Increased Proteinase Inhibitor Activity in Response to Infection of Resistant Tomato Plants by *Phytophthora infestans*

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ABSTRACT

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Proteinase inhibitor activity was measured in tomato plants following infection with compatible and incompatible races of *Phytophthora infestans*. Inhibitor activity in plant extracts was assayed for its inhibition of chymotrypsin and trypsin esterolytic activity. Proteinase inhibitor activity increased rapidly and remained at a high level in tomato plants following infection with an incompatible race of *P. infestans*. Inhibitor activity in tomato plants infected with compatible races either increased initially and then declined or it decreased from the time of infection. In both cases, the inhibitor activity declined to below the initial level as the disease developed. Infection of a single bottom leaf induced proteinase inhibitor activity in noninoculated upper leaves. The increase in upper leaves was about two times greater after inoculation with the incompatible race than with compatible races.

Additional key words: chymotrypsin inhibitor, trypsin inhibitor, disease resistance.

Proteinase inhibitors are widely distributed in the plant kingdom. Vogel et al. (33) listed members of Leguminosae, Solanaceae, Gramineae, Chenopodiaceae, and a few microorganisms in which proteinase inhibitors are known to occur. Most of the work on proteinase inhibitors of plant origin has been done in relation to their effect on mammalian proteinases. The majority of plant proteinase inhibitors are proteins with molecular weights in the range of 5,000 to 60,000 (17, 26, 33). They generally inhibit proteinases with trypsin-like or chymotrypsin-like specificities. There are a few cases known in which they inhibit endogenous proteolytic enzymes of the plant from which they are derived (26).

Proteolytic enzymes are produced by many microorganisms including plant pathogenic fungi (3, 9, 15, 21, 23) and bacteria (12, 20, 25, 31). A role for extracellular proteolytic enzymes of plant pathogenic microorganisms in pathogenesis has been suggested by many workers, because proteins are known to occur in plant cell walls, middle lamellae, and plasma membranes (1, 6, 16). In fact, an increase in proteolytic enzyme activity occurs in tissues of several plants following infection by fungal (9, 14, 19, 22, 30, 32) and bacterial (4, 13, 25) pathogens.

Potato tubers are known to contain several proteolytic inhibitors (26, 33). One of the inhibitors isolated from potato tubers, chymotrypsin inhibitor I, occurs in potato leaves (27) and accumulates in detached potato and tomato leaves (28). More recently, Green and Ryan (7) found inhibitor I accumulated throughout potato and tomato plants after mechanical wounding or after Colorado potato beetles fed on single leaves. This led to the suggestion that accumulation of proteinase inhibitors has a function in the plant's protective mechanism against insects and microorganisms.

The present study was initiated to determine (i) if the proteinase inhibitor activity is altered in tomato plants infected with *Phytophthora infestans* (Mont.) deBary and (ii) if changes in the inhibitor activity could be correlated with resistance or susceptibility to the fungus.

MATERIALS AND METHODS

Sibling tomato (*Lycopersicon esculentum* Mill.) selections TS 19 and TS 33 used in this study were derived from breeding line 386-1-5 obtained from M. E. Gallegly, West Virginia University. The two tomato races of *P. infestans,* isolate WV 75 (race 0) and isolate WV 33 (race 1), also were obtained from M. E. Gallegly (5). Selection TS 19 gives a compatible (susceptible) reaction to both fungal races 0 and 1; TS 33 gives an incompatible (hypersensitive resistant) reaction to race 0 and a compatible reaction to race 1.

Plants were propagated from seed planted in vermiculite saturated with modified Hoagland's solution (11). Except when indicated, the plants were grown in a growth chamber with a day temperature of 25 C and a night temperature of 18 C. They were illuminated 14 hours daily with 1.5×10^4 lx (1,400 ft-c) of fluorescent and incandescent light.

