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Xenobiotic Responsiveness of *Arabidopsis thaliana* to a Chemical Series Derived from a Herbicide Safener^{*[S]}

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Mark Skipsey^{†1}, Kathryn M. Knight^{§1,2}, Melissa Brazier-Hicks^{†1,3}, David P. Dixon^{†1}, Patrick G. Steel[§], and Robert Edwards^{†4}

From the [†]School of Biological and Biomedical Sciences and the [§]Department of Chemistry, Durham University, Durham DH1 3LE, United Kingdom

Plants respond to synthetic chemicals by eliciting a xenobiotic response (XR) that enhances the expression of detoxifying enzymes such as glutathione transferases (GSTs). In agrochemistry, the ability of safeners to induce an XR is used to increase herbicide detoxification in cereal crops. Based on the responsiveness of the model plant *Arabidopsis thaliana* to the rice safener fenclorim (4,6-dichloro-2-phenylpyrimidine), a series of related derivatives was prepared and tested for the ability to induce GSTs in cell suspension cultures. The XR in *Arabidopsis* could be divided into rapid and slow types depending on subtle variations in the reactivity (electrophilicity) and chemical structure of the derivatives. In a comparative microarray study, *Arabidopsis* cultures were treated with closely related compounds that elicited rapid (fenclorim) and slow (4-chloro-6-methyl-2-phenylpyrimidine) XRs. Both chemicals induced major changes in gene expression, including a coordinated suppression in cell wall biosynthesis and an up-regulation in detoxification pathways, whereas only fenclorim selectively induced sulfur and phenolic metabolism. These transcriptome studies suggested several linkages between the XR and oxidative and oxylipin signaling. Confirming links with abiotic stress signaling, suppression of glutathione content enhanced GST induction by fenclorim, whereas fatty acid desaturase mutants, which were unable to synthesize oxylipins, showed an attenuated XR. Examining the significance of these studies to agrochemistry, only those fenclorim derivatives that elicited a rapid XR proved effective in increasing herbicide tolerance (safening) in rice.

Plants have a remarkable ability to elicit selective signaling pathways after exposure to low molecular weight natural products. Such inducing agents include plant hormones, salicylate and jasmonate derivatives, allelochemicals, and endogenous

elicitors released during infection (1). In addition, plants also recognize and mount a specific stress response to a range of synthetic compounds (xenobiotics) including drugs, pollutants, and agrochemicals (2). This xenobiotic response (XR),⁵ involves the coordinated up-regulation of genes encoding a group of proteins that detoxify foreign compounds, collectively termed the xenome (3). The best known inducible xenome components are the cytochrome P450 mixed function oxidases (CYPs), family 1 glucosyltransferases, glutathione transferases (GSTs), and ATP binding cassette transporter proteins (4–6). Together, their enhanced expression allows plants to accelerate the metabolism and sequestration of toxic chemicals. In agriculture, this response is exploited using safeners, a group of crop protection agents that increase the rates of detoxification of herbicides in cereal crops, thereby enhancing the selectivity of graminicides used to control competing grass weeds (7). Over the last 30 years a diverse range of safener chemistries have been developed, with each compound used in partnership with a herbicide for use in a specific crop (4, 8). Although safening activity toward herbicides is only observed in cereals and some non-domesticated grasses (9), the ability of these compounds to selectively induce xenome enzymes also extends to dicotyledonous plants, such as poplar and *Arabidopsis thaliana* (6, 10–12). In particular, the safener fenclorim (4,6-dichloro-2-phenylpyrimidine; Fig. 1A), which is used in rice to increase tolerance to chloroacetanilide herbicides, was found to be a potent and selective inducer of GSTs in root and suspension cultures of *Arabidopsis* (12). Based on the apparent conservation in safener recognition and xenome induction in *Arabidopsis* and cereals (10–12), the use of this model plant with all the associated molecular genetic tools and available mutants offers a powerful route to unraveling these hitherto intractable signal transduction pathways associated with xenobiotic sensing.

Arabidopsis contains 54 GSTs, which based on sequence identities can be divided into the phi, tau, lambda, theta, zeta, tetrachlorohydroquinone dehalogenase and dehydroascorbate reductase classes (13). Previous proteomic and transcriptomic studies have shown that only a subset of these proteins are induced by safeners in root and suspension cultures, notably the xenobiotic-conjugating phi (F) GSTF8 and tau (U) GSTU19 and GSTU24 enzymes (10–12, 14). These GSTs are also known

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables 1–6 and Figs. 1 and 2.

The microarray data have been deposited at the NCBI gene expression and hybridization array data repository, GEO accession number GSE28431.

¹ These authors contributed equally to this work.

² Present address: Croda Europe, Crop Care, Cowick Hall, Snaith, East Yorkshire, DN14 9AA, UK.

³ Present address: Centre for Novel Agricultural Products, Department of Biology, University of York, York, GL10 5YW, UK.

⁴ To whom correspondence should be addressed: Food and Environment Research Agency, Sand Hutton, York, YO41 1LZ, UK. Tel.: 44-1904-462415; Fax: 44-1904-462111; E-mail: robert.edwards@fera.gsi.gov.uk.

⁵ The abbreviations used are: XR, xenobiotic response; RXR, rapid XR; SXR, slow XR; BSO, l-buthionine sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; CMPP, 4-chloro-6-methyl-2-phenylpyrimidine; CYP, cytochrome P450 oxidoreductase; OPDA, 12-oxo-phytyldienoic acid; Ph, phenyl.

to be induced by a range of other chemical treatments (15), including exposure to natural product allelochemicals (5), xenobiotic pollutants (10), and copper salts (14). Similar responses have also been determined in cereal crops, although exposure to these nonspecific toxic chemicals does not result in the enhanced herbicide tolerance observed with safeners (5, 6, 12). This observation suggests that the changes caused by general xenobiotics in plants must be distinguishable at a signaling level from those caused by safeners such as fenclorim, which cause no discernable phytotoxicity on application.

