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Isolation and characterization of wound-inducible carboxypeptidase inhibitor from tomato leaves

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Abstract

In a previous report [Mol. Gen. Genet. 228 (1991) 281], carboxypeptidase inhibitor protein (CPI) mRNA was found to accumulate in leaves of wounded tomato plants, but CPI protein could not be detected. In contrast, we found that CPI protein does accumulate in tomato leaves in response to wounding, and also in response to treatment with either systemin, methyl jasmonate (MeJ), oligogalacturonic acid, or chitosan. Identification of CPI protein was confirmed by its inhibition of metallo-carboxypeptidase A (CPAase), which was used as an assay during purification of the inhibitor from leaves of MeJ-treated tomato plants. Amino acid sequence analysis and mass spectroscopic analyses of the pure protein confirmed its identity as CPI. The pure protein inhibited CPAase in a 1:1 stoichimetric interaction. Time course analyses of the induction of CPI mRNA in tomato leaves in response to wounding indicated that the gene is a member of the group of "late genes" that code for defensive proteins synthesized in leaves in response to herbivore attack.

Keywords: *Lycopersicon esculentum*; cv Castlemart; Solanaceae; Tomato; Protein chemistry; Metallo-carboxypeptidase inhibitor

1. Introduction

Wounding the leaves of tomato and potato plants causes the systemic induction of proteinase inhibitors that have specificities toward digestive enzymes of animals and insects (Ryan, 2000). Several families of proteinase inhibitors are found in plants, many at high levels, usually in storage organs such as seeds and tubers (Ryan, 1971, 1981; Garcia-Olmeda et al., 1987; Pearce et al., 1988; Pouvreau et al., 2003), where they are present as storage proteins that can help protect against predators and pests. The inhibitor proteins are also found in fruit (Pearce et al., 1988) and are wound-inducible in leaves (Green and Ryan, 1973). Several families of proteinase inhibitors are found in potato tubers with individual specificities against all four known classes of animal digestive enzymes (Ryan, 1981; Garcia-Olmeda et al., 1987). Among the inhibitors is a small, 4 kDa protein (carboxypeptidase inhibitor (CPI)) that inhibits metallo-carboxypeptidases A and B (Ryan, 1971; Ryan et al., 1974), and is the only example in plants of a proteinaceous inhibitor with this specificity. CPI has been studied extensively for its multiple biological effects. It is a T-knot protein (Blanco-Aparicio et al., 1998) that has been shown to possess potent anti-tumor and anti-carcinogenic activities induced by tumor promoters, including X-ray and UV-B irradiation (Billings et al., 1989). The primary structure of the potato tuber and tomato fruit CPIs have been determined (Hass et al., 1975; Hass and Hermodson, 1981) and the three-dimensional structure of the potato tuber CPI has been resolved by X-ray crystallography (Rees and Lipscomb, 1980).

CPI protein is wound inducible in potato leaves (Graham and Ryan, 1981; Villanueva et al., 1998) and, together with other wound-inducible proteinase inhibitors, it is considered a component of the antinutrient

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proteins that help defend against attacking herbivores (Ryan, 1981). In leaves of tomato plants, a wound-inducible CPI mRNA was detected in tomato leaves but was not accompanied by the accumulation of CPI protein (Martineau et al., 1991). However, CPI protein is found in tomato fruit (Hass and Ryan, 1980), and only a single CPI gene is present in the tomato genome that accounts for the mRNA found in both leaves and fruit (Martineau et al., 1991). The reasons for the absence of wound-inducible CPI protein in tomato leaves was not determined, but it was hypothesized that either the CPI mRNA was untranslated, or that it was translated and the protein rapidly degraded following CPI synthesis.

We have reinvestigated the reported absence of CPI protein in wounded tomato leaves and have found that the protein is synthesized in response to wounding. The inhibitor protein is also induced in response to other inducers of defensive proteinase inhibitor genes in tomato leaves, including systemin, methyl jasmonate (MeJ), oligogalacturonic acid (OGA), and chitosan. We have confirmed the presence of CPI protein in tomato leaves by assaying its inhibitory activity toward bovine metallo-carboxypeptidase and by purifying the protein and analyzing its molecular mass and its amino acid sequence. CPI mRNA was synthesized in tomato leaves in response to wounding with the same temporal induction as other wound inducible proteinase inhibitors that defend the plants against attacking herbivores.