The cultures of *P. infestans* were maintained on lima bean agar medium (5). Inoculum was prepared by washing sporangia from 9-day-old lima bean agar culture plates with 10 ml of water. The suspension was diluted to 45,000 sporangia per ml of water and incubated at 12 C for 2 hours to induce zoospore liberation. Three-week-old tomato plants were

inoculated by atomizing the sporangia-zoospore suspension onto the entire plant or a single bottom leaf, depending on the experiment being conducted. Following inoculation, the plants were kept in a dark chamber with a water-saturated atmosphere for 22 to 24 hours, after which they were returned to the growth chamber.

For each treatment within an experiment, three replicate crude proteinase inhibitor extracts were prepared by grinding 1 g of fresh tomato leaflet tissue in a mortar with 0.2 ml of a solution containing 1 M NaCl and 1 M ascorbic acid and 3 ml of 0.05 M, pH 8.2 Tris-HCl buffer. The homogenate was strained through six layers of cheesecloth and centrifuged at 105,000 *g* at 4 C for 1 hour. The supernatant solution was retained and held at 4 C until it was assayed for inhibitor activity.

Crude plant extracts were assayed spectrophotometrically for proteinase inhibitor activity by measuring their inhibition of the esterase activity of α-chymotrypsin (Calbiochem) and trypsin (Sigma Chem. Co.). The chymotrypsin assayed by the method of Schwert and Takenaka (29) using tyrosine ethyl ester hydrochloride (TEE) (Mann Res. Lab.) as a substrate had a specific activity of 7.5 units per mg. The trypsin assayed by the method of Hummel (10) using *p*-tosyl arginine methyl ester hydrochloride (TAME) (Mann Res. Lab.) as a substrate had a specific activity of 40 units per mg.

Assays for proteinase inhibitors were initiated by incubating 0.05 ml of plant extracts with 0.05 ml of chymotrypsin (800 μg/ml in 1.0 mM HCl) or trypsin (50 μg/ml in 1.0 mM HCl) for 5 minutes at room temperature (about 24 C). The assay for chymotrypsin inhibitors consisted of 2.5 ml of 1.0 mM TEE in 0.05 M phosphate buffer at pH 7.0, plus 0.1 ml of pre-incubated "chymotrypsin-plant extract" in the reaction cuvette. The control cuvette contained L-tyrosine instead of TEE. Decrease in absorbance at 240 nm was plotted for 5 minutes. The assay for trypsin inhibitors consisted of 2.5 ml of 1.0 mM TAME in 0.04 M Tris-HCl buffer with 0.01 M $CaCl_2$ at pH 8.1, plus 0.1 ml of pre-incubated "trypsin-plant extract" in the reaction cuvette. Autoclaved trypsin was used in place of active trypsin in the control cuvette. Increase in absorbance at 247 nm was plotted for 3 minutes. Percentage reduction in proteinase activity by plant extracts was determined by comparing the rate of change of absorbance over the linear portion of the plot with control assays in which the enzymes without leaf extract were added to their respective substrates.

Data in this paper are reported as inhibitor units per mg of tissue protein. Inhibitor units were calculated by the method suggested by Vogel et al. (33); i.e., "the quantity of inhibitor that reduces the rate of conversion of substrate by 1 μmole/minute under standard conditions is equal to one international inhibitor unit (IU)." The protein content of extracts was determined by the method of Lowry et al. (18).

RESULTS

Assays were made for chymotrypsin inhibitor activity (CTIA) and trypsin inhibitor activity (TIA) in well-expanded terminal leaflets from TS 19 and TS 33 plants following inoculation of the entire plant with races 0 and 1 of *P. infestans*. Tests were made with plants grown at 21 and 25 C day temperature with an 18 C night temperature in both cases. Results were similar; therefore, only the 25 C results are reported. These data represent the average of four separate experiments. Chymotrypsin inhibitor activity increased 40% within 24 hours after inoculation of TS 33 with race 0 (incompatible), and the level of inhibition remained high during the 4 days after inoculation in which assays were made (Fig. 1-A). A slight increase in CTIA occurred at 24 hours when TS 33 and TS 19 were inoculated with race 1 (compatible reactions), followed by a decline during the next 3 days to a level below the initial activity. There was a decline from the

