned type I (theta class) maize GSTs and were most similar to the auxin-regulated GST family (type III or tau class GSTs) previously only identified in dicotyledonous species. The cloned GSTs were expressed as recombinant proteins in *E. coli*, allowing further characterisation, including detailed kinetic analysis for recombinant *Zm* GST I-I and *Zm* GST V-V.

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David Peter Dixon

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University of Durham

Department of Biological Sciences

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Declaration

No material presented here has previously been submitted for any other degree. Except where acknowledged, all material is the work of the author.

Statement of Copyright

Abbreviations

BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BITC	Benzyl isothiocyanate
bp.	Base pairs
BSP	Bromosulphophthalein
CDNB	1-Chloro-2,4-dinitrobenzene
cv.	Cultivar
Da	Daltons (kDa = kiloDaltons)
DCNB	1,2-Dichloro-4-nitrobenzene
DIG	Digoxigenin
EA	Ethacrynic acid
EDTA	Ethylenediaminetetraacetic acid, disodium salt
ENPP	1,2-Epoxy-3-(*p*-nitrophenoxy) propane
EPTC	*S*-Ethyl dipropylthiocarbamate
GSH	Glutathione (reduced)
GST	Glutathione transferase
h	Hour(s)
HIC	Hydrophobic interaction chromatography
IAA	Indole-3-acetic acid
kat	Katals (moles of product formed per second)
LB	Luria-Bertani
Mr	Molecular mass
NAA	α-Naphthaleneacetic acid
NBC	*p*-Nitrobenzyl chloride
NBT	Nitro blue tetrazolium
NPB	*p*-Nitrophenethyl bromide
PAGE	Polyacrylamide gel electrophoresis
PBO	*trans*-4-Phenyl-3-buten-2-one
pfu	Plaque-forming units
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl)aminomethane

Chemical Names

Alachlor	2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine
BAS 145138	1-dichloroacetyl-hexahydro-3,3,8a-trimethylpyrrolo(1,2-a)pyrimidin-6-(2H)-one
Benoxacor	4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine
CGA-133205	*O*-(1,3-dioxolan-2-yl-methyl)-2,2,2-trifluoro-4´-chloroacetophenone-oxime
Chlorimuron-ethyl	2-(4-chloro-6-methoxypyrimidin-2-ylcarbamoylsulphamoyl) benzoic acid ethylester
Cyanazine	2-(4-chloro-6-ethylamino-1,3,5-triazin-2-ylamino)-2-methyl-propionitrile
Dichlormid	*N*,*N*-diallyl-2,2-dichloroacetamide
Ethacrynic acid	[2,3-dichloro-4-(2-methylene-butyryl)phenoxy]acetic acid
Fenoxaprop	(±)-2-[4-[(6-chloro-2-benoxazolyl)-oxy]phenoxy]propanoic acid
Fluorodifen	4-nitrophenyl α,α,α-trifluoro-2-nitro-*p*-tolyl ether
Flurazole	phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate
Glyphosate	(N-phosphonomethyl)glycine
Metolachlor	2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide
Naphthalic anhydride	1*H*,3*H*-naphtho(1,8-*cd*)-pyran-1,3-dione
Oxabetrinil	α-[(1,3-dioxolan-2-yl-methoxy)-imino]benzeneacetonitrile
R-29148	3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone
Simazine	6-chloro-*N*,*N*´-diethyl-1,3,5-triazine-2,4-diamine
Tridiphane	(R,S)-2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane

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Publications

Publications resulting entirely from this work:

Dixon, D, Cole, DJ, and Edwards, R (1997). Characterisation of multiple glutathione transferases containing the GST I subunit with activities toward herbicide substrates in maize (*Zea mays*). *Pesticide Science* **50** 72-82.

Dixon, DP, Cole, DJ, and Edwards, R (1998). Purification, regulation and cloning of a glutathione transferase (GST) from maize resembling the auxin-inducible type-III GSTs. *Plant Molecular Biology* **36** 75-87.

Dixon, DP, Edwards, R, Robinson, NJ, Fordham-Skelton, AP, and Cole, DJ (1995). Spectrum of herbicide reactive glutathione transferases in maize. In *Brighton Crop Protection Conference - Weeds* 255-260 (meeting proceedings).

Dixon, DP, Cole, DJ, and Edwards, R (1996). Spectrum of glutathione transferase activities in maize. In *Proceedings of the International Symposium on Weed and Crop Resistance to Herbicides* (R De Prado, L Garciá-Torres, and J Jorrín, eds.) 72-74, Kluwer Academic Publishers, The Netherlands (meeting proceedings).

Dixon, DP, Cole, DJ, and Edwards, R (1997). Characterization and regulation of multiple glutathione transferases in corn (*Zea mays*). *Plant Physiology* **114** 746 (meeting proceedings).

Dixon, DP, Edwards, R, and Cole, DJ (1997). Regulation of maize glutathione transferases during development and their induction by xenobiotics. In *Brighton Crop Protection Conference - Weeds* 759-764 (meeting proceedings).

Dixon, DP, Edwards, R, and Cole, DJ (1997) Glutathione transferases in maize. *Journal of Experimental Botany* **48s** 22 (meeting proceedings).

Publications resulting partly from this work:

Hatton, PJ, Dixon, D, Cole, DJ, and Edwards, R (1996). Glutathione transferase activities and herbicide selectivity in maize and associated weed species. *Pesticide Science* **46** 267-275.

Cole, DJ, Cummins, I, Hatton, PJ, Dixon, D, and Edwards, R (1997). Glutathione transferases in crops and major weeds. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants* (KK Hatzios, ed) 139-154, Kluwer Academic Publishers, The Netherlands.

1. Introduction

Glutathione transferases, also referred to as glutathione *S*-transferases (GSTs) (EC 2.5.1.18), are enzymes which catalyse the conjugation of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) via the sulphydryl group of the cysteine residue with a wide range of electrophilic, often hydrophobic, substrates. These substrates include organic (alkyl or aryl) halides, esters, ethers, epoxides, lactones, quinones and activated alkenes (Wilce and Parker, 1994). The conjugation reaction usually involves nucleophilic substitution, although addition reactions can also occur. Examples of some of these reactions are shown in Figure 1, and structures of some herbicide and GST substrates used in colorimetric assays are shown in Figure 2 and Figure 3 respectively. GST activity is often assayed by following the conjugation of glutathione with the artificial substrate CDNB (1-chloro-2,4-dinitrobenzene, Figure 3) since many, though not all, GSTs show high activity towards this substrate and the reaction can be conveniently followed spectrophotometrically.

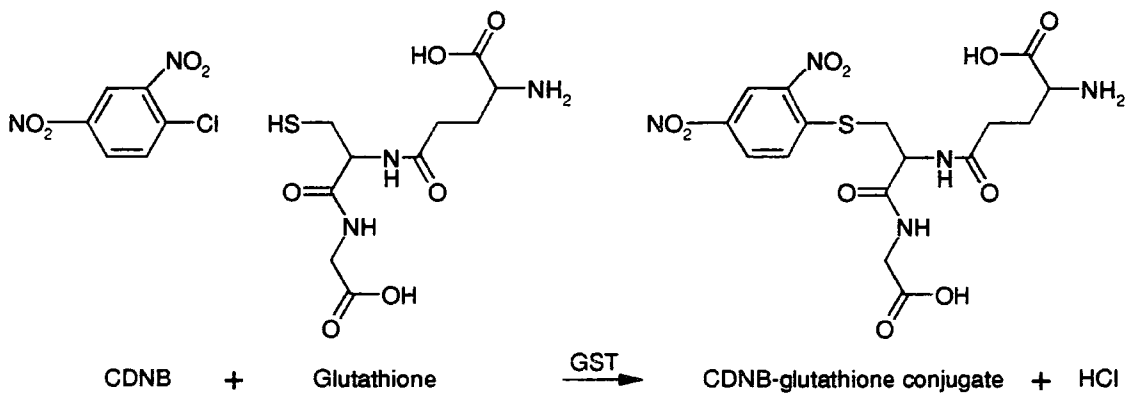
GSTs are usually very specific with respect to glutathione, although some have been shown to be able to use other related thiols, for example homoglutathione (γ-glutamyl-cysteinyl-β-alanine) (Habig *et al.*, 1974, Adang *et al.*, 1989, Skipsey *et al.*, 1997).

Interest in GSTs is mainly due to their ability to detoxify a wide range of xenobiotic compounds, including drugs, pesticides and environmental contaminants. GSTs were first identified in rat liver (Booth *et al.*, 1961) and have since been found in nearly all organisms examined including mammals (Booth *et al.*, 1961), fish (Ramage *et al.*, 1986), insects (Clark *et al.*, 1986), plants (Marrs, 1996), fungi (Cohen *et al.*, 1986), yeast (Tamaki *et al.*, 1991) and bacteria (Arca *et al.*, 1990). Major reviews include the function and regulation of plant GSTs (Marrs, 1996), the enzymology of GSTs from non-vertebrates (Clark, 1989), crystal structures of GSTs (Dirr *et al.*, 1994b) and structure, regulation and function of mammalian GSTs (Pickett and Lu, 1989, Rushmore and Pickett, 1993). In general, much more work has been done on mammalian GSTs than plant and other non-mammalian GSTs.

Halide substrates, including CDNB, atrazine and alachlor:

$$GSH + R\text{-}X \rightarrow GS\text{-}R + X^- + H^+$$

E.g. for CDNB:



Ether substrate (e.g. fluorodifen):

$$GSH + R\text{-}O\text{-}R' \rightarrow GS\text{-}R + R'\text{-}OH$$

Alkene substrate addition reaction (e.g. 4-vinylpyridine):

$$GSH + R\text{-}CH{=}CH\text{-}R' \rightarrow R\text{-}CH(SG)\text{-}CH_2\text{-}R'$$

Figure 1. Examples of reactions catalysed by GSTs.

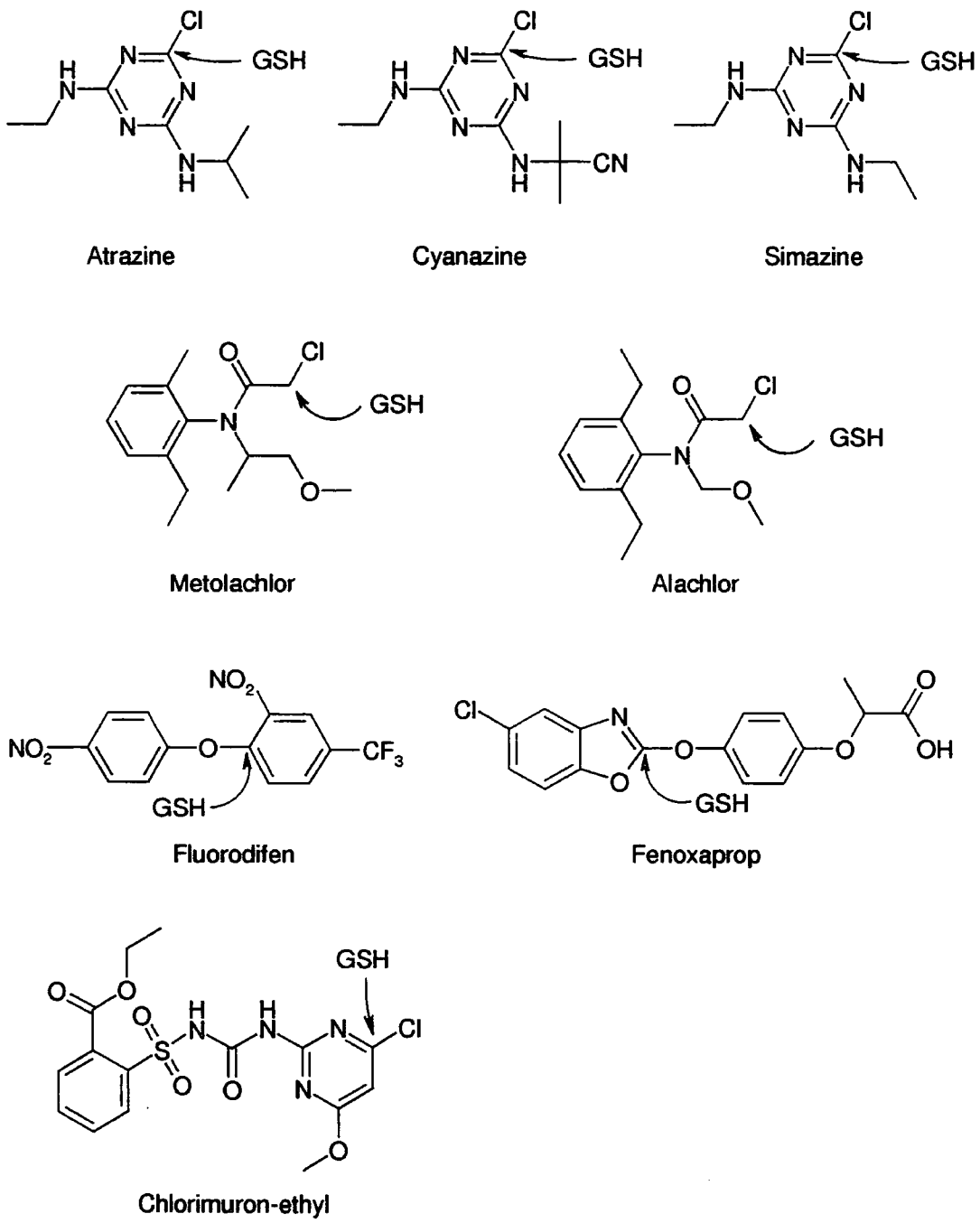
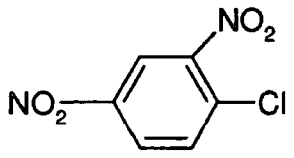
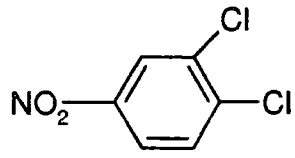


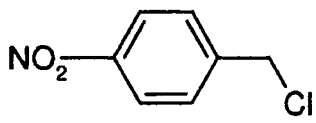
Figure 2. Structures of herbicidal GST substrates, showing positions of GSH substitution.



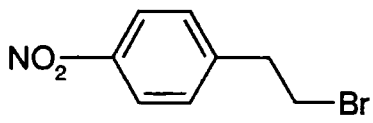
1-Chloro-2,4-dinitrobenzene



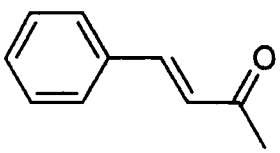
1,2-Dichloro-4-nitrobenzene



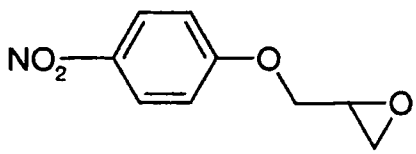
p-Nitrobenzyl chloride



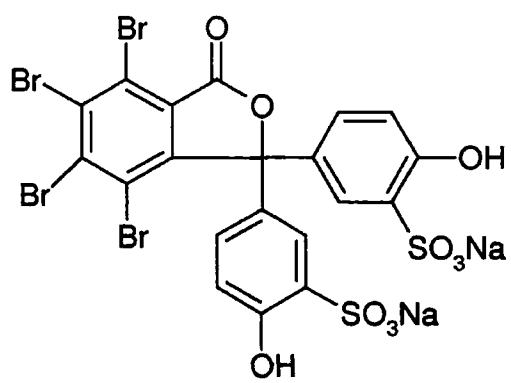
p-Nitrophenethyl bromide



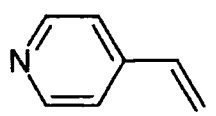
trans-4-Phenyl-3-buten-2-one



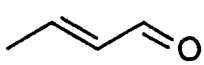
1,2-Epoxy 3-(p-nitrophenoxy) propane



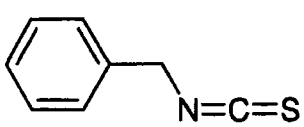
Bromosulphophthalein



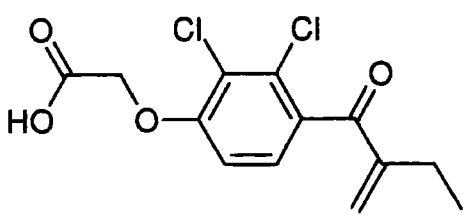
Vinylpyridine



Crotonaldehyde



Benzyl isothiocyanate



Ethacrynic acid

Figure 3. Structures of general GST substrates.

GST Families and Nomenclature

Mammalian GST subunits have been divided into five major classes based on their evolutionary relatedness: alpha, mu, pi, theta and microsomal (Mannervik *et al.*, 1985, Meyer *et al.*, 1991a, Morgenstern *et al.*, 1985). Typically, members of the same class share similar physical properties and substrate specificities, and early classification was based on such properties, together with partial amino acid sequence data. DNA sequencing of the full coding sequences of these GSTs has since confirmed these groupings and GSTs are now classified based on sequence similarity. All GSTs in eukaryotes described to date are composed of two subunits with all dimers forming between subunits of the same class rather than from subunits of different classes (Armstrong *et al.*, 1995). When comparing GSTs from different organisms, subunits within a class typically show high similarity between amino acid sequences (60 to 80% identity) while the sequence similarity between subunits of different classes is much lower, often no more than 30% identity at the amino acid level (Mannervik and Danielson, 1988). Until recently only theta class GSTs were identified outside mammalian species, being present in a diverse range of organisms including plants, insects, fungi and bacteria. It was presumed that this class is most similar to ancestral GSTs from which the remaining mammalian GST classes diversified.

In mammalian species, pi and theta class GSTs appear to be represented by single genes, while alpha and mu class GSTs have been shown to be represented by multiple genes (Dirr *et al.*, 1994b). Since dimerisation is only observed to occur between subunits of the same class, pi and theta class GSTs occur as homodimers while alpha and mu class GSTs can occur as homodimers and heterodimers.

Early work on mammalian GSTs by different groups resulted in multiple names for equivalent enzymes and no consistent nomenclature, resulting in considerable confusion. Jakoby *et al.* (1984) therefore proposed an extensible system for rat GSTs where dimers were named according their subunit composition, with each subunit being given a number. This nomenclature has been widely adopted and extended to include information about GST class, so that mammalian GSTs have the nomenclature aGST BX-Y, where a is a one letter code for the species (h = human, r = rat, p = pig, m =

mouse, etc.), B is a one letter code for the class of GST (A = alpha, M = mu, P = pi, T = theta), and X and Y are numbers corresponding to the identities of the two subunits making up the dimer. For example hGST A1-2 is a heterodimeric human alpha-class GST containing subunit 1 and subunit 2. No similar unified system of GST nomenclature exists for non-mammalian enzymes, although we have adopted a similar system for maize GSTs, as described later. A further class of GSTs has been identified in cephalopods, where they function as lens *S*-crystallin proteins, and such GSTs have been classified as a new sigma class (Buetler and Eaton, 1992).

Plant GSTs were originally all placed into the theta class. However, as plant GST sequence data accumulated and a number of auxin-regulated plant proteins were shown to be GSTs, it became clear that plant GSTs could be divided into distinct classes not found in mammalian systems. Droog *et al.* (1993) initially proposed splitting plant GSTs into three types (I, II and III) based on sequence similarity and gene exon structure. Type I GSTs include maize GST subunits I, II and III and represent the first identified plant GSTs. In each case where the gene structure of a type I GST has been examined, two introns in conserved locations have been identified. In contrast type II GSTs, until recently only represented by related genes from carnation, have genes with nine introns. This gene structure is similar to that found in mammalian alpha class GSTs, although no sequence homology exists between alpha class and plant type II GSTs. The remaining plant GSTs, including most of the GSTs originally identified as auxin-responsive and auxin-binding proteins, are grouped into type III. These GSTs are encoded by genes with a single intron.

More recently Droog (1997) proposed a reclassification of plant GSTs, based on the three types previously identified. In this new classification type I plant GSTs remain in the theta class, since this type most closely resembles other theta class GSTs, while type III plant GSTs are placed in a new tau class, recognising the significant differences between these GSTs and type I GSTs. Droog (1997) did not classify type II GSTs as they only consisted of two very similar sequences, both from carnation, so there was not enough data to merit creation of a new GST class.

Recently, a new, zeta class of GST has been proposed, following the cloning of a human GST which did not fit into any of the previously identified GST classes (Board *et al.*, 1997). Sequence similarity searches showed homology with plant GSTs previously described as type II GSTs by Droog *et al.* (1993), so these type II plant GSTs should now be classified as members of the zeta class.

In summary, numerous GST classes have been proposed, based on enzyme activity and sequence similarity. To date, alpha, mu and pi class GSTs remain confined to mammalian species, sigma class GSTs to cephalopods and tau class GSTs to plants. Theta class GSTs have a very wide species range and zeta class GSTs have representatives in mammals and plants. In addition, a distinct class of microsomal GSTs also exists, though to date this class of GST has only been characterised in mammals.

GST Protein Structure

GSTs exist as homo- or hetero-dimers with subunits of 24-30 kDa. (210-300 amino acids). Each dimer has two independent active sites (Danielson and Mannervik, 1985), with each active site having a highly specific glutathione-binding site (G-site) and a less specific co-substrate binding site (hydrophobic site or H-site). Some GSTs are also known to bind a range of hydrophobic xenobiotic and endogenous substrates at alternative sites not involved in catalysis (Mannervik, 1985), although it is unclear whether this binding is physiologically significant.

Wilce and Parker (1994) showed that 6 residues were conserved in the protein sequence of all known mammalian GSTs: Tyr-7, Pro-53, Asp-57, Ile-68, Gly-145 and Asp-152 (numbering based on the human placental pi class GST), and that the consensus motif [Ser or Thr]-[Arg or Asn]-Ala-Ile-Leu centred around residue 67 appeared to be characteristic of, but not exclusive to, GSTs. While this may be true for most mammalian GSTs sequenced to date, a large proportion of non-mammalian GST sequences contain only a limited subset of these conserved residues, and there appear to be few, if any, residues conserved in all GSTs.

The first 3 dimensional structure of a crystallised GST was elucidated by X-ray diffraction in 1991, and since then a number of other GST structures have been resolved.

Most work has concentrated on mammalian GSTs and has resulted in the crystallisation and structural determination of alpha-class GSTs (human GST A1-1: Cameron *et al.*, 1995, Sinning *et al.*, 1993), pi class GSTs (porcine GST P1-1: Reinemer *et al.*, 1991, Dirr *et al.*, 1994a, human GST P1-1: Reinemer *et al.*, 1992, mouse GST P1-1: García-Sáez *et al.*, 1994) and mu class GSTs (rat GST M1-1: Ji *et al.*, 1992, human GST M2-2: Raghunathan *et al.*, 1994). More recently, crystal structures of some non-mammalian GSTs have been determined, including theta class GSTs from *Schistosoma japonicum* (Lim *et al.*, 1994, McTigue *et al.*, 1995), *Lucilia cuprina* (Wilce *et al.*, 1995) and *Arabidopsis thaliana* (GST AtPm24; Reinemer *et al.*, 1996), and a sigma class GST from squid (Ji *et al.*, 1995). Preliminary X-ray data has also been collected for chicken liver alpha class GST CL 3-3 (Lin *et al.*, 1996), *Proteus mirabilis* GST (Feil *et al.*, 1996), *E. coli* GST (Nishida *et al.*, 1996), and *Schistosoma mansoni* GST (Trottein *et al.*, 1992).

Most GSTs have been co-crystallised with either glutathione or a GST inhibitor, and structural data obtained from such crystals can be used to determine where these co-crystallants bind to the GSTs, providing very good evidence for the location of the active site and the orientation of substrates within this site. Other studies have served to complement this data, for example Cooke *et al.* (1994) used photoaffinity labelling to identify the glutathione binding site of rat and human GSTs and were able to locate potential sites for GSH binding, and relate these to crystal structures defined by X-ray diffraction. Similarly, Colman (1994) used the compound *S*-(4-bromo-2,3-dioxobutyl) glutathione to irreversibly modify rGST M3-3, then digested the enzyme with trypsin and sequenced fragments with the attached glutathione derivative, to show that modification of tyrosine-115 was responsible for inactivating the enzyme and therefore this residue may be located at the active site.

From a combination of X-ray crystallographic and covalent modification studies, along with other methods such as site-directed mutagenesis, it has been possible to determine the structure of GSTs and locate active sites, and from such data suggest mechanisms for GST catalysis.

Structural data has shown that although sequence conservation between GST classes is typically low (usually less than 30% amino acid identity), each class of GST for which a structure exists contains a similar overall pattern of polypeptide folding, with two domains per subunit. The smaller, N-terminal domain consists of a mixture of α-helices and β-sheets, typically forming the structure β-α-β-α-β-β-α, and is highly conserved between GSTs (Dirr *et al.*, 1994b). This domain is also similar in structure (but not sequence) to the glutathione binding domains of thioredoxin and glutaredoxin with almost all the residues interacting with glutathione residing in this domain. The larger, C-terminal domain consists almost entirely of 5 amphipathic α-helices and is much less conserved, and contains most of the residues involved in hydrophobic substrate binding. While the two active sites in a GST dimer are thought to act independently, dimerisation is needed for activity not least because a small part of the glutathione binding site of one subunit is made up of residues from the other subunit (Dirr *et al.*, 1994b).

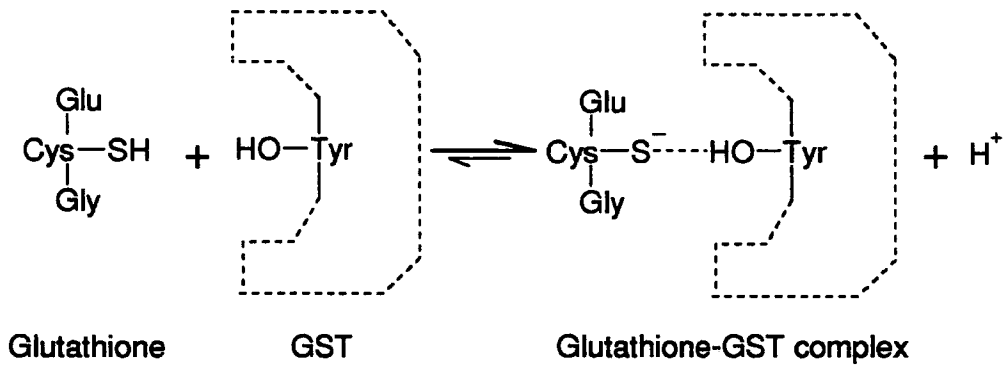


Figure 4. Activation of glutathione by alpha, mu and pi class GSTs.
Ionisation of the sulphydryl group of glutathione is stabilised in part by hydrogen bonding with an active site tyrosine residue, to give the reactive thiolate anion.

The catalytic activity of GSTs is thought to be due in part to their ability to stabilise the thiolate anion of glutathione, which is then able to react with an electrophilic centre in the co-substrate. In alpha, mu and pi class GSTs an active site tyrosine residue, along with help from other nearby residues, seems to serve to stabilise this thiolate anion by forming hydrogen bonds (Figure 4), thus lowering the glutathione pK_a by at least 2 pH units (Dirr *et al.*, 1994b). Plant GSTs and mammalian theta class GSTs do not seem to have a tyrosine residue capable of interacting with glutathione, but other residues are thought to play an equivalent role.

Overview of GST Functions

GSTs detoxify endogenous and exogenous chemicals, with the glutathione conjugates being usually non-toxic and water-soluble, making excretion (in animals) or vacuolar storage (in plants) easier. Rarely, glutathione conjugates have however been shown to be more toxic than the corresponding unconjugated compound (Pickett and Lu, 1989). In mammals glutathione conjugation is the first step in the mercapturic acid pathway in which glutathione conjugates are further metabolised via glutamate and glycine cleavage, and are then acetylated at the amino group on the cysteinyl residue to give a mercapturic acid derivative which can be excreted (Habig *et al.*, 1974). In plants, glutathione conjugation is thought to promote vacuolar sequestration due to the action of an ATP-dependent pump in the vacuolar membrane which specifically transports glutathione conjugates (Martinoia *et al.*, 1993) The glutathione conjugates are then sequentially degraded by a carboxypeptidase, to remove the glycine residue, and a γ-glutamyl transpeptidase to release the *S*-cysteinyl derivative, which can then undergo further metabolism (Lamoureux and Bakke, 1984).

Although much is known about the role of GSTs in conjugating xenobiotics very little is known about the natural functions of GSTs, or what the endogenous substrates, if any, of GSTs are, especially in plants. Endogenous metabolic roles have been suggested for some mammalian GSTs. For example some mammalian GSTs have been shown to be involved in the isomerisation of 3-ketosteroids (Benson *et al.*, 1977) and the synthesis of leukotrienes (Tsuchida *et al.*, 1987). Some GSTs have also been shown to bind with high affinity non-substrate compounds including heme, bilirubin and steroids (Mannervik, 1985). In some cases this binding seems to occur at a site other than the active site. This binding function may be involved in the intracellular transport of tetrapyrroles and led to these GSTs being originally termed ligandins. Koonin *et al.* (1994) showed that the eukaryotic translation elongation factor 1-gamma had significant homology to some GSTs and suggested that GSTs may have a role in the regulation of protein assembly and folding.

In plants, Takahashi *et al.* (1995) determined that the tau class tobacco GST parA was localised in the nucleus, showing that GSTs did not reside solely in the cytosol. Since

the *parA* gene, like other similar GST genes, showed moderate similarity to an *E. coli* protein thought to bind RNA polymerase, Takahashi *et al.* (1995) proposed that parA might function in transcription rather than functioning as a GST. However, there is as yet no published evidence to support this intriguing idea.

A number of GSTs, from a variety of sources, have been shown to have glutathione peroxidase activity towards hydroperoxides such as cumene hydroperoxide and lipid hydroperoxides (Berhane *et al.*, 1994, Zettl *et al.*, 1994, Mosialou *et al.*, 1995). These GSTs may therefore be involved in detoxifying endogenously generated products of oxidative stress.

Mammalian GSTs

GSTs were first purified from rat liver by Fjellstedt *et al.* (1973). Since then many more mammalian GSTs have been purified and characterised.

Location and Function

GSTs are very abundant in the mammalian liver, representing for example approximately 10% of soluble protein in rat liver (Habig and Jakoby, 1981), where they are involved in detoxification of endogenous and xenobiotic substrates. Other mammalian tissues rich in GSTs include the kidney, placenta, erythrocytes, breast, lung and prostate gland (Wilce and Parker, 1994). Different classes of GST are found in different tissues. As well as this spatial regulation, mammalian GST expression can also be regulated by a number of other factors. Xenobiotics can induce some mammalian GSTs, for example phenobarbital has been shown to induce GSTs in rat liver (Pickett *et al.*, 1982). Also some GST classes are also often overexpressed in tumours, where they can detoxify anti-cancer drugs (Coles and Ketterer, 1990).

Plant GSTs

Overview

To date GSTs have been characterised from many plant species, as summarised in Table 1. GST activity has also been detected in other plant species including chickpea (Hunaiti and Ali, 1991), oat (Singh and Shaw, 1988) and various weeds of maize (Anderson and Gronwald, 1991, Hatton *et al.*, 1995) as well as a wide range of lower plants (Pflumacher *et al.*, 1995).

Species	Main references
Maize	Mozer *et al.* (1983), O'Connell *et al.* (1988), Timmerman (1989), Fuerst *et al.* (1993), Irzyk and Fuerst (1993), Holt *et al.* (1995).
Wheat	Williamson and Beverley (1988), Anderson *et al.* (1993), Mauch and Dudler (1993), Edwards and Cole (1996).
Tobacco	Droog *et al.* (1993).
Silene cucubalus	Kutchan and Hochberger (1992).
Carnation	Meyer *et al.* (1991b).
Pea	Diesperger and Sandermann (1979), Edwards (1996).
Rice	Han and Hatzios (1991), Wu *et al.* (1996).
Sugarcane	Singhal *et al.* (1991).
Hyoscyamus muticus	Bilang *et al.* (1993).
Sorghum	Gronwald *et al.* (1987), Dean *et al.* (1990).
Soybean	Flury *et al.* (1995), Skipsey *et al.* (1997), Andrews *et al.* (1997).
Pumpkin	Fujita *et al.* (1995).
Giant foxtail	Hatton *et al.* (1996).
Dwarf pine	Schröder and Rennenberg (1992).
Norway spruce	Schröder and Wolf (1996).

Table 1. Summary of main references for characterisation of GSTs in plant species.

Expression of plant GSTs varies considerably. Some GSTs are constitutively expressed throughout the plant while others are only expressed in certain tissues or at a particular developmental stage. Expression of many plant GSTs can be induced or enhanced by various internal and external factors, such as tissue damage, senescence and infection (Jepson *et al.*, 1994), plant development (Sari-Gorla *et al.*, 1993), responses to plant hormones including auxins and ethylene (Droog *et al.*, 1993), heavy metals (Uotila *et al.*, 1994) and the application of herbicide safeners (see below).

The Role of GSTs in Herbicide Tolerance

Herbicides can be classified based on their structure and mode of action. The most important classes of herbicide of relevance to this work are mentioned below, together with their target sites as described in Kirkwood (1991). Herbicide structures are illustrated in Figure 2.

The triazines, such as atrazine, cyanazine and simazine, are important selective herbicides in maize, and act by inhibiting electron transport in photosystem II. The chloroacetanilides are another class of important herbicides used to control maize weeds, and include alachlor and metolachlor. Their target site is not well understood, but may involve disruption of lipid synthesis. Other classes of herbicide include the diphenyl ethers such as fluorodifen, which inhibit protoporphyrinogen oxidase, the aryloxyphenoxypropionates such as fenoxaprop-ethyl, which inhibit acetyl-CoA carboxylase, and the sulphonylureas such as chlorimuron-ethyl, which inhibit acetolactate synthase, and therefore inhibit branched-chain amino acid biosynthesis.

In general, herbicides are effective due to their phytotoxic effect on crop weeds, while being tolerated by the target crop plant. This implies that the crop plant is more tolerant to the herbicide compared with the controlled weeds, and this tolerance can be due to one or more of a number of potential factors. Such factors include firstly uptake, where tolerance is due to limited uptake of the herbicide, secondly translocation, where the herbicide is moved away from, or prevented from reaching, its target site, thirdly target site insensitivity, where an altered target site reduces herbicide effectiveness by e.g. lowering binding affinity, and lastly metabolism, where the herbicide is detoxified more quickly (or activated more slowly), lowering the plant's exposure to the active herbicide.

In many cases, GSTs are involved in the detoxification of a variety of herbicide classes and are part of a complex detoxification system. This system, which is analogous to that in animals, is typically broken down into three phases; activation (phase 1), conjugation (phase 2) and immobilisation (phase 3). Phase 1 activation adds a reactive functional group to the molecule to be detoxified, to facilitate further metabolism. Activation usually involves reactions such as hydroxylation, oxidation and dealkylation, and is often mediated by cytochrome P450 enzymes. Phase 2 enzymes typically use this new reactive centre for conjugation with hydrophilic groups such as glucosides, oligosaccharides, amino acids and glutathione to the activated molecule. Phase 3 immobilises the resulting compounds, usually either by incorporating them into the cell wall, or by transporting them into the vacuole, where they can be stored or processed further. In animals, phase 3 detoxification involves further metabolism followed by excretion of the target molecule. GSTs are involved in phase 2 of the detoxification process, although in many cases their target molecules are sufficiently reactive to undergo glutathione conjugation without requiring a phase 1 activation step. In plants, glutathione-conjugated molecules are recognised by a specific vacuolar membrane ATP-driven pump which transports the conjugate into the vacuole for further processing (Martinoia *et al.*, 1993) while in animals glutathione conjugates are metabolised and excreted via the mercapturic acid pathway, as described earlier.

Plant GSTs often play an important role in herbicide tolerance, since many herbicides are detoxified by glutathione conjugation. As described above, herbicide tolerance can be due to factors other than detoxification, such as limited uptake into the plant, lack of translocation to the active site and target-site insensitivity, however metabolism of the herbicide to a less toxic product is often the main factor in herbicide tolerance (Cole, 1994). In addition to glutathione conjugation, herbicides may be metabolised by a number of different routes including hydrolysis, glycosylation, oxidation and dealkylation, so the presence of GSTs in a herbicide tolerant plant does not necessarily mean that they are responsible for the tolerance. The action of GSTs have nevertheless been shown to be the major factor in some cases of tolerance to herbicides. For example in maize, detoxification of the herbicides atrazine and related chloro-*s*-triazines, alachlor and other chloroacetanilides is mainly due to conjugation with glutathione, and the susceptibility of maize cultivars to these herbicides is inversely proportional to their GST

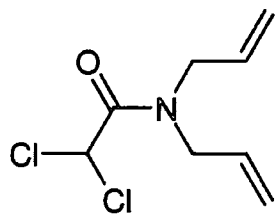
activities (Shimabukuro *et al.*, 1970). Similarly, GST activity towards herbicide substrates in seedlings of maize and competing weeds was found to correlate well with their sensitivity to herbicides (Hatton *et al.*, 1996b).

Some reports have suggested that herbicide conjugation with glutathione in plants *in vivo* does not require GSTs, as some herbicides (e.g. chloroacetanilide herbicides) are sufficiently reactive to conjugate with glutathione spontaneously (Jablonakai and Hatzios, 1993). However, at physiological pH the presence of GSTs considerably increases the rate of detoxification.

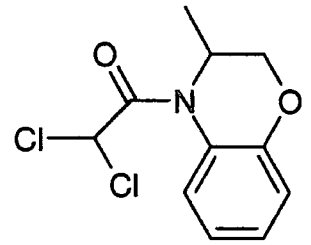
Herbicide Safeners

Herbicide safeners (Figure 5), also referred to as antidotes, are compounds applied with or before herbicide application to differentially increase the tolerance of cereal crops to herbicides while having little effect on competing weeds. They seem to act at least in part by increasing the activity of herbicide-detoxifying enzymes in the crop (Hatzios, 1991, Farago *et al.*, 1994). Several safeners have been shown to increase GST activity towards herbicide substrates in maize and sorghum and this has been shown to be due to *de novo* synthesis of GSTs (Dean *et al.*, 1990). Safeners often also increase the levels of glutathione present, but this is not thought to be a major factor in increasing herbicide metabolism since glutathione levels are rarely limiting in the crop (Farago *et al.*, 1994). Application of low concentrations of some herbicides have also been shown to have a subsequent safening effect by inducing GSTs. For example application of metolachlor has been shown to induce metolachlor-detoxifying GSTs in sorghum (Dean *et al.*, 1990), wheat, pea and maize GST levels were increased by glyphosate application (Uotila *et al.*, 1995) and maize GST activity was enhanced by application of chloroacetanilide herbicides and atrazine (Alla, 1995).

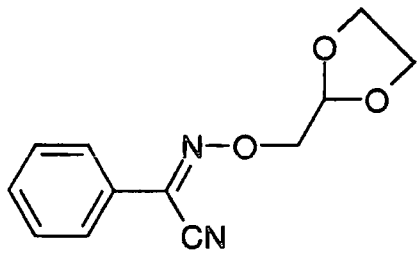
The mechanism of GST induction by safeners is poorly understood. However, recent work has begun to shed some light on the processes involved. One possibility is that



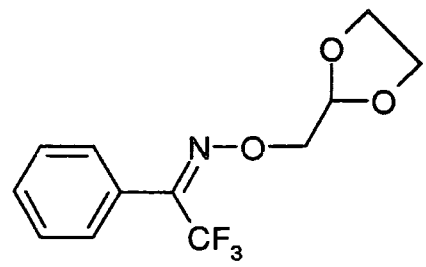
Dichlormid



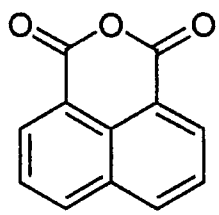
Benoxacor



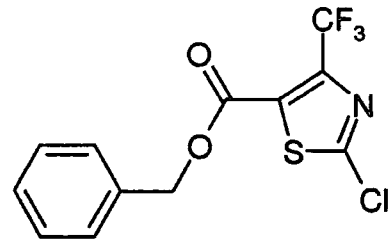
Oxabetrinil



CGA-133205



Naphthalic anhydride



Flurazole

Figure 5. Chemical structures of various herbicide safeners.

safeners such as the dichloroacetamides dichlormid and benoxacor may act as GST substrates, with the resulting glutathione conjugates acting as signals to enhance GST expression. Miller *et al.* (1996) showed that maize suspension cultures conjugated one or two glutathione molecules to the safener benoxacor, and it was then suggested that these conjugates activated GST expression using a currently undetermined signalling pathway. Alternatively, unmodified safeners may be recognised and activate GST induction. Supporting this idea, Walton and Casida (1995) identified a high-affinity dichloroacetamide-binding protein in maize extracts, with affinity for different safeners broadly corresponding to their effectiveness as safeners. Interestingly, this binding protein also recognised chloroacetanilide and thiocarbamate herbicides, providing a partial explanation for the ability of such herbicides to act as safeners.

Wheat GSTs

Despite the high levels of GSTs present in wheat plants (Anderson *et al.*, 1993), these GSTs have not been characterised nearly as well as those of maize. Williamson and Beverley (1988) purified a homodimeric GST from wheat flour with an estimated subunit molecular weight of 27.5 kDa which showed activity towards CDNB and also some glutathione peroxidase activity. No activity could be detected towards ethacrynic acid. Anderson *et al.* (1993) reported that wheat extracts had very high CDNB conjugating activity, with the CDNB-active GSTs accounting for about 4% of the total extractable protein from etiolated wheat shoots. Resolution of GST isoenzymes using DEAE anion exchange chromatography showed three CDNB-conjugating isoenzymes, with the most active isoenzyme being a homodimer with a subunit molecular weight of 26.2 kDa. Seven distinct GST subunits were resolved, with relative molecular masses of between 26.2 kDa and 35.2 kDa.

Dudler *et al.* (1991) cloned a wheat gene corresponding to the *wir5* cDNA isolated as a transcript induced by fungal infection (Schweizer *et al.*, 1989). Sequence analysis of this gene showed it to have high homology and a similar exon pattern to maize theta class GSTs, and this gene was named *GstA1*. Expression of the *GstA1* gene product in *E. coli* gave a 29 kDa polypeptide which had low GST activity towards CDNB, and which was named GST29 (Mauch and Dudler, 1993). An antibody was raised against GST29 and

used to study the induction of this GST. In addition an antiserum reported to have been raised against a combination of maize GST isoenzymes with subunits of 26 kDa and 25 kDa was also used to monitor GST expression. Polypeptides with molecular weights of 25 kDa (GST25) and 26 kDa (GST26) were recognised by the maize antiserum. GST25 and GST26 were strongly induced by cadmium and herbicides such as paraquat, atrazine, alachlor and metolachlor, while GST29 was not induced by these (Mauch and Dudler, 1993). Other workers have shown wheat GSTs to be inducible by cadmium (Uotila *et al.*, 1994) and glyphosate (Uotila *et al.*, 1995).

Herbicide-conjugating GSTs in wheat have not been well studied, although Tal *et al.* (1993) showed that detoxification of the grass herbicide fenoxaprop-ethyl in wheat was due to rapid conjugation with glutathione. This is likely to be partly due to non-enzymic conjugation with high levels of glutathione, but may also be due to the presence of a GST with activity towards this herbicide (Edwards and Cole, 1996). Scarponi *et al.* (1991) showed that wheat possessed glutathione conjugating activity towards a range of chloroacetanilide herbicides including alachlor, metolachlor and acetochlor.

Recent work on wheat GSTs has revealed an increasingly complex array of herbicide-active GSTs, with isoenzymes showing widely differing regulation (Cummins *et al.*, 1998). Wheat GST activities toward the herbicides fenoxaprop, metolachlor and fluorodifen were reported by Edwards and Cole (1996), who also demonstrated that GST activities toward different herbicides were differentially induced by safeners, with different safeners enhancing activity towards different substrates. Riechers *et al.* (1996a) showed that in wheat, the chloroacetamide herbicide dimethanamid was initially detoxified by glutathione conjugation, and this was enhanced by treatment with the safener fluxofenim. GST activity towards dimethanamid increased by application of fluxofenim or cloquintocet-mexyl (Riechers *et al.*, 1996b). A safener-inducible GST from the wheat progenitor *Triticum tauschii* has recently been purified and cloned (Riechers *et al.*, 1997).

Sorghum GSTs

Gronwald *et al.* (1987) examined the effect of the herbicide safeners oxabetrinil (CGA-92194), flurazole, dichlormid and naphthalic anhydride on GST activities in sorghum and

found that these compounds slightly enhanced GST activity towards CDNB but considerably enhanced GST activity towards metolachlor.

Dean *et al.* (1990) treated sorghum with the safeners oxabetrinil, CGA-133205, naphthalic anhydride, flurazole and dichlormid and then analysed the number of GST isoenzymes present using anion exchange chromatography. Treatment with flurazole gave rise to the greatest number of isoenzymes, increasing the number of GSTs with activity towards CDNB or metolachlor from two to seven. Oxabetrinil treatment resulted in a 25-fold increase in GST activity towards metolachlor, and treatment with the other safeners also resulted in significant increases in GST activity towards metolachlor. The safener enhancement of GSTs was prevented by treating the sorghum with cycloheximide, showing that this induction was due to the *de novo* synthesis of GST isoenzymes.

Gullner *et al.* (1995) showed that GST activity in sorghum was induced in response to viral infection, but only in cultivars resistant to the virus. Susceptible cultivars showed a decrease in GST activity following viral infection.

Soybean GSTs

Czarnecka *et al.* (1988) characterised a divergent heat-shock protein encoded by the gene *Gmhsp26-A*. The same gene was further characterised by Ulmasov *et al.* (1995), where it was referred to as *GH2/4*. The *GH2/4* promoter was found to be activated by a wide range of chemical treatments including plant hormones, methyl jasmonate, salicylic acid, cadmium and glutathione. An antibody raised against the *GH2/4* protein showed that the protein was localised in the cytosol and its expression was enhanced by auxin treatment. Sequence analysis showed that GH2/4 had homology with tau class GSTs and expression of recombinant *GH2/4* protein in *E. coli* gave a protein which could be purified using glutathione-agarose and which had GST activity towards CDNB. Recently, the *Gmhsp26-A* / *GH2/4* gene has been renamed *GSTGm1* in the light of its identification as a GST gene (Skipsey *et al.*, 1997), with the corresponding polypeptide being named GST*Gm*1. Skipsey *et al.* (1997) showed that GST*Gm*1 expressed as a recombinant protein in *E. coli* had GST activity towards a wide range of xenobiotics, and also had activity as a glutathione peroxidase. This GST was able to use

homoglutathione, the major thiol found in soybean, as well as glutathione as a co-substrate, and interestingly the specificity of the enzyme towards glutathione and homoglutathione was dependent on the xenobiotic substrate used.

Flury *et al.* (1995) purified a GST from soybean with activity towards CDNB and metolachlor, but which showed no activity towards atrazine, fluorodifen and *trans*-cinnamic acid. The GST was a homodimer of 26 kDa subunits and N-terminal peptide sequence showed that the polypeptide was related to tau-class GSTs. Using antibodies raised against this protein, it was shown that 2,4-D, but not IAA or NAA, enhanced expression of this GST. Using the published N-terminal sequence, a degenerate oligonucleotide probe was synthesised and used to isolate a full-length soybean cDNA, named *GSTS2*, encoding a tau class GST (Andrews *et al.*, 1997). The deduced N-terminal amino acid sequence of the GSTS2, the protein encoded by *GSTS2*, was identical at all residues except one to the sequence published by Flury *et al.* (1995). Purified, recombinant GSTS2 expressed in *E. coli* had GST activity toward a range of xenobiotics and also possessed glutathione peroxidase activity. However, while the GST purified by Flury *et al.* (1995) had no detectable GST activity towards fluorodifen, GSTS2 had high activity towards this substrate, suggesting that the two GSTs may be distinct, despite their nearly identical N-terminal amino acid sequences.

Flury *et al.* (1996) described a further soybean GST which was located in the apoplast and which was only detectable following treatment with 2,3,5-triiodobenzoic acid.

Pea GSTs

Frear and Swanson (1973) showed that pea contains a GST with high specificity for fluorodifen, and later work (Diesperger and Sandermann, 1979) showed that this GST activity towards fluorodifen was catalysed by enzymes with native molecular masses of 47 kDa and 82 kDa. Uotila *et al.* (1994) showed induction of pea GST activity in response to cadmium treatment. A number of pea GST isoenzymes have been purified by *S*-hexylglutathione affinity chromatography (Edwards, 1996). GST activity towards fluorodifen was catalysed by 49.5 kDa and 54 kDa proteins, while GST activity towards CDNB was catalysed by a 48 kDa protein. Analysis of purified GSTs by SDS-PAGE showed the presence of polypeptides of molecular masses 30 kDa, 29 kDa and 27.5 kDa.

Tobacco GSTs

Tobacco GSTs are one of the most extensively studied groups of plant GSTs. However while maize, wheat, soybean and pea GSTs have been studied due to their herbicide detoxifying activity, tobacco GSTs have been of interest mainly because of their interactions with auxins. Indeed, many tobacco GSTs were originally identified as auxin-binding or auxin-responsive proteins with unknown function and their activity as GSTs was not recognised for some time. The first tobacco auxin-regulated gene to be found to encode a GST was *Nt103*, which was originally cloned by Van der Zaal *et al.* (1987). The Nt103 protein was subsequently shown to have GST activity towards CDNB (Droog *et al.*, 1993). Other related genes have since been shown to encode functional GSTs, and genes in this family has been reclassified as type III or tau class GSTs (Droog, 1997). Further tau class GST sequences have been cloned from tobacco including Nt107 and the very similar *parC* gene (Van der Zaal *et al.*, 1987 and Takahashi and Nagata, 1992a respectively), *parA* (Takahashi *et al.*, 1989) and *C-7* (Takahashi and Nagata, 1992a). An additional GST gene, *parB*, has been cloned from tobacco (Takahashi and Nagata, 1992b), which unlike other tobacco GSTs is a theta class GST. Since most of the tobacco GST sequences were identified from screens for auxin-regulated genes, it is unsurprising that in most cases their transcription is induced by auxins. However this is not the case for the *C-7* gene, obtained by screening using *parC* as a probe (Takahashi and Nagata, 1992a), which is constitutively expressed and is not responsive to auxin treatment.

Maize GSTs

Plant GSTs have been most studied in maize due to the early identification of glutathione conjugation as an important mechanism for herbicide detoxification in this crop. The first GST activity determined in a plant was reported in maize by Frear and Swanson (1970), who were investigating atrazine detoxification. Activity towards this herbicide was observed to be largely confined to the foliage with very little activity detected in the roots. Since then a number of different GST isoenzymes have been found in maize with differing activities towards xenobiotic substrates.

A number of herbicide safeners have been shown to enhance the activity of maize GSTs, for example Ekler *et al.* (1993) showed increased acetochlor metabolism by GSTs when maize plants were treated with the safeners dichlormid, BAS-145138, AD-67, DKA-24 and MG-191. Fuerst *et al.* (1993) showed induction of metolachlor-active isoenzymes with the safener benoxacor in maize shoots while Miller *et al.* (1994) showed similar induction in cell suspension cultures. Dean *et al.* (1991) showed that benoxacor treatment increased GST activity towards CDNB, metolachlor and EPTC-sulphoxide, but had no effect on activity towards *trans*-cinnamic acid or atrazine.

Jachetta and Radosevich (1981) reported that the induction of atrazine-conjugating GST activity by pre-treatment with atrazine did not affect the conjugation rates of EPTC, alachlor, propachlor or barban, suggesting that this was due to a different isoenzyme.

Maize GST purification and nomenclature

GSTs in maize can account for up to 1-2% of the total soluble protein in the plant and so maize tissue is a relatively good source of GSTs. Mozer *et al.* (1983) reported that in etiolated leaves CDNB conjugating GST activity was 1.2-fold higher than the activity in green leaves and over 200-fold higher than the activity in *E. coli*. On the basis of enzyme activity per unit of protein, GST activities towards CDNB were found to be 2.5 to 3 times higher in roots than shoots with both activities being increased over 2-fold by treatment with the safener dichlormid. To date four GSTs from maize have been purified and at least partially characterised. These were named GSTs I to IV, based on their order of discovery (GSTs I and II were identified before GSTs III and IV) and their order of elution from an anion exchange column (GST I eluted before GST II).

This nomenclature was adequate in the original descriptions of the GSTs, however its continued use has proved problematical due to the discovery of new GSTs in maize (see later chapters). Therefore a new nomenclature for maize GSTs has been adopted for this work and hopefully future work on maize GSTs. One alternative was to classify the GSTs based on the relative molecular masses (Mr) of the component subunits. However, this could lead to some confusion in view of the similarities in Mrs of the maize GSTs described to date and the discrepancies in the Mr calculated by SDS-PAGE and the actual Mr calculated from the coding sequence. Instead a numbering system based on the

order of discovery of the individual subunits, rather than the isoenzyme dimers, has been adopted. Thus, the 29 kDa subunit is termed *Zm* GST I, the 27 kDa subunit *Zm* GST II and the 26 kDa subunit *Zm* GST III. This renumbering system, which is more flexible and less ambiguous than the existing classification and which will be referred to in the remainder of this work, is summarised for the known GSTs of maize in Table 2.

Old name	New name	Subunit size (kDa)
GST I	*Zm* GST I-I	29, 29
GST II	*Zm* GST I-II	29, 27
GST III	*Zm* GST III-III	26, 26
GST IV	*Zm* GST II-II	27, 27

Table 2. Revised nomenclature for maize GSTs.

GSTs from maize were first purified to homogeneity by Mozer *et al.* (1983), who purified two GSTs from safener treated, etiolated tissue which he named GST I and GST II (*Zm* GST I-I and *Zm* GST I-II in the revised nomenclature). Crude extracts were fractionated by ammonium sulphate precipitation and GSTs (assayed by conjugation with CDNB or alachlor) were resolved by DEAE-Sepharose ion exchange chromatography. Finally the isoenzymes were purified by glutathione-bromosulphophthalein affinity chromatography.

Similar methods have since been used by others to purify maize GSTs, for example:

Irzyk and Fuerst (1993) used shoots from etiolated, safener treated maize seedlings. Purification of *Zm* GST II-II was achieved using ammonium sulphate precipitation, mono Q-Sepharose and mono Q Superose ion exchange chromatography and *S*-hexylglutathione affinity chromatography.

Asaoka (1984) developed a purification procedure for bovine liver GSTs using the triazine dye Orange A as an affinity ligand. This procedure has since been used for maize GSTs (Timmerman and Tu, 1987) where GSTs containing *Zm* GST I and *Zm* GST III subunits bound to the column and eluted with glutathione while atrazine-conjugating

GSTs did not bind. Orange A chromatography has also been used to purify maize GSTs expressed in recombinant bacteria (Grove *et al.*, 1988).

Zm GST I-I

Zm GST I-I was first purified by Mozer *et al.* (1983) who showed that this enzyme was a homodimer of 29 kDa subunits, had high activity towards CDNB and some activity towards chloroacetanilide herbicides such as alachlor. It was also shown that *Zm* GST I-I was constitutively present in etiolated maize seedlings, but was also slightly induced by the application of herbicide safeners.

Wiegand *et al.* (1986) used a partial amino acid sequence from *Zm* GST I-I to design oligonucleotide probes and isolate a *Zm* GST I cDNA clone, the first plant GST cDNA to be cloned. The cDNA was sequenced and showed no obvious similarity to animal GSTs. Expression of the cDNA in *E. coli* gave rise to a recombinant GST with CDNB conjugating activity, confirming that the cDNA encoded an authentic GST. This cDNA clone was then used to isolate a *Zm* GST I genomic clone (Shah *et al.*, 1986). The *Zm* GST I gene was found to contain two introns of 760 bp. and 669 bp., both of which occurred towards the 5′ end of the coding region, corresponding to the N-terminal domain of the *Zm* GST I polypeptide. Southern blotting gave results consistent with the *Zm* GST I gene being present as a single copy in the inbred line Missouri 17 and as two copies in the hybrid Pioneer 3780A.

Grove *et al.* (1988) also sequenced and expressed a cDNA apparently encoding *Zm* GST I in *E. coli*; their sequence was nearly identical to that of Wiegand *et al.* (1986) and had the same deduced amino acid sequence. Expression of the coding sequence in *E. coli* using the pKK233-2 expression vector gave rise to a recombinant protein with CDNB activity. A further *Zm* GST I sequence was also expressed which was truncated at the amino terminus by three amino acids. This protein retained some activity towards CDNB indicating that these three truncated amino acids were not essential for activity.

Wosnick *et al.* (1989) used the published sequences for *Zm* GST I to chemically synthesise a *Zm* GST I cDNA and used this to express the GST product in *E. coli*, where it was reported to catalyse the conjugation of atrazine. However, the specific activity of

the recombinant *Zm* GST I-I towards various herbicide substrates was not reported in detail.

Zm GST I-II

Zm GST I-II was first purified as "GST II" by Mozer *et al.* (1983) who could only show its presence in the foliage of maize plants treated with herbicide safeners, indicating that this isoenzyme was not constitutively present but was safener-inducible. SDS-PAGE of the purified enzyme showed the presence of two major polypeptides with estimated molecular weights of 29 kDa and 27 kDa. Partial amino-terminal sequencing of the 29 kDa band identified it as the same subunit as that found in *Zm* GST I-I. However, the 27 kDa subunit gave no N-terminal sequence. The relative molecular mass of native *Zm* GST I-II was estimated to be about 50 kDa indicating that *Zm* GST I-II exists as a heterodimer of 29 kDa and 27 kDa subunits. Mozer *et al.* (1983) reported that *Zm* GST I-II had about a third the activity of *Zm* GST I-I towards CDNB and had 1.7-fold higher alachlor conjugating activity. GST assays were also performed on purified *Zm* GST I-II by Holt *et al.* (1995). This work reported that when compared to *Zm* GST I-I activity, *Zm* GST I-II had about half the GST activity towards CDNB, seven times the GST activity towards alachlor, higher activity towards fluorodifen and no detectable activity towards atrazine.

Following the initial characterisation of *Zm* GST I-II (Mozer *et al.*, 1983), Moore *et al.* (1986) suggested that "GST II" was a homodimer of 27 kDa subunits and Timmerman (1989) postulated that contamination of "GST II" with *Zm* GST I-I had led Mozer *et al.* (1983) to believe it was a heterodimer. However Holt *et al.* (1995) purified both *Zm* GST I-I and *Zm* GST I-II and confirmed that *Zm* GST I-II was indeed a heterodimer of 27 kDa (*Zm* GST II) and 29 kDa (*Zm* GST I) subunits, and obtained internal amino acid sequence for the *Zm* GST II subunit following controlled proteolysis. Antiserum raised against the 27 kDa subunit of *Zm* GST I-II was used to immunoscreen a maize root cDNA library, resulting in the isolation of *Zm* GST II clones (Jepson *et al.*, 1994).

Zm GST III-III

Zm GST III-III was originally named GST III and was first described from molecular studies (Moore *et al.*, 1986). In these studies an oligonucleotide probe was prepared

based on the protein sequence of the amino terminus of the *Zm* GST III subunit. However, the origins of this subunit and its purification have never been adequately reported. The oligonucleotide probe was then used to identify the corresponding cDNA in a library prepared from safener-treated, etiolated maize tissue. This cDNA was expressed in *E. coli* and was found to encode a subunit of 26 kDa which formed homodimers; this recombinant *Zm* GST III-III had higher activity than *Zm* GST I-I towards alachlor, though again these activities were not reported in detail.

A GST described as containing a *Zm* GST III subunit was purified by Timmerman and Tu (1987) using Orange A dye affinity chromatography. When the affinity-purified preparation was analysed by anion exchange chromatography, two overlapping peaks were present. The first peak was *Zm* GST I-I while the second peak contained *Zm* GST I and *Zm* GST III subunits. The authors initially proposed that the two subunits were present as a heterodimer (*Zm* GST I-III) but in later work (Timmerman, 1989) postulated that the second peak was a homodimer (*Zm* GST III-III) contaminated with *Zm* GST I-I.

Grove *et al.* (1988) also cloned, sequenced and expressed a *Zm* GST III subunit (GST-26), but found 8 reading frame shifts when compared to the previously published sequence (Moore *et al.*, 1986). The resulting deduced amino acid sequence however showed considerably more homology to mammalian GSTs than did the *Zm* GST III sequence described by Moore *et al.* (1986), and was therefore assumed to more likely be correct.