2. Results and discussion

To initiate our study of the wound-inducibility of CPI protein in leaves of wounded tomato leaves, the presence of CPI in the soluble proteins from leaves of unwounded and wounded tomato plants was analyzed by SDS electrophoresis and immunoblotting. Also analyzed were leaves of plants exposed to MeJ vapor for 24 h, leaves of excised tomato plants supplied with systemin, OGA and chitosan, and leaves of plants overexpressing the prosystemin gene. Wounding and all other treatments induced an accumulation of a low molecular weight protein that was visualized using a CPI-specific antiserum (Fig. 1). MeJ, which is known to be a potent inducer of defense genes (Farmer and Ryan, 1992), caused the highest accumulation of the protein.

To confirm that the inducible CPI inhibitor protein was biologically active in inhibiting CPAase, soluble extracts of leaves of tomato plants that had been either wounded or exposed to MeJ vapors, as in Fig. 1, were assayed for their inhibition of carboxypeptidase A (CPAase) enzyme activity. Fig. 2 shows that wounding of lower leaves of the plants caused the induction of a CPAase inhibiting activity in both lower (wounded) and upper (unwounded) leaves. Exposure of the plants to MeJ strongly induced CPAase inhibitory activity. The

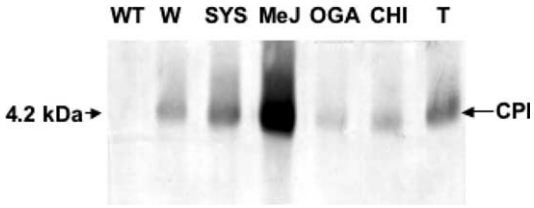

Fig. 1. Metallocarboxypeptidase inhibitor (CPI) protein accumulation in tomato leaves. Total soluble protein extracts were prepared from leaves of; wild-type unwounded plants (WT); upper unwounded leaves from plants wounded on a lower leaf (W); leaves of excised plants supplied with systemin (SYS), MeJ vapors, OGA (OGA), or chitosan (CHI), and leaves of transgenic plants overexpressing the prosystemin gene in sense orientation (T). Equal amounts of total protein extracts (100 μL) were subjected to SDS/urea–PAGE, transferred to PVDF membrane and probed using the antibody raised against potato CPI as described in Section 3.

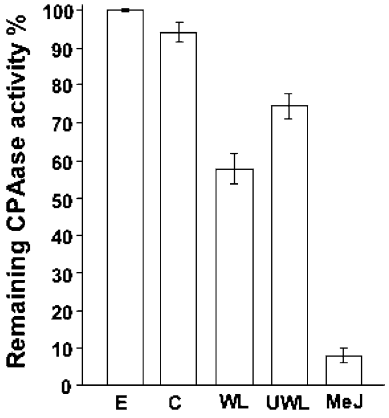

Fig. 2. Inhibition of bovine carboxypepidase A (CPAase) activity by components in soluble protein extracts from tomato leaves. CPAase activity was assayed after adding 100 μL of water (E); 100 μL of soluble extracts prepared from leaves of unwounded tomato plants (C); from lower, wounded leaves plants wounded 24 h earlier (WL); from upper, unwounded leaves from the same plants (UWL); and from leaves of plants exposed to MeJ for 24 h (MeJ). Each bar represents the average and SD of three assays.

data from Figs. 1 and 2, taken together, indicated that an active CPI protein was present in tomato leaves in response to wounding and elicitors.

