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***O*-Glucosyltransferases in wheat (*Triticum aestivum*) and the
competing weed black-grass (*Alopecurus myosuroides*)**

Melissa Catherine Brazier

PhD Thesis

University of Durham

School of Biological and Biomedical Sciences

2003

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7 NOV 2003

Melissa Catherine Brazier

***O*-Glucosyltransferases in wheat (*Triticum aestivum*) and the competing weed black-grass (*Alopecurus myosuroides*)**

Abstract

The glucosylation of xenobiotics, catalysed by UDP-glucose dependent *O*-Glucosyltransferases (OGTs), is a major route of pesticide metabolism in wheat (*Triticum aestivum* L.). OGTs have been characterised in wheat seedlings, their activities toward xenobiotics and natural products defined and their regulation by herbicide safeners studied. Corresponding OGT activities have also been studied in populations of the competing weed black-grass (*Alopecurus myosuroides*), which differ in their resistance to herbicides. OGT activities were identified in crude extracts from wheat and black-grass. Preferred substrates were 2,4,5-trichlorophenol and the flavonol quercetin. In wheat, treatment with the herbicide safeners, cloquintocet mexyl and mefenpyr diethyl enhanced OGT activities toward xenobiotic and flavonoid substrates especially in the shoots. The respective OGT activities were determined to be higher in herbicide-resistant populations of black-grass than in the herbicide-susceptible wildtype populations. A 53 kDa OGT active toward 2,4,5-trichlorophenol and quercetin was purified over 200-fold from wheat shoots using classical chromatography methods. In addition to 2,4,5-trichlorophenol the OGT also conjugated 3-hydroxyflavones. Putative GT cDNA clones were obtained from wheat and black-grass using a combination of cDNA library screens, 5' RACE and RT-PCR. The respective recombinant proteins were expressed as soluble proteins in *E.coli* but had no activity toward any xenobiotic or flavonoid substrates tested. Finally, the effects of safener treatment (wheat) and herbicide cross-resistance (black-grass) on the concentrations of endogenous glucosylated phenolic metabolites were determined. Conjugates of phenyl propanoids and C-glycosylated flavonoids were identified as major metabolites in both species. In wheat shoots, herbicide safener treatment resulted in the accumulation of conjugated ferulic acid. Herbicide-resistance in black-grass was associated with the accumulation of conjugated 4-hydroxycinnamic acid. It was concluded in both wheat and black-grass that changes in xenobiotic conjugation imposed by safeners or acquired resistance to herbicides were mirrored in changes in the accumulation of conjugated natural products.

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Declaration

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

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Publications arising from work described in this thesis

Brazier, M., Cole, D. J., Edwards, R., 2002. *O*-Glucosyltransferase activities toward phenolic natural products and xenobiotics in wheat and herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). *Phytochemistry* 59, 149-156.

Brazier, M., Cole, D. J., Edwards, R. 2001. Herbicide safeners induce glucosyltransferase activity in wheat. In *Brighton Crop Protection Conference-Weeds* 539-544 (meeting proceeding).

Brazier, M., Cole, D. J., Edwards, R. 2003. Purification and Characterisation of a 2,4,5-Trichlorophenol Detoxifying *O*-Glucosyltransferase from Wheat.

Phytochemistry. *In Press*

Publication arising from associated work

Loutre, C., Dixon, D.P., Brazier, M., Slater, M., Cole, D.J., Edwards, R. 2003.

Isolation of a glucosyltransferase from *Arabidopsis thaliana* active in the metabolism of the persistent pollutant 3,4-Dichloroaniline. *The Plant Journal*. *In press*

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Abbreviations

AMV	avian myeloblastosis virus
BCIP	5-bromo-4-chloro-3-indolyl phosphate
cDNA	complimentary DNA
CDNB	1-chloro-2,4-dinitrobenzene
cv.	cultivar
CYP	cytochrome P450 mixed function oxidase
Da	daltons (kDa = kiloDaltons)
dNTPs	deoxynucleoside triphosphates
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMF	<i>N,N</i> -dimethylformamide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GST	glutathione-S-transferase
GT	glucosyltransferase
hr	hours
HPLC	high performance liquid chromatography
IPTG	isopropyl β -thiogalactoside
LB	Luria-Bertani
min	minutes
mRNA	messenger RNA

NBT	nitro blue tetrazolium
NGT	<i>N</i> -glucosyltransferase
OGT	<i>O</i> -glucosyltransferase
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonium lyase
PCP	2,3,4,5,6-pentachlorophenol
PCR	polymerase chain reaction
Pfu	plaque forming unit
pI	isoelectric point
poly A+ RNA	polyadenylated RNA
PVPP	polyvinylpyrrolidone
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
s	seconds
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
V	Volts

1. INTRODUCTION

1.1 Xenobiotic metabolism in plants

Plants are exposed to a wide range of xenobiotic compounds in the environment, which they absorb and bioaccumulate. These potentially harmful compounds are then metabolised using a variety of mechanisms to produce non-toxic products. These reactions are of interest as they can determine the selectivity of herbicides in weeds and crops (Owen, 2000). In some cases, tolerance to a particular herbicide can be due to a number of factors including reduced absorption and translocation and different sensitivities at the target site. However, the relative rates of herbicide metabolism in crops and weeds, is recognised as a primary determinant of herbicide selectivity (Owen, 2000). Environmental regulatory authorities also monitor pesticide metabolites within food crops and this requires the identification and toxicological assessment of any significant residues. The ability of plants to metabolise industrial pollutants has also lead to research on the development of phytoremediation techniques to decontaminate polluted land (Coleman *et al.*, 1997).

Plants have always encountered challenges from microbial toxins and allelochemicals, however exposure to synthetic compounds is a relatively recent event (Coleman *et al.*, 1997). Plants metabolise synthetic compounds using enzymes that are involved in the routine endogenous metabolism of endogenous secondary metabolites (Coleman *et al.*, 1997; Cole, 1994). Overall, plants and mammals use similar routes of metabolism

to detoxify xenobiotics, the major difference between the two being that plants lack a specialised excretory system.

The process of detoxification can be divided into three phases. In Phase I reactions the parent compound is oxidised, reduced or hydrolysed resulting in the introduction of functional groups with increased reactivity and polarity. Important reactions of phase I metabolism including the hydroxylation of aromatic or alkyl groups by cytochrome P450 mixed function oxidases (CYPs), while esterases and amidases are responsible for hydrolysis reactions (Coleman *et al.*, 1997). CYPs are particularly important in phase I metabolism in plants and mammals, being membrane bound enzymes encoded by a multigene family. Phase II metabolism involves the conjugation of phase I metabolites with hydrophilic molecules such as glutathione, sugars, malonic acid or amino acids. In each case the conjugates are more water soluble and less toxic. Glutathione transferases (GSTs) catalyse the conjugation of electrophilic molecules with glutathione, and in several cases the parent compound is sufficiently reactive to undergo conjugation directly (Cole & Edwards, 2000). Glycosylation of xenobiotics, predominantly metabolites of phase I metabolism, is catalysed by the glycosyltransferases (GTs). The glycosides can then be further glycosylated to form higher saccharide derivatives (Cole & Edwards, 2000). Alternatively, the glycoside moieties are often malonylated by malonyltransferases. This malonylation of the glycoside moiety acts as a signal for the conjugate to undergo transportation into the vacuole as part of the phase III reactions in plants (Cole & Edwards, 2000). Conjugates are ultimately immobilised either in the vacuole

or the extracellular space, or undergo co-polymerisation with natural products into insoluble plant macromolecules such as lignin (Schmidt *et al.*, 1995).

1.2 Overview of glycosylation in plants

Conjugation with sugars is a major route of detoxification of many phase I metabolites of herbicides. However the conjugation of xenobiotics is poorly understood compared with the glycosylation of natural products. GTs can conjugate a variety of functional groups, including -COOH, -OH, -NH₂ and -SH moieties to give the respective carboxy ester, *O*-, *N*- and *S*- conjugates. In a limited number of cases GTs can also catalyse the formation of C-glycosides. *O*-Glycosides formed from hydroxyl and carboxyl groups, are the most frequently encountered conjugates in natural product and xenobiotic metabolism. The conjugation of amine and sulphhydryl groups has also been observed in xenobiotic metabolism (Cole & Edwards, 2000). While C-glycosides of flavonoids have been identified in several plant species (Jay, 1994), the respective C-conjugated xenobiotics have not been reported. In plants, the activated sugar uridine diphosphate glucose (UDPG) is the most common sugar donor. This is in contrast to mammals where UDP-glucuronic acid is utilised.

In the case of phenolic compounds, *O*-glycosylation with highly polar sugar moieties enhances their water solubility. In addition, glycosylation reduces their reactivity and increases their stability. Glycosylation is very common in plant secondary metabolism. For example, anthocyanins do not accumulate in plants as the aglycones

but as the respective stable glycosides. In contrast, the more stable flavonols accumulate as either glycosides or aglycones reflecting the difference in stability between these two types of flavonoid (Jones & Vogt, 2001). Salicylic acid (SA) plays an important role in plant disease resistance, however it has been found that in tobacco and other plants concentrations of SA of above 0.1 mM are phytotoxic (Lee *et al.*, 1995). Instead SA can accumulate to significant concentrations as glycosides (Lee *et al.*, 1995). In turn, SA glycosides may act as a pool of SA releasing the active aglycone to initiate and maintain plant defence responses such as systemic acquired resistance (SAR) (Lee *et al.*, 1995). Zeatin, a cytokinin involved in plant cell division and differentiation is inactivated by conjugation with xylose in bean seeds (*Phaseolus vulgaris*) while in lima bean seeds (*Phaseolus lunatus*) glucosylation occurs (Martin *et al.*, 2000a; Martin *et al.*, 2000b). Glycosylation of the side chain protects zeatin against degradative enzymes such as cytokinin oxidases and on digestion by β -glucosidase active zeatin is released (Martin *et al.*, 1999a). Many bioactive natural products are glycosylated including other plant hormones (Sembdner *et al.*, 1994) and phenylpropanoids (Lim *et al.*, 2001; Lim *et al.*, 2002). It has been presumed that the capacity of plants to glycosylate xenobiotics stems from the glycosyltransferases mistaking these foreign compounds for endogenous hydroxylated metabolites. Fortuitously, glycosylation provides an ideal mechanism for detoxifying xenobiotic compounds.

1.2.1 Role of glycosylation in the metabolism of herbicides

Herbicide metabolism has been extensively researched and provides many examples of the importance of glycosylation in the detoxification of xenobiotics (Cole & Edwards, 2000). A classic route of detoxification, which provides a good demonstration of the coordination between phase I and phase II metabolism, is the hydroxylation and subsequent *O*-glycosylation of herbicides. In the case of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), hydroxylation in *Phaseolus vulgaris* at the 4-position on the aromatic ring causes a chemical rearrangement reaction known as the 'NIH' shift which results in the production of the 2,5-dichloro-4-hydroxyphenoxyacetic acid as the major metabolite as well as 2,3-dichloro-4-hydroxyphenoxyacetic acid (Fig. 1). The hydroxylated metabolites were then further conjugated to form the respective β -D-glucosides, increasing their water solubility (Thomas *et al.*, 1964).

O-Glucosylation is also a major determinant of the selectivity of the imidazolinone and sulfonyleurea classes of herbicides, which are potent inhibitors of acetolactate synthase. In tolerant soybean (*Glycine max* L.), the imidazolinone herbicides, imazethapyr and imazamox, are hydroxylated at the 5-position and then rapidly metabolised to inactive glycosides. In herbicide susceptible maize (*Zea mays* L.), imazethapyr and imazamox are hydroxylated as in soybean, but fail to undergo glycosylation. Although hydroxylation reduces the phytotoxic activity of these herbicides, glycosylation is required for their total inactivation (Teclé *et al.*, 1997).

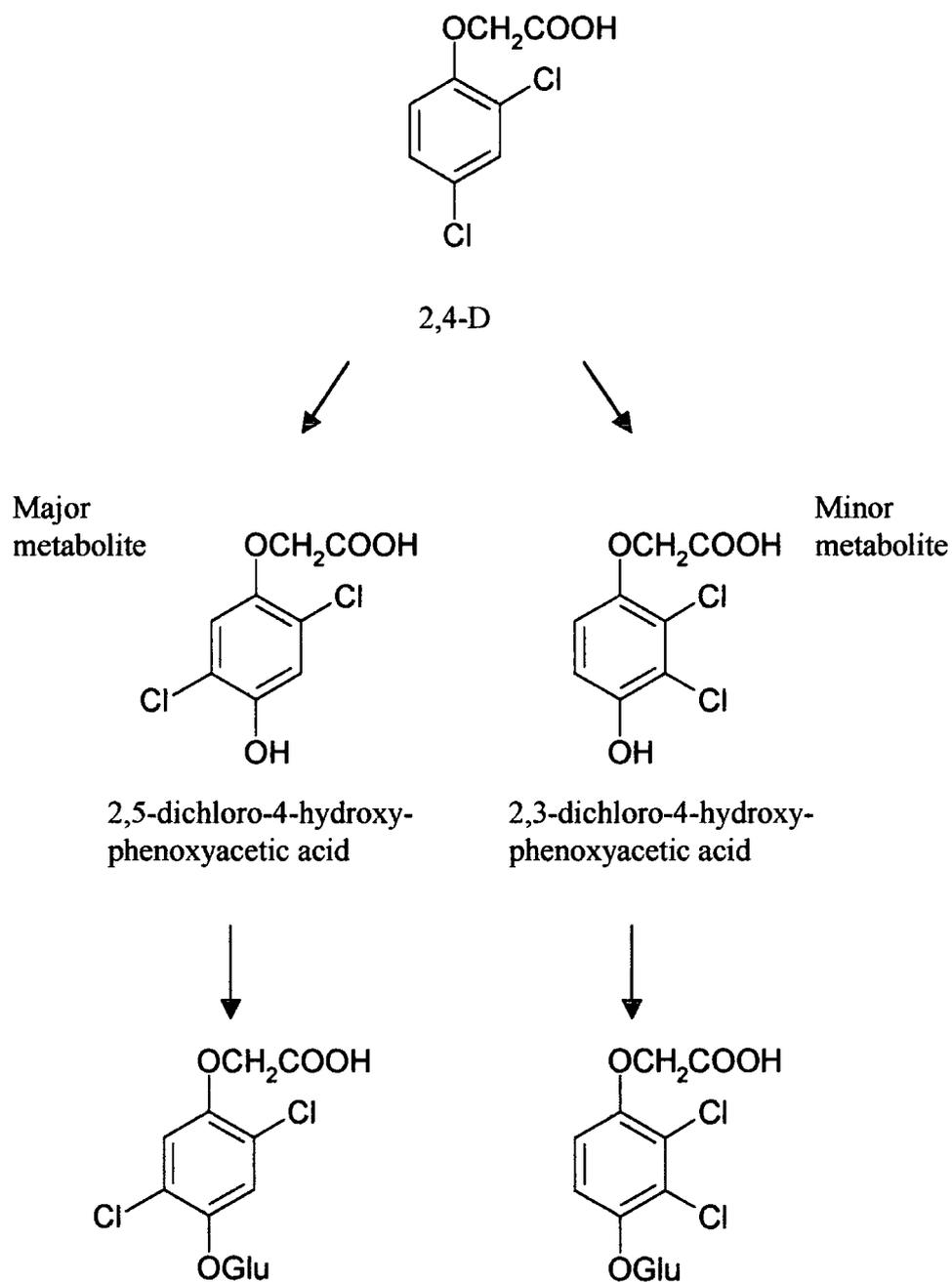


Figure 1 Pathway of 2,4-dichlorophenoxyacetic acid (2,4-D) metabolism in *Phaseolus vulgaris* (Thomas *et al.*, 1964).

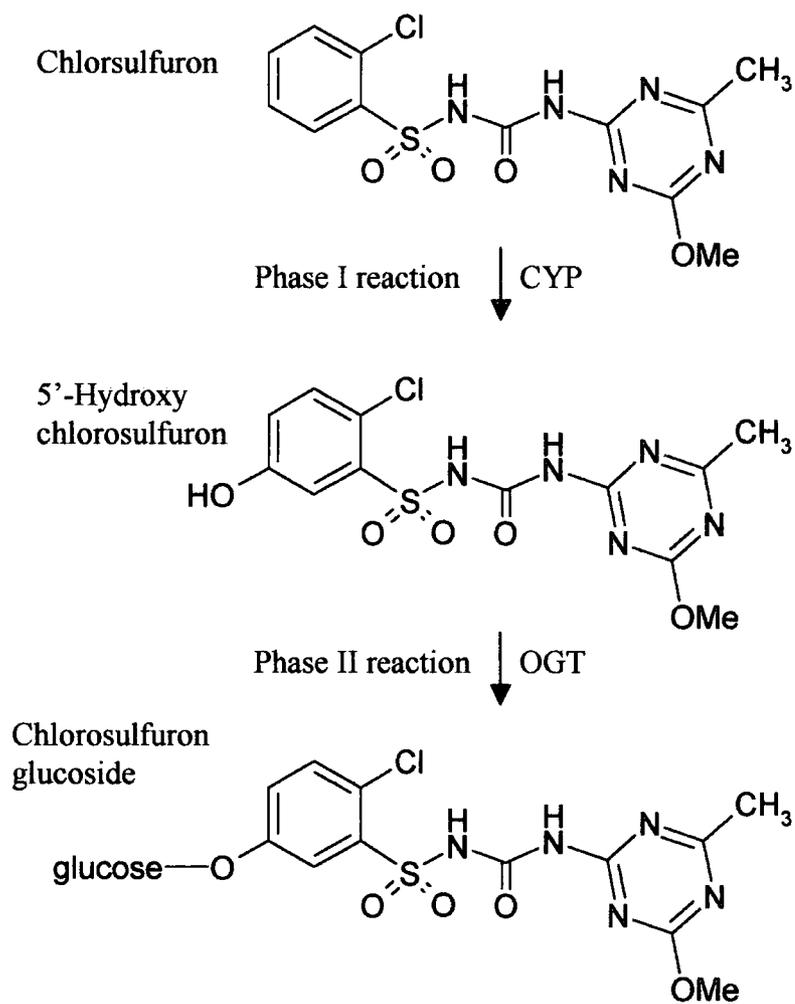


Figure 2 Metabolism of chlorsulfuron in wheat.

The sulfonylurea ALS inhibitor chlorosulfuron is used for weed control in wheat (*Triticum aestivum* L.). Chlorosulfuron is predominantly metabolised to its 5-hydroxy derivative, a potent inhibitor of ALS (Owen, 2000), with the newly formed hydroxyl group then conjugated to produce an inactive glycoside (Fig. 2). Many other sulfonylurea herbicides share the same route of detoxification and the importance of glycosylation in the selectivity of these compounds has been clearly demonstrated (Anderson & Swain, 1992). The metabolism of two closely related sulfonylurea herbicides, metasulfuron-methyl and sulfometuron-methyl, were compared. Wheat readily detoxified metasulfuron-methyl, while being intolerant of sulfometuron-methyl. Wheat seedlings treated with sulfometuron-methyl for two days were found to contain the 5-hydroxylated metabolite and its respective glycoside. When the routes and rates of metabolism were compared, metasulfuron-methyl was found to undergo greater hydroxylation after 5 hours than sulfometuron-methyl. When the conjugation of hydroxylated metabolite was compared an even greater difference between the two herbicides emerged. With metasulfuron-methyl the hydroxylated metabolite to conjugate ratio was approximately 1:8, while the sulfometuron-methyl hydroxylated metabolite to conjugate ratio was only 1:0.5 after 3 hours. The hydroxylated metabolites of both herbicides were both found to have similar inhibitory activities to the ALS of wild mustard, and it was concluded that glycosylation of the metabolites of the herbicides was the major determinant of herbicide sensitivity in each case.

Conjugation of carboxyl groups with glucose to form glucose esters is also a frequent detoxification reaction in herbicide metabolism. However, glucose esters are not generally regarded as an effective means of detoxification, as the sugar moiety is

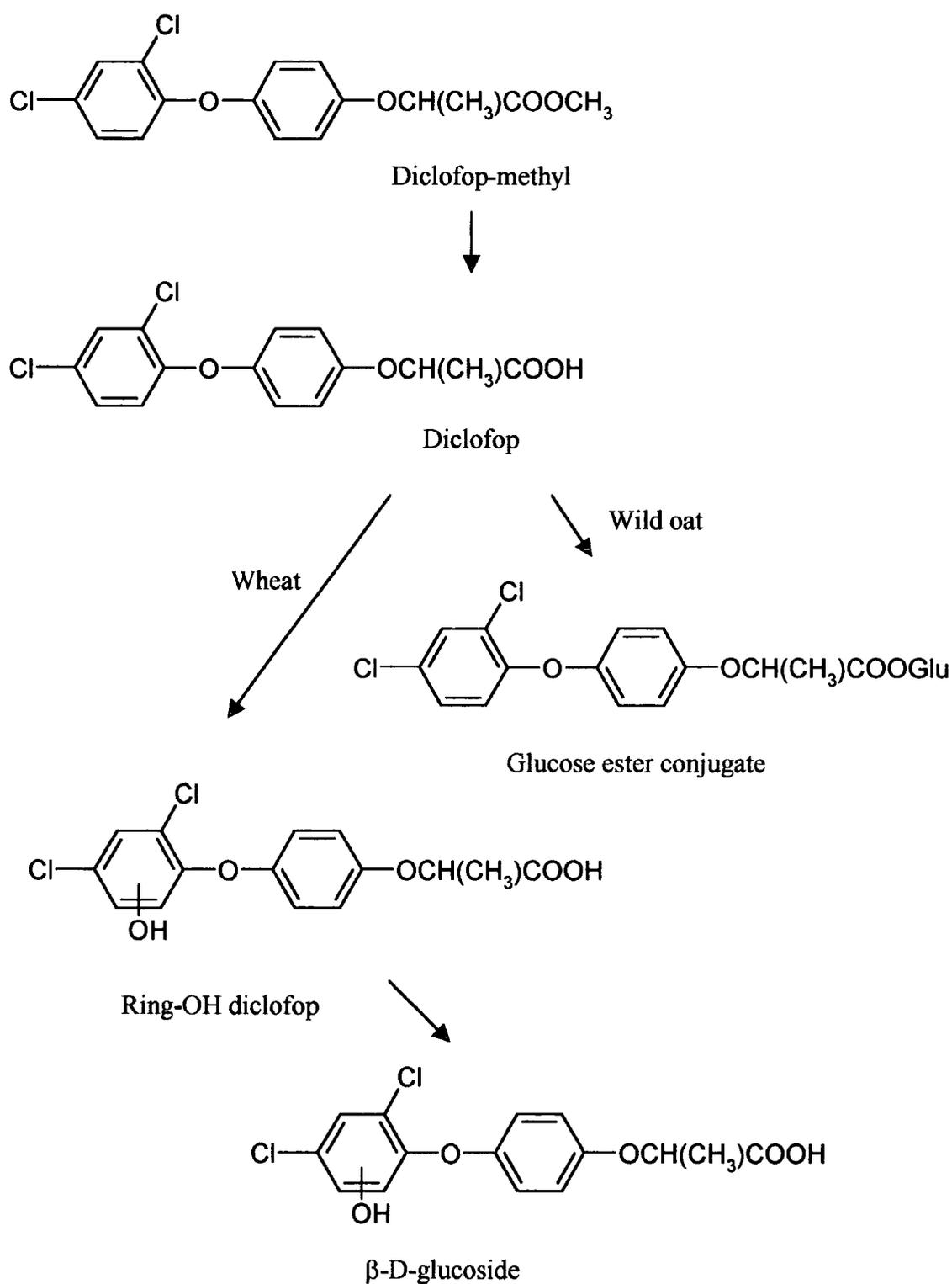


Figure 3 Metabolism of diclofop-methyl in tolerant and sensitive plants (Owen, 2000).

easily hydrolysed to release the active herbicide. A good example of this is seen with the metabolism of the aryloxyphenoxypropionate herbicide, diclofop-methyl (Shimabukuro *et al.*, 1979). Diclofop-methyl is tolerated by wheat, but not by wild oat (Fig. 3). In both wheat and wild oat diclofop-methyl was firstly de-esterified to produce the active herbicide diclofop. In tolerant wheat, the dichlorophenyl ring was rapidly hydroxylated followed by glucose conjugation to form an inactive and stable *O*-glycoside. In wild oat, the majority of the diclofop was not hydroxylated, but instead the carboxyl group was conjugated to form a glucose ester. The glucose ester was unstable and was readily turned over to produce a pool of active herbicide, with the differences in routes of metabolism accounting for the selectivity of diclofop-methyl in wheat and wild oat.

Conjugation of amino groups to form *N*-glycosides is also an important detoxification reaction. In some cases, the parent herbicide, such as picloram and chloramben, is directly glycosylated without any phase I reactions occurring (Cole & Edwards, 2000). In the case of chloramben this herbicide can also undergo glucose esterification and the two potential routes of conjugation are an important determinant in selectivity. A study by Frear *et al.*, (1978) found that in chloramben resistant morning glory and squash roots 76 % and 84 % respectively of the metabolites accumulated as *N*-glucosides compared with just 2 % and 3 % of the corresponding glucose ester. In chloramben-susceptible giant foxtail and barnyard grass the shoots contained 12 % and 23 % respectively of the metabolised herbicide as glucose esters with 50 % of the herbicide unconjugated, suggesting the combination of the lack of *N*-

conjugation and the turnover of the respective ester conjugate to re-release the parent herbicide resulted in sensitivity to chloramben.

1.2.2 Sugars utilised in the metabolism of xenobiotics

The metabolite profile of glycosides of natural products has been characterised in many plant species. A good example is seen with anthocyanins, where profiles of glycosylated anthocyanin have been established over the whole range of plant species. Sugars such as glucose, galactose, xylose, rhamnose and arabinose are utilised in many different combinations to create the respective 3-*O*- monosides, biosides and triosides of anthocyanins (Strack & Wray, 1994). In the case of herbicide metabolism, while much effort has gone in to identifying the aglycones, very little attention has been paid to the identity of the sugar moiety. What evidence there is indicates that the majority of xenobiotics are glucosylated. In tobacco (*Nicotiana tabacum* L.) plants trichloroethanol (TCOH), a metabolite of the environmental contaminant trichloroethylene, is conjugated with glucose to form a β -D-glucoside, while in mammals TCOH is conjugated with glucuronic acid (Shang *et al.*, 2001).

The glycosylation of 4-nitrophenol, a breakdown product of diphenylether herbicides such as fluorodifen, provides a good illustration of the potential glycosylation reactions for a xenobiotic. In cell suspension cultures of carrot (*Datura stramonium* L.) galactosyl- β -D-nitrophenol was identified as a minor product, while the majority of 4-nitrophenol was glucosylated (Schmidt *et al.*, 1997). After this initial glucosylation step, 4-nitrophenol was then subjected to further conjugation to form

the major conjugated product, a β -D-gentiobioside, in *Datura stramonium* L., and also in wild oat and wheat (Schmitt *et al.*, 1997; Malcherek *et al.*, 1998). Alternatively, glycosylated 4-nitrophenol was found to be malonylated at the 6-position of the glucose in soybean and corn cockle (*Agrostemma githago* L.) (Knops *et al.*, 1995; Malcherek *et al.*, 1998). In addition Malcherek *et al.* (1998) found that wheat also accumulated a 6'-O-malonylated β -glucoside of 4-nitrophenol. A metabolite of the synthetic pyrethroid insecticide, 3-phenoxybenzoic acid, has been identified conjugated with a range of saccharides including cellobiose, glucosylarabinose, glucosylxylose and triglucose reviewed by Cole & Edwards (2000).

1.2.3 Herbicide safeners

Herbicide safeners are used to protect cereal crops such as wheat, maize, sorghum (*Sorghum bicolor* L.) and rice (*Oryza sativa* L.), against selective herbicides to which they are partially tolerant (Hatzios & Wu, 1996). For example, the aryloxyphenoxypropionic acid herbicide fenoxaprop ethyl must be formulated with a herbicide safener for selective weed control in wheat (Davies & Caseley, 1999). Early herbicide safeners were applied as a seed treatment prior to sowing to avoid the safening of competing weeds. However herbicide safeners are now often applied simultaneously as a package with the respective post-emergence herbicide (Cole, 1994).

Herbicide safeners are a diverse range of chemicals, with some specific for conferring protective activity toward a particular herbicide. For example fenchlorozole ethyl,

gives protection only towards fenoxaprop ethyl in cereals (Tal *et al.*, 2000). Other herbicide safeners can confer protection toward a wide range of herbicide classes. Dichlormid can safen against five classes of herbicide in maize including chloracetanilides and thiocarbamates (Hatzios, 1991). Some herbicide safeners are structurally similar to the herbicide they protect against. Thus, the safeners dichlormid and flurazole have a similar size, shape and charge distribution as the co-formulated herbicides EPTC and alachlor respectively (Yenne & Hatzios, 1990). In sorghum, CGA 133205 was the most effective of the three safeners against metolachlor in sorghum and also possessed the most similar properties to the herbicide (Yenne & Hatzios, 1990).

Several mechanisms have been explored as a way of understanding the mode of action of safeners. The safener-mediated reduction in uptake and translocation of herbicides as well as interactions of safeners with the herbicide target sites have been investigated. The evidence has been mixed, suggesting these mechanisms are not the primary method by which safening occurs (Hatzios, 1991). Safener-induced enhancement of herbicide metabolism, reducing the amount of harmful herbicide available at the active site, has received the most attention. Safener treatment can result in enhanced GST activity and increased levels of glutathione (GSH). The GST activity towards the chloroacetamide herbicide dimethenamid was found to be increased 9-fold in wheat (*Triticum aestivum* L.) treated with the safener fluxofenim (Riechers *et al.*, 1997). Many other examples of safener-mediated increases in GST activity have been found with chloroacetamide and thiocarbamate herbicides in maize (Davies & Caseley, 1999). The effect of safener application on the enhancement of

CYP activity toward bentazon and herbicides of the aryloxyphenoxypropanionic acids, sulfonyleurea and imidazolinone classes has been reported in maize, sorghum, wheat, barley and rice (see Davies & Caseley, 1999).

Examples of enhancement of the glycosylation of herbicides, or herbicide metabolites by herbicide safeners are more limited. In maize the sulfonyleurea herbicide chlorimuron ethyl is metabolised by either hydroxylation or glutathione conjugation the hydroxychlorimuron ethyl metabolite being glycosylated (Fig. 4) (Owen, 2002).

The treatment of maize seedlings with the safener BAS 145138 resulted in a 6-fold increase in the accumulation of the β -D-glucoside (Lamoureux & Rusness, 1992). To determine whether this effect was due to enhanced hydroxylation or glycosylation, [^{14}C]–labelled hydroxychlorimuron was applied to maize in the presence and absence of BAS 145138. A doubling in the rate of glucosylation indicated that the increase in glucoside accumulation was due to enhanced rates of glucosylation. A similar result was obtained with the herbicide clodinafop propargyl and the safener cloquintocet mexyl (CGA 18072) in wheat (Kreuz et al., 1991). Clodinafop propargyl was subjected initially to ester hydrolysis to produce the herbicidal active species. This carboxylic acid could then undergo reversible glucose esterification. However, clodinafop propargyl was detoxified by hydroxylation of the pyridinyl ring or by ether cleavage with the respective hydroxy-metabolites undergoing glycosylation (Fig. 5). Treatment with the safener cloquintocet mexyl, resulted in an increase in the hydroxylated and ether cleavage metabolites formed in wheat, with the glucosidic conjugates of one of these metabolites enhanced 2-fold relative to untreated plants.

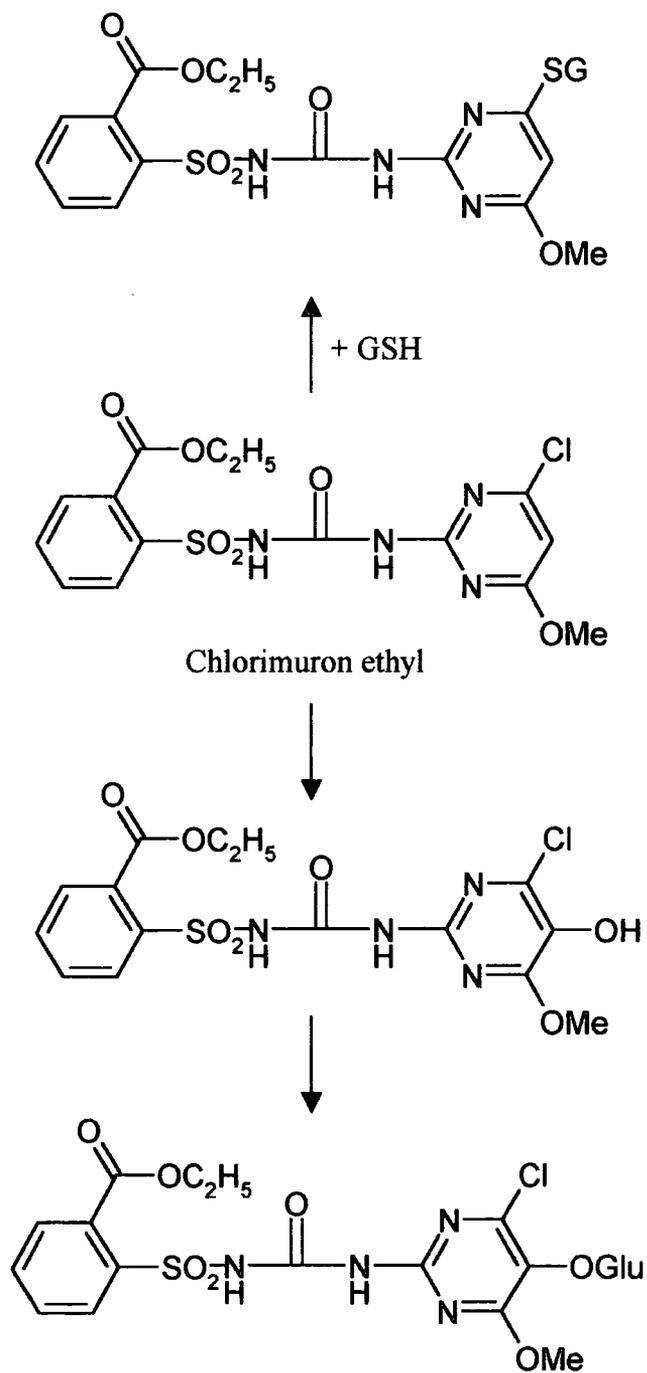


Figure 4 Metabolism of chlorimuron ethyl in maize (Owen, 2000).

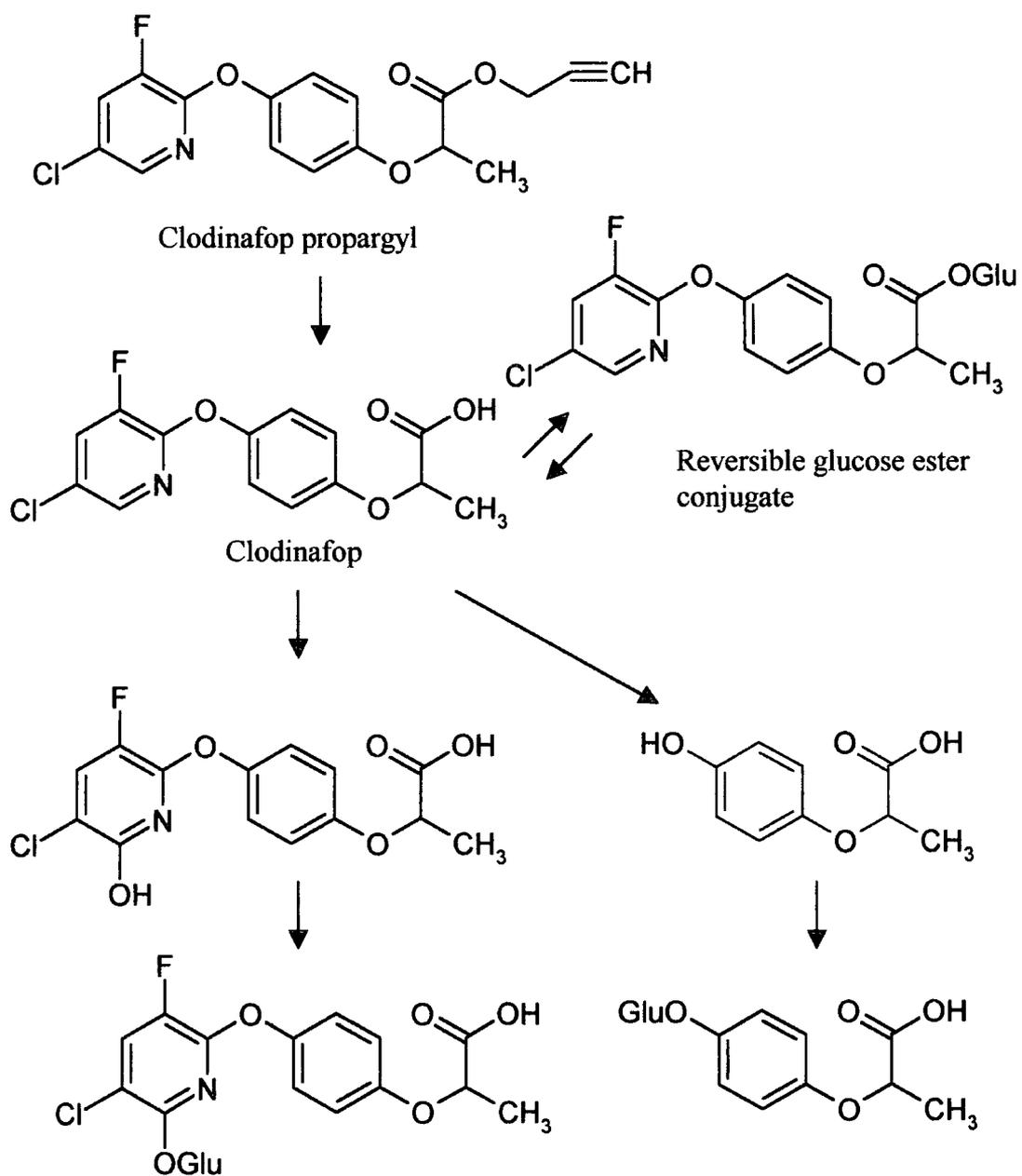


Figure 5 Pathway of clodinafop metabolism in wheat (Kreuz *et al.*, 1991).

The mechanism of the safener-mediated increase in the glycosylation of herbicide metabolites has yet to be determined.

1.2.4 Herbicide resistance in black-grass

The resistance of weeds to selective herbicides has been a growing problem since the 1970s. Repeated application of a single class of herbicide has allowed weeds to evolve resistance mechanisms. Most cases of resistance are due to selection for a mutation that renders the weeds insensitive to a particular herbicide. For example, goosegrass (*Eluesine indica*), a major weed of cotton and soybean in the USA, has developed resistance to selective dinitroaniline herbicides. Anthony *et al.* (1998) discovered the resistance was due to a single mutation in the herbicide target site, the major α -tubulin gene. In several cases, herbicide resistance has been found to result from the enhanced ability of the weed to metabolise herbicides. In the USA, populations of atrazine-resistant velvetleaf (*Abutilon theophrasti* L.) have been identified in which rates of conjugation with glutathione are elevated compared to the susceptible biotype, resulting in enhanced herbicide tolerance (Gronwald *et al.*, 1989). Such enhanced metabolism has been suggested as the probable explanation for resistance to multiple classes of herbicides (Hatizos, 1991).

Black-grass (*Alopecurus myosuroides* L.) populations that are resistant to herbicides have been found in many countries including the UK, Germany and Spain (Hall *et al.*, 1995). A black-grass population with resistance to several classes of herbicides was discovered in Peldon, Essex, UK, where winter cereal crops had received applications

of phenylurea and related herbicides annually for the previous 25 years. The Peldon biotype was found to be highly resistant to the phenylurea herbicide chlorotoluron, a photosystem II inhibitor. It was also found to be moderately resistance to the aryloxyphenylpropanionate acid herbicides, diclofop-methyl, fenoxaprop-P-ethyl and fluazifop-P-butyl, and to the dinitroaniline herbicide, tralkoxydim (Hall *et al.*, 1997).