Bieseler *et al.* (1996) filed a patent on another *Zm* GST III sequence obtained from maize cultivar Mutin, and described its use in transformed plants in detoxifying the herbicide 5-trifluoromethyl-1,3,4-thiadiazol-2-yl-oxy-acetic acid *N*-isopropyl-*N*-(4-fluorophenyl) amide. Compared to previously published *Zm* GST III sequences (Moore *et al.*, 1986, Grove *et al.*, 1988), this new sequence (GST IIIc) differed from both the previous published sequences, having frame-shifts with respect to both sequences. This new sequence gave a deduced protein sequence having higher homology to other GST sequences than those determined in previous characterisations of *Zm* GST III.

```
Moore   MAPLKLYGMPLSPNVVRVATVLNEKGLDFEIVPVDLTTGAHKQPDFLALN
Grove   MAPLKLYGMPLSPNVVRVATVLNEKGLDFEIVPVDLTTGAHKQPDFLALN
Bayer   MAPLKLYGMPLSPNVVRVATVLNEKGLDFEIVPVDLTTGAHKQPDFLALN
        **************************************************

Moore   PFGQIPALVDGDEVLFESRAINRYIASKYASEGTDLLPATASAAKLEVWL
Grove   PFGQIPALVDGDEVLFESRAINRYIASKYASEGTDLLPATASAAKLEVWL
Bayer   PFGQIPALVDGDEVLFESRAINRYIASKYASEGTDLLPATASAAKLEVWL
        **************************************************

Moore   EVESHHFYPNRVAAGVPAAREAAPGRRPDAAVVDKHAEQLAKVLDVYERT
Grove   EVESHHFHPNASPLVFQLLVRPLLGGAPDAAVVEKHAEQLAKVLDVYEAH
Bayer   EVESHHFHPNASPLVFQLLVRPLLGGAPDAAVVEKHAEQLAKVLDVYEAH
        ******* **               *  ****** **************

Moore   LARNKYLAGD-EFTLADANHASYLLYLS-KTPKAGLVAARPHVKAWWEAI
Grove   LARNKYLAGD-EFTLADANHALLPALTSARPPRPGCVAARPHVKAWWEAI
Bayer   SPATSTSPGTSSRSPTPTTR-SYLLYLS-KTPRP-ARRRPPHVKAWWEAI
                *                   *   *         **********

Moore   VARPAFQKTVAAIPLPPPPSSSA
Grove   AARPAFQKTVAAIPLPPPPSSSA
Bayer   AARPAFQKTVAAIPLPPPPSSSA
         **********************
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Figure 6. Multiple alignment of published *Zm* GST III amino acid sequences.

Moore = *Zm* GST III sequence from Moore *et al.* (1986).

Grove = *Zm* GST III sequence from Grove *et al.* (1988).

Bayer = *Zm* GST III sequence from Bayer patent (Bieseler *et al.*, 1996).

Asterisks represent residues identical in all three sequences.

A multiple alignment of *Zm* GST III amino acid sequences deduced from the published *Zm* GST III cDNA sequences described above (Figure 6) clearly illustrates the problems with published *Zm* GST III cDNA sequences. Only 74% of amino acid residues are conserved between all three sequences, with the identity of the remaining 58 residues being ambiguous. While sequences for the N-terminal half of the polypeptide are in good agreement, frame-shifts present in the cDNA sequences of at least two of the *Zm* GST III sequences result in long regions of the C-terminal half of the polypeptide for which no identity between deduced amino acid sequences exists.

The expression of GSTs containing the *Zm* GST III subunit has not been studied in detail. A GST consisting of a homodimer of 26 kDa subunits, which were described as *Zm* GST III subunits, was purified by O'Connell *et al.* (1988) who showed it to be

constitutively expressed and responsible for more than 80% of chloroacetanilide herbicide conjugation in the maize cultivar Pioneer 3320. No specific activities were reported for the purified enzyme, although apparent K_m values using alachlor or metolachlor as substrate were calculated, showing that the purified enzyme had a five-fold lower K_m for alachlor (1.69 mM) than for metolachlor (8.9 mM).

Zm GST II-II

Zm GST II-II (originally termed GST IV) was identified by Fuerst *et al.* (1993) as being distinct from previously identified GSTs. Irzyk and Fuerst (1993) supported this by partial amino acid sequencing, and went on to purify and characterise *Zm* GST II-II. Purification was achieved by anion exchange chromatography followed by *S*-hexylglutathione agarose affinity chromatography. They found *Zm* GST II-II to be induced by safener (benoxacor) treatment and composed of two 27 kDa subunits. The isoenzyme actively detoxified metolachlor (with a pH optimum between pH 7 and pH 8), had low activity towards atrazine and no activity towards CDNB or *trans*-cinnamic acid. The enzyme had an isoelectric point of pI 5.75. More recent work by the same group resulted in the isolation of the corresponding cDNA clone for the *Zm* GST II subunit (Irzyk *et al.*, 1995) indicating that the subunit is a 223 amino acid peptide with a molecular weight of 24.6 kDa. The sequence had 100% homology with the 27 kDa subunit of *Zm* GST I-II identified by Bridges *et al.* (1993) and Jepson *et al.* (1994), confirming that the 27 kDa subunit of *Zm* GST I-II was the same as the subunits of *Zm* GST II-II.

N-terminal sequencing of the *Zm* GST II subunit has been tried by a number of groups (Mozer *et al.*, 1983, Holt *et al.*, 1995, Irzyk and Fuerst, 1993) but has always failed, probably due to blocking at the N-terminus. However the identity of the DNA sequence encoding the *Zm* GST II subunit has been unambiguously confirmed by sequencing of internal peptide fragments released by controlled proteolysis (Holt *et al.*, 1995).

Atrazine-active GSTs

In contrast to *Zm* GST subunits I, II and III little is known about the atrazine-conjugating GSTs known to be present in maize.

Grogan *et al.* (1963) described two sibling strains of maize, one of which was tolerant to atrazine (GT112 RfRf) and the other which was susceptible (GT112). Scott and Grogan (1969) subsequently showed that this atrazine susceptibility of cv. GT112 was due to a single recessive gene located on the long arm of chromosome 8. The tolerance of maize to atrazine and other chloro-*s*-triazine herbicides was initially attributed to detoxification by 2-hydroxylation and *N*-dealkylation but Shimabukuro *et al.* (1970) showed that in maize shoots, but not maize roots, conjugation with glutathione was the major atrazine detoxification pathway. Further, Shimabukuro *et al.* (1971) showed that atrazine-glutathione conjugating activity in GT112 RfRf was approximately 50-fold higher than in GT112, showing that the atrazine susceptibility of strain GT112 was due to very low levels of atrazine-active GSTs.

While there is good evidence for the involvement of atrazine-detoxifying GSTs in maize, these enzymes have yet to be purified and characterised, despite numerous attempts. An atrazine-active GST was first partially purified by Frear and Swanson (1970) who used fractionation by ammonium sulphate precipitation followed by gel filtration to purify an atrazine-active GST 7.6-fold. They found that while extracts from maize leaves had good atrazine-conjugating activity, roots contained very little activity, in agreement with results obtained by Shimabukuro *et al.* (1970). The partially purified GST was reported to have a pH optimum of between 6.6 and 6.8. Guddewar and Dauterman (1979) purified an atrazine-active GST 43-fold and showed that this enzyme had a native molecular weight of about 45 kDa and was strongly inhibited by quinones. They also found a pH optimum for their atrazine conjugation assay as pH 8.0-8.5, substantially higher than that obtained by Frear and Swanson (1970). By using native PAGE the partially purified enzyme could be resolved into two atrazine-conjugating bands.

Timmerman and Tu (1987) found that atrazine-conjugating GST activity was constitutively present in shoots of maize plants, and partially purified two atrazine-active GSTs which had low activity towards CDNB. These enzymes did not bind to an Orange A affinity matrix and were found to be absent in the atrazine-susceptible GT112 line. Antibodies raised against *Zm* GST subunits I and III did not cross-react with the atrazine GSTs, confirming that these atrazine-active GSTs were distinct from previously

characterised isoenzymes. However, no further characterisation of these atrazine-active GSTs has been reported.

Mozer *et al.* (1983) assumed that the *Zm* GST I-I they purified was the same as the GST with activity towards atrazine purified by Guddewar and Dauterman (1979) and studied by Shimabukuro *et al.* (1971). This is possible as *Zm* GST I-I has been shown to possess some atrazine-conjugating activity (Wiegand *et al.*, 1986). However it seems unlikely that *Zm* GST I-I was responsible for the atrazine-conjugating activity studied by Shimabukuro *et al.* (1971), as *Zm* GST I-I is present at high levels in maize roots, but this tissue has very much lower atrazine-conjugating activity than found in maize leaves. Dean *et al.* (1991) resolved three GSTs with activity towards atrazine in etiolated maize shoots, appearing distinct from other GST activities, and *Zm* GST I-I has been purified from the atrazine-GST deficient line GT112. No detectable GST activity towards atrazine could be found in suspension cell cultures while GST activities toward chloroacetanilide herbicides and CDNB were present (Edwards and Owen, 1986), again suggesting that the major atrazine-conjugating GST was distinct from other maize GSTs. However, it is possible that the GSTs partially purified by Guddewar and Dauterman (1979) corresponded to *Zm* GST I-I and *Zm* GST I-II, which possess low atrazine-conjugating activity, rather than the distinct atrazine-conjugating isoenzymes studied by other groups.

Other GSTs

The enzymes responsible for glutathione conjugation of *trans*-cinnamic acid have been extensively studied in maize and it has been assumed until recently that these enzymes are GSTs. Dean *et al.* (1991) showed GST activity towards *trans*-cinnamic acid which was distinct from other previously identified isoenzymes. This activity was found in both microsomal and cytosolic fractions of etiolated maize shoots. Edwards and Owen (1986) also found GST activity towards *trans*-cinnamic acid in maize cell suspension cultures, but only found activity in microsomal fractions. The *trans*-cinnamic acid GST apparently required a low molecular weight activator (Dean and Machota, 1993) and could be activated after partial purification by addition of 7-hydroxycoumarin (up to 8.6-fold activation) or *p*-coumaric acid (up to 4.2-fold activation). Activation did not seem to involve covalent binding. Adding such an activator during purification enabled two

further GST isoenzymes with activity towards *trans*-cinnamic acid to be resolved. 0.05mM caffeic acid or hydroquinone completely inhibited *trans*-cinnamic acid conjugating activity, but had little effect on CDNB conjugating activity. Unusually, the enzyme catalysing *trans*-cinnamic acid conjugation appeared to be a 30 kDa monomer and was able to use cysteine as a co-substrate instead of glutathione (Dean *et al.*, 1995), while all other characterised GSTs are dimers and are very specific for glutathione. However, recent work by Dean and Devarenne (1997) showed that the enzyme responsible for the conjugation of *trans*-cinnamic acid to glutathione, while superficially acting like a GST, was instead an ascorbate peroxidase, perhaps acting by generating thiyl free radicals of glutathione able to spontaneously react with *trans*-cinnamic acid.

A further maize GST was identified by Marrs *et al.* (1995), who identified a gene (*Bronze-2*) involved in anthocyanin biosynthesis in maize, and went on to demonstrate that the product of this gene had GST activity, calculating that this enzyme accounted for up to 50% of the CDNB conjugating GST activity in 8-week old maize plants. They proposed that this GST catalysed the conjugation of glutathione with the pigment cyanidin-3-glucoside and that this conjugate is then transported into the vacuole, mediated by an ATP-dependent glutathione pump. If this is the case then this is the first maize GST with a known, endogenous function. However, the Bronze-2 protein has not been purified from maize or characterised in detail. The Bronze-2 GST amino acid sequence showed some homology with previously characterised maize GST isoenzymes, especially at the N-terminal end which is thought to be involved in glutathione binding, and was most similar to tau class plant GSTs.

In summary, three maize GST subunits (*Zm* GST I, *Zm* GST II and *Zm* GST III) have been relatively well characterised, and have been shown to associate to form the dimers *Zm* GST I-I, *Zm* GST I-II, *Zm* GST II-II and *Zm* GST III-III, each having differing activities and expression. cDNA clones corresponding to each of these three subunits have been cloned, along with the *Bronze-2* GST gene implicated in pigment synthesis. Other GSTs are known to be present in maize including atrazine conjugating isoenzymes, but these have yet to be characterised, while *trans*-cinnamic acid-conjugating activity, originally thought to be catalysed by a GST, has been shown to be catalysed by the unrelated enzyme ascorbate peroxidase.

Location and Expression of Maize GST Subunits

Zm GST I was shown to be constitutively expressed in maize roots and shoots (Fuerst *et al.*, 1993, Mozer *et al.*, 1983) and cell suspension cultures (Edwards and Owen, 1986, Miller *et al.*, 1994). Mozer *et al.* (1983) also reported that expression of this subunit was slightly increased following treatment with safener. Northern blot analysis showed that *Zm* GST I mRNA levels in etiolated tissue increased three to four fold following treatment of seedlings with dichlormid (Wiegand *et al.*, 1986). However, subsequent northern blot analyses (Jepson *et al.*, 1994) showed that *Zm* GST I mRNA was constitutively present in root, leaf, stem, endosperm, tassel and pollen samples but was not enhanced in roots by dichlormid treatment, although levels in tassels were slightly increased by dichlormid treatment.

Zm GST II was shown to be constitutively expressed at low levels in roots (Holt *et al.*, 1995) but not in shoots, and its expression was greatly enhanced in both tissues by safener treatment (Holt *et al.*, 1995, Mozer *et al.*, 1983). Edwards and Owen (1986) showed the presence of *Zm* GST I-II in cell suspension cultures indicating the constitutive expression of *Zm* GST II in this tissue. Northern blotting (Jepson *et al.*, 1994) showed that in untreated plants, *Zm* GST II mRNA was not detectable in any tissue tested except for seedling roots. However after treatment with dichlormid, *Zm* GST II mRNA was detectable in roots, leaves, silks, endosperm and embryos, illustrating the inducible expression of this GST subunit. Similar results were obtained when levels of the *Zm* GST II polypeptide in a range of tissues were analysed using western blotting (Holt *et al.*, 1995). Treatment of seedlings with wounding, salicylic acid, and ethylene did not affect levels of *Zm* GST II mRNA while treatment with 2,4-D and IAA led to a slight increase in *Zm* GST II mRNA abundance (Jepson *et al.*, 1994).

Zm GST III was found to be constitutively expressed in maize plants and cell suspension cultures (Miller *et al.*, 1994).

Project Aims and Objectives

The primary objective of the project was to identify the full complement of genes encoding GSTs in maize and to express the corresponding enzymes in recombinant bacteria with the intention of determining their substrate specificities. It was also intended to study the regulation of individual gene products.

To achieve this, the major aims of the project were to:

1. Purify and characterise both previously characterised and novel GST isoenzymes from maize.
2. Isolate cDNA clones for the subunits of the purified isoenzymes using PCR and library screening.
3. Express isolated GST clones in bacteria to produce recombinant isoenzymes in large quantities.
4. Assay recombinant GSTs for activity towards potential substrates, in particular herbicides and analogues of potential endogenous substrates.
5. Determine patterns of expression and regulation of GST subunits in maize plants.

2. Materials and Methods

Plant Material

Two cultivars of maize were used during the project. Cultivar Artus (Sharp International, Avonmouth, UK) was used for initial studies of GST activities in crude protein extracts and the development of protein purification methods. Cultivar Pioneer 3394 (Pioneer Seeds UK Ltd., Hartford, Northwich, UK) was used for later work, including enzyme purification and most molecular cloning work. For safener treatment experiments, dichlormid was dissolved in acetone to give a 5 mg/ml stock solution. Seeds were then imbibed in water containing 0.2% v/v of this dichlormid solution (giving a final concentration of 10 µg/ml dichlormid) for 1 h prior to planting in vermiculite moistened with, and subsequently watered as required with, water containing 0.1% v/v dichlormid stock solution (giving 5 µg/ml dichlormid). Control plants were imbibed in 0.2% v/v acetone for 1 h and watered with 0.1% acetone in tap water. If safener treatment was not required seeds were imbibed and seedlings watered as required with tap water only. Seedlings were grown in moist vermiculite in seed trays, while plants for studies requiring older plants were grown in pots in Levington multi-purpose potting compost. Light grown plants were grown in environmental growth chambers at 25 °C with a 16 h photoperiod and light intensity of 140 µmol/m^2/s while dark grown plants were grown in a light-proof cupboard in laboratory conditions. When required, older plants were grown in the greenhouses at Rhône-Poulenc Agriculture Ltd., Ongar, with supplementary lighting to give a 16 h photoperiod.

GST Induction in Maize Plants by Chemicals and Heat-Shock

For enzyme induction studies, 11-day old light-grown maize seedlings were carefully up-rooted and washed free of vermiculite. Plants were then divided into groups of two plants and duplicate groups exposed to chemicals by immersing their roots in treatment solution. For heat-shock treatments, maize seedlings treated with 1% v/v ethanol were placed in an incubator at 41 °C for 3 h and then harvested. For treatment with glutathione the thiol was made up to 5 mM after adjusting to pH 7, and 1% v/v ethanol

added. Treatment solutions of CDNB (1 mM), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mM) and α-naphthaleneacetic acid (NAA, 0.1 mM) were prepared by a 100-fold dilution of the respective stock solutions prepared in ethanol, while dichlormid (0.1 mM) fluorodifen (0.1 mM), alachlor (0.1 mM) and atrazine (0.1 mM) solutions were made by diluting stock solutions prepared in acetone 200-fold. Control solutions consisted either of 1% v/v ethanol or 0.5% v/v acetone. After incubation in the growth room for 24 h, the seedlings were separated into roots and shoots and homogenised in 3 v/w 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 14 mM β-mercaptoethanol in the presence of 5% w/v polyvinylpolypyrrolidone. After allowing to stand for 10 min on ice, the extracts were centrifuged (12,000 *g*, 20 min, 4 °C) and the protein concentrations of the resultant supernatants normalised to the concentration of the most dilute sample prior to analysis by SDS-PAGE and western blotting.

Maize Developmental Studies

For developmental studies, maize plants were grown in the greenhouse and harvested at 7, 13, 17, 24, 32 and 38 days after sowing by separating into roots and shoots. Mature 11 week old plants were divided into samples of stem, tassels (post-anthesis), silks (stigmas) and immature kernels. Protein was then extracted as detailed for the chemical induction study and precipitated by adding ammonium sulphate to 80% saturation. After centrifugation (13,000 *g*, 20 min, 4 °C) the resulting protein pellets were dissolved in a minimal volume of 2 mM potassium phosphate buffer pH 6.8, 2 mM DTT, and dialysed for 16 h against the same buffer. The protein contents were then normalised and assayed for GST activity, and analysed by SDS-PAGE and western blotting.

Chemicals

Analytical grade (99% purity) samples of atrazine, cyanazine, simazine, alachlor, metolachlor and dichlormid were supplied by Rhône-Poulenc Agriculture Ltd. (Ongar, Essex, UK). Analytical grade (99% purity) chlorimuron-ethyl and fluorodifen were obtained from Greyhound Chem Service Inc. (Birkenhead, UK). All other chemicals except those described below were obtained from BDH or the Sigma-Aldrich Co. Ltd.

Fenoxaprop had previously been prepared from fenoxaprop-ethyl supplied by Greyhound by mild chemical hydrolysis as described by Edwards and Cole (1996).

S-Hexylglutathione synthesis

S-hexylglutathione was synthesised as described in Mannervik and Guthenberg (1981). 3 g of glutathione was dissolved in 10 ml of distilled water. After the addition of 10 ml of 2 M NaOH, ethanol was added until the cloud point was reached. 2 g of iodohexane was added slowly with stirring and the solution allowed to stand overnight at room temperature to allow the reaction to proceed. 47% HI was then added until the pH of the solution dropped to 3.5, and the resultant suspension of precipitated *S*-hexylglutathione was stored at 4 °C overnight. The precipitate was collected by centrifugation, washed with cold water and dried over silica gel in a vacuum desiccator.

Preparation of *S*-Hexylglutathione-Sepharose

S-Hexylglutathione-Sepharose was prepared following the method of Mannervik and Guthenberg (1981). 2 g of epoxy-activated Sepharose CL-6B was added to 20 ml of distilled water and allowed to swell for 25 min. This was then washed on a sintered glass funnel with 200 ml of distilled water. 0.2 g of *S*-hexylglutathione was dissolved in a small volume of distilled water and adjusted to pH 12 with 5 M NaOH. This was added to the washed Sepharose and mixed gently for 16 h at room temperature to allow the *S*-hexylglutathione to covalently bind with the activated Sepharose. The Sepharose was then washed twice with 200 ml of distilled water as before. Any unreacted groups on the Sepharose gel were blocked by incubation with 10 ml of 2 M ethanolamine for 4 h at 30 ºC. The Sepharose was washed a further two times with distilled water as before and then washed three times each with 20 ml lots of 0.1 M sodium acetate pH 4.0 containing 0.5 M NaCl, and then 0.1 M Tris-HCl pH 8.0. Finally the gel was washed with 50 mM Tris-HCl pH 8.0 containing 0.02% w/v sodium azide and stored at -4 °C. The resultant gel, with an approximate volume of 6 ml, was then packed into a 10 mm diameter chromatography column for affinity chromatography.

Preparation of Glutathione-Sepharose

Glutathione Sepharose was prepared following the method of Simons and Vander Jagt (1981). 2 g of epoxy-activated Sepharose CL-6B was added to 20 ml of distilled water and allowed to swell for 25 min. The resultant gel was washed on a sintered glass funnel with 200 ml of distilled water, then washed with 44 mM potassium phosphate buffer, pH 7.0, resuspended in 10 ml of the same buffer and transferred to a 25 ml flask. Nitrogen was bubbled through the gel suspension for 5 min to purge any dissolved oxygen before 6 ml of 100 mM glutathione, adjusted to pH 7.0 with 1 M NaOH, was added. The gel suspension was then incubated at 37 °C with gentle shaking for 24 h to allow the glutathione to react with the activated Sepharose. The reacted gel was washed with 100 ml of water, then incubated with 1 M ethanolamine for 4 h at 30 °C to block any unreacted groups on the Sepharose, before being washed first with 100 ml 0.1 M sodium acetate pH 4.0 containing 0.5 M KCl, next with 100 ml 0.1 M sodium borate pH 8.0 containing 0.5 M KCl, and finally with 10 mM potassium phosphate pH 7.4. The coupled Sepharose was then packed into a 10 mm diameter chromatography column for affinity chromatography.

Herbicide-glutathione conjugate synthesis

Glutathione conjugates of herbicides were chemically synthesised using two different methods, depending on the water solubility of each herbicide. For the relatively insoluble triazines and chlorimuron-ethyl, trimethylamino salts were synthesised by bubbling trimethylamine through acetone until the solvent was saturated then adding this to the herbicide dissolved in a minimal volume of acetone, and leaving to stand for 3 days (Crayford and Hutson, 1972). The supernatant was then poured off to leave crystals of the trimethylamine salt, which was then dissolved in 0.1 M Tris-HCl buffer (pH 8.8) and reacted with glutathione to form the conjugate. Conjugates of the remaining herbicides were made using a reaction mixture of 10 mM glutathione and 0.5 mg/ml herbicide in 0.1 M Tris-HCl buffer at pH 8.8 and incubating this at 30 °C for 24 h. The resulting herbicide-glutathione conjugate preparations were analysed using HPLC as described later.

GST Enzyme Assays

For each of the following GST assays a stock solution of glutathione (usually 100 mM), was made up in distilled water and the pH adjusted to 7 using 1 M NaOH. Glutathione solutions were stored at -20 °C to minimise oxidation, and thawed solutions were discarded at the end of each day for the same reason.

All spectrophotometric assays were performed using an ATI Unicam UV2 dual-beam spectrophotometer. Reactions measured over 2 min or less were monitored continuously while slower reactions were monitored periodically. Absorbance measurements at wavelengths below 290 nm were performed using quartz cuvettes (1 cm path-length), while at higher wavelengths plastic cuvettes (1 cm path-length, Greiner Labortechnik Ltd., Gloucestershire, UK) were used.

Spectrophotometric Assays with General GST Substrates

Assays with the GST substrates 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA), *p*-nitrobenzyl chloride (NBC), *p*-nitrophenethyl bromide (NPB), sodium salt of bromosulphophthalein (BSP), 1,2-epoxy-3-(*p*-nitrophenoxy) propane (ENPP) and *trans*-4-phenyl-3-buten-2-one (PBO) were as described in Habig *et al.* (1974), with minor modifications. Substrates (Figure 3) were made up in ethanol (or distilled water for BSP) at forty times the concentration used in the assay. Each assay consisted of 900 µl 0.1 M potassium phosphate buffer (pH 6.5), 25 µl substrate, 50 µl 100 mM glutathione (final assay concentration 5 mM) and 25 µl enzyme solution. Assays using PBO used 50 µl 5 mM glutathione (final assay concentration 0.25 mM) to reduce the non-enzymic rate of conjugation. Assays were carried out at 30 °C and activity was measured by following the change in absorbance of the reaction mixture at the appropriate wavelength. The specific conditions for each assay are summarised in Table 3. Assay times varied depending on the reactivity of the substrate, however in each case absorbance readings were taken at multiple time points to ensure that the reaction rate remained linear over the course of the experiment. For each substrate, control incubations in which either enzyme, GSH or colorimetric substrate were omitted were also performed.

Substrate	Concentration (mM)	Assay wavelength (nm)	Extinction coefficient $\Delta\varepsilon$ ($mM^{-1}.cm^{-1}$)
CDNB	1	340	9.6
DCNB	1	345	8.5
EA	0.2	270	5
ENPP	5	360	0.5
PBO	0.05	290	-24.8
NBC	0.5	310	1.9
BSP	0.03	330	4.5
NPB	0.1	310	1.2

Table 3. Conditions for GST assays with colorimetric substrates, modified from Habig *et al.* (1974).

GST Activity assayed with Benzyl Isothiocyanate

The assay for the GST-mediated conversion of benzyl isothiocyanate (BITC) to the dithiocarbamate conjugate was taken from Kolm *et al.* (1995). Each assay consisted of 950 µl 0.1 M potassium phosphate buffer, pH 6.5, 10 µl 16 mM BITC, 25 µl enzyme solution and 10 µl 100 mM glutathione. To minimise irreversible inhibition of GSTs by BITC, the substrate was added to the reaction last and the reaction immediately transferred after mixing to the spectrophotometer. The reaction was followed by monitoring the increase in absorbance at 274 nm over 30 s and the activity calculated using an extinction coefficient for the reaction of 9.25 $mM^{-1}.cm^{-1}$. BITC was stored as a 16 mM stock in anhydrous acetonitrile and mixed thoroughly prior to dispensing. To correct for the significant chemical conjugation rate, control assays were performed where the enzyme solution was replaced with an appropriate buffer.

GST Activity assayed with Unsaturated Aldehydes

Assays for the conjugation of glutathione with the unsaturated aldehydes 4-vinylpyridine and crotonaldehyde were modified from Berhane *et al.* (1994). 1 ml assays consisted of 900 µl buffer (0.1 M potassium phosphate, pH 6.5), 10 µl 10 mM aldehyde substrate

dissolved in ethanol, 25 µl enzyme solution and 10 µl 100 mM GSH. The reaction was followed by monitoring the decrease in absorbance at 248 nm (4-vinylpyridine) or 230 nm (crotonaldehyde) over 1 min and the activity calculated using extinction coefficients for the reactions of 7.4 $mM^{-1}.cm^{-1}$ for 4-vinylpyridine and 10.7 $mM^{-1}.cm^{-1}$ for crotonaldehyde. As for other GST assays, control reactions where the enzyme was omitted were also performed.

GST Activity assayed with Herbicides

A series of HPLC based assays were developed to measure GST activity towards the following herbicides: the chloroacetanilides alachlor and metolachlor, the chloro-*s*-triazines atrazine, cyanazine and simazine, the sulphonylurea chlorimuron-ethyl, and the diphenyl ethers fluorodifen and fenoxaprop (see Figure 2 for structures).

Herbicide standards (0.1 mg/ml dissolved in acetone) and their respective glutathione conjugates prepared as described above were used to calibrate the HPLC system and identify reaction products. HPLC analysis was performed on a Gilson HPLC system using a Fisons Spherisorb ODS1 column (250 mm × 4.6 mm), equilibrated with 5% acetonitrile in 1% phosphoric acid at a flow rate of 0.8 ml/min. 50µl of each sample was injected onto the column and the following linear gradients were used to separate metabolites: 5% to 10% acetonitrile in 1% phosphoric acid over 5 min then 10% to 100% acetonitrile in 1% phosphoric acid over 35 min. The UV absorbance (at 264 nm) of the eluate was monitored, analysed and UV-absorbing peaks integrated using Gilson system controller software (version 715). The peak areas given by the herbicide-glutathione conjugates were calibrated on the assumption that the conjugates had the same extinction coefficient as the herbicide at the analysed wavelength. Since this assumption has not been tested, and although the true extinction coefficients are likely to be fairly close to the assumed coefficients, activity data from these assays should be treated as semi-quantitative.

Herbicide assays contained 50 µl buffer (100 mM potassium phosphate pH 6.8 or 50 mM glycine-NaOH buffer, pH 9.5), 120 µl enzyme solution, 10 µl 20 mM herbicide (in acetone) and 20 µl 100 mM glutathione. Assays with the triazines, chloroacetanilides and

chlorimuron-ethyl were run at pH 6.8, and assays with fluorodifen and fenoxaprop were run at pH 9.5. The enzyme solution was desalted into 2 mM potassium phosphate pH 6.8 containing 2 mM DTT prior to assay to minimise its effect on the overall assay pH. Enzyme reactions were incubated at 37 °C for 1 h and were terminated by the addition of 10 µl of 0.6 M HCl. In the event that the enzymes were assayed without desalting it was sometimes necessary to add higher concentrations of HCl, up to 3 M, to adequately lower the pH to stop the reaction. Reaction mixtures were stored at -20 °C until required. Precipitated proteins were removed by centrifugation at 12,000 *g* for 5 min prior to analysis of 50 µl of the supernatant by HPLC. All incubations were run in duplicate. To correct for the non-enzymic conjugation rate further incubations were run in the absence of the protein extracts. Further controls with the herbicide omitted from the incubations were performed to correct for any compounds co-chromatographing with the herbicide-glutathione conjugate. To ensure that the assays were linear over time, assays with and without enzyme were performed over a range of time periods up to 1 hr. In all cases (e.g. for fluorodifen, Figure 7), amount of product formed was directly proportional to assay time up to at least 1 hr for both control and enzyme assays.

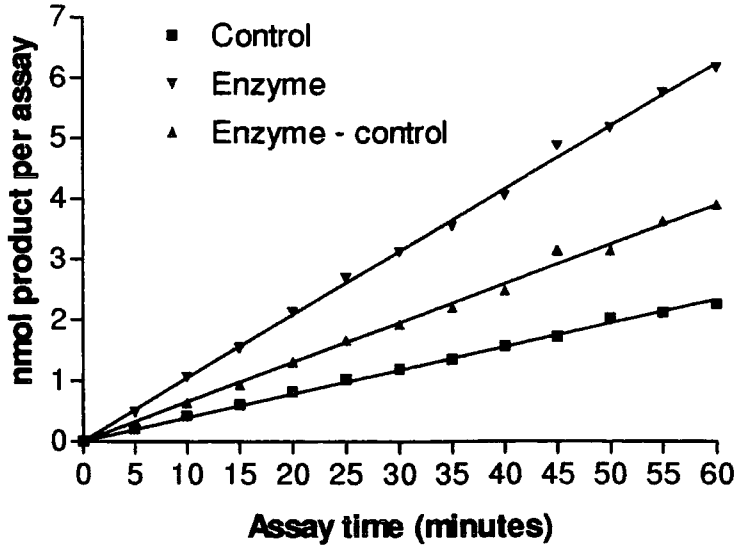

Figure 7. Graph illustrating linearity of HPLC-based GST assay for fluorodifen activity. Enzyme assays were performed using purified, recombinant *Zm* GST V-VI.

An additional assay for GST activity towards fluorodifen was used, based on the spectrophotometric measurement of the release of *p*-nitrophenol on conjugation of glutathione with fluorodifen (Edwards, 1996). While this assay was much less sensitive than the HPLC-based assay it was useful for the rapid assay of numerous samples, such as chromatography fractions. The assay consisted of 50 mM glycine-NaOH buffer, pH 9.5 (800 µl), 80 mM fluorodifen in acetone (5 µl), 100 mM glutathione (50 µl) and enzyme sample (100 µl). Assays were incubated for 60 min at 30 °C, before measuring the absorbance at 400 nm after raising the pH by the addition of 50 µl of 10 M NaOH. Assays were also carried out without glutathione, without enzyme, and without both glutathione and enzyme to correct for any chemical reaction rate and for any compounds other than *p*-nitrophenol absorbing at 400 nm. Enzyme specific activity was calculated based on an extinction coefficient for *p*-nitrophenol in alkaline solution of 20 $mM^{-1}.cm^{-1}$.

Glutathione Peroxidase Assays

Glutathione peroxidase activities were determined with the hydroperoxide substrates cumene hydroperoxide, linoleic acid hydroperoxide and hydrogen peroxide, and were performed as described in Wendel (1981) with slight modifications. The assay (1ml) consisted of 0.5 ml buffer (0.25 M potassium phosphate pH 7.0, 2.5 mM EDTA, 2.5 mM sodium azide), 0.1 ml 10mM glutathione, 0.1 ml yeast glutathione reductase (6 units/ml, where 1 unit will reduce 1.0 µmol of oxidised glutathione per min at pH 7.6 at 25 °C), 0.1 ml NADPH (2.5 mM in 0.1% $NaHCO_3$), 0.1 ml 12 mM hydroperoxide solution and 0.1 ml enzyme solution. Activity was measured by following the decrease in absorbance at 366 nm over 2 min, taking into account the chemical reaction rate, measured in the absence of enzyme.

Glyoxalase I Assay

Assays for glyoxalase I activity were performed using a spectrophotometric method (Ramaswamy *et al.*, 1983), which measures the isomerisation of the hemithioacetal adduct of methylglyoxal and glutathione to the thioester *S*-D-lactoylglutathione. Assays (1 ml), comprising 100 mM sodium phosphate buffer, pH 7.5, 16 mM $MgSO_4$, 3.5 mM methylglyoxal and 1.7 mM glutathione, were pre-incubated at 30 °C for 5 - 10 minutes

to allow formation of the hemithioacetal. The enzyme extract was then added to the assay and glyoxalase I activity was measured by following the change in absorbance at 240 nm for 30 s. Enzyme activity was then calculated assuming an extinction coefficient for *S*-D-lactoylglutathione of 3370 $M^{-1}.cm^{-1}$. Control reactions where the enzyme was omitted showed that there was no appreciable chemical rate for this reaction.

Purification of GSTs

Protein Extraction

For protein analysis and purification, plants were harvested and shoots and roots separated. Roots were washed in distilled water to remove any vermiculite or compost. All subsequent steps of the extraction were carried out at 4 °C. Each tissue was weighed, then homogenised in a blender in 3 v/w of extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 14 mM β-mercaptoethanol) with 5% w/v insoluble polyvinylpolypyrrolidone (PVPP), filtered through muslin and centrifuged at 17,000 *g* for 30 min to remove cell debris. Ammonium sulphate was slowly added to the resultant supernatant to a final concentration of 561 g/l (80% saturation). This suspension was centrifuged as before and the protein pellet formed was stored in aliquots at -80 °C until required.

Membrane and cytosolic fractions were prepared following the method of Diesperger *et al.* (1974), by addition of 25 mM $MgCl_2$ to the cell-free extract produced from the first centrifugation above. After standing at 4 °C for 1 h the extract was centrifuged at 17,000 *g* for 30 min. The membrane pellet was transferred to -80 °C while protein in the supernatant was precipitated with ammonium sulphate as described above.

Column Chromatography

Unless otherwise stated all column chromatography was performed at 4 °C using a GradiFrac (Pharmacia) low-pressure chromatography system, running at a flow rate of 1 ml/min. The elution of protein was monitored by UV absorbance at 280 nm and

recorded using a chart recorder. A flow chart summarising the chromatography steps is shown in chapter 4, Figure 10.

Crude ammonium sulphate-precipitated protein was dissolved in buffer A (10 mM potassium phosphate buffer pH 7.4, 14 mM β-mercaptoethanol) containing 0.5 M ammonium sulphate and 50 mM KCl, then centrifuged at 17,000 *g* for 30 min to remove any undissolved protein. This was loaded onto a 35 ml phenyl Sepharose column pre-equilibrated in the same buffer, then washed with the same buffer until no further protein was eluted. The column was then washed with buffer A without salt added, before elution of GSTs with 50% v/v buffer A and 50% v/v ethylene glycol, containing 2 mM glutathione, at 0.5 ml/min.

In order to remove the ethylene glycol, the active fractions were pooled and loaded at 0.5 ml/min onto a 6 ml Q Sepharose anion exchange column which had been pre-equilibrated with buffer B (20 mM Tris-HCl pH 7.8, 14 mM β-mercaptoethanol). The column was washed with buffer B until no further protein was eluted, then GSTs were recovered with buffer B containing 0.5 M NaCl.

Dye-affinity chromatography using a 5 ml Orange A agarose column (Amicon) was then used to resolve the partially purified GST preparation into two fractions. Active fractions from the Q Sepharose column were dialysed against buffer C (10 mM potassium phosphate buffer pH 6.0, 14 mM β-mercaptoethanol) for 16 h then loaded onto the Orange A column pre-equilibrated with buffer C. Unbound protein, containing GST activity, was washed from the column using buffer C and termed the pool 2 GSTs. The column was then washed with buffer D (50 mM potassium phosphate buffer pH 7.0, 14 mM β-mercaptoethanol) until no further protein was eluted, then bound GSTs were eluted using buffer D containing 2 mM glutathione to give pool 1 GSTs.

Further purification of the pool 2 GSTs present in the unbound fraction from the Orange A column was achieved using a 5 ml *S*-hexylglutathione Sepharose 6B affinity column. The sample was adjusted to pH 7.4 using 1 M NaOH and loaded onto the affinity column, previously equilibrated with buffer A. The column was washed with buffer A until no further protein was eluted. The column was then washed with buffer A

containing 0.2 M KCl, again until no further protein was eluted. Bound GSTs were recovered from the column by application of buffer A containing 0.2 M KCl and 5 mM *S*-hexylglutathione.

Purified fractions with CDNB-conjugating activity from pools 1 and 2 were dialysed for 16 h against 10 mM Tris-HCl pH 7.8, 14 mM β-mercaptoethanol. GST isoenzymes from each pool were then resolved by anion exchange chromatography using a 1 ml HiTrap Q column (Pharmacia) equilibrated in buffer B. After loading the sample onto the column and washing with buffer B, isoenzymes were eluted with a 25 ml 0 M - 0.25 M NaCl gradient in buffer B. The column was subsequently washed free of any remaining protein using buffer B containing 0.5 M NaCl.

Analysis of GST Isoenzymes in Crude Protein Extracts

Crude protein samples of maize roots and shoots from dichlormid-treated and control plants, containing similar amounts of protein, were loaded onto a Q Sepharose FF column (6ml total volume) in buffer B and eluted with a linear gradient of increasing NaCl concentration up to 0.25 M in the same buffer; fractions were assayed for GST activity towards CDNB.

Purification of Maize Glyoxalase I

Maize glyoxalase I was partially purified using methods adapted from Paulus *et al.* (1993). A crude cell-free extract was prepared from the shoots of dichlormid-treated maize seedlings, as described for the purification of GSTs. Protein precipitating between addition of ammonium sulphate to 40% saturation and addition to 80% saturation was collected by centrifugation (17,000 *g*, 30 min, 4 °C) and re-dissolved in 0.1 M sodium phosphate buffer, pH 7.0. This solution was desalted into the same buffer using PD10 gel filtration columns, then applied to an *S*-hexylglutathione affinity column previously equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The column was then washed using loading buffer, and specifically-bound protein was eluted using this buffer containing 5 mM glutathione. Fractions with glyoxalase I activity were pooled and dialysed against 16 mM Tris-HCl pH 7.8 for 16 h. Following dialysis, affinity-purified proteins were resolved by anion exchange chromatography using 1 ml HiTrap Q columns

as described for GST purification, except that β-mercaptoethanol was omitted from all buffers.

Immunological Methods

Production of Antibodies

Purified GST *Zm* I-II and GST *Zm* V-VI from the pool 1 and pool 2 GSTs respectively were used to raise polyclonal antibodies in female New Zealand white rabbits. Purified, lyophilised protein samples were resuspended in TBS (Tris-buffered saline pH 7.4) and emulsified with an equal volume of Freund's adjuvant. The emulsion (3 ml) was then injected both subcutaneously and intramuscularly. For the initial immunisations 600 µg of GST *Zm* I-II and 110 µg of GST *Zm* V-VI respectively were individually administered in Freund's complete adjuvant. In the booster injections at 26 days and 59 days, Freund's incomplete adjuvant was used. For the rabbit immunised with GST *Zm* I-II the booster inoculations of GST *Zm* I-II consisted of 600 µg protein followed by 280 µg protein, while for the immunisations with GST *Zm* V-VI a second injection of 110 µg of the pure protein was followed by a further 110 µg. Serum samples were prepared 20 days after the final injection by incubating blood samples at 37 °C for 1 h, then chilling at 0 °C for 16 h to allow red blood cells to coagulate. Serum was separated from cells by centrifugation (17,000 *g*, 20 min) and stored in aliquots at -20 °C after addition of sodium azide (0.1% w/v).

Western Blotting

For western blotting studies polypeptides were resolved by SDS-PAGE (see later) and then transferred onto Immobilon-P PVDF membrane (Millipore, Watford, UK) or Hybond-C nitrocellulose membrane (Amersham International plc, Bucks., UK) using a tank electroblotter (mini Trans-Blot cell, Bio-Rad) and the manufacturer's protocol. The electroblotting buffer was 16 mM Tris, 120 mM glycine (pH 9.5), and electroblotting was performed at 100V for 30 min. After rinsing in TBS, membranes were blocked in TBS containing 3% w/v non-fat milk powder for 30 min. The anti-GST serum was then added to the blocking solution at a dilution of 1:5,000 and the membrane incubated for

30 min. The blot was then washed twice for 10 min with TBS containing 0.1% Triton-X 100 and once for 10 min with TBS. The membrane was then incubated with Goat anti-rabbit IgG coupled to alkaline phosphatase at a 1:5,000 dilution in TBS containing 3% w/v non-fat milk powder for 30 min followed by three 10 min washes as before. Alkaline phosphatase activity was detected using 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ containing 0.3% v/v of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 50 mg/ml) dissolved in *N,N*-dimethylformamide and 0.3% v/v nitro blue tetrazolium (NBT; 100 mg/ml) dissolved in 70% v/v *N,N*-dimethylformamide. For quantification, blots were digitised using a Hewlett Packard ScanJet IIcx and the integrated density of the stained bands was measured using the free UTHSCSA *ImageTool* program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu).

Tissue Immunoblotting

Tissue immunoblotting was performed using published methods (Cassab and Varner, 1987). A nitrocellulose membrane was prepared for blotting by soaking in 0.2 M $CaCl_2$ for 30 min and was then dried between paper towels. Sections of maize tissue (approximately 1 mm thick) were cut using a razor, immediately blotted gently on paper towels to remove any excess liquid, then pressed firmly onto the membrane for 30 s, using folded paper towels to apply pressure. The membrane was allowed to dry, then stained with Ponceau S (0.1% in 5% acetic acid) for 2 min to visualise total protein. The membrane was then destained in TBS, then blocked, probed with serum (pre-immune serum, anti-*Zm* GST I-II serum or anti-*Zm* GST V-VI serum) and developed as described for western blotting to determine the location of immunodetectable GSTs in each section.

Preparation of Tissue for Immunohistochemistry

Tissue for immunohistochemistry was selected from freshly harvested maize plants, cut into small pieces and immediately placed into immunofix solution (3% paraformaldehyde diluted from a freshly made 12% aqueous stock solution, 1.25% glutaraldehyde, 0.05 M phosphate buffer pH 7.0) at 4 °C for 16 h. Samples were then rinsed in 0.05 M

phosphate buffer pH 7.0 for 45 min before being dehydrated using a series of aqueous solutions of increasing % v/v ethanol (12%, 25%, 50%, 75%, 95%, 100% and 100% by volume), incubating samples in each solution for 1 h, with gentle mixing. Samples were then transferred to 50% v/v LR White epoxy resin (medium grade resin, London Resin Company, Reading, UK) in ethanol and incubated with gentle mixing for 16 h. Resin infiltration was completed by incubating the resulting samples in 100% LR White resin for 5 days, replacing the resin with fresh solution every 12 h. Finally, resin-infiltrated samples were placed in plastic vials filled with resin, which were then placed in a 60 °C oven for 24 h to allow the resin to polymerise. Polymerised samples were stored at room temperature until required.

Protein Analysis

SDS-PAGE

Protein samples were prepared for electrophoresis by heating at 100 °C in an equal volume of 2× loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% SDS and 25 µg/ml bromophenol blue) for 5 min and then allowing to cool.

Protein SDS-PAGE was performed using a Mini-PROTEAN II electrophoresis kit and recommended protocol (Bio-Rad) using the system of Laemmli (1970) with 0.75 mm-thick gels. Resolving gels were polymerised from 12.5% acrylamide, 0.33% *N'N'*-bis-methylene-acrylamide, 0.1% ammonium persulphate, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS and 0.05% TEMED (*N,N,N',N'*-tetramethylethylenediamine). Stacking gels were polymerised from 4% acrylamide, 0.11% *N'N'*-bis-methylene-acrylamide, 0.05% ammonium persulphate, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS and 0.05% TEMED. For electrophoresis the running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Following electrophoresis (150 V for 45 - 60 min) proteins were visualised after fixing in water containing 40% v/v methanol and 10% v/v acetic acid, and stained either with the Coomassie brilliant blue substitute B/T Blu (BT Scientific Technologies, Carlsbad, California, USA) or with silver stain (Bio-Rad), in each case following manufacturer's protocols.

Non-denaturing PAGE and Native Molecular Mass Determination

Non-denaturing PAGE was performed using similar gels, buffers and conditions to those used for SDS-PAGE, except that SDS was omitted from all the buffers and β-mercaptoethanol was omitted from the loading buffer and protein samples were not heated (Bollag and Edelstein, 1991). Protein samples were prepared by adding an equal volume of 2× loading buffer; samples were kept at 4 °C until required.

Native molecular masses were determined from non-denaturing gels as described in Sigma technical bulletin No. MKR-137. Identical protein samples were run on four gels of different acrylamide concentration (6%, 7%, 9% and 10% acrylamide), together with molecular weight standards from Sigma (bovine α-lactalbumin - 14.2 kDa, bovine carbonic anhydrase - 29 kDa, chicken egg albumin - 45 kDa, and bovine serum albumin monomer and dimer - 66 kDa and 132 kDa respectively). After electrophoresis, gels were stained with B/T Blu and the relative mobility (R_f) of each protein on each gel was measured, taking the bromophenol blue dye front to have an R_f of 1.0. The change in mobility with changing gel concentration was calculated, and a calibration curve was constructed using this slope vs. molecular mass for the protein standards. The molecular masses for the other proteins was then determined from the standard curve.

Polypeptide Analysis by SDS-PAGE following Partial Proteolysis

Polypeptide analysis following partial proteolysis (peptide mapping) was performed using a commercially available kit and supplied protocols (Promega), following the method of Cleveland *et al.* (1977). Tests using purified recombinant *Zm* GST V-V were used to determine the optimal protease type and concentration, using a range of concentrations of the supplied proteases (alkaline protease, endoproteinase Glu-C and endoproteinase Lys-C). Analyses of multiple protein samples were performed using 0.04 µg alkaline protease and 2 µg protein sample per gel lane. The digested peptides were resolved using 20% acrylamide SDS-PAGE, then visualised using silver staining.

Protein Determination

Protein content of solutions was determined using a commercial dye-binding assay (Bio-Rad) based on the method of Bradford (1976), using bovine γ-globulin as a standard, following the manufacturer's protocols.

Protein Sequencing

Pure proteins in solution were transferred onto PVDF membrane using ProSpin columns (Applied Biosystems). When sequencing polypeptides following SDS-PAGE, thioglycollic acid (2 mM) was added to the electrophoresis anode buffer and the gel pre-run for 30 min before sample addition to remove chemicals which could potentially N-terminally block polypeptides. Following electrophoresis as described earlier, polypeptides were transferred onto PVDF membrane as described for western blotting, except that ProBlott (Applied Biosystems) PVDF membrane was used to increase protein binding to the membrane, and CAPS-methanol buffer (10 mM 3-[cyclohexylamino]-1-propanesulphonic acid-NaOH, pH 11, 10% v/v methanol) was used as the transfer buffer. After blotting, membranes were rinsed with distilled water then methanol. Membranes were briefly (60 s) stained with 0.1% Coomassie Blue R-250 in 40% methanol, 1% acetic acid, then destained with 50% methanol, to locate polypeptide bands. After washing with distilled water and drying, bands of interest were excised from the membrane and used for protein sequencing. Proteins were sequenced using an ABI 477A protein sequencer.

Molecular Biology Methods

Unless otherwise stated, the methods used for cloning and expression were as described in Sambrook *et al.* (1989).

Custom oligonucleotides were synthesised either in-house, or commercially by PE-Applied Biosystems UK.

Bacterial Culture

For routine work, liquid bacterial cultures were grown in Luria-Bertani (LB) broth (1% w/v bacteriological peptone, 1% w/v NaCl, 0.5% w/v yeast extract) from a single colony picked from a fresh agar plate. Liquid cultures were grown at 37 °C on an orbital shaker (200 rpm). For bacterial culture on agar, a base of 1.5% bacteriological agar in LB broth was used, and inoculated plates were incubated at 37 °C. Where appropriate, antibiotics for selection were added to culture media.

RNA extraction and cDNA synthesis for RT-PCR

RNA was extracted from maize tissue using TRIZOL reagent (GIBCO BRL) and the accompanying protocol. This RNA was used as a template for cDNA synthesis using reverse transcriptase M-MuLV (Promega) and supplied protocols. The primer used for this was either dT_{15} or og2 (used to select for mRNA) or a specific primer to selectively synthesise cDNA from the desired RNA species.

RNA Denaturing Agarose Gel Electrophoresis

For electrophoresis, RNA samples (10 µg) were made up in 0.1 × TPE (3.6 mM Tris, 3 mM NaH_2PO_4, 0.2 mM EDTA, pH 7.8) containing 60% v/v formamide. Samples were heated to 60 °C for 5 min then cooled on ice before adding 6 µl of 50% glycerol containing 0.25% bromophenol blue to each sample. Samples were loaded onto a 1.3% agarose gel containing 50% v/v formamide and 0.1 × TPE after filling wells with 0.1 × TPE containing 60% v/v formamide. Gels were run gel at 20 mA until the tracking dye had migrated 2/3 of the way down the gel. Gels were then stained with 1.0 µg/ml ethidium bromide to visualise RNA using a UV transilluminator.

PCR (Polymerase Chain Reaction)

PCR was performed using standard conditions. Each reaction (20-100 µl) contained reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, dTTP, two oligonucleotide primers (each 1 µM), template and *Taq* DNA polymerase (1-5 units, where 1 unit catalyses the incorporation of 10 nmol of dNTP into acid-insoluble form in

30 min at 74 °C). Typically, PCR amplifications consisted of 30 cycles (for amplification from a cDNA population) or 15 cycles (for amplification from plasmid) of the conditions: 94 °C (90 s), 50 °C (120 s), 72 °C (120 s). Reaction tubes were heated to 94 °C before addition of *Taq* DNA polymerase to minimise mis-priming and subsequent non-specific amplification of non-target sequences. Reactions were carried out using a Techne Progene thermal cycler.

DNA Ligation

Ligation of DNA fragments were usually performed in 10 µl reactions, using commercially available T4 DNA ligase and appropriate buffers (Promega). Digested, purified vector and insert were added in an approximate ratio of 1:3, and the reaction was made up to 10 µl with sterile distilled water. Ligations were incubated for between 3 and 16 h at 14 °C prior to transformation into *E. coli*.

Transformation of *E. coli*

For routine cloning work competent *E. coli* cells were prepared using calcium chloride solution (Sambrook *et al.*, 1989). A liquid culture (100 ml) was inoculated with a single bacterial colony and allowed to incubate until the cell turbidity increased the absorbance of the media by 0.6 AU at 600 nm. Cells were then chilled to 4 °C and harvested by centrifugation (5000 *g*, 10 min). The cell pellet was resuspended in 20 ml of chilled sterile 100 mM $CaCl_2$ solution, then re-centrifuged as before. The cell pellet was then gently resuspended in 4 ml of 85 mM $CaCl_2$, 15% glycerol, dispensed into 100 µl aliquots in chilled 1.5 ml centrifuge tubes, frozen in liquid nitrogen and stored at -80 °C until required. For applications requiring higher transformation efficiencies, for example PCR product cloning, commercially prepared cells (Promega), were used.

Transformation of DNA into chemically competent cells was performed using standard procedures (Sambrook *et al.*, 1989). Tubes containing competent cells were removed from -80 °C storage and thawed on ice. DNA for transformation (1-10 µl) was added to the cells and the tubes mixed very gently. After leaving the cells on ice for 30 min, cells were heat-shocked by placing the tubes in a 42 °C water bath for 90 s, then placing the tubes back on ice for 1-2 min. LB broth (0.9 ml) was then added to each tube, and tubes

were incubated at 37 °C for 45 min. The resulting cell suspension was selected for transformants by plating out aliquots (usually 200 µl and 800 µl) of the suspension on LB-agar plates containing ampicillin. When transforming cells with a plasmid allowing blue-white colour selection, 40 µg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) and 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) were added to the agar plates. Plates were incubated for 16 h at 37 °C.

Plasmid Purification

Single colonies of bacteria containing the appropriate plasmid were cultured overnight under ampicillin selection (100 µg/ml) in 10 ml of LB broth. Typically, 3 ml of the resulting stationary phase culture was used for plasmid purification using Wizard miniprep (Promega) reagents and the supplied protocol. Purified plasmids were dissolved in 50 µl of sterile distilled water and stored at -20 °C.

DNA Digestion with Restriction Enzymes

Restriction enzyme digests were performed using commercially available enzymes and buffers (Promega, New England Biolabs, Boehringer Mannheim, MBI Fermentas). Typically, digests were performed using 0.5 - 2.0 µg of plasmid DNA and 2 - 10 U (where 1 U will completely digest 1 µg of DNA in a total volume of 50 µl in 1h under optimal conditions) of each restriction enzyme. Digests were incubated at 37 °C for 1 - 3 h before analysis and fragment purification using agarose gel electrophoresis.

DNA Agarose Gel Electrophoresis

Electrophoresis of DNA samples was performed using 1% agarose, TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.6) gels, with TAE as buffer. 1.0 µg/ml ethidium bromide was added to both gel and buffer to allow DNA to be visualised under UV light. DNA samples were prepared by adding one-fifth volume of loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF). Gels were run horizontally at 50 - 100 V. Gels were analysed under UV light and documented using a Bio-Rad Gel Doc 1000 video documentation system.

Purification of DNA from Agarose Gels

DNA was routinely purified from agarose gels using silica fines to bind DNA. The DNA band of interest was excised from the gel in as small a volume of gel as possible, minimising exposure to UV light to prevent damage to the DNA. The gel fragment was placed in a 1.5 ml tube and at least 4 volumes (usually 0.5 ml) of gel solublising buffer (90.8% w/v sodium iodide solution, saturated with sodium sulphite) added. The mixture was heated to 65 °C with shaking until the gel fragment had completely dissolved. After cooling, 10 µl of a 50% v/v suspension of silica fines was added and the tube gently mixed for 10 min. The tube was centrifuged briefly to pellet the fines and bound DNA, and the supernatant was discarded. The pellet was washed twice by resuspending it in 0.5 ml of 70% v/v ethanol, centrifuging briefly and discarding the supernatant. The pellet was then partially dried using a gentle stream of air and resuspended in between 10 µl and 30 µl of sterile distilled water. The suspension was incubated at 37 °C for 45 min to release DNA bound to the silica, then the silica was removed by centrifugation.

Before use, silica fines (Sigma, 0.5 - 10 micron particle size) were prepared by allowing the larger particles to sediment out, then boiling the remaining silica suspension for 5 min in 50% conc. nitric acid. The suspension was pelleted by centrifugation and the silica resuspended in sterile water multiple times, until the pH of the silica suspension was neutral. The suspension was then centrifuged again, the silica pellet resuspended in an equal volume of sterile water and stored in aliquots at -20 °C.

Recombinant Expression of GSTs

Expression in pKK233-2

To express *Zm* GST I using the expression vector pKK233-2 (Amann and Brosius, 1985), a single *E. coli* colony transformed with the plasmid of interest was used to inoculate a 5 ml liquid culture. After incubation for 8 h, this culture was used to inoculate 500 ml of nutrient broth containing 1 mM IPTG, which was then incubated for a further 16 h to allow protein expression. The cells were then harvested by centrifugation (10,000 *g*, 4 °C, 10 min), resuspended in a small volume of cold extraction buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 250 mM KCl, 10% v/v glycerol, 0.5 mM DTT) and ground up in liquid nitrogen. The resulting powder was made up to 20 ml

with extraction buffer and allowed to thaw before sonicating for three 15 s pulses with 1 min breaks between pulses. The extract was then centrifuged (17,000 *g*, 4 °C, 20 min); the pellet was discarded and ammonium sulphate was added slowly to the supernatant at 4 °C to a give an 80% saturated solution. After stirring slowly for 1 h this was centrifuged as before and the resulting protein pellet was stored at -80 °C until required.

Expression in pET vectors

To express GST sequences cloned into pET vector constructs, a single colony of *E. coli* strain BL21(DE3) containing the construct of interest was grown in LB broth under antibiotic selection overnight. This culture was diluted 1:100 into similar, pre-warmed broth and cultured for about 3 h, until the absorbance of the broth at 600 nm increased by 0.5. 1 M isopropyl ß-D-thiogalactopyranoside (IPTG) was then added to give a final concentration of 1 mM and the cells allowed to grow for a further for 3 h. Cells were pelleted by centrifugation (1,000 *g*, 10 min) and resuspended either in extraction buffer as used for expression in pKK233-2 (see above), or in the appropriate loading buffer for chromatography if the expressed protein was to be purified. Cells were then lysed by sonication, as described above. Cell debris was removed by centrifugation (10,000 *g*, 20 min). Purification of protein from the resultant cell-free extract was performed following the protocols described for affinity chromatography of partially purified GSTs from plant tissue.

