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Inhibition of Colorado potato beetle larvae by a locust proteinase inhibitor peptide expressed in potato

Mihály Kondrák[1], János Kutas[2], Borbála Szenthe[3], András Patthy[1,3], Zsófia Bánfalvi[1], Miklós Nádasy[2], László Gráf[3] & Bence Asbóth[1,*]
[1]*Institute of Plant Biology, Agricultural Biotechnology Center, 2101 Gödölló̋, P.O. Box 411, Hungary*
[2]*Entomological Laboratory of the Plant Protection, Institute of Georgikon Faculty of Agricultural Sciences of Veszprém University, 8360 Keszthely, Deák F. Str. 57, Hungary*
[3]*Department of Biochemistry, Eötvös Loránd University, 1117 Budapest, Pázmány Str. 1/C, Hungary*
**Author for correspondence (Fax: +36-28-526145; E-mail: asboth@abc.hu)*

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Abstract

The cDNA for a 73-mer peptide containing two locust serine proteinase inhibitors was cloned, fused to the constitutive CaMV35S promoter and introduced into potato by *Agrobacterium*-mediated transformation. From 23 independent transgenic lines, three with high mRNA level and proteinase inhibitory activity were propagated *in vitro* and transferred to pots. The peptide from the leaves was identified by its *N*-terminal sequence and by K_i values against chymotrypsin and trypsin. Colorado potato beetle larvae reared on transgenic plants grew slightly but significantly more slowly than those on control plants. This supports the notion that expression of multifunctional proteinase inhibitors of insect origin might be a good strategy to improve insect resistance in plants.

Introduction

Proteases control a number of important biological processes, such as digestion, blood coagulation, reproduction and the activation of enzymes and hormone precursors. Their inhibitors are often produced by plants to impede digestive enzymes of their pests. Introduction of heterologous protease inhibitors into crops has often increased their resistance towards pests. However, in general, pests become resistant towards inhibitors by synthesising enzymes that are insensitive to the inhibitors (Brunelle *et al.* 2004, and references therein).

Colorado potato beetles (*Leptinotarsa decemlineata*) are the most destructive pests of potato. The major proteolytic activity in their guts is a cysteine protease with some serine, metallo-, and carboxy-peptidases also contributing to digestion (Novillo *et al.* 1997). Research first concentrated on the inhibition of cysteine protease activity, but with little success (Brunelle *et al.* 2004, and references therein). Of inhibitors of other protease families used recently, equistatin, which inhibits both cysteine and aspartyl proteases (Strukelj *et al.* 2000), was very effective when included in an artificial diet (Gruden *et al.* 1998).

We have isolated and characterised two small serine protease inhibitor peptides (*Schistocerca gregaria* chymotrypsin inhibitor, SGCI, and *S. gregaria* trypsin inhibitor, SGTI) from the haemolymph of locust *S. gregaria* (Malik *et al.* 1999). SGCI proved to be a very good inhibitor of bovine chymotrypsin whereas SGTI was a reasonable inhibitor of bovine trypsin. Since the latter inhibited two arthropodal trypsins by five

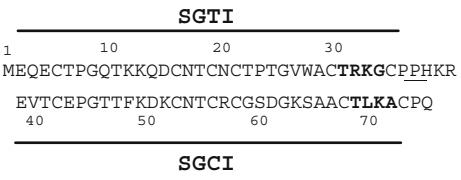

Fig. 1. Amino acid sequence of the double-headed locust inhibitory peptide. Amino acids at the active site are in bold, linker dipeptidyl moiety is underlined. The two individual peptides, *Schistocerca gregaria* chymotrypsin inhibitor, SGCI, and *S. gregaria* trypsin inhibitor, SGTI are indicated.