With an interest in investigating the chemical basis of the XR in plants in greater detail, we have studied the selective induction of GSTs in *Arabidopsis* using a series of fenclorim derivatives to investigate the chemical features that determine GST induction. We have then performed global transcriptome studies in *Arabidopsis* with closely related derivatives that elicit different XRs to identify the associated changes in gene expression and potential metabolic pathways underpinning the different responses. Finally, relating these studies in *Arabidopsis* back to herbicide safening in cereals, we have then tested a subset of the fenclorim derivatives that elicit distinct XRs in *Arabidopsis* for their ability to enhance herbicide tolerance in rice.

EXPERIMENTAL PROCEDURES

Chemicals—Pretilachlor was obtained from Greyhound/Chem Service, whereas fenclorim and its derivatives were synthesized as detailed in the [supplemental Methods](#) using published methods where appropriate (16–20). In each case, compound identities were confirmed by mass spectrometry (MS) and NMR.

Chemical Treatments of Plant Tissues—The *Arabidopsis fad3-2/fad7-2/fad8* triple knock-out line was obtained from John Browse (Washington State University). For the cell suspension studies, *Arabidopsis* Col-0 cultures were grown in the dark in MS medium and used 5 days after subculturing (21). Root cultures were grown in the dark in Gamborg's B5 medium and used 14 days after initiation (21). For studies with whole plants, *Arabidopsis* was grown in greenhouse conditions, with 16 h of light using supplementary lighting. Excised rosette leaves were then floated on treatment solutions diluted in water. For the studies with cultures and leaves, the inducing chemical treatments were prepared as 100 mM stocks in acetone and added to the medium as a 1:1000 dilution. Control treatments consisted of 0.1% v/v acetone. For thiol depletion studies, *Arabidopsis* root cultures were treated with 1 mM l-buthionine sulfoximine (BSO) 5 days before standard chemical treatment. For herbicide safening trials, rice seedlings (*Oryza sativa* ssp. *japonica* cv. Nipponbare) were germinated and grown in magenta vessels (Sigma) on 0.3% agar containing pretilachlor (10 μ M) and safener treatments (1 or 10 μ M). In each case, the chemical treatments were added to the molten agar in a total volume of 2% v/v acetone. Safening activity was assessed by measuring the protective effect on shoot and root growth. All treatments were performed in biological triplicate and assayed in duplicate.

RNA Microarray Analysis—Safener compounds were added to *Arabidopsis* root cultures for 4 and 24 h periods before RNA extraction from biological triplicate samples using Tri reagent

(Sigma). Total RNA was further purified using the RNeasy Midi kit (Qiagen, Crawley, West Sussex, UK) before submission to the NASC Microarray service for probing against Affymetrix ATH1 *Arabidopsis* genome arrays. The raw microarray data were normalized to extract comparable probe intensities using the RMA algorithm in J-Express 2009 (molmine), giving 15,500 genes called as present. The triplicate results were further analyzed using SAM Version 3.09 Excel plugin (22) to identify transcripts showing statistically significant changes in abundance between treatments. The plugin default δ value was used, and data were filtered for transcripts showing at least a 3-fold change in abundance. These cut-offs resulted in a calculated expected false discovery rate of 0 in each case.

Real-time PCR Analysis—Equal amounts of RNA, isolated from *Arabidopsis* using TRI-reagent (Sigma), were used to synthesize cDNA using Moloney murine leukemia virus reverse transcriptase (Promega) and an oligo-dT primer. For real-time PCR, the primers were designed using Primer 3 so that each primer pair spanned an intron, had an optimum annealing temperature of 60 °C, and produced a product sized between 199 and 219 bp. The housekeeping genes used as controls were GAPDH (At1g13440) and ubiquitin-conjugating enzyme 21 (At5g25760). Real time PCR was performed in a Rotorgene 3000 (Qiagen) using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma). Analysis was carried out using rotor gene 6.0 software by comparative quantification with expression of the gene of interest normalized against the mean of the housekeeping genes GAPDH or ubiquitin-conjugating enzyme 21 (both gave similar results). Primer sequences can be found in [supplemental Table 1](#).

GST Activity Analysis—GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined as described (23). Crude plant protein was extracted in 2 vol/wt of 50 mM Tris-Cl, pH 7.5, 1 mM DTT, and desalted using Zeba desalting columns (Pierce) before activity assays.

RESULTS

GST Induction in *Arabidopsis* on Treatment with Xenobiotics—To first define differences in the XR to chemicals that show safening in cereals with those that are general xenobiotics, *Arabidopsis* cell cultures were treated with fenclorim and the non-specific electrophilic chemical CDNB. Although CDNB is a well known inducer of GSTs in plants, its use does not enhance herbicide tolerance (12, 15). To define biomarkers for the XR in *Arabidopsis*, the cells were assayed for the accumulation of *GSTF8*, *GSTU19*, and *GSTU24* transcripts, which are classically associated with the XR in this species. In addition, transcripts encoding the lambda enzyme *GSTL1* were included, as this gene is also known to be responsive to a wide range of chemicals and abiotic stress treatments (15). In all cases transcript abundance was determined over the 60-min period immediately post-treatment (Fig. 1B). After a 20-min treatment with fenclorim, *GSTF8*, *GSTU19*, and *GSTU24* transcripts began to accumulate, whereas their levels were unaffected by exposure to CDNB over the full 60-min period. *GSTL1* transcripts were not induced by either treatment over the 60-min period even though this gene is known to be induced by chemicals over longer exposure periods (15). This simple experiment demonstrated that the xenobiotic responses could be divided

