Differential Induction of Glutathione Transferases and Glucosyltransferases in Wheat, Maize and *Arabidopsis thaliana* by Herbicide Safeners

Robert Edwards[a,*], Daniele Del Buono[b], Michael Fordham[a], Mark Skipsey[a], Melissa Brazier[a], David P. Dixon[a], and Ian Cummins[a]

[a] School of Biological & Biomedical Sciences, University of Durham, Durham, DH1 3LE UK. Fax: 0044 191 334 1201. E-mail: Robert.Edwards@durham.ac.uk

[b] Dipartimento di Scienze Agroambientali e della Produzione Vegetale, Università degli Studi di Perugia, Borgo XX Giugno 72, 06121, Perugia, Italy

* Author for correspondence and reprint requests

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By learning lessons from weed science we have adopted three approaches to make plants more effective in phytoremediation:

1. The application of functional genomics to identify key components involved in the detoxification of, or tolerance to, xenobiotics for use in subsequent genetic engineering/breeding programmes.
2. The rational metabolic engineering of plants through the use of forced evolution of protective enzymes, or alternatively transgenesis of detoxification pathways.
3. The use of chemical treatments which protect plants from herbicide injury.

In this paper we examine the regulation of the xenome by herbicide safeners, which are chemicals widely used in crop protection due to their ability to enhance herbicide selectivity in cereals. We demonstrate that these chemicals act to enhance two major groups of phase 2 detoxification enzymes, notably the glutathione transferases and glucosyltransferases, in both cereals and the model plant *Arabidopsis thaliana*, with the safeners acting in a chemical- and species-specific manner. Our results demonstrate that by choosing the right combination of safener and plant it should be possible to enhance the tolerance of diverse plants to a wide range of xenobiotics including pollutants.

Key words: Herbicide Safeners, Phase 2 Detoxification, Phytoremediation

Introduction

Differential rates of detoxification are a primary determinant of herbicide selectivity in crops and weeds, with metabolism occurring in four phases (Owen, 2000). In phase 1, functional groups are either introduced or revealed in the herbicide by hydrolases (Cummins and Edwards, 2004) or cytochrome P450 mixed function oxidases (CYPs) (Werck-Reichhart and Feyereisen, 2000), respectively. In phase 2, herbicides or their phase 1 metabolites are conjugated with either glucose by the action of glucosyltransferases (GTs) (Loutre *et al.*, 2003) or with the tripeptide glutathione as catalysed by the glutathione transferases (GSTs) (Edwards and Dixon, 2000). In phase 3, conjugates are then actively transported into the vacuole (Rea *et al.*, 1998) prior to phase 4 metabolism consisting of incorporation into bound residues (Skidmore, 2000). By considering these tiers of metabolism as part of a concerted process we have evolved the concept of the 'xenome', defining it as 'the biosystem responsible for the detection, transport and detoxification of xenobiotics'. Because of its importance in determining herbicide tolerance, there have been many reports of xenome manipulation in crops to improve selectivity. One successful approach has been to use genetic engineering to increase the expression of key xenome components. Using recombinant technology detoxifying CYPs (Inui *et al.*, 1999; Siminszky *et al.*, 1999), GSTs (Milligan *et al.*, 2001) and transporter proteins (Rea *et al.*, 1998) have all been over-expressed in plants and shown to confer increased tolerance to herbicides and other toxic xenobiotics. In addition, foreign xenome components have also been introduced into plants from bacteria to effect detoxification reactions not normally found in plants (reviewed by Cole and Rodgers, 2000).

While these experiments in engineering herbicide resistance have been technically successful, their reliance on genetic modification technology has prevented their universal adoption. In contrast, the use of chemicals which manipulate herbicide metabolism and enhance selectivity is in com-

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mon use world-wide, notably through the use of safeners, formerly known as antidotes (Hatzios, 2003). In cereal crops, safeners enhance tolerance to herbicides by increasing the rates of their detoxification (Davies and Caseley, 1999). This is achieved through the induction of key components of the xenome, notably proteins involved in the first three phases of metabolism (Davies and Caseley, 1999; Theodoulou *et al.*, 2003). Interestingly, safeners do not protect dicotyledonous crops or non-domesticated grass weeds form herbicide-imposed phytotoxicity, and this has led to the assumption that the induction of xenome components is only seen in large grained cereals (Davies and Caseley, 1999).

