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Intracellular Proteases and Resistance in Plants

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ABSTRACT

In order to understand the potential role of intracellular proteolytic enzymes in the mechanism of herbicide and insect resistance in plants, we have compared the levels of activity of a representative range of protease types (acid, neutral & alkaline proteinases & peptidases) in plant tissues from herbicide resistant and susceptible biotypes of the weed species *Chenopodium album, Amaranthus retroflexus and Lolium rigidum* and insect resistant (Rathu Heenati) and susceptible (TN1) cultivars of rice (*Oryza sativa*). The activities of many protease types were significantly higher (typically by 1.5-3 fold) in both herbicide and insect resistant plant cultivars, suggesting that these enzymes may play a significant role in the defence against stress in plants.

Key Words: *Chenopodium album*; *Amaranthus retroflexus*; *Lolium rigidum*; *Oryza sativa*; Weeds; Herbicides

INTRODUCTION

Resistance in organisms of economic and health importance is often defined as the ability of individuals of a species to survive conditions or treatments while other susceptible individuals would not. In this way, selection pressure on a population resulting from lethal stresses gives rise to subsequent generations expressing genes that have an adaptive advantage. Alternatively, such resistance may be introduced to individuals or populations, especially of crop plants, by selective breeding or directly by genetic modification.

Resistance in plants may be seen in weeds exposed to herbicides for many generations and many genetically controlled mechanisms such as reduced sensitivity of the site of action and enhanced metabolism of the herbicide are predominant (Shaner, 1995). For example, reduced sensitivity of acetolactate synthase and acetyl coenzyme A carboxylase has been identified for a number of herbicide types. Similarly, increased effectiveness of cytochrome P450, glutathione transferase and acyl arylamidase are known in resistant biotypes (Preston *et al*., 1996; Milner *et al*., 2001). A combination of resistance mechanisms may operate in field generated weed biotypes.

Sources of resistance to a range of crop pests (insects, mites, nematodes, fungi) have been a major part of plant breeding but usually with an inadequate understanding of the mechanisms operating. Where known, an enhancement of the production of response triggered secondary plant substances has a role in the plant's defence. Examples include phenols, alkaloids, and glucosinolates (Levin, 1976).

In the case of resistance in rice to insect herbivores such as the brown planthopper *Nilaparvata lugens* antifeeding substances are present (Liu *et al*., 1994). Similar mechanisms are likely to operate in other crops and are derived from the co-evolution of plants and their herbivores and pathogens. In some plant species, proteases and/or protease inhibitors are induced locally and distally by signals generated in response to herbivory (or pathogen) infestation (Bowles, 1990; Ryan, 1992). Research in genetic modification for crop insect resistance has focussed on the production of plant protease inhibitors, lectins and protein insecticides such as BT toxins.

Previous reports have suggested a possible role for proteases in the response of insects to environmental stress and in insecticide resistance status. Exposure to fenitrothion in *Bombyx mori* resulted in depleted protein content and raised protease activities (Nath *et al*., 1997) and in *Musca domestica* changed the activities of a range of intracellular proteases (Ahmed *et al*., 1998). Elevated protease activity levels were seen in malathion-resistant strains (compared to susceptible) of *M. domestica* and of *Tribolium castaneum* (Shakoori *et al*., 1994).

In order to further elucidate the potential role of proteolytic enzymes in the development of herbicide resistance, we have undertaken a comparison of the levels of activity of a range of protease types in herbicide resistant and susceptible biotypes of several plant species. In addition for comparative purposes, levels of intracellular proteases were also compared in insect resistant and susceptible varieties of rice, in order to determine whether these enzymes may also be involved in the development of plant resistant to herbivorous insects.

