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Expression of proteinase inhibitors I and II in transgenic tobacco plants: Effects on natural defense against *Manduca sexta* larvae

(plant defense/potato/tomato)

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ABSTRACT **Genes containing the cauliflower mosaic virus 35S promoter fused to open reading frames coding for tomato proteinase inhibitor I, tomato inhibitor II, and potato inhibitor II were expressed in transgenic tobacco plants. Inhibitor I and II proteins were identified by immunoblotting and quantified by immunoradial diffusion. Both inhibitors exhibited the molecular weights found for the native proteins in their natural environments. Extracts of leaves from transformed plants contained inhibitory activities against trypsin and chymotrypsin that reflected the levels of inhibitor I or II protein present. The results demonstrate that in tobacco leaves the introns of both inhibitor I and inhibitor II genes were excised correctly and that pre and prepro inhibitor I and II proteins were correctly processed. Growth of *Manduca sexta* larvae (tobacco hornworms) feeding on leaves of transgenic plants containing inhibitor II, a powerful inhibitor of both trypsin and chymotrypsin, was significantly retarded, compared to growth of larvae fed untransformed leaves. Levels of inhibitor II protein as low as 50 μg/g of tissue moderately affected larval growth, whereas levels above 100 μg/g severely reduced growth. The presence of tomato inhibitor I protein, a potent inhibitor of chymotrypsin but a weak inhibitor of trypsin, in transgenic tobacco leaves had little effect on the growth of the larvae. These experiments indicated that trypsin inhibitory activity, but not chymotrypsin inhibitory activity, was mainly responsible for the inhibition of larval growth.**

Potato and tomato plants contain two small multigene families that code for two powerful inhibitors of serine proteinases, called inhibitor I (monomer M_r 8100) and inhibitor II (monomer M_r 12,300) (1). Inhibitor I is an inhibitor of chymotrypsin that only weakly inhibits trypsin at its single reactive site (1), whereas inhibitor II contains two reactive sites, one of which inhibits trypsin and the other of which inhibits chymotrypsin (1). Members of both gene families are expressed in leaves in response to chewing insects or other severe mechanical damage (2). Both inhibitors are synthesized as precursors and undergo posttranslational modification (3–5) to form the mature proteins, which are sequestered in the vacuole (6). These inhibitors are thought to help defend the plant, by reducing the digestibility and nutritional quality of the leaves, against insect predators (7). Both cDNAs (4, 5) and genes (8, 9) that encode inhibitors I and II have been isolated and characterized. These are now being employed to further investigate the role of proteinase inhibitors in plant defense.

It was previously shown (10) that transformation of tobacco plants with a gene encoding a cowpea trypsin inhibitor was able to confer increased resistance against predation by *Heliothis virescens* larvae. In order to assess the potential of inhibitor I and inhibitor II for increasing the natural defenses of crop plants through transformation, genes encoding these inhibitors were stably introduced into tobacco plants. *Manduca sexta* larvae (tobacco hornworms) were fed on leaves of the transgenic plants. We herein report the differential effects of the two inhibitor families on larval growth.

MATERIALS AND METHODS

Plasmid Construction. Plasmids were constructed by standard recombinant DNA techniques (11). pJN1 was constructed by cloning a 2.5-kilobase-pair (kbp) *Hgi* I–*Sal* I fragment from TPI-I (8), containing the coding region and terminator of a tomato inhibitor I gene, into the *Pst* I and *Sal* I sites of pGA617 (12). This latter plasmid was derived from pUC19 by converting the *Ssp* I site located in the promoter region of the β-lactamase (*bla*) gene to a multiple cloning site (*Kpn* I–*Xho* I–*Stu* I). The 600-bp fragment separating the two multiple cloning sites in pJN1 was removed by digestion with *Sph* I and *Stu* I, creation of blunt ends with T4 DNA polymerase, and religation to give the plasmid pJN2. Plasmid pGA643 (13), which contains the cauliflower mosaic virus (CaMV) 35S promoter (nucleotides −418 to +1 of the strain CM1841) and the *Agrobacterium* T-DNA borders, was then digested with *Kpn* I and ligated with *Kpn* I-digested pJN2 to give pJN3. This plasmid contained the coding region for tomato inhibitor I under the control of the CaMV 35S promoter, terminated with the tomato inhibitor I terminator.

