Role of a Carboxylesterase in Herbicide Bioactivation in Arabidopsis thaliana

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Arabidopsis thaliana contains multiple carboxylesterases (AtCXEs) with activities toward xenobiotics, including herbicide esters that are activated to their phytotoxics acids upon hydrolysis. On the basis of their susceptibility to inhibition by organophosphates, these AtCXEs are all serine hydrolases. Using a trifunctional probe bearing a fluorophosphonate together with biotin and rhodamine to facilitate detection and recovery, four dominant serine hydrolases were identified in the proteome of Arabidopsis. Using a combination of protein purification, capture with the trifunctional probe and proteomics, one of these hydrolases, AtCXE12, was shown to be the major carboxylesterase responsible for hydrolyzing the pro-herbicide methyl-2,4-dichlorophenoxyacetate (2,4-D-methyl) to the phytotoxic acid 2,4-dichlorophenoxyacetic acid. Recombinant expression of the other identified hydrolases showed that AtCXE12 was unique in hydrolyzing 2,4-D-methyl. To determine the importance of AtCXE12 in herbicide metabolism and efficacy, the respective tDNA knock-out (atcxe12) plants were characterized and shown to lack expression of AtCXE12 and have greatly reduced levels of 2,4-D-methyl-hydrolyzing activity. Young atcxe12 seedlings were less sensitive than wild type plants to 2,4-D-methyl, confirming a role for the enzyme in herbicide bioactivation in Arabidopsis.

A diverse range of synthetic compounds enter plants as pollutants or crop protection agents and undergo four phases of metabolism; namely, the introduction of reactive functional groups (phase 1), bioconjugation with polar natural products (phase 2), conjugate transport into the vacuole (phase 3), and finally phase 4 mineralization or incorporation into macromolecules (1). We have termed this xenobiotic detoxifying system the xenome and are currently functionally characterizing its components in a range of plants (2). An important group of phase 1 enzymes that have received very little attention in plants are the xenobiotic-hydrolyzing carboxylesterases (CXEs). These plant enzymes detoxify persistent pollutants (3) and insecticides (4), as well as hydrolyzing pro-herbicide esters to their bioactive free acids (5, 6). In the latter case, many major classes of herbicides are applied as esters to facilitate penetration into the leaf. Ester hydrolysis within the leaf is then required to bioactivate the herbicide, and the rate of cleavage is an important determinant of selective action in crops and weeds (7, 8). Using a classification system based on the sensitivity of hydrolases to inhibition by organophosphate insecticides, herbicide-active CXEs in wheat and competing grass weeds are of the B-type (9, 10). B-class CXEs use a catalytic serine, with this residue undergoing irreversible covalent modification by organophosphates on binding. The modified catalytic serine residue is part of a conserved Ser-His-Asp catalytic triad that is found in a large number of hydrolytic enzymes in both prokaryotes and eukaryotes, most classically in the α/β-hydrolase-fold proteins (10, 11). Arabidopsis contains several superfamilies of α/β-hydrolase-fold proteins, the best characterized being the serine proteases (12). Interestingly, this well characterized active site chemistry has been recruited for multiple functions in plant metabolism, notably the hydrolysis of amide and carboxylic ester bonds, dehydrations, transacylations, and lyase functions (13).

With respect to the hydrolases active toward xenobiotic carboxylic esters (see Fig. 1A), some progress has recently been made in identifying xenobiotic-hydrolyzing serine hydrolases in tobacco (14), black-grass (Alopecurus myosuroides L.) (5), and rice (15). These studies have demonstrated that the xenobiotic-hydrolyzing enzymes described are from distinct protein families. Thus, whereas the CXEs from rice and tobacco are both classic α/β-hydrolases (14, 16), the black-grass esterase was homologous to the unrelated microbial GDS hydrolase superfamily (16). This example of convergent functional evolution is in contrast to all the other enzymes of xenobiotic metabolism in plants. Thus, the phase 1 cytochrome P450 mixed-function oxidases (17), the phase 2 glutathione transferases (18) and glucosyltransferases (19) and the phase 3 ATP-binding cassette transporters (20), are each derived from divergent superfamilies.

