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Caroline Loutre

Glucosylation of xenobiotics in maize, soybean and *Arabidopsis thaliana*.

ABSTRACT

Many natural products and xenobiotics become glucosylated in the course of their metabolism in plants. This reaction is catalysed by type 1 UDP-glucose-dependent glucosyltransferases (GTs), a super-family of enzymes which differ in their substrate specificity and which are able to glucosylate hydroxy, amino and carboxylic acid groups to form conjugates with altered bioactivities as compared with the parent aglycones.

This study has focused both on *NGT* and *OGT* enzymes active towards amino and hydroxy groups, respectively, present in natural products and pesticide metabolites in two major crops (*Zea mays* and *Glycine max*) and the model plant *Arabidopsis thaliana*. A sensitive radioactive enzyme assay was developed to monitor conjugating activity *in vitro* and the substrate specificity of *N*-GTs and *O*-GTs determined in the three plant species with respect to xenobiotic detoxification. 3,4-Dichloroaniline was found to be the optimal *N*-GT substrate and 2,4,5-trichlorophenol the preferred *O*-GT substrate in all the species tested. In addition, *O*-GT activities were also determined with other phenols of both natural and synthetic origin. To confirm the importance of *N*-GTs and *O*-GTs in xenobiotic detoxification, plant metabolism studies were carried out with [¹⁴C]-*p*-nitrophenol and [¹⁴C]-3,4-dichloroaniline. In each case, *O*- / *N*-glucosylation was found to be a major route of detoxification respectively.

To determine whether or not herbicide-safeners could enhance the glucosylation of xenobiotics as had been demonstrated for the *S*-glutathionylation of herbicides in cereals; soybean, maize and *Arabidopsis* were treated with a range of safeners. The plants were then either fed with radiolabelled [¹⁴C]-3,4-dichloroaniline, or extracted and assayed for *O*-GT and *N*-GT activities. In all species, safener treatment had no significant effect on the rate of uptake of radioactivity following feeding with [¹⁴C]-3,4-dichloroaniline. However, specific safeners were found to enhance *N*-GT and *O*-GT activities in etiolated

shoots, roots or suspension cultures in all species where tested. Several attempts were made to clone GT enzymes from soybean and maize based on a combination of bioinformatic and PCR approaches, the latter using conserved blocks of sequence in type 1 plant GTs to amplify up partial cDNAs. Using PCR and analysis of soybean expressed tagged accessions, it was possible to assemble a full-length cDNA from soybean which encoded a GT resembling an arbutin synthase GT from *Rauvolfia serpentina*. Although the resulting GT (*GmGT32_1*) could be expressed as a recombinant polypeptide in *E.coli*, the resulting protein was inactive and accumulated in the insoluble inclusion bodies. In the case of maize, a GT termed *ZmRP* was identified as a random sequenced clone from a proprietary maize cDNA library. However, *ZmRP* could not be translated into protein using bacterial expression systems.

Instead, an alternative proteomics approach at isolating plant GTs involved in xenobiotic detoxification was undertaken in *Arabidopsis*, using suspension cultured cells as the starting material. The major *N*-GT conjugating activity towards 3,4-dichloroaniline was purified 9552-fold using a combination of hydrophobic interaction, ion exchange, and affinity chromatographies. The resulting 50 kDa polypeptide was digested with trypsin and the peptide fragments analysed by MALDI TOF MS. Database analysis unambiguously identified the *Arabidopsis* protein as UGT72B1 (NM 116337).

Following the completion of the Ph.D. programme the activity of GT72B1 towards 3,4-dichloroaniline, 2,4,5-trichlorophenol and other xenobiotics was confirmed and an account of the studies carried out on *Arabidopsis* GTs published (Lao, *et al.*, 2003), (Loutre, *et al.*, 2003).

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GLUCOSYLATION OF XENOBIOTICS

IN MAIZE, SOYBEAN AND *ARABIDOPSIS THALIANA*

Caroline Loutre

PhD Thesis

University of Durham

School of Biological and Biomedical Sciences

2004



- 4 OCT 2004

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Declaration

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Abbreviations

[¹⁴ C]	Carbon-14 labelled
ATP	adenosine triphosphate
3,4-DCA	3,4-dichloroaniline
2,4,5-TCP	2,4,5-trichlorophenol
bp	base pairs
cDNA	complementary DNA
Ci	Curie
cv.	cultivar
Da	daltons (kDa = kiloDaltons)
dNTPs	deoxynucleotide triphosphates
DEPC	diethylpyrocarbamate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EST	Expressed sequence tag
GSH	glutathione
GST	glutathione- <i>S</i> -transferase
GT	glucosyltransferase
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
IAA	Indole-3-acetic acid
IPTG	isopropyl β-thiogalactoside
KPi	Potassium phosphate
LB	Luria-Bertani
mRNA	messenger ribonucleic acid

ND	not determined
NGT	<i>N</i> -glucosyltransferase
OD	optical density
OGT	<i>O</i> -glucosyltransferase
PAGE	polyacrylamide gel electrophoresis
PCP	2,3,4,5,6-pentachlorophenol
PCR	polymerase chain reaction
<i>pI</i>	isoelectric point
<i>p</i> -np	<i>p</i> -nitrophenol
polyA mRNA	polyadenylated RNA
PSPG	Plant secondary product glucosyltransferase
PVPP	polyvinylpyrrolidone
RACE	rapid amplification of cDNA ends
RNAasin	RNAse inhibitor
RT-PCR	Reverse transcriptase – polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TdT	terminal deoxynucleotidyl transferase
TEMED	<i>N, N, N', N'</i> -tetramethylethylenediamine
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UGT	UDPG-dependent glucosyltransferase
UDPG	uridine diphospho glucose
UL	uniform labelling

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Publications arising from the work described in this thesis

Loutre C, Edwards R, Cole DJ (2001) Glucosyltransferases active in pesticide metabolism in Soybean, Maize and *Arabidopsis* In *Brighton Crop Protection Conference- Weeds 2*: 533-539 (meeting proceedings)

Loutre C, Dixon DP, Brazier M, Slater M, Cole DJ, Edwards R (2003) Isolation of a glucosyltransferase from *Arabidopsis thaliana* active in the metabolism of the persistent pollutant 3,4-Dichloroaniline. *The Plant Journal* 34: 485-493

Lao SH, Loutre C, Brazier M, Coleman JOD, Cole DJ, Edwards R and Theodoulou FL (2003) 3,4-Dichloroaniline is detoxified and exported via different pathways in *Arabidopsis* and soybean. *Phytochemistry* 63: 653-661

Chapter 1 : Introduction

1.1 Importance of glucosylation

The reaction catalysed by glucosyltransferases (GTs) is based on the transfer from the activated sugar UDP-glucose (uridine diphospho glucose) as a glucose donor to various types of small acceptors described as aglycones. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzyme-Catalysed Reactions (NC-IUBMB) (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>) recommends the classification of these hexosyltransferases (EC 2.4.1) in the wider group forming the glycosyltransferases (EC 2.4), enzymes capable of transferring various sugar residues.

Most studies on the conjugation of secondary products have concentrated on glucose as the donor sugar as it has been shown to be the most common sugar transferred by GTs. However, other sugar residues can also be used including galactose, xylose and rhamnose (Hösel, 1981). The large glycosyltransferase family catalysing these conjugation reactions is composed of a large class of enzymes found in all kingdoms including algae, plants, animals and bacteria (Campbell, *et al.*, 1997). The enzymes carry out a basic reaction of transfer of sugar onto aglycones (Figure 1.1). The GT group contains members shown to take part in the conjugation of a range of molecules including endogenous products and xenobiotics (see later section).

The fixation of the glucose residue was also dependent on the group acceptor including molecules such as oxygen in an alcohol or a carboxyl functional

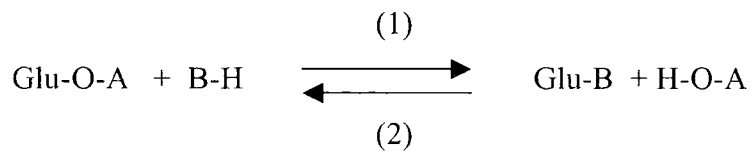
group, nitrogen and more rarely a sulfur or carbon molecule; they are then classified as *O*-GT, *N*-GT, *S*-GT or *C*-GT. *O*-GT are the most documented enzymes regarding secondary metabolites. *N*-GT has mainly been investigated in regards to pesticide metabolism as several pesticide residues, derived from the amines families, were shown to be metabolised through glucosylation (Figure 1.2).

Glucosylation regulates many properties of natural products in plants including their bioactivity, solubility and transport, both intracellularly and systemically (Mackenzie, *et al.*, 1997; Paquette, *et al.*, 2003; Ross, *et al.*, 2001; Vogt and Jones, 2000). In particular glycosylation is important in controlling the bioactivity and bioavailability of plant hormones, phytotoxins of natural and synthetic origins and secondary metabolites with roles in defence against stress. In most cases, the formation of glucose conjugates represents an efficient means to accumulate a compound in higher concentration as an inactive form which is otherwise toxic to the cell. It therefore follows that GTs have an important role in maintaining the correct concentrations of hormones which if allowed to accumulate as the aglycones could interfere with development.

The glycosylation of natural products is widely observed in the plant kingdom and is known to regulate the bioactivity and subcellular localisation of secondary metabolites in plant secondary metabolism (Lim, *et al.*, 2002).

Genomic studies demonstrated that the GT family is composed of many members in plants suggesting that individual enzymes could be specific for conjugating a single type of compound of either natural or synthetic origin (Sandermann, *et al.*, 1991). Biochemical investigations concerning this point are somewhat ambiguous, showing some GTs to have high substrate specificity while others will act on several substrates (Vogt and Jones, 2000). Irrespective of acceptor substrates, the glucosylation reaction is described in Figures 1.1 and 1.2.

Following glucosylation, further sugar residues can be attached to the glycoside moiety through a sequential mechanism using UDP-activated sugars as further donors (Hösel, 1981).



In the case of the forward reaction

A : nucleoside diphosphate with glucose

B : sugar acceptor

The reaction is reversible and while the forward reaction is carried out by transferases (1) the reverse reaction is hydrolytic (A = H) occurring through the action of β -glucosidases (2).

Figure 1.1 : Reaction of glucosylation.

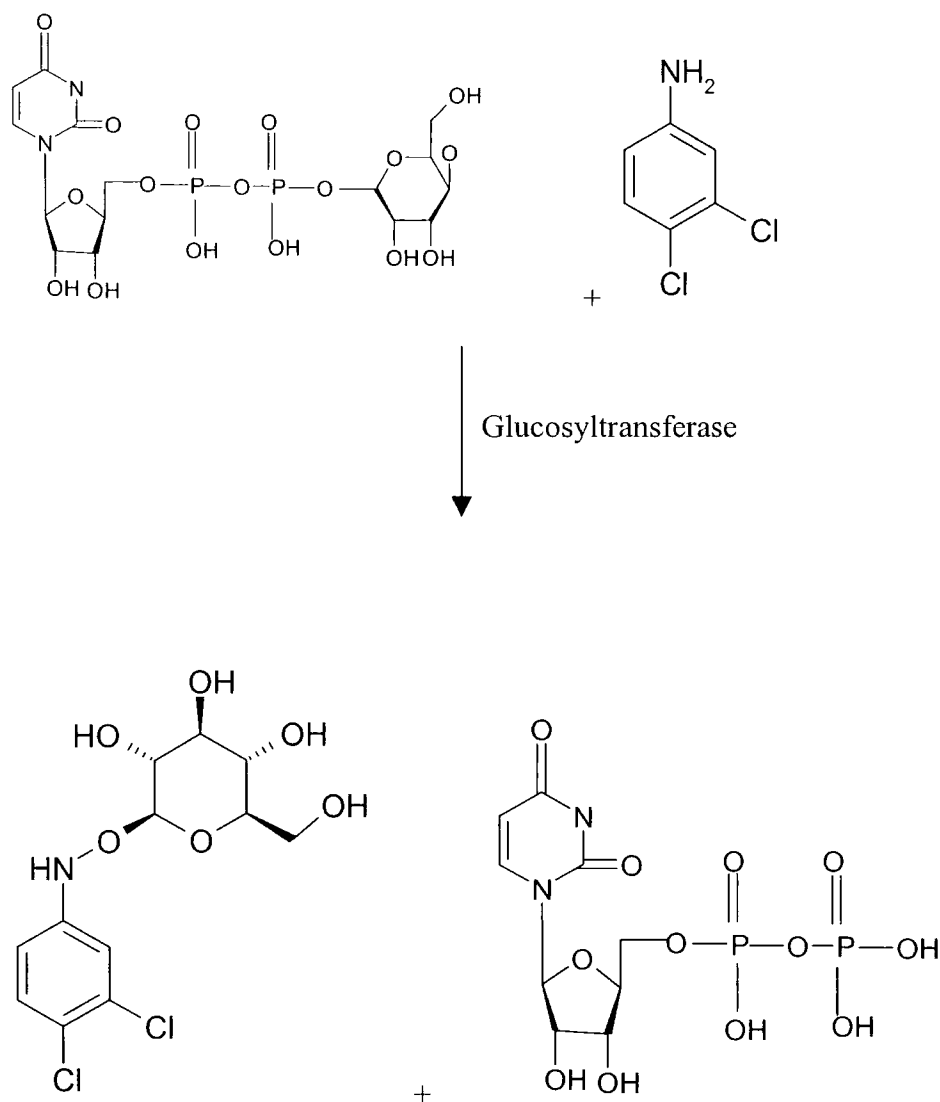


Figure 1.2 : *N*-glycosylation of 3,4-dichloroaniline.

1.1.1 *O*-glucosylation of natural products in plants.

The enzymes responsible for the *O*-glucosylation of plant secondary metabolites have been among the best studied members all of the superfamily-1 UDP-sugar dependent glucosyltransferases. Early studies focussed on some GT activity towards phenolics (Pridham, 1965) and the acid moiety of hydroxycinnamic acids (Macheix, 1977) and benzoic acids (Schlepphorst and Barz, 1979). In particular, glucosylation has been shown to be important in regulating the availability of phenyl-propanoid precursors in the lignin biosynthetic pathway in higher plants (Ibrahim, 1977).

In parallel with the early studies on simple hydroxycinnamic and benzoic acids it became apparent that flavonoids also undergo *O*-glucosylation.

The glucosylation of flavonoids was first reported in parsley cell cultures (Sutter and Grisebach, 1973). Early studies highlighted the specificity of GTs to preferentially glucosylate at specific positions in the flavonoid structure. Thus, in *Silene dioica* two distinct GTs were shown to be involved in the respective glucosylation of the 3-position and 5-position position of cyanidin pigments (Hösel, 1981).

Further examples of natural products undergoing glucosylation in plants include the formation of cyanogenic glucosides linamarin in flax seedlings (Hahlbrock and Conn, 1970) and the glucosylation of quinones (Müller and Leistner, 1978).

1.1.2 Preferences towards the sugar donor

In micro-organisms glucosyltransferases have been shown to be active towards a range of aglycones and sugar donors used in antibiotic synthesis (Mendez and Salas, 2001). Originally, it was believed that plant GTs were specific for their sugar donors, but recent studies have challenged this assumption. Thus, a flavonoid glucosyltransferase from apple skin was able to use three sugar donors in the glycosylation of quercetin and cyanidin showing a preference for UDP-galactose over UDP-glucose and UDP-xylose (Lister, *et al.*, 1997). Similarly, the expression of a tobacco (*Nicotiana tabacum*) glucosyltransferase in *E.coli* highlighted the adaptability of the enzyme to either use UDP-glucose or UDP-xylose to glycosylate scopoletin (7-hydroxy-6-methoxycoumarin) to form its glucoside-conjugate scopolin, with a preference shown for UDP-glucose as the sugar-donor (Taguchi, *et al.*, 2001). The use of alternative sugar donors has also been studied in enzymes involved in the glycosylation of cytokinin in bean and lima bean (Martin, *et al.*, 1999a; Martin, *et al.*, 1999b; Martin, *et al.*, 2000). In particular, isolation, cloning and heterologous expression allowed for the better characterisation of two of the enzymes involved. One of them, termed zog1 was shown to be able to utilise both UDP-glucose and UDP-xylose, showing a preference for UDP-glucose. The other enzyme, zox1, was only able to use UDP-xylose as a substrate donor, despite the sequences of the two enzymes being very similar. However, sequence comparisons alone have not been able to unambiguously identify the protein domain involved in determining sugar binding.

In some instances specific sugar conjugates may accumulate by alternative mechanisms other than the sugar selectivity of the respective GT. In the steroid glucosylation in potato (*Solanum tuberosum*) two enzymes were identified, one of capable of galactosylation at a lower rate (Zimowski, 1991). However, purification of the respective GT showed that UDP-galactose would not serve as a sugar donor, even though the galactoside accumulated *in planta*. It was suggested that an epimerase was required to convert the glucose moiety to galactose post-conjugation (Stapleton, *et al.*, 1991). In other cases however

specific galactosyltransferases have been identified, for example an UFGaIT from apple peel involved in the biosynthesis of anthocyanins (Ju, *et al.*, 1995) and a flavonol galactosyltransferase in *Petunia hybrida*, which was shown to be specific for UDP-galactose alone (Miller, *et al.*, 1999; Miller, *et al.*, 2002). Similarly, in *Arabidopsis thaliana*, a UDP-rhamnose:flavonol-3-*O*-rhamnosyltransferase was identified based on a functional genomic approach using T-DNA knockouts. The UGT78D1 identified as selectively used UDP-rhamnose to conjugate quercetin and kaempferol with a specificity for the hydroxyl group at the position 3 (Jones, *et al.*, 2003).

1.1.3 Regulation of bioactivity of plant secondary metabolites by *O*-glucosylation

Glucosylation is generally considered to be a detoxification process resulting in compounds having reduced biological activity, but this is not always the case as evidenced in the following examples.

1.1.3.1 Role in regulating the bitterness in citrus species

Limonin is a major glycosidic conjugate of citrus and responsible for the bitterness of citrus, orange and grapefruit juice (Berhow and Smolensky, 1995). Controlling the concentration of the glucosides is therefore particularly important in regulating bitterness in citrus species. A limonin-active GT was first identified and cloned in Satsuma (*Citrus unshiu* Marc.) by Kita (2000). A 55 kDa enzyme responsible for the formation of limonin was subsequently identified in pommelo (Karim and Hashinaga, 2002) with additional related enzymes identified in orange (Hasegawa, *et al.*, 1997). Another GT with a MW of 57.5 kDa was identified in satsumas based on its co-accumulation with limonoid-glucosides. In turn, the level of the transcripts encoding the enzyme appeared to directly regulate the level of glucosides in the plant (Kita, *et al.*, 2000).

1.1.3.2 Role in the stabilisation of cyanogenic glucosides

Cyanogenic glucosides are important protective antifeedants in many plants including crops such as cassava and *Sorghum bicolor*. Thus the cyanogenic glycosides represent a good example of the role played by GTs in plants in response to ruminant attack. Cyanogenic glucosides are stored in the vacuole as inactivated and stabilised glucosyl conjugates. During grazing, animals break open the cells and the vacuoles, mixing endogenous cytoplasmic glucosidases and the glucoside conjugates, ultimately leading to the release of HCN as a feeding deterrent following the action of nitrilases (Jones, 1999; Vogt and Jones, 2000). The last step in the formation of cyanogenic glucosides involves glucosylation, which stabilises the compounds which are otherwise labile. In the case of cassava, it was demonstrated that two enzymes were needed for the reaction (Mederacke, *et al.*, 1996) and in sorghum, the respective GT was cloned and its conjugating activity towards cyanogenic substrates determined (Jones, *et al.*, 1999). The sorghum GT had a narrow substrate specificity directed mainly at *p*-hydroxymandelonitrile and mandelonitrile, and when the benzoyl-substituted derivatives were tested, affinity was even lower. Surprisingly, activity towards the non-related terpene substrate geraniol was obtained (Jones, *et al.*, 1999).

1.1.3.3 Role in the cellular concentration of hormones

GTs are classically linked to the regulation of plant hormone activity because of their role in conjugation. Szerzen (Szerzen, *et al.*, 1994) reported that the glucoside of indole acetic acid (IAA) was inactive when formed in maize by an IAA-GT. The homologous IAA-GT was identified in *Arabidopsis*, cloned and overexpressed to determine its substrates. Several *Arabidopsis* GTs, notably UGT84B1 were identified as being able to glucosylate IAA. The most active IAA-GT was subsequently shown to be related to a limonoid GT even though derived from a different group (Milkowski, *et al.*, 2000a). GTs are also involved in regulating the levels of other plant hormones. Thus, an elevation of mRNA

levels of a GT in adzuki bean seedlings (*Vigna angularis*) was shown to be due to its induction by abscisic acid associated with water-stress or wounding and was associated with the accumulation of ABA-glucosides. The recombinant GT enzyme was subsequently overexpressed in *E.coli* and its activity towards abscisic acid determined (Xu, *et al.*, 2002).

The glycosyl conjugates of the cytokinin zeatin are also considered important in protecting these labile plant hormones from oxidation. For example, the concentration of the inactive zeatin *O*-glycosides increase in cold stressed plants of maize and bean and this was correlated with an increase of zeatin *O*-glucosyltransferase activity (Li, *et al.*, 2000). The enzyme UDP-glucose:zeatin *O*-glucosyltransferase (EC 2.4.1.203) was previously isolated from *Phaseolus lunatus* seeds, with 51.4 kDa recombinant protein efficiently converting zeatin to *O*-glucosylzeatin (Martin, *et al.*, 1999a). These enzymes and other homologous in maize are specific for *cis*-zeatin and not *cis*-ribosylzeatin, *trans*-zeatin, or *trans*-ribosylzeatin (Martin, *et al.*, 2001).

1.2 Inducibility of conjugation of natural products in plants

In addition to their substrate selectivity for acceptors and sugars donors, another characteristic of plant GTs is their enhancement in expression in response to stress with multiple treatments shown to induce specific GTs. For example, a flavonoid 3-*O*-GT was cloned by subtractive hybridisation from groundnut grown under standard or drought and light-stressed conditions (Gopalakrishna, *et al.*, 2001) with the respective enzyme shown to be induced in response to these biotic stresses. GTs are also associated with the accumulation of the toxic glycoalkaloid solanidin in potato tubers early after wounding associated with the increased expression of a solanidine glucosyltransferase (Moehs, *et al.*, 1997). Similarly, after pathogen attack, increased levels of salicylic acid and O₂⁻ in *Arabidopsis thaliana* were associated with the induction of UGT73B5 (Mazel and Levine, 2002).

The cloning of a tobacco enzyme involved in the stress response led to the identification of a GT with activity towards hydroxycoumarins and hydroxycinnamic acids with potential roles in transporting and cross-linking conjugates to the cell-wall (Fraissinet-Tachet, *et al.*, 1998). A connection between salicylic acid metabolism and GT expression was further suggested by the observation that salicylic acid-2-*O*-β-D-glucoside and glucosyl salicylate accumulated in tobacco leaves following pathogen attack. The UDP-glucose: salicylic acid glucosyltransferase capable of forming both conjugates was purified, characterised and partially sequenced. The 48 kDa enzyme was able to glucosylate salicylic acid and phenolics. The enzyme was subsequently shown to be induced both by salicylic acid and incompatible pathogens (Lee, *et al.*, 1999). Also in tobacco, treatment with salicylic acid was found to induce the expression of two GT enzymes NtGT1a and NtGT1b. The recombinant enzymes had activities towards flavonoids, coumarins and 2-naphthol with UDP-glucose and UDP-xylose used as sugar donors (Taguchi, *et al.*, 2001).

In other cases GT expression is actually suppressed in response to stress. In red clover leaves treated with fungal elicitor, glucosyltransferase and malonyltransferase activities declined, while the activities of the respective β -glucosidases and malonylsterases activities remained unchanged resulting in the overall accumulation of isoflavone aglycones (Tebayahsi, *et al.*, 2001). Similarly, the exposure of sunflower (*Helianthus annuus*) to sucrose solution resulted in the accumulation of the phytoalexin scopoletin after the inhibition of its glucosylation to the less antifungal scopolin (Gutierrez, *et al.*, 1994). In tobacco, it was shown that as the scopoletin GT expression decreased, the formation of scopoletin-glucosides also declined leading to more free aglycone to accumulate. It was proposed that scopoletin played a role as an antiviral and a role in buffering the reactive oxygen intermediates (ROI) as scopoletin is known to induce oxidative stress (Chong, *et al.*, 1999; Chong, *et al.*, 2002). Thus, in this case the GT was actually determining the balance between the relative concentrations of scopoletin its inactive glucoside.

1.3 The glucosyltransferase superfamily in plants

GTs are numerous and divergent and can only be classified as a large multigene family. Based on sequence similarities, 54 families of glycosyltransferases have been identified from living organisms (Paquette, *et al.*, 2003). All the natural product and xenobiotic-conjugating UGTs, are members of the type 1 family identified in plants, animals, bacteria, fungi and viruses (Li, *et al.*, 2001; Mackenzie, *et al.*, 1997; Ross, *et al.*, 2001). They all have a common conserved UGT-defining motif near the C-terminus and all use UDP-activated sugar moieties as a donor molecule (Paquette, *et al.*, 2003).

Following the analysis of GT phylogenetic trees, two other minor clades or groups of sequences sharing a unique ancestor and a series of characteristics that appear in its members but not in the other forms it diverged from, include sterol UGTs (e.g. UGT80A2 involved in the synthesis of sterolglucosides, characteristic lipids of the eukaryotic membrane) and lipid UGTs (e.g. UGT81A1 being the monogalactosyldiacylglycerol synthase catalysing the transfer of galactose from UDP-galactose to diacylglycerol, the major glycolipid constituent of the chloroplast) which are more related to non-plant UGT clades even though evolved from a common ancestor (Paquette, *et al.*, 2003).

Additional classes of GTs are responsible for conjugating sacchararides, proteins, lipids and nucleic acids. UDP-glucose is used as the dominant sugar donor by the type 1 UGTs, with the presence of at least two phospho-diester bonds required to drive the reaction (Hösel, 1981). More rarely, other nucleotide moieties (adenine, cytosine or guanine) and alternative sugars (xylose, rhamnose, galactose or mannose) have been reported as donor co-substrates. The acceptor molecules are much more variable, though used selectively by each UGT.

The basis of the current classification system is sequence similarity of the domain involved in binding the NDP-sugar donor, which is a conserved feature among GTs. GTs are then catalytically classified according to the NDP-sugar used and the acceptor substrate used in the reaction, along with the position of

glucosylation and the type of residue they glycosylate. The reaction catalysed by GTs alters the conformation of the carbon involved in the binding of the sugar. Most plant GTs are soluble enzymes with only a minority membrane-bound (Paquette, *et al.*, 2003). Type-1 GTs are monomeric, being composed of a polypeptide of 43 to 60 kDa (Vogt, *et al.*, 2000).

1.3.1 UGTs in *Arabidopsis*

Using the UGTs from *Arabidopsis thaliana* as a model system, analysis of the genome has identified 120 UGT genes with 8 pseudo-genes. More than half (58/112) have no introns and 44 sequences have a single intron. The surprisingly low number of introns and the presence of the conserved domain suggest that UGTs have a monophyletic origin (Paquette, *et al.*, 2003). The first analysis regarding a complete genome sequence was attempted in *Arabidopsis* where sequences sharing >70% identity (on the protein level) were placed in the same group to give 14 groups (labelled from A to N). Based on this classification, characterised GTs from plant species were added to phylogenetic trees (Lim, *et al.*, 2003). Interesting data on the substrate specificities can be obtained. A dendrogram of the major groups is presented in Figure 1.3.

Exhaustive information obtained from the release of the *Arabidopsis thaliana* genome sequence allowed to group the putative GT sequences based on sequence similarity. Biochemical characterisations of GTs from various plant species reveal useful information on the substrate specificity in the group containing closely related sequences are starting to reveal useful information for the prediction of putative substrates.

In group A, two 3-*O*-GT sequences were characterised in *Petunia hybrida* (GenBank™ AF165148) (Miller, *et al.*, 1999) suggest the enzymes could be involved in the anthocyanidin pathways and shared similarities with group A *Arabidopsis* sequences. Group D include *Arabidopsis* GTs close to other published sequences glucosylating betanidin and flavonoids with the isolation of

a *Dorantheanthus bellidiformis* (B5GT) (GenBank™ Y18871) (Vogt, *et al.*, 1999), two tobacco GTs (TOGT1 and TOGT2) involved in conjugating coumarins (GenBank™ U32644) (Horvath, *et al.*, 1996), and a potato SGT involved in the glucosylation of solanidine (GenBank™ U82367) (Moehs, *et al.*, 1997). The group F correlates with GTs involved in the conjugation of anthocyanidin and flavonol GTs in *Gentiana triflora* (GenBank™ D85186) (Tanaka, *et al.*, 1996), and *Vitis vinifera* (GenBank™ AF000372) glucosylating anthocyanidins and flavonols (Ford, *et al.*, 1998).

A *Sorghum bicolor* GT (GenBank™ AF199453) (Jones, *et al.*, 1999) involved in the glucosylation of *p*-hydroxymandelonitrile was reported to be closely related to the group G in *Arabidopsis*.

In group L, plant sequences characterised to date involve a *Brassica napus* sequence involved in the glucosylation of phenylacetylthiohydroximate (GenBank™ A62529) (Marillia, *et al.*, 2001) involved in the conjugation of phenyl acetyl thiocarbamate, a *Citrus unshiu* (GenBank™ AB033758) (Kita, *et al.*, 2000) involved in the glucosylation of limonoid, a tobacco sequence involved in the conjugation of *t*-cinnamic, benzoic and salicylic acid (Lee, *et al.*, 1999), a *Perilla frutescens* involved in the conjugation of anthocyanidin-3-*O*-GT (GenBank™ AB002582) (Gong, *et al.*, 1997) and the maize *iaglu* involved in the synthesis of IAA (GenBank™ L34847) (Szerszen, *et al.*, 1994).

The basis of the grouping of the sequences resides on sequence similarities and not on the substrate specificity or the catalytic specificity. All type-1 UGTs contain conserved sequences at the carboxy-terminus involved in binding the UDP moiety of the sugar donor (Hughes and Hughes, 1994; Ross, *et al.*, 2001). Sequence similarities highlight a more conserved C-terminal domain suggesting it is involved in binding the sugar residue. The amino-terminus area is more flexible and could represent the binding area for more variable aglycones. It consists of a protein cleft rich in positively charged amino acids (Ünlügil and Rini, 2000). To date however, no crystal structures of type 1 UGTs have been reported. The presence of the conserved sequence corresponding to the UDP-binding motif, termed the Plant Secondary Product Glycosyltransferase

(PSPG) motif (Campbell, *et al.*, 1997; Hughes and Hughes, 1994; Kapitonov and Yu, 1999; Mackenzie, *et al.*, 1997; Vogt and Jones, 2000), has been used to identify putative UGTs in a range of plant species (Hughes and Hughes, 1994) and is represented by a series of amino acids including HGCWNS highly conserved in the β -group of GTs (Vogt and Jones, 2000).

Analysis of these sequences suggest that UGTs have evolved through conservation of specific modules to integrate new substrate specificities. (Henrissat and Davies, 2000).

The classification of the sequences is described in Table 1.1 and Figure 1.3.

1.3.2 UGTs in maize

Apart from *Arabidopsis thaliana*, the other species which has attracted major attention with respect to its type-1 GTs is maize. Indeed, the first type-1 GT involved in plant secondary metabolism which was cloned was a flavonol 3-*O*-GT from maize. The cloning was based on identifying the site of insertion of a transposable controlling activator element (Ac) in the sequence of the 3-*O*-GT which inactivated the gene involved in the flavonol pathway, giving rise to the Bronze 1 phenotype of purple seeds (Fedoroff, *et al.*, 1984). The effect of point mutation, insertion and deletion in the bronze locus were subsequently studied (Ralston, *et al.*, 1987), with three Bronze-1 GT sequences reported (Furtek, *et al.*, 1988).

IAA (indole-3-acetic acid) mainly accumulated as its glucoside conjugate in maize plants, which represents an inactive form of the hormone in the plant. The conjugation is catalysed by a uridine 5'-diphosphate-glucose:indol-3-ylacetyl- β -D-glucosyl transferase with the activity confirmed following expression of the recombinant enzyme (Szerszen, *et al.*, 1994). As well as the IAA conjugating GT, enzymes active in glucosylating other hormones, such as kinetin, have been identified in maize and then cloned and expressed (Martin, *et al.*, 1999; Martin, *et al.*, 2001).

The benzoxazinones are a group of glycosylated plant defence compounds with the toxic compounds stored as glucoside conjugates. Two GTs catalysing their formation have been isolated in young maize seedlings. The analysis of the sequences of the purified enzymes revealed they were closely related enzymes which were distinct from all other groups of GTs (von Rad, *et al.*, 2001). The affinity towards the most commonly found benzoxazinoid compound 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBAO) was confirmed. The presence of the DIMBOA glucoside peaked in young maize seedlings and corresponded to the expression of the highest activities of the enzyme in plants. The expression of the 2 GTs in *Arabidopsis thaliana* confirmed the detoxification of these substrates in a heterologous plant host (von Rad, *et al.*, 2001).

A UDP-glucose:dolichol-phosphate glucosyltransferase was purified from maize endosperm microsomes by chromatography on columns of hydroxyl apatite, diethylaminoethyl-Sephacel, concanavalin A-Sepharose and uridine-diphosphate-hexanamine-agarose. The only acceptors for the enzyme were lipids, suggesting that this GT was distant and possibly not involved in plant secondary metabolism (Miernyk and Riedell, 1991).

Group	Number of sequences based on sequence alignment	Group	Number of sequences based on sequence alignment
A	14	H	19
B	3	I	1
C	3	J	2
D	13	K	2
E	22	L	17
F	3	M	1
G	6	N	1

Classification of *Arabidopsis* GTs and description of the code

Clone name	Superfamily	Family	Subfamily	Individual gene
UGT 72 B 1	UGT	72	B	1

Table 1.1 : UGT classification according to sequence homologies based on the work from Ross (Ross, *et al.*, 2001).

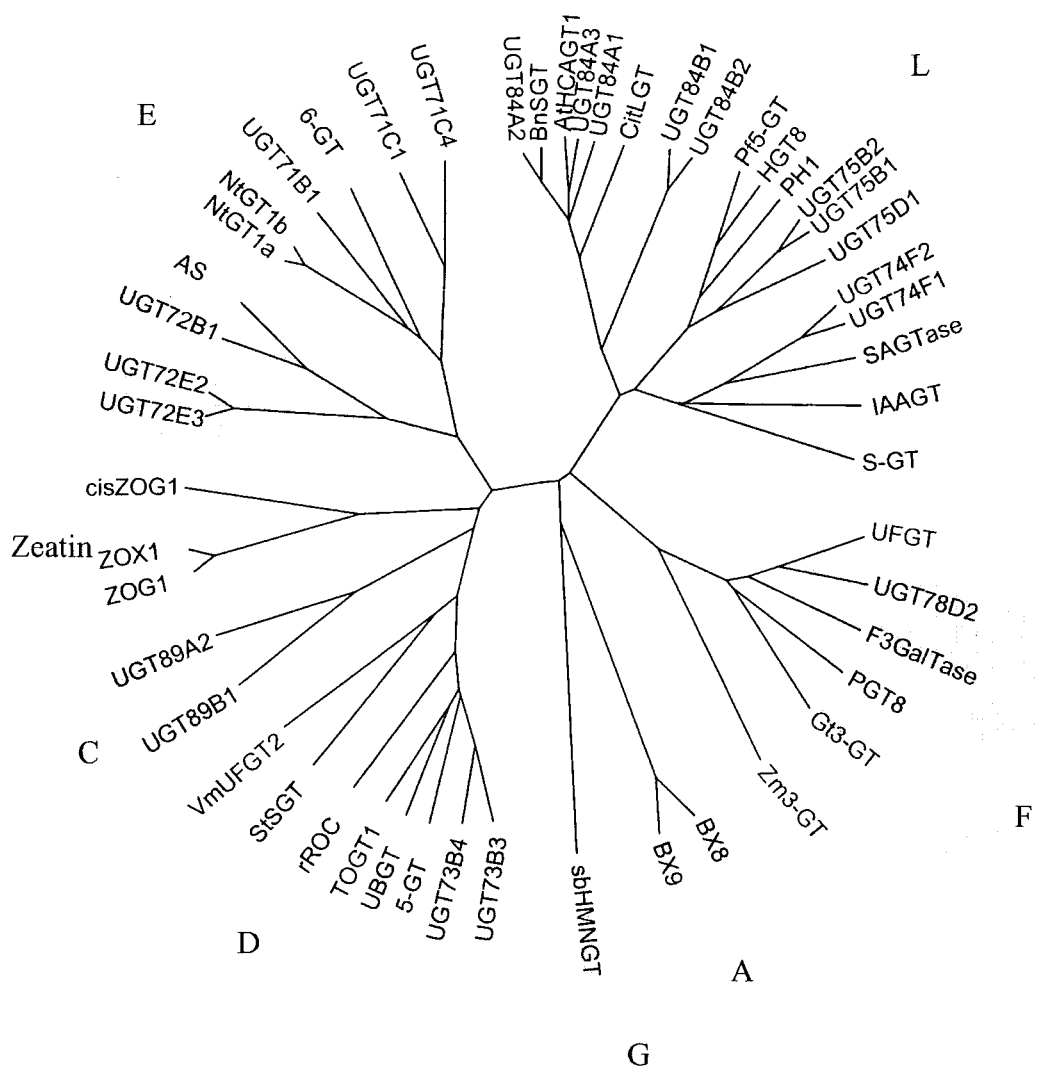


Figure 1.3 : Phylogenetic tree of higher plant GTs, showing clustering of the eight major clusters from the fourteen as defined by (Ross, *et al.*, 2001).

1.4 Metabolism of foreign compounds in plants

Plant metabolism can be broadly divided into primary and secondary metabolism. Those catabolic and anabolic pathways, essential for energy production, transport and the assimilation/degradation of essential metabolites are termed primary metabolism. In contrast, secondary metabolism is derived from primary metabolism and produces end point metabolites which have no strictly definable essential function in the plant (Lea and Leegood, 1999).

Secondary products are diverse bioactive compounds with multiple roles in conferring predator and stress protection, flower colour, scents and flavours.

Over the last 100 years plants have been exposed to an increasing number of synthetic compounds, which have entered the environment either as pollutants or as crop protection agents. While some of these compounds have proved to be highly persistent, for example some of the polychlorinated aromatic compounds, the majority have been metabolised to innocuous products by a combination of abiotic chemical degradation (e.g.; photolysis) and metabolism by microorganisms and plants. Plants have a particularly important role to play in the detoxification of herbicides, fungicides and insecticides, which are applied to crops as pre-emergence or post-emergence treatments. These compounds enter plants either through the roots or directly through the foliage, acting directly at the site of uptake after systemic transport. A primary determinant of their biological activity in crop protection is determined by their relative rate of detoxification. This is most important in determining the selectivity of herbicides, as with a few exceptions, these compounds show similar patterns of translocation in different plants and are equally active at the respective target sites in crops and weeds (Cole, 1994; Cole, *et al.*, 1995). Thus, herbicides are rapidly metabolised and inactivated in crops and generally more slowly detoxified in weeds, accounting for species-dependent differences in their toxicity (Owen 2000).

Although the mode of action and cellular specificity of these pesticide molecules are important, there are many other factors which can alter the herbicide optimal activity. The chemical formulation can include the addition of co-solvents or surfactants to allow mixing, dilution, application, and stability of the active ingredient for uniform spreading and good penetration through the waxy cuticle. Other formulation ingredients include pH adjusters, buffers, milling aids, antifoaming agents, acid scavengers, dyes, suspending agents, preservatives, dispersants, emulsifiers, densifiers, antifreeze, and crystal promoters (Tominack, 2000). The optimisation of the dose, the time of spray, the formulation and the encapsulation might also be considered.

Over the last 40 years intensive effort has been made in understanding the biochemistry and physiology of plants, which underpin their ability to detoxify xenobiotics, which has been driven by a combination of the importance of these processes in crop protection and more recently the use of plants in phytoremediation (Harvey, *et al.*, 2002; Schröder, *et al.*, 2001)). Drawing parallels with what is known about drug metabolism in mammals, the detoxification of foreign compounds in plants can be considered to occur in four phases.

1.4.1 Overview of phases of metabolism

The metabolism of toxic compounds has been well documented and four major phases can be described. Phase 1 metabolism of xenobiotics occurs either in the apoplast or more commonly within the cytoplasm of the plant cell and involves the action of oxidoreductases, most importantly cytochrome P450 mixed function oxidases (CYPs; Schuler and Werck-Reichhart, 2003) or hydrolytic enzymes, such as esterases or amidases. The action of these phase 1 enzymes is to introduce, or reveal, chemically functional groups (OH, NH₂, COOH) which can undergo further reactions with biomolecules (Cole and Edwards, 2000). In phase 2, the functionalised xenobiotic becomes conjugated with endogenous products such as amino acids, sugars, organic acids or the tripeptide glutathione. All of these reactions are catalysed by transferases, generally using high-energy precursors to drive the conjugation reactions. In the case of amino acid conjugation, the xenobiotic acid is bioactivated by the formation of a CoA ester, whereas sugars and acids utilise the bioactivated natural products, namely NDP-sugars, CoA derivatives or 3'-phosphoadenosine 5'-phosphosulphate (PAPS). The exceptions are the glutathione transferases (GSTs), which make use of the reactive sulphhydryl group of glutathione to react with electrophilic compounds (Dixon, *et al.*, 2002; Marrs, 1996). For a long time, this second phase was considered as the final detoxification step due to the effect of conjugation on the biophysical and biological properties of the xenobiotics. The most commonly encountered phase 2 reaction in plants is catalysed by the type-1 glycosyltransferases (GTs), with the UDP-glucose dependent GTs being the most important. Once conjugated, the glucose residue can then undergo a further phase 2 reaction by 6''-O-conjugation with malonic acid, as catalysed by the malonyl-CoA-dependent malonyltransferases (MTs; Lao, *et al.*, 2003). Phase 2 metabolites then undergo phase 3 metabolism which consists of ATP-dependent transport into the vacuole. Translocation across the membrane is driven by a number of different transporters most notably the ATP-binding cassette (ABC) proteins (Klein, *et al.*, 1996). The conjugates in the vacuole can then undergo further processing or be re-exported back into the cytoplasm for catabolism and

in some cases incorporation in macromolecules. These well-defined terminal stages of metabolism are collectively termed phase 4 metabolism.

Observing the similarity in detoxification systems in mammals and plants, and realising the importance of these processes in protecting the environment, Heinrich Sandermann has collectively termed the ability of plants to degrade xenobiotics as acting as a 'green liver' (Sandermann, *et al.*, 1994), and these detoxification phases in a plant are shown in Figure 1.4. While there are many similarities in the detoxification of xenobiotics in animals and plants there are also key differences. Thus, in animals, conjugates are exported from the cell for excretion while in plants these compounds are imported into the vacuole. As such, these vacuolar metabolites may undergo further catabolism prior to transfer into the cytoplasm and incorporation into macromolecules, or bound residues. In addition, whereas animals use glucuronic acid for conjugating xenobiotics, plants tend to use glucose or more rarely xylose or galactose. Thus, in mammals, the GT enzymatic reaction differs in the use of UDP-glucuronic acid in place of UDP-glucose as the activated sugar (King, *et al.*, 2000). However, the type 1 GTs involved are distantly related and have allowed a consensus sequence similar to be identified in plants which in turn has allowed a large multigene family of plant GTs to be identified (Li, *et al.*, 2001).