A 1.5-kbp *Pst* I–*Sca* I fragment from pRT8 (9), which contained the inhibitor II-K coding region and terminator, was ligated with *Pst* I/*Hin*cII-digested pUC13 to give pRJ9. Plasmid pRJ9 was digested with *Xba* I and *Eco*RI and ligated with *Xba* I/*Eco*RI-digested pGA643 to give pRJ13. This plasmid contained the coding region and terminator of potato inhibitor II-K under the control of the CaMV 35S promoter.

pGA875 was constructed by inserting the 700-bp *Spe* I–*Bam*HI fragment of pT2-47 (5), carrying a cDNA clone of tomato proteinase inhibitor II, into the *Xba* I and *Bgl* II sites of pGA643.

Transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) plants were obtained as described (14, 15).

Assays for Inhibitors I and II. Levels of inhibitor I and inhibitor II in leaves were assayed by immunological radial diffusion of juice from crushed leaves (16, 17). Rabbit anti-potato inhibitor I or anti-potato inhibitor II serum was employed, using purified inhibitors as standards. Total leaf protein was determined by the method of Bradford (18).

Preparation of Leaf Extracts. Leaf tissue (10 g from each plant) was frozen at −70°C and ground in a mortar and pestle with 10 ml of an extraction buffer containing 0.1% ascorbic acid, 0.1% cysteine, 0.5 M sucrose, and 0.1 M Tris (pH 7.0). All steps were carried out at 0–5°C. The resulting macerates were centrifuged at 10,000 × *g* for 10 min and the supernatants were again centrifuged at 10,000 × *g* for 10 min. The light-green supernatants were brought to 80% saturation with

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Abbreviation: CaMV, cauliflower mosaic virus.

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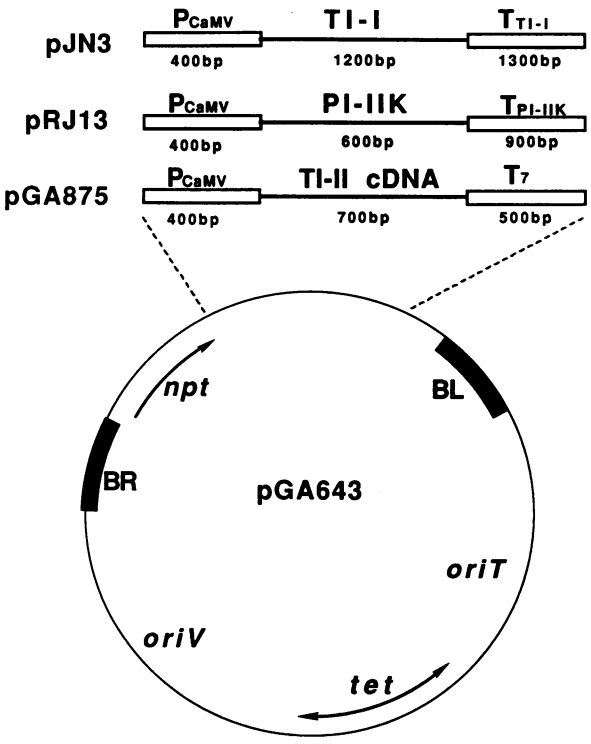

FIG. 1. Transformation plasmids pJN3, pRJ13, and pGA875. P_{CaMV}, CaMV 35S promoter; TI-I, coding region from a tomato inhibitor I gene; T_{TI-I}, tomato inhibitor I terminator; PI-IIK, coding region from the potato inhibitor II-K gene; TI-II cDNA, coding region from a tomato inhibitor II cDNA; T_7, terminator of the transcript-7 gene from *Agrobacterium tumefaciens* T-DNA; BR, T-DNA right border; BL, T-DNA left border; *npt*, a chimeric *nos–npt* (nopaline synthase–neomycin phosphotransferase) fusion that serves as a selectable marker in plants; *tet*, tetracycline-resistance gene; *oriV*, pRK2 origin of replication; *oriT*, pRK2 origin of conjugative transfer.

ammonium sulfate and allowed to stand at 0°C for 1 hr. The precipitates were collected by centrifugation at 2500 × *g* for 10 min and were taken up in 5 ml of 50 mM ammonium bicarbonate, lyophilized, and taken up in 1 ml of distilled water. Any remaining brown color was removed by addition of a small amount of Dowex-1 resin. After centrifugation at 10,000 × *g* for 10 min, the supernatants were concentrated to 3 mg of protein per ml. The clear extracts could then be fractionated by electrophoresis without interference from brown phenolic material.

