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Phytochemistry 67 (2006) 2561–2567
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Carboxylesterase activities toward pesticide esters in crops and weeds

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Received 19 June 2006; received in revised form 18 September 2006

Dedicated to the memory of Professor John R. Bowyer (1955–2006) and his many contributions to herbicide research and plant biochemistry.

Abstract

Proteins were extracted from maize, rice, sorghum, soybean, flax and lucerne; the weeds *Abutilon theophrasti*, *Echinochloa crus-galli*, *Phalaris canariensis*, *Setaria faberii*, *Setaria viridis*, *Sorghum halepense* and the model plant *Arabidopsis thaliana* and assayed for carboxylesterase activity toward a range of xenobiotics. These included the pro-herbicidal esters clodinafop-propargyl, fenoxaprop-ethyl, fenthioprop-ethyl, methyl-2,4-dichlorophenoxyacetic acid (2,4-D-methyl), bromoxynil-octanoate, the herbicide-safener cloquintocet-mexyl and the pyrethroid insecticide permethrin. Highest activities were recorded with α-naphthyl acetate and methylumbelliferyl acetate. Esters of *p*-nitrophenol were also readily hydrolysed, with turnover declining as the chain length of the acyl component increased. Activities determined with model substrates were much higher than those observed with pesticide esters and were of limited value in predicting the relative rates of hydrolysis of the crop protection agents. Substrate preferences with the herbicides were typically 2,4-D-methyl > clodinafop-propargyl > fenthioprop-ethyl, fenoxaprop-ethyl and bromoxynil-octanoate. Isoelectric focussing in conjunction with staining for esterase activity using α-naphthyl acetate as substrate confirmed the presence of multiple carboxylesterase isoenzymes in each plant, with major qualitative differences observed between species. The presence of serine hydrolases among the resolved isoenzymes was confirmed through their selective inhibition by the organophosphate insecticide paraoxon. Our studies identify potentially exploitable differences between crops and weeds in their ability to bioactivate herbicides by enzymic hydrolysis and also highlight the usefulness of Arabidopsis as a plant model to study xenobiotic biotransformation.

Keywords: Arabidopsis; Detoxification; Herbicide bioactivation; Maize (*Zea mays* L.); Pesticides; Rice (*Oryza sativa*); Sorghum (*Sorghum bicolor*); Soybean (*Glycine max*); Serine hydrolases; Weeds; Xenobiotics

1. Introduction

Several major classes of herbicides are applied in the field as inactive esters which are then hydrolysed within plant tissues to release the phytotoxic acid or alcohol (Hassall, 1990). Good examples of this metabolic activation are seen with the methyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D-methyl, **1**), bromoxynil-octanoate (**5**) and esters of the aryloxyphenoxypropionates (AOPPs) such as clodinafop-propargyl (**2**), fenthioprop-ethyl (**3**) and fenoxaprop-ethyl (**4**) (Fig. 1). Additionally, other agents used in crop protection, such as the herbicide safener cloquintocet-mexyl (**7**) and the pyrethroid insecticide permethrin (**8**) (Fig. 1), can also undergo ester hydrolysis as a route of primary metabolism. In the case of pyrethroids, hydrolysis following application on the crop abolishes their activity as insecticides (Preiss et al., 1988). It can therefore be seen that the hydrolysis of crop protection agents in plants is important in determining their bioactivity by mediating either their metabolic activation or detoxification (Hassall, 1990).

