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# Differential expression of a chimeric CaMV-tomato proteinase Inhibitor I gene in leaves of transformed nightshade, tobacco and alfalfa plants

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## Abstract

The open reading frame and terminator region of a wound-inducible tomato Inhibitor I gene, regulated by the CaMV 35S promoter, was stably integrated into the genomes of nightshade (*Solanum nigrum*), tobacco (*Nicotiana tabacum*), and alfalfa (*Medicago sativa*), using an *Agrobacterium*-mediated transformation system. The expression of the foreign Inhibitor I gene in leaves of each species was studied at the mRNA and protein levels. The levels of Inhibitor I protein present in leaves of each species correlated with the levels of mRNA. The average levels of both mRNA and Inhibitor I protein were highest in leaves of transgenic nightshade plants (over 125 μg of Inhibitor I per g tissue), less in tobacco plants (about 75 μg/g tissue), and lowest in leaves of transgenic alfalfa plants (below 20 μg/g tissue). Inhibitor I protein was observed in all tissues throughout transgenic plant species, but inhibitor concentration per gram of tissue was 2–3 times higher in young developing leaf tissues and floral organs. The differences in the expression of the CaMV-tomato Inhibitor I gene among the different plant genera suggests that either the rate of transcription of the foreign gene or the rate of degradation of the nascent Inhibitor I mRNA varies among genera. Using electron microscopy techniques, the newly synthesized pre-pro-Inhibitor I protein was shown to be correctly processed and stored as a mature Inhibitor I protein within the central vacuoles of leaves of transgenic nightshade and alfalfa. The results of these experiments suggest that maximal expression of foreign proteinase inhibitor genes, and perhaps other foreign defense genes, may require gene constructs that are fashioned with promoters and terminators that allow maximum expression in the selected plant species.

## Introduction

Serine proteinase inhibitor proteins are common to many plant species [24] and are considered to be part of the arrays of chemicals that defend plants against predators and pathogens [25]. In addition to directly inhibiting the activity of proteolytic digestive enzymes of animals, the inhibitors can also interfere with the activation of proteases from their inactive proenzyme precursors [2]. The presence of trypsin inhibitors in animal guts can trigger a feedback mechanism that results in hypersecretion of digestive enzymes and a decrease in appetite, causing the depletion

and eventual loss of essential amino acids that can result in protein starvation [6, 7, 9, 25]. Previous studies have shown that tobacco plants expressing foreign proteinase inhibitor genes, regulated by the CaMV 35S promoter, can exhibit increased resistances toward lepidopteran insects [13, 15].

Comparative studies on the accumulation and distribution of a single plant protein within the tissues of different transgenic plant species, under the regulation of the CaMV-35S promoter, are rare, and little is known concerning the levels of expression of any specific proteinase inhibitor gene driven by the CaMV 35S in different plant genera and families. To study differential expression of a tomato Inhibitor I gene in two genera of Solanaceae and one genus of Fabaceae, these plants were transformed with a fused CaMV 35S-tomato Inhibitor I gene with its own terminator. The levels of tissue-specific expression at the mRNA and protein levels in transformants of each genera were observed. In the study reported here, the tissue-specific pattern of the 35S-tomato Inhibitor I gene expression was found to be similar among the three plant genera, but the levels of expression of the chimeric Inhibitor I gene, monitored by both mRNA and protein, were strikingly different.

## Materials and methods

### *Construction of the CaMV-Tomato Inhibitor I gene*

The coding region of a wound-inducible tomato chymotrypsin Inhibitor I gene and terminator was fused with the CaMV 35S promoter as described previously [15]. The physical map of this chimeric gene and details on its construction have been reported.