The presence of multiple esterase gene families in the plant xenome is further complicated by the lack of correlation between activities of individual enzymes toward "model" xeno-

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biotic esters and “real” pollutants and pesticides. Thus, the major esterase in wheat with activity toward the general colorimetric substrates p-nitrophenyl acetate (pNA, Fig. 1A) and α-naphthyl acetate (αNA) had negligible activities toward herbicides used in this crop (9). Conversely, a 40-kDa esterase from the weed black-grass, which hydrolyzed aryloxyphenoxypropionate esters such as clodinafop-propargyl (Fig. 1A) to the respective herbicidal acids had little activity toward substrates used in colorimetric assays (5).

Based on these fragmentary data derived from multiple species, there was a need to develop new tools to functionally characterize the large numbers of CXEs in plants. Based on approaches applied to functional proteomics in animal cells (21), we now report on the use of chemical probes to identify CXEs involved in xenobiotic detoxification in Arabidopsis. The trifunctional probe used had a fluorophosphonate group that covalently modified reactive serine residues in the active sites of expressed AtCXEs, a biotin tag to facilitate recovery of labeled proteins and a fluorophore (rhodamine) for detection and quantification (see Fig. 2A). Using a combination of classic enzyme purification and this “chemotyping” probe, we have isolated the CXE in Arabidopsis plants responsible for the selective hydrolysis and hence bioactivation of the pro-herbicide methyl 2,4-dichlorophenoxyacetate (2,4-D-methyl) to the phytotoxic 2,4-D acid (Fig. 1A).

**EXPERIMENTAL PROCEDURES**

*Arapidopsis Plants and Cultures—Arapidopsis* (Columbia) suspension cell cultures and root cultures were maintained and harvested as previously detailed (22). Seed from the SAIL tDNA insertion mutant SAIL_445_G03 was obtained from the Nottingham *Arapidopsis* Stock Centre (Nottingham, UK). *Arapidopsis* knock-out and wild-type (Columbia) plants were maintained as described previously (23). For the toxicity studies with young plants (28 days), treatments of 2,4-D-methyl or 2,4-D were spray-applied in 0.1% (v/v) Tween 20 at 0, 2, 5, or 10 mg liter⁻¹ each at a rate of 360 ml m⁻², equivalent to field applications of 36, 18, and 7.2 g of active ingredient Ha⁻¹, respectively. Plants were then assessed for injury at timed intervals, with all treatments carried out in triplicate.

**Analysis of tDNA Knockouts—**Homozygous plants were selected using the primers: left product (LP), ACCAAGATCC-ACTAAAATTCATC; right product (RP), GATGTTTGTCTGACTGTC; and SAIL left border (LB), TTCATAACCATGGATTCCGAGATCGCCG-3'; and SAIL right product (RP), GATGTTTGCTCGACTGTC. The site of tDNA insertion was then selected using the primers: left product (LP), ACCAAGATCC-ACTAAAATTCATC; and SAIL right product (RP), GATGTTTGCTCGACTGTC. The site of tDNA insertion was then sequenced by the PCR products after an inhibitory pre-treatment to solubilize the biotinylated polypeptides.

**CXE Assay—**Esterase assays with pNA were colorimetrically determined, whereas the hydrolysis of the pesticide esters was monitored by high-performance liquid chromatography (9). All activities were determined in duplicate and corrected for non-enzymic hydrolysis by using boiled protein controls. CXE activity toward αNA was visualized following isoelectric focusing of protein preparations after an inhibitory pre-treatment ± 0.1 mM paraoxon (9).

**CXE Purification and Proteomic Analysis—**AtCXEs were purified from *Arapidopsis* suspension cultures using the protocol previously described, using sequential chromatography of active fractions on DEAE, butyl-Sepharose, and Mono Q fast-protein liquid chromatography (5). CXE activity was monitored in fractions using pNA and 2,4-D-methyl as substrates. In the final stages, the TriFP was used to label active CXEs for concentration by streptavidin affinity chromatography prior to resolution of the polypeptides present by SDS-PAGE using 12.5% gels (5). Proteins labeled with the TriFP fluorescence tag were visualized using a Fuji FLA-3000 Imager, and the respective bands were then heated to 90 °C in 20 μl of SDS-PAGE loading buffer to solubilize the biotinylated polypeptides.

