Catabolism of Glutathione Conjugates in *Arabidopsis thaliana*
ROLE IN METABOLIC REACTIVATION OF THE HERBICIDE SAFENER FENCLORIM*[S]

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The safener fenclorim (4,6-dichloro-2-phenylpyrimidine) increases tolerance to chloroacetanilide herbicides in rice by enhancing the expression of detoxifying glutathione *S*-transferases (GSTs). Fenclorim also enhances GSTs in *Arabidopsis thaliana*, and while investigating the functional significance of this induction in suspension cultures, we determined that these enzymes glutathionylated the safener. The resulting *S*-(fenclorim)-glutathione conjugate was sequentially processed to *S*-(fenclorim)-γ-glutamyl-cysteine and *S*-(fenclorim)-cysteine (FC), the latter accumulating in both the cells and the medium. FC was then either catabolized to 4-chloro-6-(methylthio)-phenylpyrimidine (CMTP) or *N*-acylated with malonic acid. These cysteine derivatives had distinct fates, with the enzymes responsible for their formation being induced by fenclorim and FC. Fenclorim-*N*-malonylcysteine was formed from FC by the action of a malonyl-CoA-dependent *N*-malonyltransferase. A small proportion of the fenclorim-*N*-malonylcysteine then underwent decarboxylation to yield a putative *S*-fenclorim-*N*-acetylcysteine intermediate, which underwent a second round of GST-mediated *S*-glutathionylation and subsequent proteolytic processing. The formation of CMTP was catalyzed by the concerted action of a cysteine conjugate β-lyase and an *S*-methyltransferase, with the two activities being coordinately regulated. Although the fenclorim conjugates tested showed little GST-inducing activity in *Arabidopsis*, the formation of CMTP resulted in metabolic reactivation, with the product showing good enhancing activity. In addition, CMTP induced GSTs and herbicide-safening activity in rice. The bioactivated CMTP was in turn glutathione-conjugated and processed to a malonyl cysteine derivative. These results reveal the surprisingly complex set of competing catabolic reactions acting on xenobiotics entering the *S*-glutathionylation pathway in plants, which can result in both detoxification and bioactivation.

Safeners are widely used agrochemicals, which selectively enhance herbicide tolerance in cereal crops, such as wheat, rice, sorghum, and maize, thereby increasing selectivity in weed control (1). A wide range of safener chemical classes have been developed, with each used individually for specific crop applications in combination with a herbicide partner (2). Safeners work by enhancing the activity of herbicide-detoxifying enzymes, such as cytochrome P450 mixed function oxidases, glutathione transferases (GSTs),[3] and glycosyltransferases, as well as xenobiotic transporters, such as ATP-binding cassette proteins (1–3).

Although the role of safeners in inducing herbicide metabolism in cereals has been known for many years (1–3), the specific mechanism of how these compounds exert their activity is unclear. With an interest in defining how safeners work, we are using the model plant *Arabidopsis thaliana*, as a tool to identify associated regulatory pathways. Although there are no reports of any dicotyledonous plants showing enhanced tolerance to existing selective herbicides when exposed to safeners (4), a range of xenobiotics, safeners, and herbicides have been shown to enhance the expression of GSTs (4–6), glycosyltransferases (7), and ATP-binding cassette proteins (8) in *Arabidopsis*. The conservation in chemical signaling pathways has also been demonstrated by showing that the promoter of a safener-responsive gene from maize is similarly inducible in *Arabidopsis* (9). Thus, whereas *Arabidopsis* cannot be used to determine the downstream protective effects of safeners in preventing herbicide injury, this model plant with its associated genetic resources is an attractive system to study the respective conserved signaling events leading to the induction of detoxifying enzymes (6).

In previous studies, we demonstrated that fenclorim (4,6-dichloro-2-phenylpyrimidine (Table 1), a safener developed to protect rice from chloroacetanilide herbicides (10), was an effective inducer of GSTs and glycosyltransferases in *Arabidopsis* suspension cultures (5). Thus, of eight safener compounds tested, fenclorim provided the best GST-enhancing activity in *Arabidopsis* suspension cultures, with similar activities reported in rice seedlings (10). With an interest in determining

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[3] The abbreviations used are: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; CMTP, 4-chloro-6-(methylthio)-2-phenylpyrimidine; F, fenclorim; FAC, *S*-(4-chloro-2-phenylpyrimidin-6-yl)-*N*-acetylcysteine; FACC, *S*-(4-*N*-acetylcysteine-2-phenylpyrimidin-6-yl)-cysteine; FACG, *S*-(4-*N*-acetylcysteine-2-phenylpyrimidin-6-yl)-glutathione; FC, *S*-(4-chloro-2-phenylpyrimidin-6-yl)-cysteine; FG, *S*-(4-chloro-2-phenylpyrimidin-6-yl)-glutathione; FγEC, *S*-(4-chloro-2-phenyl-pyrimidin-6-yl)-γ-glutamyl-cysteine; FMC, *S*-(4-chloro-2-phenyl-pyrimidin-6-yl)-*N*-malonylcysteine; MPMC, *S*-(4-(methylthio)-2-phenylpyrimidin-6-yl)-*N*-malonylcysteine; HPLC, high pressure liquid chromatography; NMT, *N*-malonyltransferase; SAM, *S*-adenosyl-L-methionine; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry; kat, katals.

how fenclorim induces detoxification systems in plants, we have studied its uptake and metabolic fate in *Arabidopsis*. In contrast to herbicides, relatively little has been published on the metabolism of safeners, and it is unknown whether the biotransformation of a compound like fenclorim would result in a gain or loss in function of its GST-inducing activities. We have therefore determined the metabolic fate of fenclorim in suspension cultures, identified the primary biotransforming enzymes, and examined their regulation. Finally, the GST-inducing activity of the major fenclorim metabolites has been tested in *Arabidopsis*, and the consequence of any metabolic reactivation of safener activity has been determined by testing these metabolites for their ability to protect rice from herbicide injury.

EXPERIMENTAL PROCEDURES

Chemicals—Fenclorim was obtained from Greyhound/Chem Service and was also routinely synthesized (11). Melting points were recorded using a Gallenkamp capillary melting point apparatus (Design 889339) with values quoted to the nearest 0.5 °C. ^1H and ^{13}C NMR spectra were measured on a Varian Inova 500 or a Varian VNMRS-700 instrument, with *J* values quoted in Hz and chemical shifts calibrated with reference to the residual proton and carbon resonances of the solvent (DMSO-d_6: $\delta H = 2.50$, $\delta C = 39.4$ ppm; CDCl$_3$: $\delta H = 7.26$, $\delta C = 77.0$ ppm). Assignment of spectra was carried out using COSY, HSQC, and HMBC experiments. Low and high resolution mass spectral analyses were recorded on a Micromass LCT and a Thermo Finnigan LTQ-FT mass spectrometer, respectively, using electrospray ionization operating in positive ion mode.

Synthesis of Conjugates—The *S*-conjugation of fenclorim and its derivatives with glutathione, γ-glutamylcysteine, cysteine, and *N*-acetylcysteine used essentially the same procedure. To a solution of the fenclorim derivative (0.1 mmol) in ethanol/acetonitrile (1:1 (v/v), 4 ml) was added thiol (0.2 mmol) in distilled water (1 ml). After adjusting to pH 9–10 with triethylamine, the mixture was diluted with distilled water to a final volume of 6 ml and then incubated for 12–18 h at 25 °C. Samples of the product (1 ml) were injected onto a preparative HPLC column (250 × 10 mm; RPC18; Phenomenex Luna) and eluted using the mobile phases 0.5% (v/v) formic acid in water (A) and 0.5% (v/v) formic acid in acetonitrile (B). A linear gradient was applied, starting at 5% B and increasing to 100% B over 30 min, at a flow rate of 2 ml min^{-1}. Elution of reaction products was monitored using UV absorbance ($\lambda = 260$ nm), and the purified compounds were concentrated to dryness *in vacuo*. Compounds were analyzed by NMR and high resolution MS. Chemical data on all of the fenclorim metabolites characterized are given in the supplemental materials.