GSTs are the best studied inducible xenome components and can be divided into six classes, with the lambda (GSTL), phi (GSTF) and tau (GSTU) classes all associated with safener induction in wheat (Cummins *et al.*, 1997, 2003; Pascal and Scalla, 1999; Riechers *et al.*, 1997; Theodoulou *et al.*, 1999) and maize (Dixon *et al.*, 1998; Hershey and Stoner, 1991; Jepson *et al.*, 1994). In a recent elegant proteomics study, a comprehensive set of GSTFs, GSTLs and GSTUs were all shown to be upregulated in the coleoptiles of seedlings of *Triticum tauschii* treated with the safener fluxofenim (Zhang and Riechers, 2004). Of the safener-inducible GSTs, the GSTUs and GSTFs have been the most studied due to their roles in herbicide detoxification, reviewed by Edwards and Dixon (2000). In addition to *Triticum* species and maize, safener-inducible GSTUs and GSTFs have also been reported in barley (Scalla and Roulet, 2002), rice (Deng and Hatzios, 2002) and sorghum (Gronwald and Plaisance, 1998).

We now report on the relative induction of GSTFs and GSTUs in different plant species by the diverse safeners which have been developed for use in major cereal crops. In most of the induction studies in cereals cited above, single safeners were used and the enhancement of specific GSTs studied. We have investigated whether or not the induction of GSTs by a given safener is species-specific and determined the relative enhancement of GSTUs and GSTFs in each case. To extend this study of differential induction of phase 2 xenome enzymes, we have also studied the enhancement of specific GT activities, having recently reported that the sugar conjugation of pesticide metabolites is induced in extracts from safener-treated wheat plants (Brazier *et al.*, 2002). The design of the study reported here has been to take 8 safeners developed for use in maize, sorghum, rice and wheat, and determine their effect on the enhancement of GSTs and GTs in seedlings of two cereal species (maize and wheat) and cultures of *Arabidopsis thaliana.* Recent studies have shown that GSTs in *Arabidopsis* are responsive to safeners, most notably benoxacor (DeRidder *et al.*, 2002; Smith *et al.*, 2004) and we have been interested in further studying the effect of safeners on the xenome of this model dicotyledonous plant using both plants and suspension cultured cells.

Materials and Methods

Safeners were prepared as 40 mM stock solutions in acetone (Loutre *et al.*, 2003), and applied to the cereals after a 1000-fold dilution with distilled water, giving a final treatment concentration of 40 μM. For treatment of root or suspension cultures (50 ml), stock solutions were again diluted 1000-fold on addition to the medium. Seeds of wheat (*Triticum aestivum* L. cv. Hunter = *Ta*) and maize (*Zea mays* L. DK 250 = *Zm*) were obtained from Aventis and treated with safener and grown in an environmental growth chamber as described previously (Cummins *et al.*, 1997). Shoots were harvested on day 6 (wheat) and day 7 (maize). Plant cultures of *Arabidopsis thaliana* (Ecotype Columbia) and suspension cultures were initiated and maintained as described previously (Loutre *et al.*, 2003). In both cases cultures were treated with the safener benoxacor for 24 h.

On harvest, plant tissue was blotted (plants), or filtered under vacuum (cultures) to remove excess water, weighed, frozen in liquid nitrogen and stored at – 80 °C. Frozen tissue was homogenised to a powder using a mortar and pestle and extracted in 3 v/w of extraction buffer (50 mM Tris-HCl, pH 7.5) containing 2 mM EDTA, 1 mM dithiothreitol (DTT) and 2% (w/v) polyvinylpolypyrrolidone. After filtering through nylon mesh (pore size 120 μm), the homogenate was centrifuged (11000 × *g*, 20 min, 4 °C) and the supernatant collected. The protein concentration was determined using BioRad dye-binding reagent and then normalised prior to assay for GST activity and polypeptide composition. The protein in the remaining supernatant was precipitated by the addition of $(NH_4)_2SO_4$ to 80% saturation and recovered by centrifugation (13000 × *g*, 20 min, 4 °C).

GST activity was determined in crude plant extracts using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Dixon *et al.*, 1998). After desalting, the ammonium sulphate precipitated proteins were used to assay for GT activity toward 3,4-dichloroaniline (DCA) and 2,4,5-trichlorophenol (TCP) as described previously (Brazier *et al.*, 2002). Concentrated protein preparations were also used to assay for GST activity toward the chloroacetanilide herbicide metolachlor and benoxacor using the HPLC-based assay described by Hatton *et al.* (1996).

Protein extracts were analysed by SDS-PAGE with each gel Western blotted with antisera raised to either the maize phi *Zm*GSTF1–2 (Dixon *et al.*, 1998) or the wheat tau *Ta*GSTU1–1 (Cummins *et al.*, 2003). Western blots were digitised for quantification and the integrated density across the bands measured using Gel scanning software (Quantity One, Bio-Rad Laboratories, USA). Band density was expressed as a proportion of the intensity of the corresponding polypeptide determined in the control treatment.