orders of magnitude more strongly than the mammalian one (Patthy *et al.* 2002), it is expected to affect mammals much less than insects if used as an insecticide. In preliminary feeding trials, both SGCI and SGTI painted onto *Solanum tuberosum* leaf discs showed appreciable anti-feedant activity on Colorado potato beetle larvae (our unpublished result). Thus we decided to introduce and express both inhibitors in potato as a single polypeptide (Figure 1). This form, termed locust inhibitory peptide (LIP), offered both pragmatic and theoretical advantages. Firstly, LIP is a precursor peptide of the two locust protease inhibitors, SGCI and SGTI, in locust (Kromer *et al.* 1994), thus it is relatively easy to clone their coding regions together. Secondly, a larger peptide is usually less prone to degradation during expression in hosts. Thirdly, due to the very tight binding of the SGCI domain to bovine chymotrypsin, it could be purified on an immobilised chymotrypsin column by affinity chromatography. Last but not least, it is a double-headed form capable of inhibiting proteases of different specificity.

Materials and methods

Enzymes and chemicals

Bovine trypsin and chymotrypsin were obtained from Sigma. Enzyme activity was determined in a 50 mM Tris/HCl buffer, pH 8, containing 0.3 M KCl and 10 mM $CaCl_2$ (activity buffer). The *p*-nitroanilide (pNA) derivatives of Suc-Ala-Ala-Pro-Phe and Z-Gly-Pro-Arg were obtained also from Sigma and their hydrolysis was followed at 405 nm. Protein concentrations of the plant extracts were calculated from A_{280} and A_{260} values.

Plants and bacterial strains

Solanum tuberosum cv. Désirée was vegetatively propagated from single-node stem segments in tissue culture at 24 °C under a 16 h light/8 h dark regime. Transgenic potato lines were generated by leaf transformation according to Dietze *et al.* (1995). Plants for biochemical analysis and the bioassays were cultivated in pots in the greenhouse for 2 months.

Escherichia coli strain DH5α and BL21, and *Agrobacterium tumefaciens* strain C58C1 containing pGV2260 were grown by standard techniques.

Standard molecular biological techniques

Locust inhibitory peptide was cloned in the *Escherichia coli* expression vector pET-17b (Novagen) as a PCR fragment (Szenthe *et al.* 2004) and re-cloned into the binary vector pCP60 (kindly provided by P. Ratet) by *Eco*RI-*Xba*I.

Total RNA was extracted, separated on a formaldehyde-agarose gel and blotted onto the nylon membrane Hybond N (Amersham). Hybridisation was carried out according to Sambrook *et al.* (1989). The full-length *LIP* cDNA was used for labelling reaction with random primers.

Rapid screening protease inhibitor assays

For rapid screening of potato lines, stem and leaves of *in vitro* plants were weighed, frozen in liquid N_2 and ground in a mortar. The resulting powder was suspended in activity buffer approximately tenfold to the wet weight. After centrifugation 50 μl was added to 2.9 ml activity buffer (50 mM Tris/HCl, 10 mM $CaCl_2$, 0.3 M KCl) containing 10^{-8} M chymotrypsin. The reaction was started by the addition of a stock solution of Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide to give 10^{-5} M, and first-order rate constants proportional to active enzyme concentration were determined and compared to those obtained in the absence of extract.

Purification of the locust inhibitory peptide from transgenic plants

Leaves from plants grown in the greenhouse were harvested in the morning and in a typical purification, 5 g leaves were frozen in liquid N_2 and ground in a mortar. The resulting fine powder was extracted with 15 ml activity buffer for 3–4 h at room temperature. The suspension was centrifuged at 13 000 × *g* for 15 min, and total protein in the supernatant determined. Saturated $(NH_4)_2SO_4$ solution was added to achieve 48% saturation, and the solution was stirred gently for 1 h at 4 °C. The suspension was centrifuged at 13 000 × *g* for 15 min. The supernatant was dialysed versus 15 mM Tris/HCl, 15 mM NaCl, pH 7.6 buffer, and then applied to a 1 ml α-chymotrypsin-Sepharose 4B column in the same buffer. After washing, the bound protein was eluted with 20 mM HCl and further purified in a Waters HPLC system. Chromatographic conditions: Aquapore Brownlee OD S18 (4.6 × 210 mm) reversed-phase column; flow rate, 1 ml/min; initial conditions, (A) 0.1% (v/v) TFA in water; final conditions, (B) 0.08% (v/v) TFA in acetonitrile/water (4:1, v/v). Linear gradient from (A) to (B) in 30 min. Detection was at 220 nm.