In tolerant wheat, chlorotoluron is metabolised by aryl ring hydroxylation to yield non-toxic hydroxychloroturon. A minor pathway produces a mono-demethylated metabolite, which retains 50-70 % of the phytotoxicity of the parent compound. This is then metabolised to form an *N*-demethylated hydroxymethylphenyl metabolite (Fig. 6). Conjugation with glucose occurs in both routes of metabolism (Hall *et al.*, 1995). In the herbicide-susceptible biotypes of black-grass, equal amount of the hydroxylated aryl ring and the mono-demethylated metabolites are produced. Whereas in the herbicide-resistant Peldon population, the non-phytotoxic hydroxylated aryl ring is the major metabolite. As the cytochrome P450 inhibitor, 1-aminobenzotriazole, reduced the accumulation of the non-phytotoxic metabolite, resistance to chlorotoluron in the Peldon population is probably due partly to enhanced CYP activity (Hyde *et al.*, 1996). In the case of fenoxypop-P-ethyl glutathione conjugation rather than hydroxylation is the primary mechanism of detoxification. Cummins *et al.* (1999) isolated a GST (*AmGST2*) that was expressed at higher levels in herbicide-resistant Peldon population than in the wildtype plants. It was found that rather than enhancing herbicide metabolism, *AmGST2* reduced oxidative injury by acting as a glutathione peroxidase reducing organic hydroperoxides to the respective less toxic alcohols.

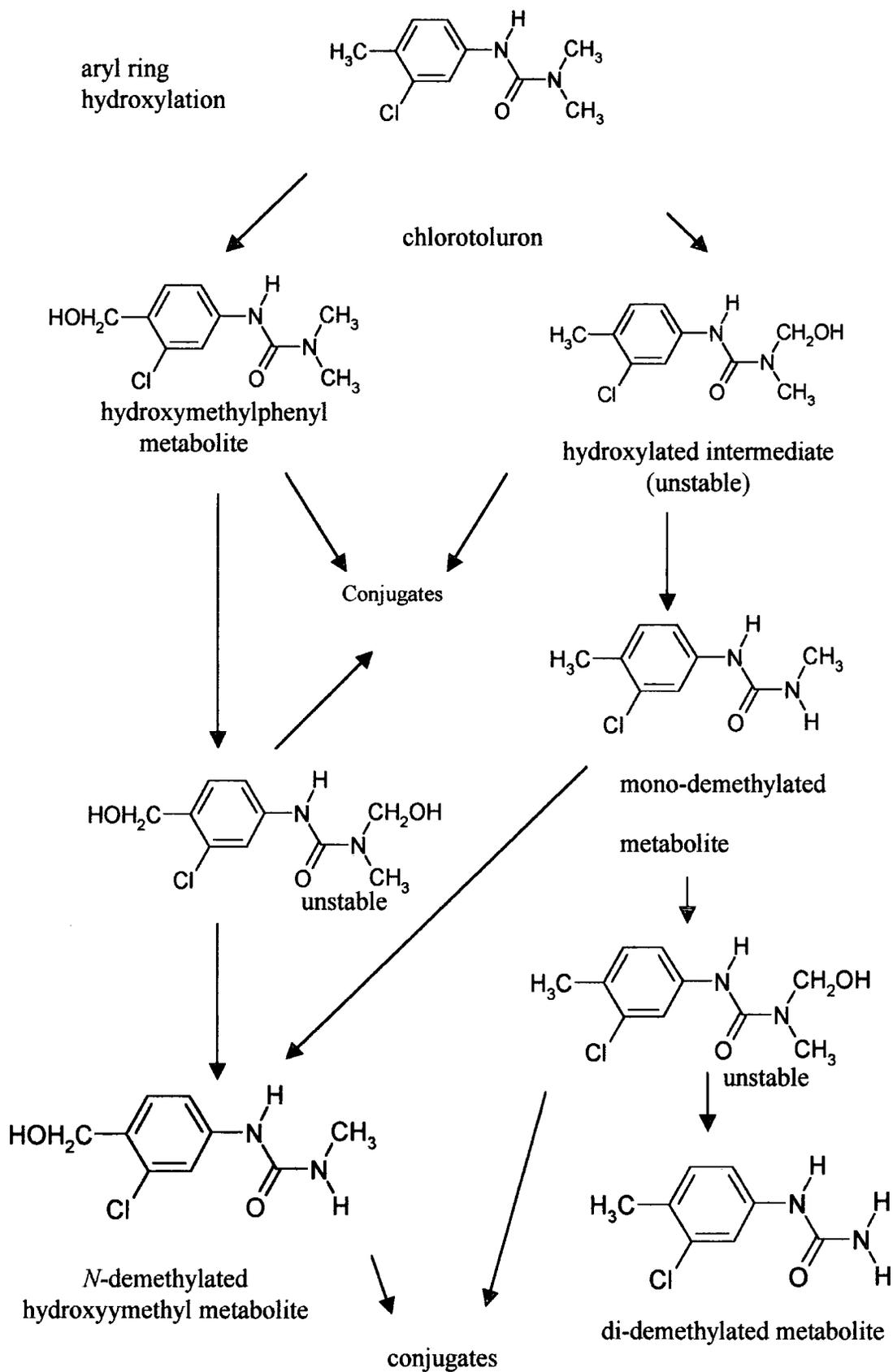


Figure 6 Chlorotoluron degradation pathway (Hall *et al.*, 1995).

Herbicides with differing modes of action led to an increase in organic hydroperoxide accumulation (Cummins *et al.*, 1999), so an increase in glutathione peroxidase activity could allow the Peldon population to tolerate multiple herbicides by reducing the level of resulting oxidative injury.

1.3 Glycosyltransferase activities towards xenobiotics in plants

Herbicide metabolism studies have shown that many synthetic phenol compounds are glycosylated *in planta*, however few of the enzymes involved have been fully characterised. The majority of the research on GT activities toward xenobiotics has been carried out in soybean, with UDP-glucose used as the sugar donor. A study measuring the GT activity towards fourteen herbicides and herbicide metabolites in soybean cell cultures (c.v. Merr Corsoy 79) demonstrated the presence of a range of GT activities in crude plant extracts (Gallandt & Balke, 1995). Significant levels of OGT activity were found toward the hydroxylated herbicide metabolites, 5-hydroxydiclofop, 6-hydroxybentazone and 8-hydroxybentazone. In the case of 6-hydroxybentazone two GT activities toward the substrate could be resolved from soybean seedlings (Leah *et al.*, 1992). GT activity was also detected toward the environmental contaminants, 2,4-dichlorophenol (the optimal substrate in the study), 3,4-dichloroaniline, and the herbicide chloramben, the latter two forming *N*-glucosides in soybean (Gallandt & Balke, 1995). 2,4-Dichlorophenol was determined to be the optimal substrate for a GT isoenzyme purified 1000-fold from soybean based on its ability to glucosylate pentachlorophenol (PCP) (Sandermann *et al.*, 1991). NGT activity toward 3,4-dichloroaniline has also been found in soybean and wheat cell

suspension cultures (Schmidt *et al.*, 1995) and it is known that the herbicide chloramben actively forms a *N*-glucoside in soybean (Frear, 1968). Using crude extracts from soybean suspension cultures no GT activity was determined with the pesticides metribuzin, PCP, or 2,2-bis-(4-chlorophenyl)-acetic acid (DDA), a metabolite of the insecticide DDT (Gallandt & Balke, 1995). However isoenzymes with limited GT activities toward these compounds have been partially purified from soybean (Frear, 1968; Sandermann *et al.*, 1991; Wetzel & Sandermann, 1994). This illustrates the problem of assaying GT, for activity toward a range of substrates using crude enzyme extracts. Potential sources of variation in the GT activities determined may arise from the extraction procedures, or from the conditions under which the assay was performed. The particular cultivar used in an experiment can also affect the levels of activity. A seven-fold difference in specific activity was found between four soybean cultivars when each was assayed for GT activity toward 6-hydroxybentazone (Gallandt & Balke, 1995).

The broad range of GT activities toward xenobiotics are not restricted to soybean or other crop plants. A total of fifty nine plants species including ferns, mosses, and algae were assayed for *OGT* activity, toward PCP, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol and DDA; *NGT* activity, toward 3-chloroaniline and 3,4-dichloroaniline and *SGT* activity, toward 4-chlorothiophenol. GT activities toward all substrates, except PCP were spread across the whole range of species, including algae. PCP was glucosylated in higher plants, but not in mosses, liverworts or algae. Levels of GT activity toward 2,3,4-trichlorophenol and 2,4,5-trichlorophenol were very

similar across the whole spread of species, suggesting that a group of related enzymes were responsible for both activities (Pflugmacher & Sandermann, 1998).

1.3.1 Purification of glucosyltransferases

It is not clear if the broad range of glucosylating activities towards xenobiotics observed in crude plant extracts are due to a small number of isoenzymes with broad substrate specificities or if many GTs are responsible, each possessing narrow substrate specificities. A number of GTs that glucosylate xenobiotics, have been partially purified, mainly from soybean.

OGTs with activity toward PCP have been isolated on several occasions. Schmitt *et al.* (1985) partially purified a GT from cell cultures of wheat and soybean with activity toward PCP using a combination of anion-exchange and gel permeation chromatography. The estimated relative molecular masses of the isoenzymes were 43 kDa and 47 kDa from wheat and soybean respectively. The PCP-conjugating GT isoenzyme from soybean was further purified (Sandermann *et al.*, 1991), using hydrophobic interaction chromatography, chromatofocussing and affinity chromatography as additional purification steps. The 1005-fold purified enzyme demonstrated GT activity toward 2,4-dichlorophenol, 2,4,5-trichlorophenol and other trichlorophenols and was determined to be a 47 kDa polypeptide by SDS-PAGE. The identical enzyme was also partially purified, while isolating a GT from soybean culture with activity toward DDA (Wetzel & Sandermann, 1994). Hydrophobic interaction chromatography resolved the GT activities toward DDA, PCP and the

flavonol quercetin. The DDA conjugating activity was subsequently purified 367-fold and found to be associated with a 50 kDa enzyme. The GT activity toward PCP and quercetin was associated with a 47 kDa polypeptide as determined by SDS-PAGE gel, and it was suggested that quercetin may be glucosylated by the same isoenzyme as conjugated PCP.

Several glucosyltransferases have been purified with activity toward 6-hydroxybenzotriazole from different cultivars of soybean. A membrane bound OGT with activity towards 6-hydroxybenzotriazole and *p*-hydroxyphenylpyruvic acid was purified from etiolated soybean (c.v. Fiskerby V). The native molecular weight of the GT was determined to be 53 kDa but run as 28 kDa polypeptides when analysed on a SDS-polyacrylamide gel suggesting it existed as a dimer (Leah *et al.*, 1992). The authors also reported the 115-fold purification of a 44 kDa soluble OGT. In addition to being active toward 6-hydroxybenzotriazole, the soluble OGT was active toward a range of other substrates, including kaempferol and quercetin. The K_M toward kaempferol was found to be twenty times lower than for 6-hydroxybenzotriazole. A second soluble OGT with activity toward 6-hydroxybenzotriazole was subsequently purified from soybean (c.v. Corsoy 79) cell suspension culture (Gallandt & Balke 1993). This OGT did not have any activity toward kaempferol or quercetin but had a molecular weight of 44 kDa. It was not clear from these reports whether or not the same enzyme was purified in both cases or whether multiple enzymes with activity toward 6-hydroxybenzotriazole were present in soybean. Assessing the activities of these enzymes as recombinant proteins in *E.coli* or yeast would facilitate the unambiguous

determination of the substrate specificity of these GT. Unfortunately none of the OGTs purified with activities toward xenobiotics have yet been cloned from soybean.

The purification of GTs with activities toward natural products has recently been reported. Many different plant species have been used as the starting material. The majority of GTs have been partially purified using a combination of ammonium sulphate precipitation, anion-exchange and hydrophobic interaction chromatography, chromatofocusing and gel filtration. Affinity chromatography using UDP-glucuronic acid or similar affinity columns has also been successfully used on a number of occasions including during the purification of a limonoid-*O*-GT from *Citrus sinensis* (Hasegawa *et al.*, 1997) and a 7-*O*-hydroxycoumarin GT from tobacco (Taguchi *et al.*, 2000). The enzymes were eluted from the columns using UDP-glucose, resulting in a final purification of 1176-fold for the 7-*O*-hydroxycoumarin GT. However, several reports have suggested that UDP-glucuronic acid affinity columns did not bind their GT of interest (Lee & Raskin, 1999), or provided no further increase in purification (Parry & Edwards, 1994). Dye ligand chromatography has also been employed in the purification of GTs. Reactive Yellow 3 was used in the purification of the UF3GT and 6-GT from *Dorotheanthus bellidiformis* cell suspension cultures (Vogt *et al.*, 1997) and in the isolation of the hydroxymandelonitrile-*O*-GT from *Sorghum bicolor* (Jones *et al.*, 1999). The latter two GTs were purified to the extent of allowing N-terminal Edman sequencing. The purification of GT, active toward cyclic hydroxamic acid from wheat and maize used blue sepharose as the first column chromatography step (Sue *et al.*, 2000; Ebisui *et al.*, 2001).

The extent of the purification of individual GTs varied, with most GTs purified between several hundred and several thousand fold. A hydroxybenzoate GT from cell suspension cultures of *Lithospermum erythrorhizon* was purified 27306-fold (Li *et al.*, 1997). The majority of GTs purified so far have been identified as single polypeptides on SDS-polyacrylamide gels. With the advent of MALDI-TOF and MS-MS technology many of these low abundance pure GTs can now potentially be sequenced and the respective genes identified. The physical properties of purified GTs are similar, with most expressed as monomeric polypeptides of between 40 kDa to 60 kDa. A few GTs have been reported as possible dimers, including the membrane bound *p*-hydroxyphenylpyruvic acid GT from soybean (Leah *et al.*, 1992) and a crocetin GT from cell suspension cultures of *Crocus sativus* (Cote *et al.*, 2000) with molecular masses of 28 kDa and 26 kDa reported for the monomers respectively. The isoelectric points of all GT purified to date that have been determined are in the range *pI* 4.0 to *pI* 5.5, excepting an indoxyl-UDPG-GT from *Baphicacanthus cusia* which had a *pI* of pH 6.5 (Marcinek *et al.*, 2000). In all cases the optimal pH for GT activity ranged from between pH 6.0 to pH 8.0.

1.4 Sequence analysis and recombinant expression of GTs

Analysis of GT sequences has led to the classification of 65 families of the respective genes (<http://afmb.cnrs-mrs.fr/CAZY/>). GTs of plant secondary metabolism belong to family 1, along with GTs from viruses, bacteria, fungi and animals (Campbell *et al.*, 1997). GTs from family 1, possess a conserved domain of forty-four amino acids in the C-terminal region which have been designated as a glucosyltransferase signature

Many putative GT have been identified from a wide range of plant species. The sequencing of the *Arabidopsis thaliana* genome has allowed the identification of 107 putative GT sequences (Ross *et al.*, 2001). *Arabidopsis* GTs are identified using a nomenclature system defined by Li *et al.* (2001). UGT identifies a putative UDP-dependent glycosyltransferase and a number from 71 to 100, designates a UGT family of plant origin, with the same number used where sequences have a greater than 45 % identity. The third component consists of a letter representing a subfamily whose members share greater than 60 % identity and finally a number is used to identify an individual gene. Analysis of 88 *Arabidopsis* GTs revealed that 34 contained single introns and three contained two introns. Nine separate insertion events were responsible for the introns (Li *et al.*, 2001). The GT genes were spread throughout the *Arabidopsis* genome with clusters of highly related GT genes in groups of two to seven genes (Li *et al.*, 2001).

Construction of phylogenetic trees using the putative GT sequences from *Arabidopsis* revealed the presence of 14 groups of genes, that have been labelled A to N (Ross *et al.*, 2002). Twenty-two of the GTs from *Arabidopsis* when expressed as recombinant proteins, in either *E.coli* or yeast, demonstrated activities toward phenolic substrates (Jackson *et al.*, 2001; Lim *et al.*, 2001; Lim *et al.*, 2002; Schäffner *et al.*, 2002). GT activity has also been observed with 29 recombinant GTs from plant species other than *Arabidopsis* (Table 1). The majority of the 29 non-*Arabidopsis* GT sequences are spread between four of the major groups identified by (Ross *et al.*, 2001) in *Arabidopsis*, with another 3 sequences falling into two further groups (Fig. 8). Three

GTs from maize, *Phaseolus lunatus* and *Phaseolus vulgaris* with activity toward the cytokinin zeatin were identified as members of a separate group (Li *et al.*, 2001).

Although the majority of the 51GTs demonstrated to have GT activity as recombinant proteins have not been tested against a comprehensive range of substrates, some relationships between sequence and regio-specificity are apparent. In group F, five of the six GTs glycosylate anthocyanins and flavonols at the 3-hydroxy position. The sixth member, UGT78D2, has not been reported as having been tested with flavonoid substrates. The majority of the members of group L esterify phenylpropanoids, benzoates or indole-3-acetic acid (IAA) with glucose. Glucose esters are only produced by members of group L, with the exception of TOGT1 from tobacco, which is a member of group D. Also clustered together in group L are three GTs that glucosylate at the 5-hydroxy position of anthocyanin-3-*O*-glycosides to form diglucosides. Any relationship between sequence and regio-specificities is much less obvious in the other two main groups, labelled D and E. Vogt *et al.* (1999) suggested that members of group D may glucosylate the 7-hydroxy position of flavonoids. However this activity has only been confirmed with a GT from *Dorotheanthus bellidiformis* (Vogt *et al.*, 1999) and an UDP-glucose: baicalein 7-*O*-GT (UBGT) from *Scutellaria baicalensis* (Hirotsani *et al.*, 2000). The majority of activities observed with members of group E were toward hydroxyphenylpropanoids, benzoates

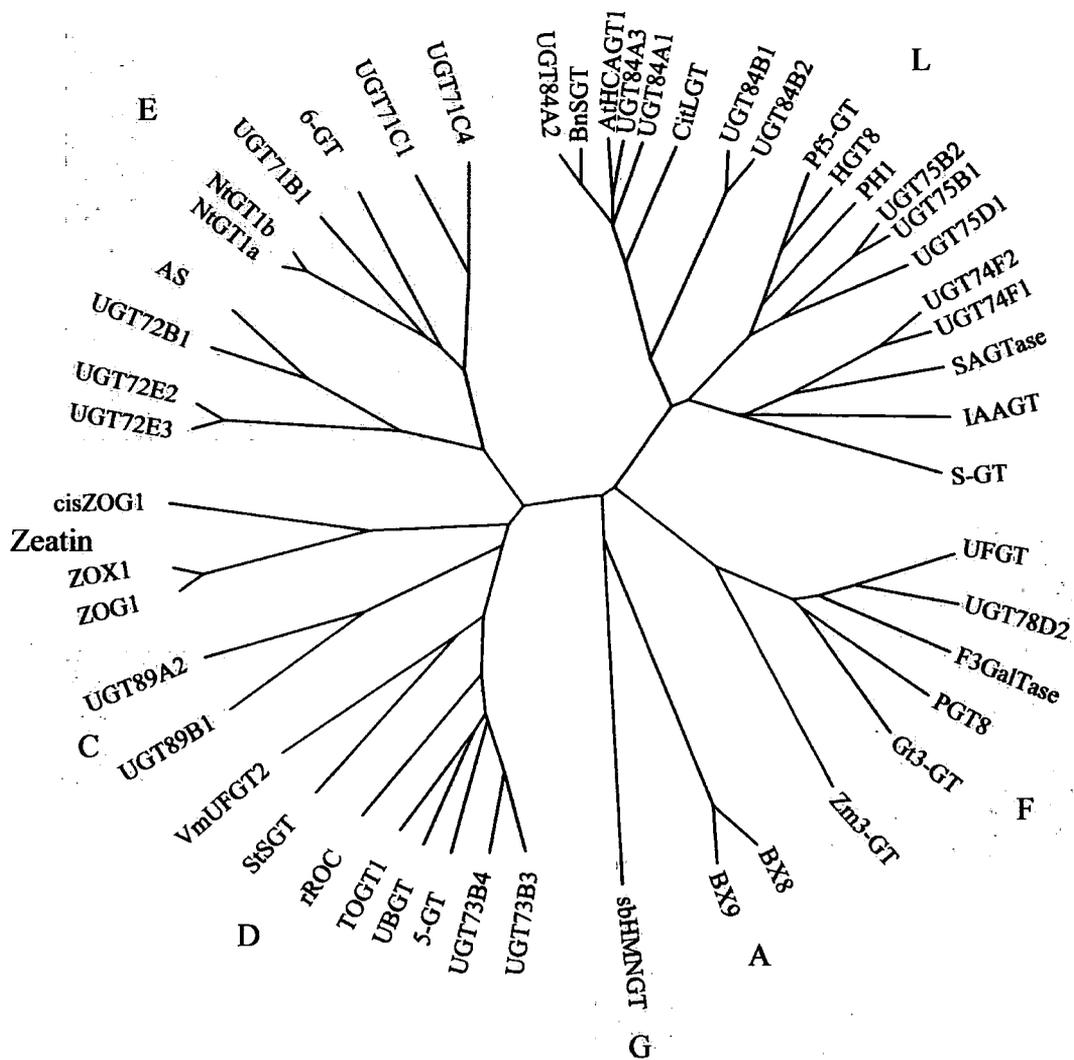


Figure 8 Phylogenetic tree of higher plant GTs, showing clustering of sequences into eight clusters as defined by (Ross *et al.*, 2002). The tree was generated using the phylogeny package PHYLIP (Felsenstein, 1989). Sequence labels are as described in Table 1.

Table 1 Recombinant GTs expressed in *E. coli* or yeast with investigated substrate specificities.

	Genbank		Putative substrate			Reference
	Name	accession no.	Source	Acceptors	Donor	
L	AthSGT1	AL161541	<i>Arabidopsis thaliana</i>	Phenylpropanoids (glucose esters)	UDP-glucose	(Milkowski et al., 2000)
L	UGT84A1	Z97339	<i>Arabidopsis thaliana</i>	Phenylpropanoids (glucose esters)	UDP-glucose	(Lim et al., 2001)
L	UGT84A2	AB019232	<i>Arabidopsis thaliana</i>	Phenylpropanoids (glucose esters)	UDP-glucose	(Lim et al., 2001)
L	UGT84A3	Z97339	<i>Arabidopsis thaliana</i>	Phenylpropanoids (glucose esters)	UDP-glucose	(Lim et al., 2001)
E	UGT72E2	AB018119	<i>Arabidopsis thaliana</i>	Phenylpropanoid (4-O-glc)	UDP-glucose	(Lim et al., 2001)
E	UGT72E3	AF077407	<i>Arabidopsis thaliana</i>	Phenylpropanoid (4-O-glc)	UDP-glucose	(Lim et al., 2001)
L	UGT84B1	AC002391	<i>Arabidopsis thaliana</i>	IAA (glucose esters)	UDP-glucose	(Jackson et al., 2001)
L	UGT84B2	AB019232	<i>Arabidopsis thaliana</i>	IAA (glucose esters)	UDP-glucose	(Jackson et al., 2001)
L	UGT75B2	AC005106	<i>Arabidopsis thaliana</i>	Benzoates (glucose ester), IAA	UDP-glucose	(Lim et al., 2002)
L	UGT75B1	AC005106	<i>Arabidopsis thaliana</i>	Benzoates (glucose ester), IAA	UDP-glucose	(Lim et al., 2002)
L	UGT74F1	AC002333	<i>Arabidopsis thaliana</i>	Benzoates (2-O-glc)	UDP-glucose	(Lim et al., 2002)
L	UGT74F2	AC002333	<i>Arabidopsis thaliana</i>	Benzoates (glucose ester)	UDP-glucose	(Lim et al., 2002)
F	UGT78D2	AL391141	<i>Arabidopsis thaliana</i>	Benzoates (4-O-glc)	UDP-glucose	(Lim et al., 2002)
E	UGT71C1	AC005496	<i>Arabidopsis thaliana</i>	Benzoates (3-O-glc)	UDP-glucose	(Lim et al., 2002)
E	UGT71C4	AC067971	<i>Arabidopsis thaliana</i>	Benzoates (3-O-glc)	UDP-glucose	(Lim et al., 2002)
E	UGT71B1	AB25634	<i>Arabidopsis thaliana</i>	Benzoates (4-O-glc)	UDP-glucose	(Lim et al., 2002)
E	UGT72B1	AC067971	<i>Arabidopsis thaliana</i>	Benzoates (3-O-glc)	UDP-glucose	(Lim et al., 2002)
D	UGT73B3	AL161584	<i>Arabidopsis thaliana</i>	Benzoates (4-O-glc)	UDP-glucose	(Lim et al., 2002)
D	UGT73B4	AC006248	<i>Arabidopsis thaliana</i>	Benzoates (3-O-glc & 4-O-glc)	UDP-glucose	(Lim et al., 2002)
C	UGT89B1	AC016662	<i>Arabidopsis thaliana</i>	Benzoates (4-O-glc)	UDP-glucose	(Lim et al., 2002)
C	UGT89A2	AL162751	<i>Arabidopsis thaliana</i>	Benzoates (3-O-glc)	UDP-glucose	(Lim et al., 2002)
L	UGT75D1	Z97339	<i>Arabidopsis thaliana</i>	Xenobiotics, IAA	UDP-glucose	(Schaffner et al., 2002)
L	<i>Bn</i> SGT	AF287143	<i>Brassica napus</i>	Sinapate	UDP-glucose	(Milkowski et al., 2000)
L	S-GT	AF304430	<i>Brassica napus</i>	Phenylacetothiohydroximate	UDP-glucose	(Marillia et al., 2001)

Table continued overpage

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Group	Name	Genbank accession no.	Source	Putative substrate			Reference
				Acceptors	Donor	Reference	
L	CitLGT	AB033758	<i>Citrus unshiu</i>	Limonate A-ring lactone	UDP-glucose	(Kita <i>et al.</i> , 2000)	
D	5-GT	Y18871	<i>Dorotheanthus bellidiformis</i>	Betandin Flavonols (7-O-glc)	UDP-glucose	(Vogt <i>et al.</i> , 1999)	
E	6-GT	AF374004	<i>Dorotheanthus bellidiformis</i>	Betandin (6-O-glc) Flavonols(3-O-glc)	UDP-glucose	(Vogt <i>et al.</i> , 1999)	
F	Gf3-GT	D85186	<i>Gentiana triflora</i>	anthocyanidins (3-O-glc)	UDP-glucose	(Tanaka <i>et al.</i> , 1996)	
E	NtGT1a	AB052557	<i>Nicotiana tabacum</i>	Coumarins	UDP-glucose	(Taguchi <i>et al.</i> , 2001)	
E	NtGT1b	AB052558	<i>Nicotiana tabacum</i>	Coumarins	UDP-glucose	(Taguchi <i>et al.</i> , 2001)	
L	SAGtase	AF190634	<i>Nicotiana tabacum</i>	Benzoates	UDP-glucose	(Lee <i>et al.</i> , 1999)	
D	TOGT1	AAK28303	<i>Nicotiana tabacum</i>	Phenylpropanoids	UDP-glucose	(Fraissinet-Tachet <i>et al.</i> , 1998)	
L	Pf5-GT	AB013596	<i>Perilla frutescens</i>	Anthocyanin-3-O-glucoside (5-O-glc)	UDP-glucose	(Yamazaki <i>et al.</i> , 1999)	
F	F3GalTase	AF165148	<i>Petunia hybrida</i>	Flavonols (3-O-glc)	UDP-galactose	(Millar <i>et al.</i> , 1999)	
L	PH1	AB027455	<i>Petunia x hybrida</i>	anthocyanidin 3-acylrutinoside (5-O-glc)	UDP-glucose	(Yamazaki <i>et al.</i> , 2002)	
F	PGT8	AB027454	<i>Petunia x hybrida</i>	anthocyanidins flavonols (3-O-glc)	UDP-glucose	(Yamazaki <i>et al.</i> , 2002)	
Zeatin	ZOG1	AF101972	<i>Phaseolus lunatus</i>	Zeatin	UDP-glucose	(Martin <i>et al.</i> , 2000)	
Zeatin	ZOX1	AF116858	<i>Phaseolus vulgaris</i>	Zeatin	UDP-xylose	(Martin <i>et al.</i> , 2000)	
E	AS	AJ310148	<i>Rauvolfia serpentina</i>	Hydroquinone, xenobiotics	UDP-glucose	(Hefner <i>et al.</i> , 2002)	
D	UBGT	BAA83484	<i>Scutellaria baicalensis</i>	Flavonoids (7-O-Glc)	UDP-glucose	(Hirofani <i>et al.</i> , 2000)	
D	St SGT	U82367	<i>Solanum tuberosum</i>	Solanidines	UDP-glucose	(Moeh <i>et al.</i> , 1997)	
G	sbHMNGT	AF199453	<i>Sorghum bicolor</i>	p-Hydroxymandelonitrile	UDP-glucose	(Jones <i>et al.</i> , 1999)	
L	HGT8	AB013598	<i>Verbena hybrida</i>	Anthocyanin-3-O-glucoside (5-O-glc)	UDP-glucose	(Yamazaki <i>et al.</i> , 1999)	
D	rROC	AB065190	<i>Vigna angularis</i>	Abcisic acid	UDP-glucose	(Xu <i>et al.</i> , 2002)	
D	VmUFGT2	AB012114	<i>Vigna mungo</i>	Flavonoids (3-O-glc)	UDP-galactose	(Mato <i>et al.</i> , 1998)	
F	UFGT	AF000371	<i>Vitis vinifera</i>	Anthocyanins (3-O-glc)	UDP-glucose	(Ford <i>et al.</i> , 1998)	
Zeatin	cisZOG1	AF318075	<i>Zea mays</i>	cis-Zeatin	UDP-glucose	(Martin <i>et al.</i> , 2001)	
H	BX8	AF331854	<i>Zea mays</i>	Benzoxazinoids	UDP-glucose	(von Rad <i>et al.</i> , 2001)	
H	BX9	AF331855	<i>Zea mays</i>	Benzoxazinoids	UDP-glucose	(von Rad <i>et al.</i> , 2001)	
L	IAAGT	L34847	<i>Zea mays</i>	IAA	UDP-glucose	(Szerszen <i>et al.</i> , 1994)	
F	Zm 3-GT	X13501	<i>Zea mays</i>	Flavonols (3-O-glc)	UDP-glucose	(Furket <i>et al.</i> , 1988)	

and coumarins resulting in the formation of ether glucosides. All but two of the 51 GTs with known substrate specificities utilise UDP-glucose as their primary sugar donor. The 3-*O*-galactosyltransferase from *Petunia hybrida* (Millar *et al.*, 1999) and the ZOX1 from *P. vulgarisi*, which utilises UDP-xylose (Martin *et al.*, 1999b), are members of group F and the zeatin group respectively and are closely related to the glucose specific GTs in those groups. This suggests that sugar specificity has evolved after the divergence in GT sequences.

Only two of the 51 active GTs are reported to have any activity toward xenobiotic substrates, mainly because most of these enzymes have only been assayed with putative endogenous substrates. The arbutin synthase (AS) from *Rauwolfia serpentina* (Hefner *et al.*, 2002) and UGT75D1 from *Arabidopsis* (Schäffner *et al.*, 2002) share only 22 % amino acid residue identity and belong to group E and group L respectively. The endogenous substrate of the AS was found to be the simple phenolic hydroquinone. The recombinant protein of AS was tested for activity toward a total of 74 natural and synthetic phenols and two cinnamyl alcohols, of these 45 substrates were accepted, although the majority were poor substrates. The synthetic compounds 4-chloro-8-hydroxyquinoline and 4-chloro-2-methylphenol gave 58 % and 19 % respectively of the maximal velocity seen with the hydroquinone, suggesting that quite substantial additions to the basic hydroquinone structure could be tolerated by AS. In addition to simple phenols, flavonoids, including apigenin and quercetin, and two coumarins were accepted by the AS although less than 5 % of the activity toward hydroquinone was observed (Hefner *et al.*, 2002).

The recombinant UGT75D1 from *Arabidopsis* was expressed in yeast and GT activity was observed toward several endogenous substrates, including IAA, coniferyl alcohol and salicylic acid, with both hydroxyl and carboxyl groups conjugated. Activity toward the synthetic phenol, 2,4,5-trichlorophenol, was ten-fold higher than towards the optimal endogenous substrate identified, IAA. Activity was also observed toward 3,4-dichloroaniline and 4-chlorothiophenol revealing that UGT75D1 was also able to glucosylate amino and sulphhydryl groups (Schäffner *et al.*, 2002). While a salicylic acid-inducible GT from tobacco (TOGT1) was found to glucosylate both carboxyl and hydroxyl groups (Frassinetti-Trachet *et al.*, 1998) this is the first demonstration of a recombinant GT able to glucosylate carboxyl, hydroxyl, amino and sulphhydryl functional groups (Schäffner *et al.*, 2002).

As the majority of recombinant GTs were only assayed with a relatively small range of substrates it is not clear whether or not the broad range of activities observed with the AS and UGT75D1 is typical of members of the GT family and how important regiospecificity is in the glycosylation of substrates. It is also difficult to determine the physiologically relevant substrates for an individual GT *in planta* from activity assays performed *in vitro*. Genetic transformations to produce antisense plants or new pathways in species have been carried out with several GTs. Tobacco plants with depleted amounts of a salicylic acid-inducible GT (TOGT) were found to show enhanced oxidative stress and weakened resistance when infected with tobacco mosaic virus (Chong *et al.*, 2002). The entire pathway for the synthesis of the cyanogenic glucoside dhurrin, including the *p*-hydroxymandelonite utilising GT (sbHMNGT), was transferred from *Sorghum bicolor* to *Arabidopsis* conferring

resistance against a natural pest of *Arabidopsis* (Tattersal *et al.*, 2002). More studies on GT activities *in vitro* and *in planta* are necessary to elucidate the exact role of GTs in xenobiotic metabolism and the relationship between amino acid sequence and regiospecificity toward substrates.

Summary of aims and objectives

O-Glucosylation, catalysed by *O*-GTs, is known to be a major route of phase II metabolism of herbicides in wheat, however little is known about the enzymes involved or of the factors that regulate their activities. The role of *O*-GTs in the resistance to multiple classes of herbicides in several populations of black-grass, an agronomically important weed of wheat, has also not been investigated. The aims of the project were therefore:

1. The characterisation of OGT activities in wheat and black-grass toward xenobiotic and natural product substrates.
2. To determine whether OGT activity can be induced in wheat and black-grass by herbicide safener treatment.
3. To compare OGT activities in herbicide-susceptible and herbicide-resistant populations of black-grass.
4. To purify major OGTs from wheat.
5. To determine whether changes in xenobiotic conjugation produced by herbicide safener treatment of wheat and herbicide-resistance in black-grass can result in changes in the accumulation of conjugates of endogenous phenolics.

2. MATERIALS AND METHODS

2.1 Assay methods

2.1.1 Plant studies

Wheat seeds (*Triticum aestivum* L. cv. Hunter) were obtained from Plant Breeding International, Cambridge. Seeds were imbibed in water for 1 hr prior to sowing on vermiculite, where they were grown for ten days at 25 °C with a 16 hr photoperiod at a light intensity of 110 $\mu\text{Em}^{-2}\text{s}^{-1}$. Shoot and root material was harvested separately, frozen in liquid nitrogen and stored at -80 °C until required.

Herbicide-resistant (Peldon) and herbicide-susceptible (wild-type) populations of black-grass (*Alopecurus myosuroides*) were obtained from Herbiseed, Wokingham, UK. Seeds were placed on moist tissue paper for three days in the light and then grown in Arthur Bower's Multi-Purpose compost for 21 days under the conditions as used for wheat. Shoot material was harvested and then frozen.

Aventis Crop Science Ltd supplied the safeners benoxacor, CMPI, fenclorim, flurazole, isoxadifen, mefenpyr diethyl, oxabetrinil and R-29148. Cloquintocet mexyl was obtained from Fluka (Poole, Dorset) and Dichlormid from ChemService Inc. (Merseyside, UK). Safeners were prepared as 10 mg ml⁻¹ stock solution in acetone and diluted 1000-fold with water for plant treatment. Control treatments consisted of 0.1 % v/v acetone.

2.1.2 Protein extraction

Tissue was homogenised in liquid nitrogen using a pestle and mortar. All further steps were carried out on ice. Protein was extracted using 3 ml of extraction buffer (0.2 M Tris-HCl, pH 8.0 containing 2 mM dithiothreitol (DTT) and 5 % (w/w) polyvinylpyrrolidone) per g of fresh weight tissue. The resulting extract was filtered through two layers of miracloth (Calbiochem, Nottingham, UK). After centrifugation (8,500 g, 15 min, 4 °C), protein in the supernatant was precipitated by the addition of ammonium sulphate to 70 % saturation and recovered by further centrifugation (8,500 g, 30 min, 4 °C). Pellets were stored at -20°C and desalted prior to use on a Sephadex G-25 column (PD-10; Amersham Biosciences, Little Chalfont, Bucks, UK).

2.1.3 Protein determination

Protein content was determined following the method devised by Bradford (1976), using dye-binding reagent from Biorad and γ -globulin as a reference protein.

2.1.4 Glucosyltransferase assay

All substrates were supplied by the Sigma-Aldrich Company Ltd (Poole, Dorset, UK), except luteolin, coumesterol and cyanidin hydrochloride, which were obtained from Apin Chemicals Ltd (Abingdon, Oxon, UK). Desalted protein (40-100 μ g) in 0.2 M Tris-HCl, pH 8.0, 2 mM DTT (65 μ l) was mixed with 5 μ l substrate (acceptor) prepared in methanol to give a final assay concentration of 66 μ M. The reaction was initiated by the addition of 5 μ l of UDP-[¹⁴C-glucose] (50,000 dpm, specific activity

205 mCi mmol⁻¹, ICN, Costa Hesta, CA, USA) and incubated at 30 °C for 20 min. To measure the activity due to the conjugation of natural products present in the crude protein preparation incubations were carried out with methanol in place of acceptor substrate. The reaction was stopped by the addition of 125 µl of 0.3 M HCl and partitioned with 200 µl of water-saturated ethyl acetate. The radioactive conjugates (100 µl) from the upper organic phase were then quantified by liquid scintillation counting (Parry & Edwards, 1994).

2.1.5 Glutathione transferase assay

GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was measured using a published spectrophotometric assay (Habig *et al.*, 1974) with minor modifications. The assay mixture containing 900 µl of 0.1 M potassium phosphate buffer pH 6.5 and 25 µl ethanolic 40 mM CDNB was incubated at 30 °C for 5 min prior to adding 25 µl of enzyme sample and 50 µl of 100 mM glutathione. The increase in absorbance at 340 nm was monitored over 30 s. As the reaction had significant spontaneous rate incubations were carried out with the enzyme omitted. The extinction coefficient for the reaction product was 9.6 mM⁻¹ cm⁻¹.

