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Structure activity studies with xenobiotic substrates using carboxylesterases isolated from *Arabidopsis thaliana*

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Abstract

Carboxylesterases (CXEs) catalyse the hydrolysis of xenobiotics and natural products radically altering their biological activities. Whereas the substrate selectivity of animal CXEs, such as porcine liver esterase (PLE) have been well studied, the respective enzymes in plants have yet to be defined and their activities determined. Using *Arabidopsis thaliana* (*At*) as a source, five representative members of the α/β hydrolase *At*CXE family of proteins have been cloned, expressed and the purified recombinant proteins assayed for esterase activity with xenobiotic substrates. Two members, *At*CXE5 and *At*CXE18 were found to be active carboxylesterases, though *At*CXE5 proved to be highly unstable as a soluble protein. *At*CXE18 and the previously characterised *S*-formylglutathione hydrolase from Arabidopsis (*At*SFGH) were assayed against a series of esters based on methylumbelliferone in which the acyl moiety was varied with respect to size and conformation. The same series was used to assay crude esterase preparation from Arabidopsis plants and the results compared with those obtained with the commonly used PLE. With straight chain esters, *At*CXE18 behaved like PLE, but the Arabidopsis hydrolases proved less tolerant of branched chain acyl components than the mammalian enzyme. While none of the enzyme preparations accurately reflected all the activities determined with crude Arabidopsis protein extracts, the plant enzymes proved more useful than PLE in predicting the hydrolysis of the more sterically constrained esters.

Keywords: *Arabidopsis thaliana*; Carboxylesterase; Ester series; α/β Hydrolases; Methylumbelliferone

1. Introduction

Serine hydrolases are a diverse group of enzymes, which catalyse hydrolytic and transacylating reactions with both natural and synthetic substrates (Heikinheimo et al., 1999). In the case of drugs and pesticides, hydrolysis radically alters biological activity and trans-membrane transport and so the respective hydrolases are key proteins in determining bioavailability and bioactivity (Satoh and Hosokawa, 1998). In microorganisms, the hydrolases active toward synthetic amides, carboxyesters and thioesters have been studied in some detail due to their importance in the biodeterioration of polymers and the bioremediation of pollutants (Ro et al., 2004). Similarly, in mammals and insects these enzymes have attracted attention due to their roles in drug and insecticide metabolism, respectively (Satoh and Hosokawa, 1998; Oakeshott et al., 1999). In contrast, the corresponding enzymes in plants have received far less attention. This is surprising, given their importance in determining the uptake and biological activity of important classes of herbicides, fungicides and insecticides (Cummins et al., 2001; Haslam et al., 2001). For example, insecticides such as the pyrethroids are detoxified by plant esterases, limiting their bioavailability in crop protection (Preiss et al., 1988). Esterases also bioactivate pro-herbicides to their phytotoxic alcohols or acids, with the differential rates of hydrolysis in different plants contributing to selective weed control (Cummins and Edwards, 2004; Haslam et al., 2001). The relationship between chemical structure and the rates of pesticide hydrolysis in plants would therefore

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be a useful tool in agrochemistry for predicting the relative rates of pesticide detoxification/bioactivation in crops and weeds. A greater knowledge of these enzymes would also be useful in determining their largely uncharacterized roles in plant secondary metabolism.

Currently, porcine liver esterase (PLE) is used as a model hydrolase to predict the likely rates of cleavage of synthetic esters in both the pharmaceutical and agrochemical industries (Redinbo and Potter, 2005). While this model enzyme may usefully reflect the activities of drug-metabolising hydrolases in animals, its utility in accurately predicting the activity of esterases in plants has not been reported. It would therefore be useful to identify 'model' esterases in plants and compare their structure–activity profiles with those of PLE. PLE is a classic serine hydrolase with a characteristic α/β structural fold and the catalytic motif G × S × G, associated with this class of protein (Heikinheimo et al., 1999). Informatic analysis based on the presence of the catalytic motif and predicted α/β structural modules shows that plants contain multiple classes of serine hydrolases. After eliminating those classic serine hydrolases with known functions in hydrolysing proteins and carbohydrates, two classes of enzymes with potential roles in biotransforming small bioactive metabolites have been identified. These are the hydroxynitrile lyase (hnl)-like (Wäspi et al., 1998) and the hsr203J-like proteins, the latter originally being identified in tobacco as being linked to the hypersensitive response to microbial pathogens (Baudouin et al., 1997). Members of both classes of enzymes have been shown to hydrolyse xenobiotic esters (Baudouin et al., 1997; Wäspi et al., 1998). The model plant *Arabidopsis thaliana* (*At*) contains both types of putative hydrolases, with the hsr203J-like proteins being encoded by a group of 20 genes termed the carboxylesterase (CXE) family (Marshall et al., 2003). Based on sequence relatedness, the Arabidopsis *At*CXE family can be arrayed into five clades (Fig. 1). CXE-like sequences have subsequently been determined in a wide variety of plants, suggesting they are of widespread importance (Marshall et al., 2003). The CXE class of esterases therefore represent a useful example of a novel plant-specific carboxylesterases with which to compare activities with PLE.