### *Preparation of leaf extracts*

Leaf extracts were prepared by homogenizing ca. 10 g of leaves from wild-type and transgenic plants [18] in a Sorvall omnimixer in 20 ml of ice-cold 10 mM sodium citrate buffer containing 5 g/l of sodium hydrosulfite (1 g of tissue per 2 ml of buffer). The extracts were poured through 4 layers of cheesecloth and centrifuged at $10\,000 \times g$ for 20 min at 4 °C. The supernatants were filtered through Whatman No. 4 paper and frozen at −70 °C. After thawing, the extracts were centrifuged at $15\,000 \times g$ for 15 min at 4 °C. The clear supernatants were brought to 80% saturation by slow addition of solid ammonium sulfate, stirred for 30 min at 4 °C and cooled on ice for another 30 min with stirring. The precipitates were recovered by centrifugation at $15\,000 \times g$ for 15 minutes at 0 °C. The pellet was resuspended in 20 ml distilled water, dialyzed exhaustively against 50 mM ammonium hydrogen carbonate at 4 °C for 24 h, lyophilized, and resuspended in 2 ml distilled water or 0.1 M KCl, 10 mM Tris-HCl pH 8.1. A 1/10 w/v of Dowex-1 resin, equilibrated to pH 8.1 with 0.1 M KCl, 10 mM Tris-HCl buffer, was added to the supernatant, to eliminate phenolics. Protein extracts were concentrated by vacuum evaporation to a volume of 0.1–0.5 ml (50–100 μg of Inhibitor I protein per ml) and stored at −20 °C. Protein concentrations were determined by the method of Bradford [5].

### *Immunological assays*

Inhibitor I protein was quantified in tissue extracts using an immunoradial diffusion assay [22, 23, 29]. Juice was expressed from different tissue samples taken from control (wild type) and transgenic plants. Inhibitor I concentration was expressed in either μg per g of plant tissue, or μg per mg of total soluble protein in the tissue.

### *Proteinase inhibition assays*

Chymotrypsin inhibitor activities present in leaf extracts were measured spectrophotometrically by the method of Hummel [14], using benzoyl-*L*-tyrosine ethyl ester as substrate. The active enzyme in each preparation was determined by active-site titration as previously described [15].

*Protein immuno-blotting*

Protein extracts were analyzed by electrophoresis in polyacrylamide gels (13%) in the presence of 0.1% SDS and 8 M urea [28]. The protein bands were transferred from the gels to nitrocellulose paper (0.2 μm), using an electroblotter Hoeffer model TE-50 at 0.3 mA. The Inhibitor I protein was immuno-detected using polyclonal potato Inhibitor I antibodies and anti-IgG second antibodies conjugated with alkaline phosphatase [22].

*RNA isolation and blot hybridization*

Total RNA was extracted from young leaves of flowering wild-type and transgenic plants. As a positive control, total RNA was also isolated from wounded young tomato plants (8 h after wounding) [10]. The leaves were frozen in liquid nitrogen, ground to find powder and immediately phenol-extracted and ethanol-precipitated, as previously described [16]. The final yield of total RNA was 250–500 μg per each 100 mg of leaf tissue. Aliquots of total RNA were electrophoresed in formaldehyde-1.4% agarose gels and transferred to nitrocellulose filters, according to Maniatis *et al.* [17]. Filters were hybridized to 0.1 μg of nick-translated $^{32}$P-labelled cDNA fragments, isolated after digestion of pTI-24 [11] with *Hin*d III and *Eco* RI. After washing under highly stringent conditions, the filters were exposed to films for 2–7 days at −70 °C to detect the Inhibitor I mRNA.

*Immunocytochemical analyses*

Tissue samples (1mm × 5 mm) were taken from young (half-expanded) leaves of 10-week-old control and transgenic nightshade and alfalfa plants, fixed in 2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde in 50 mM PIPES pH 7.2, and incubated overnight at room temperature. Tissues were washed three times with PIPES buffer pH 7.2, dehydrated with 10% graded series of ethanol (10 min each, starting at 30% and going up to 100%), and embedded in L.R. White resin (Ted Pella, Inc.), in a stepwise manner in combination with ethanol (1:4, 1:2, 1:1, 2:1, 4:1, pure two times, resin to ethanol; 16–20 h each). Resin was polymerized at 65 °C for 17 h within gelatin capsules. Thick (0.5 μm) and ultrathin sections (0.06 – 0.1 μm) obtained using glass knives were mounted respectively on gelatin-coated glass slides or 200 mesh nickel grids. Sections were bathed for 1 h in TBST buffer (10 mM Tris, 500 mM NaCl, 0.3% Tween 20 pH 7.2) containing 1–2% (w/v) bovine serum albumin (BSA), and then incubated for 3 h with proteinase Inhibitor I or II antisera [22], or with preimmune serum (as a control) diluted with TBST-BSA (1:200). Grids and slides were washed six times with TBST-BSA and then incubated one hour with 10 nm diameter protein-A gold diluted (1:50) with TBST-BSA, followed by washes with TBST-BSA, TBST alone, and distilled water, two times each. Ultrathin sections for transmission electron microscope (TEM) analysis were post-stained with 2% aqueous uranyl acetate, followed by 1% Reynolds' lead citrate, 15–30 min each, and finally examined and photographed using a Hitachi HS-8 electron microscope operated at 50 KV.