2.1.6 Phenylalanine ammonium lyase (PAL) assay

Tissue was extracted in 50 mM Tris-HCl, pH 8.5 and 80 % (NH₄)₂SO₄ pellets were prepared using the method detailed in section 2.1.2 and desalted prior to use. Crude protein (1 mg) in a 100 µl volume was mixed with 900 µl of 12.1 mM L-phenylalanine in 50 mM Tris-HCl, pH 8.5 and incubated at 40 °C. PAL activity was monitored by measuring the increase in absorbance at 290 nm every 30 mins over 2

hr. Changes in absorbance that were not due to PAL activity were corrected for using D-phenylalanine in the place of L-phenylalanine as the D isomer is not a PAL substrate. Activity was calculated as $\mu\text{kat}/\text{kg}$ using the equation (Edwards & Kessman, 1992):

$$\frac{27780 \times (\Delta A_{290} \text{ L-Phe/ 60 min} - \Delta A_{290} \text{ D-Phe/ 60 min})}{\mu\text{g protein per incubation}}$$

2.1.7 Radio-HPLC of reaction products

A scaled up version of the glucosyltransferase assay was carried out using 1 mg of desalted protein in total volume of 700 μl and the reaction started by addition of 50 μl of 66 μM unlabelled UDP-glucose. Reaction conditions and extraction methods was as for standard assay except that the reaction mixture was partitioned with 2 ml of ethyl acetate. 100 μl of the ethyl acetate phase from a reaction carried out in parallel with UDP-[^{14}C -glucose] was used to spike the 1.5 ml of the organic phase from the unlabelled co-substrate reaction. The combined mixture was then dried down using a vacuum centrifuge and resuspended in 30 μl of methanol.

HPLC analyses of reaction products were performed with a Beckman System Gold[®] liquid chromatograph, equipped with a Gilson 234 auto injector and a Beckman 171 radioisotope detector. A precolumn and a Phenosphere column (250x4.60mm) both packed with 5 μm C18 matrix (Phenomenex, Macclesfield, Cheshire) were used to resolve the reaction products. The mobile phases were solvent A, 1% formic acid and solvent B, acetonitrile. The program of elution was as follows: a linear increase of 20 % to 60 % B from 0 to 45 mins and followed by isocratic elution with 100% B for a

further 15 min. A volume of 20 μl of sample was injected. The flow rate was 0.8 ml min^{-1} and eluant monitored for UV-absorbing metabolites at 287 nm.

2.2 Metabolite analysis

2.2.1 Extraction of natural products and cellulase digestion

Plant samples were sequentially extracted with ice-cold acetone (10 v/w) followed by 10 v/w acetone: methanol (1:1) using a pestle and mortar with acid-washed sand as an abrasive. After filtration the combined extract was concentrated to near dryness under reduced pressure (Edwards & Kessman, 1992). Samples were then resuspended in 0.15 M citrate phosphate buffer pH 5.0, in the presence or absence of 1 mg ml^{-1} cellulase. After incubation at 30 $^{\circ}\text{C}$ for 12 hr, samples were partitioned with ethyl acetate, dried down and resuspended in methanol.

2.2.2 LC-MS analysis of natural products

LC-MS (liquid chromatography mass spectrometry) analysis of natural products was performed with a Waters 2790 HPLC chromatography. A pre-column and a Synergi Polar-RP (250 x2 mm) column (Phenomenex, Macclesfield, Cheshire) were used to separate metabolites. The eluents were solvent A, 1 % formic acid and solvent B, acetonitrile. The program of elution was as follows: a linear increase of 5 % to 100 % B from 0 to 42 min, followed by isocratic elution with 100 % B for a further 6 min. A volume of 10 μl of sample was injected. The flow rate was 0.2 ml min^{-1} and the eluant was monitored for UV-absorbing metabolites between 200 nm and 400 nm using a Waters 996 photodiode array detector. This was followed by ESI-TOF MS

on a Micromass LCT mass spectrometer operating in negative ion mode with the cone voltage set at 20 V, desolvation temperature at 200 °C and sample cone heated to 120 °C.

2.3 Protein purification methods

All chemicals and columns were from Amersham Biotech unless otherwise stated. Chromatography steps were either carried out at 4 °C using the GradiFrac (Pharmacia) low-pressure system (DEAE, phenyl sepharose and Q sepharose) or at room temperature using the HRLC[®] system from Biorad, Hemel Hempstead, Herts (Mono P, Superdex 200 and Mono Q). Protein elution was measured as UV absorbance at 280nm.

2.3.1 Anion exchange using DEAE sepharose

Protein preparations were desalted in 25 mM imidazole-HCl, pH 7.5, 2 mM DTT. Protein (total 120 mg) was applied to a 5 ml DEAE (diethylaminoethyl) Fast Flow column pre-equilibrated with the above buffer at 1.0 ml min⁻¹. The column was washed with 12 ml of imidazole buffer and then the protein eluted with an increasing linear concentration up to 0.4 M NaCl over 30 min. Fractions (1ml) were collected and 30 µl assayed for OGT activity.

2.3.2 Hydrophobic interaction chromatography on phenyl sepharose

Protein preparations were taken up in 50 ml of 25 mM imidazole-HCl, pH 7.5, 1 M (NH₄)₂SO₄, 2 mM DTT and centrifuged at 17,000 g for 20 min at 4 °C. The

supernatant was applied to pre-equilibrated column (60 ml) of Phenyl SepharoseTM (Fast Flow, low sub). After washing the column with 120 ml of the above buffer at 4 ml min⁻¹ the OGT activity was eluted from the column by decreasing the (NH₄)₂SO₄ concentration from 1 M to 0 M over 32.5 min followed by a further 10 min wash with buffer minus (NH₄)₂SO₄. 8ml fractions were collected and assayed and active fractions pooled and then precipitated with (NH₄)₂SO₄ prior to storage at -20°C.

2.3.3 Chromatofocusing using Mono P

Pooled active fractions derived from phenyl sepharose chromatography were desalted in 25 mM Bis-Tris- HCl, pH 6.0, 2 mM DTT using a Sephadex G-25 column (PD-10). The sample was applied to a Mono P HR 5/20 column (4ml) and washed with 10ml of 25 mM Bis-Tris buffer at 1 ml min⁻¹. Bound protein was then eluted with 50 ml of 10 % Polybuffer 74, pH 4.0, 2 mM DTT and collected in 1 ml fractions.

2.3.4 Anion exchange chromatography on Q sepharose

Protein preparations were desalted in 20 mM Bis-Tris-HCl, pH 6.0, 2 mM DTT using Sephadex G-25 gel filtration chromatography (PD-10 columns). The sample was applied to a Q Sepharose 4 Fast Flow column (20ml) and washed with 45 ml of the above buffer at 2 ml min⁻¹. The bound proteins were then eluted in 2 ml fractions using a linearly increasing concentration of 0 to 500 mM NaCl in a total volume of 80 ml. After collection, active fraction were pooled and stored as (NH₄)₂SO₄ pellets at 4°C.

2.3.5 Gel filtration chromatography using Superdex 200

(NH₄)₂SO₄ precipitated proteins were resuspended in 50 mM potassium phosphate buffer, pH 7.2, 0.15 M NaCl, 2 mM DTT when running protein molecular weight standards or 0.2 M Tris/ HCl, pH 7.5, 2 mM DTT during protein purification. Proteins (10 mg) were injected in 0.2 ml on to a Superdex 200 HR (24 ml vol.) column for each run. The protein was eluted at 0.5 ml min⁻¹ and 0.5 ml fractions collected.

2.3.6 Anion-exchange using Mono Q

Proteins were applied to a mono Q (1 ml) column in 50 mM Tris/ HCl, pH 7.5, 2 mM DTT. After washing the bound protein was then eluted with a linear gradient of 80 mM to 157 mM NaCl at 1ml min⁻¹ and collected in 1 ml fractions.

2.3.7 Large scale protein extraction

For large scale extractions of protein, to avoid oxidation of the extract, 10 mM ascorbic acid, 10 mM sodium metabisulphite plus 5 mM DTT was added to the 0.2 M Tris-HCl pH 8.0 buffer prior to extraction.

2.3.8 Determination of apparent K_M

To determine kinetic constants toward UDPG with the aglycones 2,4,5-trichlorophenol and quercetin, GT assays were carried out using 0.2 M Bis-Tris/HCl,

pH 7.2, 2 mM DTT. The aglycones (66 μM) were incubated with a range of concentrations of unlabelled UDPG containing 1 μM (50,000 dpm) of radiolabelled UDP-[^{14}C -glucose] to give total UDPG concentrations of 100 μM , 50 μM , 25 μM , 10 μM , 5 μM and 2 μM .

The K_M towards 2,4,5-trichlorophenol and quercetin was determined using 150 μM UDPG (containing 100,000 dpm; UDP-[^{14}C -glucose]) with the aglycones at concentrations of 50 μM , 25 μM , 10 μM , 5 μM , 2 μM and 0.5 μM . The aglycones at 50 μM were found to be at a saturating concentration. Desalted protein (100 μg) from the 40-60% $(\text{NH}_4)_2\text{SO}_4$ precipitated crude wheat extracts was added to start the reactions which followed the procedure described for the standard GT assay (section 2.1.4). A correction was made to account for the dilution of UDP-[^{14}C -glucose] by unlabelled UDPG when calculating product formation.

2.3.9 Catalytic efficiency

The catalytic efficiency was determined on purified OGT from Mono P using sub-saturating conditions of substrate based on the relationship:

$$\frac{K_{cat}}{K_M} = \frac{v}{[E][S]}$$

The assay velocity ($\text{M}\cdot\text{s}^{-1}$) was determined toward 2,4,5-trichlorophenol and quercetin at a substrate concentrations well below the respective K_{MS} . The enzyme concentration was then calculated from total protein in fraction (g/L) divided by the

MW (OGT) of 50,000 to give [E]. The substrate concentration [S] used was 5×10^{-7} M.

2.4 Protein analysis

2.4.1 SDS-PAGE

SDS-PAGE (sodium dodecyl sulphate- polyacrylamide gel electrophoresis) gels were prepared using the mini-PROTEAN II kit from Biorad according to the method of Laemmli (1970). Resolving gels were polymerised from 10 % or 12.5 % acrylamide/ *bis*-acrylamide (Sigma) in 375 mM Tris/ HCl, pH 9.0 containing 0.1 % (v/v) TEMED (Promega, Madison, WI, USA), 0.1 % (w/v) SDS and 0.1 % (w/v) ammonium persulphate. The stacking gel was polymerised from 4 % acrylamide/ *bis*-acrylamide, 126 mM Tris/ HCl, pH 6.8, 0.1% (v/v) TEMED, 0.1 % (w/v) SDS and 0.05 % (w/v) ammonium persulphate. Protein samples were diluted with an equal volume of 2 X SDS loading buffer (100 mM Tris-HCl, pH 6.8, 20 % glycerol, 200 mM DTT, 4% w/v SDS, 0.2 % w/v bromophenol blue) and incubated at 95 °C for 5 min prior to loading on to gel. The samples were loaded into the wells and electrophoresed in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) at 100 V for the first 10 min and then at 200 V until the dye front reached the bottom of the gel. Gels were washed thoroughly with water to remove the SDS and then if required, stained with Coomassie blue reagent (0.01 % w/v coomassie brilliant blue, 5 % v/v 95 % ethanol:water (95:5 v/v) and 10 % v/v phosphoric acid:water 85:15 v/v).

2.4.2 Western blotting and immunodetection

Polypeptides were separated by SDS-PAGE (see above) and then electroblotted onto a PVDF membrane (Hybond-P, Amersham Biotech) using a mini Trans-Blot cell (Biorad) according to manufacturer's instructions. After blotting the membrane was blocked with 3 % skimmed milk powder in Tris-buffered saline (TBS; 1 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 hr. Antisera (rabbit) raised to specific GSTs were then added at a 1: 2000 to 1: 5000 dilution and incubated at 1- 2 hr at room temperature or overnight at 4 °C. The membrane was then washed twice for 10 min with TBST (TBS plus 0.1 % v/v Triton X-100) and then for a further 10 min with TBS. Secondary antibody (anti-rabbit alkaline phosphatase conjugate from Sigma) was incubated at a 1: 5000 dilution in 3 % milk powder in TBS for 1 hr at room temperature and then the membrane was washed as before. The membrane was rinsed in 100 mM Tris/ HCl, pH 9.5 and then developed in 0.3 % (v/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 50 mg ml⁻¹) dissolved in *N,N,N',N'*-dimethylformamide (DMF) and 0.3 % (v/v) nitro blue tetrazolium (NBT; 100 mg ml⁻¹) dissolved in 70 % DMF.

To detect blotted recombinant poly-histidine-tagged fusion proteins the INDIA™ HisProbe-HRP was used in conjunction with the SuperSignal® West Pico Chemiluminescent substrate according the manufacturer's instructions (Pierce, Rockford, IL, USA).

2.4.3 Mini format 2-dimensional SDS-PAGE

All reagents were obtained from Amersham Biotech. The protein sample was pre-treated with an equal volume (62.5 μ l) of 9 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 1 % (w/v) DTT and 2 % (v/v) pH 3- pH 10 ampholytes and pipetted into a well of an ImmobilineTM Drystrip reswelling tray. The ImmobilineTM Dry Strip pH 4-pH 7 (7 cm) was rehydrated overnight by placing the strip in the well over the sample after covering with ImmobilineTM DryStrip cover fluid. The rehydrated IPG strip was washed with water and placed in the running tray of a MultiPhor® II tank (Pharmacia). The electrodes were assembled over the DryStrip and cover fluid poured over the top. The gel was run at 2000 V for 1 hr 30 min and then at 3500 V for a further 1 hr 30 min. The DryStrip was then equilibrated for 15 min in 10 ml of 50 mM Tris/ HCl, pH 8.8, 6M Urea, 30 % (v/v) glycerol, 10 % (w/v) SDS, 0.1 % (w/v) DTT, containing a trace of bromophenol blue dye. The second dimension run was performed using the standard SDS-PAGE kit, except that 1 mm spacers were used when casting the gel. A 12% acrylamide gel was polymerised and the DryStrip laid across the top of the resolving gel and filled with agarose sealer (0.1 % low melting point agarose in SDS-PAGE running buffer containing a trace of bromophenol blue). The gel was then run in SDS-PAGE running buffer at 150 V.

2.4.4 Silver staining

Silver staining of proteins in SDS-PAGE gels was carried using the method devised by Heukeshoven & Dernick (1985). Gels were fixed in 40 % (v/v) ethanol, 10 % (v/v) acetic acid for 15 min then treated with 30 % (v/v) ethanol, 0.2 % (w/v) sodium

thiosulphate and 6.8 % (w/v) sodium acetate for 30 min. The gels were washed three times with water, each for 5 min, and then immersed in 0.25 % (w/v) silver nitrate for 20 min. The gels were briefly washed and then developed in 2.5 % (w/v) sodium carbonate, 0.04% (v/v) formaldehyde until proteins could be visualised, the gels were then washed with 1.46 % (w/v) EDTA.

2.5 Isolation of RNA

To ensure any buffers and water used in extraction of the RNA were RNAase free, the solutions were treated with 0.1% (v/v) diethylpyrocarbamate (DEPC) for at 1 hr at 37 °C and then autoclaved for 15 min. The pestle and mortar were baked at 200 °C for at least 8 hr prior to use.

2.5.1 Extraction of total RNA

Total RNA was extracted from 50-100 mg of wheat and black-grass shoots using TRI REAGENT™ (Sigma) and the accompanying protocol. The final RNA pellets were resuspended in 25 µl of H₂O and stored at -80°C. The concentration of the RNA was determined by measuring the OD₂₆₀ (OD₂₆₀ 1.0 ≡ 40 µg /ml RNA).

2.5.2 Purification of Poly A+ RNA

Oligo (dT)₂₅ Dynabeads® mRNA (DYNAL® (UK) Ltd., Wirral, UK) were used to purify poly A+ RNA from total RNA in batches of 75 µg of total RNA at a time, with the beads reused up to three times for the same crude extract (Jakobsen, *et al.*, 1990).

2.6 cDNA isolation

Custom oligonucleotides were obtained from MWG-biotech AG (Ebersberg, Germany). The sequences of the primers used in the PCR reactions are detailed in Chapter 6.

2.6.1 Synthesis of cDNA using reverse transcription

Total RNA (5 µg) or poly A⁺ RNA (0.5 µg) was mixed with 10 pmol of either an oligo dT primer (Og2) or a specific primer and heated at 70 °C for 10 min. The sample was placed on ice and 5x AMV (avian myeloblastosis virus) reverse transcriptase buffer, dNTP mix (1 mM), RNasin[®](40 U), 10 U AMV reverse transcriptase (all Promega) and H₂O added to a total volume of 20 µl. Reverse transcription was carried out at 45 °C for 45 min following by a further incubation at 55 °C for 30 min.

2.6.2 PCR (polymerase chain reaction) amplification

Standard PCR reactions were performed in a total volume of 20 to 50µl. The buffer used in standard reaction was prepared from 0.5 M Tris/ HCl, pH 8.8, 50 mM MgCl₂, 76 mM 2-mercaptoethanol, 50 µM EDTA, 11 µM each of dATP, dCTP, dTTP and dGTP, 12.5 % (w/v) bovine serum albumin (BSA). PCR reactions were carried out using a 1:11 dilution of PCR buffer, two or three oligonucleotides (1 µM), appropriate amount of template and *Taq* DNA polymerase (2-5 U, where 1 U catalyses the incorporation of 10 nmol of dNTP into acid-insoluble material in 30 min at 74°C).

After denaturation at 94 °C for 2 min the incubations were subjected to 20-30 cycles at 94 °C for 30 s, 50-65 °C for 45 s and 72 °C for 45 s to 1 min 30 s. If the PCR products were to be cloned a 10 min extension cycle at 72 °C was included at the end. For PCR requiring the use of a proof reading DNA polymerase, *Pwo* DNA polymerase (2.5 U), 10x Buffer *Pwo* incomplete, 0.5-1.5 mM MgSO₄ (all from Hybaid Ltd, Ashford, Middlesex, UK), 0.2 mM dNTP mix and a suitable quantity of template were used. *Pwo* DNA polymerase was added to the reaction mix after the initial 2 min denaturing cycle at 94 °C. All PCR reactions were carried out in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany).

2.6.3 5' RACE (rapid amplification of cDNA ends)

5'RACE was employed to extend the 5'ends of truncated cDNAs. For each cDNA, two antisense oligonucleotides were designed 400 bp (primer 1) and 300 bp (primer 2) from the truncated 5' end of the cDNA with a sense tester oligonucleotide designed to the 5' end (primer 3). cDNA was synthesised from either 5 µg total RNA or 0.5 µg of poly A+ RNA using primer 1 as detailed in section 2.6.1. The cDNA was purified (see section 2.7.7) and a homopolymer tail added at the 3' end using terminal deoxynucleotidyl transferase (TdT). The cDNA (17 µl) was mixed with 2 mM dATP and 10 X NE buffer 4 (New England Biolabs, Beverly, MA, USA) and denatured at 70 °C for 10 min. The mixture was then placed on ice and 20 U of TdT was added and incubated on ice for 1 hr prior to transferring to 37 °C for 15 min. The TdT was then heat inactivated. The tailed cDNA (5 µl) was then amplified by PCR using the primer 1 and an oligo dT adaptor primer (5- CTT ATA CGG ATA TCC TGG CAA TTC GGA CTT TTT TTT TTT TTT TTT V -3') with 5 cycles at an annealing

temperature at 45 °C followed by 30 cycles at 55 °C. A 1:20 dilution of the PCR product was prepared and then subjected to a second round of PCR using the nested primers adaptor primer (5'- CTT ATA CGG ATA TCC TGG CAA TTC GGA CTT - 3') and primer 2. 25 to 30 cycles of PCR were carried out.

2.7 Cloning methods

2.7.1 Ligation reactions

For direct cloning of PCR products produced using *Taq* DNA polymerase, the pGEM[®]-T Easy vector system I (Promega) was used according to the manufacturer's instructions. For cloning into other vectors, digested insert and vector were mixed in a 3:1 ratio with the appropriate buffer and 5 U of T4 DNA ligase (MBI Fermentas, Tyne and Wear, UK) in a 10µl volume. The reaction was incubated at room temperature for 1 hr and then the ligase was heat inactivated at 70 °C for 10 min.

2.7.2 Preparation and electrotransformation of competent *E.coli*

Electrocompetent cells were prepared for routine transformation of *E.coli* using a method modified from Tung & Chow (1995). Luria-Betani (LB; 1% w/v bacterial peptone, 0.5 % w/v yeast extract, 1 % w/v NaCl, pH 7.0) medium (500 ml- 2 L) was inoculated with 5-20 ml of overnight culture and incubated at 37 °C with vigorous shaking. When the OD₆₀₀ reached 0.6, the culture was chilled on ice for 30 min. Cells were harvested at 4000g for 10 min at 4 °C, washed once with an equal volume (500 ml-2 L) of ice-cold water and again with half volume of ice-cold water followed by 5-20 ml GYT medium (10 % v/v glycerol, 0.125 % w/v yeast extract, 0.25 % w/v

trytone). Cells were then flash frozen in liquid nitrogen and stored at -80°C until required.

Prior to transformation 1 μl of DNA plasmid was mixed with 50 μl of thawed cells and transferred to a pre-chilled 2 mm electroporation cuvette (Molecular BioProducts, San Diego, CA, USA). Electrotransformation was performed using a Gene Pulser (BioRad) according to the manufacturer's instructions (25 μF , 200 Ω , 2.5 kV). Immediately after electrotransformation 1 ml of SOC medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 10 mM MgSO_4 , 10 mM MgCl_2) was added and the cells incubated at 37°C for 1 hr. Cells were then spread on selective agar plates and incubated at 37°C .

2.7.3 Bacterial culture

Liquid bacterial cultures were inoculated from a single colony in LB medium and grown in LB medium overnight at 37°C on an orbital shaker at 200 rpm. Bacterial cultures were also grown on plates made of 1.5 % agar and LB medium at 37°C overnight. If required, an antibiotic was added at an appropriate concentration (ampicillin; $100\ \mu\text{g ml}^{-1}$, chloramphenicol; $34\ \mu\text{g ml}^{-1}$, tetracycline; $12.5\ \mu\text{g ml}^{-1}$).

2.7.4 Plasmid preparation

Plasmid was prepared from 3 ml of liquid bacterial culture using the Wizard[®] Plus SV minipreps DNA purification system (Promega) according to manufacturer's instructions.

2.7.5 DNA agarose gel electrophoresis

Agarose gels were prepared by microwaving TAE (4.84 % w/v Tris base, 1.14 % v/v glacial acetic acid) and 0.8-1.5 % w/v molecular biology grade agarose (Helena BioSciences) until the agarose had melted. The gel mix was cooled to approximately 60 °C and ethidium bromide added at a concentration of 10 µl L⁻¹. The gel was poured into the casting tray of HORIZON[®] 58 horizontal gel electrophoresis apparatus (GibcoBRL[®], Paisley, UK). Samples were prepared by diluting with 6X loading buffer (0.25 % w/v bromophenol blue, 0.25 % v/v xylene, 15 % w/v Ficoll) and the gel loaded with a lane of markers (1Kb DNA ladder, GibcoBRL[®]) prior to electrophoresis TAE buffer at 120 V for 20 min.

2.7.6 Digestion of DNA by restriction enzymes

Restriction digests were carried out using restriction enzymes obtained from Promega, Roche (Lewis, East Sussex, UK) and MBI Fermentas. Generally, digests were performed on 0.5- 2 µg of plasmid DNA and 2-10 U of enzyme (where 1U of enzyme will digest 1µg of substrate DNA in a total reaction volume of 50µl using the optimal buffer) of restriction enzyme in the appropriate buffer. Digests were incubated at 37 °C for 1 - 16 hr before analysis of the reaction products by agarose gel electrophoresis.

2.7.7 Purification of DNA

PCR reactions or digested plasmid DNA were separated by electrophoresis and the required PCR product or digested DNA fragment excised from the gel. The gel slice was dissolved at 55 °C in 3 v/w binding buffer (6M Sodium Perchlorate, 50mM Tris/HCl, 10mM EDTA, pH 8.0). Resuspended silica fines (166 mg ml⁻¹; Superfine superfloss, World Mineral (UK) Ltd, Hessle, East Yorkshire, UK) were added (10 µl) to the dissolved gel and incubated at room temperature for 5 min. The sample was microfuged for 30 s at maximum speed and resulting pellet washed with 400 mM NaCl, 2 mM EDTA, 2 mM Tris/ HCl, pH 7.5 and then 70% ethanol. The pellet was then resuspended in 15 µl H₂O and incubated at 50 °C for 5 min. The sample was recentrifuged and the supernatant transferred to a clean tube.

2.8 cDNA libraries

The cDNA libraries used to obtain GT sequences had been constructed in the bacteriophage λ Uni-ZAP™ XP vector (Stratagene, La Jolla, CA, USA) and were prepared from 10 day old wheat shoots treated with the herbicide safener fenchlorozole ethyl (Thom *et al.*, 2002). Two libraries were prepared from 21 day old herbicide resistant (Peldon population) black-grass shoots containing either inserts of between 0.5-1.5 kb or inserts greater than 1.5 kb.

2.8.1 Mass excision of cDNA libraries

Prior to excision of the amplified libraries the titer of the bacteriophage was determined. Mass excised amplified libraries contained 10 times more bacteriophage than present in the primary library to ensure the statistical representation of excised clones. XL1-MRF' cells and ExAssist helper phage were incubated with the amplified libraries for 15 min at 37 °C and then 20 ml of LB was added. Samples were shaken for 3 hr at 37 °C followed by heating at 65 °C for 20 min and centrifugation.

The concentration of bacteriophage in the resulting supernatant was determined. Ten times as much phage as in the primary library was added to SOLR™ (non-suppressing) cells and incubated at 37 °C for 15 min. This was then used to inoculate 100 ml of LB containing 100 mg L⁻¹ and grown overnight with shaking at 37 °C. The culture was harvested and the respective plasmids purified.

2.8.2 Labelling of DIG probe

PCR was used to label cDNAs with DIG (digoxigenin; Roche) to produce probes for cDNA library screening. PCR was carried under the conditions described for *Pwo* DNA polymerase (section 2.6.2), except that 1 µl of PCR DIG synthesis mix (2mM dATP, dCTP, dGTP and 1.3mM dTTP and 0.7mM DIG-11-dUTP; Roche) was added to the 50 µl reaction.

2.8.3 cDNA library screening

The titer of bacteriophage in the library was first determined. To screen the library, large NZY agar plates were prepared two days prior to use from 0.5 % w/v NaCl, 0.2 % w/v $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 % w/v yeast extract, 1 % w/v NZ amine, 1.5 w/v agar. The library was then prepared from 150,000 plaque-forming units (pfu) combined with 600 μl of host cells (XL1-MRF²; $\text{OD}_{600} = 0.5$) by shaking at 37 °C for 15 min. NZY top agar (22.5 ml; NZY + 0.7 % agarose) at 48 °C was combined with library and host cells and poured over the large NZY agar plate. The plate was incubated overnight at 37 °C and then chilled at 4 °C for 2 hr. The plaques were transferred on to a nitrocellulose membrane (Hybond-N, Amersham Biosciences) and then denatured by submerging in 1.5 M NaCl and 0.5 M NaOH solution for 2 min. After washing for 5 min in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) the filters were rinsed for 30 s in 0.2 M Tris/ HCl pH 7.5 and 2x SSC (1.75 % w/v NaCl, 0.88 % w/v sodium citrate) buffer solution. The DNA was then cross linked to the membrane using 150 mJoules of UV illumination.

2.8.4 Hybridisation

Each membrane was pre-incubated in 12 ml of standard hybridisation buffer, 5x SSC, 0.1 % (w/v) *N*-lauroylsarcosine, 0.02 % (w/v) SDS and 1 % blocking solution (Roche), at 68 °C for 2-4 hr. Hybridisation with denatured DIG probes (5 μl) and 6ml of fresh standard hybridisation buffer was carried out overnight at 68 °C.

2.8.5 Stringency washes and colourimetric detection

After hybridisation, the membranes were washed in 2x SSC, 0.1% (w/v) twice at room temperature for 5 min and then a further two times at 68 °C for 15 min. Membranes were cooled to room temperature and then pre-equilibrated in maleic acid buffer (100 mM maleic acid/ NaOH, 150 mM NaCl, pH 7.5) plus 0.3 % (v/v) Tween-20 for 1 min. They were then treated for 30 min with 1 % blocking solution (Roche) in maleic acid buffer. A 1:5000 dilution of anti-digoxigenin-AP Fab fragments (Roche) were added to the blocking solution for a further 30 min. The membranes were then washed twice for 15 min in maleic acid buffer plus 0.3 % (v/v) Tween-20. Prior to detection, the membranes were incubated in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl for 2 min to which 0.3 % v/v NBT and BCIP were added. When positive plaques were clearly visible the colourimetric reaction was stopped by washing the membrane in water. The corresponding positive plaques were cored from the agar plate using a sterile Pasteur pipette and stored in 500 µl of simple medium (0.58 % w/v NaCl, 0.2 mM MgSO₄(7H₂O), 50 mM Tris-HCl, pH 7.5, 0.01 % w/v gelatin) containing 20 µl of chloroform at 4 °C.

2.9 Sequence analysis

2.9.1 DNA sequencing

Double stranded cDNAs were sequenced using automated florescent sequencers Applied Biosystems 377 DNA sequencer XL or the 373 DNA sequencer using the University of Durham sequencing service.

2.9.2 DNA and protein analysis

DNA sequences were edited, translated into three frames and restriction sites determined using the DNA sequence editing and analysis program DNA for Windows 2.4.0 (software written by Dr D. P. Dixon, School of Biological and Biomedical Sciences, University of Durham). DNA and protein sequences were aligned using CLUSTALW (Thompson *et al.*, 1994) and sequence similarities were determined using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990).

2.9.3 Phylogenetic tree

The phylogeny package PHYLIP (Felsenstein, 1989) was used to infer a phylogenetic tree for GT protein sequences. Firstly, multiple full-length protein sequences were aligned using the CLUSTALW program. This output was computed by PRODIST to produce the distance measure between paired sequences. The distance matrix method NIEGHBOR was used to produce an unrooted tree. DRAWTREE was used to plot the phylogeny.

2.10 Recombinant expression

2.10.1 Induction of recombinant proteins

To express cDNAs the open reading frame was cloned into the pET32a vector (Novagen, Madison, WI, USA) so that the sequence was in frame with the Trx●Tag™ thioredoxin protein at the N-terminal end and with a His-tag at the C-terminal end. The constructs were electroporated into *E.coli* strain Rosetta (DE3) and a single

colony used to inoculate 10 ml of LB. Antibiotic selection (100 $\mu\text{g ml}^{-1}$ ampicillin, 34 $\mu\text{g ml}^{-1}$ chloramphenicol) and 1 % glucose to suppress expression of the recombinant proteins, were added. The culture was grown overnight at 30 $^{\circ}\text{C}$ with shaking. This culture was diluted 1:1000 into fresh LB with appropriate antibiotic and glucose and grown at 30 $^{\circ}\text{C}$ until the absorbance at 600 nm was approximately 1.0. The culture was then transferred to a shaker at 15 $^{\circ}\text{C}$ for 1 hr. The culture was then induced with 0.1 mM isopropyl β -thiogalactoside (IPTG) for 16 hr. The culture was then pelleted at 4,300g for 8 min and stored at -20°C for at least 1 hr.

2.10.2 Purification of recombinant protein

Bacterial pellets prepared from 5 ml of induction culture were resuspended in 20 mM Tris-HCl, pH 8.0, 0.05 % Tween-20, 10 mM Imidazole. 1 mgml^{-1} of lysozyme was added and the sample incubated on ice for 30 min. The cells were then sonicated on ice (6 times for 10 s each time with 5 s pauses between) to lyse cells. Lysate was cleared by centrifugation at 10,000 x g for 30 min at 4 $^{\circ}\text{C}$. The recombinant protein was then purified using Ni-NTA magnetic agarose beads according to the manufacturers instructions (Qiagen Ltd, Crawley, West Sussex, UK).

3. OGT ACTIVITY IN WHEAT AND BLACK-GRASS

Introduction

To understand better the role of *O*-glucosyltransferases (OGTs) in herbicide metabolism in wheat and black-grass, OGT activities in these species toward a range of phenolic substrates were established. Initial work concentrated on optimisation of assay conditions and analysis of reaction products. A panel of xenobiotic and natural product substrates, representing a range of differing chemistries, were screened for activity in wheat shoots and roots. The effect of herbicide safener application on OGT activity in wheat was also investigated. While herbicides safeners have been shown to enhance glutathione transferase (GST) and cytochrome P450 mixed function oxidase (CYP) activities in wheat (Davies & Caseley, 1999), the effect on OGT activities have not been reported. Herbicide-susceptible and herbicide-resistant populations of black-grass were also screened with the panel of substrates for OGT activities. In the herbicide-resistant population, Peldon, specific GSTs and CYPs are expressed at higher levels than the herbicide-susceptible wildtype plants (Hyde *et al.* 1996; Cummins *et al.*, 1999), however it was not known whether or not OGT activities were similarly enhanced.

In wheat, a major mechanism for the detoxification of phenylcarbamate, phenylurea, sulfonylurea and imidazolinone herbicides is by oxidation by CYPs followed by glycosylation of the oxidised herbicide (Davies & Caseley, 1999). For example, the sulfonylurea herbicide metasulfuron-methyl is first hydroxylated and then *O*-

glycosylated in wheat (Anderson & Swain, 1992). A number of OGT activities towards xenobiotic substrates have been identified in wheat suspension cultures and a 43 kDa OGT isoenzyme with conjugating activity towards the herbicide and fungicide pentachlorophenol (PCP) partially purified (Schmitt *et al.*, 1985). Wheat suspension cultures were also tested by Pflugmacher *et al.* (1998) with four OGT xenobiotic substrates. OGT activity was found towards the substrates PCP, 2,3,4-trichlorophenol and 2,4,5-trichlorophenol, although very little activity was detected towards 2,2-(bis)-4-chlorophenylacetic acid. OGT activity has also been observed towards 4-nitrophenol, a metabolite of several pesticides, including the diphenyl ether fluorodifen. In wheat cell cultures, 4-nitrophenol was rapidly converted to its respective β -D-glucoside (Schmidt *et al.* 1993).

A limited amount of work on the activities of OGTs towards natural product substrates has been reported in wheat. Analysis of the flavonoid content of wheat leaves has revealed a range of flavonoid glucosides, primarily conjugates of the flavones luteolin and apigenin and related derivatives (Estiarte *et al.*, 1999). High concentrations of cyclic hydroxamic glucosides have also been found in germinating wheat (Nakagawa *et al.*, 1995). A glucosyltransferase with activity towards the cyclic hydroxamates 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its 7-methyl derivative (DIMBOA) was subsequently purified from 48 hour old wheat seedlings (Sue *et al.*, 2000).

Results

3.1 Optimisation and evaluation of assay conditions

OGT activity in wheat and black-grass was characterised by incubating protein extracts with UDP-[¹⁴C-glucose] and a range of phenolic substrates. The radiolabelled reaction products were partitioned into ethyl acetate and quantified by liquid scintillation counting. Preliminary experiments found OGT activity towards several simple phenol and flavonoid compounds. Two of these substrates 4-nitrophenol and the flavonol quercetin were used to optimise assay conditions. In previous studies OGT isoenzymes have been found to have optimal activities at pH 6.3 for an OGT active in conjugating kaempferol from soybean (Leah *et al.*, 1992) to pH 8.0- pH 8.5 for an anthocyanin 5-*O*-GT in *Perilla frutescens* (Yamazaki *et al.*, 1999). Using bis-Tris buffers between pH 6.4 and pH 7.4 or Tris-HCl buffers for pH 7.5 to pH 9.0 OGT activities towards 4-nitrophenol and quercetin were assayed in crude extracts from wheat (Fig. 9). OGT activities towards quercetin were maximal between pH 6.4 and pH 8.5 with this broad pH optimum suggesting more than one isoenzyme was present with activity towards the substrate. OGT activity toward 4-nitrophenol was maximal between pH 6.4 and pH 7.4. For assay of a wide range of substrates Tris-HCl buffered to pH 8.0 was used in all subsequent assays.

Thiol reducing agents have been found to enhance the stability of OGT isoenzymes, with the addition of DTT or EtSH found to stimulate OGT activity two-fold in extracts from alfalfa (Parry & Edwards, 1994). The addition of 2 mM DTT to

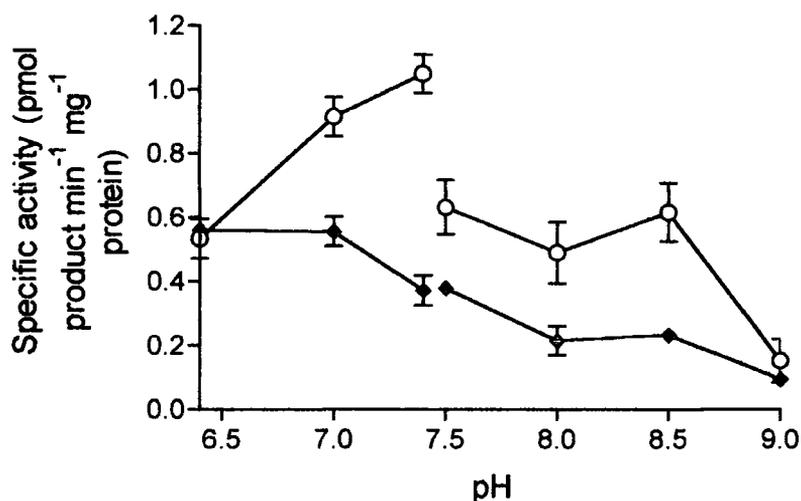


Figure 9 OGT activity towards 4-nitrophenol (◆) and quercetin (○) in crude wheat extracts over a range of pHs, determined using Bis-Tris/ HCl buffers (pH 6.3–pH 7.5) and Tris-HCl buffers (pH 7.5–pH 9.0). Error bars represent the variation in duplicate determination.

Table 2 OGT activity in 10 day wheat shoots extracted and assayed in presence and absence of 2mM DTT. All values are the mean of reaction duplicates \pm variation with the background activity subtracted

Substrate	OGT activity pmol product min ⁻¹ mg ⁻¹ protein	
	plus DTT	minus DTT
4-Nitrophenol	0.48 \pm 0.01	0.03 \pm 0.00
Quercetin	2.27 \pm 0.18	0.17 \pm 0.00

extraction and assay buffers was found to be essential in order to reliably determine OGT activity. Activity toward 4-nitrophenol was enhanced sixteen-fold and toward quercetin thirteen-fold following addition of 2 mM DTT relative to activities determined in the absence of thiol reagent (Table 2).

The formation of radioactive conjugates of 4-nitrophenol were linear over 30 minutes at 30 °C with both extracts from wheat shoots (Fig. 10A) and roots (Fig. 10B). Activity toward quercetin was not linear over the forty min duration of the assay. This may have been due the solubility of the substrate. For subsequent assays, carried out at 30 °C, reactions were stopped after 20 minutes. Between a limited range of protein concentration reaction product formation was found to be dependent on protein concentration. In extracts from wheat shoots (Fig. 11A), activity increased linearly over the range of 40 µg to 200 µg protein per assay with either 4-nitrophenol or quercetin as substrates. Below 40 µg protein per assay the activity was not strictly dependent on protein content. In wheat roots (Fig. 11B), OGT activity toward 4-nitrophenol increased linearly from 20 µg up to 100 µg protein per assay. In all future experiments 40 µg to 100 µg of wheat shoot protein and 40 µg of root protein was used per reaction to ensure strict protein dependence.

The radiolabelled reaction products formed when UDP-[¹⁴C-glucose] was incubated with either 4-nitrophenol, quercetin or 2,4,5-trichlorophenol were analysed by radio-

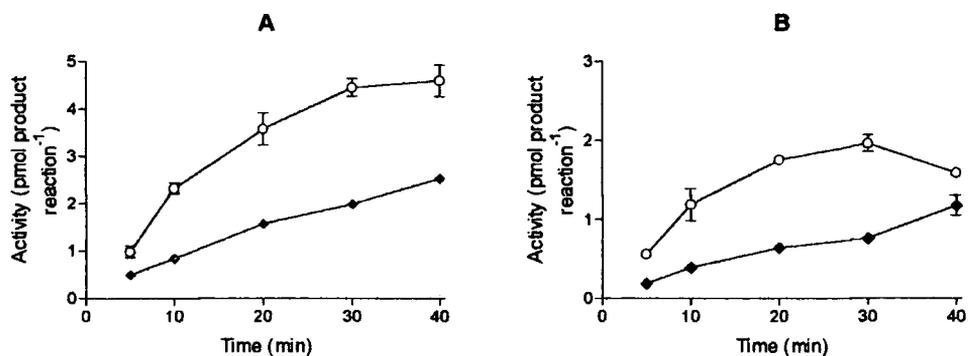


Figure 10 OGT activity towards 4-nitrophenol (◆) and quercetin (O) over a range of incubation times at 30 °C in crude protein extracts from wheat shoots (A) and wheat roots (B). Error bars represent the variation in duplicate determination.