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doi:10.1016/j.phytochem.2006.09.019

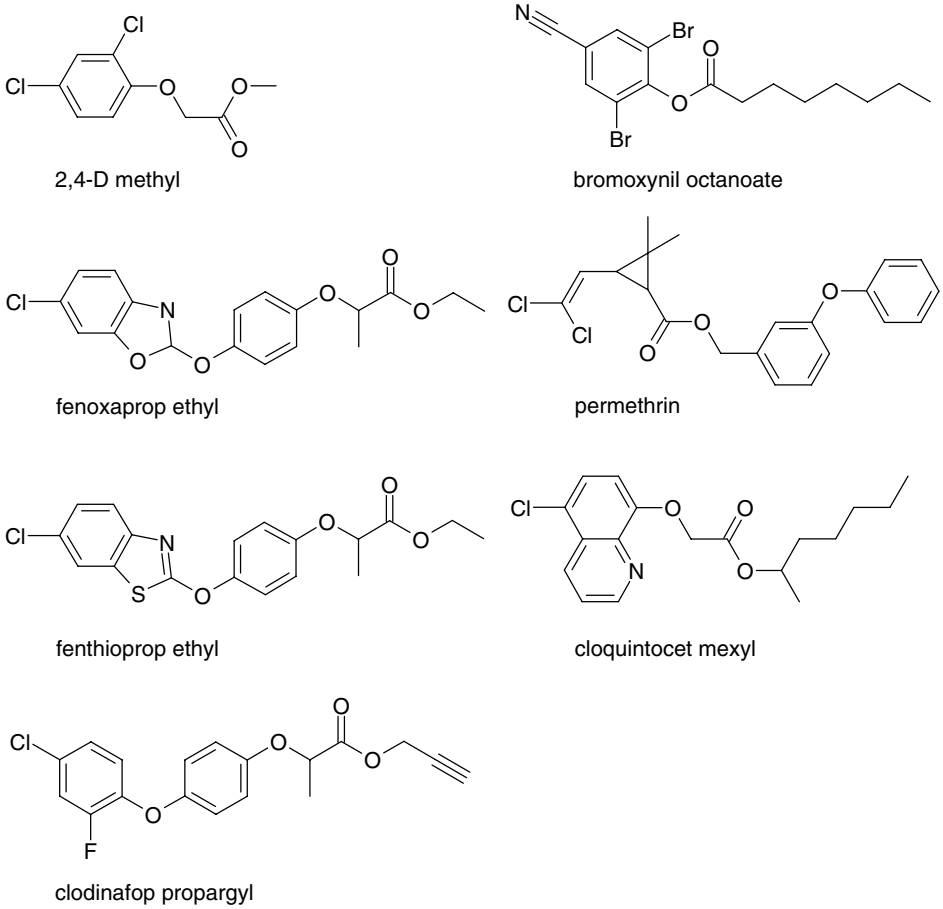

Fig. 1. Pesticide esters used in this study. Compounds are numbered in the text as **1**, 2,4-D-methyl; **2**, fenoxaprop-ethyl; **3**, fenthioprop-ethyl; **4**, clodinafop-propargyl; **5**, bromoxynil-octanoate, **6**, permethrin; **7**, cloquintocet-mexyl.

The AOPPs represent one of the best described examples of hydrolysis-mediated activation of a pro-herbicidal ester. Thus, whereas wild oats (*Avena fatua* L.) rapidly hydrolyse flamprop-isopropyl to the phytotoxic flamprop acid, this reaction is much slower in barley (*Hordeum vulgare* L.), allowing the crop to safely detoxify the herbicide (Jeffcoat and Harries, 1975). In contrast, the rapid build up of active flamprop in wild oats leads to toxicity, with this bioactivation contributing to the selective graminicidal action of this herbicide. More recent studies with the sulphonyl urea herbicide imazamethabenz-methyl have also demonstrated the importance of relative rates of hydrolysis in determining herbicide-resistance in wild oat (Nandula and Messersmith, 2000). Thus, susceptible populations hydrolysed imazamethabenz-methyl to the phytotoxic imazamethabenz acid much more rapidly than herbicide-resistant wild oat.