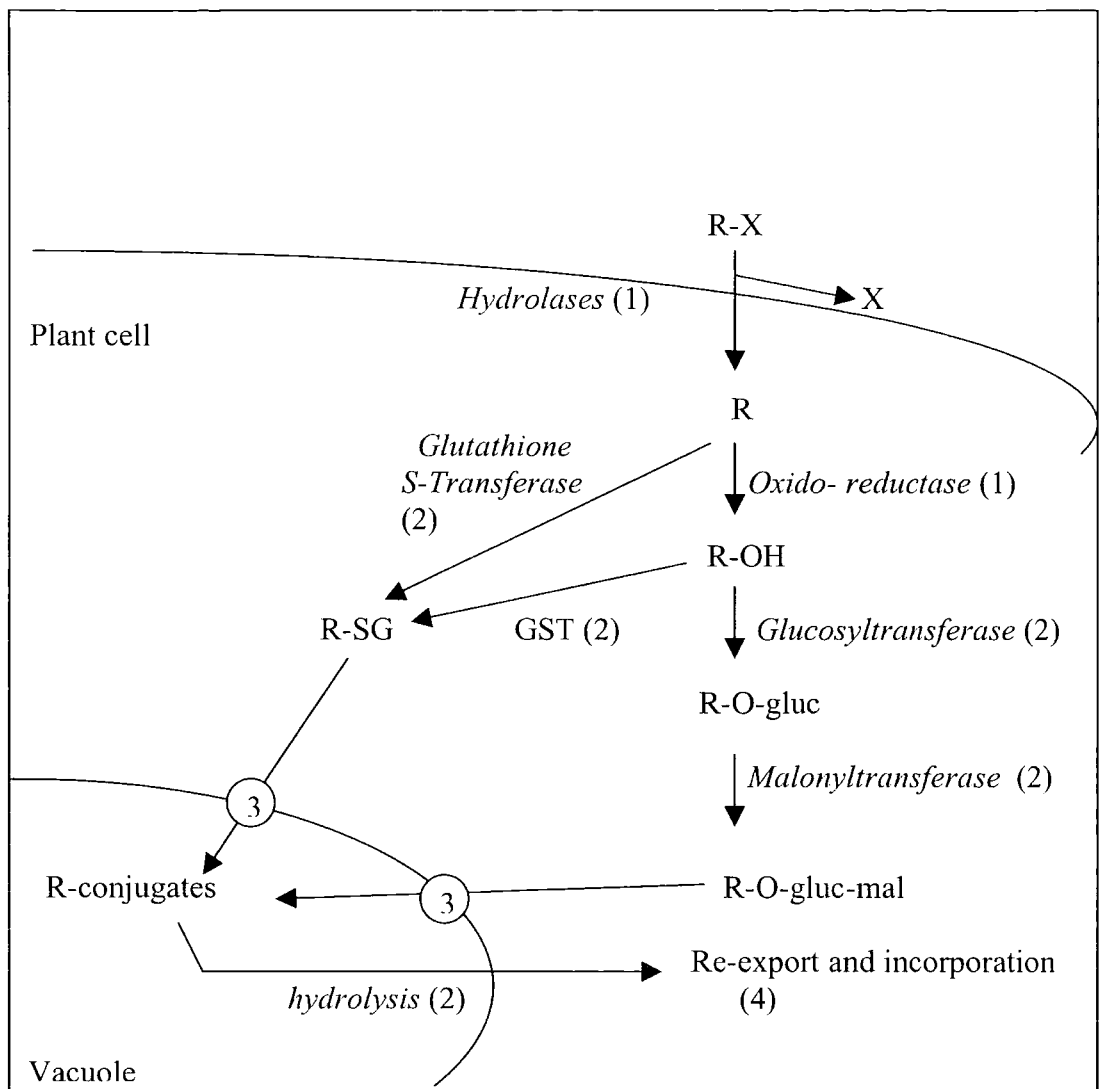


Figure 1.4 : Conjugation of xenobiotic compounds in plant.

The different phases of metabolism are represented as numbers in parentheses.

R = xenobiotics.

1.4.2 Processing and vacuolar import of conjugates

Whereas animals produce acidic glucosidic conjugates directly through their use of glucuronic acid, in plants this is a two step process, first involving glucosylation and then derivatisation of the sugar with malonic acid. The significance of this reaction is not entirely understood, though malonylation appears to act as a tag directing the conjugates for vacuolar import (Mackenbrock, *et al.*, 1992).

The malonylation reaction is carried out by malonyltransferases which can conjugate compounds containing amino (*N*-malonylation) or hydroxy residues (*O*-malonylation), (Sandermann, *et al.*, 1991). Malonylation also appears to prevent digestion by glucosidases (Schmitt, *et al.*, 1985) and may also facilitate conjugates transport across the plasma membrane (Höhl and Barz 1995; Winkler and Sandermann 1989). The malonylation of cyanidin glucosides compounds has been extensively studied in maize (Harborne and Self, 1987) and malonylation is also known to be involved in the metabolism of xenobiotics. For example, the glucosidic conjugates of pentachlorophenol formed in soybean and wheat undergo malonylation (Schmitt, *et al.*, 1985) with 3,4-dichloroaniline undergoing *N*-malonylation in soybean and wheat (Schmidt, *et al.*, 1995). In each case, the transport of the newly-formed conjugates was shown to be routed towards the vacuole.

Although malonylation of glucosides is important in directing their import into the vacuole, it is also clear that the glucosides themselves can undergo vacuolar deposition. Glucoside conjugates of a flavonoid and herbicide derivative were compared in barley (Klein, *et al.*, 1996). The transport of the flavonoid conjugates appeared to be pH dependent while uptake of the herbicide conjugate was strongly stimulated by MgATP. It was concluded that the tonoplast presents a Δ pH-dependent uptake mechanism for specific endogenous flavonoid-glucosides but requires directly energized mechanisms for abiotic glucosides, probably through the intervention of ABC transporters (Klein, *et al.*, 1996).

1.4.3 Herbicide safeners

Herbicide safeners, also known as antidotes, are chemicals that when applied before or with herbicides, selectively increase the tolerance of cereal crop to chemical herbicide injury (Hatzios, 1991).

The mode of action of safeners has yet to be determined. Some hypotheses are based on the safener acting as structural mimic of the herbicides thereby acting as antagonist at the site of action of the herbicide in the plant (Hatzios, 1991). However, as several safeners can protect crops against multiple classes of herbicides, it is more likely that several protective cellular factors are enhanced by safeners (Hatzios and Wu, 1996). The evidences remain elusive and have not yet been elucidated. These include a reduction in uptake of herbicides thereby reducing the likelihood of cellular toxicity. However, based on uptake and metabolism studies *in planta*, this seems unlikely (Lamoureux and Rusness, 1992). Instead it has been observed that safener treatment can enhance the rate of herbicide detoxification with several conjugates of herbicides shown to have been increased in concentration following safener treatment. This would favour a mode of action based on safeners enhancing metabolism. In support of this hypothesis, the activities of several detoxifying enzymes have been shown to be increased including the GSTs, the Cytochromes P-450s and GTs (Davies and Caseley, 1999). Despite interest in safeners, their activities have mainly been demonstrated in monocotyledonous crops, notably maize, wheat, sorghum and rice. Recently, the induction of detoxifying enzymes was also demonstrated in dicotyledonous plants such as *Arabidopsis thaliana* (DeRidder, *et al.*, 2002).

The chemical structures of safener are species selective, with alachlor and metolachlor being examples of chloroacetanilide herbicide which can be 'safened' by benoxacor and R-29148 in maize. (Hatzios and Wu, 1996). In sorghum, oxabetrinil, CMPI and flurazole protect against these compounds, while fenclorim performs best in safening in rice. In wheat, the aryloxyphenoxypropionates clodinafop propargyl and fenoxaprop ethyl are selectively safened by cloquintocet mexyl and mefenpyr diethyl respectively.

In addition, thiocarbamate herbicides such as EPTC and butylate can be safened in maize by dichlormid, while the sulfonylurea foramsulfuron is protected

against by isoxadifen-ethyl in maize (Hatzios and Wu, 1996). The chemical structures for the safeners are described in Chapter 2 Figure 2.2 page 55.

The selective induction of herbicide detoxifying enzymes associated with the action of the safeners identified above has been demonstrated for Cytochromes P-450, GSTs and UGTs in maize, wheat, sorghum, rice and *Arabidopsis* (DeRidder, *et al.*, 2002; Davies and Caseley, 1999; Lamoureux and Rusness, 1992; Werck-Reichhart, *et al.*, 2000). In some cases, enhancement of detoxifying enzymes was associated with an increase in co-substrate availability. Thus, GST activities towards carbamate and chloroacetanilide herbicides were enhanced by safener applications in maize and this was associated with an increase in the concentration of glutathione (GSH; Hatzios, 1991). Similarly, the increased concentration of GSH was demonstrated in maize seedlings following benoxacor treatment (Farago and Brunold, 1994a).

In all cases studied to date, the effect of safeners is likely to be mediated at the level of transcriptional activity of the respective genes. Recent studies have confirmed that UGTs active in detoxifying xenobiotics are enhanced in wheat plants treated with safeners (Brazier, *et al.*, 2002). This confirms earlier observations which reported an enhanced accumulation of glucoside conjugates in maize following BAS 145138 safener treatment (Lamoureux and Rusness, 1992).

1.5 *O*-Glucosylation of xenobiotics

While several UGTs active in the metabolism of plant hormones and secondary metabolites have been identified, the enzymes active in the detoxification of xenobiotics have received far less attention (Cole and Edwards, 2000). It would appear that these activities have not arisen as a response to exposure to pollutants or pesticides, but rather are due to UGTs with roles in natural product metabolism mistaking foreign compounds for endogenous substrates. However the relationship between the conjugation of foreign compounds and secondary metabolites is not understood. The role of GTs as part of the 'green liver' of plant detoxification emerged as the glucosylation of multiple types of synthetic compounds has been reported (Sandermann, 1992). Since then, their involvement as a class of detoxifying enzymes has been reinforced and they are likely to play a role of similar importance to other detoxifying classes of enzymes in phytoremediation processes in the future (Harvey, *et al.*, 2002; Schröder, *et al.*, 2001).

As for endogenous substrates, *O*-glucosylation of xenobiotics can occur on various residues including hydroxyl and carboxyl residues, with some substrates having multiple sites for glucosylation. Individual plants species appear to be able to glucosylate diverse xenobiotics with the glucosylation of chlorinated phenols reported in soybean tissues (Sandermann, *et al.*, 1991).

Soybean *O*-GT activities were subsequently identified towards 6-hydroxybentazone, 8-hydroxybentazone, 5-hydroxydiclofop, chloramben and 2,4-dichlorophenol. In a small number of cases, the GTs were partially purified. Thus an *OGT* active towards 6-hydroxybentazone was characterised from soybean (Gallandt and Balke, 1995).

The conjugation of hydroxybentazon was studied in soybean and highlighted the involvement of 2 *OGT* enzymes capable of selectively glucosylating the 6- or

the 8-hydroxybentazon respectively (Leah, *et al.*, 1992). A distinct *OGT* involved in the detoxification of the pesticide DDT was also subsequently reported in soybean (Wetzel and Sandermann, 1994).

An example of *OGT* conjugation is presented in Figure 1.5 showing the route of metabolism of chlorsulfuron in the tolerant broadleaf and in the sensitive graminaceous species. The *O*-conjugation of xenobiotics was carried out as a comparative study in wheat and its major weed black-grass (Brazier, *et al.*, 2002). Consequently, a flavonoid 3-*OGT* enzyme involved in the conjugation of 2,4,5-TCP was partially purified and characterised in wheat (Brazier, *et al.*, 2003).

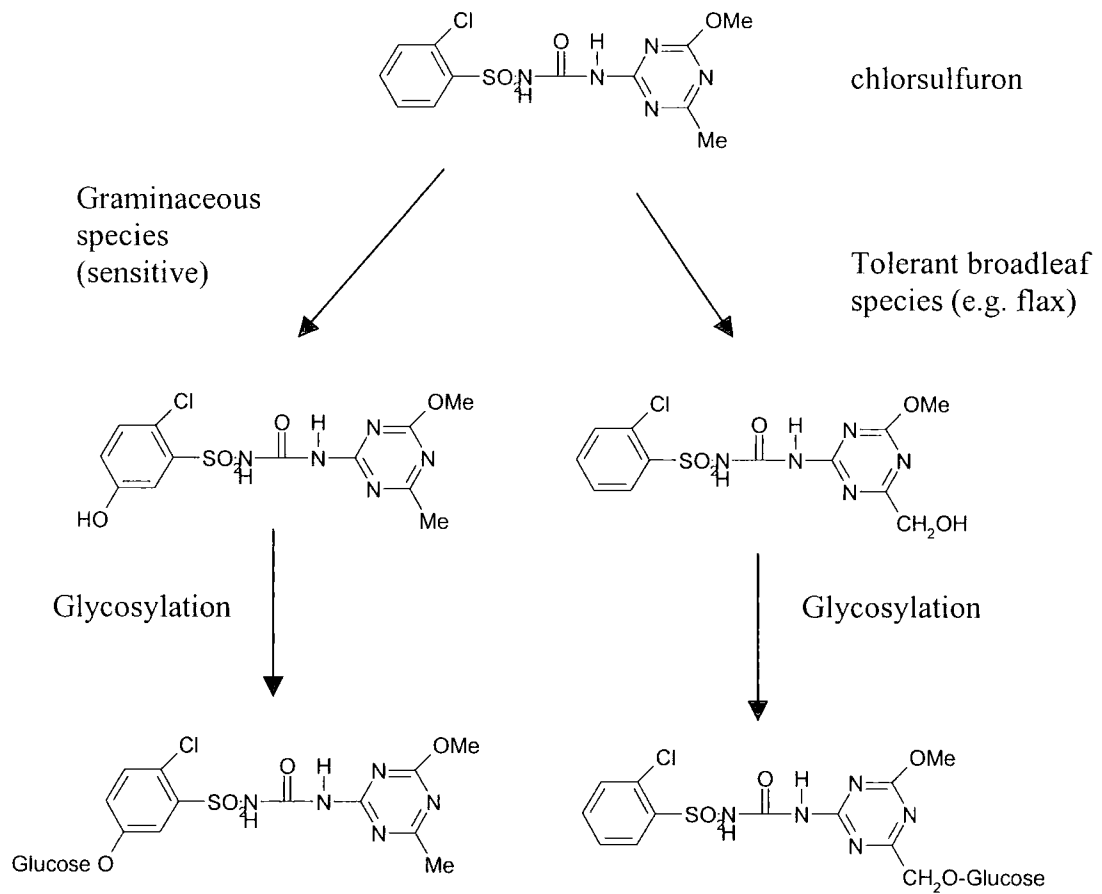


Figure 1.5 : Route of chlorsulfuron metabolism in tolerant and sensitive species (Owen, 2000)

1.6 *N*-Glucosylation of xenobiotics

Several pesticides, such as the herbicides chloramben and pyrazon are directly detoxified by *N*-glucosylation with this reaction reported to greatly affect their translocation and phytotoxicity. In addition, toxic chlorinated aniline metabolites are released from amide herbicides such as propanil in the course of their metabolism and these are also metabolised by *N*-glucosylation. In some instances, such as with chloramben and pyrazon, the *N*-glucosides appear to be end points of metabolism, while the *N*-glucoside of 3,4-dichloroaniline is further metabolised to xylose, fructose and glucose conjugates prior to being converted to products which become incorporated into lignin (Still, 1968). In contrast to *O*-glucosides, *N*-glucosides are stable to hydrolysis by β -glucosidases and are thus much more stable end point detoxification products.

N-glucosylation has been reported in several examples mainly directed at detoxification of pesticide residues. *N*-glucosides were amongst the conjugates identified as metabolites from picloram in leafy spurge (Frear, *et al.*, 1984). The herbicide metribuzin was also shown to undergo *N*-glucosylation in soybean (Figure 1.6; Frear, *et al.*, 1983a; Frear *et al.* 1985) and in tomato (Davis, *et al.*, 1991). Some characterisation of the enzyme tolerance to herbicide was investigated by Bieseler (1992).

With respect to natural product substrates of *N*-glucosylation, *N*-(β -D-glucopyranosyl) nicotinic acid has been identified in tobacco cells (*Nicotiana tabacum* XD-6). The properties of the enzyme involved in the formation of the conjugates were investigated and involved the formation of *N*-glucosylated nicotinic acid conjugates (Taguchi, *et al.*, 1997). Subsequently, a glucosyltransferase involved in the metabolism of naphthol in tobacco was also analysed (Taguchi, *et al.*, 2002) and characterised to be a UDP-glucose: nicotinic acid-*N*-GT (Taguchi, *et al.*, 2003).

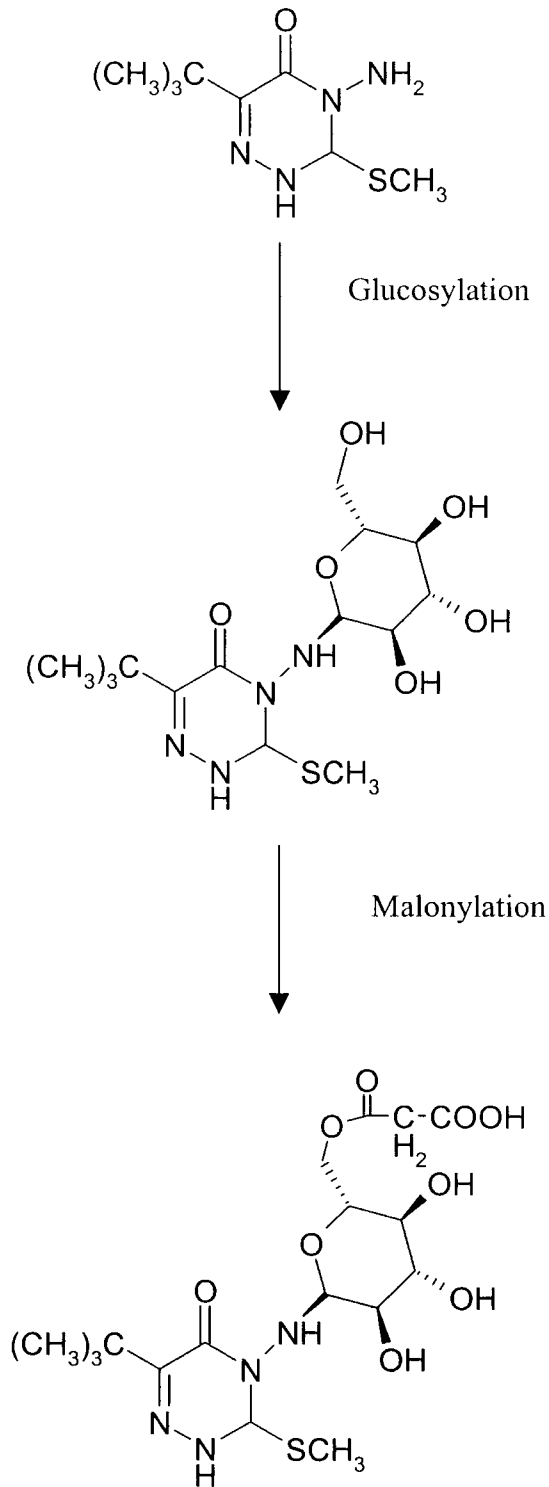


Figure 1.6 : Possible pathway of metribuzin metabolism through conjugation and malonylation (Owen, 2000).

In soybean, an *N*-glucosyltransferase active towards a broad array of substrates derived from arylamines was reported (Frear, 1968). Later, soybean and wheat were investigated for their *N*-glucosylation and *N*-malonylation of xenobiotics as representing two major conjugation systems. Subsequently, *N*-GT activities were detected in soybean cotyledons and wheat roots (Schmidt, *et al.*, 1995).

One of the most studied example of *N*-glucosylation is represented by the conjugation of 3,4-dichloroaniline. When 3,4-dichloroaniline was fed to wheat and soybean the conjugates *N*-(β -D-glucopyranosyl)-3,4-DCA, *N*-malonyl-3,4-DCA and 6'-*O*-malonyl-*N*-(β -D-glucopyranosyl)-3,4-DCA were identified (Bockers, *et al.*, 1994). Metabolism studies with radioalabelled 3,4-DCA resulted in the distribution to be determined among the fractions in extractable and non-extractable residues. The wheat tissues predominantly accumulated radioactivity recovered in roots where equal proportion of the *N*-glucoside and *N*-malonyl conjugates were formed. In soybean, more of the radioactivity was recovered from aerial parts with the *N*-malonate dominating as the glucoside conjugates accumulated in the root system. Non-extractable residues were important in both species with 23% to 35% of the dose in soybean and 45% of the dose in wheat becoming insolubilised (Bockers, *et al.*, 1994). These observations correlated with those made in soybean excised leaves and cell-suspension cultures culture (Gareis, *et al.*, 1992), in carrot suspension cultures and in other studies in wheat and soybean (Schmidt, *et al.*, 1994; Schmidt, *et al.*, 1995).

Aims and Objectives of the project

From reviewing the literature it is clear that plants contain a range of *O*-GT and *N*-GT activities. A major aim of this project was to compare in a coordinated study the OGT and NGT activities towards natural products and xenobiotics in maize (a monocotyledonous species) with soybean and *Arabidopsis* (dicotyledonous species).

It was also hypothesised that *N*-GT and *O*-GT activities could be regulated in response to chemical treatments, especially herbicide safeners, in an analogous manner to that determined with other phase II detoxifying enzymes. Based on these aims/hypotheses the following objectives were identified.

- 1- To determine how model phenols and anilines were detoxified by sugar conjugation in maize, soybean and *Arabidopsis* and identify the respective *N*-GT and *O*-GT activities.
- 2- To determine how these GT activities are regulated in response to chemical treatments with natural products, pollutants and herbicide safeners.
- 3- To purify a GT active in the detoxification of a model xenobiotic (3,4-dichloroaniline) from a representative plant species (*Arabidopsis thaliana*).
- 4- To clone and express representative members of GT gene families from soybean and maize and assay the recombinant enzymes for activity towards xenobiotics.

Chapter 2 : Materials and Methods

2.1 Chemicals

2.1.1 Xenobiotics, herbicides and safeners

A literature search was carried out to identify putative GT substrates. An extensive list of synthetic and natural substrates was acquired from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK), Riedel-de Haën[®] (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) and Fluka (Poole, Dorset, UK). The putative substrates listed in Figure 2.1 were prepared as 1 mM stock solutions in methanol and kept at 4°C.

The safeners used in all the experiments were purchased from Chem Service Inc (Merseyside, UK) or kindly donated by Aventis Crop Science (Ongar, UK) (Figure 2.2). All were prepared as stock solutions (10 mg ml⁻¹ in acetone) and kept at 4°C. Safeners were used at a dose of 10 mg l⁻¹ in the watering solution. pH of diluted safener solutions ranged from pH = 6.06 for fenclorim to pH = 6.78 for benoxacor; pH of aqueous solution of acetone in tap water (1/1000; v/v) was 8.42.

Chemical inducers (2,4,5-trichlorophenol, 3,4-dichloroaniline and salicylic acid) were prepared as 5 mM stock solutions in methanol and diluted 1:100 in water prior to use.

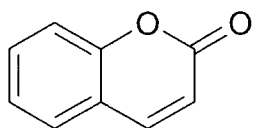
2.1.2 Radiochemicals

Specific activities of the substrate UDP-[¹⁴C-UL-glucose] (Figure 2.3 B) were 9.25 to 12.2 GBq mmol⁻¹ depending on the batch and manufacturer (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, UK; ICN, Costa Mesa, CA, USA).

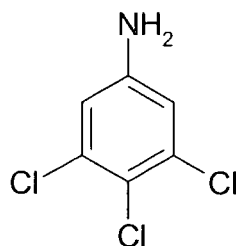
Radioactive xenobiotics were obtained from Sigma-Aldrich Company Ltd with a specific activity of 236.8 MBq mmol⁻¹ for [UL-¹⁴C]-*p*-nitrophenol, and 817.7 MBq mmol⁻¹ for 3,4-dichloroaniline-[UL-¹⁴C]. Radioactive [¹⁴C]-malonyl-CoA and as donated by Miss Si-Houy Lao through collaborative studies.

Figure 2.1 : Chemical structures of the substrates used in GT assays.

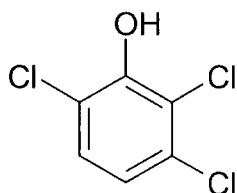
Coumarin



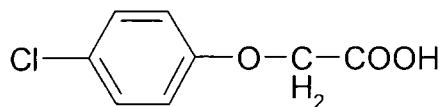
3,4,5- trichloroaniline



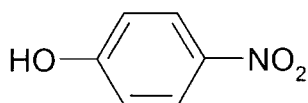
2,3,6-trichlorophenol



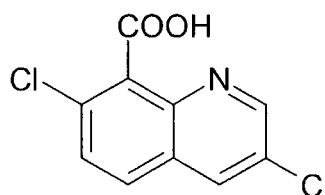
4-chlorophenoxyacetic acid



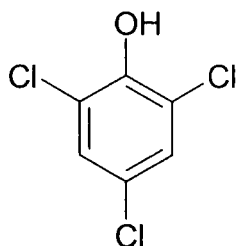
p-nitrophenol



Quinclorac



2,4,6-trichlorophenol



Chloramben

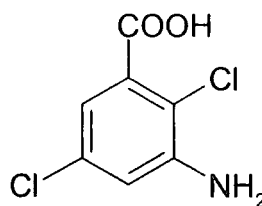
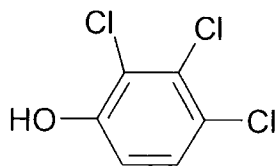
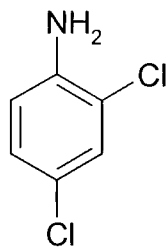


Figure 2.1 (continued)

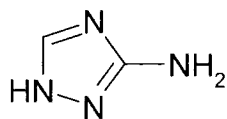
2,3,4-trichlorophenol



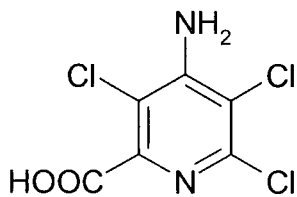
2,4-dichloroaniline



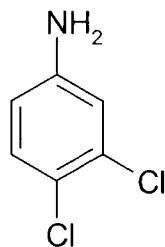
Amitrol



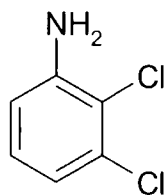
Picloram



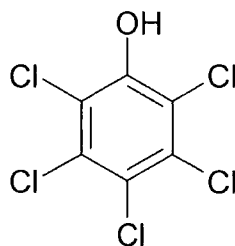
3,4-dichloroaniline



2,3-dichloroaniline



Pentachlorophenol



2,4,5-trichloroaniline

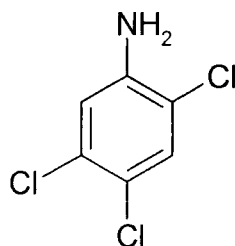
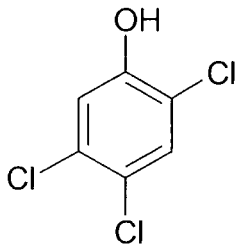
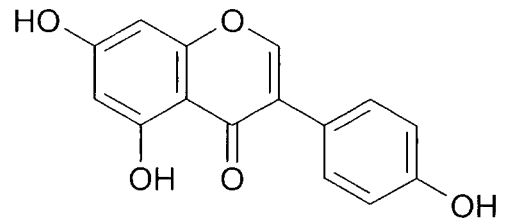


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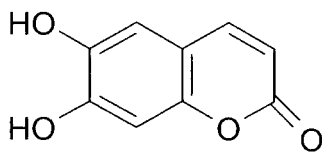
2,4,5-trichlorophenol



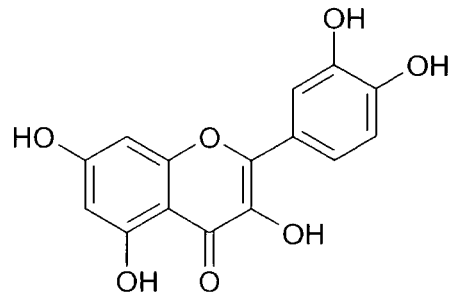
Genistein



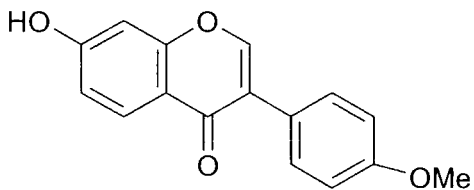
Esculetin



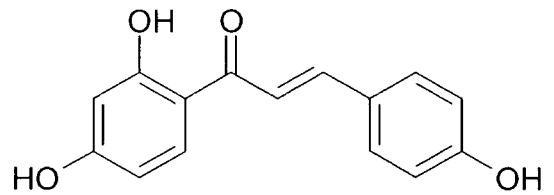
Quercetin



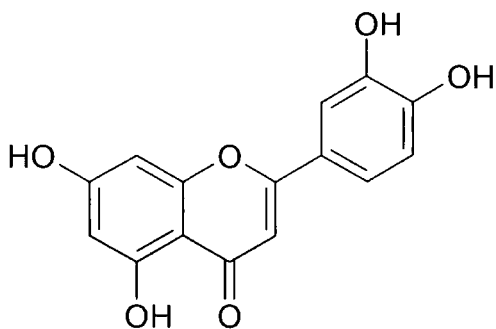
Formononetin



Isoliquirtigenin



Luteolin



Phenanthrenequinone

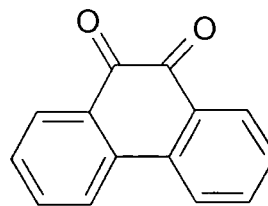
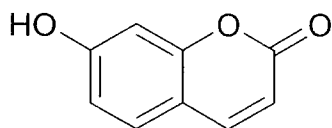
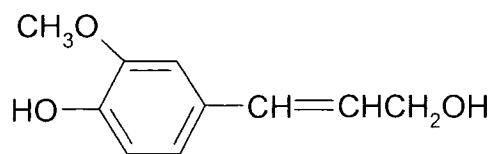


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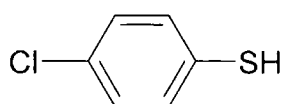
Umbelliferone



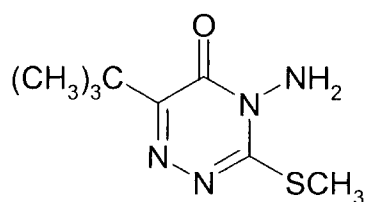
Coniferyl alcohol



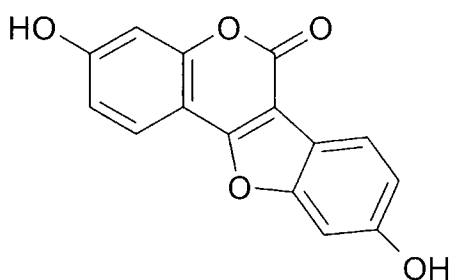
4-chlorothiophenol



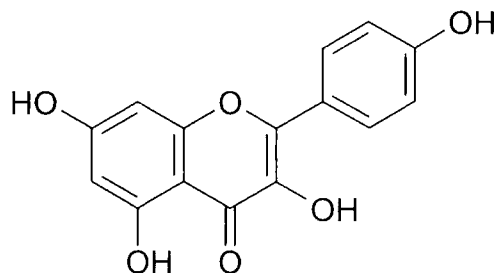
Metribuzin



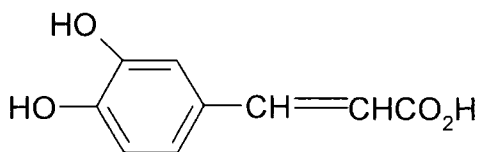
Coumestrol



Kaempferol



Caffeic acid



Maleic hydrazide

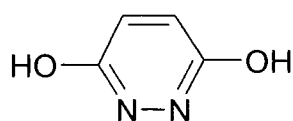
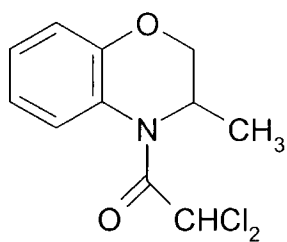


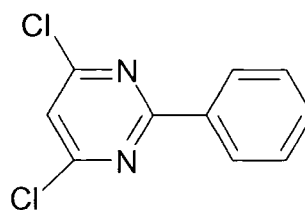
Figure 2.2 : Chemical structures of the safeners used for treatment.

Safeners were dissolved in acetone as a stock solution of 5 mM and applied to plants in the watering solution at 10 mg l⁻¹ (unless stated otherwise).

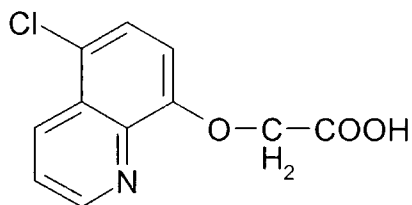
Benoxacor



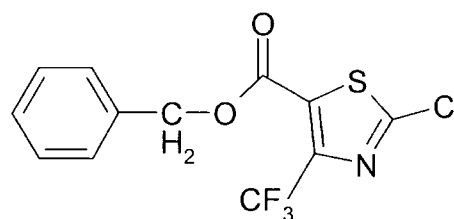
Fenclorim



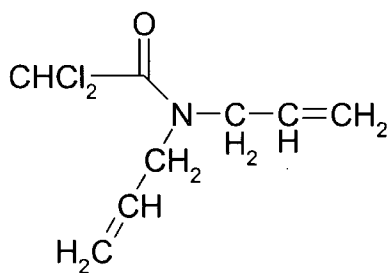
Cloquintocet mexyl



Flurazole



Dichlormid



Isoxadifen

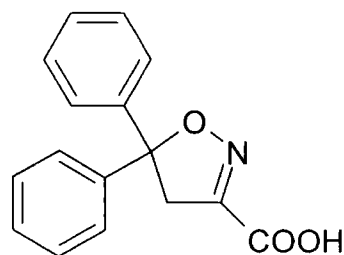
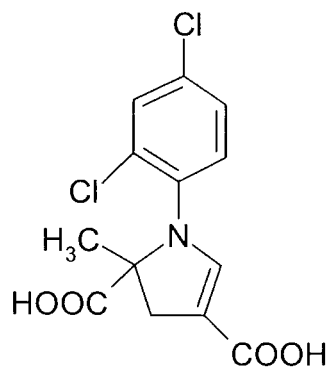
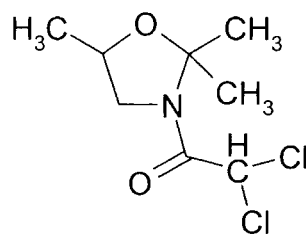


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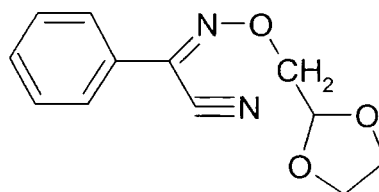
Mefenpyr



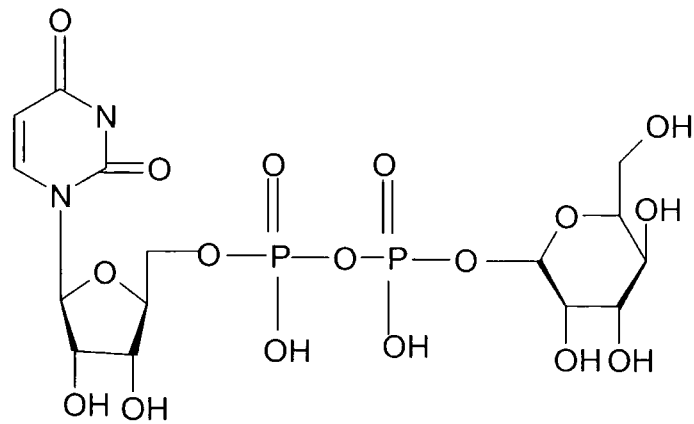
R-29148



Oxabetrinil



A



B

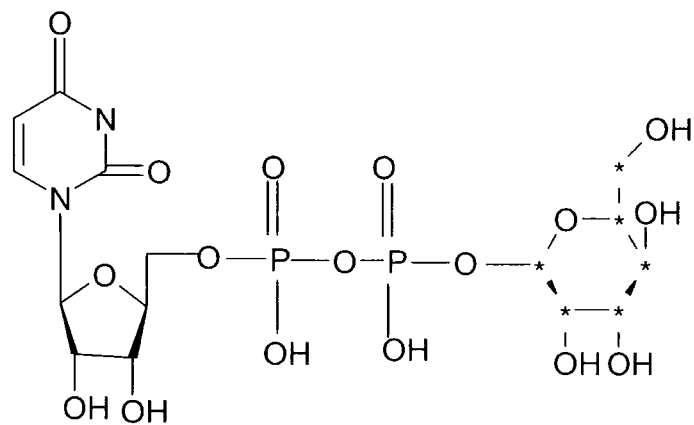


Figure 2.3 : Structure of Uridine diphospho-D-glucose.

Uridine diphospho-D-glucose (A) and uridine diphospho-D-[UL-¹⁴C]-glucose (B), showing location of the [¹⁴C] labelling.

2.2 Plant material

2.2.1 Maize and Soybean

Maize (*Zea mays* (L.) var. Cecilia) and soybean (*Glycine max* (L.) var. Chapman) were kindly donated by Aventis CropScience.

For light grown plants, seeds were imbibed for one hour in 1 l of tap water before being sown onto water-saturated vermiculite. Safener treatment involved 100 mg l⁻¹ safener during the hour soaking and 10 mg l⁻¹ on watering the plants throughout their growth. No nutrient solution was given.

Trays were then transferred to a growth chamber under standard conditions of 22°C with a 16-hour photoperiod (110 μE m⁻² s⁻¹) with pre-set humidity of 60 ± 5%. For studies with etiolated plants, seeds were imbibed in water for one hour before transfer to a tray covered with water-soaked Whatman paper. Seeds were then germinated and grown for 5 to 10 days (refer to result section) in complete darkness at 25°C.

A soybean cell culture was obtained through our collaboration with IACR-Rothamsted (Miss Si-Houy Lao) and grown in Schenck and Hildebrandt medium (Sigma) supplemented with 3% (w/v) sucrose, 1 g l⁻¹ myo-inositol, 5 mg l⁻¹ thiamine, 5 mg l⁻¹ nicotinic acid, 0.5 g l⁻¹ pyridoxine, 0.5 mg l⁻¹ 2,4-D and 0.1 mg l⁻¹ of filter-sterilised kinetin (the latter added after autoclaving), with the final solutions adjusted to pH 5.8.

Suspension cultures (50 ml) were grown at 24°C on an orbital shaker at 90 rpm with a 16-hour photoperiod (60 μE·m⁻² s⁻¹). Suspension cultures were sub-cultured every 7 days by transferring 10 ml of the suspension into fresh medium (50 ml).

2.2.2 *Arabidopsis thaliana* tissue

2.2.2.1 *Arabidopsis* suspension culture

An initial culture was donated by Dr. Steven Chivasa (University of Durham) originating from the line described by (May and Leaver, 1993). A 10% (v/v) inoculum of culture was sub-cultured weekly into fresh Murashige and Skoog Basal salts with minimal organics medium (MSMO) (Linsmaier and Skoog, 1965) (Sigma) supplemented by 30 g l⁻¹ sucrose, 0.5 mg l⁻¹ of naphthalene acetic acid and 0.05 mg l⁻¹ of kinetin. The pH was adjusted to pH 5.7 before autoclaving.

2.2.2.2 *Arabidopsis* root culture

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-O) wild type (Lehle, Round Rock, USA) were sterilised after collecting into a Miracloth™ pocket (CalBiochem, Merck Biosciences, Ltd, Beeston, UK) to ease handling. Seeds were first sterilised in aqueous ethanol (70% v/v) for one minute before transferring to bleach solution (Domestos, DiverseyLever, Ireland; 20% in distilled water) for 10 minutes. Several washes with sterilised distilled water were necessary to remove all traces of bleach, which could inhibit growth. Seeds (10) were transferred under sterile conditions to a 250 ml flask containing 50 ml of Gamborg-B5 medium supplemented with 1% sucrose and adjusted to pH 5.8 (Reiter, *et al.*, 1992). Cultures were grown in total darkness with constant agitation for up to three weeks.

2.2.2.3 *Arabidopsis* whole plants

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-O) wild type (Lehle) were sterilised as above and 5 sterile seeds added to the wells of a 25-well plate each filled with 3ml of ½MS + 1% sucrose medium (Murashige

and Skoog, 1962). Seedlings were grown for 10 days at a constant temperature of 23°C in a photoperiod of 16 hours under a light intensity of $40 \mu\text{E m}^{-2} \text{s}^{-1}$.

2.3 Radioisotope feeding studies

2.3.1 Treatment and harvesting of plants

Seedlings or sterile cultures which had been treated for 48 hours with or without safener (10 mg ml^{-1}) as described in section 2.2.1, were treated with $[\text{UL-}^{14}\text{C}]\text{-}p\text{-nitrophenol}$ ($238 \text{ MBq mmol}^{-1}$) and $[\text{UL-}^{14}\text{C}]\text{-}3,4\text{-dichloroaniline}$ ($817 \text{ MBq mmol}^{-1}$) (Sigma) as additions to the media.

At timed intervals, the plant material and respective media were harvested, weighed and frozen. Frozen plant tissues were extracted with a mortar and pestle in methanol (10:1, w/v) to give the soluble fraction. Culture medium was partitioned with ethyl acetate (1:1, v/v) and the organic phase concentrated under reduced pressure in a rotor-evaporator prior to redissolving the residue in methanol.

The insoluble fraction from the plant tissues containing the bound radioactive residues was incubated in a sealed vial with 1 ml of 2 N NaOH at 37°C overnight. After cooling and neutralisation with concentrated HCl ($173 \mu\text{l}$) (Gareis, *et al.*, 1992), the residue was washed with $100 \mu\text{l}$ of 0.1 M Tris pH 7 and the combined extract transferred to a scintillation vial containing 5 ml of Uniscint BD scintillation fluid (National Diagnostics, Hesse, UK), and the sample assayed for radioactivity on a scintillation spectrometer (Liquid scintillation Analyzer 1600TR, Canberra Packard, Didcot, UK). It was found to be necessary to allow 48 hours between the addition of the digest to the scintillant and the counting to reduce chemiluminescence. For all scintillation counting, an external standard was used to correct for quenching.

2.3.2 Digestion of glycosides by cellulase

To release the xenobiotic metabolites from their conjugated forms as extracted from plant tissues or media, 50 μl of the concentrated extracts was incubated with 450 μl of 1 mg ml^{-1} of cellulase from *Trichoderma viride* (Sigma) in 0.1 M phosphate citrate buffer (pH 5.2) for 16 hours at 30°C. Digested samples were partitioned with 0.2 ml ethyl acetate and the organic phase concentrated to dryness, before resuspension in 50 μl methanol and application of the samples onto TLC plates.

2.3.3 Cross-feeding study

A cross feeding experiment was performed to compare the ability of *Arabidopsis* root cultures and soybean seedlings to take up metabolites which had been excreted into the media by the respective plants. *Arabidopsis* root cultures were grown for 17 days. Soybean seedlings (grown by Miss Si-Houy Lao) and *Arabidopsis* cultures were then treated for 48 hours with [UL- ^{14}C]-3,4-dichloroaniline and the radioactive metabolites in the medium extracted by partitioning with ethyl acetate. After concentration, the organic phase was then taken up in a small volume of methanol, analysed by LSC (liquid scintillation counting) and the nature of the radioactivity present analysed by thin-layer chromatography (TLC) prior to adding the radioactive residues to the medium of *Arabidopsis* root cultures or soybean.