MATERIALS AND METHODS

Weed biotypes. The plant species and biotypes/varieties used are given in below. Seeds of the weed species *Chenopodium album*, *Amaranthus retroflexus* and *Lolium rigidum* (susceptible and resistant to atrazine & fluazifop) were procured from Herbiseed, Berkshire, UK, and sown in the glasshouse according to supplier's instructions. Resistant (VRL 69) and susceptible biotypes (VR 1) of *Lolium rigidum* were provided by Dr. S.B. Powles, University of Western Australia, Australia. All weed biotypes originated from agricultural sites. Seeds of all weeds germinated within one week of sowing and two weeks old plants and in case of

four weeks old plants only leaves were used for subsequent protease assays. Experiments were carried out in triplicate, with each experiment comprising 1-3 individual plants (which were measured for height & weight), depending upon size of each species.

Rice varieties. Rathu Heenati cultivar was selected for high degree of resistance to an important insect pest and compared with a standard reference susceptible, TN1. Rathu Heenati is highly resistant to the rice brown planthopper (*Nilaparvata lugens* stal., Hemiptera; Delphacidae) and is used as a donor to introduce a planthopper resistant gene into improved rice cultivars in resistance breeding programs. The rice brown planthopper secreted 22 times less honeydews on Rathu Heenati than on TN1.

All reagents (including protease substrates) were obtained from Sigma, UK.

Extraction of plant tissues. Plant tissues (leaves & roots) were chopped up manually prior to homogenization using an Ultra Turrax T 25 homogenizer (2 X 10 sec at 15000 rpm) (all at ice cold temperatures). A 1:5 (wet weight of plant / volume of extraction solution) extract was prepared; for neutral (cytoplasmic) proteases the extraction solution comprised 50 mM Tris-acetate buffer pH 7.5, 1 mM dithiothreitol (DTT), 0.15 M NaCl and 3 mM Na azide, whilst for acidic (vacuolar) proteases the extraction solution was similar but the buffer replaced with 50 mM acetate pH 5.5. The homogenates were centrifuged at 3000 g for 10 min at 6°C and supernatants retained for protease assays.

Protease assays. Plant homogenate (0.05 mL supernatant) was incubated with the appropriate assay medium (total volume 0.3 mL) at 37°C for 10-120 min and the reaction terminated by addition of 0.6 mL of ethanol. The fluorescence of the liberated 7-amino-4-methylcoumarin (AMC) was measured at λex 370nm, λem 430 nm. Assay blanks were run with assay medium minus homogenate. The stock substrate solutions (2.5 mM) were prepared in 10% ethanol. Assays were carried out for the following enzymes, the reaction media for which are given below**:**

Neutral (cytoplasmic) proteases

Alanyl aminopeptidase. mM Tris-acetate buffer pH 7.5, 5mM CaCl2, 1 mM DTT, 0.25 mM Ala-AMC; a**rginyl aminopeptidase**: 50 mM phosphate buffer pH 6.5, 0.15 M NaCl, 1 mM DTT, 0.25 mM Arg-AMC; **leucyl aminopeptidase**: 50 mM glycine-NaOH buffer pH 9.5, 5 mM $MgCl_2$, 1 mM DTT, 0.25 mM Leu-AMC; **dipeptidyl aminopeptidase IV**: 50 mM Tris-acetate buffer pH 7.5, 1 mM DTT, 0.25 mM Gly-Pro-AMC; **tripeptidyl aminopeptidase**: 50 mM Tri-acetate buffer pH 7.5, 2 mM DTT, 0.25 mM Ala-Ala-Phe-AMC; **proline endopeptidase**: 50 mM Tris-acetate buffer pH 7.5, 2 mM DTT, 0.25 mM CBZ-Gly-Pro-AMC.

Acid (vacuolar) proteases

Dipeptidyl aminopeptidase I: 50 mM actate buffer pH 5.5, 2 mM DTT, 0.25 mM Gly-Arg-AMC; **dipeptidyl aminopeptidase II**: 50 mM acetate buffer pH 5.5, 2 mM DTT, 0.25 mM Lys-Ala-AMC; **cathepsin B and cathepsin L**: 50 mM acetate buffer pH 5.5, 2mM DTT, 0.25 mM CBZ-Phe-AMC (Cathepsin L) or 0.25 mM CBZ-Arg-Arg-AMC (Cathepsin B only); **cathepsin H**: 50 mM phosphate buffer pH 6.0, 1 mM DTT, 0.5 mM puromycin, 0.25 mM Arg-AMC.

