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TRYPTIC INHIBITORY ACTIVITY IN WILD AND CULTIVATED CRUCIFERS

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Key Word Index—Cruciferae; wild crucifers; cultivated crucifers; host-plant resistance; trypsin inhibitors.

Abstract—Tryptic inhibitory activity was detected in all species and cultivars of crucifers examined. When comparing the three groups of crucifers tested [(i) foliage of cultivated crucifers, (ii) foliage of wild crucifers and (iii) storage organs of cultivated crucifers], the foliage of cultivated crucifers contained significantly higher levels of tryptic inhibitory activity than the other two groups.

INTRODUCTION

Proteinase inhibitors are proteins or polypeptides that occur in a wide variety of plants [1]. The proteinase inhibitors that have been most extensively studied are those that inhibit serine proteinases (e.g. trypsin, chymotrypsin), which are common digestive enzymes in animals and micro-organisms, but are not present in plants. Since plants do not contain serine proteinases, the presence of serine proteinase inhibitors in plants suggests that they are acting as a defence against herbivory and/or pathogenesis [2, 3]. Plant proteinase inhibitors will inhibit the proteinases common in many herbivorous insects [4–6] and plant pathogens [7–9], and have the potential to act as defensive agents in plants against these biological stresses [10–15].

Plant proteinase inhibitors are usually products of a small complex of genes [16, 17], and the presence and expression of these genes in plants is determined by the ancestral genetic material, environmental and developmental signals, and (in the case of cultivated lines) the breeding process. Traditionally, plant breeders have selected cultivars that have high quality and/or yield, and are resistant to specific environmental and/or biological stresses. However, this selection process has been relatively imprecise. In general, plant breeders have no information about the biochemical factors that influence the majority of yield and resistance traits. While selecting cultivars that display one desirable character, the breeders may inadvertently lose other potentially important characters. Previous studies have compared the levels of serine proteinase inhibitory activity in cultivated lines with their ancestral or wild progenitors. In the case of *Cicer* [18] and tomato [19], the wild/ancestral species contain significantly higher levels of proteinase inhibitor than the cultivated lines. For wheat [20] and barley [21], the cultivated lines have more inhibitory activity than the wild species. This information suggests that, because the traditional breeding process selects for an overall high quality/resistant crop (not for high levels of specific resistance factors), some potential resistance factors may be lost, enhanced, or remain unchanged during the breeding process.

Proteinase inhibitory activity has been reported for some cultivated crucifers [22–25]. Partial characterization of proteinase inhibitors in the seeds of kale, Japanese radish, broccoli and radish indicate that these are small proteins (M_r 2 500–19 000) that have the ability to inhibit a number of different proteinases, including trypsin, chymotrypsin, cathepsin and subtilopeptidase A. However, a survey of the proteinase inhibitory activity in Cruciferae has not been performed. The present study measures tryptic inhibitory activity in 19 varieties of cultivated crucifers and 13 species of wild crucifers.

RESULTS AND DISCUSSION

Proteinase inhibitory activity was detectable in all 32 species/cultivars of crucifer examined. The level of tryptic inhibitory activity in the foliage from the selected crucifers are presented in Table 1 (foliage from cultivated crucifers) and Table 2 (foliage from wild crucifers). The means $\pm$s.e. of the inhibitory activity for the foliage from cultivated and wild crucifers are 529 ± 52 and 250 ± 39 μg trypsin inhibited/mg protein, respectively. Since environmental factors may significantly influence the level of proteinase inhibitors in crucifers, the suitability of the environmental conditions used for this study were tested against the tomato plant, which contains environmentally inducible proteinase inhibitors that have been extensively studied [26]. The level of tryptic inhibitory activity in the tomato foliage, *Lycopersicon esculentum* cv. Glamour (Seedway Inc., Hall, NY), maintained and experimentally treated under the same controlled conditions as the crucifers, was 295 ± 4 μg trypsin inhibited/mg protein, indicating that the environmental conditions were appropriate for induction of proteinase inhibitors. There is no significant difference between the tryptic inhibitory activity in the foliage from wild crucifers and tomato ($p > 0.05$). However, the tryptic inhibitory activity in the foliage of cultivated crucifers is significantly higher ($p < 0.001$) than that for wild crucifers or tomato foliage. In addition, there is as much as a 10-fold difference between cultivated *Brassica* and the most primitive species [27] of the genus (i.e. cultivated Chinese pak choi $= 1220 \pm 8$ vs *Brassica adpressa* $= 133 \pm 5$ μg trypsin inhibited/mg protein). It is unknown whether this elevated level of inhibitory activity in commercial cultivars was (i) actively selected for because this character confers enhanced resistance to the