Peptide analysis

The HPLC-homogeneous sample was reduced and pyridylethylated and subjected to *N*-terminal sequencing by automated Edman degradation using a model 494 Applied Biosystems protein sequencer.

Growth of insects and larvae

Experiments to study the effect of LIP expressing transgenic potato plants on the growth of larvae were performed with a modified method of Sáringer (1967). Field-collected adult Colorado potato beetles were reared in the laboratory and their eggs were collected. Young third instar larvae used for the bioassay were hatched from eggs laid by one female. The bioassay was conducted in a climatic chamber at 21 °C and under 20 h/4 h light/dark photoperiod. Data sets were analysed for significant differences using independent samples *T*-test (SPSS 9.0 for Windows).

Results

Cloning of the LIP

For their expression in transgenic plants, cloning of the inhibitory peptides was done as described in detail by Szenthe *et al.* (2004). Briefly, total RNA was isolated from the fat body of the locust and the gene encoding both SGTI and SGCI on a single chain in SGTI-Lys-Arg-SGCI form, termed *LIP*, was amplified and the PCR product was ligated into the pET-17b expression vector. Success of the construction was checked by sequencing.

The plasmid containing the *LIP* insert was used for the transformation of *E. coli* BL21 cells. After induction by IPTG, these produced the protein in sufficient quantities in a soluble form. After isolation and purification (Szenthe *et al.* 2004), the double-headed inhibitor displayed K_i values both versus bovine chymotrypsin and trypsin identical to those obtained earlier for the individual peptides (Malik *et al.* 1999). This result indicated that the peptide was correctly folded in *E. coli*.

Isolation of transgenic potato lines expressing the LIP

The *LIP* was cloned into a binary vector behind the constitutive CaMV35S promoter and used for *Agrobacterium*-mediated transformation of 105 potato leaves. From the approximately 300 calli formed, 222 independent, kanamycin resistant regenerated potato plants were isolated and 23 analysed for both expression on a northern blot (Figure 2) and chymotrypsin inhibition displayed in the rapid screening assay. The rapid assay was repeated with plants of the northern positive lines and then lines LIP7, 9, and 13 were chosen for further analysis based on their significantly increased inhibitory activity compared to the untransformed control plants in both tiers (Table 1).

Purification of LIP peptide from transgenic potato plants

Leaves of 2-months-old *LIP* transgenic potato lines LIP7, 9, and 13 grown in pots were harvested in the morning and after measuring wet

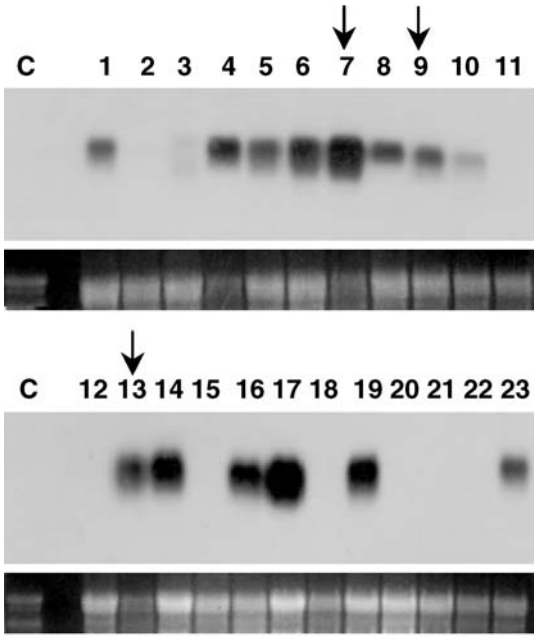

Fig. 2. Northern blot analysis of the *S. tuberosum* cv. Désirée lines transformed with the locust inhibitory peptide construct. C, non-transformed control; the numbers indicate individual transgenic lines. Arrows show the transgenic lines selected for further experiments. Ethidium bromide-stained rRNA bands are shown as loading controls.

