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Wound-induced Accumulation of Trypsin Inhibitor Activities in Plant Leaves

SURVEY OF SEVERAL PLANT GENERA[1]

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ABSTRACT

Proteinase inhibitor-inducing factor (PIIF)-induced accumulation of trypsin inhibitory activity was assayed in leaves of 23 species of plants representing 10 agriculturally important genera. Inhibitory activity was assayed in extracts from attached leaves or from excised leaves supplied through the cut petioles for 30 minutes with extracts containing the wound hormone PIIF, obtained from either tomato leaves or from the leaves of each plant under study. During subsequent incubation in light for 72 hours, PIIF-induced trypsin inhibitory activity accumulated in significant quantities in 10 of the 23 species. Alfalfa accumulated the highest levels of inhibitory activity (340 μg trypsin inhibited/ml leaf juice), followed by tobacco, tomato, potato, strawberry, cucumber, squash, clover, broadbean, and grape. It is suggested that the inhibitors might be classed as allelochemics that are present in certain plants and not others in response to environmental pressures during their evolution.

Wound-induced accumulation of proteinase inhibitors in potato and tomato leaves is mediated by a wound hormone, called PIIF[3] (6, 7), that is released from severely wounded tissues and travels through the vascular system to both nearby and distal tissues where it initiates the accumulation of proteinase inhibitors I and II (2, 15, 17). The response is thought to be a primitive immunological response to protect the plant tissues from proteinases of invading pests (19). In a previous communication (14), we demonstrated that PIIF, or PIIF-like substances, were present in a wide variety of plants. An active PIIF-like activity that could induce proteinase inhibitors in tomato leaves was found in all but two of 39 species selected from among 20 families of the four major divisions of plants. The presence of the inducing factor(s) in the tissue extracts was not sufficient evidence to establish whether endogenous PIIF or tomato PIIF could, in fact, induce accumulation of proteinase inhibitors in other plants besides tomato. Therefore, in this communication we examine leaves of 23 plant species from 10 genera for their capacity to produce trypsin inhibitors when leaves are detached and supplied with a solution of the wound hormone PIIF derived from either tomato or extracts from the plant's own leaves.

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[3] Abbreviations: PIIF, proteinase inhibitor-inducing factor; TAME, tosyl-L-arginine methyl ester.

MATERIALS AND METHODS

All plants except cherry, peach, larch, apple, and strawberry were grown from seed under summer greenhouse conditions. Cherry, peach, larch, apple, and strawberry leaves were obtained from plants growing on the Washington State University campus. The young, expanding leaves of each plant were used for the assays.

Trypsin was assayed spectrophotometrically by the method of Hummel (11). Bovine trypsin (twice crystallized) was purchased from Worthington and TAME from Sigma. The trypsin was determined to be 54% pure as measured by the method of Chase and Shaw (3). Trypsin inhibitor activity was measured after preincubating for 2 min varying volumes (0.01-0.15 ml) of leaf extracts with 0.5 to 1.0 μg trypsin in 0.001 M HCl, and 0.046 M tris buffer (pH 8.1) containing 0.0115 M $CaCl_2$, to give a final volume of 0.2 ml. The assay was initiated by adding to the preincubation mixture 2.5 ml tris buffer and 0.3 ml of the substrate TAME (0.01 M). The rate of hydrolysis was measured at 247 nm. The percentage of trypsin activity remaining was plotted against the amount of extract added, and the quantity of extract at 50% inhibition of trypsin was determined by extrapolating from the linear portion of the plot. The total inhibitory activity in the extracts was expressed in μg trypsin inhibited per ml leaf juice.

Crude plant PIIF from each type of plant was prepared from detached leaves that had been autoclaved for 15 min, lyophilized, and ground to a powder. Leaf powder was mixed with water (1 g/20 ml) and then centrifuged at 1,000*g* for 5 min. The supernatant (final pH adjusted to 5.5-5.7) was employed as a crude PIIF solution.