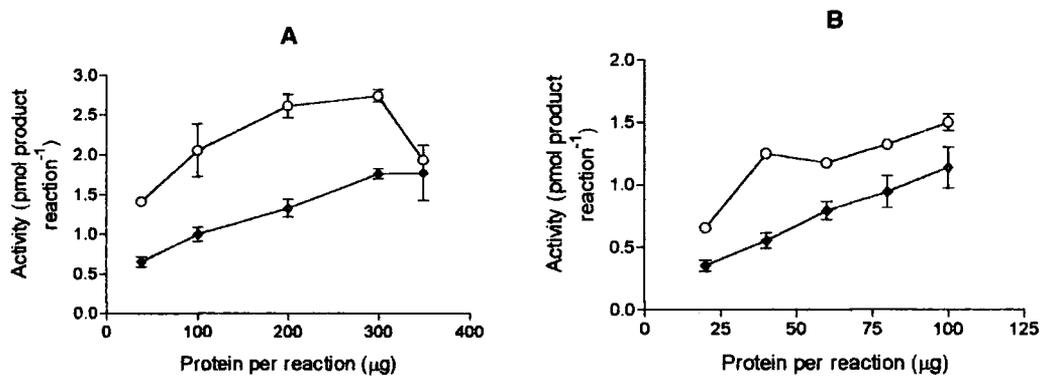


Figure 11 OGT activities towards 4-nitrophenol (◆) and quercetin (O) with increasing amounts of crude protein extracts from 10 day old wheat shoots (A) and roots (B). Error bars represent the variation in duplicate determination.

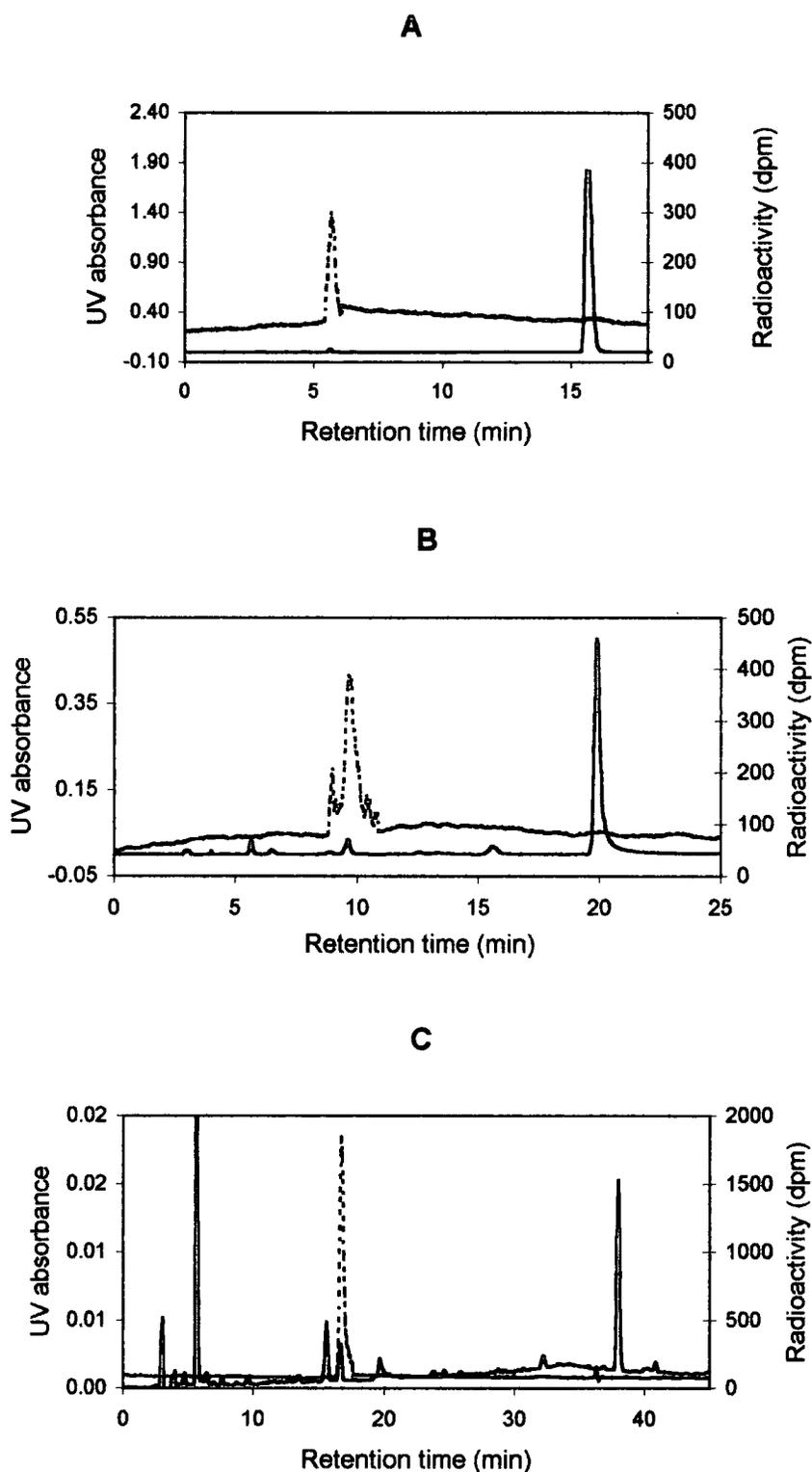


Figure 12 Analysis by HPLC of [^{14}C -glucosylated] reaction products of 4-nitrophenol (A), quercetin (B) and 2,4,5-trichlorophenol (C). The metabolites were monitored for both UV absorbance at 287nm (—) and radioactivity (-----).

HPLC (Fig. 12). The metabolites were monitored for their UV absorbance at 287nm with parent 4-nitrophenol, quercetin and 2,4,5-trichlorophenol eluting at 16.4 mins, 21.8 mins and 38 mins respectively. In all three cases, the more polar glucosylated reactions products eluted prior to the aglycones and were detected as both UV-absorbing and radioactive metabolites. Single peaks were identified for both glucosylated 4-nitrophenol and 2,4,5-trichlorophenol at 5.5 min and 16.7 min respectively. With quercetin as the substrate one major peak was observed at 9.5 mins although several minor radioactive peaks were also visible suggesting the possibility of the formation of multiple reaction products.

3.2 Spectrum of OGT activities in wheat

Wheat shoots and roots were screened with a broad range of natural products and xenobiotics (Table 3). Natural products included a range of hydroxybenzoic acids, coumarins, phenylpropanoids and flavonoids. Xenobiotics assayed included the pesticides chloramben, 2,4-dichlorophenoxyacetic acid, pentachlorophenol and the pesticide metabolites 4-nitrophenol and 2,4,5-trichlorophenol. OGT activities in shoot and root extracts from 10 day old light-grown and 6 day old dark-grown wheat were compared to see whether or not seedling age and illumination affected glucosylating activities toward the different substrates.

After correcting for 'endogenous' activity using methanol in place of substrate, appreciable activity was detected with phenols 4-nitrophenol and 2,4,5-trichlorophenol as well as the flavonoids quercetin, luteolin, genistein and coumestrol in all samples tested (Table 3). In light grown shoots two further

Table 3 OGT activities in extracts from wheat shoots and roots of 10 day old light and 7 day old dark grown plants. Product formation determined with solvent carrier methanol indicates the level of background conjugation of endogenous metabolites present in the protein preparation.

Substrates	OGT activity pmol product min ⁻¹ mg ⁻¹ protein			
	Light grown (10 day)		Dark grown (7 day)	
	Shoots	Roots	Shoots	Roots
Methanol	0.36 ± 0.05	0.53 ± 0.06	0.58 ± 0.02	0.74 ± 0.08
Phenol	0.47 ± 0.00	0.65 ± 0.09	0.58 ± 0.15	1.00 ± 0.03
4-Nitrophenol	1.37 ± 0.01	1.26 ± 0.00	1.62 ± 0.06	1.18 ± 0.06
2,4,5-Trichlorophenol	2.55 ± 0.14	1.83 ± 0.06	1.99 ± 0.04	1.63 ± 0.01
Benzoic acid	0.37 ± 0.00	2.19 ± 0.00	0.68 ± 0.01	0.92 ± 0.05
Salicylic acid	0.35 ± 0.03	1.44 ± 0.32	0.31 ± 0.00	0.86 ± 0.01
Umbelliferone	0.30 ± 0.02	0.97 ± 0.03	0.63 ± 0.03	0.81 ± 0.02
4-Hydroxycinnamic acid	0.24 ± 0.01	0.92 ± 0.03	0.51 ± 0.11	1.04 ± 0.11
Caffeic acid	0.32 ± 0.01	0.78 ± 0.00	0.32 ± 0.02	0.63 ± 0.06
Ferulic acid	0.34 ± 0.01	0.73 ± 0.10	0.78 ± 0.06	0.96 ± 0.11
Conferyl alcohol	0.37 ± 0.06	0.67 ± 0.06	1.09 ± 0.02	1.12 ± 0.10
Isoliquirtigenin	0.41 ± 0.04	0.73 ± 0.01	0.81 ± 0.05	1.20 ± 0.04
Naringenin	0.40 ± 0.01	0.81 ± 0.05	0.97 ± 0.10	0.99 ± 0.05
Quercetin	2.59 ± 0.40	2.27 ± 0.29	1.88 ± 0.01	2.94 ± 0.06
Luteolin	1.03 ± 0.09	1.37 ± 0.04	0.68 ± 0.01	2.30 ± 0.00
Genistein	0.85 ± 0.03	0.75 ± 0.00	0.98 ± 0.10	1.31 ± 0.10
Coumestrol	1.49 ± 0.12	1.49 ± 0.00	1.19 ± 0.01	3.25 ± 0.14
Cyanidin chloride	1.02 ± 0.09	0.77 ± 0.05	0.86 ± 0.07	1.30 ± 0.08
2,4-Dichlorophenoxyacetic acid	0.36 ± 0.01	0.55 ± 0.01	0.61 ± 0.03	0.64 ± 0.03
4-Hydroxyphenylpyruvic acid	0.37 ± 0.06	0.62 ± 0.04	0.49 ± 0.13	0.72 ± 0.19
Chloramben	0.41 ± 0.02	0.68 ± 0.08	0.74 ± 0.03	1.41 ± 0.10
Picloram	0.49 ± 0.01	0.57 ± 0.08	0.68 ± 0.03	0.82 ± 0.15

Values represent the means of duplicate determination ± range in the replicates.

flavonoids, the isoflavone genistein and the anthocyanin cyanidin chloride also gave significant levels of activity. In roots but not shoots, activity toward benzoic acid and salicylic acid was found to be high in light grown extracts. In all samples, little activity was found toward the hydroxybenzoic acids, coumarins or phenylpropanoids although activity was two-fold higher in root tissue than that determined in the respective shoot tissue. In dark-grown tissue the background conjugation rates were found to be slightly higher than determined in light grown tissue. Enhanced activity toward flavonoids was observed in extracts from roots from dark grown seedlings particularly towards the isoflavonoid coumesterol.

3.2.1 Effect of herbicide safener treatment on OGT activity

Light-grown seedlings were treated with one of four safeners which were applied, all at 10 mg L^{-1} , by imbibing the seeds prior to sowing and then watering the plants with the respective safener for 10 days. Two of the safeners used were normally applied to maize, namely dichlormid, which enhances tolerance to chloroacetanilide herbicides (Kreuz *et al.*, 1991), and the new safener isoxadifen. The other two safeners were used in wheat and were the mefenpyr-diethyl and cloquintocet mexyl. Six substrates including xenobiotics and natural products were chosen to measure the effect of herbicide safener treatment on OGT activity (Table 4).

Treatment with the two wheat safeners, mefenpyr diethyl and cloquintocet mexyl lead to a greater enhancement of activity toward all substrates, than did treatment with the maize safeners. In shoot extracts, the greatest increases in activity were observed with cloquintocet mexyl treatment, while in roots activity toward 4-nitrophenol, 2,4,5-

Table 4 Comparison of OGT activity in extracts from 10 day old light grown wheat shoots (A) and roots (B) treated with four different safeners. The background conjugation in the absence of added phenolic substrate has been corrected for in each case. Results are the means of duplicated extractions \pm range in the duplicates.

A					
OGT activity pmol product min ⁻¹ mg ⁻¹ protein					
Substrates	Control	Dichlomid	Isoxadifen	Mefenpyr diethyl	Cloquintocet mexyl
Methanolic control	0.35 \pm 0.04	0.38 \pm 0.01	0.44 \pm 0.00	0.46 \pm 0.00	0.79 \pm 0.09
4-Nitrophenol	1.02 \pm 0.00	1.31 \pm 0.13	1.30 \pm 0.10	1.59 \pm 0.01	2.44 \pm 0.03
2,4,5-Tri-chlorophenol	2.00 \pm 0.01	3.15 \pm 0.16	2.46 \pm 0.15	3.59 \pm 0.16	4.25 \pm 0.13
Quercetin	4.14 \pm 0.17	2.52 \pm 0.14	3.68 \pm 0.45	6.53 \pm 0.21	7.90 \pm 0.42
Luteolin	0.71 \pm 0.02	0.80 \pm 0.01	0.85 \pm 0.02	1.18 \pm 0.10	2.11 \pm 0.08
Genstein	0.88 \pm 0.01	1.07 \pm 0.00	1.06 \pm 0.00	1.23 \pm 0.06	2.16 \pm 0.07
Coumesterol	1.36 \pm 0.06	2.02 \pm 0.19	2.02 \pm 0.11	2.99 \pm 0.05	4.53 \pm 0.31

B					
OGT activity pmol product min ⁻¹ mg ⁻¹ protein					
Substrates	Control	Dichlomid	Isoxadifen	Mefenpyr diethyl	Cloquintocet mexyl
Methanolic control	0.65 \pm 0.13	0.82 \pm 0.02	0.85 \pm 0.02	0.88 \pm 0.00	1.08 \pm 0.04
4-Nitrophenol	1.53 \pm 0.13	1.83 \pm 0.07	1.70 \pm 0.01	3.04 \pm 0.29	2.38 \pm 0.22
2,4,5-Tri-chlorophenol	2.08 \pm 0.13	2.53 \pm 0.15	2.18 \pm 0.01	4.41 \pm 0.10	3.51 \pm 0.28
Quercetin	4.46 \pm 0.08	6.13 \pm 0.34	5.72 \pm 0.75	7.17 \pm 0.49	8.68 \pm 0.62
Luteolin	1.29 \pm 0.10	2.31 \pm 0.06	2.53 \pm 0.04	3.94 \pm 0.58	3.56 \pm 0.21
Genstein	0.71 \pm 0.01	1.21 \pm 0.01	1.38 \pm 0.07	1.41 \pm 0.01	2.22 \pm 0.15
Coumesterol	1.52 \pm 0.08	4.77 \pm 0.08	4.51 \pm 0.31	6.68 \pm 1.09	7.82 \pm 0.41

trichlorophenol and luteolin was highest following treatment with mefenpyr diethyl. Dichlormid and isoxadifen treatment produced only minor enhancement of activities toward most substrates in shoots and with quercetin actually decreased activity. In the roots the two maize safeners gave a modest enhancement of OGT activity. Activity toward coumesterol was strongly enhanced by all safeners in shoots and roots with the increase being greatest following treatment with cloquintocet mexyl > mefenpyr diethyl > dichlormid > isoxadifen.

A second set of treatments were carried out using nine different safeners used commercially to safen wheat, maize, sorghum and rice crops. In these experiments, crude extracts of wheat shoots were assayed at 6 days and 12 days after germination using the xenobiotic substrate 2,4,5-trichlorophenol and the flavonol quercetin (Fig. 13). As found previously, the highest increase in activity was found with the two wheat safeners, cloquintocet mexyl and mefenpyr diethyl. Activity toward 2,4,5-trichlorophenol was also significantly enhanced by treatment with CMP1, fenclorim, oxabetrinil and R-29148, but not by benoxacor or flurazole. With quercetin as the substrate of non-wheat safened plant extracts only CMP1 and oxabetrinil gave any significant enhancement in OGT activity.

3.2.2 Treatment of wheat seedlings with natural products and 2,4,5-trichlorophenol

Wheat seedlings were treated with 2,4,5-trichlorophenol, coumesterol and luteolin to see if xenobiotic and natural product OGT substrates could also enhance OGT

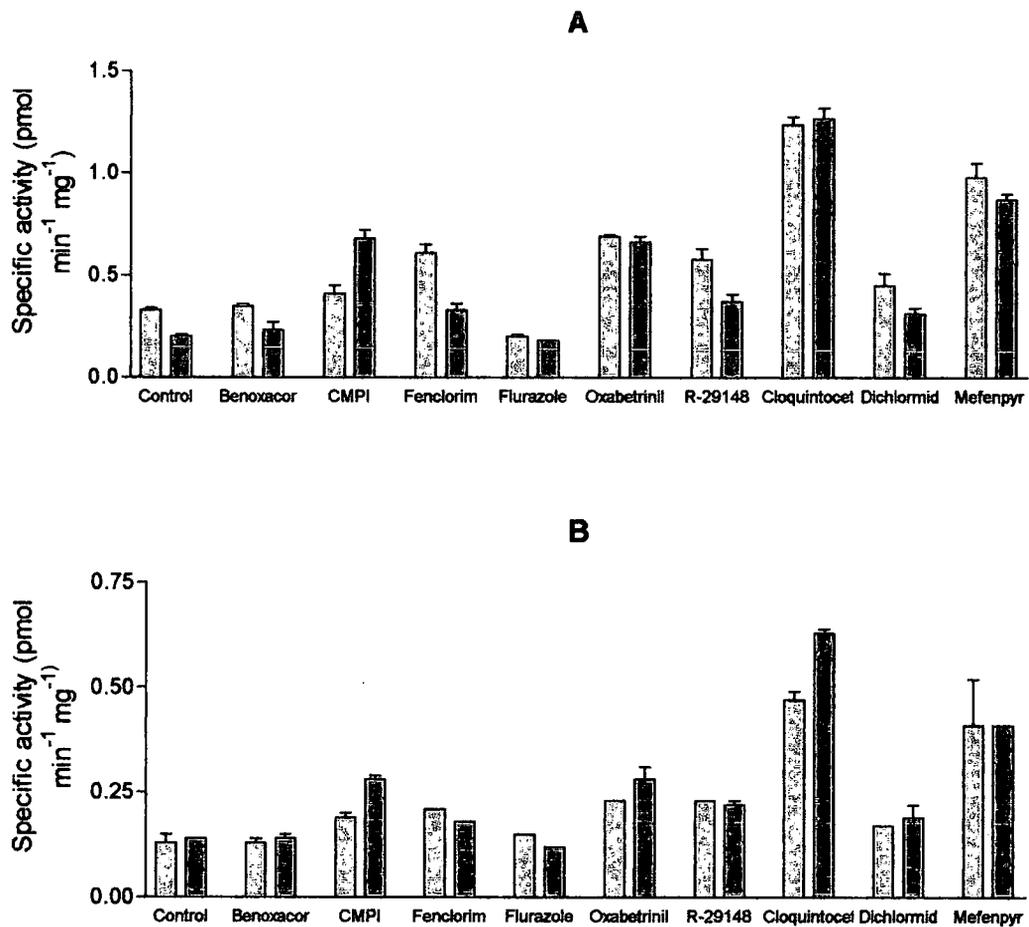


Figure 13 Comparison of OGT activity toward 2,4,5-trichlorophenol (A) and quercetin (B) in 6 day (▨) and 12 day (■) old wheat shoots treated with nine different safeners. Results represent the means of duplicate determinations with error bars showing the variation in the replicates.

activities. Wheat seeds were imbibed and then watered with 2,4,5-trichlorophenol, coumesterol or luteolin, all at 50 μ M, until the plants were 10 days old. In addition GST activity toward CDNB was also determined as a classic marker for the enhancement of detoxifying activities in wheat by chemical treatments (Edwards & Cole, 1996). The effect of these treatments on OGT and GST activities is shown in Table 5.

GST activity toward CDNB was enhanced by all treatments (Table 5). In shoots, treatment with the natural products coumesterol and luteolin increased activity more than 3-fold and 2.5-fold respectively. In roots, the enhancement of activity was more modest, with the greatest increase in GST activity seen in 2,4,5-trichlorophenol treated roots. OGT activities towards the six model substrates were measured. All treatments produced a slight increase in activity toward 2,4,5-trichlorophenol, except in roots treated with luteolin (Table 5). In the roots, treatment with 2,4,5-trichlorophenol also enhanced activity towards quercetin. However the chemical treatments had little effect or even repressed OGT activity toward luteolin or coumesterol.

3.3 Spectrum of OGT activities in herbicide-susceptible and herbicide-resistant black-grass

Only the OGT activities in the shoots of the herbicide-susceptible wildtype and the herbicide-resistant Peldon populations of black-grass were investigated.

In the wildtype black-grass, as in wheat shoots, activity toward the xenobiotics, 4-nitrophenol and 2,4,5-trichlorophenol, and toward the flavonoids quercetin, luteolin

Table 5 OGT activities and GST activity toward CDNB in extracts from shoots (A) and roots (B) of wheat seedlings treated with 2,4,5-trichlorophenol or the natural products coumestrol, or luteolin. All values are mean \pm range of replicates in duplicated determination with the background conjugation rates corrected for.

Substrates	Treatment			
	Control	2,4,5-Trichlorophenol	Coumestrol	Luteolin
OGT activity pmol product min ⁻¹ mg ⁻¹ protein				
Methanolic control	0.68 \pm 0.05	0.72 \pm 0.03	0.75 \pm 0.01	0.56 \pm 0.01
4-Nitrophenol	1.16 \pm 0.00	1.50 \pm 0.03	1.49 \pm 0.09	1.22 \pm 0.01
2,4,5-Trichlorophenol	2.71 \pm 0.05	3.51 \pm 0.08	3.78 \pm 0.09	3.51 \pm 0.36
Quercetin	4.34 \pm 0.23	3.41 \pm 0.21	3.13 \pm 0.17	1.38 \pm 0.12
Luteolin	0.94 \pm 0.01	1.02 \pm 0.05	1.11 \pm 0.10	0.98 \pm 0.19
Genistein	1.13 \pm 0.02	0.96 \pm 0.01	1.16 \pm 0.01	1.09 \pm 0.00
Coumestrol	1.99 \pm 0.01	1.61 \pm 0.02	2.12 \pm 0.07	1.66 \pm 0.00
GST activity nkat mg ⁻¹ protein				
CDNB	3.58 \pm 0.25	5.48 \pm 0.26	14.13 \pm 0.05	9.50 \pm 0.62

Substrates	Treatment			
	Control	2,4,5-Trichlorophenol	Coumestrol	Luteolin
OGT activity pmol product min ⁻¹ mg ⁻¹ protein				
Methanolic control	0.54 \pm 0.06	0.77 \pm 0.01	0.66 \pm 0.06	0.59 \pm 0.03
4-Nitrophenol	1.33 \pm 0.02	1.83 \pm 0.07	1.24 \pm 0.05	1.14 \pm 0.12
2,4,5-Trichlorophenol	1.86 \pm 0.05	2.42 \pm 0.26	2.27 \pm 0.08	1.63 \pm 0.04
Quercetin	3.27 \pm 0.17	4.54 \pm 0.45	3.62 \pm 0.16	2.98 \pm 0.13
Luteolin	0.90 \pm 0.02	1.69 \pm 0.17	1.11 \pm 0.05	0.98 \pm 0.00
Genistein	0.58 \pm 0.03	1.01 \pm 0.02	0.65 \pm 0.07	0.57 \pm 0.07
Coumestrol	1.41 \pm 0.08	2.65 \pm 0.14	1.71 \pm 0.09	1.48 \pm 0.08
GST activity nkats mg ⁻¹ protein				
CDNB	17.11 \pm 1.13	26.70 \pm 0.39	22.50 \pm 0.38	21.08 \pm 0.81

Table 6 OGT activities in shoots of 21 day old herbicide-susceptible (wildtype) and herbicide-resistant (Peldon) populations of black-grass. Control values indicate the endogenous activity without added substrate. Values represent mean \pm range in the replicates.

Substrates	OGT activity pmol product min ⁻¹ mg ⁻¹ protein	
	Wildtype	Peldon
Methanolic control	0.34 \pm 0.06	0.73 \pm 0.08
Phenol	0.45 \pm 0.00	0.87 \pm 0.03
4-Nitrophenol	0.91 \pm 0.05	1.63 \pm 0.01
2,4,5-Trichlorophenol	1.49 \pm 0.03	3.27 \pm 0.27
Benzoic acid	0.47 \pm 0.06	0.85 \pm 0.01
Salicylic acid	0.35 \pm 0.04	0.70 \pm 0.02
Umbelliferone	0.38 \pm 0.06	0.70 \pm 0.01
4-Hydroxycinnamic acid	0.39 \pm 0.06	0.66 \pm 0.01
Caffeic acid	0.35 \pm 0.01	0.67 \pm 0.01
Ferulic acid	0.50 \pm 0.01	0.68 \pm 0.05
Conferyl alcohol	0.45 \pm 0.08	0.82 \pm 0.08
Isoliquirtigenin	0.49 \pm 0.05	0.81 \pm 0.08
Naringenin	0.79 \pm 0.03	1.58 \pm 0.21
Quercetin	9.65 \pm 0.51	12.31 \pm 0.20
Luteolin	2.16 \pm 0.08	3.81 \pm 0.02
Genistein	0.45 \pm 0.03	1.76 \pm 0.12
Coumesterol	0.86 \pm 0.00	2.39 \pm 0.14
Cyanidin chloride	0.44 \pm 0.02	1.20 \pm 0.03
2,4-Dichlorophenoxyacetic acid	0.31 \pm 0.02	0.76 \pm 0.04
4-Hydroxyphenylpyruvic acid	0.28 \pm 0.04	0.72 \pm 0.06
Chloramben	0.37 \pm 0.08	0.85 \pm 0.05
Picloram	0.41 \pm 0.04	0.83 \pm 0.04

and coumesterol was determined (Table 6). The OGT activities determined in the wildtype black-grass shoots were slightly lower than those determined in wheat shoots with most substrates, except in the case of quercetin and luteolin, where conjugation rates were substantially higher. As compared with the wildtype OGT activities were considerably higher in the Peldon herbicide-resistant black-grass toward all substrates tested, with most activities being doubled.

3.3.1 Effect of safener treatment on herbicide-susceptible and herbicide-resistance populations of black-grass

Wildtype and Peldon black-grass seeds were imbibed and watered with the maize safener dichlormid and with cloquintocet mexyl which is used to safen wheat. GST activity toward CDNB was determined to establish any enhancement of activity as a result of safener treatment.

In both wildtype and Peldon shoots dichlormid gave a very slight enhancement in GST activity, while cloquintocet mexyl treatment activity resulted in a decrease in activity (Fig. 14). When OGT activities toward 4-nitrophenol, 2,4,5-trichlorophenol and flavonoid substrates were determined, little difference in activity was observable between control and safener-treated extracts (Fig. 15).

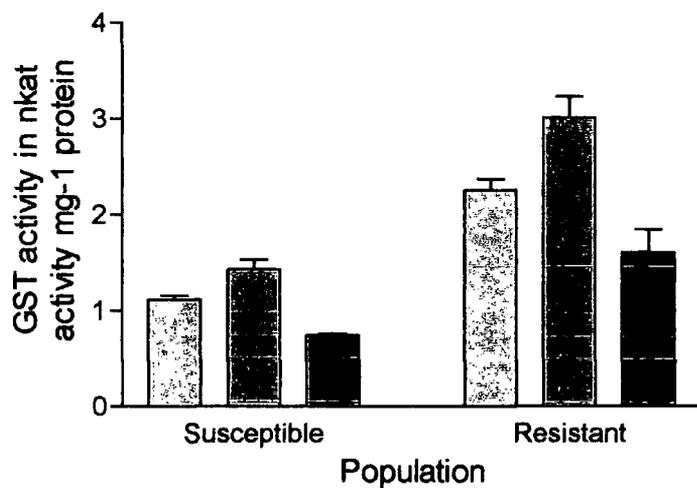


Figure 14 GST activities towards 1-chloro-2,4-dinitrobenzene (CDNB) in herbicide-susceptible (wildtype) and herbicide-resistant (Peldon) populations of black-grass. Seedlings were either untreated (□) or treated with dichlormid (▨) or cloquintocet mexyl (■). Error bars represent the range in the duplicate determinations.

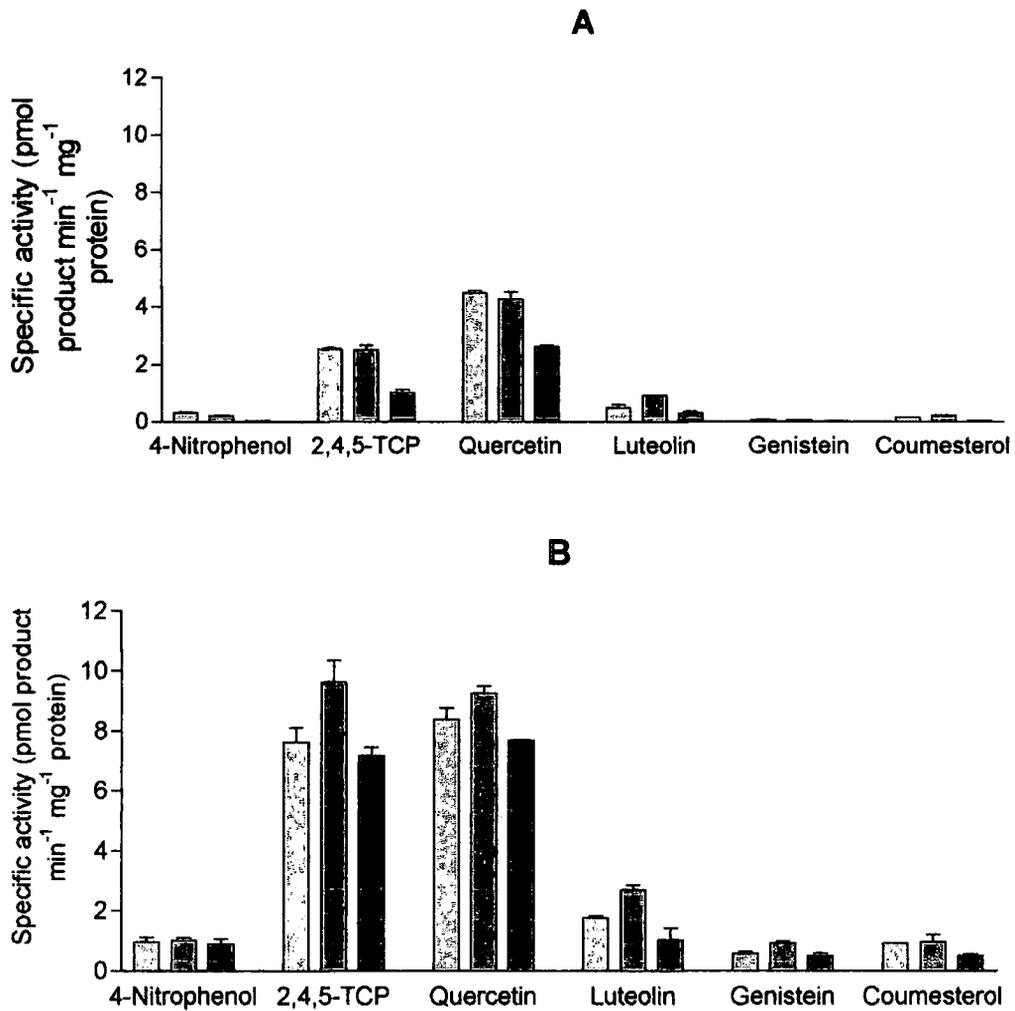


Figure 15 OGT activities in herbicide-susceptible (A) and herbicide-resistant (B) black-grass populations treated with the herbicide safeners dichlormid (▨) or cloquintocet mexyl (■) and untreated control (□). Error bars represent the variation in the replicates.

Discussion

OGT activities in wheat

In wheat, OGT activity was observed with the pesticide metabolites 2,4,5-trichlorophenol and 4-nitrophenol as reported previously (Pfumacher *et al.*, 1998; Schmitt *et al.*, 1993). OGT activities toward the wheat flavone luteolin as well as other flavonoids not found in this species were also determined. In contrast to the glucosylation of phenolic hydroxyl groups, the conjugation of carboxyl groups in wheat of benzoic acids and phenylpropanoids or the pesticides chloramben and picloram was not observed.

Treatment of wheat with cloquintocet mexyl has been found to increase the glycosylation of the herbicide clodinafop (Kreuz *et al.*, 1991). However the reasons for this increased conjugation were not determined. Our results suggest that safener-mediated increases in the conjugation of herbicides may result from enhanced activities of the conjugating OGT. Thus, treatment of wheat with the wheat safeners, cloquintocet mexyl and mefenpyr diethyl increased OGT activity towards both xenobiotic substrates and natural products. These safeners also enhanced GST activity suggesting a coordinated induction of phase II conjugating enzymes. However, although the natural products luteolin and coumesterol enhanced GST activity toward CDNB they were unable to increase OGT activity in wheat seedlings. This observation suggests that OGTs and GSTs must be under subtly different regulation by chemical inducers.

OGT Activity in black-grass

A similar range of OGT activities toward xenobiotic and natural products was observed in black-grass as seen in wheat, although activity towards quercetin and luteolin was greater in the weed. As compared to the herbicide-susceptible wild-type plants, the herbicide resistant Peldon populations contained significantly higher activities toward all substrates. This elevation of GT activities, along with the increase in activity of specific GSTs (Cummins *et al.*, 1999) and CYPs (Hyde *et al.*, 1996) provides further evidence that multiple detoxifying enzymes are up-regulated in the herbicide-resistant Peldon population. Herbicide safener treatment with dichlormid gave a slight enhancement in GST activities, as reported previously with herbicide-susceptible black-grass treated with the wheat safener fenchlorozole ethyl (Cummins *et al.*, 1999) and in addition resulted in a modest enhancement of OGT activities. However treatment with cloquintocet mexyl led to a slight decrease in both GST and OGT activities.

4. PURIFICATION OF A WHEAT O-GT WITH ACTIVITY TOWARD 2,4,5-TRICHLOROPHENOL

Introduction

Purification of glucosyltransferases involved in the conjugation of xenobiotic and natural products in wheat was carried out as part of the investigation into the relationship between conjugating activities toward naturally occurring and synthetic phenols in this species. The aim was to isolate the enzyme(s) responsible for the conjugation of the xenobiotic substrate 2,4,5-trichlorophenol, which has been used as both fungicide and bactericide and is a major metabolite of the herbicide 2,4,5-trichlorophenoxyacetic acid. Although not endogenous to wheat, quercetin was chosen as the natural product substrate as (chapter 3) had identified this flavonol as a preferred substrate for conjugation.

Results

Previously, comparison of the specific activities toward 2,4,5-trichlorophenol and quercetin in wheat shoots and roots had shown that these tissues contained high OGT activities toward these substrates (Chapter 3). As large amounts of wheat foliage could be harvested with relative ease, shoots were used as the starting material for the purification of the respective OGT activities. OGT activity was found to be stable in the form of $(\text{NH}_4)_2\text{SO}_4$ precipitates for at least six months and this method was used to store extracted protein prior to chromatography. Differential precipitation with $(\text{NH}_4)_2\text{SO}_4$ resulted in the majority of extracted OGT activity recovered between 40 to 60 % saturation. Activity was slightly enriched by this procedure with a 2-fold purification toward 2,4,5-trichlorophenol and 3-fold enhancement toward quercetin determined. When the purification was scaled up, recovery of total activity following $(\text{NH}_4)_2\text{SO}_4$ precipitation fell to 30 %, as compared with 60-80 % with the small-scale extraction. The addition of 5 mM DTT, 10 mM ascorbic acid and 10 mM sodium metabisulphite to the extraction buffer resulted in an improved 60 % recovery of activity at this stage.

O-Glucosyltransferase activity has previously been found to be inhibited by the salts used in purification procedures (Parry & Edwards, 1994). Addition of 0.5 M NaCl to the OGT assay buffer resulted in a 8-fold and a 5-fold reduction of activity toward 2,4,5-trichlorophenol and quercetin respectively. Similarly, 0.5 M $(\text{NH}_4)_2\text{SO}_4$ produced a decrease in activity of 12.5-fold toward 2,3,4-trichlorophenol and 5.5-fold toward quercetin. As part of the same experiment, MgSO_4 and MnCl_2 were added to

the basic assay buffer. The addition of 2 mM MgSO₄ stimulated activity very slightly towards both substrates. More interestingly, the inclusion of 2 mM MnCl₂ in the assay resulted a slight decrease in activity toward 2,4,5-trichlorophenol, while abolishing activity toward quercetin. Subsequent investigations revealed that this effect was probably due to the Mn⁺⁺ ions forming complexes with quercetin and hence preventing the substrate being available for glucosylation.

4.1 Effect of herbicide safener treatment on the OGT activities resolved by ion exchange chromatography

Previously, it was determined that treatment of wheat seedlings with the herbicide safener cloquintocet mexyl resulted in an increase in OGT activity of at least two-fold in shoot tissue relative to untreated controls. To examine the enhancement of OGT activity in safener treated shoots in more detail, (NH₄)₂SO₄ precipitates of untreated shoots and shoots treated with cloquintocet mexyl were desalted and equal amounts of protein applied to a DEAE sepharose anion exchange column (Fig. 16). As well as measuring the activity toward 2,4,5-trichlorophenol and quercetin, activity toward luteolin and coumesterol was also monitored. Activity towards all four substrates co-eluted in a broad peak along with the majority of protein in extracts from both untreated and cloquintocet mexyl treated shoots. Activity towards all four substrates was clearly enhanced in cloquintocet mexyl treated shoots. Chromatography using the DEAE sepharose was unable to resolve distinct OGT isoenzymes. However the broad elution of the peaks suggested the presence of multiple OGT isoenzymes, active toward 2,4,5-trichlorophenol, quercetin, luteolin and coumesterol respectively. In all

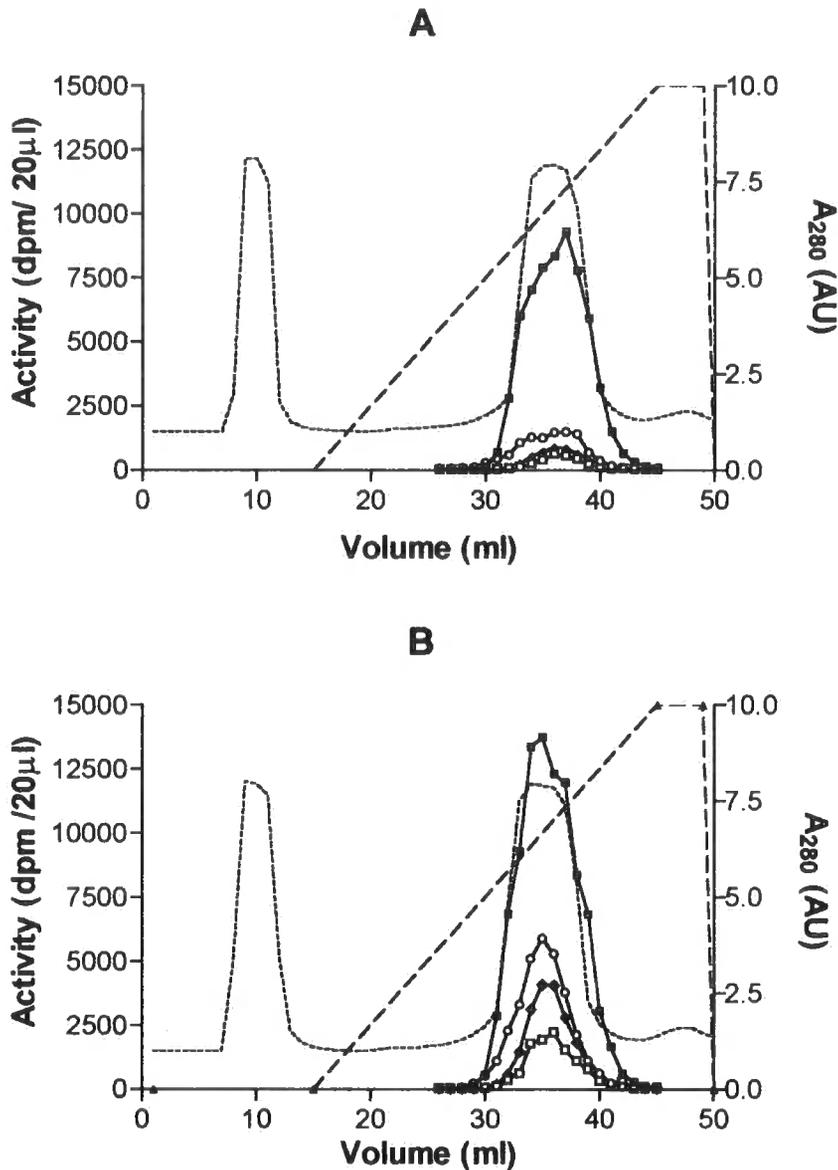


Figure 16 DEAE anion exchange chromatography of identical amounts of protein for extracts of 10 day old wheat shoots which were (A) untreated or (B) treated with cloquintocet mexyl. Proteins were eluted with a linear gradient of 0M to 400mM NaCl (----) and their absorbance monitored at 280nm as shown (.....). Fractions (1 ml) were collected and assayed for OGT activity toward 2,4,5-trichlorophenol (■), quercetin (○), coumesterol (◆) and luteolin (□). Activity is quoted by dpm of reaction product partitioned into 200 µl of water saturated ethyl acetate after assay of 20 µl of the fraction.

subsequent studies, safener treated wheat shoots were used as the starting material for OGT purification. Typically, these shoots contain $3.88 \text{ pmol product min}^{-1}\text{mg}^{-1}$ of crude protein toward 2,4,5-trichlorophenol as compared with $2.42 \text{ pmol product min}^{-1}\text{mg}^{-1}$ of crude protein in non-safener shoots.