Soybean seedlings were similarly treated with [^{14}C]-3,4-DCA and the medium used in feeding extracted studies with the two plant species. This was to determine whether the exported radioactivity from the soybean and *Arabidopsis* feeding studies was imported back into the roots of either *Arabidopsis* or soybean. After a 24-hour feeding study, the distribution of radioactivity between plant tissue and medium was determined in each case.

2.3.4 TLC analysis

Radioactive metabolites were separated using one of the following solvent mixtures: A: ethyl acetate: formic acid: water (70:4:4; v/v) or B: chloroform: methanol: water (60:35:8; v/v) or C: ethyl acetate : acetic acid : water (63:1:2; v/v) using thin-layer chromatography (TLC) plates coated with silica gel containing F₂₅₄ fluorescent indicator (Sigma). Following development, UV-absorbing metabolites were identified using a UV-lamp.

TLC plates were then exposed to X-ray film (Kodak, Hemel Hempstead, UK) at -80°C for up to 2 weeks and the films developed using an X-ograph Compact X4 X-ray developer (Imaging Systems, Tetbury, UK). Alternatively, TLC plates were exposed to a phosphorimager screen (BioRad GS-525; BioRad, Hemel Hempstead, UK) and the images of radioactive metabolites analysed using the software Multi-Analyst Version 1.1 (Bio-Rad).

Metabolites were identified by co-chromatography with authentic standards where available. In the case of the *p*-nitrophenol feeding studies this included the following conjugates *p*-nitrophenyl- β -D-mannopyranoside, *p*-nitrophenyl- β -D-galactopyranoside, *p*-nitrophenyl- β -D-glucopyranoside, *p*-nitrophenyl- β -D cellobioside and *p*-nitrophenyl- β -D-maltopyranoside which were all obtained from Sigma.

2.4 OGT and NGT GT assay

The GT assay protocol was based on a method developed to detect OGT activity towards flavonoids and phenolic metabolites in alfalfa (Parry and Edwards, 1994) but was optimised regarding certain parameters. The method was also modified for the detection of NGT activity.

2.4.1 Protein extraction

All steps were performed on ice or at 4°C, unless stated otherwise. Plant tissues (25 g) were ground into powder in a mortar and pestle under liquid nitrogen. The homogenised plant material was extracted in 4 volumes (w/v) of extraction buffer (0.2 M Tris-HCl pH 8 + 2 mM DTT) with polyvinylpolypyrrolidone (PVPP) (5% w/w) added. The crude preparation was filtered through Miracloth[®] (Calbiochem) and centrifuged (8,512 x g, 15 minutes). The supernatant was decanted and the proteins precipitated using ammonium sulphate (0-70% saturation). Proteins were pelleted by centrifugation (8,512 x g, 30 minutes) and the precipitates stored for up to several months at -20°C with negligible loss of activity.

Protein pellets were resuspended in reaction buffer and the solution loaded onto PD-10[®] Sephadex G25M (Amersham Pharmacia Biotech, Chalfont St.Giles, UK) desalting columns in 2.5 ml batches following the manufacturer's instructions. For smaller volumes, Sephadex G25M (Amersham Pharmacia Biotech) was packed in pierced microfuge tubes and the protein extract loaded on top of the column and desalted centrifugation for 2 minutes at 2,000 x g.

2.4.2 Protein determination

This method was based on the shift in absorbance of the acidic dye reagent Coomassie Brilliant Blue G-250 (BioRad protein assay kit) on binding to proteins (Bradford, 1976). The procedures were followed according to the manufacturer's instructions with the absorbance determined at 595 nm. A standard curve was determined using the reference protein bovine γ -globulin (0.5 mg ml^{-1}) diluted in the range of 5, 10, 20, 30, 40, 50 $\mu\text{g ml}^{-1}$ in extracting buffer and the standard curve data plotted using GraphPad Prism software, Version 2.01 (GraphPad Software Incorporated).

2.4.3 Assay with radiolabelled UDP-[^{14}C -glucose]

The assays (75 μl) consisted of crude protein extracts dissolved in 0.2 M Tris-HCl buffer pH 8 containing 2 mM DTT together with 5 μl of xenobiotic dissolved in methanol (67 nM final concentration). The reaction was started by the addition of 5 μl of uridine diphospho-D-[UL- ^{14}C -glucose] (962 Bq ICN, 11.8 GBq mmol^{-1}). After a twenty-minute incubation at 30°C , the reaction was stopped by the addition of 125 μl of 0.3 M HCl for the OGT assays, or 125 μl of reaction buffer for the NGT assays, was added before partitioning with 200 μl of water-saturated ethyl acetate. After vortex-mixing the reaction tubes were centrifuged for 2 minutes at full speed in a microfuge tube to separate the phases. An aliquot of the organic phase (100 μl) containing the polar [^{14}C -glucoside]-conjugate was then transferred to a scintillation vial containing 4 ml of scintillation cocktail (Ecoscint TM A, National Diagnostics) for assay on a scintillation spectrometer (Liquid Scintillation Analyzer 1600TR, Canberra Packard, Pangbourne, UK). Assays were carried out in duplicate with the amount of radioactivity in the organic phase averaged and the variation in the replicates determined. The enzyme activity in pmoles of product formed towards each substrate was calculated from the specific activity of the radiolabelled UDP-[^{14}C -glucose]. It was calculated as pmole of glycosylated product formed

per minute per mg of crude protein. Radioactive assays were carried out as duplicate determination expect during purification procedures. Specific activities against a substrate were determined on crude protein extract on two different plant populations (grown in standard conditions but not necessarily at the same time).

2.4.4 Data analysis

Calculation of specific activities.

During the assay, a methanol control was done in duplicate to determine a background level, as it was used to dissolve the substrates tested. Radioactive assays were done in duplicate as contamination with lower phase while transferring the 100 μ l aliquot of the ethyl-acetate usually explained major differences in dpm counts after LSC. The dpm counts corresponding to the 'methanol control' was subtracted to the dpm value obtained for each substrate. The enzyme activities determined were expressed as mean \pm standard deviation of duplicate. When further analysis were carried, two populations of tissues (unless stated otherwise) were used for accurate determination of the specific activities present.

Replication in the design of the feeding studies.

Graphs represent the mean of duplicate or triplicate individual samples (depending on experimental settings) with error bars presenting the standard deviation.

Statistical tests were to carry out to determine whether safener treatment had an effect or not on the general distribution of the xenobiotic over time.

Samples were expected to follow a Gaussian distribution and were subjected to parametric tests for small samples or '*t*-tests'. The parameters for the analysis used were based on the hypothesis that the safener could only have a positive effect on the uptake and distribution of the xenobiotic applying a one-tailed test. An interval of confidence of 95% was applied in all tests.

P values were determined on the plant extracts both for treated and untreated tissues to avoid having to correct for multiple testing.

The hypothesis was identical for all the feeding studies and was formulated as follow :

H_0 : The populations tested are identical i.e. safener-treatment has no effect on the uptake of the xenobiotic and distribution over the time course.

The hypothesis was accepted or not based on the *P* value obtained in the 95% confidence interval.

2.5 Purification of GT

Plant tissues were ground to a powder under liquid nitrogen and then extracted in 3 : 1 (v/w) 0.2 M Tris-HCl pH 8, 5 mM DTT in the presence of 5% (w/w) PVPP. After filtering through Miracloth™, the slurry was centrifuged (10,000 x g, 15 min) and the supernatant decanted, prior to precipitation with ammonium sulphate between 40% and 60% saturation.

Unless stated otherwise, all steps of purification were carried out at 4°C using a GradiFrac low pressure chromatography system (Pharmacia Biotech) with the UV absorbance monitored at 280 nm and recorded on a chart recorder.

2.5.1 Hydrophobic interaction chromatography using Phenyl Sepharose™

The protein fraction corresponding to the 40-60% ammonium sulphate cut was resuspended in buffer A (50 mM phosphate buffer, pH 7.2, 1 mM DTT) containing 1 M ammonium sulphate. The fraction was loaded onto a 60 ml Phenyl Sepharose™ CL-4B Fast Flow (Pharmacia Biotech) column conditioned with buffer A. The protein was then eluted using in a gradient of 1 M to 0 M ammonium sulphate in buffer A in a total volume of 120 ml at a flow rate of 4 ml min⁻¹.

Individual fractions were assayed for NGT activity using 3,4-DCA as substrate and the 7 most active fractions were pooled and dialysed overnight against 5 l of buffer B (20 mM Tris-HCl, pH 7.8, 1 mM DTT).

2.5.2 Anion-exchange chromatography using Q-Sepharose™

A Q-Sepharose™ 4 Fast Flow column (20 ml) was equilibrated in buffer B (20 mM Tris-HCl, pH 7.8, 1 mM DTT) before the dialysed protein sample was applied, with the activity present eluted using a linear 200 ml-gradient of 0 to 1 M NaCl in buffer B at a flow rate of 4 ml min⁻¹. The most active fractions were pooled and dialysed overnight against 5 l of buffer B.

2.5.3 Blue Sepharose chromatography

A Blue Sepharose™ CL-6B (Amersham Pharmacia) column (10 ml) was equilibrated in buffer B and the dialysed active protein from the anion exchange run applied. The column was then washed with buffer B and the UV trace (280 nm) monitored until all unbound proteins were washed off. Proteins of interest were then eluted using 15 ml of 20 mM UDP-glucose in buffer B at a flow rate of 0.5 ml min⁻¹.

2.5.4 Anion Exchange chromatography using a mono-Q column HR 5/5 column

The proteins were finally resolved on an anion exchange chromatography column using a Mono-Q column HR 5/5 column (1 ml) (Amersham Pharmacia). The elution regime consisted of buffer B with a linear NaCl concentration gradient of 0 to 0.25 M over 50 ml.

2.6 Gel electrophoresis of proteins

Acrylamide gels (0.75 mm-thick) were cast in a slab in a Mini Protean II electrophoresis kit (Bio-Rad) assembled as detailed by the manufacturer and derived from the method of Leammili (Leammili, 1970). For most applications, 12.5% (w/v) acrylamide gels were used, containing 0.33% (w/v) *N,N'*-bis-methylene-acrylamide, 0.1% (w/v) ammonium persulphate, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% TEMED (*N,N,N',N'*-tetramethylethylenediamine) was used for the resolving part of the gel and 3.9 % 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 in aqueous solution for the stacking gel.

Prior to loading, protein samples (5 μ l) were mixed with 2 x SDS-PAGE loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β -mercaptoethanol, 4% SDS and 25 μ g ml⁻¹ bromophenol blue) and heated at 100°C for 5 minutes. Samples were then loaded onto the SDS-PAGE gels (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subjected to electrophoresis using 1 x SDS running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) at an applied potential of 150 V for 60 minutes.

The gels were carefully removed from the glass plates and rinsed 3 times with distilled water over 10 minutes before being immersed in a Coomassie Brilliant Blue based dye. Several stains were used, notably the commercial stains EZBlue (Sigma) and Gel Code Blue (Pierce, Perbio Science UK Ltd., Tattenhall, UK) and a home-made dye (100 mg l⁻¹ of Coomassie Brilliant Blue G250 dissolved in 5% (v/v) 95% ethanol, 10% (v/v) orthophosphoric acid (85%). After staining for an hour, the gels were transferred to distilled water overnight for destaining.

For silver staining, gels were fixed by two separate immersions in 40% ethanol, 10% acetic acid in aqueous solution (each for 5 minutes). The gels were then

treated for 30 minutes in a 50 ml solution containing 15 ml ethanol, 2 ml 5% sodium thiosulphate and 3.4 g of sodium acetate in aqueous solution before being washed three times in water for 5 minutes each. Staining was performed over 20 minutes in a water-bath containing 125 mg of silver nitrate in 50 ml water. The gels were washed again twice for 1 minute each in water and then developed for 2 to 5 minutes in a 50 ml solution containing 1.25 g of sodium carbonate and 20 μ l of formaldehyde. The development was stopped using a wash with 0.73 g EDTA in 50 ml water for at least 10 minutes.

2.7 Proteome analysis

Polypeptides of interest contained in the silver-stained gel were excised with a scalpel and treated with trypsin *in situ* after reductive alkylation (Chivasa, *et al.*, 2002). The peptide digest was then eluted from the gel with acidified acetonitrile, mixed with α -cyano-4-hydroxycinnamic acid and then analysed on a Voyager DE-STR Applied Biosystems MALDI-TOF mass spectrometer (Chivasa, *et al.*, 2002). The analysis was carried out by Dr. JW Simon using the facilities of the Biological and Biomedical Sciences Department.

The resulting peptide mass ions were used to screen a non-redundant protein database using Mascot (<http://www.matrixscience.com/>). To determine the molecular mass of the parent GT polypeptides, the pure protein was injected directly into a Micromass LCT time-of-flight (TOF) mass spectrometer, using electrospray ionisation at a flow rate of 0.1 ml min^{-1} . Operating in positive ion mode, mass ions were collected in the mass range of 500 Da to 2,000 Da and analysed using the supplied MassLynx software, with multiple charged peaks de-convoluted using the MaxEnt1 plugin after calibration with horse-heart myoglobin. The analysis were carried out by Dr. Ian Cummins and Dr. David Dixon.

2.8 Molecular biology procedures

2.8.1 Standard procedures

Procedures were developed using protocols supplied by the various manufacturers of cloning reagents and solutions or following standard procedures described by Sambrook (Sambrook, *et al.*, 1989).

2.8.2 mRNA isolation and synthesis of cDNA

mRNA from fresh or frozen tissue was extracted using TRI[®] Reagent (Sigma) following the manufacturer's instructions. The glassware was previously washed and treated with 1% (v/v) diethylpyrocarbamate-treated water for one hour before being autoclaved for 15 minutes. Mortar and pestles were kept in foil at 200°C for at least 8 hours prior to the extraction. Both qualitative and quantitative tests on RNA content were carried out by determining the OD₂₆₀ and OD₂₈₀ with an OD₂₆₀ = 1 corresponding to a concentration of 40 µg ml⁻¹ of RNA. The quality of the RNA in the extraction being judged as good if a ratio OD₂₈₀/OD₂₆₀ of 1.9 or higher was obtained. RNA was also analysed by agarose electrophoresis.

2.8.3 Electrophoresis

A 1%-agarose (Sigma) gel was cast in a tray Horizon[®] 58 gel electrophoresis apparatus and run in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.6) tank (Gibco BRL, Paisley, UK) at 90 V for 45 minutes and the resolved DNA fragments checked under UV irradiation using ethidium bromide staining.

In the case of mRNA, the tank was cleaned with DEPC-treated water prior to running the gel. RNA samples were kept at -80°C to ensure stability.

In the case of DNA material, digestion products were mixed with 6 x loading buffer (Promega) and loaded in the agarose gel and run in a tank filled with 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.6) in an electric field of 90 V for 30 minutes. DNA fragments were visualised under UV due to the ethidium bromide staining. The sizes of the DNA fragments were compared to DNA size markers (1kb ladder, GibcoBRL®).

2.8.4 PCR amplification

The method of RT-PCR (Reverse Transcriptase - Polymerase Chain Reaction) was based on a two-step process starting with an initial reverse transcription of total mRNA into cDNA before a PCR amplification of specific DNA fragments. The retro-transcription used the AMV reverse transcriptase (Promega, Southampton, UK) with *Taq* polymerase (Promega) used for the PCR. The reverse transcription step was achieved using total mRNA (5 µg) with the desired primer (2.5 µg) in a final volume of 15 µl. The mix was heated for 10 minutes at 70°C before being placed back on ice. The latter mixture was supplemented by a 5 x AMV (avian myoblastosis virus) reverse transcriptase buffer, dNTP mix (1 mM), RNAsin® (40 U), 10 U AMV reverse transcriptase up to 20 µl (all reagents by Promega). The mix was placed at 45°C for 45 minutes followed by a further 55°C incubation for 30 minutes.

PCR reactions were performed on maize dichlormid-treated root cDNA libraries (Dixon, *et al.*, 1998) (kindly donated by Dr. David Dixon) or cDNA freshly prepared from mRNA isolated from fresh tissue sources (*Arabidopsis thaliana* and maize tissues). The design of primers included the insertion of restriction enzyme sites at the flanking regions of the gene.

PCR reactions were carried out under standard conditions (Table 2.1) in a Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) using appropriate primers (MWG-Biotech, Ebersberg, Germany).

The PCR mix was constituted by 10 x PCR buffer supplemented by the four dNTPs (200 μ M each), Mg^{2+} (1.5 mM) and 2-5 U *Taq* DNA polymerase (all from Promega). For specific amplifications, a proof reading DNA polymerase was used which was provided as a High Expand Fidelity PCR System kit from Roche (Lewes, UK) following manufacturer's instructions.

Table 2.1: Standard PCR programme for amplification of DNA.

Step	Temperature (°C)	Time (s)	Description
1	94	60	Warming of the sample
Addition of the <i>Taq</i> DNA polymerase			
2	94	30	Separation of the strands
3	50-55	60	T _m (melting temperature) of the primers (variable). The primers stick to the DNA template
4	72	60-180	Variable, depending on the size of the template (30 s per kb to be amplified)
Repeat Steps 2 to 5 for up to 30 times			
5	72	600	Termination of amplification of all unfinished strands
6	4		For stability of the sample until storage at -20°C

2.8.5 Ligation procedure

The addition of an extra A residue on the amplified fragments by *Taq* polymerase during PCR allowed direct ligation of the products into a sub-cloning vector containing T-overhang residues at the insertion site. The sub-cloning protocol was followed according to manufacturer's instructions using either pGEM[®]-T Easy Vector I (Promega) or the TOPO[™] TA Cloning kit (Invitrogen, Paisley, UK).

In the case of the ligation of digested fragments, 5 U of T4 DNA ligase (Promega) was added to the appropriate ligation buffer with a ratio of 3 : 1 insert : vector and incubated at 4°C overnight or 1 hour at room temperature following manufacturer's instructions. The inactivation of the T4 DNA ligase was done at 70°C for 10 min.

2.8.6 Digestions with appropriate restriction enzymes

Restriction digestions were generally performed at 37°C for 1 hour in a total volume of 20 µl including appropriate enzyme buffer and 1-10 Units of restriction enzyme following manufacturer's instructions (Promega or New England BioLabs, Inc, Beverly, US). When buffers were incompatible, digestions were performed sequentially. Ethanol precipitation was carried out to remove salt residues by adding 1/10 volume of 3 M sodium acetate and 2.5 volumes of pure ethanol. The mixture was then left on ice for one hour and centrifuged. The DNA pellet was washed with 70% ethanol and dried for 15 minutes at room temperature before resuspension in distilled water.

2.8.7 Purification of DNA fragments from agarose slices

Fragments of the correct size were excised from agarose gels using a razor blade and purified using the commercial kit Prep-A-Gene[®] DNA Purification Systems (BioRad). Pieces of agarose were added to 3 volumes (1:3 w/v) of binding buffer (6 M sodium perchlorate, 50 mM Tris pH 8, 10 mM EDTA). The mixture was incubated at 65°C or until the agarose melted and 10 µl of powdered silica added. Silica was then sedimented using a microcentrifuge (30 seconds) and the supernatant discarded. The DNA bound to the silica fines was then washed repeatedly with 1 ml of wash buffer (400 mM NaCl, 20 mM Tris pH 7.5, 2 mM EDTA pH 7.5; 50% ethanol (v/v)) followed by 1 ml of 70% ethanol. Beads were dried for 15 minutes at 37°C before the DNA fragments were released with 15 to 20 µl of sterile water.

2.8.8 Transformation into bacterial host

Transformation was carried out in NovaBlue Competent Cells (Novagen) or DH5α (Qiagen) *E.coli* strains based on the heat-shock method (Sambrook, *et al.*, 1989). When electrocompetent cells were chosen, transformation was achieved using a transient high electric field in a Gene Pulser II Electroporator (Bio-Rad) following manufacturer's instructions. Either of the following transformations were performed routinely usually depending on the characteristics of the batches of competent cells.

Transformation into chemically competent cells.

An aliquot of competent cells was left on ice for 30 minutes before 2 µl of the ligation product was added and mixed by pipetting. The mixture was thawed for a further 15 minutes on ice before being transferred at 42°C for 30 seconds. After 2 minutes back on ice, 900 µl of SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 10 mM MgSO₄, 10 mM MgCl₂) were

added to the cells, allowing optimal recovery following the heat shock treatment. The cells were then incubated at 37°C for at least 30 minutes and then plated on Luria-Betani medium (LB; 1% w/v peptone, 0.5% w/v yeast extract, 1% w/v NaCl, pH 7) + Agar containing an antibiotic as selective marker and incubated overnight at 37°C.

Transformation into electrocompetent cells.

A 500 ml flask was inoculated with 5 ml of an overnight bacterial culture derived from a single colony. The 500 ml culture was kept at 37°C under gentle agitation until the OD reached 0.8 and was then chilled on ice for 30 minutes before the cells were recovered by centrifugation (5,000 x g for 10 minutes). The bacterial pellet was washed twice with 1 mM sterile HEPES (dextrose 1 g l⁻¹, HEPES 5 g·l⁻¹, KCl 0.37 g l⁻¹, NaCl 8 g l⁻¹, Na₂HPO₄·2H₂O 0.13 g l⁻¹) prior to a wash in 45 ml of GYT medium (10% (v/v) glycerine, 0.125% (w/v) yeast extract, 0.25% (w/v) casein peptone and 0.02% (v/v) Tween 80) and resuspension in 4 ml GYT. The cells were then aliquoted and frozen at -80°C.

An aliquot of 1 µl of salt-free plasmid preparation was mixed to 50 µl of ice-thawed competent bacteria. The mixture was transferred to a 2-mm electroporation cuvette (Molecular BioProducts, San Diego, USA) which was subjected to an electrotransformation in a Gene Pulser (BioRad) according to the manufacturer's instructions (25 µF, 200 Ω, 2.5 kV). The cells were immediately mixed with a 950 µl SOC medium and incubated for 1 hour at 37°C under constant shaking. The mixture was spread on LB-agar supplemented with the appropriate selective marker.

For overexpression studies, more specialised strains such as BL21(DE3) Competent Cells (Stratagene) or Rosetta™(DE3) Competent Cells (Novagen) were used, using similar procedures.

2.8.9 Bacterial cultures

A single bacterial colony was used to inoculate a liquid culture of LB medium supplemented by appropriate selective antibiotics (ampicillin, 100 $\mu\text{g ml}^{-1}$, chloramphenicol, 34 $\mu\text{g ml}^{-1}$, tetracycline, 12.5 $\mu\text{g ml}^{-1}$ routinely used). The culture was then grown overnight to the appropriate density.

When the bacteria were grown on plates, LB medium was supplemented by 1.5% (w/v) of agar (Sigma) before being poured on Petri dishes.

2.8.10 Plasmid preparation

Plasmids were extracted from the bacterial host (overnight cultures in LB medium plus appropriate selective marker) using a commercially available Wizard[®] Plus SV Miniprep DNA purification system kit (Promega) following the manufacturer's instructions.

2.8.11 Checking the transformants

Colonies were screened for the correct construct after extraction of the plasmid by a restriction digest to confirm the presence of the putative correct insert. The efficiency of transformation was determined from the number of colonies present on the plates after an overnight incubation at 37°C.

2.8.12 Sequencing of DNA fragments

The sequencing of the insert was done systematically in order to verify the integrity of the sequence in particular the right insertion into the vector and any point mutation resulting of errors inserted by the polymerase. The sequencing of DNA fragments was carried out with universal T3/T7 primers on either ABI 377

XL DNA sequencer or ABI 373 Stretch DNA sequencer with Big Dye terminator chemistry according to PE Biosystems protocols by the Sequencing Service, School of Biological and Biomedical Sciences, University of Durham. The bioinformatic software DNA for Windows 2.4 (Dr. David Dixon, University of Durham) was used to edit and analyse the sequences; they were aligned using CLUSTALW (Thompson, *et al.*, 1994) and were compared to the public databases (www.ncbi.nlm.nih.gov/BLAST/) using BLAST (Basic Local Alignment Search Tool) (Altschul, *et al.*, 1990).

2.8.13 Expression of recombinant proteins

According to the cloning strategy, the construct corresponding to the gene of interest was inserted in an expression pET vector (pET Expression System Novagen) or pGEX vector (Amersham) and transformed into a bacterial strain of *E.coli* BL21(DE3) competent cells (Novagen), or Rosetta(DE3) competent cells (Novagen).

Overnight 10 ml cultures were grown with appropriate selective marker and 2% (w/v) of the expression repressor glucose used to inoculate fresh larger pre-warmed LB Broth cultures (100 ml) which were grown at 30°C with shaking. When OD₆₀₀ reached 0.8, 1 mM isopropyl β-thiogalactoside (IPTG) was added to the cultures initiating the induction lasting up to 3 hours under constant shaking depending on the expression strategy. Bacterial cultures were harvested at time intervals, pelleted (4,000 x g, 10 minutes) and frozen at -20°C. Optimisation of expression were attempted by varying incubation temperatures (10, 20, or 25°C).

An aliquot of the bacterial pellet was sonicated and centrifuged in order to separate soluble from insoluble fraction prior to analysis on SDS-PAGE gel electrophoresis.

2.8.14 Phylogenetic analysis

DNA sequences to be analysed were subjected to grouping procedures based on sequence similarities. Specialised software for comparison of sequences (CLUSTALW) was used to achieve the comparison of sequences. The phylogeny package PHYLIP (Felsenstein, 1989) helped design a phylogenetic tree of the related sequences. The PRODIST output obtained was further processed using NEIGHBOR and the final tree plotted using DRAWTREE.

Chapter 3 : Glucosyltransferases active towards xenobiotics in maize

3.1. Introduction

In 1988, Furtek and colleagues identified the *bronze-1* mutation in maize resulting in the phenotype of purple-pigmented seeds. The enzyme responsible for this phenotype was cloned and found to be involved in the anthocyanin biosynthetic pathway, being a UDP-glucose: 3-*O*-glucosyltransferase (Furtek *et al.* 1988). With growing interest in elucidating the importance of GTs in plant secondary metabolism, the maize *bronze-1* sequence was used to demonstrate the presence of homologues in other plant species (Brazier, *et al.*, 2003; Ford, *et al.*, 1998; Kroon, *et al.*, 1994; Miller, *et al.*, 1999; Wise, *et al.*, 1990). Maize GTs involved in the conjugation of plant hormones including indole-3 acetic acid (IAA) (Szerszen, *et al.*, 1994) and zeatin (Martin, *et al.*, 1999) have also been the subject of detailed study. In the latter case, a characterised bean (*Phaseolus lunatis*) sequence (Martin, *et al.*, 1999) was used to isolate an homologue of a zeatin-*O*-GT involved in the storage and regulation of cytokinins. The sequences were over 90% similar between maize and bean. A further closely related zeatin *O*-GT of 51.1 kDa has since been identified in maize (Veach, *et al.*, 2003) which featured a high degree of identity (98.3% on the nucleotide level) and had a similar activity profile to the first zeatin-*O*-GT isolated. The enzymes were highly specific to *cis*-zeatin reinforcing the concept of high specificity of the GT enzymes towards their substrates.

With its importance as a worldwide cultivated crop, maize has been the focus of much effort in the crop protection industry and as such has formed the basis of many metabolism studies with pesticides. Many studies with selective herbicides highlighted that maize plants are capable of detoxifying xenobiotics more efficiently than other plant species (Sandermann, *et al.*, 1991). As described in Chapter 1, all

four major classes of detoxification enzymes have been demonstrated to be present in maize (Farago, *et al.*, 1994; Lamoureux, *et al.*, 1992). However, of the xenobiotic-detoxifying enzymes, the GTs have received relatively little attention.

In this chapter, attention is focussed on the ability of maize plants to detoxify xenobiotics using GT-mediated conjugation, with the activity of the respective enzymes identified in crude plant preparations.

In addition, using maize seedlings, radiolabelled *p*-nitrophenol and 3,4-DCA were individually used as model substrates to follow glucosylation in metabolism studies *in vivo*, with the resulting conjugates identified when possible. It was of interest to detect whether safener could alter the uptake or the conjugation rate of xenobiotic as an increase in GT specific activity had been demonstrated following safener treatment.

GT activities towards a range of putative GT substrates was also carried out using extracts from a range of maize tissues in order to determine a good tissue source for GT activities. Finally, attempts were made to enhance the specific activities of GTs using a wide range of safeners, as GTs had been shown to be chemically inducible in maize previously (Woo, *et al.*, 1999).

3.2 Metabolism of radiolabelled xenobiotics in maize

3.2.1 Metabolism of *p*-nitrophenol in maize seedlings

[UL-¹⁴C]-*p*-nitrophenol was fed to 10 day-old maize seedlings which had been treated with or without the safener dichlormid (10 mg l⁻¹) for 24 hours in the watering solution. The roots were cut from the leaves with a razor blade in water to avoid air bubble entering and blocking the vascular system. The rootless seedlings were transferred to microfuge tubes containing the radiolabelled xenobiotic homogenised in the feeding solution (Figure 3.1). The uptake and metabolism of [¹⁴C]-*p*-nitrophenol was monitored over time after the xenobiotic entered *via* the cut stems.

Fractions corresponding to the plant tissues, the medium and when possible the bound residues were extracted separately based on the partition of the feeding medium with Ethyl Acetate (1:1 v/v); by methanol extraction for the plants extracts (10:1, w/v) and based on the use of 2 N NaOH for the bound residues (see Materials and Methods section).

Radiolabelled metabolites in the plants were resolved by TLC separation and the conjugates identified by co-chromatography with a range of authentic *O*-glycosidic conjugates of *p*-nitrophenol. To confirm their identity, co-chromatography was carried out under different solvent systems and after enzymatic digestion with cellulase to confirm the presence of a glycoside. Samples were incubated in the presence of cellulase or in solely buffer and profiles were compared after migration on TLC plates.

In a preliminary study (Figure 3.2), the time course of the experiment was optimised to last 3 days with 24 hour intervals between samplings. In each case, the initial dose of *p*-nitrophenol was 97 µM present in 1 ml treatment solution, with seedlings harvested in triplicate, except after 48 hours, where samples were harvested in duplicate. The radioactivity present in the medium rapidly decreased from

97 μM to 47 μM in the control seedlings (48% of the initial dose) after 24 hours. Similar uptake was observed with safener-treated plants with 53 μM (55% of the initial dose) remaining in solution after one day. The radioactivity in the media of the untreated plants remained constant for the next 24 hours with 46 μM (47%) recovered while in the safener-treated plants the label compound continued to be taken up, albeit slowly. Radioactivity in the medium was decreased to 42 μM after 48 hours with 60 μM recovered from the plant. The recovery data are presented in Table 3.1 and show that almost all of the dose was recoverable. These results suggested that safener treatment might slow the rate of [^{14}C]-*p*-nitrophenol uptake in the maize seedlings, though after 72 hours the overall distribution of label compound between plants and media was effectively identical in both safener-treated and untreated medium.

A statistical test was carried out on the two plant populations and the *P* value obtained was 0.3094 with $P < 0.05$. There was no significant difference between the two populations tested. The hypothesis H_0 described in Chapter 2 section 2.4.4 had to be accepted. The treatment with safener did not affect the rate of uptake and the distribution of xenobiotic over the time course.

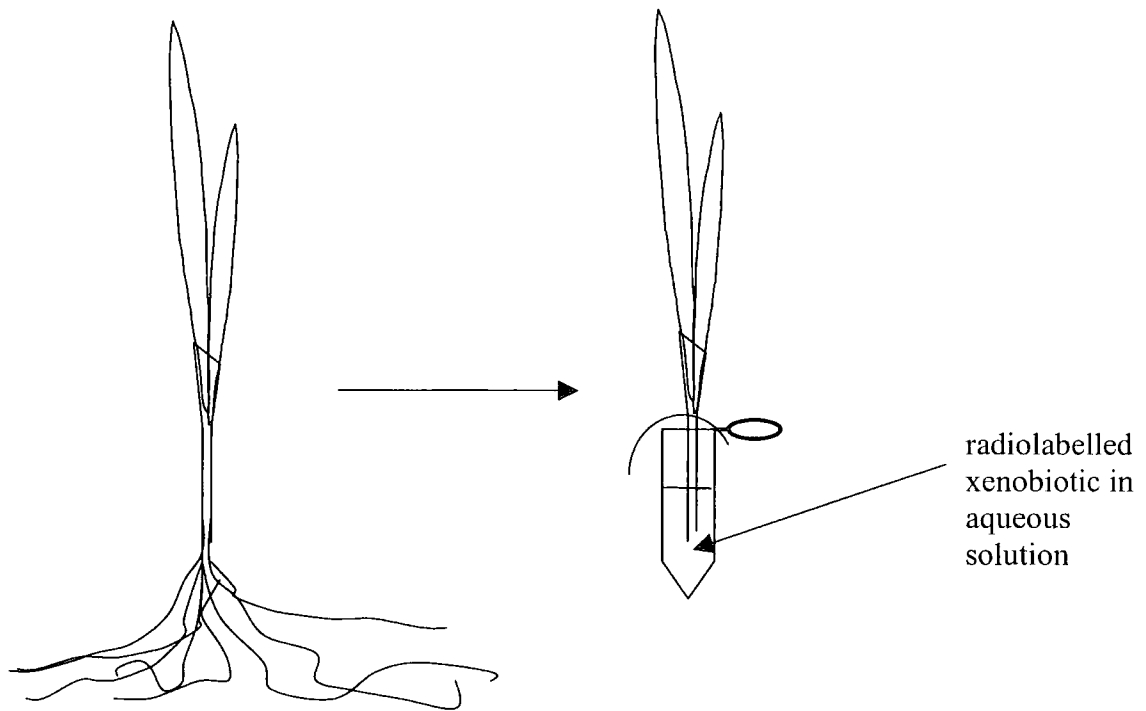


Figure 3.1 : Detail of feeding study experimental.

Maize seedlings were grown in vermiculite for 10 days. The leaves were separated from the root system and immersed in the feeding solution composed of the radiolabelled xenobiotic (dissolved in 5 μ l of 20% ethanol) homogenised in 1 ml of water. The tubes were sealed with Parafilm[®] to avoid evaporation and to allow the plant to stand in the tube. Samples were harvested at time intervals.

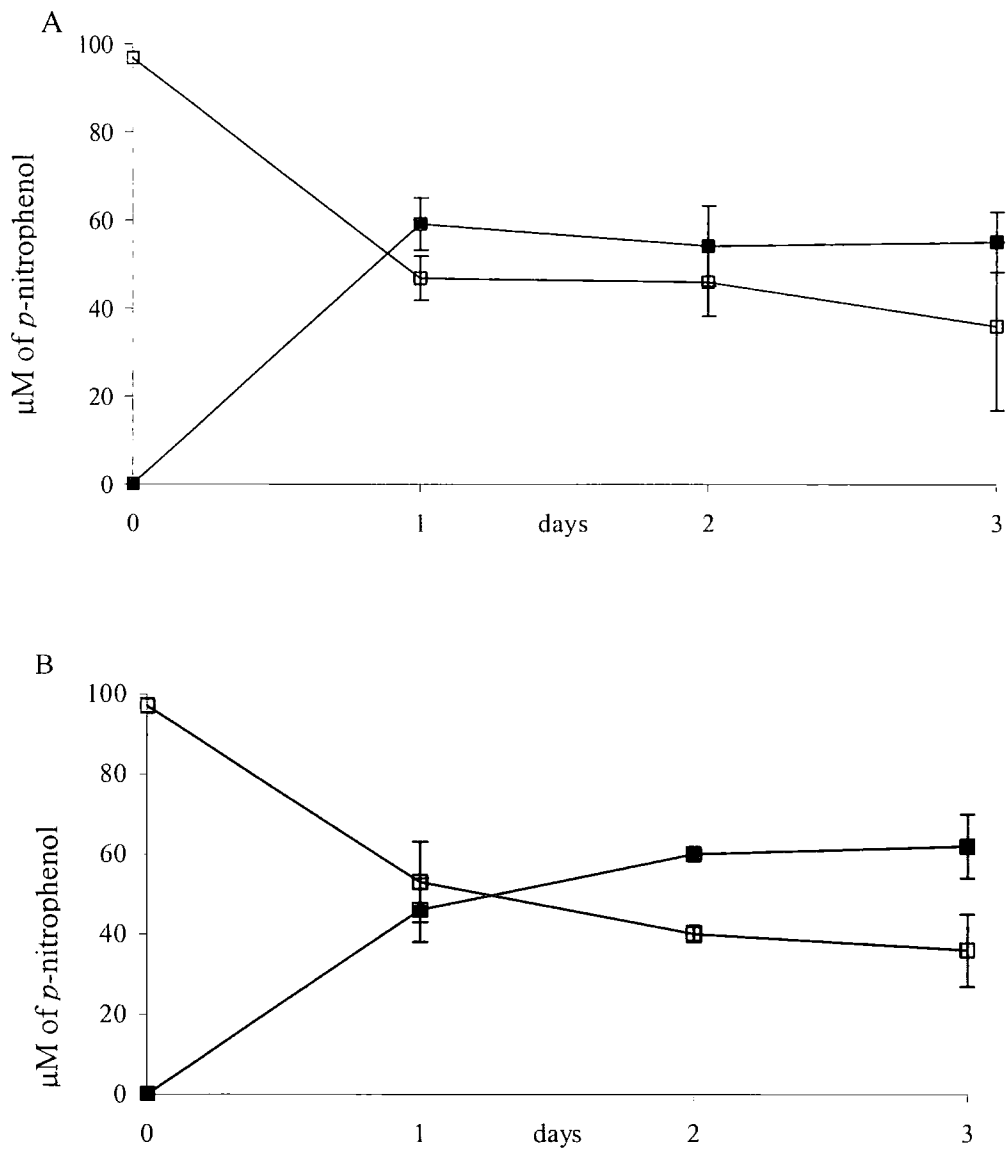


Figure 3.2 : Uptake of $[^{14}\text{C}]$ -*p*-nitrophenol in maize seedlings ($97 \mu\text{M}$).

Distribution of radioactivity in the plant (■) and in the medium (□) following a three-day treatment with a fixed concentration of $97 \mu\text{M}$ of $[^{14}\text{C}]$ -*p*-nitrophenol to maize seedlings in the presence (B) or absence (A) of the safener dichlormid (10 mg l^{-1}). The safener treatment was 10 mg l^{-1} dichlormid or the equivalent amount of solvent carrier (acetone) dissolved in the watering solution. Data are the mean \pm standard deviation ($n=3$) except for time point 48h where $n=2$ for the safener-treated tissues.

Table 3.1 : Percentage total recovery of applied radioactivity after feeding [¹⁴C]-*p*-nitrophenol (97 μM) to maize seedlings.

The recoveries are the result of the summation of the radioactivity present in the plants and residual feeding solution from metabolism study in which maize seedlings which had been previously exposed ± dichlormid, were treated with [¹⁴C]-*p*-nitrophenol. Data are the mean ± standard deviation (n=3) except for time point 48h where only duplicate values were analysed in the safener-treated tissues.

Day	Recoveries (%)	
	Non treated	Treated
D+1	105.7 ± 5.4	99.3 ± 8.6
D+2	100.0 ± 8.5	99.0 ± 1.4
D+3	91.7 ± 12.7	97.7 ± 8.1

To confirm the results obtained in the initial feeding study, the experiment was repeated with a higher initial dose of radioactive *p*-nitrophenol administered.

Two points were to be investigated: 1) to confirm the dynamics of uptake and 2) to observe more closely any potential action of the safener treatment when the plants were exposed to higher concentrations of xenobiotics. A similar experiment was set up and samples analysed again at daily intervals.

The initial dose was almost twice that used in the previous experiment, consisting of 188 μM of radioactive *p*-nitrophenol per ml of treatment solution (Figure 3.3). In the untreated plants, the radioactivity in the medium decreased drastically within 24 hours with less than a quarter of the initial dose remaining (44 μM , 23% of the dose). The radioactive content of the medium then stabilised over the next 24 hours before decreasing to 34 μM (18%) after 72 hours. In the safener-treated tissues, the trend was similar to that previously determined; the uptake of label compound seemed slower than in the untreated plants. However, by 72 hours the distribution of label compound between plant extract and medium was identical to that seen in the untreated plants (Figure 3.3).

The majority of uptake of *p*-nitrophenol occurred during the first 24 hours, with absorption being slower in the safener-treated plants. There was however no significant difference between treated *versus* non-treated tissues with P value = 0.1613 with $P < 0.05$. The hypothesis that the population were identical had to be accepted and resulted in no difference in uptake and distribution of radiolabelled over the time course in the plant tissues. Recovery data are presented in Table 3.2.

A further more detailed short-term study was carried out with maize seedlings treated with or without dichlormid and fed with [^{14}C]-*p*-nitrophenol over 24 hours (Figure 3.4). In this case, plants and media were sampled after 4, 8, 12 and 24 hours. The results of this study showed that after 4 hours and 12 hours, the safener-treated plants had actually taken up more radioactivity than the untreated plants (Figure 3.3). There was however no visual difference in uptake in the samples after 8 hours or 24 hours and the difference in the uptake is not significant regarding the whole study $P = 0.0714$ with $P < 0.05$. The hypothesis that the populations were identical was accepted. Recoveries of radioactivity were high for all time points (Table 3.3).

From the results obtained from these three feeding studies it was concluded that there was no significant effect of safener on the uptake and localisation of [¹⁴C]-*p*-nitrophenol and relative conjugates in maize.

The hypothesis that the populations treated or not with safeners were behaving similarly towards the uptake and distribution of the xenobiotic had to be accepted for the three feeding studies. In these studies, the regime of treatment with safener was constant. It would be of interest to vary the concentrations applied and the conditions of treatment before excluding with certainty the effect of safener.

