HERBICIDE BIOACTIVATION IN ARABIDOPSIS THALIANA: ROLE OF A CARBOXYLESTERASE

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Running title: ‘2,4-D-methyl bioactivation in Arabidopsis’

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Arabidopsis thaliana contains multiple carboxylesterases (AtCXEs) with activities toward xenobiotics, including herbicide esters that are activated to their phytotoxic acids upon hydrolysis. On the basis of their susceptibility to inhibition by organophosphates, these AtCXEs are all serine hydrolases. Using a trifunctional probe (TriFP) 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 TriFP and proteomics, one of these hydrolases, AtCXE12, was shown to be the major carboxylesterase responsible for hydrolysing the pro-herbicide methyl-2,4-dichlorophenoxyacetate (2,4-D-methyl) to the phytotoxic acid 2,4-D. 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 T-DNA 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 hydrolysing 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, 6). 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 super-families 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-hydrolysing enzymes described are from distinct protein families. Thus, whereas the carboxylesterases from rice and tobacco were both classic \( \alpha/\beta \)-hydrolases (14, 16), the black-grass esterase was homologous to the unrelated microbial GDS hydrolase super-family (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 super-families.

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’ xenobiotic esters and ‘real’ pollutants and pesticides. Thus, the major esterase in wheat with activity toward the general colorimetric substrates \( p \)-nitrophenyl acetate (\( p \)NA, Fig 1A) and \( \alpha \)-naphthyl acetate (\( \alpha \)NA) had negligible activities toward herbicides used in this crop (9).

Conversely, a 40 kDa esterase from the weed black-grass, which hydrolyzed aryloxyphenoxypropionate (AOPP) esters such as clodinafop-propargyl (Fig 1A) to the respective herbicidal acids had little activity toward substrates used in colorimetric assays (5).

Based on this 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 labelled proteins and a fluorophore (rhodamine) for detection and quantification (see Fig. 2A).

Using a combination of classical enzyme purification and this ‘chemotyping’ probe, we have isolated the CEX 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

Arabidopsis plants and cultures- Arabidopsis (Columbia) suspension cell cultures and root cultures were maintained and harvested as previously detailed (22). Seed from the SAIL T-DNA insertion mutant SAIL_445_G03 was obtained from the Nottingham Arabidopsis Stock Centre (NASC: Nottingham, UK). Arabidopsis 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 L\(^{-1}\) each at a rate of 360 ml m\(^{-2}\), equivalent to field applications of 36, 18 and 7.2 g active ingredient Ha\(^{-1}\) respectively. Plants were then assessed for injury at timed intervals, with all treatments carried out in triplicate.

Analysis of T-DNA knockouts- Homozygous plants were selected (http://signal.salk.edu/tdnaprimers.2.html) using the primers: Left product (LP): ACCAAGATCCACTAAAATTCATC; Right product (RP): GATGTTTGCTCCTGACCAAGATCCACTAAAATTCATC.

The generation of the trifunctional fluorophosphonate (TriFP) probe utilized the modular synthetic strategy described for the synthesis of the corresponding sulfonate ester probes (21). Firstly, the \( N \)-hydroxysuccinimide (NHS) ester of 10-(fluoroethoxyphosphinyl)decanoic acid was prepared from 10-(ethoxyhydroxyphosphinyl)decanoic acid (23). Separately, \( \alpha \)-(9-fluorenylmethoxycarbonyl-(FMOC))-lysine conjugated with biotin through its carboxy function and with tetramethylrhodamine through its \( \epsilon \)-amine function was prepared (21). The FMOC protecting group was removed by treating (14
mg, 5 μmol) of the bifunctional linker with 5 ml 4N HCl in dioxane for 60 min at room temperature. After drying under a stream of N₂, the deprotected intermediate was dissolved in methanol (3 ml) containing NaHCO₃ (5 mg) and reacted with freshly prepared 10-(fluoroethoxyphosphinyl)-N-hydroxysuccinyl) decanamide (1.9 mg, 5 μmol). After reacting under argon for 4h at room temperature, solvent was evaporated under vacuum and the TriFP purified by preparative reversed phase HPLC C18 column using an increasing linear gradient of water: MeCN (95:5 v/v) to MeCN. The final product (0.2 μmol) was analyzed by MALDI-ToF MS m/z = 1134.38 (C₅₃H₅₂F₈N₄O₁₀PS requires 1134.38 Da).