Immunoblots. Leaf extract proteins were separated by electrophoresis in 13% acrylamide/0.6% *N,N'*-methylenebisacrylamide gels by the method of Swank and Munkres (19) in the presence of 0.1% SDS and 8 M urea. After electrophoresis, the proteins in the gels were transferred electrophoretically to nitrocellulose with a Hoefer model T-50 electroblotter. Proteins blotted onto the nitrocellulose were identified with anti-inhibitor I or anti-inhibitor II serum as described (20).

Proteinase Inhibitor Assays. Inhibitory activity against trypsin and chymotrypsin was determined by titrating the enzymes with increasing amounts of tobacco leaf extract (21). One microgram of trypsin or 3 μg of chymotrypsin was used in each assay. The substrate for trypsin was N^{α}-tosyl-L-arginine methyl ester and for chymotrypsin was N^{α}-benzoyl-L-tyrosine ethyl ester.

Insect Feeding Experiments. *M. sexta* eggs were obtained from Carolina Biological Supply. The eggs were soaked in 1% bleach for 15 min, thoroughly washed with deionized water, and dried on paper towels. The eggs were incubated at 25°C in sterile Petri dishes until hatching. The larvae were fed an artificial diet (22) before use in the experiments. For the feeding trials, excised tobacco leaves were placed on top of moist Whatman paper in sterile Petri dishes. First-instar larvae weighing about 3–4 mg each were placed in the dishes and incubated at 25°C. Each day, the larvae were transferred to new dishes containing fresh leaves.

RESULTS

Transformation of Tobacco Plants and Expression of Proteinase Inhibitors. Three plant transformation plasmids were constructed containing either the inhibitor I or the inhibitor II coding region from the genes or cDNA, under the control of the CaMV 35S promoter (Fig. 1). Plants were transformed with these plasmids, and leaves from the transgenic plants were assayed immunologically for the presence of inhibitors I and II. Transformed plants expressing at least 50 μg of inhibitor per gram of leaf tissue were selected. Several plants exhibited >200 μg/g of tissue. The levels of the two inhibitor proteins in these transgenic leaves were within the range that is routinely induced by wounding in leaves of tomato and potato plants (23, 24). The identity of the foreign potato and tomato proteinase inhibitors present in leaves of the transgenic plants was confirmed by immunoblotting analysis following electrophoretic separation in SDS and by assays for proteinase inhibitory activity.

Extracts from plants transformed with pJN3 contained a protein of M_r 8000 that comigrated with an inhibitor I standard and that reacted strongly in immunoblotting analysis when probed with anti-inhibitor I antibodies (Fig. 2*A*). Similarly, plants transformed with pRJ13 or pGA875 contained a protein of M_r 12,000 that comigrated with an inhibitor II standard and that bound strongly to anti-inhibitor II antibodies (Fig. 2*B*).

Leaf extracts from tobacco plants transformed with pRJ13 (inhibitor II gene) strongly inhibited both trypsin and chymotrypsin (Fig. 3). Extracts from leaves of untransformed tobacco plants contained little trypsin inhibitory activity and no detectable chymotrypsin inhibitory activity. Extracts from plants transformed with pGA875 (inhibitor II cDNA)

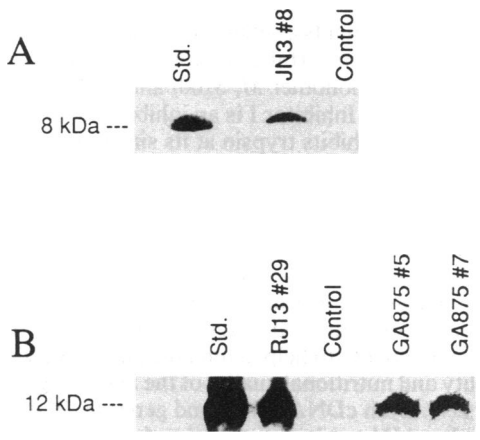

FIG. 2. Expression of inhibitor I (*A*) and inhibitor II (*B*) in transgenic tobacco plants. Leaf extracts were prepared as described in *Materials and Methods*, and proteins were separated by electrophoresis and transferred to nitrocellulose filters. (*A*) Gel lanes were loaded with 20 μl of leaf extract from control untransformed tobacco plants, 20 μl of leaf extract from transformant JN3-#8, or 5 μg of purified inhibitor I from potato tubers as a standard (Std.). The blotted proteins were probed with anti-inhibitor I antibodies. (*B*) Gel lanes were loaded with 5 μg of inhibitor II isolated from potato tubers as a standard (Std.) or with 20 μl of leaf extract from transformant RJ13-#29, GA875-#5, or GA875-#7 or from an untransformed control tobacco plant. The blotted proteins were probed with anti-inhibitor II antibodies.