Table 1. Chymotrypsin inhibition of potato lines by the rapid assay.

Extract added	Chymotrypsin activity
None	100%
Désirée	95 ± 4%
LIP7	71 ± 5%
LIP9	77 ± 8%
LIP13	85 ± 5%

The first-order rate constant of hydrolysis of 10^{-5} M Suc-Ala-Ala-Pro-Phe-pNA determined at $[E]_0 = 10^{-8}$ M, 0.007 s^{-1}, is 100% chymotrypsin activity. Assays were performed in duplicates; averages of two independent trials are shown.

weight, plant material was worked up as described in Materials and methods. In samples purified by affinity chromatography on the α-chymotrypsin-Sepharose 4B column, a band corresponding to the synthetic LIP in size was visible on SDS-PAGE. Similarly, when the affinity-purified samples were applied to a reverse-phase HPLC column, in the elution profiles of all three lines a peak eluted with the same retention time as intact LIP from *E.coli*.

From line LIP9, protein of this peak (0.7 μg/mg soluble protein) was collected from several runs in sufficient quantity for peptide sequencing and for K_i determinations as described earlier (Malik *et al.* 1999). *N*-terminal sequence was found to be MEQECTPGQTK, identical to the expected *N*-terminal (Figure 1), and inhibitory constants against chymotrypsin and trypsin were practically identical with that determined with the individual natural peptides (Table 2).

Feeding trials with transgenic lines LIP7, 9 and 13

All three transgenic lines used for HPLC analysis were used in bioassay. Results obtained with line LIP9 are shown in Figure 3. A repeat of this experiment and that with lines LIP7 and LIP13 showed similar results.

Figure 3a indicates that the feeding of potato beetle larvae was slightly but significantly inhibited by LIP. The average daily weights of control larvae fed with untransformed potato leaves were higher than the weight of larvae fed with line LIP9 potato leaves from the first day till the day of pupation (5th day). Note that weight loss of control larvae occurred on the 5th day because most of them reached prepupal stage on the 4th day (and after it they consumed less food), but the experimental conditions did not let them pupate. Significant differences ($p = 0.05$) were observed between the weights of control and LIP consuming larvae on the 2nd and 4th days. Three larvae fed on LIP transgenic plants died during the bioassay (the first one died on the 3rd day, the second on the 4th and the third larva died on the last day).

During the bioassays the weight of consumed leaves was also measured. These data showed

Table 2. Inhibitory constants versus chymotrypsin and trypsin.

Inhibitor	K_i versus chymotrypsin ($\times 10^{12}$) M	K_i versus trypsin ($\times 10^{7}$) M
LIP from line LIP9	8.0 ± 1.4	3.0 ± 0.6
SGCI	6.2 ± 1.5[a]	
SGTI		2.1 ± 0.4[a]

K_i values for LIP from line LIP9 were determined as described earlier (Malik *et al.* 1999).
[a]Data from Malik *et al.* (1999).