cDNA Library Construction

Total RNA was isolated from the roots of 10-day etiolated dichlormid-treated maize seedlings using TRIZOL (GIBCO BRL) and the manufacturer's protocol. RNA was enriched for poly(A)$^+$ RNA using Dynabeads Oligo $(dT)_{25}$ (Dynal) following the manufacturer's protocol, and used to construct a unidirectional cDNA library in bacteriophage λ Uni-ZAP XR vector (Stratagene). Library construction followed the supplied protocol, except that no radiolabelled nucleotides were used during cDNA synthesis, and therefore the synthesis of first and second strand cDNA was not followed by gel electrophoresis.

cDNA Library Screening

cDNA library titering and plating were performed following protocols supplied by Stratagene. Primary screens were carried out using 24 cm x 24 cm bio-assay dishes (Life Technologies Ltd., Renfrewshire, Scotland) while subsequent screens were carried out using petri dishes (90 mm diameter). In each case, phage-infected *E. coli* (strain XL1-Blue MRF') was mixed with molten NZY top agar and plated out onto dishes containing NZY agar which had been previously stored at 4 °C for at least 2 d to ensure good adhesion of the top agar. Primary screens were performed with approximately 170,000 pfu per large dish and subsequent screens were performed with 40 - 200 pfu per petri dish. Libraries were screened using antibody or DNA probes as described below, and regions of the plated library containing putative positive clones were identified. These regions were cored using a 200 µl pipette tip and each was transferred to 0.5 ml of SM buffer containing 25 µl of chloroform. This phage stock was titered and used for subsequent rounds of screening until single positive plaques could be isolated.

Antibody Screening

Immunoscreening was performed using published procedures (Sambrook *et al.*, 1989). Dishes containing phage-infected *E. coli* were incubated at 37 °C either until plaques were visible (for primary screening) or overnight (for secondary and tertiary screens). Nitrocellulose membrane, pre-soaked in 10 mM IPTG and dried, was carefully placed onto the plaques and the dish was incubated for a further 4 - 6 h at 37 °C to allow time for protein expression. The dish was then chilled at 4 °C for 30 min before carefully lifting off the membrane, to minimise transfer of top agar to the membrane. The membrane was gently washed in TBS to remove any adhering top agar, then probed with the primary antibody and developed using protocols as described for western blotting.

DNA Screening using DIG-Labelled Probes

For cDNA library screening with DNA probes, DIG (digoxigenin) labelling was used as an alternative to radiolabelling. PCR was used to generate large amounts of the probe sequence and after agarose gel electrophoresis the specific PCR product was excised from the gel and purified. Approximately 1 µg of this DNA was DIG-labelled, using the

DIG High-Prime labelling system (Boehringer Mannheim UK, East Sussex, UK) to incorporate DIG-11-dUTP, following the supplied protocol.

Library screening with DIG-labelled probes was carried out following supplied protocols (Boehringer Mannheim). Dishes containing phage-infected *E. coli* were incubated at 37 °C until plaques were visible, then chilled at 4 °C for 30 min. A positively charged nylon membrane (Boehringer Mannheim) was laid onto the agar and left for 1 min, then carefully removed after marking membrane and dish to allow re-alignment once positive plaques had been identified on the membrane. Throughout the screening protocol the membrane was placed with the contact side uppermost. The membrane was briefly blotted on dry Whatman 3MM paper, then blotted on 3MM paper soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 5 min. The membrane was again blotted briefly on dry 3MM paper, then blotted on 3MM paper soaked in neutralisation solution (1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl) for 15 min. Following a further brief blot on dry 3MM paper, then membrane was blotted on 3MM paper soaked with 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) and once again blotted briefly on dry 3MM paper. DNA bound to the membrane was then crosslinked using UV light (150 mJ, Bio-Rad GS Gene Linker UV chamber). To remove any contaminating protein the membrane was subsequently treated with 0.2 mg/ml proteinase K solution (Boehringer Mannheim) for 1 h at 37 °C. The treated membrane was blotted between sheets of 3MM paper soaked in distilled water to remove any remaining cellular debris. The prepared membrane was then probed, using a Techne hybridiser HB-1D for all incubations to maintain the correct temperature and agitation. The membrane was first pre-hybridised at 68 °C for 1 h using hybridisation buffer consisting of 0.75 M NaCl, 75 mM sodium citrate, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% blocking reagent (Boehringer Mannheim), pH 7.0. The DNA probe was then denatured by heating to 100 °C for 5 min, then added to hybridisation buffer pre-warmed to 68 °C. The membrane was hybridised in this solution for 16 h at 68 °C. The hybridisation solution was then poured off and stored at -20 °C for re-use, and the membrane was washed to remove weakly bound probe. Washing consisted of two washes with 2 × SSC containing 0.1% SDS for 5 min at 20 °C, followed by a further two washes with 0.5 × SSC (75 mM NaCl, 7.5 mM sodium citrate pH 7.0) containing 0.1% SDS for 15 min at 68 °C. All subsequent steps were performed at room temperature. The membrane was then washed in maleic acid

buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 0.3% v/v Tween 20 for 1 min, then treated with blocking solution (maleic acid buffer containing 1% blocking reagent) for 1 h. Anti-DIG-alkaline phosphatase antibody (Boehringer Mannheim) was then added to this blocking solution at a dilution of 1:5,000, and the membrane incubated for 30 min. The membrane was then washed twice with maleic acid buffer containing 0.3% v/v Tween 20 for 15 min each. Finally, the membrane was rinsed with development buffer and developed using BCIP and NBT as described above for western blots.

Clone excision

After three rounds of screening pBluescript phagemids were isolated from positive plaques by *in vivo* excision using ExAssist helper phage (Stratagene) and supplied protocols.

DNA Sequencing

DNA sequencing was performed on double-stranded DNA using ABI 373A or ABI 377A automated fluorescent sequencers.

DNA and Protein Sequence Analysis

Routine DNA sequence analysis including sequence translation, restriction enzyme analysis and analysis of DNA sequencing chromatograms was performed on software written by the author for the Windows '95 operating system. Multiple sequence alignments were performed using CLUSTALW (Thompson *et al.*, 1994). Database similarity searches were performed using the BLAST suite of programs (Altschul *et al.*, 1990) on the National Center for Biotechnology Information's BLAST WWW Server.

Phylogenetic Analysis

Phylogenetic analysis was performed using the multiple sequence alignment program CLUSTALW (Thompson *et al.*, 1994) and the phylogeny package PHYLIP (Felsenstein, 1993). Protein sequences of plant GSTs were aligned with each other using CLUSTALW with default parameters, and the results output to a PHYLIP format file.

A phylogenetic tree was constructed from this alignment using the PHYLIP package programs PROTDIST (to calculate evolutionary distances between each pair of proteins) and NEIGHBOR (to construct a tree from distance data using neighbour-joining), and was visualised using the PHYLIP program DRAWTREE. To assess the significance of groupings of sequences in this tree, the original sequence alignment was reanalysed following multiple resampling of the data. The alignments were resampled 100 times by delete-half-jack-knifing, where for each sample, residues at half of the sequence positions (chosen at random) of each sequence were deleted, using the PHYLIP program SEQBOOT. The resulting 100 data sets were then analysed using the PROTDIST and NEIGHBOR programs as before, and the resulting 100 trees were analysed by the PHYLIP program CONSENSE to determine which of the groupings present in the original tree were present in a significant proportion of the 100 trees constructed from the jack-knifed data sets.

3. GST Activities in Crude Maize Extracts

Introduction

Before starting on the purification of multiple GSTs from maize plants, it was important to determine the best organ source for purification by establishing the distribution of GST activity not only towards the model substrate CDNB, but also towards important herbicides. Initial work focused on developing sensitive assays for the determination of GST activity towards herbicides, as described in chapter 2. These activities were then determined in preparations from root and shoot tissue of maize seedlings. Since herbicide safeners had previously been shown to enhance GST activity in maize seedlings, protein from tissues of maize plants treated with safeners were also assayed for GST activity.

Several studies have reported GST activities in different maize organs and the effect of safener treatment, but in most cases a limited range of GST substrates was used, usually CDNB and/or chloroacetanilide herbicides. Ekler *et al.* (1993) showed that treating maize with a range of safeners enhanced GST activity towards acetochlor, and to a lesser extent towards CDNB. Edwards and Owen (1988) reported that following treatment with 15 µM dichlormid for 24 h, maize seedling GST activity doubled towards metolachlor, while activity towards atrazine was not affected. Immunodetectable GST levels did not however correlate with metolachlor activity, suggesting that the immunoreactive GST (probably *Zm* GST I) was not the major metolachlor-active GST. Studies on isoenzymes in crude extracts showed that treatment of maize seedlings with the safeners benoxacor (Fuerst *et al.*, 1993) or CGA-154281 (Dean *et al.*, 1991) induced or enhanced levels of GSTs with activity towards CDNB or metolachlor.

While these studies examined GST activity towards a limited number of substrates it was clear that safener treatment only enhanced activity of certain GST isoenzymes in maize, and that different GST isoenzymes had different substrate specificities. It was therefore of interest to determine GST activity with a wider range of potential substrates and determine their relative enhancement by safener treatment. This has been achieved by

investigating the effect of safener treatment on GST activity towards structurally diverse xenobiotic substrates where activity can be monitored spectrophotometrically and three important herbicide classes. With the herbicides, the effect of safener treatments on GST enhancement has also been studied in roots and shoots in different cultivars.

Results

Development of Assays for GST Activity towards Herbicides

Assays for GST activity towards herbicides were performed using the HPLC-based method described in chapter 2. To calibrate this system samples of the herbicides, and the crude herbicide-glutathione conjugates prepared as described in chapter 2, were analysed to give the retention times and peak area calibrations shown in Table 4. Figure 8 shows typical HPLC traces for the resolution of atrazine and the chemically synthesised glutathione conjugate of atrazine; similar resolution was shown for the other herbicides used. In most cases, both the herbicide and the respective herbicide-glutathione conjugate gave a single major UV-absorbing peak. However, only minor peaks other than the herbicide were present in the chlorimuron-ethyl conjugate preparation so the retention time of the conjugate could not be identified with confidence.

Herbicide	Herbicide retention time (min)	Herbicide conjugate retention time (min)	Peak area per nmol herbicide
Atrazine	23.3	13.2	228000
Cyanazine	20.0	13.5	296000
Simazine	20.6	11.6	245000
Alachlor	27.7	19.8	68000
Metolachlor	28.2	17.5	39000
Fenoxaprop	26.2	17.7	265000
Fluorodifen	28.6	17.6	682000
Chlorimuron-ethyl	26.3	Not determined	908000

Table 4. Calibration data for HPLC-based GST assays.

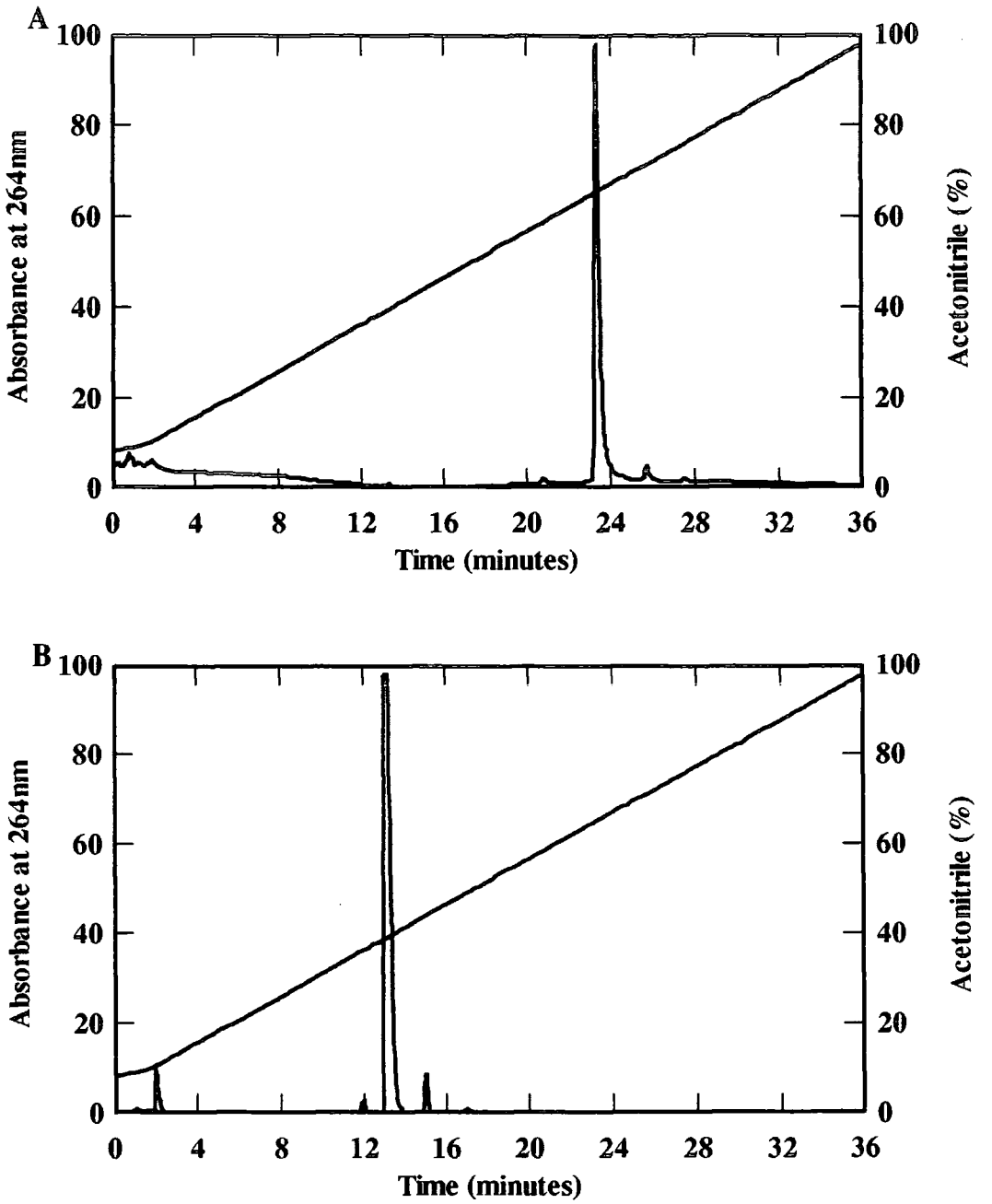

Figure 8. HPLC traces showing resolution of atrazine (A) from the chemically synthesised glutathione conjugate of atrazine (B).

The mobile phase acetonitrile concentration is shown.

GST Activities in Safener-Treated and Control Maize Seedlings

As discussed in chapter 1, many herbicide safeners are thought to at least in part work by inducing herbicide-detoxifying enzymes. It was therefore of interest to examine the range of GST activities which were enhanced by safener treatment in maize seedlings. In initial studies the maize cultivar Artus was used. Maize plants were grown in the dark for 11 days either with or without safener treatment (see chapter 2).

The extracts from safened and control root and shoot tissue were fractionated into cytosolic and $MgCl_2$-precipitated membrane extracts. The soluble protein extracts from the shoots of dichlormid-treated and control seedlings were assayed for GST activity towards a range of colorimetric substrates. Extracts from both tissues had highest GST activity towards CDNB, with progressively lower activities towards ethacrynic acid, NBC and DCNB (Table 5). No significant activity could be detected towards the substrates 4-vinylpyridine, crotonaldehyde, benzyl isothiocyanate, BSP, ENPP, PBO or NPB. Safener treatment resulted in a doubling of activity towards CDNB, ethacrynic acid and NBC, but did not significantly alter the activity towards DCNB.

	Enzyme activity* (pkat/mg protein)	
Substrate	- Dichlormid	+ Dichlormid
CDNB	2483 ± 67	5533 ± 500
DCNB	4 ± 1	4 ± 1
Ethacrynic acid	137 ± 45	300 ± 167
NBC	87 ± 5	173 ± 5

Table 5. GST activities toward xenobiotic substrates in crude extracts from shoots of untreated and dichlormid-treated etiolated maize (cv. Artus) seedlings. * Values given are means of duplicate determinations ± the variations between the mean and the replicates.

In contrast to the cytosolic preparations, in all cases the $MgCl_2$-precipitated membrane extracts, which contained approximately 2% of the total extracted protein, were found to have negligible activity towards CDNB (data not shown). Likewise, subsequent work

found that no activity towards any of the herbicides used could be detected in the $MgCl_2$-precipitated membrane extracts.

Having shown that dichlormid induced GST activity towards several xenobiotics in shoots, the root and shoot protein extracts were then assayed for the effect of dichlormid on GST activity towards a range of herbicides. The herbicides used were the chloro-*s*-triazine herbicides atrazine, cyanazine and simazine, the chloroacetanilide herbicides alachlor and metolachlor, the diphenyl ether herbicide fluorodifen, the aryloxyphenoxypropionate herbicide fenoxaprop and the sulphonylurea herbicide chlorimuron-ethyl. The HPLC-based assays described in chapter 2 were used to quantify the extent of enzyme-catalysed glutathione conjugation for each herbicide. As there was also evidence in the literature that GST activities can vary between maize cultivars (Timmerman, 1989), it was of interest to determine the relative GST specific activities toward the herbicides, and their respective sensitivities to safener treatment, in two differing varieties. Therefore in addition to cv. Artus, the cultivar Pioneer 3394 was also used and the results of this study are shown in Table 6. No GST activity could be detected towards the herbicides simazine, cyanazine, chlorimuron-ethyl and fenoxaprop in any of the preparations, and these herbicides were omitted from subsequent assays.

In untreated plants the relative distribution of GST activities toward the herbicides were similar in the roots and shoots in both cultivars, with the exception of atrazine-conjugating activity. GST specific activity towards atrazine was considerably higher in Pioneer 3394 than in Artus, especially in root tissue. In untreated shoots activities were in the order metolachlor = atrazine = alachlor > fluorodifen. In the roots the order was alachlor = metolachlor > atrazine > or = fluorodifen. Interestingly, the GSTs with activities toward chloroacetanilides were more highly expressed in the roots than in the shoots, while the opposite appeared to be the case for the activity toward atrazine, at least for the cultivar Artus. In contrast, the specific activities of the GST conjugating fluorodifen were similar in the two organs. Treatment of the seedlings with dichlormid revealed some interesting differences in the relative enhancements of the different activities. In the shoots of both cultivars safener treatment increased the activity towards alachlor three to four-fold, the activity towards metolachlor and fluorodifen approximately two-fold and had a negligible effect on atrazine-conjugating activity. In

the roots of both cultivars dichlormid treatment increased activities toward alachlor and metolachlor, but to a considerably lesser extent to that observed in shoots. Activity toward fluorodifen in the roots of both cultivars also increased on application of dichlormid, this increase was similar to that in shoots. However, the activity toward atrazine was only increased significantly by dichlormid treatment in Pioneer 3394 suggesting that varietal differences exist with respect to the inducibility of GST activities toward herbicides. Collectively these observations regarding the differential expression of the GST activities depending on organ type and safener treatment suggest that the regulation and substrate specificity of GST isoenzymes in maize was very complex, supporting earlier genetic (Rossini *et al.*, 1995) and biochemical (Dean *et al.*, 1991, Fuerst *et al.*, 1993) studies.

Cultivar and tissue	(-/+) dichlormid	Enzyme activity* (pkat/mg protein) Atrazine	Alachlor	Metolachlor	Fluorodifen
Artus					
shoot	-	1.7 ± 0.3	1.3 ± 0.4	2.4 ± 0.9	0.6 ± 0.2
	+	1.9 ± 0.3	4.6 ± 2.0	5.5 ± 0.9	1.5 ± 0.1
root	-	0.8 ± 0.2	14.1 ± 1.5	9.2 ± 1.9	0.7 ± 0.3
	+	0.5 ± 0.2	16.1 ± 1.7	17.2 ± 3.2	2.0 ± 0.2
Pioneer 3394					
shoot	-	2.4 ± 0.2	1.5 ± 1.4	3.8 ± 0.4	0.3 ± 0
	+	3.6 ± 0.8	5.9 ± 2.5	6.5 ± 0.6	0.7 ± 0.1
root	-	2.3 ± 0.4	15.3 ± 2.5	12.0 ± 1.2	0.5 ± 0.1
	+	5.3 ± 0.3	20.5 ± 1.0	18.0 ± 1.5	1.2 ± 0.1

Table 6. GST activities toward herbicide substrates in the roots and shoots of etiolated seedlings of the cultivars Artus and Pioneer 3394 treated with and without dichlormid. * Values given are means of duplicate determinations ± the variations between the mean and the replicates.

Effect of dichlormid on GST isoenzymes in maize roots and shoots.

GST activities from different plant organs and treatments were resolved using anion exchange chromatography to investigate the distribution of GST isoenzymes. 10 day etiolated maize seedlings (cv. Artus) treated with or without dichlormid were used in this study. Crude protein extracts from roots and shoots of these seedlings, containing identical amounts of total protein, were analysed by ion exchange chromatography on Q Sepharose to resolve major GST isoenzymes with activity towards CDNB (Mozer *et al.*, 1983, Dean *et al.*, 1991, Fuerst *et al.*, 1993, Miller *et al.*, 1994, Holt *et al.*, 1995). When fractions were assayed for activity towards CDNB both roots and shoots appeared to contain two major forms of GST (Figure 9). The elution of the isoenzymes was extremely similar to that obtained with extracts from dichlormid-treated maize on DEAE Sepharose (Mozer *et al.*, 1983) and Mono Q FPLC (Holt *et al.*, 1995). Thus the isoenzyme eluting first has been previously identified as *Zm* GST I-I, while the second isoenzyme is *Zm* GST I-II. On comparing the elution profiles under conditions of identical protein loading both untreated and dichlormid-treated roots contained higher activities toward CDNB than did the respective shoot extracts. The variation in the elution profile of *Zm* GST I-I in unsafened and safener-treated shoot extracts resulted from the relatively low resolution of the anion-exchange column and was not otherwise significant. In the shoot extract from untreated plants, as described in previous studies (Holt *et al.*, 1995), *Zm* GST I-II made a minor contribution to the total GST activity eluting from the column in untreated extracts but represented some 30% of the total activity in extracts from dichlormid treated plants. These results also showed that safener treatment resulted in an increase in *Zm* GST I-I as well as increasing *Zm* GST I-II in both the shoots and roots, with the relative induction being greatest in the roots. In untreated roots approximately 40% of the total GST activity toward CDNB was associated with *Zm* GST I-II, demonstrating that this isoenzyme is constitutively expressed in the roots.

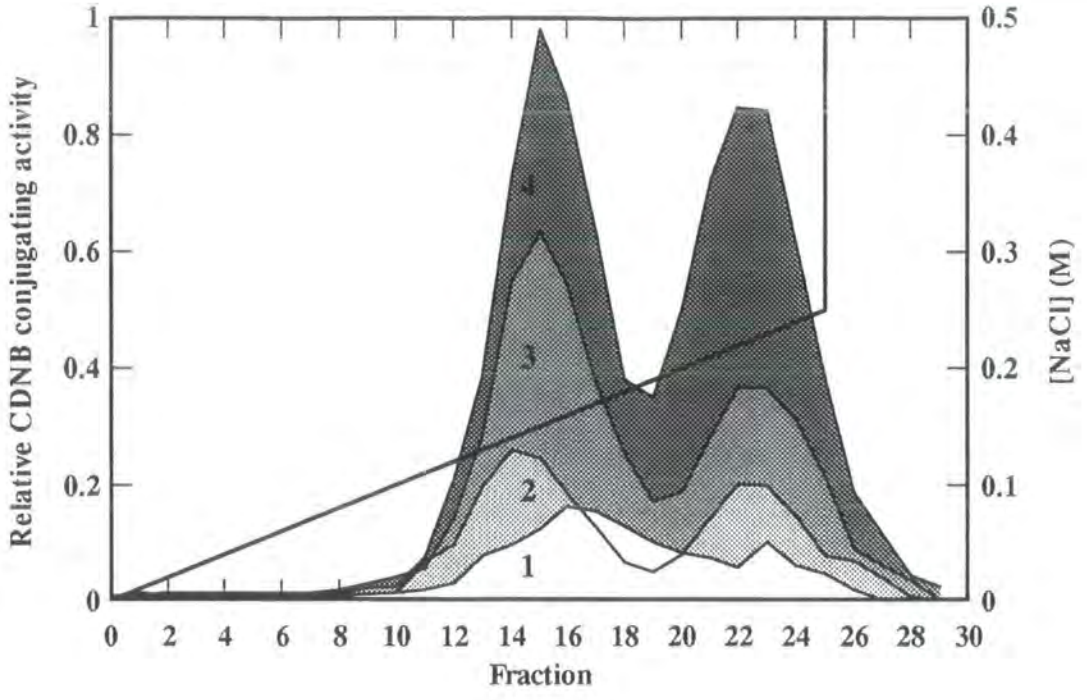

Figure 9. Anion exchange chromatography of GST isoenzymes in crude maize extracts. Chromatograms show profiles of GST activity towards CDNB in 1. untreated shoots, 2. dichlormid-treated shoots, 3. untreated roots and 4. dichlormid-treated roots. The first peak corresponds to *Zm* GST I-I and the second to *Zm* GST I-II. The increasing concentration of NaCl used to elute the isoenzymes is shown.

Discussion

GST Activity in Crude Extracts

Data for activities toward the xenobiotics used in the colorimetric assays in this study have been published for animals (e.g. Habig *et al.*, 1974, Asaoka, 1984, Clark, 1989) and sugarcane (Singhal *et al.*, 1991). However, apart from reporting GST activity towards CDNB no previously published data could be found on the activity of maize GSTs towards the colorimetric substrates used. Comparison of the data for maize with data for other species shows considerable differences, and illustrates the diversity of GST activities between species. For example, comparison with data from assays of crude sugarcane extracts (Singhal *et al.*, 1991) shows that while maize has double the activity towards CDNB, it has half the activity towards ethacrynic acid and 1% of the activity towards DCNB.

Analysis of cytosolic extracts and $MgCl_2$-precipitated membrane extracts showed that only the cytosolic preparation contained GST activity towards the substrates tested. It was therefore assumed that maize GSTs are essentially soluble enzymes. However, the membrane and cytosolic preparations were not assayed for marker enzyme activities, so the quality of these preparations was not known. Further work would be needed to show conclusively that GST activity was absent in maize membrane preparations.

Previous studies have shown that several safeners can enhance GST activities in maize (Kreuz *et al.*, 1989, Dean *et al.*, 1991), however most of these studies have concentrated on a single GST substrate and have not looked at activities towards different substrates. This study shows that treatment with the safener dichlormid roughly doubles the GST activities in maize towards CDNB, ethacrynic acid and NBC but did not affect the DCNB-conjugating activity.

With respect to herbicide substrates, GST induction studies with safeners have tended to concentrate on a single herbicide or class of herbicide, and have concentrated on shoot extracts. Both shoots and roots of untreated plants contained appreciable GST activity towards the herbicides atrazine, alachlor, metolachlor and fluorodifen. Safener treatment significantly increased GST activity towards alachlor and metolachlor

(chloroacetanilides) and the diphenyl ether herbicide fluorodifen in both roots and shoots. In cv. Artus, atrazine conjugating activity was not significantly changed by safener treatment while in cv. Pioneer 3394 the activity was modestly enhanced. Significantly, atrazine-conjugating activity in the roots and shoots of untreated Pioneer 3394 seedlings was much higher than in untreated Artus seedlings, suggesting that there were significant differences in the regulation of atrazine-active GSTs in the two cultivars.

Isoenzyme Induction

The elution profiles of CDNB-conjugating GSTs for shoot tissue obtained by anion exchange chromatography broadly agree with those published (Mozer *et al.*, 1983, Fuerst *et al.*, 1993) which showed that *Zm* GST I-I is the major GST in untreated shoot tissue and is slightly induced by safeners. *Zm* GST I-II was only expressed in shoots following dichlormid treatment, but was present constitutively in roots, which is in general agreement with earlier observations (Holt *et al.*, 1995). However the current studies gave novel insights into the inducibility of GST isoenzymes by herbicide safeners in the roots. Thus safener-treated roots of Pioneer 3394 contained the highest GST activities of any maize source tested. Therefore, in subsequent studies dichlormid-treated roots of Pioneer 3394 were used as the starting material for the further characterisation of GST isoenzymes and for the isolation of the respective cDNA clones.

4. Purification of Maize GSTs

Introduction

One of the major aims of this project was to purify and characterise the full complement of GSTs in maize, so it was important to develop a robust method to purify to homogeneity as many maize GSTs as possible. To achieve this, CDNB assays were routinely used to monitor purification, but since not all GSTs are necessarily active towards CDNB, fractions to be discarded from the purification were assayed for activity towards a selection of herbicides to ensure that no appreciable activity was lost.

Frear and Swanson (1970) were the first to publish a method for partially purifying maize GSTs active against the herbicide atrazine, and their use of ammonium sulphate precipitation and gel filtration resulted in a 7.6-fold purification of the enzyme activity. Later work by Guddewar and Dauterman (1979) described the 43-fold purification of atrazine-active GSTs using ion exchange, gel filtration and hydroxylapatite chromatography; it was unclear whether these GSTs were the same as those partially purified by Frear and Swanson (1970). Surprisingly, the major atrazine-active GSTs have yet to be purified to homogeneity and characterised.

GSTs from maize were first purified to homogeneity by Mozer *et al.* (1983), who purified 2 GSTs from safener-treated, etiolated tissue which he named GST I and GST II (*Zm* GST I-I and *Zm* GST I-II respectively, in our revised terminology). Crude extracts were fractionated by ammonium sulphate precipitation and two major CDNB-active GST activities were resolved by DEAE Sepharose ion exchange chromatography of the active fraction. *S*-bromosulphophthalein-glutathione affinity chromatography was then used to purify to near homogeneity the GST responsible for each peak of activity. Further maize GSTs have since been purified including *Zm* GST II-II, purified from the shoots of safener-treated maize seedlings by Irzyk and Fuerst (1993), using ion exchange chromatography and *S*-hexylglutathione affinity chromatography, and *Zm* GST III-III, purified by O'Connell *et al.* (1988), using *S*-alachlor-glutathione as an affinity ligand.

In all of the above cases, affinity chromatography was used to purify GSTs and this powerful technique has allowed GSTs to be purified from numerous other species, often giving pure or nearly pure GST preparations, sometimes with just a single-step purification. GST affinity chromatography typically uses a glutathione derivative to selectively bind GSTs and the same derivative, or glutathione, to specifically elute the GSTs. The most widely used affinity ligands have been *S*-hexylglutathione Sepharose and glutathione Sepharose, since they are commercially available or relatively easy to synthesise, and bind a large number of GSTs from a diverse range of source materials.

S-Hexylglutathione matrices have been used to purify GSTs from species including soybean (Flury *et al.*, 1995), pea (Edwards, 1996) and wheat (Williamson and Beverley, 1988), while glutathione matrices have been used to purify GSTs from numerous plant species including broccoli (Lopez *et al.*, 1994), sugar cane (Singhal *et al.*, 1991), *Hyoscyamus muticus* (Bilang *et al.*, 1993) and *Arabidopsis thaliana* (Watahiki *et al.*, 1995), and also from many non-plant sources, such as *E. coli* (Iizuka *et al.*, 1989, Arca *et al.*, 1990). Other less common affinity matrices used to purify GSTs include *S*-bromosulphophthalein-glutathione matrices, used to purify *Zm* GST I-I and *Zm* GST I-II (Mozer *et al.*, 1983, Edwards ad Owen, 1988), *S*-alachlor-glutathione, used to purify *Zm* GST III-III (O'Connell *et al.*, 1988), and Orange A agarose, used to purify *Zm* GST I-I and a further GST (probably *Zm* GST I-III) (Timmerman and Tu, 1987). Each of these affinity ligands typically only bind a subset of the GSTs present. For example, Mauch and Dudler (1993) showed that in wheat, a GST containing a 25 kDa subunit described as GST25, but not a 29 kDa subunit-containing GST called GST29, bound to an *S*-hexylglutathione affinity column while GST29, but not GST25, bound to Cibacron blue 3GA-agarose. Thus, a variety of different affinity ligands would have to be employed to purify as many GSTs as possible from a particular source.

While a number of maize GSTs have already been successfully purified, there are good reasons for trying to extend these studies. Firstly, the GSTs previously purified from maize remain relatively poorly characterised, with little data reported on substrate specificity and in some cases conflicting evidence of enzyme subunit composition and activity. There is also good evidence for the presence of as yet uncharacterised GSTs in maize. For example, anion exchange elution profiles of crude protein from safener

treated seedlings have shown unidentified peaks of activity towards CDNB and herbicides in addition to peaks corresponding to previously purified GSTs (Dean *et al.*, 1991, Fuerst *et al.*, 1993).

Results

Development of a Purification Method

To facilitate the purification of maize GSTs, a method was developed to first purify all the GSTs as a single pool from the majority of contaminating proteins, followed by fractionation of the individual GST types by affinity chromatography. A selection of purification techniques were initially assessed for their suitability for GST purification. A summary of the results of these initial studies is presented below.

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) using a phenyl Sepharose column was used to fractionate crude maize protein extracts. Samples were loaded onto the column and the unbound proteins removed by washing with 0.5M ammonium sulphate in buffer A (10 mM potassium phosphate buffer pH 7.4, 14 mM ß-mercaptoethanol). Under these conditions 100% of the applied GST activity was retained. After washing with buffer A 20% of GST activity towards CDNB was eluted. The majority (80%) of GST activity was eluted using 50% ethylene glycol, 50% buffer A and 2 mM glutathione, showing that the majority of maize GSTs with activity towards CDNB were unusually hydrophobic. This procedure typically gave a 6-fold purification of CDNB-conjugating activity with good yield.

Dye Affinity Chromatography

Affinity chromatography using an agarose-linked triazinyl dye (Amicon Orange A) was also tested as an initial step in maize GST purification, since this matrix had been previously used to purify bovine liver GSTs (Asaoka, 1984), two maize GSTs, probably *Zm* GST I-I and *Zm* GST I-III (Timmerman and Tu, 1987), and recombinant *Zm* GST I-I (Grove *et al.*, 1988) from *E. coli* crude extracts. GSTs were purified from crude maize protein extracts following the method of Asaoka (1984). The GST activity, which eluted

as a broad peak with 1 mM glutathione, was purified 14-fold, with 50% recovery of enzyme activity.

Ion Exchange Chromatography

Ion exchange chromatography using a Q Sepharose Fast Flow column was used to separate different GST isoenzymes with activity towards CDNB. Samples were loaded onto the column in low salt buffer (20 mM Tris-HCl pH 7.8, 14 mM β-mercaptoethanol) and eluted with a gradient of increasing NaCl concentration up to 0.5 M in the same buffer; fractions were assayed for activity towards CDNB. No CDNB-conjugating GST activity was detected in the column flow through on washing with low salt buffer, and 100% of the applied GST activity was recovered over the salt gradient, eluting in two distinct major peaks. No further GST activity was detected on washing the column in buffer containing 1 M NaCl.

Combined Purification Procedure

Since phenyl Sepharose is relatively inexpensive and robust, and could be used to purify a high proportion of the GST activities present, it was chosen as the initial purification step (Figure 10). Since samples were loaded in buffer containing ammonium sulphate from the previous precipitation step, this also meant that the proteins did not have to be desalted before chromatography. It was decided not to use affinity chromatography as the first stage in purification since such affinity matrices are often selective in their retention of GST isoenzymes, unstable and often do not work well with crude protein samples due to high levels of protein and inhibition of specific binding by contaminants in the extract. Following HIC on phenyl Sepharose, anion exchange chromatography on Q Sepharose was chosen as the next purification step, as the eluant from the HIC, containing 50% ethylene glycol and glutathione, could be loaded directly onto the column (Figure 10). After washing to remove ethylene glycol and glutathione, which might have interfered with subsequent chromatographic steps, GSTs could be eluted by application of buffer containing 0.5 M NaCl. After dialysis, dye-affinity chromatography using Orange A agarose was then used to further purify the GSTs. Assays on protein that did not bind the affinity column showed that although Orange A agarose bound a large proportion of the GST activity towards CDNB, some activity remained unbound. In addition, a substantial amount of herbicide conjugating activity also did not bind,

indicating that some GSTs were not being purified by this method. Initially, little work was done on these unbound GSTs, however in later studies, after Orange A chromatography the GSTs present in the unbound protein were purified using *S*-hexylglutathione affinity chromatography (Figure 10). Following affinity chromatography individual GSTs were resolved using anion exchange chromatography, since this method has been extensively used to successfully resolve maize GST isoenzymes in the past (Dean *et al.*, 1991, Fuerst *et al.*, 1993).

Summaries of the purification of GSTs from extracts of the roots of etiolated, dichlormid-treated maize cv. Artus (Table 7) and cv. Pioneer 3394 (Table 8) are shown.

Sample	Protein (mg)	CDNB activity (nkat/mg)	Purification (-fold)	Recovery (%)
Ammonium sulphate precipitate	420	10.3	1	100
Phenyl Sepharose	51	49.3	4.8	58
Q Sepharose	18	113.1	11	46
Orange A agarose	1.24	806.4	78	23

Table 7. Purification of GSTs from safener-treated Artus maize roots.

Sample	Protein (mg)	CDNB activity (nkat/mg)	Purification (-fold)	Recovery (%)
Ammonium sulphate precipitate	280	25	1	100
Phenyl Sepharose	45	147	5.8	93
Q Sepharose	18	277	11.0	71
Orange A agarose	2.2	1422	56.6	44

Table 8. Purification of GSTs from safener-treated Pioneer 3394 maize roots.

Purification of GST isoenzymes in maize.

Almost all GSTs purified from maize previously have been purified from the shoot tissue of seedlings, often after safener treatment to increase the levels and diversity of GSTs.

However, experiments have shown that for maize seedlings, roots consistently contain significantly higher GST activities toward both CDNB and herbicides when compared to the corresponding shoot tissue (Mozer *et al.*, 1983 and Table 6). It was therefore decided to use roots as the major source for GST purification, since this should give higher yields of GSTs and include GSTs not present in shoot tissue and therefore not previously studied. Dichlormid was used to increase levels of GSTs by both imbibing the seed and watering the plants with the safener solution as required with. Roots were then harvested from 10-day etiolated seedlings.

The overall purification method is shown in Figure 10. Using phenyl Sepharose chromatography, the majority of proteins were not retained under the loading conditions used, while all the detectable GST activity was bound to the column and eluted in two discrete fractions (Figure 11). The less hydrophobic fraction, representing 20% - 25% of the total GST activity towards CDNB recovered from the column, was eluted in the absence of ammonium sulphate. This fraction was termed the polar GST fraction. The majority of the GST activity towards CDNB could only be eluted in the presence of 50% ethylene glycol and this was termed the hydrophobic GST fraction. The further purification of the maize GSTs concentrated on the hydrophobic fraction, although the polar fraction was also analysed, as described towards the end of this chapter.

After removing the ethylene glycol by anion exchange chromatography the hydrophobic GSTs were affinity purified using Orange A agarose chromatography (Figure 12). With CDNB as substrate 8% of the applied activity was unretained on the column (unbound fraction) while 51% was recovered in the presence of glutathione. Reapplication of the unbound fraction confirmed that no further GST activity was retained on the Orange A agarose and so the unbound fraction contained GSTs with differing affinities to the ligand than were present in the bound fraction.

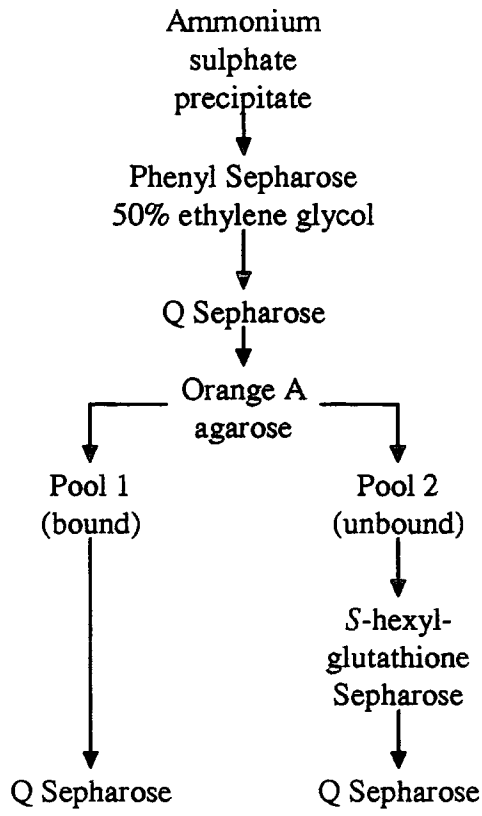

Figure 10. Overview of typical purification procedure for maize GSTs.

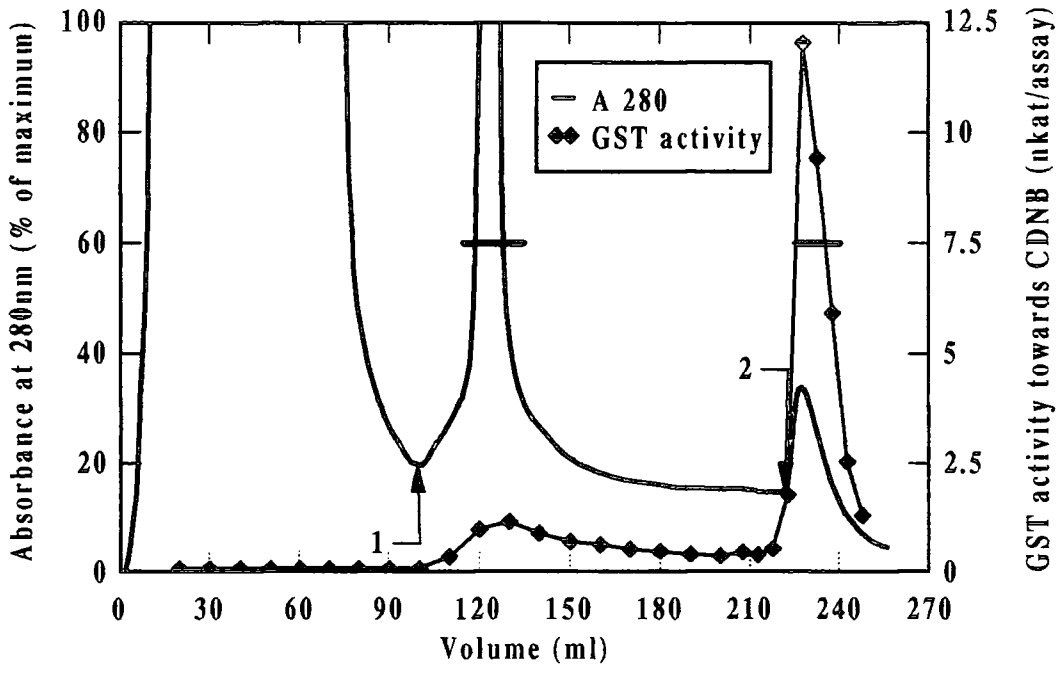

Figure 11. Purification of GST activity towards CDNB from dichlormid-treated maize roots by hydrophobic interaction chromatography using phenyl Sepharose. Absorbance at 280 nm and GST activity toward CDNB are shown. The arrows show (1) application of buffer A and (2) application of 50% buffer A, 50% ethylene glycol, 2mM glutathione. Horizontal bars indicate fractions pooled for further analysis.

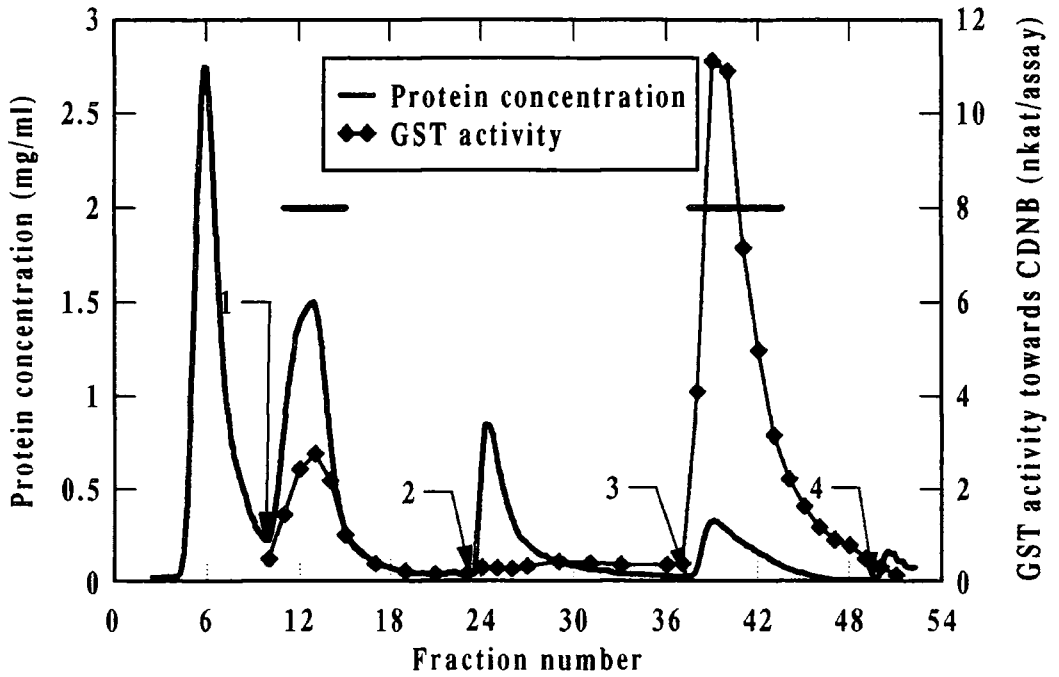

Figure 12. Purification of GST activity from dichlormid-treated maize roots by dye-affinity chromatography using Orange A agarose. Protein content and GST activity towards CDNB are shown. Arrows indicate (1) re-application of sample, (2) application of buffer D, (3) application of buffer D containing 2 mM glutathione and (4) application of buffer D containing 2 mM glutathione and 1 M NaCl. Horizontal bars indicate fractions pooled for further analysis.

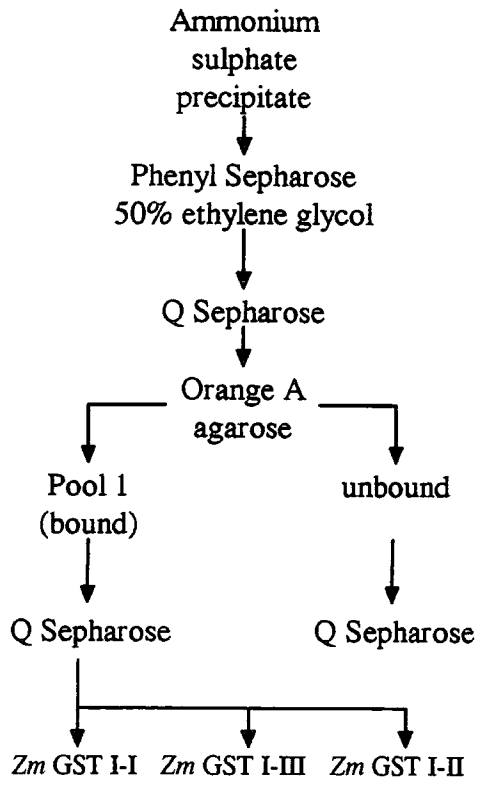

Figure 13. Overview of initial purification procedure for pool 1 GSTs

The bound fraction was termed pool 1 and accounted for the majority of the GST activity toward the herbicides alachlor, metolachlor, atrazine and fluorodifen applied to the Orange A column. Purification of pool 1 GSTs is summarised in Figure 13. Pool 1 GSTs were characterised in greater detail by resolving the GSTs present by anion exchange chromatography on Q Sepharose using a 1 ml HiTrap Q cartridge (Figure 14). Chromatography of the pool 1 GSTs showed the presence of three distinct UV-absorbing peaks and each fraction was assayed for activity towards CDNB and the herbicides atrazine, metolachlor and fluorodifen. These three herbicides were selected as each represents a major class of herbicides which are detoxified by GSTs in plants (Cole *et al.*, 1987), with atrazine representing the chloro-*s*-triazines, metolachlor the chloroacetanilides and fluorodifen the diphenyl ethers. These assays showed that the two major UV-absorbing peaks were clearly associated with distinct activities toward CDNB and atrazine. However, assays of fractions with metolachlor and fluorodifen suggested that the minor peak eluting at the trailing edge of the first major peak contained a discreet isoenzyme with high activities toward these herbicides.

To improve the resolution of this minor isoenzyme fractions 11 and 12 were dialysed and reapplied to the HiTrap Q cartridge. In the presence of increasing salt two UV absorbing peaks associated with GST activity were clearly resolved (Figure 15). The polypeptide composition of the individual fractions arising from anion exchange chromatography was analysed by silver staining following SDS-PAGE (Figure 16).

The first major UV-absorbing peak showing GST activity in Figure 14 and Figure 15, which eluted between 100 mM and 125 mM NaCl, contained a single polypeptide with a molecular mass (Mr) of 29.5 kDa (Figure 16). The respective isoenzyme appeared to be identical to the *Zm* GST I-I purified in earlier reports (Mozer *et al.*, 1983, Holt *et al.*, 1995) and this was confirmed by N-terminally sequencing a 5 µg sample of the protein. The amino acid sequence obtained, APMKLY, confirmed the presence of the *Zm* GST I subunit (Holt *et al.*, 1995).

The second UV-absorbing peak, eluting between 130 mM and 150 mM NaCl, was shown to contain 29.5 kDa and 28.5 kDa polypeptides (Figure 16). However, the two peaks still overlapped slightly, so the 29.5 kDa polypeptide in this second peak could

have been a contaminant from the first *Zm* GST I-I peak. To discount this, anion exchange fractions between the two peaks were also analysed by SDS-PAGE (Figure 16). This showed that the concentration of the 29.5 kDa polypeptide decreased after the first peak, before increasing again over the second peak. The 29.5 kDa polypeptide found in the second peak was therefore not a contaminant and was most likely present as a heterodimer with the 28.5 kDa polypeptide. Sequence analysis confirmed that the 29.5 kDa peptide was identical to that determined in *Zm* GST I, but no sequence was obtained for the 28.5 kDa subunit. Timmerman (1989) reported that the GSTs which bound to an Orange A affinity column contained an isoenzyme composed of 29 kDa and 26 kDa subunits, with the smaller subunit being identified as *Zm* GST III. We therefore termed this heterodimer *Zm* GST I-III.

The final peak of activity shown in Figure 14 which eluted in fractions 13 - 18 (165 - 200 mM NaCl) contained 29.5 kDa and 29 kDa subunits (Figure 16) and appeared to be *Zm* GST I-II. The identity of the 29.5 kDa GST I subunit was confirmed by N-terminal sequencing. As reported in earlier accounts no sequence was obtained from the 27 kDa *Zm* GST II subunit, which is N-terminally blocked in maize (Holt *et al.*, 1995).

The Mr values determined for the *Zm* GST subunits I and II were higher than previously reported (Timmerman, 1989, Holt *et al.*, 1995, Irzyk and Fuerst, 1993), probably due to differences in gel composition, and were significantly higher than the deduced molecular masses based on the published nucleotide sequences for the *Zm* GST I and II subunits (23.7 kDa and 24.4 kDa respectively) (Jepson *et al.*, 1994, Irzyk *et al.*, 1995). Purified *Zm* GST I-II was then used to raise antibodies in rabbits for further characterisation of the pool 1 GSTs, as described in chapter 5.

Anion exchange chromatography of the unbound fraction from the Orange A column gave the profile shown in Figure 17, showing that several distinct peaks of GST activity towards CDNB and fluorodifen were present in this fraction. Therefore, in subsequent purification runs the GSTs unretained on the Orange A column were applied to an *S*-hexylglutathione Sepharose column. 61% of the recovered GST activity towards CDNB bound to the *S*-hexylglutathione Sepharose column and was eluted with 5 mM *S*-hexylglutathione, and this fraction was termed the pool 2 GSTs, while 17% of the activity did not bind the column and 22% of the activity was non-specifically bound and was eluted from the column with 0.2 M KCl.

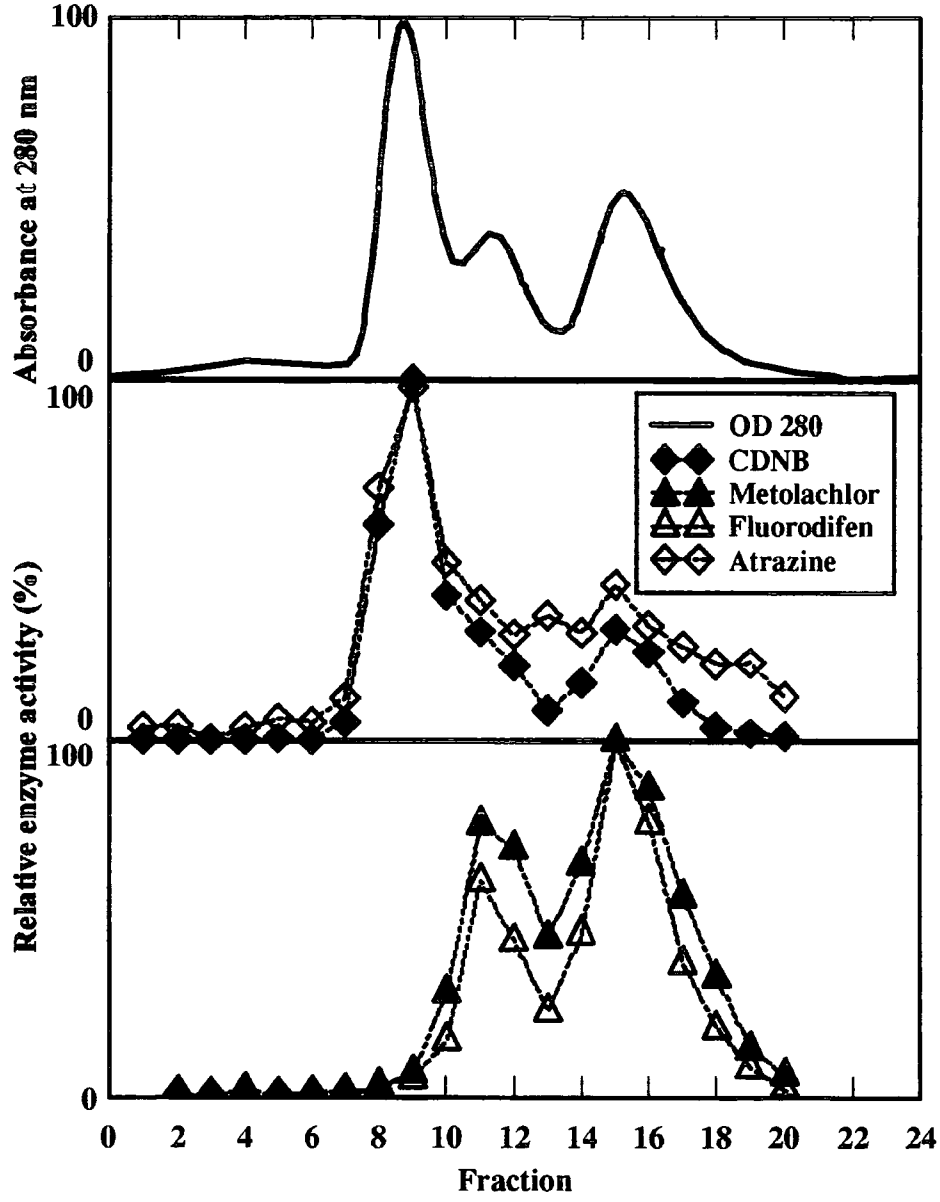

Figure 14. Resolution of pool 1 GSTs purified from dichlormid-treated maize roots by anion exchange chromatography using a 1 ml HiTrap Q column. Enzyme activities are expressed as a percentage of the maximum activity determined toward each substrate.

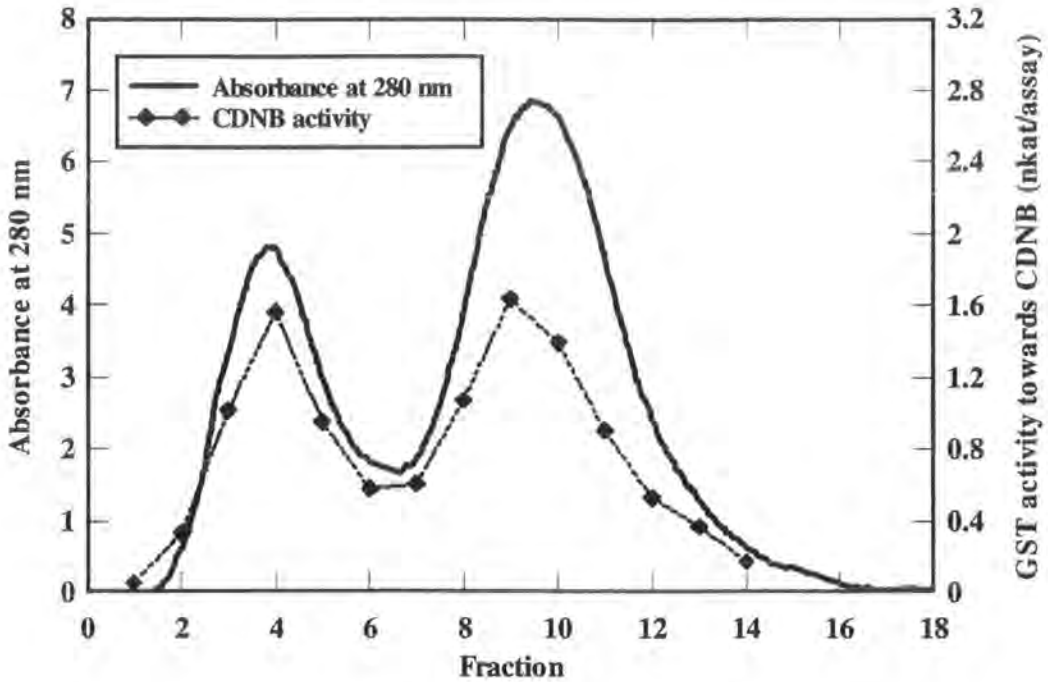

Figure 15. Re-chromatography of fractions 11 and 12 from the anion exchange chromatography of pool 1 GSTs (Figure 14). Proteins were eluted using a linear gradient of NaCl, from 100 mM at fraction 0 to 190 mM at fraction 18.

Figure 16. Silver-stained SDS-PAGE gel showing pool 1 GSTs resolved by anion exchange chromatography. Lanes 1 to 4 show fractions from the re-chromatography of fractions 11 and 12 from anion exchange chromatography of pool 1 GSTs (see Figure 15); lane 1 = fraction 4 (*Zm* GST I-I), lane 2 = fraction 6, lane 3 = fraction 8 and lane 4 = fraction 10 (*Zm* GST I-III). Lanes 5 and 6 show different protein loadings of *Zm* GST I-II and lane 7 shows molecular weight markers, with their molecular masses indicated.

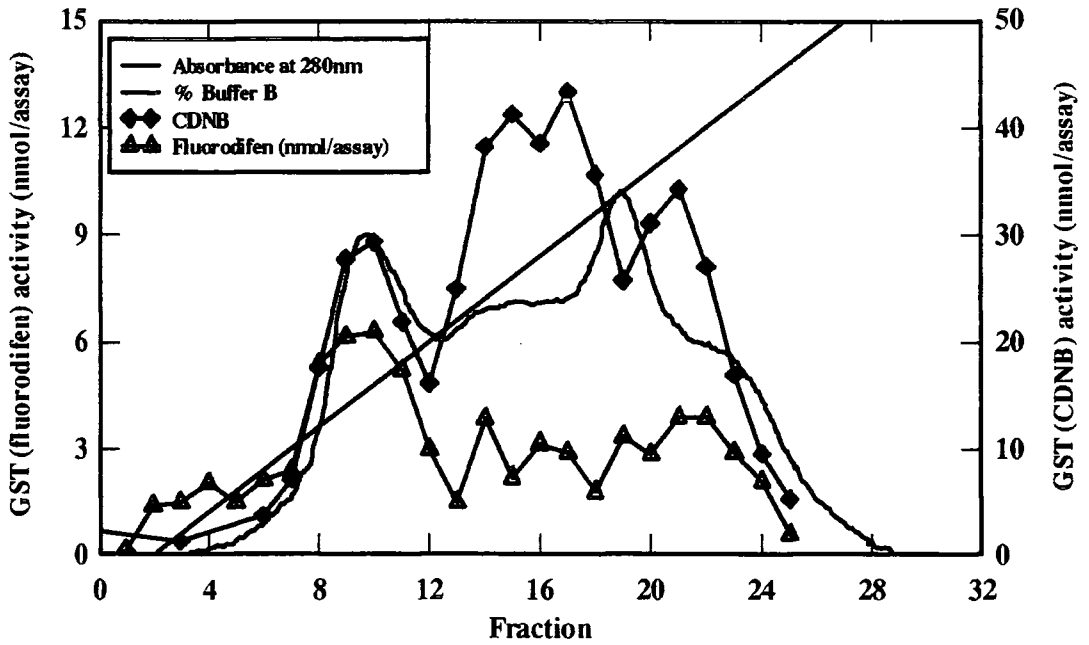

Figure 17. Anion exchange chromatography of protein unretained on Orange A agarose. Fractions were assayed for GST activity towards CDNB, and also toward fluorodifen using the colorimetric assay. Peaks of activity towards both substrates are present showing that a number of GST isoenzymes do not bind to the Orange A column.

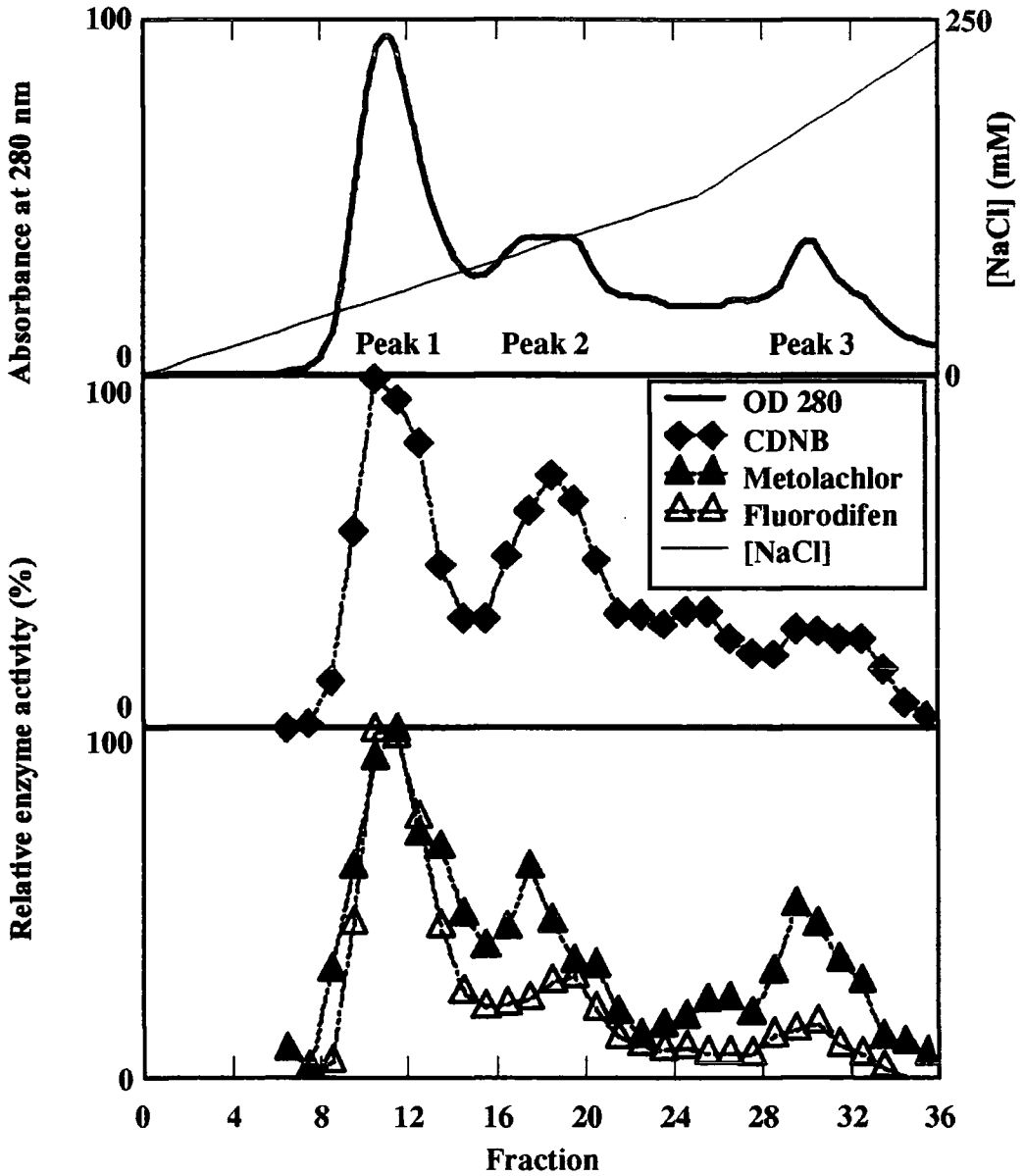

Figure 18. Resolution of pool 2 GSTs purified from dichlormid-treated maize roots by anion exchange chromatography using a 1 ml HiTrap Q column. Enzyme activities are expressed as a percentage of the maximum activity determined towards each substrate.

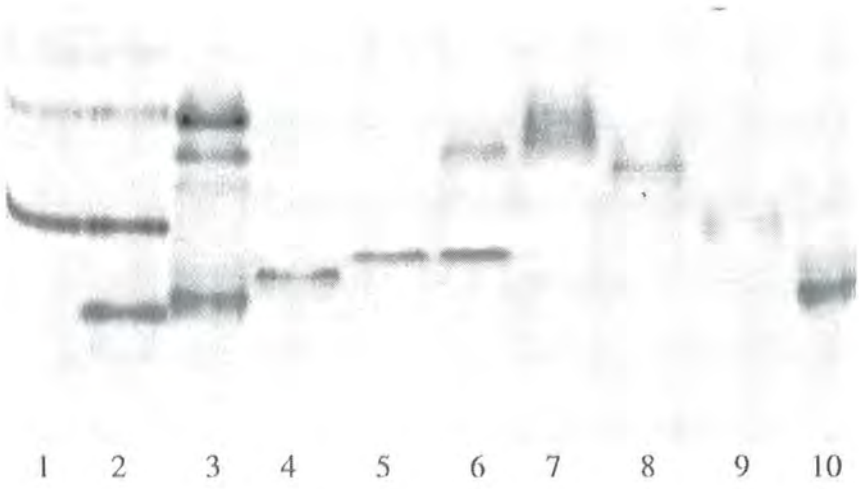

Figure 19. B/T-Blo-stained 10% acrylamide non-denaturing PAGE gel of purified maize GSTs. Lanes 1, 2, 3 and 10 show molecular weight standards; lane 1 = bovine serum albumin, lane 2 = bovine serum albumin & α-lactalglobulin, lane 3 = chicken egg albumin & carbonic anhydrase and lane 10 = chicken egg albumin. Lane 4 = *Zm* GST I-II and lane 5 = *Zm* GST I-I. For reference, the remaining lanes, discussed in later work, are: lane 6 = recombinant *Zm* GST I-I and *Zm* GST V-V, lane 7 = *Zm* GST V-V, lane 8 = *Zm* GST V-VI and lane 9 = putative *Zm* GST V-VII. Approximately 2 µg of each protein sample was loaded per lane.

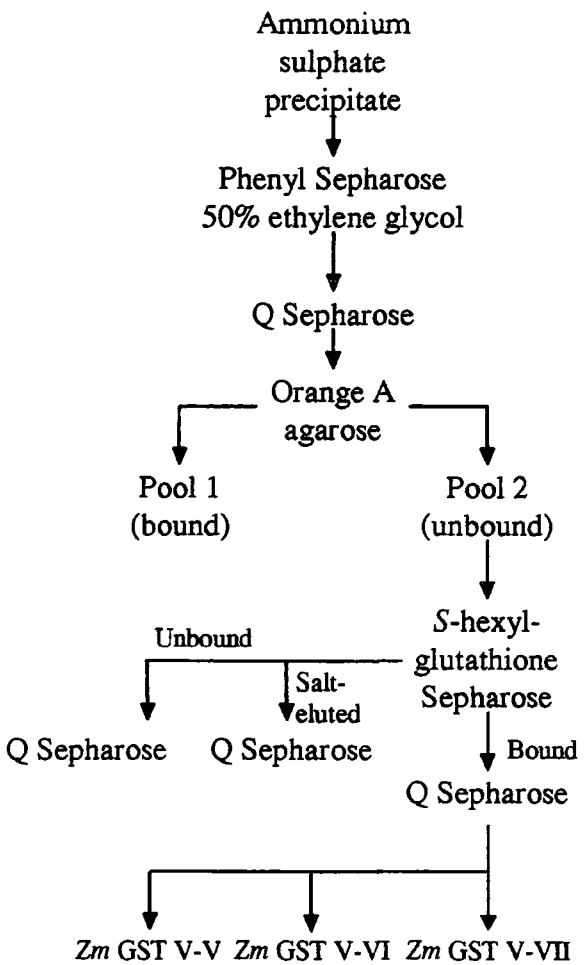

Figure 20. Overview of purification procedure for pool 2 GSTs.

An overview of the purification of pool 2 GSTs is shown in Figure 20. GSTs present in pool 2 which bound to *S*-hexylglutathione were resolved by anion exchange chromatography using a 1 ml HiTrap Q column, and were assayed for GST activities toward CDNB and the herbicides atrazine, fluorodifen and metolachlor (Figure 18). Following chromatography, three major UV-absorbing peaks were observed with the first two peaks, peak 1 and peak 2, being coincident with peaks of GST activity towards CDNB. Peaks 1 and 2 also coincided with peaks of activity toward fluorodifen and metolachlor. Peak 3 was associated with a minor peak of activity towards CDNB, metolachlor and fluorodifen. None of the fractions showed detectable GST activity towards atrazine.

When the pool 2 GSTs were analysed by SDS-PAGE, peak 1 was found to run as a single 28.5 kDa polypeptide, while peak 2 contained equal amounts of a 28.5 kDa polypeptide and a 27.5 kDa polypeptide (Figure 22). Peak 3 contained at least 4 different stained bands, with two major 29.5 kDa and 28.5 kDa polypeptides, and 2 minor polypeptides of 30 kDa and 27.5 kDa (Figure 23).

Non-denaturing PAGE was then used to determine the purity and native molecular weight of the proteins in peaks 1 and 2 (Figure 18), and also *Zm* GSTs I-I and I-II from pool 1. In this method relative molecular mass is determined from the mobility of protein during electrophoresis on gels with different acrylamide concentrations (Bollag and Edelstein, 1991). The stained polypeptides determined from one of these analyses is shown in Figure 19. Both *Zm* GST I-I and *Zm* GST I-II gave a single band, with estimated molecular masses of 48 kDa and 52 kDa respectively, thus showing that both proteins were pure and existed as dimers, as previously reported (Mozer *et al.*, 1983). Peak 1 was found to contain two major proteins running close to each other. Both proteins had a calculated molecular mass of 57 kDa. Peak 2 consisted of a major protein and a slightly faster running minor protein, both with a calculated Mr of 55 kDa.

Collectively, these results suggested that peak 1 contained two GSTs, each composed of 28.5 kDa subunits and with native molecular masses of approximately 57 kDa, while peak 2 contained a 55 kDa GST composed of a 28.5 kDa subunit and a 27.5 kDa subunit. As will be discussed later, an antibody raised to the heterodimer containing the

28.5 kDa and 27.5 kDa polypeptides (peak 2) recognised the 28.5 kDa polypeptide in the homodimer in peak 1 and this subunit therefore appeared to be common to both isoenzymes. Assuming that any multiple polypeptides present in peak 1 were similar enough to be grouped together, we termed the 28.5 kDa polypeptide *Zm* GST V and the 27.5 kDa polypeptide *Zm* GST VI. Using our nomenclature the GST in peak 1 was described as *Zm* GST V-V and the peak 2 isoenzyme *Zm* GST V-VI. Attempts to sequence the N-terminus of *Zm* GST V and *Zm* GST VI subunits by Edman degradation following SDS-PAGE and electroblotting of the purified polypeptides proved unsuccessful, suggesting that both subunits were N-terminally blocked. However, protein sequencing of *Zm* GSTs V-V and V-VI after storage of the pure proteins at 4 °C for 3 months both gave the amino acid sequence ENPVLK, which probably resulted from sequencing of degradation products following proteolysis. The sequences may have come from contaminating peptides, however this seemed unlikely since the same sequence was obtained from *Zm* GST V-V and *Zm* GST V-VI, and no such sequences were obtained when identically purified, fresh proteins were used for sequencing. This suggested that while both subunits were N-terminally blocked, degradation of the *Zm* GST V subunit gave rise to a cleavage product giving the above N-terminal amino acid sequence. This sequence did not correspond to any published sequences for maize GSTs or other proteins.

The results of a typical purification of GSTs from dichlormid-treated roots are summarised in Figure 21, which shows the distribution of GST activity towards CDNB in the various partially purified fractions and in the individual purified GST isoenzymes.

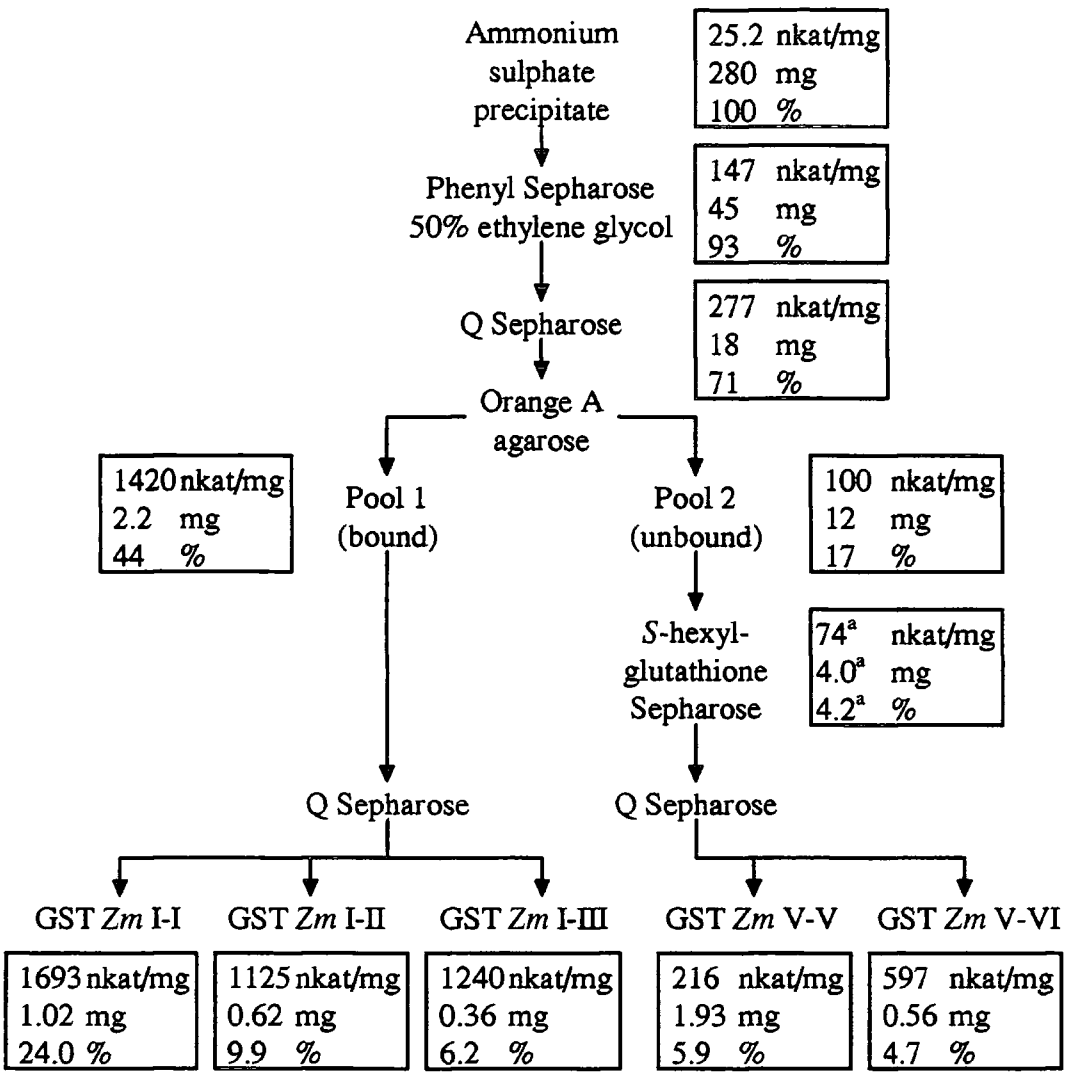

Figure 21. Flow chart showing the purification of pool 1 GSTs (*Zm* GST I-I, *Zm* GST I-II and *Zm* GST I-III) and pool 2 GSTs (*Zm* GST V-V and *Zm* GST V-VI), with the activity towards CDNB (nkat/mg), protein content (mg) and % recovery of enzyme activity towards CDNB (%) indicated. [a] = Activity inhibited by *S*-hexylglutathione.

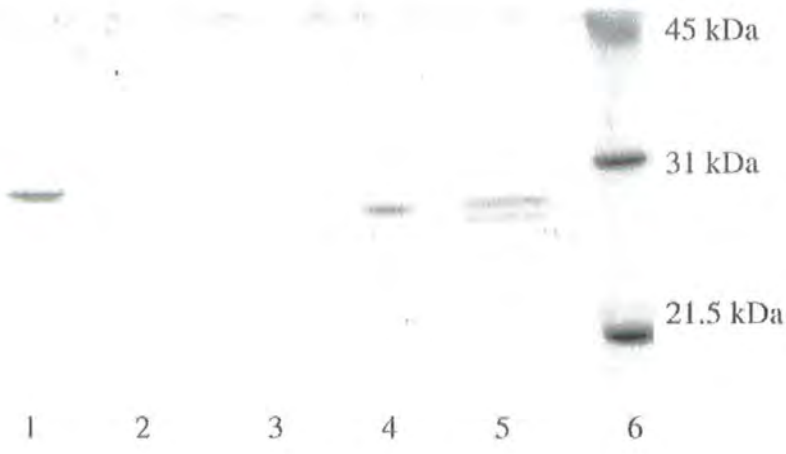

Figure 22. B/T Blu-stained SDS-PAGE gel of pool 1 and pool 2 GSTs. Lane 1 = *Zm* GST I-I, lane 2 = *Zm* GST I-II, lane 3 = *Zm* GST I-I (with slight contamination with *Zm* GST III subunit), lane 4 = *Zm* GST V-V, lane 5 = *Zm* GST V-VI and lane 6 = molecular weight standards, as labelled.

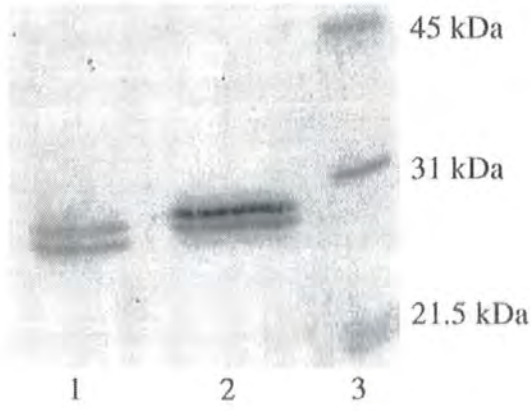

Figure 23. Silver-stained SDS-PAGE gel of peptides present in peak 3 of pool 2 GSTs. Lane 1 = *Zm* GST V-VI, lane 2 = polypeptides present in peak 3 (see Figure 18) and lane 3 = molecular weight standards, as labelled.

Purified *Zm* GST V-VI was then used to raise antibodies in rabbits for further characterisation of the pool 2 GSTs, as described in chapter 5. The antibody raised against *Zm* GST V-VI was used to examine the subunit composition of the GSTs present in peak 3 (Figure 18 and Figure 23). Western blotting following SDS-PAGE showed that the 28.5 and 27.5 kDa polypeptides present in peak 3 reacted strongly with antibody raised against *Zm* GST V-VI suggesting that these subunits were the same as those found in *Zm* GST V-VI. However, the 30 and 29.5 kDa polypeptides which were also present in peak 3 did not cross-react with either *Zm* GST I-II or *Zm* GST V-VI antibodies (Figure 31). Peak 3 was also analysed by non-denaturing PAGE, both by staining and by western blotting. Staining showed two closely spaced protein bands migrating in front of *Zm* GST V-V and *Zm* GST V-VI (Figure 19). Western blotting revealed that both these bands were strongly recognised by anti-*Zm* GST V-VI serum, and so both these proteins probably contained the *Zm* GST V subunit. Taken together these results suggested that the major protein present in peak 3 was a heterodimer of an unknown 29.5 kDa subunit and *Zm* GST V. N-terminal protein sequencing of the 29.5 kDa polypeptide gave the sequence SPXVKILGHY, which was similar to amino acid sequences of other plant GSTs. Since the 29.5 kDa polypeptide had an N-terminal amino acid sequence consistent with being a GST, appeared to form dimers with *Zm* GST V and was associated with GST activity, the polypeptide was assumed to be a previously uncharacterised GST subunit and was named *Zm* GST VII. The proposed heterodimer between this subunit and *Zm* GST V was thus called *Zm* GST V-VII.

Since maize contained multiple GSTs with similar ranges of activities, it was difficult to correct for the losses in *Zm* GST V-V and *Zm* GST V-VI maize during purification. However, 1.2 mg of *Zm* GST V-V was purified from 170 mg of total protein so assuming that *Zm* GST V-V was recovered quantitatively it would represent 0.7% of the total soluble protein in dichlormid-treated maize roots, while *Zm* GST V-VI would represent 0.2% of the total soluble protein. Similar calculations for the major pool 1 GSTs showed that *Zm* GST I-I would represent 0.4% of the total soluble protein and *Zm* GST I-II would represent 0.2% of the total soluble protein in dichlormid-treated maize roots.

Spectrum of GST activities associated with purified isoenzymes.

Pool 1 GSTs

The purified pool 1 GST isoenzymes *Zm* GST I-I, *Zm* GST I-III and *Zm* GST I-II were assayed with a range of xenobiotic and herbicide substrates and the specific enzyme activities are shown in Table 9. Each of the isoenzymes was shown to have distinct preferences for the xenobiotic substrates tested. *Zm* GST I-I had appreciable activity toward atrazine and was active toward alachlor, but showed little activity toward metolachlor and fluorodifen. *Zm* GST I-I also showed broad ranging activities toward the other xenobiotic substrates but had no detectable activity as a glutathione peroxidase. *Zm* GST I-III and *Zm* GST I-II showed a similar spectrum of activities toward the various substrates, though *Zm* GST I-II typically was more efficient in conjugating fluorodifen and alachlor. As compared with *Zm* GST I-I both *Zm* GST I-III and *Zm* GST I-II were less efficient in conjugating atrazine and the non-herbicide substrates and far more active toward fluorodifen and metolachlor. Interestingly, the *Zm* GST I-II fraction also had activity as a glutathione peroxidase with cumene hydroperoxide as substrate. As judged from SDS-PAGE analysis the *Zm* GST I-II preparation did not contain any contaminating polypeptides which could account for this peroxidase activity (Figure 16). As the *Zm* GST I subunit did not possess glutathione peroxidase activity it appeared that the activity was associated with *Zm* GST II and that this subunit was active as both a GST and glutathione peroxidase, as has been determined for GSTs in wheat flour (Williamson and Beverley, 1988), pea (Edwards, 1996), soybean (Skipsey *et al.*, 1997) and *Arabidopsis thaliana* (Bartling *et al.*, 1993).

It was possible to calculate the individual contribution made by the pool 1 isoenzymes in conjugating CDNB and each of the herbicides, assuming each isoenzyme was purified with similar recoveries. A summary of the distribution of GST activities toward CDNB and the herbicides in the purified isoenzymes from pool 1 is shown in Table 10.

Substrate	Enzyme activity (nkat/mg protein)		
	Zm GST I-I	*Zm* GST I-III	*Zm* GST I-II
Atrazine	0.11	0.07	0.07
Alachlor	1.47	1.25	3.20
Metolachlor	0.04	1.93	1.72
Fluorodifen	0.01	0.19	0.26
CDNB	1693	1240	1125
DCNB	1.10	0.18	0.24
NBC	58.17	18.67	21.00
NPB	0.57	0.28	0.31
Ethacrynic acid	27.0	19.7	25.0
Cumene hydroperoxide	ND	NT	3.16*

Table 9. Specific activities of GSTs purified from dichlormid-treated maize roots toward herbicide and xenobiotic substrates.

ND = None detected. NT = Not tested. *Activity expressed as change in absorbance at 366nm/min/mg protein.

	Activity (% of total recovered activity)		
Substrate	*Zm* GST I-I	*Zm* GST I-III	*Zm* GST I-II
CDNB	53.3	18.3	28.4
Atrazine	53.1	17.5	29.4
Alachlor	32.1	12.7	55.2
Metolachlor	1.5	39.4	59.1
Fluorodifen	2.3	28.9	68.8

Table 10. Activities of pool 1 GSTs purified from safener-treated roots.

The activities are shown as percentage of total activity recovered as the affinity bound fraction from the Orange agarose A column.

Pool 2 GSTs

The two major pool 2 GSTs, *Zm* GST V-V and *Zm* GST V-VI, were assayed for activity towards CDNB and the herbicides atrazine, metolachlor and fluorodifen (Table 11). Compared with activities for the pool 1 GSTs, *Zm* GST V-V and *Zm* GST V-VI had much lower activity towards CDNB, partly explaining why these GSTs have not previously been characterised. Unlike GSTs containing the *Zm* GST I subunit, neither *Zm* GST V-V nor *Zm* GST V-VI had detectable GST activity towards atrazine, however both these enzymes had moderate GST activity towards metolachlor. Both *Zm* GST V-V and *Zm* GST V-VI had high GST activity towards fluorodifen, which in both cases was nearly double that of *Zm* GST I-II, the pool 1 GST with the highest activity towards this substrate. It is therefore likely that these pool 2 GSTs contribute significantly to the total GST activity towards fluorodifen present in the roots of dichlormid-treated maize seedlings.

	Specific activity (nkat/mg protein)	
Substrate	*Zm* GST V-V	*Zm* GST V-VI
CDNB	216	597
Atrazine	ND	ND
Metolachlor	0.49	0.88
Fluorodifen	0.47	0.49

Table 11. Specific activities of purified *Zm* GST V-V and *Zm* GST V-VI.
ND - No activity detected.

Purification of GSTs from untreated roots

GSTs present in roots of maize seedlings which had not been treated with dichlormid were purified using the methods used for GSTs from dichlormid-treated roots. The fractions from anion exchange chromatography of the pool 1 and pool 2 GSTs purified by Orange A and *S*-hexylglutathione chromatography respectively were analysed by SDS-PAGE (Figure 24). The fractions with GST activity towards CDNB corresponding to *Zm* GSTs I-I, I-II and I-III were all present in untreated roots, but at lower levels than found in treated roots. In particular, although the *Zm* GST III subunit was visualised by

SDS-PAGE, no obvious protein or CDNB activity peaks corresponding to *Zm* GST I-III were visible following anion exchange chromatography of pool 1 GSTs (data not shown). This contrasted with the well defined peak of *Zm* GST I-III observed following anion exchange chromatography of pool 1 GSTs from dichlormid-treated roots, and suggested that levels of *Zm* GST I-III were enhanced more than *Zm* GST I-I and *Zm* GST I-II following dichlormid treatment. This observation would suggest that *Zm* GST III expression was enhanced by safener treatment. However, levels of *Zm* GST I-III purified from dichlormid-treated roots varied considerably between purification runs and was often much lower than shown in Figure 14. The low level of purified *Zm* I-III detected in untreated roots may therefore be due to inefficient purification, and the apparent substantial induction of *Zm* GST I-III by dichlormid treatment may be an artefact of variable yield on purification. GST *Zm* V-V and GST *Zm* V-VI were barely detected in untreated roots, suggesting that both these GSTs are considerably safener-induced.

Purification of GSTs from dichlormid-treated shoots