For the benoxacor-induction studies, *Arabidopsis* cell suspension cultures were treated with the safener (final concentration 100 μM) 4 days after sub-culturing. In the controls, solvent carrier alone was added (0.5 ml acetone). After 24 h, cultures were further treated with benoxacor (100 μM), CDNB (50 μM) or the chloroacetanilide herbicide metolachlor (10 μM). At timed intervals, the medium was collected by vacuum filtration and 10 ml applied to a C-18 solid phase extraction cartridge (500 mg) which had been pre-washed with methanol (10 ml) followed by water (10 ml). After washing with water (2 ml) and water/methanol (4:1, v/v, 1 ml) parent compounds were recovered in methanol (2 ml) and analysed by HPLC (Hatton *et al.*, 1996).

For the proteomic analysis of safener induced GSTs, crude protein from *Arabidopsis* cell cultures was applied to a glutathione affinity column (Cummins *et al.*, 2003). Affinity-purified proteins were acetone-precipitated, then redissolved in 340 μl IEF buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM DTT, 0.8% pH 4–7 NL ampholytes, 0.002% w/v bromophenol blue), and subjected to IEF on 7 cm pH 4–7 NL Immobiline DryStrips using a Multiphor II flatbed electrophoresis system (Amersham). Following IEF, strips were washed in 2nddimension buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue) containing 1% w/v DTT (15 min, 20 °C) followed by 2nd dimension buffer containing 2.5% w/v iodoacetamide (15 min, 20 °C) prior to electrophoresis on an ExcelGel 2-D homogeneous 12.5% acrylamide gel (Amersham). Gels were silver stained and major polypeptide spots picked, digested with trypsin and analysed on an Applied Biosytems Voyager DE-STR MALDI-TOF mass spectrometer (Chivasa *et al.*, 2002). Resulting peptide mass ions were used to screen a non-redundant protein database using Mascot (http://www.matrixscience.com/).

For HPLC-MS analysis of benoxacor and its glutathione conjugates, reference conjugates were prepared by incubating benoxacor (2 mM) with glutathione (10 mM) in 0.1 M Tris-HCl, pH 8.8. Products were separated by HPLC (Phenonemex Luna ODS2, 150 mm × 2 mm, 3μm) and analysed by diode array detection (210–400 nm) and electrospray-ionisation mass spectrometry (ESI-MS; Micromass LCT) as described previously (Loutre *et al.*, 2003). To determine benoxacor metabolites in *Arabidopsis*, suspension cultures were treated with 100 μM benoxacor for 18 h. Cells were extracted with 5 vol of cold (– 20 °C) methanol and after centrifugation, the debris re-extracted with 1 vol of methanol and the combined solvent concentrated to dryness under reduced pressure. The concentrate was then dissolved in 2 ml methanol and analysed by HPLC-ESI-MS.

Results

Differential enhancement of GST and GT activities by safeners in maize, wheat and Arabidopsis

Seedlings of wheat, maize and sterile plant cultures of *Arabidopsis* were individually exposed to safeners used in maize, rice, wheat and sorghum, respectively, at identical concentrations. The enhancement in GST and GT activity was then determined as compared with plants exposed to solvent carrier alone (Table I). GST activity was determined with the general substrate CDNB, while *O*-glucosyltransferase (*O*GT) activity was determined with TCP and *N*-glucosyltransferase (*N*GT) activity with DCA. In wheat, cloquintocet-mexyl proved to be the most effective xenome inducing agent, enhancing all the GST and GT activities tested. In contrast all safeners except fenclorim gave some enhancement of GST activity but had no effect on *N*GT or *O*GT activities. In maize, with the exception of a modest enhance-

Table I. Effect of safener treatment on GST activities (nkat mg^{-1}protein) and *O*GT and *N*GT activities (pmol product $min^{-1}mg^{-1}$) in crude extracts of wheat and maize shoots and *Arabidopsis* plant cultures. Values are the average of duplicate treatments (± SEM).