Assay for cathepsin D. Assay of cathepsin D activity was based on the spectrophotometric procedure of Pennington (1977): 50 mM acetate buffer pH 3.5, 1 mM DTT, 3 mg mL^{-1} acetic acid denatured haemoglobin substrate (total assay volume 0.5 mL). The reaction was terminated by addition of 0.6 mL 10% perchloric acid (PCA); the sample centrifuged at 2000 g for 5-10 min and absorbance of acid soluble peptides was determined from UV absorption at 280 nm. Assay blanks were run as above.

Determination of soluble protein. Soluble protein levels in the supernatants used for assays of the above proteases were determined by the method of Bradford (1976).

RESULTS

Morphological differences between resistant and susceptible forms of the various plant species 15 days after germination, with resistant plants being shorter and more branched than that corresponding susceptible plant (Table I). These morphological differences persisted up to 4-weeks in *C. album*, but morphological differences were not found in 4-week old plants of *L. rigidum*.

Comparisons of the levels of protease activities in resistant and susceptible biotypes of the three weed species *Chenopodium album*, *Amaranthus retroflexus* and *Lolium rigidum* are shown in Tables II-IV, respectively. The assays for various individual protease types listed above are based on specific fluorometric methods originally developed for higher animals (Faiz *et al*., 1995) and selected on the basis of experience and on providing a good range of protease types. The levels of activity for these proteases in the plant species investigated were, in general terms, found to be similar to those in higher animals, although the absolute activity levels for specific enzyme varied with plant species, cultivar and/or developmental stage (Tables II-IV). The enzyme activities in two species of plants had a contrasting

Table I. Plant characteristics of resistant and susceptible weed biotypes

	C. album			
Plant age	**R**		**S**	
	Height (cm)	leaves (number)	Height (cm)	leaves (number)
2-week	10.8±0.3*	8.6±0.2*	19.2±1.5	7.0±0.4
4-week	26.0±1.0*	10.8±0.3*	44.6±2.2	7.5±0.3
	L. rigidum (Herbiseed biotypes)			
2-week	12.3±0.7*	3.5±0.2*	16.7±0.8	2.2±0.2
4-week	19.8±0.9	4.2±0.2	20.8±0.7	4.0±0.3

Values are means±SE (n=3). Differences in plant height and number of leaves between resistant and susceptible plants was determined by One Way of ANOVA. * significantly different at P<0.05. R, resistant; S, susceptible.

Table II. Protease activities in resistant and susceptible biotypes of *C. album*

Enzyme type	Activity (μ moles h^{-1} mg^{-1} protein)			
Cytoplasmic	**4-week old plant**		**2-week old plant**	
	R	**S**	**R**	**S**
Alanyl aminopeptidase	2.53±0.1*	1.61±0.17	4.02±0.32*	1.86±0.05
Arginyl aminopeptidase	0.59±0.07	0.30±0.03	1.35±0.33	0.54±0.05
Leucyl aminopeptidase	0.60±0.1	0.20±0.1	No activity	No activity
Dipeptidyl aminopeptidase IV	0.07±0.00*	0.04±0.00	0.08±0.00*	0.03±0.00
Tripeptidyl aminopeptidase	1.21±0.22	0.78±0.08	0.67±0.07*	0.20±0.01
Proline endopeptidase	0.20±0.07	0.15±0.07	No activity	No activity
Vacuolar				
Dipeptidyl aminopeptidase I	0.05±0.00	0.04±0.00	0.09±0.02	0.05±0.01
Dipeptidyl aminopeptidase II	No. activity	0.29±0.04	No activity	No activity
Cathepsin L	0.09±0.05	0.10±0.04	0.56±0.03*	0.36±0.05
Cathepsin B	0.24±0.04	0.19±0.03	0.30±0.02*	0.13±0.01
Cathepsin H	0.31±0.02	0.29±0.02	0.10±0.00	0.06±0.01
Cathepsin D[1]	270±50	180±60	40±20	10±0

Protease activity values listed are means±SE (n=3). Differences in enzyme activities between resistant (R) and susceptible (S) biotypes were determined by One Way of ANOVA (*significant at P<0.05).