CPI protein was isolated from leaves exposed to MeJ and its structural and inhibitory characteristics were characterized. The purity of the protein was confirmed using HPLC (Fig. 3), and its molecular mass of 4096 Da was established using mass spectrometry. This value was identical to the known mass of tomato fruit CPI protein, based on its cDNA sequence (Martineau et al., 1991). The inhibitory activity of the pure protein toward bovine CPAase is shown in Fig. 4, in which the titration of CPAase was nearly linear over a range of 0–90% inhibition. At 50% inhibition (in the linear region of the plot), one molecule of CPI ($M_r = 4$ kDa) was estimated to inhibit one molecule of CPAase (35 kDa).

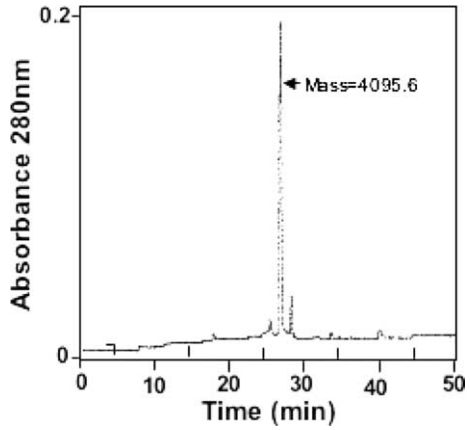

Fig. 3. HPLC analysis of purified CPI. HPLC chromatography and mass spectrometry was performed as described in Section 3. The major protein peak corresponding to tomato fruit CPI had a major ion of 4095.6 when this peak was analyzed by mass spectrometry as described in Section 3. Potato CPI ($M_r = 4.2$ kDa) was used as a molecular weight marker.

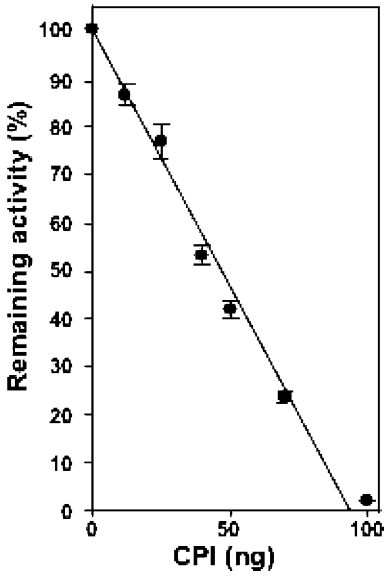

Fig. 4. Titration of bovine carboxypeptidase A (CPAase) activity by purified CPI from tomato. Increasing quantities of inhibitor were added to a constant amount of enzyme activity (1 μg CPAase) and the remaining activities were measured spectrophotometrically. Each point is the average of three independent assays.

Amino acid sequencing of the pure tomato leaf CPI by Edman degradation failed. The C-terminus was blocked, as previously found with both potato CPI and tomato fruit CPI. In these latter two proteins, the N-terminal glutamate is cyclized to form a pyroglutamate residue that lacks a primary amine and cannot undergo Edman degradation. However, an Asp–Pro couple is predicted in tomato leaf CPI at positions #4 and #5, based on the sequences of both potato and tomato fruit CPIs (Hass and Ryan, 1981). This bond is labile at acid pH, causing an Asp–Pro cleavage of both potato CPI and tomato fruit CPI to produced a new N-terminus at residue #5 (Hass and Ryan, 1981). Incubation of the pure tomato leaf CPI in 0.1% trifluoroacetic acid (TFA) at a pH of 2.5 for several days caused an Asp-Pro cleavage, and an N-terminal sequence of 11 amino acids could be obtained, which was the limit of the sequencer with the small sample available after cleavage. Every residue of the tomato leaf CPI protein, from residue #5 through residue #16, was identical to those of tomato fruit CPI protein (Hass and Hermodson, 1981). These data, together with the mass spectral analyses in which the masses of the fruit and leaf CPI proteins are identical, confirm that the peptide was the product of the single tomato CPI gene.