Chemical Treatment of Arabidopsis Cell Suspension Cultures—*Arabidopsis* cell suspension cultures (Columbia) were grown in the dark in MS medium and used 5 days after subculturing (7). Rice suspension cultures *Oryza sativa* L. Tsukinohikari (12) were maintained on N1 medium composed of 4.4 g/liter ChuN6 salts and vitamins (Duchefa Biochemie, The Netherlands), 0.3 g/liter casamino acids, 1 mg/liter 2,4-dichlorophenoxyacetic acid, and 30 g/liter sucrose. Cells were used 5

days after subculture. Fenclorim was added to the cell cultures in acetone, with its polar derivatives prepared in dimethyl sulfoxide. In each case, chemicals were diluted 1:1000 upon addition to cultures, with additions of the respective solvents (0.1%) alone used as control treatments. On harvest, cells were separated from the growth medium by filtration prior to weighing, freezing in liquid nitrogen, and storage at −80 °C. Medium samples were analyzed directly.

Herbicide and Safener Trials in Rice—Rice seedlings (*Oryza indica japonica* cv. Nipponbare) were germinated and grown in 25-cm^2 pots for 9 days on potting compost at 25 °C at a light intensity of 100 microeinsteins m^{-2} s^{-1}. The herbicide pretilachlor and fenclorim were dissolved in acetone and diluted 100-fold in 0.1% Tween 20. Treatments were applied in three 1-ml sprays, such that a total of 0.075 mg of pretilachlor and 0.025 mg of fenclorim were delivered per pot. Plants were grown on for 12 days, and then the foliage was harvested and weighed. Treatments were carried out in triplicate.

Metabolite Analysis—*Arabidopsis* cells (1 g) were powdered under liquid nitrogen using a pestle and mortar and extracted with 10 ml of methanol/acetone (1:1, v/v). After centrifugation (3,000 × g, 5 min), the supernatant was concentrated to 1 ml *in vacuo*. Metabolites were extracted from the growth medium by adding 1 volume of ice-cold methanol and centrifuging to remove cell debris. Extracts (10 μl) were injected onto an Acquity UPLC^{TM} BEH C18 (1.7 μM; 2.1 × 100 mm) column at a flow rate of 0.2 ml min^{-1} and eluted with a gradient starting at 20% B rising to 100% B over 9 min. The eluent was analyzed using a Waters Q-TOF Premier mass spectrometer after electrospray ionization (capillary 2.55 kV, sample cone 41 kV, extraction cone 5.0 kV, source 100 °C with desolvation at 180 °C). Samples were analyzed in positive ion mode, initially with a low collision energy imposed at 10 V, subsequently ramped to 30 V to induce fragmentation (13). Identification of metabolites was based on co-chromatography with authentic standards and comparing MS fragmentation spectra. Metabolites were quantified by reference to known amounts of their respective synthetic standards using UV absorbance at 264 nm. MS fragmentation data for all standards are given in the supplemental materials. The glutathione and cysteine content of the cells was determined by HPLC after reduction and derivatization with monobromobimane (14).

Enzyme Activity Determinations—Crude protein extracts were prepared from frozen cells (5), and enzyme activities were determined using HPLC-based assays. GST activity toward fenclorim was determined by incubating 120 μl of crude enzyme extracts (120 μg of protein) with 50 μl of 0.1 M Tris-HCl, pH 8.0, 10 μl of fenclorim (10 μM in acetone), and 20 μl of reduced glutathione (100 mM) for 10 min at 37 °C. The reaction was stopped by the addition of 200 μl of methanol. *N*-Malonyltransferase (NMT) activity and C-S lyase/*S*-methyltransferase activity toward the fenclorim-cysteine (FC) conjugate were determined by suspending 13 μl of crude protein (25 μg) in 0.1 M Tris-HCl, pH 8.0, containing 2 mM dithiothreitol and 1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride-HCl. After incubating at 37 °C for 5 min, the reaction was initiated by adding
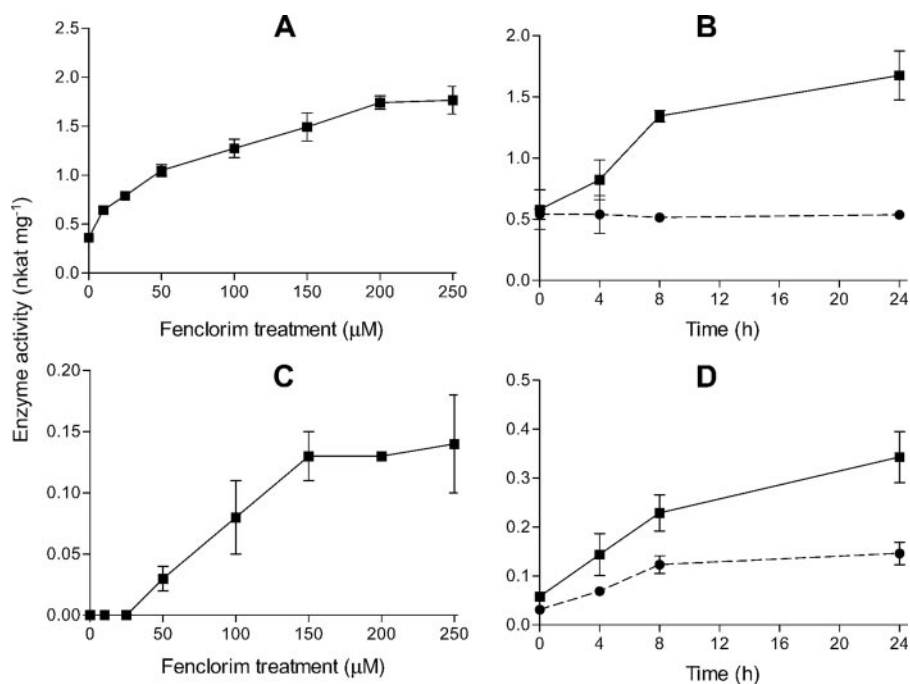
FIGURE 1. **The induction of GST activity in *Arabidopsis* cell cultures exposed to fenclorim.** Crude protein was extracted from cultures treated with fenclorim (■) or control (●) cultures, and GST activity was determined toward the substrates CDNB (*A* and *B*) and fenclorim (*C* and *D*), with respect to varying dose and then harvesting after a 24-h exposure (*A* and *C*) and time dependence from the addition of 100 μM safener (*B* and *D*). Results refer to mean values ± S.D. (*n* = 2), with the differences in absolute specific activities determined in the time course and dose-response experiments arising from two different cell batches.

tgaggtgattcttctg-3′ and 5′-gcgcgc-gtcgacttactccaacccaagtttcttc-3′ for *At*GSTU24 (accession BT012184) and cloned into the custom vector pET-Strp3 (15).

RESULTS

Induction of GSTs in Arabidopsis Cell Cultures by Fenclorim—To determine a suitable treatment regime, a range of concentrations of fenclorim were tested for their ability to increase GST activity toward the model substrate CDNB over a 24-h period in *Arabidopsis* cell suspension cultures (Fig. 1). Increasing the concentration of fenclorim over the range 10–200 μM resulted in a steady enhancement in GST activity, although higher concentrations gave no further induction (Fig. 1*A*). The wide range of concentrations resulting in enzyme induction was surprising, in view of the limited solubility of fenclorim in aqueous solution (~10 μM). Closer examination of the bioavailability of fenclorim in the conditioned medium from suspension cultures showed that at a nominal treatment rate of 100 μM, the concentration of the free safener determined in solution after filtration was just 7 μM. It was concluded that the safener was partitioning between the aqueous medium and insoluble matrices in the culture flasks, thereby limiting its true bioavailability. To avoid variations in cell culture composition perturbing the concentrations of freely soluble fenclorim available to the cells when dosed at lower concentrations, the safener was routinely used at 100 μM in all further studies, which approximates delivering fenclorim at its solubility limit in water. Under these conditions, an increase in GST specific activity was determined in cell extracts 4 h after dosing (Fig. 1*B*).