In addition to looking at plant-specific esterases, we have also been interested in the possibility that the hydrolysis of synthetic esters can be conserved in plants and animals. Thus, *S*-formylglutathione hydrolase (SFGH), is an example of a serine hydrolase which is highly conserved in both prokaryotes and eukaryotes (Kordic et al., 2002; Haslam et al., 2002), where it performs a key role in C-1 metabolism (Fig. 1). Interestingly, irrespective of their source SFGHs are also known to be highly active in hydrolysing xenobiotic carboxylic esters including methylumbelliferyl acetate (Kordic et al., 2002; Cummins et al., 2006). We have therefore used SFGH from Arabidopsis (*At*SFGH) as an example of a serine hydrolase which will be found in plants as well as animals. To test their diversity in substrate usage, we have prepared a custom series of esters of methylumbelliferone (MU) and used these to test for carboxylesterase activities in crude plant preparations from Arabidopsis. The profile of activities has then been compared with those determined with commercially available PLE, a representative member of the *At*CXE superfamily and *At*SFGH.

2. Results

2.1. Cloning and expression of *At*CXEs from Arabidopsis

Representatives from five of the six clades of the *At*CXEs were cloned from Arabidopsis foliage by PCR, namely *At*CXE5, *At*CXE13, *At*CXE16, *At*CXE17, *At*CXE18 and *At*CXE20 (Fig. 2). The sequences were then sub-cloned into the pET24 plasmid for the expression of

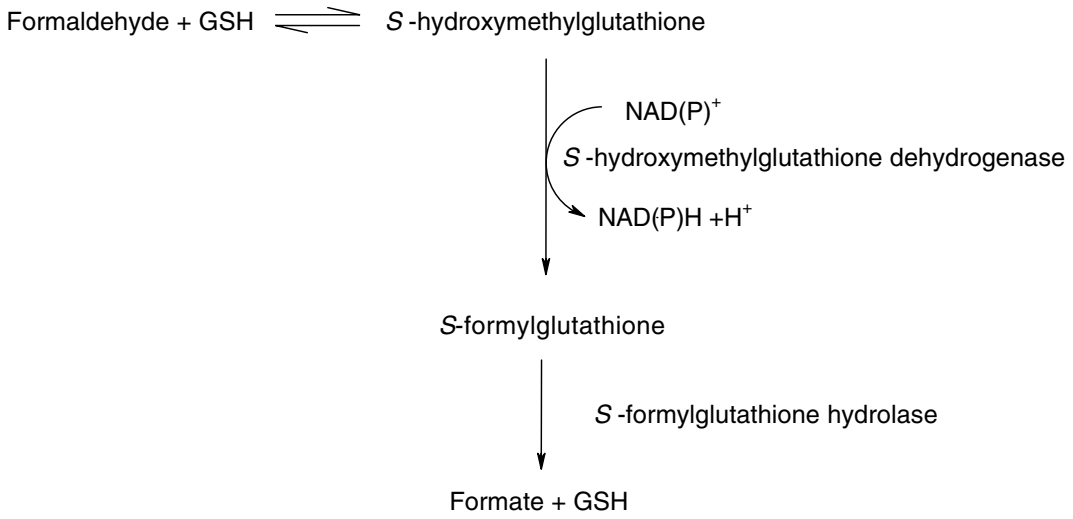


Fig. 1. Role of *At*SFGH in C-1 metabolism. After the spontaneous reaction of formaldehyde with glutathione to form *S*-hydroxymethylglutathione, the conjugate is selectively oxidised to *S*-formylglutathione, which is then hydrolysed by *At*SFGH to release formic acid and glutathione.