Zm GSTs I-I, I-II, V-V and V-VI were also purified from dichlormid-treated shoots of maize (cv. Pioneer 3394) etiolated seedlings. Resolution of pool 1 GSTs by anion exchange chromatography gave the profile shown in Figure 25. This profile was essentially similar to that obtained from dichlormid-treated roots, except that an additional early-eluting peak (fractions 6 to 8) was present. Analysis of the three peaks by SDS-PAGE and western blotting (Figure 26) confirmed that the second, large peak and the third smaller peak were *Zm* GST I-I and *Zm* GST I-II respectively. The first peak consisted of a 28.5 kDa polypeptide which was not recognised by anti-*Zm* GST I-II serum but was strongly recognised by anti-*Zm* GST V-VI serum (Figure 26). This peak therefore appeared to be *Zm* GST V-V, even though this protein had never previously bound to an Orange A affinity column. It is unclear why *Zm* GST V-V, or possibly a protein very similar to *Zm* GST V-V, should bind to an Orange A column when purified from shoot tissue but not when purified from root tissue.

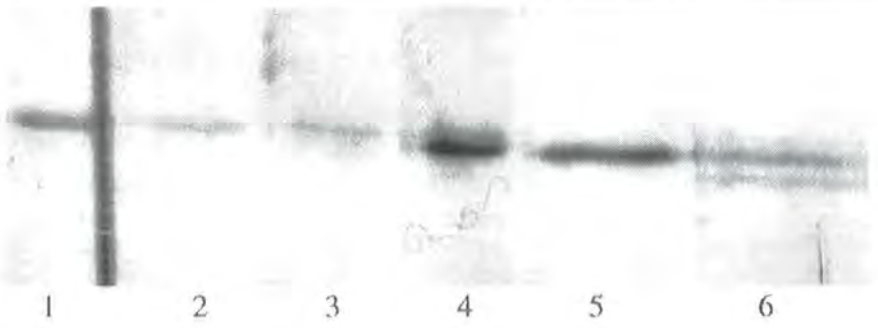

Figure 24. Silver-stained SDS-PAGE gel showing GSTs purified from roots of untreated etiolated maize seedlings. Lane 1 = *Zm* GST I-I, lane 2 = *Zm* GST I-I + *Zm* GST I-III, lane 3 = *Zm* GST I-III + *Zm* GST I-II, lane 4 = *Zm* GST I-II, lane 5 = *Zm* GST V-V and lane 6 = *Zm* GST V-VI.

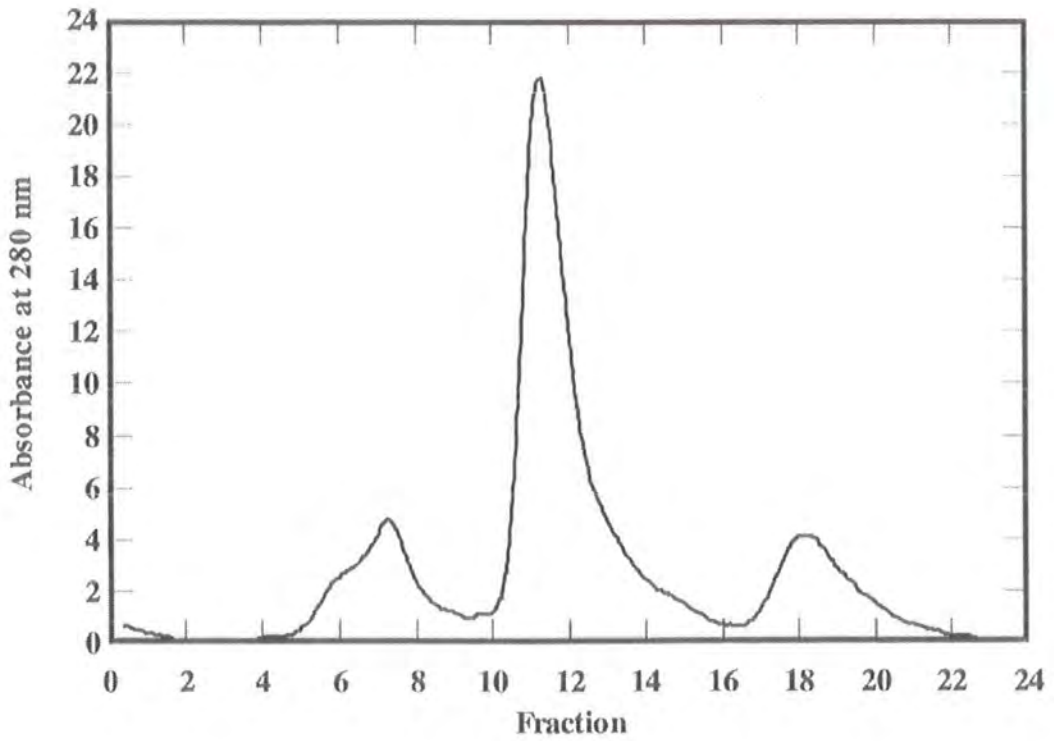

Figure 25. Resolution by anion exchange chromatography of pool 1 GSTs purified from shoots of dichlormid treated maize seedlings.

The major peak is *Zm* GST I-I, the early peak appears to be *Zm* GST V-V and the late peak is *Zm* GST I-II.

Anion exchange chromatography of pool 2 GSTs purified from dichlormid-treated shoots showed a more complicated profile than that found for similar GSTs purified from roots, with numerous overlapping protein peak visible (Figure 27). SDS-PAGE analysis of resolved protein peaks from pool 2 showed the presence of GST *Zm* V-V and GST *Zm* V-VI, but many of the fractions also contained a 29.5 kDa polypeptide, which remains to be identified but may be *Zm* GST VII (Figure 28). Western blotting confirmed the presence of the *Zm* GST V subunit (Figure 26), and also showed that the unidentified 29.5 kDa polypeptide was not recognised by either anti-*Zm* GST I-II serum or anti-*Zm* GST V-VI serum.

GSTs present in the HIC polar fraction

Most purification work concentrated on the hydrophobic GSTs recovered from the HIC column with ethylene glycol, since this fraction contained the majority of GST activity towards CDNB. However, 20 - 25% of GST activity towards CDNB was eluted in the polar fraction and it was of interest to determine whether the polar GSTs were similar to those present in the hydrophobic fraction or were distinct isoenzymes. Following HIC of extracts from the shoots of dichlormid-treated seedlings, GSTs in the polar fraction were further purified using Q Sepharose anion exchange chromatography followed by Orange A affinity chromatography, as described for purification from the hydrophobic fraction. Of the GST activity towards CDNB in the polar fraction, approximately 30% of the applied activity was retained on the Orange A column and specifically eluted with glutathione, suggesting the presence of pool 1 GSTs. Analysis of the polar pool 1 fraction by anion exchange chromatography (Figure 29) showed the presence of a single major CDNB-active protein peak, eluting in a similar salt concentration to *Zm* GST I-II and with a specific activity towards CDNB of 1060 nkat/mg, compared with a specific activity of 1125 nkat/mg for *Zm* GST I-II. Analysis of this protein by SDS-PAGE (Figure 30) showed that it consisted of equal amounts of two polypeptides, each running identically to the corresponding subunits of *Zm* GST I-II. This protein therefore appeared to be a heterodimer with very similar characteristics to *Zm* GST I-II. Protein sequencing of this heterodimer gave a single sequence (APMKLY), consistent with being the *Zm* GST I subunit. The lack of a second sequence suggested that the second polypeptide was N-terminally blocked. Considering all these results, it is very likely that

this protein purified from the polar fraction was *Zm* GST I-II. An additional UV-absorbing peak was also resolved by anion exchange chromatography from the polar pool 1 GSTs, which eluted earlier than *Zm* GST I-II (fraction 7, Figure 29). This peak had no detectable activity towards CDNB and no polypeptides could be visualised by silver staining following SDS-PAGE. It is unclear whether this peak was a GST, and further characterisation was not attempted.

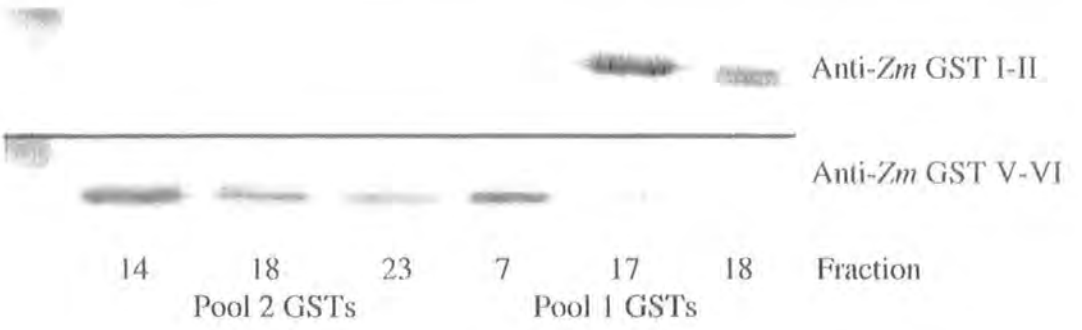

Figure 26. Western blots following SDS PAGE of purified pool 1 and pool 2 GSTs from shoots of dichlormid treated maize seedlings resolved by anion exchange chromatography. The blots were probed with antisera as indicated. For both blots, the left three lanes show pool 2 GST fractions (see Figure 27) and the right three lanes show pool 1 fractions (see Figure 25).

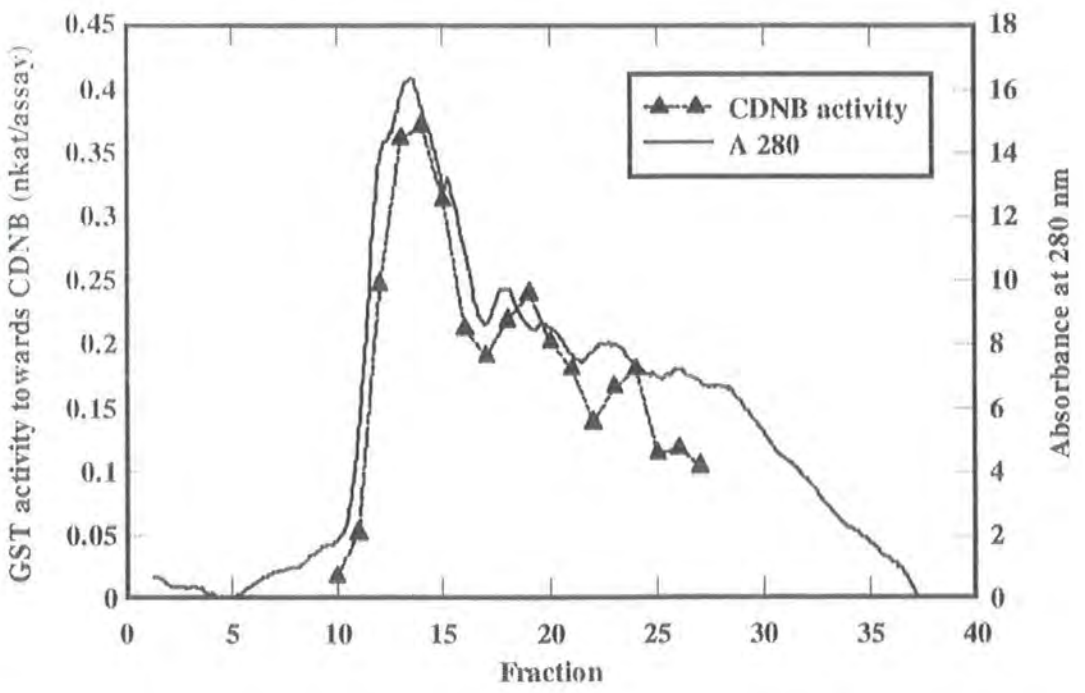

Figure 27. Resolution of purified pool 2 GSTs from shoots of dichlormid treated maize seedlings by anion exchange chromatography.

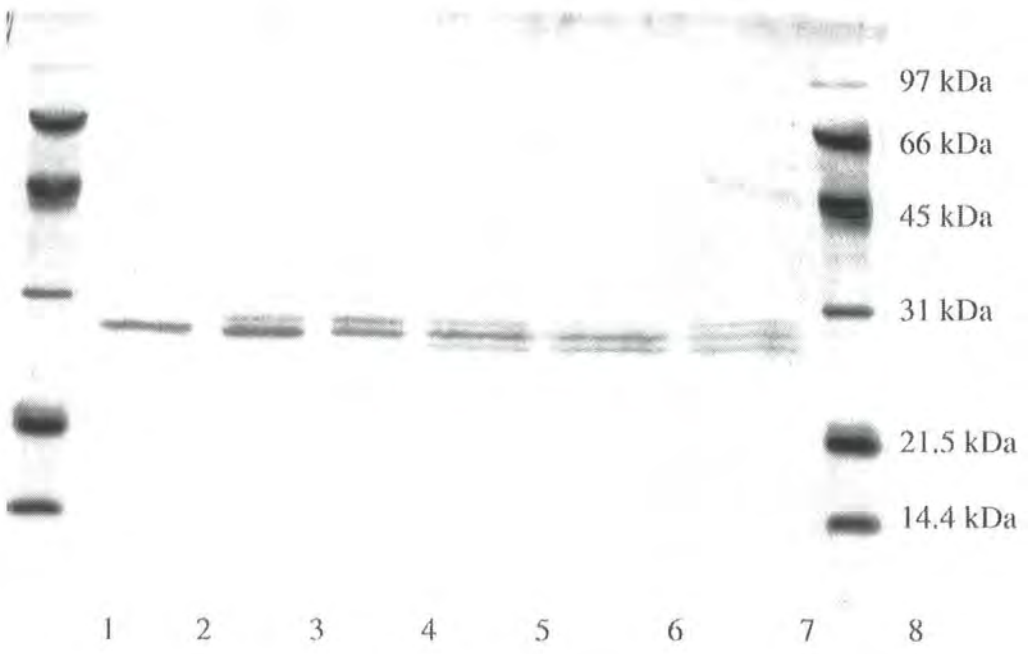

Figure 28. Silver-stained SDS-PAGE gel showing pool 2 GSTs purified from shoots of dichlormid treated maize seedlings, following resolution by anion exchange chromatography. Lanes 1 and 8 show molecular weight standards and lanes 2 to 7 show fractions eluting from the anion exchange column (Figure 27), with lane 2 = fraction 12, lane 3 = fraction 14 (*Zm* GST V-V), lane 4 = fraction 16, lane 5 = fraction 18 (*Zm* GST V-VI), lane 6 = fraction 20 and lane 7 = fraction 23.

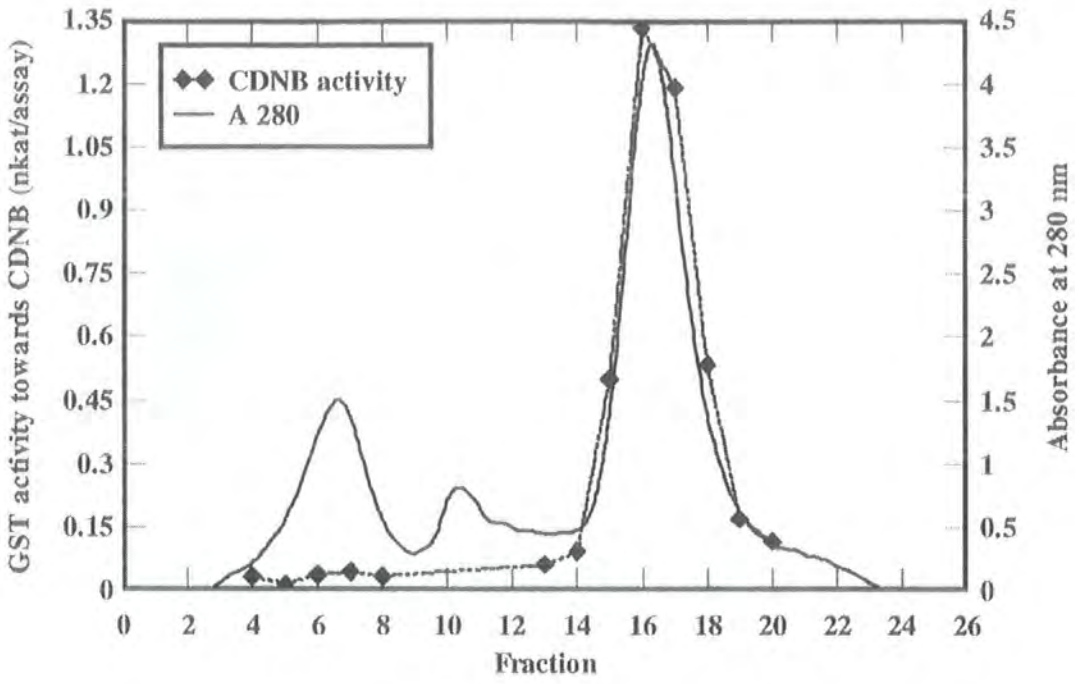

Figure 29. Resolution of purified polar pool 1 GSTs from shoots of dichlormid treated maize seedlings by anion exchange chromatography.

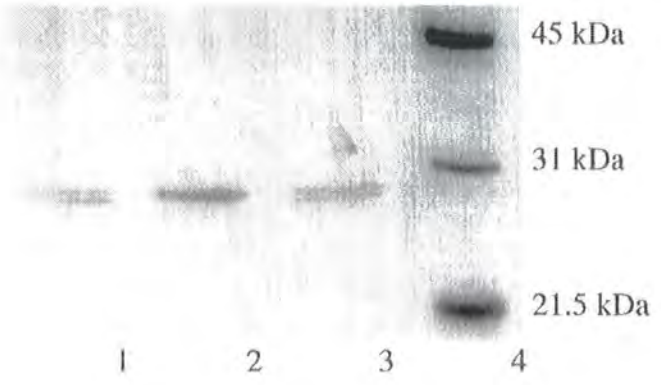

Figure 30. Silver-stained SDS-PAGE gel showing the major polar pool 1 GST.
Lane 1 shows the polypeptide composition of fraction 16 from Figure 29 which represents the major pool 1 GST present in the polar fraction (eluting in no salt buffer from a phenyl Sepharose column). Lane 2 = reference *Zm* GST I-I, lane 3 = reference *Zm* GST I-II and lane 4 = molecular weight standards as shown.

Discussion

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography using phenyl Sepharose CL-4B has previously been used to purify GSTs from wheat flour (Williamson and Beverley, 1988) and pea seedlings (Edwards, 1996). As found in maize, wheat and pea GSTs with activity towards CDNB were rather hydrophobic, but unlike maize GSTs did not require ethylene glycol for elution. However, in both cases it is possible that further GST activity would have been recovered from the HIC column if ethylene glycol had been used, as was the case for the majority of the GST activity in maize. For example in wheat, only 24% of the GSTs with activity towards CDNB were recovered from an HIC column without the use of ethylene glycol, which is much lower than the nearly quantitative recovery of maize GSTs from an HIC column achieved when 50% ethylene glycol was used as the final eluant.

Pool 1 GSTs

Orange A dye affinity chromatography has been used previously to purify maize GSTs (Timmerman and Tu, 1987, Timmerman, 1989), but characterisation of the purified enzymes was not reported in detail. In these earlier studies, following affinity chromatography two enzymes were purified using a Mono Q anion exchange column. The isoenzyme which eluted first was a homodimer of 28 kDa subunits (*Zm* GST I-I in our terminology) and the second isoenzyme consisted of the same 28 kDa subunit (*Zm* GST I) and an additional, slightly smaller subunit which was identified as the *Zm* GST III subunit. However, from the work of Timmerman and Tu (1987) it was unclear whether the later eluting GST composed of the *Zm* GST I and *Zm* GST III subunits was present as the *Zm* GST I-III heterodimer or as homodimers of *Zm* GST I-I and *Zm* GST III-III. The work presented here has shown a similar isoenzyme profile for GSTs purified from safener-treated maize roots, with *Zm* GST I-I eluting first, and the *Zm* GST III subunit present in a second peak eluting as a shoulder on the first peak (Figure 14). The results presented show conclusively that *Zm* GST III is present in this second peak as a heterodimer with *Zm* GST I to give the *Zm* GST I-III heterodimer, rather than as *Zm*

GST III-III contaminated with *Zm* GST I-I (Figure 15). A third enzyme peak identified as *Zm* GST I-II was also resolved, eluting after *Zm* GST I-III. Timmerman and Tu (1987) did not report such an isoenzyme, presumably as they used untreated shoot tissue as a protein source, which does not contain the *Zm* GST II subunit (Holt *et al.*, 1995) rather than safener-treated roots, as used in this study.

All three proteins purified from safener-treated maize roots using Orange A chromatography (i.e. pool 1 GSTs, see Figure 16) were GSTs containing the *Zm* GST I subunit, while other GSTs previously identified in maize (*Zm* GST III-III, O'Connell *et al.*, 1988, and *Zm* GST II-II, Irzyk and Fuerst, 1993) were not detected in this affinity-purified fraction. It therefore seems that under the conditions used, the Orange A column specifically bound isoenzymes containing the *Zm* GST I subunit. Interestingly Orange A, a triazinyl dye, has some structural similarity with atrazine and may act by binding at the enzyme active site. This is particularly appealing as *Zm* GST I is the only well characterised maize GST subunit shown to have activity towards atrazine. However other, uncharacterised atrazine-active GSTs do not bind to the Orange A column (Timmerman and Tu, 1987), and the reasons for the specificity towards the *Zm* GST I subunit remain unclear.

Pool 2 GSTs

In contrast to Orange A-agarose columns, *S*-hexylglutathione columns have been used to purify many different GSTs and other glutathione-dependent enzymes, such as glyoxalase I, from a diverse range of organisms (Mannervik and Guthenberg, 1981, Edwards, 1996, Paulus *et al.*, 1993), and might be expected to bind a number of different maize GST subunits. However, all the proteins purified by *S*-hexylglutathione in this study (pool 2 GSTs) appeared to contain the *Zm* GST V subunit, suggesting that in maize, this matrix is specific for a single subunit.

While there have not been earlier reports on the purification of *Zm* GST V-V, there is evidence in the literature that this protein has been previously observed in crude protein extracts. For example, Mono-Q anion exchange chromatography of total protein from benoxacor treated seedlings (Dean *et al.*, 1991) showed the presence of metolachlor-conjugating activity in early eluting fractions which was not present in similar analyses of

untreated seedlings (identified as peak 2 in Figure 2 from Dean *et al.*, 1991). A similar experiment by Fuerst *et al.* (1993) also identified an early-eluting peak of metolachlor-conjugating activity in extracts from safener-treated seedlings (peak A in Figure 1). A low level of CDNB-conjugating activity was also associated with peak A, in agreement with the GST activity observed for *Zm* GST V-V. Miller *et al.* (1994) reported the presence of a similar peak of GST activity in extracts from safener-treated maize suspension cultures. These peaks of activity correspond well with data obtained for *Zm* GST V-V in this study in terms of GST activity, behaviour on anion exchange columns and safener-inducibility. Since this study has shown that *Zm* GST V-V appears to be the most abundant previously uncharacterised maize GST in safener-treated maize seedlings it is highly likely that its presence gave rise to the peaks of GST activity described above.

It was also possible that pool 1 GSTs were also present in some of the fractions eluting from the *S*-hexylglutathione column. Some GST activity towards CDNB either did not bind the *S*-hexylglutathione column, or was eluted on washing with 0.2 M KCl. Preliminary studies using anion exchange chromatography showed that the unretained GSTs in this fraction resembled *Zm* GST I-I and *Zm* GST I-II (data not shown), and it seems likely that the majority of CDNB activity not purified by *S*-hexylglutathione was due to a small proportion of these contaminating pool 1 GSTs which did not bind to the Orange A column. If necessary this could be tested by probing western blots of the appropriate fractions with anti-*Zm* GST I-II serum.

Other GSTs

While the purification methods used were able to purify and resolve numerous GST isoenzymes, some GSTs known to exist in maize were not purified. The atrazine-specific GSTs partially purified by Frear and Swanson (1970) were not purified in this study. This is almost certainly due to their low levels of expression in root tissue, from which GSTs were routinely purified (Hatton *et al.*, 1996). *Zm* GST II-II, purified by Irzyk and Fuerst (1993), should have been present in the tissue used for purification, since the *Zm* GST II subunit was present in the *Zm* GST I-II heterodimer. The failure to locate *Zm* GST II-II was probably due to its negligible activity towards CDNB, as this activity was used to identify the GSTs in this study. *Zm* GST II-II has previously been purified using

S-hexylglutathione affinity chromatography from benoxacor-treated maize (Irzyk and Fuerst, 1993) and therefore if it were present in the pool 2 GSTs which were unretained on the Orange A column it would be expected to be found in the pool 2 GSTs, giving rise to a late-eluting peak when subsequently analysed by anion exchange chromatography. As no such peak was observed (Figure 18), it is possible that *Zm* GST II-II was not present in the tissue used for purification. One potential explanation is since this GST was not in the unretained fraction from the Orange A column it may have been loosely bound to the dye column and eluted in the salt wash which was not further analysed in this study. Proteins in the salt wash were shown to have negligible CDNB activity (Figure 12) and were therefore not investigated further, however *Zm* GST II-II has little or no CDNB activity (Irzyk and Fuerst, 1993) and may have been present. Another potential explanation for the absence of this homodimer, despite the presence of the *Zm* GST II subunit in heterodimers, was suggested by Rossini *et al.* (1996). Their work showed that in the alachlor-susceptible maize inbred line H99, *Zm* GST I-II was present but *Zm* GST II-II was absent, while in other lines tested *Zm* GST II-II was present. They suggested that line H99 may contain an altered gene coding for *Zm* GST II, resulting in a subunit that can form heterodimers with *Zm* GST I, but cannot form homodimers with other *Zm* GST II subunits. It is possible that Pioneer 3394, the source of material for purification, also contained such *Zm* GST II subunits impaired in their ability to homodimerise.

Similarly, *Zm* GST III-III was not purified from Pioneer 3394, although it should have been present. It is likely that it did not specifically bind either of the affinity matrices used, and was present in either the flow-through or salt wash from the *S*-hexylglutathione column. Previous work has identified *Zm* GST III-III as co-chromatographing with *Zm* GST I-II when crude extracts were analysed on anion exchange columns (Fuerst *et al.*, 1993). Thus *Zm* GST III-III may have been co-chromatographing with *Zm* GST I-II in the non-specifically bound fractions from *S*-hexylglutathione chromatography.

Non-denaturing PAGE

Analysis of purified GSTs by non-denaturing PAGE (Figure 19) showed that *Zm* GST I-I, *Zm* GST I-II and *Zm* GST V-VI gave a single major band, as expected. However, *Zm* GST V-V gave a less well defined smear, consisting of at least two resolved bands. Thus, the purified *Zm* GST V-V preparation was not homogenous, but consisted of at least two slightly different proteins. These proteins therefore probably represent either isoenzymes of different charge or distinct proteins.

GST Substrate Specificity

Comparison of the substrate specificities of the maize GSTs purified in this work with those of other plant GSTs is difficult due to the limited published data available. Singhal *et al.* (1991) used a similar range of non-herbicide substrates in assays on GST purified from sugarcane leaves and showed, like the maize enzymes, that this GST had activity towards a number of different substrates, although activities were generally significantly lower than those determined for *Zm* GST I-I, *Zm* GST I-II and *Zm* GST I-III. The purified maize enzymes had a similar range of activities as has been determined for a number of rat GSTs (Habig *et al.*, 1974), with comparable values obtained for specific activities.

Zm GST I-II, and to a lesser extent *Zm* GST V-V, showed significant glutathione peroxidase activity towards cumene hydroperoxide, a model organic hydroperoxide. It is unlikely that this activity was due to contamination by other enzymes since the activity was reproducible, the enzyme preparations appeared pure as judged by SDS-PAGE and in the case of *Zm* GST V-V the activity was also present when the enzyme was expressed in *E. coli* and purified (see chapter 8). Also, no activity was detected towards hydrogen peroxide, which should have been present in enzyme preparations contaminated with conventional glutathione peroxidases. The presence of this activity is interesting, firstly, because the reaction mechanism must be different to that for GST activity. While the transferase activity involves a substitution reaction requiring a single glutathione molecule, the peroxidase activity involves the oxidative coupling of two glutathione molecules together with the reduction of the peroxide substrate to its corresponding alcohol and the release of one water molecule:

$$R\text{-}OOH + 2\ GSH \rightarrow R\text{-}OH + GS\text{-}SG + H_2O$$

Secondly, the observed glutathione peroxidase activity may well play a role in protection against oxidative stress, giving these GSTs a natural function in plant metabolism. Plants under oxidative stress produce lipid and other hydroperoxides which must be detoxified to prevent damage to the plant. Other peroxidases are no doubt important in this respect but it is unclear whether GSTs with peroxidase activity significantly enhance the detoxification of peroxides.

5. Immunological Studies on Maize GSTs

Introduction

The study of the regulation of expression of GSTs in plants has evoked considerable interest and has provided some tantalising clues as to the endogenous functions of these enzymes. For example, GSTs which are expressed in stressed plants are likely to play roles in protecting the plant or recovering from the stress. While the purification techniques described in the previous chapter are useful in characterising GST isoenzymes, their use would be limited when studying GST expression, since large amounts of plant material and time are needed to analyse a single sample. An alternative approach, measuring the activity towards a particular substrate for a range of samples, is more practical but is not very specific, since multiple GSTs may have similar activities. One very effective way of analysing protein expression is the use of antibodies and western blotting, allowing the regulation of individual isoenzymes to be investigated in many samples, each derived from a small amount of material.

The expression of a number of theta class plant GSTs have been studied, and although protein sequence is reasonably well conserved in this group the expression patterns have been shown to vary considerably. For example, *Zm* GST I is expressed constitutively throughout maize plants, while *Zm* GST II only shows constitutive expression in young roots and is expressed in other tissues in response to safener treatment and senescence (Holt *et al.*, 1995, Jepson *et al.*, 1994). The *Arabidopsis thaliana* GSTs ERD11 and ERD13 are inducible by drought stress, but not by auxins (Kiyosue *et al.*, 1993), while wheat GST29 expression is induced by pathogen (*Erysiphe graminis*) attack and abiotic elicitors such as glutathione, but not by xenobiotics (Mauch and Dudler, 1993). An mRNA encoding HmGST-1 in *Hyoscyamus muticus* is induced by 2,4-D but not by IAA, NAA or metolachlor (Bilang and Sturm, 1995).

Tau class plant GSTs were originally identified as genes encoding auxin-induced or auxin binding proteins (Takahashi *et al.*, 1989). Many of these GSTs are induced in response to auxin treatment. However not all tau class GSTs are auxin responsive, and many are

induced by a variety of other treatments. For example, wheat GST25 and GST26 proteins, which are immunologically related to a tau class GST from maize (Cummins *et al.*, 1998), were expressed at enhanced levels following treatment with cadmium, atrazine, paraquat or alachlor (Mauch and Dudler, 1993). In tobacco, transcription of *parA* (Takahashi *et al.*, 1995) and *parC* (Takahashi and Nagata, 1992a) genes was strongly induced by the auxin 2,4-D. However, no such induction was observed for the closely related gene *C-7* (Takahashi and Nagata, 1992a), demonstrating the very different regulation of otherwise very similar genes. Soybean HSP26a mRNA was induced by auxins, and also by heavy metals, heat shock, salicylic acid, jasmonic acid, glutathione and DTT (Ulmasov *et al.*, 1995). *Arabidopsis thaliana GST5* mRNA was unaffected by auxin treatment, but was induced by heat shock or wounding (Watahiki *et al.*, 1995). Potato *prp1-1* (pathogenesis related protein 1) mRNA was induced by the pathogen *Phytophthora infestans* (Hahn and Strittmatter, 1994). From this short account it is clear that tau class GSTs cannot be considered to be exclusively induced by auxins.

Antibodies were therefore raised against purified pool 1 and pool 2 GSTs and were used to study the expression of these GST isoenzymes both in healthy tissue during plant development, and when plants were subjected to various treatments. The availability of antibodies also allowed other studies to be undertaken, including the investigation of GST dimerisation and immunocytochemistry.

Results

Characterisation of antibodies raised to different classes of maize GSTs

The major GST heterodimers purified from the pool 1 and pool 2 GSTs, *Zm* GST I-II and *Zm* GST V-VI (see chapter 4), were used to raise polyclonal antibodies in rabbits. Sera from test bleeds were used to characterise the specificity of the antibodies by probing western blots of both purified, and crude, maize GST preparations following SDS-PAGE (Figure 31). It was confirmed that pre-immune sera did not recognise any polypeptides in crude maize extracts (data not shown). The lanes containing purified GSTs showed that the antibody raised to *Zm* GST I-II recognised both the *Zm* GST I

and *Zm* GST II subunits, and reacted weakly with the *Zm* GST III subunit, but did not recognise the *Zm* GST V or *Zm* GST VI subunits. The anti-*Zm* GST V-VI serum recognised the *Zm* GST V subunit and to a lesser extent the *Zm* GST VI subunit, but not the *Zm* GST I, II and III subunits. When crude maize root extracts were probed with *Zm* GST I-II antibody only polypeptides similar in size to *Zm* GST subunits I, II and III were recognised by the antibody. The antibody also recognised smaller polypeptides, but since these polypeptides were also present in the lane containing purified *Zm* GST I-II it is likely that these are degradation products of one of the *Zm* GST subunits, so this antibody appeared to be highly specific for *Zm* GST subunits I, II and III. Crude protein samples probed with *Zm* GST V-VI antibody showed that the major reactive polypeptides corresponded to the *Zm* GST V and VI subunits. Again, immunoreactive lower molecular mass polypeptides were probably GST degradation products. A number of high molecular weight polypeptides also cross-reacted with the antibody (not shown). The identity of these was unclear, although they may have been high molecular weight proteins immunologically related to GSTs or GSTs covalently attached to other cellular components. The *Zm* GST V-VI antibody therefore seemed to specifically recognise *Zm* GST V and *Zm* GST VI subunits.

From these studies it was demonstrated that the anti-*Zm* GST V-VI serum did not recognise *Zm* GST subunits I, II or III, and the anti-*Zm* GST I-II serum did not recognise the *Zm* GST V or VI subunits. The pool 1 GSTs (*Zm* GST subunits I, II and III), purified by Orange A chromatography, were thus immunologically distinct to the pool 2 GSTs (*Zm* GST subunits V and VI), purified by *S*-hexylglutathione chromatography.

Changes in GST expression in response to plant age and development.

Changes in the relative expression of the *Zm* GST I, *Zm* GST II and *Zm* GST V subunits during the growth and development of maize were monitored by western blotting following SDS-PAGE using antibodies raised against *Zm* GST I-II and *Zm* GST V-VI (Figure 32). Using the anti-*Zm* GST I-II serum the 29.5 kDa *Zm* GST I and 29.0 kDa *Zm* GST II subunits were visualised, as well as an additional 27 kDa polypeptide. This 27 kDa polypeptide had not been recognised by the antiserum in previously analysed plant extracts (see Figure 31) and appeared to be due to the limited proteolysis of *Zm* GST I

which occurred during the dialysis of the crude extracts. Thus, the relative levels of the immunodetected 27 kDa polypeptide degradation product related well with those of *Zm* GST I in all tissues.

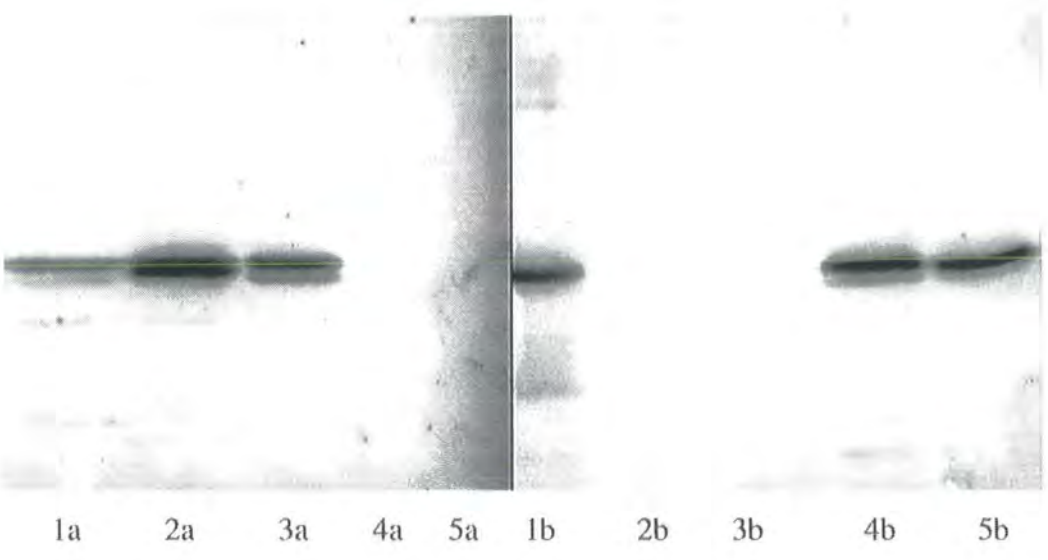

Figure 31. Western blots of polypeptides resolved by SDS-PAGE probed with a = anti-*Zm* GST I-II serum and b = anti-*Zm* GST V-VI serum. Lane 1 = total protein from 10-day etiolated maize roots, lane 2 = purified *Zm* GST I-II, lane 3 = purified *Zm* GST I-III, lane 4 = purified *Zm* GST V-VI and lane 5 = purified *Zm* GST V-VII (peak 3 from pool 2 GSTs; see Figure 23).

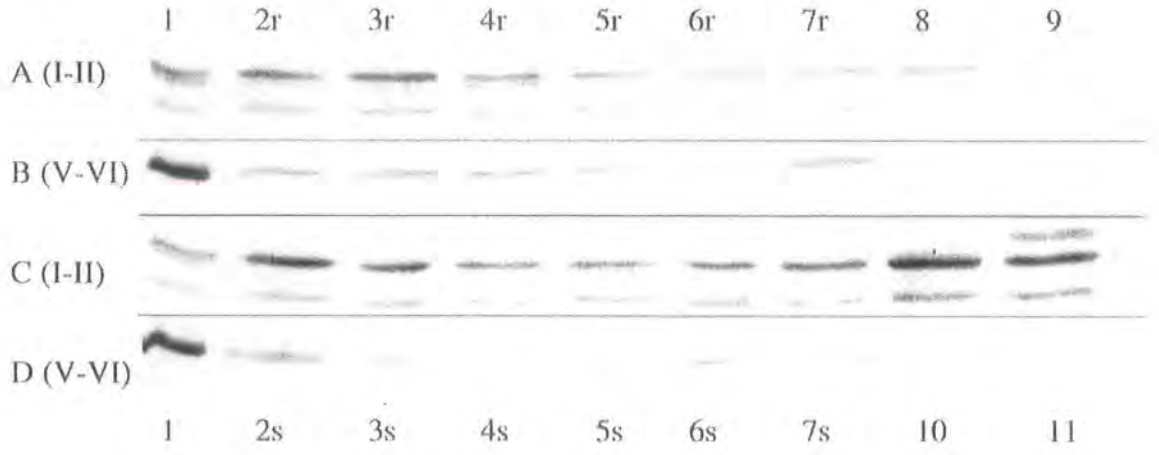

Figure 32. Composite image of western blots of polypeptides from developing maize plants following resolution of crude protein extracts by SDS-PAGE. Lane 1 is 12 µg of total protein from 10-day old dichlormid-treated etiolated maize roots, lane 2 = 7-day tissue (r = roots, s = shoots), lane 3 = 13-day tissue, lane 4 = 17-day tissue, lane 5 =24-day tissue, lane 6 = 32-day tissue, lane 7 = 38-day tissue, lane 8 = tassels, lane 9 = mature stem, lane 10 = silks and lane 11 = immature kernel. Blots A and B show similar samples probed with anti-*Zm* GST I-II serum and anti-*Zm* GST V-VI serum respectively and blots C and D show similar samples also probed with anti-*Zm* GST I-II serum and anti-*Zm* GST V-VI serum respectively. Except for lane 1, each lane on blots A and B represents 12 µg of total protein while each lane on blots C and D represents 60 µg of total protein.

Analysis of samples using anti-*Zm* GST I-II serum facilitated the monitoring of pool 1 GST subunits (Figure 32). Levels of immunodetectable *Zm* GST I decreased slightly in both root and shoot with age. At all time points, on the basis of % extracted protein, immunodetectable *Zm* GST I was five times more abundant in root tissue compared with shoot tissue. In mature plants *Zm* GST I was also present in male and female floral tissues, immature kernels and mature stems. *Zm* GST II could only be detected in the roots of young seedlings, while *Zm* GST III, which reacted poorly with the antibody used, could only be detected in 13-day roots. Immature kernel tissue also contained a larger polypeptide (about 32 kDa molecular mass) which cross-reacted with the antibody; this protein was not observed in other tissues.

Using anti-*Zm* GST V-VI serum, expression of *Zm* GST V remained constant throughout development in roots and shoots (Figure 32). Again on the basis of % of extracted protein *Zm* GST V was five-fold more abundant in root tissue than shoot tissue. *Zm* GST V was barely detectable in male floral tissue and stem tissue but was present in higher amounts in female floral tissue and immature kernels. The *Zm* GST VI subunit, which was poorly recognised by the antibody, was not detected in any of the samples.

Induction of GSTs by heat shock and chemical exposure

The production of antibodies which were specific to two major classes of maize GSTs also facilitated the study of the differential regulation of *Zm* GST I, *Zm* GST II and *Zm* GST V subunits in maize seedlings exposed to heat-shock or a variety of chemical treatments. Following SDS-PAGE of crude extracts from experimental plants, the resolved polypeptides were analysed by western blotting using either the antibody raised to *Zm* GST I-II, or to *Zm* GST V-VI. The experiments were carried out in duplicate, and both replicates are shown in Figure 33. Table 12 shows a summary of the treatments and the inducibility of *Zm* GST subunits when seedlings were subjected to these treatments. Anti-*Zm* GST V-VI serum identified the *Zm* GST V subunit as being expressed in control shoots and roots exposed to water containing the solvents used to dissolve the treatments. Although recent studies have shown that exposure to 1% v/v ethanol, but not 0.5% acetone, can elevate GST expression in wheat (Cummins *et al.*,

1998), the use of solvent carriers did not appear to enhance the expression of *Zm* GST I, *Zm* GST II or *Zm* GST V subunits in maize, except possibly for a slight induction of *Zm* GST II in shoots by 1% ethanol. Relative to controls, *Zm* GST V expression in the roots was unaffected by all treatments except dichlormid, which caused a minor increase in immunodetectable polypeptide. In the shoots, dichlormid treatment resulted in a major increase in *Zm* GST V, with CDNB and heat shock also giving a slight enhancement of expression.

			Subunit induction †					
			Zm GST I		*Zm* GST II		*Zm* GST V	
Treatment	Concentration	Solvent*	R‡	S‡	R	S	R	S
Ethanol control	-	E	-	-	-	-	-	-
Acetone control	-	A	-	-	-	-	-	-
Glutathione	5 mM	E	-	-	-	+	-	-
CDNB	1 mM	E	-	-	-	++	-	+
2,4-D	100 µM	E	-	-	-	+++	-	-
NAA	100 µM	E	-	-	+	++	-	-
Dichlormid	5 µg/ml	A	-	-	+	++	++	+++
Fluorodifen	100 µM	A	-	-	+	+	-	-
Alachlor	100 µM	A	-	-	+	+	-	-
Atrazine	100 µM	A	-	-	-	-	-	-
Heat-shock	-	E	-	-	-	-	-	+

Table 12. Summary of inducibility of *Zm* GST subunits in 11 day old seedlings by heat shock and a range of chemical treatments. * A = 0.5% v/v acetone, E = 1% v/v ethanol. † (-) = no induction, (+) = slight induction, (++) = moderate induction and (+++) = substantial induction. ‡ R = Root tissue, S = Shoot tissue.

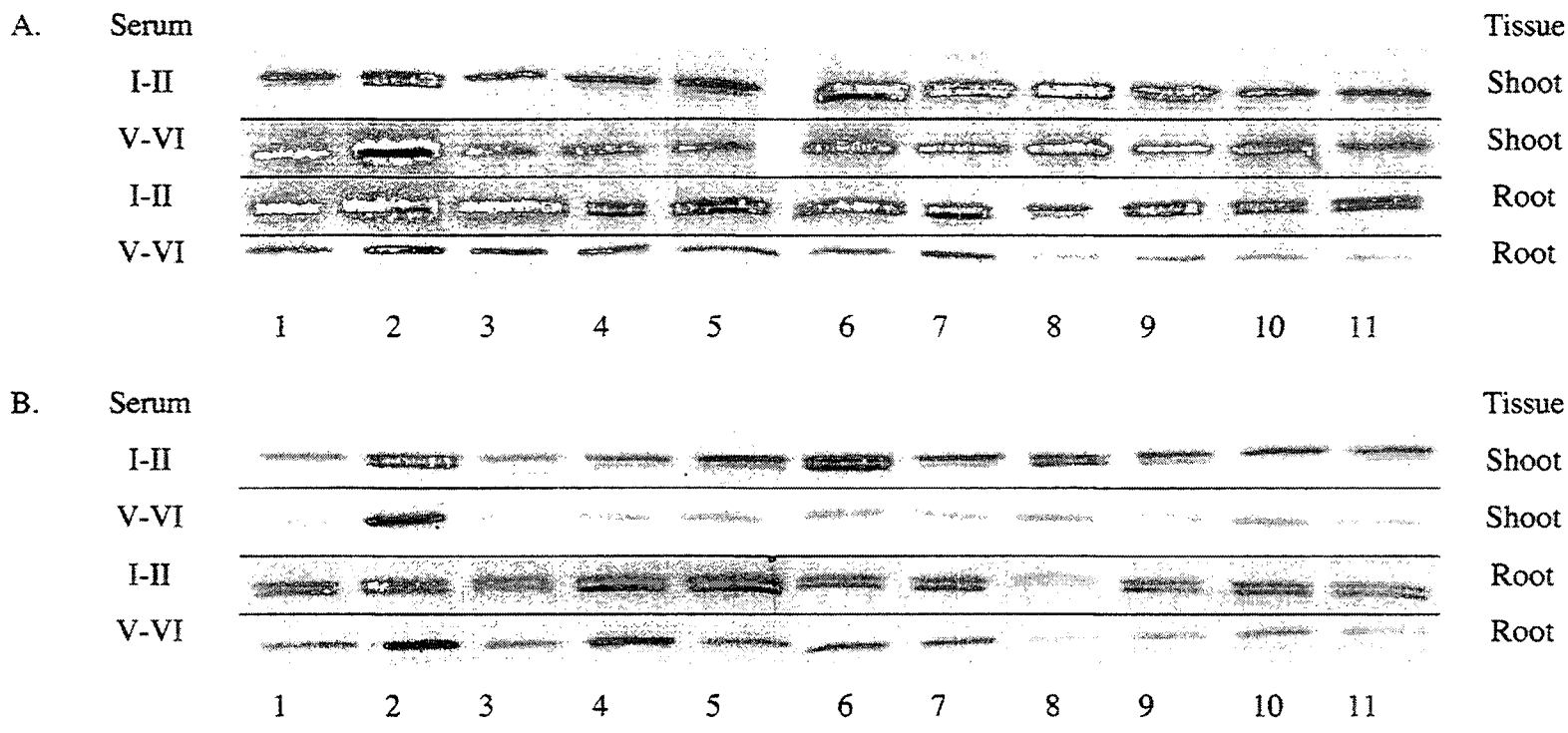

Figure 33 A and B. Composite images showing western blots of polypeptides resolved by SDS-PAGE of identical amounts of crude protein isolated from the roots and shoots of maize seedlings exposed for 24 h to: 1 = 0.5% acetone (control), 2 = 0.1 mM dichlormid, 3 = 0.1 mM atrazine, 4 = 0.1 mM alachlor, 5 = 0.1mM fluorodifen, 6 = 0.1 mM 2,4-D, 7 = 0.1 mM NAA, 8 = 1 mM CDNB, 9 = 5 mM glutathione, 10 = heat shock and 11 = 1% ethanol (control). Bands labelled I-II were probed with anti-*Zm* GST I-II serum and bands labelled V-VI were probed with anti-*Zm* GST V-VI serum. For simplicity, only the bands corresponding to immunodetectable GSTs are shown. A and B are identical replicates.

As reported in previous studies, using the anti-*Zm* GST I-II serum both *Zm* GST I and *Zm* GST II subunits were observed in untreated roots, while only the *Zm* GST I subunit was observed in the untreated shoots. Expression of *Zm* GST I in the roots was unaffected by heat shock or any of the chemical treatments, while *Zm* GST II expression was enhanced in response to treatment with NAA, alachlor, fluorodifen and dichlormid. In the shoots, *Zm* GST I expression was again unaffected by any of the treatments. The *Zm* GST II subunit accumulated in the shoots following treatment with 2,4-D, dichlormid, NAA and CDNB, and to a lesser extent following treatment with glutathione and fluorodifen. Despite the low affinity of the *Zm* GST I-II antibody for *Zm* GST III, western blots detected this subunit as a very faint band in all of the samples, and no obvious induction of *Zm* GST III by any of the treatments was observed. In roots, treatment with CDNB appeared to reduce the levels of *Zm* GST subunits I, II and V to below control levels. This may be as a result of the phytotoxicity of CDNB at the concentration used.

Induction of GSTs over time by Dichlormid Treatment

Since the maize safener dichlormid caused a significant accumulation of both *Zm* GST V and *Zm* GST II subunits a more detailed induction study was carried out with dichlormid in dark-grown seedlings. Whole seedlings (roots + shoots) were extracted and quantitatively analysed for changes in GST activities toward CDNB and the herbicides atrazine, fluorodifen and metolachlor, together with changes in immunodetectable polypeptides, giving results summarised in Table 13. Using the *Zm* GST I-II antibody, the *Zm* GST I subunit was found to be expressed at similar levels at all the time points, and in extracts from whole seedlings, did not accumulate following dichlormid treatment. The *Zm* GST II subunit was not detected in seeds, but was present in extracts of whole seedlings, with dichlormid treatment increasing its expression, particularly in the 4 to 6-day old plants. Probing with the *Zm* GST V-VI antibody showed that the *Zm* GST V subunit was present in untreated tissue, and was significantly induced by application of dichlormid, particularly after 8 to 10 days. When changes in GST activities were related to changes in immunodetectable GST subunits (Table 13), it was apparent that the small increase of GST activity towards CDNB in the dichlormid-treated seedlings reflected maintenance of a constant level of the *Zm* GST I subunit, which is responsible for the

majority of CDNB-conjugating activity in maize, as shown in chapter 4. The minor increase in CDNB-conjugating activity which was observed was therefore probably due to the major enhancement of *Zm* GST V and other tau class subunits, which show limited activity toward CDNB. Compared with GST activities in the seeds at day 0, in the presence of dichlormid GST activity toward metolachlor increased six-fold during the 10 day study and only three-fold during the same period in untreated plants. Over the first 6 days of the study these increases correlated well with the increase in the *Zm* GST II subunit, which is highly efficient in detoxifying this herbicide (Holt *et al.*, 1995, Irzyk and Fuerst, 1993). After day 6, the increase of *Zm* GST V also appeared to be making a contribution to metolachlor detoxification. Following dichlormid treatment, GST activity toward fluorodifen doubled within 4 days and this could be accounted for by the rapid enhancement of both *Zm* GST II and *Zm* GST V subunits, both of which are associated with high fluorodifen activity. Atrazine activity, which is partly due to *Zm* GST I subunits, but mainly due to other, unidentified GSTs, did not change significantly over time and was unaffected by dichlormid treatment, showing that like *Zm* GST I, atrazine-active GSTs are not induced in response to safener treatment. The values obtained for 10 day old seedlings in general agree well with results from earlier studies in this work for safener-treated and untreated maize seedlings (see chapter 2, Table 6). However, whereas GST activity towards atrazine remained unchanged in dichlormid-treated seedlings in this study, the earlier work showed that in cultivar Pioneer 3394, but not in cultivar Artus, activity towards atrazine doubled in response to treatment with dichlormid. It is unclear why there should be such a difference in the inducibility of atrazine-conjugating activity between the two studies.

Day	Dichlormid	GST activities (pkat/mg protein)				Immunodetectable GSTs (%)*		
	(-/+)	CDNB	Atraz	Met	Flu	*Zm* I	*Zm* II	*Zm* V
0		5470	1.37	3.27	1.04	104	0	24
4	-	5640	1.29	5.22	1.40	100	100	100
4	+	5840	1.62	7.93	2.54	85	250	151
6	-	4840	1.15	5.41	0.98	101	198	118
6	+	7000	1.51	14.37	2.76	108	358	176
8	-	3580	1.23	7.38	0.61	73	167	120
8	+	7210	1.58	19.12	2.70	71	248	215
10	-	5200	1.58	9.16	0.90	106	238	132
10	+	7490	1.79	16.45	2.58	83	220	322

Table 13. Effect of dichlormid treatment on GSTs in 0 to 10-day old seedlings, as measured by enzyme activity towards CDNB and the herbicides atrazine (Atraz), metolachlor (Met) and fluorodifen (Flu), and by western blotting using anti-*Zm* GST I-II and anti-*Zm* GST V-VI sera. * Integrated density of respective western blot band, expressed as % of relative value obtained for the respective GST subunit in 4-day old untreated tissue.

Western Blotting following Non-denaturing PAGE

While western blotting following SDS-PAGE was useful in determining the presence or absence of particular immunoreactive GST subunits, it could not provide information on the composition of the respective GST dimers. Since non-denaturing PAGE does not split these dimers, this can be used prior to western blotting to investigate the composition of immunoreactive subunits in the GST dimers. Since all the GST dimers are likely to have approximately the same molecular mass, non-denaturing PAGE will resolve the dimers based mainly on their charge at pH 8.8, the pH used for electrophoresis. Previous studies have shown that the pool 1 and pool 2 GSTs could be resolved as dimers on the basis of their charge during anion exchange chromatography at pH 7.8 (chapter 4). It was therefore anticipated that non-denaturing PAGE should result in a similar resolution of GST dimers.

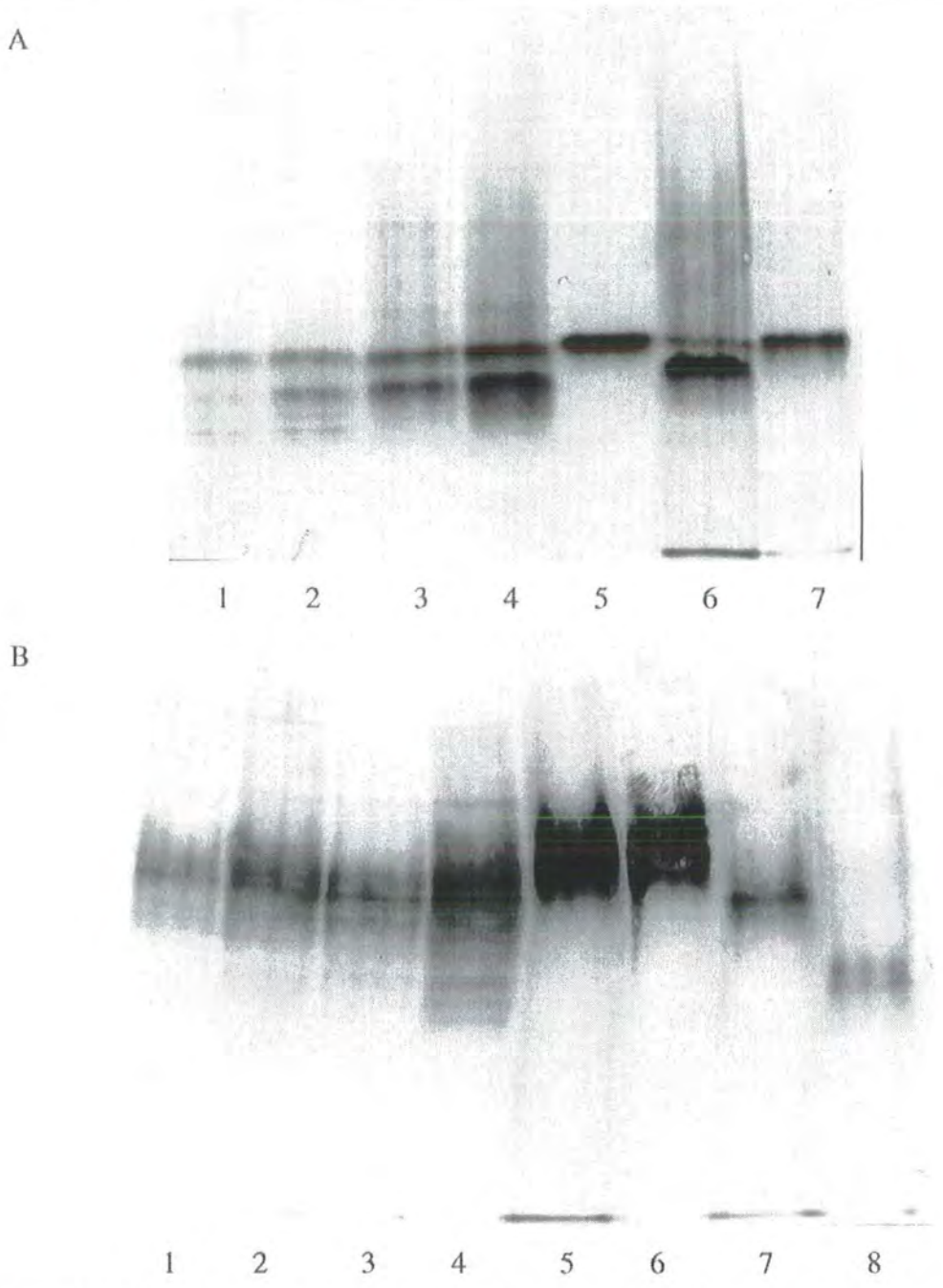

Figure 34. Western blots following non-denaturing PAGE of purified GSTs, and equal quantities of crude protein extracts.

A. Blot probed with anti-*Zm* GST I-II serum, with lane 1 = protein from untreated shoots, lane 2 = protein from dichlormid-treated shoots, lane 3 = protein from untreated roots, lane 4 = protein from dichlormid-treated roots, lane 5 = *Zm* GST I-I, lane 6 = *Zm* GST I-II and lane 7 = *Zm* GST I-I + *Zm* GST I-III.

B. Blot probed with anti-*Zm* GT V-VI serum, with lanes 1 to 4 as for A., lane 5 = *Zm* GST V-V, lane 6 = recombinant *Zm* GST V-V, lane 7 = *Zm* GST V-VI and lane 8 = *Zm* GST V-VII.

Figure 34 shows western blots following non-denaturing PAGE of purified GSTs, together with the analysis of equal quantities of crude protein from control and safener-treated etiolated maize seedling roots and shoots. Figure 34 A, showing a western blot probed with anti-*Zm* GST I-II serum, clearly showed that there were two major immunoreactive bands in each of the crude samples. By comparing band mobilities with those of purified GSTs, the upper of these two bands was identified as *Zm* GST I-I, while the lower band was identified as *Zm* GST I-II. Analysis of a mixture of *Zm* GST I-I and *Zm* GST I-III gave a single band so it seemed that *Zm* GST I-III co-migrated with *Zm* GST I-I. *Zm* GST II-II should have reacted strongly with the antiserum but no respective band was visible in any of the samples, so either the dimer was not present or it migrated very close to *Zm* GST I-II, as has been reported during anion exchange chromatography (Fuerst *et al.*, 1993). In addition to the two major bands, a number of minor bands were also visible. Three faint bands, all migrating faster than *Zm* GST I-II, were present in protein from treated and untreated shoots. These bands may have been artefacts caused by partial degradation of *Zm* GSTs I-I and I-II, but this seemed unlikely since the bands were sharp, there was no smearing indicative of degradation (as found with purified *Zm* GST I-II) and these bands were not found in either of the root samples. It is therefore possible that these bands represent as yet unidentified GSTs, either containing the *Zm* GST I or *Zm* GST II subunit, or immunologically related proteins. A number of slow-migrating bands were also visible in protein from the two root samples which again may be unidentified GST dimers, or possibly aggregates of GSTs or GSTs associated with other proteins. As expected from data presented earlier, immunoreactive GST dimers were more abundant in roots than in shoots and their levels were increased by safener treatment. This inducibility was particularly pronounced for the *Zm* GST I-II band.

Figure 34 B shows a similar non-denaturing gel probed with anti-*Zm* GST V-VI serum. Since this antiserum appeared to be specific for the *Zm* GST V subunit, with limited activity towards the *Zm* GST VI subunit, each of the bands on this western blot should correspond to a GST dimer containing the *Zm* GST V subunit. Each of the four crude protein samples contained a large number of immunoreactive bands. Purified *Zm* GST V-V and recombinant *Zm* GST V-V were detected as slow-migrating smears which corresponded to similar smears in each of the crude samples, which were therefore

probably *Zm* GST V-V. The reasons for the poor resolution of this isoenzyme are unknown. *Zm* GST V-VI gave a much sharper major band and similar bands were again present in each of the crude samples. Bands co-migrating with the putative *Zm* GST V-VII protein (see chapter 4) were also present in each of the crude samples. All of the immunoreactive proteins were more abundant in root samples than shoot samples, and were enhanced in dichlormid-treated samples compared with untreated samples, correlating well with previously presented data. In each of the crude samples there were numerous bands which did not correspond to the purified GST isoenzymes and these were probably uncharacterised dimers. It was clear that GSTs recognised by the anti-*Zm* GST V-VI serum formed a much more complex family than those recognised by the anti-*Zm* GST I-II serum. This agreed with data from anion exchange chromatography of affinity-purified GST pools, where pool 1 GSTs, recognised by the anti-*Zm* GST I-II serum, gave three distinct protein peaks, while pool 2 GSTs, recognised by the anti-*Zm* GST V-VI serum, gave three main peaks, but with many smaller, overlapping peaks also being present.

Immunohistochemistry

To get a rough idea of the distribution of GSTs in maize stems and leaves, a simple tissue-blotting technique was used to transfer protein from thin sections onto nitrocellulose membranes, followed by detection of GSTs using anti-GST sera as described in chapter 2. When probed with either *Zm* GST I-II or *Zm* GST V-VI antibodies, in both cases immunoreactive GSTs were visualised in all parts of each section, with staining intensity correlating with levels of total blotted protein, as visualised using Ponceau S staining. Figure 35 shows two blots, using the *Zm* GST V-VI antibody. A similar experiment with pre-immune serum did not recognise any protein, showing that the results obtained with anti-GST sera were due to the specific binding of the antibody rather than being due to a non-specific effect. The results suggest that both pool 1 and pool 2 GSTs recognised by each antibody are distributed evenly throughout stem and leaf tissue.

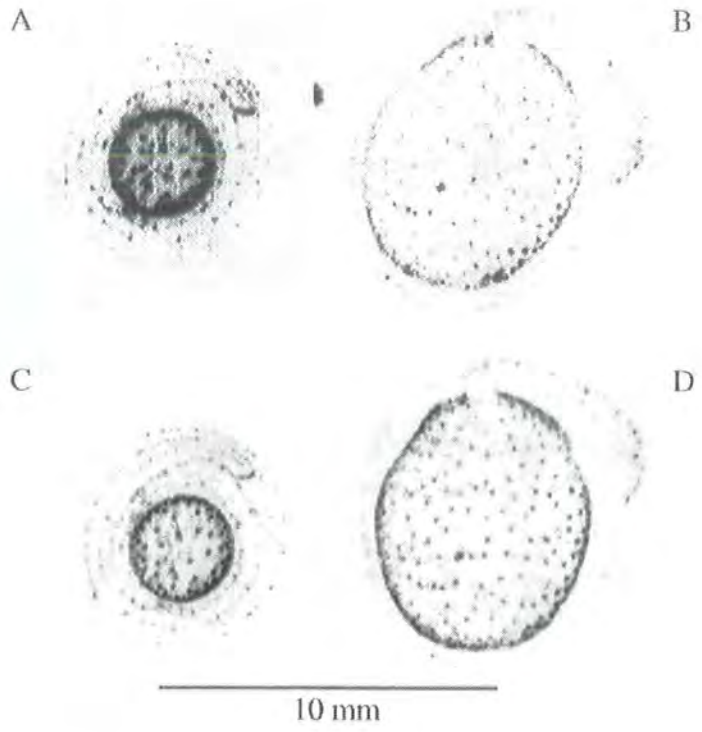

Figure 35. Maize stem tissue blots showing immunoreactivity with anti-GST sera.
Blots A and B were stained for total protein with Ponceau S while blots C and D were the same blots as A and B respectively, but stained for protein recognised by anti-*Zm* GST V-VI serum. All four blots show transverse stem sections from a mature maize plant; blots A and C show sections from young stem with numerous young leaves surrounding it, while blots B and D show sections of older stem with a single peripheral leaf.

Discussion

Distribution of GSTs

Previous studies have examined the expression of maize GST subunits *Zm* GST I and *Zm* GST II either in young seedlings or in tissues of mature plants (Jepson *et al.*, 1994, Holt *et al.*, 1995). The current studies have considerably extended earlier work, following the expression of these maize GST subunits and the newly characterised *Zm* GST V subunit throughout vegetative tissue in maize plants over time, and also in selected reproductive tissues in mature plants (Figure 32).

Holt *et al.* (1995) examined the distribution of *Zm* GST I and *Zm* GST II subunits in 50 day old plants and showed that *Zm* GST I was present in leaf, stem, root and tassel tissue while *Zm* GST II was only present in low levels in root tissue. Jepson *et al.* (1994) investigated the distribution of *Zm* GST I and *Zm* GST II mRNA in tissues from mature plants, with results complementing the work with the respective proteins of Holt *et al.* (1995). *Zm* GST I mRNA was present in all the tissues tested (root, stem, leaf, endosperm, tassel and pollen) while *Zm* GST II mRNA was not detected in these tissues unless plants were treated with safener. These results agree well with data presented here, where *Zm* GST I was found throughout the plant while *Zm* GST II was only detected in young roots. In addition, this study has shown that levels of immunodetectable *Zm* GST subunits I and V decrease slowly with plant age, and are much higher in the roots than in shoots.

In immature kernels, the anti-*Zm* GST I-II serum recognised a 32 kDa polypeptide, in addition to *Zm* GST I (Figure 32). This immunoreactive polypeptide was not detected in any other tissues so appears to have a very specific expression pattern. Since the anti-*Zm* GST I-II serum is very specific for GST subunits it is reasonable to presume that this 32 kDa polypeptide is also a GST. However it is unclear whether this polypeptide is a modified form of *Zm* GST I or a previously uncharacterised related polypeptide. It would therefore be of great interest to further study this polypeptide, perhaps by protein sequencing following purification using immunoprecipitation.

It would also be of interest to determine the localisation of GSTs in plant organs and at the subcellular level in greater detail. To this end, root and shoot samples from maize seedlings along with a number of different tissues from mature plants have been resin-embedded in preparation for immunohistochemistry for continuing studies on maize GSTs. Both light and electron microscopy will be used to localise immunoreactive GSTs.

Induction of GSTs by heat shock and chemical exposure

The results illustrated in Figure 33 showed that immunodetectable levels of each of the three *Zm* GST subunits studied (*Zm* GST I, *Zm* GST II and *Zm* GST V) were affected very differently by chemical treatment of maize seedlings. Levels of *Zm* GST I were unchanged by any of the treatments. Previous studies have shown that the *Zm* GST I subunit is constitutive and unresponsive to safener-treatment (Jepson *et al.*, 1994, Holt *et al.*, 1995), in agreement with the data presented here. However, interestingly Weigand *et al.* (1986) demonstrated that levels of *Zm* GST I mRNA were enhanced following safener treatment. This work has also shown that *Zm* GST I levels are also unaffected by a range of previously untested treatments including herbicides, auxins, CDNB, glutathione and heat-shock.

Zm GST II was induced by a wide range of treatments. It is likely that the *Zm* GST II subunit has a role in general stress responses since it is constitutively expressed only in young roots, and its expression is induced by a variety of chemical treatments. The wide range of treatments able to elicit *Zm* GST II induction means that it is unlikely that this induction is the result of a specific signal recognition pathway, but rather a more general stress-induced signal. Previous work has shown that expression of the *Zm* GST II subunit is induced by the safeners dichlormid and R-29148 (Holt *et al.*, 1995). The corresponding mRNA was also induced in response to safeners, along with slight induction on treatment with high levels of auxins but no induction following wounding or treatment with salicylic acid or ethylene (Jepson *et al.*, 1994). The results presented here agree well with these previous studies, except that auxin treatment elicited a much greater increase in levels of the *Zm* GST II polypeptide than was observed previously for the *Zm* GST II mRNA (Jepson *et al.*, 1994). In addition, it has also been demonstrated

that *Zm* GST II expression is also induced in response to treatment with the herbicides fluorodifen and alachlor (both *Zm* GST II substrates), glutathione and CDNB.

In contrast to *Zm* GST II, *Zm* GST V expression was specifically and highly induced by dichlormid, with no other treatments tested producing a similar response. It is unlikely therefore that this induction is a response to general stress, and it suggests that induction is instead due to a much more specific signalling process. Evidence for the existence of a specific receptor, required for such a signalling pathway, has been suggested by Walton and Casida (1995). They showed the presence of a soluble protein in etiolated maize seedlings with a high binding affinity for the dichloroacetamide safener R-29148 (K_d = 0.12 µM). Dichlormid inhibited this binding with an IC_{50} of 0.01 µM, and binding was also inhibited to a lesser extent by other related safeners such as benoxacor (IC_{50} = 0.74 µM), chloroacetanilide herbicides such as alachlor (IC_{50} = 0.07 µM) and thiocarbamate herbicides. Reasonable correlation was demonstrated between safening activity and binding inhibition, suggesting that this safener-binding protein may play a role in the response of maize to dichloroacetamide safeners. This unidentified safener-binding protein may therefore be at the start of a specific signalling pathway and this, or a similar, pathway may be responsible for induction of expression of *Zm* GST V. In order to elucidate the nature of this pathway, it would be of interest to sequence and characterise the promoter of the *Zm* GST V gene, since identification of promoter elements might identify transcription factors involved in specifically regulating gene transcription by safeners.

These different responses suggest that each GST subunit has a different functional role, as GSTs with similar roles would be more likely to be co-ordinately regulated.

Induction of GSTs over time by Dichlormid Treatment

Previous reports have shown that *Zm* GST I is constitutively present and only modestly responsive to safener treatment (Wiegand *et al.*, 1986), while *Zm* GST II is strongly induced in response to safener treatment (Holt *et al.*, 1995, Jepson *et al.*, 1994). This study has confirmed these previous findings and has been able to provide further information about the relative importance of different subunits in detoxifying various xenobiotics, both in untreated and safener-treated seedlings. In addition, the anti-*Zm*

GST V-VI serum has shown that the *Zm* GST V subunit is likely to be important in herbicide detoxification. By examining GST activity and immunodetectable GST levels over time, it was clear that while induction of *Zm* GST II contributed to the increased GST activity towards metolachlor, other GSTs, in particular *Zm* GST V, also played an important role in this enhancement since moderate metolachlor-conjugating activity was detected in seeds where *Zm* GST II could not be detected. Also, in 8-10 day safener-treated seedlings GST activity towards metolachlor rose over time, as did *Zm* GST V expression, even though *Zm* GST II levels appeared to decrease.

Non-denaturing PAGE Profiles

Sari-Gorla *et al.* (1993) used non-denaturing PAGE in conjunction with gel staining for CDNB activity to investigate the distribution of GST isoenzymes in different tissues and inbred lines, although no attempt was made to identify them. In this study several GSTs, responsible for the majority of CDNB-conjugating activity in dichlormid-treated maize roots, have been purified, and antibodies raised which recognise most of these isoenzymes. Therefore the non-denaturing western blot analyses from the present studies should be useful in interpreting the activity gels of Sari-Gorla *et al.* (1993).

Sari-Gorla *et al.* (1993) found that two fast-migrating proteins accounted for the majority of GST activity towards CDNB, especially in roots, and that these proteins were present in most tissues examined. These proteins were however absent in two of the maize lines they were examining, B37 and B83. These fast-migrating bands correspond well with the bands identified as *Zm* GST I-I and *Zm* GST I-II on non-denaturing western blots, and this agrees with earlier data showing that *Zm* GSTs I-I and I-II account for the majority of CDNB activity in maize roots and shoots. From this data it is likely therefore that inbred lines B37 and B83 do not express *Zm* GST I. This was confirmed by Rossini *et al.* (1995), who then speculated on why a single null mutation should remove two distinct isoenzymes, postulating that either one of the isoenzymes was a modified *Zm* GST I-I homodimer, or that at least one of the isoenzymes was a heterodimer of *Zm* GST I and another subunit. Our work suggests that the latter is true, with the slower-migrating band being *Zm* GST I-I and the faster-migrating band being *Zm* GST I-II.

Sari-Gorla *et al.* (1993) also described the presence of between 2 and 5 slow-migrating CDNB-active isoenzymes in each maize line, whose electrophoretic mobilities varied between lines. It is likely that these isoenzymes correspond to the pool 2 GSTs, recognised as slow-migrating proteins by the anti-*Zm* GST V-VI serum when analysed by non-denaturing western blotting, and provides more evidence for the relative complexity of this family of maize GSTs.

6. Cloning of Theta Class Maize GSTs

Introduction

Three theta class (type I) GST cDNAs have been cloned from maize, encoding the *Zm* GST I subunit (Grove *et al.*, 1988, Shah *et al.*, 1986), the *Zm* GST II subunit (Jepson *et al.*, 1994, Irzyk *et al.*, 1995), and the *Zm* GST III subunit (Grove *et al.*, 1988, Moore *et al.*, 1986, Bieseler *et al.*, 1996). A *Zm* GST I genomic clone has also been isolated (Shah *et al.*, 1986). Although these GSTs have already been cloned the characterisation of the respective recombinant enzymes has not been described in detail and it was considered useful to re-isolate similar cDNA clones for a number of reasons. Clones were required for expression of recombinant GSTs, allowing large quantities of pure GSTs to be obtained without the risk of co-purifying other similar GSTs with differing activities. These pure recombinant GSTs would then be useful for substrate specificity studies, antibody production and crystallisation prior to structural determination. Clones could also be used as probes in northern and Southern hybridisation experiments to determine transcriptional regulation and gene copy number. Also, while the reported multiple sequences for *Zm* GST I and *Zm* GST II clones are in close agreement, the three sequences for *Zm* GST III cDNAs give rise to very different deduced polypeptide sequences mainly due to numerous frame-shifts between sequences, and so this required clarification.

Results

Cloning of *Zm* GST I

Since *Zm* GST I had already been cloned and its sequence reported by Grove *et al.* (1988) and Shah *et al.* (1986), it was decided to use a PCR-based strategy to isolate *Zm* GST I clones, using primers designed from the published sequences to specifically amplify the *Zm* GST I coding region.

Total RNA was extracted from roots and shoots of light grown maize (cv. Artus) seedlings and visualised by 50% formamide denaturing agarose gel electrophoresis to check RNA purity and lack of degradation. Reverse transcriptase was used to make first strand cDNA from root and shoot total RNA, using either oligo $dT_{(15)}$ (to selectively synthesise cDNA from mRNA species) or the *Zm* GST I primer Pmr-1b (Table 14; to selectively synthesise *Zm* GST I cDNA, see chapter 2) as oligonucleotide primers. In each case, an additional control reaction was performed in which the reverse transcriptase was omitted, to test for contamination of the RNA by genomic DNA. Each of the reverse transcription and control reaction products were used as templates for a PCR using oligonucleotide primers Pmr-1a and Pmr-1b (Table 14). Pmr-1a was designed to the 5′ end of the *Zm* GST I coding sequence and contained an *Nco*I restriction site to facilitate subsequent cloning, while Pmr-1b was designed to the reverse-complement of the 3′ end of the *Zm* GST I coding sequence and contained a *Hind*III restriction site. PCRs were performed for 30 cycles and each reaction product was visualised following agarose gel electrophoresis (Figure 36).

Primer	Nucleotide sequence (restriction sites in bold)
Pmr-1a	GAG AGG TTG GGT CTG GGA CA**C CAT G**
Pmr-1b	CC**A AGC TT**A TTG ACA GGG CAA CCG TTT C
Pmr-1c	CC**G GAT CC**A TTG ACA GGG CAA CCG TTT C
Pmr-2a	GTG CAG AGA ACA GGA **CAT ATG**
Pmr-2b	CC**G GAT CC**T TCG ATC GCT TCA TCG TCA C
Pmr-3a	**CCA TGG** CGC CTC TGA AGC TGT AC
Pmr-3b	**GGA TCC** ACG AGC AAA TGC AAG ACA GG

Table 14. Sequences of oligonucleotide primers used to clone theta class GSTs.
Primers Pmr-1a, Pmr-2a and Pmr-3a were designed to recognise the start of the respective cDNA coding region, with the 'ATG' of the highlighted restriction sites recognising the start codon. Underlined bases in Primers Pmr-1b, Pmr-1c and Pmr-2b correspond to the respective cDNA stop codons. Primer Pmr-3b was designed to recognise a region 73 bp. to 92 bp. downstream of the cDNA stop codon.

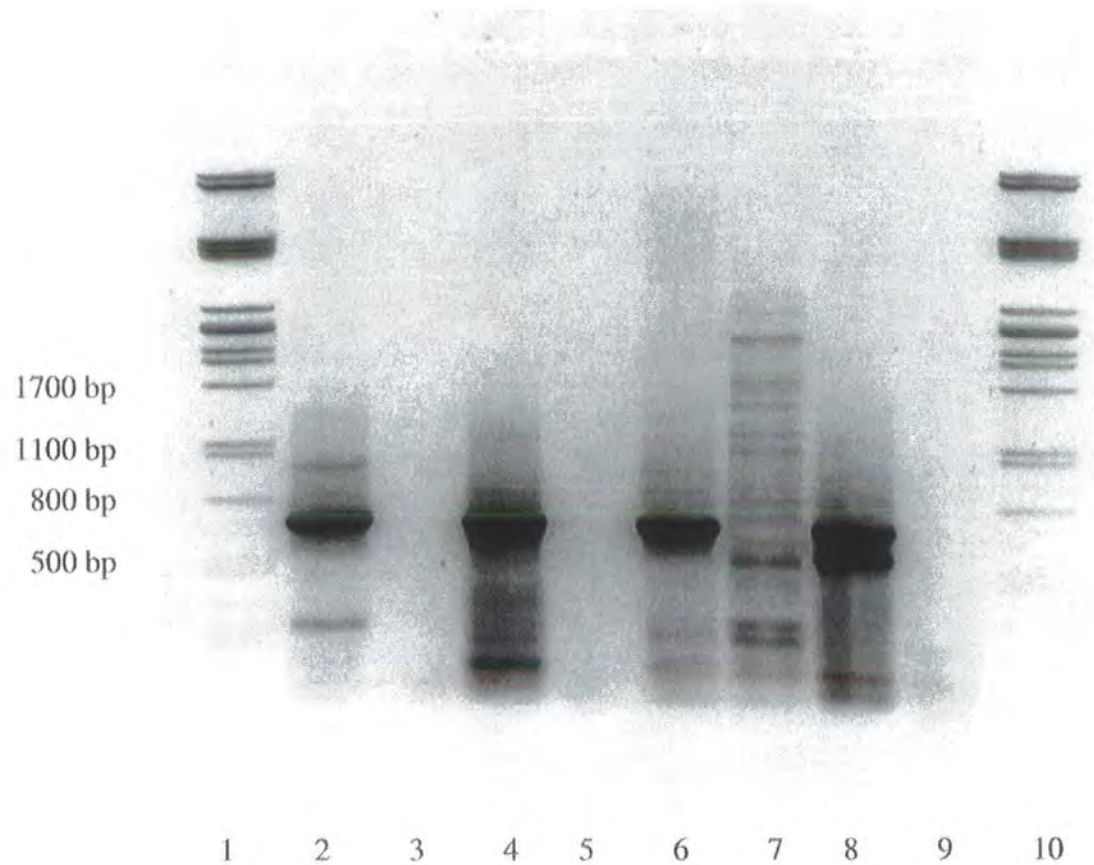

Figure 36. Agarose gel electrophoresis showing *Zm* GST I cDNA PCR products. Templates were the product of reverse transcriptase reactions using maize total RNA, either with or without reverse transcriptase, and using the oligonucleotide primer as described below.

Lanes 1 & 10 = λ-PstI DNA size standards,

2 = Root RNA + dT_{15} primer + Reverse transcriptase,

3 = Root RNA + dT_{15} primer - Reverse transcriptase,

4 = Root RNA + Pmr-1b primer + Reverse transcriptase,

5 = Root RNA + Pmr-1b primer - Reverse transcriptase,

6 = Shoot RNA + dT_{15} primer + Reverse transcriptase,

7 = Shoot RNA + dT_{15} primer - Reverse transcriptase,

8 = Shoot RNA + Pmr-1b primer + Reverse transcriptase,

9 = Shoot RNA + Pmr-1b primer - Reverse transcriptase.

After incubating with reverse transcriptase, each RNA sample produced a similar 680 bp. product after PCR, which was the expected size for amplified *Zm* GST I cDNA. RNA samples not incubated with reverse transcriptase did not produce a similar product following PCR, showing that these products were not due to contaminating genomic DNA. cDNA synthesised from shoot RNA primed with Pmr-1b primer also gave rise to a prominent 550 bp. product. The 680 bp. PCR products amplified from root cDNA synthesised using oligo $dT_{(15)}$, and from shoot cDNA synthesised using Pmr-1b, were excised from the gel, purified and cloned using the pGEM-T cloning system (Promega) to give clones DD1r and DD1s respectively. The 550 bp. PCR product amplified from shoot cDNA synthesised using Pmr-1b was similarly cloned, to give clone DDu1.

Sequencing of clones DD1r (Figure 37) and DD1s showed that both were *Zm* GST I clones. DD1r, the root sequence, differed slightly from the published *Zm* GST I sequences (clone pMON9000 from Shah *et al.*, 1986, clone pGTC2 from Grove *et al.*, 1988). Comparison of nucleotide coding sequences showed 6 substitutions between pGTC2 and DD1r, at positions 43 (T to G), 225 (T to G), 333 (T to C), 363 (T to G), 528 (C to T) and 625 (C to T), numbered from the start of the coding sequence. All but one of these substitutions were silent, so that comparison of the deduced amino acid sequences revealed a single substitution of leucine to valine at the 15th residue, corresponding to the nucleotide substitution at base 43. This difference is unlikely to be important since other *Zm* GST I sequences, like DD1r, code for valine at the 15th residue, including sequence from Shah *et al.* (1986) and partial sequences from Grove *et al.* (1988). DD1s, the shoot sequence, was identical to DD1r except for two nucleotide substitutions at positions 14 (A to G) and 344 (T to C). Both of these substitutions altered the deduced amino acid sequence, changing lysine to arginine at the 5th residue and changing phenylalanine to serine at 115th residue. Neither of these substitutions was found in any other *Zm* GST I sequence reported and it was assumed that they resulted from errors introduced during PCR. Clone DD1r was therefore chosen for later use in the construction of a *Zm* GST I heterologous expression system, as it appeared to be error-free.