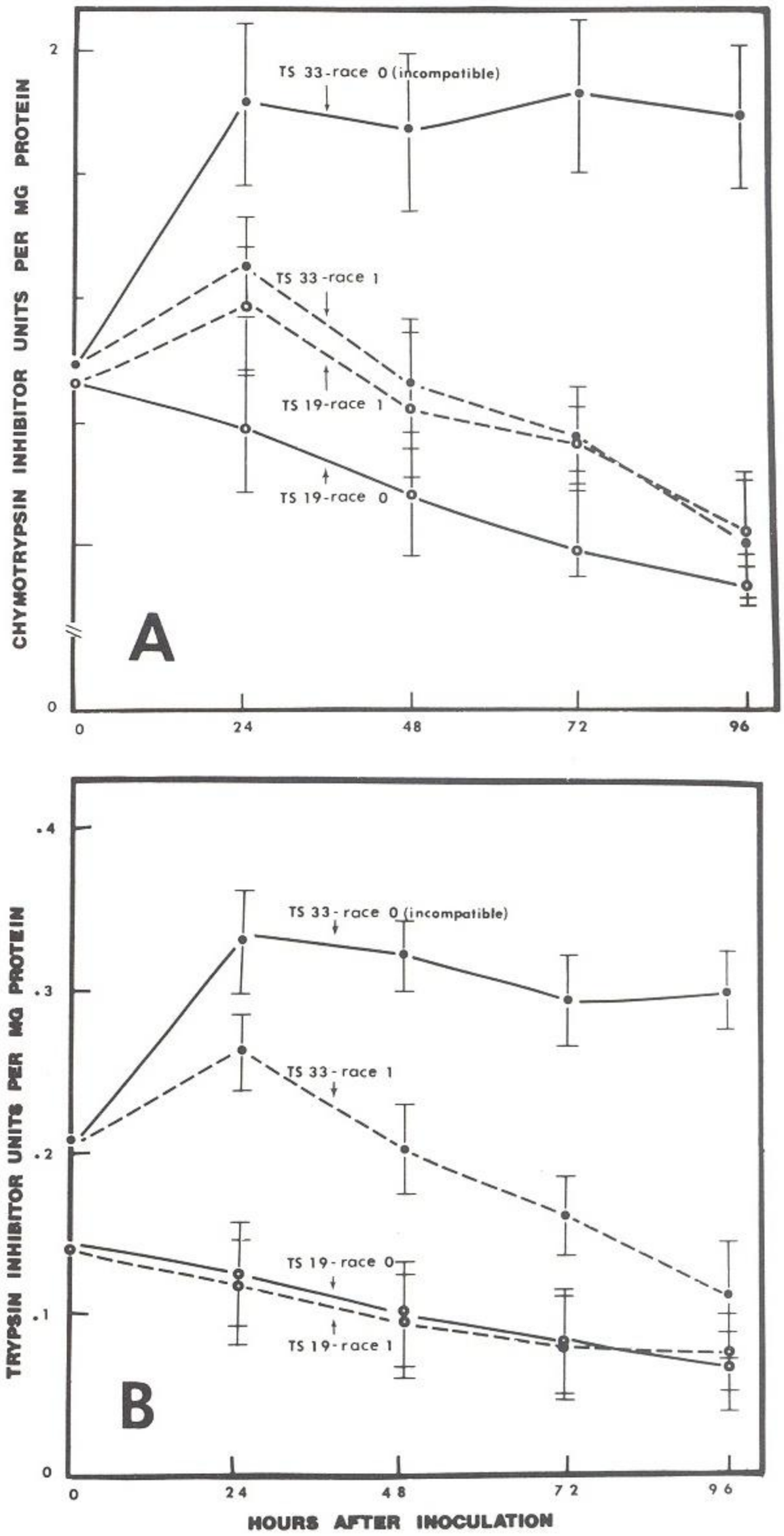

Fig. 1-(A, B). Proteinase inhibitor activity in terminal tomato leaflets following inoculation of the entire plant with *Phytophthora infestans* spores. Tomato selection TS 33 inoculated with race 0 is incompatible (resistant). All other host-fungus combinations are compatible (susceptible). Bars show standard errors of the means for four determinations of values. **A**) Chymotrypsin inhibitor activity. **B**) Trypsin inhibitor activity.

initial level of CTIA in TS 19 following inoculation with race 0 (compatible).