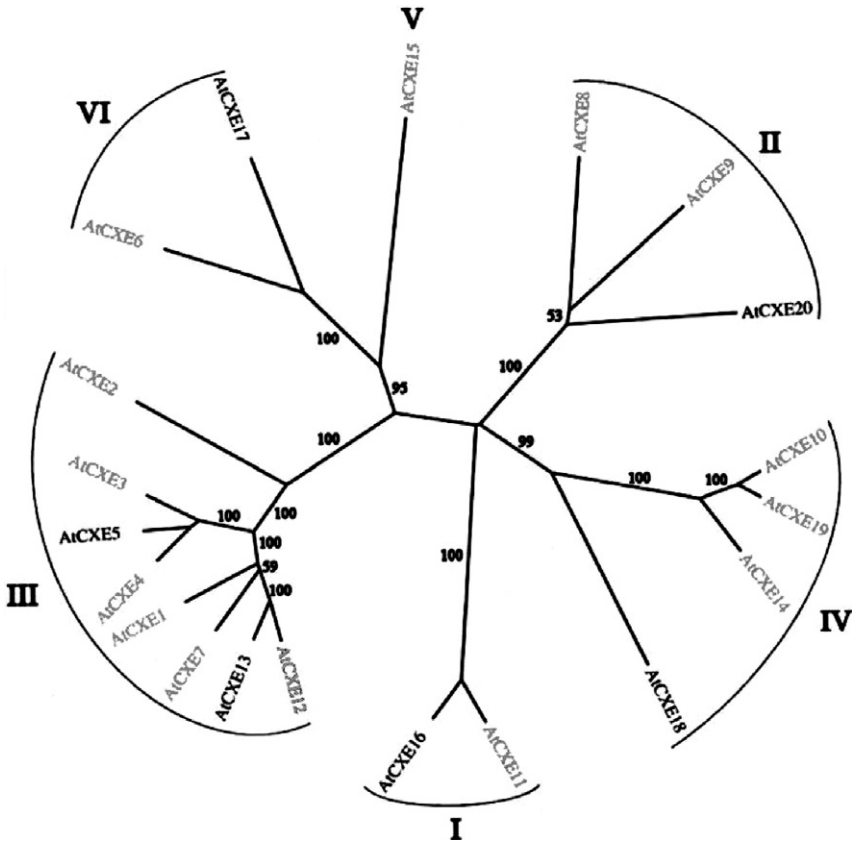


Fig. 2. Dendrogram of the *At*CXE family in Arabidopsis as determined by sequence identity/similarity with the values showing the number of bootstrap replicates (of 100) that concurred at each branch-point. Clade numbers are shown as assigned by Marshall et al. (2003). Family members selected for cloning and expression are shown in bold.

the respective C-terminal His-tagged proteins in *Escherichia coli*. As determined by SDS–PAGE, recombinant protein expression was not seen with *At*CXE13, *At*CXE16 and *At*CXE17. With the other three clones, the expressed recombinant proteins largely accumulated in the insoluble fraction (Fig. 3). Attempts to enhance the expression of these proteins in the soluble fraction by growing the induced bacteria at low temperature proved unsuccessful. When the respective soluble fractions of these cultures were analysed for hydrolytic activity toward *p*-nitrophenyl acetate (*p*NA), *At*CXE5 and *At*CXE18 were found to show high levels of carboxyesterase activity after correcting for the endogenous activity present in *E. coli*. No activity was found in extracts from *At*CXE20, even though soluble protein was detected following SDS–PAGE (Fig. 3). Soluble proteins were purified by Ni-chelate affinity chromatography and all found to migrate as 40 kDa polypeptides when analysed by SDS–PAGE, with a trace of contaminating higher molecular weight polypeptides (Fig. 3). No carboxylesterase activity could be determined with purified *At*CXE20. When assayed with *p*NA, activity was determined with freshly prepared pure *At*CXE5 (6.5 μkat mg^{-1} protein) and *At*CXE18 (3.9 μkat mg^{-1}). Both enzymes were unstable, *At*CXE5 being particularly labile with the respective enzyme activity having a half-life of only 30 min when stored on ice. As determined by gel permeation chromatography, instability was due to aggregation to high molecular weight inactive complexes (data not shown). Attempts to stabilize the activity by adding reducing agents, salts or glycerol all proved ineffective and further characterization of *At*CXE5 was not attempted. Instead, studies focussed on *At*CXE18 which was analysed by time-of-flight mass spectrometry after electrospray ionisation and shown to have a parent mass of 38,649 Da. Based on the predicted mass of 38,754 Da this suggested that the N-terminal methionine of *At*CXE18 had been cleaved during expression in *E. coli*.