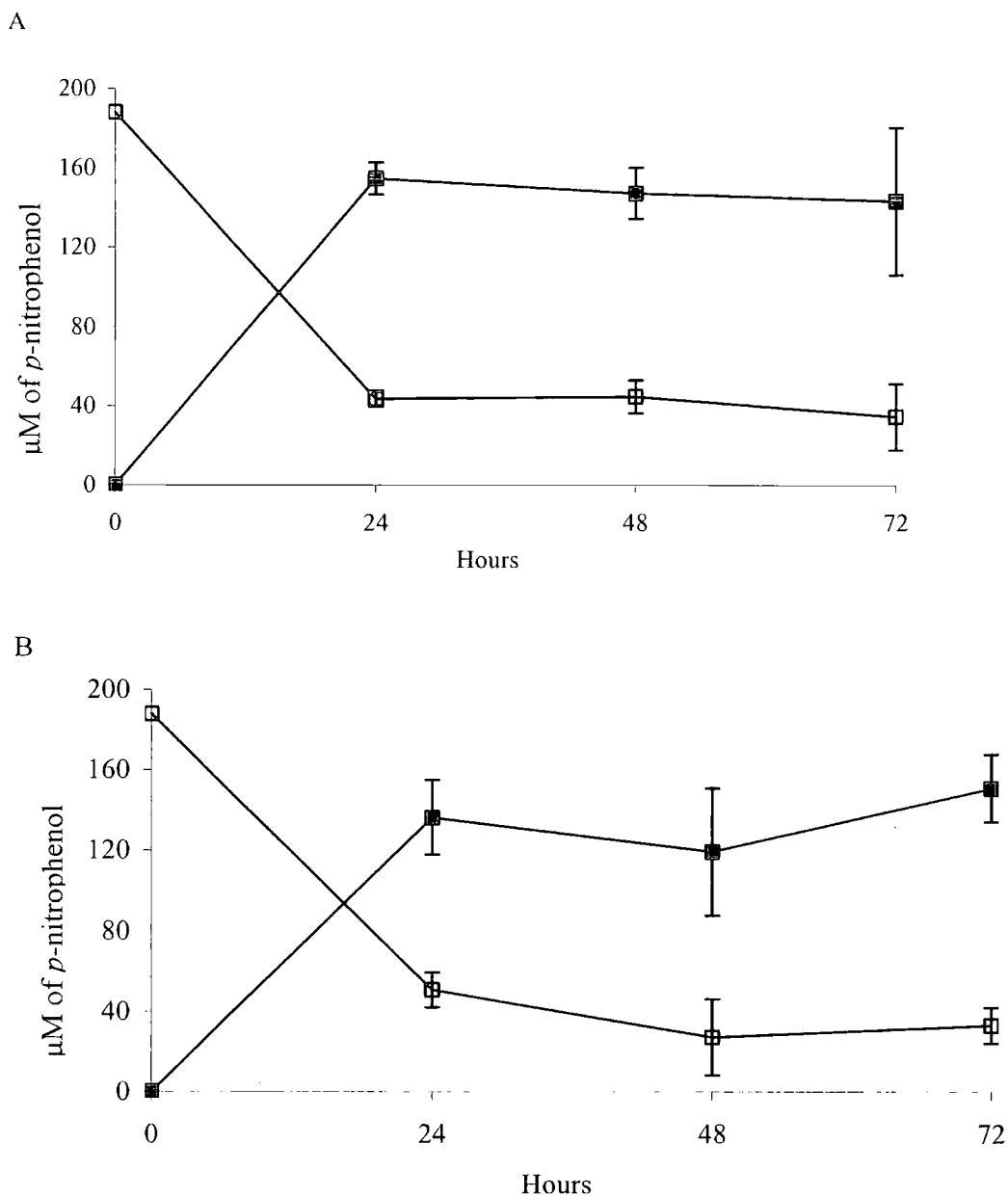


Figure 3.3 : Uptake of [^{14}C]- p -nitrophenol in maize seedlings (188 μM).

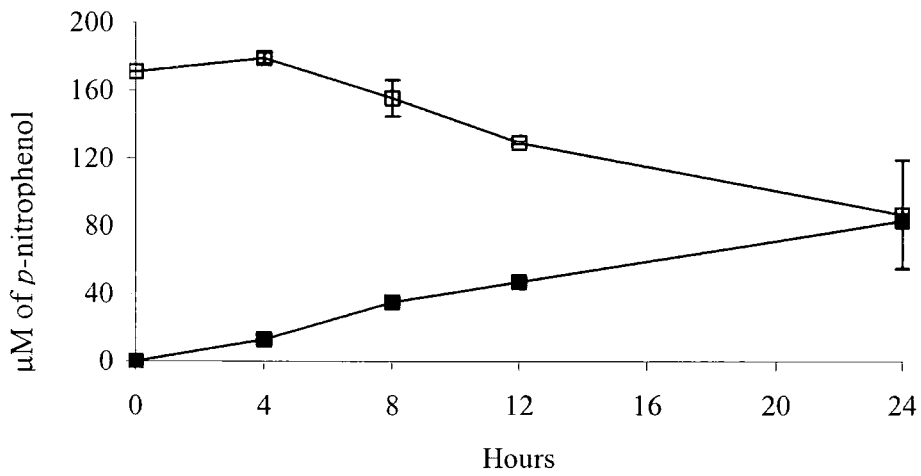
Distribution of radioactivity in the plant (\blacksquare) and in the medium (\square) following a three-day treatment of [^{14}C]- p -nitrophenol (188 μM) to maize seedlings in the presence (B) or absence (A) of the safener dichlormid. The safener treatment was 10 mg l^{-1} dichlormid or equivalent amounts of the solvent carrier (acetone) in watering solution. Data are the mean \pm standard deviation ($n=3$).

Table 3.2 : Percentage total recovery of applied radioactivity after feeding [¹⁴C]-*p*-nitrophenol (188 μM) to maize seedlings.

Plant tissues were previously treated with or without the safener dichlormid and total recoveries determined by summing the radioactivity present in the plants and treatment solutions. Data are the mean ± standard deviation (n=3).

Day	Recoveries (%)	
	Non treated	Treated
D+1	105 ± 3	100 ± 7
D+2	102 ± 6	78 ± 14
D+3	95 ± 15	98 ± 1

A



B

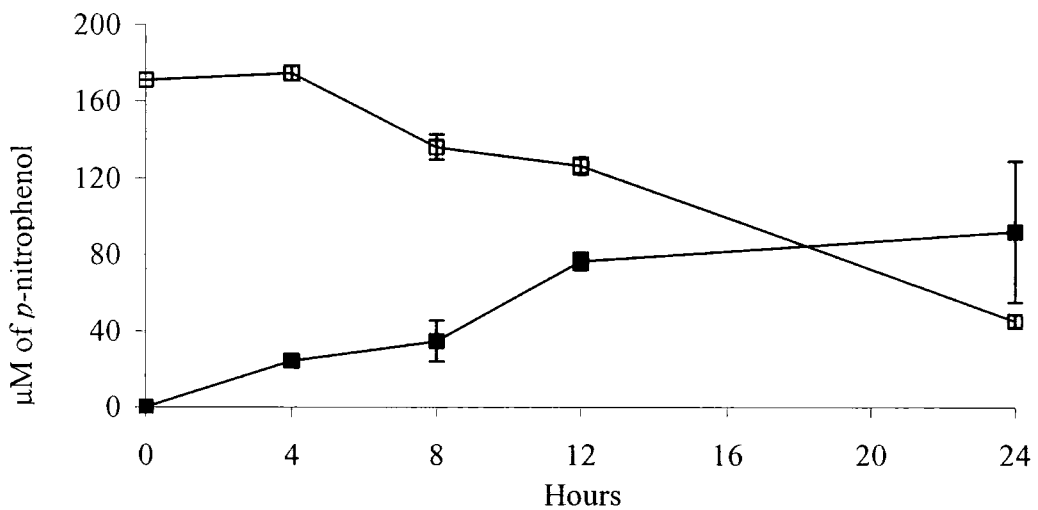


Figure 3.4: Uptake of [^{14}C]-*p*-nitrophenol in maize seedlings (163 μM).

Distribution of radioactivity in the plant (■) and in the medium (□) following a three-day treatment of [^{14}C]-*p*-nitrophenol (163 μM) to maize seedlings in the presence (B) or absence (A) of the safener dichlorimid. The safener treatment was 10 mg l^{-1} dichlorimid or equivalent amounts of the solvent carrier (acetone) in watering solution. Data are the mean \pm standard deviation ($n=3$).

Table 3.3 : Percentage total recovery of applied [^{14}C]-*p*-nitrophenol (163 μM) to maize seedlings.

The recoveries are the result of the summation of the radioactivity present in the plants and residues feeding solution from metabolism study in which maize seedlings which had been previously exposed \pm dichlormid, were treated with [^{14}C]-*p*-nitrophenol. Data are the mean \pm standard deviation (n=3).

Time (h)	Recoveries (%)	
	Control	Dichlormid treated
4	110 \pm 0	114 \pm 4
8	109 \pm 6	98 \pm 2
12	101 \pm 0	116 \pm 0
24	98 \pm 18	79 \pm 20

3.2.2 Identification of the conjugates by co-chromatography using 2 different solvent systems.

Radioactive metabolites present in the media and plant extracts were analysed by TLC and identified by co-chromatography with commercially available sugar-conjugates of *p*-nitrophenol using two solvent systems. The R_f values of the reference conjugates, the parent *p*-nitrophenol and the radioactive metabolites identified in the plant extracts, are shown in Table 3.4. When the media were analysed, the only radioactive entity present was the parent [¹⁴C]-*p*-nitrophenol. In the plant extracts two major radioactive polar conjugates were observed (Figure 3.5). The conjugate identified as 'Metabolite 1' (M1) had a R_f value of 0.37 in the EFW solvent system (Ethyl Acetate : Formic Acid : Water 70:4:4 v/v) and 0.56 in BAW (Butanol : Acetic Acid : Water 80:20:20 v/v). By comparison, the synthetic *p*-nitrophenol glucoside derivative ran at a R_f value of 0.32 in EFW and 0.57 in BAW. Having established the R_f values for the two metabolites in two different solvent system, the 'Metabolite 1' formed after *in vivo* conjugation was identified as the *p*-nitrophenyl glucoside conjugate (*p*-nitrophenyl-β-D-glucopyranoside). The conjugate named 'Metabolite 2' (M2) was found to have a R_f value of 0.31 in EFW and 0.46 in BAW. These values are very similar to those associated with the *p*-nitrophenyl galactoside conjugate (*p*-nitrophenyl-β-D-galactopyranoside) with a R_f value of 0.25 in EFW and 0.49 in BAW. No further metabolite identification using HPLC or mass-spectrometry was attempted.

To further characterise these conjugates, an enzymatic digestion was carried out to release the sugar moiety from the aglycone using cellulase. A fraction (5 μl) of the plant extract corresponding to *in vivo* metabolites was incubated with or without 1 mg ml⁻¹ cellulase in citrate buffer pH 5.2 for 30 hours at 16°C. The comparison of the profiles obtained after the digested samples alongside the parent *p*-nitrophenol were run on a TLC plate later exposed to film. The release of the parent compound when the sample had been incubated with cellulase compared to a similar profile as shown in Figure 3.X in the case of the sample incubated in citrate buffer only was confirmed. These experiments further confirmed that M1 and M2 were

glycosidic conjugates of *p*-nitrophenol based on their lability to cellulase (β -glucosidase) and release of the parent phenol.

Table 3.4 : Determination of the nature of the conjugates formed *in planta*.

Rf values of commercially available *p*-nitrophenyl conjugates and *in planta* metabolites formed by [¹⁴C]-*p*-nitrophenol fed to maize seedlings run under 2 solvent systems.

	Rf values	
	Ethyl acetate: Formic acid: Water EFW (70:4:4)	Butanol: Acetic acid:Water BAW (80:20:20)
<i>p</i> -nitrophenyl- β -D-cellobioside	0.06	0.44
<i>p</i> -nitrophenyl- β -D-galactopyranoside	0.25	0.49
<i>p</i> -nitrophenyl- β -D-glucopyranoside	0.32	0.57
<i>p</i> -nitrophenyl- β -D-maltopyranoside	0.07	0.45
<i>p</i> -nitrophenyl- β -D-mannopyranoside	0.21	0.52
<i>p</i> -nitrophenol	0.78	0.72
Metabolite 1 (M1)	0.37	0.56
Metabolite 2 (M2)	0.31	0.46

p-nitrophenol

M1

M2



Figure 3.5 : Isolation of conjugates of [^{14}C]-*p*-nitrophenol by TLC.

Thin-layer chromatogram showing extracts from maize seedlings fed with [^{14}C]-*p*-nitrophenol for 24 hours. The formation of two conjugates was not dependent on safener treatment. Samples were run in a solvent system (Ethyl acetate : formic acid : water 70:4:4 v/v) and the radioactive spots then identified using autoradiography. The upper spot was co-chromatographed with the authentic parent compound *p*-nitrophenol while two more polar metabolites were also identified which were subsequently identified as the glucoside (M1) and the galactoside (M2) of *p*-nitrophenol.

3.2.3 Uptake and metabolism of [UL-¹⁴C]- 3,4-DCA in maize seedlings.

For many years, 3,4-DCA has been of concern as a persistent chemical resulting from the breakdown of several pesticides. Conjugation with glucose was found to be a common mechanism for detoxification of this compound in plants (Bockers, *et al.*, 1994; Gareis, *et al.*, 1992; Schmidt, *et al.*, 1994; Schmidt, *et al.*, 1995) and the interest focused on whether 3,4-DCA was metabolised by sugar conjugation in maize and whether or not safeners could accelerate this detoxification process. Dichlormid was chosen as the safener as it was often shown to be active in inducing other detoxification systems in maize (Hatzios and Wu, 1996).

Maize shoots were excised from their root system and placed in microfuge tubes containing [¹⁴C]-3,4-DCA and the plant samples harvested at timed-intervals with the radioactivity monitored in the media and plant tissues as described in Figure 3.1. In untreated (= no dichlormid) plants, the uptake of label was constant. The radioactivity present in the media decreased steadily over the course of the 48 hour-period from an initial 3 mM to a final level of 610 µM after 48 hours. Associated with this decrease, the extractable and non-extractable radioactive residues in the plants increased over the course of the experiment. However, this uptake could not account for all the radioactivity lost from the medium (Table 3.5); it was clear that there was a net loss of recoverable radioactivity from the experiment, most likely as volatile residues.

The distribution of radioactivity in the safener-treated plants over time showed one major difference to that determined in the untreated plants, i.e. an apparent major influx of label compound into the plants 4 hours after feeding. During the next 4 hours (4-8 hours) the radioactivity in the plant rapidly declined and after 8 hours, was virtually identical to that determined in the untreated plants. Over the next phase 8-48 hours, the proportion of the radiolabel compound recovered in the plant tissues was slightly lower in the safener-treated tissues than in the untreated plants but overall loss of radioactivity from the medium was enhanced in these plants relatively to the untreated. Unfortunately, due to the limited time available, the

nature of the radioactivity in the plants and media was not characterised and as such the meaning of the transient increase in 3,4-DCA uptake after 4 hours is difficult to explain. If the label compound had been metabolised in the plant in the first 4 hours, this did not lead to its efflux back into the media or its incorporation into bound residues. Overall, it seemed most likely that this transient uptake was due to an artefact of measurement. In any event, this interesting result would need to be repeated before concluding that safening had altered 3,4-DCA uptake/metabolism in maize.

Based on statistical evidence however, the hypothesis that the two plants populations were similar had to be accepted based on the P value = 0.2426 with $P < 0.05$. The safener treatment did not affect the uptake and the distribution of xenobiotic over the time course in the treated population compared to the control.

There was no attempt to characterise the metabolites formed to a lack of time to optimise a reliable HPLC-based method.



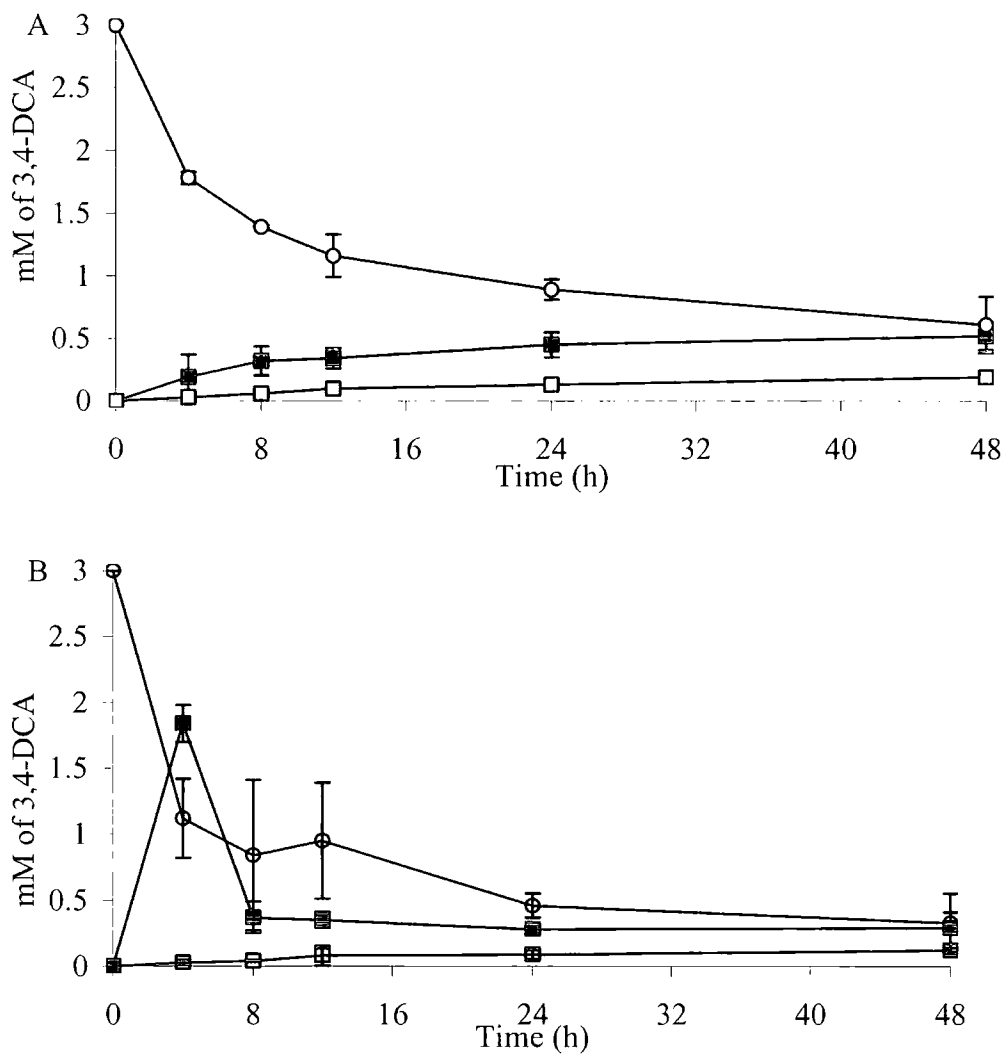


Figure 3.6 : Uptake of $[^{14}\text{C}]$ -3,4-DCA in maize seedlings.

Seedlings were untreated (A) or treated (B) with the safener dichlormid (10 mg l^{-1}). The distribution of radioactivity was determined in plant extracts (■), medium (○) and the non-extractable fraction (□) from the seedlings. Data are the mean \pm standard deviation (n=2).

Table 3.5 : Percentage total recovery of applied radioactivity after feeding maize with [¹⁴C]-3,4-DCA (3 mM).

Total recoveries were determine by summing together the radioactivity present in the media + soluble + insoluble fraction in the plant. Data are the mean \pm standard deviation (n=2).

Hours (h)	Recoveries (%)	
	Control tissues	Dichlormid treatment
4	67 \pm 3	67 \pm 6
8	63 \pm 0	59 \pm 15
12	65 \pm 2	53 \pm 20
24	42 \pm 1	49 \pm 1
48	37 \pm 8	44 \pm 4

3.3 GT activities in maize

A range of xenobiotic substrates was tested as potential glucose acceptors in GT assays using crude protein extracts from maize seedlings. Compounds were selected based on published accounts of their glucosylation in metabolism studies, or previously demonstrated activity in *in vitro* assays with plant GT preparations (Cole and Edwards, 2000). The range of OGT substrates included picloram, maleic acid, quinchlorac, pentachlorophenol, metribuzin, *p*-nitrophenol, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol, phenol and the flavonoids quercetin and luteolin. All substrates were prepared in methanol as 1 mM stock solutions and 5 μ l used in *in vitro* assays. For NGT assays, 3,4-DCA was used as a model substrate. The assay conditions for the detection of NGT activity were modified using buffer in place of the acidic stop solution and were immediately partitioned using ethyl acetate.

Etiolated and light-grown maize shoots were grown under standard conditions as described in Materials and Methods section. 10 g of fresh tissues were extracted and used in GT assays. After the protein concentration had been determined, a fixed quantity was used to determine the specific activity of the extract.

The enzymes were stable at -20°C for up to 6 months when precipitated with 70% ammonium sulphate (Sambrook, *et al.*, 1989; data not shown). Of the substrates tested, only *p*-nitrophenol, quercetin, 3,4-DCA, 2,4,5-TCP and coumestrol were found to act as glucose acceptors in the assays (Table 3.6).

GT activities in the roots from light-grown maize plants were not detected, or were too low to be quantified. In light-grown maize shoots, 0.07 pmol product $\text{min}^{-1}\text{mg}^{-1}$ protein was detected with 2,4,5-TCP as a substrate, and 0.11 pmol product $\text{min}^{-1}\text{mg}^{-1}$ protein with quercetin as a substrate. As compared with light-grown plants, etiolated tissues showed higher GT activities towards all substrates tested, with the exception of quercetin as a substrate where the activity was not seen in etiolated plants.

In the etiolated seedlings, OGT activity was generally higher in shoot tissues than roots, thus activity towards *p*-nitrophenol was 0.30 pmol product $\text{min}^{-1}\text{mg}^{-1}$ protein in etiolated roots compared to 1.58 pmol product $\text{min}^{-1}\text{mg}^{-1}$ protein in etiolated

shoots, representing higher activity than the shoot system. The difference observed for the substrate 2,4,5-TCP was even greater found in the shoots as compared with the roots. Activity towards coumestrol was identical in all the tissues tested (0.4 pmol product min⁻¹ mg⁻¹ of protein). NGT activity towards 3,4-DCA was also detected in etiolated plants with activities of 0.20 and 3.08 pmol product min⁻¹mg⁻¹protein determined in roots and shoots respectively.

These results demonstrated that OGT and NGT activities towards xenobiotics were much higher in etiolated as compared with light-grown maize seedlings, and that activities in the shoots were greater than in the roots. These observations are in accordance with literature (Coleman, *et al.*, 1997) and suggest that these enzymes may either be under developmental regulation or that a co-extractive in the light-grown plant extracts inhibited GT activity.

Having established that GT activity was low in light-grown tissues, etiolated maize seedlings were treated with safeners to determine if conjugation activity would be enhanced as had been previously demonstrated for detoxifying enzymes GSTs in similarly treated tissues (Holt, *et al.*, 1995). In particular, dichlormid has been used as a successful safener in maize and was therefore chosen for treatment. The determination of active pool of safeners was to obtain some comparative data in various crop and active substances on detoxifying enzymes present in maize and already studied in regards to safener treatment (unpublished data).

Maize seeds were imbibed in dichlormid (10 mg l⁻¹) and germinated on Whatman paper which had been soaked in the safener solution. After 14 days growth in the dark, the seedlings were harvested and assayed along with seedlings which had been treated with 1% acetone solution (solvent carrier) (Table 3.7).

GT activities determined in the shoots of the untreated plants were broadly similar to those recorded previously. In the roots though, activities were higher than measured in the first studies. In the shoots, dichlormid treatment resulted in an enhancement in OGT activity towards *p*-nitrophenol and trichlorophenol (2,3,4 and 2,4,5 isomers), with some activity towards luteolin, and phenol being determined in the safener-treated shoots; the latter activity being absent in the corresponding

control tissues. In the roots, all activities were actually suppressed by dichlorimid treatment as compared with those determined with the untreated controls (Table 3.6). The results obtained through this approach identified the GT activities present in maize. The data obtained constitute an useful basis for further purification work that could be attempted in maize.

Table 3.6 : OGT and NGT activities in various maize tissues.

Data are the mean \pm standard deviation (n=2). Specific activities are reported as pmol product min⁻¹mg⁻¹ protein.

ND not detected.

GT enzyme activity in maize seedlings (pmol product min ⁻¹ mg ⁻¹ protein)					
Tissue sources	Substrates				
	<i>p</i> -nitrophenol	2,4,5-TCP	Coumestrol	Quercetin	3,4-DCA
Light-grown shoots	ND	0.07 \pm 0.02	ND	0.11 \pm 0.01	ND
Etiolated roots	0.30 \pm 0.15	0.37 \pm 0.04	0.43 \pm 0.17	ND	0.20 \pm 0.09
Etiolated shoots	1.58 \pm 0.01	2.34 \pm 0.47	0.42 \pm 0.07	ND	3.08 \pm 0.05

Table 3.7 : GT activities in maize roots and shoots.

OGT activities in the shoots and roots of etiolated maize plants (14 days-old) treated with or without dichlormid (10 mg l⁻¹). Data are the mean ± standard deviation (n=2).

Substrates	GT enzyme activity (pmol product min ⁻¹ mg ⁻¹ protein in maize)			
	Tissue source			
	Shoots Not treated	Shoots Dichlormid	Roots Not treated	Roots Dichlormid
<i>p</i> -nitrophenol	0.40 ± 0.07	0.75 ± 0.19	2.02 ± 0.10	0.65 ± 0.15
Phenol	ND	0.17 ± 0.08	0.24 ± 0.14	ND
2,3,4-TCP	0.10 ± 0.04	0.25 ± 0.03	0.46 ± 0.02	ND
2,4,5-TCP	0.43 ± 0.06	0.59 ± 0.05	0.89 ± 0.13	0.18 ± 0.01
Quercetin	1.29 ± 0.02	1.40 ± 0.05	2.06 ± 0.21	0.55 ± 0.05
2-chlorothiophenol*	ND	0.20 ± 0.07	ND	ND
Luteolin	ND	0.03 ± 0.00	0.43 ± 0.03	ND
Picloram	0.28 ± 0.10	ND	0.40 ± 0.09	0.11 ± 0.02

* : Substrate tested for S-GT activity.

3.4 Effect of different safeners on GT activities in maize

Based on the apparent induction of xenobiotic-detoxifying GT activities in etiolated maize following treatment with the safener dichlormid (Table 3.7), a comparative study was carried out on dark-grown maize seedlings treated for 14 day-growth with 10 mg l^{-1} of different safeners. The safeners used represented a diverse set of chemistries, all showing activities in maize (dichlormid, isoxadifen-ethyl, R-29148, benoxacor), rice (fenclorim) and wheat (cloquintocet mexyl). The aim was to determine pools of active substances and to ultimately compare them with profile obtained for GST activities in similar plant tissues. This would help to hypothesise on whether one safener is species-dependent or if it can selectively act on enzyme classes. Unfortunately, comparison between the profile of action on GT *versus* GST was not finalised for the GSTs.

Maize seedlings were extracted and protein preparations derived from crude ammonium sulphate precipitates analysed for OGT activities towards 2,4,5-TCP and quercetin, the optimal xenobiotic and flavonoid GT substrates in maize determined from the assay of over 40 substrates (Table 3.6).

In the absence of comparative data, it was interesting however to determined that benoxacor and dichlormid were the highest inducers of the GT activity while R-29148, oxabetrinil and cloquintocet mexyl also exhibited some action on GT specific activity.

Table 3.8: GT activities in etiolated maize seedlings treated with a range of safeners.

Safener was added to etiolated seedlings over 14 days in the watering solution (10 mg l⁻¹). Specific activities were determined towards quercetin and 2,4,5-TCP.

Data are the mean ± standard deviation (n=2). In chapter 6, Table 6.4, the influence of the solvent control was not significant (*P* value = 0.0973 with *P*<0.05) compared to the water control for the *Arabidopsis* root cultures.

Safener treatment	Specific activity (pmole min ⁻¹ mg ⁻¹ protein)	
	Quercetin	2,4,5-TCP
Solvent control	0.93 ± 0.03	0.24 ± 0.05
Benoxacor	1.21 ± 0.05	0.67 ± 0.03
CMPI	0.96 ± 0.06	0.24 ± 0.01
Fenclorim	0.86 ± 0.03	0.53 ± 0.01
Flurazole	0.43 ± 0.01	0.58 ± 0.04
Oxabetrinil	0.87 ± 0.02	0.74 ± 0.01
R-29148	0.99 ± 0.04	0.66 ± 0.02
Cloquintocet mexyl	0.99 ± 0.02	0.71 ± 0.06
Dichlormid	1.18 ± 0.01	0.85 ± 0.01
Isoxadifen-ethyl	0.72 ± 0.01	0.42 ± 0.01

3.5 Discussion

Radiolabelled *p*-nitrophenol was fed to maize seedlings and found to be readily taken up and metabolised to the respective β -D-glucoside and to a lesser extent to the β -D-galactoside. Overall recovery profiles were in accordance with published studies by Malcherek (Malcherek, *et al.*, 1998) on related species, identifying the formation of polar conjugates mainly present in the plant tissues (root and aerial parts in wild oat and corn cockle) and a low formation of bound residues. Similarly, 3,4-DCA a xenobiotic also known to be primarily metabolised by *N*-glycosylation in wheat (Bockers, *et al.* 1994; Schmidt, *et al.*, 1995), was also rapidly taken up in maize. However, in the case of 3,4-DCA we were unable to confirm the nature of the metabolites.

Although the feeding studies with *p*-nitrophenol and 3,4-DCA shared no significant effect of safening on the rates of uptake into maize seedlings based on statistical analysis (*t*-test), studies with etiolated seedlings showed that *OGT* activities were enhanced by applications of the safeners dichlormid, benoxacor, oxabetrinil and cloquintocet mexyl. Similarly, studies with wheat seedlings have revealed that cloquintocet mexyl in particular is a strong enhancer of *OGT* activities towards similar substrates (Brazier, *et al.*, 2003). It would therefore be of interest to carry out metabolism studies in maize plants \pm safener treatment with some of the substrates shown to conjugate with greater activity following treatment with oxabetrinil or cloquintocet mexyl.

Standardised assays carried out on different tissue sources established that maize crude protein extracts contained enzymes capable of conjugating both natural and xenobiotic compounds using UDP-glucose as a substrate in *in vitro* assays. *GT* activities were found to be higher in shoot tissues compared to roots and also higher in etiolated tissues compared to light-grown ones. The substrate specificity being unpredictable, a range of substrates was used routinely to screen for optimal activities in the maize tissues tested. A limited list of substrates was found to be

active including a natural flavonol quercetin, and pesticide metabolites including *p*-nitrophenol, 2,4,5-TCP and 3,4-DCA. These observations highlighted that GTs were equally able to conjugate compounds from natural and xenobiotic origins with similar specific activities and were in accordance with xenobiotic preferred substrates (Pflugmacher, *et al.*, 1998). It was however not possible to confirm whether the enzymes involved in the glucosylation of xenobiotics and natural products were similar or not. Based on the differential enhancement of OGTs activities by the range of safeners, it would appear that the enzymes responsible for conjugating quercetin and 2,4,5-TCP cannot be identical. Determining the relationship between the GTs previously characterised in maize as conjugating phenolic secondary metabolites or plant hormones and the GTs involved in xenobiotic detoxification will be of particular interest.

Chapter 4 : Glucosyltransferases active towards xenobiotics in soybean

4.1 Introduction

The important worldwide cultivated crop soybean has been the subject of many pesticide metabolism studies and an abundant pool of data is available on the formation of conjugates of pesticides, some of which are reported to be glucosides. Soybean is well known to metabolise herbicide metabolites arising from phase I metabolism by *O*- and *N*-glucosylation. Back in 1968, an UDP-glucose arylamine *N*-glucosyltransferase having a broad substrate specificity towards arylamines was isolated from soybean by purification and some of its properties elucidated (Frear, 1968). Subsequently, the triazinone herbicide metribuzin was reported to be glucosylated by an *NGT* in soybean (Bieseler, *et al.*, 1992; Frear, *et al.*, 1983). Similar observations on the formation of *N*-glucosides with the herbicide were reported in tomato cell cultures (Davis, *et al.*, 1991). *N*-glucosylation of the pesticide metabolite 3,4-DCA has also been reported in soybean excised leaves and cell suspension cultures where it was a minor route of metabolism as compared with *N*-malonylation (Gareis, *et al.*, 1992). The slow formation of 3,4-DCA-*N*-glucosides could be accounted for the low *NGT* activities as compared with the high *N*-malonyltransferase activities towards 3,4-DCA (Schmidt, *et al.*, 1995).

The metabolism of the diphenyl ether herbicide acifluorfen has been shown to involve glucosylation. Following cleavage of the diphenyl ether bond, the phenolic entity released undergoes glucose conjugation (Frear, *et al.*, 1983). The isolation of glucosides and malonyl-conjugates has also been reported for the xenobiotic pentachlorophenol in soybean (Schmitt, *et al.*, 1985) and an acid metabolite of the chlorinated organic DDT (Wetzel and Sandermann, 1994). The glucosylation of *p*-nitrophenol has also been studied in excised soybean leaves and cell suspension cultures (Schmidt, *et al.*, 1993). The glycosidic conjugates formed by the

biotransformation were subsequently identified as 1-(*O*- β -D-glucopyranosyl)-4-nitrobenzene by TLC, HPLC, ^1H - and ^{13}C -NMR spectroscopy in soybean suspension cultures (Knops, *et al.*, 1995).

NGT and *OGT* activity towards xenobiotics has been reported in soybean cell cultures. Substrates included 6- and 8-hydroxybentazone, 5-hydroxydiclofop, as well as the herbicide chloramben and contaminants such as 3,4-DCA and 2,4-dichlorophenol (Gallandt and Balke, 1993). A UDP-glucose 6-hydroxybentazone glucosyltransferase which also showed activity towards the herbicide chloramben were subsequently partially purified from soybean (Gallandt and Balke, 1995). In a separate study, two GT enzymes involved in the conjugation of 6-hydroxybentazone was reported in soybean culture, one isoenzyme also having activity towards the flavonol kaempferol and the other towards *p*-hydroxyphenylpyruvic acid. From this, it was concluded that in soybean, several GTs with overlapping specificity were involved in the conjugation of herbicide derivatives and natural compounds (Leah, *et al.*, 1992).

In vitro conjugation by *NGT* and *OGT* was determined against chlorinated phenols and anilines in soybean (Sandermann, *et al.*, 1991). A screen of *in vitro* GT activities against a range of substrates including chlorinated phenols was carried out on a wide range of plant species (Pflugmacher and Sandermann, 1998) and the formation cleavage of conjugates of chlorinated anilines studied in soybean (Winckler and Sandermann, 1992).

In this chapter the role of *NGTs* and *OGTs* in the detoxification of xenobiotics in soybean has been examined. This has included a seedling feeding study with [^{14}C]-*p*-nitrophenol as a *OGT* model substrate. The effect of safener on the uptake and distribution of *p*-nitrophenol *in planta* was also tested. GT activities in soybean were studied *in vitro* after optimisation of the respective enzyme assay. Several soybean tissues were then tested with a range of putative substrates in order to gain knowledge of the substrate profile and optimal sources of GT activities. Finally, soybean plants have been exposed to treatments with chemical compounds and safeners in order to test the inducibility of the GT enzymes in this dicotyledonous species.

4.2 Glucosylation of radiolabelled xenobiotics in soybean

The xenobiotic *p*-nitrophenol was used as a model substrate to study its *O*-glycosylation in plants. Soybean seedlings were grown for a fortnight on vermiculite and watered with and without the addition of the safener oxabetrinil (10 mg l⁻¹). The safener was chosen as it exhibited the strongest effect on GT specific activity (Figure 4.9) The leaves above the cotyledons (see Figure 4.1) were detached and used in stem feeding studies with [¹⁴C]-*p*-nitrophenol (33.3 kBq corresponding to 150 µM of *p*-nitrophenol). The seedlings were harvested after 4, 8, 12, 24 and 48 hours and analysed for radioactivity. Analyses focused on 3 fractions: the plant extracts, the medium and the non-extractable residues. For each time point, two applied batches of tissues were collected. In each case, recoveries of applied radioactivity were determined and dpm values converted to the concentration of *p*-nitrophenol equivalent based on the specific activity of the administered radiolabel and the volume.

Over the first 4 hours, only half of the initial radioactive dose (78 µM; 52% of the initial dose) was still present in the medium with 72 µM (48%) recovered in the plant extract. Only a minor fraction (1 µM, <1%) was recovered in the non-extractable residues (Figure 4.2A). Similar distributions of radioactivity were determined in the oxabetrinil-treated plants with 81 µM recovered in the medium (54%), 66 µM in the plant extracts (44%) and 1 µM (<1%) in the non-extractable residues (Figure 4.2B).

After 8 hours, only 3 µM from the initial 150 µM were still present in the bathing medium of the control plants. As compared with maize feeding studies, soybean seedlings took up the whole solution and microfuge tubes had to be refilled to avoid seedling to dry during the time course. In contrast, the uptake of radiolabel in the safener-treated plants was lower. In the untreated plants, after 8 hours the radioactivity was largely recovered in the extractable fraction of the leaf tissue.

In the safener-treated plants, uptake continued until 12 hours before equilibrium in the distribution of label between plants and media was obtained. In all cases, the

quantity of label associated with the non-extractable residues remained low over the whole time course.

From this, it was concluded that excised soybean seedlings rapidly took up [¹⁴C]-*p*-nitrophenol, largely through transpiration with the entire label absorbed within 12 hours. This efficient uptake, resulting in the whole dose to enter the plant, was not associated with any visible signs of phytotoxicity. At all time points, very little of the radioactivity became associated with the insoluble fraction, with a high percentage of the administered label being recovered in the extractable fraction. Recovery data obtained after summation of the radioactivity collected for each fraction are presented in Table 4.1. When analysed by autoradiography, the incorporated radioactivity was seen to be concentrated in stems and vascular tissues of the leaves (Figure 4.3).

Statistical analysis comparing the plant tissues treated or not with safener did not result in a significant difference in the uptake and distribution of the xenobiotic over time with *P* value of 0.0903 with $P < 0.05$. The hypothesis (H_0) tested was accepted. The treatment with safener did not affect the uptake and distribution of the xenobiotic during the time course.

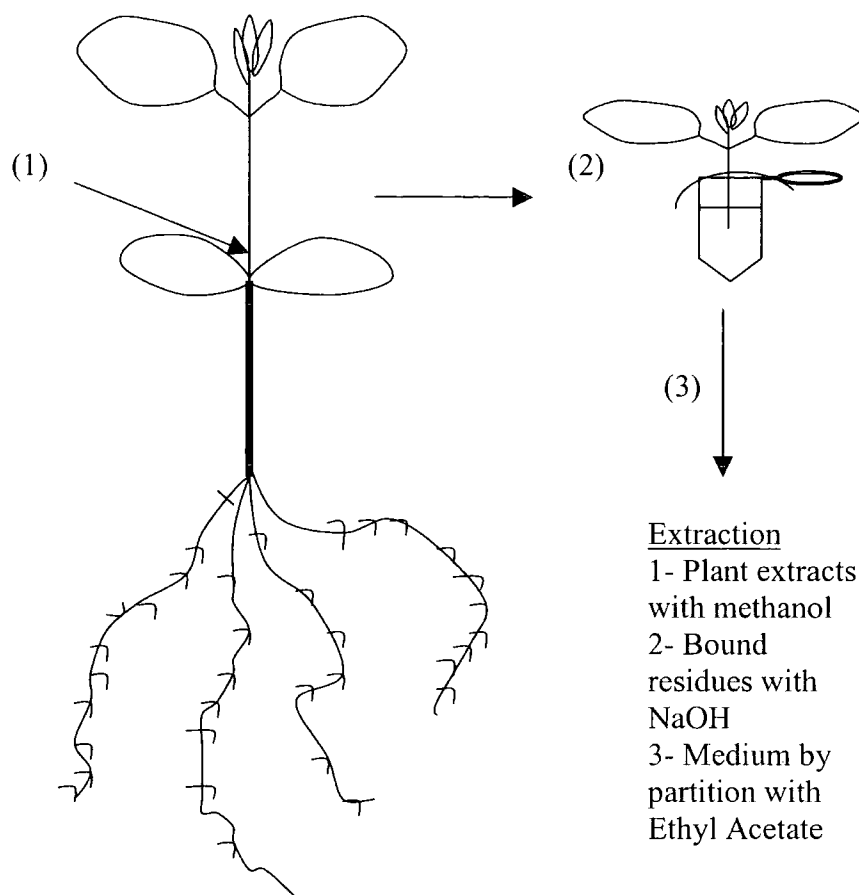


Figure 4.1 : Detail of the feeding study experimental

Soybean seedlings were grown in vermiculite for 10 days. The leaves were separated from the root system and immersed in the feeding solution composed of the radiolabelled xenobiotic (dissolved in 5 μ l of ethanol) homogenised in 1ml of water. The tubes were sealed with Parafilm[®] to avoid evaporation and to allow the plant to stand in the tube. Samples were harvested at time intervals.

(1) : The upper part of the stem was separated above the cotyledons with a blade in water to avoid any air-bubble to enter the vascular system

(2) : The stem was transferred to the feeding solution in a microfuge tube closed with parafilm.

(3) : During the feeding period, the plants were kept under constant light in a fume hood with aspiration prior to harvest and further extraction.

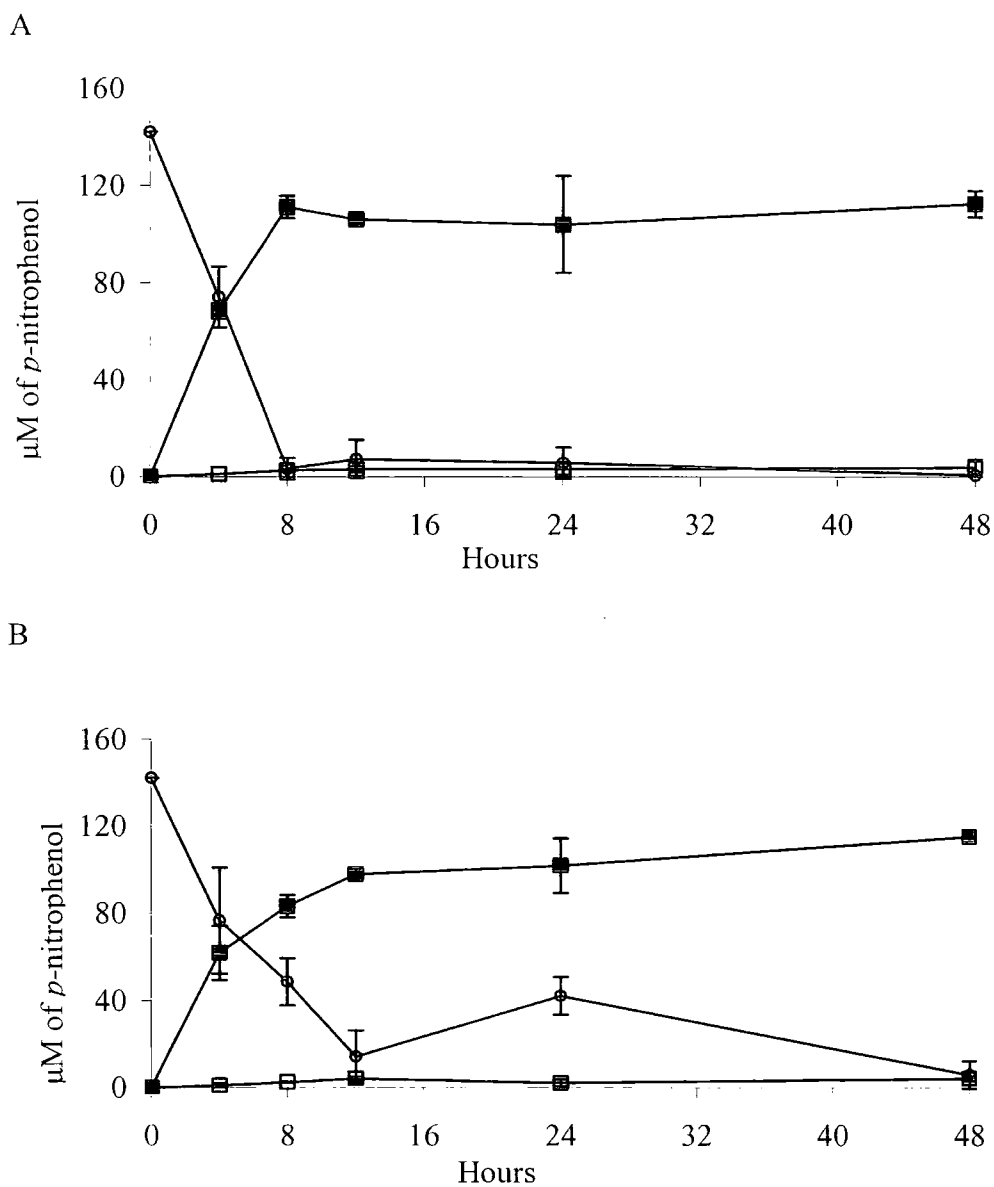


Figure 4.2 : Uptake of [^{14}C]-*p*-nitrophenol in soybean leaves.