Table III. Protease activities in resistant and susceptible biotypes of *A. retroflexus*

Enzyme type	Activity (μ moles h^{-1} mg^{-1} protein)			
Cytoplasmic				
	4-week old plant		**2-week old plant**	
	R	**S**	**R**	**S**
Alanyl aminopeptidase	6.50±0.2	6.27±0.14	1.94±0.01*	1.20±0.00
Arginyl aminopeptidase	3.35±0.40	2.18±0.12	0.70±0.00*	0.04±0.01
Leucyl aminopeptidase	0.58±0.03*	0.35±0.00	0.11±0.00*	0.07±0.00
Dipeptidyl aminopeptidase IV	0.63±0.03	0.47±0.02	0.08±0.00	0.06±0.00
Tripeptidyl aminopeptidase	6.78±0.08*	5.15±0.20	0.97±0.07	0.61±0.06
Proline endopeptidase	1.31±0.04*	0.84±0.08	0.28±0.02	0.25±0.01
Vacuolar				
Dipeptidyl aminopeptidase I	0.25±0.00*	0.21±0.00	0.07±0.00*	0.04±0.00
Dipeptidyl aminopeptidase II	No. activity	No. activity	No. activity	No. activity
Cathepsin L	2.08±0.04	1.83±0.05	0.03±0.00	0.02±0.00
Cathepsin B	1.05±0.14	1.28±0.31	0.06±0.00*	0.04±0.00
Cathepsin H	1.04±0.03	1.03±0.03	0.05±0.00	0.03±0.00
Cathepsin D	112±0.04	100±0.02	60±0.00	50±0.00

Protease activity values listed are means±SE (n=3). Differences in enzyme activities between resistant (R) and susceptible (S) biotypes were determined by One Way of ANOVA (*significant at P<0.05).

combination, like DAPI and DAPII activities were not detected in the *C. album* but DAPI was present in the *L. rigidum*. The susceptible plants of *L. rigidum* lacked in proline endopeptidase. Activity of leucyl aminopeptidase in *C. album* was significantly higher than resistant counterpart. The activities of enzymes had a significant difference in resistant and susceptible plants of *L. rigidum*. The resistant plants of *L. rigidum* had a comparable total activity of cytoplasmic proteases with resistant plants of *C. album*. Although difference in activities of enzyme in *C. album* was not significant as compared to *L. rigidum* but it had still higher activities for arginyl - and tripeptidyl aminopeptidases. The leaves of *C. album* collected from one month old plants showed significantly higher activity of cytoplasmic proteases altogether and alanyl-and dipeptidyl aminopeptidases IV in resistant plants than susceptible ones. The leaves of resistant *L. rigidum* plants not only had total cytoplasmic proteases higher than susceptible one but also lysosomal proteases as well. *L. rigidum* too shows similar trend in one month old plants. Fifteen days old plants of resistant *A. retroflexus* plants were significantly different in alanyl -, arginyl - and leucyl aminopeptidase activities from cytoplasmic group and dipeptidyl aminopeptidase I and cathepsin B from lysosomal group than susceptible plants. The leaves of one month old resistant Amaranthus plants exhibited such similar trend as in two other resistant plant species.

In comparing herbicide resistant and susceptible biotypes of weed species, in general terms the activity levels of both neutral and acidic proteases were significantly higher typically by a factor of 1.5-3 fold in the resistant (versus susceptible) biotypes of the three weed species, although not for every combination of weed species/developmental stage/protease type (Table II-IV).