2.2. Structure activity studies with an MU ester series

*At*CXE18, *At*SFGH and PLE were subjected to structure activity studies with a range of MU carboxylesters prepared from the respective acid chlorides (Fig. 4). For reference, these activities were compared to those determined with crude protein extracts obtained from Arabidopsis plants. With the straight chain acyl ester series, extension from C2 to C5 (compounds **1**–**4**) was poorly tolerated by the carboxylesterases present in the plant extracts. Interestingly, the hexanoate ester (**5**) was an excellent substrate for the Arabidopsis esterase preparation, while further extension of the acyl moiety resulted in a total loss in activity. The substrate preferences observed with the

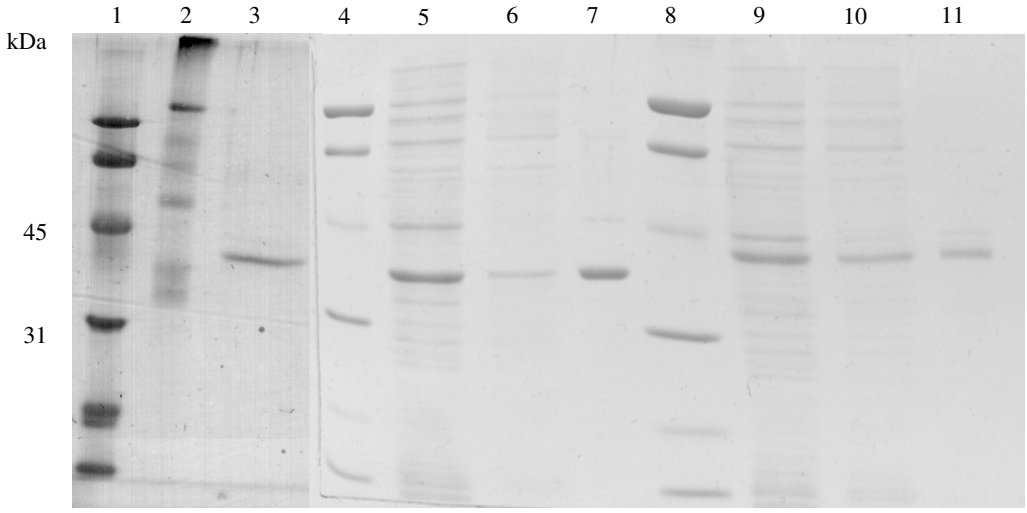


Fig. 3. SDS–PAGE of recombinant *At*CXEs showing expression in *E. coli* with molecular mass reference markers shown in lanes 1, 4 and 8 (lane 2 = pre-stained Mr standards mixture). *At*CXE18, the family member used in the structure activity studies is shown as the purified recombinant enzyme in lane 3. The other two *At*CXEs which were not analysed further are also shown for reference. For *At*CXE5, the analysis of the respective total *E. coli* pellet is shown in lane 5, the soluble crude protein in lane 6 and the purified enzyme in lane 7. Similarly for *At*CXE20 the protein samples are total extract (lane 9), soluble fraction (lane 10) and purified protein (lane 11).

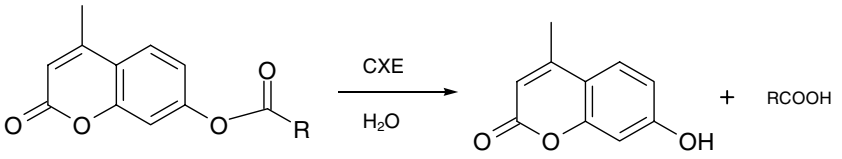
straight chain ester series in the plant extracts were quite different from those determined with the individual Arabidopsis esterases, which were far more tolerant of the longer acyl chains. Of the two Arabidopsis enzymes, *At*CXE18 behaved most like PLE showing high activities with acyl components up to C8 (**6**), albeit with 10-fold lower specific activities than the mammalian carboxylesterase. With all enzyme preparation the hexanoate ester (**5**) proved to be an excellent substrate.