There is a long history of xenobiotic-hydrolysing carboxyesterases being described in plants using model colorimetric substrates such as α-naphthyl acetate. Although these old studies did not ascribe specific functions to these enzymes, major differences in esterase isoenzyme content between plant species and even within cultivars were demonstrated (Cherry and Katterman, 1971). Esterase expression has also been recorded to change during plant development (Frossard and Voss, 1978; Chandra and Toole, 1977), and in response to stress and infection (Muarlidharan et al., 1996; Baudouin et al., 1997; Wäspi et al., 1998). While these qualitative changes in expression are suggestive of important functions for these enzymes in plant growth, development and stress tolerance, very few studies have been directed at their functional characterisation.

We are interested in identifying esterases in crops and weeds which are active in pesticide metabolism. In earlier studies, we demonstrated esterase activities toward the herbicides diclofop-methyl, binapacryl and bromoxynil-octanoate in wheat (*Triticum aestivum* L.), and the competing weeds black-grass (*Alopecurus myosuroides* L.) and wild oat (Cummins et al., 2001). Esterase activities toward the herbicides were significantly higher in the weeds than in wheat, most markedly with the AOPP herbicide diclofop-methyl. The respective enzymes have been shown to be expressed in the apoplast in both wild oat (Holl et al., 1986) and wheat (Haslam et al., 2001) and have recently been identified and cloned from black-grass (Cummins and Edwards, 2004). Based on sequence analysis, the 40 kDa esterase from black-grass showed

homology to a large group of ill-characterised hydrolases found in micro-organisms and plants termed the GDSL lipases (Brick et al., 1995). Subsequently, the plant enzymes were renamed the GDS hydrolases and shown to be present in large gene families in a range of crops (Cummins and Edwards, 2004).

From this brief review of the literature it is clear that based on the widespread occurrence of GDS-hydrolases in nature and the results of herbicide metabolism and toxicity studies conducted *in planta*, that many esterases in crops and weeds have the potential to hydrolyse crop protection agents. Since these hydrolyses can contribute to pesticide efficacy, a greater knowledge of the capacity of plants to carry out these reactions on different substrates could be a useful predictive tool in crop protection. To this end we have taken seven pesticide ester chemistries (Fig. 1), which were previously established as being actively hydrolysed by plant extracts (Cummins et al., 2001), together with five colorimetric/fluorometric model esterase substrates and used these to screen monocotyledonous and dicotyledonous crops and weeds for carboxylesterase activities. For each species the numbers of xenobiotic-hydrolysing esterase present were then assessed using isoelectric focussing (IEF) with activity staining. In each case the presence of carboxylesterases using a catalytic serine was determined based on the sensitivity of such hydrolases to inhibition by organophosphates (Cummins et al., 2001).

2. Results

2.1. Esterase activities toward agrochemical substrates

Esterase activities toward the pesticide esters (Fig. 1) in the crops and weeds were determined by HPLC (Cummins et al., 2001). In all the species tested, the herbicide 2,4-D-methyl (**1**) was the preferred substrate, notably in Arabidopsis (Table 1). Uniquely, maize extracts were also highly active toward the herbicide bromoxynil-octanoate (**5**) and the safener cloquintocet-mexyl (**7**), suggesting that this would be a useful cereal crop species to further study diverse pesticide-hydrolysing esterase activities. In most species, after 2,4-D-methyl (**1**), the next best substrates were the AOPP esters. In all plants, the insecticide permethrin (**6**) proved to be the poorest substrate, with Arabidopsis, *Echinochloa crus-galli* and *Phalari canariensis* unable to hydrolyse this pyrethroid at all. This was consistent with the slow rates of ester hydrolysis of pyrethroid insecticides determined in whole plant metabolism studies (Lee et al., 1988).