Soybean leaves from seedlings which had been pre-treated in the presence (B) or absence (A) of the safener oxabetrinil were fed with [^{14}C]-*p*-nitrophenol (33.3 kBq) and the level of radioactivity (in nmoles) monitored in the feeding solution (○), the extractable fraction from the leaves (■) and the insoluble fraction (□). Data are the mean \pm standard deviation (n=2).

Table 4.1: Percentage total recovery of applied radioactivity after feeding [^{14}C]-*p*-nitrophenol (150 μM) to soybean seedlings.

Recoveries were obtained after summing the radioactivity present in each of the three fractions recovered from the metabolism study. Data are the mean \pm standard deviation (n=2).

Hours (h)	Recoveries (%)	
	Untreated tissues	Oxabetrinil-treated tissues
4	102.5 \pm 6.4	98.7 \pm 8.1
8	82.3 \pm 0.4	95.8 \pm 4.3
12	82.1 \pm 4.0	82.2 \pm 6.9
24	79.3 \pm 11.2	103.5 \pm 1.8
48	82.2 \pm 1.2	88.1 \pm 5.2



Figure 4.3: Localisation of radioactive residues in soybean leaves fed with [^{14}C]-*p*-nitrophenol.

Autoradiograph of soybean seedlings each fed with 33.3 kBq of [^{14}C]-*p*-nitrophenol for 2 days prior to exposure to film.

4.3. GT activities in soybean

4.3.1 Whole plant studies

GT activities were determined in soybean seedlings based on a method developed for the detection of GTs in alfalfa (Parry and Edwards, 1994).

As a preliminary study, soybean seedlings were grown for 15 days in the presence or absence of the safener dichlormid, which was administered 24 hours prior to harvest at a concentration of 10 mg l⁻¹. The tissues were separated into root and shoot tissues and assayed separately. All studies were carried out on duplicate treatments. Enzyme specific activities were determined after quantifying the protein content and correcting for the specific activity of the UDP-[¹⁴C-UL-glucose] to express results as product formation in pmole min⁻¹ mg⁻¹ protein. OGT activity was determined towards a panel of synthetic and natural product phenolic substrates.

From the 20 substrates tested, only a limited number gave measurable GT activities under the conditions described for the assay. Thus, seven substrates including phenol, *p*-nitrophenol, 2,4,5-TCP, quercetin, luteolin, genistein, and coumestrol were all identified as GT substrates in soybean plants (Table 4.2).

Unfortunately, due to a fault in the setting for the scintillation counter the GT activities in the control shoots were not comparable to those determined in the safener treatments and have had to be omitted from the results, as this change in counting conditions was only determined some time after the experiment was performed. In the safener-treated shoots, as determined in maize, the optimal substrates were 2,4,5-TCP and quercetin. In the roots, activity towards phenol, genistein and coumestrol could not be detected in any of the samples with activities towards luteolin and *p*-nitrophenol being much lower in roots than shoots. However activity towards quercetin was higher in roots than in shoots. Interestingly, safener

treatment enhanced GT activities in the roots, notably towards 2,4,5-TCP, with the corresponding activity not being detected in the untreated roots.

OGT activities were determined based on an existing method (Parry and Edwards, 1994). In the case of NGT activities using 3,4-DCA as substrate, it was necessary to omit adding the acid at the end of incubations to promote efficient recovery of the 3,4-DCA glucoside into the organic phase.

In all cases, conditions for GT assays in soybean tissues had to be optimised regarding the quantity of crude protein extract, the time, the temperature and the presence of salt ions. In particular, the low concentration of UDP-[¹⁴C-UL-glucose] in the assay required careful control of the protein content and incubation time to ensure strict dependence of product formation on the amount of enzyme present.

As a first step, the dependence of product formation on the content of crude protein was determined for each assay. Two substrates were assayed in the protein dependence experiments, the OGT substrate quercetin and the NGT substrate 3,4-DCA. The product formation for 3,4-DCA as a substrate was 0.460 ± 0.00 dpm/ μ g of protein and 2.76 ± 0.01 dpm/ μ g of protein for quercetin. Activities towards 3,4-DCA were low and product formation was shown to be dependent on protein content up to 200 μ g of crude protein per assay. In the case of quercetin, product formation was dependant on protein content between 80 and 160 μ g, with signs of product formation becoming saturating at higher protein contents. It was therefore decided to use 100 μ g of crude protein extract in all further assays unless stated otherwise (Figure 4.4).

Since the glucose acceptors were not water soluble, it was possible that proteins present in the assay may have been binding substrates and thereby altering their availability for glucosylation. To test this, an increasing quantity of crude protein extract was used in the assay, supplemented with or without 1 mg ml⁻¹ of bovine serum albumin as a source of protein carrier for the substrate (Figure 4.5). The similarity in results obtained with GT activities determined towards quercetin and 3,4-DCA supplemented with or without BSA confirmed that the protein dependence of the assay was due to the presence of varying amounts of enzyme catalysts rather than being due to protein binding effects.

Table 4.2: GT activities determined in soybean tissues.

Soybean seedlings (15 day-old) were treated with or without dichlormid (10 mg l⁻¹) 24 hours prior to harvest. Data are the mean ± standard deviation (n=2).

ND = activity not determined.

	Specific activity in (pmole min ⁻¹ mg ⁻¹ protein)		
	Treated shoots	Control Roots	Treated roots
Phenol	0.08 ± 0.00	ND	ND
<i>p</i> -nitrophenol	0.12 ± 0.00	0.08 ± 0.04	0.25 ± 0.07
2,4,5-TCP	0.68 ± 0.02	ND	0.43 ± 0.00
Quercetin	0.87 ± 0.00	1.75 ± 0.01	1.97 ± 0.09
Luteolin	0.18 ± 0.02	0.06 ± 0.03	0.11 ± 0.00
Genistein	0.13 ± 0.00	ND	ND
Coumestrol	0.16 ± 0.02	ND	ND

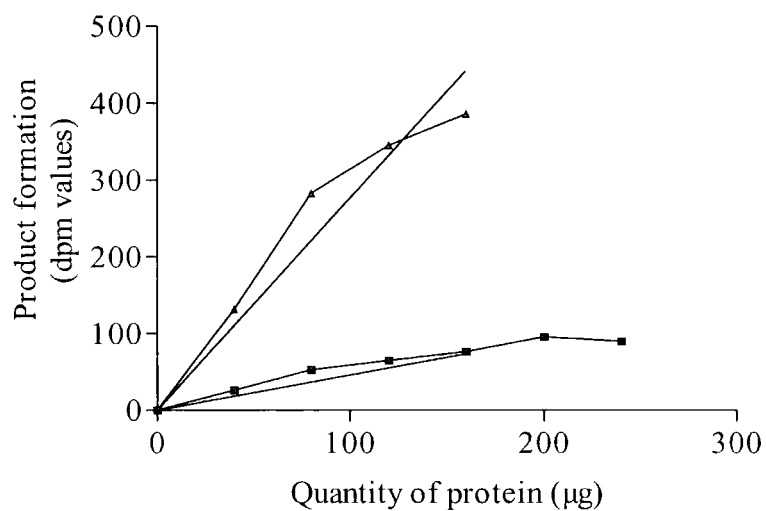


Figure 4.4: Protein dependence on GT activity.

GT activities were determined in crude soybean extracts using quercetin and 3,4-DCA as substrates. Product formation using quercetin (▲) and 3,4-DCA (■) as substrates is given as dpm of radioactive product formed; per assay in the presence of increasing protein quantity. All other components of the assay were as described in the standard protocol.

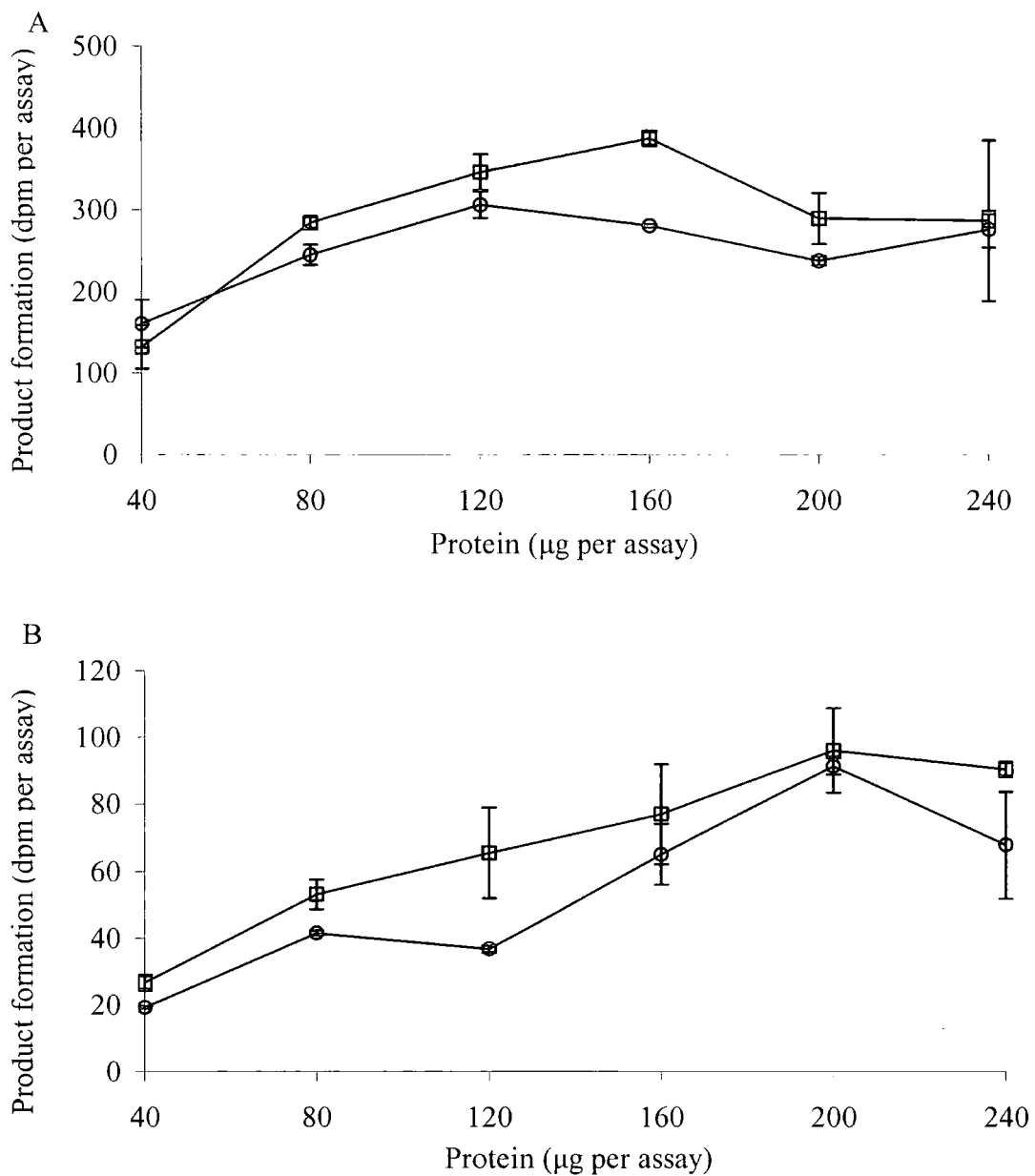


Figure 4.5: Effect of the presence of carrier proteins on the GT assays.

Dependence of product formation (dpm product formed per assay) on increased crude protein quantity in the presence (\square) or absence (\circ) of added BSA (1mg ml^{-1}). GT activity was determined with quercetin (A) and 3,4-DCA (B) as substrates. Crude protein extracts were obtained from 15 day-old soybean tissues.

The optimisation of the pH of the reaction and the dependence on assay buffer were determined using extracts from shoot tissues assayed in Tris-HCl buffer ranging from pH of 7 to 9.5, with Bis-Tris buffers ranging from a pH 6 to pH 7 and Tricine buffer ranging from pH 7.5 to pH 8.5. Optimum pH was determined to be pH 8 for Tris-HCl buffer and pH 7.5 for Bis-Tris or Tricine buffer (Figure 4.6).

To ensure linearity between product formation and the time of incubation, an assay was carried out over 90 minutes at 30°C. The substrates tested were quercetin and *p*-nitrophenol with product formation monitored at timed intervals. Product formation with both substrates increased linearly with time up to 40 minutes. Quercetin formation followed a linear regression of $2.5 \times 10^{-2} \pm 0.0$ pmole of product formed/min with $r^2 = 0.963$ but a non linear regression for *p*-nitrophenol where $r^2 = 0.209$ with a regression of $10^{-4} \pm 0.00$ pmole of product formed/min. However, for all further assays, a 20-minute incubation period was found to be a good compromise to ensure adequate product formation which was strictly dependent on incubation time (Figure 4.7).

In order to gain information on the stability of the enzyme, its resistance to heat treatment was checked by incubating the crude protein extract for 30 minutes in a water bath at 30°C, 37°C and 50°C prior to assaying for activity using quercetin as substrate (Table 4.3). A sample of crude protein extract kept on ice and its activity representing the control level in this experiment. OGT activity towards quercetin was significantly decreased as the temperature of pre-incubation of the crude protein sample increased. This experiment revealed the vulnerability of GT enzymes to high temperatures. Procedures were therefore designed to ensure the protein samples were kept at 4°C on ice for optimal stability of the enzyme outside of the time of the assay.

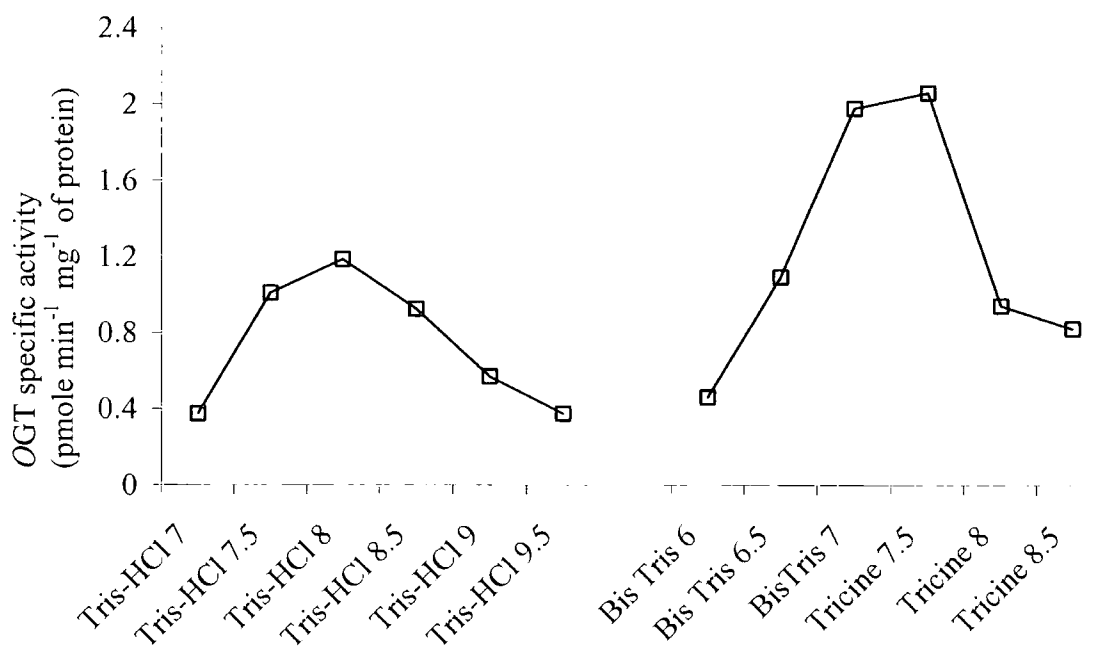


Figure 4.6: Influence of the reaction buffer at various pHs on the GT assay.

Determination of soybean *OGT* specific activity towards quercetin in the presence of different buffers and pH.

Crude protein extracts were obtained from 15 day-old soybean tissues.

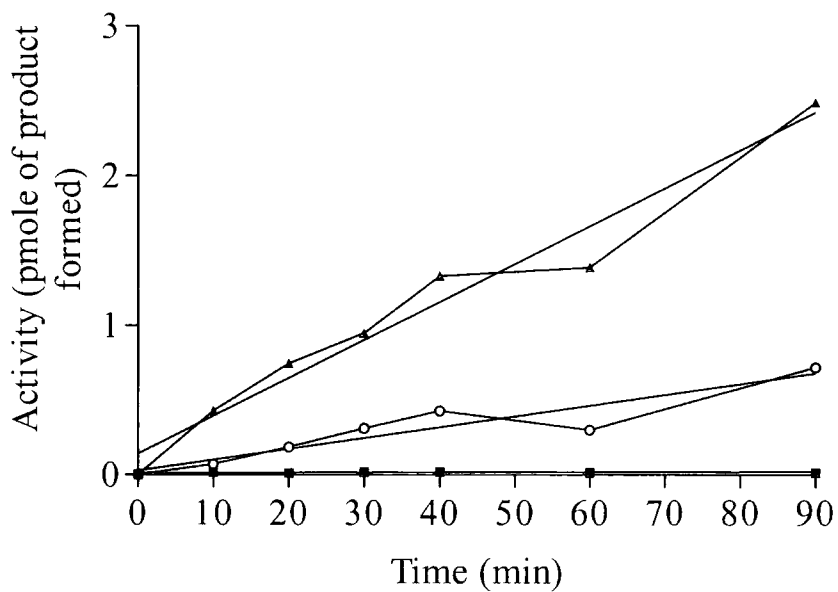


Figure 4.7: Effect of time of product formation in assay of GTs.

Product formation was determined in pmole using quercetin (▲) and *p*-nitrophenol (▽) as substrates. Radioactive products formed in the absence of added acceptor are shown for reference (■).

Crude protein extracts were obtained from 15 day-old soybean tissues.

Table 4.3: Effect of heat on GT activities extracted from soybean foliage.

Percentage of *OGT* activity towards quercetin remaining after a 30 min heat treatment as compared to a control sample kept on ice and assayed in standard conditions. The stability of the enzyme was tested at 30°C, 40°C and 50°C.

Temperature pre-treatment (30 min)	Percentage activity *
30°C	60 ± 6
40°C	45 ± 0
50°C	11 ± 1

* Compared with control protein extract (protein extract kept on ice until the time of assay).

In order to gain background information for purification work and to identify potential GT inhibitors, assays were carried out after adding divalent cations, and increasing the ionic concentrations of the assays (Table 4.4). GT activity was moderately inhibited by the addition of EDTA, calcium ions (Ca^{2+}) or magnesium ions (Mg^{2+}). The impact on GT activity was most significantly affected by the addition of manganese ions (Mn^{2+}) with a knockdown of more than 80% of the specific activity. However, it was subsequently determined that the Mn^{2+} was binding to quercetin, effectively reducing the availability of the substrate (Brazier, *et al.*, 2002). Increasing salt concentrations also inhibited the activity.

The experiment highlighted the sensitivity of the enzyme to manganese ions and to the presence of salt and moderate sensitivity to EDTA, Ca^{2+} and Mg^{2+} . During the purification process, it was therefore concluded that desalting would be required prior to accurately determining OGT specific activities.

Table 4.4: Effect of the presence of ions or salt on GT activities.

GT activities were determined towards quercetin in assays supplemented by various ions or increasing salt concentration using crude extracts from soybean shoots (15 day-old).

Addition to assay	Specific activity ($\text{pmole min}^{-1} \text{mg}^{-1} \text{protein}$)
Standard assay	1.87
2 mM EDTA	1.30 ± 0.02
2 mM Ca^{2+}	1.35 ± 0.14
2 mM Mg^{2+}	1.23 ± 0.24
2 mM Mn^{2+}	0.32 ± 0.05
0.1 M NaCl	0.51 ± 0.06
0.2 M NaCl	0.38 ± 0.08
0.5 M NaCl	0.22 ± 0.00

4.3.2 Cell culture

Soybean cell suspension cultures were obtained through collaboration with IACR-Rothamsted and tested as a source of GT activities. Cultures (7 days after sub-culturing) were extracted after a 24 hour treatment with either the isoflavone formononetin or 3,4-DCA. Formononetin was used as it had been shown to enhance GT activity towards flavonoids in chickpea (Mackenbrock, *et al.*, 1993). It was also of interest to determine whether the presence of increased concentration of GT substrates, such as 3,4-DCA, could have an effect on the conjugating activity of the enzyme.

OGT specific activities were then determined in the protein preparations from the cells using quercetin and 2,4,5-TCP as substrates. As compared with soybean plants, specific activities were higher than in the soybean suspension cultures. OGT activity towards quercetin was found to be enhanced in the cell cultures treated with either formononetin or 3,4-DCA (Table 4.5). Similarly, activity towards 2,4,5-TCP was also enhanced by the chemical treatments of the suspension cultures.

Soybean cell cultures featured higher GT activities and were shown to be inducible by chemical treatment. Unfortunately, technical problems relating to the maintenance of the cultures did not allow further experiments on this type of tissue.

Table 4.5: GT activity extracted from soybean cell cultures.

OGT activities were determined in 7 day-old cultures after a 24 hour-treatment with 500 μM formononetin or 500 μM 3,4-DCA. Data are the mean \pm standard deviation (n=2).

Treatment	Enzyme activity ($\text{pmol product min}^{-1} \text{mg}^{-1} \text{protein}$)	
	Substrates	
	Quercetin	2,4,5-TCP
Untreated	1.05 \pm 0.64	1.90 \pm 0.85
Formononetin (500 μM)	2.30 \pm 0.00	3.55 \pm 0.35
3,4-DCA (500 μM)	4.00 \pm 0.28	5.60 \pm 0.00

4.3.3 Assays on etiolated soybean

Cell cultures had higher *OGT* specific activity as compared to light-grown whole plant tissues (Table 4.5 and Table 4.2), but in the absence of reliable sources of cultures, alternative sources of enhanced GT activity in soybean were screened for. Soybean seeds were grown in darkness and the etiolated seedlings harvested after 6 days and the proteins extracted following standard procedures. GT activities were then determined using a wide range of substrates of which the most preferred are presented in Table 4.6. The optimal substrate by far was determined to be 2,4,5-TCP being 10 times more actively conjugated than any other substrate. The specific activities against all substrates were greater in etiolated soybean than those determined in light-grown plants.

In order to establish the chemical inducibility of GT enzymes in etiolated soybean, a similar experiment as the one carried out on cell cultures was initiated. Etiolated soybean seedlings were grown for 4 days before being transferred onto Petri dishes containing 50 μM inducing solutions. The seedlings were then kept in the dark for 48 hours.

In the first experiment, the ability of the safener dichlormid to enhance *OGT* activities was tested. In the untreated plants, *OGT* activity towards 2,4,5-TCP was virtually identical to that determined in the earlier study while activity towards quercetin and *p*-nitrophenol was higher. With all substrate treatments dichlormid actually reduced, rather than increased, *OGT* activities (Table 4.7). No statistical data are relevant in this case as the only purpose was to gain information on the enzyme activity.

Table 4.6: GT activity in extracts from etiolated soybean (6 day-old).

Data are the mean \pm standard deviation (n=2).

Substrates	Specific activity ($\text{pmole min}^{-1}\text{mg}^{-1}\text{ protein}$)
2,4,5-TCP	9.17 ± 0.11
Quercetin	0.83 ± 0.15
<i>p</i> -nitrophenol	0.08 ± 0.01
Phenol	0.33 ± 0.03
Cyanidin chloride	0.15 ± 0.02

Table 4.7: Optimisation of chemical induction of OGT activities in etiolated soybean seedlings.

OGT enzyme activity in extracts from etiolated soybean (6 day-old) treated with 50 μM inducing treatment for 48 hours prior to harvest.

Data are the mean \pm standard deviation (n=2).

Substrates	Specific activity ($\text{pmole min}^{-1} \text{mg}^{-1} \text{protein}$)	
	Untreated tissues	Dichlormid-treated tissues
<i>p</i> -nitrophenol	0.2 ± 0.1	0.1 ± 0.0
Quercetin	2.8 ± 0.1	1.6 ± 0.3
2,4,5-TCP	8.8 ± 3.1	3.2 ± 0.9

Experiments were then performed with etiolated soybean seedlings treated with synthetic and endogenous substrates of *OGTs*, namely 2,4,5-TCP, salicylic acid, formononetin, and 3,4-DCA for *NGT*. The treatments consisted of exposing the seedlings to 500 μM treatments of the chemicals for 48 hours prior to harvest. The results demonstrated that formononetin enhanced *OGT* activity towards both 2,4,5-TCP and quercetin, 2 fold and 3 fold respectively. All of the other chemical treatments gave no induction of *OGT* activities (Table 4.8).

Finally, a modified protocol was applied to test the ability of a broader range of safeners to induce *GT* activity in etiolated soybean seedlings. Etiolated soybean seedlings (7 day-old) were cut at the bottom of the stem and stood in a tube containing a small volume of inducing solution (10 mg l^{-1}) for 2 hours before harvest of the tissues. Two populations, each of 20 seedlings, were treated and assayed separately and their specific activities listed in the Table 4.9.

The experiment of induction of *GTs* on soybean revealed that safener treatment had a limited effect on *NGT* activity towards 3,4-DCA with activities being low in all cases and any enhancement falling within the limit of variation of the replicates.

In the case of the substrate quercetin, isoxadifen-ethyl repressed the activity to 0.3 $\text{pmole min}^{-1} \text{mg}^{-1}$ protein. CMPI and cloquintocet mexyl left the activity unchanged. An enhancement was seen with dichlormid, fenclorim and flurazole. Benoxacor and mefenpyr-diethyl also enhanced the specific activity. Oxabtrinil and R-29148 were found to be optimal inducers. *OGT* activities with 2,4,5-TCP proved less responsive to safeners, with only oxabtrinil > isoxadifen ethyl > R-29148 giving a modest enhancement in activity.

This study illustrated the effect of safeners on the *GT* specific activity. The hypothesis that safeners could enhanced the *GT* activity was based on similar studies carried out on known inducible detoxifying *GST* enzymes; this work was part of this systematic approach to characterise the effect of safeners on detoxifying enzymes (data notshown).

Table 4.8: Chemical induction of *OGT* activities from etiolated soybean.

OGT activities towards quercetin and 2,4,5-TCP determined on 6 day-old etiolated soybean tissues treated for 48 hours with 500 μ M inducing solutions of formononetin, salicylic acid, 3,4-DCA or 2,4,5-TCP.

Data are the mean \pm standard deviation (n=2).

Inducing treatment	Specific activity ($\text{pmole min}^{-1} \text{mg}^{-1} \text{protein}$)	
	Substrates	
	Quercetin	2,4,5-TCP
Control sample	2.48 \pm 0.02	1.81 \pm 0.10
Formononetin	7.90	3.85 \pm 0.14
Salicylic acid	2.40 \pm 0.05	1.87 \pm 0.10
3,4-DCA	1.53	2.59 \pm 0.20
2,4,5-TCP	0.86 \pm 0.00	1.34 \pm 0.21

Table 4.9: GT activities in etiolated soybean seedlings treated with a range of safeners.

Seedlings were grown for 7 days and treated for 24 hours with safeners prior to harvest. Data are the mean \pm standard deviation (n=2) except with the safener benoxacor, where activities were determined only on a single population, due to the loss of a treatment replicate.

Safener treatment	Specific GT activity ($\text{pmole min}^{-1} \text{mg}^{-1} \text{protein}$)		
	Substrates		
	3,4-DCA	Quercetin	2,4,5-TCP
Acetone control	0.2 ± 0.1	1.0 ± 0.4	5.1 ± 0.6
Benoxacor	0.2	3.0	6.2
Cloquintocet mexyl	0.2 ± 0.0	1.3 ± 0.2	5.7 ± 0.3
CMPI	0.2 ± 0.0	1.0 ± 0.1	6.5 ± 0.6
Dichlormid	0.2 ± 0.1	2.0 ± 1.4	6.2 ± 0.8
Fenclorim	0.3 ± 0.0	2.4 ± 0.6	5.4 ± 0.6
Flurazole	0.3 ± 0.1	2.4 ± 1.1	6.5 ± 1.4
Isoxadifen-ethyl	0.3 ± 0.1	0.3 ± 0.8	7.9 ± 1.3
Mefenpyr-diethyl	0.2 ± 0.1	3.2 ± 0.7	5.1 ± 0.5
Oxabetrinil	0.4 ± 0.3	4.1 ± 1.3	9.0 ± 1.6
R-29148	0.3 ± 0.0	4.1 ± 0.2	7.2 ± 1.6

4.4 Discussion

Metabolism studies with *p*-nitrophenol highlighted that the soybean plants took the compound up quickly, though the nature of the metabolites formed in the plants was not undertaken as previous studies have shown that this compound is rapidly glucosylated in soybean cell suspension cultures (Knops, *et al.*, 1995), in aseptically cultivated soybean plants (Malcherek, *et al.* 1998) and soybean excised leaves and cell-suspension cultures (Schmidt, *et al.*, 1993). The metabolism of *p*-nitrophenol has also been extensively studied in plants of wheat, wild oat and corn cockle (Malcherek, *et al.* 1998), wheat cultures (Schmidt, *et al.*, 1993), *Datura* (Schmitt, *et al.*, 1997) and carrot (Schmidt, *et al.*, 1994). In all cases studied, *p*-nitrophenol-conjugates did not constitute a long-term storage form of this xenobiotic in plants with the associated residues being transported to the aerial parts of the plants in wild oat and corn cockle (Malcherek, *et al.*, 1998).

NGT, OGT, NMT and OMT activities have previously been reported in soybean cell cultures (Pflugmacher and Sandermann, 1998; Sandermann, *et al.*, 1991; Schmidt, *et al.*, 1995). An OGT enzyme involved in the detoxification of the pesticide DDT has also been isolated and characterised (Wetzel and Sandermann, 1994) representing one of the few examples of a GT enzymes involved in pesticide detoxification being purified in a crop plant. Other examples of GT activities which have been partially purified including those active in the glucosylation of hydroxybentazone in soybean (Leah, *et al.*, 1992) and in *Avena sativa* (Thomas, *et al.*, 1964).

In vitro assays using extracts from soybean tissues confirmed high GT specific activities towards 2,4,5-TCP and the natural substrate quercetin. In contrast, NGT activity was found to be higher in dark-grown tissues compared to light-grown ones. Although most studies reporting GT activities in soybean used cell cultures (Pflugmacher and Sandermann, 1998; Schmidt, *et al.*, 1995), technical difficulties of maintaining cell cultures did not allow routine assays to be carried out on this tissue

source and so the discovery of etiolated seedlings material as a GT source was useful.

The optimisation of assay conditions were carried out testing for protein dependence, pH of the buffer, time of incubation, resistance of the enzyme to heat treatment and presence of salts, EDTA, and ions. Standardised assays were determined to be carried out for 20 minutes in Tris-HCl pH 8, with 100 µg of crude protein extract with the enzymes kept on ice prior to the assay. These assay conditions were kept throughout the studies in all species tested.

Specific GT activities suggested a possible safener effect. The induction of detoxifying enzymes by safeners is much more commonly associated with cereals rather than dicotyledonous crops (Davies and Caseley, 1999). However, gene expression has been shown to be modified following exposure to safener treatment (De Veylder, *et al.*, 1997) and recent studies have shown that GSTs are induced by selected safeners in *Arabidopsis* (DeRidder, *et al.*, 2002). Interestingly, we have determined that OGT activities towards quercetin were the most responsive to safeners in soybean with the greatest induction seen with oxabetrinil and R-29148. These safeners also were the most active in inducing OGT activities towards 2,4,5-TCP, though it was clear that the GT activities towards the two substrates were independently regulated by the panel of safeners tested, suggesting different enzymes were responsible for the two activities. The optimisation of the safener regime might overcome the moderate variation observed in the specific activities following the exposure to safener. The study presented in this chapter constituted an initial study to identify and confirm the presence GT specific activities *in vitro* assays. The safener treatment applied to seedlings prior feeding the xenobiotic did not result in a modification in the profile of uptake and distribution of model xenobiotic *in vivo*.

Chapter 5 : Molecular cloning and expression of maize and soybean GTs in *E.coli*

5.1 Introduction

The cloning and functional expression of GTs in bacterial hosts is a useful mean of characterising these labile enzymes, due to the difficulties of purifying respective proteins to homogeneity from plants. A number of type-1 GT sequences have now been cloned from several plants and have been shown to have various substrate specificities. The first successful cloning of a GT was achieved in maize with the cloning of the bronze-1 3-*O*-GT (Federoff, *et al.*, 1984). Later, an homologue in grape was isolated and its substrate specificity determined (Ford, *et al.*, 1998). Since that time, the publication of the *Arabidopsis* genome has been particularly useful in obtaining information on the diversity of type 1 GTs in plants. It has allowed the first exhaustive alignments of all the putative GTs and their grouping based on sequence homologies (Bowles, 2002; Lim, *et al.*, 2003; Paquette, *et al.*, 2003; Ross, *et al.*, 2001). The phylogenetic trees obtained have highlighted several groups of related sequences, though this has not always meant that substrate preferences are shared. Based on searches for putative GTs in the *Arabidopsis* genome using the conserved domains, 107 type 1 GT-like sequences can be identified. The cloning and testing for activity of these 107 GTs is currently in progress, though there have been few attempts reported to clone and characterise *Arabidopsis* GTs active in xenobiotic metabolism (Meßner, *et al.*, 2003).

The present chapter concentrates on the molecular cloning of GTs from the two crop species maize (*Zea mays*) and soybean (*Glycine max*). Several substrates were shown to be glucosylated both *in vivo* and *in vitro* (Chapter 3 and 4). The strategy of cloning developed in this chapter is based on the cloning of GTs and their overexpression in bacteria prior screening for substrate specificity using

biochemical analysis. Biochemical studies demonstrated that glucose conjugation was present in crude protein extracts. Feeding studies with the substrates (*p*-nitrophenol and 3,4-DCA) confirmed uptake and glucose conjugation in *in vivo* studies. The cloning strategy was aimed at identifying, cloning and determine the substrate specificity of some of the enzymes involved. Sequence comparisons were also used to predict putative GT sequences and were aimed at collect data for future prediction of sequences with substrate specificity of interest.

The first report of a successful cloning of a GT from maize dates back to 1984 with the cloning of the bronze-1 locus (Federoff, *et al.*, 1984). Related sequences were consequently analysed and compared (Ralston, *et al.*, 1988). Since then, there have been other reports of cloned GTs from maize involved in the storage and regulation of the endogenous cytokinin, namely *cis*-zeatin glucosyltransferase (Martin, *et al.*, 2001). A maize *iaglu* gene was also was identified (Szerszen, *et al.*, 1994) which encoded an enzyme involved in the conjugation of the plant hormone indole-3-acetic acid (IAA). However, the respective recombinant protein was not isolated or biochemically characterised.

To date, there have been no reports of the cloning and expression of soybean GTs. This is in contrast with other dicotyledonous crops with several reports of active GTs being cloned and expressed including a sinapate GT in rape (Milkowski, *et al.*, 2000), a solanidine GT in potato (Moehs, *et al.*, 1997), an abscissic acid GT from adzuki bean (Xu, *et al.*, 2002) and an *S*-GT from *Brassica napus* (Marillia, *et al.*, 2001). In most cases, GTs have proved particularly difficult to express in bacteria as soluble active enzymes.

In order to clone GTs from maize and soybean, EST and genome databases were searched for conserved motifs or annotated sequences of type 1 plant GTs. In addition, it was also possible to use public EST databases to identify partial GT sequences and then assemble full-length sequences from the fragments. This proved particularly fruitful in maize and soybean through search of their relatively extensive EST databases.

Although this molecular approach represented an undirected search for xenobiotic-conjugating GTs, it did offer a powerful method to potentially isolate functional GTs as compared with conventional biochemical approaches. After isolating full-length GT sequences the intention was then to express the recombinant enzymes in *E.coli* and using them for GT activity towards natural and xenobiotic -OH and -NH₂ containing substrates.

5.2 Results

5.2.1 Maize *bronze-1* GT

The first GT reported to have been cloned was the *bronze-1* GT from maize (*Zea mays*) with specificity towards the 3-*O*-position of flavonoids (Federoff, *et al.*, 1984). The original cloning was based on identifying the site of insertion of a transposon in the locus of the *bronze-1* gene. Subsequently, several studies have reported the cloning of homologues to the *bronze-1* enzyme in related species including a partial sequence obtained in *Vitis vinifera* (Sparvoli, *et al.*, 1994) *Antirrhinum majus* (Sparvoli, *et al.*, 1994). Boss (Boss, *et al.*, 1996) based their cloning strategy on the *Vitis vinifera* partial sequence to isolate another *Vitis vinifera* GT from a different variety that was shown to influence the colours of berries depending on the expression of the enzyme (Boss, *et al.*, 1996). Conflicting information arose from various studies aimed at determining whether the same enzymes could be responsible for both the conjugation of flavonol and cyanidin compounds. While some enzymes could glucosylate both chemistries, Do proved that the partially purified grape GT had activity solely towards members of the anthocyanin family and not towards flavonols (Do, *et al.*, 1995). The GT was subsequently purified to homogeneity and cloned and its preference for anthocyanin over flavonoids substrates proved (Ford, *et al.*, 1998). The cloning of the Bz-1 enzyme was of interest as there was no report of investigation for its wider substrate specificity particularly towards glucosylating xenobiotics as such attempts were made to first clone and express Bz-1 from maize and test it for activities towards a diverse range of substrates.

Primers were designed based on the published sequences of *bronze-1* X13501 sequence. The primers were degenerate as they were designed to amplify Bronze-1 from any monocotyledonous species (Figure 5.1). PCR amplifications were attempted on cDNA prepared from 2 week-old maize seedlings grown

hydroponically under standard conditions. mRNA was extracted with TRI Reagent (Sigma) and its purity was checked on an electrophoresis gel prior to RT-PCR.

Despite repeated attempts, it was not possible to amplify any products using these primers even in more favourable conditions consisting in lowering the annealing temperature for the binding of the primers. It was concluded that the level of expression was probably too low in seedlings.

The initial report on the isolation of the *bronze-1* gene had used developing maize kernels (Fedoroff, *et al.*, 1984), a tissue technically more difficult and less abundant to obtain. In view of other developments in the lab, further attempts to PCR *bronze-1* were postponed in favour of using degenerate 'generic' GT primers to amplify a more diverse range of plant GTs.

Table 5.1 : Primer sequences used to amplify the *bronze-1* gene in maize.

Primer sequences	
Bz1-1	d(CA(C,G) ACG ATC TC(C,G) ACG AA(C,G) C(G,T)G T(A,C or G)G AAG TTC TT)
Bz1-2	d(GT(C,G) (A,T)(C,G)(C,G) TGC GTS GTS GGS GAC GCS TTC GTS TGG)

5.2.2 Cloning attempts using degenerate primers

63 GT sequences were aligned from various plants and the highly conserved domain, known as the PSPG box in the literature, identified as signatures of GT sequences (Hughes and Hughes, 1994). The PSPG box is located about 500 bp from the 3' end of the sequence and the amplification of DNA fragments from the PSPG box to the 3' end represented an ideal size of product for amplification using Polymerase Chain Reaction (PCR). Sequence analysis of the PSPG motif revealed 2 groups of sequences in which one amino acid would differ, which identified the need for the design of a second degenerate primer to cover all possibilities (Table 5.2).

Four partial sequences were obtained from maize and soybean after PCR amplification, using a mix of both the degenerate primers and the Og2 primer designed to bind the respective polyA tail of any mRNA. The sequences obtained were BLAST against the databases to check for homologies with GT sequences, focusing on the respective maize and soybean EST databases in order to obtain specific species-related comparisons. Although EST sequences are anticipated to have a relatively high error rate, they are sufficiently accurate to unambiguously identify the originating gene in most cases.

The maize libraries used in the following amplifications were kindly donated by Dr. David Dixon, who also assisted the alignments and primer design. Based on this PCR strategy, 4 partial sequences were amplified and sequences namely CL55 (Figure 5.1), CL33 (Figure 5.2), CL 34 (Figure 5.3) and CL32 (Figure 5.4).

Table 5.2 : Primer sequences for the degenerates primers.

Sequences were designed towards the 3' end of the GT sequence based on conserved domains highlighted after aligning 63 GT sequences from various sources. The Og2 sequence is a poly-T derivative primer to bind the mRNA poly-A tail. GT1 a and GT1 b were designed by Dr. David Dixon.

Name	Sequence
Og2 (<i>Bam</i> HI)	GAG AGA GGA TCC TCG AGT TTT TTT TTT TTT TTT T
GT1 a	TT(C,T) 5T5 (A,T)C5 CA(C,T) TG(C,T) GGT GGA A
GT1 b	TT(C,T) 5T5 AG(C,T) CA(C,T) TG(C,T) GGT GGA A

5 = inosine. Brackets refer to choice to base option.

The conserved motifs identified in the 63 GT sequences are represented by 3 major motifs as described below:

F	I	S	H	C	G	W	N	S
F	V	T	H	C	G	W	N	S
F	L	T	H	C	G	W	N	S

The comparison of the partial sequences against the databases positively identified them as GTs based on the presence of the conserved domains, particularly the PSPG box represented by the motif 'WNS' as described in Table 5.2 and underlined as amino-acid sequence in the respective figures (Figures 5.1, 5.2, 5.3 and 5.4).

A continuous sequence of DNA that has been assembled from overlapping cloned DNA fragments to build a contig was unsuccessful for 3 of the partial sequences namely for CL33, CL34, CL55. However, all these sequences had high homologies to GT sequences and their top hits after a BLASTX search are summarised in Table 5.3.

The isolation of only a limited number of ESTs for each of the 3 partial sequences only allowed an extension of several hundred base-pairs without full confidence of the accuracy of the sequence. The 5' rapid amplification of cDNA ends (RACE) method was therefore attempted to extend the sequence, but resulted in the loss of signals after further amplification rounds both for CL33 and CL34.

<u>TTCGTGAGTC</u>	<u>ATTGCGGGTG</u>	<u>GAACTCGGCG</u>	ACGGAGGCCA	TGGCGTCCGG
F V S	H C G W	N S		
CGTGCCCATC	GTCGGTGTGC	CCAACATGTT	CGACCAGCCG	ACGAACATGT
ACTTGGTCGA	GGAAGAGTGG	GGGGTCGGCG	TCAGAGGAGA	GCGAGACGCC
GATGGCGTGC	TGACAGGGGC	GGAGCTGGCA	AGGTGCATTG	AGCTGGTCAT
GGGTGATGGC	GCAGGAGCAG	TGGCGATCAG	GGAAAGGGCA	AAGGCCTTGC
AAGAGACAGC	GCAGGCGGCG	GCCAGTGCAG	CAGGCGGGCC	CGCCCGAGAC
AAACCTTTGT	CACCTCGTCA	AGACGATGTC	AAAATAATTA	TCAAAGCTGT
CGTAGAAAAT	TCAGTCTGCA	GGTCCATTCA	TGACAAGGAC	TTCTTTCAAT
AAAAAAAAAA	AAAAAAA			

Figure 5.1 : Partial nucleotide sequence of CL55.

The amplification from maize library used a mix of the two degenerate primers and the Og2 binding to the mRNA poly A tail. The presence of the conserved motif is highlighted underneath with the conserved amino acid identified.