Xenobiotic Responsiveness in *Arabidopsis*

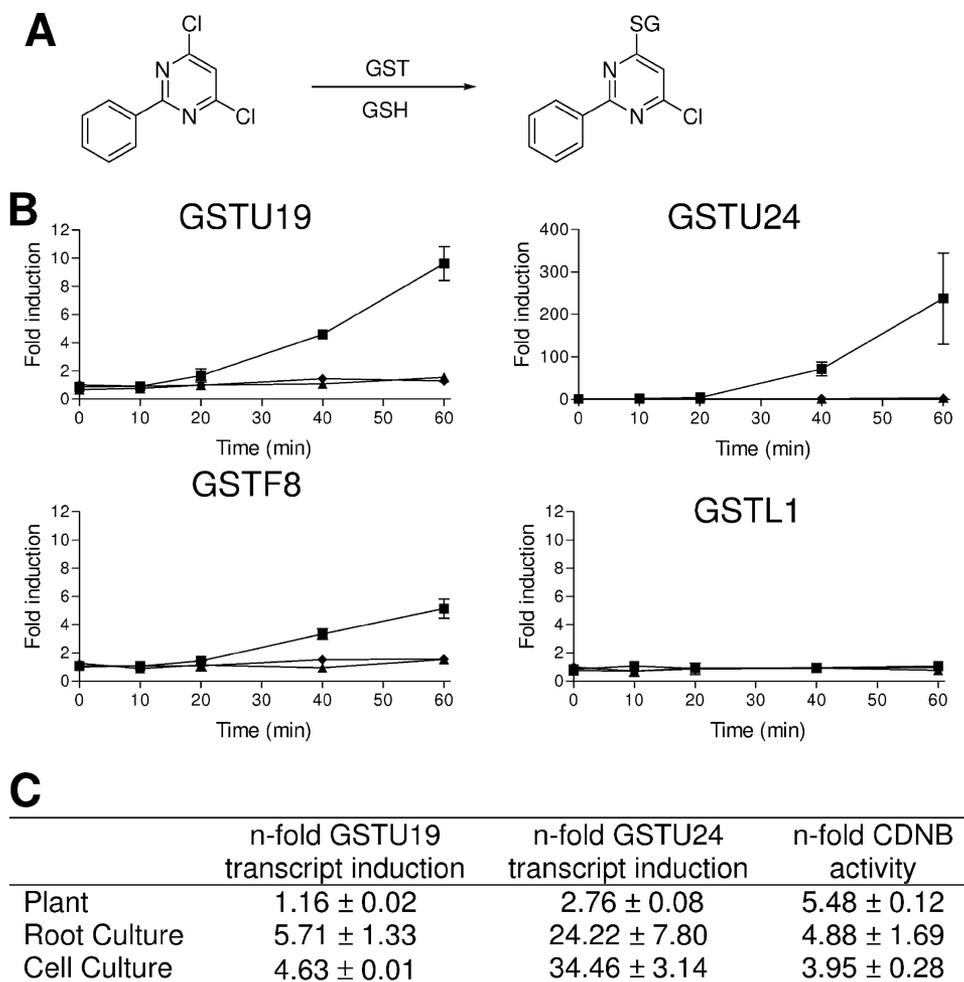


FIGURE 1. **The glutathionylation and xenome inducing activity of fenclorim in *Arabidopsis*.** *A*, on entering plant cells the safener undergoes GST-mediated glutathionylation. *B*, shown is induction of *GSTU19*, *GSTU24*, *GSTF8*, and *GSTL1* transcripts by fenclorim and CDNB in *Arabidopsis* cell suspension cultures (■, fenclorim; ▲, CDNB; ◆, acetone) over 60 min post-treatment. *C*, the induction of *GSTU19* and *GSTU24* was also determined in different *Arabidopsis* plant tissues after 60 min along with GST activity toward CDNB 24 h post-treatment.

into rapid and slow XR types as invoked by fenclorim and CDNB, respectively. The induction of *GSTU19* and *GSTU24* mRNAs by fenclorim was then monitored in other *Arabidopsis* tissues, namely root cultures and plant leaves (Fig. 1C). In all cases induction of the *GST* genes was observed, confirming that all these *Arabidopsis* tissue types were responsive to the safener. The induction observed in the suspension and root cultures was greater than that determined in the intact leaves. Based on previous metabolism studies, it appeared most likely that this difference in response was due to the very rapid uptake of the safener in plant cultures as compared with the foliage (20).

Screening of a Fenclorim Analog Series for Their Associated XRs in *Arabidopsis*—Having established the relative rates of enhancement of *GSTU19* and *GSTU24* transcripts induction as a biomarker of the type of XR, a series of fenclorim derivatives were prepared and tested for their ability to induce GST transcripts in suspension cultures. The synthetic strategy adopted to generate the fenclorim series was based on varying the reactivity and molecular size of the safener (Table 1). Thus, the electrophilicity of the substituted pyrimidine ring was altered by varying potential leaving groups (1–3, 8–17), with substitu-

tions at the 2-position (4–7, 19) introducing steric variation. The core structure was also modified through the generation of isosteric analogues such as phenyl pyridine (20) and biphenyls (21 and 22) in which the pattern of electrophilic centers and steric bulk are retained but with differing reactivities and binding requirements (Table 1). This series also encompassed a known metabolite of fenclorim (12) formed in *Arabidopsis* and rice plants treated with the safener *in vivo*, which was known to be an inducer of GSTs and to undergo glutathionylation despite having a reduced electrophilicity compared with the parent safener (20). Each compound was tested for its ability to increase *GSTU19* and *GSTU24* transcript abundance over 60 min and enhance GST enzyme activity toward CDNB over a 24-h period. The latter screen was included as a classic measure of the XR in *Arabidopsis* and other plants (10, 12, 15, 20, 24).

This screen confirmed that the xenobiotic responses in *Arabidopsis* could be divided into a slow (S) SXR and a rapid (R) RXR. In the SXR invoked by compounds 5–15, 17, 19, 20, and 22, GST enzyme activity was significantly enhanced over 24 h, without a rapid (within 60 min) induction of the respective transcripts. In the RXR shown with compounds 1–4, 16, and 18, the enzyme enhancement at 24 h was associated with rapid

TABLE 1

A series of fenclorim derivatives was prepared and assayed for the ability to induce GSTs in suspension cultures of *Arabidopsis*

Each compound was administered at 100 μM , and the -fold enhancement in GST-mediated conjugation of CDNB was determined after 24 h as compared with the enzyme activities determined in acetone-treated cultures of *Arabidopsis* (1.11 ± 0.10 nanotials mg^{-1}). As compared with controls, the induction of GST activity for *Arabidopsis* cell cultures treated with 100 μM CDNB was measured at 1.34 ± 0.18 nanotials mg^{-1} . In *Arabidopsis* the induction of *GSTU19* and *GSTU24* transcripts was measured over 60 min by quantitative PCR using ubiquitin-conjugating enzyme 24 as a control. Results shown are the means of three determinations \pm S.D.