**Cloning of AtCXEs—**The AtCXEs identified by proteomics were cloned using selective forward (F) and reverse (R) primer combinations. For AtCxE5 (At1g49960), F = 5'-TCTCATATTGGAATCTCGAACATGCTGTCC-3' and R = 5'-TCTCGAGACC-AATAAATTGACCTG-3'; for AtCXE2 (At3g48690), F = 5'-TACAACTACATGATTCCGAGATCGCCG-3' and R = 5'-CGGCTCAGGGTTCCCTCCTTTATAAACCC-3'; for AtCXE20 (At5g62180), F = 5'-TCATATGGCCGCCGCTCG-3' and R = 5'-TCCTCGAGCAACACAGAGATATGAA-3'. cDNA was prepared from total RNA from the aerial tissues of flowering *Arapidopsis* plants and used as the template for PCR, with the amplification products cloned first.
into pGEMT and then pET24 for the transformation of Escherichia coli strain ROSETTA DE3 (pLysS, Novagen) as described (25). Expression and purification of the recombinant AtCXEs was carried out essentially as previously described (25), except cultures were cooled and maintained at 10 °C when induced with isopropyl 1-thio-D-galactopyranoside.

Accession Numbers—Sequence data from this article can be found in the GenBank™/EMBL data libraries under the following accession numbers: AtCXE5, At1g49660; AtCXE12, At3g48690; and AtCXE20, At5g62180.

RESULTS

CXE Activities in Arabidopsis—To evaluate the usefulness of Arabidopsis as a model plant to study the hydrolytic bioactivation of herbicides, extracts from the foliage, roots, and suspension cultures were assayed against 2,4-D-methyl and the aryloxyphenoxypropionate herbicide clodinafop-propargyl (Fig. 1A). The model CXE substrate pNA was also included (Fig. 1A) for reference. With the herbicide substrates, all of the tissues tested had a different range of hydrolytic activities (Fig. 1C). 2,4-D-Methyl was always the preferred substrate, particularly in suspension cultures, with lower activities being determined with clodinafop-propargyl, a herbicide used in the selective control of grass weeds in cereal crops (5). These results demonstrated that Arabidopsis plants and cultures were able to catalyze the hydrolytic bioactivation of herbicide esters, particularly 2,4-D-methyl, a compound designed to be used in the control of dicotyledous weeds. To study the diversity of CXEs present, crude extracts from Arabidopsis foliage, roots and suspension cultures were resolved by isoelectric focusing and visualized by incubating with α-naphthyl acetate, with and without a prior treatment with the organophosphate inhibitor paraoxon (Fig. 1B). In total, 10 CXEs could be visualized by this method, all of which were sensitive to inhibition by paraoxon, demonstrating that these were all serine hydrolases (Fig. 1B).

Identifying Serine Hydrolases in Arabidopsis—The zymogen analysis showing the presence of multiple CXEs sensitive to inhibition by organophosphates suggested that this labeling chemistry could be used to identify the respective proteins using a directed proteomics approach. The method adopted involved the preparation of a customized trifunctional probe bearing a fluorophosphonate labeling group, a biotin recovery tag, and a rhodamine fluorescent reporter function (Fig. 2A). The TriFP probe was synthesized and purified, and its identity was confirmed by MALDI-TOF MS prior to use. Total protein extracts from Arabidopsis plants and cell cultures were incubated with the TriFP, and the resulting labeled proteins recovered using streptavidin affinity chromatography. The tagged proteins were then resolved by SDS-PAGE, and the dominant