*Tobacco transformation*

Leaf segments taken from one-month-old *in vitro* grown seedlings of *Nicotiana tabacum* var. Xanthi were wounded with a syringe needle and co-cultivated for three days with the diluted culture ($5 \times 10^8$ cells/ml) of *Agrobacterium*. The explants were rinsed three times with liquid MS medium [19], blot-dried with sterile filter paper, and plated on selective medium containing the MS salts, B5 vitamins [8], m-inositol (100 mg/l), sucrose (3%), BAP (1 mg/l), IAA (0.1 mg/l), adenine (40 mg/l), MES (0.5 g/l), cefotaxime (250 mg/l), carbenicillin (500 mg/l), kanamycin (200 mg/l), and phytoagar (8 g/l). The explants were subcultured to fresh medium after three days and then subcultured at weekly intervals. After 10 days, they were transferred to the same media lacking IAA. Fi-

nally, shoots (3–4 cm high) were transferred to MS medium without IAA, containing the antibiotics vancomycin (500 mg/l) and kanamycin (50 mg/l). Thirty primary transformants expressing the tomato Inhibitor I gene in leaves were identified immunologically.

*Nightshade transformation*

Detached cotyledons from *in vitro* germinated 8-day-old nightshade seedlings (*Solanum nigrum*) were treated in a similar way as tobacco explants, and incubated in the same media lacking IAA. Shoots were also rooted in the same medium as tobacco, but supplemented with NAA (0.1 mg/l) Twenty primary transformants were identified immunologically that were expressing the tomato Inhibitor I gene in leaves.

*Alfalfa transformation*

Cotyledons from *in vitro* germinated 10-day-old alfalfa seedlings (*Medicago sativa,* clones 93–21 and 93–13, a generous gift from D.H. Mitten, Plant Genetics, Inc.) were pre-conditioned by incubating them for two days on in tobacco feeder plates [20]. The cotyledons were wounded with a sterile syringe needle, co-cultivated for 30 min with the diluted culture of *Agrobacterium* ($5 \times 10^8$ cells/ml), blotted with sterile filter paper, and incubated for two days in the feeder plates. Thereafter, the explants were washed three times with liquid MS medium. The last rinse contained cefotaxime (500 mg/ml). The tissues were blotted with sterile filter paper and planted in selective (SH) medium consisting of the SH salts [26], m-inositol (100 mg/l), sucrose (3%), NAA (4.6 mg/l), KIN (2.15 mg/l), cefotaxime (250 mg/l), carbenicillin (500 mg/l), and kanamycin (50 mg/l). When the first calli appeared (after 3–4 weeks), they were transferred to the same medium, containing 2,4-D (11 mg/l), and KIN (1.07 mg/l) as the unique plant hormones, and incubated for three days to induce somatic embryos [27, 30]. Embryos were transferred to a third SH medium, modified by introducing $NaH_2PO_4$ (358.8 mg/l), and $(NH_4)_2S0_4$ (1.321 g/l), to provide a $NH_4^+$ concentration of 20 mM, to favor development of embryos [30]. This medium was also supplemented with casein hydrolysate (2 g/l) and the respective antibiotics, but did not contain any hormones. Finally, the well differentiated embryos were transferred to the same modified SH medium, but containing $GA_3$ (0.1 mg/l), for the complete development of embryos into plants. Twenty-five primary transformants were identified immunologically that were expressing the tomato Inhibitor I gene.

## Results and discussion

Constitutive expression of the chimeric 35S-Inhibitor I gene, pJN-3, had previously been demonstrated in transformed tobacco plants [15], with Inhibitor I levels reaching over 100 $\mu$g protein per gram leaf tissue.

Following transformation of nightshade, tobacco and alfalfa plants with pJN-3, kanamycin-resistant plants were regenerated from calli of the three species (25 nightshade, 34 tobacco and 29 alfalfa plants) and the leaves of each plant were assayed for levels of tomato Inhibitor I protein. More than 80% of regenerated plants from each species were found to express the foreign tomato Inhibitor I gene in leaves.