Derivative	Substitution	n-fold CDNB	n-fold U19 transcript induction	n-fold U24 transcript induction		
R ¹	R ²	R ³				
Acetone						
1	Ph	Cl	Cl	1.00 \pm 0.04	1.00 \pm 0.13	1.00 \pm 0.57
2	Ph	Br	Br	3.49 \pm 0.01	5.58 \pm 0.82	26.18 \pm 0.64
3	Ph	F	F	2.47 \pm 0.18	5.49 \pm 0.23	20.56 \pm 1.52
4	nButyl	Cl	Cl	3.48 \pm 0.33	5.44 \pm 0.51	20.04 \pm 1.84
5	PhCH ₃	Cl	Cl	2.67 \pm 0.24	1.87 \pm 0.39	2.01 \pm 0.94
6	PhCH ₂ N ₃	Cl	Cl	2.12 \pm 0.10	1.56 \pm 0.12	0.94 \pm 0.14
7	4'-COCH ₃ Ph	Cl	Cl	3.00 \pm 0.00	2.57 \pm 0.62	7.79 \pm 1.32
8	Ph	H	H	1.64 \pm 0.34	1.32 \pm 0.22	2.57 \pm 0.88
9	Ph	Cl	H	1.64 \pm 0.06	1.21 \pm 0.20	0.55 \pm 0.03
10	Ph	Cl	CH ₃	1.91 \pm 0.05	1.45 \pm 0.23	1.12 \pm 0.48
11	Ph	OCH ₃	H	1.76 \pm 0.32	1.43 \pm 0.11	1.15 \pm 0.64
12	Ph	Cl	SCH ₃	2.74 \pm 0.38	1.70 \pm 0.04	2.11 \pm 1.14
13	Ph	SCH ₃	SCH ₃	2.04 \pm 0.28	0.80 \pm 0.60	0.70 \pm 0.08
14	Ph	Cl	OCH ₃	2.18 \pm 0.27	0.69 \pm 0.16	1.32 \pm 0.38
15	Ph	OCH ₃	OCH ₃	2.61 \pm 0.57	1.74 \pm 0.43	1.49 \pm 0.23
16	Ph	F	SCH ₃	2.35 \pm 0.07	5.95 \pm 0.58	36.98 \pm 14.43
17	Ph	NHEt	Cl	2.74 \pm 0.53	2.14 \pm 0.15	2.98 \pm 0.11
18	Cl	Cl	Ph	1.79 \pm 0.02	4.54 \pm 0.05	31.37 \pm 8.49
19	H	Cl	Cl	1.73 \pm 0.27	1.26 \pm 0.06	1.29 \pm 0.15
20	2,4-Dichloro-6-phenylpyridine			1.43 \pm 0.04	1.69 \pm 0.19	3.96 \pm 0.72
21	3,5-Dichloro-2,6-dinitrobiphenyl			0.94 \pm 0.02	1.23 \pm 0.19	1.55 \pm 0.43
22	3,5-Dichloro-biphenyl			1.11 \pm 0.03	1.38 \pm 0.13	1.10 \pm 0.05

transcriptional *GST* activation. Compound **21** did not induce any response and, therefore, exhibited neither a SXR nor a RXR. The results suggested that the observed differences in the XRs invoked were dependent on both the electrophilicity and the size of the derivative. Based on the SXR determined on treatment with compounds **8–11**, it was concluded that two good leaving groups are required to induce the RXR. Moreover, to elicit an RXR, the pyrimidine ring had to be sufficiently electron deficient, as shown by the activity of **1–4**, **16**, and **18** as compared with **20** and **22**. In addition, evidence for a steric requirement could be identified, in that modulating the bulk of the 2-phenyl substituent (**5–7** and **19**) resulted in the modified fenclorim losing RXR activity, suggesting a need for more specific binding requirements in safener recognition. This notion was reinforced through the comparison of compounds **1** and **18**, in which the relative position of the nitrogen atom between Cl and Ph-substituted carbons was conserved, whereas varying the second, ring-activating, nitrogen atom did not cause any significant loss in RXR activity. Finally, although the pattern of electrophilic sites, leaving groups, and C-2 substituent size is maintained in **21**, this compound was ineffective in promoting any XR.

Microarray Study with Compounds Invoking Rapid and Slow Xenobiotic Responses—Having identified two classes of response, a more detailed study of the differences between RXR and SXR was performed. A microarray study was carried out to determine whether compounds invoking an RXR in *Arabidopsis* induced different subsets of genes to closely related chemicals that only caused an SXR. Fenclorim (**1**) was selected as the

classic RXR inducer, whereas 4-chloro-6-methyl-2-phenylpyrimidine (CMPP; **10**) was chosen as a very closely related derivative of the parent safener that did not induce an RXR. After treatment with the chemicals, *Arabidopsis* cell cultures were harvested at 4 and 24 h for transcriptome analysis. These time points were chosen to distinguish between the RXR and SXR but also to allow enough time at the early time point to allow both sensitive detection of early events and at 24 h to allow secondary events to be monitored.

