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S. Marchetti · M. Delledonne · C. Fogher · C. Chiabà
F. Chiesa · F. Savazzini · A. Giordano

Soybean Kunitz, C-II and PI-IV inhibitor genes confer different levels of insect resistance to tobacco and potato transgenic plants

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Abstract In modern, highly intensive agriculture, the control of insect pests is basically achieved with the application of chemical pesticides. Heavy reliance on this sole strategy is associated with several drawbacks, and the development of alternative or complementary methods to chemical control is desirable. In this work, three soybean genes (*KTi_3*, *C-II* and *PI-IV*) coding for serine proteinase inhibitors were isolated by PCR and transferred to *Agrobacterium tumefaciens* EHA 105, which in turn was used for transforming tobacco leaf and potato tuber discs. Biochemical assays confirmed that transgenic plants synthesized serine proteinase inhibitors; rates of expression varied among plants. The level of insect resistance (tested with *Spodoptera littoralis* Boisduval) was particularly high in tobacco, where many plants caused the death of all larvae. In potatoes, larval mortality was much less frequently achieved, but the results were still encouraging in that larval weight gain was reduced by 50% in the presence of adequate amounts of inhibitor. When 8-day-old larvae were fed different *KTi_3*-expressing tobacco plants, a highly significant (*$P<0.01$*) correlation was observed between inhibitor content and larval live weight. Larval weight gain was found to be dependent on midgut proteolytic activity. On the basis of the evidence collected, it is suggested that further work is required to identify more specific inhibitors for the main proteinases of the target insect.

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S. Marchetti (✉) · C. Chiabà · A. Giordano
Dipartimento di Produzione Vegetale e Tecnologie Agrarie,
Università di Udine, Via delle Scienze 208, 33100 Udine, Italy
Fax: +39 0432-558603

M. Delledonne · C. Fogher · F. Savazzini
Istituto di Botanica e Genetica Vegetale,
Università Cattolica del S. Cuore, Via Emilia Parmense 84,
29100 Piacenza, Italy

F. Chiesa
Dipartimento di Biologia Applicata alla Difesa delle Piante,
Università di Udine, Via delle Scienze 208, 33100 Udine, Italy

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Introduction

Proteinase inhibitors of plants are involved in a number of functions, including the control of endogenous proteolytic enzymes (Richardson 1977), the reserve of ammonia and sulphur amino acids within the storage organs (Pusztai 1972; Lorensen et al. 1981; Tan-Wilson et al. 1985), and the defence against insect and nematode attack (Sijmons 1993; Urwin et al. 1995). In tomato and tobacco plants, proteinase inhibitors have been found to accumulate in response to infection by pathogenic microorganisms (Peng and Black 1976; Rickauer et al. 1989).

Proteinase inhibitors form stoichiometric complexes with specific proteolytic enzymes, thus preventing their catalytic function. Several inhibitors against serine-, cysteine-, aspartic- and metallo-proteinases have been identified in both the animal and plant kingdoms. Since proteinase inhibitors are primary gene products, they are excellent candidates for engineering pest resistance into plants. Inhibitor genes of plant origin are particularly promising. When inserted into other plant genomes, little or no problems are met in their expression. Compared to animal analogues, their acceptance as transgenes is notably higher. Large amounts of inhibitors are normally present in many raw foods of plant origin, but their antinutritional effect is drastically reduced with simple technological treatments such as cooking. The oral administration of small quantities of certain inhibitors (e.g. soybean Bowman-Birk) has a protective role against carcinogenesis in the esophagus, duodenum and colon (St. Clair et al. 1990; Kennedy 1994).

This article deals with three soybean genes encoding inhibitors specific for serine-proteinases, which are the main digestive proteinases in most Lepidopteran insects (Boulter 1993). In particular, the aim of the investigation was to demonstrate the level of resistance achievable with

tobacco and potato transgenic plants against *Spodoptera littoralis* Boisduval, a noctuid whose digestive enzymes were characterized in a previous study (Marchetti et al. 1998).

Materials and methods

Cloning of genes

Genes were isolated by PCR (polymerase chain reaction; Saiki et al. 1988) from soybean (*Glycine max* Merrill) genomic DNA (Table 1). Template DNA was extracted from soybean seedlings according to Doyle and Doyle (1987). Forward and reverse primers were designed on the basis of published sequences (Table 1). Specific 6-mer recognition sites for different restriction enzymes were added in order to permit gene isolation during subcloning (Table 1). Primers were the following:

KTi_3

forward:
5'-GGATCCATGAAGAGCACCATCTTCTTTCTCTTTC-3'
reverse:
5'-CAGCTGTCACTCACTGCGAGAAAGGCCATG-3'

C-II

forward:
5'-GGATCCATGGAACTGAACCTCTTCAAAAGTGAT-3'
reverse:
5'-GAGCTCCTAGTCATCATCTTCATCACTGGA-3'

PI-IV

forward:
5'-GGATCCATGTGTATTCTGAGCTTCCTCAAAAGT-3'
reverse:
5'- GAGTCTCTAGTCATCTCTGGACTTGCAAGG-3'.