With the AOPP esters the most commonly observed substrate preference for hydrolysis corresponded to clodinafop-propargyl (**4**) > fenthioprop-ethyl (**3**) = fenoxaprop-ethyl (**2**) (Table 1). Based on the similarity in structure of fenthioprop and fenoxaprop, it was not surprising that most species showed similar activities toward the two herbicide substrates. However, there were some notable exceptions. Thus, soybean and *Echinochloa*

Table 1
Carboxylesterase activity toward pesticide esters in crude protein extracts from crops and weeds

	Species	Rate of carboxylester hydrolysis (pkat/mg extracted protein)						
		2,4-D methyl	Clodinafop propargyl	Fenthioprop ethyl	Fenoxaprop ethyl	Bromoxynil octanoate	Permethrin	Cloquintocet mexyl
Dicots								
Crops	*Glycine max* (Soybean)	118.7 ± 4.3	63.91 ± 7.20	ND	3.50 ± 0.42	2.69 ± 0.76	0.3 ± 0.0	2.72 ± 0.14
	Linum usitatissimum (Flax)	187.1 ± 26.4	18.13 ± 0.28	7.07 ± 1.25	5.82 ± 1.15	3.84 ± 0.02	3.7 ± 0.5	7.02 ± 0.80
	Medicago sativa (Lucerne)	162.4 ± 32.7	119.0 ± 13.1	18.01 ± 5.62	15.80 ± 8.14	4.80 ± 0.81	1.5 ± 0.2	4.28 ± 0.62
Weeds	*Abutilon theophrasti* (Velvetleaf)	127.8 ± 2.9	20.35 ± 0.65	7.03 ± 0.16	6.09 ± 0.22	5.29 ± 0.06	6.3 ± 0.4	0.97 ± 0.33
	Arabidopsis thaliana	365.2 ± 54.8	44.99 ± 0.00	22.47 ± 0.23	11.25 ± 0.24	2.09 ± 0.31	ND	4.82 ± 1.29
Monocots								
Crops	*Oryza sativa* (Rice)	103.3 ± 1.4	8.77 ± 1.06	2.47 ± 0.32	ND	7.46 ± 0.10	0.8 ± 0.2	3.40 ± 0.22
	Sorghum bicolor (Sorghum)	236.9 ± 9.1	25.79 ± 0.55	8.57 ± 0.69	9.09 ± 0.16	7.04 ± 1.91	2.3 ± 0.2	5.06 ± 0.03
	Zea mays (Maize)	403.2 ± 15.9	18.13 ± 0.28	7.77 ± 1.43	7.44 ± 0.11	23.46 ± 1.25	7.1 ± 0.2	9.63 ± 1.72
Weeds	*Echinochloa crus-galli* (Barnyard grass)	54.0 ± 3.7	4.71 ± 0.51	ND	4.31 ± 0.00	0.92 ± 0.10	ND	ND
	Phalaris canariensis (Canary grass)	65.8 ± 6.3	10.06 ± 0.51	5.86 ± 0.85	5.27 ± 0.16	1.78 ± 0.11	ND	2.60 ± 0.58
	Setaria faberii (Giant Foxtail)	82.2 ± 0.5	19.56 ± 1.15	1.83 ± 0.90	4.23 ± 0.08	9.95 ± 0.58	2.9 ± 0.4	4.30 ± 0.10
	Setaria viridis (Green Foxtail)	149.5 ± 11.1	20.30 ± 0.05	7.32 ± 0.56	7.42 ± 0.08	8.80 ± 0.24	2.7 ± 0.2	5.07 ± 0.22
	Sorghum halepense (Johnson grass)	189.5 ± 32.2	8.27 ± 0.78	4.55 ± 1.03	5.90 ± 0.69	4.11 ± 0.19	2.5 ± 0.2	6.49 ± 0.08

Values represent means ± variation in biological replicates ($n = 2$).
ND, not detected.

crus-galli were able to cleave fenoxaprop-ethyl (**2**), but not fenthioprop-ethyl (**3**), while the converse was the case with rice. These species-dependent differences in activities toward the AOPP esters demonstrated even minor structural changes in these herbicide substrates can highlight major variations in esterase activities. In the monocots assayed, activities toward the AOPP esters were of a similar order of magnitude in crops and weeds. This in contrast to the studies with temperate cereals and grass weeds, where much higher levels of AOPP-hydrolysing activities were determined in black-grass and wild oat as compared with wheat and barley (Cummins et al., 2001; Jeffcoat and Harries, 1975).