4.2 Development of purification method

Small-scale hydrophobic interaction chromatography was carried out using 5 ml columns of phenyl-, butyl- and octyl- sepharose. The 40-60 % $(\text{NH}_4)_2\text{SO}_4$ protein precipitate dissolved in 1M $(\text{NH}_4)_2\text{SO}_4$ was applied to a phenyl sepharose column and the bound protein recovered by reducing the $(\text{NH}_4)_2\text{SO}_4$ concentration down to 0 M followed by the application of 50 % ethylene glycol to remove hydrophobic proteins. The majority of activity towards 2,4,5-trichlorophenol and quercetin co-eluted in the 50% ethylene glycol fractions suggesting that the OGTs are highly hydrophobic proteins. Butyl- and octyl- sepharose columns were also tested using step wise elutions of high salt (1 M $(\text{NH}_4)_2\text{SO}_4$), no salt and ethylene glycol. The majority (65-90 %) of both activities were eluted in the low salt fraction along with the bulk of the protein. To remove the ethylene glycol step, a second phenyl sepharose column was employed with a lower binding capacity. In this case, the majority of the activity then eluted in the low salt fraction after the bulk of the protein had eluted, removing the necessity of an ethylene glycol wash. The partially purified OGT preparation from the phenyl sepharose was then pooled and used to investigate other purification steps including gel-filtration, anion-exchange chromatography and chromatofocusing.

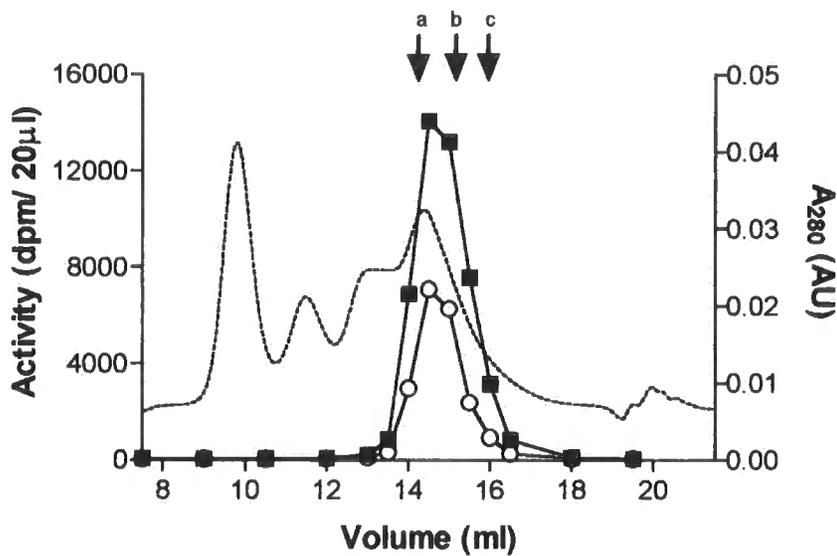


Figure 17 Gel filtration chromatography on Superdex 200 HR. A protein preparation extracted from wheat shoots treated with cloquintocet mexyl and partially purified by phenyl sepharose chromatography was applied onto a pre-calibrated superdex 200 HR column. Protein was monitored by UV absorbance at A_{280} (—) and fractions (0.5 ml) collected and 20 μ l assayed for OGT activity towards 2,4,5-trichlorophenol (■) and quercetin (○) and the reaction products partitioned into 200 μ l of ethyl acetate. The position of the calibrating protein of known molecular mass are indicated by the arrows in the upper part of the figure: (a) bovine serum albumin; 66 kDa, (b) ovalbumin; 45 kDa and (c) carbonic anhydrase; 29 kDa.

Gel filtration was used as a means of estimating the molecular weight of the OGTs responsible for the activity towards 2,4,5-trichlorophenol and quercetin. Partially purified protein was applied to a 24 ml Superdex 200 column. The OGT activities co-eluted as a single peak (Fig. 17). Calibration using bovine serum albumin, ovalbumin and carbonic anhydrase suggested an estimated molecular weight of 51.5 to 60 kDa for the OGT.

Anion-exchange chromatography was also carried out using Q Sepharose in place of DEAE Sepharose. Whereas the protein from the DEAE column was eluted in one sharp peak (Fig. 16) the UV absorbing protein from the Q sepharose column was eluted in a broader band. Activity eluted from the Q sepharose column between 200 mM and 400 mM NaCl, with only a modest enrichment (2-fold) in activity being observed.

Chromatofocusing using a Mono P column was tested. A partially purified OGT preparation was applied to the column at pH 6.0 and the activity eluted with 10 % polybuffer reducing the pH 6.0 to pH 4.0 (Fig. 20). Two separate peaks of OGT activity toward 2,4,5-trichlorophenol were observed. The first peak eluted at pH 4.8 and coincided with a peak in quercetin activity. The second peak at pH 4.6 was not associated with any quercetin activity.

A full-scale purification employing chromatofocusing as a key step was then carried out. A total of 900 g of fresh weight shoots were extracted, the protein precipitated

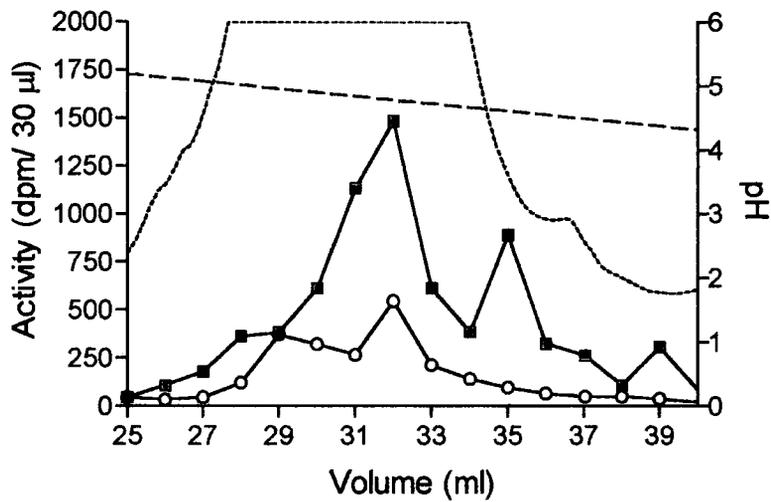


Figure 18 Chromatofocusing of wheat shoot protein, previously partially purified on a phenyl sepharose column. Protein was applied to a Mono P column starting at pH 6.0 followed by elution with 10% polybuffer 74 (pH 4.0). OGT activity was measured in dpm of reaction product produced from 30 µl of each of the 1ml fractions with 2,4,5-trichlorophenol (■) and quercetin (○) used as substrates. Protein levels were measured at 280nm (-----).

with 40 % - 60 % $(\text{NH}_4)_2\text{SO}_4$ and sequentially purified using to phenyl sepharose, Q sepharose, gel filtration and Mono P columns. In the final Mono P step only 6.9 mg of protein was applied, and it was necessary to add 50 μg of BSA to each assay reaction to ensure the accurate determination of OGT activity due to the low concentration of protein in the collected fractions. Chromatofocussing resolved two peaks of activity toward 2,4,5-trichlorophenol. The first OGT peak was enriched for activity toward 2,4,5-trichlorophenol by 120-fold and 60-fold toward quercetin. The second peak with activity toward 2,4,5-trichlorophenol was purified 52-fold. Fractions were lyophilised and subjected to SDS-PAGE. Silver staining could not detect polypeptides in any of the OGT active fractions. As it appeared that the presence of the ampholytes in the polybuffer was causing the problem, a second hydrophobic interaction step was tried after the Mono P using a 1 ml phenyl sepharose column. However due to the low concentration of applied protein very poor recovery of active enzyme was obtained.

As the aim of the purification was to obtain a purified OGT polypeptide that could be identified on a SDS-PAGE gel other chromatographies were investigated to replace the final Mono P step. A hydroxyapatite column failed to bind the majority of applied wheat protein including the OGT activity under any conditions tested. However a second anion-exchange step using a 1 ml Mono Q column was found to be suitable for a large-scale purification.

4.3 Large scale purification of OGTs

A scheme for the large-scale purification of OGTs was devised using four chromatographic steps (Fig. 19).

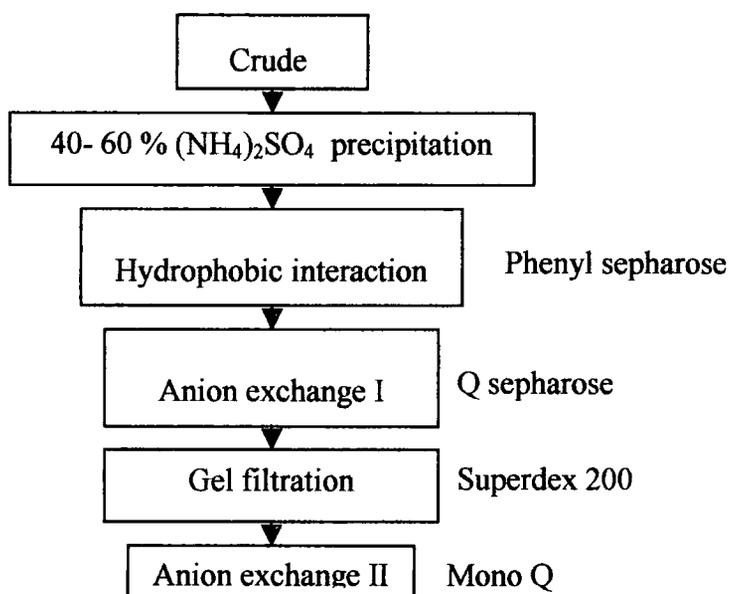


Figure 19 Scheme of large scale purification of OGTs.

After extraction and precipitation with 40-60 % (NH₄)₂SO₄ the protein was applied to the phenyl sepharose column (Fig. 20A) followed by the first anion-exchange step (Fig. 20B). As with the DEAE sepharose chromatography (Fig. 16) a broad peak of activity for each substrate could be discerned. Protein from the active fractions was concentrated by precipitation with (NH₄)₂SO₄ prior to gel filtration. As an anion-exchange column was to be used in the final step the buffer was exchanged for 0.2 M Tris-HCl, pH 7.5 during gel filtration. The two activities assayed co-eluted in a single peak (Fig. 20C) with activity toward 2,4,5-trichlorophenol enriched by 3.8-fold

compared, to 1.3-fold for quercetin relative to the specific activities of the protein applied. The second anion-exchange step employed a shallow gradient of 80 mM to 157 mM NaCl over a total volume of 37 ml (Fig. 20D). The main peak of activity toward both 2,4,5-trichlorophenol and quercetin eluted with ~ 125 mM NaCl. In contrast to the gel filtration step, the greatest fold purification was seen with quercetin as substrate with the specific activity increasing by 38-fold over the previous step. Overall the final purification of the OGT provided very similar levels of enrichment toward the two activities with a 215-fold increase in activity toward 2,4,5-trichlorophenol and a 249-fold toward quercetin (Table 7). The purified fractions were found to be labile. After the addition of 10 % (v/v) glycerol the enzyme preparation was stored at -20°C for 16 hr. The sample was then re-assayed with a 40 % drop in activity towards both 2,4,5-trichlorophenol and quercetin observed.



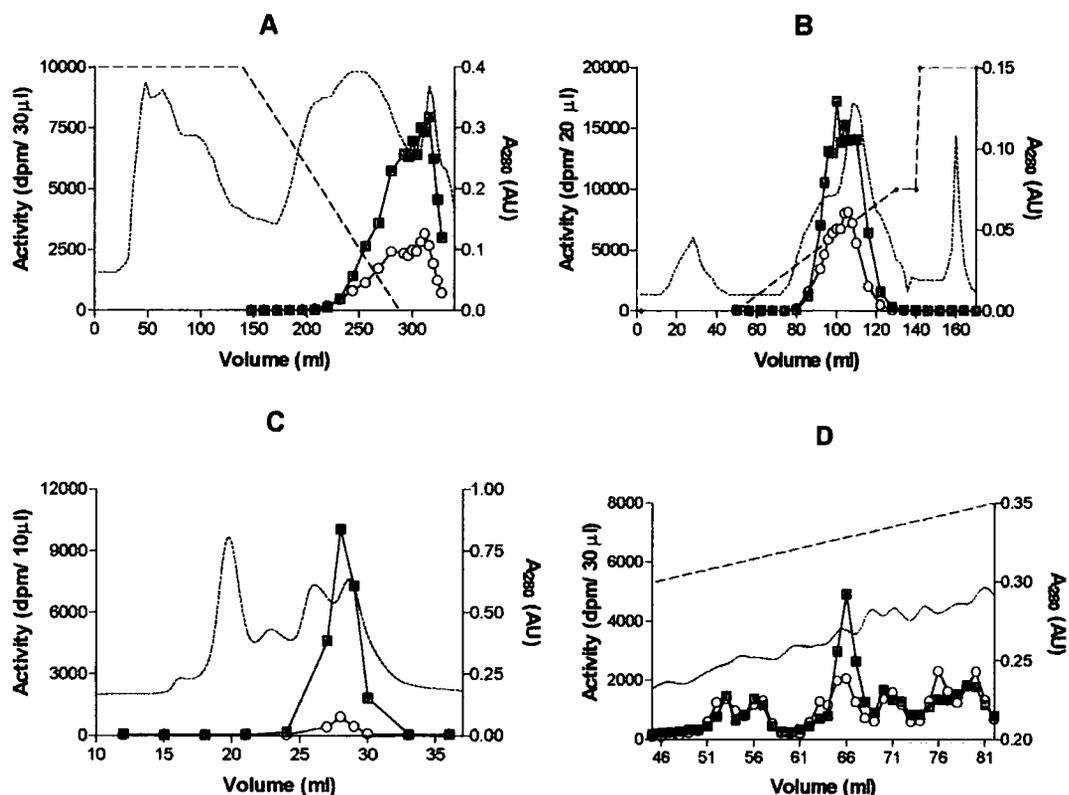


Figure 20 Sequential purification of OGTs by (A) Hydrophobic interaction chromatography on phenyl sepharose column eluted with a decreasing linear gradient of 1 M to 0 M (NH₄)₂SO₄ (----); (B) Anion-exchange chromatography on Q sepharose eluted with a linear gradient of 0 M to 0.5 M NaCl (----); (C) Gel filtration on a Superdex 200 column; (D) Anion-exchange on Mono Q column eluted with a linear gradient of 80 mM to 157 mM NaCl. OGT activity determined using with 2,4,5-trichlorophenol (■) and quercetin (○) as substrates as dpm of reaction product produced. Protein content was monitored using UV absorbance (A₂₈₀) as shown (.....). Fractions equivalent to volumes 296 ml to 324 ml for A, 96 ml to 110 ml for B, 27 ml to 29 ml for C and 65 ml to 67ml for D were combined after each chromatography step.

Table 7 Purification of an *O*-glucosyltransferase with activity toward 2,4,5-trichlorophenol and quercetin.

Purification step	Substrates	Total protein (mg)	Total activity (pmol product min ⁻¹)	Specific activity (pmol product min ⁻¹ mg ⁻¹ protein)	Purification (n-fold)	Recovery (%)
Crude	2,4,5-Trichlorophenol	4610	30725	6.7	1	100
	Quercetin		12647	2.7	1	100
40-60% (NH ₄) ₂ SO ₄	2,4,5-Trichlorophenol	2428	20578	8.5	1.3	67
	Quercetin		6973	2.9	1.1	55
Phenyl sepharose	2,4,5-Trichlorophenol	168	4398	26	3.9	14.3
	Quercetin		1232	7	2.7	9.7
Q sepharose	2,4,5-Trichlorophenol	27	1396	53	7.9	4.5
	Quercetin		382	14	5.3	3.0
Superdex 200	2,4,5-Trichlorophenol	3.7	743	204	30.5	2.4
	Quercetin		64	18	6.4	0.5
Mono Q	2,4,5-Trichlorophenol	0.04	52	1432	215	0.2
	Quercetin		26	716	249	0.2

4.3.1 SDS-PAGE analysis

Fractions from the Mono Q step were analysed by SDS-PAGE (Fig. 21). The purified OGT was not homogeneous in the three fractions (65-67) associated with the OGT activity. However one polypeptide was observed on three separate purification attempts whose abundance mirrored changes in the respective OGT activities. This polypeptide had an estimated molecular weight of 53 kDa, which was in agreement with the gel filtration data, suggesting that the purified OGT was a monomer.

As many contaminating polypeptides were also present, the OGT preparation was further resolved using mini-format two-dimensional gel electrophoresis. The first dimension employed an IEF gel (pH 4.0 to pH 7.0) and the second dimension a 12 % SDS-polyacrylamide gel. The purified OGT (Fig. 22; indicated by arrow) had a *pI* of pH 5.0 along with five other proteins between 40 and 60 kDa. The *pI* is similar to the pH (pH 4.8) associated with the elution of the OGT using chromatofocusing. In addition, one 55 kDa protein was observed which was considerably more basic than the rest with *pI* of pH 6.0 along with three polypeptides of *pI* 5.5 with masses between 25 and 30 kDa. As gel filtration was used in the purification procedure the smaller proteins present in this final preparation must have formed dimers under native conditions. A different silver staining technique was used to stain the 2-D gel to allow MALDI-TOF analysis of the polypeptides. This may explain why the putative OGT spot on the 2-D gel is less intensely stained than on the original 1-D gels.

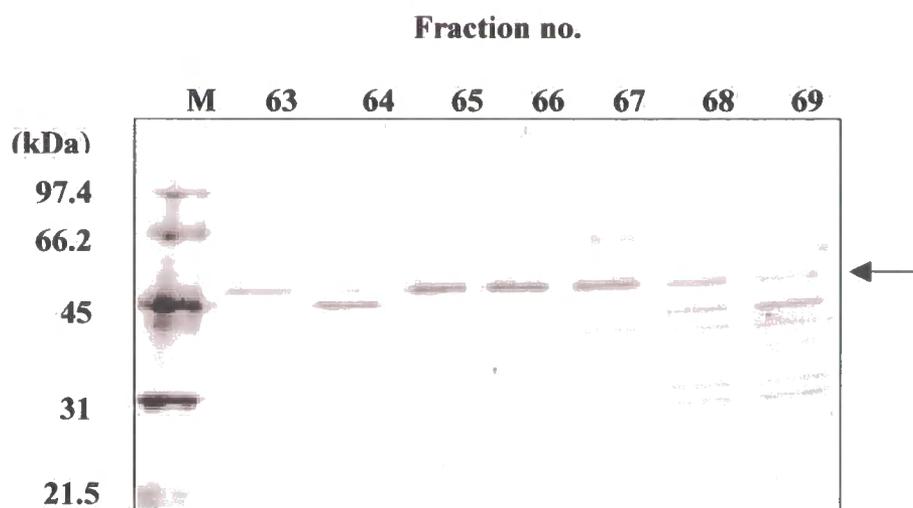


Figure 21 Silver stained SDS-PAGE of fractions from OGT purification. Lanes correspond to fractions from the final Mono Q chromatography with fractions 65, 66 and 67 containing the active OGT (see Fig. 20D).

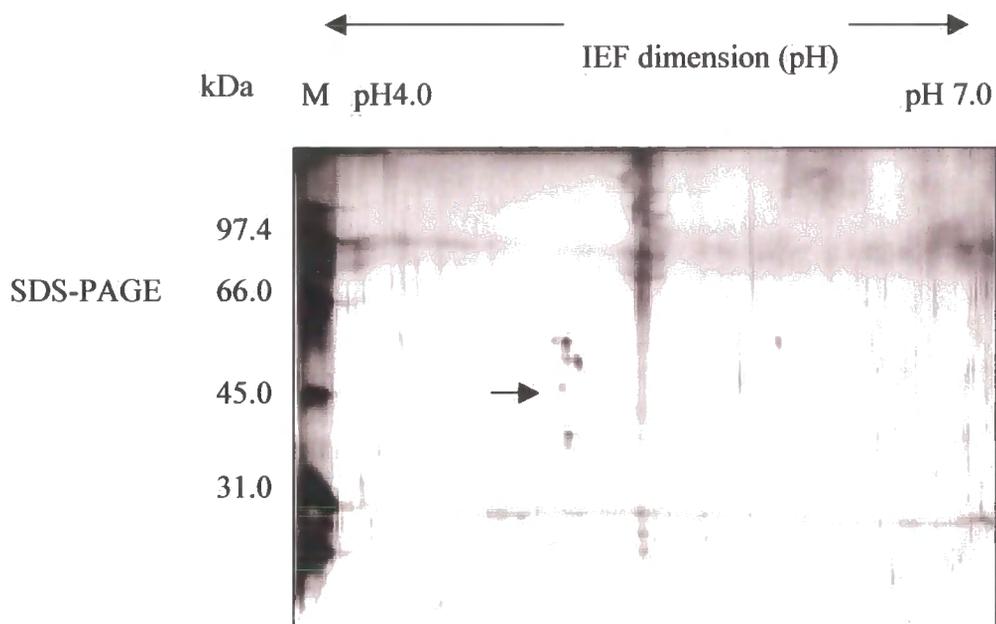
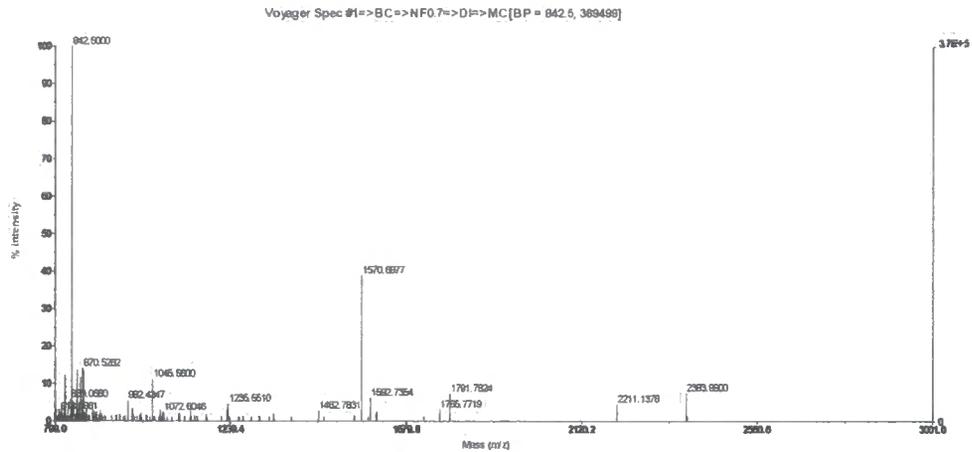


Figure 22 Silver stained mini format two-dimensional gel electrophoresis resolved purified OGT (see arrow). Purified protein from mono Q chromatography was run on a 7 cm IEF gel pH 4.0 - 7.0 (left to right) and then subjected to electrophoresis on a 12 % SDS-PAGE gel. Molecular weight markers (lane M) were run on the SDS-PAGE dimension and the gel silver stained.

A



B

Mass ions (Da)				
825.0812	870.5262	994.1469	1232.6186	1608.5897
839.068	899.5593	1016.5599	1235.551	1765.7719
855.0408	903.4099	1065.5496	1349.657	1791.7824
856.5166	905.5185	1072.6046	1462.7831	2211.1376
864.4801	913.5446	1111.6353	1570.6977	2383.99
868.517	982.4347	1140.801	1592.7354	

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839.068	899.5593	1016.5599	1235.551	1765.7719
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856.5166	905.5185	1072.6046	1462.7831	2211.1376
864.4801	913.5446	1111.6353	1570.6977	2383.99
868.517	982.4347	1140.801	1592.7354	

Figure 23 MALDI-TOF analysis of purified OGT from 2-D gel. (A) The mass spectrum of the tryptic digests of the putative OGT and (B) the summary of resolved mass ions.

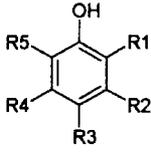
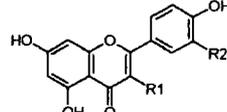
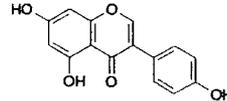
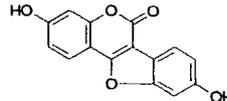
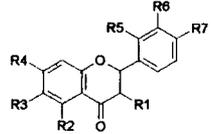
The putative OGT polypeptide was subjected to MALDI-TOF analysis using the Durham proteomics facility (Dr W. Simon), after digesting the excised spot from the 2-D gel with trypsin. A total of 29 mass ions of low intensity were obtained (Fig. 23).

The putative OGT polypeptide was subjected to MALDI-TOF analysis using the Durham proteomics facility (Dr W. Simon), after digesting the excised spot from the 2-D gel with trypsin. A total of 29 mass ions of low intensity were obtained (Fig. 23). The peptide mass ions were analysed using the program MASCOT (www.matrixscience.com). *Viridiplantae* (green plants), and the NCBI proteome databases for *Arabidopsis thaliana* and rice were searched. All of the sequences retrieved were below a meaningful probability score for identification of a single coding sequence. The mass ions were also analysed using the EST databases (prospector.UCSF.com) of wheat, *Arabidopsis* and rice and again the retrieved sequences were all below the probability score.

4.3.2. Spectrum of activities and kinetic analysis of the purified OGT

The purified OGT was tested for activity toward xenobiotic and natural product substrates (Table 8). 2,4,5-Trichlorophenol was found to be the optimal substrate tested for the OGT with 2.4-fold higher activity than with quercetin when assayed under standard conditions. Several other chlorinated phenols were tested with 2,3,6-trichlorophenol used as substrate, while 2,4,6-trichlorophenol and pentachlorophenol (PCP) were not glucosylated. Activity was also observed towards 4-nitrophenol, although activity toward 2,4-dinitrophenol was very low. Several of the flavonoid compounds that were previously found to be glucosylated in crude wheat shoots were also tested for activity. Activity was highest towards quercetin, closely followed by coumestrol. Only low levels of activity were observed toward luteolin, apigenin and the isoflavone genistein. The purified OGT was tested for activity toward a range of mono and di-substituted hydroxyflavones to establish the site of glucosylation. The

Table 8 Spectrum of xenobiotic and natural product substrates accepted by the purified OGT and their chemical structures.

Structure	Substrate						Specific activity *		
		R1	R2	R3	R4	R5			
	Xenobiotic								
	2,4,5-Trichlorophenol	-Cl	-H	-Cl	-Cl	-H	1199 ± 64		
	2,3,6-Trichlorophenol	-Cl	-Cl	-H	-H	-Cl	113 ± 0		
	2,4,6-Trichlorophenol	-Cl	-H	-Cl	-H	-Cl	59 ± 0		
	Pentachlorophenol	-Cl	-Cl	-Cl	-Cl	-Cl	46 ± 3		
	4-Nitrophenol	-H	-H	-NO ₂	-H	-H	274 ± 6		
	2,4-Dinitrophenol	-NO ₂	-H	-NO ₂	-H	-H	29 ± 1		
	Phenol	-H	-H	-H	-H	-H	29 ± 2		
		R1		R2					
	Flavonoid								
	Quercetin	-OH		-OH			399 ± 10		
	Luteolin	-H		-OH			135 ± 1		
	Apigenin	-H		-H			105 ± 3		
	Isoflavonoid						89 ± 6		
	Genistein								
	Coumestan						389 ± 14		
	Coumesterol								
		R1	R2	R3	R4	R5	R6	R7	
	Mono & di substituted hydroxyflavone								
	3-Hydroxyflavone	-OH	-H	-H	-H	-H	-H	-H	509 ± 41
	5-Hydroxyflavone	-H	-OH	-H	-H	-H	-H	-H	33 ± 3
	6-Hydroxyflavone	-H	-H	-OH	-H	-H	-H	-H	287 ± 15
	7-Hydroxyflavone	-H	-H	-H	-OH	-H	-H	-H	58 ± 5
	2'-Hydroxyflavone	-H	-H	-H	-H	-OH	-H	-H	183 ± 14
	4'-Hydroxyflavone	-H	-H	-H	-H	-H	-H	-OH	48 ± 1
3',4'-Dihydroxyflavone	-H	-H	-H	-H	-H	-OH	-OH	99 ± 9	

* Mean specific activity ± variation in duplicates was measured as pmol product produced min⁻¹ mg⁻¹ protein.

highest level of activity was observed toward 3-hydroxyflavone (ie: a flavonol) with some activity towards the 6-hydroxyflavone and the 2'-hydroxyflavone. It was postulated that the 3-hydroxyflavone was a better substrate than quercetin due to reduction in steric hindrance by other hydroxy-substituents.

The pH optimum of the purified OGT towards 2,4,5-trichlorophenol and quercetin was established. Activity was determined between pH 6.3 - 7.2 in Bis-Tris-HCl and from pH 7.2 - 9.0 in Tris-HCl. Activity towards 2,4,5-trichlorophenol and quercetin was optimal between pH 7.0 and pH 7.5 (Fig. 24). Activity was significantly higher using the Bis-Tris-HCl buffer.

Kinetic constants with 2,4,5-trichlorophenol and quercetin as substrates were determined. As the purified OGT was only available in small quantities, the apparent K_M was measured using protein from the $(NH_4)_2SO_4$ precipitation step (Fig. 25 & 26). The apparent K_M toward 2,4,5-trichlorophenol was 12.4 μM and 10.6 μM toward UDPG and with quercetin 10.1 μM and 61.8 μM for UDPG. However the purified OGT was used to determine catalytic efficiency. The K_M values obtained with the crude enzyme were used to calculate a K_{cat}/K_M for 2,4,5-trichlorophenol of 3900 $M^{-1}.s^{-1}$ and 2180 $M^{-1}.s^{-1}$ with quercetin as substrate.

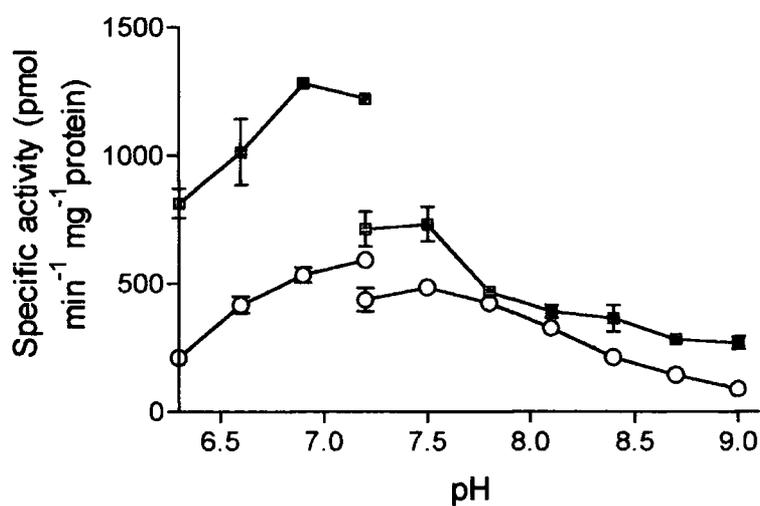
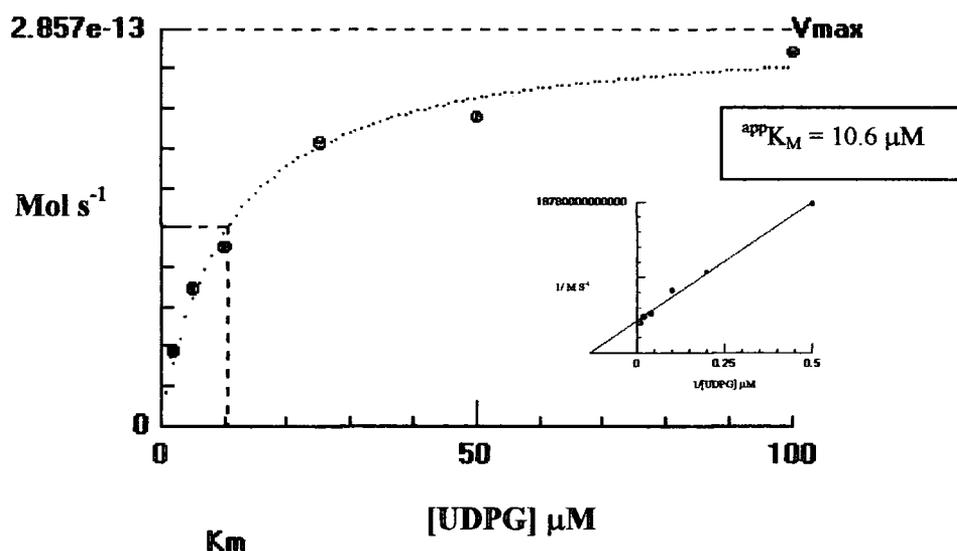


Figure 24 Determination of optimal pH of purified OGT toward the substrates 2,4,5-trichlorophenol (■) and quercetin (○). Buffer from pH 6.3 to pH 7.2 was 0.2 M Bis-Tris-HCl and from pH 7.2 to pH 9.0 was 0.2 M Tris-HCl.

A



B

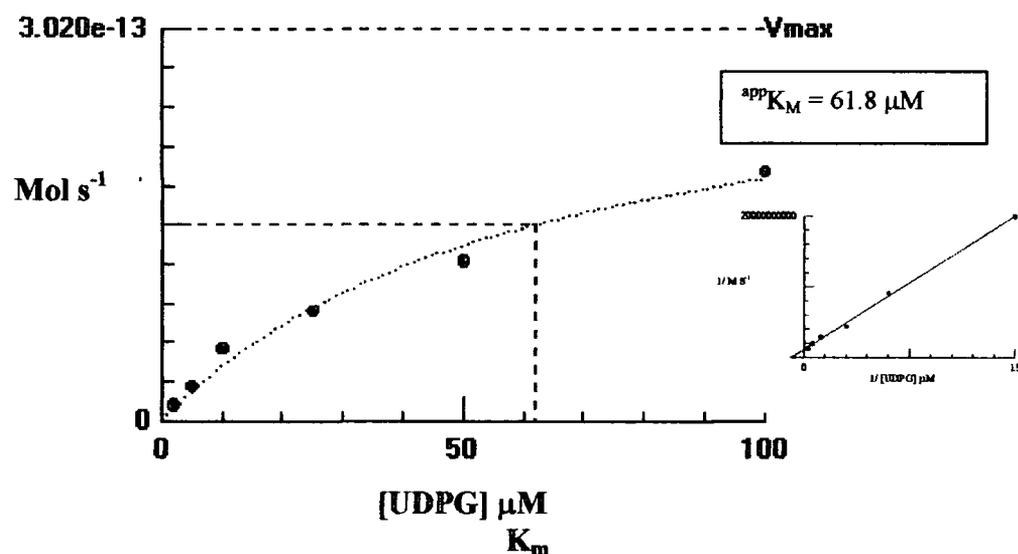
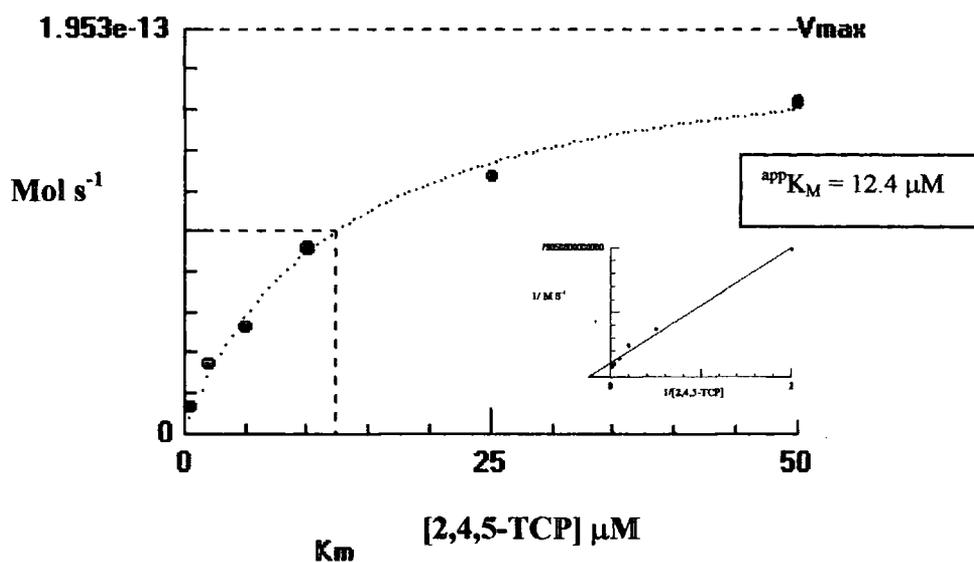


Figure 25 The effect of varying UDPG concentration on the activity of the crude OGT toward (A) 2,4,5-trichlorophenol (66 μM) and (B) quercetin (66 μM). The inserts show the respective Lineweaver-Burk plots.