FIGURE 1. Hydrolysis of xenobiotics and herbicides in Arabidopsis plants and cultures. A, the structures of the pro-herbicides 2,4-D-methyl and clodinafop-propargyl and the model esterase substrate pNA. Sites of hydrolysis are denoted as arrows. B, resolution of esterases active in hydrolyzing pNA from Arabidopsis foliage (1 and 4), roots (2 and 5) and suspension cultures (3 and 6). Samples 4–6 were incubated with the serine hydrolase inhibitor paraoxon prior to electrophoresis, whereas samples 1–3 were not treated. C, hydrolytic activities toward the substrates shown in A in crude extracts from plants and cell cultures of Arabidopsis and in purified recombinant AtCXEs. Activities are in picokatal mg⁻¹ protein and are means of duplicates ± the variation in the replicates. ND, no activity detected.
fluorescently labeled polypeptides excised and subjected to MALDI-TOF MS-based proteomics (Fig. 2B). Four polypeptides were identified as the major expressed serine hydrolases, namely prolyl-oligopeptidase (At1g76140), and three serine hydrolases of unknown function: a 46-kDa putative GDS-type hydrolase, a 36-kDa putative CXE previously termed AtCXE12 (At3g48690, Marshall et al. 26), and a 27-kDa lysophospholipase-like CXE (At5g20060). The screen was useful for defining the relative abundance of expressed serine hydrolases in Arabidopsis and confirmed the diversity of proteins bearing this catalytic mechanism. The utility of the TriFP in identifying serine hydrolases in crude plant extracts suggested that it would also be a useful tool in helping purify and identify low abundance AtCXEs associated with specific hydrolytic activities. Thus, a chemotyping probe with fluorophosphonate functionality could be employed in the final stages of enzyme enrichment to unambiguously identify serine hydrolases present and affinity-concentrate them for MS-based proteomics.

**Purification of AtCXEs**—The CXEs responsible for the hydrolysis of 2,4-D-methyl in Arabidopsis suspension cultures were purified from a crude protein extract using a combination of ammonium sulfate precipitation and separation based on anion exchange and hydrophobic interaction chromatographies (Table 1). First, proteins precipitated between 40 and 80% ammonium sulfate saturation were resolved using a DEAE-Sepharose column. CXE activity toward 2,4-D-methyl eluted in two pools, with the majority recovered in peak DEAE1 (supplemental Fig. S1A). The DEAE2 peak was applied onto a butyl Sepharose column, where it was resolved into peaks butyl 1 and 2 (supplemental Fig. S1B). These fractions were stored separately, with the major pool (butyl 2) applied onto a Mono Q fast-protein liquid chromatography column, where the activity eluted in a single sharp peak (supplemental Fig. S1C). Overall, the 2,4-D-methyl-hydrolyzing activity was purified 305-fold with 3.3% recovery using this protocol (Table 1). In contrast, when the final purified preparation was assayed with clodinapropargyl, the enrichment was only 30-fold in 0.3% yield, demonstrating that the CXE activity isolated was selectively enriched for the hydrolysis of 2,4-D-methyl rather than other classes of herbicide.

The polypeptides present at each stage of the purification were monitored by SDS-PAGE (Fig. 3A). When analyzed for total protein content it was difficult to identify a single polypeptide that was being enriched by sequential purification, with the final preparation containing multiple polypeptides. This was to be expected based on the low selectivity of the chromatography steps employed and is a common feature of purifying low abundance enzymes of secondary metabolism. However, by labeling proteins from each stage of the purification with the TriFP and then using the biotin recognition tag, the identification of the active serine hydrolases in each fraction was immediately clarified, with a single 36.6-kDa polypeptide identified in the final preparation. Use was then made of the biotin affinity tag to purify the TriFP-labeled protein from the final enriched fraction using streptavidin affinity chromatography, purifying and concentrating the polypeptide for proteomic analysis in a single step (Fig. 3B). Tryptic digests of the purified protein were analyzed by MALDI-TOF MS and analysis of the peptide fragments identified AtCXE12 as the active hydrolase (Table 2 and supplemental Fig. S2).