The enzymes were then assayed with branched chain and unsaturated acyl esters (Fig. 4b, compounds **7**–**11**). As with the longer chain derivatives, with the crude protein extracts from Arabidopsis, turnover was low or below the limit of detection. Generally, the introduction of an alkene (**10**, **11**) resulted in lower turnover, consistent with the general reactivity of α,β-unsaturated carbonyl compounds. The effect of branching identified clear differences in the three carboxylesterases. Compound **9** was not tolerated by any of the enzymes, whereas compounds **7** and **8** probed subtle differences in their respective active sites. Thus, whereas *At*CXE18 was intolerant of both esters, they were turned over with similar activities by PLE, whereas *At*SFGH only acted on **7**. To extend the series of sterically hindered acyl esters, a set of cyclic containing substrates moieties were then tested (Fig. 4b, compounds **12**–**14**). These gave more varied results but some basic trends were observed. The two cyclopentane esters (**12**, **13**) were hydrolysed by the plant extracts at similar rates. This would not have been predicted from the studies conducted with any of the enzyme preparations, with compound **12** being a much better substrate than **13** with PLE, *At*CXE18 and *At*SFGH, respectively. Interestingly, given the similar steric volumes (*A*-values) of an ^{i}Pr (2.15), cyclopentyl (~2.1) and phenyl group (2.7) an alternate odd-even trend is observed in the effect of the position of the bulky acyl group along the series such that **12** > **7** < **13** > **14**. Finally, whilst the aryl containing ester substituted **14** was hydrolysed by all the preparations, it was a particularly good substrate for *At*CXE18.

To examine the reasons for the differences in activity observed with the MU ester series, kinetic analyses were performed with compounds **1**–**14** using *At*SFGH as a test-bed (Table 1). Based on the k_{cat} and K_m determinations, it could be seen that binding affinity for the MU esters was little affected by varying the acyl group. Instead, varying the structure had a more profound effect on turnover. For example, when comparing the simple C5 ester (**4**) with the branched C5 (**7**) and the unsaturated C5 (**11**), K_m values of 50 μM, 80 μM and 60 μM were very similar, while k_{cat}/K_m values (s^{-1} mM^{-1}) were 291, 103 and 9, respectively. Similar detailed kinetic analysis was not conducted with *At*CXE18. However, preliminary studies with outlying members of the MU ester series showed that differences in activity were due to altered k_{cat} rather than K_m values, as had been determined with *At*SFGH.

3. Discussion

Representative members of the *At*CXE superfamily from Arabidopsis were cloned and expressed in *E. coli*, with *At*CXE5 and *At*CXE18, being shown to be active carboxylesterases when assayed with xenobiotic substrates. All 20 members of the *At*CXE superfamily are classic serine hydrolases (Marshall et al., 2003), with the potential of being catalytically active. Therefore, it is quite possible that the other *At*CXEs expressed were also active hydrolases, but their activity was not apparent due to their insta-



Compound #	R		Specific Activity (nkat mg^{-1} protein)			
	Theoretical mass	Observed mass	PLE	*At*CXE18	*At*SFGH	*At* Protein Extract
1	CH_3 218.2	*m/z*+1 219.2	2120 ± 33	238 ± 4	588 ± 12	15 ± 2
2	C_2H_5 232.2	*m/z*+1 233.2	1796 ± 78	218 ± 14	399 ± 9	2 ± 0.1
3	246.3	*m/z*+1 247.2	3566 ± 124	225 ± 13	437 ± 11	7 ± 2
4	260.3	*m/z*+1 261.2	4135 ± 228	338 ± 32	330 ± 8	1 ± 0.25
5	274.3	*m/z*+1 275.2	8645 ± 241	474 ± 9	604 ± 15	21 ± 2
6	302.4	*m/z*+1 303.4	1374 ± 62	105 ± 2	162 ± 2	0
7	260.3	*m/z*+1 261.2	1989 ± 42	8 ± 0.2	230 ± 3	2 ± 0.5
8	260.3	*m/z*+1 261.2	1948 ± 67	4 ± 0.3	9 ± 1	0
9	274.3	*m/z*+1 275.2	12 ± 2	0	12 ± 3	0
10	244.3	*m/z*+1 245.1	794 ± 13	45 ± 4	34 ± 4	1 ± 0.35
11	258.3	*m/z*+1 259.2	220 ± 27	7 ± 0.5	11 ± 1	0.2 ± 0.1
12	272.3	*m/z*+1 273.2	1971 ± 54	344 ± 28	226 ± 1	6 ± 1
13	300.4	*m/z*+1 301.2	158 ± 18	63 ± 2	76 ± 0	6 ± 3
14	324.3	*m/z*+1 325.1	988 ± 112	272 ± 34	184 ± 6	3 ± 0.2