The previous report of the wound-inducibility of the CPI mRNA (Martineau et al., 1991) did not examine its temporal synthesis in response to wounding. To establish whether the wound induction of CPI correlates with "early" or "late" genes (Ryan, 2000), a time course of the localized and systemic synthesis of CPI mRNA in leaves in response to wounding was performed. Figs. 5A and B shows the time course of the wound-inducibility of CPI mRNA in leaves of young wounded tomato plants. Leaves of unwounded plants exhibited very low levels of CPI mRNA in leaves, whereas in leaves of the wounded plants, CPI mRNA could be detected at 2 h and continued to increase through 10 h. This time course is consistant with its identity as a "late gene", a class which includes the two defensive proteinase inhibitors I and II, in contrast to early genes whose mRNAs maximally accumulate at about 4 h and are associated with early signaling events during the wound

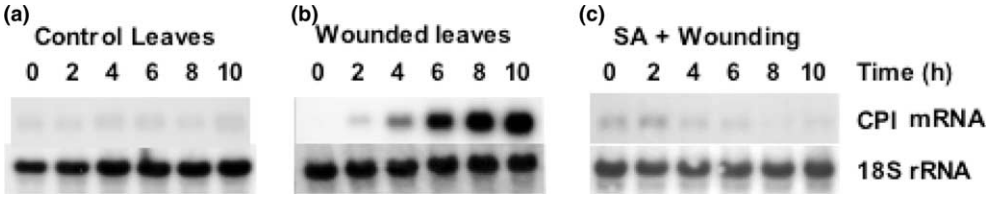

Fig. 5. Time course analysis of CPI mRNA accumulation in leaves of 14 days old tomato plants in response to wounding, assayed using *CPI* cDNA as a probe. (a) mRNA from leaves of control, unwounded plants, (b) mRNA from leaves of wounded plants. (c) mRNA from leaves of plants excised at the base of the main stem and supplied with 1 mM SA for 1 h before wounding. Leaves were collected for RNA extracted at the times indicated and the RNA extacted as described in Section 3. Fifteen μg of total RNA from each extraction were subjected to mRNA blot analysis. Tomato *CPI* cDNA was used as a probe; *18S* ribosomal RNA was used as a loading control.

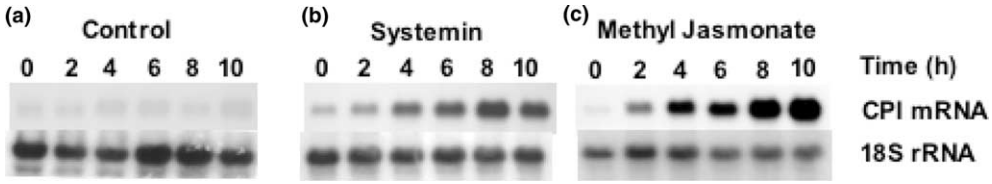

Fig. 6. Time course analysis of CPI mRNA accumulation in leaves of tomato plants in response to systemin and MeJ. (a) Control plants supplied with 10 mM phosphate buffer (pH 6.5). (b) Excised plants supplied with systemin (12.5 nM, final concentration in buffer). (c) Excised plants exposed to MeJ vapors as described in Section 3. CPI mRNA was assayed as in Fig. 5.

response (Ryan, 2000). Also, like the inhibitor I and II genes, the CPI gene is induced by systemin and MeJ (Fig. 6) and OGA and chitosan (data not shown), with MeJ producing the highest levels of CPI mRNA.

Salicylic acid (SA) is a potent inhibitor of the wound-induction of inhibitors I and II in tomato leaves (Doares et al., 1995) was assayed for its ability to inhibit the accumulation of CPI mRNA in response to wounding. In Fig. 5C, SA can be seen to be a powerful inhibitor of the wound-inducible synthesis of CPI mRNA. This indicates that the cross-talk between SA and the systemin signaling pathway that regulates proteinase inhibitor I and II synthesis (Doares et al., 1995) is similarily regulating CPI synthesis.

The reason or reasons for the lack of detection of the tomato CPI protein in leaves (Martineau et al.) is not clear, but it was not likely due to the assays, since antibodies used in the previous studies could detect the CPI protein at very low levels. Green and Ryan (1973) have shown that growing tomato plants under low light or low temperatures can suppress the wound response, which may have contributed to the lack of wound inducibility in the previous studies, in which conditions of plant growth were not reported. The data presented here, however, establish through physical, enzymatic, and immunological data that the CPI protein is synthesized in tomato leaves in response to wounding in a manner consistent with its role as a defensive protein.