**TABLE 1**

| Fraction | Total protein | Specific activity | Purification | Total activity | Total recovery |
|----------|---------------|------------------|--------------|----------------|----------------|
| Mono Q1  | 0.22          | 87900            | 304.5        | 19.33          | 3.3            |
| Mono Q2  | 0.91          | 48930            | 169.5        | 44.3           | 7.5            |
| DEAE     | 66            | 6070             | 21           | 401            | 67.7           |
| 40–80% (NH₄)₂SO₄| 664 | 940 | 3.3 | 626 | 106 |
cation runs that there were additional CXEs with this activity present. Thus the 2,4-D-methyl-hydrrolyzing activity could be resolved into distinctly eluting pools both at the stage of DEAE-anion exchange chromatography (supplemental Fig. S1A) and on butyl-Sepharose (supplemental Fig. S1B). Attempts to isolate CXEs from the minor DEAE 1 pool proved unsuccessful due to the instability of the respective enzymes. However, when the butyl 1 fraction was applied to a Mono Q column, a broad peak of CXE activity was recovered (supplemental Fig. S3).

Using the TriFP, the peak was subsequently shown to contain three distinct serine hydrolases that were analyzed by MALDI-based proteomics. All the proteins were identified as members of the AtCXE family (26), namely AtCXE5 (At1g49660) with lesser amounts of AtCXE20 (At5g62180) and AtCXE12 (At3g48690) also being determined (Table 2). The identification of this “secondary” minor source of AtCXE12 was made with a lower degree of confidence than was the case with the other two CXEs, but the polypeptide did have the same molecular mass as the AtCXE12 protein derived from the major butyl 2 fraction. The apparent presence of AtCXE12 in both chromatographic fractions would suggest that the protein has undergone different routes of post-translational processing to produce isoforms that differed in their hydrophobicities.

The coding sequences of AtCXE5, AtCXE12, and AtCXE20 were PCR-amplified from cDNA prepared from Arabidopsis plants and subcloned into pET24 plasmids for expression of the C-terminal His-tagged proteins in E. coli. When analyzed by SDS-PAGE, AtCXE5 and AtCXE20 accumulated large amounts of recombinant protein in the inclusion bodies, even when the induced cultures were grown at low temperatures. AtCXE12 expression was less problematic, with significant amounts of soluble recombinant enzyme being produced. Sufficient amounts of each recombinant enzyme were purified to allow them to be analyzed by SDS-PAGE (Table 2) and assayed for CXE activity (Fig. 1C). Recombinant AtCXE20 showed no measurable CXE activity toward the substrates tested. Both AtCXE5 and AtCXE12 were similarly active in hydrolyzing pNA, whereas AtCXE12 was unique in rapidly hydrolyzing 2,4-D-methyl. AtCXE5 was unstable when stored in solution, tending to aggregate into high molecular weight complexes and was not characterized further.

**Characterization of AtCXE12 Knock-out Arabidopsis Plants**
To characterize the role of AtCXE12 in planta, tDNA Express was used to identify transposon insertions that could disrupt AtCXE12 expression. Line SAIL_445_G03 was identified in the SAIL tDNA insertion line collection (27) and homozygous lines were selected. Analysis of the insertion site by PCR resulted in two products of sizes ~400 and ~600 bp, with the larger produced using primers directed to the right product (RP) and left border (LB), whereas PCR with primers to the left product (LP) and left border (LB) produced the smaller fragment. This suggested there might be a double, back-to-back tDNA insertion, and left border (LB) produced the smaller fragment. This suggested there might be a double, back-to-back tDNA insertion, an event subsequently confirmed by sequencing of the PCR products (supplemental Fig. S4).