It was then of interest to determine whether fenclorim itself was a substrate of the GSTs in *Arabidopsis*, as previously shown in rice (10). Crude protein extracts from untreated *Arabidopsis* were incubated with glutathione and fenclorim, and the reaction products were analyzed by HPLC with the eluate monitored by UV diode array and mass spectrometry (MS). A novel UV-absorbing metabolite (λ_{max} 260 nm) was observed with a retention time of 5.98 min, which on MS analysis gave a parent molecular ion at 496 Da, with a characteristic single $^{35/37}$Cl isotope pattern. Based on these MS data, the metabolite was tentatively identified as the glutathione conjugate of fenclorim (6-*S*-(4-chloro-2-phenylpyrimidinyl)-glutathione (FG); *m/z* 496 [M + H]$^+$, ^{35}Cl), arising from the substitution of one of the chlorine atoms with the tripeptide glutathione (Table 1). This identification was supported by the observation that upon ionization, FG underwent the neutral loss of the 129-Da fragment, which is consistent with the characteristic loss of a pyroglu-

0.1 M Tris-HCl, pH 8.0 (36 μl), containing FC (10 nmol) and co-substrate (15 nmol). In the case of NMT activity, malonyl coenzyme A was added, whereas for C-S lyase/*S*-methyltransferase, *S*-adenosyl-L-methionine (SAM) was used. Control reactions were also run where co-substrates were omitted. After 10 min, the reaction was stopped by the addition of 50 μl of methanol, and after centrifugation (12,000 × *g*, 3 min), products (10 μl) were analyzed by analytical HPLC as described above, using a linear gradient starting at 5% B and increasing to 100% B over 9 min. The identity of the reaction products was confirmed using the reference metabolites. GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined using a spectrophotometric assay (5).

Proteomic Analysis, Cloning, and Expression of GSTs—Crude protein extracts were applied to a glutathione-Sepharose column, and the affinity-bound polypeptides were recovered and resolved on miniformat two-dimensional SDS-polyacrylamide gels (5). After Coomassie staining, polypeptides were digested with trypsin, with the resulting peptides analyzed on an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer, and the identities of the parent proteins were determined using MASCOT proteomics software (5). The GSTs identified were amplified from cDNA from *Arabidopsis* leaf tissue using primers 5′-gcgcgccatatgggagcaattcaagctcg-3′ and 5′-gcgcgcgtcgac-tactgcttctggaggtca-3′ for *At*GSTF8 (accession NM_180148), primers 5′-gcgcgccatatggcggagagatcaaattcag-3′ and 5′-gcgcgc-gtcgactcaagcagatttgatattgag-3′ for *At*GSTU7 (accession AY086358), primers 5′-gcgcgccatatggcgaacgaggtgattcttc-3′ and 5′-gcgcgcgtcgacttactcaggtacaaatttcttcc-3′ for *At*GSTU19 (accession NM_106485), and primers 5′-gcgcgccatatggcaga-

TABLE 1

Quantification of fenclorim metabolites formed 0–24 h after treating *Arabidopsis* suspension cultures (100 ml) with 100 μM safener or conjugated metabolites

Peak numbers refer to the chromatogram and compound structures shown in Fig. 3. Quantities refer to mean values $\pm$ S.D. ($n = 3$). Fenclorim was recovered from the growth medium at 0 h (721 ± 31 nmol) and 4 h (101 ± 55 nmol) and FC at 4 h (1001 ± 238 nmol).

Peak	Metabolite	Quantity of metabolites extracted from cells at four time points			
		0 h	4 h	8 h	24 h
		nmol	*nmol*	*nmol*	*nmol*
1	FACC		2 ± 1	9 ± 6	64 ± 32
2	FACG		6 ± 3	18 ± 6	30 ± 11
NP	Natural product	122 ± 11	98 ± 13	87 ± 22	87 ± 5
3	FC		284 ± 57	63 ± 8	21 ± 11
4	FG		414 ± 89	588 ± 60	147 ± 37
5	FγEC		125 ± 46	168 ± 14	80 ± 17
6	Unknown			14 ± 2	23 ± 4
7	FMC		206 ± 53	544 ± 65	629 ± 149
8	MPMC		6 ± 1	19 ± 2	28 ± 2
9	Unknown				10 ± 5
F	Fenclorim	325 ± 175	242 ± 94	38 ± 5	
10	CMTP		52 ± 30	66 ± 9	35 ± 28

tamyl moiety from a glutathione conjugate (13). The identification of FG was further confirmed by synthesizing an authentic glutathione conjugate of fenclorim that had identical properties when analyzed by HPLC and MS (see supplemental materials). Having identified FG as a reaction product, it was apparent that in untreated cell cultures, the GST-catalyzed rate of *S*-glutathionylation was relatively low (0.03 nkat/mg total protein), compared with the nonenzymatic conjugation rate of 0.02 nkat. Based on the known time and dose-dependent enhancement of GST activity toward CDNB following safener treatment (Fig. 1, *A* and *B*), it was of interest to determine whether a similar induction was observed with fenclorim as substrate. GST activity toward fenclorim was found to be dose-dependent (Fig. 1*C*) and time-dependent (Fig. 1*D*), confirming that exposure to the safener could potentially accelerate its own metabolism in *Arabidopsis.*

To identify the safener-responsive GSTs, proteins present in cultures exposed to 100 μM fenclorim were analyzed by two-dimensional gel electrophoresis after affinity chromatography using glutathione-Sepharose (4, 5). Eleven affinity-purified polypeptides could be resolved in the protein extracts from cells exposed to the safener, whereas only one stained spot was discernable in the control cultures (Fig. 2*A*). Proteomics by MALDI-TOF MS identified that the 11 polypeptides were derived from four GSTs. Based on the accepted nomenclature for *Arabidopsis* GSTs (3), three of these were tau (U) class enzymes, *At*GSTU7, *At*GSTU19, and *At*GSTU24, and one from the phi (F) class, *At*GSTF8 (Fig. 2*B*). The cDNAs encoding the GSTs were cloned and expressed in *Escherichia coli*, and the pure fusion proteins were assayed for conjugating activity toward CDNB and fenclorim (Fig. 2*C*). All four GSTs showed conjugating activity toward the two xenobiotics in the order *At*GSTU24 > *At*GSTU19 > *At*GSTF8 $\gg$ *At*GSTU7, with CDNB being the preferred substrate in each case.

Metabolism of Fenclorim in Arabidopsis Cultures—Having identified *S*-glutathionylation as a potential route of biotransformation *in vitro*, the metabolism of the safener was investigated *in vivo* in cultures treated with 100 μM fenclorim. After a
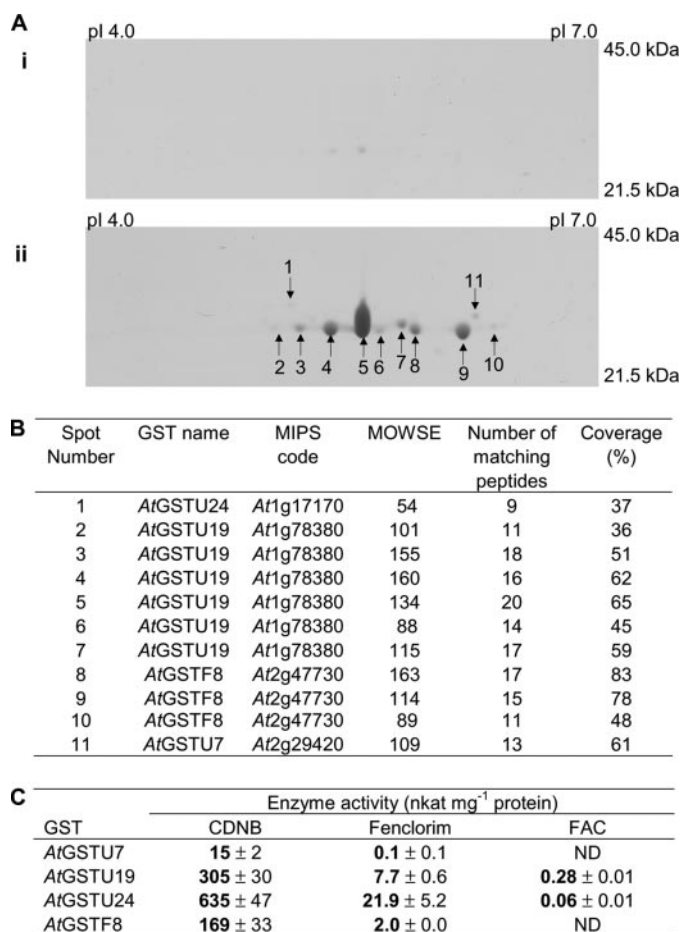

FIGURE 2. **Identification of GSTs and associated activities of GSTs induced by fenclorim treatment in *Arabidopsis* cell suspension cultures.** *A*, proteomic analysis of GSTs induced by 24-h treatment with 100 μM fenclorim (*A*, *ii*) as compared with control treatment (*A*, *i*), following enrichment using glutathione affinity chromatography. *B*, polypeptides were then excised, digested with trypsin, and identified by MALDI-TOF MS proteomics. *C*, GST activities of recombinant GSTs toward the substrates, CDNB, fenclorim, FAC, and CMTP. *ND*, not determined. Results refer to mean values $\pm$ S.D. ($n = 3$).