<u>TGGTGGA</u> ACT	<u>CGGTG</u> TTGGA	AGCGATTTGT	GAAGGGGTTTC	CAATGGTGGC
W N	S			
GTGGCCTTTG	TACGCGGAGC	AGAAGCTGAA	TAGGGTGATT	TTGGTGGAGG
AAATGAAGGT	GGGGTTGGCG	GTGGAGCAGA	ACAATAACGG	GTTAGTGAGT
TCCACCGAGT	TGGGTGACCG	AGTCAAGGAG	CTCATGAACT	CGGATAGGGG
AAAAGAGATT	AGACAGAGGA	TTTTCAAAAT	GAAAAACAGT	GCCACCGAAG
CAATGACTGA	AGGTGGATCC	TCAGTAGTTG	CATTGAATAG	GTTGGTAGAA
ATATGGAGGG	AGCATTAAATG	GTATAGAACT	TGAGAATGAG	AGAATGATTT
AGAAATGTGC	ATGAGATTCA	GTATAAGAAC	TGAATTTCTT	TCTCAATAAA
GAATGAGCAC	GAGTTAAGTT	GTTGTAAATT	ATCGATGTTC	TGAATATGAA
TTCGGTCGTG	ATATTTATTA	CTATCTAAAA	AAAAAAAAAAA	AAAA

Figure 5.2 : Partial nucleotide sequence of CL33.

The amplification from etiolated soybean used a mix of the two degenerate primers and the Og2 binding to the mRNA poly A tail. The presence of the conserved motif is highlighted underneath with the conserved amino acid identified.

<u>TGGTGGA</u> ACT	CCATCCTAGA	GGCAGTGGCC	GCGGGCGTGC	CTATGCTCAC	ATGGCCAAAG
W N	S				
TTTAATGACC	AATTCGTCAA	CGAGAGGCTA	ATCAATGATG	TGCTAGGAAT	TGGGCACCGA
CTGTGGCCGC	ATGGCGCTGG	CTTACGGAGC	GAGGATTACG	AAAAACATGA	GCTGATCCCG
GCCGACGATG	TGGCCCGGGC	ATTGCTCACA	TTCATGCATC	CTGGAGGACC	TGGGGACGTT
ATGAGGACCA	GGGTAATGGA	CCTCGCCTCA	AAGTCTCATG	GCGCCCTCGC	AGAAGGAGGC
TCCTCGCAGC	AAGATTTGCA	CCGCCTCGTC	AACGATCTCA	TGGCAGCAAA	GGAGGGGCGG
AACTAGCTAG	CTTGCTAGCT	GCCGTGCATG	AGTTAATCGA	ACTCATCGCA	CCAAAGTAAA
TCATTCCTGT	TGCAATGCAC	GACGTATATA	TGTTTCCGTG	TAGTATTTGT	TTTGACATTC
AGTCTTTTCT	ACTTGCCTGT	TTTTTCTTCA	ATAAATAAAA	TAAAA	

Figure 5.3 : Partial nucleotide sequence of CL34 amplified from maize library.

The amplification from maize library used a mix of the two degenerate primers and the Og2 binding to the mRNA poly A tail. The presence of the conserved motif is highlighted underneath with the conserved amino acid identified .

Table 5.3 : Partial sequences amplified in crops and their closest hits.

Partial sequences were obtained after PCR amplification on various tissue sources using the combination of the degenerate primers with a primer designed to bind the polyA tail.

Clone Reference	Tissue source	Closest hit (Blast x)	Further cloning
CL32	Soybean tissues (15 d-old)	Hydroquinone glucosyltransferase <i>Rauvolfia serpentina</i> GenBank™ AJ310148	Cloned and overexpressed
CL33	4 days-old etiolated soybean	UDP-glycose:flavonoid glucosyltransferase <i>Vigna mungo</i> GenBank™ AB070743	5' RACE
CL34	Maize library	putative immediate-early salicylate-induced glucosyltransferase <i>Oryza sativa</i> AAP13007	5' RACE
CL55	Maize library	UDP-glucose:anthocyanin 5-O-glucosyltransferase <i>Perilla frutescens</i> GenBank™ AB013596	No further cloning attempt

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AATTCACTAG TGATTTTCGT GTCGCACTGC GGGTGGAACT CTATTCTGGA
          F V   S H C   G W N   S
GAGCGTGGTG AACGGTGTGC CTTTCATAGC CTGGCCTCTG TTCGCGGAGC
AGAGGACCAA CGCCTTTATG CTCACGCATG ACGTCAAGGT GGCCTGAGG
CCCAACGTTG CAGAGAGTGG CTTGGTGGAG AGACAGGAGA TAGCCAGCCT
AGTCAAGTGC CTCATGGAAG GTGAACAAGG GAAGAAGCTT CGTTACCGAA
TCAAGGATAT CAAGGAGGCT GCGGCTAAGG CTCTCGCTCA ACATGGTTCC
TCCACCACCA ACATCTCCAA TTTGGCTCTC AAGTGGACCC ACAAATTCAA
CTAGTAGCTT CTTCTTTTCA ATTATCTGCA CTTAACTTAT CAAATGTCNC
TTTGCATACA TGACATTAAT AAAATAATTG GGTAAGTGTA CGTAAATTTT
AAAAAAAAAA AAAAAAAAAA

```

Figure 5.4 : Partial nucleotide sequence of CL32 amplified from soybean.

The amplification from soybean tissues (15 days old) used a mix of the two degenerate primers and the Og2 binding to the mRNA poly A tail. The presence of the conserved motif is highlighted underneath with the conserved amino acid identified .

The same primer combinations were then used to amplify up GT sequences from soybean cDNA (15 day-old). A single product (Figure 5.4) was obtained with analysis of the sequence of soybean fragment CL32 identifying a number of soybean ESTs represented on a diagram (Table 5.4 and Figure 5.5) selecting for soybean ESTs eventually lead to the construction of a full-length contig based on soybean partial sequences only. The full-length cDNA was isolated after designing primers (Table 5.6) from the start and stop codons of the putative contig (Figure 5.6). The result of the search for homologues with the CL32 partial sequence revealed a high number of closely related EST sequences. A series of BLAST searches using the furthest EST towards the 5', allowed to extend the sequence from the 3' end to the 5' end of the gene. Sequences were manually checked, orientated and any inappropriate sequences discarded. They were then assembled in order to form a contig of overlapping sequences. The references for the 26 soybean ESTs used for the construct of the contig derived from CL32 are listed in Table 5.4 and schematic presented in Figure 5.5. The putative contig formed by the overlapping sequences allowed the confident prediction of an open reading frame and the location of the start and stop codons that would match standard GT criteria (Figure 5.6).

The number of sequences involved in the overlapping of fragments was accurate enough to predict a full-length ORF of 1431 bp (Figure 5.6) in accordance with average GT sizes reported in the literature. The predicted size for the resulting protein would be of 52.463 kDa (Figure 5.7) being composed of 476 amino acids with a theoretical pI of pH 5.60.

Although there was no predicted substrate for the putative enzyme, the closest hit after similarity based database searches revealed high homology to an arbutin synthase isolated from cell cultures of *Rauvolfia serpentina* which accession number was Q9AR73 (Arend, *et al.*, 2000; Hefner, *et al.*, 2002). Hefner (Hefner, *et al.*, 2002) demonstrated that the enzyme from *Arabidopsis* had the ability to conjugate both natural and xenobiotic compounds. This resulted in attempts to clone the CL32 contig from soybean into an expressing host.

PCR primers were designed to the predicted sequence of the 3' and 5' ends of the full-length contig, termed *GmGT1_32*, which included convenient restriction sites for subsequent cloning procedures (Table 5.5). Using these primers the GT was amplified from cDNA prepared from 14 day-old soybean tissue. A 1.4 kb fragment

was amplified and cloned into the His-Tag expression vector pET-24 (Novagen) prior to transformation into *E.coli* BL21(DE3) for expression. Attempts to overexpress the recombinant protein under standard conditions of IPTG induction and growth temperature resulted in the majority of the putative GT being expressed in the insoluble fraction (Figure 5.8). Varying the growth temperature of the bacterial cultures to 20°C, 30°C or 37°C and the induction time for expression (1, 2 and 3 hours) did not significantly alter the pattern of expression of the putative recombinant GT between the soluble and insoluble fractions.

The sequence of the *GmGT1_32* clone revealed two point mutations (Figure 5.6). However, it seemed unlikely that the point mutations could explain the expression of the respective polypeptide as an insoluble protein as it only changed two amino acids of the protein sequence. Many reports refer to difficulties in optimising plant GT expression in bacteria. In further attempt to express *GmGT1_32* as a soluble protein, the sequence was sub-cloned into the pGEX plasmid with the expression of an *N*-terminal GST fusion; such an expression system has previously been reported to successfully express *Arabidopsis* GTs which were otherwise prone to insoluble (Lim, *et al.*, 2001). However, the new expressing system did not result in higher soluble expression of the protein *GmGT1_32* (data not shown).

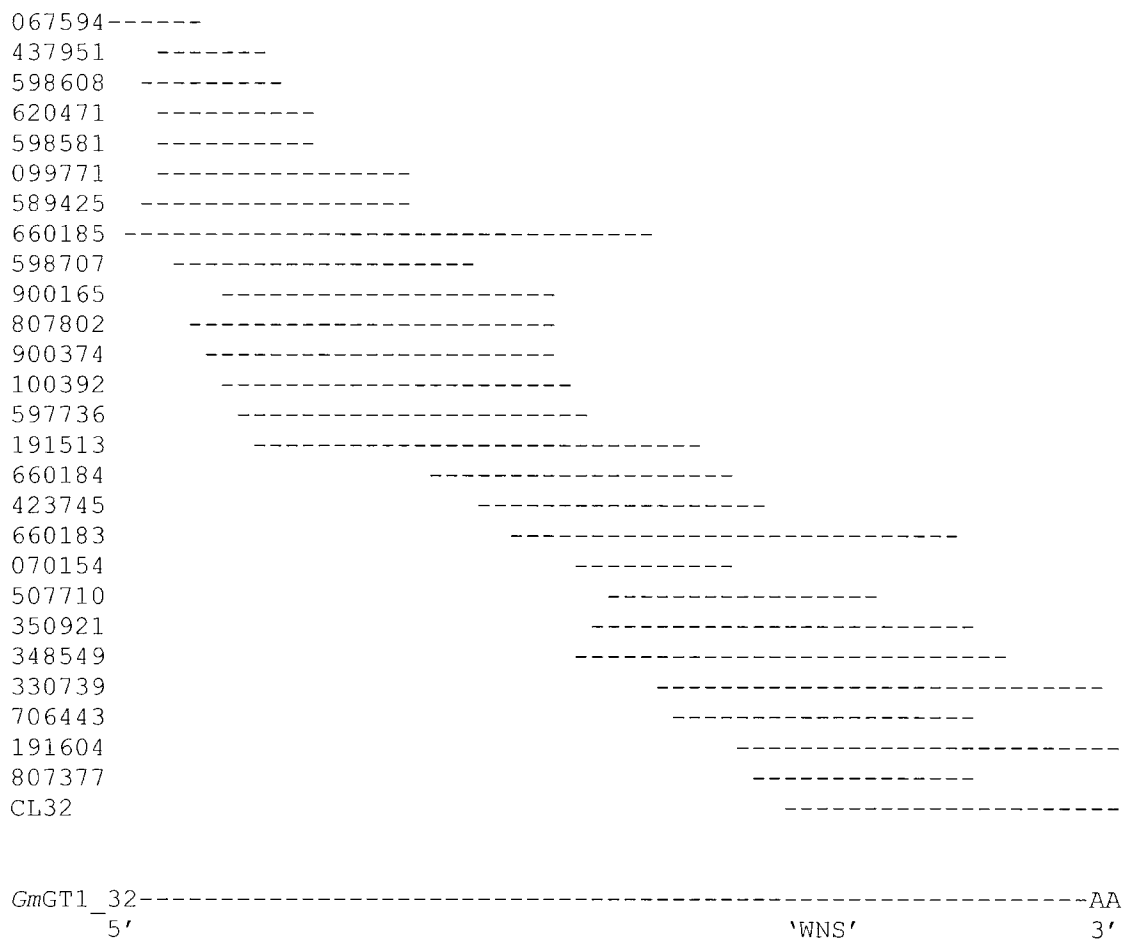


Figure 5.5 : Construct of the contig CL32.

Diagram representing the construct of the contig CL32 after isolation, orientation and alignment of 26 partial EST sequences obtained from the soybean EST database.

Table 5.4: Description of the soybean ESTs used to make the contig.

The sequences were obtained from GenBank™, EMBL and DDBJ databases.

GenBank™ Accession	dbEST Id	EST name	EST source	bp
BE807377	6081132	ss32c05.y1	Mature flowers of field grown plants	385
BE191604	4795754	sn78e11.y1	Senescing leaves, greenhouse grown	530
AW706443	4155715	sj58d12.y1	'Desloy 5710' seedling roots	495
AW330739	3712847	707043E04.x1	Mixed adult tissues	649
AW348549	3730658	GM210002B12 H12	Root	715
AW350921	3733030	GM210010B10 C8	Root	680
AI507710	2322901	sb10f10.y1	Root	409
BF070154	6428088	st07h12.y1	Germinating shoots	397
BE660183	5917060	678	Seed coats	798
BF423745	6884528	sr38e02.y1	Floral meristematic mRNA	588
BE660184	5917061	561	Seed coats	550
BE191513	4795663	sn77e05.y1	Senescing leaves, greenhouse grown	643
AW597736	4030586	sj97c12.y1	Seed coats of greenhouse grown plants	483

AI900374	3021908	sc04g11.y1	Apical shoot tips, 9-10 days old etiolated seedlings	307
BE807802	6081549	ss30d07.y1	Mature flowers of field grown plants	522
AI900165	3021699	sc01g07.y1	Apical shoot tips, 9-10 day old etiolated seedlings	477
AW598707	4031557	sj94h12.y1	Seed coats of greenhouse grown plants	521
BE660185	5917062	733	Seed coats	772
AW598425	4031275	sj91c11.y1	Seed coats of greenhouse grown plants	499
AW099771	3238988	sd31c06.y2	Apical shoot tips, 9-10 day old etiolated seedlings	479
AW598581	4031431	sj93d05.y1	Seed coats of greenhouse grown plants	470
AW620471	4055079	sj05e12.y1	Cotyledons of 8-day-old 'Williams' seedlings	
AW598608	4031458	sj93h08.y1	Seed coats of greenhouse grown plants	468
AI437951	2252766	sa41g04.y1	Root	441
AW706443	4155715	sj58d12.y1	'Desloy 5710' seedling roots	496
BF067594	6424901	st66e08.y1	Whole seedling, 3 week old, greenhouse grown	553

Table 5.5 : Primer sequences used to amplify the *GmGT1_32*.

The cloning strategy was based on the cloning of a partial sequences using the mix of degenerate primers and the polyA binder sequence. A putative full-length clone was identified from EST alignment. The gene was first amplified with CL106 and CL107 designed for direct cloning into the pET vector.

GmGT1a and *GmGT1b* were subsequently designed for cloning into the pGEX plasmid system.

	Primer sequence
CL106 (<i>Nco</i> I)	d(GGG ATT TCC ATG GAA GTC TC)
CL107 (<i>Sal</i> I)	d(GCG CGC GTC GAC GTT GAA TTT GTG GGT CC)
<i>GmGT1a</i>	d(CAT GGA AGT CTC AAA AGA AGA AGC TC)
<i>GmGT1b</i> (<i>Not</i> I)	d(CGC GCG CGG CCG CTA GTT GAA TTT GTG GGT CCA CTT)

```

1  ATGGAAGTCT  CAAAAGAAGA  AGCTCGAGTG  GCACCCGCAC
41  CGGCCCTAGT  GGCGATGCTG  CCATCCCCTG  GCATGGGCCA
81  CCTAATCCCA  ATGATCGAGT  TCGCAAAGCG  AGTGGTGTGC
121  TACCATAACC  TGGCGGTGAG  CTTCGTGATC  CCCACTGACG
161  GCCCACCTTC  AAAAGCCCAA  AAGGCAGTTC  TGGAGGCCCT
201  TCCGGACTCA  ATTTCCCACA  CCTTCCTCCC  TCCGGTCAAT
241  CTCTCCGACT  TCCCGCCGGA  CACCAAATC  GAAACCCTGA
281  TCTCCCACAC  CGTCCTCCGC  TCCCTTCCTT  CCCTCCGCCA
321  AGCATTCCAC  TCCCTCTCCG  CCACAAACAC  CCTCTCCGCC
361  GTCGTCTAG  ACCTCTTCTC  CACCGACGCC  TTCGACGTCG
401  CCGCCGAATT  CAATGCCTCC  CCCTACGTCT  TCTATCCCTC
441  CACTGCCACC  GTCCTCTCCT  TGTTCTTCCA  CCTCCCCACC
481  CTCGACCAGC  AGGTCCAGTG  CGAGTTCCGA  GACCTCCCAG
521  AACCGGTCAG  CATCCCCGGT  TGCATTCTC  TTCCCGGCAA
561  GGACCTGTTG  GACCCGGTTC  AGGACCGCAA  AAACGAGGCC
601  TACAAGTGGA  TCCTACACCA  CTGCAAGCGA  TACAAAGAAG
641  CCGAGGGGAT  AATCGGGAAC  AGCTTCGAGG  AGCTCGAACC
681  GGGAGCCTGG  AACGAGCTGC  AAAAGGAAGA  ACAAGGGAGG
721  CCTCCGGTTT  ACGCGGTGGG  ACCGCTTGTA  AGAATGGAAG
761  CAGGTCAGGC  CGACTCGGAG  TGCTTGAGGT  GGCTGGACGA
801  GCAGCCACGT  GGAAGTGTTC  TGTTTGTTTC  CTTGCGAAGC
841  GGTGGGACAC  TCTCGAGTGC  ACAGATCAAC  GAGCTGGCTC
881  TCGGGTTGGA  AAAAAGTGAG  CAACGGTTTC  TGTGGGTGGT
921  GAAGAGCCCA  AACGAAGAAA  TAGCCAACGC  GACGTACTTC
961  AGCGCCGAGA  GCCAAGCGGA  TCCCTTGCAG  TTCTTACCGG
1001  AAGGGTTCGT  GGAGAGAACA  AAAGGAAGAG  GGTTTTTGGT
1041  TCAGTCTTGG  GCTCCGCAGC  CTCAGGTATT  GGGCCATCCA
1081  TCCACGGGTG  GGTTCTTGAC  CCACTGCGGC  TGGAACTCTA
1121  TTCTGGAGAG  CGTGGTGAAC  GGTGTGCCTT  TCATAGCCTG
1161  GCCTCTGTTC  GCGGAGCAGA  GGACCAACGC  CTTTATGCTC
1201  ACGCATGACG  TCAAGGTGGC  GCTGAGGCC  AACGTTGCAG
1241  AGAGTGGCTT  GGTGGAGAGA  CAGGAGATAG  CCAGCCTAGT
1281  CAAGTGCCTC  ATGGAAGGTG  AACAAGGGAA  GAAGCTTCGT
1321  TACCGAATCA  AGGATATCAA  GGAGGCTGCG  GCTAAGGCTC
1361  TCGCTCAACA  TGGTTCCTCC  ACCACCAACA  TCTCCAATTT
1401  GGCTCTCAAG  TGGACCCACA  AATTCAACTA  G

```

Figure 5.6 : *GmGT1_32* DNA sequence from soybean.

The contig constructed from EST alignments matched a 1431 bp-sequence which was subsequently amplified in soybean tissues. The point mutations arising from the PCR amplification are shown in bold (289: A mutated in T and 299: G mutated in A).

```

1 MEVSKEEARV PAPALVAMPL PGMGHLIPMI FAKRVVCYHN
41 AVSFVIPTDG PSKAQKAVLE LPDSISHTFL PVNLSDFPPD
81 KIETLISHT RSLPSLRQAF SLSATNTLSA VVDLFSTDAF
      S H
121 VAAEFNASPY FYPSTATVLS FFHLPTLDQQ QCEFRDLPEP
161 SIPGCIPLPG DLLDPVQDRK EAYKWILHHC RYKEAEGIIG
201 SFEELEPGAW ELQKEEQGRP VYAVGPLVRM AGQADSECLR
241 LDEQPRGSVL VSGSGGTLS AQINELALGL KSEQRFLWVV
281 SPNEEIANAT FSAESQADPL FLPEGFVERT GRGFLVQSWA
321 QPQVLGHPST GFLTHCGWNS LESVVNGVPF AWPLFAEQRT
361 AFMLTHDVKV LRPNVAESGL ERQEIASLVK LMEGEQGKKL
401 YRIKDIKEAA KALAQHGSST NISNLALKWT KFN

```

Figure 5.7 : Predicted protein sequence for the *GmGT1_32*.

The protein sequence is composed of 476 amino acids and results in a 52.4 kDa protein. The sites of the mutations are shown in bold (T replaced by S and R replaced by H).

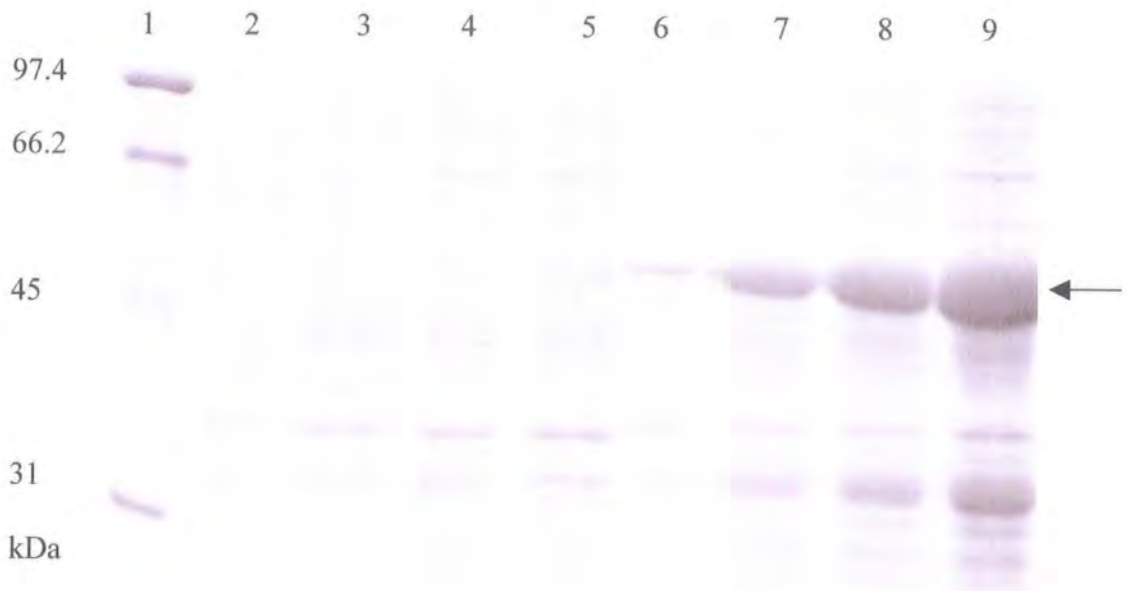


Figure 5.8 : SDS-PAGE analysis of recombinant expression of *GmGT1_32*.

Lane 1 : Standard molecular weights (kDa)

Lane 2-5 : soluble expression of pET24-*GmGT1_32* with no induction (2),
1 hour induction (3), 2 hour-induction (4) and 3 hour-induction (5)

Lane 6-9 : insoluble expression of pET24-*GmGT1_32* with no induction (6),
1 hour induction (7), 2 hour-induction (8) and 3 hour-induction (9)

The putative recombinant *GmGT1_32* polypeptide is arrowed.

5.2.3 Cloning of *ZmRP* in maize

As part of the collaborative work with Aventis Crop Science, a search of the company's internal databases identified a putative full length GT sequence from maize later renamed as *ZmRP*. The cDNA was eventually cloned from a maize library following cloning standard procedures using the set of primers described in Table 5.6.

The GT sequence obtained revealed no close homology to previously characterised GTs. The closest *Arabidopsis* sequences were determined to be the UGT85A2 and UGT85A1. From phylogenetic alignments, the maize sequence belonged to a group of GTs involved of which one GT has been characterised and was found to be involved in cyanogenesis in plants (Li, *et al.*, 2001). Overall, the protein had a predicted molecular mass of 55.089 kDa (Figure 5.12) and pI of pH 5.89.

The amplified 1,446 bp of *ZmRP* (Figure 5.9) had not been described previously in maize so there was no means of establishing whether the product contained errors due to PCR (Figure 5.9) although a stop codon was identified at 805 bp from the start. The sequence was not available when the sub-cloning was carried out. Sub-cloning was attempted as a fragment of the correct size was amplified by PCR (Figure 5.10) and all other characteristics found for this GT suggested it could represent a full-length. The initial clone identified in the database was missing the internal sequence which would have not been helpful to determine whether this triplet corresponding to the stop codon was an artefact or not. The *ZmRP* amplification product was sub-cloned directly into the pET24 expression plasmid and transformed into *E.coli* BL21(DE3) cells. When the soluble and insoluble fractions from the bacteria were analysed after induction with IPTG, no recombinant polypeptide corresponding to the *ZmRP* GT could be identified (Figure 5.11). The coding sequence was then sub-cloned into the pGEX vector as detailed for *GmGT1_32* but again no transgenic expression was observed. Finally, Rosetta™ cells (containing rare codons supplied by tRNA genes for AGG, AGA, AUA, CUA, CCC, GGA on a plasmid) were used for the transformation studies with the two vectors as the unusual dosage of rare codons in the *ZmRP* sequence might have adversely affected expression of the respective protein in a conventional *E.coli*

strain. However, as determined with the other attempts no expression of the recombinant polypeptide was observed.

Table 5.6 : Primer sequences used to amplify the *ZmRP* from maize.

The cloning strategy was based on EST alignments. The prediction of a putative gene matching GT criteria resulted in the design of primers. 2 sets of primers were designed for subsequent cloning in pET vector (*ZmRP1* and *ZmRP3*) and in pGEX vector (*ZmGT1a* and *ZmGT1b*).

Primer sequences	
<i>ZmRP1</i> (<i>NdeI</i>)	d(CGC GCG CAT ATG GGT TCG CTG CCG)
<i>ZmRP3</i> (<i>XhoI</i>)	d(CGC GCG CTC GAG GGC TAG GAG CAC CTC TTC)
<i>ZmGT1a</i> (<i>EcoRI</i>)	d(GCG CGC GAA TTC ATG GGT TCG CTG CCG CCG G)
<i>ZmGT1b</i> (<i>XhoI</i>)	d(CGC GCG CTC GAG CTA GGC TAG GAG CAC CTC)
<i>ZmRP4</i>	d(CTC GTC GAG CGT GGT ATG GTC)

```

1 ATGGGTTCGC TGCCGCCGGT GGACGGGCAG AGACGCCCCC
41 CACACGTTGT GATGATCCCG TACCCGGCGC AGGGCCACAT
81 CACGCCGATG CTGCAGTTTG CCAAGCTCCT CCATACTCGC
121 GGCTTCCACG TCACCTTCGT CAACAACGAG TTCAATCATC
161 GACGCCACCT GCGTGCGCGA GGGCCCAACG CGCTCGACGG
201 CACGGATGGG TTTCGCTTCG CCGCCATCGA CGACGGTCTC
241 CCGCTCTTTG AAGCGGACGC CACCCAGGAC ATCCCCGCGC
281 TCTGCCACTC CACCTTGACC ACCTGCCTGC CCCGGTTTAA
321 GGATCTCATC GCCAGGATTA ACGCCGAGGC TGAGGCCGAG
361 GGACAACCCA CCGTGACATG CGTCGTCCGC GACAGCACCA
401 TGACCTTCGC GCTCCGCGCC GCGCGAGAGC TCGGCCTCCG
441 CTGCGCTACA CTCTGGACCG CCAGCGCCTG CGGCTTCATC
481 GGCTACTTCC ACTACCGCCA CCTCGTTCGAG CGTGGTATGG
521 TCCCCCTCAA GAACGAGGAG CAGCTCACGG ACGGGTACCT
561 AGACACCATC GTCGACTGGA TCCCGGGCGC GCCCAAGGAC
601 CTGCGGCTCC GGGACTTCCC GAGCTTCGTG CGCACCACGG
641 ATCCGAACGA CGTCATGCTC AACTTCTTCA TTCACGAGAC
681 GGAGGGCATG TCGCAGGCGT CGGCGGTGGT CATCAACACC
721 TTCGACGAGC TCGACGCGAC GCTTGCTTCG CTTGCCATGG
761 CGAAGCTTCC TTGCCGCCA TCTACACCGT GGGCCCCTC
801 CAGCTTAGCGG TGCGGAACAA CGTGCAGCGA ACAGTCCTGT
841 CGTTGCTATT GGGTCCAACC TGTGAAGAAG CAGAAGGCGC
881 CGCTCCGGTG GCTCAACGGC CGTGCGCCGC GCTCCGTTGT
921 GTATGTCAAC TTCGGGAGCA TCACGGTGAT GTCCAACGAG
961 CAGCTGGTCC AGTTCGCGTG GGGGCTGGCC AACACCGGCT
1001 ACTACTTCCT ATGGAACGTA CGCCCGGACC TCGTCAAGAG
1041 TGGCGACTCC GCCGGGCTGC CGCCAGAGTT CTCAGCTGCG
1081 ACAGAGGGAC GAAGCATGCT GTCTACGTGG TGCCCGCAGG
1121 CAGCTGTGCT GGAGCATGAT GCCGTGGGGG TGTTCTGAC
1161 GCACTCCGGG TGGAACTCCA CGCTGGAGAG TATTTGCGGC
1201 GCGTTCCTCA TGCTGTGCTG GCCCTTCTTC GCCGAGCAGC
1241 AGACCAACTG CCGATACAAG TGCACCGAGT GGGGCATCCG
1281 AAAAGAGATC GGTGACGATG TACAGCGGGG CGAGGTAGAG
1321 TCGCTCATAA GGGAGGCCAT GGAGGGGGAG AAAGGGCAAG
1361 AGATGCTCCG GCGCGTACT GAACTCAGGG ACAGCGCCGT
1401 GGCCGCTGCA GGGCCTGACG GACGGTCTAT GCGCAACGTA
1441 GACAGGCTCA TCGAAGAGGT GCTCCCTCGA GCACCACCAC
1481 CACCACCACT G

```

Figure 5.9 : *ZmRP* sequence obtained from amplification in maize library.

The gene sequence gave a stop codon at position 804 (shown in bold) due to a PCR error.

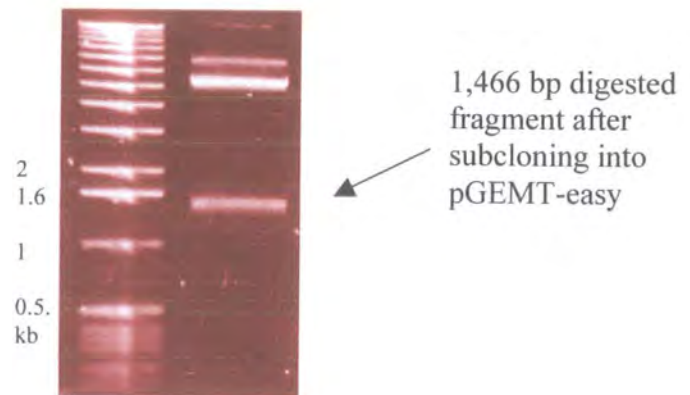


Figure 5.10 : *ZmRP* gene released from its plasmid after restriction digest.

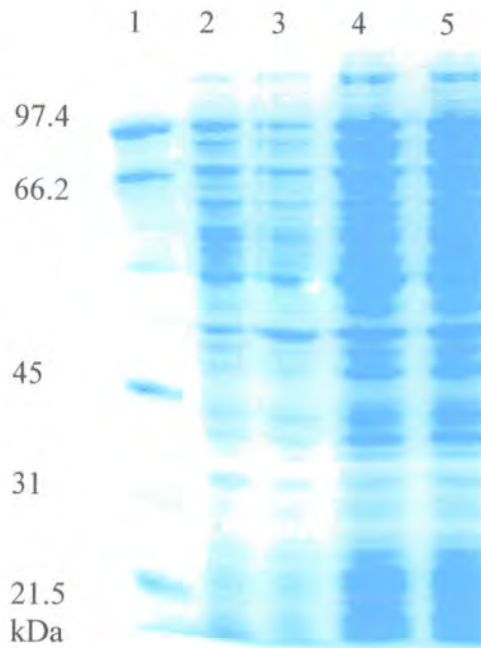


Figure 5.11 : SDS-PAGE analysis of expression of recombinant *ZmRP*.

The expression occurred in BL21(DE3).

Lane 1 : Standard molecular weights (kDa)

Lane 2 : soluble expression of pET24-*ZmRP* with no induction (2),

Lane 3 : soluble expression of pET24-*ZmRP* with 3 hour-induction (3)

Lane 4 : insoluble expression of pET24- *ZmRP* with no induction (4),

Lane 5 : insoluble expression of pET24- *ZmRP* with 3 hour-induction (5)

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1  MGSLPPVDGQ RRPPHVVMIP YPAQGHITPM LQFAKLLHTR
41  GFHVTFVNNE FNHRRHLRAR GPNALDGTDG FRFAAIDDGL
81  PLFEADATQD IPALCHSTLT TCLPRFKDLI ARINAEAEAE
121 GQPTVTCVVG DSTMTFALRA ARELGLRCAT LWTASACGFI
161 GYFHyrHLVE RGMVPLKNEE QLTDGYLDTI VDWIPGAPKD
201 LRLRDFPSFV RTTDPNDVML NFFIHETEGM SQASAVVINT
241 FDELDTLAS LAMAKLPCRP STPWARSSRC GTTCSEQSCR
281 CYWVQPVKKQ KAPLRWLNGR APRSVVYVNF GSITVMSNEQ
321 LVEFAWGLAN TGYyFLWNVR PDLVKSGDSA GLPPEFSAAT
361 EGRSMLSTWC PQAAVLEHDA VGVFLTHSGW NSTLESICGG
401 VPMLCWPFFA EQQTNCRYKC TEWGIGKEIG DDVQGEVES
441 LIREAMEGEK GQEMLRrvTE LRDSAVAAAG PDGRSMRNVD
481 RLIEEVLpRA PPPPPL

```

Figure 5.12 : Predicted protein sequence for *ZmRP*.

5.3 Discussion

Based on PCR, a number of approaches have been employed in an attempt to isolate cDNAs encoding functional GTs from soybean and maize. Initial attempts focussed on the classic Bz-1 GT from maize, which although well characterised as a UDP-glucose 3-*O*-flavonol glucosyltransferase has never been tested for its activity towards xenobiotics. The potential role of *bronze-1* GTs in xenobiotic metabolism was considered to be an interesting question, as a flavonol 3-OGT from wheat has been associated with activity towards chlorinated phenols in wheat (Brazier, *et al.*, 2003). Unfortunately, it was found to be impossible to amplify Bz-1 from the maize tissues available, probably due to the exclusive expression of this gene in developing maize kernels, a tissue source which was not available in the course of the work.

Using a PCR-based strategy centred on conserved blocks of coding sequence of type-1 plant GTs, coupled with an associated screen of the EST databases it was possible to amplify a specific GT sequence from soybean and then use existing sequences to extend to the full-length contig. The resulting full length clone *GmGT1_32* was then recovered from soybean cDNA by PCR and sub-cloned into pET-24 and pGEX for the expression of the recombinant nature and GST fusion protein respectively. Unfortunately, the resulting recombinant protein was totally insoluble and inactive as a GT. The soybean sequence *GmGT1_32* which resembled an arbutin synthase from the medicinal plant *Rauvolfia serpentina* was cloned in *E.coli*. The arbutin synthase was found to be active in conjugating a diverse range of phenolic natural products and several xenobiotics although showing preference to hydroquinone as a substrate. The enzyme demonstrates a broad spectrum of activity both towards endogenous and xenobiotic origin (Hefner, *et al.*, 2002). The *rGmGT1_32* might be expected to show a similarly diverse range of conjugating activities towards foreign compounds and natural products. However, the technical problem resulting in expression as insoluble enzyme could not lead to any further determination of substrate specificity.

3 other partial GT sequences were obtained after PCR amplification using a similar mix of primers. Sequence analysis revealed close hits with GT sequences. Unfortunately, searches in public databases for the respective GTs tools did not allow us to extend the sequences of the PCR product to obtain full-length clones. The use of the method of 5' RACE aimed at extending sequences from the 5' end directing was attempted instead. This method was applied to the clones CL33 from soybean tissues CL34 from maize. However both attempts were unsuccessful with the loss of products after a few amplification experiments. Further RACE work on the CL55 sequence was not attempted due to the difficulties encountered with CL33 and CL34.

Finally, an informatics approach led to the isolation of a type-1 GT from maize through a collaboration with Rhône-Poulenc Agriculture Limited which resulted in the isolation of a putative full-length clone in maize. The sequence, labelled *ZmRP*, was successfully cloned into expression vector but overexpression studies of the recombinant enzyme in *E.coli* failed to express the protein solubly or insolubly. The maize sequence was Blasted in the databases and the closest hits to putative *Arabidopsis* sequences. According to the classification by Ross (Ross, *et al.*, 2001), the closest hits revealed all belonged to the G group of type 1 GTs notably UGT85A1, UGT85A7 and UGT85A5. *ZmRP* was closely related to a previously cloned and overexpressed UDP-glucose:*p*-hydroxymandelonitrile-*O*-glucosyltransferase from *Sorghum bicolor*. This enzyme was shown to be involved in the last step of the synthesis of the cyanogenic glucose dhurrin (Jones, *et al.*, 1999). The sorghum enzyme was tested and showed to have activity towards a limited number of substrates including *p*-hydroxymandelonitrile, mandelonitrile, benzyl alcohol, and benzoic acid (Jones, *et al.*, 1999). The enzyme was subsequently termed UGT85A1 based on the nomenclature established by UGT Nomenclature Committee (Mackenzie, *et al.*, 1997). The *in vitro* substrate specificity of UGT85A1 was tested as a recombinant protein and revealed that it was able to glucosylate a range of substrates including cyanohydrins and aliphatic and aromatic groups (Hansen, *et al.*, 2003). The regioselectivity shown by UGT85A1 was distinct from that of any of the *Arabidopsis* UGT tested. Since maize does not contain cyanogenic

glucosides the presence of the UGT85A1 homologue in its tissues suggests that it must have an alternative substrate selectivity. It would be interesting however to determine the activity of the recombinant *ZmRP* enzyme towards *p*-hydroxymandelonitrile since none of the 107 type 1 *Arabidopsis* GTs could catalyse the formation of durrhin (Tattersall, *et al.*, 2001).

Chapter 6 : Purification and Cloning of *N*-GT in *Arabidopsis* cell cultures

6.1 Introduction

Over the last few years, much genetic data has been gathered on the model plant *Arabidopsis thaliana* due to its small genome, ease of handling, transformation/mutagenesis and short life cycle. As such, *Arabidopsis* is often used in systematic approaches for the analysis of gene families or superfamilies such as the GTs.

Based on highly conserved domains in the protein sequence represented by 42 amino acids, including a conserved 'WNS' motif located at the C-terminal end (Vogt & Jones, 2000) also known as the PSPG box (Hughes and Hughes, 1994) and involved in the binding of UDP-glucose, multiple members of the type 1 GT superfamily have been identified in the *Arabidopsis* genome (Li, *et al.*, 2001). In total, 107 putative GT sequences and 10 pseudogenes have been confirmed which, based on conserved amino acids can be grouped into 14 evolutionary groups labelled from A to N (Ross, *et al.*, 2001). Although half of the GT genes did not have any introns, the remaining members of the superfamily had up to two introns. The predicted proteins encoded by the GT genes were composed of between 435 to 507 amino-acids, with 9 conserved domains identified (Ross, *et al.*, 2001). Unlike the mammalian type-1 GTs which are associated with the endoplasmic reticulum and utilise UDP-glucuronic acid in place of UDP-glucose, the plant GTs lack the amino terminal leader sequence which target these enzymes to the membranes. In the absence of other putative targeting sequences, it seems likely that the GTs in *Arabidopsis* have a cytoplasmic location.

In this chapter, three approaches have been employed to study the GTs involved in xenobiotic detoxification in *Arabidopsis*. The first approach was concentrated on the effect on GT activities in plants following chemical and safener treatments. As part of this work, the optimal tissue source for the purification of the GT activities of interest was also determined.

In the second approach, attention was focused on the glucosylation of xenobiotics, the formation of conjugates *in planta* and the characterisation of the metabolites formed. The emphasis of this work was directed at studying the detoxification of 3,4-DCA, a compound known to undergo different routes of conjugation in soybean and wheat (Bockers, *et al.*, 1994; Schmitt, *et al.*, 1985).

Finally, the NGT active in 3,4-DCA detoxification was purified and sequenced using MALDI TOF MS after tryptic digestion. Following the completion of the project, this GT was subsequently cloned, expressed and functionally characterised, giving the first unambiguous identification of a major xenobiotic detoxifying GT in higher plant (Loutre, *et al.*, 2003).

6.2 Regulation and activities of GTs in *Arabidopsis* root and suspension cultures

It was of interest to determine the range of GT activities present in *Arabidopsis* plants and tissue cultures, and the optimal tissue sources which could be used for purification of the respective GTs. The other main interest was to determine whether the GTs in this dicotyledonous model plant were enhanced in a similar way to that determined in wheat in response to safener or chemical induction (Brazier, *et al.* 2002). In particular, herbicide safeners were of great interest due to their well-known ability to enhance herbicide selectivity by increasing the expression of xenobiotic detoxification enzymes in cereals (Davies and Caseley, 1999). In addition, GSTs were enhanced in *Arabidopsis* showing that phase II detoxifying enzymes were also responsive to safeners in dicotyledonous plants (DeRidder, *et al.*, 2002).

Putative GT substrates were selected based on the results of the GT studies carried out in maize and soybean in Chapters 3 and 4. The substrates were used for screening GT activities in *Arabidopsis* tissues (Table 6.1) using the conditions optimised in the work described in Chapter 4. Tissue sources tested included *Arabidopsis* plants, suspension cultures and root cultures. The substrates tested included maleic hydrazide, 2,3,6-trichlorophenol, *p*-nitrophenol, quinchlorac, chloramben, coumarin, 2,4,6-trichlorophenol, 4-chlorophenoxyacetic acid, 3,4,5-trichloroaniline, 2,3,4-trichlorophenol, picloram, pentachlorophenol, amitrol, 2,4-dichloroaniline, 3,4-dichloroaniline, 2,3-dichloroaniline, 2,4,5-trichloroaniline, 2,4,5-trichlorophenol, formononetin, esculetin, luteolin, umbelliferone, genistein, quercetin, isoliquirtigenin, coumestrol, phenanthrenequinone and 4-chlorothiophenol.

Arabidopsis plants displayed low GT activities towards a limited list of substrates including 3,4-DCA, quercetin, 2,4,5-trichlorophenol, coumestrol, cyanidin chloride, luteolin and *p*-nitrophenol respectively and 4-chlorothiophenol. No activity was determined towards the other substrates tested.

Root cultures were tested with a similar range of substrates, with 3,4-DCA, quercetin and 2,4,5-trichlorophenol identified as preferred substrates, while *Arabidopsis* suspension cultures exhibited higher specific activities towards these three substrates due to the low GT activities determined in the whole plants, subsequent studies on *Arabidopsis* GTs concentrated on root and suspension cultures (Table 6.1).