			Safener treatment								
	Enzyme	Substrate	Control	Benoxacor	CMPI	Fenclorim	Flurazole	Oxabetrinil	R-29148	Cloquinto-cet-mexyl	Dichloromid
Wheat	*N*GT	3,4-DCA	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.6 ± 0.0	0.3 ± 0.1
	*O*GT	2,4,5-TCP	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.2	0.1 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	1.4 ± 0.2	0.6 ± 0.1
	GST	CDNB	2.7 ± 0.1	3.2 ± 0.1	3.1 ± 0.3	2.3 ± 0.3	3.2 ± 0.5	3.1 ± 0.3	4.2 ± 0.1	4.5 ± 0.3	3.4 ± 0.3
Maize	*N*GT	3,4-DCA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	*O*GT	2,4,5-TCP	0.2 ± 0.0	0.7 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.9 ± 0.0
	GST	CDNB	1.6 ± 0.1	3.3 ± 0.2	1.8 ± 0.1	1.5 ± 0.1	2.9 ± 0.1	1.9 ± 0.2	4.8 ± 0.2	1.7 ± 0.2	4.1 ± 0.6
Arabidopsis	*N*GT	3,4-DCA	6.0 ± 0.4	7.4 ± 0.6	9.2 ± 0.4	6.1 ± 0.6	7.5 ± 0.9	6.9 ± 0.5	6.0 ± 0.2	7.3 ± 0.7	7.1 ± 0.3
	*O*GT	2,4,5-TCP	6.6 ± 0.5	9.2 ± 1.1	11.1 ± 0.8	10.1 ± 0.6	9.0 ± 0.5	7.8 ± 0.3	6.8 ± 0.2	9.7 ± 1.8	11.1 ± 0.4
	GST	CDNB	0.7 ± 0.1	2.7 ± 0.4	2.6 ± 0.1	5.2 ± 0.1	2.7 ± 0.5	1.1 ± 0.1	1.1 ± 0.1	1.8 ± 0.1	1.0 ± 0.1

n.d., not determined.

ment of GST activity determined with flurazole only the maize safeners benoxacor, dichloromid and R-29148 increased conjugating activity toward CDNB. However, all safeners except CMPI enhanced *O*GT activity. Thus, whereas in wheat the greatest chemical selectivity in safening was seen in GT induction, this selective induction was seen with the GSTs in maize. *Arabidopsis* plant cultures were more responsive to induction by safeners than either of the cereals. Enhancement of GST activity was greatest with the rice safener fenclorim, with flurazole and CMPI (used in sorghum) and benoxacor (maize) also giving significant increases. Enhancement of *O*GT activities was also seen, with the safeners most active in inducing GSTs also giving the largest increase in glucosylation. One notable difference was the induction of *O*GT activity by dichloromid, with this compound showing negligible GST enhancing activity in this species. *N*GT activity was more selectively induced by safeners. For example CMPI induced both *O*GT and *N*GT activity, whereas fenclorim only increased *O*GT activity.

Selective induction of GSTUs and GSTFs by herbicide safeners

The availability of class-specific antisera raised to tau and phi GSTs provided a further tool to dissect the responsiveness of the GST protein family to the 8 safeners. Protein extracts from safener-treated wheat, maize and *Arabidopsis* plants were resolved by SDS-PAGE and then Western-blotted using antisera raised to either *Zm*GSTF1–2 or *Ta*GSTU1–1, which are specific for phi and tau GSTs, respectively, in both wheat and maize (Cummins *et al.*, 2003). These antisera had not been employed previously in *Arabidopsis*, so for reference, the recombinant tau *At*GSTU19, which had been identified and cloned as a major safener-inducible GST in this model plant (DeRidder *et al.*, 2002; Smith *et al.*, 2004), was included. The antisera to the tau GST recognised *At*GSTU19, while the antibody raised to the more distantly related maize phi GST did not, thus confirming the class specificity of the respective antisera (Fig. 1). To demonstrate the changes in GST polypeptide composition in each species on safening, extracts from the plants treated with their optimal GST-inducing chemical treatment are shown as Western blots (Fig. 1). For comparative purposes, the individual immuno-recognised polypeptides were digi-

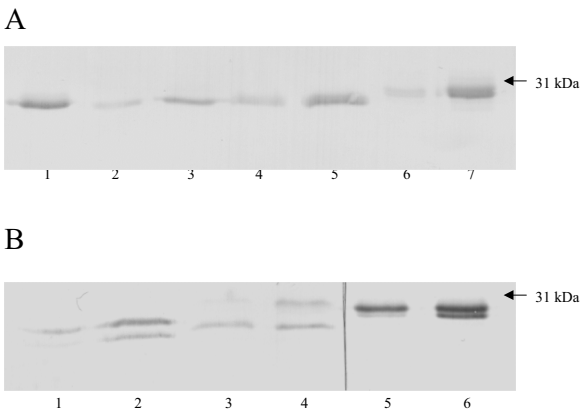

Fig. 1. Western blotting of polypeptides resolved by SDS-PAGE using antisera raised to (A) the tau class wheat *Ta*GSTU1–1 and (B) the phi class maize *Zm*GSTF1–2. Lane 1, *At*GSTU19; lanes 2/3, *Arabidopsis* roots untreated/fenclorim-treated; lanes 4/5, wheat shoots untreated/cloquintocet-mexyl-treated; lanes 6/7, maize shoots untreated/benoxacor-treated.