```
ATGGCTCCGATGAAGCTGTACGGGGCGGTGATGTCGTGGAACGTGACGAG  50
 M  A  P  M  K  L  Y  G  A  V  M  S  W  N  V  T  R

GTGCGCAACGGCGCTGGAGGAGGCTGGCTCCGACTACGAGATCGTGCCCA 100
  C  A  T  A  L  E  E  A  G  S  D  Y  E  I  V  P

TCAACTTCGCCACCGCCGAGCACAAGAGCCCCGAGCACCTCGTCCGCAAC 150
I  N  F  A  T  A  E  H  K  S  P  E  H  L  V  R  N

CCGTTTGGTCAGGTTCCAGCTCTGCAGGATGGTGACTTGTACCTCTTCGA 200
 P  F  G  Q  V  P  A  L  Q  D  G  D  L  Y  L  F  E

ATCACGAGCAATCTGCAAGTACGCGGCTCGCAAAAACAAGCCAGAGCTGT 250
  S  R  A  I  C  K  Y  A  A  R  K  N  K  P  E  L

TGAGGGAAGGAAACCTCGAGGAGGCAGCAATGGTGGATGTTTGGATCGAG 300
L  R  E  G  N  L  E  E  A  A  M  V  D  V  W  I  E

GTGGAGGCTAACCAGTACACCGCTGCACTGAACCCCATCCTCTTCCAGGT 350
 V  E  A  N  Q  Y  T  A  A  L  N  P  I  L  F  Q  V

CCTCATCAGTCCGATGCTTGGGGGAACCACCGACCAGAAAGTTGTGGATG 400
  L  I  S  P  M  L  G  G  T  T  D  Q  K  V  V  D

AGAACCTTGAGAAGCTGAAGAAGGTGCTAGAGGTGTACGAGGCACGCCTG 450
E  N  L  E  K  L  K  K  V  L  E  V  Y  E  A  R  L

ACCAAGTGCAAGTACCTTGCTGGAGACTTCCTCAGCCTCGCCGACCTGAA 500
 T  K  C  K  Y  L  A  G  D  F  L  S  L  A  D  L  N

CCATGTGTCTGTCACTCTCTGCCTGTTTGCTACGCCCTACGCATCTGTGC 550
  H  V  S  V  T  L  C  L  F  A  T  P  Y  A  S  V

TCGACGCCTACCCGCATGTGAAGGCCTGGTGGTCTGGTCTGATGGAGAGG 600
L  D  A  Y  P  H  V  K  A  W  W  S  G  L  M  E  R

CCGTCTGTCCAGAAGGTCGCTGCCTTGATGAAGCCATCTGCTTGA      645
 P  S  V  Q  K  V  A  A  L  M  K  P  S  A  *
```

Figure 37. Coding region of *Zm* GST I PCR product, clone DD1r.

Partial sequencing of clone DDu1 gave a nucleotide sequence that showed 99.6% identity with a portion of a ps1A2 gene from maize encoding a component of the chloroplast photosystem 1 complex (GenBank accession number X86563), as shown in Figure 38. Similarly the deduced amino acid sequence from DDu1 showed high identity to the photosystem 1 P700 apoprotein A2 amino acid sequence. Analysis of DDu1 showed that the template it had been amplified from had been primed with Pmr-1b at both ends. When the Pmr-1b primer was compared to the nucleotide sequence of photosystem 1 A2 (Figure 38), it showed there was considerable similarity at both sites where the primer had annealed. Pmr-1b could therefore have annealed to these partially matching regions either during cDNA synthesis or during PCR, thus explaining why this product was amplified during the PCR. Since the sequence of Pmr-1b did not match perfectly with that of the PS1 A2 gene, using the oligo $dT_{(15)}$ primer for cDNA synthesis and increasing the annealing temperature used for the PCR would probably have prevented the formation of this mis-primed PCR product.

Construction of a Safener-Induced Maize cDNA Library

To isolate cDNA clones corresponding to the GSTs purified from maize, a cDNA library was constructed, using RNA from the roots of dichlormid-treated maize seedlings so that cDNA clones representing all the GSTs purified from this tissue should be present in the library. 400 µg of total RNA was isolated from 1 g of roots from dichlormid-treated maize (cv. Pioneer 3394) seedlings, giving 3.6 µg of mRNA following poly $(A)^+$ enrichment. A unidirectional cDNA library was synthesised from this mRNA, using a ZAP-cDNA synthesis kit (Stratagene). cDNA was size-fractionated using Sephacryl S-500, and aliquots of each fraction were analysed by non-denaturing PAGE (Figure 39). Equal volumes of fractions 2 and 3 (giving a cDNA size range of between about 5,000 and 500 bp.) were combined and ligated into the Uni-ZAP XR vector. A fifth of this ligated cDNA was packaged into phage particles to give a primary library with approximately four million independent clones. 500,000 of these clones were subsequently amplified to give a high-titre secondary library, which was stored in aliquots at -80 °C.

```
DDu1                                                ccaagcttattgacagggca
ps1_A2    tttctgccgcttcttctccctctgaatcaaactttttttgccataatgt

DDu1      accgtttctattattaccaagtatatggttctaatcctagatagaaaaat
ps1_A2    gccgttcctattattaccaagtatatggttctaatcctagatagaaaaat
                 ****************************************

DDu1      aaatagcaaaaatctaaaaaggcggatcctccctctccatcaagagtaat
ps1_A2    aaatagcaaaaatctaaaaaggcggatcctccctctccatcaagagtaat
          **************************************************

DDu1      gaactgggttctgatacagtacaaaaaaaaataactaaattaaccaaact
ps1_A2    gaactgggttctgatacagtacaaaaaaaaataactaaattaaccgaact
          ********************************************** ****

DDu1      tgcctgatgttgaggcaatcaagaaagctgcataagtgaatatataaccc
ps1_A2    tgcctgatgttgaggcaatcaagaaagctgcataagtgaatatataaccc
          **************************************************

DDu1      acggaaaagtgggctaatccgaccaatcttgcttgcacaatggaaagagc
ps1_A2    acggaaaagtgggctaatccgaccaatcttgcttgcacaacggaaagagc
          ****************************************** *********

DDu1      cacgggcttatctctccagcgaattaaattagccaaaggtgtccgttcat
ps1_A2    cacgggcttatctctccagcgaattaaattagccaaaggtgtccgttcat
          **************************************************

DDu1      gagcccatgctaaagtctcaattaattcctgccaatatccacgccaggaa
ps1_A2    gagcccatgctaaagtctcaattaattcctgccaatatccacgccaggaa
          **************************************************

DDu1      attaagaacataaatcctgtagcccaaacaagatgtccaaataagaacat
ps1_A2    attaagaacataaatcctgtagcccaaacaagatgtccaaataagaacat
          **************************************************

DDu1      ccaagcccatactgataaactattcatcccaaaaggattatatccattaa
ps1_A2    ccaagcccatactgataaactattcatcccaaaaggattatatccattaa
          **************************************************

DDu1      taagttgtgaagagtttaaccataggtaatctcttaaccatcccatcaaa
ps1_A2    taagttgtgaagagtttaaccataggtaatctcttaaccatcccatcaaa
          **************************************************

DDu1      taagtggaggattcattaaattgtgaaacggttgccctgtcaataagctt
ps1_A2    taagtggaggattcattaaattgtgaaacg-ttgccctgccataatgtaa
          ************************

DDu1      gg
ps1_A2    tgtgtttccaatgccaataaaaagtaacccat
           *
```

Figure 38. Sequence alignment showing similarity between DDu1 and photosystem 1 P700 apoprotein A2.

DDu1 = Clone DDu1 nucleotide sequence, ps1_A2 = Photosystem 1 P700 apoprotein A2 sequence (GenBank X86563). Identical bases in the two sequences are indicated with asterisks, bases corresponding to the primer Pmr-1b are highlighted in bold and bases in ps1_A2 complementary to Pmr-1b are underlined.

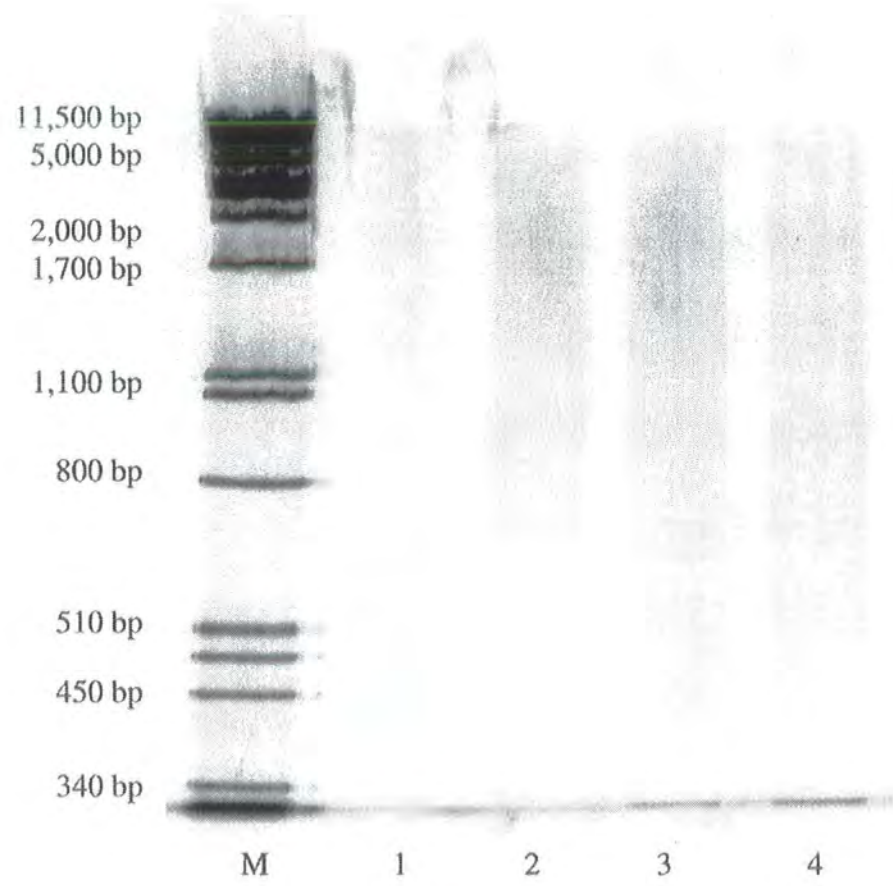

Figure 39. Silver-stained non-denaturing PAGE gel showing size fractionated cDNA. cDNA was obtained from roots of dichlormid-treated maize seedlings, for use in cDNA library synthesis. Lane M shows DNA size standards (PstI-digested λ DNA) with approximate sizes indicated in base-pairs, and lanes 1 to 4 show 5 µl (one twelfth of the total) of fractions 1 to 4 from size-fractionation of cDNA by Sephacryl S-500 chromatography.

Library Screening using *Zm* GST I-II Antibody

Library screening using anti-*Zm* GST I-II serum was used to isolate full length, error-free *Zm* GST I clones, and also *Zm* GST II clones since PCR-based approaches had previously failed to isolate any such clones (see below). The expression library synthesised from mRNA from the roots of dichlormid-treated maize seedlings (see above) was screened using the antibody raised against *Zm* GST I-II. 40 putative positive plaques were identified after screening approximately 170,000 clones from the primary library. Four of these plaques were subsequently purified by two further rounds of screening. Plasmids containing the putative GST inserts were excised from the purified plaques and the 5′ end of each of the inserts was sequenced.

All of the four clones recognised by *Zm* GST I-II antibody were identified as *Zm* GST I clones by sequence identity based on sequencing of the 5′ ends of the clones. Of these, three gave identical sequences while the fourth was also identical except for a 9-base truncation at the 5' end. No clones corresponding to *Zm* GST II were identified, which may have been due to chance, or the presence of an in-frame stop codon just upstream of the start codon present in previously identified clones (Irzyk *et al.*, 1995, Jepson *et al.*, 1994), preventing expression of *Zm* GST II as a fusion protein.

Cloning of *Zm* GST III

In order to clone the coding sequence for *Zm* GST III, a similar strategy to that successfully used for *Zm* GST I was initially used. Total RNA isolated from the shoots of dichlormid treated maize seedlings (cv. Pioneer 3394) was used as a template for first strand cDNA synthesis using reverse transcriptase primed with oligo $dT_{(15)}$ and this was used as a template for PCR using *Zm* GST III-specific primers (Pmr-3a and Pmr-3b, Table 14). These primers were designed using sequences of clones from Moore *et al.* (1986) and Grove *et al.* (1988) and since these sequences differed considerably, care was taken to ensure that primers were designed to sequences completely conserved between the clones. Using these primers a PCR product of the expected size was generated, then cloned using the pGEM-T cloning system. Two of the resulting clones were sequenced and both showed very high homology to the published *Zm* GST III sequences (Moore *et*

al., 1986, Grove *et al.*, 1988). The two sequences were identical except for one nucleotide, which gave rise to a change in the deduced amino acid sequence. To ensure that these sequences did not contain any PCR-generated errors, a maize root cDNA library was then screened using DIG-labelled *Zm* GST III PCR product as a probe. Using this method, four cDNA clones were isolated, all of similar length. Sequencing of each of these clones showed that all were full-length *Zm* GST III clones, however they were not all identical. Since parts of the cDNA clones were only sequenced in a single direction (see Appendix) there remains a small chance that the sequences may contain errrors; however all the differences described below appeared unambiguous. Two sequences were identical to each other apart from the position of their polyadenylation sites, and had a coding sequence identical to one of the sequences isolated by RT-PCR (Figure 40). The longer clone of the two was named DD3a. The two remaining sequences were identical to each other, but differed slightly from DD3a, with their coding sequence giving rise to a deduced protein sequence with 4 amino acid residues different from those derived from DD3a. These clones were named DD3b (Figure 41). When the sequences of the cDNA clones were aligned with the published sequences for *Zm* GST III, a number of differences were observed (Figure 42), which could be grouped into three categories:

a) Single nucleotide insertions/deletions within the coding sequence.

When compared with the coding regions of the sequences of Moore *et al.* (1986) and Grove *et al.* (1988), the coding regions of both DD3a and DD3b showed a number of single nucleotide insertions/deletions, all present towards the 3´ end. Compared with the sequence from Moore *et al.* (1986), both DD3a and DD3b contained 2 deletions and 5 insertions, while a similar comparison with the sequence from Grove *et al.* (1988) gave 3 deletions. All but one of these changes occurred within a run of 5 or more G/C bases, which can be difficult to sequence accurately. Examination of sequence runs for DD3a and DD3b clones showed no ambiguities in any of these regions and it was likely that all the previously reported insertions and deletions were sequencing artefacts resulting from inaccurate sequencing. Further evidence for this came from translation of the nucleotide sequences, showing that the deduced amino acid sequences of DD3a and DD3b were

more similar than the other *Zm* GST III sequences to the amino acid sequence of other plant GSTs. Further, a more recent *Zm* GST III sequence published as part of a patent application (Bieseler *et al.*, 1996) contained no insertions or deletions when compared with DD3a or DD3b.

b) Differences in the untranslated regions.

The 3′ and 5′ untranslated regions of each of the sequences were very similar, suggesting that all the clones corresponded to the same gene. However, some differences were present. When all the sequences were compared with each other, there were insertions/deletions of between 1 and 8 nucleotides at six locations in the 3′ and 5′ untranslated regions, and 9 nucleotide substitutions. While it was possible that some of the substitutions and short insertions/deletions were again sequencing artefacts, it was very unlikely that such artefacts could account for the larger insertions/deletions observed.

c) Substitutions within the coding region.

The coding region of DD3a contained a single nucleotide substitution (G to A, nucleotide 218) which was not present in any of the published clones. This resulted in a change in the deduced amino acid sequence from alanine in all of the published sequences to threonine in DD3a. Apart from this change the amino acid sequence of DD3a was identical to that of Moore *et al.* (1986), if the presumed sequencing artefacts were corrected. The coding region of DD3b contained two nucleotide substitutions (T to C, nucleotide 272 and G to A, nucleotide 398) which were not present in any of the published clones. Neither of these substitutions altered the deduced amino acid sequence, which was identical to that of the *Zm* GST III sequence reported by Bieseler *et al.* (1996) except that at residue 108, a tyrosine residue (as found in DD3a and Moore's sequence) replaced a histidine residue. Comparison of the coding region of DD3a with that of DD3b gave 7 nucleotide substitutions, giving rise to 3 amino acid substitutions, at residues 48, 134 and 199.

```
ctcccactttactcctatccactgcggcctggacgcgtgcgagaggcttg  50

accaagcagcagcagcagcagcagcgATGGCGCCTCTGAAGCTGTACGGG 100
                            M  A  P  L  K  L  Y  G

ATGCCGCTGTCCCCCAACGTGGTGCGCGTGGCCACCGTGCTCAACGAGAA 150
 M  P  L  S  P  N  V  V  R  V  A  T  V  L  N  E  K

GGGCCTCGACTTCGAGATCGTCCCCGTCGACCTCACCACCGGCGCCCACA 200
  G  L  D  F  E  I  V  P  V  D  L  T  T  G  A  H

AGCAGCCCGACTTCCTCACCCTCAACCCTTTCGGCCAGATCCCGGCTCTC 250
K  Q  P  D  F  L  T  L  N  P  F  G  Q  I  P  A  L

GTCGACGGAGACGAAGTCCTCTTCGAGTCCCGTGCGATCAACCGGTACAT 300
 V  D  G  D  E  V  L  F  E  S  R  A  I  N  R  Y  I

CGCCAGCAAGTACGCGTCGGAGGGCACGGACCTGCTCCCCGCGACGGCGT 350
  A  S  K  Y  A  S  E  G  T  D  L  L  P  A  T  A

CGGCGGCGAAGCTGGAGGTGTGGCTAGAGGTGGAGTCGCACCACTTCTAC 400
S  A  A  K  L  E  V  W  L  E  V  E  S  H  H  F  Y

CCGAACGCGTCGCCGCTGGTGTTCCAGCTGCTCGTGAGGCCGCTCCTGGG 450
 P  N  A  S  P  L  V  F  Q  L  L  V  R  P  L  L  G

CGGCGCCCCCGACGCGGCGGTGGTGGACAAGCACGCGGAGCAGCTCGCCA 500
  G  A  P  D  A  A  V  V  D  K  H  A  E  Q  L  A

AGGTGCTCGACGTGTACGAGGCGCACCTCGCCCGCAACAAGTACCTCGCC 550
K  V  L  D  V  Y  E  A  H  L  A  R  N  K  Y  L  A

GGGGACGAGTTCACGCTCGCCGACGCCAACCACGCGTCCTACCTGCTCTA 600
 G  D  E  F  T  L  A  D  A  N  H  A  S  Y  L  L  Y

CCTCAGCAAGACCCCCAAGGCCGGGCTCGTCGCCGCCCGCCCCCACGTCA 650
  L  S  K  T  P  K  A  G  L  V  A  A  R  P  H  V

AGGCCTGGTGGGAGGCCATCGTCGCCCGCCCCGCGTTCCAGAAGACCGTC 700
K  A  W  W  E  A  I  V  A  R  P  A  F  Q  K  T  V

GCCGCCATCCCCTTGCCCCCGCCGCCCTCCTCCTCGGCTTGAcctcgcct 750
 A  A  I  P  L  P  P  P  P  S  S  S  A  *

tgcgttgcgccgttgcctgggtcgcggatgcgtcggagccccgagtcgaa 800

taaaagaggcagcatcctgtcttgcatttgctgctgcgccatgtgttaac 850

agcctgtgtaataaacactgttgctttcgtgtgtgttcaaaaaaaaaaaa 900

aaaaaa                                              906
```

Figure 40. Nucleotide sequence of *Zm* GST III cDNA clone DD3a, with deduced amino acid sequence.

```
ctcctatccactgcggcctggacgcgtgcgagaggcttgaccaagcagca  50

gcagcagcagcagcgATGGCGCCTCTGAAGCTGTACGGGATGCCGCTGTC 100
               M  A  P  L  K  L  Y  G  M  P  L  S

CCCCAACGTGGTGCGCGTGGCCACCGTGCTCAACGAGAAGGGCCTCGACT 150
  P  N  V  V  R  V  A  T  V  L  N  E  K  G  L  D

TCGAGATCGTCCCCGTCGACCTCACCACCGGCGCCCACAAGCAGCCCGAC 200
F  E  I  V  P  V  D  L  T  T  G  A  H  K  Q  P  D

TTCCTCGCCCTCAACCCTTTCGGCCAGATCCCGGCTCTCGTCGACGGAGA 250
 F  L  A  L  N  P  F  G  Q  I  P  A  L  V  D  G  D

CGAAGTCCTCTTCGAGTCCCGCGCGATCAACCGGTACATCGCCAGCAAGT 300
  E  V  L  F  E  S  R  A  I  N  R  Y  I  A  S  K

ACGCGTCGGAGGGCACGGACCTGCTCCCCGCGACGGCGTCGGCGGCGAAG 350
Y  A  S  E  G  T  D  L  L  P  A  T  A  S  A  A  K

CTGGAGGTGTGGCTGGAGGTGGAGTCGCACCACTTCTACCCGAACGCATC 400
 L  E  V  W  L  E  V  E  S  H  H  F  Y  P  N  A  S

GCCGCTGGTGTTCCAGCTGCTCGTGAGGCCGCTCCTGGGCGGCGCCCCCG 450
  P  L  V  F  Q  L  L  V  R  P  L  L  G  G  A  P

ACGCGGCGGTGGTGGAGAAGCACGCGGAGCAGCTCGCCAAGGTGCTCGAC 500
D  A  A  V  V  E  K  H  A  E  Q  L  A  K  V  L  D

GTGTACGAGGCGCACCTGGCCCGCAACAAGTACCTCGCCGGGGACGAGTT 550
 V  Y  E  A  H  L  A  R  N  K  Y  L  A  G  D  E  F

CACGCTCGCCGACGCCAACCACGCGTCCTACCTGCTCTACCTCAGCAAGA 600
  T  L  A  D  A  N  H  A  S  Y  L  L  Y  L  S  K

CCCCCAAGGCCGGGCTCGTCGCCGCCCGCCCCCACGTCAAGGCCTGGTGG 650
T  P  K  A  G  L  V  A  A  R  P  H  V  K  A  W  W

GAGGCCATCGCCGCCCGCCCCGCGTTCCAGAAGACCGTCGCCGCCATCCC 700
 E  A  I  A  A  R  P  A  F  Q  K  T  V  A  A  I  P

CTTGCCCCCGCCGCCCTCCTCCTCGGCTTGAcctcgccttgcgctgcctg 750
  L  P  P  P  P  S  S  S  A  *

ggtcgcggatgcgtcggagccccgagtcgaataaaagaggccgcatcctg 800

tcttgcatttgctgctgcgccatgtgctataacagcctgtgtaataaaca 850

ctgttgctttcgtgtgtgttcattgccttttggttggttggtccttgcat 900

atcctactagtgctgatctttttgtaaaaaaaaaaaaaaaaaaa       944
```

Figure 41. Nucleotide sequence of *Zm* GST III cDNA clone DD3b, with deduced amino acid sequence.

```
Moore                                                    agacttgaccaagcagc  17
DD3a    ctcccactttactcctatccactgcggcctggacgcgtgcgagaggcttgaccaagcagc  60
DD3b              ctcctatccactgcggcctggacgcgtgcgagaggcttgaccaagcagc  49
Grove     cccactttactcctatccactgcggcctggacgcgtgcgagaggcttgaccaagcagc  58
Bayer

Moore   agcagcagg------gATGGCGCCTCTGAAGCTGTACGGGATGCCGCTGTCCCCCAACGT  71
DD3a    agcagcagcagcagcgATGGCGCCTCTGAAGCTGTACGGGATGCCGCTGTCCCCCAACGT 120
DD3b    agcagcagcagcagcgATGGCGCCTCTGAAGCTGTACGGGATGCCGCTGTCCCCCAACGT 109
Grove   agcagcagcagcagcgATGGCGCCTCTGAAGCTGTACGGGATGCCGCTGTCCCCCAACGT 118
Bayer                   ATGGCGCCTCTGAAGCTGTACGGGATGCCGCTGTCCCCCAACGT  44

Moore   GGTGCGCGTGGCCACCGTGCTCAACGAGAAGGGCCTCGACTTCGAGATCGTCCCCGTCGA 131
DD3a    GGTGCGCGTGGCCACCGTGCTCAACGAGAAGGGCCTCGACTTCGAGATCGTCCCCGTCGA 180
DD3b    GGTGCGCGTGGCCACCGTGCTCAACGAGAAGGGCCTCGACTTCGAGATCGTCCCCGTCGA 169
Grove   GGTGCGCGTGGCCACCGTGCTCAACGAGAAGGGCCTCGACTTCGAGATCGTCCCCGTCGA 178
Bayer   GGTGCGCGTGGCCACCGTGCTCAACGAGAAGGGCCTCGACTTCGAGATCGTCCCCGTCGA 104

Moore   CCTCACCACCGGCGCCCACAAGCAGCCCGACTTCCTCGCCCTCAACCCTTTCGGCCAGAT 191
DD3a    CCTCACCACCGGCGCCCACAAGCAGCCCGACTTCCTCACCCTCAACCCTTTCGGCCAGAT 240
DD3b    CCTCACCACCGGCGCCCACAAGCAGCCCGACTTCCTCGCCCTCAACCCTTTCGGCCAGAT 229
Grove   CCTCACCACCGGCGCCCACAAGCAGCCCGACTTCCTCGCCCTCAACCCTTTCGGCCAGAT 238
Bayer   CCTCACCACCGGCGCCCACAAGCAGCCCGACTTCCTCGCCCTCAACCCTTTCGGCCAGAT 164

Moore   CCCGGCTCTCGTCGACGGAGACGAAGTCCTCTTCGAGTCCCGTGCGATCAACCGGTACAT 251
DD3a    CCCGGCTCTCGTCGACGGAGACGAAGTCCTCTTCGAGTCCCGTGCGATCAACCGGTACAT 300
DD3b    CCCGGCTCTCGTCGACGGAGACGAAGTCCTCTTCGAGTCCCGCGCGATCAACCGGTACAT 289
Grove   CCCGGCTCTCGTCGACGGAGACGAAGTCCTCTTCGAGTCCCGTGCGATCAACCGGTACAT 298
Bayer   CCCGGCTCTCGTCGACGGAGACGAAGTCCTCTTCGAGTCCCGTGCGATCAACCGGTACAT 224

Moore   CGCCAGCAAGTACGCGTCGGAGGGCACGGACCTGCTCCCCGCGACGGCGTCGGCGGCGAA 311
DD3a    CGCCAGCAAGTACGCGTCGGAGGGCACGGACCTGCTCCCCGCGACGGCGTCGGCGGCGAA 360
DD3b    CGCCAGCAAGTACGCGTCGGAGGGCACGGACCTGCTCCCCGCGACGGCGTCGGCGGCGAA 349
Grove   CGCCAGCAAGTACGCGTCGGAGGGCACGGACCTGCTCCCCGCGACGGCGTCGGCGGCGAA 358
Bayer   CGCCAGCAAGTACGCGTCGGAGGGCACGGACCTGCTCCCCGCGACGGCGTCGGCGGCGAA 284

Moore   GCTGGAGGTGTGGCTAGAGGTGGAGTCGCACCACTTCTACCCGAACCGGGTCGCCGCTGG 371
DD3a    GCTGGAGGTGTGGCTAGAGGTGGAGTCGCACCACTTCTACCCGAAC-GCGTCGCCGCTGG 419
DD3b    GCTGGAGGTGTGGCTGGAGGTGGAGTCGCACCACTTCTACCCGAAC-GCATCGCCGCTGG 408
Grove   GCTGGAGGTGTGGCTGGAGGTGGAGTCGCACCACTTCCACCCGAAC-GCGTCGCCGCTGG 417
Bayer   GCTGGAGGTGTGGCTGGAGGTGGAGTCGCACCACTTCCACCCGAAC-GCGTCGCCGCTGG 343

Moore   TGTTCCAGCTGCTCGTGAGGCCGCTCCTGGGCGGCGCCCC-GACGCGGCGGTGGTGGACA 430
DD3a    TGTTCCAGCTGCTCGTGAGGCCGCTCCTGGGCGGCGCCCCCGACGCGGCGGTGGTGGACA 479
DD3b    TGTTCCAGCTGCTCGTGAGGCCGCTCCTGGGCGGCGCCCCCGACGCGGCGGTGGTGGAGA 468
Grove   TGTTCCAGCTGCTCGTGAGGCCGCTCCTGGGCGGCGCCCCCGACGCGGCGGTGGTGGAGA 477
Bayer   TGTTCCAGCTGCTCGTGAGGCCGCTCCTGGGCGGCGCCCCCGACGCGGCGGTGGTGGAGA 403

Moore   AGCACGCGGAGCAGCTCGCCAAGGTGCTCGACGTGTACGAG-CGCACCTCGCCCGCAACA 489
DD3a    AGCACGCGGAGCAGCTCGCCAAGGTGCTCGACGTGTACGAGGCGCACCTCGCCCGCAACA 539
DD3b    AGCACGCGGAGCAGCTCGCCAAGGTGCTCGACGTGTACGAGGCGCACCTGGCCCGCAACA 528
Grove   AGCACGCGGAGCAGCTCGCCAAGGTGCTCGACGTGTACGAGGCGCACCTGGCCCGCAACA 537
Bayer   AGCACGCGGAGCAGCTCGCCAAGGTGCTCGACGTGTACGAGGCGCACCTGGCCCGCAACA 463
```

Figure 42 (continued).