2.2. Esterase activities toward other xenobiotic substrates

Activities toward the colorimetric and fluorometric substrates were much higher than those determined with the agrochemical esters, with 4-methylumbelliferyl acetate and α-naphthyl acetate being the preferred substrates (Table 2). In studies with porcine liver esterase and soil microbial esterase preparations, fluorescein diacetate was found to be a good model substrate in predicting the relative rates of hydrolysis of fenoxaprop-ethyl (2) (Zablotowicz et al., 2000). However, in our study, which used a wider range of substrates and enzyme sources very limited correlations were observed in relative activities toward colourimetric substrates and the crop protection agents across the plant species tested. This would suggest that there is limited usefulness in extrapolating from results obtained with any single xenobiotic when predicting likely rates of hydrolysis of pesticides in crops and their associated weeds. For example, *p*-nitrophenyl acetate is a common model esterase substrate and when used to determine relative rates of hydrolysis in maize and the associated weeds *A. theophrasti* and *S. faberi* defined their relative hydrolysing activities as being in the ratio of 1.5:1:1. While this ratio would reasonably predict the relative activities of these plants toward clodinafop-propargyl (**4**), it would not reflect the rates of hydrolysis of 2,4-D-methyl (**1**) in maize, *A. theophrasti* and *S. faberi* (5:3:1). This is an important observation, as it challenges the view that the hydrolysis of pesticides is carried out by low-specificity esterases which act on a wide range of xenobiotics (Preiss et al., 1988).

The studies with the *p*-nitrophenyl esters gave some useful information as to the species-dependent differences in tolerance of the acyl component. Most plants showed a decrease in activity as the chain length of the acid moiety was increased from C-2 to C-12. Partial tolerance to increasing acyl chain length was observed in maize which hydrolysed up to C-8 with no decline in activity, while the extracts from *E. crus-galli* and *P. canariensis* showed no activity beyond C-2. Interestingly, the tolerance of acyl chains up to C-8 in maize was also seen with the herbicide

Table 2
Carboxylesterase activity toward model xenobiotic esters in crude protein extracts from crops and weeds

	Species	Rate of carboxylester hydrolysis (pkat/mg extracted protein)						
		α-Naphthyl acetate	Methylumbelliferyl acetate	Fluorescein diacetate	*p*-Nitrophenyl acetate	*p*-Nitrophenyl butyrate	*p*-Nitrophenyl caprylate	*p*-Nitrophenyl laurate
Dicots								
Crops	*Glycine max* (Soybean)	6376 ± 36	2574 ± 252	48 ± 7	510 ± 20	426 ± 34	382 ± 10	29 ± 0
	Linum usitatissimum (Flax)	2246 ± 145	3636 ± 8	33 ± 1	559 ± 59	260 ± 44	172 ± 25	34 ± 15
	Medicago sativa (Lucerne)	6159 ± 580	2843 ± 147	35 ± 6	578 ± 29	387 ± 64	470 ± 39	ND
Weeds	*Abutilon theophrasti* (Velvetleaf)	1159 ± 145	2133 ± 760	29 ± 6	480 ± 69	25 ± 25	113 ± 5	74 ± 44
	Arabidopsis thaliana	2246 ± 507	3728 ± 256	29 ± 3	990 ± 127	490 ± 10	294 ± 10	10 ± 10
Monocots								
Crops	*Oryza sativa* (Rice)	942 ± 290	1969 ± 63	73 ± 13	382 ± 20	59 ± 10	15 ± 15	ND
	Sorghum bicolor (Sorghum)	1739 ± 217	2172 ± 192	30 ± 7	721 ± 44	142 ± 5	83 ± 44	20 ± 30
	Zea mays (Maize)	2464 ± 217	3998 ± 4	30 ± 4	789 ± 5	691 ± 25	779 ± 5	49 ± 10
Weeds	*Echinochloa crus-galli* (Barnyard grass)	616 ± 36	937 ± 27	23 ± 3	289 ± 5	ND	ND	ND
	Phalaris canariensis (Canary grass)	6485 ± 109	2401 ± 113	39 ± 1	343 ± 39	ND	ND	ND
	Setaria faberii (Giant Foxtail)	760 ± 254	2595 ± 38	30 ± 7	505 ± 54	142 ± 5	83 ± 44	ND
	Setaria viridis (Green Foxtail)	2355 ± 181	6848 ± 42	32 ± 6	873 ± 49	113 ± 5	98 ± 59	ND
	Sorghum halepense (Johnson grass)	3913 ± 72	3531 ± 222	46 ± 3	1127 ± 10	824 ± 49	461 ± 0	ND