TriFP (5 μM) was incubated with protein (1 ml) in 0.1M Tris-HCl buffer (pH 7.2) for 60 min at 37 °C. Labelled proteins were then either analyzed directly by SDS-PAGE, or subjected to affinity chromatography by mixing with streptavidin Sepharose (40μl of 50% slurry) in the presence of SDS (0.2% w/v) on an end-over-end mixer (60 min). The streptavidin sepharose was pelleted by centrifugation and washed twice with 1 ml SDS (0.2% w/v), followed by 2 x 1ml distilled water. The beads were then heated to 90°C in 20µl SDS-PAGE loading buffer to solubilize the biotinylated polypeptides.

**CXE assay**- Esterase assays with p-NA were colorimetrically determined while the hydrolysis of the pesticide esters was monitored by HPLC (Cummins et al., 2001). All activities were determined in duplicate and corrected for non-enzymic hydrolysis by using boiled protein controls. CXE activity toward α-NA was visualized following isoelectric focussing (IEF) of protein preparations after an inhibitory pre-treatment ± 0.1 mM paraoxon (9).

**CXE purification and proteomic analysis**- AtCXEs were purified from Arabidopsis suspension cultures using the protocol previously described, using sequential chromatography of active fractions on DEAE, butyl-sepharose and Mono Q FPLC (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 labelled with the TriFP fluorescent tag were visualized using a Fuji FLA-3000 Imager and the respective bands excised and digested with trypsin prior to analysis on a Voyager DE-STR MALDI-ToF mass spectrometer (Applied Biosystems, Warrington, UK) as described (22). The resulting peptide mass ions were used to screen a non-redundant Arabidopsis protein database using Mascot (http://www.matrixscience.com/).

**Cloning of AtCXEs**- The AtCXEs identified by proteomics were cloned using selective forward (F) and reverse (R) primer combinations. For AtCXE5 (At1g49660); F = 5’TCATATGGAATCTGAAATCGCCTCCG 3’ and R = 5’TCTCGAGACCAATAAAAACTGACG 3’; for AtCXE12 (At3g48690); F = 5’TCAACTAACCATGGATTCGAGATTCCG 3’ and R = 5’TGGCTCAGGCTTAAAACTCCCTCCCTTAATAAACCC 3’; for AtCXE20 (At5g62180), F = 5’TCATATGTCGAAACCAAGTCCCAATC 3’ and R = 5’TCTCGAGACCAAGAAGATATGAA 3’. cDNA was prepared from total RNA from the aerial tissues of flowering Arabidopsis plants and used as the template for PCR, with the amplification products cloned first into pGEMT and then pET 24 for the transformation of E. coli strain ROSETTA DE3 (pLysS, Novagen) as described (Kordic et al., 2002). 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 IPTG.

**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; 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 AOPP herbicide clodinafop-propargyl (Fig. 1A). The model carboxylesterase substrate p-nitrophenyl acetate (p-NA) 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 dicotyledenous weeds. To study the diversity of CXEs present, crude extracts from Arabidopsis foliage, roots and suspension cultures were resolved by isoelectric focussing (IEF) 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). Similarly, the hydrolytic activities toward the pro-herbicides were also sensitive to inhibition by the organophosphate. The differences in esterase expression between the tissues were relatively subtle. For example, a CXE with a basic pI was present at higher levels in root cultures and foliage than in suspension culture, while the converse was true for an enzyme with a pI of pH 5.1. Based on these results it was concluded that Arabidopsis cell cultures were an excellent source of CXEs with activity toward 2,4-D-methyl and that the respective enzymes were serine hydrolases.

Identifying serine hydrolases in Arabidopsis- The zymogen analysis showing the presence of multiple CXEs sensitive to inhibition by organophosphates suggested that this labelling 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 labelling group, a biotin recovery tag and a rhodamine fluorescent reporter function (Fig. 2A). The trifunctional fluorophosphonate probe (TriFP) was synthesized, purified and its identity 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 labelled proteins recovered using streptavidin affinity chromatography. The tagged proteins were then resolved by SDS-PAGE and the dominant fluorescently-labelled 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 carboxylesterase previously termed AtCXE12 (At3g48690, Marshall et al., 2003) and a 27 kDa lysophospholipase-like carboxylesterase (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). Firstly, proteins precipitated between 40-80% 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 DEAE 2 (Supplemental Fig 1A). The DEAE 2 peak was applied onto a butyl sepharose column, where it was resolved into peaks butyl 1 and 2 (Supplemental Fig. 1B). These fractions were stored separately, with the major pool (butyl 2) applied onto a Mono-Q FPLC column, where the activity eluted in a single sharp peak (Supplemental Fig 1C). 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 clodinafop-propargyl, 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 labelling 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-labelled 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 analysed by MALDI-ToF-MS and analysis of the peptide fragments identified AtCXE12 as the active hydrolase (Table 2 and Supplemental Fig. 2).