3. Experimental

3.1. Plant material

Tomato plants (*Lycopersicon esculentum* cv Castlemart) were grown in an environmental chamber with 17-h days of 300 μEinsteins/m/s of light at 28 °C and 7 h nights at 18 °C.

3.2. Enzyme inhibition assays

Analysis of CPAase activity was based on the procedure described by Folk and Schirmer (1963). Bovine CPAase (Sigma, St. Louis, MO) was dissolved in molarity to a final concentration of molarity. Seventeen μL of diluted enzyme corresponding to 1 μg CPA was added to the substrate solution (1 M hippuryl-L-phenylalanine (Sigma, St. Louis, MO) in 25 mM Tris–HCl, pH 7.5 with 0.5 M NaCl) to a final volume of 500 μL. Inhibition assays were performed incubating enzyme and inhibitor together for 3 min at 4 °C before adding the substrate. The mixture was immediately placed into a 500 μL quartz cuvette, with the absorbance increase at 262 nm recorded every 30 s at 25 °C using a Hewlett–Packard 8453 UV–Vis spectrophotometer.

3.3. Protein assays

Protein was assayed using the Bradford reagent (Sigma, St. Louis, MO) with bovine serum albumin as a standard.

3.4. Wounding and elicitor treatments

Wounding and elicitor treatments were performed with two-week-old tomato plants, except when otherwise indicated. Wounding was performed by crushing leaves with a hemostat across the main vein (Stratmann and Ryan, 1997). MeJ was applied to intact plants by placing 1 μL of the chemical on a cotton wick in a closed container for 24 h as described (Bergey and Ryan, 1999). Systemin (28 nM), oligogalacturonic acid (prepared as in Bishop et al., 1981) (250 μg/mL), and chitosan (100 μg/mL), each dissolved in 10 mM phosphate buffer (pH 6.5), were supplied to tomato plants for 1 h through their cut stems. The plants were excised with a razor blade by cutting the main stem just below the cotyledons. SA (1 mM) was supplied to the plants through the cut stems for 1 h and the plants were wounded on the lower leaves. CPI mRNA was detected by gel blot analysis 24 h after wounding.

3.5. Purification of tomato leaf CPI

Four-week-old tomato plants were sprayed initially with MeJ (125 μL of MeJ in 500 mL 0.05% [w/v] Triton X-100) and again 24 h later. The entire aerial parts of the plants were harvested 24 h after the second application. Tissues were frozen in liquid N_2 and ground to a fine powder using a mortar and pestle. Approximately 120 g of ground tissue was homogenized with 360 mL of

extraction buffer (84 mM citric acid, 32 mM Na_2HPO_4, and 15 mM mercaptoethanol, pH 2.8) in a blender, filtered through 2 layers of miracloth and clarified by centrifugation at 10,000*g* for 30 min at 4 °C. A total volume of 480 mL of clarified supernatant was obtained (crude extract) containing 159 mg protein. Tomato CPI was monitored measuring the inhibitory activity against CPAase as described above. By titration with CPAase, 651 ug of CPI protein was estimated to be in the crude extract.

Ammonium sulfate (35% saturation) was slowly added to the crude extract and the extract was stirred for 10 min at 4 °C. After centrifugation at 10,000*g* for 10 min at 4 °C, the precipitate was recovered and redissolved in 70 mL water with 15 mM mercaptoethanol. The solution was heated to 80 °C for 5 min, cooled in an ice bath, and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was dialyzed against water (below 6000 molecular weight retention), and lyophilized. The dry powder was resupended in distilled water (4 mL), adjusted to 80% ethanol by adding absolute ethanol, stirred gently for 15 min at room temperature, and then centrifuged at 10,000*g* for 10 min at 4 °C to remove debris. Ethanol was removed from the clear supernatant by dialysis for 8 h against distilled water, and the solution was lyophilized.