```
Moore   AGTACCTCGCCGGGGACGAGTTCACGCTCGCCGACGCCAACCACGCGCTCCTACCTGCTC 549
DD3a    AGTACCTCGCCGGGGACGAGTTCACGCTCGCCGACGCCAACCACGCG-TCCTACCTGCTC 598
DD3b    AGTACCTCGCCGGGGACGAGTTCACGCTCGCCGACGCCAACCACGCG-TCCTACCTGCTC 587
Grove   AGTACCTCGCCGGGGACGAGTTCACGCTCGCCGACGCCAACCACGCGCTCCTACCTGCTC 597
Bayer   AGTACCTCGCCGGGGACGAGTTCACGCTCGCCGACGCCAACCACGCG-TCCTACCTGCTC 522

Moore   T-ACCTCAGCAAGACCCC-AAGGCCGG-CT-CGTCGCCGCCCGCCCC-ACGTCAAGGCCT 604
DD3a    T-ACCTCAGCAAGACCCCCAAGGCCGGGCT-CGTCGCCGCCCGCCCCCACGTCAAGGCCT 656
DD3b    T-ACCTCAGCAAGACCCCCAAGGCCGGGCT-CGTCGCCGCCCGCCCCCACGTCAAGGCCT 645
Grove   TCACCTCAGCAAGACCCCCAAGGCCGGGCTGCGTCGCCGCCCGCCCCCACGTCAAGGCCT 657
Bayer   T-ACCTCAGCAAGACCCCCAAGGCCGGGCT-CGTCGCCGCCCGCCCCCACGTCAAGGCCT 580

Moore   GGTGGGAGGCCATCGTCGCCCGCCCCGCGTTCCAGAAGACCGTCGCCGCCATCCCCTTGC 664
DD3a    GGTGGGAGGCCATCGTCGCCCGCCCCGCGTTCCAGAAGACCGTCGCCGCCATCCCCTTGC 716
DD3b    GGTGGGAGGCCATCGCCGCCCGCCCCGCGTTCCAGAAGACCGTCGCCGCCATCCCCTTGC 705
Grove   GGTGGGAGGCCATCGCCGCCCGCCCCGCGTTCCAGAAGACCGTCGCCGCCATCCCCTTGC 717
Bayer   GGTGGGAGGCCATCGCCGCCCGCCCCGCGTTCCAGAAGACCGTCGCCGCCATCCCCTTGC 640

Moore   CCCCGCCGCCCTCCTCCTCGGCTTGAcctcgccttgcgttgcgccgttgcctgggtcgcg 724
DD3a    CCCCGCCGCCCTCCTCCTCGGCTTGAcctcgccttgcgttgcgccgttgcctgggtcgcg 776
DD3b    CCCCGCCGCCCTCCTCCTCGGCTTGAcctcgccttgcg--------ctgcctgggtcgcg 757
Grove   CCCCGCCGCCCTCCTCCTCGGCTTGAcctcgccttgcg--------ttgcctgggtcgcg 769
Bayer   CCCCGCCGCCCTCCTCCTCGGCTTGA                                    666

Moore   gatgc-tcggagccccgagtcgaataaaagaggcagcatcctgtcttgcatttgctcgtg 783
DD3a    gatgcgtcggagccccgagtcgaataaaagaggcagcatcctgtcttgcatttgctgctg 836
DD3b    gatgcgtcggagccccgagtcgaataaaagaggccgcatcctgtcttgcatttgctgctg 817
Grove   gatgcgtcggagccctgagtcgaataaaagaggccgcatcctgtcttgcatttgctcgtg 829
Bayer

                                                      a          a
Moore   cgccatgtgtta--acagcctgtgtaataaacactgttgcctttcgtgtgtgttcattgc 841
DD3a    cgccatgtgtta--acagcctgtgtaataaacactgttgc-tttcgtgtgtgttca     889
DD3b    cgccatgtgctataacagcctgtgtaataaacactgttgc-tttcgtgtgtgttcattgc 876
Grove   cgccatgtgctataacagcctgtgtaataaacactgttgcctttcgtgtgtgttcattgc 889
Bayer

              a            a                              a
Moore   ctttt----ggttggtctttgc                              859
DD3a
DD3b    cttttggttggttggtccttgcatatcctactagtgctgatcttttgta 926
Grove   ctttttgttggttggtctttgca                            912
Bayer
```

Figure 42. Nucleotide sequence alignment of *Zm* GST III clones DD3a and DD3b with similar published sequences. `Moore` = *Zm* GST III (b) sequence, Moore *et al.* (1986), GenBank X04455; `Grove` = *Zm* GST III sequence, Grove *et al.* (1988), GenBank X06755; `Bayer` = *Zm* GST III patent sequence, Bieseler *et al.* (1996); `DD3a` = DD3a *Zm* GST III sequence, this work; `DD3b` = DD3b *Zm* GST III sequence, this work. Nucleotide differences with respect to DD3b are highlighted in bold, polyadenylation sites are indicated by 'a' above the alignment.

Cloning of *Zm* GST II

To try to clone the coding region of the *Zm* GST II gene, a similar approach to that used for *Zm* GST III was used. Oligonucleotide primers, containing restriction sites to facilitate cloning, were designed using published sequences for *Zm* GST II (Jepson *et al.*, 1994, Irzyk *et al.*, 1995), to give the primers Pmr-2a and Pmr-2b (Table 14). Total RNA isolated from the shoots of dichlormid-treated maize seedlings, or poly $(A)^+$-enriched RNA isolated from the roots of similar seedlings were used as templates for reverse transcriptase reactions. In both cases, when using the products of these reactions as templates and Pmr-2a and Pmr-2b as primers, PCR failed to amplify a product of the correct size.

Discussion

Cloning of *Zm* GST I

cDNA clones encoding *Zm* GST I have been successfully isolated using both RT-PCR and library immunoscreening. The nucleotide sequences of the resulting clones were identical, and together were very similar to previously published *Zm* GST I cDNA and genomic sequences (Shah *et al.*, 1986, Grove *et al.*, 1988). Shah *et al.* (1986) showed that the inbred maize line Missouri 17 possessed a single *Zm* GST I gene, while the hybrid Pioneer 3780A possessed two such genes. The amino acid sequence of *Zm* GST I, as deduced from the various published clones and those described here, is practically identical in each case. Further evidence for this lack of variation in *Zm* GST I comes from the work of Sari-Gorla *et al.* (1993). By analysing non-denaturing PAGE gels of crude samples from multiple maize lines for GST activity, they showed the presence of two major GSTs with activity towards CDNB, which almost certainly corresponded to *Zm* GST I-I and *Zm* GST I-II (see chapter 5). While the relative mobility of other GSTs varied between maize lines, the relative mobility of the two major isoenzymes remained unchanged, suggesting that *Zm* GST I and *Zm* GST II subunits did not vary in the maize lines analysed. Primer extension of *Zm* GST I mRNA (Shah *et al.*, 1986) showed that the 5′ untranslated regions of the two genes identified in the same work were identical except that one contained a 5 bp. insertion. This insertion was not present in pGTC2

(Grove *et al.*, 1988) but was present in each of the *Zm* GST I clones isolated by immunoscreening in this study. This insertion increased the number of pentameric (AGAGG) repeats present in the 5′ untranslated region of *Zm* GST I mRNA from 6 to 7. It is unclear whether this repeated sequence is functionally important, although Shah *et al.* (1986) commented that a similar but longer repeat in a chicken clone was susceptible to cleavage by S1 nuclease. These repeats may therefore be involved in regulating mRNA turnover.

Cloning of *Zm* GST III

Library screening isolated two similar, but distinct, *Zm* GST III clones which on translation gave rise to slightly different polypeptide products. It is most likely that these two clones represent allelic variants of a *Zm* GST III gene rather than two genes, since the 5′ and 3′ untranslated regions of the clones, which usually differ between gene copies, are almost identical. Previous studies (Moore *et al.*, 1986), showed that the *Zm* GST III gene is present at a single or low copy number in the maize genome. Thus, in the cultivar Pioneer 3394, which is a hybrid and is therefore likely to be highly heterozygous, two distinct alleles, corresponding to clones DD3a and DD3b, are both present. It is likely that the single nucleotide difference between the second *Zm* GST III PCR product sequenced and other *Zm* GST III sequences was due to an error introduced during PCR.

Cloning of *Zm* GST II

While PCR was successfully used to amplify *Zm* GST I and *Zm* GST III cDNA sequences, this method consistently failed to amplify *Zm* GST II cDNA sequences. It is unlikely that the lack of a PCR amplification product was due to poor synthesis of cDNA template since cDNA from the same reactions were successfully used to amplify *Zm* GST III sequences and to construct a cDNA library. The PCR may therefore have failed due to the absence of cDNA sequences recognised by the primers, either because of the *Zm* GST II mRNA was absent, or because the *Zm* GST II cDNA sequence differed over one or both of the regions used for primer design. However, the PCR could also have failed for a number of other reasons, including inefficient primers, cDNA secondary structure

stalling strand extension, or sub-optimal PCR cycling conditions. Further work is necessary to resolve why the PCR failed; this could involve PCR under different conditions, or using primers designed to different regions of the *Zm* GST II sequence. It is very unlikely that *Zm* GST II mRNA was not present in the total RNA from dichlormid-treated roots since the RNA was isolated from tissue known to contain high levels of the *Zm* GST II subunit. Furthermore, *Zm* GST II was originally cloned from a cDNA library prepared from maize plants exposed to dichlormid, confirming that the *Zm* GST II transcript must be present in the RNA used as the source for PCR amplification (Jepson *et al.*, 1994).

7. Cloning of Tau Class Maize GSTs

Introduction

Plant GSTs can be classified into two major groups, based on DNA sequence homology and exon position (Droog *et al.*, 1995, Droog, 1997). All the well characterised maize GSTs with known sequence are members of the type I, or theta class, including the *Zm* GST I and *Zm* GST III sequences, described in the previous chapter. The other major group, type III or tau class GSTs, have no well characterised members from maize or any other grass species. The maize *Bronze2* gene appears to belong to the tau class, however the polypeptide it encodes has not been well studied (Marrs *et al.*, 1995). Most tau class GSTs have been identified as auxin-regulated or stress-induced genes and their function as GSTs was not initially recognised. It is only recently that these cDNA clones have been expressed and found to have GST activity (Droog *et al.*, 1993). Very little work has been done on the substrate specificities of these GSTs, and in particular their ability to detoxify herbicides and protect the plant from these and other xenobiotics.

As detailed in earlier chapters, purification and subsequent characterisation of *Zm* GST subunits V, VI and VII (see page 108) showed that they were different from the theta class *Zm* GST subunits I, II and III, and did not form heterodimers with theta class subunits. *Zm* GST subunits V, VI and VII were also different from *Zm* GST subunits I, II and III in terms of their properties during purification, their range of activities, their immunological characteristics and their expression. All these data suggested that *Zm* GST subunits V, VI and VII belonged to a distinct class of GSTs to those previously characterised in maize. Therefore, the cDNA clones corresponding to *Zm* GST subunits V, VI and VII were cloned, both to obtain sequence data for these novel, herbicide-active maize GSTs and to see how these sequences were related to other plant GST sequences.

Results

Immunological Screening for *Zm* GST V and *Zm* GST VI Clones

To isolate clones corresponding to *Zm* GST V and *Zm* GST VI subunits, the *Zm* GST V-VI antibody was used to screen a maize cDNA expression library prepared from maize roots treated with the herbicide safener dichlormid. Approximately 170,000 pfu from the unamplified library were used for the primary screen. The *Zm* GST V-VI antibody detected 10 putative positive plaques, although cross-reactivity of the antiserum with an *E. coli* protein made positives difficult to identify, due to a high background of reactivity with all plaques. Five of the putative positives were taken through to a secondary screen; three of these subsequently gave positive plaques which were put through a third round of screening to allow single plaque isolation. Positive, well isolated plaques were cored, and plasmids containing the putative GST inserts were excised from the phage to give pBluescript SK plasmids containing the inserts of interest. These plasmids were grown up in *E. coli*, purified and their inserts sequenced.

Two of the three clones encoding polypeptides recognised by the *Zm* GST V-VI antibody were fully sequenced in both directions, and were termed DD5 and DD6a (Figure 43 and Figure 44). The third clone was partially sequenced and appeared to be almost identical to DD6a, but with a longer 3' untranslated region and 2 base changes within the coding sequence (C to G at base 512 and G to A at base 615), resulting in an amino acid change from valine to isoleucine at amino acid residue 178. These differences are underlined in Figure 44. This clone was termed DD6b.

```
ctaacgccggaggcgagaacaagaaaaagctcgacATGGCCGAGGAGAAG   50
                                    M  A  E  E  K

AAGCAGGGCCTGCAGCTGCTGGACTTCTGGGTGAGCCCATTCGGGCAGCG  100
 K  Q  G  L  Q  L  L  D  F  W  V  S  P  F  G  Q  R

CTGCCGCATCGCGATGGACGAGAAGGGCCTGGCCTACGAGTACCTGGAGC  150
   C  R  I  A  M  D  E  K  G  L  A  Y  E  Y  L  E

AGGACCTGGGGAACAAGAGCGAGCTGCTCCTCCGCGCCAACCCGGTGCAT  200
Q  D  L  G  N  K  S  E  L  L  L  R  A  N  P  V  H

AAGAAGATCCCCGTGCTGCTGCACGACGGCCGCCCCGTCTGCGAGTCCCT  250
 K  K  I  P  V  L  L  H  D  G  R  P  V  C  E  S  L

CGTCATCGTGCAGTACCTCGACGAGGCGTTCCCGGCGGCGGCGCCGGCGC  300
   V  I  V  Q  Y  L  D  E  A  F  P  A  A  A  P  A

TGCTCCCCGCCGACCCCTACGCGCGCGCGCAGGCCCGCTTCTGGGCGGAC  350
L  L  P  A  D  P  Y  A  R  A  Q  A  R  F  W  A  D

TACGTCGACAAGAAGCTCTACGACTGCGGCACCCGGCTGTGGAAGCTCAA  400
 Y  V  D  K  K  L  Y  D  C  G  T  R  L  W  K  L  K

GGGGGACGGCCAGGCGCAGGCGCGCGCCGAGATGGTCGAGATCCTCCGCA  450
   G  D  G  Q  A  Q  A  R  A  E  M  V  E  I  L  R

CGCTGGAGGGCGCGCTCGGCGACGGGCCCTTCTTCGGCGGCGACGCCCTC  500
T  L  E  G  A  L  G  D  G  P  F  F  G  G  D  A  L

GGCTTCGTCGACGTCGCGCTCGTGCCCTTCACGTCCTGGTTCCTCGCCTA  550
 G  F  V  D  V  A  L  V  P  F  T  S  W  F  L  A  Y

CGACCGCTTCGGCGGCGTCAGCGTGGAGAAGGAGTGCCCGAGGCTGGCCG  600
   D  R  F  G  G  V  S  V  E  K  E  C  P  R  L  A

CCTGGGCCAAGCGCTGCGCCGAGCGCCCCAGCGTCGCCAAGAACCTCTAC  650
A  W  A  K  R  C  A  E  R  P  S  V  A  K  N  L  Y

CCGCCCGAGAAGGTCTACGACTTCGTCTGCGGGATGAAGAAGAGGCTGGG  700
 P  P  E  K  V  Y  D  F  V  C  G  M  K  K  R  L  G

CATCGAGTAGagcatccatcggtcggccggtggctggccgggagtaataa  750
   I  E  *

tgacgaaccaattatctagttttggtttgagtgtgctcagcagagcagtt  800

cgtgttcatgagttcgtcgtcgttgtattttctattgtcagcggtggcag  850

cgccgtacgtgttgcctcgtacaccacaaccgaataaggggtgtttggt  900

ttgcccctcctaaaatttagcccctatcaaaaaaaaaaaaaaaaaaa    947
```

Figure 43. DD5 cDNA sequence showing coding region and deduced protein sequence.

```
ctctaatccatttcggcatttccaacgccttcgccctaccagccacgtcg   50

cttcgaggccgatcgaccgagcagctggtggcaATGGCGGCGGCGGCGGA  100
                                  M  A  A  A  A  E

GGTCGTGCTGCTGGACTTCTGGGTGAGCCCCTTCGGGCAGCGCTGCCGGA  150
  V  V  L  L  D  F  W  V  S  P  F  G  Q  R  C  R

TCGCGCTGGCGGAGAAGGGCGTGGCCTACGAGTACCGCGAGCAGGACCTC  200
I  A  L  A  E  K  G  V  A  Y  E  Y  R  E  Q  D  L

CTGGACAAGGGCGAGCTGCTCCTCCGCTCCAACCCCATCCACAAGAAGAT  250
 L  D  K  G  E  L  L  L  R  S  N  P  I  H  K  K  I

CCCCGTCCTGCTCCACGCCGGCAGGCCCGTCTGCGAGTCGCTCGTCATCC  300
  P  V  L  L  H  A  G  R  P  V  C  E  S  L  V  I

TCCAGTACATCGACGAGGCCTGGCCGGACGTCGCGCCGCTCCTCCCCAAG  350
L  Q  Y  I  D  E  A  W  P  D  V  A  P  L  L  P  K

GACGACCCCTACGCCCGCGCGCAGGCGCGTTTCTGGGCCGATTACATCGA  400
 D  D  P  Y  A  R  A  Q  A  R  F  W  A  D  Y  I  D

CAAGAAGATCTATGACAGCCAGACTCGGCTGTGGAAGTTCGAGGGCGAGG  450
  K  K  I  Y  D  S  Q  T  R  L  W  K  F  E  G  E

CGCGGGAGCAGGCGAAGAAGGACCTGGTGGAGGTCCTGGAGACCCTGGAG  500
A  R  E  Q  A  K  K  D  L  V  E  V  L  E  T  L  E

GGGGAGCTCGCCGACAAGCCTTTCTTCGGCGGCGGCGCCCTCGGCTTCGT  550
 G  E  L  A  D  K  P  F  F  G  G  G  A  L  G  F  V

GGACGTGGCTCTGGTGCCCTTCACGTCCTGGTTCCTCGCCTACGAGAAGC  600
  D  V  A  L  V  P  F  T  S  W  F  L  A  Y  E  K

TGGGCGGGTTCAGCGTCCAGGAGCACTGCCCCAGGATCGTGGCCTGGGCC  650
L  G  G  F  S  V  Q  E  H  C  P  R  I  V  A  W  A

GCGCGCTGCAGGGAGCGGGAGAGCGTGGCCAAGGCCATGTCCGACCCTGC  700
 A  R  C  R  E  R  E  S  V  A  K  A  M  S  D  P  A

CAAGGTGCTCGAGTTCGTCCAGTTCCTCCAGAGCAAGTTCGGGGCCAAGT  750
  K  V  L  E  F  V  Q  F  L  Q  S  K  F  G  A  K

GAtcggaagcattgcgtgtgctgctagcctgatatgccctatgcaggcca  800
*

ggctggtgctttgatctgctcgatcagctctatgcccatgctagcgttgc  850

atagcgcagttgatgtgtgatgtgtctggttggttgtagctgctctttgc  900

ctggtttcgtacgtcagtgtaaggtttcaggttttcagtgtctggggtag  950

ctctgcgttgcccttgcccctgccccctacctagcggctcttgagctctt 1000

cggctcgccagcaataaagttgcagaggctttagctaaaagtttctgtat 1050

tttttagttgacgattattggtccaatgtattcgggaattttgttctctc 1100

taaaaaaaaaaaaaaaaaaa                                1121
```

Figure 44. DD6a cDNA sequence showing coding region and deduced protein sequence. Bases and residues in bold show location of differences when compared to DD6b; see text for details.

Characterisation of Clone DD5

The nucleic acid sequence of DD5 is shown in Figure 43, along with the deduced polypeptide sequence. This 947 bp. clone contained a single potential open reading frame long enough to encode a GST (675 bp.), and appeared to be full length, with a 35 bp. 5′ untranslated region, a 218 bp. 3′untranslated region and a 19 bp. polyadenylated tail. Initial database searches showed high homology between DD5 and plant GST sequences, and there was good evidence that this sequence coded for the *Zm* GST V subunit. As will be discussed in chapter 8, expression of DD5 gave a protein which shared numerous characteristics with *Zm* GST V, including chromatographic behaviour, mobility in SDS and non-denaturing PAGE, enzyme activity and peptide digestion patterns. From this evidence it was assumed that DD5 encoded one of the *Zm* GST V variants found in maize, and this GST sequence was termed GST5.

The sequence contained a single putative polyadenylation signal (AATAAG, starting at base 833), and was polyadenylated starting at base 929. The coding region of GST5 showed an exceptionally high preference for G or C bases at the third position of each codon, with 99.1% (i.e. all but two) of codons ending in G or C, compared with a calculated value of 51.6% for a similar sequence without codon bias. In general, coding regions of maize mRNA show some bias towards C or G bases at the third position of each codon (Fennoy and Bailey-Serres, 1993), however the bias shown by GST5 was extreme. A bias towards G or C was also evident at the first position of the codons. Here, bias is only possible at codons for leucine and arginine, and of 41 such codons in GST5, 39 started with G or C (in unbiased sequences the expected frequency would have been 27).

GST5 encoded a polypeptide of 224 amino acid residues which had a calculated molecular mass of 25,123 Da. Protein database sequence similarity searches using the BLAST program (Altschul *et al.*, 1990) showed that this polypeptide most closely resembled the type III, or tau class GSTs originally identified as auxin-regulated clones (Droog *et al.*, 1995, Droog, 1997), with highest homology to the tobacco *parC* and *C-7* clones, and a eucalyptus auxin-induced protein, as described later.

Characterisation of DD6a

DD6a was a 1121 bp. clone containing a single open reading frame of 669 bp., with an 83 bp. 5′ untranslated region, a 349 bp. 3′ untranslated region and a 20 bp. polyadenylated tail. A putative polyadenylation signal (AATAAA) was located starting at base 1013. The nucleotide sequence of DD6a is shown in Figure 44, along with the deduced polypeptide sequence, and since evidence (chapter 8) strongly suggested that this sequence coded for *Zm* GST VI, the sequence was subsequently termed GST6a.

As shown for GST5, the coding region of GST6a showed a very strong bias for C and G nucleotides, with 96.4% of codons ending with G or C, compared with a calculated expected frequency of 51.1% in the absence of any bias. This bias was also evident at the first position of codons, with 35 of 38 potentially biased first positions being G or C.

Translation of the open reading frame gave a deduced polypeptide of 222 amino acids with a calculated molecular mass of 25,126 Da. This polypeptide sequence was found to be very similar to that of GST5, with 149 (67%) identical amino acid residues. Database searches showed that like GST5, GST6a was most similar to tobacco *parC* and *C-7* clones, and a eucalyptus auxin-induced protein.

Correlation of GST5 and GST6a Sequences with *Zm* GST V Protein Sequence

Internal peptide sequence had been obtained for *Zm* GST V-V and *Zm* GST V-VI (chapter 4), and this was compared with the deduced polypeptide sequence of GST5. The exact peptide sequence was not present in GST5 or GST6a, however a similar sequence was found:

Zm GST V fragment	E N P V L K
GST5	A N P V H K
GST6a	S N P I H K

Reanalysis of the *Zm* GST V protein sequencing results showed that a minor alanine (A) peak was consistently present in the first cycle and similarly a minor histidine (H) peak

was present in the fifth sequencing cycle. This suggested that two variants of the *Zm* GST V subunit were present in the samples submitted for protein sequencing, giving rise to differing amino acid residues on sequencing where the two polypeptide sequences differed. The protein sequence of one of these variants was present in GST5, so it is likely that GST5 encoded one of the two *Zm* GST V variants. Since the *Zm* GST V-VI antibody was probably raised against protein containing both *Zm* GST V variants it should recognise both variants, and further library screening with the antibody would be likely to isolate clones for the currently uncharacterised variant.

Comparison of GST5 and GST6a Sequences with Homologous Sequences

A multiple alignment of deduced polypeptide sequences was constructed for the two new maize GSTs coded for by GST5 and GST6a, and homologous polypeptide sequences deduced from GenBank database entries (Figure 45). This showed high homology between all the polypeptides throughout the alignment, showing that these proteins are structurally very similar.

Interestingly, GST5 and GST6a polypeptide sequences also showed high homology (47% and 52% amino acid identity respectively) to a soybean glyoxalase I clone (Paulus *et al.*, 1993). Glyoxalase I catalyses the isomerisation of a hemithioacetal, derived from the spontaneous reaction of glutathione with methylglyoxal, to give *S*-lactoylglutathione (Thornalley, 1990), and the apparent homology between this enzyme from soybean and GSTs 5 and 6a suggested that these GSTs might also have glyoxalase I activity, giving them an endogenous function. However, GSTs 5 and 6a had no detectable sequence similarity when compared with a glyoxalase I sequence cloned from tomato (Espartero *et al.*, 1995). Later work (see chapter 8) showed that when expressed as recombinant proteins GSTs 5 and 6a did not produce proteins with glyoxalase I activity.

```
ZM GST5   MAEEKKQGLQLLDFWVSPFGQRCRIAMDEKGLAYEYLEQDLG-NKSELLLRANPVHKKIP
ZM GST6a  MAAAAE--VVLLDFWVSPFGQRCRIALAEKGVAYEYREQDLL-DKGELLLRSNPIHKKIP
EG PAR    MAEE----VILLDFWPSPFGMRAKIALREKGVHFDLREEELLSNKSPLLLQMNPVHKKIP
NT parC   MANEE---VILLDFWPSMFGMRLRIALAEKEIKYEYKQEDLR-NKSPLLLQMNPIHKKIP
NT C7     MADE----VVLLDTYVSMFGVSVRIALAEKGIQYEYKEQDLL-NKTPLLLQMNPIHKKIP
          **       : *** : * **   :**: ** : ::  :::*  :*  ***: **:*****

ZM GST5   VLLHDGRPVCESLVIVQYLDEAFPAAAPALLPADPYARAQARFWADYVDKKLYDCGTRLW
ZM GST6a  VLLHAGRPVCESLVILQYIDEAWPDVAPLLPKDDPYARAQARFWADYIDKKIYDSQTRLW
EG PAR    VLIHNGKPVCESHIIVQYIDETWGPESP-LLPSEPHERARARFWADYVDKKIFPAGRAAW
NT parC   VLIHNGKPICESIIAVEYIEEVWKDKAPSLLPSDPYDRAQARFWADYIDKKLYDFGRKLW
NT C7     VLIHNGKPICESLIIVEYIDEVWKDKSP-FMPSDPYKRAQARFWADYVDKKIYESGKKMW
          **:* *:*:*** : ::*::*.:   :* :   :*: **:*******:***::      *

ZM GST5   KLKGDGQAQARAEMVEILRTLEGALGDGPFFGGDALGFVDVALVPFTSWFLAYDRFGGVS
ZM GST6a  KFEGEAREQAKKDLVEVLETLEGELADKPFFGGGALGFVDVALVPFTSWFLAYEKLGGFS
EG PAR    RSTGEAQEAAKKEYIEGLKMLEGELGDTPYFGGERFGFLDVSLIPFYSWFYAVETLTGCS
NT parC   ATKGEEQEAAKKDFIECLKVLEGALGDRPYFGGESFGFVDIALIGFYSWFYAYETFGNFS
NT C7     TSKVEDQEAANKEFIECLKLLEGELGDKPYFGGERFGFVDMALMPYYSWFPSYEKFGNFS
              : :  *. : :* *. *** *.* *:***  :**:*::*: : *** : : : . *

ZM GST5   VEKECPRLAAWAKRCAERPSVAKNLYPPEKVYDFVCGMKKRLGIE
ZM GST6a  VQEHCPRIVAWAARCRERESVAKAMSDPAKVLEFVQFLQSKFGAK
EG PAR    FEEECPKLVGWAKRCMQRESVARSLPDQHKVYDFSRRSGRRFRRK
NT parC   TEAECPKFVAWAKRCMQRESVAKSLPDQPKVLEFVKVLRQKFGLE
NT C7     IEAECPKIVEWAKKCVQKESVSKSLADPDKVYDYIVMARQKWGIA
           : .**::. ** :* :: **:: :    ** ::      :
```

Figure 45. Multiple alignment of GST5 and GST6a deduced polypeptide sequences with homologous plant GST sequences.

Sequence labels are as described in Table 15.

* = Residues identical in all sequences.

: = Residues in one of the groups STA; NEQK; NHQK; NDEQ; QHRK; MILV; MILF; HY; FYW in all sequences (i.e. strongly conservative substitutions).

. = Residues in one of the groups CSA; ATV; SAG; STNK; STPA; SGND; SNDEQK; NDEQHK; NEQHRK; FVLIM; HFY in all sequences (i.e. weakly conservative substitutions).

GST5 and GST6a also showed significant similarity to the maize Bronze2 GST involved in anthocyanin metabolism (Marrs *et al.*, 1995). However it is unlikely that these GSTs play a similar role since *Zm* GST V, and probably *Zm* GST VI also, appeared to be constitutively expressed throughout the plant (see chapter 5) and if these GSTs, like Bronze2, showed activity towards cyanidin-3-glucoside, their activity in kernels and other pigmented tissue would rescue the *bz2* phenotype. This does not occur, however application of abscisic acid to *bz2* kernels causes an increase in anthocyanin accumulation, suggesting that an auxin-inducible GST has weak activity towards cyanidin-3-glucoside (Walbot *et al.*, 1994).

Isolation of *Zm* GST VII cDNA

As described in chapter 4, anion exchange chromatography of pool 2 GSTs resolved a 29.5 kDa polypeptide eluting towards the end of the salt gradient which was tentatively identified as a GST subunit and named *Zm* GST VII. Western blotting showed that this polypeptide was not recognised by either anti-*Zm* GST I-II serum or anti-*Zm* GST V-VI serum, and so appeared to be a novel GST subunit. N-terminal protein sequencing of this 29.5 kDa polypeptide gave the sequence S P X V K I L G H Y (chapter 4), which aligned well with published N-terminal amino acid sequences of a number of plant GSTs, but was different from any other maize GST sequence. A degenerate oligonucleotide primer was therefore designed using this protein sequence to enable PCR amplification and subsequent cloning of the cDNA for this polypeptide. A fully degenerate oligonucleotide primer coding for the amino acid residues underlined above was initially designed as follows:

5′ GTN AA(A/G) AT(A/C/G) (C/T)TN GGN CA(C/T) TA(C/T) 3′

This however had a 2304-fold degeneracy, which was considered too great to successfully amplify the correct cDNA. An attempt was made to decrease this degeneracy by using a codon usage table based on all the full-length maize genes in the GenBank database (Nakamura *et al.*, 1997) to bias the primer towards the codons found most frequently in maize. However, this table did not show enough codon bias to reduce the primer degeneracy sufficiently, so an alternative codon usage table was

constructed based on only those maize sequences which coded for known GSTs, since codon usage is known to vary between gene families. The resulting table (Figure 46) showed a very strong bias towards C and G nucleotides at the third position of each codon (91.2% C or G, compared with 67.9% C or G for the GenBank database codon usage table) and this enabled the design of the following oligonucleotide primer, Pmr-7a, with only 16-fold degeneracy and an estimated 70% chance of containing the correct sequence:

Pmr-7a: 5′ GT(C/G) AAG ATC CT(C/G) GG(C/G) CA(C/T) TAC 3′

RNA extracted from roots of dichlormid-treated maize seedlings was used as a template for a reverse transcriptase reaction, using the oligonucleotide og2 (Table 19) to prime the synthesis of cDNA. The resultant cDNA was used as a template for PCR, using the primer Pmr-7a (see above) and the non-specific primer og9 (Table 19). Agarose gel electrophoresis of the PCR product showed the presence of three major bands with estimated sizes of 1250, 820 and 650 bp. The 820 bp. band was of the expected size, and so was purified, but did not contain enough DNA for cloning. An aliquot of the purified product was therefore used as a template for a further PCR using the same primers as before, which gave a single, intense 820 bp. band which was subsequently cloned, sequenced and found to have high homology to other plant GSTs. To obtain a full-length cDNA clone, this PCR product was DIG-labelled and used to probe the safener-treated maize root cDNA library. After three rounds of screening three positive clones were isolated. On sequencing two of these clones were found to be identical to each other and very similar to eukaryotic translation initiation factor sequences, while the third clone was a full-length clone identical to the cloned PCR product sequence, and was termed DD7. Analysis of the translation initiation factor sequence did not reveal any obvious sequence similarity to DD7 so it was unclear why two such sequences were isolated. The most likely explanation is that the PCR product used as a probe was contaminated with an additional PCR product corresponding to a fragment of the initiation factor clones and that this contaminant was of the same size as the GST PCR product, resulting in it being overlooked and then co-purified with the GST PCR product.

Amino Acid	Codon	Number	Usage	Amino Acid	Codon	Number	Usage
A	GCA	11	0.06	P	CCA	6	0.07
A	GCC	79	0.44	P	CCC	39	0.44
A	GCG	70	0.39	P	CCG	37	0.42
A	GCT	21	0.12	P	CCT	7	0.08
C	TGC	20	1.00	Q	CAA	0	0.00
C	TGT	0	0.00	Q	CAG	42	1.00
D	GAC	67	0.91	R	AGA	0	0.00
D	GAT	7	0.09	R	AGG	17	0.23
				R	CGA	1	0.01
E	GAA	7	0.07	R	CGC	38	0.52
E	GAG	90	0.93	R	CGG	11	0.15
				R	CGT	6	0.08
F	TTC	56	0.97				
F	TTT	2	0.03	S	AGC	25	0.42
				S	AGT	1	0.02
G	GGA	7	0.09	S	TCA	3	0.05
G	GGC	49	0.61	S	TCC	16	0.27
G	GGG	20	0.25	S	TCG	9	0.15
G	GGT	4	0.05	S	TCT	5	0.08
H	CAC	24	0.83	T	ACA	0	0.00
H	CAT	5	0.17	T	ACC	17	0.46
				T	ACG	18	0.49
I	ATA	1	0.03	T	ACT	2	0.05
I	ATC	35	0.97				
I	ATT	0	0.00	V	GTA	1	0.01
				V	GTC	43	0.39
K	AAA	3	0.04	V	GTG	60	0.55
K	AAG	71	0.96	V	GTT	5	0.05
L	CTA	3	0.02	W	TGG	25	1.00
L	CTC	82	0.49				
L	CTG	75	0.45	Y	TAC	40	0.98
L	CTT	3	0.02	Y	TAT	1	0.02
L	TTA	0	0.00				
L	TTG	4	0.02	STOP	TAA	1	0.17
				STOP	TAG	1	0.17
M	ATG	23	1.00	STOP	TGA	4	0.67
N	AAC	25	0.96				
N	AAT	1	0.04				

Figure 46. Maize GST codon usage table.

The table was constructed using the nucleotide sequences for the following GST subunits: GST *Zm* I (Grove *et al.*, 1988, GenBank X06754), GST *Zm* II (Irzyk *et al.*, 1995, GenBank U12679), GST *Zm* III (Bieseler *et al.*, 1996), GST *Zm* V (see Figure 43), GST *Zm* VI (see Figure 44) and BZ2 (Nash *et al.*, 1990, GenBank U14599). `Number` refers to number of occurrences of a codon, while `Usage` refers to the frequency of occurrence of the codon within its synonymous family (i.e. all the codons coding for the same amino acid).

```
gccaaccaacgagtagcaggaaacATGTCTCCGCCCGTCAAGATCCTCGG   50
                         M  S  P  P  V  K  I  L  G