For PCR reactions, AmpliTaq polymerase (Perkin Elmer Cetus Corp) was used. Aliquots of the amplified DNAs were digested with suitable endonucleases (Boehringer Mannheim) to verify the occurrence of the expected restriction pattern (Table 1).

The linear phagemid pGEM-T (Promega) was used as the cloning vector; genes were inserted directly through ligation by T4 DNA ligase (Promega) following the manufacturer's instructions. Host strain JM 101 of *Escherichia coli* was transformed with the engineered phagemid by calcium chloride treatment (Sambrook et al. 1989), grown in ampicillin and selected using the X-gal assay. DNAs purified by Magic™ Minipreps (Promega) from at least two independent clones were sequenced in both strands using modified versions of Sanger's method (Sanger et al. 1977) (Table 1).

Genes were then subcloned in the binary vector pBI 121 (Clontech) in place of the *uidA* (GUS) gene. For this purpose, pBI 121 was extracted from *E. coli* JM 101 with a maxiprep (Qiagen Plasmid Purification kit), digested with *Bam*HI and *Sac*I to remove the *uidA* locus, run at 3.5 V cm^{-1} for 3 h on a 0.5% agarose TAE gel, recovered with the GeneClean protocol (Bio 101) and finally purified by ethanol precipitation. In the case of *KTi_3* subcloning, the *Sac*I site was eventually blunted by T4 DNA polymerase to generate a *Pvu*II-compatible end. Both the promoter (35 S of CaMV) and the polyadenilation signal (*nos* ter) of the GUS cassette were maintained in the new pBI 121-derived plant expression vectors.

Plant transformation

Recombinant *E. coli* cells were used as the donor strain in a triparental mating (Hood et al. 1986). Overnight selective cultures of recombinant *E. coli*, *Agrobacterium tumefaciens* recipient strain EHA 105 and *E. coli* helper strain JM 101 (pRK2013) were centrifuged and resuspended in saline solution before being mixed. The mating mixture was spread on LB agar plates without antibiotics and incubated for 20 h; *Agrobacterium* transconjugant cells were then selected on LB medium containing kanamycin and rifampicin.

Leaf discs of tobacco (*Nicotiana tabacum* L. cv. Xanthi) and tuber discs of potato (*Solanum tuberosum* L. cv. Desirée) were transformed according to Horsch et al. (1985) and Sheerman and Bevan (1988), respectively. Several plantlets were regenerated in both species under selective conditions (200 mg l^{-1} kanamycin). Putative transgenic plants were potted in peat and hardened in a greenhouse together with controls (plants of the donor cultivars raised *in vitro* from uninfected discs). Potato plants were multiplied vegetatively while tobacco plants were selfed, and the resulting seed was surface-sterilized with 2% sodium hypochlorite. Seed was germinated on an agar medium consisting of Murashige and Skoog (1962) mineral solutions and 150 mg l^{-1} kanamycin. After selection, 20 green plantlets of each progeny were potted in peat, hardened in a greenhouse, tested for the presence of the transgene and used for assaying insect resistance.

The stable integration and expression of the foreign genes in the regenerated plants as well as in their progenies was verified using PCR, RT-PCR (reverse transcriptase-polymerase chain reaction, Perkin-Elmer Cetus Corp), Southern and Northern blotting. Genomic DNAs were extracted from tobacco and potato plants according to Doyle and Doyle (1987), whereas total RNAs were extracted with the RNAfast system (Qiagen). Molecular analyses were done using standard protocols (Sambrook et al. 1989) and full length genes as probes. Transgenic plants and their corresponding controls were also analysed to determine the trypsin inhibitory activity (TIA) of the crude sap (Smith et al. 1980; Lorenzoni et al. 1990). TIA was expressed as milligrams SBTI-A_2 protein per milliliter crude sap. Crude sap was obtained by squeezing tobacco or potato leaf tissue with a leaf-juice press based on a roller system (Erich Pollähne, Germany).