tised, scanned and the total intensity of each band quantified for all safener treatments (Fig. 2). While the anti-GSTU serum recognised single polypeptide species in each plant, the anti-GSTF-serum recognised multiple GST subunits in each case (Fig. 1).

Quantification of the blots provided defined the relative induction of GSTs by each safener. In *Arabidopsis*, fenclorim proved to be the optimal enhancer of both *At*GSTUs and *At*GSTFs, consistent with the enzyme activity induction data (Table I). *At*GSTUs were generally more safener-inducible than the *At*GSTFs following treatment with benoxacor, CPMI and flurazole. Interestingly, cloquintocet-mexyl enhanced the content of *At*GSTUs while having no effect on *At*GSTFs. In wheat, in agreement with the enzyme data, cloquintocet-mexyl was shown to be the optimal inducer of *Ta*GSTFs. However, when considering the enhancement of *Ta*GSTUs, the maize safeners dichloromid and R-29148 showed similar inducing activity, even though these compounds were ineffective in increasing CDNB-conjugating activity. These results suggest that in wheat safener-inducible GSTUs must have relatively little activity in conjugating CDNB. In maize, both *Zm*GSTUs and *Zm*GSTFs showed virtually identical patterns of induction, with the maize safeners dichlormid and R-29148 giving optimal enhancement of both classes.

Safening of Arabidopsis cell cultures by benoxacor

Recent studies have reported that the safener benoxacor induces the expression of *At*GSTUs and *At*GSTFs in *Arabidopsis* plant cultures (Smith *et al.*, 2004). To determine the mechanism and functional significance of this induction in greater detail, *Arabidopsis* suspension cultures were pre-treated ± 100 μM benoxacor and then fed with xenobiotics, which undergo *S*-glutathionylation as a primary route of metabolism. The xenobiotics selected were the well characterised GST substrates CDNB and the chloroacetanilide herbicide metolachlor (Edwards and Dixon, 2000) and benoxacor, which is known to rapidly metabolise to glutathionylated conjugates in maize cell cultures (Miller *et al.*, 1996a,b). As the xenobiotics were only available in unlabelled form, the rates of detoxification were determined by monitoring the disappearance of parent compound from the medium over a 24 h period. Pre-treatment with safener significantly increased the rate of uptake of both metolachlor and benoxacor, leading to a doubling in the uptake rate over the first 8 h (Table II). With CDNB, uptake was so rapid in the untreated cells that any effect due to the safener was not detected. Interestingly, the uptake of benoxacor by the cells which had not been pre-treated with safener largely occurred between 8–24 h. This was consistent with the compound acting to induce its own detoxification after an 8 h lag period.

The feeding studies confirmed that a pre-treatment with benoxacor increased the rate of uptake of the safener in *Arabidopsis* suspension cultures, but did not address whether or not this disappearance involved the metabolism of the parent compound. In maize cell suspension cultures benoxacor is rapidly metabolised by conjugation with glutathione (Miller *et al.*, 1996a,b). Using the published data from these metabolism studies, reference benoxacor metabolites were synthesised and characterised by HPLC-MS. The most abundant products derived from this chemical conjugation were the formylcarboxamide derivative ([MH^+] = 206.2 Da) together with a compound ([MH^+] = 529.1 Da) corresponding to a mono-glutathione conjugate of 4-(chloroacetyl)-3,4-dihydro-3-methyl-*2H*-1,4-benzoxazine which contained two additional oxygen atoms (Miller *et al.*, 1996b) . Using these reference benoxacor metabolites, extracts from the suspension cultures treated with the safener for 16 h were then analysed by HPLC-ESI-MS. No parent benoxacor was identified, demon-