Fig. 4. (a) The enzymic hydrolysis of the MU ester series. (b) The acyl (R) series used with compound numbers referred to in the text and predicted and obtained ($m/z + 1$) parent molecular masses of the pure ester substrates shown. Rates of hydrolysis for each of the enzymes are shown as means of duplicate determinations with the error bars showing the variation in the replicates.

Table 1
Kinetic constants determined with SFGH using the MU ester series as numbered in Fig. 4b

	*At*SFGH K_m (mM)	*At*SFGH K_{cat}/K_m (s^{-1} mM^{-1})
1	0.08	283
2	0.08	202
3	0.07	264
4	0.05	291
5	0.09	243
6	0.04	217
7	0.08	103
8	0.1	4
9	0.09	5
10	0.08	19
11	0.06	9
12	0.04	224
13	0.05	68
14	0.08	97

bility as soluble recombinant proteins or their inability to hydrolyse the limited range of MU ester substrates tested. *At*CXE18, *At*SFGH and PLE were incubated with the MU ester series with the intention of comparing their activities to one another as well as identifying which enzymes, if any, could predict the spectrum of carboxylesterases present in crude Arabidopsis protein extracts. With the straight chain acyl esters (compounds **1**–**6**), the pure proteins proved of limited value in predicting the likely rates of hydrolysis in the crude plant extracts, with the exception of highlighting the rapid hydrolysis of the hexanoate ester (**5**). Interestingly, the results of the current study with carboxylesterases are quite different from those obtained in earlier studies with MU esters incubated with lipase preparations from wheat germ, castor bean and peanut, where the hydrolysis of the acyl moiety was sustained to C9 and found to be identical to that determined with a mammalian lipase (Jacks and Kircher, 1967). This confirms that the Arabidopsis hydrolases selected were indeed CXEs likely to act on small esters rather than classic lipases which due to their active site architecture show a preference for long chain acylated substrates (Grabulkeda et al., 1997). With the more sterically constrained branched chain and cyclised esters, the Arabidopsis enzymes did prove to be more useful in reflecting the restricted activities seen in the plant extracts. This was particularly apparent with compounds **7** and **8**, which were good substrates of PLE but poorly tolerated by the plant enzymes. Since PLE would tend to over-estimate the rates of cleavage of esters bearing sterically constrained acyl functions, this result suggests that the incorporation of plant esterases into screens of carboxylesterase-mediated metabolism of pesticide esters would be potentially useful predicting rates of hydrolysis in crops and weeds. Importantly, when assayed with MU-derived substrates, both Arabidopsis enzymes and PLE were true carboxylesterases. Thus, when incubated with a series of thioesters and amides based on the same substitutions shown in Fig. 3, negligible activities with any substrate were determined after correcting for non-enzymic hydrolysis (data not shown). In the case of *At*SFGH, this result was somewhat surprising, as the endogenous substrate of this enzyme is the thioester *S*-formylglutathione, with the enzyme performing an important role in formaldehyde detoxification (Fig. 1). When the related *S*-acetylglutathione was assayed as a substrate, no thiohydrolase activity was determined with *At*CXE18 or PLE (data not shown), whereas this thioester was actively hydrolysed by *At*SFGH. From this it was concluded that the thioesterase activity of *At*SFGH was strictly dependent on the substrate being a thioester of glutathione alone, reflecting its conserved essential role in C-1 metabolism in both prokaryotes and eukaryotes (Fig. 1). In fact, thioesters of MU such as the hexanoate derivative actually acted as competitive inhibitors of carboxylesterase activity with substrate **1**, with a K_i of 0.31 mM being determined.