Trypsin inhibitor activity increased 63% within 24 hours in the TS 33-race 0 combination and it remained at about the same elevated level during the 4 days assays were made (Fig. 1-B). A 30% increase in the TIA was observed in the TS 33-race 1 combination at 24 hours, followed by a decline to below the initial level during the next 3 days. A decline from the initial level of TIA was measured in TS 19 plants following inoculation with race 0 or 1.

The ability of a compatible and incompatible combination of *P. infestans* to induce increased levels of CTIA and TIA in upper noninoculated plant parts was tested by inoculating a single bottom leaf on individual plants and then excising the inoculated leaves at 0, 4, 8, 12, and 24 hours. Terminal leaflets from the upper noninoculated leaves were assayed 48 hours after inoculation. These data represent the average of four separate experiments. Chymotrypsin inhibitor activity and TIA increased by about 85 and 106%, respectively, in the TS 33-race 0 incompatible combination (Table 1, 2). Near maximum accumulation of CTIA was induced even when inoculated leaves were excised 4 hours after inoculation, but most of the induction of TIA did not occur until 8 hours after inoculation. In the compatible combination, TS 33-race 1, CTIA and TIA were increased by about 46 and 44%, respectively (Table 1, 2). Even though some increase in the levels of inhibition was induced within the first 4 hours in the susceptible combination, maximum induction of CTIA and TIA occurred when the inoculated leaves were left attached for 8 hours. Little or no further induction occurred if the inoculated leaf remained attached for periods longer than 8 hours.

In a separate experiment, assays were made to determine inhibitor activity in the "lesion area" and the adjacent "uninvaded area" of infected tomato leaflets. Tissue was cut with a cork borer from rapidly expanding lesions and adjacent tissue 4 days after the entire plants were inoculated with spores of compatible races of *P. infestans*. These data represent an average of two separate experiments. In all cases, both the CTIA and the TIA were less in the lesion area than in the uninvaded area or similar leaf tissue from noninoculated control plants (Table 3). The CTIA was considerably higher in the uninvaded area of both TS 33 and TS 19 infected with race 1 but less in TS 19 infected with race 0, when compared with noninoculated control plants.

The influence of temperature on CTIA accumulation in response to wounding was studied in an attempt to distinguish the wound response from the pathogenic attack response. A single bottom leaf on TS 19 and TS 33 plants was wounded by cutting seven, 5-mm diameter holes with a cork borer. The terminal leaflets of upper leaves were assayed to determine the effect of wounding on accumulation of CTIA. In separate experiments on plants grown at day temperatures of 21, 25, and 30 C, the influence of temperature on CTIA was determined. The data presented here represent two replicate experiments at 21 C and three replicate experiments at 25 and 30 C. Maximum accumulation of CTIA occurred in plants

TABLE 1. The effect of detachment of an individual bottom tomato leaf inoculated with *Phytophthora infestans* on the stimulation of chymotrypsin inhibitor activity in the terminal leaflets of noninoculated leaves on the same plant

Time detached after inoc. (hours)	Chymotrypsin inhibitor[a]			
	TS 33 inoc. with race 0		TS 33 inoc. with race 1	
	IU[b]	Increase (%)	IU	Increase (%)
0	1.02 A	. . .	1.02 A	. . .
4	1.74 B	71	1.20 A	18
8	1.74 B	71	1.49 A	46
12	1.77 B	74	1.46 A	43
24	1.78 B	75	1.48 A	45
Not detached	1.89 B	85	1.45 A	42

[a]All assays were done at the same time 48 hours after inoculation of the bottom leaf. IU = inhibitor units per milligram protein.
[b]Means of four determinations; those followed by the same letter in each column do not differ significantly, $P = 0.05$.