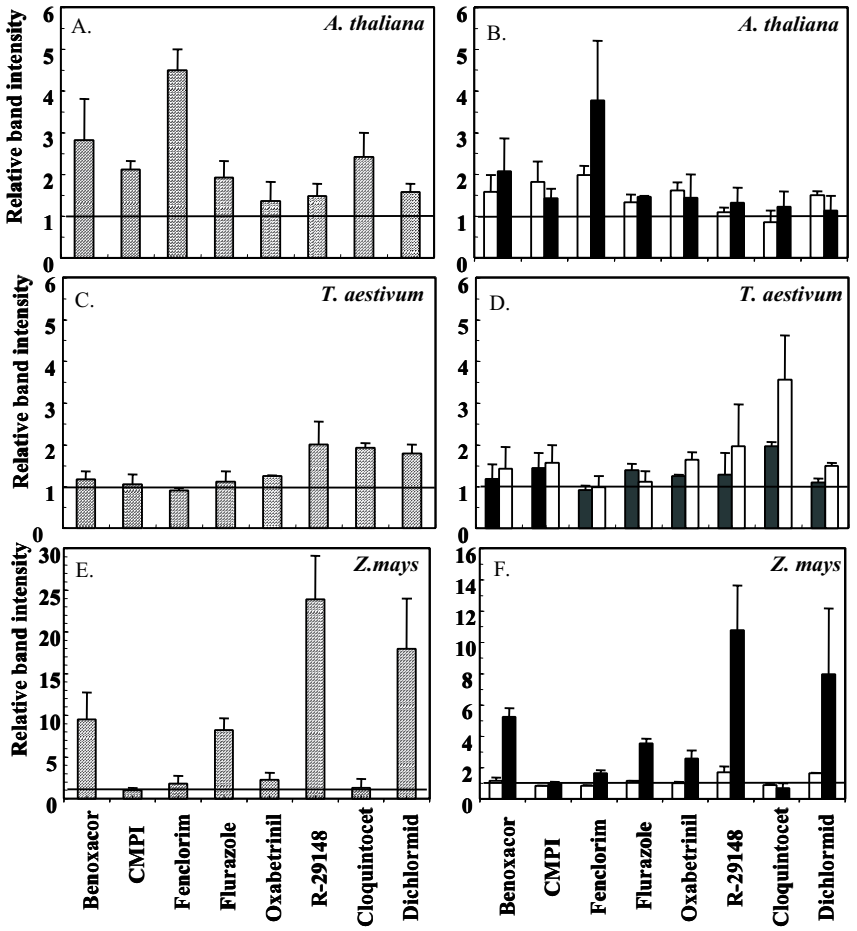

Fig. 2. Quantification of tau and phi class GSTs in *Arabidopsis*, wheat and maize following safener treatment. The immunodetected polypeptides shown in Fig. 1 were digitally quantified following SDS-PAGE and Western blotting using antisera raised to the tau *Ta*GSTU1–1 (A, C, E) and the phi *Zm*GSTF1–2 (B, D, F). With the anti-*Zm*GSTF1–2 serum it was possible to resolve and quantify two polypeptide subunits; high molecular weight (open symbol) and lower molecular weight (closed). Values are the means of duplicate treatments (± SEM).

Table II. Effect of a 24 h pre-treatment with benoxacor on xenobiotic uptake in *Arabidopsis* suspension cultures. Owing to differences in phytotoxicity determined in preliminary studies, cultures were treated with 50 μM CDNB, 10 μM metolachlor or 100 μM benoxacor. Values represent means of duplicated studies ± SEMs.

Xenobiotic	Benoxacor pre-treated (+/–)	% parent in medium 0 h	4 h	8 h	24 h
CDNB	–	100	11 ± 2	10 ± 1	ND
	+	100	9 ± 1	ND	ND
Metolachlor	–	100	100 ± 8	74 ± 15	66 ± 1
	+	100	88 ± 16	38 ± 3	30 ± 1
Benoxacor	–	100	93 ± 2	83 ±	ND
	+	100	52 ± 4	28 ±	ND

ND, not detected.

strating that the safener had been metabolised. While the glutathionylated conjugates of the safener could not be detected, the formylcarboxyamide metabolite which is formed as a consequence of *S*-glutathionylation (Miller *et al.*, 1996b), was observed in benoxacor-treated cultures but not in the controls. This confirmed that benoxacor was being rapidly conjugated with glutathione in *Arabidopsis* as demonstrated in maize cell cultures (Miller *et al.*, 1996a,b). To test if *S*-glutathionylated benoxacor could induce GST activity, the safener was incubated with glutathione for 72 h and after removing any unreacted parent compound, the conjugate added to suspension cultures to give an equivalent 100 μM safener treatment. This resulted in no induction in GST activity, suggesting that either the glutathione conjugates were not the active safening entity, or that these metabolites were not able to enter the cells.