Table 1. The level of tryptic inhibitory activity in the foliage of cultivated crucifers

Species	μg trypsin inhibited‡ mg protein
Brassica oleracea L.	
subsp. *gongylodes* cv. Kolpak kohlrabi*	757±0
subsp. *botrytis*	
subvar. *cymosa* cv. Packman broccoli*	508±9
subsp. *gemmifera* cv. Oliver Brussel sprouts*	535±9
subsp. *acephala*	
subvar. *medullosa* cv. Champion collards*	438±5
subvar. *millecapitata* cv. Winterbor kale*	320±9
subsp. *capitata*	
cv. Superpack (F1) cabbage*	515±0
cv. NY2528 cabbage†	209±0
cv. Round-up cabbage†	181±4
Brassica rapa (syn. *campestris*) L.	
subsp. *chinensis* cv. Chinese pak choi*	1220±8
subsp. *rosularis* cv. WR Green Chinese cabbage*	556±21
subsp. *rapifera* cv. Market Express turnip*	322±0
Brassica napus L.	
subsp. *napobrassica* cv. Laurentian rutabaga*	811±30
subsp. *oleifera* cv. rapeseed*	652±0
Brassica juncea (L.)	
cv. Green Wave mustard greens*	530±22
cv. Burgonde brown mustard*	207±16
Sinapis alba (L.) cv. Tilney yellow mustard*	125±50
Raphanus sativus L.	
cv. Marabelle radish*	1190±22
cv. Miyashige Daikon radish*	215±3
Nasturtium officinale R. Br. cv. watercress*	786±7

*Seeds provided by Johnny's Selected Seed, Albion, Maine.

†Seeds provided by Dr M. H. Dickson, NYS Agric. Exp. Stn, Geneva, NY.

‡Plant juice diluted 1:3 with pH 8 buffer; incubated 1:1 with trypsin for 10 min prior to the enzyme assay; data = mean ± s.e. of three replicates.

Table 2. The level of tryptic inhibitory activity in the foliage of wild crucifers

Species	μg trypsin inhibited¶ mg protein
*Brassica adpressa** Ad-114	133±5
Brassica kaber†	449±0
Arabidopsis thaliana‡ Turk Lake CrGC113	285±9
Greenville CrGC111	247±8
Lepidium virginicum‡ CrGC107	128±17
Lepidium campestre‡ CrGC106	94±7
Sinapis alba ex Emergo‡ CrGC103	137±14
Sinapis arvensis‡ CrGC104	193±9
Capsella bursa-pastoris‡ CrGC105	836±0
Descurainia pinnata†	352±5
Thlaspi arvense‡ CrGC110	114±4
Hesperis matronalis§	90±3
Sisymbrium officinale‡ CrGC109	196±8

*Seeds provided by Dr. Carlos Quiros, U.C., Davis, CA.

†Seeds provided by Valley Seed Service, Fresno, CA.

‡Seeds provided by Crucifer Genetics Cooperative, University of Wisconsin, Madison, WI.

§Seeds provided by Dr. A. A. Renwick, Boyce Thompson Institute, Ithaca, NY.

¶ Plant juice diluted 1:3 with pH 8 buffer; incubated 1:1 with trypsin for 10 min prior to the enzyme assay; data = mean ± s.e. of three replicates.