The lyophilized powder was dissolved in 50 mM sodium acetate and passed through a column (2×10 cm) of Sulfopropyl-Sephadex (Amersham Biosciences, Piscataway, NJ) equilibrated by washing with 50 mM sodium acetate, pH 5. Fractions containing the inhibitory activity eluted at the void volume (10 mL) and were desalted by dialysis. The dialyzed solution was applied to a 2×10 cm column of QAE–Sepharose (Pharmacia, Piscataway, NJ) and eluted as above. Again, all of the inhibitory activity was recovered in the fractions at void volume, yielding 35 μg CPI protein, based on titration with pure CPAase).

HPLC separation was performed in an analytical reversed-phase C18 column (218TP54, 5 μm, 4.6×250 mm, Vydac, Hesperia, CA). Seven μg of crude tomato CPI was loaded onto the column that had been equilibrated in 0.1% TFA/water. After 2 min, a gradient of 0–100% TFA/acetonitrile was performed over a 60 min time period at a flow rate of 1 mL/min with absorbance was monitored at 280 nm. Fractions (1 mL) were collected with the major absorbance peak at 27.6 min analyzed by MALDI-MS.

3.6. Mass spectroscopic analyses

MALDI spectra were obtained using a PerSeptive Biosystems Voyager time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm) (Framington, MA). α-Cyano-4-hydroxycinnamic acid was used as the matrix.

3.7. Amino acid sequence analyses

Amino acid sequencing from the N-terminus was performed on an Applied Biosystems (Foster City, CA) Procise Model 492 protein sequencer using Edman chemistry.

3.8. Isolation and sequencing of cDNA clones

Tomato *CPI* cDNA was amplified from a tomato 35S:PROSYS cDNA library using RT-PCR (Heitz et al., 1997). A specific primer 5′-GGTTATTGCTGCTCAAGATG-3′ was designed from the known tomato *CPI.* GenBank sequence (Accession No. X59282) in combination with the vector-based primer T7 (5′-GTAATACGACTCACTATAGGGC-3′; UniZap XR; Stratagene, La Jolla, CA).

The amplified cDNA fragment was identical to the tomato *CPI* oligonucleotide sequence deposited in the GenBank and was used to screen the library (Heitz et al., 1997) for other isoforms. Probes were radiolabeled using the DNA random priming method according to the manufacturer's instructions (Ambion, Austin, TX).

3.9. RNA blot analyses

Tomato leaves were collected at 0, 2, 4, 6, 8, 10 h after treatment, immediately frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted using the TRIzol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Fifteen μg of total RNA was separated in agarose gels, and RNA blot analysis was performed as previously described (Moura and Ryan, 2001). Images were obtained using the Phosphoimager and the Molecular Analyst Software (Bio-Rad, Hercules, CA). Gel blots were hybridized with *18S* rRNA cDNA probe as loading control. All experiments were repeated at least three times.

3.10. Immuno-blot analyses

Tomato leaves were harvested 24 h after treatments and ground using a mortar and pestle. The leaf juice was collected, heated to 80 °C and maintained at that temperature for 5 min, centrifuged at 14,000*g* for 5 min and the clear supernatant was stored at −80 °C. Proteins present in the supernatant were subjected to SDS/urea–PAGE. Following electrophoresis, the proteins were electrotransferred to polyvinylidenedifluoride (PVDF) membranes. Filters were blocked for 30 min at room temperature with 5% dry milk in TTBS (20 mM Tris–HCl pH 7.5, 500 mM NaCl, 0.05% Tween 20) and were incubated overnight with potato CPI antibody (1:1000 dilution in 2% dry milk TTBS). Protein blots were developed using an alkaline phosphatase conjugate substrate kit following the manufacturer's instructions

(Bio-RAD, Hercules, CA). Purified tomato fruit CPI ($M_r = 4$ kDa, Hass and Ryan, 1981) was used as a standard.

Rabbit anti-potato CPI was prepared as previously described (Ryan et al., 1974).

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