24-h treatment, the cell extracts were analyzed by HPLC and found to contain 10 novel UV-absorbing peaks in addition to one peak corresponding to an unknown natural product also present in the controls (Fig. 3*A*). When analyzed by MS, six of these novel compounds were found to have the characteristic isotopic signature of monochlorine-containing metabolites, strongly suggesting that they were substitution products derived from the dichlorinated parent (fenclorim). The new compounds were labeled **1–10** on the basis of their retention time (Fig. 3*A*) and changes in their relative abundance in the cells and medium determined over time (Table 1). The concentration of bioavailable fenclorim in the medium declined rapidly, with the parent compound cleared from the cultures within 8 h (Table 1). Based on their relative order of appearance and abundance over time, metabolite **4** was identified as the likely major primary metabolite, since it accumulated steadily over the first 8 h and then declined (Table 1). Metabolite **4** was identified as FG, with its rapid appearance consistent with its formation through GST-catalyzed conjugation.

By searching for other UV-absorbing compounds that followed a similar transient accumulation over 8 h (Table 1), other

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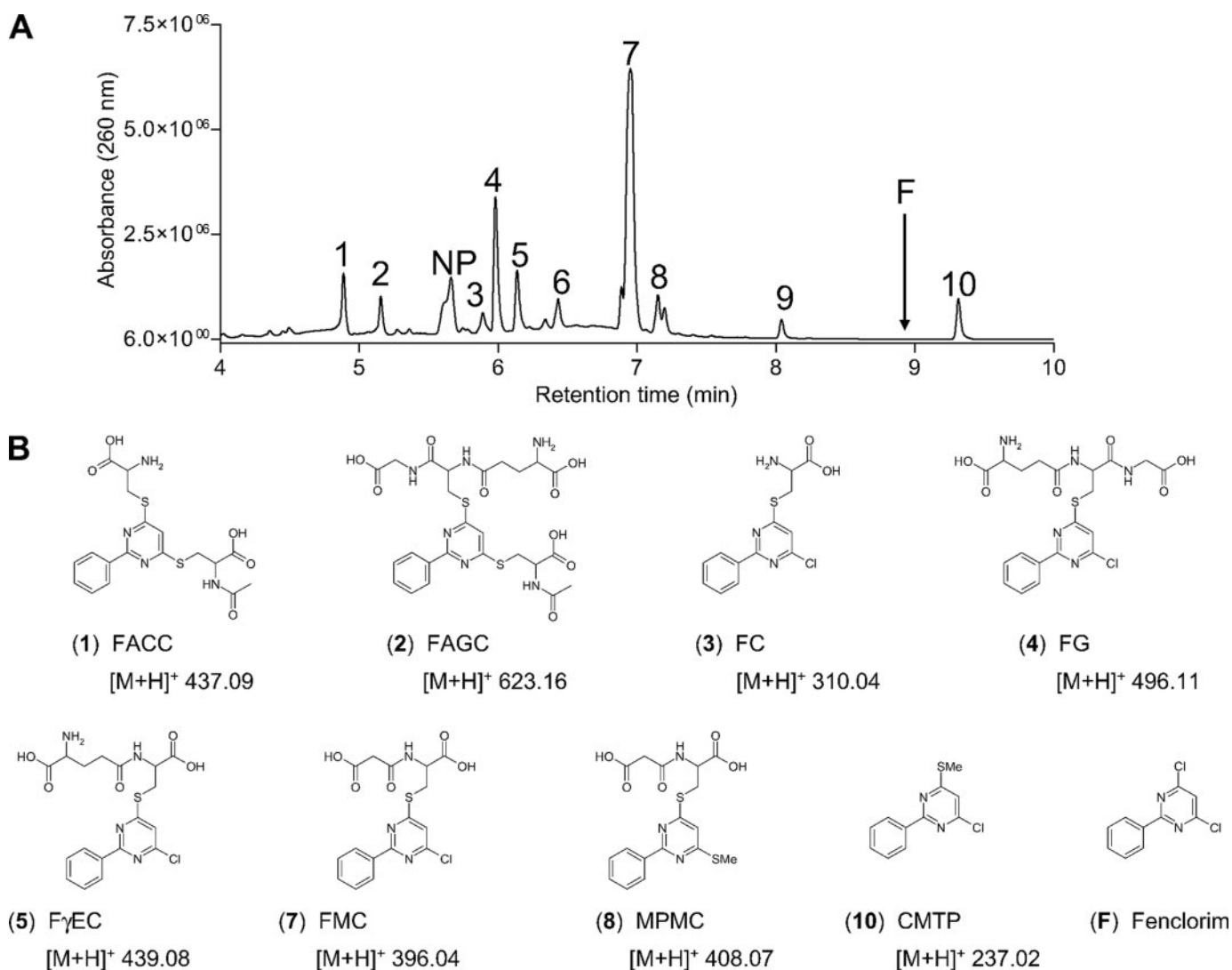
FIGURE 3. **Metabolites derived from the *S*-glutathionylation of the safener fenclorim in *Arabidopsis*.** Fenclorim was fed to *Arabidopsis* cell suspension cultures, and after 24 h the metabolites present in the cells were extracted with methanol and then resolved by HPLC (*A*). The retention time of fenclorim is indicated by the *arrow*. The identities of metabolites are shown with associated chemical structures and molecular ions (*B*). Additional information on characterization of the respective synthetic standards is given in the supplemental materials. The structures of natural product (*NP*) and metabolites **6** and **9** were not determined.

likely primary metabolites of fenclorim were highlighted. These compounds were then systematically identified using a combination of the literature describing the processing of glutathione conjugates in plants (reviewed in Ref. 16), MS data, and comparisons with independently synthesized and characterized reference metabolites (see supplemental materials).

The first step in the further metabolism of FG in the cell cultures was its hydrolytic processing to metabolite **5** (Table 1), which was identified as 6-*S*-(4-chloro-2-phenylpyrimidinyl)-γ-glutamylcysteine (FγEC; Fig. 3*B*). The FγEC was then catabolized to metabolite **3** (Table 1), which corresponded to 6-*S*-(4-chloro-2-phenylpyrimidinyl)-cysteine (FC; Fig. 3*B*). Although FγEC accumulated steadily over 8 h in cells only, FC reached maximal concentrations at 4 h, when it was found in both the cells and medium (Table 1). The rapid appearance of high concentrations of FC suggested that the conjugate could arise from the direct reaction of fenclorim with free cysteine rather than through the catabolism of FG. To test this possibility, the con-