Values represent means ± variation in biological replicates ($n = 2$).
ND, not detected.

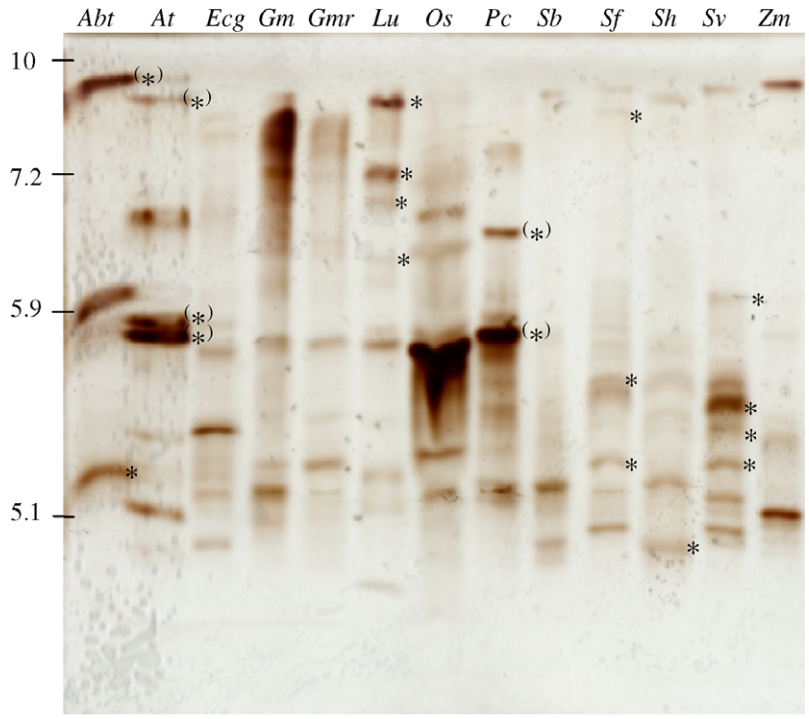

Fig. 2. Resolution of plant carboxylesterases present in the foliage and active toward α-naphthyl acetate by polyacrylamide gel electrophoresis using isoelectric focussing. The pH values for reference proteins are shown on the left-hand side and the plant extracts are in the following order: *Abutilon theophrasti* (*Abt*), *Arabidopsis thaliana* (*At*), *Echinochloa crus-galli* (*Ecg*), *Glycine max* (*Gm*), *Linum usitatissimum* (*Lu*), *Oryza sativa* (*Os*), *Phalaris canariensis* (*Pc*), *Sorghum bicolor* (*Sb*), *Setaria faberii* (*Sf*), *S. halepense* (*Sh*), *S. viridis* (*Sv*) and *Zea mays* (*Zm*). The suffix ''r'' indicates protein from root material. * To the right of a band denotes a protein not inhibited by paraoxon treatment and (*) indicates partial inhibition.

bromoxynil-octanoate (**5**) (Table 1), suggesting that in this species similar lipase-like enzymes were involved in xenobiotic and herbicide metabolism.