Tomato Inhibitor I protein was detected in all tissues of the transgenic tobacco, nightshade and alfalfa plants, but the levels were tissue-specific. The highest levels of Inhibitor I per g/tissue were found in leaves and reproductive tissues (Fig. 1). However, roots in all the three transgenic plant species exhibited the highest percentage of Inhibitor I, based on total soluble protein (up to 5% in transgenic nightshade plants). Reproductive organs also showed a high percentage of Inhibitor I in its soluble protein (especially in the case of transgenic tobacco), whereas, upper younger leaves had the lowest percentage of protein (Fig. 1). A higher percentage of Inhibitor I protein (about 1% of the soluble protein on average) was also found in apical tissue of younger to-

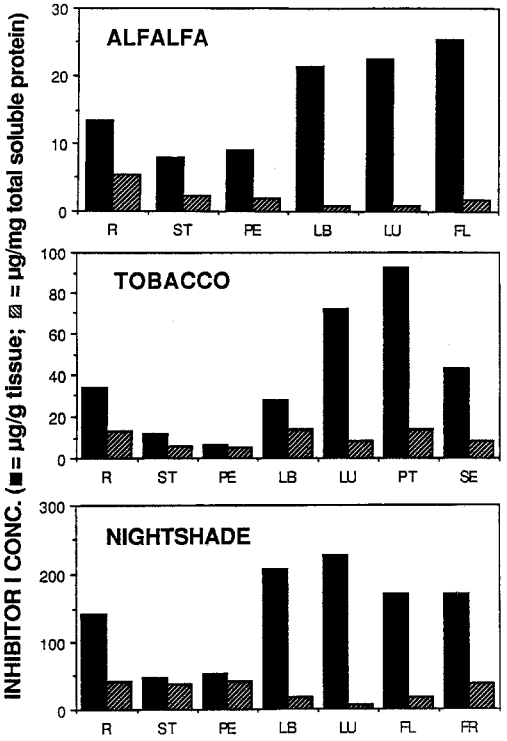



*Fig. 1.* Inhibitor I protein accumulation in tissues from different transgenic plant species. Values represent the average of three different initial transformants for each species. R, roots; ST, stem; PE, petiole; LB, basal leaves; LU, upper leaves; SE, sepals; PT, petals; FL, whole flowers; FR, fruits.

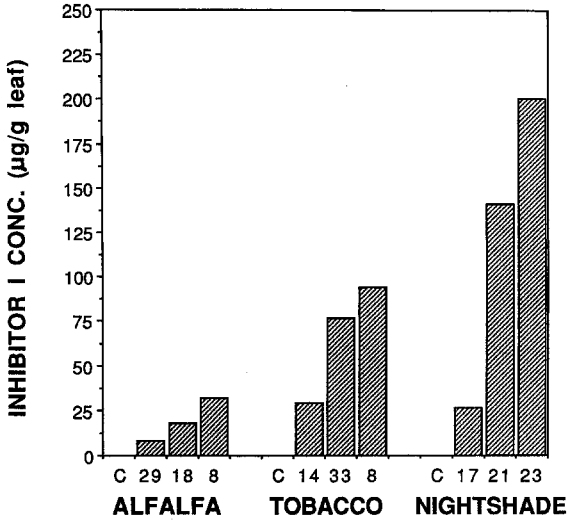



*Fig. 2.* Comparisons of the accumulation of tomato Inhibitor I protein in leaves of different plant species transformed with a CaMV 35S-tomato Inhibitor I chimeric gene. In each comparison C is the untransformed control. Each bar represents the levels of Inhibitor I in a selected transformant, designated by a number at the base. The lowest and highest expressing transformants selected from over 20 individually transformed plants are presented together with a transformant that represents the average of each group.

bacco leaves, compared with basal tissues from the same leaves (about 0.5% on average). The unusually long half-lives of the proteinase inhibitors [12] probably account for the higher Inhibitor I protein levels in older tissues, based on the total soluble protein. However, although the 35S promoter is expressed in all plant cell types [21], it has also been observed that this viral promoter is more active in some tissues or cell types (e.g., roots, stems and petioles) than in others [3, 4, 21].