Comparing the microarray data for responses 4 h after treatment with fenclorim or CMPP, it was clear that as compared with the controls, both chemical treatments had a marked effect on transcription. In each case, large numbers of mRNAs were induced, some very strongly, whereas a smaller number of transcripts were down-regulated. Comparison of the two treatments (Fig. 2) showed a strong positive linear correlation ($R^2 = 0.73$) between the two responses with respect to the entire transcriptome. Overall, fenclorim appeared a slightly better modulator of transcript abundance than CMPP, giving on average a 10% higher response. Treatments with both fenclorim and CMPP rapidly and strongly induced many genes involved in xenobiotic detoxification including GSTs, glucosyltransferases, CYPs, and ATP binding cassettes (Fig. 2; supplemental Tables 2–4). In particular, *GSTU24* transcripts were up-regulated 56-fold by CMPP and 168-fold by fenclorim in the microarrays, confirming the relative strength of the inductions observed in quantitative PCR studies. After the 4-h treatment of the 100 genes most strongly up-regulated by fenclorim, 6 were GSTs, 12 were glucosyltransferases, 7 were CYPs, 14 were transporters, and 15 were redox-catalyzing enzymes, with these xenome components representing half of the most responsive transcripts. In contrast, a large number of cell wall-modifying gene transcripts were down-regulated by both fenclorim and CMPP treatment, indicating a rapid reduction in the expression of genes associated with cell growth. In addition to the commonality of responses to the two chemical treatments, it was also clear that a subset of genes showed a strong deviation from this correlation, and these differences were studied in more detail. Statistical analysis of the 4-h microarray data were used to extract transcripts showing significant modulation by (a) fenclorim versus carrier, (b) CMPP versus carrier, and (c) fenclorim versus CMPP (supplemental Tables 2–4). A number of genes specific for the RXR induced by fenclorim were linked to a sulfur starvation response (25). These included thioglucosidase, a high affinity sulfate transporter, storage proteins, 5'-adenylyl phosphosulfate reductase, sulfite reductase, and 5'-adenylyl sulfate reductase. Similarly, phenolic secondary metabolism appeared to be differentially up-regulated by fenclorim as compared with CMPP, with multiple genes encoding phenylalanine ammonium lyase, 4-coumarate-CoA ligase, caffeoyl-CoA methyltransferase, *N*-hydroxycinnamoyl benzoyltransferase, and a range of CYPs all induced. These RXR-specific transcriptional responses closely match the changes in metabolism observed during safening in cereals, which include increased levels of glutathione (GSH) and flavonoids (26–28).

After 24 h of treatment, it was anticipated that the SXR would be fully deployed and effectively more closely match the RXR. However, compared with the 4-h results, the transcriptome

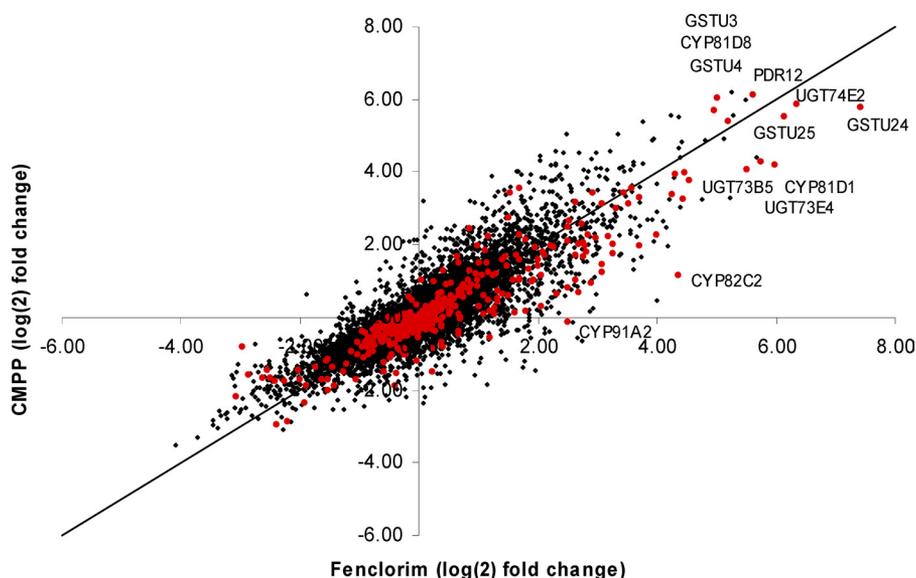


FIGURE 2. Cluster plot showing the correlation between -fold induction over solvent control of transcripts after 4 h of treatment with either fenclorim or CMPP, derived from averages of triplicate microarray analyses. Each microarray probe is represented by a black point, whereas genes associated with xenobiotic detoxification (GSTs, glucosyltransferases, CYPs, and the ATP binding cassette transporter PDR12) are shown as red dots with the most highly induced transcripts labeled. CMPP and fenclorim responses are highly correlated, and both induce substantial changes in the transcriptome, with xenobiotic detoxifying genes overrepresented among the most highly induced transcripts.

responses to fenclorim and CMPP were more divergent after 24 h. Although a positive linear correlation remained between the two treatments, it was much reduced ($R^2 = 0.52$). Many of the genes that were strongly up-regulated by both treatments at 4 h had declined significantly in abundance in the fenclorim-treated cells by 24 h. Of the 200 genes most highly up-regulated after 4 h by both treatments, by 24-h transcript abundance was decreased by 59% in the cultures exposed to fenclorim, whereas for CMPP this decrease was only 18% (Fig. 3). Thus, not only were the RXR transcripts more rapidly induced by fenclorim, but their induction was also more transient as compared with the SXR invoked by CMPP.

The XR and Glutathione Metabolism—Reasoning that the XR in plants must be related to “natural” abiotic or biotic stress responses, the microarray data were interrogated for patterns of response that would suggest coordinated biochemical changes associated with defense. The clear link between the RXR response and activation of sulfur-starvation pathways coupled with the known glutathione-mediated metabolism of fenclorim (20) suggested that changes in GSH metabolism could play an active role in the safener response. Similarly, changes in GSH content and the relative abundance of its oxidized disulfide derivative GSSG have been found to be involved in redox stress signaling in a number of plants (29). Both CDNB and fenclorim are known to be rapidly glutathionylated when fed to *Arabidopsis* cells and, therefore, have the potential to elicit their XR through a rapid perturbation of thiol homeostasis (12, 15). To determine the effect of adding these xenobiotics, the GSH pool was determined 60 min after dosing *Arabidopsis* root cultures with either 100 μM fenclorim or 100 μM CDNB. These studies showed that whereas fenclorim reduced GSH content by 40%, CDNB treatment led to a 93% depletion in the thiol (Table 2). From this result it was concluded that although GSH depletion was a common feature in both responses, the RXR could not be explained by the scale of the perturbation, as