TABLE 2. The effect of detachment of an individual bottom tomato leaf inoculated with *Phytophthora infestans* on the stimulation of trypsin inhibitor activity in the terminal leaflets of noninoculated leaves on the same plant

Time detached after inoc. (hours)	Trypsin inhibitor[a]			
	TS 33 inoc. with race 0		TS 33 inoc. with race 1	
	IU[b]	Increase (%)	IU	Increase (%)
0	0.18 A	. . .	0.18 A	. . .
4	0.23 A	28	0.20 A	11
8	0.34 B	89	0.26 A	44
12	0.33 B	83	0.25 A	39
24	0.35 B	94	0.24 A	33
Not detached	0.37 B	106	0.24 A	33

[a]All assays were done at the same time 48 hours after inoculation of the bottom leaf. IU = inhibitor units per milligram protein.
[b]Means of four determinations; those followed by the same letter in each column do not differ significantly, $P = 0.05$.

grown at the highest temperature. During the 4 days after wounding, CTIA increased to more than 50% above the initial level at 30 C (Table 4). In plants grown at 25 C, there was an increase of a little more than 20% over the same period. There was no increase in CTIA at 21 C following wounding. Tomato selection TS 33 and TS 19 plants behaved similarly in response to wounding; however, the level of CTIA was slightly lower in TS 19 in all comparable measurements.

DISCUSSION

The results of this study show a correlation between an increase of proteinase inhibitor activity in tomato plants following infection and resistance of those plants to *P. infestans*. The proteinase inhibitor activity increased rapidly and persisted in tomato plants following infection with an incompatible race of the fungus. But, in compatible host-fungus combinations, the proteinase inhibitor activity either (i) declined following infection or (ii) increased slightly soon after inoculation and then declined to a level below that measured at the time of infection.

The earliest assay for increased proteinase inhibitor activity in tomato leaflets following inoculation of entire plants was made at 24 hours. At this time the maximum increase in activity had already occurred. Therefore, how soon the increase in inhibitor activity was initiated after infection and at what time the maximum level was reached was not determined. It was shown in another experiment by excising the single inoculated bottom leaf from plants at various times after inoculation that within 4 hours sufficient inducer had been translocated from a single leaf inoculated with the incompatible race to elicit a near maximum CTIA response by the host. If it is assumed that infection is a prerequisite to induction and that germination and penetration is a 2-hour process (24), the majority of the induction process must occur within the first 2 hours after infection.

The TS 19-race 0 combination results in a compatible reaction that is indistinguishable from TS 19- and TS 33-race 1 combinations based on disease symptoms, but there is a difference in the proteinase inhibitor response. There is a general decline of the inhibitor activity in the TS 19-race 0 combination, whereas race 1 elicits a small increase in inhibitor activity during the first 24 hours after infection followed by a decline. These data suggest that the decline in the inhibitor activity is a result of invasion by the fungus as it grows in the susceptible tomato leaf tissue. This suggestion is strengthened somewhat by assays that show less inhibitor activity in lesions than in uninvaded portions of infected leaves or leaves from healthy plants.

The large increase in CTIA in uninvaded leaf tissue immediately adjacent to developing lesions from race 1 of *P. infestans* was somewhat of a surprise. Corresponding experiments with the incompatible combination seemed desirable but did not appear feasible because of the very small lesion area associated with the incompatible reaction. It should be pointed out that the same compatible combinations that elicited the increase in CTIA in tissue surrounding lesions also elicited a small increase in CTIA in the terminal leaflets of spray-inoculated tomato plants at the 24-hour measurement.

Since proteinase inhibitor activity increases in tomato plants in response to wounding (7, 8), we were concerned that the response to infection by *P. infestans* also might be a wound response. Green and Ryan (8) assayed wounded tomato plants for levels of chymotrypsin inhibitor I and found that it accumulates optimally in plants at 35 C, only a little at 25 C, and none at 20 C. Our assays with infected tissue were for total CTIA rather than chymotrypsin inhibitor I levels, so we determined the effect of temperature on CTIA in wounded plants. Results of our assays for CTIA in wounded plants at 21, 25, and 30 C followed a pattern similar to that established by Green and Ryan for chymotrypsin inhibitor I levels; i.e., no increase at 21 C, a slight increase at 25 C, and a substantial increase at 30 C. All of our experiments with *P. infestans*-infected plants were conducted at 21 C or 25 C; therefore, we believe the increase in proteinase inhibitor activity following wounding and fungal infection to be distinct responses.