Large variations in Inhibitor I levels (µg/g fresh tissue) were observed among the transformed plants within each species, and the average levels of foreign Inhibitor I protein also differed strikingly among the three transformed species. In Fig. 2 are shown the lowest and highest expressing transformants from each species along with a transformant that represented the average. The highest average levels of the tomato Inhibitor I were observed in leaves of transgenic nightshade plants, whereas the lowest levels of the inhibitor protein were found in transgenic alfalfa plants, with tobacco being intermediate (Fig. 2). The average levels of Inhibitor I protein that were found in leaves of transgenic nightshade plants (125 µg/g leaf tissue), were about 2 and 5 times higher than the highest levels observed in leaves

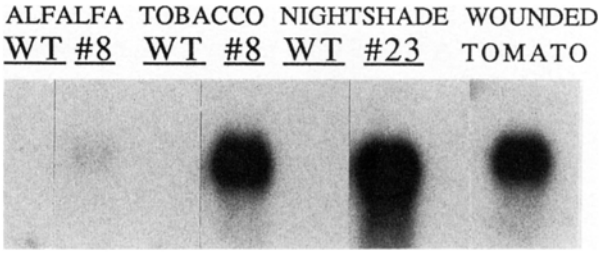



*Fig. 3.* Northern blot analysis of total RNA extracts obtained from wild type (WT) and transgenic alfalfa, tobacco and nightshade plants compared with native wound-inducible tomato Inhibitor I mRNA.

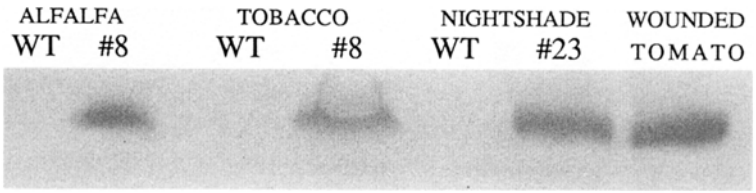



*Fig. 4.* Immunoblot detection of proteinase Inhibitor I protein in leaf extracts from transgenic plants. Leaf extracts (20 $\mu$l) from wild type (WT) and transgenic plants were separated by electrophoresis and the proteins were blotted and visualized with anti-Inhibitor I serum. The blots are compared with wound-inducible tomato Inhibitor I protein.

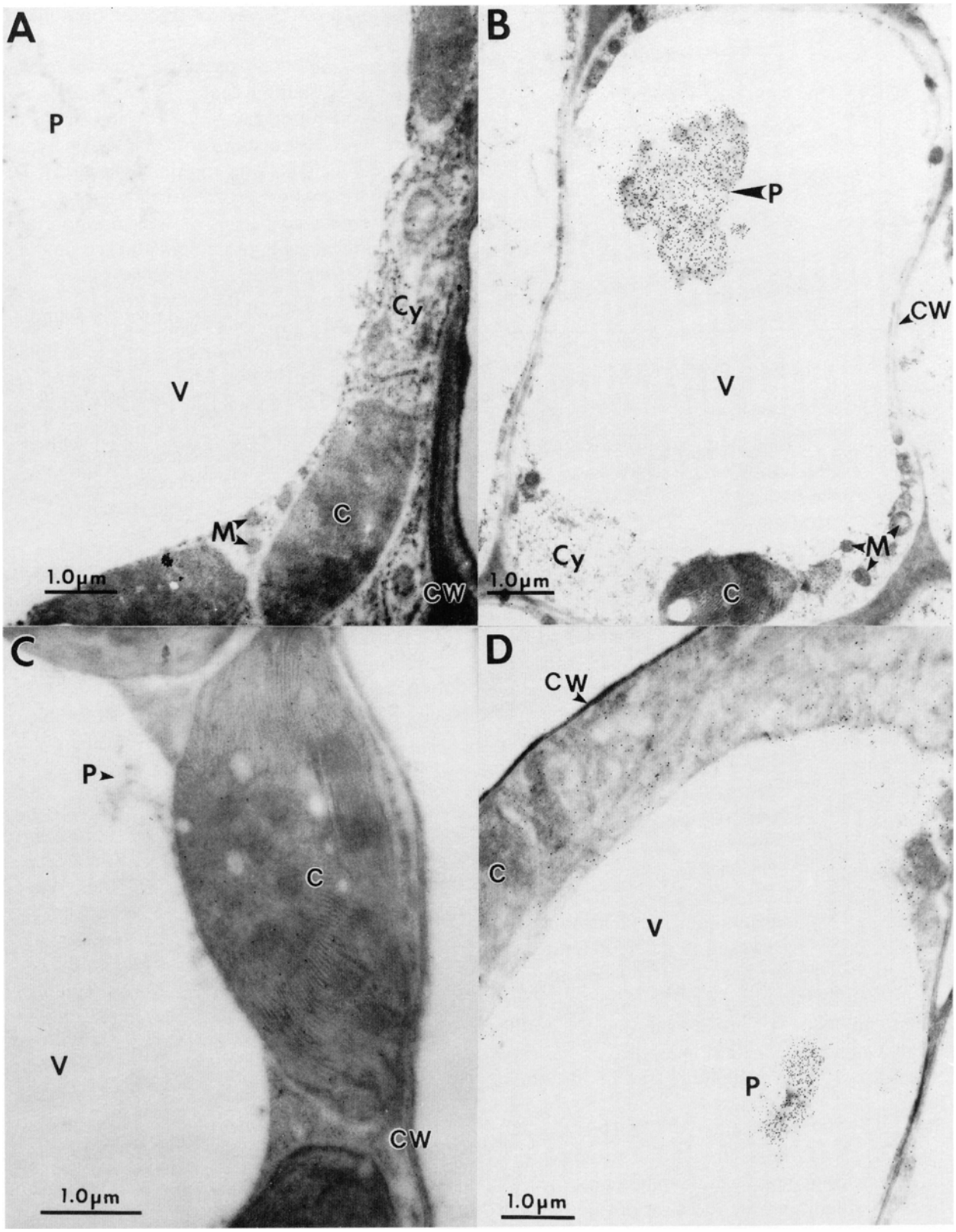
of transgenic tobacco and alfalfa plants, respectively.