A



B

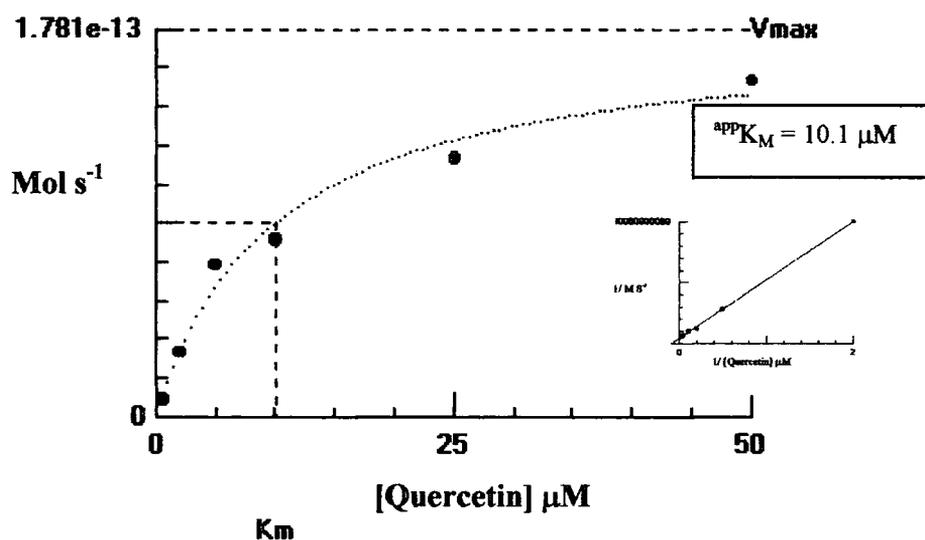


Figure 26 The effect of substrate concentration on the activity of the purified OGT. The substrates were (A) 2,4,5-trichlorophenol and (B) quercetin and in both cases the concentration of UGPG was $150 \mu\text{M}$. The inserts show their respective Lineweaver-Bulk plots.

Discussion

The purification of the OGT from wheat shoots resulted in an overall enrichment of activity of 215-fold and 249-fold toward 2,4,5-trichlorophenol and quercetin respectively. Similar purification has been obtained for other glucosyltransferases utilizing similar chromatography techniques, though greater levels of purification has been obtained using affinity chromatography. Reactive yellow 3 was used to purify a betanidin 6-GT 2700-fold from *Dorotheanthus bellidiformis* cell cultures (Vogt *et al.*, 1997). However a more modest enrichment of activity by several 100-fold was enabled the identification of purified plant glucosyltransferase by SDS-PAGE in other studies (Arend *et al.*, 2000; Stapleton *et al.*, 1991; Leah *et al.*, 1994).

Hydrophobic interaction chromatography has been utilised as a purification step with a number of glucosyltransferases. As with this study, the OGT activity has either eluted in the presence of low salt or ethylene glycol indicating that these enzymes are hydrophobic (Wetzel & Sandermann, 1994; Parry & Edwards, 1994; Rasmusen & Rudolph, 1997). Interestingly, (Sanderman *et al.*, 1991) found that a soybean NGT with activity toward 3,4-dichloroaniline was much less hydrophobic than an OGT with activity toward pentachlorophenol allowing the separation of the two activities.

A combination of gel filtration and SDS-PAGE analysis revealed that the purified OGT was a monomeric ~ 53 kDa enzyme. The molecular weights of purified glucosyltransferases have ranged from 38 kDa for a solanidine glucosyltransferase (Stapleton *et al.*, 1991) to 59 kDa for the betanidin 5-GT from *Dotheanthus bellidiformis* cell cultures (Vogt *et al.*, 1997). The glucosyltransferases purified from wheat cell cultures with activity toward pentachlorophenol had a molecular mass of

43 kDa (Wetzel & Sandermann, 1994) and two OGTs active toward cyclic hydroxamic acids isolated from wheat seedlings had Mr 47-49 kDa and 47 kDa respectively (Sue *et al.*, 2000).

The isoelectric point of the purified OGT (*pI* 4.8) was found to be similar to other glucosyltransferases exhibiting activity towards xenobiotic substrates. In soybean glucosyltransferases with activity toward DDA and PCP had *pI*s of pH 4.9 and pH 4.7-4.9 respectively (Wetzel & Sandermann, 1994; Sanderman *et al.*, 1991). It is possible that more than one was in the final mono Q fractions. As *Triticum aestivum* is hexaploid, derived from three genomes, it would not be surprising to observe multiple activities toward the same substrate, derived from multiple isogenes.

The spectrum of activities observed in the purified OGT are similar to an *O*-glucosyltransferase purified 1005-fold towards pentachlorophenol (PCP) from soybean cell cultures (Sandermann *et al.*, 1991). When assayed with UDP-[¹⁴C-glucose] using a similar method as in this study the optimal substrate was found to be 2,4,5-trichlorophenol for the soybean OGT.

Very little activity was observed toward PCP and 2,4,6-trichlorophenol in the wheat OGT fractions from this purification and of the trichlorophenols tested only 2,4,6-trichlorophenol was glucosylated by the soybean OGT to a significant level. During a subsequent purification of an OGT with activity towards the DDT metabolite DDA from the same soybean cell suspension cultures, the purification of the PCP OGT was also monitored (Wetzel & Sandermann, 1994). While the activity toward PCP could be resolved from the activity toward DDA, the PCP conjugating activity co-purified at

each step with the activity toward quercetin. Activity toward quercetin in the final partially purified soybean OGT preparation was approximately 20 times higher than for PCP. Quercetin conjugating activity was also associated with an OGT active toward 6-hydroxybentazone which had been purified from a cultivar of soybean tolerant to the parent herbicide bentazone (Leah et al., 1992). The K_M for 6-hydroxybentazone was found to be approximately 20 times higher than toward kaempferol, which was the optimal flavonol substrate for this enzyme. In wheat, the apparent K_M for 2,4,5-trichlorophenol and quercetin was found to be very similar, 12.4 μM and 10.1 μM respectively. However as quercetin is not endogenous to wheat it might be anticipated that the 'true' flavonol substrate would have a much lower K_M . From this review of the literature we can conclude that OGT activity toward quercetin has been associated with three separate OGT activities toward xenobiotic substrates. This suggests that OGTs with primary roles in flavonol metabolism can be used by different plants to detoxify synthetic phenolic compounds.

5. ISOLATION OF cDNAs ENCODING PUTATIVE GTs FROM WHEAT AND BLACK-GRASS

Introduction

The first cDNA sequence of a GT involved in plant secondary metabolism to be cloned was the *Bronze-1* gene from maize. A molecular approach using transposon tagging resulted in the isolation of the *Bronze-1* locus and the identification of a putative flavonol 3-*O*-GT (Fedoroff et al., 1984). Subsequently, many cDNAs encoding flavonoid GTs have been isolated from a range of plant species based on their homology to the maize *Bronze-1* gene. A 3-*O*-GT was isolated from an *Antirrhinum majus* cDNA library using the *Bronze-1* gene as a probe (Martin et al., 1991). The *Antirrhinum* 3-*O*-GT was then in turn used as a probe to isolate another 3-*O*-GT from *Gentiana triflora*, a baicalein 7-*O*-GT from *Scutellaria baicalensis* and six putative GTs from cassava (Tanaka et al., 1996; Hirotsani et al., 2000; Hughes & Hughes, 1994).

Isolation of the six putative GTs from cassava resulted in the identification of a characteristic sequence motif, the PSPG box, in glucosyltransferases involved in secondary product metabolism (Hughes & Hughes, 1994). DNA probes designed around this motif subsequently facilitated the isolation of full length GT sequences that encoded enzymes with activity toward non-flavonoid substrates. In *Brassica napis* a degenerate sense primer directed to the 'PSPG box' was used with 3' RACE techniques to isolate five sequences of 400-700 nucleotides long (Milkowski et al.,

2000b). These partial cDNAs were then used as probes to identify the full length sequences following screening of a cDNA library, resulted in the cloning of a UDP-glucose:sinapate GT (SGT). A similar approach was used by (Xu *et al.*, 2002) in the isolation of a GT with activity toward abscisic acid (ABA) from Adzuki bean (*Vigna angularis*) seedlings. Two degenerate antisense primers derived from the 'PSPG box' were used to produce partial PCR products and a combination of 5' and 3' RACE then utilised to obtain the full length sequence.

To date there have been no reports of the isolation and characterisation of any GT sequences from wheat or black-grass. A strategy for the cloning of full-length GT sequences from wheat and black-grass was devised utilising the 'PSPG box'. Degenerate primers were designed to the 'PSPG box' by Dr D. P. Dixon and PCR used to generate partial cDNAs. The partial cDNAs were then used as probes in the screening of wheat and black-grass cDNA libraries to obtain full-length putative GT sequences.

Results and discussion

5.1 Cloning of putative GTs from wheat and black-grass

To facilitate the design of degenerate primers the amino acid sequences of 63 glycosyltransferase sequences were aligned at the 'PSPG box' (Fig. 33). Four amino acids were found to be identical between all 63 sequences and a further two were 100 % conserved. Conservation of amino acid residues between amino acid 16 and 24 of the 'PSPG box' was found to be 80 %, or greater, between the sequences. Two degenerate primers were required to accommodate the most frequently reported eight residues between amino acid 16 and 24 (Fig. 27).

e.g

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 primer GT-1A was designed for PCR sequences so as to contain the nucleotide bases encoding for threonine (i.e. ACT, ACC, ACA and ACG) and serine (i.e. TCT, TCC, TCA and TCG). To accommodate the degeneracy of the genetic code for serine (i.e. AGT and AGC) a second primer, GT-1B was also designed (Table 9).

5.1.1 Cloning of a full length putative GT from wheat

Firstly, a cDNA library prepared from wheat shoots treated with the herbicide safener fenchlorozole ethyl (Thom et al., 2002) was subjected to mass excision. The degenerate primers were then used for PCR amplification using the mass excised

Table 9 Sequences of oligonucleotides used in the cloning of putative GTs from wheat and black-grass.

Name of oligonucleotide	Description		Direction	Sequence
	Experiment	GT clone		
GT-1A	3' RACE	Partial GTs	Degenerate	5' - TTY XTX WCX CAY TGY GGT GGA A -3'
GT-1B	3' RACE		Degenerate	5' - TTY XTX AGY CAY TGY GGT GGA A -3'
WB1	5' RACE	7aGT	Sense	5' - TTG CGG CCC TAC AAA GTA GG -3'
WB2	5' RACE		Sense	5' - CAG CTT GCA TGG AAT GAA CC -3'
WB3	5' RACE		Antisense	5' - CTC GAC GGA ATC CCT GAT GC -3'
WB4	Full length		Antisense	5' - GCG CGC CCA TGG CTT CTT CTA CCA CTA CCA CTA GCA ACG -3'
WB7	Full length		Antisense	5' - CGC GCG CTC GAG GTT CGC CCC CTC CTT TGC -3'
BGA1	5' RACE	AmGT	Sense	5' - GAC CTC ACC ATC GCA TCT CAG CTC C -3'
BGA2	5' RACE		Sense	5' - AGT TGG GTA GGG AAC CCT GGG ATC G-3'
BGA3	5' RACE		Sense	5' - CAG AAG CCG ATA AAG GAG AGC CTG G -3'
BGA4	5' RACE		Antisense	5' - CGA GAA CCT CGA CAT GAT CCA GTC C -3'
BGA5	Full length		Sense	5' - GCG CGC CAT ATG ATC TTC GGC GGC AAC -3'
BGA6	Full length		Antisense	5' - GCG CGC CTC GAG GCC GCA TGC GTT TGC C -3'
Og2	Oligo dT	General	Antisense	5' - GAG AGA GGA TCC TCG AGT TTT TTT TTT TTT T -3'
Adaptor	5' RACE			5' - CTT ATA CCG ATA TCC TGG CAA TTC GGA CTT -3'
Adaptor dT	5' RACE			5' - CTT ATA CCG ATA TCC TGG CAA TTC GGA CTT TTT TTT TTT TTT TTT D -3'
T7	Sequencing			5' - TAA TAC GAC TCA CTA TAG GG -3'
T3	Sequencing			5' - AAT TAA CCC TCA CTA AAG GG -3'
SP6	Sequencing			5' - CGT ATT TAG GTG ACA GTA TAG -3'
M13 forward	Sequencing			5' - CGT ATT TAG GTG AGA CTA TAG -3'

X = inosine W = A or T Y = C or T D = A or G or T

cDNA library as template to ensure that any partial clones generated would be represented in the library. PCR was carried out using both degenerate primers (GT-1A and GT-1B) together with the oligo dT antisense primer Og2 (Table 9). At least four products of between 500 bp and 750 bp were obtained. The products were cloned and analysed by restriction digest. Six of the resulting clones were sequenced and four of these clones (W100.5, W100.6, W100.7 and W100.10) were each found to have unique nucleotide sequences in the putative open reading frame (Fig. 28). Partial cDNAs W100.5 and W100.6 were 88 % identical at the amino acid level, with an overall identity of only 31 % to 33 % determined between the four partial cDNAs. Searches of the predicted amino acid sequence using Blast P (Altschul et al., 1990) revealed that all four partial sequences were most similar to putative GTs from rice (*Oryza sativa*).