Two other lines of evidence indicated that the inhibitor responses to wounding and infection are distinct. They are: (i) light requirements and (ii) rate of response. Green and Ryan (8) showed that induction and accumulation of chymotrypsin inhibitor I is light-dependent. In our experiments, plants were kept in a dark moisture chamber for 22 hours after inoculation and returned to the light

TABLE 3. Distribution of chymotrypsin and trypsin inhibitor activity in tomato leaves 4 days after infection with a compatible race of *Phytophthora infestans*

	Inhibitor units/mg protein		
	TS 33 inoc. with race 1	TS 19 inoc. with race 1	TS 19 inoc. with race 0
Chymotrypsin			
Lesion area	0.68[a]	0.71	0.51
Uninvaded area	2.09	1.53	0.64
Healthy plants	1.17	1.09	1.09
Trypsin			
Lesion area	0.11	0.06	0.05
Uninvaded area	0.17	0.07	0.07
Healthy plants	0.18	0.12	0.12

[a]Each value average of two experiments.

TABLE 4. The effect of daytime temperature on the stimulation of chymotrypsin inhibitor activity in terminal leaflets of upper leaves following wounding[a] of a single bottom leaf on tomato plants

Hours after wounding	Chymotrypsin inhibitor units/mg protein[b]					
	TS 33			TS 19		
	21 C	25 C	30 C	21 C	25 C	30 C
0	1.12	1.16	1.18	0.97	1.01	1.03
24	1.14	1.26	1.13	0.98	1.12	1.15
48	1.10	1.29	1.50	0.98	1.15	1.47
72	1.09	1.45	1.80	1.00	1.28	1.52
96	1.15	1.29	1.87	0.99	1.19	1.58

[a]Seven, 5-mm diameter holes were cut with a cork borer in a single bottom leaf.

[b]Data for 25 and 30 C average of three experiments and for 21 C are an average of two experiments.

chamber only 2 hours prior to the 24-hour assay. The fact that maximum measured activity was in the 24-hour assay suggests that induction and accumulation of proteinase inhibitor activity in response to infection can occur in the dark. This statement cannot be made unequivocally because of the 2-hour light exposure. The rate of CTIA increase in tomato plants following wounding is much slower than the increase following fungal infection. Chymotrypsin inhibitor activity only increased slightly by 24 hours after wounding and generally continued to increase through the 96-hour measurement period (Table 4). In comparison, the CTIA increase was near its maximum by 24 hours after inoculation with the incompatible fungal race and remained near that level through the 96-hour measurement (Fig. 1-A).

The association of a rapid increase of proteinase inhibitor activity with hypersensitive resistance of tomato to *P. infestans* suggests a role for the increased inhibitor activity in the host's resistance mechanism. What role the inhibitor activity may play in resistance only can be speculated. It was pointed out by Ryan (26) that many plant proteinase inhibitors are known to inhibit extracellular proteinases of microorganisms and therein could be a mechanism of disease resistance. Certainly this area needs investigation to determine whether the increased inhibitor activity in inoculated tomato plants can inhibit extracellular proteinases of *P. infestans*.

We have not determined what class of compound is responsible for the increase in proteinase inhibitor activity in infected tomato plants. However, since most proteinase inhibitors from plants are proteins (17, 26, 33), we suspect the increased inhibitory activity is due to accumulation of protein proteinase inhibitor(s). If the increase of inhibitory activity in tomato plants following infection is due to accumulation of protein proteinase inhibitor(s), a question then arises as to whether the accumulated inhibitor(s) is distinct or an increase in the level of that already present.

The questions raised here as to the nature of the increased inhibitor activity can be answered only by isolation and identification of the inhibitor(s) involved. A recent study showed that potato tubers have at least 13 distinct inhibitors of trypsin and chymotrypsin (2). Therefore, it would not be surprising if several inhibitors are involved in the increased CTIA and TIA found in tomato plants following inoculation with an incompatible race of *P. infestans*.

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