Table 3. The level of tryptic inhibitory activity in the storage organs of selected cultivated crucifers

Species*	μg trypsin inhibited† / mg protein
Brassica oleracea	
subsp. *gongylodes* cv. Kolpak kohlrabi stem	163 ± 1
Brassica rapa (syn. *campestris*)	
subsp. *rapifera* cv. Market Express turnip root	264 ± 0
Raphanus sativus cv. Marabelle radish root	339 ± 14
cv. Miyashige Daikon radish root	306 ± 6

*All seeds provided by Johnny's Selected Seed, Albion, NY.

†Plant juice diluted 1:3 with pH 8 buffer; incubated 1:1 with trypsin for 10 min prior to the enzyme assay; data = mean ± s.e. of three replicates.

plant against herbivores and/or pathogens, or (ii) inadvertently selected for because it is genetically linked to some other desirable character.

In addition to the foliage, proteinase inhibitors are also commonly found in storage organs of plants, such as tubers, seeds and unripe fruit [1, 29, 28]. The present study demonstrates that there is tryptic inhibitory activity in the storage organs of selected cultivated crucifers (Table 3). However, in general, the inhibitory activity in these organs is significantly less than the proteinase inhibitory activity in the foliage of the respective plants. This difference in the proteinase inhibitory activity in the different organs (i.e. leaf vs storage organ) may originate from (i) different concentrations of the same protein, or (ii) the presence of different proteins in the different organs.

EXPERIMENTAL

Seeds. All seeds used for this study were provided as gifts by: Johnny's Selected Seeds, Albion, Maine; Valley Seed Service, Fresno, CA; Crucifer Genetics Cooperative, Univ. of Wisconsin, Madison, WI; Dr A. A. Renwick, Boyce Thompson Institute, Ithaca, NY; Dr M. H. Dickson, Horticultural Science, NYS Agricultural Experiment Station, Geneva, NY; and Dr C. F. Quiros, Vegetable Crops, U. C. Davis, CA.

Seeds were germinated in Cornell mix [29] in 13 × 20 × 6 cm plastic flats (1 flat/cultivar, thinned to 6 seedlings/flat). The plants were maintained under 1 kW metal halide lamps in 26° greenhouse. The plants were watered daily, and at 6-weeks-old, fertilized with a water-soluble nutrient mix (16-32-16), and subsequently fertilized once every 3 weeks. The plants used for the following analyses were 8–10 weeks old.

Isolation of proteinase inhibitors. To optimize trypsin inhibitor levels in the foliage, each leaf used in the experiments was wounded by crushing the periphery with hemostats, then the plant was exposed to 1 kW metal halide light (900 fc) for 16 hr. The wounded leaves were then ground, using a pestle and mortar, and the leaf juice was collected for determination of the quantity of protein and the level of inhibitory activity against trypsin. Protein concentration was estimated using bicinchoninic acid (BCA) reagent (30) (Pierce). Bovine serum albumin (Sigma) was used as a standard.

Trypsin inhibitor assays. A standard spectrophotometric assay [31] was used to determine the ability of leaf juice to inhibit tryptic activity. Bovine trypsin (0.04 mg/ml, Sigma), was mixed (1:1) with leaf juice (diluted 1:3 with buffer), and incubated at room temp. for 10 min. Then 50 μl of the mixture was added to 2.9 ml of buffer (0.04 M Tris, pH 8.1, 0.01 M $CaCl_2$) containing 1.04 M *p*-toluene-sulphonyl-L-arginine methyl ester (Sigma). Tryptic activity was monitored at 247 nm for 3 min. Soybean trypsin inhibitor was used as a standard. The lower detectable limit of soybean tryptic inhibitory activity was 1 μg inhibitor/μg trypsin. Each sample was replicated at least three times.

Statistics. Significant differences between groups of plants were determined (at the 95% confidence level) by analysis of variance followed by orthogonal comparisons, using the statistical computer software, Systat (Systat Inc, Evanston, IL).