Northern analyses of total RNA showed a good agreement between the quantity of message produced and the amount of Inhibitor I protein present in leaves of the three transgenic plant species (Fig. 3). In wild-type tomato leaves, Inhibitor I mRNA levels had previously been shown to correlate with the levels of nascent Inhibitor I protein in response to wounding [10]. In leaves of unwounded transgenic nightshade, tobacco and alfalfa, only one RNA band was found to hybridize with the tomato Inhibitor I cDNA probe. Untransformed control plants did not exhibit the Inhibitor I mRNA in leaves of unwounded plants. The mRNA species co-migrated exactly with the Inhibitor I mRNA produced in tomato plants in response to wounding. This indicated that correct processing (i.e., splicing) of the Inhibitor I initial transcript occurred in cells of all three transgenic species.

Western blot analysis of protein extracts isolated from control (wild-type) and transgenic plant tissues revealed the presence of Inhibitor I protein only in tissues of transgenic plants (Fig. 4). Thus, both northern analyses of Inhibitor I mRNA (Fig. 3) and protein immunoblots of Inhibitor I protein (Fig. 4) in leaf protein extracts obtained from leaves of all three transgenic species indicated that the nascent tomato Inhibitor I mRNA and protein were processed to mature forms identical in size and immunological reactivity to the mature mRNA and proteins found in leaf-cell vacuoles of wounded tomato plants. The amino acid sequence analysis of Inhibitor I protein purified from transgenic nightshade fruits (data not shown), confirmed that the first 40 N-terminal amino acids corresponded with the known amino acid sequence of tomato Inhibitor I protein [11]. Additionally, the tomato Inhibitor I protein that accumulated in leaf cells of both transgenic nightshade and alfalfa plants was found in the vacuoles (Fig. 5), as in wounded, wild-type tomato. The amount of gold-labelling of proteinase inhibitors within the cell vacuoles of all transgenic plant species was consistent with the levels of inhibitor protein previously detected within the leaves (Fig. 2). The nascent tomato inhibitor I protein was therefore being correctly processed and compartmentalized in the cells of nightshade and alfalfa plants.