CCACTACGCGAGCCCGTACTCGCACCGCGTCGAGGCCGCTCTGCGGCTCA  100
  H  Y  A  S  P  Y  S  H  R  V  E  A  A  L  R  L

AGGGCGTGCCGTACGAGCTGGTCCAGGAAGACCTGGGCAACAAGAGCGAG  150
K  G  V  P  Y  E  L  V  Q  E  D  L  G  N  K  S  E

CTGCTGCTCGCCAAGAACCCTGTCCACAAGAAGGTGCCCGTGCTCCTCCA  200
 L  L  L  A  K  N  P  V  H  K  K  V  P  V  L  L  H

TGGCGACAGGGCCGTCTGCGAGTCCCTCCTCATCGTCGAGTACGTCGACG  250
  G  D  R  A  V  C  E  S  L  L  I  V  E  Y  V  D

AGGCCTTCGACGGGCCGTCCATCCTGCCGGCCGACCCCCACGACCGTGCC  300
E  A  F  D  G  P  S  I  L  P  A  D  P  H  D  R  A

GTCGCCCGTTTCTGGGCGAACTTCTTGGACACCAAGTTCTCCCAGCCGTT  350
 V  A  R  F  W  A  N  F  L  D  T  K  F  S  Q  P  F

CTGGCTGGCGTACTGGGCGGAGGGCGAGGCGCAGAAGGCCGTGGTGAAGG  400
  W  L  A  Y  W  A  E  G  E  A  Q  K  A  V  V  K

AGGCCAAGGAGAACCTGGCGCTCCTGGAGGCGCAGCTCGGCGGGAAGAGC  450
E  A  K  E  N  L  A  L  L  E  A  Q  L  G  G  K  S

TTCTTCGGCGGCGACACGCCCGGGTACCTCGACATAGCCGCGTGCGCGTT  500
 F  F  G  G  D  T  P  G  Y  L  D  I  A  A  C  A  L

GGGTCCTTGGATCGGCGTGCTCGAGGAGGTGACTGGAGTGGCCTTGCTGG  550
  G  P  W  I  G  V  L  E  E  V  T  G  V  A  L  L

ACGCCGACGAGTTCCCCGCTCTATGCCAGTGGGCCAGGGACTACAGCTCC  600
D  A  D  E  F  P  A  L  C  Q  W  A  R  D  Y  S  S

AGTGAAGCGCTCAGGCCATGCCTGCCGGACAGGGACCGACTCGTTGCCTA  650
 S  E  A  L  R  P  C  L  P  D  R  D  R  L  V  A  Y

CTTCACCGAGAACAAGGAGAAGTACAAGACATTTGCCAAGGCAACGTTGC  700
  F  T  E  N  K  E  K  Y  K  T  F  A  K  A  T  L

ATCAGTAGctgctagttgggtgcaaaccgcttctttatctctgtgtggaa  750
H  Q  *

taatgtatacgtacgtgctccctcgatatcaaataaatcagctaccggag  800

ttgactgtagtcaaattgctaaaaaaaaaaaaaaaaaaa             840
```

Figure 47. DD7 cDNA nucleotide and deduced amino acid sequences.

The novel GST clone (DD7; Figure 47) was 840 bp. in length, with a putative 684 bp. open reading frame encoding a 227 amino acid polypeptide, a 24 bp. 5′ untranslated region, a 112 bp. 3′ untranslated region with a putative polyadenylation signal (AATAA) at base 832 and a 20 bp. polyadenylated tail. The deduced protein sequence had the N-terminal sequence M S P P V K I L G H Y, in agreement with the results of protein sequencing, consisting with DD7 encoding *Zm* GST VII. Those amino acid residues conserved between plant GSTs were all present in this polypeptide, confirming its identity as a GST, and the corresponding gene was named *GST VII*. Database searches showed that the protein sequence showed maximum homology (40% amino acid identity) with the tobacco *Nt103*, *Nt110* and *Nt111* GSTs, potato pathogenesis-related protein 1 and soybean HSP26-A.

Discussion

Maize tau class GST clones

Apart from work presented here, the only maize tau class GST to be reported previously has been the *Bronze2* (*Bz2*) gene, which although a tau class GST, is rather divergent when compared to other members of the class (Figure 48). When Schmitz and Theres (1992) screened a maize cDNA library using *Bz2* as a probe, a weakly-hybridising clone was discovered, which was named CT24. CT24 was found to be expressed throughout plant, and at particularly high levels in roots of etiolated seedlings and in necrotic leaf sheath tissue. Although the sequence of CT24 was not reported its deduced polypeptide sequence was 41% identical (61% similar) to that of *Bz2*. These values are very close to those obtained when GST5 is compared with *Bz2*, and since *Bz2* is more similar to GST5 than to any other maize sequence available to date it is highly likely that screening with a *Bz2* probe would identify *Zm* GST V clones, and CT24 may well be a *Zm* GST V homologue.

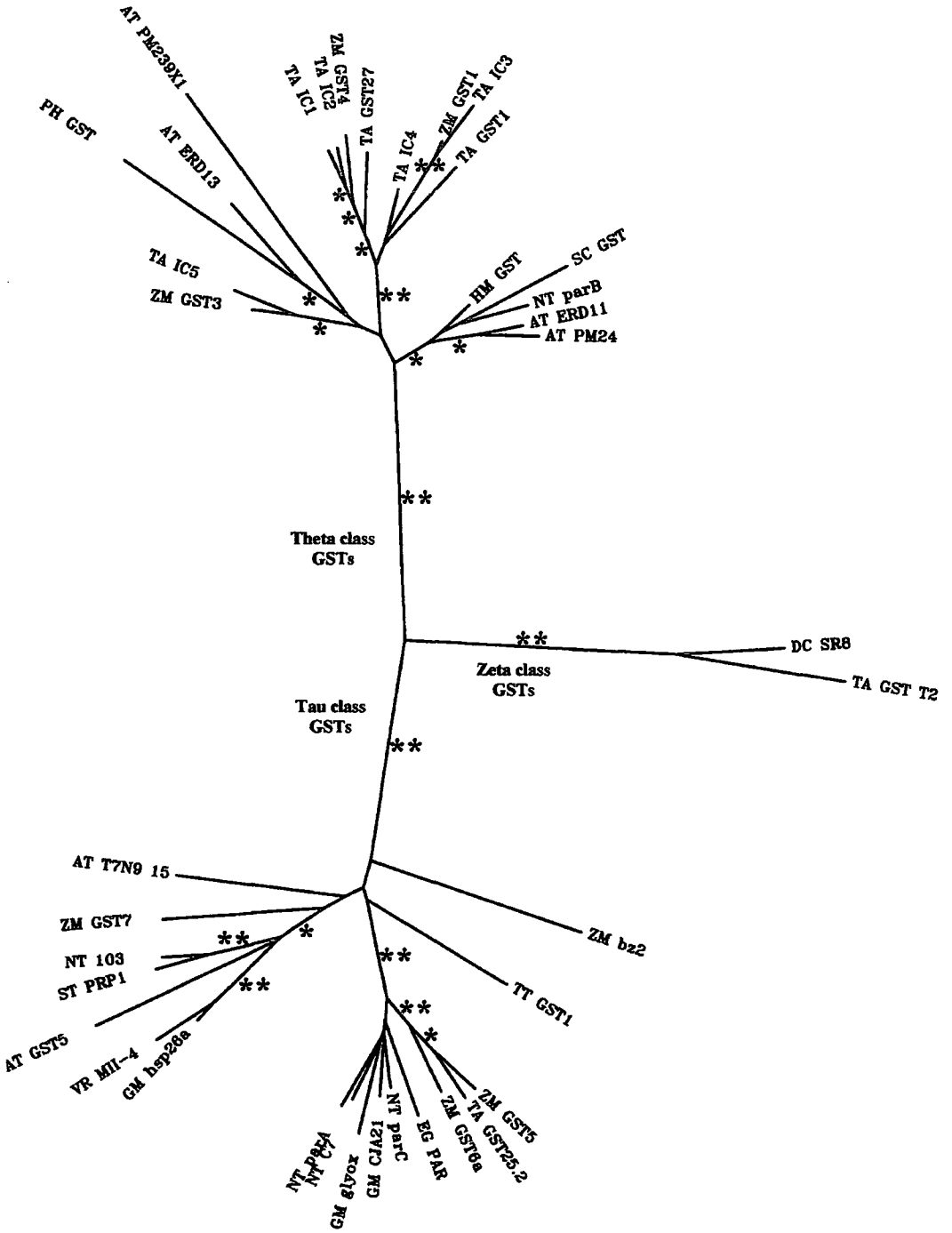

Figure 48. Phylogenetic tree of higher plant GSTs as inferred from protein sequence alignments, showing clustering of sequences into three classes as indicated.

Sequence labels are as described in Table 15. Asterisks represent significant groupings found in the consensus tree, as described in the text, with (*) representing groupings occurring in at least 80% of calculated trees and (**) representing groupings occurring in at least 95% of calculated trees. Line lengths are proportional to calculated evolutionary distances between sequences.

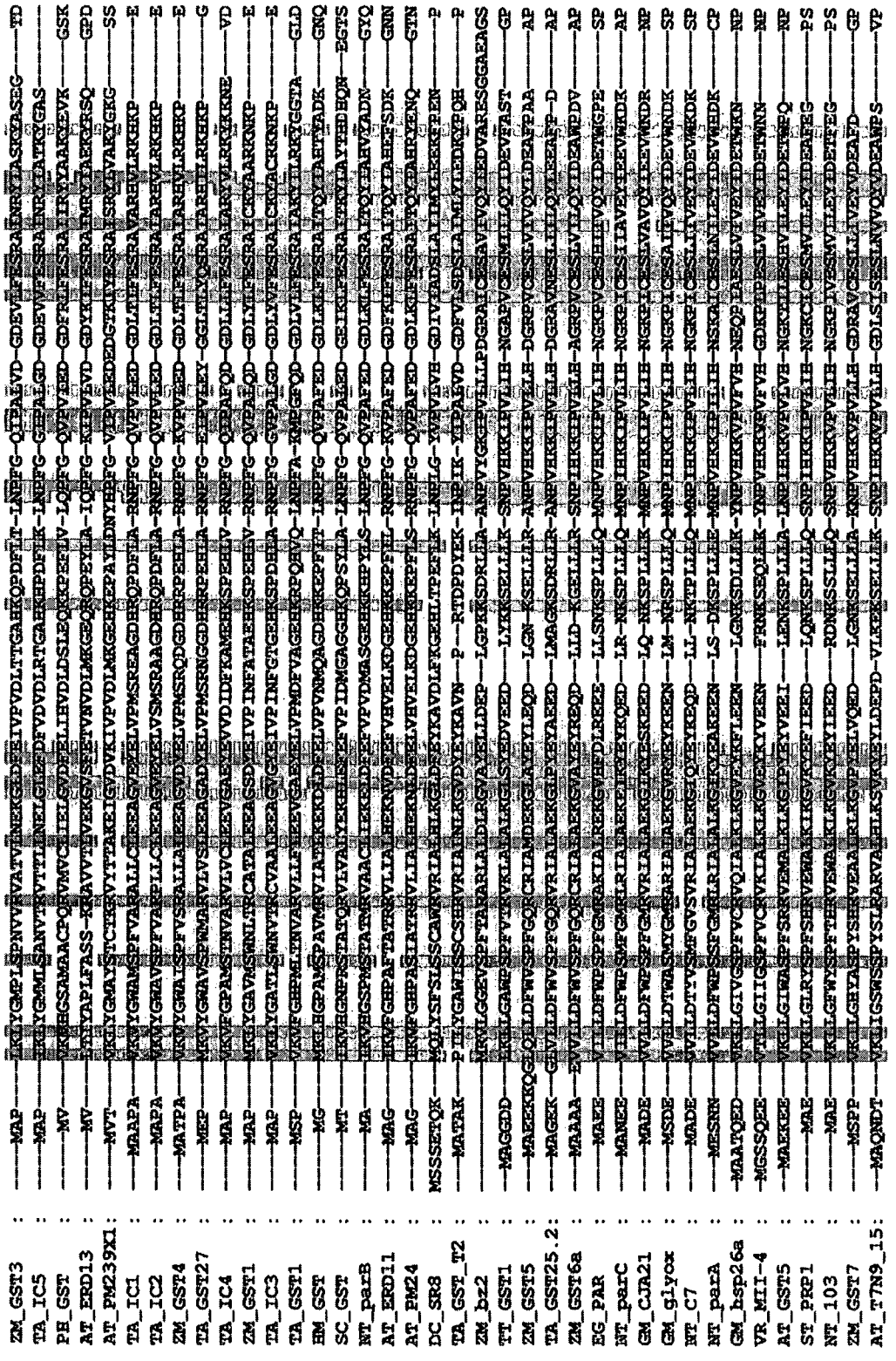

Figure 49 (continued).

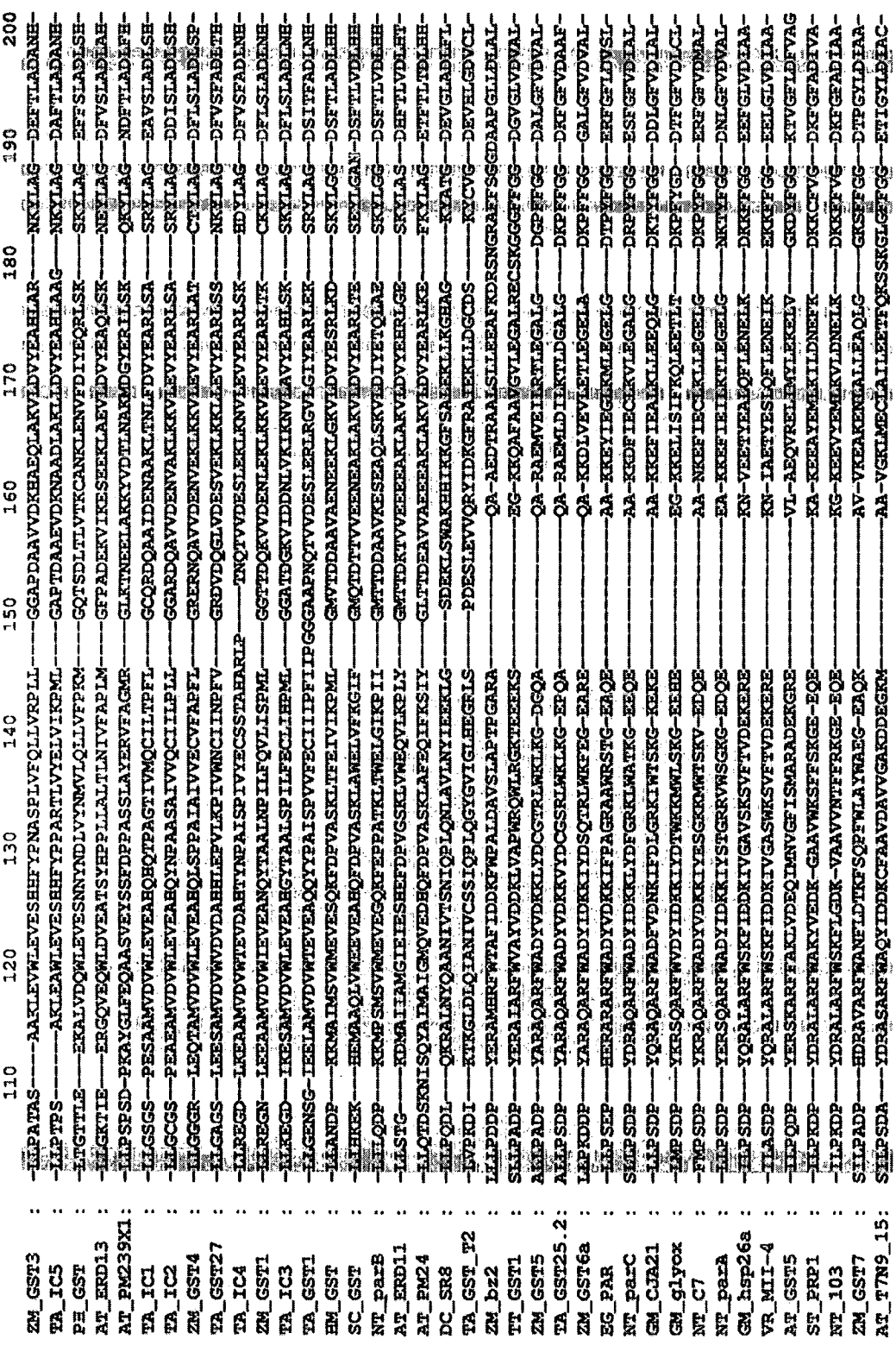

Figure 49 (continued).

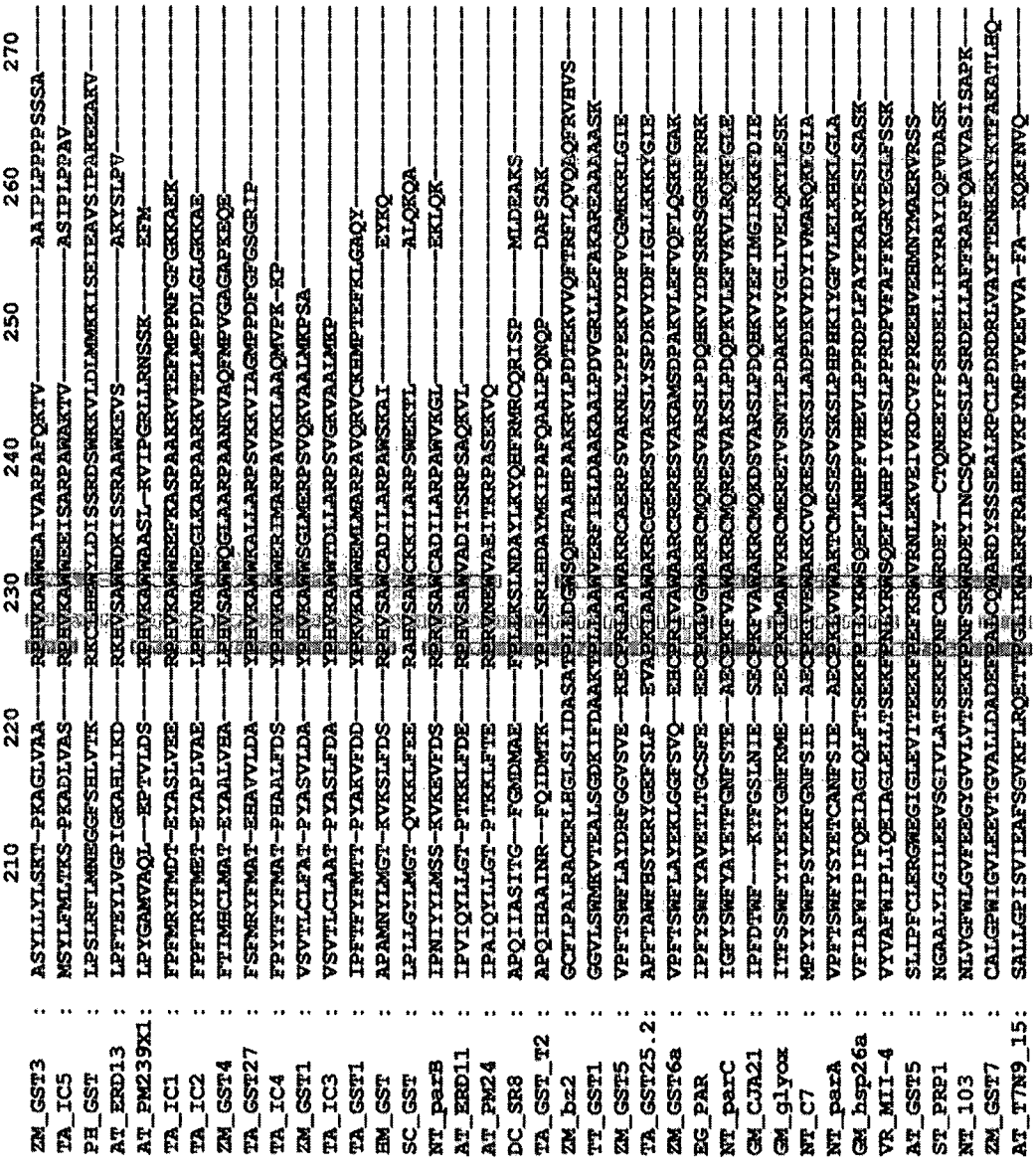

Figure 49. Multiple alignment of plant GST amino acid sequences.

Sequence labels are described in Table 15, and sequences are arranged with similar sequences grouped together. Amino acid residues conserved, or replaced by conservative substitutions, between a high enough proportion of sequences are highlighted; residues conserved in at least 90% of sequences are shaded in dark grey while residues conserved in 75% - 90% of sequences are shaded in light grey.

Codon Bias in Maize GSTs

In eukaryotes, codon bias is thought to result from the location of the gene within a stretch of DNA biased towards certain bases, or from the evolutionary selection for codons with corresponding tRNAs expressed at high levels, allowing for efficient mRNA translation (Fennoy and Bailey-Serres, 1993). The extremely strong C/G bias at the third position of codons in GST5 suggests that there has been considerable selective pressure to produce and maintain this bias in maize tau class GST genes. The reasons for this codon bias are unknown but may be linked to efficiency of transcription and translation. For example, all other maize GSTs sequenced to date also show strong bias towards C/G at the third codon position, varying from 74.0% C/G for *Zm* GST I to 96.4% C/G for *Zm* GST VI. The corresponding mRNAs might therefore be expected to be efficiently transcribed, and this may indeed be the case for *Zm* GST I and *Zm* GST II mRNAs, where the presence of these mRNAs at 0.1% of the total mRNA pool leads to accumulation of the corresponding protein to 1-2% of the total soluble protein (Wiegand *et al.*, 1986, Jepson *et al.*, 1994). However, other factors may also play an important role, such as mRNA and protein stability and turnover.

Classification of GSTs

To visualise the relationship between GSTs cloned during this work and other plant GSTs, multiple alignment and phylogenetic analysis was used to compare sequence data. Initially all available full-length protein sequences for higher plant GSTs were used to construct a single multiple alignment (not shown). However, a number of sequences were removed, for reasons described below, to give the multiple alignment shown in Figure 49. The database sequence for VR MII-4 (*Vigna radiata* auxin-induced GST; see Table 15) appeared to contain a frame shift error close to the N-terminal end of the sequence resulting in a divergent N-terminal sequence. The frame was therefore altered by one base to give the sequence used for the alignment, which showed much greater homology to other GST sequences. As has been observed previously for GSTs from plants (Droog, 1997) and animals (Wilce and Parker, 1994), highest homology between sequences occurred in the N-terminal half of the alignment, with only a small number of

highly conserved residues in the C-terminal domain. Structural data from *Arabidopsis thaliana* GST Atpm24 (Reinemer *et al.*, 1996) showed that many of the N-terminal conserved residues are involved in glutathione binding, thus explaining the high conservation in this region. It is likely that the remaining conserved residues found throughout the sequence are required to allow correct folding of the polypeptide. Since in general these conserved residues are present in all three classes of plant GST is likely that each of these classes has the same overall structure. However, Figure 49 also shows that there are obvious sequence differences between classes, especially between theta class GSTs at the top of the alignment and tau class GSTs at the bottom of the alignment. These differences no doubt account for immunological differences between classes, and may prevent inter-class dimerisation. These differences may also affect the catalytic activity of GST classes, with certain classes preferring particular substrates, however there is not sufficient activity data from a wide enough range of GSTs to date to test this.

Figure 48 shows a phylogenetic tree derived from the multiple alignment shown in Figure 49. This clearly shows the three distinct classes of plant GST, with the theta class represented by 18 sequences, the tau class by 18 sequences and the zeta class by 2 sequences. Each of these three classes contains sequences from both monocotyledonous and dicotyledonous plants. Examining the tau class GST sequences, the *Zm* GST V and *Zm* GST VI sequences were closely related, while *Zm* GST VII was much more divergent, although it was still a tau class GST. Despite this divergence between *Zm* GST V and *Zm* GST VII (with only 37% amino acid identity), purification studies in maize showed that *Zm* GST VII formed heterodimers with *Zm* GST V, presumably due to complementary residue charges and topology at the dimer interface. This suggests that the ability of *Zm* GST V and *Zm* GST VII to form heterodimers has been conserved, and that the *Zm* GST V-VII heterodimer plays a role which cannot be replaced by the two corresponding homodimers. Otherwise, such divergent subunits would not be expected to be able to dimerise. Tau class GSTs may also form heterodimers in species other than maize, however almost all other work on these GSTs has concentrated on molecular approaches in which these GST genes have been cloned rather than the

enzymes purified from plants, and it is therefore unknown whether or not such heterodimers are present.

Label	Description (database accession No.)	Reference
AT ERD11	*A. thaliana* ERD11 GST (P42760)	Kiyosue *et al.*, 1993
AT ERD13	*A. thaliana* ERD13 GST (P42761)	Kiyosue *et al.*, 1993
AT GST5	*A. thaliana* GST5 (D44465)	Watahiki *et al.*, 1995
AT PM239X1	*A. thaliana* PM239x14 GST (P42769)	Bartling *et al.*, 1993
AT PM24	*A. thaliana* PM24 (p46422)	Zettl *et al.*, 1994
AT T7N9 15	*A. thaliana* BAC clone (AC000348)	Database entry only
DC SR8	Carnation GST 1 (P28342)	Meyer *et al.*, 1991b
EG PAR	*Eucalyptus globulus* auxin-induced GST (U80615)	Database entry only
GM CJA21	Soybean GST (Y10820)	Andrews *et al.*, 1997
GM glyox	Soybean glyoxalase I (P46417)	Paulus *et al.*, 1993
GM hsp26a	Soybean HSP-26a (P32110)	Czarnecka *et al.*, 1988
HM GST	*Hyoscyamus muticus* auxin-binding GST (P46423)	Bilang and Sturm, 1995
NT 103	Tobacco *Nt103* (Q03664)	van der Zaal *et al.*, 1991
NT C7	Tobacco *C-7* (X64399)	Takahashi and Nagata, 1992a
NT parA	Tobacco *parA* (P25317)	Takahashi *et al.*, 1989
NT parB	Tobacco *parB* (P30109)	Takahashi and Nagata, 1992b
NT parC	Tobacco *parC* (P49322)	Takahashi and Nagata, 1992a
PH GST	Petunia GST (Y07721)	Database entry only
SC GST	*Silene cucubalus* GST (Q04522)	Kutchan and Hochberger, 1992
ST PRP1	Potato pathogenesis-related protein (P32111)	Taylor *et al.*, 1990
TA GST T2	Wheat GST (AF002211)	Database entry only
TA GST1	Wheat GST A1 (P30110)	Dudler *et al.*, 1991
TA GST25.2	Wheat GST25.2	I. Cummins, unpublished
TA GST27	Wheat GST27	I. Cummins, unpublished
TA IC1	Wheat IC1 sequence	I. Cummins, unpublished
TA IC2	Wheat IC2 sequence	I. Cummins, unpublished
TA IC3	Wheat IC3 sequence	I. Cummins, unpublished
TA IC4	Wheat IC4 sequence	I. Cummins, unpublished
TA IC5	Wheat IC5 sequence	I. Cummins, unpublished
TT GST1	*Triticum tauschii* GST TSI-1 (AF004358)	Riechers *et al.*, 1997
VR MII-4	*Vigna radiata* auxin-induced GST (U20809)	Database entry only
ZM bz2	Maize *bronze2* (U14599)	Nash *et al.*, 1990
ZM GST1	Maize GST I (P12653)	Grove *et al.*, 1988
ZM GST3	Maize GST III, clone DD3a	This work
ZM GST4	Maize GST IV (*Zm* GST II subunit) (P46420)	Irzyk and Fuerst, 1993
ZM GST5	Maize GST V, clone DD5 (Y12862)	This work
ZM GST6a	Maize GST VI, clone DD6a	This work
ZM GST7	Maize GST VII, clone DD7	This work

Table 15. Summary of full-length plant GST sequences used for multiple alignments and phylogenetic analysis. Database entries, where available, are for entries in either the Swiss-Prot protein database or the GenBank DNA database.

A number of plant GSTs were omitted from this tree and the sequence alignment. A rice cold-induced protein (Binh and Oono, 1992) similar to tau class GSTs was omitted since its sequence was considerably divergent when compared to other plant GSTs. Numerous other GST sequences were omitted since identical or very similar sequences were already present. For example tobacco GST apic (Ezaki *et al.*, 1995) was very similar to NT parB, *Nicotiana plumbaginifolia* msr-1 (Dominov *et al.*, 1992) was very similar to NT parA, tobacco Nt107 (van der Zaal *et al.*, 1991) was very similar to NT parC, tobacco Nt111 and Nt110 (van der Zaal *et al.*, 1991) were very similar to NT 103, and *A. thaliana* 103-1a (van der Kop *et al.*, 1996) was identical to AT GST5.

The phylogenetic tree inferred in Figure 48 agrees well with previously constructed trees (Droog *et al.*, 1995, Droog, 1997, Marrs, 1996) and shows the same three clusters of sequences. In addition this tree extends previous work as it includes a number of new sequences, allowing new conclusions to be drawn. As noted by Droog (1997), most plant GSTs fall into two major groups, which have been named the theta class and the tau class. In addition, a single GST sequence from carnation was found to fall outside these two classes, but since no homologues existed it could not be classified with any certainty. However, a further plant GST sequence with homology to this carnation GST has been isolated from wheat (GenBank AF002211), increasing evidence for the existence of a third major plant GST class. Additionally, partial sequences similar to carnation GST have been isolated as ESTs from *Arabidopsis thaliana* (GenBank T88643) and rice (GenBank D41906, D46089 and D40558), with deduced polypeptides of these ESTs having a 45-55% identity when compared with the carnation polypeptide sequence. Surprisingly another similar sequence was identified from human tissue, and this sequence was placed into a newly created zeta class of GST (Board *et al.*, 1997). The related carnation and wheat GSTs shown in Figure 48 as DC SR8 and TA GST T2 have therefore also been classified here as zeta class GSTs, and the *A. thaliana* and rice ESTs also presumably belong to this class. The diversity of species apparently possessing zeta class GSTs makes it likely that many other plant species also contain such GSTs, in which case it is surprising that so few clones have been isolated. Details of the expression pattern of the wheat zeta class GST have not been reported, but the carnation SR8 mRNA was found to be induced by ethylene in senescing flower petals,

and if this tight regulation is mirrored for other plant zeta class GSTs, such GSTs may only have a limited spatial and temporal distribution, decreasing the chances of isolating a cDNA clone. Since so little work has been done on plant zeta class GSTs it would be of great interest to express their cDNA clones to enable the substrate specificities of the proteins to be assayed. Such proteins may have a very different spectrum of activity compared to theta and tau class GSTs. It would therefore be of interest to determine their potential role in herbicide detoxification. Determining the substrate specificities and patterns of expression of zeta class plant GSTs may also help to determine the endogenous function of these enzymes.

8. Expression of Recombinant Maize GSTs

Introduction

The purification of GSTs from maize seedlings, as described in chapter 4, allowed various GST isoenzymes to be characterised. However, purification was very time consuming and practical considerations allowed purification of at most 1-2 mg of purified enzyme, and in some cases much less, of a particular GST isoenzyme. Also, while in most cases the purified GSTs appeared to be pure there was a possibility of contamination by related proteins. Importantly, maize contains multiple GSTs with similar properties, as illustrated by the co-purification of GSTs containing variants of the *Zm* GST V subunit. It would therefore be very useful to have a system which was known to express only one particular GST subunit, with this expression being at very high levels to facilitate purification of large quantities of active enzyme. The most convenient system for achieving this is to clone the cDNA encoding the GST of interest and using an appropriate expression vector, express the GST of interest at high levels in *E. coli*. *E. coli* was a particularly useful host as it contains very low levels of endogenous GST activity (Sheehan and Casey, 1993) and any endogenous GSTs present are likely to be very different from maize GSTs, so recombinant maize GSTs purified from *E. coli* should be free from contaminating bacterial GSTs. There are however a number of potential problems with this method. Firstly, not all proteins are expressed at useful levels in *E. coli*. This problem can sometimes be alleviated by using alternative expression vectors and host strains, though the causes of poor expression are often unclear. Some proteins expressed in *E. coli* do not fold properly or become insolublised as inclusion bodies, and while there are protocols for resolublising and refolding such proteins, success is not guaranteed. Eukaryotic proteins that require post-translational modification will also not be expressed in *E. coli* in their correct form. However, there is no evidence of post-translational processing of GSTs in plants and several plant GSTs, including *Zm* GST I and *Zm* GST III, have been expressed successfully as active, recombinant protein in *E. coli* (Wiegand *et al.*, 1986, Moore *et al.*, 1986). The substrate specificities of these recombinant GSTs were not reported in detail, therefore to expand

on these earlier studies *Zm* GST I-I and *Zm* GST III-III have been expressed as recombinant enzymes and assayed with a diverse range of xenobiotics including herbicides. In addition the tau class GSTs have also been expressed and characterised.

Results

Expression of Recombinant *Zm* GST I

Expression using the vector pKK233-2

Recombinant expression of *Zm* GST I-I was attempted, to enable the production of large amounts of *Zm* GST I-I, free from contamination with other GSTs. *E. coli* was chosen as the host for expression, due to the relatively straightforward procedures involved. pKK233-2 was chosen as the expression vector due to its availability, and because it had been used previously to successfully express other plant-derived GST sequences (Takahashi and Nagata, 1992b).

The *Zm* GST I insert obtained from root mRNA (see chapter 6) was subcloned into the plasmid expression vector pKK233-2 and the resultant plasmid, named pKK-DD1, was transformed into *E. coli* JM101 for expression. Protein expression was induced using 1 mM IPTG in the culture medium, and cells were harvested 16 h after induction. Extracts from *E. coli* JM101 containing either pKK233-2 or pKK-DD1 were assayed for GST activity using CDNB as substrate; bacteria with vector containing the insert had approximately 20 times the conjugating activity of the control bacteria without the *Zm* GST I insert. However, the specific activity of the recombinant GST in the crude protein preparation (1.03 nkat/mg), equated to only 0.06% of the specific activity of *Zm* GST I-I purified from maize roots.

Analysis by SDS-PAGE showed no visible differences between *Zm* GST I-expressing and control bacteria in the polypeptide compositions of either soluble or total protein extracts after induction. Extracts from bacteria transformed with pKK233-2 (control) or pKK-DD1 were then applied to an Orange A affinity column, and proteins eluting with glutathione were analysed by SDS-PAGE. The protein purified from *Zm* GST I-

expressing bacteria had an extra polypeptide with an estimated molecular weight of 29.5 kDa as compared with the control (data not shown).

These results showed that active, soluble *Zm* GST I-I was being expressed in this system, but at very low levels. To try to increase expression, different *E. coli* host strains were tested, since host strain may affect protein expression. For example different host strains may differ in the types and levels of proteases present, resulting in differing stability of expressed proteins. pKK-DD1 was transformed into *E. coli* strains JM109, DH5α and Y1090ZL. Expression of *Zm* GST I was then induced in pKK-DD1-transformed colonies; the resulting bacteria were harvested, lysed by sonication and analysed for the presence of the *Zm* GST I polypeptide, by SDS-PAGE analysis of soluble and insoluble protein fractions, and the presence of GST activity towards CDNB. Results from SDS-PAGE analysis of crude protein fractions from the expressing bacteria showed that no obvious additional polypeptide of 29.5 kDa was evident in any of the pKK-DD1-transformed hosts. However significantly higher GST activity was determined in all transformed lines, as compared with control, non-transformed bacteria. The highest specific activity was detected from *E. coli* strain Y1090ZL; this strain was then used for a larger-scale induction experiment where recombinant *Zm* GST I-I was purified using glutathione Sepharose affinity chromatography. CDNB-conjugating activity in the crude protein extract from expressing *E. coli* was 1.05 nkat/mg total protein as compared with 25 nkat/mg in a maize crude extract. GST activity towards CDNB for the purified recombinant *Zm* GST I-I was 1071 nkat/mg, as compared with 1693 nkat/mg for *Zm* GST I-I purified from maize. However, as less than 0.2 mg of recombinant *Zm* GST I-I would be purified from a litre of bacterial culture, it was concluded that the pKK233-2 expression vector was not suitable for further work with recombinant *Zm* GST I-I.

Expression using the vector pET-11d.

Since *Zm* GST I expression using pKK-DD1 was very low in all the bacterial strains tested, an alternative expression vector, pET-11d, was used in an attempt to increase levels of expression. Since it was not possible to transfer the *Zm* GST I coding sequence from the previously used vectors directly into pET-11d, the coding sequence for *Zm* GST I was re-amplified using PCR, using the oligonucleotide primers Pmr-1c (similar to

Pmr-1b but designed to provide a *BamH*1 restriction site, compatible with the pET-11d vector; see **Table 14**), and Pmr-1a, the primer used in the initial PCR amplification (chapter 6). The PCR product was purified and cloned using the pGEM-T cloning system, then sub-cloned into the vector pET-11d to give the construct pET-DD1. This was transformed into *E. coli* strain BL21(DE3) for protein expression.

To determine the optimal induction time, cultures of transformed bacteria were induced using 1 mM IPTG and harvested at 0, 0.5, 1, 2 and 3 h after induction. Total soluble extracts from each sample were analysed by SDS-PAGE. Figure 50 shows that after induction a polypeptide with a relative molecular mass of 29.5 kDa accumulated at very high levels. When a similar gel was used for western blotting with anti-*Zm* GST I-II serum, a single intense band in each lane containing protein from induced bacteria, corresponding to the 29.5 kDa polypeptide was determined (data not shown). The soluble bacterial extracts were also assayed for GST activity towards CDNB. Non-induced samples showed negligible activity while induced samples showed very high activity. Highest activity was found 2 hours after induction, such that the GST specific activity in the protein from the bacterial lysate was 62% of that found for pure *Zm* GST I-I from maize. Induction of a 320 ml culture followed by purification of the expressed *Zm* GST I-I by Orange A dye affinity and anion exchange chromatography resulted in recovery of 27 mg of purified recombinant *Zm* GST I-I, equating to a recovery of 85 mg of pure recombinant *Zm* GST I-I per litre of bacterial culture transformed with pET-DD1.

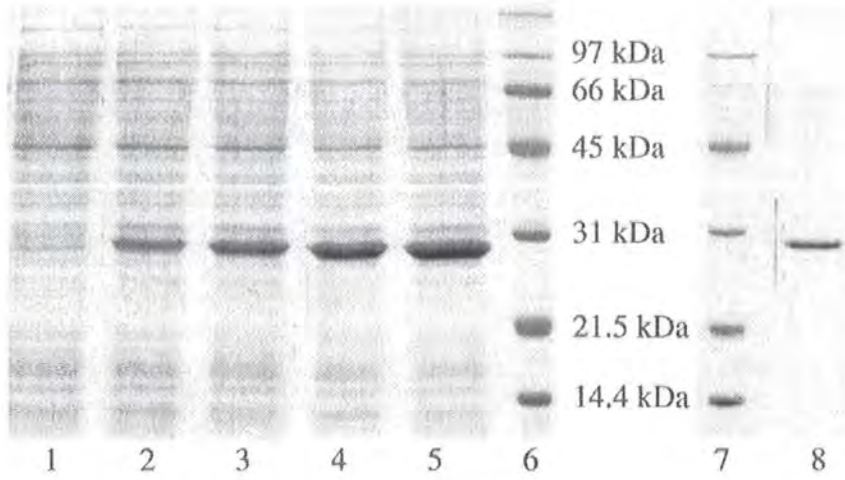

Figure 50. Expression of recombinant *Zm* GST I-I over time following induction with IPTG using *E. coli* BL21(DE3) containing the vector pET-DD1. Lane 1 = uninduced control, lane 2 = 0.5 h after induction, lane 3 = 1 h after induction, lane 4 = 2 h after induction, lane 5 = 3 h after induction, lanes 6 and 7 = molecular weight standards and lane 8 = purified recombinant *Zm* GST I-I.

	Specific activity* (nkat/mg)	
Substrate	Maize *Zm* GST I-I	Recombinant *Zm* GST I-I
CDNB	1693 ± 5	1662 ± 50
Ethacrynic acid	27 ± 8	31 ± 3
Atrazine	0.085 ± 0.013	0.11 ± 0.03
Alachlor	1.47 ± 0.22	0.59 ± 0.02
Metolachlor	0.032 ± 0.080	0.061 ± 0.006
Fluorodifen	0.0055 ± 0.0003	0.0055 ± 0.0003

Table 16. GST activities of purified *Zm* GST I-I from maize and *E. coli*.

* Values given are means of duplicate determinations ± the variations between the mean and the replicates.

Purified recombinant *Zm* GST I-I was assayed for GST activity towards a range of substrates and the resulting activities were compared to those found for *Zm* GST I-I purified from maize (Table 16). The activities of the recombinant protein were very similar to those of *Zm* GST I-I from maize, with the only apparent difference being the higher activity towards alachlor for maize *Zm* GST I-I. This may have been due to contamination of the maize protein with *Zm* GST I-III, which has high activity towards chloroacetanilide herbicides. The generally lower errors for the recombinant enzyme activities reflected the higher concentration of the enzyme in the assays resulting in more accurate values.

Expression of Recombinant *Zm* GST III

The coding regions of the *Zm* GST III clone DD3a (chapter 6) was introduced into the pET-11d expression vector and the resulting insert in the pET vector resequenced to confirm its identity. The resulting vector was designated pET-DD3a. On induction of expression in *E. coli*, a polypeptide of the correct molecular mass (as judged by SDS-PAGE) accumulated in the soluble protein extract of bacteria transformed with pET-DD3a (Figure 51). Expression of the clone gave a protein extract with significantly higher CDNB activity (5.52 nkat/mg) than a non-induced bacterial protein extract or an extract from induced bacteria containing pET-11d without insert (less than 0.5 nkat/mg). Attempts to affinity purify the over-expressed pET-DD3a protein using Orange A agarose, *S*-hexylglutathione Sepharose and glutathione Sepharose showed that none of these matrices specifically bound this GST. Therefore crude, rather than purified, protein extracts from bacteria expressing pET-DD3a were used for GST assays. Moore *et al.* (1986) reported that crude *E. coli* extracts inhibited the activity of recombinant *Zm* GST III towards alachlor, therefore crude extracts were desalted using Sephadex G-25 (Pharmacia PD10 column) prior to enzyme assays. Low but significant GST activity was demonstrated towards CDNB and the herbicides alachlor, metolachlor and fluorodifen (Table 17).

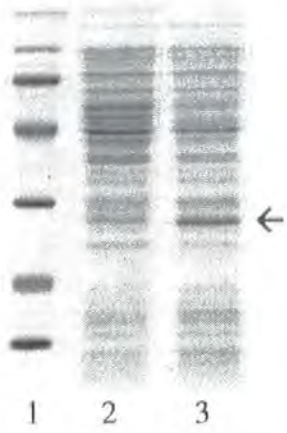

Figure 51. B/T Blu-stained SDS-PAGE gel showing expression of recombinant *Zm* GST III in *E. coli*. Lane 1 = Molecular weight standards, 2 = pET-DD3a uninduced control and 3 = pET-DD3a, 3 h after induction, showing induced 28.5 kDa polypeptide (arrowed).

Substrate	Specific Activity (pkat/mg)
CDNB	5520
Alachlor	4.94
Metolachlor	1.22
Fluorodifen	0.29

Table 17. Specific activities of crude extract from *E. coli* expressing *Zm* GST III-III. Similar extracts from *E. coli* containing vector only showed no significant activity towards these substrates.

Since *Zm* GST III-III was expressed at about 30% of the total soluble protein in this system as judged by SDS-PAGE, the specific activities of pure recombinant *Zm* GST III-III should be about 3-fold higher than those in Table 17. Even when taking this into account, the activities toward the herbicides are all 100-fold lower than for *Zm* GST I-III purified from maize, where it is assumed that the *Zm* GST III subunit is responsible for almost all the herbicide activity of the heterodimer since *Zm* GST I-I has very low activity towards herbicides. It therefore seems highly likely that the *Zm* GST III subunit purified from maize is not the same as the *Zm* GST III subunit expressed in *E. coli* (see discussion).

Interestingly, Pioneer 3394 appears to contain at least two distinct sequences coding for *Zm* GST III, as described in chapter 6. The first, corresponding to clone DD3a has very low GST activity when expressed as a recombinant protein, possibly due to a substitution at amino acid 48. The second sequence, corresponding to clone DD3b, differs slightly from the first, and does not have the substitution at amino acid 48. This sequence has not yet been expressed as a recombinant protein so it is not yet known whether the protein has different GST activities when compared to DD3a.

Expression of Recombinant Tau Class GSTs as Fusion Proteins

The antibody screening procedure which identified *Zm* GST V and *Zm* GST VI clones relied on the presence of expressed protein, which if full-length, could be assayed for GST activity without the need for subcloning. The proteins were expressed as fusions with the N-terminal portion of β-galactosidase and it was unclear if this fusion would affect GST activity. To rapidly test if DD5, DD6a and DD6b encoded functional enzymes, the GSTs were expressed as fusion proteins using the plasmid derived from the recombinant phage (pBluescript SK$^-$) in *E. coli* strain SOLR, along with a control consisting of β-galactosidase fused to a truncated DD6b sequence. Small scale cultures containing ampicillin and IPTG were grown up overnight. The cells were then harvested and soluble protein extracts prepared by sonication and centrifugation, which were then assayed for GST activity towards CDNB (Table 18). All three full-length cDNAs encoded active GSTs, which were active when expressed as fusion proteins.

Sample	CDNB activity (nkat/mg)
Truncated DD6b (control)	None detected
DD5	8.0
DD6a	10.9
DD6b	9.1

Table 18. GST activity towards CDNB in bacterial lysates from GST clones recognised by the anti-*Zm* GST V-VI serum.

Due to the high homology between these the tau class GST cDNA clones and a glyoxalase I isolated from soybean (Paulus *et al.*, 1993), it was of interest to determine whether maize tau class GSTs possessed glyoxalase I activity. However, crude extracts from bacteria expressing clones DD5, DD6a and DD6b, although possessing GST activity towards CDNB (Table 18), had no detectable glyoxalase I activity. To further confirm that maize tau class GSTs were not glyoxalases, purified *Zm* GSTs V-V and V-VI from maize were also tested for glyoxalase I activity but again no activity could be detected, even though glyoxalase I activity could be readily determined in partially purified maize extracts (see chapter 9), confirming that the assay worked. It was therefore concluded that the maize tau class GSTs are not glyoxalases, despite the high sequence similarity shown between maize tau class GST clones and a cDNA clone reported to be a glyoxalase I in soybean.

Expression of Recombinant *Zm* GST V

To enable expression of the native protein encoded by DD5 and to confirm that DD5 encoded *Zm* GST V, a similar strategy to that used to express recombinant *Zm* GST I was used. PCR was used to amplify the coding and 3′ untranslated regions of the cDNA clone DD5 using a specific 5′ primer (Pmr-5a; Table 19) to introduce an *Nco*I restriction site at the translation initiation codon and a poly(A)-specific 3′ primer (og2) to introduce a *BamH*I restriction site at the 3′ end of the sequence. The resulting PCR product was sub-cloned using the pGEM-T system and transferred to the expression vector pET-11d utilising the engineered restriction sites to give the vector pET-GST5. DNA sequencing

was used to confirm that the PCR had not introduced any errors into the sequence to be expressed.

Primer	Oligonucleotide sequence (restriction sites in bold)
Pmr-5a	G**CC ATG G**CC GAG GAG AAG A
Pmr-6a	G**CC ATG G**CG GCG GCG GC
Pmr-7b	GG**C ATA TG**T CTC CGC CCG TCA AG
og2	GAG AGA **GGA TCC** TCG AGT TTT TTT TTT TTT TTT T
og9	CGC ACT GAG AGA **GGA TCC** TCG AG

Table 19. Sequences of oligonucleotide primers used to express tau class GSTs.

Following induction of expression of recombinant protein in bacteria transformed with pET-GST5, a 28.5 kDa polypeptide was determined by SDS-PAGE in the lysate of the host bacteria, representing about 25% of the total soluble protein, which was not present in the uninduced control (see Figure 55). Relative to extracts from bacteria containing the pET vector without an insert, the lysate contained greatly elevated levels of GST activity towards CDNB showing that an active GST was being expressed. To confirm that the recombinant polypeptide was the *Zm* GST V subunit, the corresponding active enzyme was purified from the *E. coli* lysate by *S*-hexylglutathione affinity chromatography and anion exchange chromatography (Figure 53) under identical conditions to that used for the *Zm* GST V-V and *Zm* GST V-VI enzymes from maize (chapter 4). Using this procedure 17 mg of pure *Zm* GST V-V was purified from 100 ml of bacterial culture. Recombinant *Zm* GST V-V co-chromatographed with *Zm* GST V-V purified from maize on both *S*-hexylglutathione and Q Sepharose columns. Analysis of the resultant purified recombinant *Zm* GST V-V by SDS-PAGE showed that the recombinant GST consisted of a single 28.5 kDa polypeptide band which co-migrated with *Zm* GST V-V (Figure 52, lanes 1a to 4a), while electrophoresis under non-denaturing conditions showed a single 59 kDa protein, confirming that the enzyme was active as a dimer (**Figure 19**; chapter 4). Western blotting showed that the recombinant polypeptide was recognised by the antibody raised to *Zm* GST V-VI (Figure 52, lanes 1b to 4b). *E. coli* crude extracts also contained an endogenous 44 kDa polypeptide

recognised by anti-*Zm* GST V-VI serum, however this polypeptide did not co-purify with recombinant *Zm* GST V-V. To further confirm the identity of the recombinant GST as *Zm* GST V the respective subunits were analysed by *in situ* partial proteolytic digestion in SDS-PAGE gels using alkaline protease (Cleveland *et al.*, 1977). Following electrophoresis the recombinant polypeptide was degraded into a pattern of fragments similar to those observed with *Zm* GST V-V (Figure 54).

Substrate specificity of recombinant *Zm* GST V-V

Purified recombinant *Zm* GST V-V was assayed for activities as a GST and a glutathione peroxidase using a variety of substrates (Table 20). GST activity toward fluorodifen was very similar to that determined with the plant enzyme (0.490 nkat/mg). However, the *Zm* GST V-V preparation from plants showed higher specific activities toward CDNB (216 nkat/mg) and metolachlor (0.47 nkat/mg), suggesting that the plant preparation may have been contaminated with a further GST activity. Relative to *Zm* GST I-I and *Zm* GST I-II, recombinant *Zm* GST V-V had much lower activities toward CDNB, NBC and NPB. Also, unlike the other maize GSTs containing the *Zm* GST I subunit, *Zm* GST V-V showed no activity toward atrazine. However, *Zm* GST V-V was more active than *Zm* GST I-I in detoxifying metolachlor and, under conditions of saturating substrate concentrations, more efficient at detoxifying fluorodifen than any other characterised GST from maize. *Zm* GST V-V also conjugated the toxic alkenal derivatives crotonaldehyde, 4-vinylpyridine and ethacrynic acid and showed some activity as a glutathione peroxidase, able to reduce cumene hydroperoxide, but not linoleic acid hydroperoxide, to the corresponding non-toxic alcohols.

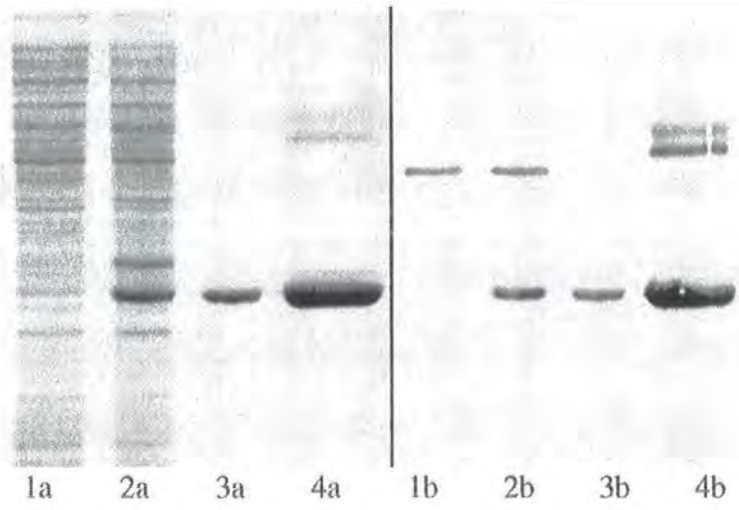

Figure 52. Composite image showing expression and purification of recombinant *Zm* GST V-V.

Lanes 1a to 4a show samples analysed by SDS-PAGE and stained with B/T Blu, lanes 1b to 4b show equivalent samples analysed by western blotting using anti-*Zm* GST V-VI serum following SDS-PAGE. In both cases, lane 1 shows a crude extract from induced bacteria containing pET-11d, lane 2 = crude extract from induced bacteria containing pET-GST5, lane 3 = recombinant *Zm* GST V-V purified *from E. coli* and lane 4 = lyophilised, reconstituted *Zm* GST V-V purified from maize. The minor high molecular weight polypeptides present in maize *Zm* GST V-V appear to be artefacts caused by lyophilisation, since freshly purified protein (e.g. **Figure 22**) did not contain these polypeptides.

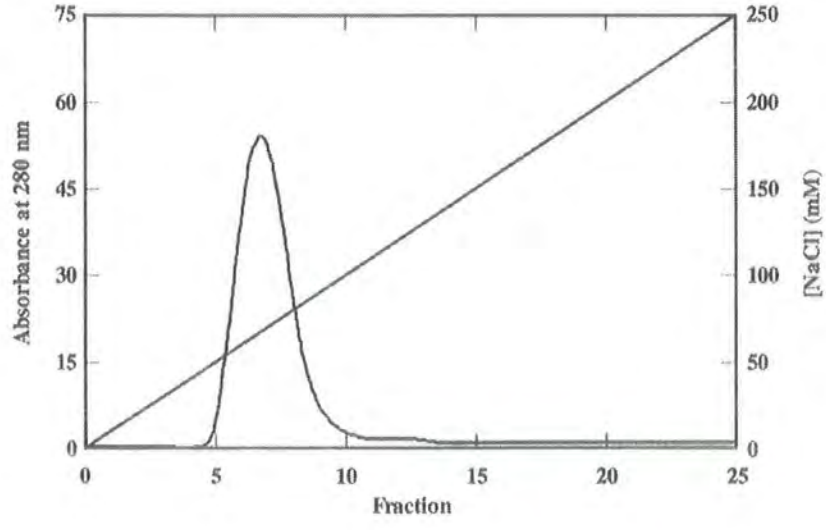

Figure 53. Anion exchange chromatography of recombinant *Zm* GST V-V purified by *S*-hexylglutathione chromatography.

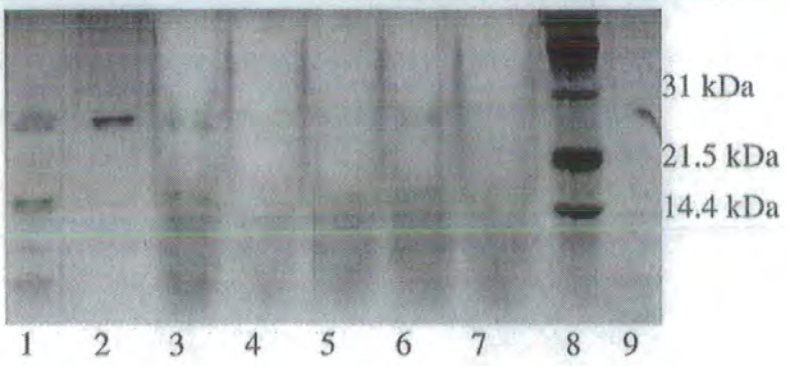

Figure 54. Partial proteolytic digests of GST subunits resolved by SDS-PAGE.
Lane 1 = r (recombinant) *Zm* GST V, Glu-C digested, 2 = r *Zm* GST V, Lys-C digested, 3 & 4 = r *Zm* GST V, alkaline protease digested, 5 = *Zm* GST V-V alkaline protease digested, 6 = *Zm* GST V-VI 28.5 kDa subunit (GST V) alkaline protease digested, 7 = *Zm* GST V-VI 27.5 kDa subunit (GST VI) alkaline protease digested, 8 = molecular weight standards and 9 = r *Zm* GST V, undigested. Approximately 2 µg of protein was loaded in each lane.

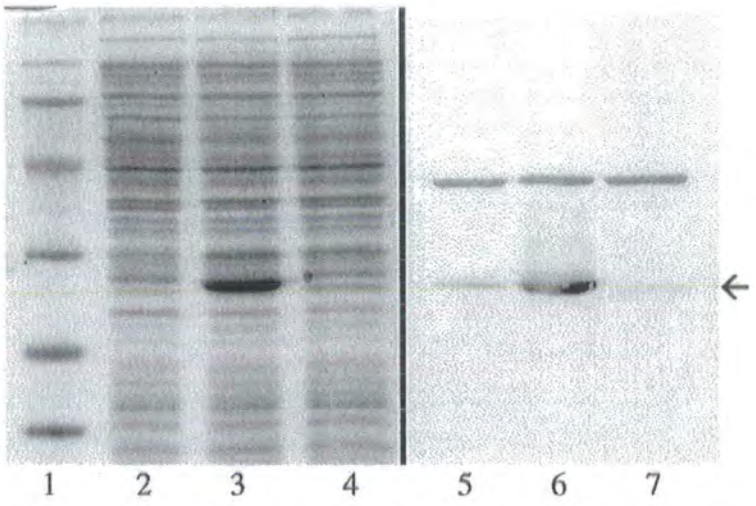

Figure 55. SDS-PAGE analysis of expression of recombinant *Zm* GST VI.
Lanes 1 to 4 show lanes stained with B/T Blu, with lane 1 = molecular weight standards, lanes 2 = soluble protein from uninduced bacteria containing pET-GST5, lane 3 = soluble protein from induced bacteria containing pET-GST5 and lane 4 = soluble protein from induced bacteria containing pET-GST6a. Lanes 5, 6 and 7 show a western blot probed with anti-*Zm* GST V-VI serum, containing equivalent samples to lanes 2, 3 and 4 respectively. Lanes 4 and 7 show the low level expression of *Zm* GST VI as a faint band (arrowed) running slightly faster than *Zm* GST V (lanes 3 and 6).

Substrate	Activity (nkat/mg)
CDNB	90.7
1,2-Dichloro-4-nitrobenzene	0.41
p-Nitrobenzyl chloride	3.03
Ethacrynic acid	4.92
p-Nitrophenethyl bromide	ND
1,2-Epoxy-3-(nitrophenoxy) propane	ND
trans-4-Phenyl-3-buten-2-one	ND
Benzyl isothiocyanate	4.28
Atrazine	ND
Metolachlor	0.253
Fluorodifen	0.518
4-Vinylpyridine	0.99
Crotonaldehyde	1.56
Cumene hydroperoxide	0.137*
Linoleic acid hydroperoxide	ND
Hydrogen peroxide	ND

Table 20. Activity of recombinant *Zm* GST V-V towards a wide range of potential substrates.

* = activity expressed as change in absorbance at 366 nm/min/mg.

ND = no activity detected.

Expression of Recombinant *Zm* GST VI

To allow recombinant expression of *Zm* GST VI, the coding sequence and 3′ untranslated region of the cDNA clone DD6a was introduced into pET-11d to give pET-GST6a using a PCR-based approach as described above for *Zm* GST V, using the oligonucleotide primers Pmr-6a and og2 (Table 19). Following induction of expression in *E. coli* containing pET-GST6a, much lower levels of active GST were produced than found for pET-GST5, with the GST activity towards CDNB in the crude lysate being 5.3 nkat/mg. As determined by SDS-PAGE (Figure 55) the recombinant 27.5 kDa

polypeptide only accounted for 1 - 2% of the total protein. Different induction conditions were tried in an attempt to increase this expression but these proved unsuccessful. Analysis by SDS-PAGE and western blotting showed that the 27.5 kDa recombinant polypeptide was recognised by anti-*Zm* GST V-VI serum (Figure 55). In view of its molecular mass and recognition by the anti-*Zm* GST V-VI serum it appeared most likely that this polypeptide corresponded to the *Zm* GST VI subunit purified from maize. The recombinant protein did not bind to an *S*-hexylglutathione affinity column and further purification was not attempted at this time.

Expression of Recombinant *Zm* GST VII

In view of the difficulties in purifying GSTs containing the *Zm* GST VII subunit it was of interest to express recombinant *Zm* GST VII for further characterisation. Purification studies (chapter 4) had shown that *Zm* GST VII dimerised with *Zm* GST V and that this heterodimer had GST activity, however this activity could have been due to the *Zm* GST V subunit alone. To express recombinant *Zm* GST VII, the coding sequence of the clone GST7, apparently encoding *Zm* GST VII (see chapter 7), was sub-cloned into the pET-11a expression vector to give the construct pET-GST7, following PCR using the oligonucleotide primers Pmr-7b and og2 (Table 19) to engineer appropriate restriction sites as described for construction of pET-GST5. Induction of expression of this GST in *E. coli* resulted in the appearance of a low but measurable CDNB-conjugating activity in the crude bacterial lysate. A very faint additional 29.5 kDa polypeptide band was observed in this lysate when compared to uninduced bacterial lysate using SDS-PAGE (Figure 56: soluble protein, 3 h time point). This gave further confirmation that GST7 encoded a functional GST subunit, however this GST was not well expressed in *E. coli* using the pET expression system. Total protein and soluble protein samples were prepared from *E. coli* containing pET-GST7 at various time points after induction of protein expression to determine optimal induction time to increase protein yield. Analysis of these samples by SDS-PAGE (Figure 56) showed that in total protein extracts, a 29.5 kDa polypeptide, which was probably *Zm* GST VII, accumulated over time, with highest levels 21 h after protein induction. However in soluble protein extracts, this polypeptide was most abundant 3½ h after induction and its levels declined

considerably by 21 h. This suggested that the polypeptide was either being slowly insolublised, probably due to formation of inclusion bodies, or degraded.

Expression of Recombinant *Zm* GST V-VI Heterodimers

Since *Zm* GST V and *Zm* GST VI subunits dimerise together in plants to give *Zm* GST V-VI it was of interest to see whether such a heterodimer could be produced from recombinant GST subunits. A system was designed to enable the co-expression of the GSTs encoded by both these clones in *E. coli* so that the two subunits could heterodimerise as well as forming homodimers. If GST5 and GST6a coded for the *Zm* GST V and *Zm* GST VI subunits respectively any heterodimer produced would correspond to *Zm* GST V-VI. To express such heterodimers, a vector was constructed from pET-GST5 and pET-GST6a (described earlier in this chapter), utilising an *Nde*I restriction site in the 3′ untranslated region of the GST6a insert, such that the GST5 coding sequence together with the pET-11d ribosome binding site from pET-GST5 were inserted immediately downstream of the GST6a coding sequence in pET-GST6a to give the construct shown in Figure 57, termed pET-DD56. On induction of expression from pET-DD56, transcription by T7 polymerase should synthesise poly-cistronic mRNA containing GST5 and GST6a sequences. Following ribosome binding at the two binding sites in this mRNA, both cistrons should then be co-translated to produce *Zm* GST V and *Zm* GST VI subunits which should then dimerise to form a mixture of heterodimers and homodimers.

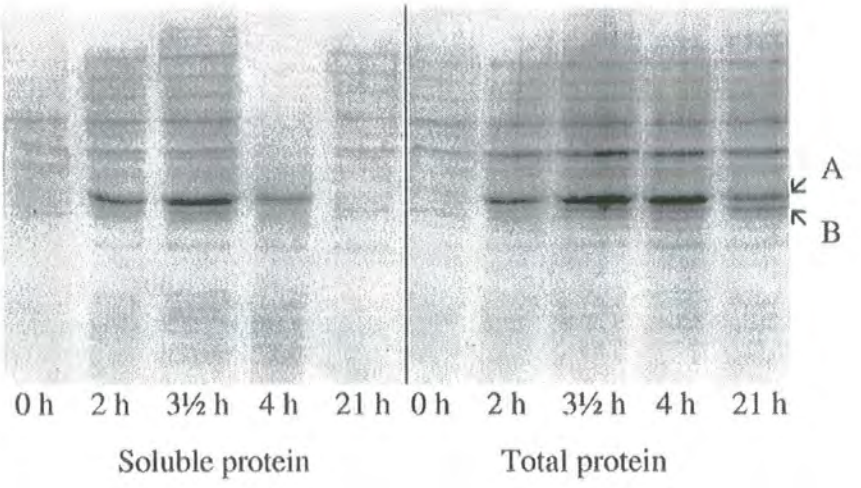

Figure 56. Time course showing expression of *Zm* GST VII in *E. coli*.

Times indicate length of time following induction of expression.

A = β-lactamase polypeptide band (31 kDa)

B = *Zm* GST VII polypeptide band (29.5 kDa)

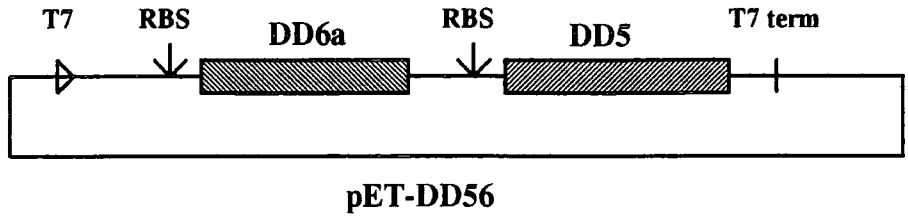

Figure 57. Overall structure of vector pET-DD56, for recombinant expression of GST5 and GST6a polypeptides as heterodimers.
T7 = T7 polymerase promoter sequence, RBS = ribosome binding site and T7 term = T7 polymerase transcription terminator sequence.

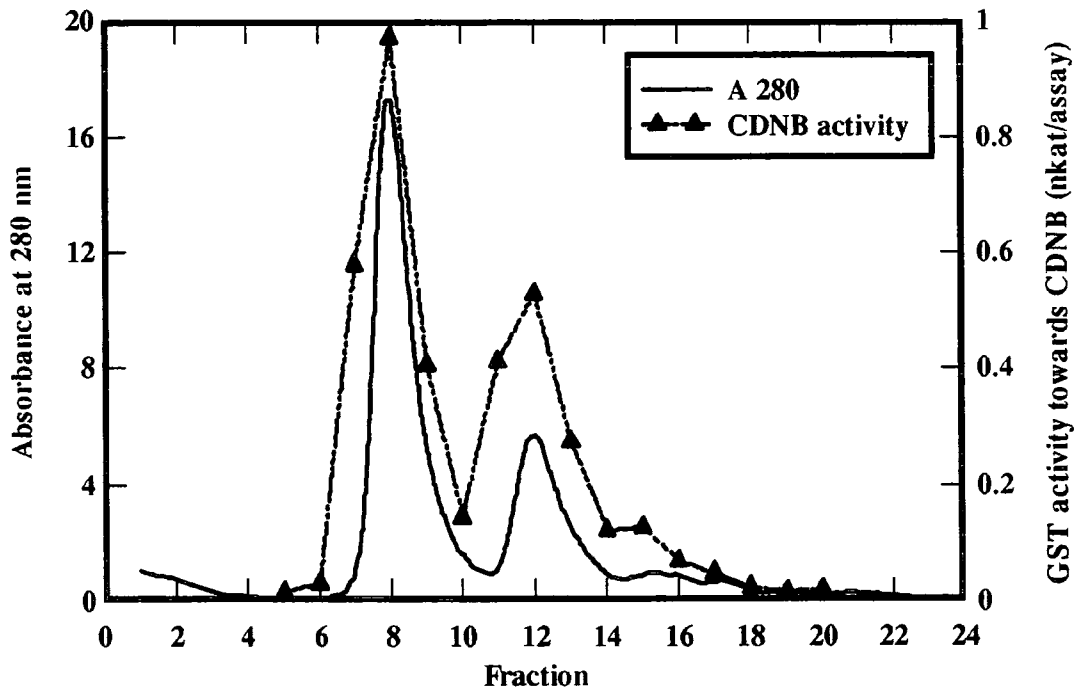

Figure 58. Anion exchange chromatography of recombinant *Zm* GSTs V-V and V-VI. Both recombinant proteins were derived from expression in *E. coli* of the pET-DD56 construct.

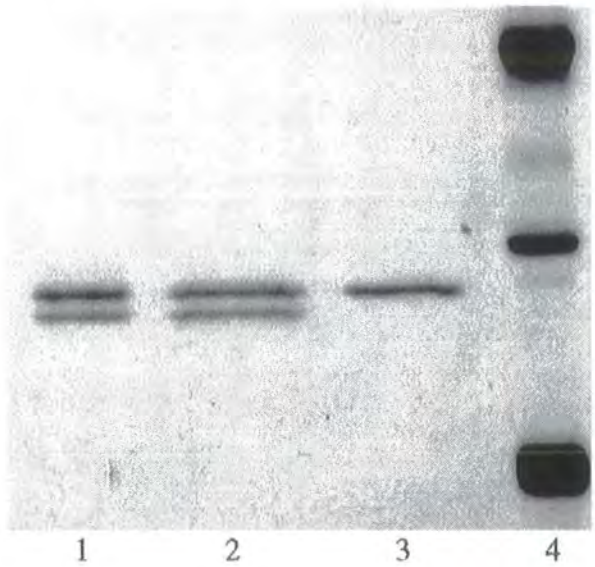

Figure 59. Silver-stained SDS-PAGE gel showing purified recombinant *Zm* GST V-VI. Lane 1 = purified *Zm* GST V-VI from maize, lane 2 = purified recombinant *Zm* GST V-VI (fraction 12, Figure 58), lane 3 = purified recombinant *Zm* GST V-V (fraction 8, Figure 58) and lane 4 = molecular mass standards.

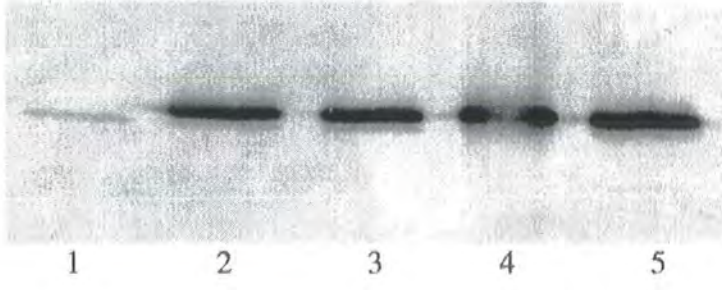

Figure 60. Western blot showing expression of recombinant *Zm* GST V-VI.
Lane 1 = crude extract from *E. coli* expressing pET-GST6a (*Zm* GST VI), lane 2 = crude extract from *E. coli* expressing pET-DD56 (*Zm* GST V + *Zm* GST VI), lane 3 = protein from *E. coli* expressing pET-DD56 purified by *S*-hexylglutathione chromatography, lane 4 = purified recombinant *Zm* GST V-V (fraction 8, Figure 58) and lane 5 = purified recombinant *Zm* GST V-VI (fraction 12, Figure 58).

The subunits encoded by this vector were expressed using the same induction methods as for pET-GST5. The recombinant GSTs in the resultant *E. coli* protein extract containing the *Zm* GST V subunit were purified using *S*-hexylglutathione affinity chromatography and the isoenzymes then resolved by anion exchange chromatography using a 1ml HiTrap Q column (chapter 4). Two protein peaks were eluted from this column (Figure 58). The first eluted at the same position in the salt gradient as both maize and recombinant *Zm* GST V-V, and had CDNB-conjugating activity. The second peak, approximately a third the size of the first peak, eluted at the same position as the *Zm* GST V-VI heterodimer from maize. The second peak had higher specific activity towards CDNB than the first peak, corresponding to similar results obtained with *Zm* GST V-V and *Zm* GST V-VI purified from maize roots (Figure 27). Analysis by SDS-PAGE (Figure 59) showed that the first peak only contained 28.5 kDa polypeptides and appeared identical to *Zm* GST V-V. The second peak contained 28.5 kDa and 27 kDa polypeptides and appeared identical to *Zm* GST V-VI. Western blotting using anti-*Zm* GST V-VI serum (Figure 60) showed that both subunits in the second peak were recognised by the antiserum, and the difference in antiserum binding between the subunits was similar to that found for *Zm* GST V-VI. In addition to demonstrating that the GST subunits can form heterodimers in recombinant hosts, this study also gave further evidence that DD6a encoded the 27.5 kDa *Zm* GST VI subunit.

Enzyme Kinetics of Recombinant *Zm* GST I-I and *Zm* GST V-V

The efficient expression and purification of recombinant *Zm* GST I-I and *Zm* GST V-V meant that large quantities of pure, uncontaminated, GSTs from both of the two main plant GST classes were available, allowing various comparative studies to be performed using these two proteins. As reported above, both GSTs were assayed for GST activity towards a range of potential substrates and the results showed that the two GSTs had markedly different substrate specificities. To further investigate and compare the properties of these GSTs, purified recombinant *Zm* GST I-I and *Zm* GST V-V were used to study various aspects of their catalysis of CDNB conjugation.

K_M and V_{max}

The kinetics of CDNB conjugation catalysed by *Zm* GST I-I and *Zm* GST V-V were studied using varying concentrations of CDNB and glutathione to determine apparent K_M and V_{max} values for both substrates. Since kinetic parameters for each substrate were only determined using a single fixed concentration of the co-substrate, actual kinetic values could not be determined, however the apparent values shown here give a reasonable indication of the enzymes' kinetic characteristics. The results, shown in Figure 61, are summarised in Table 21. From their relative apparent K_M values, *Zm* GST V-V had a 2.5-fold greater binding affinity for CDNB compared with *Zm* GST I-I, but had a 4-fold lower binding affinity for glutathione. *Zm* GST I-I had a much higher apparent V_{max} than *Zm* GST V-V. The V_{max} values obtained for CDNB are more likely to reflect the true catalytic efficiency of the GSTs as GSH was held at saturating concentrations (5 mM) while V_{max} (GSH) was obtained when CDNB concentrations were non-saturating. The apparent turnover number (k_{cat}) could also be calculated for the pure proteins from V_{max} (CDNB) data to give k_{cat} (*Zm* GST I-I) = 145 s^{-1} and k_{cat} (*Zm* GST V-V) = 5.0 s^{-1}.

Enzyme	K_M (CDNB) mM	V_{max} (CDNB) nkat/mg	K_M (GSH) mM	V_{max} (GSH)* nkat/mg
Zm GST I-I	2.51 ± 0.25	5790 ± 340	0.70 ± 0.06	1970 ± 60
Zm GST V-V	1.28 ± 0.08	199 ± 6	2.82 ± 0.28	140 ± 7

Table 21. Enzyme kinetics for recombinant *Zm* GST I-I and *Zm* GST V-V.
Each value is shown ± standard error. * V_{max} (GSH) was calculated using a non-saturating concentration of CDNB (1.0 mM), therefore values are much lower than for V_{max} (CDNB), where glutathione was at saturating levels (5 mM).

Enzyme Inhibition Studies

Singh and Shaw (1988) showed that tetrapyrroles were potent inhibitors of oat (*Avena sativa*) GSTs, and it was therefore of interest to determine the sensitivity of maize GSTs to these compounds. The tetrapyrroles haemin and chlorophyllin were chosen due to their availability. Initially, these compounds were tested for their ability to inhibit the

activity of recombinant GSTs *Zm* GST I-I and *Zm* GST V-V in standard CDNB assays. 2.5 µg/ml chlorophyllin inhibited *Zm* GST I-I activity 36% and inhibited *Zm* GST V-V activity 66%, while 2.5 µg/ml haemin did not significantly inhibit *Zm* GST I-I activity and inhibited *Zm* GST V-V activity 22%. *Zm* GST V-V was therefore considerably more sensitive to inhibition by tetrapyrroles than *Zm* GST I-I, and for both enzymes chlorophyllin was a more potent inhibitor than haemin. To further characterise the inhibition of *Zm* GST V-V by these tetrapyrroles, kinetic studies were performed where enzyme reactions were carried out at varying concentrations of CDNB in the presence or absence of a fixed concentration of inhibitor (2.5 µg/ml chlorophyllin or 5.0 µg/ml haemin). The results, shown in Figure 62 A and Figure 62 B, showed that chlorophyllin and haemin appeared to act as noncompetitive inhibitors, since for each tetrapyrrole, best fit lines on a Lineweaver-Burk plot for reactions with and without inhibitor intersected at $x < 0$ (i.e. to the left of the y-axis). Thus, it is likely that these tetrapyrroles bind GSTs away from the CDNB binding site, and on binding, induce a conformational change in the enzyme which decreases enzyme activity.

The amino acid sequence of *Zm* GST V-V was similar to that of a number of other plant GSTs which have been shown to be inhibited by auxins (Droog *et al.*, 1995, Watahiki *et al.*, 1995, Hahn and Strittmatter, 1994, Bilang and Sturm, 1995), so recombinant *Zm* GST V-V was tested for such inhibition. Initial tests to determine a suitable concentration of 2,4-D to use (data not shown) showed that 2.5 mM 2,4-D inhibited *Zm* GST V-V activity 50% in a standard CDNB assay. The CDNB activity of *Zm* GST V-V was assayed in the presence or absence of 2.5 mM 2,4-D at a range of CDNB concentrations to determine the kinetics of inhibition. Analysis of the results (Figure 62 C) showed that 2,4-D did act as an inhibitor of CDNB activity. 2.5 mM 2,4-D inhibited *Zm* GST V-V CDNB activity with a K_i of 2.23 mM. This inhibition appeared competitive with respect to CDNB, as best fit lines of double-reciprocal plots of CDNB versus enzyme velocity for samples with and without 2,4-D present intersected at $x \approx 0$ (i.e. close to the y-axis), indicative of competitive inhibition. This suggested that 2,4-D was inhibiting *Zm* GST V-V activity by binding at the CDNB binding site.

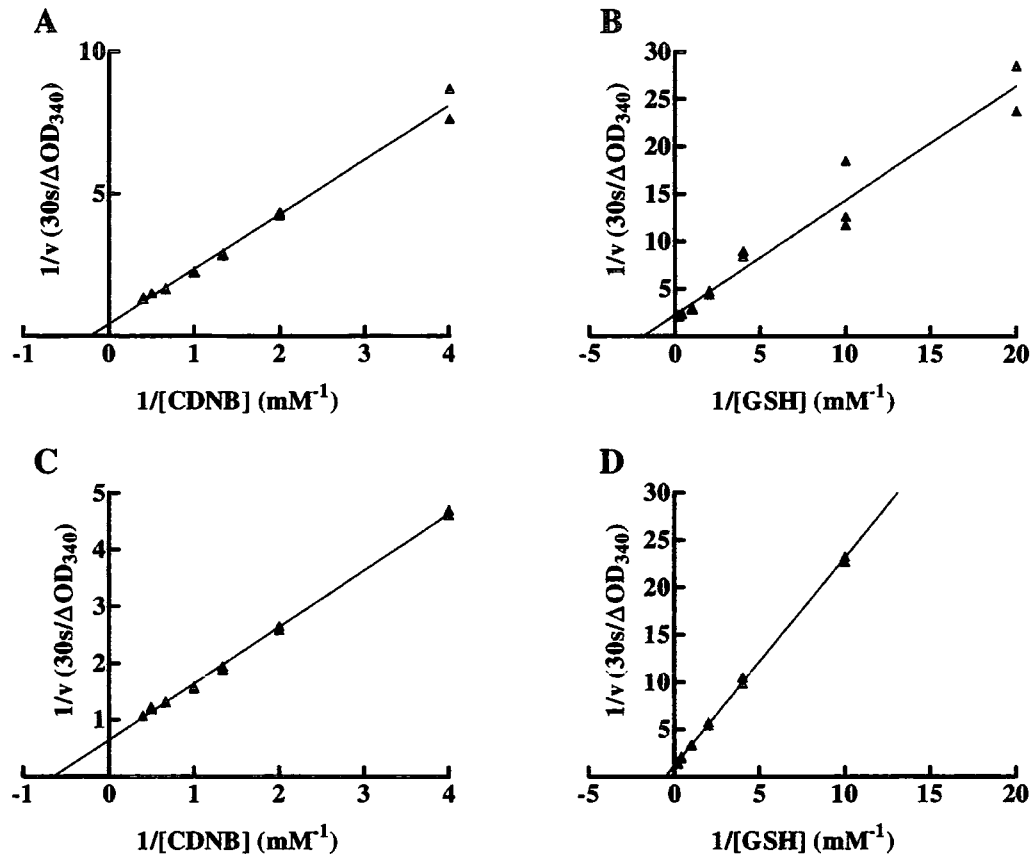

Figure 61. *Zm* GST I-I and *Zm* GST V-V CDNB conjugation kinetics.

Lineweaver-Burk plots showing kinetics of CDNB conjugation for recombinant GSTs *Zm* GST I-I (graphs A and B) and *Zm* GST V-V (graphs C and D). Graphs A and C show reaction velocity at varying [CDNB], with [GSH] = 5 mM. Graphs B and D show reaction velocity at varying [GSH], with [CDNB] = 1 mM.

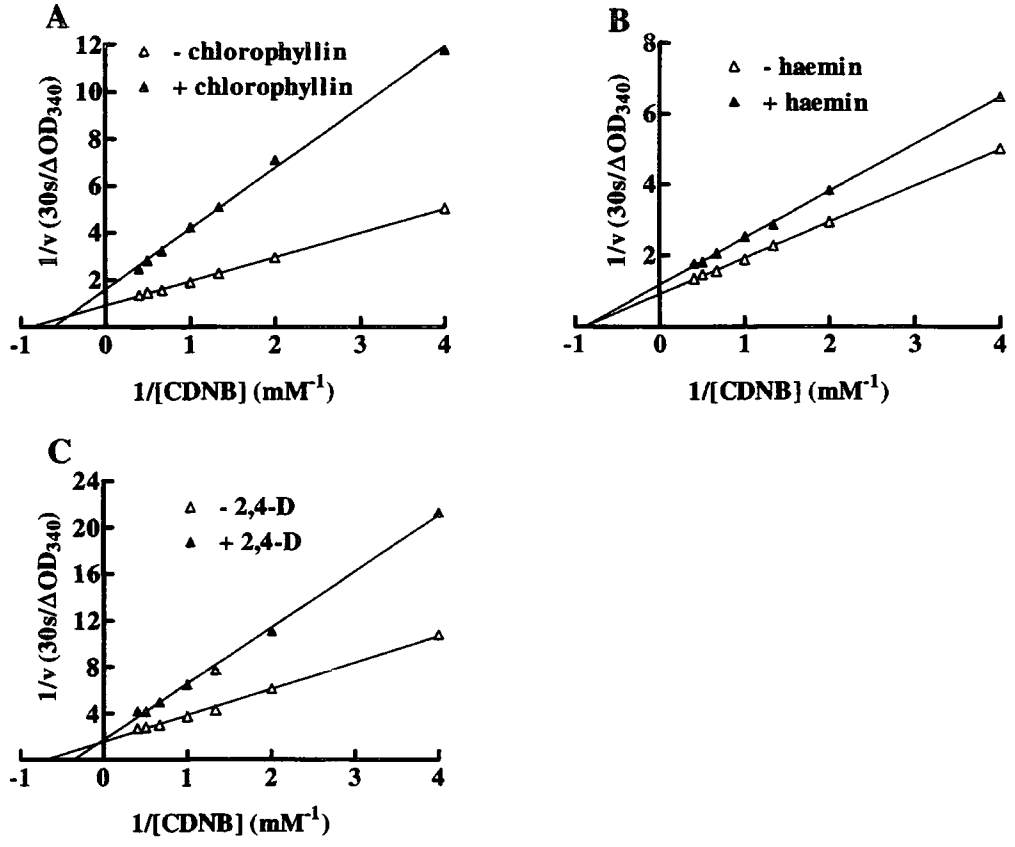

Figure 62. *Zm* GST V-V inhibition kinetics.

Lineweaver-Burk plots showing recombinant *Zm* GST V-V inhibition by: A) chlorophyllin, B) haemin and C) 2,4-D. Lines are best fit lines, calculated by linear regression analysis.

pH Sensitivity

To determine pH sensitivity, both enzymes were assayed for CDNB activity over the pH range 6.0 - 8.8, using two buffer systems to enable this range to be covered (0.1M potassium phosphate or 0.075M Tris-HCl). Assays were similar to the standard CDNB assay, except that glutathione was added to a final concentration of 1 mM instead of 5 mM, to reduce the non-enzymic reaction rate. The non-enzymic reaction rate increased exponentially with increased pH (data not shown), making it difficult to obtain accurate enzymic rates above pH 8.8. Figure 63 shows the activity of both enzyme at each pH, after correcting for the non-enzymic rate of reaction. *Zm* GST I-I activity increased up to pH 7.0, and was then more or less constant up to pH 8.8, while *Zm* GST V-V activity continued increasing from pH 6.0 to pH 8.8.

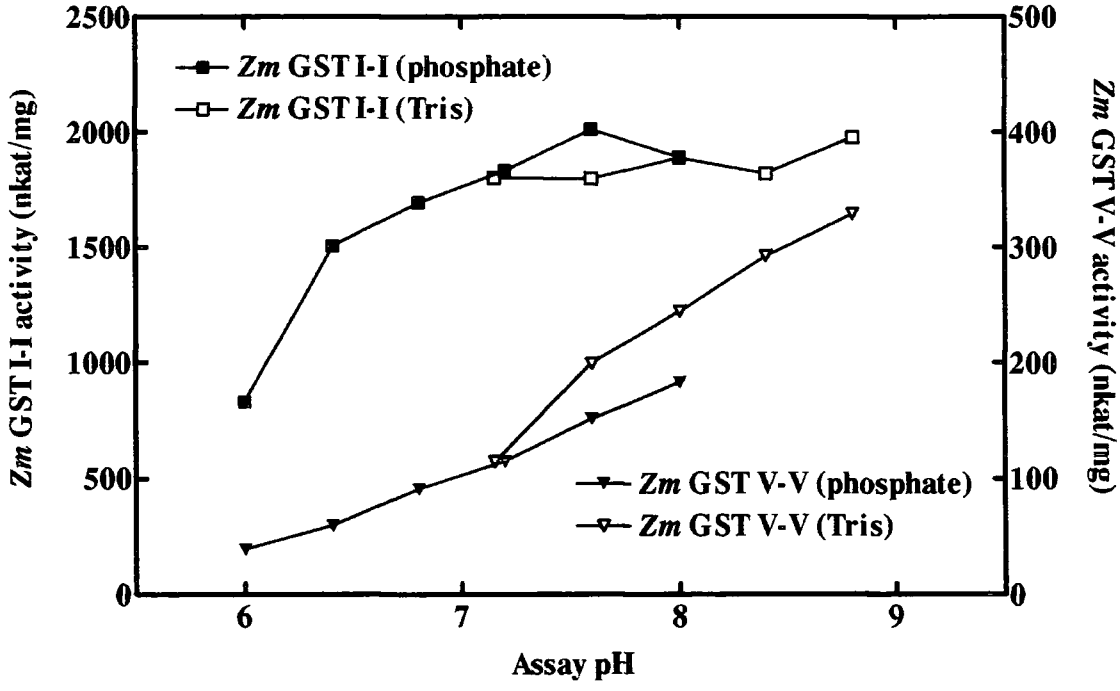

Figure 63. CDNB-conjugating activity of recombinant maize GSTs *Zm* GST I-I and *Zm* GST V-V at varying pH.

Values were corrected for the spontaneous chemical rate, which increased exponentially with increasing pH.

Discussion

Expression of Recombinant Theta Class GSTs

Two major theta class maize GSTs, *Zm* GST I and *Zm* GST III, have been successfully expressed as recombinant protein in this study. Recombinant expression of *Zm* GST I produced a dimeric protein with GST activities very similar to those of *Zm* GST I-I purified from maize. This indicated that *Zm* GST I subunits were able to fold correctly in *E. coli* and spontaneously dimerise to form *Zm* GST I-I. Recombinant *Zm* GST I-I appeared identical in all characteristics to maize *Zm* GST I-I, so it is unlikely that *Zm* GST I-I undergoes major post-translational modification in maize.

Recombinant expression of *Zm* GST III produced high levels of protein, however this protein had low GST activity towards herbicides, with levels much lower than those anticipated when compared with activities of maize *Zm* GST I-III. Furthermore, *Zm* GST III-III has been reported to have appreciable GST activity towards chloroacetanilide herbicides when expressed in *E. coli* (Moore *et al.*, 1986). It is however difficult to assess whether the GST activities for *Zm* GST III-III presented here agree with those previously published, both for the recombinant enzyme and for *Zm* GST III-III purified from maize, as the literature is not at all clear on these points. Moore *et al.* (1986) expressed *Zm* GST III-III in *E. coli* and assayed the resulting crude bacterial lysate for GST activity. This lysate had GST activity towards CDNB (19 nkat/mg) and alachlor (1.8 pkat/mg), however no indication of the level of expression of *Zm* GST III-III was given making it impossible to estimate specific activity for the recombinant enzyme. O'Connell *et al.* (1988) purified *Zm* GST III-III from maize and used the purified enzyme to determine enzyme kinetics but omitted to show the specific activity of the enzyme. However, based on approximations from the published kinetics graphs, at a substrate concentration of 1 mM the purified enzyme had a specific GST activity of 1100 pkat/mg towards metolachlor and 2500 pkat/mg towards alachlor. This value for GST activity towards alachlor is 1000-fold higher than that obtained by Moore *et al.* (1986), raising doubts that the same enzyme was used in the two studies. Unfortunately it is very

difficult to confirm the identity of the enzyme purified by O'Connell *et al.* (1988) as almost no data on the purification and characterisation of this enzyme was shown.

There is no direct evidence, such as protein sequencing, that the *Zm* GST I-III identified from maize in this study contains a *Zm* GST III subunit. However it would seem likely, as its identity was assigned on the basis of subunit molecular mass, correlation of its chromatographic behaviour with that found in previous studies (Timmerman, 1989) and its substrate specificity.

The restricted conjugating activity of recombinant *Zm* GST III may be due one of a number of possibilities, which will need to be tested. For example, sequencing showed that there were two *Zm* GST III variants in cv. Pioneer 3394 (chapter 6), of which only one has been expressed in *E. coli*. Even though the second variant has a very similar deduced amino acid sequence to the first, expression may yield a protein with high GST activities, although this has yet to be tested. Alternatively, recombinant *Zm* GST III may not correspond with *Zm* GST III from maize; this could be tested by expressing the *Zm* GST I-III heterodimer as a recombinant protein and comparing its properties with those of maize *Zm* GST I-III. Another less likely possibility is that *Zm* GST III may only be active as a heterodimer, and again, this could be tested by expressing and assaying recombinant *Zm* GST I-III. Finally, *Zm* GST III may not fold or dimerise correctly when expressed in *E. coli*, drastically reducing its activity as a GST.

Expression of Recombinant Tau Class GSTs

Three tau class maize GSTs, *Zm* GST V, *Zm* GST VI and *Zm* GST VII, were expressed as recombinant proteins, however only *Zm* GST V was expressed at high enough levels to be further purified and characterised, and was shown to correspond to *Zm* GST V from maize. Protein sequence from maize *Zm* GST VII also enabled the putative *Zm* GST VII clone to be positively identified without the need for high level expression of the recombinant protein. It was much more difficult to conclusively show that DD6a encoded the *Zm* GST VI subunit since no protein sequence for this subunit was available, and the expression of DD6a gave low yields of recombinant protein, which were not able to be purified. However, co-expression in *E. coli* of the putative *Zm* GST VI clone with

Zm GST V produced a heterodimer which could be purified, and which behaved identically to *Zm* GST V-VI from maize. This provided good evidence that DD6a did indeed encode *Zm* GST VI, and demonstrated that recombinant expression of GST heterodimers was both possible and useful.

Enzyme Activity

Substrate	Specific activity (nkat/mg protein)		
	Zm GST I-I	*Zm* GST V-V	GST *Gm*1-1
CDNB	1690	91	122
Ethacrynic acid	27.0	4.9	3.7
NBC	58.2	3.0	0.9
ENPP	ND[a]	ND[a]	0.4
BITC	NT[b]	4.3	31.8
VP	NT[b]	1.0	0.5
Metolachlor	0.04	0.25	0.23
Fluorodifen	0.01	0.52	0.40
Cumene hydroperoxide	ND[a]	0.14*	0.44*

Table 22. Comparison of activities of purified plant GSTs.
Activities for the maize isoenzymes *Zm* GST I-I and *Zm* GST V-V are those determined in this study; activities for the soybean enzyme GST *Gm*1-1 are from Skipsey *et al.* (1997).

* Activity measured as abosrbance change at 366 nm/min/mg protein.

[a] ND = No activity detected. [b] NT = Activity not tested.

The production of purified recombinant *Zm* GST I-I and *Zm* GST V-V allowed the specific activities of these GSTs to be studied in detail, as shown in Table 16 and Table 20. **Table 9** also shows activity of *Zm* GST I-I purified from maize towards a range of substrates. Very few other plant GSTs have been assayed for activity towards such a wide range of substrates, so comparisons are difficult. However, recombinant soybean HSP26-a (GST *Gm*1-1) was assayed towards a similar range of substrates as for *Zm*

GST V-V, using identical protocols (Skipsey *et al.*, 1997). The assays are therefore comparable and show that GST *Gm*1-1 and *Zm* GST V-V have a very similar range of activities (Table 22). In contrast *Zm* GST I-I has very different substrate specificity when compared with that of *Zm* GST V-V, showing much higher activity towards CDNB, ethacrynic acid and NBC, but much lower activity towards metolachlor and fluorodifen. Since *Zm* GST V-V and GST *Gm*1-1 are tau class GSTs while *Zm* GST I-I is a theta class GST it is tempting to suggest that the two classes of plant GST have differing substrate specificities, with GSTs within a class having similar substrate specificities. This however is not the case, at least for theta class plant GSTs. For example while *Zm* GST I-I has high activity towards CDNB and very low activity towards metolachlor and fluorodifen, the related theta class GST *Zm* GST II-II has negligible activity towards CDNB and high activity towards metolachlor and fluorodifen (Irzyk and Fuerst, 1993), thus having similar activities as *Zm* GST V-V. Further substrate specificity studies on a wider range of tau class and theta class plant GSTs should ascertain whether there are any links between enzyme activity and classification, as seems to be the case for mammalian GSTs (Mannervik *et al.*, 1985).

Glyoxalase activity

Despite the sequence similarity between maize tau class GST cDNA clones and soybean glyoxalase I (Paulus *et al.*, 1993), none of the proteins encoded by the clones had detectable glyoxalase I activity. One explanation is that the sequence published for the soybean glyoxalase actually codes for a GST rather than glyoxalase I. To date there are no published data on the enzyme activities of expressed soybean glyoxalase cDNA, and while the sequence shows very high homology to some plant GST sequences, it shows no homology to a glyoxalase I sequence from tomato (Espartero *et al.*, 1995), which is similar to glyoxalase I cDNA sequences from mammals. Preliminary studies in maize have shown that the *S*-hexylglutathione agarose affinity purification of glyoxalase I using the method used to purify soybean glyoxalase I (Paulus *et al.*, 1993) would result in the co-purification of GSTs. The affinity-purified soybean glyoxalase I was used to raise antibodies which were then used to immunoscreen a cDNA expression library for glyoxalase I clones. Our studies would suggest that this antibody raised by Paulus *et al.*

(1993) must have recognised GST as well as glyoxalase clones and that the soybean glyoxalase sequence reported most likely encodes a GST. The problem of the specificity of the anti-glyoxalase serum may be exacerbated since more hydrophobic proteins generally elicit a greater immune response when injected into rabbits since these proteins tend to be more stable. Since GSTs are in general highly hydrophobic, while soybean glyoxalase has been described as highly hydrophilic (Paulus *et al.*, 1993), it would be likely that an antibody raised against a mixture of glyoxalase I and GST would more strongly recognise the GST.

Expression of Recombinant GST Heterodimers

The co-expression of *Zm* GST V and *Zm* GST VI subunits in *E. coli* to form *Zm* GST V-VI has demonstrated that it is possible to synthesise recombinant GST heterodimers. There appear to be no other reports of recombinant expression of GST heterodimers in the literature.

Enzyme Kinetics

K_M and V_{max}

Data for the kinetics of *Zm* GST I-I-catalysed conjugation of CDNB have been previously published, and broadly agree with data presented here (Table 21). Mozer *et al.* (1983) estimated the K_M (CDNB) for *Zm* GST I-I to be 3.0 mM, compared to 2.51 mM estimated here. Edwards and Owen (1986) reported significantly higher K_M (CDNB) values for two unidentified GSTs, both of which were probably *Zm* GST I-I, partially purified from maize leaves (K_M = 6.7 mM) and cell cultures (K_M = 13.3 mM). Most arthropod GSTs have a K_M (CDNB) of between 0.1 and 1.0 mM (Clark, 1989), while rat GSTs have K_M (CDNB) of 0.06 - 0.1 mM (Habig *et al.*, 1974) and K_M (CDNB) values for plant GSTs include 9.8 mM for potato prp-1 (Hahn and Strittmatter, 1994), 0.2 mM for tobacco Nt107 and 0.86 mM for tobacco Nt103 (Droog *et al.*, 1995). Therefore K_M (CDNB) for *Zm* GST I-I appears quite high when compared to other GSTs, even though the enzyme has a high V_{max} for the conjugation of CDNB. Conversely, *Zm* GST V-V has a much lower K_M (CDNB), but also a much lower V_{max}.

Inhibition Studies

GSTs from animals and plants (Singh and Shaw, 1988) have been shown to bind tetrapyrroles, and it was of interest to determine whether the tetrapyrroles haemin and chlorophyllin would bind to, and inhibit, recombinant maize GSTs. Results showed that both tetrapyrroles were very potent inhibitors, particularly towards *Zm* GST V-V. Comparison with similar experiments performed on impure oat GSTs (Singh and Shaw, 1988) showed that *Zm* GST V-V was considerably more sensitive to inhibition by tetrapyrroles than CDNB-active oat GSTs, presumably due to either a higher-affinity tetrapyrrole binding site, or to a greater decrease in enzyme activity on tetrapyrrole binding.

As some tau-class GSTs have been shown to be inhibited by auxins, recombinant *Zm* GST V-V was tested for sensitivity to the artificial auxin 2,4-D. This auxin acted as a competitive inhibitor of *Zm* GST V-V with respect to CDNB, with K_i = 2.23 mM. These results are comparable with published results obtained using other tau class plant GSTs. For example *Hyoscyamus muticus* Hmgst-1 activity towards CDNB was inhibited competitively by 2,4-D, with K_i = 3.9 mM (Bilang and Sturm, 1995). Tobacco GST1-1 and GST2-1 were similarly inhibited by 2,4-D but at much lower auxin concentrations, with K_i values for 2,4-D of 0.08 mM and 0.2 mM respectively (Droog *et al.*, 1995). *A. thaliana* GST 5 CDNB activity was also inhibited by 2,4-D with 50% inhibition occurring at a 2,4-D concentration of 0.24 mM (Watahiki *et al.*, 1995). Despite the common occurrence of GST inhibition by auxins, it is unclear whether this has any functional sugnificance, and whether auxins can act as GST substrates. However, it is unlikely that auxins substantially affect GST activity *in planta*, since they are present in considerably lower concentrations than when used in the studies mentioned above (Watahiki *et al.*, 1995).

pH Sensitivity

As shown in Figure 63, when assayed for CDNB activity *Zm* GST I-I had a broad pH optimum of 7.2 to at least 8.8, while *Zm* GST V-V activity steadily increased up to at least pH 8.8. The pH optimum of *Zm* GST I-I has not previously been reported, however pH optima have been determined for a number of other plant GSTs. Maize

atrazine-active GSTs have been reported to have a pH optimum of 6.6 - 6.8 (Frear and Swanson, 1970) while later work determined this optimum to be pH 8.0 - 8.5 (Guddewar and Dauterman, 1979). Activity of *Zm* GST II-II to metolachlor had a pH optimum of 7 - 8 (Irzyk and Fuerst, 1993). pH optima in other plants have similar values, with pea GSTs being most active at pH 9.3 - 9.5 (Fear and Swanson, 1973) or pH 9.0 (Edwards, 1996), and wheat GSTs being most active at pH 8.5 - 9.5 (Edwards and Cole, 1996). In species other than plants pH optima for GSTs vary, but tend to be between pH 7.0 and 9.5.

Structural and catalytic studies on GSTs have suggested that GST catalyse glutathione conjugation in part by stabilising the thiolate anion of glutathione, which is the chemically active form of glutathione. High pH favours the formation of the thiolate anion, explaining why spontaneous glutathione conjugation reaction rates increase with increasing pH. By stabilising the thiolate anion of glutathione, GSTs effectively increase the pH at the active site. Therefore GST activity should increase with increasing pH due to the increasing stability of the thiolate anion of glutathione, until the pH is high enough to affect protein stability, when GST activity should start to decrease as the active site becomes denatured. If this is so, the broad pH optimum observed for *Zm* GST I-I may be due to the ability of this enzyme to stabilise the thiolate anion nearly completely above pH 7.2, and to remain stable and active up to at least pH 8.8. The steady increase of *Zm* GST V-V activity with increasing pH suggests that between pH 6.0 and 8.8, the proportion of glutathione molecules present as thiolate anions at the *Zm* GST V-V active site increases, so it appears that *Zm* GST V-V is not as efficient at activating glutathione as is *Zm* GST I-I. Since the pH optimum of *Zm* GST V-V activity is at least pH 8.8, it appears that this enzyme remains stable and in an active conformation up to at least pH 8.8, and therefore is relatively stable. However, other factors such as the charge-dependent binding of glutathione to the GST active site will also play a role in determining enzyme activity with varying pH, so the nature of the responses to pH shown by *Zm* GST I-I and *Zm* GST V-V remain to be conclusively explained.

9. Additional Work

Glyoxalase I Purification

As described in chapters 7 and 8, cDNA sequences for *Zm* GST V and *Zm* GST VI were very similar to that for a sequence described as being glyoxalase I (Paulus *et al.*, 1993). However, no glyoxalase I activity could be detected when recombinant *Zm* GST V-V and *Zm* GST V-VI were assayed. To confirm that the assay for glyoxalase I activity worked, an attempt was made to partially purify glyoxalase I from dichlormid-treated maize shoots, using methods similar to those used to purify the enzyme from soybean (Paulus *et al.*, 1993). This protocol differed substantially from the method used to purify *Zm* GSTs V-V and V-VI (see chapter 2), however it did use the same *S*-hexylglutathione Sepharose matrix for affinity chromatography. Using this, the resulting affinity-purified fraction had both glyoxalase I activity (confirming that the assay did work) and low GST activity towards CDNB. To try to resolve the GST and glyoxalase activities present in the affinity-purified fraction, a Q Sepharose anion-exchange column was used. Fractions were eluted from this column with a gradient of NaCl and were assayed for both GST activity towards CDNB and glyoxalase I activity (Figure 64). GST activity towards CDNB was detected in very low amounts across a broad range of fractions while glyoxalase I activity was confined to a single sharp peak, providing good evidence that the two activities are distinct and due to different proteins. SDS-PAGE analysis (Figure 65) and western blotting of fractions across this peak showed the presence of the *Zm* GST V subunit and other probable GST subunits in all the samples. An extra band with an estimated molecular mass of 24.5 kDa was present only in fractions with glyoxalase I activity, and the intensity of this band appeared roughly proportional to the glyoxalase I activity of the corresponding fraction. This 24.5 kDa polypeptide was therefore likely to be glyoxalase I.

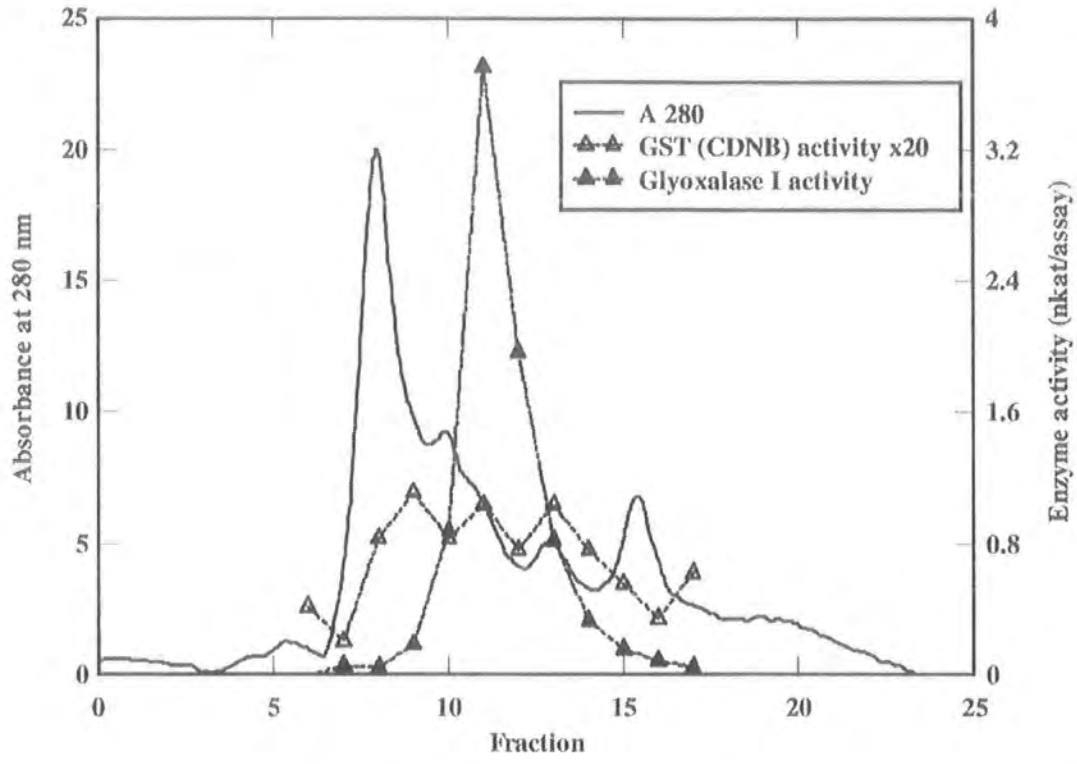

Figure 64. Anion exchange chromatography of partially purified maize glyoxalase I. Following purification from maize extracts on *S*-hexylglutathione agarose, GST and glyoxalase I activities were resolved using a linear gradient of NaCl from 0 mM at fraction 0 to 250 mM at fraction 25.

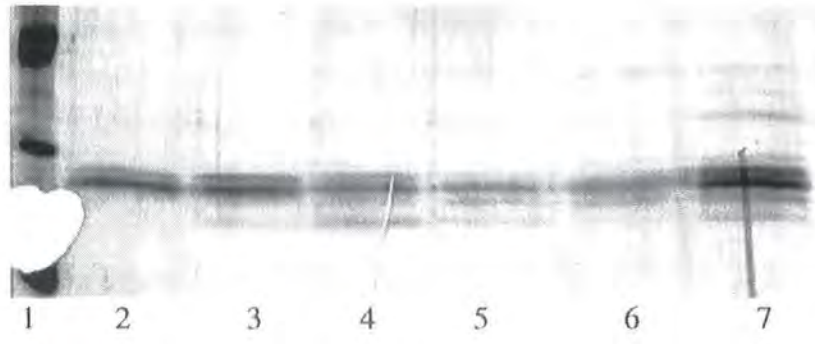

Figure 65. Silver-stained SDS-PAGE showing fractions from glyoxalase I purification. Lane 1 = molecular weight standards (top to bottom - 45 kDa, 31 kDa and 21.5 kDa). Lanes 2 to 6 show fractions eluting from Q-Sepharose column following affinity chromatography as numbered in Figure 64, with lane 2 = fraction 8, lane 3 = fraction 10, lane 4 = fraction 11, lane 5 = fraction 13 and lane 6 = fraction 16. Lane 7 = affinity-purified protein before Q-Sepharose chromatography. The major 28.5 kDa band present in all the samples corresponded to *Zm* GST V.

cDNA Library Screening using Antibiotic Resistance

In drug-resistant strains of bacteria, some antibiotics are thought to be detoxified by GSTs. In particular, one group presented evidence that resistance to the epoxide antibiotic fosfomycin (1,2-epoxypropylphosphonic acid) in one bacterial strain was due to the presence of a 25 kDa GST capable of detoxifying this compound by conjugating it with glutathione. It was therefore decided that it may be possible to screen a maize cDNA library in bacteria for clones containing novel maize GST sequences by their ability to detoxify, and therefore protect the host bacteria from fosfomycin, while all other clones should be killed by the antibiotic.

A kill curve was determined for fosfomycin toxicity towards *E. coli* in liquid culture and as shown in Figure 66 it was found that the antibiotic completely inhibited growth of *E. coli* at concentrations down to 0.5 µg/ml, and considerably inhibited growth at 0.2 µg/ml. Therefore, 0.5 µg/ml fosfomycin was chosen for antibiotic selection, which should inhibit growth of most bacteria but allow at least some growth of bacteria with increased tolerance to the antibiotic. To select for maize cDNAs encoding proteins conferring fosfomycin resistance, the cDNA library was excised from λ phage into pBluescript plasmid vectors in *E. coli* before plating out the cells onto nutrient agar containing ampicillin (to select for cells carrying phagemid), IPTG (to induce expression of protein encoded by the plasmid) and 0.5 µg/ml fosfomycin, to select for antibiotic resistance. Only a small number of plasmid-containing clones grew, however a negative control consisting of untransformed *E. coli* cells grown on IPTG and 0.5 µg/ml fosfomycin also produced a number of resistant colonies. It was therefore likely that many of the plasmid-containing resistant clones were resistant for reasons other than due to the presence of a plasmid-encoded resistance gene. To select for plasmid-based resistance, a pool of plasmids was isolated from resistant clones and used to transform fresh *E. coli* cells. Of these transformed cells, only about 5% became fosfomycin-resistant, so at least 95% of the original resistant clones had developed non-plasmid encoded resistance. Six resistant clones were selected and the maize cDNA inserts in each of their plasmids were partially sequenced.

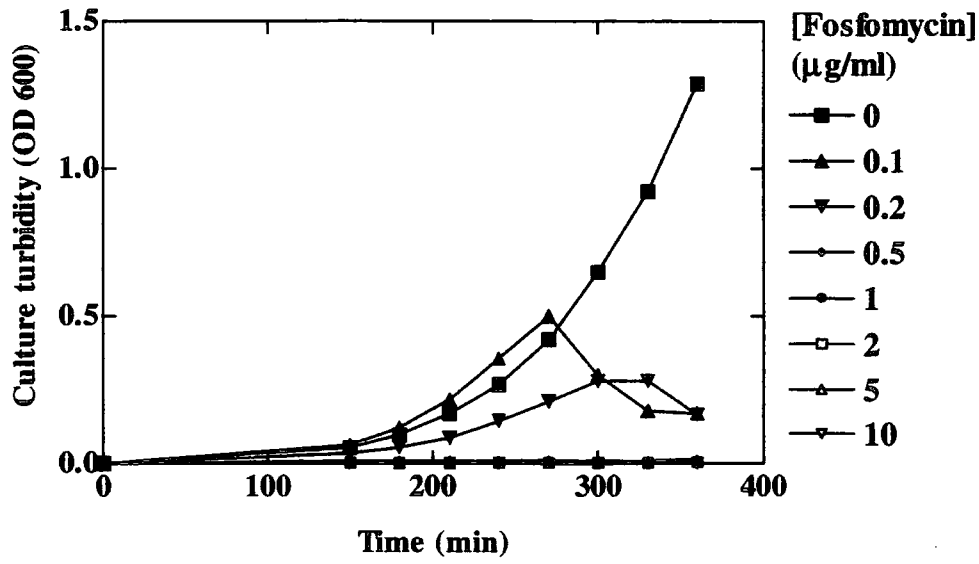

Figure 66. Effect of fosfomycin at varying concentrations on growth of *E. coli* in liquid culture, as measured by increase in medium turbidity over time.

None of the six sequenced putative fosfomycin resistance genes showed any homology to known GST sequences. Three sequences had no significant homology to any database sequence, while the remaining three sequences showed high homology to a histone protein, a 60S ribosomal protein and an adenosine kinase. It seems likely that all six sequences were false positives. The adenosine kinase may be of interest as it appeared to be full-length except for 2 nucleotides, and a sequence for this protein has not previously been isolated from any plant species.

Since carrying out the fosfomycin screen, recent work (Bernat *et al.*, 1997) has shown that "GST-based" fosfomycin resistance in bacteria was actually due to a 16 kDa manganese metalloglutathione transferase enzyme similar in sequence to glyoxalase I, and not as previously thought a typical bacterial GST. It is therefore perhaps not surprising that no GST sequences were isolated by the screen. Activity screening is a very powerful tool for isolating sequences for detoxifying enzymes and the methods described here may work in different systems, for example screening using alternative antibiotic compounds known to be detoxified by GSTs.

Cellular and Sub-Cellular Localisation of Maize GSTs

To determine the cellular and sub-cellular distribution of GSTs throughout maize plants, work was started to produce thin sections of maize tissues suitable for immunolocalisation of GSTs and observation by light and electron microscopy. Samples of roots and shoots from 10 day old light-grown seedlings, and anthers from mature plants, were dehydrated and embedded in epoxy resin in preparation for sectioning, but no time was available to complete this work.

Expression of Other Recombinant GST Heterodimers

The work described in chapter 8 showed that GST heterodimers could be successfully expressed by introducing two GST coding sequences in tandem into the pET-11d expression vector. However this approach could not be immediately applied to produce a wider range of GST heterodimers since only the pET-GST6a construct contained the

appropriate restriction sites to enable the straightforward insertion of an additional GST sequence. To solve this problem the pET-11d vector was modified to allow many more pairs of GST sequences to be easily expressed together. To construct this vector, a 316 bp. fragment of maize adenosine kinase cDNA (see earlier in this chapter), containing an internal *Nhe*I site, was released by digestion with *Pst*I and ligated into *Pst*I-digested pBluescript KS. This modified plasmid was then digested with *BamH*I and *Xho*I to release the adenosine kinase fragment, along with part of the vector polylinker. This fragment was ligated into pET-GST6a previously digested also with *BamH*I and *Xho*I, to give the expression vector pET-*Nhe*I, shown in Figure 67. For expression of heterodimers, the first GST sequence ("GST a" in Figure 67) was inserted as an *Nco*I-*Xho*I fragment into pET-*Nhe*I digested with *Nco*I and *Xho*I. The second GST sequence ("GST b" in Figure 67), which had been cloned into pET-11a or pET-11d to provide an efficient ribosome binding site, was inserted as an *Xba*I-*BamH*I fragment into the pET-*Nhe*I plasmid containing the first GST, following digestion with *BamH*I and *Nhe*I, to give DNA ends compatible with *Xba*I-cut ends. The resulting tandem expression vectors had a similar overall structure to pET-DD56 (Figure 57), with a single transcription promoter and terminator and two ribosome binding sites, one just upstream from the translation start site of each GST sequence.

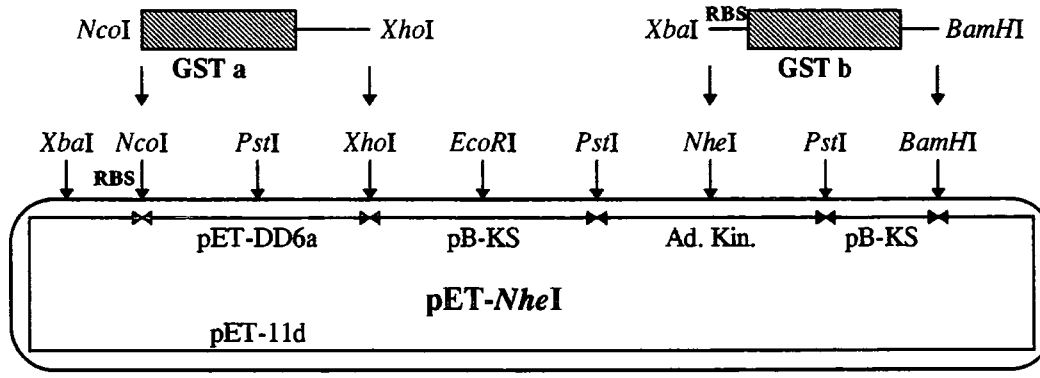

Figure 67. Plasmid map of vector pET-*Nhe*I for heterodimer expression.

The origins of each of the pET-*Nhe*I fragments are shown, as well as the important restriction sites. The positions of insertion of the two GST sequences to be expressed are indicated, as well as the nature of these inserts; hatched boxes represent the coding regions of the clones. pB-KS = pBluescript KS, Ad. Kin. = fragment of maize adenosine kinase cDNA and RBS = ribosome binding site.

Pairs of GST subunit clones were introduced into this vector to allow expression of the pairs of GST subunits listed below. All the clones mentioned were as described in this and previous chapters except for *Ta* GST 27, which was a wheat clone homologous to *Zm* GST II (I. Cummins, unpublished), shown in Figure 49.

Zm GST I + *Zm* GST III	(pETN-DD13)
Zm GST I + *Zm* GST V	(pETN-DD15)
Zm GST I + *Zm* GST VI	(pETN-DD16)
Zm GST I + *Ta* GST 27	(pETN-DD1W)
Zm GST V + *Zm* GST VI	(pETN-DD56)
Zm GST V + *Zm* GST VII	(pETN-DD57)
Zm GST V + *Ta* GST 27	(pETN-DD5W)

Each of these constructs was transformed into *E. coli* and expressed, and in each case significant GST activity towards CDNB was detected. To determine whether any of these constructs were expressing heterodimers, protein was extracted from expressing bacteria and used for western blotting following non-denaturing PAGE. Using this system, homodimers of immunoreactive subunits should be resolved from heterodimers containing an immunoreactive subunit. However the results from this were inconclusive, so further work will be needed to determine whether any of these constructs can produce GST heterodimers.

The construction of the general tandem expression vector pET-*Nhe*I will make expression of many different combinations of GST subunits relatively straightforward. There are problems with this system, for example many of the constructs contain GST sequences which do not express well in pET vectors, such as *Zm* GST VI and *Zm* GST VII, which may make it more difficult to detect and purify heterodimers. However, preliminary data from some of the tandem expression studies have suggested that expression of certain combinations of subunits may provide some intriguing results. For example, initial studies showed that expression of pETN-DD13 produced high levels of both *Zm* GST I and *Zm* GST III subunits, and future purification of the resulting dimers using Orange A and anion exchange chromatography should determine whether any *Zm* GST I-III dimers produced correspond to *Zm* GST I-III purified from maize. Also of

interest is the construct pETN-DD1W, which should express *Zm* GST I and *Ta* GST 27, which originate from two different species. Both these theta class GST polypeptides express well on their own so should express well together, and it is of great interest to determine whether these GST subunits from two distinct species will heterodimerise to form a chimeric GST, and if so whether the resulting heterodimer has any unique properties. It is important to determine how GST subunits from different species interact since genetic engineering of crop plants with foreign GST sequences has been proposed as a way to introduce or increase herbicide tolerance (Bridges *et al.*, 1993, Bieseler *et al.*, 1996), so it is a real possibility that chimeric GSTs could form in such engineered plants, and may be commercially important. Co-expression of closely related GST subunits from different species may also help to determine whether heterodimeric GSTs are important *in vivo*. If such similar subunits from differing species do not form heterodimers, while less similar subunits from the same species do, it may suggest that GST subunits are "designed" to heterodimerise, and therefore that heterodimers are important.

10. Discussion

As detailed in the previous chapters, most of the original aims and objectives of the project (see chapter 1) have been achieved. Six GST isoenzymes have been purified from maize and full-length cDNA sequences for five of the subunits making up these isoenzymes have been cloned and expressed as recombinant proteins. The substrate specificities of GST isoenzymes purified from both maize and bacteria expressing recombinant protein have been assayed for a wide range of herbicide and non-herbicide substrates. Antibodies raised to two maize GST heterodimers have enabled the expression and regulation of a number of the major GST subunits in maize to be characterised.

The purification of a number of GST isoenzymes from maize and subsequent assays of these enzymes for activity towards a wide range of potential substrates has allowed the examination of maize GST diversity and substrate specificity to be performed in much more detail than has been possible for any other plant species to date. It is now apparent that in maize the family of GST isoenzymes is much more complex than has been previously determined. In particular, this work has shown that in addition to the theta class GSTs previously described in maize, a second family of GSTs similar to the tau class GSTs of dicotyledonous plants is also present. This work has resulted in the identification and cloning of three of these tau class GSTs, however there is evidence, such as the numerous bands present on a non-denaturing PAGE gel probed with anti-*Zm* GST V-VI serum (Figure 34), that maize contains a number of other as yet uncharacterised tau class GSTs. Phylogenetic analysis of plant GSTs (Figure 48) clearly showed the existence of a third, zeta class of plant GSTs identified in wheat and carnation, and searches of expressed sequence tag (EST) databases revealed homologues in rice. It is therefore not unreasonable to assume that maize contains one or more zeta class GSTs, although there is as yet no direct evidence for their existence. Intriguingly, further EST database searches located two ESTs from *Arabidopsis thaliana* (GenBank entries T46668 and Z35742) which were similar to mammalian theta class GSTs but very

different from other plant GST sequences. It therefore seems that a fourth class of plant GST exists whose members fall into the mammalian theta class GST family. If so this will necessitate the renaming of the plant theta class, which has quite low homology to the mammalian theta class, and it should probably become the phi class, a term already used by many mammalian GST workers to describe plant GSTs. The probable existence of at least four distinct classes of plant GST, with multiple isoenzymes present in at least the tau and plant theta classes, means that the complexity of plant GST isoenzymes is likely to rival that found in mammals.

The regulation of plant GSTs is also very complex, as illustrated by the results obtained in this study with maize GSTs (Figure 33). Some GST subunits, such as *Zm* GST I, are constitutively present and unresponsive to any of the treatments tested, while others, such as *Zm* GST II and *Zm* GST V, are undetectable or present at low levels in healthy tissue, but are induced in response to chemical treatments. This induction response is not the same for all GST subunits however; for example while *Zm* GST II is induced by a wide range of chemicals *Zm* GST V is only significantly induced by the safener dichlormid. The inducibility of a subunit does not seem to correlate well with the classification of the subunit, with both inducible and constitutively present isoenzymes being found in both the theta and tau classes.

While mammalian GSTs can often be classified with some confidence based on their substrate specificities toward certain substrates no such correlation between enzyme class and substrate specificity has been identified for plant GSTs. Closely related plant GST subunits can possess very different activities (e.g. *Zm* GST I and *Zm* GST II subunits, Table 9). This complexity of isoenzyme classes and families, regulation and substrate specificity found in plant GSTs and exemplified by the maize GSTs in this study strongly suggests that the total complement of GSTs in a plant plays a number of different roles. For example it is likely that isoenzymes such as *Zm* GST II which are highly induced in response to chemical treatment play a role in stress tolerance and may be involved in minimising oxidative damage. It is however difficult to envisage a similar role for *Zm* GST I, which is present at high levels in healthy plants. Some constitutively present isoenzymes probably have endogenous functions, for example the Bronze-2

maize GST is involved in anthocyanin metabolism, but it remains unclear whether this is true for all plant GSTs or just a limited subset. It is even uncertain whether the primary activity *in vivo* of some GSTs is glutathione conjugation. Some plant GSTs, such as *Zm* GST I-II and *Zm* GST V-V, possess appreciable glutathione peroxidase activity and there is evidence that some GSTs can act as isomerases (Niklaus *et al.*, 1996). This work has also shown that plant GSTs bind tightly to auxins and tetrapyrroles (chapter 8), and this binding may play an as yet undetermined role, such as for example transport. While this and other studies have provided much information about plant GST sequences, activity towards exogenous compounds and regulation of expression the natural functions of these enzymes have still not been conclusively determined and this remains one of the major goals of plant GST research.

11. Appendix - DNA Sequencing Strategies

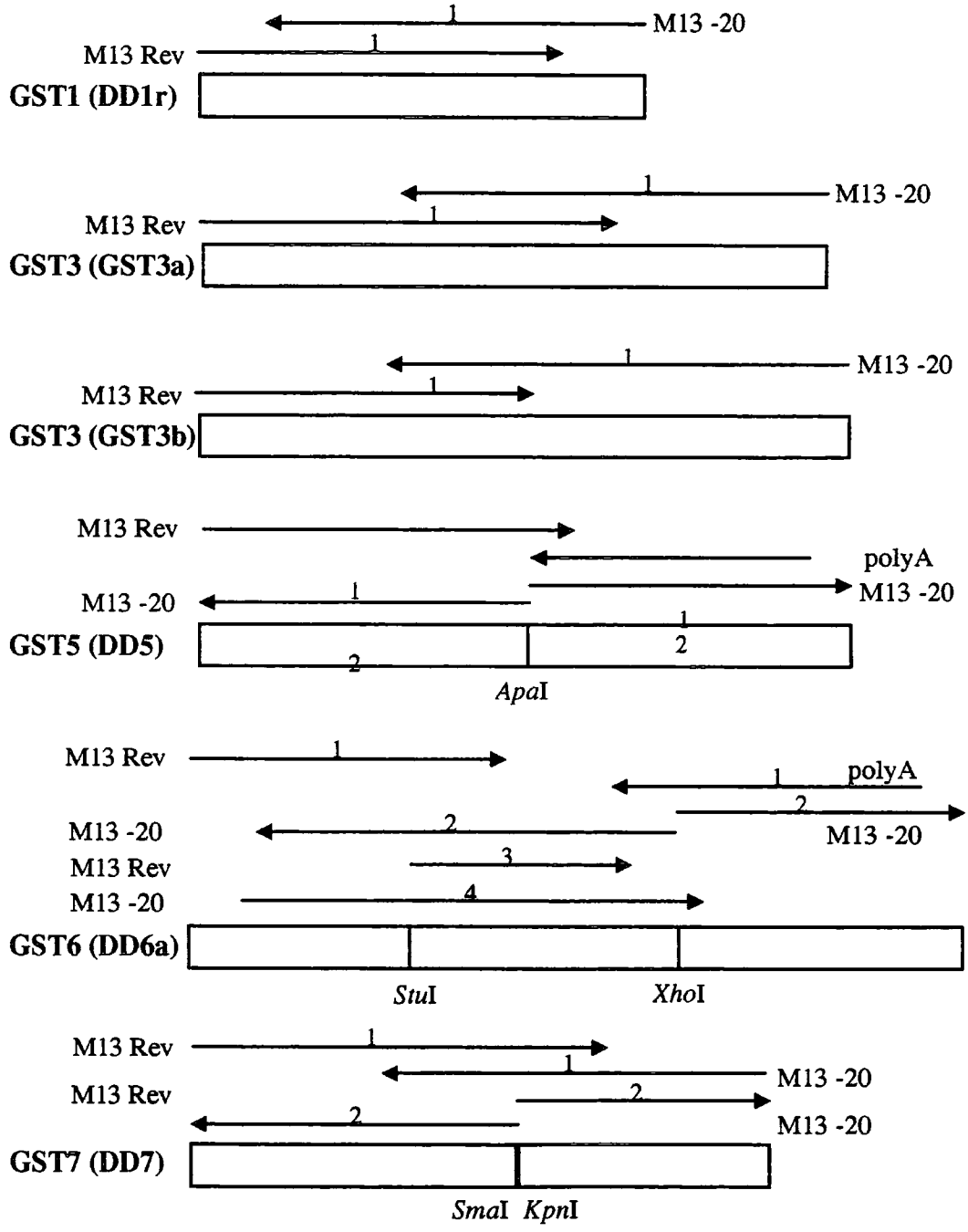

Figure 68. DNA sequencing strategies for GST cDNA clones.

All clones illustrated with 5′ end at left. Diagrams to scale (1 cm = 100 bp.). Arrows represent direction and length of reliable sequence information and the sequencing primer used; numbers correspond to those in the text.

GST Clone Sequencing Strategies

GST1

1) GST1 PCR clones (eg. DD1r; 645 bp.) were sequenced from both ends with M13 Rev and -20 primers. The sequences overlapped to give full length contigs. Both strands were not fully sequenced as GST1 had previously been sequenced by others (the deduced polypeptide sequence of DD1r was as expected from previous reports), and data for singly-sequenced regions were unambiguous.

GST3

1) GST3 cDNA library clones (up to 950 bp.) were sequenced from both ends with M13 Rev and -20 primers. The pairs of sequences each overlapped to give full length contigs. It was not considered necessary to sequence both strands for the same reasons as stated for GST1; in addition multiple clones were isolated and sequenced for each GST3 variant so each region was sequenced multiple times, albeit in the same direction.

GST5

1) The clone was sequenced from both ends (5 Rev, 5 polyA); the sequences overlapped to give a near full-length contig.
2) Fragments either side of an internal ApaI site were sub-cloned and sequenced: the 5' end (5L; 476 bp.) was fully sequenced (5L -20) and the 3' end (5S; 471 bp.) was also fully sequenced (5S -20).
3) A contig was assembled from these four sequences to give a 947 bp. full-length sequence, fully sequenced in both directions except for 33 bp. of the 3' untranslated region and poly(A) tail, which gave high-quality sequence from one run.

GST6

1) The clone was sequenced from both ends (6 Rev, 6 polyA); sequences did not overlap.

2) Fragments either side of a sequenced internal XhoI site were sub-cloned. The 5' end (6L; 710 bp.) was partially sequenced (6L -20) and the 3' end (6S; 411 bp.) was fully sequenced (6S -20)

3) A long 3' fragment was cloned using an internal StuI site and was partially sequenced (6SS Rev).

4) An NcoI site was introduced at the start codon by PCR; and the coding region sub-cloned and fully sequenced (Not shown on contig assembly - see map).

5) A contig was assembled from the five sequences to give a 1121 bp. full-length sequence, fully sequenced in both directions except for 27 bp. of the 3' untranslated region and poly(A) tail, and 51 bp. of the 5' untranslated region, both of which gave high-quality unambiguous sequence from one run.

GST7

1) The clone sequenced from both ends (7 Rev, 7 -20); sequences overlapped to give a full-length contig.

2) The 5' end was sub-cloned using an internal KpnI site and was fully sequenced (7K -20; 475 bp.).

3) The 3' end was sub-cloned using an internal SmaI site and was fully sequenced (7S Rev; 369 bp.).

4) A contig was assembled from the four sequences to give a 840 bp. full-length sequence, fully sequenced in both directions.

GST5 Contig Assembly