To create probes to use in the screening of the cDNA library, all four partial cDNA were labelled with digoxigenin (DIG). As partial cDNAs W100.5 and W100.6 were very similar to one another, the two probes were used in combination. Each probe or probe mixture was screened against 150,000 plaque-forming units (pfu). The primary screen resulted in the identification of 50 positive plaques, 26 positive plaques derived from screening with the W100.5/W100.6 probe mixture, 3 positive plaques from the W100.7 probe and 21 positive plaques from the W100.10 probe. All of the positive plaques were isolated and then cDNAs excised. Analysis by restriction digest suggested the 50 positive clones consisted of two different sequences derived from

```

W100.5 _____ TTTGTGTCGCATTGCGGGTGGAACTCGACGCTGGAGGGCATCACGGCAGGGT-TGCCGCT
W100.6 _____ TTTGTGTCGCATTGTGGGTGGAACTCGACACTGGAGGGCATCACGGCAGGGT-TGCCGCT
W100.7 _____ TTCGTGTCGCATTGTGGGTGGAACTCCATCCTAGAGGCAGTGGCCGCG-GGCGTGCCTAT
W100.10 _____ TTTGTGTCGCACCTGCGGGTGGAACTCGACCGTGGNTGGCGT-GCTGCACGGCGTGCCTGT
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W100.5 _____ GCTGTGCTGGCCGCTGTACGCGGAGCAG-----AAGGTGAA-----CAAGG-TGCACA
W100.6 _____ GCTGTGCTGGCCGCTGTACGCGGAGCAG-----AAGGTGAA-----CAAGG-TGCACA
W100.7 _____ GCTCACATGGCCAAAGTTTCATGACCAATTCGTCAACGAGAGGCTAATCAATGATGTGCT
W100.10 _____ CCTGTGCGGGNCTACTTCGCGGACCAATTCTGCACCNGAG-----CTATG-TGTGCA
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W100.5 _____ TAGTGGAGGGAATGAA-----GCTCGGGTGGAGATGAGAGGCTA-----CAATGA
W100.6 _____ TAGTGGAGGGAATGAA-----GCTCGGGTGGAGATGAGAGGCTA-----CAATGA
W100.7 _____ AGGAATTGGGCACCGACTGTGGCCGATGGCGCTGGCTTACGGAGCGAGGATTACGAAAA
W100.10 _____ ACGTGTGGGCGAC-----CGGCGCAAGCTCCGGCGAGA-----CGAGCG
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W100.5 _____ A---AAGGTTGTTAAGGCCGAGGA---GGTGGAGGAGAAGGTCAGATGGGTTATGGCGTC
W100.6 _____ A---GAGGTGGTTAAGGCTGAGGA---GGTGGAGGAGAAGGTCAGATGGGTTATGGCGTC
W100.7 _____ ACATGAGCTGATCCCGCCGACGATGTGGCCCGGCATTGCTCACATTCAT----GCATC
W100.10 _____ A---GGGTCGTCTCGAAGGAGGA---GGTCCAGAGGAAGGTCGCGCGGCT----GCT
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W100.5 _____ CGAGGGAGGAGGCAGGGCAGCTCAGGGTGCGGGTAGCGGCAGCGAAGGATGCAGCGG----
W100.6 _____ CGAGGGAG---GCACGGCAGCTCAGGGTGCGGGTAGCGGCAGTGAAGGATGCAGCGG----
W100.7 _____ CT---GGAGGACCTGGGGACGTTA---TGAGGACCAGGGTAATGGACCTCGCCTCAAAGTC
W100.10 _____ C---GGAG---ACGGGGAGGTCAGGGCAGGGCGGCGACGTGGAAGGAGGTCGCG----
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W100.5 _____ CTGAGGC-GC-TCCA-GGAAGGGGGCTCGTCCAAC TTGGCGTTTGCCCAATTCCTAAAAG
W100.6 _____ CTGAGGC-GC-TCCA-GGAAGGGGGCTCGTCCAAC TTGGCGTTTGCCCAATTCCTAAAAG
W100.7 _____ TCATGGC-GCCCTCGCAGAAGGAGGCTCCTCGCAGCAAG-ATTTGCACCGCTCGTCAAC
W100.10 _____ -TGTGGCAGCATTCG-GGAGGGTGGTCCTCGCA-TGGGAATTTGCTCAAGCTTGTGACG
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W100.5 _____ ----ACATGGACATTTC--CAAGGTCGCA-----ACCATGCA-----C
W100.6 _____ ----ACGTGGATGTG----AAGCTTGTA-----AACCTACT-----T
W100.7 _____ GATCTCATGGCAGCAAAGGAGGGCGGAAC TAGCTAGCTTGCTAGCTGCCGTGCATGAGT
W100.10 _____ ----TTGCTGAGACAAC--AATGATGGCAC-----GGCCTACT-----T
* * * * *

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W100.5 _____ -GAT--T--GATGT-----G---AAGC---TTGTAAACCC---T
W100.6 _____ -AAT--GCCGGATTTTCT-----TTTTAG---AAACACACTGCAGACGC---A
W100.7 _____ TAATCGAACTCATTCACCAAAGTAAATCATTCTGTTGCAATGCACGCGTATATATGT
W100.10 _____ TGAC--AAAGGATCTCGC-----TGCCAGTGCAGGCGCGGAGAGGCAT---T
* * * * *

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W100.5 _____ ----ACTTAGTGT----TGATTTCCTTTT-----TAGAAGC-----
W100.6 _____ CAAAACCCAG-----AGGATATTCCTCCATGCAGATGTATAAGCACATGAT--GGGA
W100.7 _____ TTCCGTGTAGTATTTGTTTTGACATTCAGTCTTTTCTACTGTCTGTTTTTTCATCAATA
W100.10 _____ TTGACCGCGGCAG-----GCGGCACGCA-CGACGTCGGCCGGCCGCCACCGCA--AAAA
* * * * *

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W100.5 _____ -----
W100.6 _____ ATTAACAAT-GCTCATACTTCATTC---
W100.7 _____ AATAA-AATAAAATCTTTCAGCCCTGT
W100.10 _____ ATTAATAAAAGCATTCGGTTGACACT--

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Figure 28 Alignment of nucleotide sequence of partial cDNAs obtained by PCR from the wheat mass excised cDNA library.

W100.5 and W100.7 respectively. The longest clone of each was then sequenced. The library product corresponding to W100.5 (WA) was found to be 1307 bp long of which 1144 bp consisted of open reading frame. Two stop codons were present starting at nucleotides 40 and 244 suggesting that WA was the product of a pseudogene and so this clone was discarded. The second library product, WB, was found to be derived from W100.7 and was 1323 bp long of which 1115 bp consisted of open reading frame. Analysis of the sequences using the BLAST P program suggested that clone WB was truncated by approximately 100 amino acids at the 5' end. None of the other library products were found to be any longer than WB.

Although WB was the least abundant of the GTs obtained from the library 5' RACE was employed to obtain its full-length sequence. Two nested antisense primers (WB1 and WB2) were designed at 400 bp and 300 bp from the 5' end. Reverse transcription was then carried out on poly A⁺ RNA derived from wheat shoots using the WB1 primer. A homopolymer tail was added at the 3' end of the reaction products using terminal deoxynucleotidyl transferase. The first round of PCR used the WB1 and an oligo dT (adaptor dT) primer. Primers WB2 and 'adaptor' were used in the second round of PCR. The reaction products were cloned and PCR used to identify positive products. The total length of the extension was 689 bp long and the product contained the first methionine and 316 bp of extended open reading frame sequence.

To obtain the full-length sequence of WB, primers (WB4 and WB7) were designed to allow subcloning into pET24d (Novagen) such that the sequence would contain a C

1 TTTTTTTTTTTTTTTTGACCCACAGCCTTGTGCTCCTAGTAGGAAATTAAGAAATAAAGATG
 F F F F F * P T A L C S * * E I K E I K M

64 GCTTCTTCTACCACTAGCAACGGCAGCAAGACCCTGCGTGTCTGCTATTGCCCCACTTTGCG
 A S S T T S N G S K T L R V L L L P H F A

127 ACTGGCCACATCCACCCCTTACCGAGCTTGCCGTTAGCCTCGCCGCGTCGTCTAGCCCAAAC
 T G H I H P F T E L A V S L A A S S S P N

190 GCCACCATAGAAGCCATCATCGCAGTCACGCCGGCGAACGTTCCGATCGTCCAGTCCTTGCTG
A T I E A I I A V T P A N V P I V Q S L L

253 GAGCGCCATAGCGCAGCAACTGTCAAGATAGTTACCTATCCGTTCCCAACGGTGGAGGGCCTG
 E R H S A A T V K I V T Y P F P T V E G L

316 CCCAAGGGTGTGCGAGAACCTTGGAAGGCGGCCACTCAGGCGGATTCTATGCGCATCAACATC
 P K G V E N L G K A A T Q A D S M R I N I

379 GCCGCTCGACCGAATCCCTGATGCGCCCCGCGCACGAGACGCTCGTCCGGGCGCAGTCTCCA
 A A S T E S L M R P A H E T L V R A Q S P

442 GACGCCATCATCACCGACTTGCTCTTACCTGGAGCGCCGACATCGCCGACGAGCTCGGCGTG
 D A I I T D L L F T W S A D I A D E L G V

505 CCATGTGTCACATTCCATGTACCCGGCGCCTTCTCGATGCTCGCCATGCGCCACCTCATGATG
 P C V T F H V T G A F S M L A M R H L M M

568 GAGGACGCCGCGATCGACGGAGATGACACGGTGACGGCCCTTCCGTTCCCGACCCCTCAGATA
 E D A A I D G D D T V T A L P F P T P Q I

631 CGGGCCCCGAGGACCGAGCTGCCCCGACTTATCGATATCTCGATACGTCTTACGCAAGGTTTCAT
 R A P R T E L P D L S I S R Y V F S K V H

694 TCCATGCAAGCTGCCTGCTTTCGGCCTCGCCGTC AACACATTCTCAGGCCTGGAGCAGCAGTAC
 S M Q A A C F G L A V N T F S G L E Q Q Y

757 TGCGACATGTACACGGGCCAAGGGTACGTCCAGCGCTCCTACTTTGTAGGGCCGCAACTGCAA
 C D M Y T G Q G Y V Q R S Y F V G P Q L Q

810 TCCTCTGAGAGCCCCACAGATGACTCAAAGTCACAGTACATCGGTTGGCTTGACACAAAGTCG
 S S E S P T D D S K S Q Y I G W L D T K S

873 GACCATTGCGTGTGACGTGTCCTTTGGCTCGTGCGCCCTAGTATCACATGCTCAGCTGGAC
 D H S V V Y V S F G S C A L V S H A Q L D

936 CAACTTGCTCTTGGGTTGGAGGCCTCGGGGAAGCCGTTTTTGTGGGCGGTCAGGGCGGCCGAG
 Q L A L G L E A S G K P F L W A V R A A E

999 AAGTGGACTCCACCCAAAGGGTGGGAGAAGCGTGTGCGAGGACCGGGGGTTCATCATCAGATCC
 K W T P P K G W E K R V E D R G V I I R S

1062 TGGGCTCAGACAACCTGCCATACTTGCGCACCCCGCAGTGGGCGGTTTCTGACGCACTGTGGG
 W A Q T T A I L A H P A V G A F L T H C G

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1125  TGGAACTCCATCCTAGAGGCAGTGGCCGCGGGCGTGCCTATGCTCACATGGCCAAAGTTCCAT
      W N S I L E A V A A G V P M L T W P K F H
1188  GACCAATTCGTCAACGAGAGGCTAATCAATGATGTGCTAGGAATTGGGCACCGACTGTGGCCG
      D Q F V N E R L I N D V L G I G H R L W P
1251  CATGGCGCTGGCTTACGGAGCGAGGATTACGAAAAACATGAGCTGATCCCGGCCGACGATGTG
      H G A G L R S E D Y E K H E L I P A D D V
1314  GCCCGGGCATTGCCACATTCATGCATCCTGGAGGACCTGGGGACGTTATGAGGACCAGGGTA
      A R A L P T F M H P G G P G D V M R T R V
1377  ATGGACCTCGCCTCAAAGTCTCATGGCGCCCTCGCAGAAGGAGGCTCCTCGCAGCAAGATTTG
      M D L A S K S H G A L A E G G S S Q Q D L
1430  CACCGCCTCGTCAACGATCTCATGGCAGCAAAGGAGGGGCGCAACTAGCTAGCTTGCTAGCTG
      H R L V N D L M A A K E G R N * L A C * L
1493  CCGTGCATGAGTTAATCGAACTCATCGCACCAAAGTAAATCATTCTGTTGCAATGCACGACG
      P C M S * S N S S H Q S K S F L L Q C T T
1556  TATATATGTTTCCGTGTAGTATTTGTTTTGACATTCAGTCTTTTCTACTGTTTTTTCTTCAAT
      Y I C F R V V F V L T F S L F Y C F F F N
1619  AAATAAAATAAAATTCTCTTCAGCCCTGTATTGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
      K * N K I L F S P V L P K K K K K K K K

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Figure 29 Full length nucleotide and deduced amino acid sequence of *TaGT* from wheat. The 5' untranslated region (UTR) is indicated by blue lettering and the 3' UTR by red lettering. Underlined sequence was obtained by 5'RACE. The boxes indicate the *N*-glycosylation motif with pattern N[Δ P][ST][Δ P] where [Δ P] is any amino acid residue except proline (Xu *et al.*, 2002).

terminal His-tag. RT-PCR was carried out on wheat shoot Poly A⁺ RNA and a 1434 bp product was obtained. The product was cloned into pGEM-T Easy (Promega) and sequenced prior to cloning into pET24d for expression studies. This clone was named *TaGT* (Fig. 29).

5.1.2 Cloning of a full length putative GT from black-grass

Two unidirectional cDNA libraries had previously (termed library 2 and 3 respectively) been prepared from herbicide resistant (Peldon population) black-grass shoots (Cummins *et al.*, 1999). Library 2 contained inserts which were longer than 1.5 kb and library 3 contained inserts of between 0.5 and 1.5 kb in length. Both libraries were mass excised and PCR performed using the degenerate GT-1A and GT-1B primers and the M13 forward primer. One 500 bp PCR product was produced from library 2 and several PCR products were between 500 and 750 bp were cloned from library 3. After analysis by restriction digestion one clone from library 2 and four clones from library 3 were sequenced. Three sequences were found to be unique at the nucleotide level (Fig. 30), with BG137 obtained from library 2 and BG141.2 and BG141.3 from library 3. They were found to share between 24 % and 41 % identity at the amino acid level. Analysis of the amino acid sequences using Blast P (Altschul *et al.*, 1990) revealed that all three sequences were most similar to putative GTs from *Oryza sativa*.

The three partial cDNAs were labelled with DIG to act as probes in the library screening. The probes from BG141.2 and BG141.6 were mixed and used to screen

CLUSTAL W (1.81) multiple sequence alignment

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BG137 _____ TTTGTGACGCACTGCGGGTGGAACTCAACAATAGAGGGCATCTGTGCAGGTGTGCCCATG
BG141.2 _____ TTTGTGAGCCACTGCGGGTGGAACTCGACCCCTGGAGGCCGTGAGCGCCGGTGTTCGGATG
BG141.6 _____ TTTGTGAGTCATTGCGGGTGGAACTCGACGCTGAAGAGCGTG-GCGTCGGCGTCC---G
***** ** ***** ** * ** * * * * * * * * * *

BG137 _____ ATCACGTGG--CCACACTTTGCGGAGCAGTTTTTGAACGAGAAGCTGATTGTGGATGTGC
BG141.2 _____ GTCACCTGG--CCACGGTACGCGGATCAGTTCTACAACGAGAAGCTCGTCTGGAGTTGC
BG141.6 _____ GTCGTCGGGGTCCGCAGTGGACGGACCAGGGCACC AATGCGTGGCTGGTGGAGCGGCAGC
** ** ** * * **** ** * * * * * * * * * *

BG137 _____ TGAAAAC TGGATTGGA-----GGTTGGAGTGAAGGAGTCACA--CAGTGGGAAACGA
BG141.2 _____ TCAAGGTGGGTGTTAG-----CATCGGAGCTAAGGACTACGCATCCCTGTGAGAGCC
BG141.6 _____ TCGGCACGGGGT CAGAGCCCGCTCAGCGACAAGGACGGCGTG---CTG--GAGCCG
* ** * * * * * * * * * * * * * * *

BG137 _____ ACAAC-----AAGAG-GTTA---TGGTTACA---AGAGATG---CTGTGGAGACAGCG
BG141.2 _____ ACGA-----GGTG-ATCA---CCG--GCG---AGGTAAT---CGCTGAATCCATCG
BG141.6 _____ ATGAGCTGCAGAGGTGCATCGGCTTCGCCACGTCGGAGATAGTGCGCCCAAGGCCA-CA
* * * * * * * * * * * * * * * *

BG137 _____ GTGTACACCC TGA-----TGGGTGAGGGG-GAGGCTGCAGAGGAGTTGAGAATGCGAGCA
BG141.2 _____ GCAGAT----TGA-----TGGGCAGCAGC-GAGGAAGGTGACGCGATACAGAAGAAGGCT
BG141.6 _____ GTATGGAGGGAGAAAGCGCGGGCAGCGCCCGCTGGGTGGTTTCGTCGGAGA-GCAATCT
* ** ** * * * * * * * * * * * * * *

BG137 _____ AAAGA-CTGTGCAGTTAAGGCAAGGAGGGCTTTCGGTAAGGAAGGTTCTTCATATAACA-
BG141.2 _____ AAAGATCTGGGT-GTGGAGGCGAGGAGCGCGGTAGAGAAGGCCGGATCTCGTACAATG-
BG141.6 _____ CAGGGCGTTTCGTCGCC--GGCAACTAGC-CAGCCGACA--GCCACAT-TGCATGCACTGC
* * * * * * * * * * * * * * * *

BG137 _____ ACG--T--AAGGCTATT-----AATT-----CAAGAAATGGGAAACAAGGCCAAACG-CA
BG141.2 _____ ATG--TTGGAAGGCTGATGGACGAGTT-----GATGGCCCGCGGAGAGCCAT----CT
BG141.6 _____ ATGGTCCCTTCGGATCGACAG--CATTTAGCAGGACAGACTGGCGTAC--GTCACGCGGCT
* * * * * * * * * * * * * * * *

BG137 _____ TG-CGGCTGATACAGATGGCAATA----AGCTCTCTTAG-----TC-TCTTTTCGTGT
BG141.2 _____ TGACAAC TATG--GATTTTCATCCCATGGCTTGTTTCG-----TC-TGTTTT--TGG
BG141.6 _____ AGGCACATTAGGGGGTAGTTCCCC---ATCTTATTCGGGGGAATCAGGCATC--TG-
* * * * * * * * * * * * * * * *

BG137 _____ AAAAA CAAGTGAAGATCCTACAGTATAAATATCATTATAAAGAATTTATCTTACAAGC
BG141.2 _____ CAATAC---TGAAGACCTCCC--TTTTGAT-TGATTATAA---TTCCCATTT-----
BG141.6 _____ CAATAT--GCAGACGATACCTTAATCCTGCT-TCAGAATGACGACCTGTCCATAGCCAAC
** * * * * * * * * * * * * * *

BG137 _____ ---
BG141.2 _____ ---
BG141.6 _____ CTC
    
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Figure 30 Alignment of nucleotide sequence of partial black-grass cDNAs obtained by PCR of mass excised Peldon cDNA library.

300,000 pfu from library 3 while the BG137 probe was used to screen 300,000 pfu from library 2. From the combined screen, library 2 was found to contain 56 positive plaques and library 3, 71 positive plaques. The positive plaques were excised and the respective clones purified and analysed by restriction digest. Only one unique sequence appeared to be present. A representative clone of ~1.4 kb (BGA) was sequenced and found to contain the sequence of BG137. Clone BGA was 1344 bp long, of which 1223 bp consisted of open reading frame. Analysis using Blast P (Altschul *et al.*, 1990) revealed BGA to be truncated by approximately 90 amino acids at the N-terminal end. As with the truncated wheat clone, 5' RACE was employed to obtain the full-length putative black-grass GT. Three antisense primers (BGA1, BGA2 and BGA3) were designed 400 bp, 300 bp and 200 bp from the 5' end. Reverse transcription was carried out using the BGA1 primer on total RNA prepared from Peldon shoots. The reaction products were polyA tailed at the 3' end and then two rounds of PCR carried out firstly using the BGA2 and adaptor dT primers and then using the BGA3 and adaptor primers. One product of approximately 500 bp was obtained and it was then cloned into pGEM-T Easy (Promega). The new clone provided 265 bp of extended open reading frame sequence including the putative first methionine.

The new sequence data obtained from the 5' RACE was used to design primers for cloning the full length BGA into the expression vector pET24a (Novagen) for expression of the carboxy terminal His-tagged fusion protein. RT-PCR was carried out on total RNA prepared from Peldon shoots and a 1488 bp long product obtained. This was then cloned into pGEM-T Easy for sequencing and named *AmGT* (Fig. 31).

1 TTTTTTTTTTTTTCCAACAGTAGCCACTGCCACTTACCTCATCAAGCTTGACTGACTCCAC
 F F F F F P T V A T A T Y L I K L D * L H

64 CACCTCACAGCCACCATGATCTTCGGCGGGCGGAACAATGGCCAGATCGGCTCCACCAAGGCA
 H L T A T M I F G G G N N G Q I G S T K A

127 CACTTCGTGCTGGTTCCAATGATGGCGCAGGGCCACACCATCCCCATGACAGACATGGCGCGC
 H F V L V P M M A Q G H T I P M T D M A R

190 CTCCTGGCCGAGAATGGCGCCCAAGTCAGCTTCATCACCACGCCGGTGAACGCCTCCAGGTTG
 L L A E N G A Q V S F I T T P V N A S R L

253 GCAGGCTTCGCCGCCGATGTGGAGCGAGTAGGTCTGGCGATCCAGCTTGTGGAGCTCCGCTTC
 A G F A A D V E R V G L A I Q L V E L R F

316 CCAGCCGCCGAGTTTCGGCCTACCGGACGGGTGCGAGAACCTCGACATGATCCAGTCCAAGGGT
 P A A E F G L P D G C E N L D M I Q S K G

379 CTGTTCTGAACTTCATGGAGGCTTGCGCCGCGCTCAGGGAGCAGTTAATGGCGCACCTCTGT
 L F L N F M E A C A A L R E Q L M A H L C

442 GAGCAGCAGGGCTTGCCCTCCGAGCTGCATCATATCTGACATGATGCACCTGGTGGACCGGTGAC
 E Q Q G L P P S C I I S D M M H W W T G D

505 ATCGCAAGGGATCTCGGTATCCCCAGGCTCTCCTTTATCGGCTTCTGTGGTTTCTCGTCCCTT
 I A R D L G I P R L S F I G F C G F S S L

568 GTCAGGCACACCATCTCTCACAACAGATTGCTGGAAAACGTCACAGATGAAAATGAGCTCATC
 V R H T I S H N R L L E N V T D E N E L I

631 GCGATCCCAGGGTTCCCTACCCAAC TAGAGTTGCCGAAGGATAAATGTCCCAGGAAAGCCTTTGC
 A I P G F P T Q L E L P K D K C P G S L C

694 GTTCCAGGTATGGAGAAAATCCGTGAGAAGATGATCGAGGAGGAGCTGAGATGTGATGGTGAG
 V P G M E K I R E K M I E E E L R C D G E

757 GTCATTAACAGCTTCCAAGAGCTTGAGACATTGTACATTGAATCCCTTGAGCAAGTGACAAGG
 V I N S F Q E L E T L Y I E S L E Q V T R

810 AAGAAGGTCTGGGCGATCGGCCCAATGTGCCTCTGCCACCGAGACAGCAACACAATGTCCGCA
 K K V W A I G P M C L C H R D S N T M S A

873 AGAGGAAACAAGGCATCAATGGATGAGGCCCTGTGTTTGCAGTGGCTTGATTCAATGAAGCCA
 R G N K A S M D E A L C L Q W L D S M K P

936 GGCTCAGTAATCTTTGTGAGCTTTGGCAGCCTCGCTGCCACTACACCTGAACAGCTTGTTGAG
 G S V I F V S F G S L A A T T P E Q L V E

999 CTGGGGCTGGGACTTGAAGCCTCCAAGAAACCGTTTATTTGGGTGATCAAAGCAGGGGCTAAG
 L G L G L E A S K K P F I W V I K A G A K

1062 TTTCCAGAAGTTGAGGAATGGCTCGCGGATGGGTTTGGAGGCGTGTCAAGGACAGAGGCATG
 F P E V E E W L A D G F E E R V K D R G M

1125 ATCATCAGAGGCTGGGCGCCACAGGTAATGATCTTGTGGCACCAGCCATTGGAGGATTTCATG
 I I R G W A P Q V M I L W H Q A I G G F M

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1188 ACACACTGTGGGTGGAACCTCAACAATAGAGGGCATCTGTGCAGGTGTGCCCATGATCACGTGG
    T H C G W N S T I E G I C A G V P M I T W

1251 CCACACTTTGCGGAGCAGTTTTTTGAACGAGAAGCTGATTGTGGATGTGCTGAAAACCTGGATTG
    P H F A E Q F L N E K L I V D V L K T G L

1314 GAGGTTGGAGTGAAAGGAGTCACACAGTGGGGGAACGAACAACAAGAGGTTATGGTTACAAGA
    E V G V K G V T Q W G N E Q Q E V M V T R

1377 TATGCTGTGGAGACAGCAGTGTACACCCTGATGGGTGAGGGGGAGGCTGCAGAGGAGTTGAGA
    Y A V E T A V Y T L M G E G E A A E E L R

1430 ATGCGAGCAAAAGACTGTGCAGTTAAGGCAAGGAGGGCTTTCGATAAGGAAGGTTCTTCATAT
    M R A K D C A V K A R R A F D K E G S S Y

1493 AACACGTAAGGCTATTAATTCAAGAAATGGGAAACAAGGCAAACGCATGCGGCTGATACAGA
N N V R L L I Q E M G N K A N A C G * Y R

1556 TGGCAATAAGCTCTCTTAGTCTCTTTTTCGTGTA AAAACAAGTGAAAGATCCTACAGTATAAAT
W Q * A L L V S F R V K T S E R S Y S I N

1619 ATCATTATAAAGAATTTTCATTTTACAAGCAAAAAAAAAAAAAAAAAAAAA
I I I K N F I L Q A K K K K K K

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Figure 31 Full length nucleotide and deduced amino acid sequence of *AmGT* from black-grass. The 5' UTR region is indicated by blue lettering and the 3' UTR by red lettering. Underlined sequence was obtained by 5' RACE. The boxes indicate the *N*-glycosylation motif with pattern N[\wedge P][ST][\wedge P] where [\wedge P] is any amino acid residue except proline (Xu *et al.*, 2002).

5.2 Sequence analysis of *TaGT* and *AmGT*

Sequences similar to *TaGT* and *AmGT* were obtained by searching the Genbank database at NCBI. Four sequences encoding functional GTs were aligned with *TaGT* and *AmGT* using Clustal W (Thompson *et al.*, 1994) (Fig. 32). The presence of the conserved domain I at the amino terminus (Moehs *et al.*, 1997), a possible UDP-binding domain II (Yamazaki *et al.*, 2002) and the PSPG box at domain III (Hughes & Hughes, 1994) were all consistent with *TaGT* and *AmGT* being members of the GT-1 family. The deduced amino acid sequences of *TaGT* and *AmGT* contain *N*-glycosylation motifs with the pattern [N][\wedge P][ST][\wedge P], where [\wedge P] is any amino acid residue except proline (Xu *et al.*, 2002). *TaGT* contained two putative *N*-glycosylation sites towards the amino terminus while three *N*-glycosylation motifs were identified in *AmGT* indicating that both putative GTs would be predicted to be glycoproteins (Fig.29 & Fig. 31).

A phylogenetic tree (Fig. 33) was constructed to examine the relationship of *TaGT* and *AmGT* to eight of the clusters of GT-1 sequences defined in *Arabidopsis* (Ross *et al.*, 2002). *TaGT* and *AmGT* were both identified as members of group D, sharing 29 % identity at the amino acid level with other group members. A comparison of the amino acid sequences of *TaGT* and *AmGT* was made with seven GTs that were members of group D and had proven substrate specificity. The amino acid sequence of *TaGT* ranged from 30 % identity with the 3-*O*-flavonoid GT *VmUFGT2* (Mato *et al.*, 1998) to 25 % with the 7-*O*-flavonoid GT *UBGT* (Hirotsani *et al.*, 2000). *AmGT* was found to share greater identity with group D members than did the *TaGT*. The

TaGT : ---MASSITTSNGSKTTRVLLLHFATGHIHFTLAVSLVAASSSPNATIEAIIAVTPAN-VPIVQSLLEHRSAAATVKIVTYP* 80
 AmGT : MIFGGNGQIGSTKAHFVLMMAQGHITIMTDMARLLAENGAQVSFTTPVNASRLAGFAADVERV---GLAIQLVELRFAAFFGL 85
 5-GT : ---MGTHST---APDLHVFFPFLAHGMIPLSDIAKLFARCVKTIITFTPLNASMPTKAEKTRKNTETQMETEVFSRSEBAGL 83
 StSGT : ---MVATCNSGEILHVLELFLSAGHIFLVNARLFAARGVKATILFTTPHNALLFRSTTDDDDVRSIGFPFSIVTIKPSAEVGL 84
 VmUFGT2 : ---MG-----METPLKIYFLFFAQGHQIMVQLARLIA SRGQHIITLITSGNAQFTL-FQKTI DDDDI-ASGHHIRLHLKFPGTQLGL 77
 TOGT1 : ---MG-----QVHFFFPVMAHGMIITLDMAKLFA SRGVKATIIITPLNEFVFT-FSKAIQRN-KHLGIEIEIRLIKFAVENGL 76
 P a G H I P L A L A G t f P P e g L

Domain I * 120 140 160 180
 TaGT : PKGVNLGKAAATQADSMRINI ASTESLMRPAHETLVRAQDAITLLETTSAIADDEIGV CVT FHVTCGASMLAMRHLM-----M : 169
 AmGT : PDCCENIDMIQSKGLFLNFME CAALREQLMAHLCEQQGLP SCIIISMMHMWGTGRIARDIGIPELS FIFGCGSSLVPHITIS--HNRL 174
 5-GT : PLGCENLEQAMAIGANNEFFN AFTNLLKEQLENFLVKTR-NCLVAMFFTAASSTAKFNITLIVHGFSFQAQCAKEVMWRVFTKPY : 172
 StSGT : PEGISFN SATSPMPHKIFYAFTLSLLQKPMEDKIRELR-PCIFSMYFPVVIADDEHHIRILYNLSAYMCSIMHNLKVFTYREH : 173
 VmUFGT2 : PEGVNLV SATNNITAGKIHM AHFIQPFVTSVLKESP-DVFPDIITTSKMSKRLQIRLVNPISTFDVCMIOAIKAH---PE : 163
 TOGT1 : PEECRIDQIPSDKELPNFFKAVAMMQEPLFTEQLIEECR-PDCILSMFLPITTAAKFNIRIVFHGTSTFALCVENSVR--LNKPF : 163
 P g E L W D i P f

Domain II * 200 220 240 260 280 300 320 340 360
 TaGT : EDAAIDGDDTVTAL FFTPQIRAPRTELPDLS-----ISRYVFSKVHSMQAACFGGLAVITISGF--EQQCDMTGCGYVQ-RSY : 246
 AmGT : ENVT--DENELIAI GFPTQLELPKDKCPGSLCPVG-----MEKIREKMIIEELRCQGEVI--NSQEE--FTLIESLEQVTRK--KVM : 251
 5-GT : KAVS--SDTEVFSL FLPHEVKMTRLQVPESMRKGE--THFTKTERIRERLERKRSYGVFTVNSIYEI--EPDADFLRKKELGR--RAW : 254
 StSGT : KQPN-LDESQSFVV GLPDEIKFKLSQLTDLRLKSDQKTVFDELLEQVEDSEERSFTYGI VHDTYEII--PPAVDYTYQKLLKPP--KCV : 258
 VmUFGT2 : AFLS-FTDSGPYQI GLP-----HPTLLPVKPSPGFAVLTESLLEGGEDS-----HGVIVMSAEI--DABTQYKEKFTLTGRKVM : 237
 TOGT1 : KNVSFTSDSETFVV DLPHEIKLRTQVSPFERSGEE--TAMTRMIKTVRESDSKSYGVVF--NSIYEIYFTTDDVEHYTEVVLGR--RAW : 247
 P p n F e L e Y w

Domain III * 280 300 320 340 360
 TaGT : FVYQLQSSSEPTDSDS-----KSQYIGMID--TKSDHVVY--VSFGSCALVSHAADDQIALGLGEEAKKPKLMAVR----- : 313
 AmGT : AIGMCLCHRDSNTMSARGNKASMDALCQIMD--SMKREGVIF--VSFGSLAATTPEDVELGLGEEAKKPKLMAVR-----AKFP : 333
 5-GT : HIGVSLCNRSTEDKAQRGQTSIDEDCLKMNSFTKPKDVIIN--ICFGSTGHLIAPDIHEIATALEAGGDDIWA VRGD---HGQG : 338
 StSGT : HFVLSHFASKRSKELISEHN--NNEIVIDMFTNAQKPKVLYSFGSMARFPE--SWNINIAQADANVPTFVLRPN-----E : 337
 VmUFGT2 : HVVSSLMVEQIVKPKPAIVSEI--RNECLTADDSKERD--VLYICFGLSVLLSDKFTDIYELANGDASGHSVWVHRKKKEGQEEE : 322
 TOGT1 : AIGVLSMCMNRDIEDKAERGKSSIDRHECKIMD--SKKESAVFTTYVCFGSVANFTASDIHFLAMGIEAGQEIWVVR-----TEL : 328
 GP WL SV QL e a l AS Fiw

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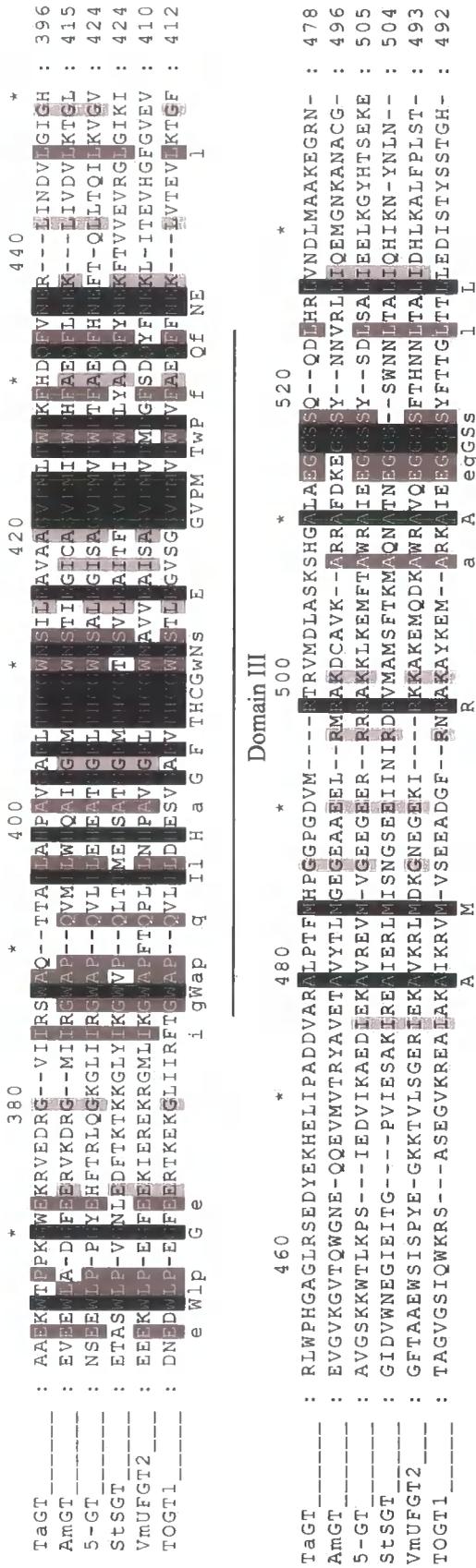


Figure 32 Multiple alignment using clustal W (Thompson *et al.*, 1994) of deduced amino acid sequence of TaGT and AmGT and related glucosyltransferases whose catalytic activities have been experimentally proven. The Genbank accession numbers and species of the aligned GTs are listed in Table 1, Chapter 1. *Black shading* shows 100 % conservation of amino acids between sequences, *dark grey shading* shows 80 % or greater conservation and *light grey shading* shows 60 % or greater conservation. Underlined sequences indicate conserved domains.

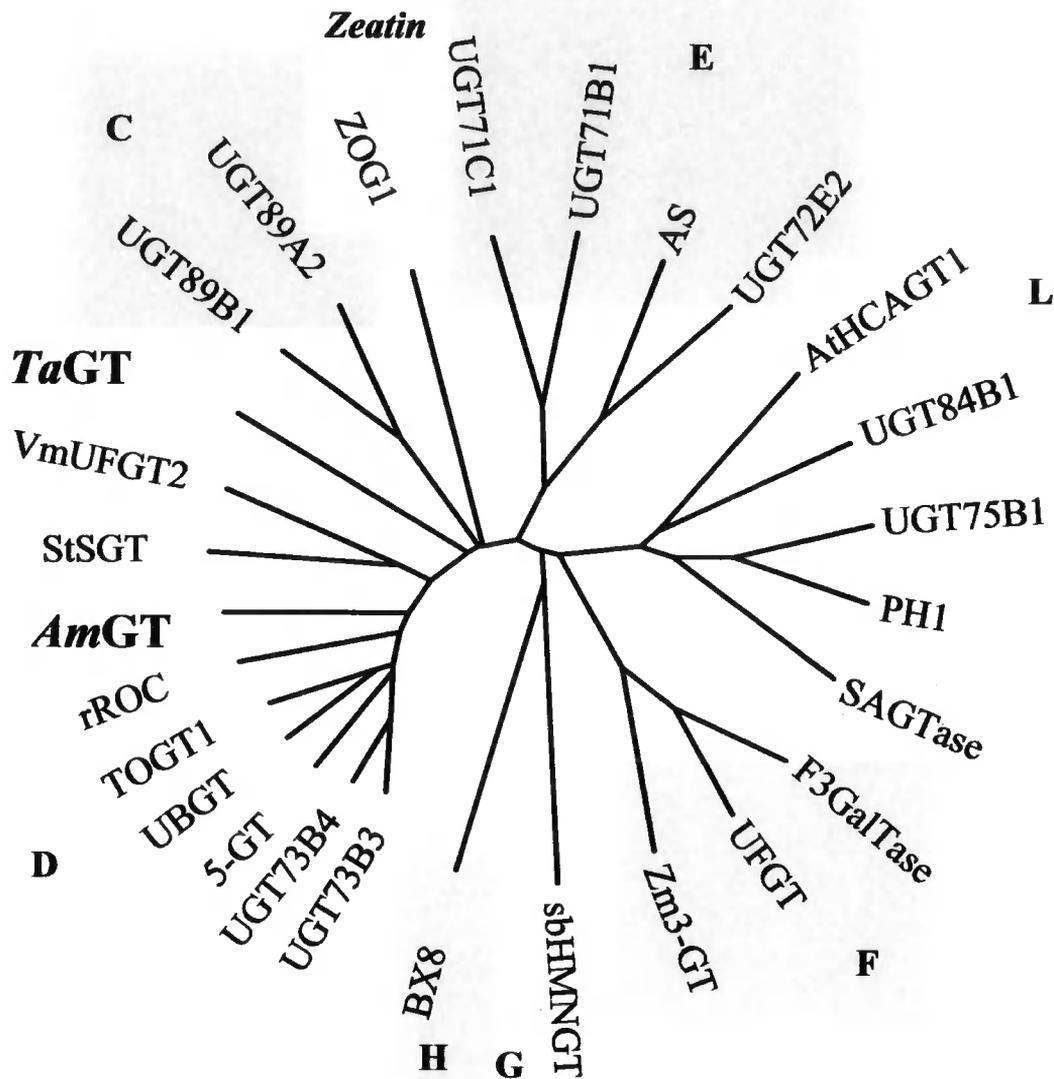


Figure 33 Phylogenetic tree of higher plant GTs, showing the relation of *TaGT* and *AmGT* to eight of the clusters defined by (Ross et al., 2002) in *Arabidopsis*. Genbank accession numbers of the GTs are listed in Table 1, Chapter 1.

Arabidopsis GTs UGT73B3 and UGT73B4 (Lim *et al.*, 2002) shared 41 % identity with *AmGT* while the STSGT from potato (Meohs *et al.*, 1997) shared just 30 % identity.

To identify potential substrates for *TaGT* and *AmGT*, the substrate specificity of the seven group D GTs was examined. Unlike the group F members, which are mainly 3-*O*-flavonol GTs (Jones & Vogt, 2000) there is very little correlation between gene sequence and substrate specificity in group D. Ether glucosides are produced by all seven members, with TOGT1 also capable of forming glucose esters. The substrate specificity of the group D members includes the 7-*O*-flavonoid GTs from *Dorotheanthus bellidiformis* (Vogt *et al.*, 1999) and *Scutellaria baicalensis* (Hirofani *et al.*, 2000), 3-*O*-flavonoid GT from *Vigna mungo* (Mato *et al.*, 1998) and the phenylpropanoid and benzoic acid utilising GTs from tobacco and *Arabidopsis* (Fraissinet-Tachet *et al.*, 1998; Lim *et al.*, 2002). In addition two further members of group D, a plant hormone conjugating GT from *Vigna angularis* and a solandine GT from potato (STSGT), have been identified (Xu *et al.*, 2002; Moehs *et al.*, 1997).

STSGT is the only member of group D for whom a putative substrate-binding domain has been proposed. Alignment of STSGT with two human estrogen specific enzymes (Genbank accession nos. P16662 and P36538) and a rat β -hydroxysteroid glucuronosyltransferase (P19488) lead to the identification of the sterol binding motif (Meohs *et al.*, 1997). This domain consisted of 34 amino acid residues, of which eight residues were identical and a further 13 residues were conserved. Comparison of the deduced amino acid sequences of *TaGT* and *AmGT* with the sterol-binding

motif found that 4 amino acid residues were conserved. However other members of group D, which have no known conjugating activity toward sterols also share this level of identity.

Group D contains a cluster of inducible GTs (Hirotsani *et al.*, 2000) including UBGT, TOGT1 and the putative GT *Twil* from tomato, whose mRNA levels increase on wounding or treatment with salicylic acid (Hirotsani *et al.*, 2000; Frassiniet-Tachet *et al.*, 1998; O'Donnell *et al.*, 1998). It is not known whether *TaGT* and *AmGT* are similarly inducible.

5.3 Overexpression of *TaGT* and *AmGT* in *E.coli*

TaGT and *AmGT* were overexpressed in *E.coli* with the aim of characterising the activities of the recombinant proteins toward xenobiotic and natural product substrates. *TaGT* and *AmGT* were cloned into the overexpression vector pET24 in frame with a carboxy terminal (his)₆ tag. The expression constructs were then electroporated into BL21(DE3) competent cells and the resulting colonies grown in LB medium at 37 °C. After three hours induction with 1 mM IPTG, cells were harvested, sonicated and the lysates analysed by SDS-PAGE. Comparison of the crude soluble fraction with the insoluble fraction suggested that both *TaGT* and *AmGT* were present only in the inclusion bodies. When the soluble fractions from both inductions were subjected to purification on a nickel chelate column, the protein present in the affinity purified fraction contained no polypeptides of the expected molecular mass of either putative GT. In an attempt to obtain soluble recombinant

protein, the inclusion bodies were solubilised in 6 M guanidine-HCl and then allowed to renature. Analysis of the refolded *TaGT* and *AmGT* by gel filtration suggested that both putative GTs appeared aggregate on refolding, suggesting they were effectively denatured proteins.

A second strategy to obtain soluble protein was followed. This involved a combination of using alternative fusion, the use of a modified host strain and alteration of the induction conditions. *TaGT* and *AmGT* were fused via their N-termini to thioredoxin using the pET32 a vector, which also contained a carboxy terminal (his)₆ tag for ease of purification. Thioredoxin is a readily expressed, soluble fusion tag consisting of 109 amino acid residues (Trx•TagTM, Novagen, Madison, WI, USA) and has been found to promote solubility of otherwise insoluble proteins (LaVaillie *et al.*, 1993). In addition, the translation of thioredoxin can enhance correct disulphide bond formation of the recombinant proteins in the cytoplasm (LaVaillie *et al.*, 1993). The fusion proteins of the putative GTs were designated Trx-*TaGT* and Trx-*AmGT*. The conditions of the expression of the fusion proteins in *E.coli* were then altered to further encourage the formation of soluble protein. A derivative of the BL21 host strain, Rosetta (DE3), which can enhance expression of eukaryotic proteins was investigated. The Rosetta (DE3) strain contains a plasmid encoding five tRNAs which bind to the AGG, AGA, CUA, CCC and GGA, codons respectively, all of which are rarely used in *E.coli*. Analysis of the nucleotide sequences in the plant GT sequences found 25 of these rare codons present in *TaGT* and 32 in *AmGT*. Low temperature induction was also used to promote the production of soluble Trx-*TaGT* and Trx-*AmGT*. Cultures were grown at 37 °C in LB medium containing 0.1 %

glucose to repress expression until the OD_{600} of the culture was approximately 0.5. The cultures were then cooled and transferred to a shaker at 15 °C, prior to induction with 0.1 mM IPTG for 16 hr.

The soluble fractions of the overexpressed putative GTs were subjected to purification using Ni-NTA magnetic beads (Qiagen Ltd). SDS-PAGE analysis of Trx-*Ta*GT induction and purification (Fig. 34A) revealed that the overexpressed protein appeared to be soluble and that inclusion bodies were not formed. Several proteins of the correct molecular weight (~ 69.8 kDa) appeared to purify using the Ni-NTA magnetic agarose beads. Immunodetection of the His-tagged Trx-*Ta*GT protein using the India His probe (Pierce) was performed (Fig. 34B). A major polypeptide at ~ 70 kDa corresponding to Trx-*Ta*GT was present in the post-induction and purified lanes. A minor immunoreactive band at ~ 60 kDa was also observed. Potentially this was a degradation product of Trx-*Ta*GT. Analysis of the Trx-*Am*GT induction and purification fractions by SDS-PAGE (Fig. 35A) also showed a clearly visible polypeptide at ~ 70 kDa. Immunodetection with the India His probe confirmed the identity of the polypeptide as Trx-*Am*GT (Fig. 35B).

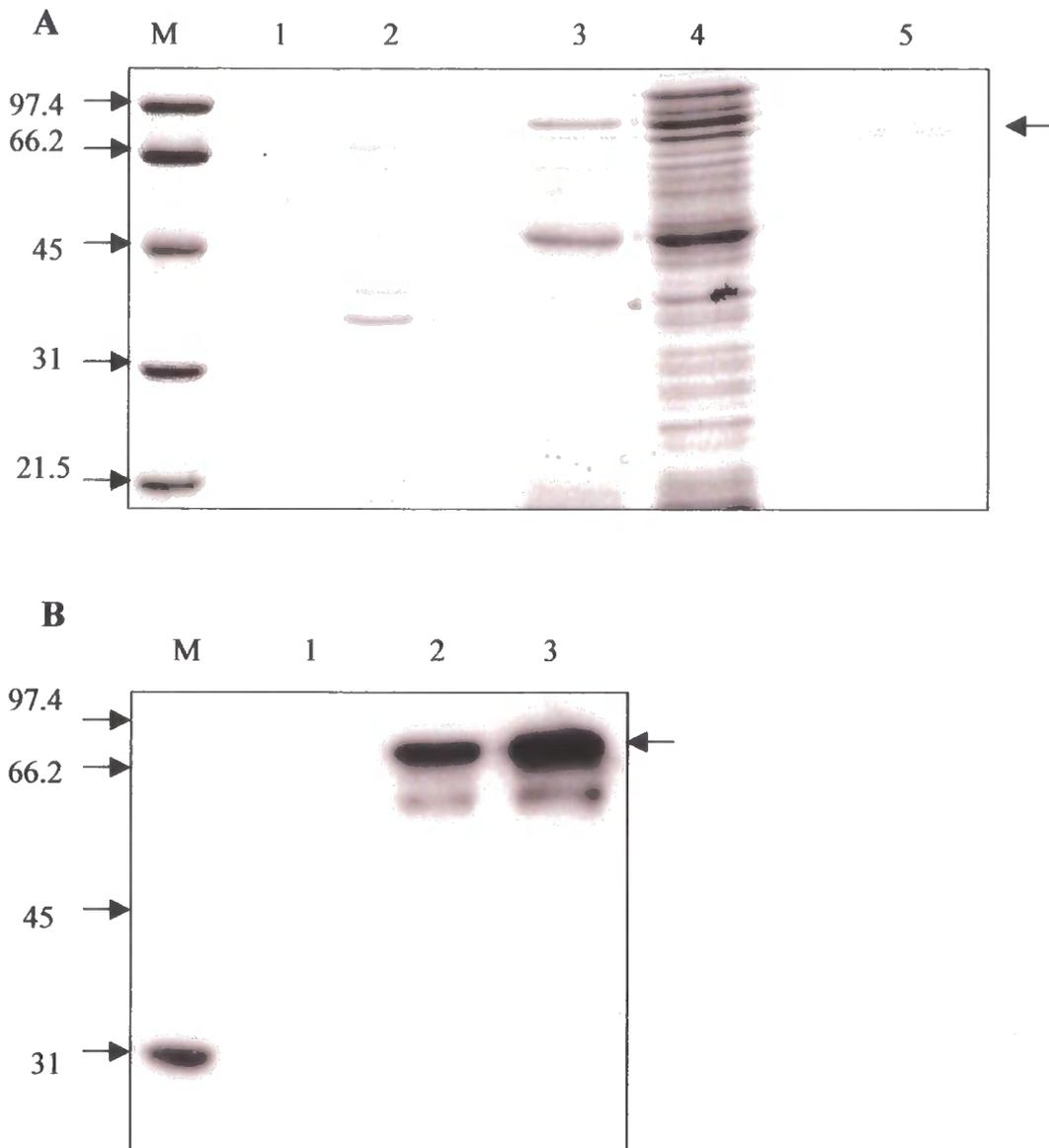


Figure 34 Analysis of purification of Trx-TaGT using Ni-NTA magnetic agarose beads. SDS-PAGE analysis (A) of fractions from purification. Lane 1, insoluble protein pre-induction; lane 2, insoluble protein 16 hr post-induction; lane 3, soluble protein pre-induction; lane 4, soluble protein 16 hr post-induction; lane 5, Ni-NTA purified protein. Immunodetection (B) of His-tagged proteins using the India His probe. Lane 1, soluble pre-induction; lane 2, soluble 16 hr post-induction and lane 3, purified protein. Arrow indicates Trx-TaGT protein. Lane M contains the molecular weight markers.

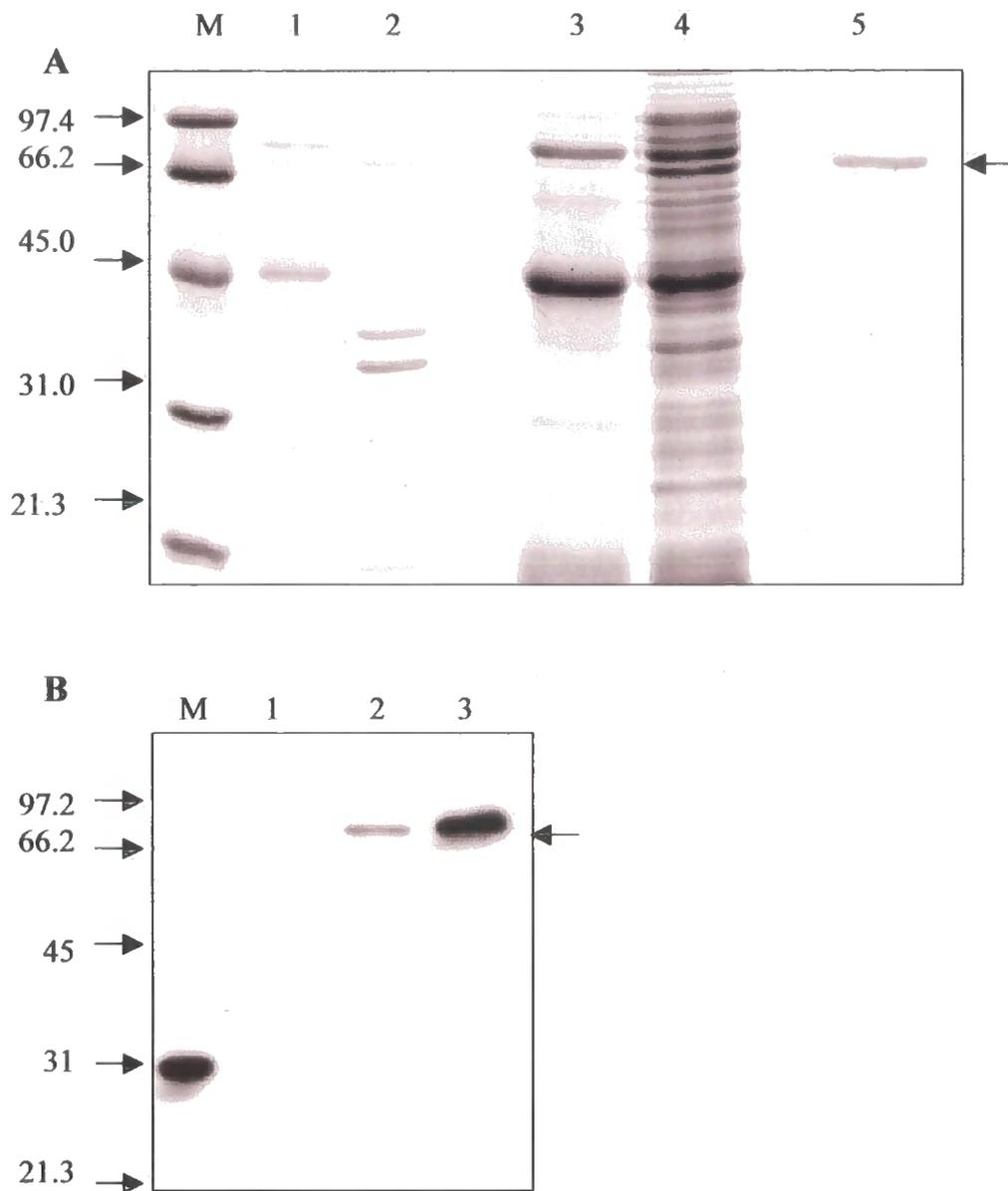


Figure 35 Analysis of purification of Trx-*AmGT* using Ni-NTA magnetic agarose beads. SDS-PAGE analysis (A) of fractions from purification. Lane 1, insoluble protein pre-induction; lane 2, insoluble protein 16 hr post-induction; lane 3, soluble protein pre-induction; lane 4, soluble protein 16 hr post-induction; lane 5, Ni-NTA purified protein. Immunodetection (B) of His-tagged proteins using the India His probe. Lane 1, soluble protein pre-induction; lane 2, soluble 16 hr post-induction and lane 3, purified protein. Arrows indicate Trx-*AmGT* protein. Lane M contains the molecular weight markers.

5.3.1 Screening of Trx-TaGT and Trx-AmGT for GT activity toward a range of substrates

Purified Trx-TaGT and Trx-AmGT were assayed for GT activity toward xenobiotic and natural product substrates. Synthetic phenols, including phenol, 4-nitrophenol, 2,4,5-trichlorophenol, pentachlorophenol and 4-chlorothiophenol, and anilines, 3,4-dichloroaniline and 4-nitroaniline, were tested for *O*-, *N*- and *S*- conjugation by the putative GTs. Trx-TaGT and Trx-AmGT were also assayed using the herbicides chloramben, picloram and amitrole as substrates. No activity was observed toward any xenobiotic substrate by either Trx-TaGT or Trx-AmGT. Trx-TaGT and Trx-AmGT were also assayed with phenylpropanoids including cinnamic acid, 4-hydroxycinnamic acid, caffeic acid, ferulic acid and coniferyl alcohol and benzoic acids including salicylic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid. Where these compounds contained carboxyl groups, the assay was also performed at a lower pH (pH 6.5) to promote the formation of glucose esters. Three hydroxycoumarin substrates, scopoletin, umbelliferone and esculetin were also tested, as was a range of flavonoid substrates including naringenin, isoliquiritigenin, quercetin, luteolin, genistein, mycetrin, and coumestrol. In total 10 xenobiotic substrates and 20 natural products were tested on the two recombinant fusion proteins but no reaction products were observed. This lack of activity could be due to a number of reasons. Firstly, Trx-TaGT and Trx-AmGT may not be catalytically active GTs. Alternatively their activity may require *N*-glycosylation during post-translational processing of the protein, a process not effected in *E.coli*. However it is more likely that in the limited time available that the endogenous substrate was not tested. There are hundreds of potential compounds that can be glucosylated in plants