A partially purified tomato PIIF was prepared from autoclaved, freeze-dried tomato leaves. The powder was fat-extracted with chloroform-methanol (2:1) and then water-extracted. The water extract was dialyzed overnight against distilled H_2O and lyophilized. This lyophilized powder is called "crude tomato PIIF," and at a concentration of 1 mg/ml is effective in inducing accumulation of inhibitors I and II in tomato leaves when supplied through the cut petiole for 15 min.

For induction of trypsin inhibitors, young excised leaves were supplied with crude PIIF from the plant under study, crude tomato PIIF, or water, for 30 min through their cut petioles. Control leaves were excised and immediately frozen at 0 C. After PIIF treatments, the leaves were rinsed and supplied with water under 1,000 ft-c for 72 hr at 31 C. At the end of the incubation, the leaves were stored frozen until assayed.

Extracts of leaves for measurement of trypsin inhibitor were prepared with 0.5 M tris buffer (pH 8.5) (1 g leaf tissue/3 ml tris). The mixture was macerated with washed sand with a ceramic mortar and pestle at 4 C, and then filtered through cheesecloth. The extracts were centrifuged under N_2 at 144,000*g*

at 0 C for 1 hr in a Spinco model L ultracentrifuge. The clear supernatant was maintained under N_2 and assayed. For extraction of cucumber, broadbeans, and kidney beans, dithioerythritol (final concentration of 5 mM) was added to the tris buffer.

RESULTS

Trypsin inhibitor activity was assayed in leaves of 23 species of plants, representing 10 genera of agriculturally important crops. Assays were performed on extracts from attached leaves and from excised leaves that were supplied with either water or partially purified tomato PIIF, or a crude solution of leaf extract obtained from the species under study. These conditions allowed the comparison of inhibitors present under growing conditions with inhibitors present after excision and incubation with water or PIIF.

Figure 1, A, B, and C, shows the patterns of trypsin inhibition found in leaves of three species representing plant genera that responded to PIIF induction by accumulating (A) significant trypsin inhibitor activity; (B) moderate trypsin inhibitor activity; and (C) only weak trypsin inhibitor activity. Attached tobacco leaves (A) contained significant trypsin inhibitory activity and responded to both tomato PIIF and endogenous PIIF by accumulating even higher levels of inhibitor. Cucumber leaves (B) contained about $^1/_{10}$ the amount of trypsin inhibitor in the leaves, but responded to both tomato and cucumber PIIF in accumulating more inhibitor. Lentil leaves (C) contained no trypsin inhibitor in leaves, but did respond to tomato or lentil PIIF in accumulating small, but detectable trypsin inhibitory activity.

A summary of these and similar experiments with leaves of 20 other species, representing 10 agriculturally important genera, is presented in Table I. It can be noted that leaves of several species contained little or no inhibitors and did not respond to any treatments by accumulating inhibitors. Several species contained measurable trypsin inhibitory activity, but they did not all respond to PIIF treatments by accumulating inhibitors. A few species, including squash, cucumber, and strawberry leaves, accumulated moderate amounts of trypsin inhibitors when treated with PIIF. All of the Solanaceae tested and alfalfa accumulated large quantities of inhibitors when treated with PIIF.

Table I. *Trypsin Inhibitor Activity in Various Plant Leaf Extracts.*

Detached leaves were supplied with either water, endogenous PIIF, or tomato PIIF for 30 min through the cut petiole, followed by incubation with water under 1,000 ft-c for 72 hr at 31 C. Attached leaves were taken directly from the plants.