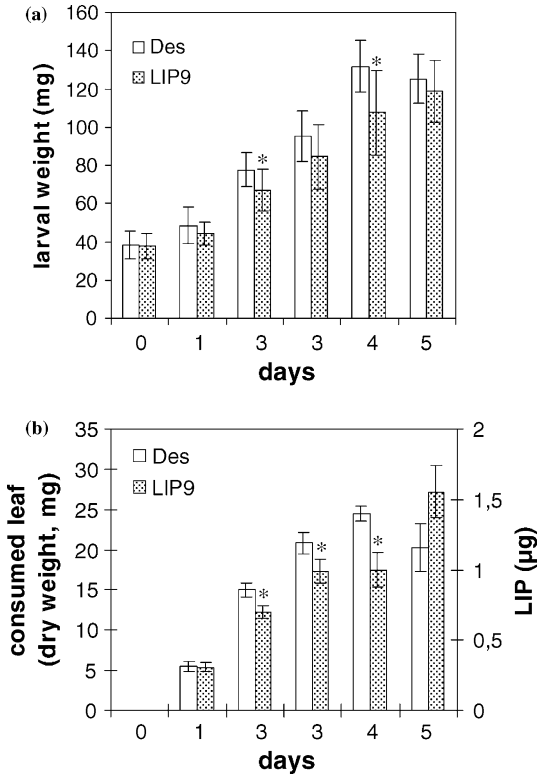

Fig. 3. Feeding trials with the transgenic potato line LIP9. Each larva was put into a glass container (12 cm in diameter, 4 cm high) half-filled with water and covered with linen with the aid of a rubber ring, with filter paper and Petri dish cover. Leaves of potato plants were cut into halves. After measuring the fresh weight of each half, one half was put into a drying chamber for 24 h at 105 °C and weighed afterwards for determining dry matter percent while the other half was put into the glass container for feeding a larva. After 24 h the remaining leaf debris (food was always provided in excess) after drying and the larva were weighed. Then the larva was put back to the container and the whole procedure was repeated. (a) Average daily live weights of 10 potato beetle larvae fed with control Désirée (hollow columns) and of 10 with transgenic line LIP9 leaves (grey columns). Day 0 represents starting weight. (b) Dry weight of leaves (left axis) and amount of LIP (right axis) consumed daily by larvae. Columns are showing mean values, vertical bars indicate standard errors. Asterisks depict differences significant at $p = 0.05$.

that feeding increased proportionally with larval growth. While the feeding behaviour seemed to be normal in the case of larvae fed with line LIP9 potato plants, these larvae consumed less food day after day than larvae fed on non-transformed Désirée leaves (Figure 3b). This can explain the results shown in Figure 3a and also conveys the suggestion that LIP has a physiologically toxic effect.

The bioassays ended when the largest larvae wanted to enter the soil for pupation. All larvae entered the soil within 3 days. After pupation adult beetles emerged from the soil and were reared in laboratory for 2 weeks. Every larvae reached adulthood and no morphological deformation could be observed on them.

Discussion

Leptinotarsa decemlineata, the major pest of potato in many areas of the world, has not yet been successfully controlled by protease inhibitors. This might be due to the fact that it is able to inactivate not only stress-induced protease inhibitors of potato's self-defence (Gruden *et al.* 2003) but also several heterologous protease inhibitors introduced into potato (Brunelle *et al.* 2004, and references therein). Ironically, when equistatin, a polypeptide inhibiting carboxypeptidase by one of its domains and cysteine protease activities by another domain was expressed in the plant, it was the potato proteases that inactivated the inhibitor (Outchkourov *et al.* 2003) although equistatin proved to be effective when added into an artificial diet (Gruden *et al.* 1998).

In the present paper we report that the natural peptide from a locust haemolymph inhibiting both trypsin and chymotrypsin with its twin heads, has been successfully cloned and introduced into potato. The presence of the active LIP protein in the leaf extracts albeit in low amounts was demonstrated. Despite of the low level of LIP the growth of the Colorado potato beetle larvae was restricted on the transgenic plants compared to that on the non-transformed control. LIP can bind to bovine chymotrypsin very tightly (Patthy *et al.* 2002) and thus inhibition of the larval growth might be due to the inhibition of the chymotrypsin-like gut proteases of potato beetle larvae (Novillo *et al.* 1997), which may contribute more significantly to digestion than previously thought. Serine proteases are known to process pro-proteins including many proteases synthesised in a proenzyme form. Such an activating protease, present probably at a very low concentration, can also be inactivated by LIP. Finally it is also possible that LIP, as a mem-

ber of the pacifastin family involved in the regulation of arthropodal immune system (Simonet *et al*. 2002) may affect larval growth and development by such a mechanism. Although further studies are needed to shed light on the abovementioned possibilities, our results lend support to the idea that expression of multifunctional protease inhibitors with insect origin in plants might be a good strategy to improve insect resistance.

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