Enhancement of GT activities was then studied using a range of safeners with the effect on OGT and NGT activity determined using quercetin, 2,4,5-TCP and 3,4-DCA in root cultures (Table 6.2) and suspension cultures (Table 6.3).

Table 6.1 : GT activity in *Arabidopsis* tissues.

Crude protein extracts of *Arabidopsis* plants (30 day-old), root cultures (20 day-old) and suspension cultures (harvested 6 days after subculture) were assayed for GT specific activities against a range of natural and xenobiotic substrates.

Data are the mean \pm standard deviation (n=2).

Substrates	Specific activity ($\text{pmole min}^{-1} \text{mg}^{-1} \text{protein}$)		
	Whole plant	Root cultures	Suspension cultures
3,4-DCA	1.5 ± 0.6	6.4 ± 0.5	12.3 ± 1.4
Quercetin	1.9 ± 0.3	1.1 ± 0.5	3.4 ± 0.5
2,4,5-TCP	1.7 ± 0.2	5.2 ± 2.1	9.2 ± 1.5
Coumestrol	0.7 ± 0.0	ND	Detected *
Cyanidin chloride	0.2 ± 0.0	ND	ND
Luteolin	0.2 ± 0.0	ND	ND
<i>p</i> -nitrophenol	0.2 ± 0.1	ND	ND
4-chlorothiophenol	0.1 ± 0.0	ND	ND

* Detected but not quantified, ND not detected.

In root cultures, the safener R-29148 showed no effect on GT activity towards the three substrates tested. NGT activities were increased with all safeners except with fenclorim and R-29148 where there was no effect. Mefenpyr-diethyl inhibited activity towards both 3,4-DCA and quercetin but exhibited some GT conjugation towards 2,4,5-TCP. Activity towards quercetin was not enhanced with dichlormid, oxabetrinil, flurazole, while benoxacor, cloquintocet mexyl, fenclorim and CMPI enhanced specific GT activities 2 fold. Optimal safener enhancement of GT activity towards 2,4,5-TCP was limited as compared with control treatments (Table 6.2).

In *Arabidopsis* suspension cultures, GT activities were enhanced by several safener treatments (Table 6.3). Benoxacor was found to be the best inducer towards 3,4-DCA, quercetin and 2,4,5-TCP. Fenclorim was also active in enhancing activity. CMPI and R-29148 inhibited specific activities towards all substrates as compared to controls samples. The other safeners showed some enhancement depending on the substrate tested.

Chemical treatments were also carried out on dark-grown roots and suspension cultures of *Arabidopsis* to determine whether treatment with GT substrates could enhance the respective detoxifying GT activities (Table 6.4). Plants and cultures were grown as described in Chapter 2 and treated for 24 hours prior to harvest with safener at a concentration of 10 mg l⁻¹ or with GT substrates (as inducers) at a final concentration of 50 µM.

Formononetin was included in the screen as this isoflavone has been reported to enhance GT activity towards flavonoids in chickpea (Mackenbrock, *et al.*, 1993).

GT activities in suspension cultures towards both OGT and NGT conjugated substrates were suppressed in the treated tissues. Root cultures showed some responsiveness to exposure to 50 µM 3,4-DCA and formononetin while quercetin and 3,4-DCA activities were increased. Specific activity towards 2,4,5-TCP was enhanced following exposure to formononetin. Chemical induction had a limited effect on NGT activity towards 3,4-DCA.

Table 6.2 : Effect of safener treatment on GT activities in root cultures.

Arabidopsis roots cultures (18 day-old) were treated with a range of safeners (10 mg l⁻¹) for 24 hours prior to harvest and the specific GT activities determined towards 3 substrates.

Data are the mean ± standard deviation (n=2). Control treatments consisted of an equivalent amount of the solvent carrier alone (acetone) added to the root medium.

Treatment	Specific activity (pmole min ⁻¹ mg ⁻¹ protein)		
	3,4-DCA	Quercetin	2,4,5-TCP
Acetone control	6.0 ± 0.6	1.4 ± 0.3	6.6 ± 0.7
Benoxacor	7.4 ± 0.9	3.3 ± 0.7	9.2 ± 1.5
Cloquintocet mexyl	7.3 ± 2.0	2.8 ± 0.9	9.7 ± 2.5
Flurazole	7.5 ± 1.3	1.7 ± 1.0	9.0 ± 0.7
Oxabetrinil	6.9 ± 0.7	1.7 ± 0.1	7.8 ± 0.4
Fenclorim	6.1 ± 0.9	3.7 ± 0.4	10.1 ± 0.9
CMPI	9.2 ± 0.6	3.1 ± 0.1	11.1 ± 1.1
Dichlormid	7.1 ± 0.6	1.5 ± 0.4	11.1 ± 0.5
R-29148	6.0 ± 0.2	1.3 ± 0.1	6.8 ± 0.3
Mefenpyr-diethyl	5.4 ± 0.7	1.0 ± 0.5	9.5 ± 1.7

Table 6.3 : Effect of safener treatment on GT activities in suspension cultures.

Arabidopsis suspension cultures (harvested 5 days-after subculturing) were treated with a range of safeners (10 mg l⁻¹) for 24 hours prior to harvest and the specific GT activities determined towards 3 substrates. Data are the mean \pm standard deviation (n=3). Control treatments consisted of an equivalent amount of the solvent carrier alone (acetone) added to the root medium.

Treatment	Specific activity (pmole min ⁻¹ mg ⁻¹ protein)		
	3,4-DCA	Quercetin	2,4,5-TCP
Acetone	1.8 \pm 1.1	3.4 \pm 0.4	1.4 \pm 1.1
Benoxacor	6.4 \pm 1.3	13.9 \pm 1.1	7.5 \pm 1.6
Cloquintocet mexyl	2.6 \pm 0.6	6.0 \pm 2.0	3.1 \pm 0.4
Flurazole	3.5 \pm 2.1	2.8 \pm 2.0	2.7 \pm 1.3
Oxabetrinil	3.6 \pm 1.0	4.2 \pm 0.6	3.1 \pm 0.3
Fenclorim	5.9 \pm 1.5	9.0 \pm 2.3	6.1 \pm 2.0
CMPI	1.1 \pm 0.1	3.0 \pm 0.1	1.1 \pm 0.2
Dichlormid	3.2 \pm 0.6	2.3 \pm 0.2	1.1 \pm 0.4
R-29148	1.2 \pm 0.5	3.5 \pm 1.5	2.0 \pm 1.0
Isoxadifen-ethyl	3.7 \pm 3.2	4.5 \pm 1.6	2.8 \pm 1.3

Table 6.4 : GT activities in root cultures following chemical induction.

Specific GT activities were determined in suspension cultures following a 24-hour treatment with 50 μ M formononetin, 50 μ M 3,4-DCA or 1% (v/v) methanol (solvent carrier).

Data are the mean \pm standard deviation (n=2).

		Substrates		
		Specific activity (pmole product min ⁻¹ mg ⁻¹ protein)		
	Treatment	Quercetin	2,4,5-TCP	3,4-DCA
Root cultures	Water control	0.7 \pm 0.4	3.7 \pm 1.4	6.7 \pm 0.1
	Methanol	0.9 \pm 0.1	5.3 \pm 0.3	7.3 \pm 0.5
	Formononetin	1.0 \pm 0.2	7.1 \pm 0.4	8.1 \pm 0.3
	3,4-DCA	1.0 \pm 0.4	5.2 \pm 0.8	7.5 \pm 0.4
Suspension cultures	Water control	3.8 \pm 0.2	8.1 \pm 1.4	11.3 \pm 1.6
	Methanol	3.9 \pm 0.2	9.1 \pm 1.7	11.3 \pm 1.5
	Formononetin	3.3 \pm 0.1	4.0 \pm 0.2	4.2 \pm 0.4
	3,4-DCA	3.7 \pm 0.2	4.4 \pm 0.3	4.3 \pm 0.5

6.3 Metabolism of 3,4-DCA by sugar conjugation in *Arabidopsis thaliana*.

3,4-DCA was determined to be the best substrate for *N*-glucosylation in *in vitro* experiments with all the tissue sources tested (Chapter 3 in Table 3.6 and Chapter 4 Table 4.9). Plant tissues were therefore fed with [^{14}C]-3,4-DCA and extracted at timed intervals to determine the distribution of radioactive material in the extractable and non-extractable fractions and the medium. As feeding studies carried out in maize tissues (Chapter 3) had revealed that conjugation occurred rapidly after dosing, time-courses of analyses were limited to a two-day period. In addition, in view of the enhancing effect of safeners on GT activities in *Arabidopsis*, feeding studies with [^{14}C]-3,4-DCA were carried out with both untreated and cloquintocet mexyl-treated cultures to determine whether or not safener treatment affected the rate of 3,4-DCA detoxification as it has been described to selectively enhance OGT activity in wheat (*Triticum aestivum* (L.) towards flavonoids and xenobiotics (Brazier, *et al.*, 2002)

The feeding studies were set up in parallel on *Arabidopsis* root cultures, suspension cultures and whole plants with each treated with, or without safener as a co-treatment. Plants and media were sampled for radioactivity over a two-day period. Further experiments were subsequently set up to follow the fate of 3,4-DCA in detail over the first 24 hours. In each case, the radioactive metabolites of [^{14}C]-3,4-DCA were determined in the medium, the plant tissues and when possible, in the non-extractable bound residues.

The rate of uptake of radioactivity following treatment with [^{14}C]-3,4-DCA was quantified in the presence and absence of safeners to test for an effect of the safener on the uptake. The total recoveries of radioactivity in the media and plant material (extractable and non-extractable) were then determined.

6.3.1 Uptake of [¹⁴C]-3,4-DCA in *Arabidopsis* suspension cultures

When 5 day-old suspension cultures were treated with [¹⁴C]-3,4-DCA, the radioactivity was rapidly taken up into the cells from the medium and within 8 hours, the pools of [¹⁴C]-3,4-DCA were evenly partitioned between the plant material and media (Figure 6.1).

Over the initial 8 hour-period of study, 41 nM of 3,4-DCA (44% of the dose) became associated with the cells. Afterwards the distribution of radioactivity in the cells stabilised over the remaining period of study, representing 39 nM (42%) of the dose at 48 hours. The radioactivity in the initial medium rapidly decreased to reach less than half of the initial dose, with 39 nM (42%) recovered in the first time interval (8 hours). Interestingly, the proportion of radioactivity in the medium then slowly increased again to reach 52 nM (56%) at the end of the study. The formation of non-extractable residues in all cases was a minor sink of metabolism representing less than 2.5% of administered radioactivity by the end of the study.

Based on statistical analysis, the safener treatment did not significantly alter the profile of uptake and distribution of xenobiotic in the plant tissues over the time course. Between the two plant populations tested, P was 0.1774 with $P < 0.05$ resulting in the hypothesis H_0 (the two populations were identical) had to be accepted.

Overall, recoveries of the administered radioactivity varied between $77 \pm 4\%$ to $99 \pm 9\%$ (Table 6.5).

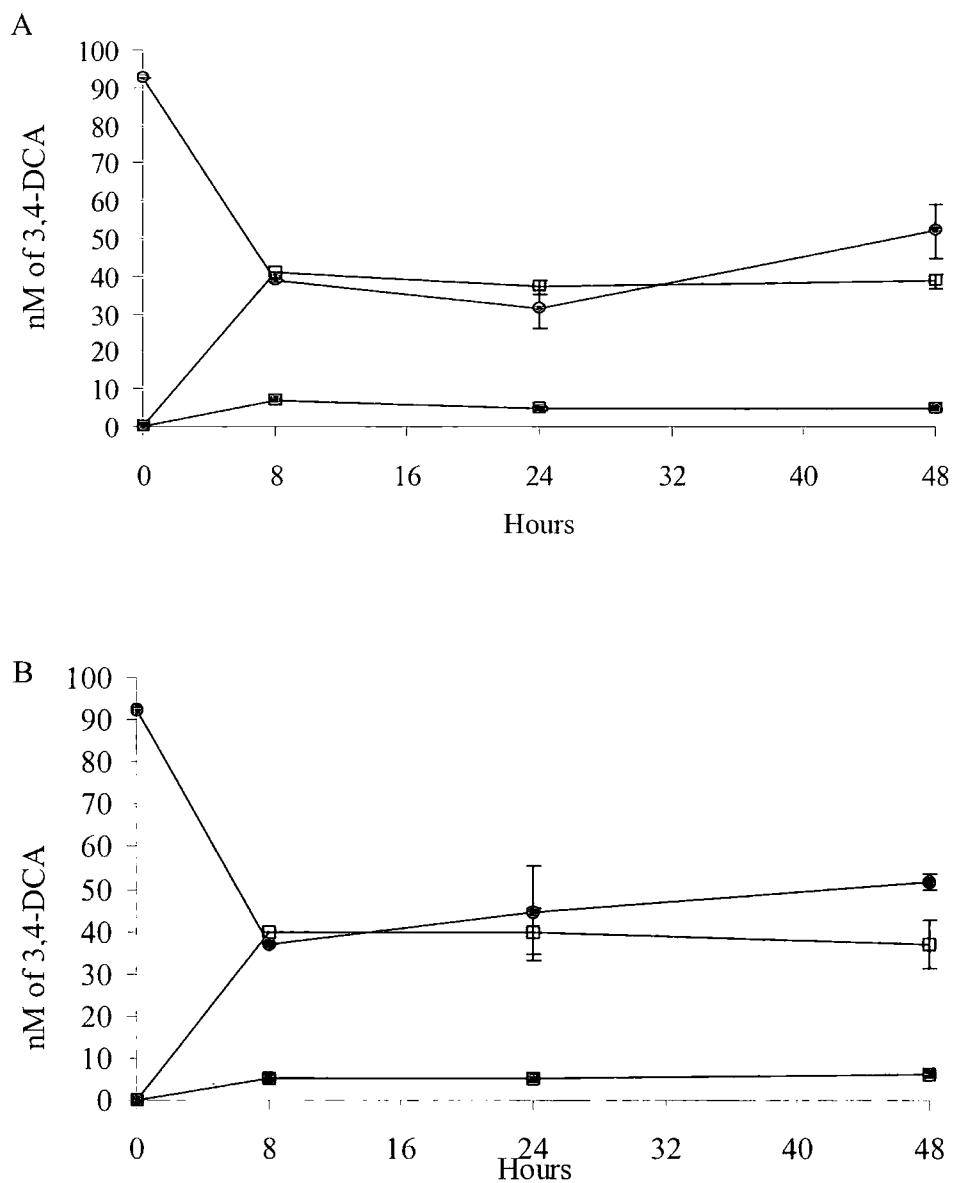


Figure 6.1 : Uptake of [^{14}C]-3,4-DCA in *Arabidopsis* suspension cultures.

Suspension cultures (20 ml) treated with (B) without (A) the safener cloquintocet mexyl (10 mg ml^{-1}) 24 hours prior to harvest were fed with an initial dose of $1,5 \text{ kBq } [^{14}\text{C}]\text{-3,4-DCA}$. Radioactivity was then monitored in the medium (●), in the extractable fraction from plant tissue (□) and the non-extractable fraction (■). Data are the mean \pm standard deviation ($n=2$).

Table 6.5 : Percentage total recovery of applied radioactivity after feeding [¹⁴C]-3,4-DCA to *Arabidopsis* suspension cultures.

Recoveries (%) obtained after summation of the radioactivity present in the plant extract, the medium and the bound residues of cloquintocet mexyl-treated or control tissues.

Values represent the means \pm variation in the duplicates.

Hours (h)	Recoveries (%)	
	Control tissues	Cloquintocet mexyl- treated tissues
8	90	86
24	77 \pm 4	95 \pm 4
48	99 \pm 9	99 \pm 5

6.3.2 Uptake of [¹⁴C]-3,4-DCA in *Arabidopsis* seedlings

Arabidopsis seedlings were grown for 10 days in plastic well plates (3 ml per well) and then after a 24 hour pre-treatment with or without cloquintocet mexyl (10 mg l⁻¹) were fed with 25.1 kBq of [¹⁴C]-3,4-DCA. Similar patterns of uptake to those obtained in suspension cultures (Figure 6.1) were observed (Figure 6.2). Thus, the radioactivity was partitioned between the media and plant tissue after 8 hours, with 332 nM (54%) of the initial radioactive dose recovered in the control tissues. The radioactive content then stabilised, with the plant tissue containing 332 nM of the dosed radioactivity (54%) after 48 hours.

Based on statistical analysis, the safener treatment affected the uptake and distribution of the xenobiotic with $P = 0.0290$ with $P < 0.05$. It resulted in the hypothesis to be rejected.

In the control cultures, radioactivity in the medium rapidly decreased in the first 8 hours to 197 nM (32%), before increasing again to 271 nM (44%) after 24 hours and finally 246 nM (40%) after 48 hours. In the safened tissues, the trend was similar with 258 nM (42%) after 24 hours and stabilising at 256 nM (42%) after 48 hours.

The formation of bound residues was not determined in this study; however, previous studies suggested they only represented a minor fraction of the administrated dose (Table 6.6).

No further attempt to confirm the effect of the significant difference obtained between the plant treated or not with safener and how this data could fit in a possible mode of action.

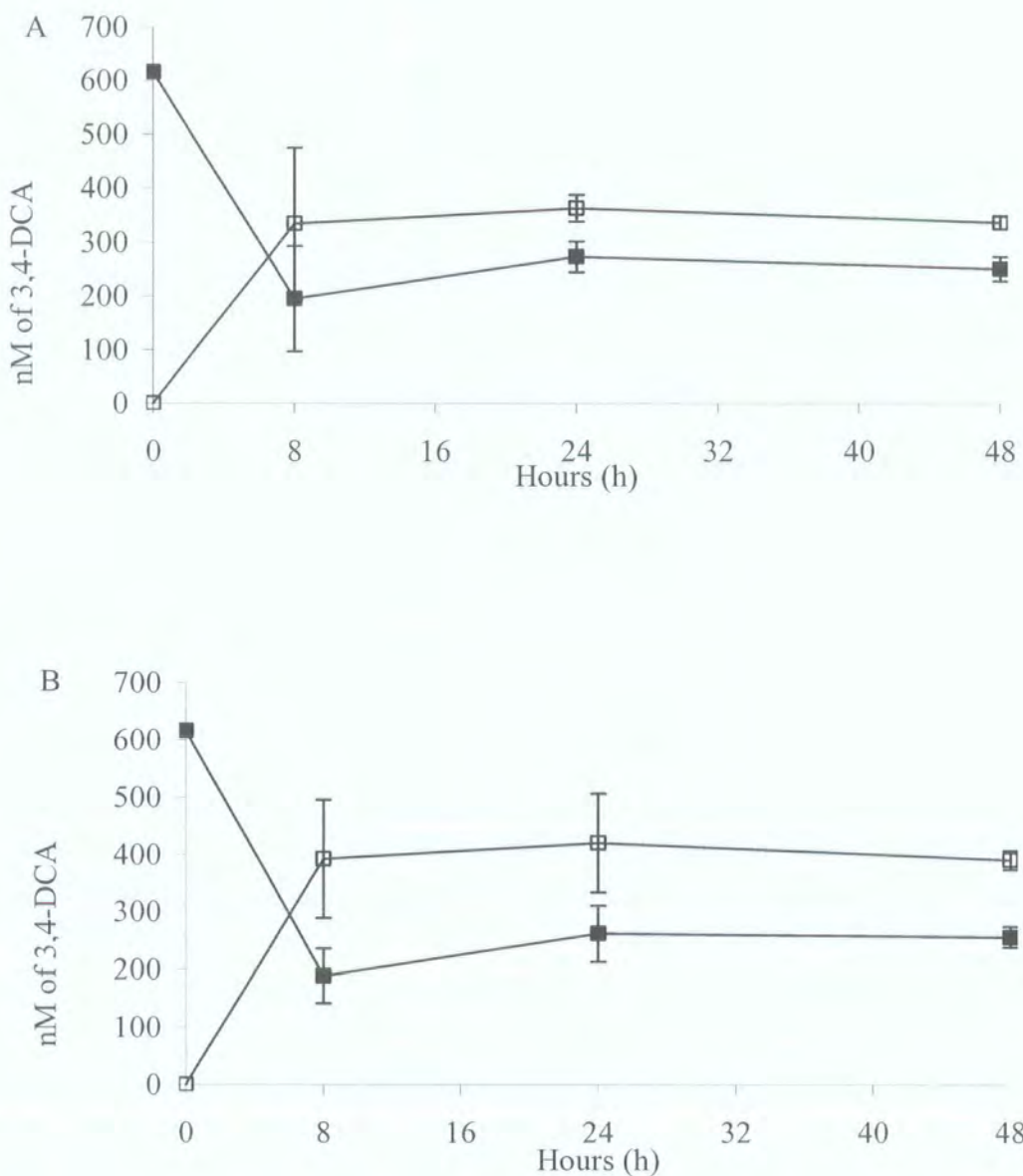


Figure 6.2 : Uptake of $[^{14}\text{C}]\text{-3,4-DCA}$ in *Arabidopsis* seedlings.

Hydroponically grown seedlings treated with (B) or without (A) the safener cloquintocet mexyl (10 mg l^{-1}) 24 hours prior to harvest were fed with an initial dose of $1.5 \text{ kBq } [^{14}\text{C}]\text{-3,4-DCA}$. The distribution of radioactivity was determined in plant extracts (\square) and medium (\blacksquare). Data are the mean \pm standard deviation ($n=2$).

Table 6.6 : Percentage total recovery of applied radioactivity after feeding [¹⁴C]-3,4-DCA to *Arabidopsis* seedlings.

Recoveries (%) were obtained by summing of the radioactivity present in the two fractions corresponding to the plant extract and the medium. Data are the mean \pm standard deviation (n=2).

Hours (h)	Recoveries (%)	
	Control tissues	Cloquintocet mexyl-treated tissues
8	95 \pm 40	105 \pm 27
24	115 \pm 9	123 \pm 24
48	106 \pm 2	116 \pm 6

6.3.3 Uptake of [¹⁴C]-3,4-DCA in *Arabidopsis* root cultures

The uptake and distribution of [¹⁴C]-3,4-DCA was determined in 21 day-old root cultures which had been previously treated for 24 hours with, or without, cloquintocet mexyl (10 mg l⁻¹; Figure 6.3). In the untreated cultures, within the first 12 hours, 236 nM (39%) of the radioactivity became associated with the roots, while in the safener-treated tissues, 121 nM (20%) were taken up in this time. It therefore appeared that safener treatment had actually reduced the initial uptake of [¹⁴C]-3,4-DCA. However, in the respective media, 327 nM (54%) of the radioactivity was determined for the non-treated plants and a quantity of 315 nM (52%) was present in the medium of safener-treated plants after 12 hours. As the incubation time increased, a difference was observed in the uptake and distribution of radioactivity in the root cultures, which had been treated with or without cloquintocet mexyl. In the safener-treated cultures, radioactivity steadily accumulated in the root cultures over the 48 hour-period. In the untreated cultures, radioactivity was taken up by the roots rapidly over the first 12 hours and then gradually declined (Table 6.7).

Based on statistical analysis, the hypothesis H₀ had to be accepted and the two populations tested were considered identical with $P = 0.2931$ with $P < 0.05$.

To determine the uptake and metabolism of [¹⁴C]-3,4-DCA in *Arabidopsis* root cultures in greater detail, untreated cultures were incubated with the radiolabel over 48 hours, with the uptake over the first 12 hours determined at 4 hours intervals (Figure 6.4). The recovery data are presented in Table 6.8. The radioactivity in the roots peaked after 4 hours treatment with 330 nM (55%) of xenobiotic entering the plant. The radioactivity recovered from the plant tissue then slowly decreased to 132 nM (20%) between 4 and 48 hours.

The rapid uptake over 0-4 hours was associated with the loss of 146 nM (22%) from the medium. The radioactivity in the medium slowly increased to reach 390 nM (65%) after 24 hours, then declined slowly to 354 nM (59%) after 2 days. Recoveries of applied radioactivity averaged $70 \pm 12\%$ and $99 \pm 14\%$ for this study for at 8 hour and 24 hour harvest respectively (Table 6.9), with the bound residue fraction accounting for only 3% of the total radioactivity.

These results suggested that after rapid uptake of the [^{14}C]-3,4-DCA over 0 to 4 hours, the radioactivity was then re-exported back into the media. It was therefore of interest to identify the radioactive metabolites of 3,4-DCA accumulating in both the roots and medium.

Overall, recoveries from the root cultures were more variable (72%-127%) than determined in the studies with the suspension cultures or seedlings, particularly with the cloquintocet mexyl-treated flasks. This suggested that there might have been a problem in the dosing regime and that the apparent differences in the uptake and distribution of [^{14}C]-3,4-DCA between untreated and safener treated root cultures were an artefact.

Methanolic root extracts and ethyl acetate partitioned radioactivity from the media was carefully concentrated and applied to TLC plates using chloroform/methanol/water (60:35:8; v/v) as developing solvent. Radioactive metabolites were then visualised by autoradiography (Figure 6.5). In addition to parent [^{14}C]-3,4-DCA, six metabolites could be resolved, which were termed M1 to M6 inclusive. A summary of TLC metabolites is given in Table 6.9.

TLC analysis revealed that the 6 metabolites were synthesised within 4 hours. Of the 6 metabolites, 2 (M1 and M2) were found to accumulate both in the roots and the medium.

In order to identify putative conjugates, [^{14}C]-3,4-DCA was incubated with UDP-glucose in the presence of a crude protein preparation from *Arabidopsis* under the optimised conditions described in Chapter 4. Following analysis of the labelled 3,4-DCA conjugates by TLC metabolite M2 was found to co-chromatograph with *N*- β -D-glucopyranosyl-3,4-DCA. Thus, this latter glucoside was found to be a dominant metabolite in both the roots and the medium. Identification of the metabolite M2 was not carried out.

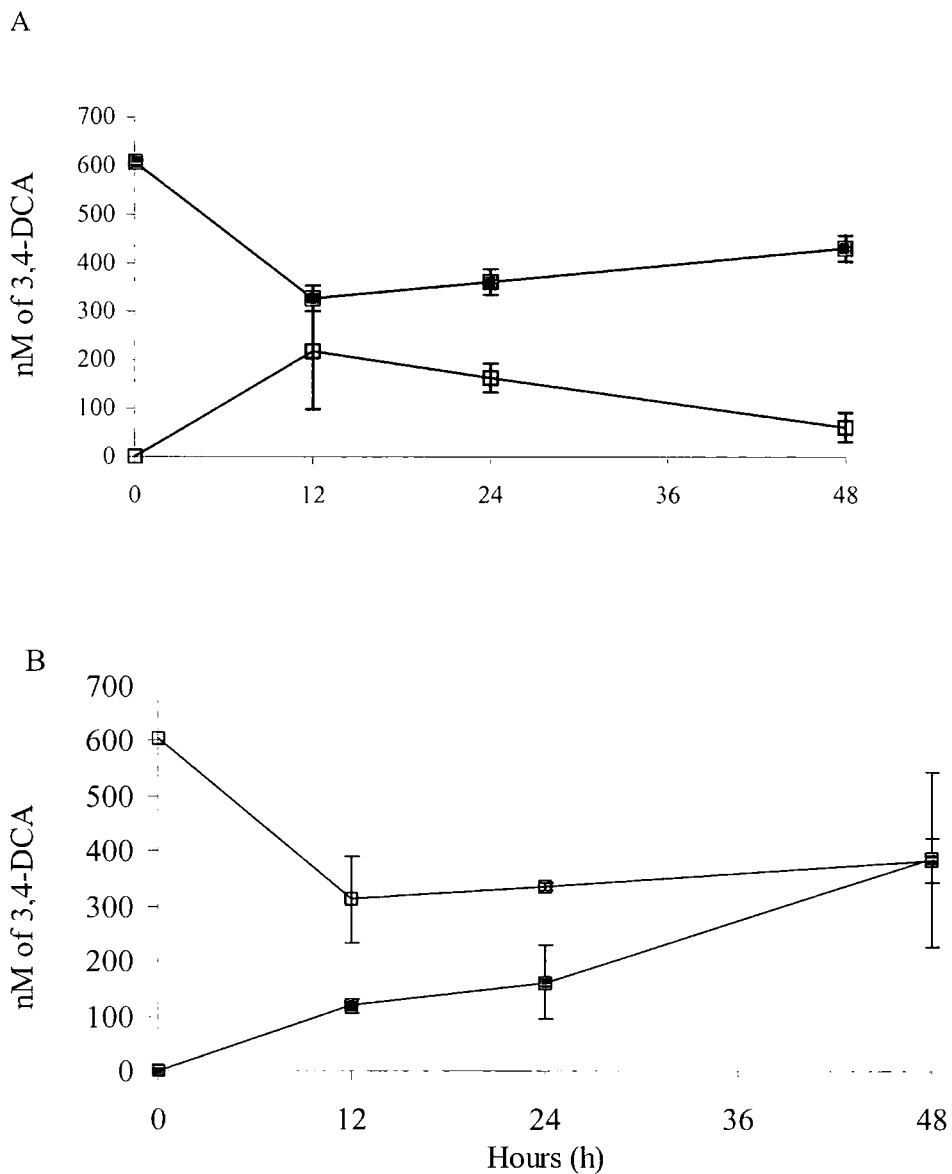


Figure 6.3 : Uptake of [^{14}C]-3,4-DCA in *Arabidopsis* root cultures.

Root cultures treated (B) or without (A) the safener cloquintocet mexyl (10 mg ml^{-1}) 24 hours prior to harvest were fed with an initial dose of $37 \text{ kBq } [^{14}\text{C}]\text{-3,4-DCA}$. Radioactivity was then monitored in the medium (■) and in the plant tissues (□). Data are the mean \pm standard deviation ($n=2$).

Table 6.7 : Percentage total recovery of applied radioactivity after feeding [¹⁴C]-3,4-DCA to *Arabidopsis* root cultures.

Total recoveries were obtained after summing of the radioactivity recovered in the plant extract and the medium.

Data are the mean \pm standard deviation (n=2).

Hours (h)	Recoveries (%)	
	Control tissues	Cloquintocet mexyl treated tissues
8	90 \pm 25	72 \pm 10
24	87 \pm 1	83 \pm 12
48	82 \pm 10	127 \pm 3

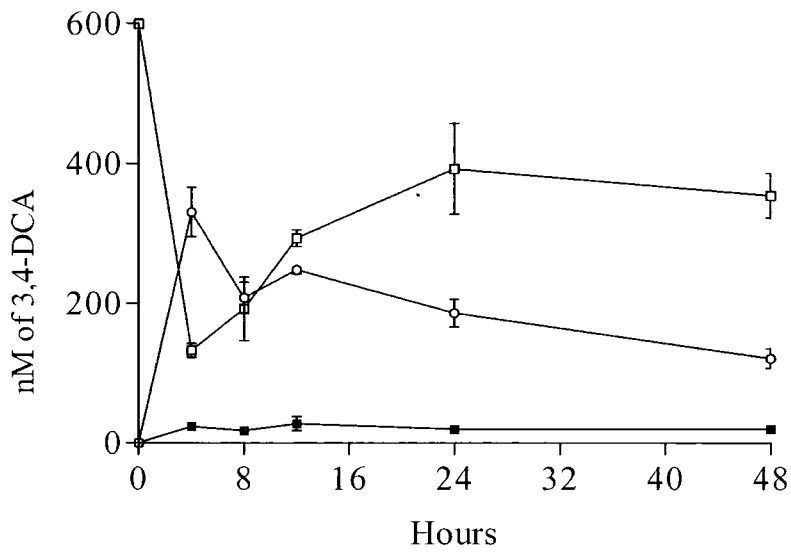


Figure 6.4 : Uptake of $[^{14}\text{C}]\text{-3,4-DCA}$ in *Arabidopsis* root cultures.

Suspension cultures were fed with an initial dose of 24,8 kBq of $[^{14}\text{C}]\text{-3,4-DCA}$ and radioactivity monitored in the extractable fraction from the plant (○), the non-extractable fraction (■) and the medium (□).

Table 6.8 : Percentage total recovery of applied radioactivity after feeding [¹⁴C]-3,4-DCA to *Arabidopsis* root cultures over 48 hours.

Recoveries (%) were determined after summing the radioactivity recovered in the plant extract, the medium and the bound residues.

Values represent the mean values ± variation in the duplicates.

Time (h)	Recoveries (%)
4	81 ± 4
8	70 ± 12
12	94 ± 3
24	99 ± 14
48	83 ± 8

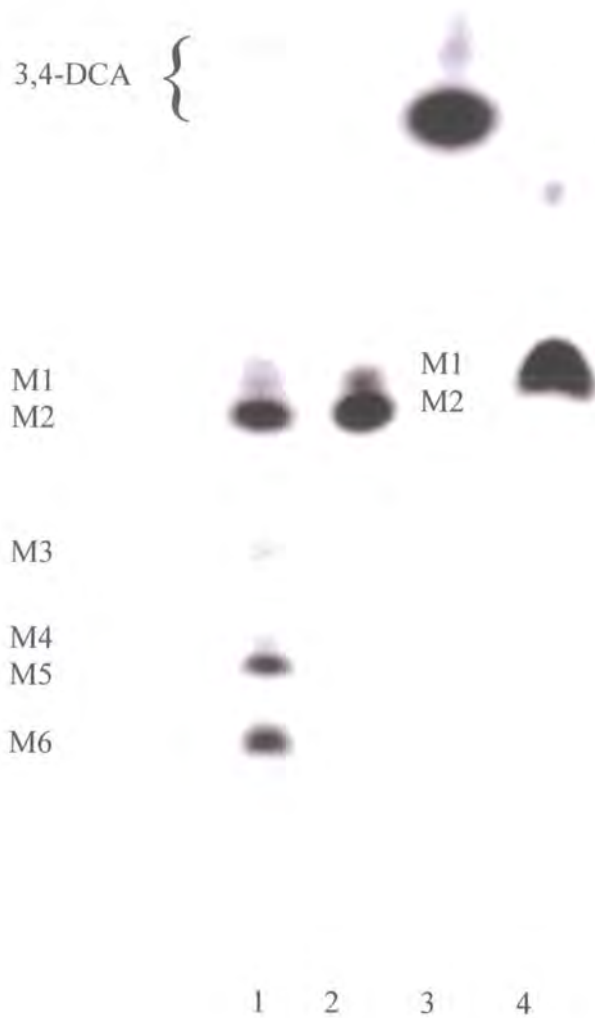


Figure 6.5 : Profile of radiolabelled metabolites determined by TLC after feeding [^{14}C]-3,4-DCA to *Arabidopsis* root cultures.

TLC showing extracts from *Arabidopsis* root culture tissue (1) and media (2), together with standards of [^{14}C]-DCA (3) and [^{14}C]-3,4-DCA glucoside (4).

Table 6.9 : Separation of metabolites formed in *Arabidopsis* root cultures fed with [¹⁴C]-3,4-DCA using TLC.

TLC plates were developed using the solvent mix : Chloroform : Methanol : Water (60:35:8; v/v), with radioactive metabolites identified using autoradiography.

6 metabolites (M1 to M6) were identified in the plant extracts and only one in the medium extract (M1).

	Metabolites	Rf
Plant extract	3,4-DCA	0.846
	M1	0.607
	M2	0.573
	M3	0.447
	M4	0.313
	M5	0.300
	M6	0.220
Medium	3,4-DCA	0.830
	M1	0.603
	M2	0.567

6.3.4 Metabolism of [¹⁴C]-3,4-DCA in *Arabidopsis* tissues

In all cases however, [¹⁴C]-3,4-DCA was rapidly taken up and the radioactivity then partitioned between the plant tissue and the media, with evidence of export of 3,4-DCA metabolites back into the surrounding medium. Very little of the radioactivity in the plants or medium was identified as unchanged 3,4-DCA at any time point in the study, confirming the rapid metabolism of this compound in *Arabidopsis*. In the root cultures, 6 metabolites were identified with 5 metabolites identified in the suspension cultures. In root cultures, further investigation revealed that the major conjugate was *N*- β -D-glucopyranosyl-3,4-DCA as determined by its co-chromatography. Several other minor conjugates were also identified on TLC, though their further characterisation was not attempted.

The fate of 3,4-DCA has previously been investigated in several plant species such as soybean, wheat and carrot suspension cultures (Bockers, *et al.*, 1994; Gareis, *et al.*, 1992; Schmidt, *et al.*, 1994; Schmidt, *et al.*, 1995; Schmitt, *et al.*, 1985; Winkler and Sandermann, 1989). Unlike these earlier studies, very little of the 3,4-DCA in *Arabidopsis* became incorporated into the insoluble fraction, with this being a major route of deposition of this chloroaniline in maize and soybean (Bockers, *et al.*, 1994; Schmidt, *et al.* 1995; Schmitt, *et al.* 1985, Winkler and Sandermann, 1989). Instead, the low rate of incorporation of 3,4-DCA into bound residues was associated with a rapid glucosylation and the export of the conjugate into the media.

Unlike soybean plants which largely metabolise 3,4-DCA to its malonyl-conjugate, *Arabidopsis* plants favour the formation of glucosyl-conjugates (Lao, *et al.*, 2003). Unlike the *N*-malonates which are stable to enzymatic digestions, the *N*-glucosides of 3,4-DCA can be hydrolysed in the soil (Winkler and Sandermann, 1989). It is surprising therefore that the plant has developed such an incomplete mechanism to conjugate the xenobiotic to

re-export it in the medium where it could easily be reversed to its original form and enter the plant again.

6.3.5 Cross-feeding experiment

3,4-DCA was shown to undergo conjugation in *Arabidopsis*, principally to the *N*-glucoside. After accumulation in the plant tissue, this major metabolite was exported in the medium where it accumulated over time. Similar studies were carried out examining the metabolism of 3,4-DCA in soybean plants (Lao, *et al.*, 2003) with several conjugates formed over time and the *N*-malonylated conjugate identified as the major metabolite being exported into the medium in a similar way to that observed in *Arabidopsis*. Having demonstrated parallel routes of 3,4-DCA export in different plants, it was then of interest to determine whether or not *Arabidopsis* could absorb the *N*-malonate and soybean the *N*-glucoside.

Both *Arabidopsis* root cultures and soybean plants were given an identical dose of [¹⁴C]-3,4-DCA (37 kBq of [¹⁴C]-3,4-DCA) and left for 48 h. An increasing quantity of metabolites accumulated in respective media (Figure 6.4 for *Arabidopsis* time course). The culture media were partitioned with ethyl acetate and concentrated before being resuspended in 50 µl of methanol. Fresh plants or cultures were then placed for 24 h in media supplemented with the concentrated extracts containing the metabolites (6.7 kBq of extracted metabolites). The distribution of radioactivity was then determined in both plants and medium.

Soybean plants were able to take an equal quantity of conjugates (13%) whether they originated from soybean or *Arabidopsis* (Table 6.10). *Arabidopsis* root cultures showed a greater capacity to take some of their own metabolites (13%) compared to the ones formed by soybean (5%). Once taken up into the roots, almost no bound residues were formed in accordance with previous time-course experiments.

With both *Arabidopsis* and soybean, 76% to 95% of the radioactivity remained excluded from the plant, irrespective of whether the roots were exposed to the 3,4-DCA *N*-glucosyl (as synthesised by *Arabidopsis*) or 3,4-DCA-*N*-malonate

(as synthesised by soybean). This observation suggested that such an export was an effective detoxification mechanism for 3,4-DCA for both species.

Table 6.10 : Percentage of administered [¹⁴C]-3,4-DCA metabolites reabsorbed after cross-feeding studies.

Source of exported metabolites	% applied radioactivity reabsorbed by plant tissue	
	<i>Arabidopsis</i>	Soybean
Soybean	5.1 ± 1.7	12.9 ± 0.2
<i>Arabidopsis</i>	13.2 ± 1.4	11.7 ± 0.4

6.4 Purification of xenobiotic metabolising GT enzyme

The major route for metabolism of 3,4-DCA in *Arabidopsis in planta* was shown to be through glucosylation, suggesting that this plant must contain a NGT capable of conjugating 3,4-DCA with glucose. It has previously been reported that GTs could glucosylate chlorinated aniline substrates in crop species such as wheat (Bockers, *et al.*, 1994; Schmidt, *et al.*, 1995; Schmitt, *et al.*, 1985) and carrot suspension cultures (Schmidt, *et al.* 1994). *N*-glucosylation of anilines has also been identified as a major route of detoxification for soybean with several reviews referring to the importance of these reactions in the remediation of aniline pollutants and the detoxification of herbicides (Bockers, *et al.*, 1994; Gareis, *et al.*, 1992 ; Frear, 1968; Harvey, *et al.*, 2002; Sandermann, 1994; Sandermann, *et al.*, 1991; Winckler and Sandermann, 1992).

The type 1 GTs of *Arabidopsis* have not been subject to investigation until very recently. The focus has been on the GTs of *Arabidopsis* involved in lignin synthesis (Lim, *et al.*, 2001) or their role in the metabolism of sterols (Warnecke, *et al.*, 1997). The GT family in *Arabidopsis* has recently been reviewed in several articles after the release of the genome sequence of *Arabidopsis thaliana* (TAGI, 2000). Most recently, GTs with activities towards caffeic acid (Lim, *et al.*, 2003), benzoic acids (Lim, *et al.*, 2002), IAA (Jackson, *et al.*, 2001) and several hydroxycinnamates (Milkowski, *et al.*, 2000) have been cloned and characterised.

Specific activities in *Arabidopsis* suspension cultures were assayed each day after subculturing to determine the optimal source for harvest and purification of the NGT activity. GT activity towards quercetin was unchanged over the time course, unlike the GT activities towards 3,4-DCA and 2,4,5-TCP, where the highest activities were found between 5 and 7 days after subculturing. Cells were subsequently harvested 6 days after subculturing. Activity towards 3,4-DCA was 13.2 ± 0.4 pmole min⁻¹ mg⁻¹ of protein and 10.24 ± 0.1 pmole min⁻¹ mg⁻¹ towards 2,4,5-TCP (Figure 6.6). Although specific GT

activities were very high on the day of subculturing, they were the result of cells freshly diluted; the weekly subculturing regime helped keeping healthy cells.

Thus, fast growth and yield were the determining factors for the choice of suspension cultured *Arabidopsis* cells as the optimal source for OGT purification. Harvesting at day 6 proved to be a good compromise between high activities and yield of cultured cells (Figure 6.6). In comparison, *Arabidopsis* root cultures yielded 5 grams (in 50 ml culture) of fresh tissues in 2 ½ weeks, while the suspension cultures produced up to 10 grams (per 50 ml culture) per week. Specific activities towards substrates of interest did not vary significantly on two populations of independent cultures as shown in Table 6.11.

GT activities were then determined in extracts from suspension cultures after precipitating the crude protein with differing quantities of ammonium sulphate. These studies showed that the optimal fraction for the recovery of GT activities towards 3,4-DCA, quercetin and 2,4,5-TCP was associated with the proteins precipitating between 40% and 60% $(\text{NH}_4)_2\text{SO}_4$. Ammonium sulphate precipitation represented a first useful step of purification with 69% of 3,4-DCA activity, 68% of quercetin activity and 70% of 2,4,5-TCP activity recovered in the 40% to 60% ammonium sulphate cut (Figure 6.7).