Extracts from the homozygous atcxe12 knock outs were assayed for esterase activity toward 2,4-D-methyl and pNA. When assayed with 2,4-D-methyl, hydrolyzing activity in the wild-type plants (511 ± 116 picokatals mg⁻¹ protein; mean ± S.D., n = 3) was 4-fold higher than that determined in the knock-outs plants (116 ± 39 picokatals mg⁻¹). The hydrolysis of pNA was less affected, with the atcxe12 plants (450 ± 40 picokatals mg⁻¹) having three quarters of the activity determined in wild types (600 ± 60 picokatals mg⁻¹). To determine if this loss of CXE activity was due to

**TABLE 2**
CXEs identified through the purification of CXE activity toward 2,4-D-methyl from Arabidopsis suspension cultures

| Fraction | CXE     | AGI gene code | Predicted mass | Observed mass | MALDI statistics |
|----------|---------|---------------|----------------|---------------|-----------------|
|          |         |               | kDa            |               |                 |
| Mono Q1  | AtCXE12 | At1g49660     | 35.4           | 35.4          |                 |
| Mono Q2  | AtCXE12 | At1g49660     | 35.4           | 35.4          |                 |
| Mono Q3  | AtCXE20 | At5g62180     | 36.4           | 36.6          |                 |
| Mono Q4  | AtCXE5  | At5g62180     | 36.4           | 36.6          |                 |

|          |          |                                     |             |               | % | Mascot score | p value |
|----------|----------|-------------------------------------|-------------|---------------|---|--------------|---------|
| Mono Q1  | 35.4     | 35.4                                | 35.4        | 35.4          | 0.0018 |
| Mono Q2  | 35.4     | 35.4                                | 35.4        | 35.4          | 0.4   |
| Mono Q3  | 36.4     | 36.6                                | 36.6        | 35.4          | 0.026 |
| Mono Q4  | 36.4     | 38.1                                | 38.1        | 35.4          | 0.004 |

**FIGURE 3. Isolation of the 2,4-D-methyl-hydrolyzing AtCXE using the TriFP.** A, purification of the major CXE-hydrolyzing 2,4-D-methyl from Arabidopsis suspension cultures as monitored by SDS-PAGE with protein staining (lanes 1–5) and by visualization of biotinylated peptides using Western blotting, probing with streptavidin-linked phosphodiesterase after labeling each fraction with the TriFP (lanes 6–10). Lane 1, M, markers; lanes 2 and 7, crude 40–80% (NH₄)₂SO₄ protein precipitate; lane 3 and 8, peak DEAE; lanes 4 and 9, peak Butyl 2; lanes 5 and 10, Mono Q peak; lane 6, streptavidin blot of the crude 40–80% (NH₄)₂SO₄ protein precipitate without prelabeling with the TriFP demonstrating the presence of endogenously biotinylated proteins in the extract. B, the CXEs present in the final Mono Q fraction (lanes 5 and 10 in A) were treated with the TriFP, and the biotinylated proteins were then recovered by streptavidin affinity chromatography and analyzed by SDS-PAGE (lane 2) and Western-blotted with streptavidin (lane 3), prior to proteomic analysis of the major 36.6-kDa polypeptide. Lane 1, M, markers. The major stained polypeptide running at the bottom of the gel is streptavidin.

| Fraction | CXE     | AGI gene code | Predicted mass | Observed mass | MALDI statistics |
|----------|---------|---------------|----------------|---------------|-----------------|
| Mono Q1  | AtCXE12 | At1g49660     | 35.4           | 35.4          |                 |
| Mono Q2  | AtCXE12 | At1g49660     | 35.4           | 35.4          |                 |
| Mono Q3  | AtCXE20 | At5g62180     | 36.4           | 36.6          |                 |
| Mono Q4  | AtCXE5  | At1g49660     | 35.4           | 38.1          |                 |
the suppression of AtCXE12 expression, crude plant extracts from wild-type and knock-out plants were treated with the TriFP, and the streptavidin-labeled proteins were analyzed for rhodamine fluorescence following resolution by SDS-PAGE (Fig. 4). The use of the probe confirmed that the AtCXE12 was selectively suppressed in the knock-out plants, unlike the other labeled CXEs.