2.3. Isoelectric focussing and organophosphate inhibitor study

Isoelectric focussing gels were run with paraoxon-inhibited and non-inhibited protein samples from twelve species of crops and weeds (Fig. 2). These gel-based assays gave an indication of the number of esterases present in each plant which could be resolved on the basis of their isoelectric points, rather than any other physical characteristics such as relative molecular mass. The IEF gel illustrated the species-dependent diversity of carboxylesterases showing activity toward α-naphthyl acetate. In addition to resolving esterases on the basis of their p*I*s, the inhibition study with the insecticide paroxon was a useful diagnostic test for serine hydrolases, as this class of hydrolase is irreversibly inactivated by organophosphates (Cummins et al., 2001). In soybean and the cereal crops all of the visualised esterases behaved as serine hydrolases, which was not the case with the majority of the weeds. Interestingly, the intensity of activity staining in the gel assays with each plant extract did not always reflect the relative hydrolyses of α-naphthyl acetate quantitatively determined in the respective spectrophotometric assays (Table 2). For example, as determined in the spectrophotometric assays, extracts from *S. halepense* possessed high esterase activity, but the respective highly stained bands were absent from the IEF gel (Fig. 2). In contrast, with rice a major activity was observed in the gel analysis, whereas when assayed with the same substrate in the spectrophotometer the respective extracts showed little activity. From this it was concluded that IEF must result in the selective activation of some esterases and the inhibition of the activity of others. This further reinforces the role of zymogen esterase assays as being useful qualitative tools for assessing esterase diversity rather than as being useful in quantifying total hydrolytic activity.

3. Discussion

Our knowledge of plant xenobiochemistry has been steadily increasing with respect to the potential diversity of pesticide metabolic chemistry (reviewed by Roberts, 1998). However, the number of plant species studied has tended to be confined to the major crops and our knowledge of the biotransforming enzymes involved is still limited. Taxonomic surveys of the ability of the plant kingdom to metabolise synthetic compounds would provide useful predictive tools in modelling how specific plants are likely to metabolise pesticides and pollutants. In recent years, a wide range of plants have been surveyed for their content of detoxifying glutathione transferases (Pflugmacher et al.,

2000) and glucosyltransferases (Pflugmacher and Sandermann, 1998). Using a selection of major crops and weeds, we have examined the biodiversity of xenobiotic-hydrolysing esterase activities which are known to regulate the biological activity of both pesticides (Jeffcoat and Harries, 1975; Nandula and Messersmith, 2000) and pollutants (Krell and Sandermann, 1984) in plants. The available evidence suggests that unlike glutathione transferases and glucosyltransferases, carboxylesterases have evolved in parallel from several distinct protein families rather than divergently from single progenitors. This may help explain the disparate activities seen in different plants and the diversity of isoenzymes resolvable by IEF electrophoresis. While this makes these enzymes even more difficult to functionally characterise, the presence of a genetically diverse group of proteins all catalysing similar reactions does give considerable scope for exploiting differences in esterase content between plants in regulating herbicide metabolism. For example, subtle differences in the expression of members of the GDS hydrolase family in wheat and competing weeds form the basis of the selective activation of AOPP herbicides (Haslam et al., 2001; Cummins and Edwards, 2004). While this basis for herbicide selectivity was originally discovered largely as a result of large scale chemical screening programmes, a more detailed appreciation of the esterase complement of plant species could be a very powerful means of targeting herbicides for selective detoxification in crops, or bioactivation in weeds. As such, expanding the scope of this study to include additional chemistries, crops and weeds will be of future benefit to both the agrochemical and bioremediation industries.