The tomato Inhibitor I protein present in extracts of the leaves of transgenic plants retained its ability to inhibit chymotrypsin with a similar kinetic profile as the natural inhibitor protein present in tomato (Fig. 6), confirming that the foreign Inhibitor I protein retained its antiproteinase activities when present in the foreign environments. The levels of the chymotrypsin inhibitory activities in nightshade and tobacco reflected accurately the levels of Inhibitor I proteins that had accumulated in the leaves, determined by immu-

*Fig. 5.* Electron micrographs showing immuno-gold labelling of Inhibitor I protein in leaf sections of transgenic nightshade (A–B) and alfalfa (C–D) plants. Most of the label is associated with material in the vacuole. A and C are control sections treated with pre-immune serum, and B and D were treated with anti-inhibitor I serum. C, chloroplast; M, mitochondrion; V, vacuole; V, vacuole; Cy, cytoplasm; CW, cell wall; P, proteinase Inhibitor I protein. A, × 10400; B, × 9960; C, × 14800; D, × 10770.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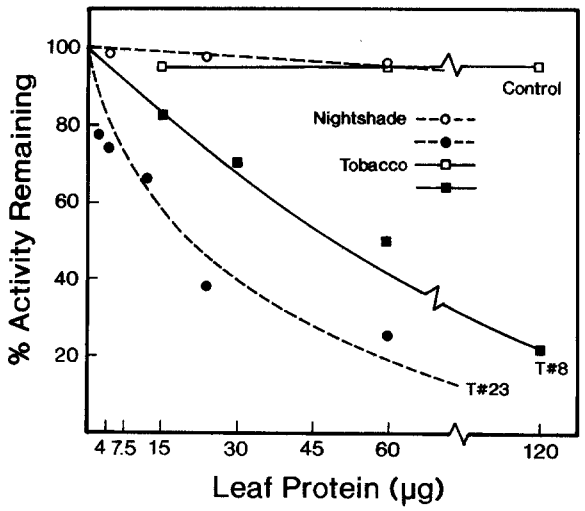


*Fig. 6.* Inhibition of chymotrypsin by leaf extracts from control untransformed and transgenic nightshade (T#23) and tobacco (T#8) plants accumulating a chymotrypsin inhibitor I protein. Chymotrypsin activity was assayed against benzoyl-*L*-tyrosine ethyl ester.

nological assays. Inhibitory activity in alfalfa extracts was so low that accurate enzymic assays were not possible.

The differential expression of the CaMV-tomato Inhibitor I gene in nightshade, tobacco and alfalfa plants reflected the evolutionary relationships of these three genera to tomato plants. Nightshade, the closest relative to tomato of the three genera, expressed the highest levels of Inhibitor I, whereas tobacco, a more distant relative in the Solanaceae family, expressed less. Alfalfa, from the distantly related Fabaceae family, expressed the gene very weakly. The expression in leaves of each species was reflected in the levels of both mRNA and Inhibitor I protein. Whether the differences are a result of differential rates of transcription or in rates of mRNA degradation must await further analyses. The 3′ region of the tomato Inhibitor II gene, a gene that is coordinately wound induced in tomato leaves, appears to enhance mRNA stability [1], and both Inhibitor I and II mRNAs exhibit relatively long half lives (ca. 12 h) after synthesis [10]. Thus, the different levels of tomato Inhibitor I mRNA in the three transgenic plant genera may reflect the differential stability of the message in the different genera, and not the rates of transcription of the gene.

Protease inhibitors, as single gene products with defensive antinutrient activities against insects and microorganisms, have been of considerable interest to transform crop plants to increase resistances against pests and pathogens [25]. To successfully employ proteinase inhibitor genes, and other defensive genes as well, to important crop genera, specific promoters and terminators that can strongly express the genes in specific tissues will be crucial. It is clear from this study that the CaMV promoter regulating the tomato Inhibitor I gene is not in itself suitable to fulfill this need among dicots. More fundamental research is needed to establish a reliable information base concerning the factors that govern the maximal expression of proteinase inhibitors genes and other useful genes in different plant species, and in particular in important crop plants.

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## References

1. An G, Mitra A, Choi HK, Costa MA, An K, Thornburg RW, Ryan CA: Functional analysis of the 3′ control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell 1: 115–122 (1989).
2. Applebaum SW: Biochemistry of digestion. In: Kerkut GA, Gilbert LI (eds.) Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 4, pp. 279–311. Pergamon, New York (1985).
3. Benfey PN, Chua NH: Regulated genes in transgenic plants. Science 244: 174–181 (1989).
4. Benfey PN, Chua NH: The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250: 959–966 (1990).

5. Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254 (1976).
6. Broadway RM, Duffey SS: Plant proteinase inhibitors: Mechanism of action and effect on the growth and digestive physiology of larval *Heliotis zea* and *Spodoptera exigua*. J Insect Physiol 32: 827–833 (1986).
7. Broadway RM, Duffey SS, Pearce G, Ryan, CA: Plant proteinase inhibitors: A defense against herbivorous insects? Entomol Exp Appl 41: 33–38 (1986).
8. Gamborg OL, Miller RA, Ojima K: Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158 (1968).
9. Gatehouse AMR, Boulter D: Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*) and other legumes on development of the Bruchid beetle *Callosobruchus maculatus*. J Sci Food Agric 34: 345–350 (1983).
10. Graham JS, Hall G, Pearce G, Ryan CA: Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta 169: 399–405 (1986).
11. Graham JS, Pearce G, Merryweather J, Titany K, Ericsson L, Ryan CA: Wound-induced proteinase inhibitors from tomato leaves. I. The cDNA deduced primary structure of pre-inhibitor I and its post-translational processing. J Biol Chem 260: 6550–6555 (1985).
12. Gustafson G, Ryan CA: Specificity of protein turnover in tomato leaves: Accumulation of proteinase inhibitors, induced with the wound hormone, PIIF. J Biol Chem 251, 7004–7010 (1976).
13. Hilder VA, Gatehouse AMR, Sheerman SE, Barker RF, Boulter D: A novel mechanism of insect resistance engineered into tobacco. Nature 330: 160–163 (1987).
14. Hummel BCW: A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. Can J Biochem Physiol 37: 1393 (1959).
15. Johnson R, Narvaez J, An G, Ryan CA: Expression of proteinase inhibitor I and II in transgenic tobacco plants: Effects on natural defense against *Manduca sexta* larvae. Proc Natl Acad Sci USA 86: 9871–9875 (1989).
16. Lawton MA, Dixon RA, Hahlbrock K, Lamb CJ: Elicitor induction of mRNA activity: Rapid effects of elicitor on phenylalanine ammonia-lyase and chalcone synthase mRNA activities in bean cells. Eur J Biochem 130: 131–139 (1983).
17. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
18. Melville JC, Ryan CA: Chymotrypsin inhibitor I from potatoes. J Biol Chem 247: 3445–3453 (1972).
19. Murashige T, Skoog F: A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15: 473–495 (1962).
20. Narváez-Vásquez J: Expression of proteinase inhibitor genes in transgenic plants: Effects on insect resistance; levels of expression in four plant species; and cellular compartmentalization. Ph.D. thesis, Washington State University, Pullman, WA, USA (1991).
21. Odell JT, Nagy F, Chua N-H: Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812 (1985).
22. Pearce G, Ryan CA, Lilegren D: Proteinase inhibitor I and II in fruit of wild tomato species: Transient components of a mechanism for defense and seed dispersal. Planta 175: 527–531 (1988).
23. Ryan CA: Quantitative determination of soluble cellular proteins by radial diffusion in agar gels containing antibodies. Anal Biochem 19: 434–440 (1967).
24. Ryan CA: Proteinase inhibitors. In: The Biochemistry of Plants, vol. 6, pp. 351–370. Academic Press, New York (1981).
25. Ryan CA: Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopath 28: 425–449 (1990).
26. Shenk RU, Hildebrant AC: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous cell cultures. Can J Bot 50: 199–204 (1972).
27. Stuart DA, Nelsen J, Strickland SG, Nichol JW: Factors affecting developmental processes in alfalfa cell cultures. In: Henke R, Huges KW, Konstantin MP, Hollaender A (eds) Tissue Culture in Forestry and Agriculture, pp. 59–73. Plenum, New York (1985).
28. Swank RT, Munkres KD: Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal Biochem 39: 462–477 (1971).
29. Trautman R, Cowan KM, Wagner GG: Data processing for radial immunodiffusion. Immunochemistry 8: 901–916 (1971).
30. Walker KA, Sato SJ: Morphogenesis in callus tissue of *Medicago sativa*: The role of ammonium ion in somatic embryogenesis. Plant Cell Tissue Organ Culture 1: 109–122 (1981).