Plants	Common Name	Trypsin Inhibitor Activity in Extracts of Leaves[1]			
		Attached	Detached and Treated With		
		No Treatment	Water	Endogenous PIIF	Tomato PIIF
Betulaceae					
Betula pubescens	Birch	0	0	0	0
Compositae					
Lactuca sativa	Lettuce	0	0	0	0
Cucurbitaceae					
Cucurbita maxima	Squash	12	25	18	18
Cucumis sativus	Cucumber	13	26	26	22
Gramineae					
Hordeum vulgare	Barley	0	0	0	0
Triticum sativum	Wheat	3	4	4	5
Zea mays	Corn	0	0	0	2
Leguminosae					
Lens culinaris	Lentil	0	3	4	4
Medicago sativa	Alfalfa	49	180	245	338
Phaseolus vulgaris	Kidney bean	0	3	4	4
Pisum sativum	Pea	4	9	4	9
Trifolium repens	Clover	0	2	7	7
Vicia faba	Broadbean	4	9	7	9
Liliaceae					
Allium cepa	Onion	0	0	0	0
Pinaceae					
Larix occidentalis	Larch	32	--	21	26
Rosoceae					
Fragaria virginiana	Strawberry	27	42	24	45
Malus pumila	Apple	16	20	18	14
Prunus serotina	Cherry	0	0	0	0
Prunus persica	Peach	0	0	0	0
Solanaceae					
Lycopersicum esculentum	Tomato	60	62	108	120
Nicotiana tobacum	Tobacco	133	133	284	284
Solanum tuberosum	Potato	37	49	68	68
Vitaceae					
Vitis vinifera	Grape	3	8	--	6

[1] µg trypsin inhibited/ml leaf juice.

DISCUSSION

Proteinase inhibitors had previously been shown to increase in tomato and potato leaves after physical wounding or after induction with the wound hormone PIIF (6, 7, 19). In this study, we have shown that leaves of plants in families other than the Solanaceae respond to PIIF by inducing proteinase inhibitors, and that leaves of a number of species respond very little or not at all (Table I). In the plants that did respond, we found that PIIF prepared from both the plant under study as well as PIIF prepared from tomato leaves was effective in inducing proteinase inhibitors. In a previous survey (14), leaf juices from a variety of plants were found to have the capability to induce accumulation of proteinase inhibitor I in excised tomato leaves.

Ten of the 23 species tested, representing five genera, responded to PIIF by accumulating trypsin inhibitors. Alfalfa leaves, which had previously been demonstrated to possess con-