Further evidence that benoxacor was inducing its own detoxification through enhancing its conjugation with glutathione was obtained by quantifying the respective GST activity toward the safener as well as CDNB and metolachlor (Table III). As reported for *Arabidopsis* root cultures (DeRidder *et al.*, 2002), benoxacor enhanced GST activity toward both CDNB and metolachlor. With benoxacor as substrate, the major GST-catalysed reaction product co-chromatographed with the oxygenated mono-*S*-glutathionylated reference conjugate. Significantly, benoxacor treatment was seen to increase the GST activity responsible for the detoxification of the safener over five fold (Table III).

Finally, the GSTs induced by safeners in *Arabidopsis* suspension cultures were identified by MALDI-ToF MS proteomics after resolving all of the proteins retained on a glutathione-Sepharose affinity column using 2D-gel electrophoresis (Fig. 3 and Table IV). In *Arabidopsis* root cultures,

Table III. Effect of the safener benoxacor (100 μM) on GST activities toward CDNB, metolachlor and benoxacor in *Arabidopsis* suspension cultures.

Treatment	Extractable GST activity[a] CDNB	Metolachlor	Benoxacor
Control	0.12 ± 0.07	0.017 ± 0.001	0.011 ± 0.002
Benoxacor	0.27 ± 0.06	0.030 ± 0.003	0.057 ± 0.106

[a] Activities refer to means of duplicated experiments ± SEM. Enzyme activity is given as nkat mg^{-1}protein with CDNB as substrate and pkat $mg^{-1}$with metolachlor and benoxacor.

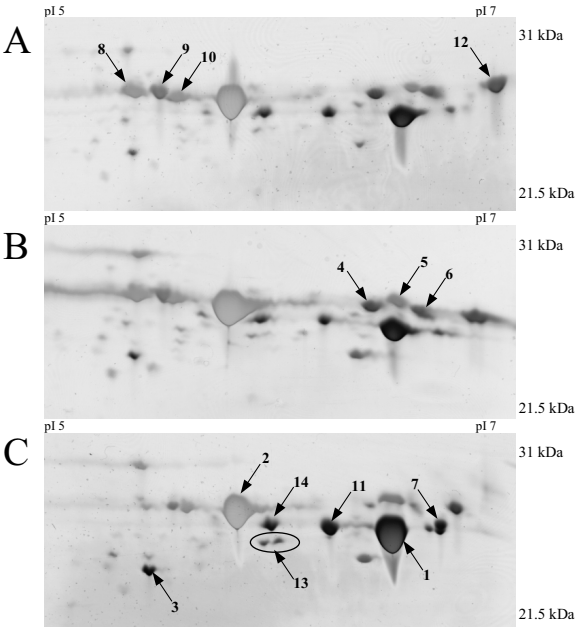

Fig. 3. Proteomic analysis of benoxacor-inducible GSTs in *Arabidopsis* by silver stained two-dimensional gels of glutathione affinity purified proteins extracted from cell cultures treated with acetone (A), 10 μM benoxacor (B) and 100 μM benoxacor (C) for 24 h. For reference, pIs (horizontal) and molecular masses (vertical) of the polypeptides are shown. Numbers associated with the spots on the gel image refer to the proteins identified by MALDI-TOF MS which are listed in Table IV.

*At*GSTU19 was the major safener-enhanced GST following treatment with benoxacor (DeRidder *et al.*, 2002), with *At*GSTF2, *At*GST6, *At*GSTF7 and *At*GSTF8 also being weakly induced (Smith *et al.*, 2004). In suspension cultured cells although *At*GSTU19 (spot 2, 8 and 10) was a major component of the GST proteome, its expression was not upregulated by safener-treatment. Instead, *At*GSTF8 (spot 1, 3, 7, 11 13 and 14) was the most safener-inducible GST, with very minor differences in expression observed for the other four identified GSTs. *At*GSTF8 is also known to be induced in suspension cultures following treatment with salicylic acid (Sappl *et al.*, 2004) and in *Arabidopsis* plants by auxins, ethylene and salicylic acid (Mang *et al.*, 2004; Wagner *et al.*, 2002).

Discussion

The results presented here demonstrate that safeners act in a species- and chemical-specific manner to differentially induce phase 2 detoxifying en-

Table IV. Identification of GST polypeptides by peptide mass fingerprinting as resolved by 2D-electrophoresis.