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REFERENCES

1. Whitaker, J. R. (1981) *Impact of Toxicology on Food Processing* (J. C. Ayres and J. C. Kirschman, ed). AVI Publishers, Westport, Conn.
2. Green, T. R. and Ryan, C. A. (1972) *Science* **175**, 776.
3. Peng, J. H. and Black, L. L. (1976) *Phytopathology* **66**, 958.
4. Broadway, R. M. and Duffey, S. S. (1986) *J. Insect Physiol.* **32**, 673.
5. Gatehouse, A. M. R., Butler, K. J., Fenton, K. A. and Gatehouse, J. A. (1985) *Entomol. Exp. Appl.* **39**, 279.
6. Sharma, B. R., Martin, M. M. and Shafer, J. A. (1984) *Insect Biochem.* **14**, 37.
7. Ries, S. M. and Albersheim, P. (1973) *Phytopathology* **63**, 625.
8. Tseng, T. C. and Mount, M. S. (1974) *Phytopathology* **64**, 229.
9. Urbanek, H. and Yirdaw, G. (1978) *Physiol. Plant Pathol.* **13**, 81.
10. Broadway, R. M. and Duffey, S. S. (1986) *J. Insect Physiol.* **32**, 827.
11. Broadway, R. M. and Duffey, S. S. (1988) *J. Insect Physiol.* (in press).
12. Broadway, R. M., Duffey, S. S., Pearce, G. and Ryan, C. A. (1986) *Entomol. Exp. Appl.* **41**, 33.
13. Cleveland, T. E. and Black, L. L. (1983) *Phytopathology* **73**, 664.
14. Gatehouse, A. M. R. and Boulter, D. (1983) *J. Sci. Food Agric.* **34**, 345.
15. Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. and Boulter, D. (1987) *Nature* **330**, 160.

16. Lee, J. S., Brown, W. E., Graham, J. S. *et al.* (1986) *Proc. Natl Acad. Sci.* **83**, 7277.
17. Thornburg, R. W., An, G., Cleveland, T. E., Johnson, R. and Ryan, C. A. (1987) *Proc. Natl Acad. Sci.* **84**, 744.
18. Smirnoff, P., Rotman, M. and Ladizinsky, G. (1981) *Biochem. System. Ecol.* **9**, 23.
19. Pearce, G., Liljegren, D. and Ryan, C. A. (1987) *Tomato Biotechnology*. Alan R. Liss.
20. Applebaum, S. W. and Konijn, A. M. (1966) *J. Insect Physiol.* **12**, 665.
21. Kirsi, M. and Ahokas, H. (1983) *Phytochemistry* **22**, 2739.
22. Chen, I. and Mitchell, H. L. (1973) *Phytochemistry* **12**, 327.
23. Hojima, Y., Pisano, J. J. and Cochrane, C. G. (1983) *Biochem. Pharmacol.* **32**, 985.
24. Ogawa, T., Higasa, T. and Hata, T. (1971) *Agr. Biol. Chem.* **35**, 712.
25. Wilimowska-Pelc, A. (1985) *Acta Biochim. Polon.* **32**, 351.
26. Ryan, C. A. (1974) Bayer-Symposium V *Proteinase Inhibitors*, pp. 667–673. Springer, Berlin.
27. Song, K. M., Osborn, T. C. and Williams, P. H. (1988) *Theor. Appl Genet.* **75**, 784.
28. Pearce, G., Sy, L., Russell, C., Ryan, C. A., and Hass, G. A. (1982) *Arch. Biochem. Biophys.* **213**, 456.
29. Boodly, J. W. and Sheldrake, R. Jr. (1977) *Cornell Information Bull.* **43**, 1.
30. Smith, P. K., Krohn, R. I., Hermanson, G. T., *et al.* (1985) *Anal. Biochem.* **150**, 76.
31. Walsh, K. A. (1970) *Methods in Enzymology*, Vol. XIX (Perlmann, G. E. and Lorand, L. eds) Chap. 4. Academic Press, New York.