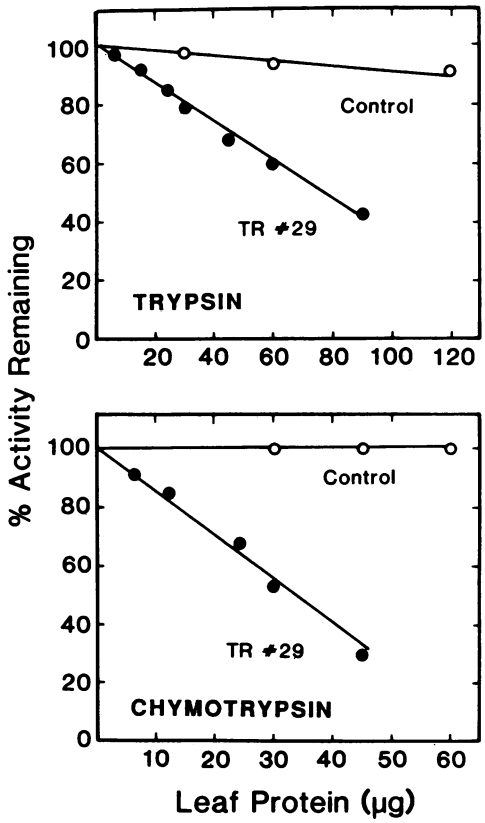

FIG. 3. Inhibition of trypsin (*Upper*) and chymotrypsin (*Lower*) by tobacco leaf extracts. Leaf extracts from a control untransformed plant (○) or from transformant (TR) RJ13-#29 (●) were incubated for 2 min with either trypsin or chymotrypsin. Trypsin activity against N^{α}-tosyl-L-arginine methyl ester or chymotrypsin activity against N^{α}-benzoyl-L-tyrosine ethyl ester was then assayed.

also inhibited both of these proteinases (data not shown). Therefore, the inhibitor II proteins expressed in the pRJ13 and pGA875 plants are active inhibitors of both trypsin and chymotrypsin, reflecting the specificities expected from the amino acid sequence at the reactive sites, i.e., Arg-Glu for the trypsin inhibitory site and Leu-Asn for the chymotrypsin inhibitory site in potato inhibitor II (9) and Arg-Glu (trypsin) and Phe-Asn (chymotrypsin) in tomato inhibitor II (4).

On the other hand, extracts from plants transformed with pJN3 (inhibitor I gene) strongly inhibited chymotrypsin (Fig. 4) but not trypsin, as predicted from the nucleotide sequence of the gene at the reactive-site residues (Leu-Asp) (8).

Effect of Inhibitors on Insect Growth. Larvae of *M. sexta* (tobacco hornworms) were fed a diet consisting only of leaves

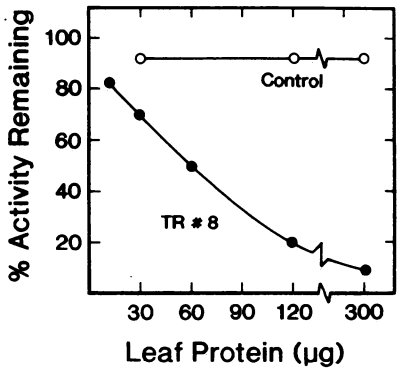

FIG. 4. Inhibition of chymotrypsin by tobacco leaf extracts. Leaf extracts were taken from a control untransformed plant (○) or from transformant (TR) JN3-#8 (●). Assays were as in Fig. 3.

from either control or transgenic tobacco plants to assess the effects of the presence of foreign proteinase inhibitors on insect growth. Larvae fed a diet of leaves from plants transformed with pRJ13 (inhibitor II gene) or pGA875 (inhibitor II cDNA) grew more slowly and consumed less leaf material than those fed on control leaves. Leaves from all of the transgenic plants that contained ≥100 μg of inhibitor per gram of tissue were very effective in inhibiting growth of the larvae. Data from two typical feeding experiments are presented in Fig. 5. Fig. 6 shows a typical experiment in which the larvae have consumed, in the same time period, much less leaf tissue from transformed plants than from the control plants.