The results of this study suggest that the acyl binding pocket of the Arabidopsis esterases studied here is more constrained than that determined for the large hydrophobic pocket of PLE (Provencher and Jones, 1994), In the case of *At*SFGH, we have recently solved the structure of the protein to a resolution of 1.7 Å (Cummins et al., 2006), but were unable to map the active site of the enzyme due to difficulties in co-crystallising the protein with xenobiotic ester substrates. We are therefore unable to rationalise why *At*SFGH showed differing activities toward the ester series due to variations in k_{cat} rather than K_m. The kinetic studies clearly demonstrate the recognition of the MU-esters is dominated by the selective binding of the alcohol rather than the acyl moiety. By extending the structure activity series with the MU esters to determine which acyl components are tolerated and therefore accommodated, it would be possible to functionally map the dimensions of the acyl binding site of the plant esterases. Currently, we would predict that the acyl binding pocket for *At*SFGH to be somewhat smaller than the $6.2 \times 4.7 \times 3.1$ $Å^3$ determined for PLE (Provencher and Jones, 1994). A large acyl-binding pocket was functionally determined with a lipase from *Pseudomonas cepacia* (Grabulkeda et al., 1997). Our results suggest that such an exercise with the plant enzymes isolated here would be of limited value, since the structure activity studies would suggest that they do not accurately reflect the hydrolyses of esters determined *in planta*. This is in contrast to mammalian tissues, where a single class of esterase can dominate the hydrolysis of xenobiotics (Rojas-Garbanzo and Mata-Segreda, 1998). In plants, this appears to be a consequence of the wide diversity of serine hydrolases recruited to catalyse a much wider diversity of hydrolytic and acyl-transfer reactions in endogenous secondary metabolism. It is therefore more likely that the development of esterases as targets for selective activation or detoxification of pesticides in crops or weeds will have to be developed in a species-specific manner, to reflect the differing ranges of hydrolases present. To this end, we are currently identifying dominant esterases in major crops and weeds and characterising their ability to hydrolyse pesticide and xenobiotic esters. Inter-

estingly, while we have recently determined major differences between plant species in their ability to hydrolyse pesticide esters, our results demonstrate that Arabidopsis possesses an unusually representative set of hydrolytic activities toward diverse chemistries (Gershater et al., 2006).

4. Materials and methods

4.1. Synthesis of MU esters

Ester substrates (approximately 50 mg) were prepared using standard synthetic chemistry procedures. Carboxylic acids were treated with oxalyl chloride (1.2 equivalents) in dichloromethane (~0.2 M solution) containing a catalytic amount of dimethylformamide. Following concentration under vacuum, acid chlorides (0.4 mmol) were dissolved in CH_2Cl_2 (1 ml) containing 0.8 mmol *N*-ethyldiisopropylamine (125 μl of stock) and reacted with 4-methylumbelliferolne (0.32 mmol) added in CH_2Cl_2 (2 ml). Similar ratios of 7-mercapto-4-methyl coumarin and 7-amino-4-methyl coumarin were used for the preparation of the respective thioesters and amides, respectively. After 16 h at ambient temperature, the reaction mixture was partitioned two times with saturated aqueous $NaHCO_3$ (1 v/v) and the organic phase dried under reduced pressure and applied to silica extraction columns (3 ml) in Et_2O (1 ml). Columns were eluted with Et_2O in 1 ml aliquots which were assessed for purity by LC–MS. Fractions judged to be >95% pure were dried under reduced pressure, weighed and then prepared as stock solutions in acetone for enzyme assays.

4.2. LC–MS

Samples of esters were injected onto a C18 column (Phenonemex, Ultrosphere 3 μM, 30 × 2 mm) and eluted at 0.2 ml min^{-1} with a linear solvent gradient starting with MeCN:H_2O (5:95 v/v) both containing 0.5% (v/v) HCOOH (5:95 v/v) and ending with 100% MeCN containing 0.5% (v/v) HCOOH over 10 min. The column eluate was sequentially analysed using a diode array detector ($\lambda = 200$–400 nm) prior to injection into a Micromass LCT time-of-flight mass spectrometer operating with electrospray ionization (positive ion mode) with a cone voltage of 25 V and nitrogen used as cone and desolvation gas. Positive mass ions were detected in the range m/z 100–1000, with the instrument calibrated using sodium iodide. The mass ions ($m/z + 1$) of the MU esters used in the study are shown in Fig. 3.