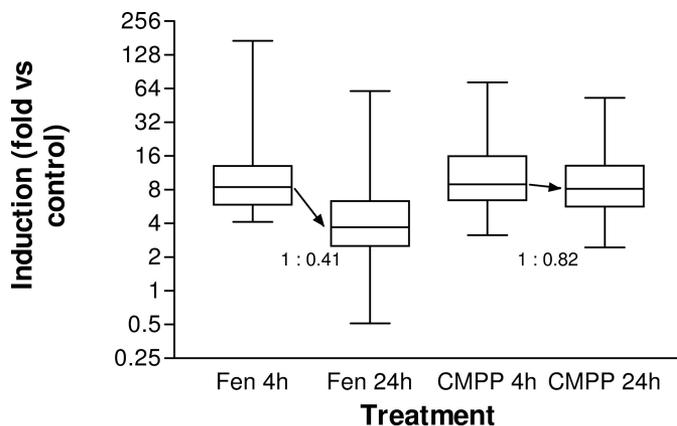


FIGURE 3. Box and whisker plot showing transcript abundance changes at 4 and 24 h for the 200 transcripts most highly induced by both fenclorim and CMPP after 4 h. The median line is shown, with boxes indicating the 25th and 75th percentiles, and whiskers indicating the total range of changes. Although both chemicals caused a major enhancement of these transcripts, this induction was much more transient in the case of fenclorim (Fen) treatment.

CDNB that caused the greater loss only elicited an SXR (Fig. 1B). To further probe the link between GSH content and the XR, the selective glutathione synthetase inhibitor BSO was used to deplete the thiol pool in *Arabidopsis* root cultures (Table 2). The BSO treatment resulted in a reduction of GSH content to barely detectable levels and caused a basal elevation in *GSTU19* and *GSTU24* transcripts in all cultures examined. When the GSH-depleted cells were treated with CDNB or fenclorim, similar XRs were determined with either chemical treatment. Lowering the GSH content, therefore, appeared to abolish the differential sensitivity of the cells to fenclorim as compared with CDNB observed in the earlier studies (Fig. 1B), showing the availability of the thiol modulated the XR.

The RXR and Oxylipin Signaling—Comparative analysis of the microarray data with other transcriptome experiments suggested a strong similarity in the XRs promoted by both

TABLE 2**The effect of depleting cellular glutathione on *GSTU19* and *GSTU24* induction in *Arabidopsis* root cultures**

Glutathione content was determined after a 60-min exposure to 100 μM fenclorim or CDNB after a 5-day pretreatment with (+) or without (–) the glutathione synthesis inhibitor BSO. Results shown are the means of three determinations \pm S.D.

Treatment	\pm BSO	GSH <i>nmol g⁻¹</i>	<i>GSTU19</i> relative transcript abundance	<i>GSTU24</i> relative transcript abundance
Control	–	12.4 \pm 1.9	1.0 \pm 0.2	1.0 \pm 0.4
	+	0.2 \pm 0.0	5.0 \pm 0.6	15.5 \pm 0.7
Fenclorim	–	7.4 \pm 0.7	5.3 \pm 1.1	23.7 \pm 3.1
	+	0.1 \pm 0.1	9.5 \pm 0.7	33.2 \pm 7.7
CDNB	–	0.8 \pm 0.6	2.5 \pm 0.7	8.0 \pm 1.1
	+	0.0 \pm 0.0	7.3 \pm 1.9	31.5 \pm 7.9

fenclorim and CMPP treatments with that determined in mixotrophic *Arabidopsis* cell cultures exposed to the phyto-prostane PPA₁ (30). PPA₁ and a group of related compounds termed oxylipins are a group of reactive electrophilic metabolites oxidatively generated from endogenous unsaturated fatty acids during plant wounding (30). Comparison of microarray data for fenclorim and CMPP treatments with that for a 4-h treatment of cell cultures with the phyto-prostane (30) showed a highly significant overlap between the responses to these treatments. For example, a global comparison of gene induction by fenclorim compared with gene induction by PPA₁ (supplemental Fig. 2) showed a strong positive correlation ($r = 0.49$), with a $p < 0.0001$, being a correlation due solely to chance. Of the 50 most PPA₁-inducible genes, 27 of these were also induced at least 2-fold by fenclorim. Conversely, of the 50 most fenclorim-inducible genes, 39 were induced at least 2-fold by PPA₁. Intriguingly, the xenome enzymes CYP710A1, CYP71A12, and CYP81F2 were strongly differentially induced by fenclorim, whereas nine heat shock protein transcripts were at least eight-fold more induced by PPA₁ as compared with fenclorim. At least some of the differences in gene induction between the two treatments will be due to differences in the experimental systems, with the remaining strong similarities in transcriptome responses pointing to fenclorim and PPA₁ inducing very similar responses. However, the response to PPA₁ in cell cultures had already been shown to be similar to the response of *Arabidopsis* seedlings to treatment either with phyto-prostanes or the oxylipin 12-oxo-phytodienoic acid (OPDA) (30), confirming the viability of test platforms when comparing plants with cultured cells. To further test the potential involvement of oxylipins in the XR, root cultures from wild type plants and mutants (*fad3-2/fad7-2/fad8*) defective in forming the oxylipin precursor linolenic acid were treated with either fenclorim or acetone carrier. Mass spectrophotometric analysis (31) of the fatty acid content of wild type and mutant plantlets confirmed that unlike wild type plants, the mutant contained no detectable linolenic (18:3) or 16:3 fatty acids incorporated into lipids, with linoleic (18:2) and 16:2 fatty acids dominating, as expected (32). This mutant line is, therefore, unable to synthesize OPDA but could form other dienoic acid-derived oxylipins. On treatment with fenclorim, the suppression in induction of GSTs showed that the RXR was markedly depressed in the *fad3-2/fad7-2/fad8* plants (Table 3), consistent with a link between the RXR response and endogenous oxylipin signaling.