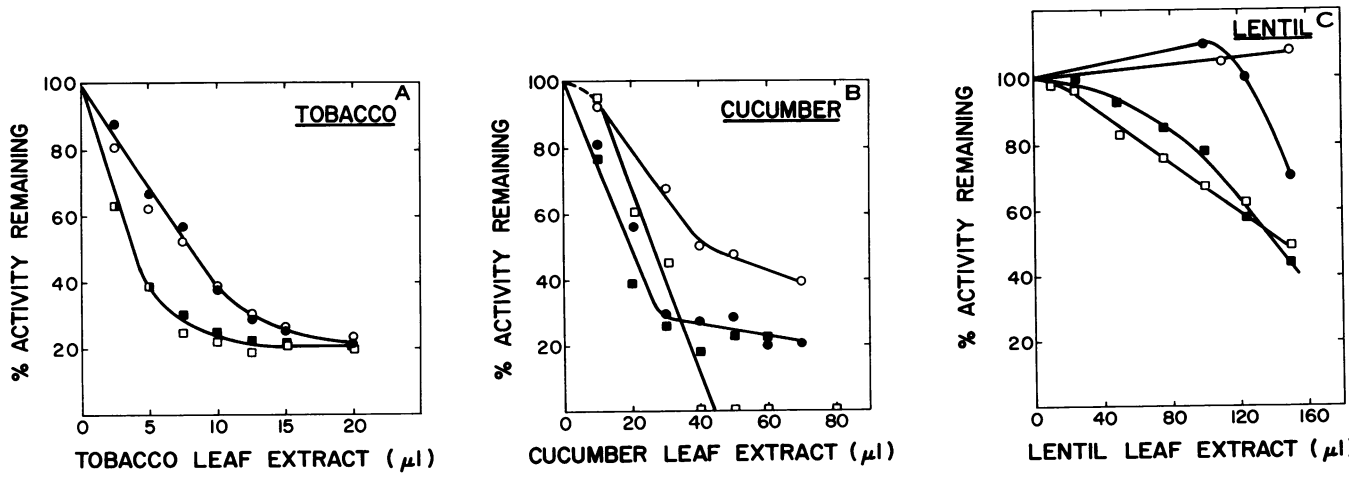

FIG. 1. Trypsin inhibitory activity in extracts prepared from (A) tobacco, (B) cucumber, and (C) lentil leaves as described in the text. Activity is shown in extracts from attached leaves (○ – – ○); and excised leaves supplied with water (● – – ●); crude PIIF extract from plant's own leaves (■ – – ■); and crude tomato PIIF (□ – – □).

siderable trypsin inhibitor activity (4), accumulated the highest levels of trypsin inhibitory activity in response to PIIF. Three Solanaceae plants, tobacco, tomato, and potato, followed in that order, while strawberry, cucumber, squash, grape, clover, and broadbean accumulated low, but significant trypsin inhibitory activity. In several species, PIIF-induced trypsin inhibitory activities could not be detected at all.

It is apparent from these studies that the wound-induced accumulation of trypsin inhibitors is not a ubiquitous property in the plant kingdom, even though PIIF or PIIF-like substances were found (14) in leaves of nearly every plant tested to date. It may be that inhibitors of proteolytic enzymes other than trypsin accumulate when plants are wounded and our assays for only trypsin may not have detected them. Chymotrypsin inhibitors accumulate in leaves of several species of the Solanaceae (8). We did attempt to measure increases in chymotrypsin inhibitors in several plants, but in attempting to survey extracts from various plants we encountered endogenous hydrolytic enzyme activity toward chymotrypsin substrates used in the spectrophotometric assays. Some chymotrypsin ester substrates are good substrates for plant carboxypeptidase (1, 13, 16). The presence of carboxypeptidase activity in many plant leaves has been reported (18), including tomato (5), and it could have accounted for the chymotryptic-like activity we encountered.

The finding that proteinase inhibitors can be induced by wounding in leaves of some plants and not in leaves of others was not wholly unexpected. Plant proteinase inhibitors can be likened to, or included in, the broad group of chemicals called "allelochemics." These are chemicals that have been defined by Whittaker and Feeny (20) as naturally occurring "chemicals by which organisms of one species affect the growth, health, behavior, or population biology of organisms of another species." Haukioja and Hakala (9) have suggested that specific chemicals (allelochemicals) are produced in some plants and not in others for chemical defense, and that the energy expanded to maintain them for specific interactions with herbivores, may depend on the pressure placed on the plants to survive. The plants may maintain the chemicals all of the time, or may produce such substances only when necessary, depending on herbivore pressures. In the latter case, energy would only be expended to produce the chemicals when environmental stimuli predict a high level of herbivore attack (9). The wound-induced accumulation of proteinase inhibitors may be an example of this latter type of response. We have suggested (19) that the wound-induced accumulation of proteinase inhibitors may be a primitive immune response directed against pests (microorganisms or insects) because the inhibitory specificities of the inhibitors are directed toward the serine proteinase class of endopeptidases to which many of the digestive enzymes of microorganisms and insects belong (18). Instead of the more complex and advanced immunoglobulin system of animals, plants may have retained and developed a broad spectrum of chemicals to perform the role of plant protection. Thus, the lack of a sophisticated memory system as is involved in animal antibody production, and the absence of a circulatory system to mobilize it, may have necessitated in plants a vast array of secondary plant compounds to deal with the multitudes of pests that attack them. Janzen (12) has recognized this problem and has suggested that a study of allelochemicals should be undertaken by "chemical ecologists" to understand fully plant-pest relationships. Not only does one need to study the array of chemicals present, and to classify them by plant genera and species, but to determine the changes that might take place in the concentrations of these chemicals during the plant's life cycle and under various stresses to the plant.

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