Having determined that AtCXE12 was a major 2,4-D-methyl-hydrolyzing CXE in Arabidopsis, it was then of interest to determine whether or not the enzyme had a role in herbicide bioactivation. If so, then suppression of its expression should result in a decreased sensitivity of Arabidopsis plants to the pro-herbicide 2,4-D but not toward 2,4-D. Four-week-old wild-type Arabidopsis and homozygous atcxe12 plants were sprayed with 0, 7.2, 18, and 26 gHa⁻¹ of 2,4-D, or 2,4-D-methyl, respectively. Both sets of plants showed identical sensitivities to 2,4-D but differed in their tolerance to 2,4-D-methyl, with the atcxe12 lines being the least affected (Fig. 5).

**DISCUSSION**

AtCXE12 was shown to be a major CXE in Arabidopsis with a key role in hydrolyzing and hence bioactivating the pro-herbicide 2,4-D-methyl to the phytotoxic acid 2,4-D in planta. Studies with herbicide-resistant and -susceptible weed populations (7) and metabolism studies with crops and wild grasses (8) have correlative pointed to the importance of esterase-mediated hydrolysis in herbicide bioactivation. Using Arabidopsis as a plant model, our molecular genetic studies demonstrate that a single CXE can confer this bioactivation trait and determine herbicide sensitivity. Interestingly, the studies in Arabidopsis have identified a completely different CXE to be involved in pro-herbicide hydrolysis than that identified in the weed blackgrass (5). The black-grass CXE was a member of the GDS family of hydrolases, which are distinct in sequence and structural fold from the classic α/β hydrolases such as the AtCXEs (17, 26). The black-grass enzyme, AmGDSH1, was active toward aryloxyphenoxypionate esters (5), whereas AtCXE12 favored the phenoxyacetate 2,4-D-methyl. However, our results demonstrate that it is not possible to ascribe substrate-specific activities to each class of hydrolase. For example, although AtCXE12 hydrolyzed 2,4-D-methyl, the related AtCXE5 did not. Interestingly, whereas the aryloxyphenoxypionate herbicides are bioactivated by apoplastic hydrolases (6), AtCXE12 was predicted to be localized to the cytoplasm. Thus, we would predict that with 2,4-D-methyl hydrolysis to the phytotoxic 2,4-D occurred within...
the cell. Although derived from a different metabolic mechanism, the intracellular bioactivation to 2,4-D in Arabidopsis has precedence, being reported as a consequence of beta-oxidation of 2,4-dichlorophenoxbutyric acid (28, 29). From this we can conclude that the paradigm that herbicide bioactivation through de-esterification is predominantly an extracellular event (6), cannot apply to all plants.

Our proteomic studies identified both GDS hydrolases and CXEs as being expressed in Arabidopsis along with two other classes of serine hydrolases. Similarly large gene families encoding GDS hydrolases, CXEs, and other classes of serine hydrolases are present in rice (15, 12). Based on the known diversification in function of the serine hydrolases in plant primary and secondary metabolism (13), it is more than likely that other classes of these enzymes are involved in the hydrolysis of other (pro)-herbicide chemistries. Although our results do not suggest a simple informatics-based approach to predict hydrolytic activities based on the class of hydrolase, they do point to the great variety in hydrolytic activation potentially available in developing new selective crop protection agents in different plants. A more detailed understanding of the expression of specific hydrolases in crops and competing weeds will be very useful in the future in designing crop protection agents that are selectively activated or detoxified by the species-specific complement of esterases present in the xenome of the respective plant.

Our studies have also identified the value of using activity-based probes for functional proteomic studies in plants. In this study we have used the TriFP to identify serine hydrolases using both a global and directed screen. In view of the large number of interesting enzymes of plant secondary metabolism that use the catalytic triad of the serine hydrolases to effect hydrolytic, acyl transfer, lyase, and dehydratase activity (30, 13, 26) this chemotyping approach will be very useful in identifying other low abundance enzymes using this catalytic mechanism. In addition, the fluorophosphonate and other chemotyping probes have proven very useful in differential proteomics studies in determining functional changes in enzyme expression under different developmental conditions in healthy and diseased mammalian cells (31). There is therefore considerable scope in the future to expand the use of such chemical biology approaches developed for applications in medicinal chemistry to define protein function in plants.

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