centrations of free thiols available for conjugation in the cultures were determined after derivatizing them to their fluorescent bimane derivatives (14). No free cysteine or glutathione was detected in the medium, confirming that thiol conjugation must only occur within the cells. When the free thiols within the cells were quantified, glutathione (423 nmol/g fresh weight) was found to be 7 times more abundant than cysteine (61 nmol/g fresh weight). Based on these relative pool sizes, we concluded that the high concentrations of FC determined at 4 h were much more likely to be derived through the processing of FG rather than direct conjugation with cysteine. This conclusion was further supported by determining changes in the free thiol pool during safener treatment, which revealed that 1 h after the addition of 100 μM fenclorim, the pool of glutathione was reduced by 40%, whereas the concentration of cysteine remained unchanged. After 4 h, FC was totally cleared from the medium and declined 4-fold in the cells (Table 1). This large scale disappearance of FC was due to its metabolism within the
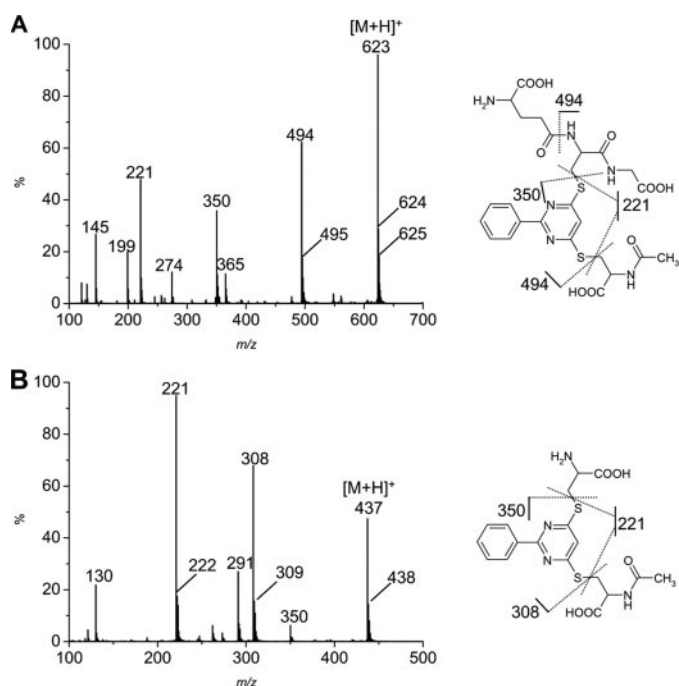
FIGURE 4. **MS/MS fragmentation and chemical structures of FACG (*A*) and FACC (*B*).**

cells, with a reference synthetic standard of the conjugate being unaffected by incubation in conditioned growth medium for 4 h.

Based on this rapid disappearance of FC in the cells, the profiles of UV-absorbing peaks were examined for compounds that showed a corresponding increase and therefore could be potential catabolites (Table 1). After eliminating FG from the search, the major increases observed between 4 and 8 h were determined with compound **7**, with modest amounts of compounds **2**, **6**, **8**, and **9** also accumulating. Compound **7** had a molecular mass consistent with a cysteine conjugate having undergone conjugation with malonic acid. The *N*-malonylation of cysteine conjugates is a well known reaction in the processing of xenobiotic glutathione conjugates in plants (16). Compound **7** was therefore tentatively identified as *S*-(4-chloro-2-phenylpryimidyl)-6-*N*-malonylcysteine (FMC; Fig. 3*B*). Subsequent esterification of purified FMC **7** with diazomethane confirmed the presence of two carboxylic acids (observed increase in M^+ of 32 mass units), consistent with *N*-malonylation (supplemental materials). Attention was then focused on the other potential FC catabolites. Compound **2** showed a gain in mass of 313 Da compared with FC, suggesting further bioconjugation of the catabolite. The MS isotope pattern indicated that the conjugate no longer contained chlorine atoms but gained two sulfurs. MS-MS fragmentation analysis of the parent ion produced a mass ion of *m/z* 221 Da, consistent with the pyrimidine ring having undergone two rounds of thiol substitution (Fig. 4*A*). In addition, compound **2** underwent the same neutral loss of the 129-Da γ-glutamyl component observed with FG, consistent with the metabolite being a glutathione conjugate. By inference, the second sulfur-containing group must therefore derive from a compound with a parent mass of 162 Da. A review of the literature on the processing of *S*-glutathionylated drugs suggested that this second sulfur-containing group was *N*-acetyl-

cysteine. Although the formation of *N*-acetylcysteine (mercapturic acid) conjugates is very common in drug metabolism in animals (17), such modifications have rarely been determined in pesticide metabolism in plants, notably as residues of the herbicide cycloate in radishes (18). The identity of compound **2** as *S*-(4-*N*-acetylcysteine-2-phenylpyrimidin-6-yl)-glutathione (FACG; Fig. 3*B*) was subsequently confirmed by synthesizing an authentic standard (supplemental materials). The identification of this pathway of secondary glutathionylation facilitated the characterization of compound **1**. MS analysis suggested that compound **1** also contained two sulfur-bound substituents, with the difference between the parent ion (*m/z* 437) and the major fragment (*m/z* 308) again consistent with a *N*-acetylcysteinyl substituent (Fig. 4*B*). Based on the known proteolytic processing of FG to the respective cysteine conjugate, we postulated that compound **1** was the respective catabolite of FACG, namely *S*-(4-*N*-acetylcysteine-2-phenylpyrimidin-6-yl)-cysteine (FACC; Fig. 3*B*). Confirmation of FACC was provided by independent chemical synthesis of the respective reference metabolite.

Attention was then focused on the remaining metabolites. Compound **10** had a different metabolic profile to the FC catabolites, accumulating rapidly over the first 4 h and then remaining at similar concentrations for the remainder of the feeding study. Elution of peak **10** during HPLC later than fenclorim suggested that it was an unusually hydrophobic metabolite, with MS analysis showing that it contained both chlorine and sulfur atoms. Based on its parent mass ion of 237 Da, it was clear that this compound had arisen from the extensive processing of the sulfur donor substituent, such that all of its polar functional groups had been removed. Careful scrutiny of the literature (18, 19) suggested that compound **10** was the *S*-methylated derivative of fenclorim, 4-chloro-6-(methylthio)-2-phenylpyrimidine (CMTP; Fig. 3*B*), and this was confirmed by chemical synthesis of an authentic standard (supplemental materials). The accumulation of *S*-methylated xenobiotics derived from the glutathione conjugation pathway has previously been reported in the course of the metabolism of the agrochemicals pentachloronitrobenzene and fluorodifen in onion roots and pine cell cultures, respectively (19, 20). Based on these studies, we would therefore predict that CMTP was derived from the sequential biotransformation of FC first by a cysteine conjugate-β-lyase to yield 4-chloro-6-thio-2-phenylpyrimidine and then an *S*-methyltransferase. Drawing parallels with secondary glutathionylation of the acetylated cysteine derivatives, the identification of CMTP directed attention to finding related further conjugation products. MS-MS analysis of compound **8** resulted in its fragmentation to a major ion (*m/z* 235), corresponding to a derivative of CMTP in which the C4 chlorine had been substituted by a sulfur atom. Based on similarities with the MS analysis of compound **7**, the difference in mass between the parent ion of compound **8** and the major fragmentation ion suggested substitution with an *N*-malonylcysteine unit. Compound **8** was therefore tentatively identified as *S*-(4-(methylthio)-2-phenylpyrimidin-6-yl)-*N*-malonylcysteine (MPMC; Fig. 3*B*). To confirm its identity, *Arabidopsis* suspension cultures were treated with 100 μM chemically synthesized CMTP for 24 h. The major metabolite identified was
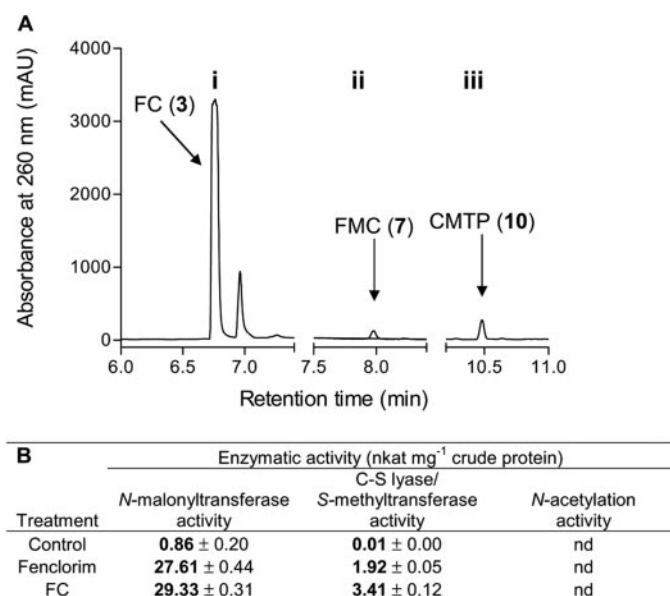
FIGURE 5. *A*, determination of the activity of enzymes acting on the FC conjugate as determined by HPLC. FC (*i*) was incubated with crude protein from *Arabidopsis* cultures in the presence of malonyl-CoA (*ii*) or *S*-adenosyl-L-methionine (*iii*), and the respective products, FMC and CMTP, were quantified by HPLC. *B*, *N*-malonyltransferase and C-S lyase/*S*-methyltransferase activities were determined in crude protein extracts from cells exposed to fenclorim or FC (100 μM) for 24 h. Results refer to mean values $\pm$ S.D. (*n* = 3). No activities were determined with boiled protein controls.