Identification, cloning and expression of AtCXEs involved in xenobiotic hydrolysis in Arabidopsis cultures- While AtCXE12 was the major 2,4-D-methyl-hydrolyzing carboxylesterase expressed in Arabidopsis, it was also apparent from the purification runs that there were additional CXEs with this activity present. Thus the 2,4-D-methyl hydrolysing activity could be resolved into distinctly eluting pools both at the stage of DEAE anion exchange chromatography (Supplemental Fig 1A) and on butyl sepharose (Supplemental Fig. 1B). 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. 3). Using the TriFP, the peak was subsequently shown to contain three distinct serine hydrolases that were analysed 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 sub-cloned 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 carboxylesterase activity toward the substrates tested. Both AtCXE5 and AtCXE12 were similarly active in hydrolyzing pNA, whereas AtCXE12 was unique in rapidly hydrolysing 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 characterise the role of AtCXE12 in planta, ‘T-DNA Express’ (http://signal.salk.edu/cgi-bin/tdnaexpress) was used to identify transposon insertions that could disrupt AtCXE12 expression. Line SAIL_445_G03 was identified in the SAIL T-DNA insertion line collection (27) and homozygous lines selected. Analysis of the insertion site by PCR resulted in two products of sizes ~400bp and ~600bp, with the larger produced using primers directed to the right product (RP) and left border (LB), while 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 T-DNA insertion, an event subsequently confirmed by sequencing of the PCR products (Supplemental Fig. 4).
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, hydrolysing activity in the wild-type plants (511 ± 116 pkat mg⁻¹ protein; mean ± SD, n = 3) was four-fold higher than that determined in the knock-outs plants (116 ± 39 pkat mg⁻¹). The hydrolysis of pNA was less affected, with the atcxe12 plants (450 ± 40 pkat mg⁻¹) having three quarters of the activity determined in wild types (600 ± 60 pkat mg⁻¹). To determine if this loss of CXE activity was due to the suppression of AtCXE12 expression, crude plant extracts from wild-type and knock-out plants were treated with the TriFP, and the streptavidin-labelled proteins analysed 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 labelled CXEs.

Having determined that AtCXE12 was a major 2,4-D-methyl hydrolysing 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 methyl, 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 hydrolysing 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 black-grass (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 AtCXE1s (17, 26). The black-grass enzyme, AmGDSH1, was active toward AOPP esters (5), whereas AtCXE12 favoured 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 AOPP-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. Though 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-dichlorophenoxybutyric 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. While 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.

REFERENCES


FOOTNOTES

MCG acknowledges the support of a Cooperative Award in Science and Engineering from the Biotechnology and Biological Sciences Research Council (BBSRC) and Syngenta. The authors thank Dr Kate Sharples of Syngenta for useful advice. The study with the chemical probes forms part of a research development fellowship awarded to RE by the BBSRC.
Figure Legends

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 α-NA 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, while 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 pkat mg⁻¹ protein and are means of duplicates ± the variation in the replicates. ND = no activity detected.

FIGURE 2. Use of TriFP to identify serine hydrolases in Arabidopsis.  
A: Structure of TriFP. When acted on by a catalytic serine, the fluorine atom is displaced leaving the probe bearing the biotin and rhodamine tags covalently bound to the hydrolase.  
B: Resolution of polypeptides from total protein extracts of Arabidopsis plants and cell cultures covalently labelled with the TriFP. The biotinylated proteins were recovered using streptavidin affinity chromatography and after SDS-PAGE the TriFP fluorescently labelled polypeptides directly visualized. Dominant polypeptides were then excised and identified by MALDI-ToF-MS based proteomics as 1. prolyl oligopeptidase (At1g76140); 2. a GDS hydrolase of unknown function (At1g76140); 3. a carboxylesterase of unknown function from the AtCXE family (Marsh et al. 2003) termed AtCXE12 (At3g48690); 4. a lysophospholipase-like carboxylesterase (At5g20060).