Spot Number	GST name	MIPS code	MOWSE	Number of matching peptides	Coverage (%)
1	*At*GSTF8	At2g47730	117	13	65
2	*At*GSTU19	At1g78380	85	10	34
3	*At*GSTF8	At2g47730	102	13	57
4	*At*GSTF2	Atg402520	159	16	85
5	*At*GSTF2	Atg402520	66	7	33
6	*At*GSTU4	At2g29420	56	8	35
7	*At*GSTF8	At2g47730	160	13	76
8	*At*GSTU19	At1g78380	109	15	52
9	*At*GSTU5	At2g29450	114	15	48
10	*At*GSTU19	At1g78380	69	11	39
11	*At*GSTF8	At2g47730	105	16	74
12	*At*GSTF9	At2g30860	100	13	60
13	*At*GSTF8	At2g47730	49	6	32
14	*At*GSTF8	At2g47730	121	14	64

Affinity purified GSTs from total protein extracts from *Arabidopsis thaliana* cell cultures treated with acetone or benoxacor (final concentration of 10 μM or 100 μM) for 24 h were separated by 2D-PAGE (see Fig. 3). MOWSE scores of > 65 are statistically significant. (Spot 6 and 13 putative identities are shown despite low MOWSE scores as the best hit for both spots were GSTs).

zymes not only in cereals, where their activity is well known, but also in *Arabidopsis*. In *Arabidopsis* cultures this induction of xenome proteins was associated with an accelerated detoxification of xenobiotics, including herbicides, in a similar manner to that determined in cereals (Davies and Caseley, 1999; Hatzios, 2003). The major distinction between safener activity in the crops and *Arabidopsis* is that increased tolerance to herbicides is only evident in cereals (DeRidder *et al.*, 2002). The failure of safeners to protect dicot plants from herbicides may relate to the tissue specificity of the induction of detoxifying enzymes. In cereals the safener-induction of herbicide-detoxifying GSTs occurs in all tissues, especially in the foliar parts of the plant which are targeted by herbicides (Jepson *et al.*, 1994; Dixon *et al.*, 1998). In fact the tissue-specific expression of safener-inducible detoxifying enzymes may be critical in imparting increased tolerance to herbicides. In *Triticum tauschii*, it has recently been demonstrated that the safener-inducible GSTU largely responsible for detoxifying dimethenamid is selectively expressed in the dermal layers around the coleoptiles, which is the site of action of the herbicide (Riechers *et al.*, 2003). In contrast, whereas herbicide-detoxifying GSTs and GTs can be induced in *Arabidopsis* root and suspension cultures, it is not clear if this induction is also seen in the foliage (DeRidder *et al.*, 2002). Earlier studies in peas demonstrated that whereas GSTs active toward herbicides were inducible in the roots this enhancement was not seen in the shoots (Edwards, 1996). Similarly, our recent studies in soybean have shown that whereas safeners can induce GSTs in the foliage that the enzymes concerned have no activity toward herbicides (Andrews *et al.*, 2005).

Although safener-responsiveness in dicots may not extend to imparting tolerance to herbicides, the fact that *Arabidopsis* (DeRidder *et al.*, 2002; Smith *et al.*, 2004), peas (Edwards, 1996) and tobacco (Yamada *et al.*, 2000) have all been demonstrated to be xenome-responsive to safeners which were developed for use in cereals suggests that this trait must be wide spread in the plant kingdom. While the 'safening' response in dicots may not be useful in crop protection, it may have applications in remediating contaminated soil and water. Thus it could be envisaged that systemic or soil applied safeners could enhance the detoxification of xenobiotics in the roots of useful phytoremediating species. This could be particularly useful in detoxifying water-borne pollutants, with the safeners fed to the plants in the water to be treated.

In the cereals, the detoxifying enzymes were most highly induced by those safeners which had been developed for use in the respective crops. In contrast, *Arabidopsis*, a plant not associated with conventional safening, proved to be the most responsive and least discerning of safener chemistry. This suggests that each safener must have a subtly different site of action, with maize and wheat having the greatest specialization in chemical inducibility and *Arabidopsis* presumably containing multiple chemical 'switches'. The differential induction of GT and GST activities by safeners in each species suggests that these two branches of phase 2 metabolism cannot be activated though one central signalling event. Rather each safener must give rise to individual transduction events,

some of which lead to the induction of both GSTs and GTs and some of which are more restricted in their activation. Based on the diversities of chemistries which show safener activity, their multiplicity in sites of action is not unexpected and this in turn suggests that there is great scope to discover new safeners. In particular, it would be very interesting to screen existing and new chemistries for their xenome-inducing activity in phytoremediating species with the premise that the detoxification potential of each plant may be most effectively enhanced by a specific companion chemical. Future studies will be directed at achieving this goal, with initial studies concentrating on the mode of action of different safeners in *Arabidopsis*.

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