MPMC, accounting for 8% of the CMTP fed to cells. Further confirmation of the glutathione conjugation pathway operating with CMTP was obtained by identifying trace amounts of the glutathionylated, cysteinylated, and γ-glutamylcysteinyl derivatives of CMTP. In contrast to the other metabolites, compounds **6** and **9**, which steadily accumulated to low levels during the course of fenclorim metabolism, could not be identified. Although compound **6** contains a chlorine atom, the lack of any chlorine atoms in compound **9** suggested that, like FACC and FACG, this metabolite had most likely arisen from two rounds of *S*-substitution.

Identification and Safener Inducibility of FC-catabolizing Enzymes—Although the enzymes involved in the processing of glutathione and γ-glutamylcysteine conjugates of xenobiotics in *Arabidopsis* have been described (21–23), very little is known about the events that occur downstream of cysteine conjugate formation. In the case of fenclorim metabolism, three distinct pathways acting on the cysteine conjugate FC could be identified, namely (i) *N*-malonylation, (ii) *N*-acetylation and subsequent secondary *S*-glutathionylation, and (iii) processing to the methylthio-derivative CMTP and downstream conjugation. Since the respective enzymes responsible for the formation of these catabolites have not been identified in plants, we wanted to establish whether or not their activities could be measured and their responsiveness to safener treatment. Crude protein extracts from untreated *Arabidopsis* cultures were incubated with FC in the presence of either malonyl-CoA or acetyl-CoA, to identify the *N*-malonyl transferase and *N*-acetyl transferase, respectively. HPLC-MS analysis identified FMC but not *S*-(fenclorim)-*N*-acetylcysteine (FAC) (Fig. 5*A*), confirming the activity of the NMT only. Although NMT activity was just measurable in extracts from control cultures, treatment with 100 μM

fenclorim for 24 h resulted in a 30-fold increase in NMT specific activity (Fig. 5*B*). In contrast, the respective acetyl transferase activity could not be determined. The results from the metabolism study showed an appreciable accumulation of FC at the early time points, followed by the rapid disappearance of the conjugate (Table 1). Based on the observed induction of GSTs by their substrate fenclorim, it was of interest to determine if NMT activity was similarly responsive to the accumulation of FC. Treatment with FC invoked an identical increase in NMT activity as seen with the safener (Fig. 5*B*), suggesting that either FC or a derived metabolite could autoregulate the rates of removal of the cysteine conjugate. Although these studies were unable to identify an *N*-acetyl transferase active toward FC, the subsequent *S*-glutathionylation of the putative FAC intermediate was an extremely unusual reaction, and it was of interest to determine whether its formation was spontaneous or the result of GST-mediated conjugation. When FAC was incubated with a crude protein extract from untreated cultures, a low but measurable protein-dependent *S*-glutathionylating activity was determined (2.03 $\pm$ 0.01 pkat/mg protein), as compared with a chemical rate of 1.63 $\pm$ 0.13 pkat. When protein extracts from cultures treated for 24 h with 100 μM fenclorim were assayed, GST activity toward FAC was increased to 3.72 $\pm$ 0.06 pkat/mg, demonstrating that the safener enhanced the conjugation of both itself and one of its conjugated catabolites. Assaying the recombinant safener-inducible GSTs showed that FAC was a substrate of both *At*GSTU19 and *At*GSTU24 (Fig. 2*C*).

Finally, the enzyme activities responsible for CMTP synthesis and conjugation were determined. Although the C-S lyase acting on FC requires no co-substrate, the methylation of the resulting free thiol would require an activated donor species, such as SAM. Crude protein extracts were therefore incubated with FC in the presence of SAM. Extracts from untreated cells showed a low but measurable rate of protein-dependent CMTP production, which fell below the limit of detection if SAM was omitted (Fig. 5*B*). The combined C-S lyase/*S*-methyltransferase activity was greatly enhanced in cells treated for 24 h with fenclorim or FC, again demonstrating the inducibility of these conjugate-processing enzymes by catabolites of the glutathione conjugation pathway (Fig. 5*B*). Intriguingly, when the safener-treated extracts were incubated with FC in the absence of SAM, no new metabolites corresponding to 4-chloro-6-thio-2-phenylpyrimidine were observed, suggesting that the C-S lyase activity was integrally dependent on *S*-methylation. With respect to its further conjugation, CMTP was found to be conjugated by crude protein extracts (2.0 pkat/mg; chemical rate = 1.1 $\pm$ 0.1 pkat) and by recombinant *At*GSTU19 and *At*GSTU24 (Fig. 2*C*).

Activity of Fenclorim Metabolites as GST-inducing Agents—The induction of the cysteine conjugate-processing enzymes by FC posed the interesting possibility that metabolites derived from the glutathione conjugation of fenclorim could be having an active role in safener induction of GSTs in *Arabidopsis*. This was not inconsistent with the very rapid metabolism of fenclorim observed in the cell cultures (Table 1), with shorter term feeding studies showing that 25% of the safener absorbed by the cells over 30 min was converted to FG and associated metabo-
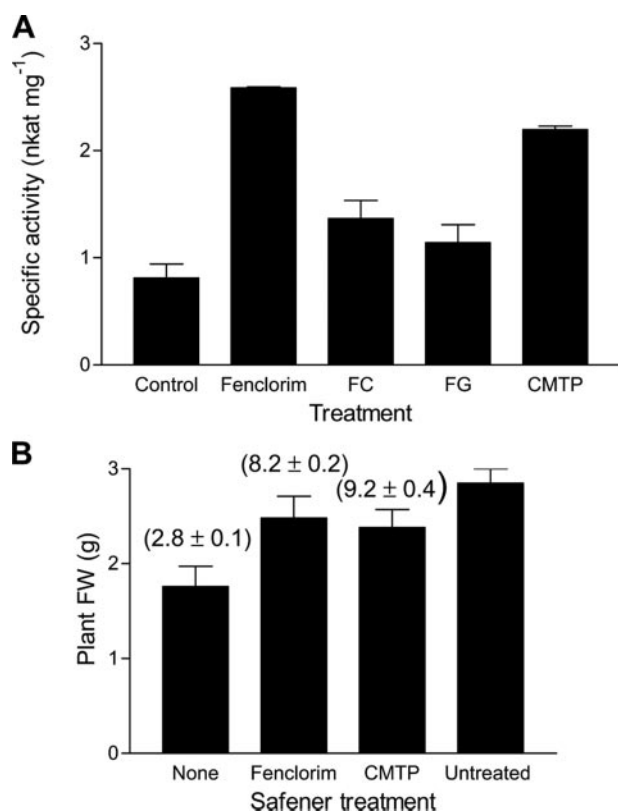
FIGURE 6. **The effect of fenclorim metabolites on GST induction in *Arabidopsis* cultures and herbicide safening in rice.** *A*, after treatment with fenclorim or related metabolites for 24 h (100 μM), crude protein was extracted from *Arabidopsis* cell suspension cultures, and GST activity toward CDNB was determined. *B*, rice plants were sprayed at rates equivalent to 300 grams active ingredient hectare^{-1} with the herbicide pretilachlor in the presence and absence of fenclorim or its metabolite CMTP (100 grams active ingredient hectare^{-1}). After a 12-day treatment, plants were weighed, and the results were compared with untreated controls. In addition, rice suspension cultures were treated with fenclorim or CMTP for 24 h (100 μM), and GST activity toward CDNB was determined, with the results shown in *parentheses above* the respective *bars* in each case. Results are mean $\pm$ S.D. ($n = 3$).

lites (data not shown). The major primary metabolites of fenclorim conjugation, FG and FC, were fed to the cells at a final concentration of 100 μM, and the induction of GST activity was determined. Compared with fenclorim, both compounds gave a modest induction of GST activity (Fig. 6*A*), although it was unclear if this attenuated enhancement was due to problems with the compounds entering the cells. Metabolism studies subsequently showed that these conjugates were absorbed, with CMTP recovered as a major metabolite.