(Jones & Vogt, 2000) and some GTs have been found to possess great substrate specificity. For example, UGT73B3 and UGT74B4 from *Arabidopsis* only glucosylate 3-hydroxybenzoic acid or 4-hydroxybenzoic acid respectively (Lim *et al.*, 2000). Trx-*Ta*GT and Trx-*Am*GT may not have been tested for activity toward their true specific substrates. Similarly, with synthetic substrates although conjugation of xenobiotic compounds has been reported for two recombinant GTs in *Arabidopsis* (Hefner *et al.*, 2002; Schäffner *et al.*, 2002), it is not yet known whether many or just a selected few GTs are able to glucosylate synthetic compounds. Finally Trx-*Ta*GT and Trx-*Am*GT were assayed only using the most common activated sugar in plants UDP-glucose. It is possible that these enzymes may use alternative UDP-sugars. Analysis of sequence data was unable to determine the most likely sugar to be utilised as evolution of sugar specificity appears to have developed later than toward the acceptor substrates (Jones & Vogt, 2002).

6. PROFILE OF PHENOLIC NATURAL PRODUCTS IN WHEAT AND BLACK-GRASS

Introduction

Plants synthesise a wide range of phenolic compounds with multiple biological functions. Many of these compounds have roles in stress tolerance in plants, with biotic and abiotic stress known to result in the accumulation of many phenylpropanoid and flavonoid compounds. For example, exposure of plants to high light or UV has been found to induce the accumulation of anthocyanins, flavones and isoflavones as a protective measure (Winkel-Shirley, 1999). Wounding of plants can also result in the induction of phenylpropanoid derivatives such as ferulate esters, wall bound phenolic acids and lignin, along with a range of other compounds (Dixon & Paliva, 1995).

The characterisation of the phenolic metabolites of wheat at different growth stages has been carried out by a number of workers. In contrast, the phenolic metabolites in competing grass weeds, such as black-grass have received little attention. In wheat leaves and bran the dominant UV absorbing metabolites present were derived from the flavones apigenin and luteolin, mainly as the respective C-glycosides, notably the C-glucoside of luteolin and isoorientin {Julian *et al.*, 1971; Michael *et al.*, 1998; Estairte *et al.*, 1999). In addition, chlorogenic acid and 4-hydroxycinnamic acid have also been identified in wheat bran (Michael *et al.*, 1998). Other phenylpropanoid metabolites, including ferulic acid, sinapic acid, 4-hydroxycinnamic acid, syringic acid, caffeic acid and vanillic acid have also been detected as components of wheat gluten and in wheat flours (Labat *et al.*, 2000).

In this study, solvent extracts have been examined to determine if safener treatment had an effect on the composition of the phenolics in wheat shoots. In addition, the phenolic content of herbicide resistant and herbicide susceptible black-grass plants have been compared. These studies were carried out as our earlier studies had shown that both safener-treatment in wheat, and herbicide resistance in black-grass was associated with increased GT activity toward phenolic natural products (Chapter 3). In addition, a number of microsomal cytochrome P450s are involved in the phenylpropanoid and flavonoid biosynthetic pathway (Winkel-Shirley, 1999), and herbicide safener treatment of wheat and herbicide resistance in black-grass is associated with enhanced cytochrome p450 activities (Davies & Caseley, 1999; Hyde *et al.*, 1996).

Results

Phenolic metabolites were sequentially extracted from wheat shoots and roots and black-grass shoots using acetone and acetone: methanol (1:1) and the combined preparation analysed by HPLC (Edwards & Kessman, 1992). Initial HPLC analysis of the metabolite profiles were carried out with UV absorbing metabolites monitored at 287nm. The phenolic profiles were then subjected to further investigation with the aim of identifying and quantifying metabolites whose content had been modified by herbicide safener treatment (wheat) or herbicide resistance (black-grass). Individual metabolites were analysed by HPLC-MS, which allowed the parent molecular mass of metabolites to be determined along with their absorbance spectrum determined using a photodiode array detector (200 nm - 400 nm). The combination of high resolution MS and UV spectral analysis then allowed for the unambiguous identification of flavonoids and phenylpropanoids by comparison with published data on these metabolites in wheat (Estairte *et al.*, 1999). To assist in their analysis, extracts were treated with β -glucosidase to remove conjugating sugars and release the aglyca. β -glucosidase treated extracts were compared with the respective undigested extracts to identify glycosylated metabolites. This analysis had an additional benefit as it became apparent that treatment with β -glucosidase was unable to hydrolyse C-glycosides, thus allowing selective diagnosis of *O*-glycosylation.

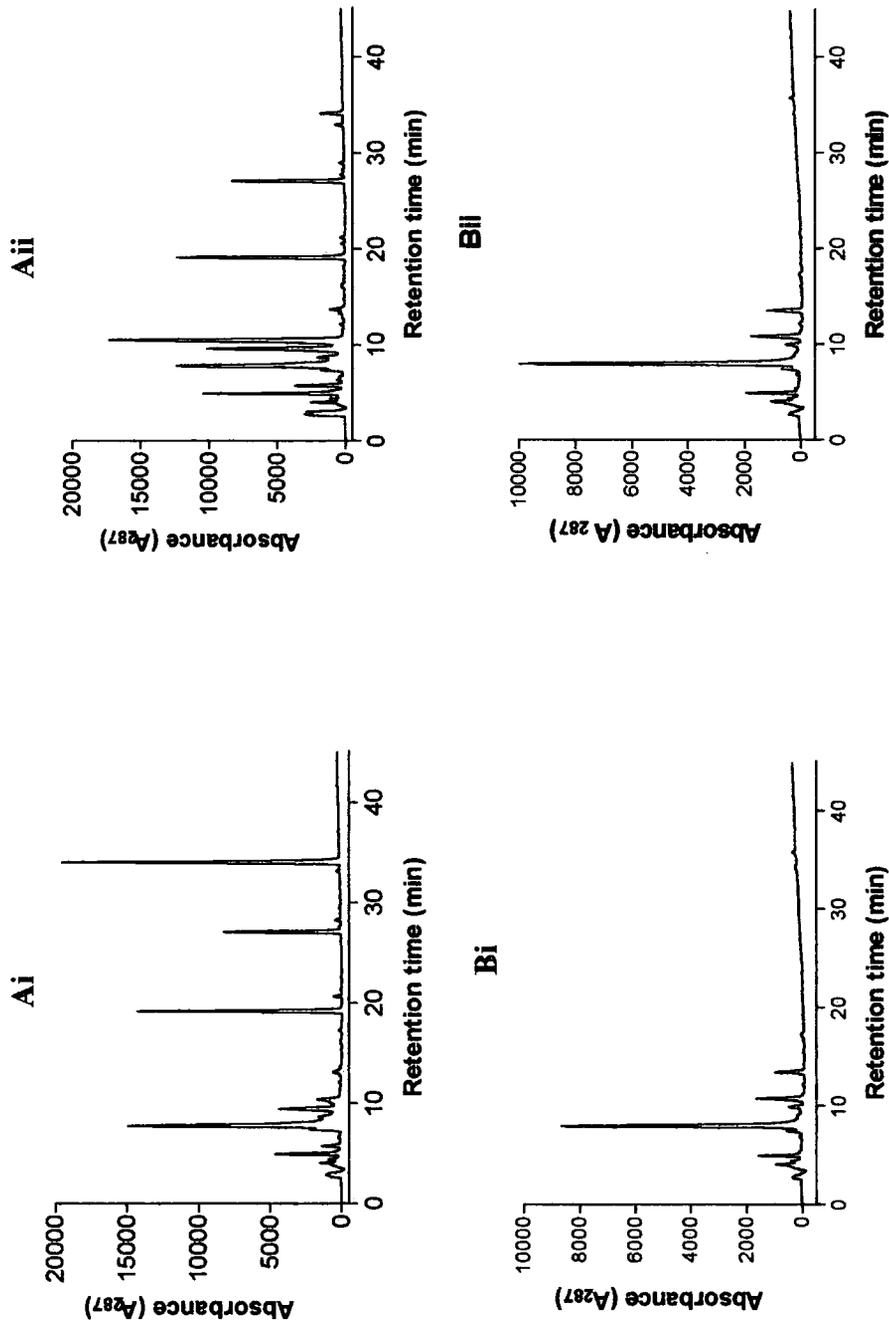


Figure 36 HPLC profiles of solvent extracts from (A) wheat shoots and (B) wheat roots. Seedling were either untreated (i) or treated with cloquintocet methyl (ii). UV absorbance was monitored at 287 nm.

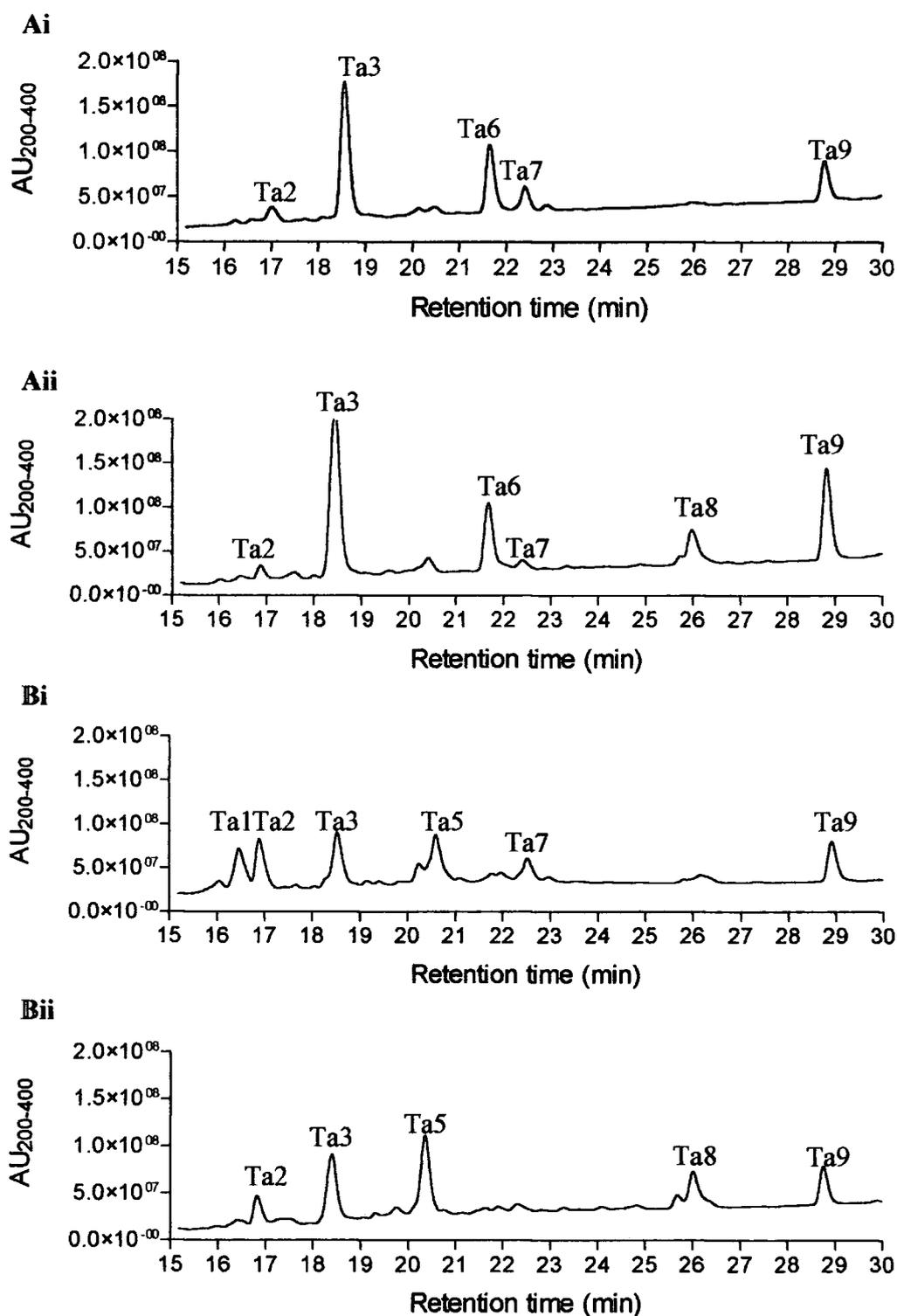


Figure 37 HPLC profile of solvent extracts from wheat shoots. UV absorbance was monitored between 200 and 400 nm and the summative spectra are displayed. Profiles are (A) untreated wheat shoots and (B) wheat shoots treated with cloquintocet mexyl. Extracts were either undigested (i) or digested with β -glucosidase (ii).

6.1 The effect of safener treatment on the phenolic metabolites of wheat shoots

In earlier studies (chapter 3) treatment of wheat seedlings with the wheat herbicide safener cloquintocet mexyl was found to produce the greatest enhancement in OGT activity towards natural products of all the safeners tested. Cloquintocet mexyl was used to treat wheat seedlings and after a 10 day exposure, the phenolic metabolites were extracted and analysed from the roots and shoots of treated and untreated plants. Initial analysis by HPLC (method as in section 2.1.7) revealed several differences in the metabolite profiles between untreated and cloquintocet mexyl-treated wheat shoots (Fig 36A). In contrast, in the roots, no major differences were apparent in the extracts between untreated and safener treated extracts (Fig 36B). In the shoots it was possible to resolve nine major UV-absorbing peaks using the Synergi Polar-RP HPLC column. The nine metabolites (Ta1 to Ta 9) eluted with RT between 16-30 min (Fig 37) and were analysed by electrospray time of flight MS after reducing the cone voltage from 30 V to 20 V to reduce fragmentation of glucosidic bonds.

6.1.1 Identification of major metabolites in extracts from wheat shoots

Seven out of the nine major UV absorbing peaks contained a single major mass ion. Six of these metabolites were identified using a combination of mass spectral data, UV spectral data and authentic standards (Table 10). Structures of identified metabolites are presented in Fig. 38.

Table 10 Compilation of retention time, UV spectral and molecular weight data from the nine major UV absorbing peaks found in acetone/methanol extracts from wheat shoots and from authentic standards and reference data.

Peak no.	Identity of peak	Retention time (min)		UV spectral data (λ_{max})		Molecular mass (Da)	
		Extract	Standard	Extract	Standard or reference*	Extract	Reference#
Ta1	Glycosylated ferulic acid	16.7	N/A	300sh, 331	N/A	356.18	356.33
Ta2	Isoorientin	17.1	N/A	255, 269, 347	242sh, 255, 271, 348*	448.13	448.39
Ta3	Not determined	18.1	N/A	216, 279, 291sh	N/A	149.07	N/A
			N/A		N/A	164.11	N/A
			N/A		N/A	211.14	N/A
Ta4	4-Hydroxycinnamic acid	19.8	19.7	295sh, 309	295sh, 309	164.13	164.16
Ta5	Ferulic acid	20.4	20.3	295sh, 319	295sh, 323	194.14	194.19
Ta6	Possibly 3,4-hydroxy-cinnamaldehyde	22.0	N/A	287	N/A	164.10	N/A
Ta7	Tricin-7-glucuronide	26.6	N/A	280, 350	N/A	506.22	506.42
Ta8	Tricin	26.4	N/A	245, 269, 300sh, 353	244, 269, 299sh, 350*	330.16	330.3
Ta9	Sinopyl malonate	29.1	N/A	216, 279	N/A	224.21	224.21
			N/A		N/A	312.24	312.28

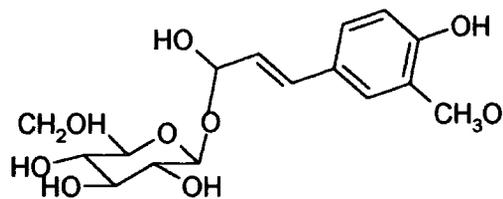
N/A indicates that authentic standards or UV spectral data was not available.

* UV spectral data in methanol from (Mabry *et al.*, 1970).

Molecular weights obtained from (Harbourne & Baxtor, 1995).

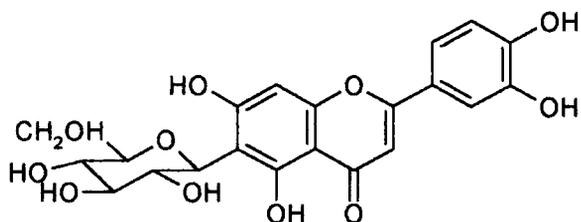
Peak Ta1

Ferulic acid glucose ester



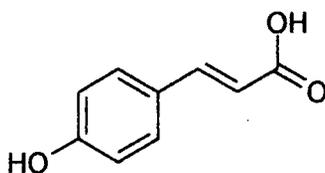
Peak Ta2

Isoorientin



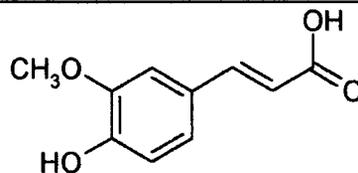
Peak Ta4

4-Hydroxycinnamic acid



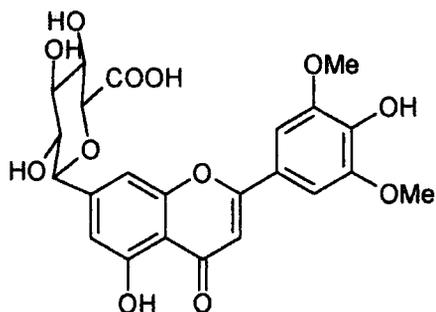
Peak Ta5

Ferulic acid



Peak Ta7

Tricin-7-glucuronide



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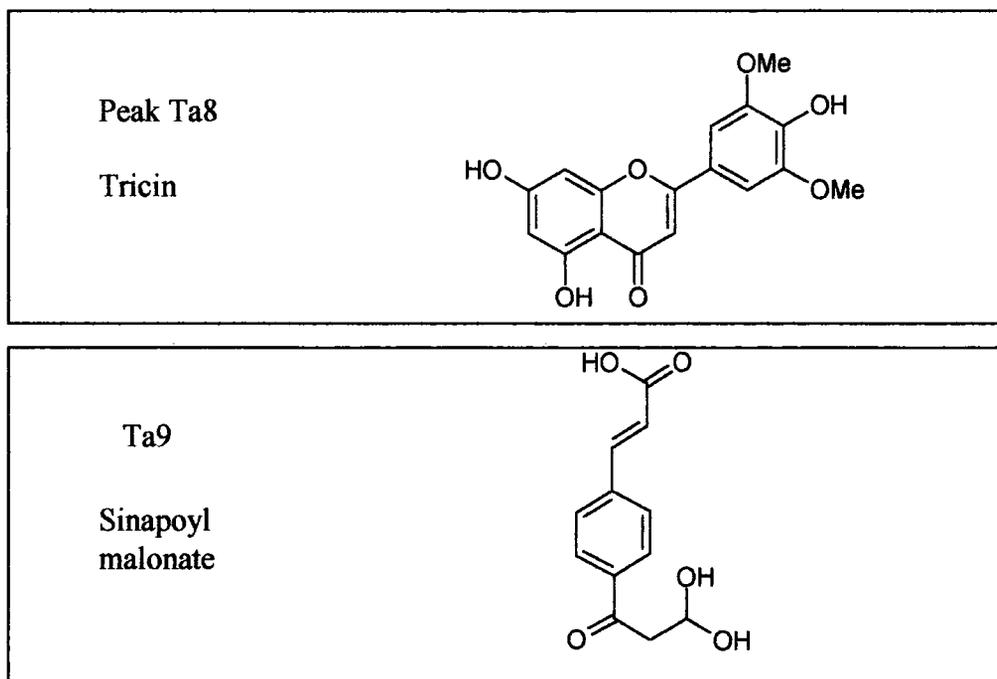


Figure 38 Metabolites identified in acetone:methanol extracts from wheat shoots.

Peak Ta1 and peak Ta5

Peak Ta1 is only present in extract not digested by β -glucosidase suggesting it is an *O*-glycoside. Theoretical removal of a sugar moiety such as glucose from the metabolite gives a molecular weight of 194, which is the same as for peak Ta5. By way of confirmation, peak Ta5 was present at much higher concentrations following β -glucosidase treatment. The UV spectrum of Ta5 consisted of a shoulder at 295 nm and a peak at 322 nm, similar to that of a phenylpropanoid. As ferulic acid has a molecular weight of 194.19, it was run as an authentic standard on the HPLC-MS resulting in a retention time of 20.4 min, compared to a retention time of 20.3 min for peak Ta5. Peak Ta5 was therefore identified as ferulic acid and peak Ta1 as its glycoside. The identity of the sugar moiety was not determined nor the position of glycosylation, however glucose esters of ferulic acid have been identified in wheat flours previously (Labat *et al.*, 2000).

Peak Ta2

Peak Ta2 was present in both β -glucosidase digested and undigested extracts. In addition increasing the sample cone voltage from 20 V to 30 V did not affect the concentration of peak Ta2, suggesting that any sugar moieties were not *O*-glycosides. The UV spectrum of peak Ta2 was characteristic of flavonoids with peaks at 255 nm, 269 nm and 347 nm. This data combined with a molecular weight of 448.13 allowed identification of the metabolite as isoorientin, a C-glycoside derivative of luteolin. No authentic standard was available to confirm retention time. Isoorientin has been

identified as a major flavonoid metabolite in wheat in a number of studies (Estairte *et al.*, 1999; Julian *et al.*, 1971).

Peak Ta3

Peak Ta3 consists of two major mass ions of 150.11 and 165.14 and a minor ion at 211.14. The intensity of all three peaks was consistent through out the UV peak suggesting the presence of one compound that has been broken down during ionisation. The identity of the compound was not determined.

Peak Ta4

The molecular mass was found to be 164.18 and the UV spectrum consisted of a shoulder at 295 nm and a peak at 309 suggesting that the metabolite was a phenylpropanoid. When 4-hydroxycinnamic acid, which has a molecular weight of 164.16, was run as an authentic standard the resulting retention time was 19.7 mins compared to peak Ta4 which eluted at 19.8 mins. The UV spectrum of the standard also matched that of peak Ta4, therefore Ta4 was identified as 4-hydroxycinnamic acid.

Peak Ta6

Peak Ta6 was found to have a mass of 165.14 and the UV absorbance was maximal at 287 nm, characteristic of the phenylpropanoids. The mass data is consistent with 3,4-dihydroxycinnaldehyde, however spectral data and standards were not available to confirm this.

Peak Ta7 and peak Ta8

Peak Ta8 was only found in extracts digested with β -glucosidase and peak Ta7 in both digested and undigested extracts. The UV spectra of both peaks had a absorbance maxima at around 350 nm suggesting that they were flavonoid type compounds. The mass data and UV spectrum of peak Ta8 was similar to that of triclin, which has previously identified as a metabolite of wheat (Estairte *et al.*, 1999). As peak Ta7 is more polar than triclin and only present in undigested extracts it must be glycosylated. The mass data suggests that the identity of peak Ta 7 is triclin-7-glucuronide, which has previously been identified in *Triticum dicoccum* (Harborne & Baxtor, 1991).

Peak Ta9

Peak Ta9 consists of two mass ions at 224.21 and 312.24. The difference in mass between the two ions is 88 suggesting that the compound was conjugated with malonic acid. The ion of 224.26 matched the mass of the phenylpropanoid sinapic acid. An authentic standard of sinapic acid was run on the LC-MS and a shorter retention time observed than for peak Ta9 confirming that sinapic acid must be conjugated with malonic acid in wheat as it is in *Arabidopsis* (Chapple *et al.*, 1992).

6.1.2 Quantification of major metabolites in extracts from wheat shoots

Initially metabolites were quantified based on their concentration in extracts that had not been digested with β -glucosidase. However, while 4-hydroxycinnamic acid was

Table 11 Quantification of UV-absorbing phenolic metabolites in extracts from wheat shoots treated \pm cloquintocet mexyl. All peaks were quantified in undigested extracts, except for peaks Ta4, Ta5 and Ta8 that were quantified from extracts digested with β -glucosidase. All values are means \pm variation in duplicated determinations.

Peak no.	Identity of peak	Concentration of metabolites in wheat shoots (nmol g ⁻¹ Fresh weight)	
		Untreated	Cloquintocet mexyl treated
Ta1	Ferulic acid glucose ester -		16.7 \pm 7.4
Ta2	Isoorientin	9.8 \pm 0.0	41.7 \pm 18.0
Ta3	Not determined	119.3 \pm 14.7	19.1 \pm 9.1
Ta4	4-Hydroxycinnamic acid -		14.9 \pm 1.3
Ta5	Ferulic acid -		61.8 \pm 4.8
Ta6	Possibly 3,4-dihydroxy-cinnamaldehyde	57.8 \pm 4.9	14.0 \pm 1.3
Ta7	Tricin-7-glucuronide	56.2 \pm 10.7	-
Ta8	Tricin	133.4 \pm 16.6	85.9 \pm 4.7
Ta9	Sinopyl malonate	40.5 \pm 7.5	13.2 \pm 6.0

detected in β -glucosidase digested extracts it was not quantifiable as identifiable conjugate(s) suggesting that it is glycosylated in multiple forms *in planta*. Similarly the quantities of ferulic acid glucose ester and triclin-7-glucuronide determined were also significantly lower than the respective aglycones quantified after β -glucosidase treatment suggesting that additional minor conjugate derivatives of the parent compounds were present. Collectively, these results suggested that it would be accurate to quantify the aglycones released on β -glucosidase treatment rather than analyse the respective polar conjugates of these compounds. To quantify the metabolites, the HPLC-MS was calibrated with a range of concentrations of available standards of 4-hydroxycinnamic acid and ferulic acid. As standards were not available for the other metabolites, ferulic acid was used to quantify the other phenylpropanoids while naringin (4',5,-dihydroxyflavanone 7-rhamnoglucoside), was used to quantify the remaining flavonoids and their glycosides.

Quantifiable concentrations of ferulic acid and 4-hydroxycinnamic acid were only determined in extracts from cloquintocet mexyl-treated wheat shoots. In these extracts the concentration of the free acid of ferulic acid was approximately four-fold higher than either its glucose ester or 4-hydroxycinnamic acid (Table 11). Of the other three phenylpropanoid metabolites, peak Ta3, peak Ta6 and sinopyl malonate were present at least three fold higher in untreated extracts than in safener extracts. Isoorientin was found in extracts from both untreated and cloquintocet mexyl treated wheat shoots, although its concentration was found to be four-fold higher in safener-treated shoots. In contrast, triclin-7-glucuronide and triclin were present at higher concentrations in extracts from untreated shoots.

6.2 Comparison of phenolic metabolites in extracts from herbicide-resistant and herbicide-susceptible black-grass

Extraction of metabolites was performed on the wildtype (herbicide-susceptible), population and the Peldon (herbicide-resistant) population. As with the determination of OGT activities in black-grass, only the shoot tissue was examined. Initial HPLC analysis by monitoring UV absorbing metabolites at 287 nm showed little difference between herbicide-susceptible and herbicide-resistant plants. However, following treatment with β -glucosidase major differences in the phenolic metabolite were determined in the two populations. As with the wheat extracts it appears that some glycosylated metabolites were not effectively resolved as defined metabolites on the column. All further identification and quantification was carried out on β -glucosidase digested extracts.

6.2.1 Identification of major metabolites in β -glucosidase digested extracts from black-grass.

Five major UV absorbing peaks eluting between 16 min and 21 min were labelled Am1 to Am5 (Fig. 39). The retention times, UV spectra and molecular mass of these five major metabolites were compared with authentic standards and reference material (Table 12). The structures of the identified metabolites are presented in Fig. 40.

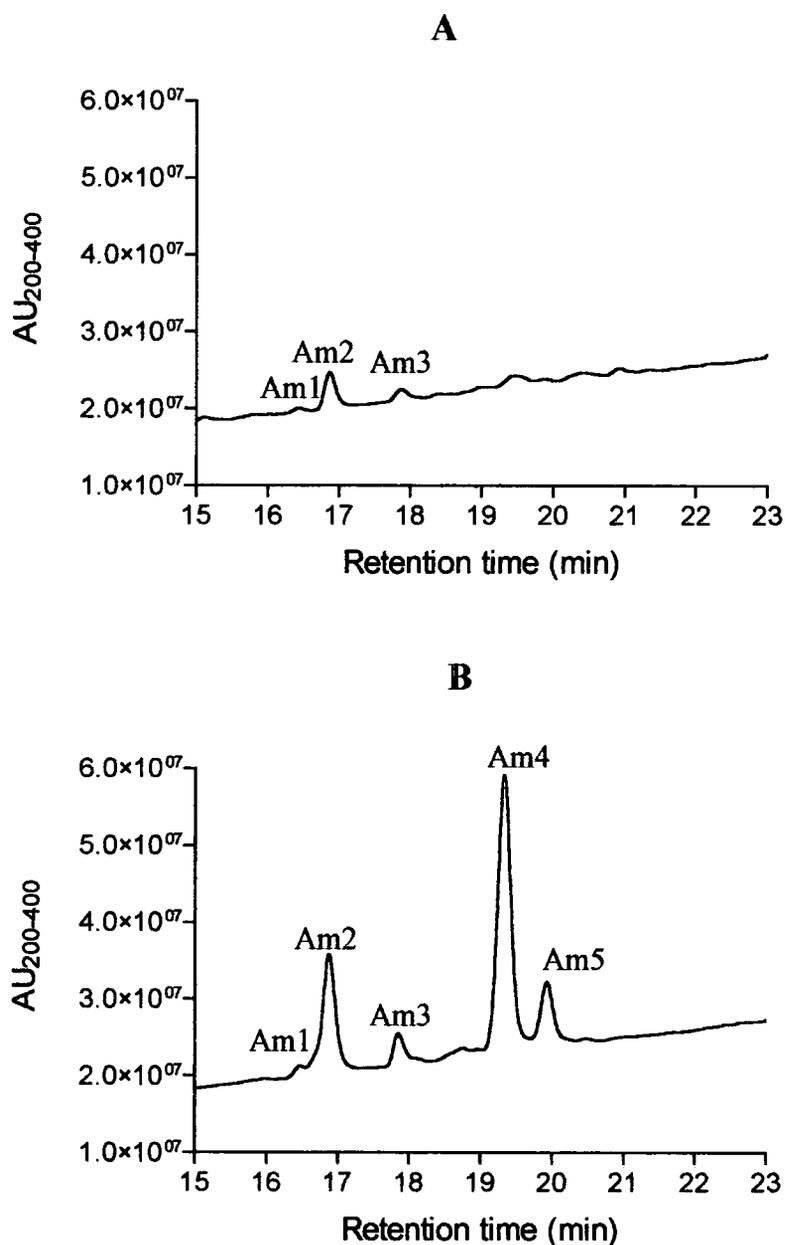


Figure 39 HPLC profiles of acetone/methanol extracts from black-grass shoots. UV absorbance was monitored between 200 nm and 400 nm and the summative spectrum is displayed. Profiles are (A) herbicide susceptible and (B) herbicide resistant black-grass. Extracts were digested with β -glucosidase.

Table 12 Compilation of retention time, UV spectral and molecular mass data for the five major UV absorbing peaks found in β -glucosidase digested acetone/methanol extracts from black-grass shoots as compared with authentic standards and reference data.

Peak no.	Identity of peak	Retention time (min)		UV spectral data (λ_{max} nm)		Molecular mass (Da)	
		Extract	Standard	Extract	Standard or reference*	Extract	Reference#
Am1	Isoorientin	16.8	N/A	255, 270, 349	242sh, 255, 271, 349*	448.22	448.34
Am2	2"-O-Xylosylvitexin	17.2	N/A	271, 300sh, 337	270, 301sh, 335*	564.28	564.50
Am3	Isovitexin	18.2	N/A	271, 337	271, 336*	432.25	432.39
Am4	4-Hydroxycinnamic acid	19.8	19.7	295sh, 310	295sh, 309	164.15	164.16
Am5	Ferulic acid	20.4	20.4	295sh, 323	295sh, 319	194.18	194.19

N/A indicates that authentic standards or UV spectral data was not available.

* UV spectral data in methanol from (Mabry *et al.*, 1970).

Molecular weights obtained from (Harborne & Baxter, 1995).

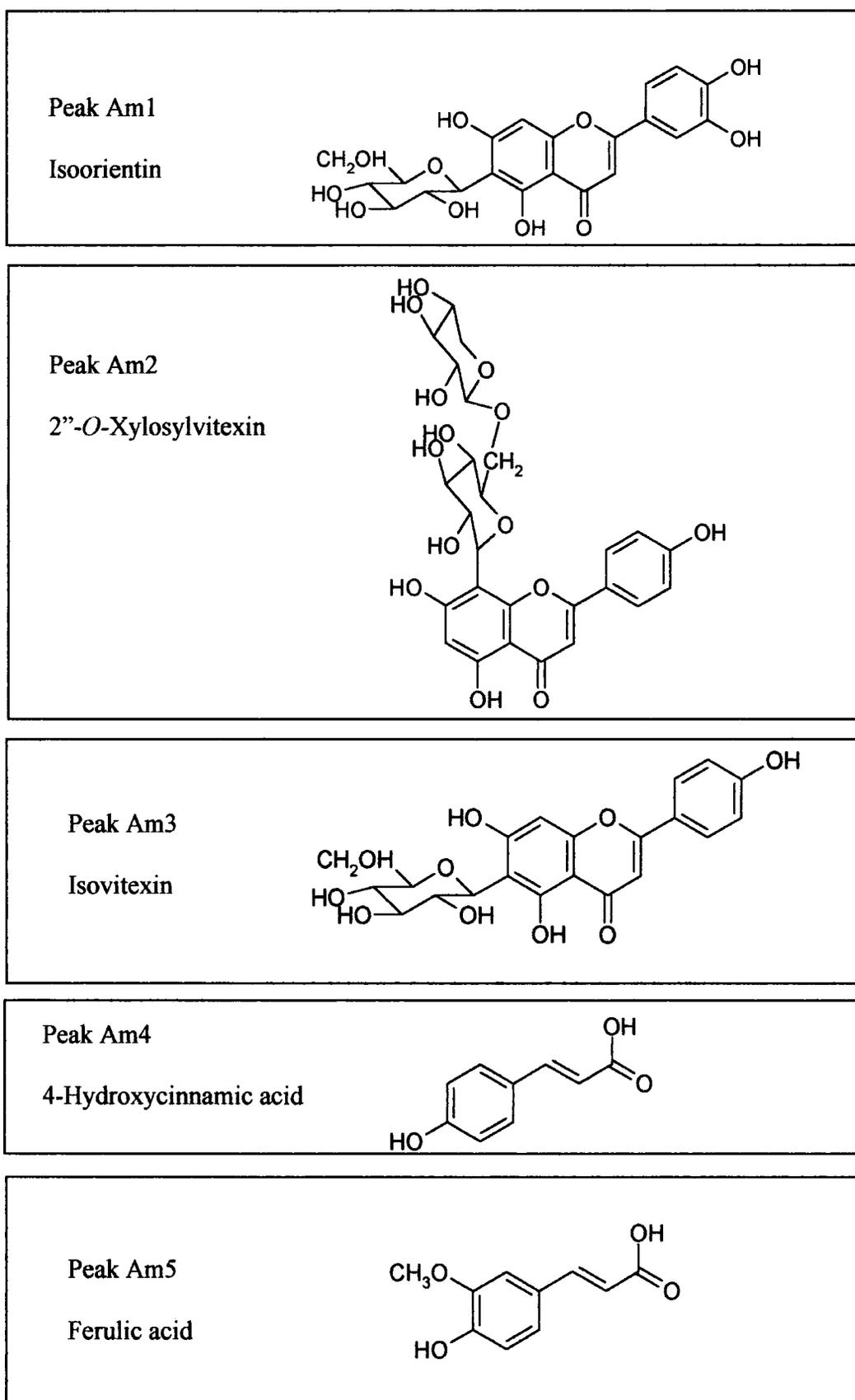


Figure 40 Structures of metabolites identified in extracts from black-grass.

Peak Am1

The concentration of peak Am1 was not affected by β -glucosidase digestion indicating it was not *O*-glycosylated. The UV spectrum and mass of peak Am1 matches that of isoorientin. The retention time of peak Am1 is similar to that of isoorientin from peak Ta2.

Peak Am2

β -glucosidase digestion did not affect peak Am2 suggesting that any sugar moieties present must be attached to the aglycone via a carbon atom. The molecular weight of 456.28 was consistent with an apigenin aglycone with a glucose or galactose and xylose attached. The UV spectrum for 2''-*O*-xylosylvitexin was compared to that for peak Am2 and found to be very similar. Peak Am2 is probably 2''-*O*-xylosylvitexin.

Peak Am3

Peak Am3 was also resistance to β -glucosidase suggesting it was not an *O*-glucoside. The molecular mass was consistent with apigenin aglycone C-conjugated with glucose or galactose. The UV spectrum of Am3 contains peaks at 271 nm and 337 nm but does not contain a shoulder at 302 nm suggesting that the identity of peak Am3 is isovitexin rather than vitexin.

Peak Am4

Peak Am4 was only present in β -glucosidase digested extracts suggesting the respective metabolites were *O*-glycosylated *in planta*. The retention time, UV

spectrum and molecular weight of peak Am4 were consistent with it being 4-hydroxycinnamic acid.

Peak Am5

The retention time, UV spectrum and molecular weight of peak Am5 suggested it is ferulic acid. It was only detected in β -glucosidase extracts and would be expected to accumulate as *O*-glycosylated derivatives.

6.2.2 Quantification of major metabolites in extracts from black-grass

To quantify metabolites in extracts from black-grass, naringin was used as a reference standard for isoorientin, 2''-*O*-xylosylvitexin and isovitexin. 4-Hydroxycinnamic acid and ferulic acid were quantified using authentic standards. Comparison of the concentration of the flavonoid derived metabolites (Am1, Am2 and Am3) revealed that all three metabolites had accumulated to higher concentrations in extracts from herbicide-resistant black-grass than in extracts from herbicide-susceptible black-grass (Table 13). Increases in concentration of 2.5-fold, 5.7-fold and 3.7-fold were observed toward isoorientin, 2''-*O*-xylosylvitexin and isovitexin respectively in herbicide-resistant extracts compared to herbicide-susceptible extracts. 4-Hydroxycinnamic acid and ferulic acid, which were abundant in the herbicide-resistant weeds, were not detected in herbicide-susceptible plants.

Table 13 Quantification of major phenolic metabolites found in extracts from herbicide-susceptible and herbicide-resistant black-grass shoots. All values are means \pm variation in duplicated determination.

Peak no.	Identity of peak	Concentration of metabolites in black-grass shoots (nmol g ⁻¹ Fresh weight)	
		Herbicide-susceptible	Herbicide-resistant
Am1	Isoorientin	13.6 \pm 2.2	35.1 \pm 1.0
Am2	2"-O-Xylosylvitexin	126.6 \pm 25.3	665.4 \pm 62.0
Am3	Isovitexin	45.4 \pm 9.1	155.6 \pm 15.1
Am4	4-Hydroxycinnamic acid	-	851.6 \pm 249.5
Am5	Ferulic acid	-	100.9 \pm 1.6

6.3 L-Phenylalanine ammonia lyase (PAL) activity in wheat shoots and black-grass

As phenylalanine ammonium lyase (PAL) is a key regulatory enzyme of both phenylpropanoid and flavonoid synthesis (Dixon & Palva, 1995) the activities of PAL were determined in the herbicide-susceptible and herbicide-resistant black-grass plants (Fig 41). The activity of PAL was also determined in cloquintocet mexyl treated and untreated wheat shoots. PAL activity was approximately 20 % lower in cloquintocet mexyl treated wheat shoot protein extracts compared with extracts from the untreated plants. In black-grass, PAL activity was about 30 % higher in herbicide-resistant crude protein extracts than in herbicide-susceptible extracts.

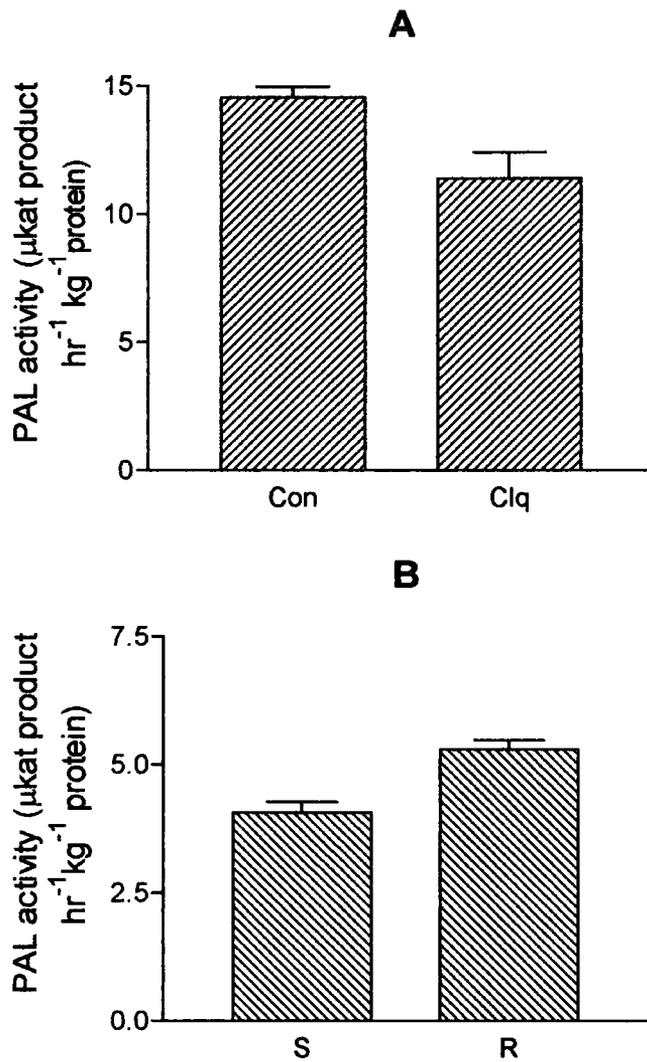


Figure 41 PAL activities in **A.** untreated (con) and cloquintocet mexyl treated (clq) 10 day old wheat shoots and **B.** herbicide susceptible (S) and herbicide resistant (R) 21 day old black-grass shoots. Values represent the means of duplicate determinations with the error bars showing the variation in the replicates.

Discussion

In wheat, four phenolic natural products and their respective glycosides were identified. The flavonoids isoorientin and triclin have been previously observed in methanolic extracts from wheat leaves (Estairte *et al.*, 1999) while the 7-C-glucuronide of triclin has been found in one of the progenitors of wheat, *Triticum dicoccum* (Harbourne & Baxtor, 1995). 4-Hydroxycinnamic acid and ferulic acid were also positively identified using authentic standards. Glycosylated ferulic acid was detected and was most probably the glucose ester as reported in wheat flour (Labat *et al.*, 2000). 4-Hydroxycinnamic must also have been glycosylated as it was only detectable in β -glucosidase digested extracts. Sinapoyl malonate was identified on the basis of fragmentation data, while Ta6 was tentively identified as 3,4-dihydroxycinnamaldehyde. Four of the five metabolites determined in black-grass have previously been observed in wheat. Thus isoorientin, 4-hydroxycinnamic acid and ferulic acid were also identified in wheat while isovitexin has previously been reported as a flavonoid metabolite in wheat leaves (Estairte *et al.*, 1999).

The first step in phenylpropanoid biosynthesis involves the conversion of L-phenylalanine into *trans*-cinnamic acid by L-phenylalanine ammonium lyase (PAL). PAL activity in response to herbicide safener treatment in wheat and herbicide resistance in black-grass was measured to see if this accounted for the accumulation of 4-hydroxycinnamic acid and ferulic acid in both plants. In wheat, PAL activity was decreased slightly in cloquintocet mexyl-treated shoots. Chemical treatments have been reported to reduce PAL activity in other plants. For example, treatment of *Arabidopsis thaliana* leave slices with heavy metals and the herbicide glyphosate has

also found to result in a decrease in PAL activity (Park *et al.*, 1999). PAL activity was 30 % greater in herbicide-resistant compared to herbicide-susceptible black-grass. It is therefore possible that this increase in PAL activity could contribute to the enhanced accumulation of 4-hydroxycinnamic acid in herbicide-resistant black-grass. Significantly, 4-hydroxycinnamic acid is an intermediate in biosynthesis of both lignin and flavonoids.

Wheat plants grown in a CO₂ enriched atmosphere have been found to accumulate increased concentrations of isoorientin in their leaves (Estairte *et al.*, 1999), suggesting that the synthesis of isoorientin can be up-regulated in response to environmental stresses. Isoorientin and to a lesser extent isovitexin have been found to have antioxidative properties in the fruits of *Boreava orientalis* (Sakushima *et al.*, 2000) as have some components of the phenylpropanoid pathway including 4-hydroxycinnamic acid (Grace & Logan, 2000). It is interesting to speculate that herbicide safener applications in wheat and the acquisition of herbicide-resistance in black-grass effectively increase the content of protective phenolic antioxidants. This in turn may contribute to the increased herbicide tolerance seen in both cases through suppression of the damage caused by herbicides (Cummins *et al.*, 1999).

7. DISCUSSION AND FUTURE WORK

Prior to this study little was known about OGTs in wheat or black-grass. OGT activities toward synthetic phenols and flavonoids substrates were observed in crude extracts from both wheat and black-grass. However it should be noted that these substrates are probably the most reactive under the assay conditions used and therefore not a true reflection of the breadth of OGT activities *in planta*. The specific activities values quoted for these substrates were also an underestimation of the catalytic efficiency of the OGTs due to the use of low concentrations of UDP-[¹⁴C-glucose] to avoid radioisotope dilution.

Purification of OGT activity toward 2,4,5-trichlorophenol resulted in the detection of one major isoenzyme, which was associated with activity toward 3-hydroxyflavone, suggesting that the purified OGT could be a 3-*O*-glucosyltransferase. As MALDI-TOF analysis was unable to identify any polypeptides from the purified OGT, overexpression and characterisation of a recombinant bronze-1 flavonoid 3-OGT homolog from wheat would be useful to confirm the substrate specificities identified in the purified OGT. Most of the 3-*O*-GTs whose catalytic activities have been demonstrated show great sequence similarity and the majority are members of group F (Jones & Vogt, 2000). The two previously reported recombinant OGTs with activities toward xenobiotics, the arbutin synthase from *Rauvolfia serpentina* (Hefner *et al.*, 2002) and UGT75D1 from *Arabidopsis* (Shäffner *et al.*, 2002), are members of group E and group L respectively. This suggests GTs with differing endogenous roles have evolved the ability to glucosylate xenobiotics in different plant species.

However, it is not known how widespread the ability to conjugate xenobiotics is throughout the GT-1 family. Systematic screening of all the members of the GT-1 family for activity toward xenobiotics in a plant species such as *Arabidopsis* would allow insight into the mechanisms used by plants to detoxify xenobiotics.

This project focused on the activity of xenobiotic-detoxifying OGTs, however with respect to natural products, in wheat the majority of flavonoids are derivatives of C-glycosides (Estairte *et al.*, 2000). Analysis of the metabolite profile of black-grass in this study has suggested that black-grass also contains C-glycosides. This may be an important factor when trying to identify the true substrate specificity of the recombinant *TaGT* and *AmGT*. Very little work has been reported on the enzymes responsible for the formation of C-glycosides in plants, although the reaction is interesting due to difficulty of the formation of the respective linkage. A C-GT has been purified from *Fagopyrum esculentum* and shown to glucosylate the 6 or 8-C atom of 2-hydroxyflavone precursors leading to the formation of vitexin or isovitexin respectively (Kersher & Franz, 1988). Activity of the C-GT is confined to the precursors of 2-hydroxyflavones indicating the importance of testing *TaGT* and *AmGT* with a wide range of potential substrates. To enhance the likelihood of identifying the true substrate specificity of *TaGT* and *AmGT*, the recombinant GTs could be assayed with metabolites extracted from wheat or black-grass, however sugars would have to be removed to form aglycones. β -glucosidases able to remove *O*-glycosides are commercially available. To encourage the correct folding and post-translational modifications *TaGT* and *AmGT* could be overexpressed in *Arabidopsis* and then purified using the His-tag. In addition, examination of the metabolite profile

of the transgenic *Arabidopsis* plants compared to that of wildtype *Arabidopsis* could lead to identification of the substrate specificity of *TaGT* and *AmGT*.

It is well established that treatment with herbicide safeners can result in enhancement of CYP and GST activities (Davies & Caseley, 1999) and can also increase the rate of glycosylation of herbicide metabolites (Kreuz *et al.*, 1991; Lamoureux & Rusness, 1992). The current work has also shown an increase in OGT activities as a result of treatment with herbicide safeners. All the activities toward the two xenobiotics and four flavonoids were increased significantly. It is not known how many isoenzymes were represented within the activities towards these six compounds and thus how many GTs activities are enhanced by safener treatment in wheat. Immunological studies or northern blotting could help determine whether a subset of GTs are safener-inducible as for the GSTs in wheat (Cummins *et al.*, 1997; Riecher *et al.*, 1997). It would also be of interest to establish if exposure to other abiotic stresses resulted in a similar enhancement of OGT activities. Some variation in the effect of chemical treatment on GT activities and GST activities was observed when wheat was treated with luteolin and coumesterol suggesting that the two different families of enzymes are under related but subtly differently regulated control. The analysis of the metabolite profile of wheat treated with safener found that the plant was accumulating antioxidant compounds, such as phenylpropanoids and the respective conjugates. This could allow the plant not only to counter the effects of oxidative stress caused by the safener, but also protect against damage caused by herbicide treatment prior to the detoxification of the herbicide.

Higher levels of OGT activity toward all substrates tested were in found in herbicide-resistant populations of black-grass as compared to the herbicide-susceptible wildtype populations of black-grass. This could be due to the natural variation in activities between different populations of black-grass. However as CYPs and GSTs activities are elevated in herbicide-resistant black-grass this suggests the enhancement of GT activities is part of a coordinated up-regulation of detoxifying enzymes in the Peldon population (Hyde *et al.*, 1996; Cummins *et al.*, 1999). A further exploration of the factors involved in herbicide-resistance in black-grass would be of interest. Construction of subtractive libraries would allow the identification of enzymes up-regulated by the acquisition of herbicide resistance. Previously, treatment of the black-grass with the wheat safener fenchlorozole ether was found to enhance the level of GST activity in the herbicide-susceptible wildtype population (Cummins *et al.*, 1999). This study found that treatment with a second wheat safener cloquintocet mexyl resulted in a minor decrease in OGT and GST activity in both herbicide-resistance and herbicide-susceptible populations. It would be interesting to determine whether the choice of safener has any impact on the control of black-grass in the field.

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