Data for comparison of the levels of neutral and acidic proteases in insect resistant and susceptible cultivars of rice (at 4 weeks only) are shown in Table V. The levels of activity of a number of neutral and acidic proteases (although not every enzyme type) again showed typical significant activity increases of 1.5-2 fold in the resistant, compared to the susceptible cultivar.

Table IV. Protease activities in resistant and susceptible biotypes of *L. rigidum.*

Enzyme type	Activity (μ moles h^{-1} mg^{-1} protein)					
Enzymes	4-weeks old plants		2-weeks old plants		2-weeks old plants	
Cytoplasmic	R	S	R	S	R (VRL)	S (VR1)
Alanyl aminopeptidase	7.20±0.13*	2.75±0.11	5.77±0.22*	3.47±0.23	2.16±0.12*	1.77±0.05
Arginyl aminopeptidase	2.38±0.04*	0.82±0.02	1.89±0.37	1.00±0.04	1.26±0.05	1.16±0.10
Leucyl aminopeptidase	0.28±0.02*	0.05±0.00	Not assayed	Not assayed	Not assayed	Not assayed
Dipeptidyl aminopeptidase IV	0.59±0.00*	0.01±0.00	0.13±0.00*	0.07±0.00	Not assayed	Not assayed
Tripeptidyl aminopeptidase	1.54±0.13*	0.48±0.03	0.48±0.03*	0.21±0.00	1.12±0.01*	0.56±0.04
Vacuolar						
Dipeptidyl aminopeptidase I	0.12±0.00*	0.07±0.00	0.13±0.02	0.04±0.01	Not assayed	Not assayed
Cathepsin L	0.66±0.04*	0.53±0.04	0.74±0.01	0.26±0.03	0.22±0.04	0.15±0.01
Cathepsin B	1.10±0.01*	0.94±0.01	0.57±0.19	0.13±0.01	0.34±0.02*	0.19±0.00
Cathepsin H	0.14±0.00*	0.04±0.00	0.14±0.00	0.09±0.00	0.12±0.00	0.11±0.00
Cathepsin D	8±0.00*	7±0.00	28±7.2	12±1.5	Not assayed	Not assayed

Protease activity values listed are means±SE (n=3). Differences in enzyme activities between resistant (R) and susceptible (S) biotypes were determined by One Way of ANOVA (*significant at P<0.05).

DISCUSSION

Intracellular proteases are ubiquitously distributed amongst living organisms, and are responsible for the processing of intracellular proteases essential for the normal functioning of all cell types. In general terms, proteases are classified principally on the basis of the pH optimum of activity (acid, neutral or alkaline), size of substrate (proteins and peptides) and nature of enzyme active site (*serine, cysteine, aspartic, metallo*). Much of what is known about the characteristics of proteolytic enzymes has been derived from work in higher animals, and question arises as to how closely the characteristics of plant proteases may parallel the latter. A number of proteases have been identified in plant tissues, the general characteristics of which appear to be similar to their counterparts in higher animals. These include plant tissue analogues of the mammalian enzymes cathepsin D, cathepsin H, aspartic proteinases, proteosomal multicatalytic protease and leucyl aminopeptidase (Vierstra, 1996; Gu *et al*., 1999).

The localization of acidic cathepsin proteases in plant vacuoles appears to be analogous to the localization of these enzymes within lysosomes in the cells of higher animals (Vierstra, 1996). The proteolytic enzymes (& their associated assay methods) investigated in the present study were therefore selected on the basis of known levels of activity and characteristics in tissues of higher animals (Faiz *et al*., 1994).