Our work also demonstrates the utility of using Arabidopsis as a useful model plant for studying the detoxification of xenobiotics. Arabidopsis plants contained esterases which hydrolysed all classes of herbicides used, with activities which were representative of those determined in the crop species. A family of xenobiotic-hydrolysing esterases has recently been reported in Arabidopsis, though their activities toward pesticides was not reported (Marshall et al., 2003). An esterase D from Arabidopsis which rapidly hydrolysed a range of colourimetric substrates was completely inactive toward herbicide esters (Kordic et al., 2001). It now appears from work carried out with AOPP herbicides in wheat (Haslam et al., 2001) and black-grass (Cummins and Edwards, 2004) that only a small sub-set of hydrolases act on crop protection agents. In this respect, Arabidopsis will prove to be extremely useful in identifying these specific herbicide-hydrolysing esterases and determining their importance in herbicide bioactivation *in planta*.

4. Methods

4.1. Plants

Seeds of maize (*Zea mays* L.), soybean (*Glycine max* L.), rice (*Oryza sativa* L.) sorghum (*Sorghum bicolor* L.), *A. theophrasti*, *E. crus-galli*, *P. canariensis*, *S. faberii*, *S. viridis*, *S. halepense* were obtained from Syngenta (Jealott's Hill International Research Station, Bracknell, Berks, UK). Seeds of *Arabidopsis thaliana* (Columbia) were purchased from Lehle Seeds (Round Rock, Texas, USA). Seeds were sown directly onto moist potting compost (John Innes number 3), except for *A. theophrasti*, where the seeds were first soaked in hot water (60 °C, 15 min). Seeds of maize and soybean were soaked in water for 60 min prior to planting, while rice was first germinated in the dark on wet tissue for 48 h at 25 °C prior to transferring to compost. Plants were maintained in growth chambers for up to 34 days as described previously (Andrews et al., 1997). At harvest, the foliage from plants was excised and frozen in liquid nitrogen prior to storage at −80 °C.

4.2. Esterase extraction and assay

Frozen plant material was powdered using a pestle and mortar and extracted with 5 v/w ice-cold 0.1 M Tris–HCl pH 7.5, containing 1 mM DTT, 2 mM EDTA and 5% w/v polyvinylpolypyrrolidone (PVP). After centrifugation (10,000 g, 15 min, 4 °C), the supernatant was decanted and ammonium sulphate added to 40% saturation prior to re-centrifugation. After discarding the pellet, ammonium sulphate was added to 80% saturation and the protein precipitate recovered by a final centrifugation. The pellet was dissolved in 0.1 M K-Pi buffer pH 7.2 and desalted using a HiTrap™ Sephadex column (Amersham Biosciences) prior to determining protein concentration using dye binding reagent (BioRad) with γ-globulin as the reference protein. Esterase assays with α-naphthyl acetate, fluorescein diacetate and the *p*-nitrophenyl esters were determined colorimetrically (Kordic et al., 2002). The hydrolysis of 4-methylumbelliferyl acetate was determined by incubating 100 μg protein extract in 0.1 M K-Pi buffer pH 7.2 in a total volume of 198 μl at 37 °C in a microtitre plate. The reaction was initiated with 2 μl 4-methylumbelliferyl acetate (0.1 mM) and the increase in fluorescence at 450 nm measured at 5 s intervals over 30 s using a Labsystems Fluoroskan Ascent, with excitation at 370 nm. Product formation was quantified from a standard prepared from 4-methylumbelliferone. Hydrolysis of the pesticide esters was determined using an HPLC based assay (Cummins et al., 2001). In all assays activities were determined in duplicate and corrected for non-enzymic hydrolysis using boiled protein controls.

4.3. IEF studies

Proteins were prepared for assay ± treatment with 0.1 mM paraoxon and then resolved by IEF on polyacrylamide gels, with esterase activity toward α-naphthyl acetate visualised using fast blue staining as described previously (Cummins et al., 2001). The p*I*s of the enzymes were approximated using a curve of best-fit applied to marker proteins of known p*I*.

Acknowledgements

This work was supported by a Co-operative award in Science and Engineering awarded to M. Gershater and jointly funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and Syngenta. The work of R. Edwards forms part of a Research Development Fellowship awarded by the BBSRC. The authors thank Dr I. Cummins for skilled scientific and technical advice.

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