```
                    *        20         *        40         *
5 Rev (+)  ctaacgccggaggcgagaacaagaaaaagctcgacatggccgaggagaag
5 PolyA (-)----------------------------------------------------
5L -20 (-) ctaacgccggaggcgagaacaagaaaaagctcgacatggccgaggagaag
5S -20 (+) ----------------------------------------------------
Contig     CTAACGCCGGAGGCGAGAACAAGAAAAAGCTCGACATGGCCGAGGAGAAG

                   60         *        80         *        100
5 Rev (+)  aagcagggcctgcagctgctggacttctgggtgagcccattcgggcagcg
5 PolyA (-)----------------------------------------------------
5L -20 (-) aagcagggcctgcagctgctggacttctgggtgagcccattcgggcagcg
5S -20 (+) ----------------------------------------------------
Contig     AAGCAGGGCCTGCAGCTGCTGGACTTCTGGGTGAGCCCATTCGGGCAGCG

                    *       120         *       140         *
5 Rev (+)  ctgccgcatcgcgatggacgagaagggcctggcctacgagtacctggagc
5 PolyA (-)----------------------------------------------------
5L -20 (-) ctgccgcatcgcgatggacgagaagggcctggcctacgagtacctggagc
5S -20 (+) ----------------------------------------------------
Contig     CTGCCGCATCGCGATGGACGAGAAGGGCCTGGCCTACGAGTACCTGGAGC

                   160         *       180         *       200
5 Rev (+)  aggacctggggaacaagagcgagctgctcctccgcgccaacccggtgcat
5 PolyA (-)----------------------------------------------------
5L -20 (-) aggacctggggaacaagagcgagctgctcctccgcgccaacccggtgcat
5S -20 (+) ----------------------------------------------------
Contig     AGGACCTGGGGAACAAGAGCGAGCTGCTCCTCCGCGCCAACCCGGTGCAT

                    *       220         *       240         *
5 Rev (+)  aagaagatccccgtgctgctgcacgacggccgccccgtctgcgagtccct
5 PolyA (-)----------------------------------------------------
5L -20 (-) aagaagatccccgtgctgctgcacgacggccgccccgtctgcgagtccct
5S -20 (+) ----------------------------------------------------
Contig     AAGAAGATCCCCGTGCTGCTGCACGACGGCCGCCCCGTCTGCGAGTCCCT

                   260         *       280         *       300
5 Rev (+)  cgtcatcgtgcagtacctcgacgaggcgttcccggcggcggcgccggcgc
5 PolyA (-)----------------------------------------------------
5L -20 (-) cgtcatcgtgcagtacctcgacgaggcgttcccggcggcggcgccggcgc
5S -20 (+) ----------------------------------------------------
Contig     CGTCATCGTGCAGTACCTCGACGAGGCGTTCCCGGCGGCGGCGCCGGCGC

                    *       320         *       340         *
5 Rev (+)  tgctccccgccgacccctacgcgcgcgcgcaggcccgcttctgggcggac
5 PolyA (-)----------------------------------------------------
5L -20 (-) tgctccccgccgacccctacgcgcgcgcgcaggcccgcttctgggcggac
5S -20 (+) ----------------------------------------------------
Contig     TGCTCCCCGCCGACCCCTACGCGCGCGCGCAGGCCCGCTTCTGGGCGGAC

                   360         *       380         *       400
5 Rev (+)  tacgtcgacaagaagctctacgactgcggcacccggctgtggaagctcaa
5 PolyA (-)----------------------------------------------------
5L -20 (-) tacgtcgacaagaagctctacgactgcggcacccggctgtggaagctcaa
5S -20 (+) ----------------------------------------------------
Contig     TACGTCGACAAGAAGCTCTACGACTGCGGCACCCGGCTGTGGAAGCTCAA
```

```
                    *       420         *       440         *
5 Rev (+)  gggggacggccaggcgcaggcgcgcgccgagatggtcgagatcctccgca
5 PolyA (-)-------------------------------------------------
5L -20 (-) gggggacggccaggcgcaggcgcgcgccgagatggtcgagatcctccgca
5S -20 (+) -------------------------------------------------
Contig     GGGGGACGGCCAGGCGCAGGCGCGCGCCGAGATGGTCGAGATCCTCCGCA

                   460         *       480         *       500
5 Rev (+)  cgctggagggcgcgctcggcgacgggcccttcttcggcggcgacgccctc
5 PolyA (-)-------------------------------------ggcgacgccctc
5L -20 (-) cgctggagggcgcgctcggcgacggg-----------------------
5S -20 (+) -------------------------ccccttcttcggcggcgacgccctc
Contig     CGCTGGAGGGCGCGCTCGGCGACGGGCCCTTCTTCGGCGGCGACGCCCTC

                    *       520         *       540         *
5 Rev (+)  ggcttcgtcgacgtcgcgctcgtgcccttcacgtcctg------------
5 PolyA (-)ggcttcgtcgacgtcgcgctcgtgcccttcacgtcctggttcctcgccta
5L -20 (-) -------------------------------------------------
5S -20 (+) ggcttcgtcgacgtcgcgctcgtgcccttcacgtcctggttcctcgccta
Contig     GGCTTCGTCGACGTCGCGCTCGTGCCCTTCACGTCCTGGTTCCTCGCCTA

                   560         *       580         *       600
5 Rev (+)  -------------------------------------------------
5 PolyA (-)cgaccgcttcggcggcgtcagcgtggagaaggagtgcccgaggctggccg
5L -20 (-) -------------------------------------------------
5S -20 (+) cgaccgcttcggcggcgtcagcgtggagaaggagtgcccgaggctggccg
Contig     CGACCGCTTCGGCGGCGTCAGCGTGGAGAAGGAGTGCCCGAGGCTGGCCG

                    *       620         *       640         *
5 Rev (+)  -------------------------------------------------
5 PolyA (-)cctgggccaagcgctgcgccgagcgccccagcgtcgccaagaacctctac
5L -20 (-) -------------------------------------------------
5S -20 (+) cctgggccaagcgctgcgccgagcgccccagcgtcgccaagaacctctac
Contig     CCTGGGCCAAGCGCTGCGCCGAGCGCCCCAGCGTCGCCAAGAACCTCTAC

                   660         *       680         *       700
5 Rev (+)  -------------------------------------------------
5 PolyA (-)ccgcccgagaaggtctacgacttcgtctgcgggatgaagaagaggctggg
5L -20 (-) -------------------------------------------------
5S -20 (+) ccgcccgagaaggtctacgacttcgtctgcgggatgaagaagaggctggg
Contig     CCGCCCGAGAAGGTCTACGACTTCGTCTGCGGGATGAAGAAGAGGCTGGG

                    *       720         *       740         *
5 Rev (+)  -------------------------------------------------
5 PolyA (-)catcgagtagagcatccatcggtcggccggtggctggccgggagtaataa
5L -20 (-) -------------------------------------------------
5S -20 (+) catcgagtagagcatccatcggtcggccggtggctggccgggagtaataa
Contig     CATCGAGTAGAGCATCCATCGGTCGGCCGGTGGCTGGCCGGGAGTAATAA

                   760         *       780         *       800
5 Rev (+)  -------------------------------------------------
5 PolyA (-)tgacgaaccaattatctagttttggtttgagtgtgctcagcagagcagtt
5L -20 (-) -------------------------------------------------
5S -20 (+) tgacgaaccaattatctagttttggtttgagtgtgctcagcagagcagtt
Contig     TGACGAACCAATTATCTAGTTTTGGTTTGAGTGTGCTCAGCAGAGCAGTT

                    *       820         *       840         *
5 Rev (+)  -------------------------------------------------
5 PolyA (-)cgtgttcatgagttcgtcgtcgttgtattttctattgtcagcggtggcag
5L -20 (-) -------------------------------------------------
5S -20 (+) cgtgttcatgagttcgtcgtcgttgtattttctattgtcagcggtggcag
Contig     CGTGTTCATGAGTTCGTCGTCGTTGTATTTTCTATTGTCAGCGGTGGCAG
```

```
                  860         *        880         *        900
5 Rev (+)  --------------------------------------------------
5 PolyA (-)cgccgtacgtgttgcctcgtacaccacaaccgaataaggggtgt-----
5L -20 (-) --------------------------------------------------
5S -20 (+) cgccgtacgtgttgcctcgtacaccacaaccgaataaggggtgtttggt
Contig     CGCCGTACGTGTTGCCTCGTACACCACAACCGAATAAGGGGGTGTTTGGT

                    *       920         *       940
5 Rev (+)  ----------------------------------------------
5 PolyA (-)----------------------------------------------
5L -20 (-) ----------------------------------------------
5S -20 (+) ttgcccctcctaaaatttagcccctatcaaaaaaaaaaaaaaaaaaa
Contig     TTGCCCCTCCTAAAATTTAGCCCCTATCAAAAAAAAAAAAAAAAAA
```

GST6 Contig Assembly

```
                    *         20          *         40          *
6 Rev (+)   ctctaatccatttcggcatttccaacgccttcgccctaccagccacgtcg
6SS Rev (+)-------------------------------------------------
6L -20 (-)  -------------------------------------------------
6 polyA (-)-------------------------------------------------
6S -20 (+)  -------------------------------------------------
Contig      CTCTAATCCATTTCGGCATTTCCAACGCCTTCGCCCTACCAGCCACGTCG

                   60          *         80          *         100
6 Rev (+)   cttcgaggccgatcgaccgagcagctggtggcaatggcggcggcggcgga
6SS Rev (+)-------------------------------------------------
6L -20 (-)  -ttcgaggccgatcgaccgagcagctggtggcaatggcggcggcggcgga
6 polyA (-)-------------------------------------------------
6S -20 (+)  -------------------------------------------------
Contig      CTTCGAGGCCGATCGACCGAGCAGCTGGTGGCAATGGCGGCGGCGGCGGA

                    *        120          *        140          *
6 Rev (+)   ggtcgtgctgctggacttctgggtgagcccccttcgggcagcgctgccgga
6SS Rev (+)-------------------------------------------------
6L -20 (-)  ggtcgtgctgctggacttctgggtgagcccccttcgggcagcgctgccgga
6 polyA (-)-------------------------------------------------
6S -20 (+)  -------------------------------------------------
Contig      GGTCGTGCTGCTGGACTTCTGGGTGAGCCCCTTCGGGCAGCGCTGCCGGA

                   160          *        180          *        200
6 Rev (+)   tcgcgctggcggagaagggcgtggcctacgagtaccgcgagcaggacctc
6SS Rev (+)-------------------------------------------------
6L -20 (-)  tcgcgctggcggagaagggcgtggcctacgagtaccgcgagcaggacctc
6 polyA (-)-------------------------------------------------
6S -20 (+)  -------------------------------------------------
Contig      TCGCGCTGGCGGAGAAGGGCGTGGCCTACGAGTACCGCGAGCAGGACCTC

                    *        220          *        240          *
6 Rev (+)   ctggacaagggcgagctgctcctccgctccaaccccatccacaagaagat
6SS Rev (+)-------------------------------------------------
6L -20 (-)  ctggacaagggcgagctgctcctccgctccaaccccatccacaagaagat
6 polyA (-)-------------------------------------------------
6S -20 (+)  -------------------------------------------------
Contig      CTGGACAAGGGCGAGCTGCTCCTCCGCTCCAACCCCATCCACAAGAAGAT

                   260          *        280          *        300
6 Rev (+)   ccccgtcctgctccacgccggcaggcccgtctgcgagtcgctcgtcatcc
6SS Rev (+)-------------------------------------------------
6L -20 (-)  ccccgtcctgctccacgccggcaggcccgtctgcgagtcgctcgtcatcc
6 polyA (-)-------------------------------------------------
6S -20 (+)  -------------------------------------------------
Contig      CCCCGTCCTGCTCCACGCCGGCAGGCCCGTCTGCGAGTCGCTCGTCATCC

                    *        320          *        340          *
6 Rev (+)   tccagtacatcgacgaggcctggccggacgtcgcgccgctcctccccaag
6SS Rev (+)-----------------cctggccggacgtcgcgccgctcctccccaag
6L -20 (-)  tccagtacatcgacgaggcctggccggacgtcgcgccgctcctccccaag
6 polyA (-)-------------------------------------------------
6S -20 (+)  -------------------------------------------------
Contig      TCCAGTACATCGACGAGGCCTGGCCGGACGTCGCGCCGCTCCTCCCCAAG
```

```
                  360         *        380         *        400
6 Rev (+)  gacgacccctacgcccgcgcgcaggcgcgtttctgggccgattacatcga
6SS Rev (+)gacgacccctacgcccgcgcgcaggcgcgtttctgggccgattacatcga
6L -20 (-) gacgacccctacgcccgcgcgcaggcgcgtttctgggccgattacatcga
6 polyA (-)-------------------------------------------------
6S -20 (+) -------------------------------------------------
Contig     GACGACCCCTACGCCCGCGCGCAGGCGCGTTTCTGGGCCGATTACATCGA

                    *       420         *       440         *
6 Rev (+)  caagaagatctatgacagccagactcggctgtggaagttcgagggcgagg
6SS Rev (+)caagaagatctatgacagccagactcggctgtggaagttcgagggcgagg
6L -20 (-) caagaagatctatgacagccagactcggctgtggaagttcgagggcgagg
6 polyA (-)-------------------------------------------------
6S -20 (+) -------------------------------------------------
Contig     CAAGAAGATCTATGACAGCCAGACTCGGCTGTGGAAGTTCGAGGGCGAGG

                  460         *        480         *        500
6 Rev (+)  cgcggga-----------------------------------------------
6SS Rev (+)cgcgggagcaggcgaagaaggacctggtggaggtcctggagaccctggag
6L -20 (-) cgcgggagcaggcgaagaaggacctggtggaggtcctggagaccctggag
6 polyA (-)-------------------------------------------------
6S -20 (+) -------------------------------------------------
Contig     CGCGGGAGCAGGCGAAGAAGGACCTGGTGGAGGTCCTGGAGACCCTGGAG

                    *       520         *       540         *
6 Rev (+)  -------------------------------------------------
6SS Rev (+)ggggagctcgccgacaagcctttcttcggcggcggcgccctcggcttcgt
6L -20 (-) ggggagctcgccgacaagcctttcttcggcggcggcgccctcggcttcgt
6 polyA (-)-------------------------------------------------
6S -20 (+) -------------------------------------------------
Contig     GGGGAGCTCGCCGACAAGCCTTTCTTCGGCGGCGGCGCCCTCGGCTTCGT

                  560         *        580         *        600
6 Rev (+)  -------------------------------------------------
6SS Rev (+)ggacgtggctctggtgcccttcacgtcctggttcctcgcctacgagaagc
6L -20 (-) ggacgtggctctggtgcccttcacgtcctggttcctcgcctacgagaagc
6 polyA (-)-------------------------------------------------
6S -20 (+) -------------------------------------------------
Contig     GGACGTGGCTCTGGTGCCCTTCACGTCCTGGTTCCTCGCCTACGAGAAGC

                    *       620         *       640         *
6 Rev (+)  -------------------------------------------------
6SS Rev (+)tgggcgggttcagcgtccaggagcactgccccaggat-------------
6L -20 (-) tgggcgggttcagcgtccaggagcactgccccaggatcgtggcctgggcc
6 polyA (-)---------------------------gccccaggatcgtggcctgggcc
6S -20 (+) -------------------------------------------------
Contig     TGGGCGGGTTCAGCGTCCAGGAGCACTGCCCCAGGATCGTGGCCTGGGCC

                  660         *        680         *        700
6 Rev (+)  -------------------------------------------------
6SS Rev (+)-------------------------------------------------
6L -20 (-) gcgcgctgcagggagcgggagagcgtggccaaggccatgtccgaccctgc
6 polyA (-)gcgcgctgcagggagcgggagagcgtggccaaggccatgtccgaccctgc
6S -20 (+) -------------------------------------------------
Contig     GCGCGCTGCAGGGAGCGGGAGAGCGTGGCCAAGGCCATGTCCGACCCTGC

                    *       720         *       740         *
6 Rev (+)  -------------------------------------------------
6SS Rev (+)-------------------------------------------------
6L -20 (-) caaggtgctc--------------------------------------------
6 polyA (-)caaggtgctcgagttcgtccagttcctccagagcaagttcggggccaagt
6S -20 (+) ----------gagttcgtccagttcctccagagcaagttcggggccaagt
Contig     CAAGGTGCTCGAGTTCGTCCAGTTCCTCCAGAGCAAGTTCGGGGCCAAGT
```

```
                760         *       780         *       800
6 Rev (+)  ----------------------------------------------------
6SS Rev (+)----------------------------------------------------
6L -20 (-) ----------------------------------------------------
6 polyA (-)gatcggaagcattgcgtgtgctgctagcctgatatgccctatgcaggcca
6S -20 (+) gatcggaagcattgcgtgtgctgctagcctgatatgccctatgcaggcca
Contig     GATCGGAAGCATTGCGTGTGCTGCTAGCCTGATATGCCCTATGCAGGCCA

                  *       820         *       840         *
6 Rev (+)  ----------------------------------------------------
6SS Rev (+)----------------------------------------------------
6L -20 (-) ----------------------------------------------------
6 polyA (-)ggctggtgctttgatctgctcgatcagctctatgcccatgctagcgttgc
6S -20 (+) ggctggtgctttgatctgctcgatcagctctatgcccatgctagcgttgc
Contig     GGCTGGTGCTTTGATCTGCTCGATCAGCTCTATGCCCATGCTAGCGTTGC

                860         *       880         *       900
6 Rev (+)  ----------------------------------------------------
6SS Rev (+)----------------------------------------------------
6L -20 (-) ----------------------------------------------------
6 polyA (-)atagcgcagttgatgtgtgatgtgtctggttggttgtagctgctctttgc
6S -20 (+) atagcgcagttgatgtgtgatgtgtctggttggttgtagctgctctttgc
Contig     ATAGCGCAGTTGATGTGTGATGTGTCTGGTTGGTTGTAGCTGCTCTTTGC

                  *       920         *       940         *
6 Rev (+)  ----------------------------------------------------
6SS Rev (+)----------------------------------------------------
6L -20 (-) ----------------------------------------------------
6 polyA (-)ctggtttcgtacgtcagtgtaaggtttcaggttttcagtgtctggggtag
6S -20 (+) ctggtttcgtacgtcagtgtaaggtttcaggttttcagtgtctggggtag
Contig     CTGGTTTCGTACGTCAGTGTAAGGTTTCAGGTTTTCAGTGTCTGGGGTAG

                960         *       980         *      1000
6 Rev (+)  ----------------------------------------------------
6SS Rev (+)----------------------------------------------------
6L -20 (-) ----------------------------------------------------
6 polyA (-)ctctgcgttgcccttgcccctgccccctacctagcggctcttgagctctt
6S -20 (+) ctctgcgttgcccttgcccctgccccctacctagcggctcttgagctctt
Contig     CTCTGCGTTGCCCTTGCCCCTGCCCCCTACCTAGCGGCTCTTGAGCTCTT

                  *      1020         *      1040         *
6 Rev (+)  ----------------------------------------------------
6SS Rev (+)----------------------------------------------------
6L -20 (-) ----------------------------------------------------
6 polyA (-)cggctcgccagcaataaagttgcagaggctttagctaaaagtttctgtat
6S -20 (+) cggctcgccagcaataaagttgcagaggctttagctaaaagtttctgtat
Contig     CGGCTCGCCAGCAATAAAGTTGCAGAGGCTTTAGCTAAAAGTTTCTGTAT

               1060         *      1080         *      1100
6 Rev (+)  ----------------------------------------------------
6SS Rev (+)----------------------------------------------------
6L -20 (-) ----------------------------------------------------
6 polyA (-)tttttagttgacgattattggtcc----------------------------
6S -20 (+) tttttagttgacgattattggtccaatgtattcgggaattttgttctctc
Contig     TTTTTAGTTGACGATTATTGGTCCAATGTATTCGGGAATTTTGTTCTCTC

                  *      1120
6 Rev (+)  ----------------------
6SS Rev (+)----------------------
6L -20 (-) ----------------------
6 polyA (-)----------------------
6S -20 (+) taaaaaaaaaaaaaaaaaaa
Contig     TAAAAAAAAAAAAAAAAAAA
```

GST7 Contig Assembly

```
                  *        20         *        40         *
7 Rev (+) gccaaccaacgagtagcaggaaacatgtctccgcccgtcaagatcctcgg
7K -20 (-)gccaaccaacgagtagcaggaaacatgtctccgcccgtcaagatcctcgg
7 -20 (-) ----------------------------------------------------
7S Rev (+)----------------------------------------------------
Contig    GCCAACCAACGAGTAGCAGGAAACATGTCTCCGCCCGTCAAGATCCTCGG

                 60         *        80         *       100
7 Rev (+) ccactacgcgagcccgtactcgcaccgcgtcgaggccgctctgcggctca
7K -20 (-)ccaatacgcgagcccgtactcgcaccgcgtcgagaccgctctgcggctca
7 -20 (-) ----------------------------------------------------
7S Rev (+)----------------------------------------------------
Contig    CCAATACGCGAGCCCGTACTCGCACCGCGTCGAGACCGCTCTGCGGCTCA

                  *       120         *       140         *
7 Rev (+) agggcgtgccgtacgagctggtccaggaagacctgggcaacaagagcgag
7K -20 (-)agggcgtgccgtacgagctggtccaggaagacctgggcaacaagagcgag
7 -20 (-) ----------------------------------------------------
7S Rev (+)----------------------------------------------------
Contig    AGGGCGTGCCGTACGAGCTGGTCCAGGAAGACCTGGGCAACAAGAGCGAG

                160         *       180         *       200
7 Rev (+) ctgctgctcgccaagaaccctgtccacaagaaggtgcccgtgctcctcca
7K -20 (-)ctgctgctcgccaagaaccctgtccacaagaaggtgcccgtgctcctcca
7 -20 (-) ----------------------------------------------------
7S Rev (+)----------------------------------------------------
Contig    CTGCTGCTCGCCAAGAACCCTGTCCACAAGAAGGTGCCCGTGCTCCTCCA

                  *       220         *       240         *
7 Rev (+) tggcgacagggccgtctgcgagtccctcctcatcgtcgagtacgtcgacg
7K -20 (-)tggcgacagggccgtctgcgagtccctcctcatcgtcgagtacgtcgacg
7 -20 (-) ----------------------------------------------------
7S Rev (+)----------------------------------------------------
Contig    TGGCGACAGGGCCGTCTGCGAGTCCCTCCTCATCGTCGAGTACGTCGACG

                260         *       280         *       300
7 Rev (+) aggccttcgacgggccgtccatcctgccggccgacccccacgaccgtgcc
7K -20 (-)aggccttcgacgggccgtccatcctgccggccgacccccacgaccgtgcc
7 -20 (-) ----------------------------------ccccccacgaccgtgcc
7S Rev (+)----------------------------------------------------
Contig    AGGCCTTCGACGGGCCGTCCATCCTGCCGGCCGACCCCCACGACCGTGCC

                  *       320         *       340         *
7 Rev (+) gtcgcccgtttctgggcgaacttcttggacaccaagttctcccagccgtt
7K -20 (-)gtcgcccgtttctgggcgaacttcttggacaccaagttctcccagccgtt
7 -20 (-) gtcgcccgtttctgggcgaacttcttggacaccaagttctcccagccgtt
7S Rev (+) ----------------------------------------------------
Contig    GTCGCCCGTTTCTGGGCGAACTTCTTGGACACCAAGTTCTCCCAGCCGTT

                360         *       380         *       400
7 Rev (+) ctggctggcgtactgggcggagggcgaggcgcagaaggccgtggtgaagg
7K -20 (-)ctggctggcgtactgggcggagggcgaggcgcagaaggccgtggtgaagg
7 -20 (-) ctggctggcgtactgggcggagggcgaggcgcagaaggccgtggtgaagg
7S Rev (+)----------------------------------------------------
Contig    CTGGCTGGCGTACTGGGCGGAGGGCGAGGCGCAGAAGGCCGTGGTGAAGG
```

```
                    *       420         *       440         *
7 Rev (+) aggccaaggagaacctggcgctcctggaggcgcagctcggcgggaagagc
7K -20 (-)aggccaaggagaacctggcgctcctggaggcgcagctcggcgggaagagc
7 -20 (-) aggccaaggagaacctggcgctcctggaggcgcagctcggcgggaagagc
7S Rev (+)-------------------------------------------------
Contig    AGGCCAAGGAGAACCTGGCGCTCCTGGAGGCGCAGCTCGGCGGGAAGAGC

                460         *       480         *       500
7 Rev (+) ttcttcggcggcgacacgcccgggtacctcgacatagccgcgtgcgcgtt
7K -20 (-)ttcttcggcggcgacacgcccgggt------------------------
7 -20 (-) ttcttcggcggcgacacgcccgggtacctcgacatagccgcgtgcgcgtt
7S Rev (+)----------------------gggtacctcgacatagccgcgtgcgcgtt
Contig    TTCTTCGGCGGCGACACGCCCGGGTACCTCGACATAGCCGCGTGCGCGTT

                    *       520         *       540         *
7 Rev (+) gggtccttggatcggcgtgctcgaggaggtgactggagtggccttgctgg
7K -20 (-)-------------------------------------------------
7 -20 (-) gggtccttggatcggcgtgctcgaggaggtgactggagtggccttgctgg
7S Rev (+)gggtccttggatcggcgtgctcgaggaggtgactggagtggccttgctgg
Contig    GGGTCCTTGGATCGGCGTGCTCGAGGAGGTGACTGGAGTGGCCTTGCTGG

                560         *       580         *       600
7 Rev (+) acgccgacgagttccccgctctatgccagtgggccagggactacagctcc
7K -20 (-)-------------------------------------------------
7 -20 (-) acgccgacgagttccccgctctatgccagtgggccagggactacagctcc
7S Rev (+)acgccgacaagttccccgctctatgccagtgggccagggactacagctcc
Contig    ACGCCGACGAGTTCCCCGCTCTATGCCAGTGGGCCAGGGACTACAGCTCC

                    *       620         *       640         *
7 Rev (+) -------------------------------------------------
7K -20 (-)-------------------------------------------------
7 -20 (-) agtgaagcgctcaggccatgcctgccggacagggaccgactcgttgccta
7S Rev (+)agtgaagcgctcaggccatgcctgccggacagggaccgactcgttgccta
Contig    AGTGAAGCGCTCAGGCCATGCCTGCCGGACAGGGACCGACTCGTTGCCTA

                660         *       680         *       700
7 Rev (+) -------------------------------------------------
7K -20 (-)-------------------------------------------------
7 -20 (-) cttcaccgagaacaaggagaagtacaagacatttgccaaggcaacgttgc
7S Rev (+)cttcaccgagaacaaggagaagtacaagacatttgccaaggcaacgttgc
Contig    CTTCACCGAGAACAAGGAGAAGTACAAGACATTTGCCAAGGCAACGTTGC

                    *       720         *       740         *
7 Rev (+) -------------------------------------------------
7K -20 (-)-------------------------------------------------
7 -20 (-) atcagtagctgctagttgggtgcaaaccgcttctttatctctgtgtggaa
7S Rev (+)atcagtagctgctagttgggtgcaaaccgcttctttatctctgtgtggaa
Contig    ATCAGTAGCTGCTAGTTGGGTGCAAACCGCTTCTTTATCTCTGTGTGGAA

                760         *       780         *       800
7 Rev (+) -------------------------------------------------
7K -20 (-)-------------------------------------------------
7 -20 (-) taatgtatacgtacgtgctccctcgatatcaaataaatcagctaccggag
7S Rev (+)taatgtatacgtacgtgctccctcgatatcaaataaatcagctaccggag
Contig    TAATGTATACGTACGTGCTCCCTCGATATCAAATAAATCAGCTACCGGAG

                    *       820         *       840
7 Rev (+) ---------------------------------------
7K -20 (-)---------------------------------------
7 -20 (-) ttgactgtagtcaaattgctaaaaaaaaaaaaaaaaaaaa
7S Rev (+)ttgactgtagtcaaattgctaaaaaaaaaaaaaaaaaaaa
Contig    TTGACTGTAGTCAAATTGCTAAAAAAAAAAAAAAAAAAAA
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