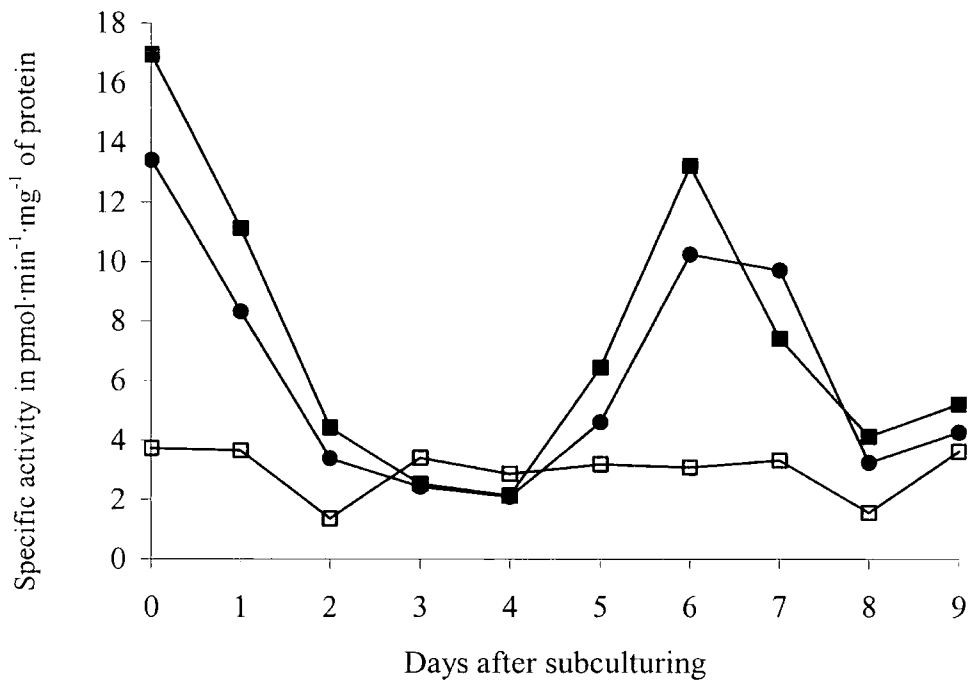


Figure 6.6 : GT specific activity determined daily after subculturing.

Specific activities were determined using 3,4-DCA (■), quercetin (□) and 2,4,5-TCP (●) as substrates.

Table 6.11 : Variation in *NGT* activity in 2 independent batches of *Arabidopsis* cell cultures and root cultures.

Specific *NGT* activities towards 3,4-DCA were determined in extracts from suspension cultured cells and root cultures grown on separate occasions.

Population	<i>NGT</i> specific activity ($\text{pmole min}^{-1}\text{mg}^{-1}$ of protein)	
	Root cultures	Suspension cultures
1	6.7 ± 0.1	11.3 ± 1.6
2	6.4 ± 0.5	12.3 ± 1.4

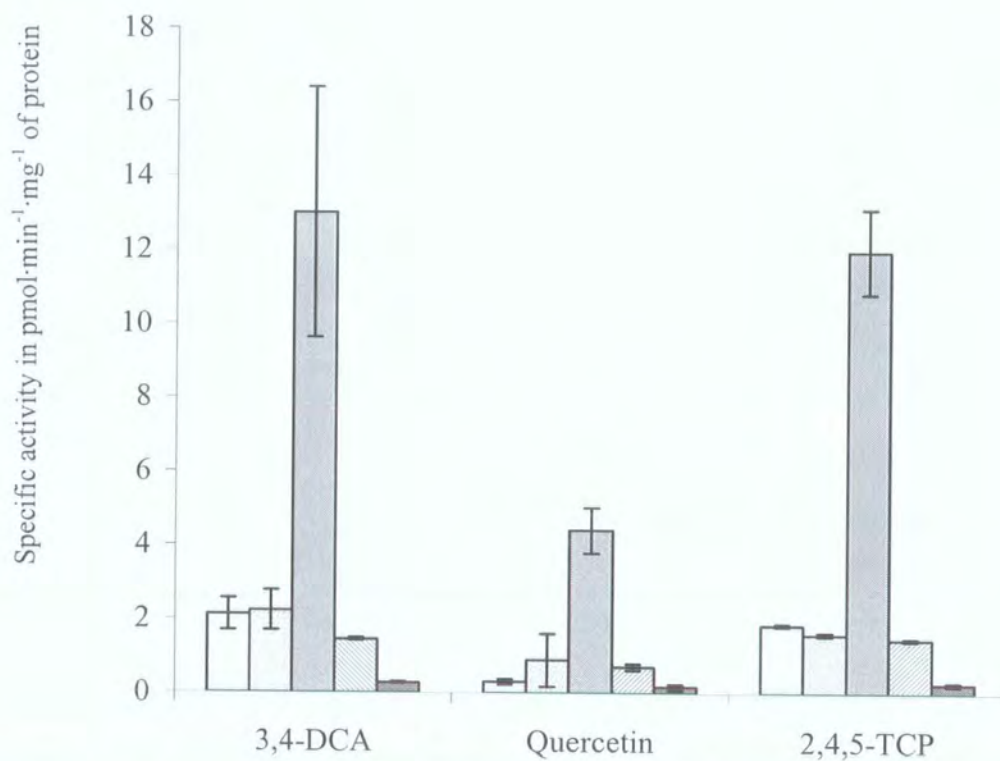


Figure 6.7 : GT specific activity in various ammonium sulphate fractions.

Specific activity towards 3,4-DCA, quercetin, and 2,4,5-TCP were determined from ammonium sulphate fractions prepared from *Arabidopsis* suspension cultures.

□ Crude extract □ 0-40% ■ 40-60% ▨ 60-80% ■ 80-100%

In crude preparations, safeners had been found to enhance the expression of GT activities in *Arabidopsis* cultures. *Arabidopsis* suspension cultures were treated with or without benoxacor for 24 hours prior to harvest. Identical quantities of crude protein extracts were then loaded on to a Q-Sepharose column previously equilibrated in 20 mM Tris-HCl, pH 8 + 1 mM DTT. The bound proteins were eluted with increasing NaCl up to 500 mM. As seen in Figure 6.8, the elution profiles of NGT activity towards 3,4-DCA were virtually identical. For simplicity non-safener-treated tissues were used as a source of the NGT enzyme in further purification steps.

The purification of the NGT active towards 3,4-DCA was achieved in 4 steps. The first purification step consisted of $(\text{NH}_4)_2\text{SO}_4$ (Figure 6.7) precipitation and this resulted in a 2.8 fold purification with 82% of the activity recovered (Table 6.12). Before the next step, the pelleted proteins were resuspended in phosphate buffer supplemented with 1 M ammonium sulphate pH 7.2. The protein was then loaded onto a 45-ml phenyl sepharose column (Phenyl Sepharose 6 Fast Flow CL-4B, Amersham Biosciences) in phosphate buffer (KPi 20 mM + 1 mM DTT pH 7.2) containing 1 M ammonium sulphate. The salt concentration was then reduced to salt-free phosphate buffer (20 mM KPi + 1 mM DTT pH 7.2). An equal mix of ethylene glycol: phosphate buffer (1:1, v/v) was then used to elute the strongly bound hydrophobic proteins at the end of the run (Figure 6.9). With respect to the UV absorbing proteins, non-bound proteins were eluted in the initial fractions (12 to 18) where no specific activity was detected as shown on Figure 6.9 before the decreasing salt content eluted the remaining hydrophobic proteins. GT activity was located in the fractions 50 to 60 coinciding with high associated protein absorbance at 280 nm. The highest NGT activity was located in fraction number 54. A compromise between recovery and purification was reached by pooling the active fractions (51 to 58) to obtain a 80 ml solution. The NGT recovered from the hydrophobic interaction step had a specific activity of $126 \text{ pmole min}^{-1} \text{ mg}^{-1}$ of protein having been purified 19 fold overall with 54% recovery of the starting activity (Table 6.12).

The pooled proteins were then dialysed overnight in 50 mM Tris pH 7.8 + 1 mM DTT and then applied onto a Q-Sepharose column (Amersham Biosciences, 10 ml volume) with the bound protein recovered with an increasing concentration of NaCl (from 0 to 1 M; Figure 6.10). The activity recovered was purified 44 fold, with a specific activity towards 3,4-DCA of 297 pmole min⁻¹ mg⁻¹ of protein being determined with a recovery of 31% of the starting NGT activity achieved (Table 6.12). The equilibrated protein pool was then further purified through an ion-exchange chromatography using a Blue Sepharose column previously equilibrated in phosphate buffer (20 mM Tris-HCl + 1 mM DTT pH 7.8) at a flow rate of 2 ml min⁻¹. The NGT was selectively eluted using 15 ml of 20 mM UDP-glucose (Figure 6.11). The active fractions corresponding to the peaks were then desalted on a HiTrap gel filtration column to remove UDP-glucose at a flow rate of 4 ml min⁻¹ and then the pooled active fractions reapplied onto a Q-Sepharose column, which was eluted with an increasing concentration of NaCl (Figure 6.12). Overall, the activity recovered was purified 9552 fold with a specific activity towards 3,4-DCA of 64,000 pmole min⁻¹ mg⁻¹ of protein with a recovery of 6% and a total of 0.01mg of protein recovered (Table 6.12).

The fractions of interest (fractions 32-38) were then individually collected and run on an SDS-PAGE gel (12%) (Figure 6.13). When analysed by SDS-PAGE a 50 kDa polypeptide was seen (Figure 6.13). This polypeptide was the only visible polypeptide in the active fractions and was therefore assumed to be the major GT with activity towards 3,4-DCA in *Arabidopsis*. Gel excision of this polypeptide from the first peak followed by trypsin digestion and peptide mass fingerprinting by MALDI-TOF MS identified the polypeptide as *Arabidopsis* glucosyl transferase UGT72B1 (Bowles, 2002; Ross, *et al.*, 2001). This GT was found to be closely related to an arbutin synthase sequence from *Rauvolfia serpentina* (GenBank™ AJ310148) (Figure 6.13).

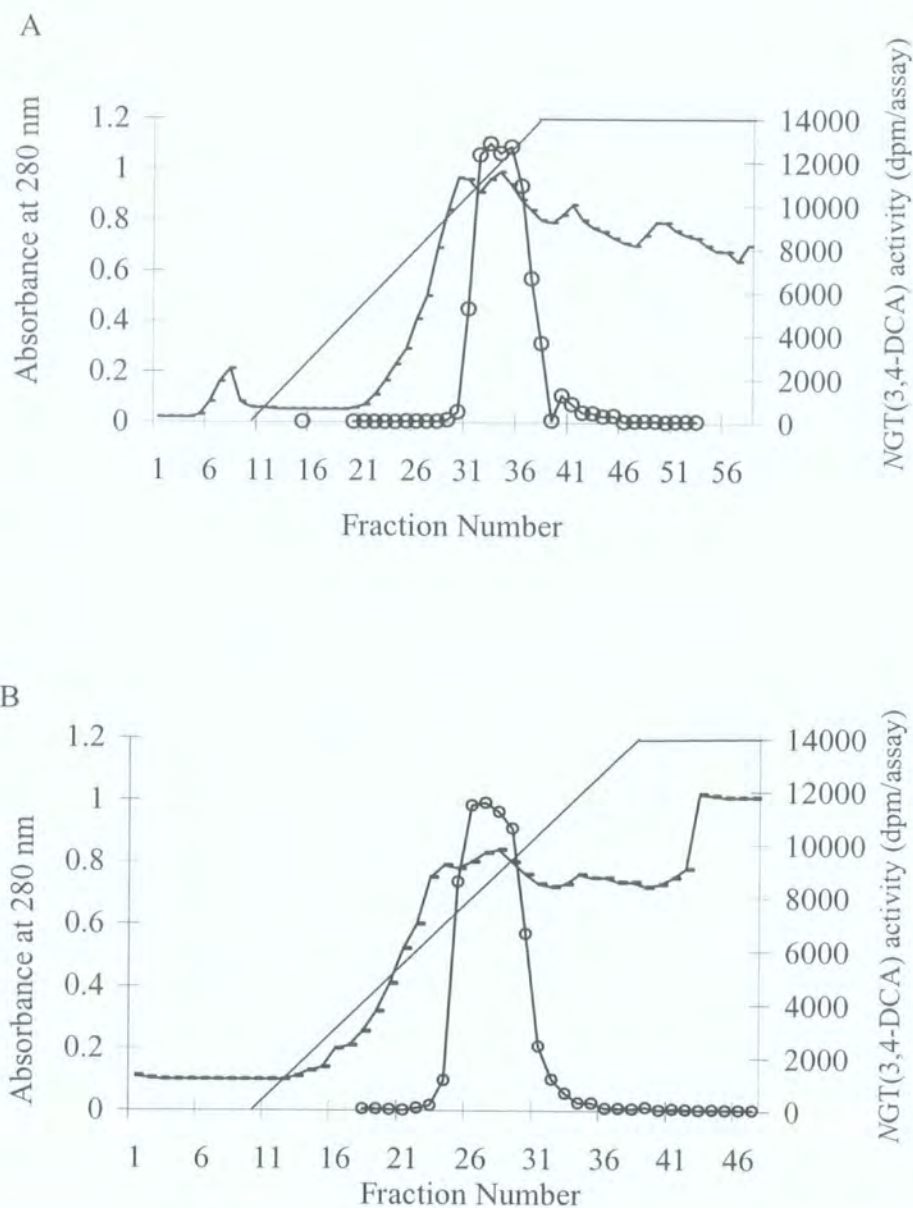


Figure 6.8 : Effect of safener-treatment of *Arabidopsis* suspension cultures on the profile of elution of NGT activity.

3,4-DCA conjugating activity was resolved from crude protein extracts of *Arabidopsis* suspension cultures, treated for 24 hours prior to harvest with (B) or without (A) benoxacor (10 mg ml^{-1}) using anion exchange chromatography (Q-Sepharose). The increasing concentration of salt used to elute the column are shown (—), along with the UV absorbance (—) and the NGT activity (\circ) of individual fractions.

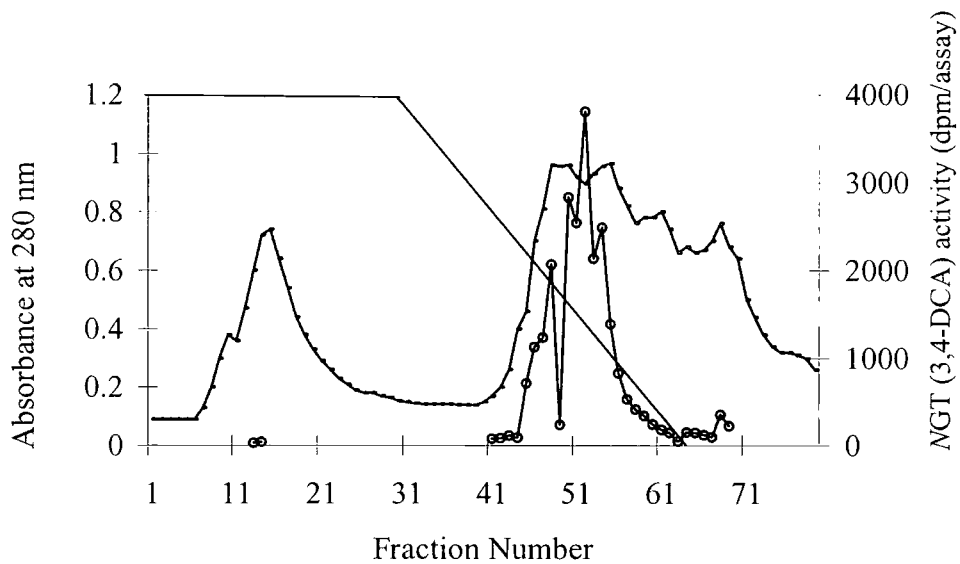


Figure 6.9 : Hydrophobic interaction chromatography of extracts of *Arabidopsis* suspension cultures.

The phenyl sepharose column was eluted with a decreasing concentration of ammonium sulphate (1 M to 0 M, —) and individual fractions monitored for UV absorbance (-) and NGT activity (○).

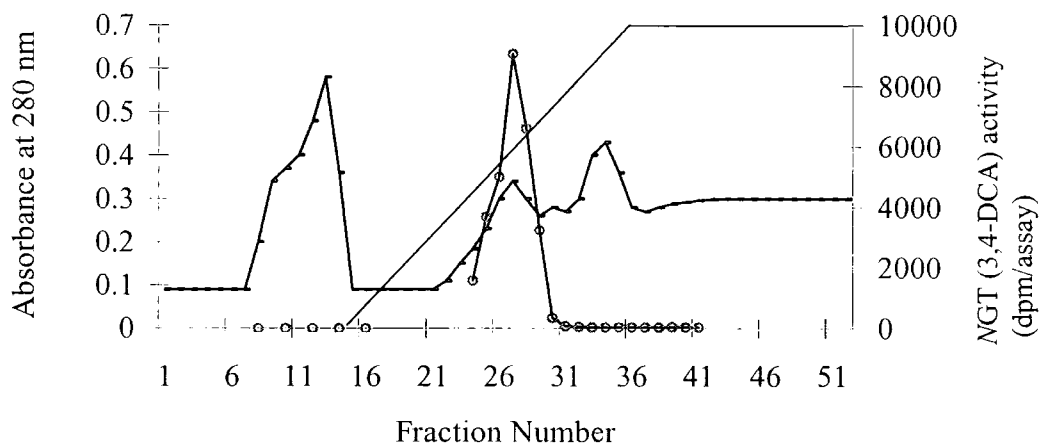


Figure 6.10 : Anion exchange chromatography of the *N*-GT partially purified protein from *Arabidopsis* suspension cultures.

Purification of *NGT* activity by anion exchange chromatography following a prior purification on the hydrophobic interaction column. Protein was eluted with an increasing concentration of salt (0 to 1 M NaCl) and fractions assayed for UV absorbance (-) and *NGT* activity (○).

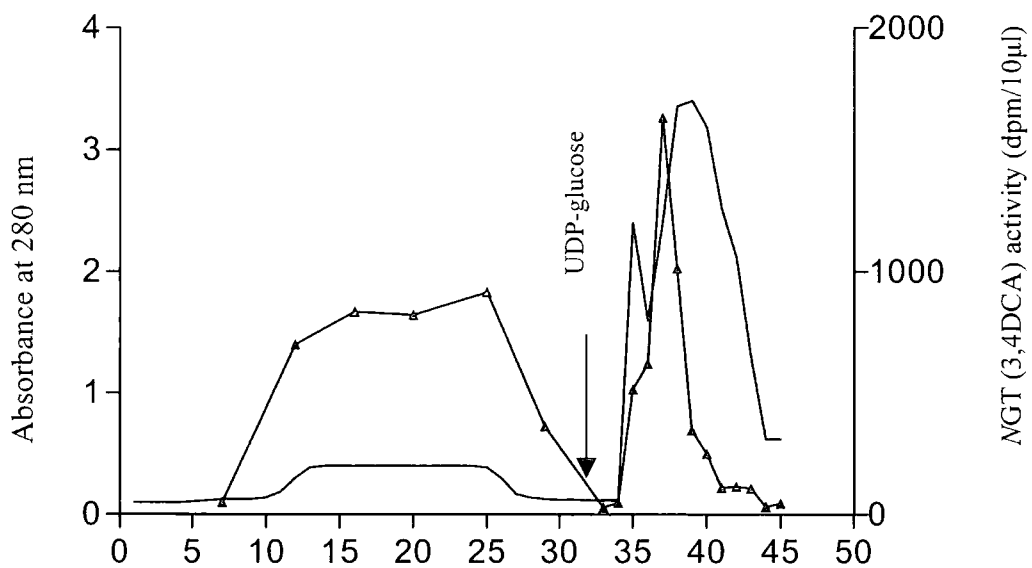


Figure 6.11 : Elution of *NGT* activity from Blue Sepharose using UDP-glucose.

The Blue Sepharose column was equilibrated in 20 mM Tris-HCl pH 7.8 + 1 mM DTT buffer. The bound proteins were eluted with 15 ml of 20 mM UDP-glucose in buffer.

Specific *NGT* activity was determined using 3,4-DCA as a substrate (▲) and protein absorbance monitored at 280 nm (-).

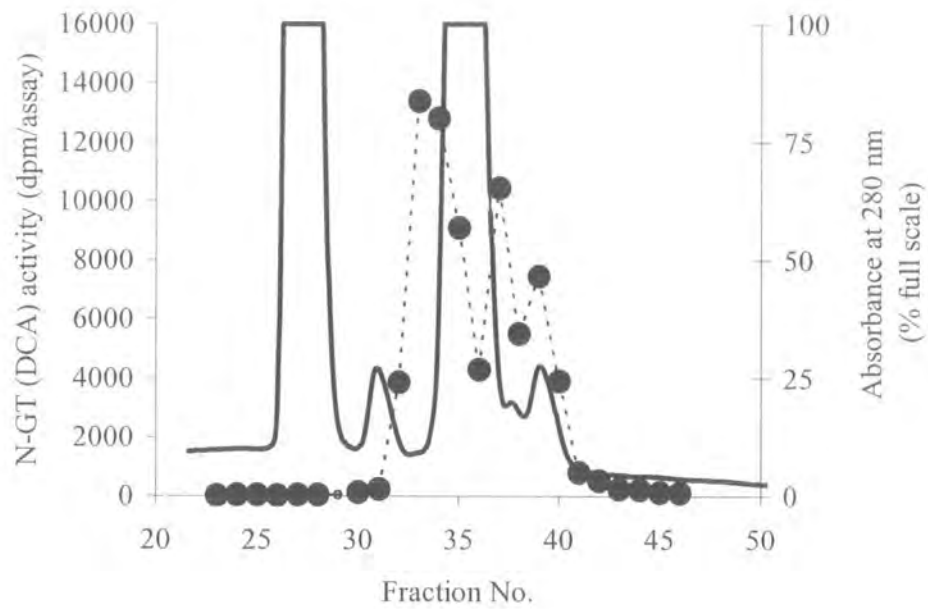


Figure 6.12 : Anion exchange chromatography of the affinity purified *N*-GT.

The elution profile represents the UV absorbance of the eluate (-) and *N*-GT activity towards 3,4- DCA (●).

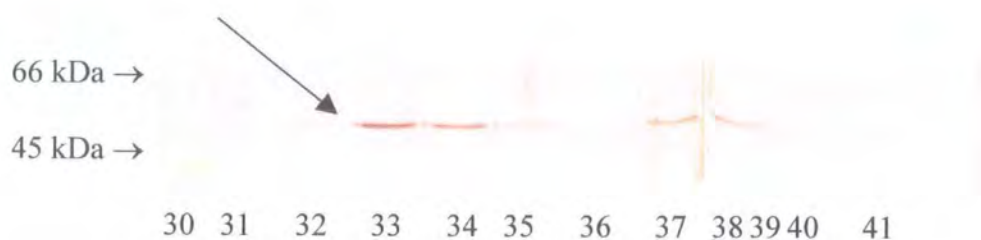


Figure 6.13 : *NGT* most active fractions after purification.

Samples of the fractions 30 to 41 were analysed by silver staining after SDS-PAGE, and the 50 kDa polypeptide in fraction 33 (arrowed) subjected to peptide mass fingerprinting analysis.

Table 6.12 : Summary of purification of NGT from *Arabidopsis* suspension cultures

Fraction	Protein (mg)	Activity (pmol min ⁻¹)	Sp. Activity (pmol min ⁻¹ mg ⁻¹)	Purification (fold)	Yield (%)
Crude	1672	11,170	6.7	-	100
40% - 60% Ammonium Sulphate	450	9,200	18.8	2.8	82
Phenyl Sephacrose	48	6,000	126	19	54
Q Sepharose	12	3,500	297	44	31
Blue Sephacrose/ MonoQ	0.01	663	64,000	9552	6

6.5 Discussion

NGT and OGT activities were determined in extracts from *Arabidopsis* in plant tissues based on optimised conditions as determined previously in soybean tissues (Chapter 4). *Arabidopsis* root and suspension cultures represented optimal sources for GT activities compared to whole plant tissues. A wide range of substrates were tested as GT substrates and a similar range of compounds found to be preferred as determined in maize and soybean including 3,4-DCA and 2,4,5-TCP and quercetin.

OGT activities have recently been shown to be enhanced by safener treatment in wheat tissues (Brazier, *et al.*, 2002). Safeners usually target monocotyledonous crops, but recent reports of the modification of GST gene expression in *Arabidopsis* (De Veylder, *et al.*, 1997) and the effect on GST activity (DeRidder, *et al.*, 2002) suggested that safener treatment might also affect GT activities in *Arabidopsis*. Using similar treatment conditions as described in maize and soybean (Chapter 3 and 4 respectively), a range of safeners was tested on *Arabidopsis* tissues and specific OGT and NGT activities determined. In root cultures, CMPI was found to be a good enhancer of NGT activity, with fenclorim found to be the best inducer of OGT activity towards quercetin. CMPI and dichlormid were both good enhancer of OGT activity towards 2,4,5-TCP in root cultures. In suspension cultures, the maize safener benoxacor and the rice safener fenclorim were found to be optimal inducers of GT specific activities.

The metabolism of 3,4-DCA in *Arabidopsis* was tested in whole plants, root cultures and suspension cultures previously treated with or without safeners. In all studies, 3,4-DCA was rapidly metabolised, with only a minor proportion of the parent 3,4-DCA recovered at any time point following dosing. Initially, an increasing quantity of radioactivity was taken up by the plant, but was then released back in the surrounding medium. Analyses of the plant extracts confirmed the presence of six conjugated metabolites of 3,4-DCA. The major conjugate found *in planta* was identified as the *N*-glucosyl-3,4-DCA derivative,

and this compound was found to be exported into the medium. These observations confirmed previous reports in carrot suspension cultures of rapid uptake of 3,4-DCA and its conjugation to more soluble compounds particularly glucosides (Schmidt, *et al.*, 1994). In carrots, the conjugated metabolites then underwent further metabolism following conjugation by malonyltransferases which was a minor reaction in *Arabidopsis* (Schmidt, *et al.*, 1994). Similar observations were made in soybean plants, with glucosides of 3,4-DCA accumulating in the roots while *N*-malonyl conjugates were transported to aerial parts of the plant tissues (Bockers, *et al.*, 1994) or excreted in the surrounding medium (Gareis, *et al.*, 1992).

The modest formation of bound residues in *Arabidopsis* representing less than 5% of the applied radioactivity found in root cultures was in accordance with the observations made in carrot suspension cultures where bound residues also represented a minor proportion (Schmidt, *et al.*, 1994). However, radioactive residues from [¹⁴C]-3,4-DCA were extensively associated with the insoluble fraction in soybean and wheat tissues (Bockers, *et al.*, 1994).

A comparative study in *Arabidopsis* and soybean confirmed that the route of detoxification of 3,4-DCA occurred predominantly through *N*-malonylation in soybean (Bockers, *et al.*, 1994; Gareis, *et al.*, 1992; Schmidt, *et al.*, 1995; Winkler and Sandermann, 1989). 3,4-DCA metabolism in *Arabidopsis* more closely resembled that seen in wheat and rice where it is rapidly *N*-glucosylated (Bockers, *et al.*, 1994; Schmitt, *et al.*, 1985; Still, 1968; Winkler and Sandermann, 1992). Both soybean and *Arabidopsis* exported their 3,4-DCA conjugates into the surrounding medium. Although soybean tissues showed an equal ability to absorb conjugates from either plant species as compared with *Arabidopsis*, the uptake of conjugates in the media was modest in both cases, with the majority of the metabolites (87 to 94% of the radioactivity) remaining in the medium throughout the feeding study. *N*-malonyl conjugates have shown to be relatively stable end products of 3,4-DCA metabolism representing an efficient detoxification step. In contrast, the efflux of *N*-glucosyl conjugates is not a long term mechanism of detoxification as the glucosyl conjugates have been shown to be labile to both non-enzymatic and microbial hydrolysis (Winkler and Sandermann, 1992).

The purification of the *N*-GT with activities towards 3,4-DCA from *Arabidopsis* suspension culture was based on four steps of chromatography using ammonium sulphate precipitation, hydrophobic interaction, anion exchange chromatography (2 rounds) and affinity chromatography using Blue Sepharose. The 9552 fold purification of the enzyme resulted in the identification of a single 50 kDa polypeptide as determined by SDS-PAGE, which following tryptic digestion and peptide mass fingerprinting using MALDI TOF was unambiguously identified the enzyme UGT72B1 (Ross, *et al.*, 2001).

Subsequent confirmation as to the identity of UGT72B1 as the *NGT* was done after the completion of my labwork by Dr David Dixon, Dr Ian Cummins and Dr Melissa Brazier (Loutre, *et al.*, 2003). The cloning of the gene from *Arabidopsis* was carried out by amplification of the sequence using primers designed specifically to the gene sequence (NM_116337). The sub-cloning in an expression vector was subsequently done in pET11d expression vector prior to its transformation in the Rosetta (DE3) competent cells. After induction with IPTG, the majority of the protein accumulated in the insoluble fraction but a minor proportion was recovered in the soluble fraction. After purification, the rGT72B1 enzyme was assayed for activity and towards 3,4-DCA and found to be highly active (10.67 ± 0.18 nkat mg⁻¹ pure protein). Several chlorinated anilines and phenols substrates were also tested and activities towards 3,4,5-trichloroaniline (1.03 ± 0.09 nkat mg⁻¹), 2,4-dichloroaniline (0.71 ± 0.01 nkat mg⁻¹), picloram (0.04 ± 0.00 nkat mg⁻¹), 2,4,5-Trichlorophenol (5.19 ± 0.33 nkat mg⁻¹), 2,3,4-trichlorophenol (0.44 ± 0.00 nkat mg⁻¹), 2,3,6-trichlorophenol (0.03 ± 0.00 nkat mg⁻¹), phenol (0.06 ± 0.00 nkat mg⁻¹), 4-nitrophenol (0.26 ± 0.00 nkat mg⁻¹), 4-hydroxyphenylpyruvic acid (0.18 ± 0.14 nkat mg⁻¹) also determined while the enzyme could *N*-glycosylate the herbicide picloram it was not active towards metribuzin, chloramben, amitrole or the pollutant 4-nitroaniline.

The identity of the *N*-glucosyl-3,4-DCA formed in the *in vitro* assays was confirmed by electrospray ionization time of flight mass spectrometry ESI-TOF

MS analysis ($m/z = 322.4$ Da in negative mode). The molecular mass of the enzyme was also confirmed by ESI-TOF MS analysis as 52,928 Da. Interestingly, the activity of UGT72B1 as an NGT was markedly dependent on the presence of DTT, or a similar thiol reducing agent, in the assay mixture and was totally inhibited by treating the recombinant enzyme with iodoacetamide. Kinetic data were also obtained for NGT (3,4-DCA) with a $K_M = 22.3 \pm 6.8 \mu\text{M}$, $V_{\text{max}} = 3.6 \pm 0.4 \text{ nkat mg}^{-1}$ with acceptor and $K_M = 4.6 \pm 2.3 \mu\text{M}$, $V_{\text{max}} = 17.5 \pm 4.1 \text{ nkat mg}^{-1}$ with UDP-glucose. The O-GT activity towards 2,4,5-TCP had a $K_M = 14.3 \pm 7.5 \mu\text{M}$, $V_{\text{max}} = 1.75 \pm 0.30 \text{ nkat mg}^{-1}$ towards acceptor and $K_M = 1.2 \pm 0.4 \mu\text{M}$, $V_{\text{max}} = 11.2 \pm 1.3 \text{ nkat mg}^{-1}$ with UDP-glucose. 3,4-DCA was the preferred substrate ($K_{\text{cat}}/K_M = 86800 \text{ M}^{-1} \text{ s}^{-1}$) as compared with 2,4,5-TCP ($K_{\text{cat}}/K_M = 54500 \text{ M}^{-1} \text{ s}^{-1}$).

Although sequence homologies of UGT72B1 and UGT72D1 (Meßner, *et al.*, 2003) were low, both enzymes have detoxifying activity towards 2,4,5-TCP and 3,4-DCA.

It was interesting that the sequence of the 72 sub-group as described by Ross (Ross, *et al.*, 2001) was associated with enzymes active in xenobiotic conjugation. Although UGT72B1 was shown to be more closely related to a *Rauvolfia serpentina* OGT (Hefner, *et al.*, 2002), it did not accept hydroquinone as a substrate. The arbutin synthase GT accepted hydroquinone as preferred substrate but also had a much broader substrate specificity towards natural and synthetic phenolic compounds (Hefner, *et al.*, 2002).

The comparison of GT sequences involved in xenobiotic detoxification in *Arabidopsis* (Meßner, *et al.*, 2003), and *Rauvolfia serpentina* (Hefner, *et al.*, 2002) reveal the complex relationship between sequence and substrate specificity which is apparent even between closely related sequences. This highlights the extreme complexity developed by plants to adapt and diversify the role played by their GTs in xenobiotic and natural product metabolism.

Overall Conclusions and Future Work

Conclusions

Metabolism studies investigated conjugation of two model xenobiotics including 3,4-DCA and *p*-nitrophenol. The plants were fed with radiochemicals and the distribution of radioactivity determined in plants, medium and non-extractable residues at timed-intervals. The plants took up the xenobiotics and on analysis of the radioactive residues, were found to conjugate these compounds to form polar metabolites. Two conjugate formed from *p*-nitrophenol accumulated in maize tissue with the major one being identified as the glucoside. 3,4-DCA was conjugated into six metabolites in *Arabidopsis* root cultures with the *N*-glucoside conjugate identified as the major metabolite present in the plant extract but also in the surrounding medium where it was shown to be exported.

Crude protein extracts prepared from maize, soybean and *Arabidopsis* were shown to exhibit *OGT* and *NGT* activities towards a range of compounds of both natural and synthetic origin. The detection of the *GT* activity was based on an assay using radiolabelled UDP-glucose (Parry, *et al.*, 1994) to monitor the formation of the radioactive glucoside, with the optimised conditions determined using extracts from soybean found to be also suitable for studies with maize and *Arabidopsis*. In all cases, both *NGT* and *OGT* activities were higher in non-photosynthetic tissues as compared to photosynthetic material. Chemical pre-treatments including *GT* substrates and safeners affected *GT* activities in all species; demonstrating that the inducing activity of safeners extends to *GTs* in both monocotyledonous and dicotyledonous plants.

N-glucosylation in *Arabidopsis* and *N*-malonylation in soybean were respectively confirmed as representing the major routes for detoxification of 3,4-DCA *in planta*. A cross-feeding experiment was set up to study the uptake of metabolites primarily exported from each species into the root medium.

In both cases re-import was found to be inefficient though soybean was able to absorb metabolites originating from soybean more efficiently than *Arabidopsis*.

OGT activities were increased by benoxacor and dichlormid in maize, by oxabetrinil and R-29148 in etiolated soybean and fenclorim *Arabidopsis* root and suspension culture while NGT activities were enhanced by CMPI in *Arabidopsis* root culture. Interestingly these results demonstrated a similarity in responsiveness of specific GTs to safeners in monocotyledonous and dicotyledonous. However, the induction of GTs activities was not associated with an increase in the conjugation of the xenobiotic *in planta*.

The cloning of GT from soybean and maize was attempted in order to establish if enzymes involved in plant secondary metabolism also had activity towards xenobiotics. Maize *bronze-1* represented a good example to test for wider substrate specificity. Unfortunately, the gene sequence could not be amplified from maize. Instead, a conserved domain present in several type 1 GTs from plants was used to design primers to amplify sequences located 500 bp towards the 3' end of GT sequences. Four partial sequences were amplified from soybean and maize tissues and in the case of soybean, sequences obtained from public databases allowed the construction of a full length GT sequence termed *GmGT1_32*. However, when expressed in *E.coli*, the recombinant *GmGT1_32* protein was found to be insoluble.

The identification of a putative full length GT clone from maize (*ZmRP*) was achieved through collaborative work with Aventis Crop Science. However, after transformation of the recombinant sequence in *E.coli*, no overexpressed protein could be obtained.

Finally, based on the optimised biochemical assay, a directed approach was used to purify a *N*-GT involved in the conjugation of the pesticide residue 3,4-DCA from the model plant *Arabidopsis thaliana*. The purification was carried out using a 4-step purification strategy including an affinity step which resulted in the isolation of a pure 50 kDa protein. This protein was subsequently identified as UGT72B1 from *Arabidopsis* using proteomics. The subsequent cloning and overexpression in bacterial host allowed to determine a similar substrate

specificity as for the purified enzyme. This was the first example of the isolation of a GT involved in the detoxification of xenobiotics.

Future work

Based on the outputs from this thesis the following studies would be of future interest.

- It would be of interest to optimise an overexpression system to detect the soluble GT activity of the full-length enzymes cloned from maize and soybean and assay these enzymes for their substrate specificity towards a wide range of substrates.
- Molecular cloning and expression of further GTs from maize and soybean are likely to reveal useful information on the evolution of the GT family in addition to what has already been achieved by studying the GT family in *Arabidopsis*.
- The study of T-DNA knockout *Arabidopsis* plants in the locus of UGT72B1 would be of interest to determine whether there are other GTs present with overlapping specificities towards 3,4-DCA and xenobiotics.

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APPENDIX 1

A putative full-length GT (DPBG GT) was identified from soybean EST searches. The sequence was characteristic of GTs although shorter than most of those described in the databases.

As such gene was found to be distinct from other type 1 GTs identified from plants.

Study of DPBG GT from soybean

A search on the EST databases isolated two soybean ESTs : AW 310 191 (figure A) and AW 233 960 (Figure B) corresponding to the sequences of the same clone (sf32e06) from both ends. The two partial sequences were analysed and found to be closely related to a dolichyl-phosphate- β -glucosyltransferase from *Arabidopsis thaliana*. The closely related Arabidopsis protein accession reference at the time of the search was Q9SLN0, now NP_181 943. The protein sequence identified was predicted to encode 336 amino acids in *Arabidopsis*, with a predicted molecular mass is 38.337 kDa and a predicted pI of pH 8.90.

Although the sequence of this DPBG GT was distant from other members of the family-1 GTs, the aim was to clone the enzyme and to determine whether it had any activity towards natural products or xenobiotics. It also aimed at gaining information of non-related sequences and at determining ability to carry out the divergent role of glucosylation of low molecular weight compounds.

PCR with the newly designed primers DPBG 1 and DPBG 2 (table A) resulted in the isolation of fragments of expected size that were subcloned into expression vectors. The restriction patterns on the amplification products in three soybean libraries (cell culture, root and shoot) (Skipsey, *et al.*, 2000) established variant restriction patterns following digestions with restriction enzymes. The fragment amplified from the cell culture library was sequenced and a frame shift mutation close to the 3' end of the sequence was outlined. This resulted in the HisTag present

in the expression vector to be out of frame. Any further purification was therefore impossible.

Following a new search in the databases, additional ESTs corresponding to DPBG GT were found and seem to correlate the restriction patterns obtained after running the amplified products on electrophoresis gels. ESTs deriving from mature flowers, 4 days-old seedlings, 14 days-old seedlings have an extra 82 bp compared to the root and cell culture sequences. The prediction of the start codon was correct for a different tissue source. Not being aware of the importance of the tissue source in the initial amplification protocol, the use of the primers on the cell culture library resulted in the amplification of a wrong fragment due to the different splicing site in cell cultures. The stop codon was misplaced. To obtain a topology of the splicing site, a new primer was designed downstream any putative splicing site named DPBG4. It was supposed to be used in combination of the primer designed towards the 3' of the gene in order to establish the splicing effect. A correct start primer was designed again to be used in the amplification programs on cell culture library.

Following cloning of the sequence into pET no expression of the respective recombinant polypeptide could be achieved in *E.coli* and further attempts at characterising these clones was abandoned.

Table A : Primer sequences used to amplify a dolichyl-phosphate- β -glucosyltransferase.

The initial amplification of the putative full-length GT from soybean was carried out with DPBG 1 and DPBG 2. DPBG3 was the modified primer to the 5' end of the gene after a splicing effect was identified. DPBG4 was designed further along the sequence to study the splicing effect and to gain information on its effect depending on the tissue source tested.

Primer sequence	
DPBG 1 (<i>Nco</i> I)	d (GGA ATA GCC ATG GAT TAT ATC TGT CTT TTT GTC)
DPBG 2 (<i>Xho</i> I)	d (GCG GCG CTC GAG GGT AGA ATT AGA AAT TCT CC)
DPBG 3 (<i>Xho</i> I)	d (CGC GCG CTC GAG GAA ATT CTC CAC ATA CCA GTC)
DPBG 4	d (TTG TTT AGA TAG GAA AAT GCC ACT TCT C)

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1  GGCTCAAGAG CTCATCAGAG GAGAAAGCTT TAGCTACAAG
41 AAAGCTGGTA CCGCAATTTT TTGATGAAGG GCTTCCATCT
81 TGTGGTTCTC TTGGCTGCTG GTCCTGGAAT TCGTGATACA
121 CAGTGTGGTT TCAAGATGTT TACTAGGGCA GCAGCCAGGA
161 AGCTTTTTTC AAATGTACGC TTAAAAAGGA TCCAAGGTGA
201 ATCTTCTGAG TATACCTAAC. ATGCTTTGGG AGCTTGTGCT
241 CATGTCTGTG GGGTACAGGA CTGGTATGTG GAGAATTTCT
281 AATTCTACCT GAGCGAGCGT GGATGATCAA CTTAACTGGG
321 AAGAGAAGTG GCATTTTCCT ATCTAAACAA GAGTCACCTC
361 TTACAAGAGA ACATATACTC TCATGCCGGG CTTAAGGGAC
401 AATCATGCAA TATTTAAAGT TTGTAGGACT AACCATACCT
441 TTCCGGGTTA TTCCCTTCAG TTTGGGATCA TAGAGGCTTT
481 TATCCTTTTT ACTTTTAGTA TTGCTATTTT TACCAGGTAA
521 AAATATATTC AACATTATGT TGTTACTGTT GGCTGGGAAT
561 AGTTTTCAAT CACATTGGTT TGTATTTTTC TTAATCAAAA
601 TATTATATAG ATAACCTTGT ATTCCTTTCA TTCCATTTGA
641 AAGACGTTAT CTAATATTAA AGAGTTAAAA AA

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Figure A : DNA sequence of the soybean EST AW 310 191.

The EST resulted from the sequencing of the clone sf32e06.x1 sequenced from roots of 'Supernod' plants used to design the contig DPBG to be amplified.

1 GGGTCGGGTT GAACTTCGAA GTCTTGATCG ACGACGTTGA
41 GCGTTCTCTC TCTTCGTTCA GCTCAATCTG GGATACGAAG
81 CTTGCAATAG CTGCAGCATC TGAATAGGAA TGGATTATAT
121 CTGTCTTTTT GTCACCTTCA CTGCGATTGC GCTCTTAGGG
161 TTTGTCTTTG TAGTGTTTTT TGAAGCATAT AAAAGAAGAA
201 ACAACCATCA GCATATCGAA GTTCCGGCTA TTTTGAAGA
241 TCCTAGCTCA TTGAAACAGG TTCCTTGCCC ACATATTGTT
281 GATCCAGCCA CAAAGTATAT TTCTTTGATT ATCCCTGCAT
321 TCAATGAGGA GCATAGGCTT CCAGGAGCTC TTGAGGAAAC
361 TATGAATTAT CTTCATCAAC GTACTTTAAA GGATTCTTCA
401 TTTACATATG AGGTTGTAAT TATTGATGAT GGAAGTGCTG
441 ATGAGACAAA NAGAGTAGCT TTTGAATTTG TGAGGAAATA
481 CACAGTAGAC AAGGTAAGGG TCATCCTTCT AGGAAGAAAT
521 CATGGCAAAG GAGAAGCAAT C

Figure B : DNA sequence of the soybean EST AW 233 960.

The EST resulted from the sequencing of the clone sf32e06.y1 sequenced from roots of 'Supernod' plants used to design the contig DPBG to be amplified in soybean.