The observation that CMTP was effectively a hydrophobic metabolite resembling the parent safener, which had the common characteristic of being formed by feeding fenclorim and its conjugated metabolites FG or FC focused attention on its possible biological activity. When the *Arabidopsis* cells were dosed with 100 μM CMTP, the metabolite promoted 85% of the GST-inducing activity seen with the parent safener (Fig. 6*A*). The biological significance of this apparent metabolic reactivation of inducing activity was then tested in rice, the crop targeted for use with fenclorim (10). First, rice seedlings were sprayed with the chloracetanilide herbicide pretilachlor formulated with either fenclorim or CMTP. The plants were then weighed after 12 days to assess growth inhibition relative to untreated con-

trols and plants exposed to herbicide alone (Fig. 6*B*). The results demonstrated that CMTP reduced herbicide injury in the crop. Second, rice suspension cultures were treated with 100 μM fenclorim or CMTP, and the induction of GST activity by the safener metabolite was confirmed (Fig. 6*B*). From these studies, it was concluded that CMTP was not only an effective inducing agent of GSTs in *Arabidopsis* but also showed identical activities in rice and that this resulted in herbicide safening.

DISCUSSION

These studies demonstrate that fenclorim induces its own metabolism in *Arabidopsis* by enhancing the expression of GSTs active in glutathionylating the safener as well as enzymes involved in processing the resulting conjugates. Fenclorim selectively induced the GSTs, enhancing the expression of four family members. Two of these enzymes, *At*GSTU19 and *At*GSTF8, have previously been identified as being induced by the safener benoxacor (4, 5). In contrast, *At*GSTU7 and *At*GSTU24 were not previously known to be inducible by safeners in *Arabidopsis* but were identified as being transcriptionally up-regulated by the allelochemical benzoxazolinone (8). In addition to conjugating fenclorim, the inducible *At*GSTU19 and *At*GSTU24 also showed activity toward its metabolite FAC, thereby acting at two points in the metabolism of this xenobiotic.

By following the biotransformations of fenclorim and its conjugates by HPLC-MS, it was possible to demonstrate that *Arabidopsis* cultures have a surprisingly complex system for bioprocessing *S*-glutathionylated xenobiotics (Fig. 7). We are confident that the observed metabolism of fenclorim in cell cultures is representative of that seen in whole plants, since we have observed very similar biotransformations of the safener in *Arabidopsis* root cultures (supplemental materials). Similarly, fenclorim is metabolized by the same glutathione conjugation and associated processing pathway in rice cultures (supplemental materials).

Recently, the initial steps of the glutathione conjugate catabolic pathway and the localization of the respective enzymes in plants have been the subject of renewed interest (21–24). Based on herbicide metabolism studies in cereal crops, it had previously been assumed that following GST action, *S*-glutathionylated xenobiotics were imported into the vacuole by ATP-binding cassette transporters (25). Once inside the vacuole, glutathione conjugates were then hydrolyzed by carboxypeptidases to form the γ-glutamylcysteine derivatives (26), which in turn were processed to the cysteine conjugates by γ-glutamyl transpeptidases (25). This hydrolytic sequence is in contrast to that determined in animals, where *S*-glutathionylated drugs are sequentially hydrolyzed by a γ-glutamyl-transpeptidase, followed by a carboxypeptidase (16). Recent biochemical and molecular genetic studies in *Arabidopsis* have challenged the model developed in cereals by identifying two parallel processing pathways. In pathway 1, glutathione conjugates are sequentially processed in the vacuole to cysteinylglycine derivatives and then to the cysteine conjugates (21–23). In pathway 2, the glutathione conjugates are first processed to the γ-glutamylcysteine derivative in the cytosol through the carboxypeptidase activity of the enzyme phytochelatin synthase (24). Subsequent conversion to the cysteine conjugate is then catalyzed by a
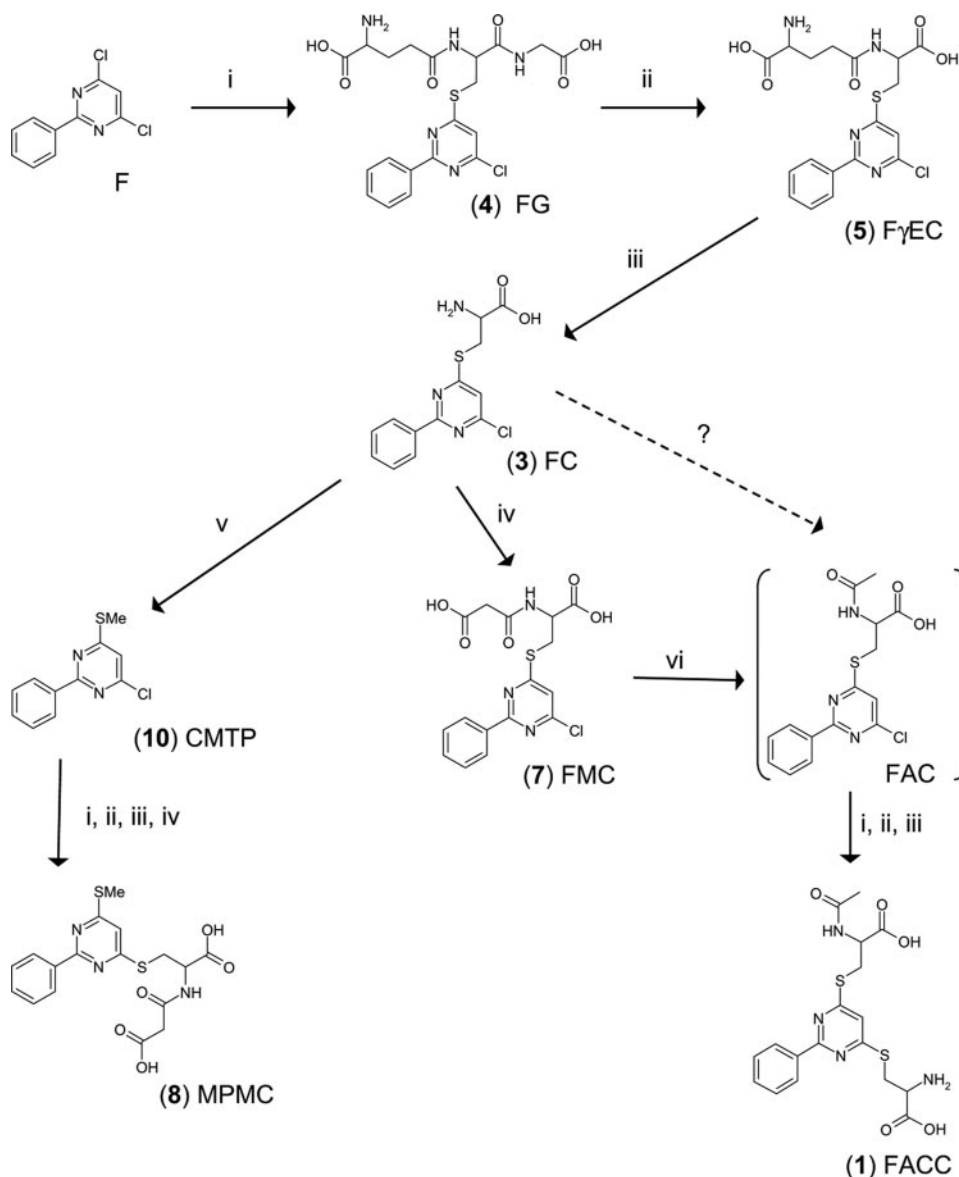
FIGURE 7. **The metabolic fate of fenclorim in *Arabidopsis* cell suspension cultures over 24 h.** The *numbered arrows* refer to the following reactions: glutathionylation (*i*), removal of a glycine moiety (*ii*), removal of a γ-glutamyl moiety (*iii*), *N*-malonylation (*iv*), C-S lyase mediated cleavage of the cysteine moiety and subsequent *S*-methylation of the exposed thiol (*v*), and decarboxylation (*vi*). The putative intermediate FAC (*S*-(4-chloro-2-phenylpyrimidin-6-yl)-*N*-acetylcysteine), which was not isolated as a metabolite, is shown in *parentheses*. The available evidence suggests that FAC is derived from FMC, but the possibility that this intermediate results from the *N*-acetylation of FC is also indicated.