In a further examination of the link between safener- and oxylipin-mediated signaling and metabolism, we also tested the

TABLE 3**Effect of linolenic acid content in *Arabidopsis* root cultures on RXR, as measured by induction of the GST transcripts *GSTU19* and *GSTU24* 1 h after treatment with either fenclorim (RXR inducer) or acetone (control)**

Wild-type plants have high levels of linolenic acid, whereas *fad3-2/fad7-2/fad8* mutants accumulate linoleic acid instead of linolenic acid. Results shown are the means of three determinations \pm S.D.

Plant	Treatment	Relative transcript abundance	
		<i>GSTU19</i>	<i>GSTU24</i>
Wild type	Acetone	1.0 \pm 0.3	1.0 \pm 0.7
	Fenclorim	5.7 \pm 1.3	24.2 \pm 7.8
<i>fad3-2/fad7-2/fad8</i>	Acetone	1.2 \pm 0.2	2.6 \pm 0.2
	Fenclorim	1.7 \pm 0.7	7.7 \pm 2.3

potential for xenobiotic conjugating GSTs to show similar detoxifying activities toward oxylipins. As such, GSTs could share a common function in modulating the availability of electrophilic signaling agents of both synthetic and natural origins. Previous studies have shown that OPDA can be conjugated by the *Arabidopsis* enzymes GSTU6, GSTU10, GSTU17, GSTU19 and GSTU25 (33) and GSTF8 (30). By testing further members of the *Arabidopsis* superfamily, we demonstrated that in total 11 GSTs can catalyze the glutathionylation of oxylipins in *Arabidopsis* (supplemental Table 5). Intriguingly, while examining further functional links between fenclorim and oxylipin detoxification, we also demonstrated that the parent safener **1** and compounds **2**, **3**, and **4**, which all elicit an RXR, lead to an inhibition of the GSTU19-catalyzed conjugation of OPDA (supplemental Table 6).

*Correlation between the XR in *Arabidopsis* and Physiological Safening in Rice*—To test whether or not the type of XR in *Arabidopsis* correlated with safening, the ability of the fenclorim series to protect rice from herbicides was determined. Rice seedlings were germinated on agar containing the chloroacetanilide herbicide pretilachlor in the presence and absence of members of the fenclorim chemical series. Two concentrations of safener were employed corresponding to a low (1 μM) and high (10 μM) treatment rate. In each case, herbicidal activity was assessed by determining root and shoot elongation relative to untreated controls (supplemental Fig. 1). When exposed to 10 μM pretilachlor, rice seedling growth was strongly arrested, with this effect largely reversed in the presence of 1 μM fenclorim (Fig. 4). A strong protective effect at this lower concentration (1 μM) of safener was also observed with the dibromo- (**2**) and difluoro- (**3**) fenclorim derivatives. Only at the higher concentration of 10 μM did compounds **4**, **9**, **12** (Fig. 4), and **18** also gave significant protection, whereas **10** and **16** were inactive.

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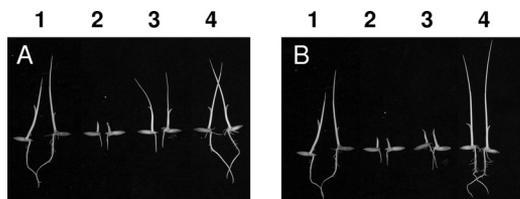


FIGURE 4. **Safening activity of fenclorim in rice.** When exposed to the herbicide pretilachlor, the normal stunting of growth of rice seedlings was ameliorated by applications of safener-active compounds such as fenclorim (**1**), whereas closely related compounds such as **12** fail to protect the plants. Lane 1, acetone control; lane 2, 10 μM pretilachlor; lane 3, 1 μM safener and 10 μM pretilachlor; lane 4, 1 μM safener. A, safener treatment fenclorim (**1**). B, safener treatment 4-chloro-6-(methylthio)-2-phenylpyrimidine (**12**).

These experiments showed a good correlation between an RXR response in *Arabidopsis* and safening in rice, with five of the RXR-activating compounds also having safening activity in rice. However, the correlation between the responses in the two plant species was not perfect, as two compounds (**9**, **12**) that showed some safening activity in the cereal only invoked an SXR in *Arabidopsis*.

DISCUSSION

Our results demonstrate that plants can respond to xenobiotics by either eliciting a rapid, or slow, XR. In the case of fenclorim, the ability to induce an RXR was found to be surprisingly sensitive to changes in both the electrophilicity of the pyrimidine ring (suggestive of an ability to selectively alkylate soft nucleophilic groups such as cysteinyl residues) and to minor variations in the phenyl ring substituent. Such structure activity relationships are typically demonstrated in protein-based recognition systems. In terms of xenobiotic recognition, such systems are not unprecedented in mammals and fungi, with the receptor protein releasing transcription factors after selective alkylation with electrophiles. For example, in mammals the keap1 protein sequesters the transcription factor Nrf2 in an inactive form in the cytoplasm (34). In the presence of stress stimuli such as electrophilic agents, cysteinyl residues on keap1 that interact with Nrf2 are modified, leading to a reduction in binding affinity and the release of the transcription factor. Nrf2 then translocates to the nucleus, where it binds to regulatory antioxidant responsive elements, which lead to the transcription of genes encoding phase II detoxifying enzymes and antioxidant stress proteins (35). To date, no ortholog of the keap1 receptor protein has been identified in plants. Alternatively, several enzymes involved in signaling events are also known to have their activity regulated by selective alkylation. For example, the active site cysteinyl residues of phosphatases involved in the regulation of protein phosphorylation can be inactivated by alkylation (36). Such selective enzyme inhibition by xenobiotics has also been demonstrated in plants with enzymes of primary metabolism, including *S*-formylglutathione hydrolase (37) and ribulose-bisphosphate carboxylase/oxygenase (38). In the current study we attempted to identify a fenclorim-binding protein using “click”-based approaches using the safener derivatized with an azido function (39). These studies were unable to demonstrate any specific binding, although as our structure activity studies evolved it became clear that this was due to the minor modification in fenclorim

chemistry required in the generation of the probe resulting in the loss of the RXR. Similarly, other attempts to identify plant proteins that bind to safeners have proved inconclusive. In studies with the radiolabeled safener dichlorimid (*N,N*-diallyl-2,2-dichloroacetamide), an apparently selective binding interaction to a methyltransferase of unknown function was determined in maize seedlings (8). However, the functional significance of this binding to eliciting safening in maize was not determined.