Larvae grown on a diet of leaves (from plants transformed with pJN3) containing tomato inhibitor I at ≤130 μg/g grew at nearly the same rate as larvae that were fed control leaves. Data from a typical feeding experiment are shown in Fig. 7.

DISCUSSION

The expression in tobacco leaves of chimeric genes encoding inhibitors I and II and driven by the CaMV 35S promoter

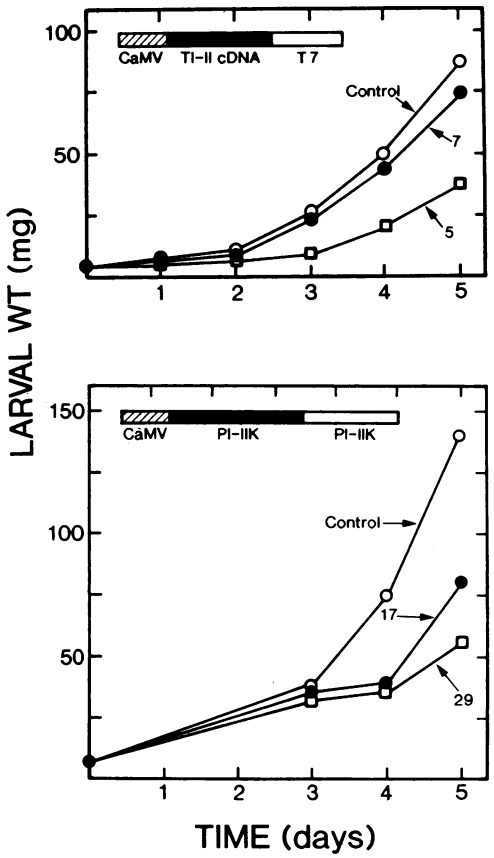

FIG. 5. Growth of tobacco hornworms on a diet of tobacco leaves. Larvae were hatched from eggs, and after 2 days of growth on an artificial diet, they were placed in Petri dishes containing the leaves for feeding trials. Constructs used to obtain transgenic plants are shown (see Fig. 1). (*Upper*) Larvae were fed leaves from a control plant, containing no inhibitor II (○), leaves from transformant GA875-#7, containing 52 μg of inhibitor II per gram (●), or leaves from transformant GA875-#5, containing 114 μg of inhibitor II per gram (□). The points shown represent the average weight of five larvae. (*Lower*) Larvae were fed leaves from a control plant, containing no inhibitor II (○), leaves from transformant RJ13-#17, containing 262 μg of inhibitor II per gram (●), or leaves from transformant RJ13-#29, containing 332 μg of inhibitor II per gram (□). The points shown represent the average weight of 24 larvae grown on the control leaves and 12 larvae grown on leaves from each of the two transgenic plants.

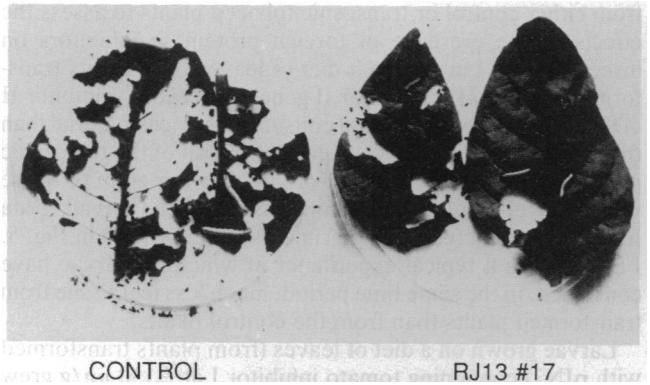

FIG. 6. Effect of tobacco hornworms on control tobacco leaves, containing no inhibitor II, or on leaves from transgenic plant RJ13-#17, containing 262 μg of inhibitor II per gram. Larvae were allowed to grow for 3 days on the tobacco leaves. They were then placed on fresh leaves and were photographed after 24 hr.

resulted in levels of these inhibitors equivalent to those found in leaves of wounded potato and tomato plants (50–300 μg/g of tissue) of many lines. The inhibitor I and II proteins that are expressed in these transgenic plants exhibit the same molecular weight (Fig. 2) and inhibitory activities (Fig. 3) as the inhibitors that are expressed in their normal environments. This indicates that tobacco cells are able to properly splice the introns present in the genes (8, 9) and to carry out the correct posttranslational modifications required to form the mature proteins. These processing events involve the removal of both pre and pro sequences from inhibitor I (3, 4) and removal of a pre sequence from inhibitor II (5).