nant in dedifferentiated callus tissues. The processing of FγEC to FC at the cell surface by the γ-glutamyltranspeptidase in the plasma membrane (23) may explain the rapid accumulation of FC in the medium as well as in the cells during the early stages of metabolism. The extracellular deposition of the FC was transient, with the conjugate then reabsorbed and processed. The speed of this process is suggestive of a specific and efficient import and recycling system.

Although the early steps of the glutathione conjugation pathway in *Arabidopsis* and other plants are relatively well studied, less attention has been directed at the enzymes that act downstream of the cysteine conjugates. The metabolism of the FC conjugate in *Arabidopsis* followed one of three pathways (Fig. 7), although it is likely that additional minor biotransformations also operated, based on the presence of other uncharacterized metabolites that behaved as terminal FG catabolites. In contrast to studies carried out in crop plants, on the basis of HPLC-MS studies, we saw no evidence that *Arabidopsis* processed FC by *N*-glucosylation or transamination to give the thiopyruvate derivative and subsequent further metabolism to the thiolactic and thioacetate derivatives (reviewed in Ref. 16).

In *Arabidopsis*, the major route of FC metabolism was *N*-malonylation (Fig. 7), a reaction previously described in the processing of glutathione conjugates of a number of herbicides (16). The respective malonyl-CoA-dependent NMT activity responsible for the derivatization of cysteine conjugates was identified for the first time. We speculate that the enzyme responsible must be related to the largely uncharacterized NMTs active toward D-amino acids and 1-aminocyclopropane-1-carboxylase (27). In turn, these NMTs are likely to be members of the large superfamily of BAHD acyltransferases in *Arabidopsis* (28).

The second route of FC metabolism involved the formation of a putative *N*-acetylated cysteine conjugate. Although the formation of such mercapturic acid conjugates is common in drug metabolism in animals (17), it is an unusual reaction in plants (18). In animals, the *N*-acetylation of cysteine is catalyzed by a specific acetyl-CoA-dependent acetyltransferase (17). In our

γ-glutamyltranspeptidase isoenzyme localized to the plasma membrane (23). The determination of which of these dichotomous pathways operates to process glutathione conjugates in *Arabidopsis* has not been fully resolved. However, on the basis of enzyme expression data, it appears that whereas pathway 1 predominates in the roots, pathway 2 functions in the foliage (23, 24). Our studies in *Arabidopsis* suspension cultures show that following its rapid *S*-glutathionylation, fenclorim is processed by pathway 2 in the cytosol (Fig. 7). We base this conclusion on the presence of FγEC as a major intermediate, with *S*-(fenclorim)-cysteinylglycine being undetectable in our feeding studies. Significantly, *S*-(bimane)-glutathione was also metabolized by the cytosolic pathway in *Arabidopsis* suspension cultures (24), suggesting that this pathway must be domi-

studies, this enzyme activity was not detected and to our knowledge has not been described in any other plant. It has been suggested that *N*-acetylcysteine derivatives of pesticides could instead arise from the decarboxylation of the respective *N*-malonylated conjugates (18). In support of this hypothesis, we observed that FMC spontaneously decarboxylated to form FAC at a slow but measurable rate of 1.6 μmol s^{-1} mol^{-1} when stored as a methanolic solution. Based on the absence of evidence of active *N*-acetylation of FC, we therefore propose that FAC is slowly generated from FMC by decarboxylation and that the intermediate once formed is immediately *S*-glutathionylated by GSTs. The reasons for plants *N*-malonylating cysteine conjugates rather than forming the *N*-acetyl derivatives, as determined in animals, is intriguing. It is possible that the formation of the acidic malonylated conjugate helps direct these cysteine derivatives for vacuolar import, as is known to be the case for phenolic glucosides (29). If so, then metabolites, such as FMC, would be physically separated from cytosolic enzymes, which could lead to further metabolism. In contrast, if the acetylated derivatives were produced, these would remain in the cytosol and be treated as other xenobiotics. In the case of FAC, this resulted in secondary GST-mediated *S*-glutathionylation. To date, such multiple rounds of conjugation of xenobiotics have rarely been recorded. A notable example is the safener benoxacor, which undergoes two rounds of glutathionylation in maize suspension cultures (30, 31).

The third route of FC catabolism involved the concerted action of a cysteine conjugate β-lyase and *S*-methyltransferase to yield CMTP. Similar biotransformations of cysteine conjugates of xenobiotics have been reported in onions (19) and spruce trees (20) and can result in the formation of residues that can be lost from the plant by volatilization (20). Enzymes with C-S lyase (32) and *S*-methyltransferase (33) activities toward xenobiotic derivatives have been individually characterized in Brassica species with their functions in endogenous secondary metabolism postulated. Our results suggest that the lyase and methyltransferase must act in a closely coordinated manner on xenobiotic cysteine conjugates to form the *S*-methyl derivatives, since we were unable to identify any trace of the thiol intermediate. In onions, these enzymes behave as a complex (19), suggesting that this processing pathway effectively channels the C-S lyase products derived from cysteine conjugates into the biogenesis of *S*-methylated metabolites.

Although it has been known for some time that the cysteine conjugates of xenobiotics can undergo multiple metabolic fates in plants, the biological significance of these pathways has received little attention, presumably because the natural substrates of this intricate catabolic network have proved elusive. The fenclorim metabolism studies provide useful clues as to the biological importance of these reactions. Thus, the competing cysteine conjugate β-lyases and associated *S*-methylation pathway gives rise to CMTP, which was found to be both a good GST-inducing agent in *Arabidopsis* and an active herbicide safener in rice. Importantly, our studies suggest that the modification of the amino group is important in directing the cysteinyl derivatives away from pathways that can metabolically reactivate them. Similarly, it has been proposed that mercapturic

acid formation is important in the mammalian kidney in preventing drug reactivation though the action of C-S lyases (17).

The glutathione conjugation of xenobiotics in plants is generally considered to result in their detoxification, although there are rare examples of reactive toxins being produced during the associated catabolism of the conjugates (16). Our studies examining the role of metabolism in determining the GST-inducing activity in *Arabidopsis* suggest that the glutathionylation pathway and the processing of the conjugates can result in both bioactivation and detoxification. Testing of the major fenclorim conjugates that accumulated during the induction period gave only a weak enhancement of GST activity, and this appeared to be due to the metabolism of FG and FC to CMTP. Based on the timing of the appearance of CMTP following fenclorim treatment as compared with the enhancement of GST activity, it seems unlikely that the metabolite is a primary inducing agent. However, the formation of CMTP may contribute to the longevity of safener action, since the levels of this metabolite were relatively stable 8–24 h after feeding, whereas by the end of the time course, the fenclorim had been totally depleted. Interestingly, CMTP is an example of a compound that having arisen from metabolic bioactivation through the glutathione conjugation pathway is then subject to a second round of conjugation leading to its inactivation again. Based on these studies, understanding how the glutathione conjugate catabolic pathway is regulated may therefore provide a novel route to bioactivating other crop protection agents. In the case of safener action, these results will now form the basis of structure activity studies in which a set of fenclorim derivatives will be prepared to investigate the essential chemical characteristics required for GST-inducing activity in *Arabidopsis* and crop plants.

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