4.3. Esterase assays

For the assay of the crude Arabidopsis proteins, 30-day-old plants were frozen in liquid nitrogen and then homogenized to a powder using a pestle and mortar, prior to extracting in 0.1 M Tris–HCl buffer, pH 7.4, containing 5 mM EDTA, 2 mM DTT and polyvinylpolypyrrolidone (5% w/v). After centrifuging (12,000*g*, 20 min) the supernatant was treated with $(NH_4)_2SO_4$ to 80% saturation and the protein pellet collected by re-centrifuging. The protein was re-dissolved in 0.1 M Tris–HCl pH 7.4 and desalted in the same buffer by gel filtration prior to assay, at a final protein concentration of 2.4 mg ml^{-1}. PLE (Sigma, Dorset, UK) was diluted to 0.2 mg ml^{-1} protein in assay buffer prior to use. The purified recombinant Arabidopsis hydrolases (0.2 mg ml^{-1}) were desalted in 100 mM K.Pi buffer, pH 7.2, prior to use. Hydrolysis of MU esters was measured continuously using fluorescence-based assays (Kordic et al., 2002). In each case the reaction rate obtained for heat-denatured enzyme was subtracted from the enzymatic rate. Kinetic data were obtained by varying substrate concentrations from 20 μM to 500 μM with K_m and V_{max} values obtained from double reciprocal plots.

4.4. Cloning and expression of AtCXEs

Members of the *At*CXE family of Arabidopsis (Marshall et al., 2003), were clustered into clades based on sequence similarity (Fig. 1). Representatives of each of the five major clades were identified and PCR forward (F) and reverse (R) primers designed for the amplification of the respective full coding sequences as follows. *At*CXE5 (At1g49660); F = 5′ TCATATGGAATCTGAAATCGCCTCC 3′ and R = 5′ TCTCGAGACCAATAATAAACTCGAC 3′. *At*CXE13 (At3g48700); F = 5′ TCATATGGATTCCGAGATCGCCG 3′ and R = 5′ TCTCGAGCTTGTCTCCCTTGATA 3′. *At*CXE16 (At5g14310); F = 5′ TCATATGCCAGGGGTAGCTGTGCTC 3′ and R = 5′ TCTCGAGGTAAGAGAACTCATGGCC 3′. For *At*CXE17 (At5g16080); F = 5′ TCATATGGCAACCATTTCCTTCTCTCAC 3′ and R = 5′ TCTCGAGTGATGGATGTATGAAGTTGTG 3′. *At*CXE18 (At5g23530); F = 5′ TCATATGGCGACAGATTCTCAACCA 3′ and R = 5′ TCTCGAGAGCGGAGAGCGAAGC 3′. *At*CXE20 (At5g62180); F = 5′ TCATATGTCCGAACCAAGTCCAATC 3′ and R = 5′ TCTCGAGCAGAACAGAGAATATGAA 3′.

Total RNA was isolated from aerial tissues of flowering *Arabidopsis* plants using Tri-Reagent (Sigma) and cDNA prepared using oligo(dT) to prime reverse transcription as the template for PCR. Amplification and cloning of products into pGEMT (Promega) prior to sub-cloning of the authenticated sequence into pET 24 (Novagen) was as described previously (Kordic et al., 2002). Following transformation of *E. coli* strain ROSETTA DE3 (pLysS)(Novagen), cultures were grown to an OD of 0.6 at 37 °C, then cooled to 10 °C prior to induction with IPTG (1 mM). Following growth at 10 °C for 16 h, bacteria were pelleted and then lysed by resuspending in 1/100 original volume B-PER (Pierce). Recombinant His-tagged proteins were then purified by nickel-chelate affinity chromatography (Kordic et al., 2002), with Triton X-100 (0.1% v/v) added to all chromatographic buffers. Polypeptide composition was

assessed by SDS–PAGE (12% gels) with coomassie staining.

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