In addition to the differential speed at which GST transcripts were induced in the RXR and SXR, the studies with fenclorim and CMPP also demonstrated that these two closely related compounds elicited subtly different effects on the *Arabidopsis* transcriptome. Much of this difference was observed in the kinetics of the respective responses. This highlights the importance of the timing of sampling in xenobiotic treatment studies, with our results suggesting that assaying at either a single time point or at time points much later than 4 h after treatment would overlook the differences in gene expression induced by a safener as compared with those determined by chemicals inducing an SXR. Overall, the profile of transcripts induced by fenclorim showed considerable overlap with those induced by other electrophilic chemicals, such as the toxic allelochemical benzoxazolin-2(3*H*)-one (5), or the reactive B₁-type phytoprostanes, such as PPA₁, released on plant wounding (40). Of the 50 most benzoxazolin-2(3*H*)-one-inducible genes, 44 were also induced by at least 2-fold by fenclorim and 34 at least 2-fold by PPA₁. Collectively these results suggest that different electrophiles lead to essentially similar transcriptional modulation but over different timescales. We postulate that general reactive electrophiles, such as CDNB, elicit an SXR after causing cellular toxicity due to the alkylation of sensitive proteins and DNA. The resulting disruption in metabolism then leads to the release of reactive endogenous signaling molecules, which on selective recognition, activate a protective XR. In contrast, electrophiles, including safeners, which mimic these endogenous stress signaling molecules, directly activate this protective receptor system and induce a relatively large but short-lived signaling response that activates cellular defenses before the xenobiotic causing extensive damage. Intriguingly, the chemical depletion of GSH content in the *Arabidopsis* cells led to an enhanced sensitivity in the XR to both CDNB and fenclorim treatments. This raises the possibility that under normal conditions GSH affords protection to electrophile-sensitive protein thiols, thereby preventing alkylation by CDNB, but that these groups remain sensitive to fenclorim modification, perhaps through a selective association of the safener at a ligand binding site proximal to key cysteinyl residues.

Following the hypothesis that safeners elicit endogenous stress signaling pathways, it was of interest that the XR of *Arabidopsis* was sensitive to perturbations in fatty acid desaturation (Table 3). As treatment of *Arabidopsis* with OPDA or phytoprostanes gives a response very similar to that observed with fenclorim (41), we speculate that the safener must be interacting with the signaling invoked by oxylipins, with these metabolites in turn derived from unsaturated fatty acids. Whereas reactive oxylipin derivatives such as OPDA are derived from linolenic acid by the concerted action of lipoxygenases, allene

oxide synthases, and allene oxide cyclase (42), the phytoprostanes are derived from the spontaneous oxidation of linolenic acid and to a lesser extent linoleic acid (43). Many of these signaling-active derivatives are unstable and will react with GSH and potentially protein-sulfhydryl groups over time (41). The fact that a reduction in unsaturated fatty acids attenuates the RXR invoked by fenclorim in *Arabidopsis* suggests that the safener must act either in parallel or upstream of oxylipin signaling, potentially through regulating the availability of these endogenous molecules. However, to date we have been unable to determine any major effects of fenclorim on OPDA or phytoprostane metabolism *in planta*, suggesting that this safener-mediated regulation involves a minor subset of these compounds, or operates in a restricted spatiotemporal manner.

Although a link between safening with oxylipin signaling has been proposed (7), a causative unifying mechanism of action is yet to be determined. Based on the observations from the current study, we can propose three potential mechanisms whereby xenobiotics could interact with endogenous stress recognition pathways to elicit an RXR. First, fenclorim or a rapidly formed downstream metabolite could selectively bind and activate a signaling protein that normally binds to and thus senses oxylipins, in both cases presumably through modification of a reactive cysteine residue. Recent studies have shown that oxylipins selectively alkylate and modify the function of a number of redox-sensitive cysteinyl-bearing proteins, some of which are implicated in signaling (41). Second, fenclorim (or a downstream metabolite) could activate a minor release of oxylipins leading to signal elicitation. A candidate enzyme for such bioactivation would be a lipase with many plants including *Arabidopsis*, accumulating relatively large amounts of esterified OPDA and phytoprostanes in lipids (44). If the safeners were to cause a selective release of such preformed stores through the up-regulation of hydrolytic enzymes, this would potentially lead to an RXR. Third, fenclorim (or a downstream metabolite) could prevent the metabolic deactivation of oxylipins, leading to their transient accumulation and resulting signal initiation. The widespread ability of GSTs to catalyze oxylipin glutathionylation (supplemental Table 5) coupled with their consistent sensitivity to inhibition by glutathionylated fenclorim and certain related compounds (supplemental Table 6) suggests that fenclorim treatment could transiently increase free oxylipin levels through inhibition of their enzyme-mediated glutathionylation. However, inconsistencies in the inhibitory *versus* safening activity of other compounds in the series do not support a simple link between interfering with GST activity and disrupting oxylipin metabolism (supplemental Table 6). Further studies are now required to establish how safeners intercede in oxylipin turnover and signaling. In view of the lack of discernable disruption in total oxylipin content on safening, one promising area may be to study the effect of fenclorim on the intracellular disposition of these endogenous signals between the cytosol, vacuole, and peroxisomes as mediated by ATP binding cassette transporters (45, 46). Although our studies raise additional questions as to how xenobiotics can selectively intercede in intracellular stress signaling pathways, the use of the fenclorim derivatives in defining structure activity relationships clearly demonstrates the subtle distinctions

between the RXR and agronomically useful herbicide safening from SXR and the more commonly encountered general xenobiotic response.

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