FIGURE 3. Isolation of the 2,4-D-methyl-hydrolyzing AtCXE using the TriFP.  
A. Purification of the major CXE hydrolysing 2,4-D methyl from Arabidopsis suspension cultures as monitored by SDS-PAGE with protein staining (lanes 1 - 5) and by visualisation of biotinylated peptides using western blotting, probing with streptavidin-linked phosphodiesterase after labelling each fraction with the TriFP (lanes 6-10). Lane 1: Mr markers, Lane 2 & 7 = crude 40 - 80% (NH₄)₂SO₄ protein precipitate, Lane 3 & 8 = peak DEAE, Lane 4 & 9 peak Butyl 2, Lane 5 & 10 = MonoQ peak, Lane 6: streptavidin blot of the crude 40 - 80% (NH₄)₂SO₄ protein precipitate without pre-labelling 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 & 10 in A) were treated with the TriFP and the biotinylated proteins 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: Mr markers. The major stained polypeptide running at the bottom of the gel is streptavidin.

FIGURE 4. Confirmation of knock-out of AtCXE12 expression in atcxe12 plants.  
Protein extracts from the wild-type and homozygous SAIL_445_G03 (atcxe12) plants were labelled with TriFP, and after affinity pull-down with streptavidin, separated by SDS-PAGE then imaged for rhodamine fluorescence. Mr as indicated in kDa with AtCXE12 arrowed.

FIGURE 5. Effect of AtCXE12 knockout on 2,4-D-methyl toxicity. 
Four-week-old wild-type and atcxe12 Arabidopsis plants were sprayed with the rates of herbicide indicated and left for four days to allow symptoms to develop before assessment for symptoms of 2,4-D toxicity. Examples of plants showing symptoms relating to toxicity scores of 2-10 are given. Dead plants were assigned a score of 0.
Table 1: Summary of the purification of the major CXE activity toward 2,4-D-methyl from *Arabidopsis* suspension cultures.

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Table 2: CXEs identified through the purification of CXE activity toward 2,4-D-methyl from *Arabidopsis* suspension cultures. Searches using data from MALDI analyses were limited to *Arabidopsis thaliana* proteins.

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<td>AtCXE12</td>
<td>At3g48690</td>
<td>35.8</td>
<td>36.6</td>
<td>33%</td>
<td>74</td>
<td>0.0018</td>
</tr>
<tr>
<td>MonoQ2</td>
<td>AtCXE12</td>
<td>At3g48690</td>
<td>35.8</td>
<td>36.6</td>
<td>30%</td>
<td>52</td>
<td>0.4</td>
</tr>
<tr>
<td>MonoQ3</td>
<td>AtCXE20</td>
<td>At5g62180</td>
<td>36.4</td>
<td>40.1</td>
<td>29%</td>
<td>63</td>
<td>0.026</td>
</tr>
<tr>
<td>MonoQ4</td>
<td>AtCXE5</td>
<td>At1g49660</td>
<td>35.4</td>
<td>38.1</td>
<td>45%</td>
<td>71</td>
<td>0.004</td>
</tr>
</tbody>
</table>
2,4-D-methyl Clodinafop-propargyl p-nitrophenyl acetate

Figure 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>2,4-D methyl</th>
<th>Clodinafop propargyl</th>
<th>p-nitrophenyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foliage</td>
<td>377 ± 57</td>
<td>45.8 ± 1.3</td>
<td>990 ± 130</td>
</tr>
<tr>
<td>Root Culture</td>
<td>251 ± 19</td>
<td>27.2 ± 0.7</td>
<td>930 ± 40</td>
</tr>
<tr>
<td>Suspension Culture</td>
<td>472 ± 0</td>
<td>8.1 ± 0.2</td>
<td>1100 ± 30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pure recombinant enzyme</th>
<th>2,4-D methyl</th>
<th>Clodinafop propargyl</th>
<th>p-nitrophenyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAtCXE5</td>
<td>12.9 ± 0.4</td>
<td>5.49 ± 0.1</td>
<td>6949 ± 355</td>
</tr>
<tr>
<td>rAtCXE12</td>
<td>1840 ± 450</td>
<td>180 ± 11</td>
<td>6230 ± 320</td>
</tr>
<tr>
<td>rAtCXE20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
Figure 4
Figure 5
Herbicide bioactivation in Arabidopsis thaliana: Role of a carboxylesterase
Markus C. Gershater, Ian Cummins and Robert Edwards

J. Biol. Chem. published online May 22, 2007

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