There have been relatively few previous reports describing the effects of herbicide treatment or insect infestation on the activity levels of proteases in plants, the activity of which may have been determined using relatively insensitive and non-specific assay methods (e. g., based on the use of azocasein type substrates). Thus Hasaneen *et al*. (1994) found increased protease activity in castor bean and maize plants following the application of metribuzin (3-10 g m^{-3}), whist protease activity levels in sweet corn seedlings were found to be inversely correlated with concentration of (25-400 mg L^{-1}) following traizine application (Khodary, 1990). The herbicides thiobencarb and butachlor caused a reduction in protease activity in the weed *Echinochloa crus-galli* at 25 ppm, whilst activities were increased at 75 ppm (Kumar & Prakash, 1994).

As far as we are aware, this is the first report describing significantly increased activity for a range of protease types in herbicide-resistant biotypes and insect-resistant plant varieties, compared to their corresponding susceptible counterparts. This increase in proteolytic activity may confer a survival advantage to resistant varieties, via an increased supply of free amino acids to the intracellular

Table V. Protease activities in insect resistant and susceptible varieties of rice plants (4-week)

Enzyme type	Activity (nmoles h^{-1} mg^{-1} protein)	
cytoplasmic	Rathu Heenati (RH)	TN1
Alanyl aminopeptidase	1542±54**	943±70
Arginyl aminopeptidase	647±2**	506±40
Leucyl aminopeptidase	79±4	69±0.8
Dipeptidyl aminopeptidase IV	21±0.4*	24±0.5
Tripeptidyl aminopeptidase	172±31	163±1
Proline endopeptidase	No activity	No activity
Vacuolar		
Dipeptidyl aminopeptidase I	99±8*	66±7
Dipeptidyl aminopeptidase II	30±2*	18±1
Cathepsin L	61±16	54±2
Cathepsin B	67±27	58±10
Cathepsin H	98±5*	69±4
Cathepsin D	15±2	13±0.2

Protease activity values listed are means±SE (n=3). Differences in activity levels between RH and TN1 varieties were determined via the LSD test (*P<0.05, *

Species	Resistance and ratio	Variety/ biotype	Source/ reference
C. album	S		Herbiseed
	R to atrazine (x 4)		-do-
A. retroflexus	S		Herbiseed
	R to atrazine		-do-
L. rigidum	S		Herbiseed
	R to fluazifop (x2)		-do-
	S	VR1	Dr. S. B. Powles
	*R to chlorotoluron, simazine (x 4)	VRL69	
O. sativa	susceptible	TN1	Taiwan
	22x feeding by N. lugens	Rathu Heenathi	Srilanka

*<0.01)

pool, either for *de novo* synthesis of known herbicide metabolising enzymes such as cytochrome P450, or for increased biosynthesis of secondary plant substances which would be even more important for compounds such as phenolics, alkaloids, glucosinolates or glycoside antifeedants derived from amino acid precursors (Zhu-Salzman & Salzman, 2001). The wounding of tomato or potato plants induces defence related proteins / enzymes such as leucine aminopeptidase, aspartic proteases and cysteine protease inhibitors, in addition to polyphenol oxidases (Walling & Gu, 1996).

Morphological differences between resistant and susceptible forms of the various plant species 15 days after germination, with resistant plants being shorter and more branched than that corresponding susceptible plants are similar to the observations of Hall *et al*. (1996) for resistance to auxinic herbicides.

Since the levels of protease activities are increased to a broadly similar degree in both herbicide and insect resistant plant varieties, it is possible that this phenomenon may be involved as part of a generalized plant defence mechanism to stress; in this regard cysteine proteases have previously been shown to accumulate in plant tissues exposed to environmental (drought) stress (Koizumi *et al*., 1993).

Schwenger-Erger & Barz (2000) found decreased rate of protein degradation in high light in low metribuzin-resistant cell lines. It is also possible that similar mechanisms operate in other resistance situations; for example, resistance in crops to pathogens and other pests, mechanisms based on genetically modified resistant varieties and in bacteria to antibiotics. However, since specific functions have yet to be established for any of the protease types investigated (either in plants or higher animals), this must remain a matter of speculation pending further experimental investigation of this phenomenon.

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