The relatively high levels of the potato and tomato inhibitors that were synthesized and accumulated in transgenic tobacco leaves provided materials for a direct test of the defensive properties of the foreign inhibitor proteins in tobacco against a common predator, *M. sexta* (tobacco hornworm). This insect pest grows and develops on tobacco plants from the tiny, newly hatched larvae (≈2 mg) to very large larvae (>500 mg) within 8–10 days. During this time the hornworms consume increasingly large quantities of leaf material, severely damaging the host plants.

The presence of the foreign tomato or potato inhibitor II in tobacco leaves at levels >100 μg/g of tissue severely retarded growth of larvae that fed on them, compared to larvae fed on untransformed plants or on transformed plants that did not express the inhibitor genes (Fig. 5). At lower levels (≈50 μg/g of tissue) the larval growth was retarded to a lesser

PLANT #	INHIBITOR I μg/g tissue	# LARVAE	AVR. WT. (mg) START	FINISH
CONTROL #1	0	20	4	303
CONTROL #2	0	20	4	272
TR #14	26	20	4	316
TR #33	72	20	4	255
TR #8	122	20	4	255

FIG. 7. Growth of tobacco hornworms on tobacco leaves expressing a tomato inhibitor I gene. Larvae were fed on control leaves or on leaves from transgenic plant JN3-#8, #14, or #33 for 6 days.

degree than at the higher levels, suggesting that there is a dose-dependent relationship between the levels of inhibitor II and larval growth. This relationship has been confirmed in several experiments using leaves of transformed plants with various levels of inhibitor II as the total diets for *M. sexta*. Data from all of the experiments with inhibitor II protein in tobacco leaves demonstrated unequivocally that the presence of this inhibitor can have significant detrimental effects on the growth and development of *M. sexta* and that severity of the effects is related to the level of inhibitor protein present.

Larvae fed on tobacco leaves containing the tomato inhibitor I protein did not show the severe growth retardation found when larvae were fed leaves containing inhibitor II (Fig. 7). The reduction in larval growth due to the presence of inhibitor I was only about 15% in the most severe cases. The protein was properly expressed and processed as judged by electrophoresis and immunoblotting. The inhibitor I protein was also shown to be a potent chymotrypsin inhibitor. Thus, the presence of strong chymotrypsin inhibitory activity in tobacco leaves did not affect growth of tobacco hornworms in the same potent manner as the trypsin/chymotrypsin inhibitory activity of inhibitor II. This implies that the trypsin inhibitory activity present in inhibitor II, perhaps supplemented with its chymotrypsin inhibitory activity, is the primary cause of the detrimental effects on larval growth. Hilder *et al.* (10) have shown that a foreign proteinase inhibitor gene expressed in transgenic tobacco, having only trypsin inhibitor specificity, effectively reduced growth of *H. virescens* larvae.

Under natural conditions the plant's constitutive and induced defensive chemicals strongly influence the survival or demise of insect populations that feed exclusively on such plants. The effects of inhibitor II on the growth of *M. sexta* is another example of how the addition of a single defensive chemical can provide enhanced protection against an important predator. The severe delay in growth and development caused by inhibitor II, if occurring in a natural setting, would provide a much longer period in which the larvae would be subject to their natural predators and pathogens. Additionally, while not addressed in this study, the full normal pattern of development of the larvae may not be possible under conditions of nutritional stress, as imposed here.

Further questions concerning the relationship between these proteinase inhibitors and the growth, development, and survival of insect species can now be addressed in transgenic plants, where effects of single gene changes can be observed. Aspects of inhibitor specificity, insect proteinase specificity, physiological effects of proteinase–inhibitor complexes or feedback mechanisms in insect digestion, and the roles of combined or synergistic effects of defensive genes can be assessed. The results presented here indicate not only the feasibility of such studies but their necessity as well.

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