Insect rearing and bioassay

Insect resistance was checked using a stabilized strain of the Egyptian cotton leafworm (*Spodoptera littoralis* Boisduval), collected in Sudan and reared under laboratory conditions since 1972 at Sipcam-Isagro (Milano, Italy). This insect was chosen because it can damage both tobacco and potato crops and because two

Table 1 Main information relative to gene cloning

	Cloned gene		
	KTi_3	*C-II*	*PI-IV*
Soybean cultivar	Columbia	Maple Arrow	Maple Arrow
Sequence source	EMBL S45092	EMBL M20732	EMBL M20733
5′ excision site	*Bam*HI	*Bam*HI	*Bam*HI
3′ excision site	*Pvu*II	*Sac*I	*Sac*I
Nuclease for RFLP	*Sac*I	*Hinf*I	*Hinf*I
Sequencing method	Thermal cycling	Labeling/termination	Labeling/termination

trypsin-like enzymes are present in its gut (Marchetti et al. 1998); larval development should therefore be affected by serine-proteinase inhibitors expressed in transgenic leaf tissue. In our laboratory, the larvae were reared at 25°C, 60% RH on a suitable artificial diet (Marchetti et al. 1998); under these conditions, the life cycle was completed in about 30 days, which is the minimum span needed in nature (Tremblay 1986). Adults laid large batches of good quality eggs. Inside the rearing boxes, mortality was trivial and restricted to the first instar and the pupal stage. For the bioassay first- and third-instar larvae were used; first-instar larvae were collected with a soft, thin brush as soon as they hatched and immediately transferred onto tobacco or potato leaf tissue; third-instar larvae were 8 days old and were reared on lettuce leaves until the beginning of the entomological test.

Tests were performed on the selfed, kanamycin-resistant and PCR-positive progeny of 63 transformed tobacco plants and 92 transformed potato clones after one vegetative cycle. In both species, the bioassay was carried out just before anthesis; controls were untransformed plants of the same variety grown in the same manner under the same environment. A 17-mm-diameter tobacco leaf disc was placed inside a 5-ml tube and artificially infested with 10 larvae; to test 1 plant, the rate of survival was determined in 6 tubes (3 replicates of 20 larvae each), 48 h after infestation. To assess insect resistance in potatoes, we used a similar procedure except for the following: i. infestation was carried out on single leaflets detached from the plants and put into 60-mm petri dishes; ii. leaflets were changed every day; and iii. data were collected 120 h after infestation by scoring the larval live weight with an analytical balance (4 replicates of 10 larvae each). These differences were introduced after the rate of survival was found to be largely inadequate to express the level of insect resistance in transgenic potatoes.

Correlation between inhibitor content and level of insect resistance

Ten tobacco plants carrying the KTi_3 gene plus an untransformed control were analysed in order to quantify the TIA of the crude sap. The phenological stage of the plants and the analytical method used were as above. Immediately after TIA was recorded, 18-cm^2 leaf discs were collected from each plant and placed in three 90-mm petri dishes, each containing three 8-day larvae. Larvae were fed *ad libitum* for 4 days; the leaf tissue was renewed every 24 h and larvae were weighed daily. Leaf consumption was evaluated with a leaf area meter. At the end of the experiment, the correlation between larval live weight (or leaf consumption) and TIA was checked.

To demonstrate that growth reduction induced by transgenic food was due to the inhibition of digestive enzymes, we determined the midgut proteolytic activity of larvae of the same weight. In particular, 5 larvae grown on each of 3 transgenic plants were weighed, anaesthetized with carbon dioxide and dissected for midgut removal. The same was done for control larvae having the same weight. Midguts extracted from larvae fed the same plant were homogenized at 0°C using 200 µl 0.1 M Tris, pH 11.0 (solution A). After centrifugation at 10,000 *g* for 10 min, 10 µl of the supernatant was pipetted into tubes containing 50 µl of solution A plus 100 µl of 2% azocasein in the same solution. After 60 min of incubation at 25°C, the reaction was stopped by adding 480 µl of 10% trichloroacetic acid. Samples were allowed to stand for 15 min at 0°C and centrifuged at 8,000 *g* for 3 min; 500 µl of supernatant was transferred to a test tube containing 580 µl 1 M NaOH. The absorbance of the samples was read at 405 nm.

Before assessing the midgut proteolytic activity in larvae fed transgenic tissue, we tested different quantities of midgut proteinase mix on a constant amount of substrate to determine the maximum concentration showing linear activity during incubation time.

Statistical analysis

Data concerning proteinase inhibitor content, leaf consumption and larval weight were submitted to one-way analysis of variance after having checked the normality of distribution and the homoscedasticity of variances with the Kolmogorov-Smirnov test and the Bartlett test, respectively. Means were compared with the new Duncan multiple range test at a probability level of $P=0.01$.

The coefficient of linear correlation (r) between larval live weight and crude sap TIA was calculated after having checked the linearity of distribution of the data. Non-linear distributions were corrected by log transformation and checked again.

The coefficient r was also calculated to verify the linear trend of substrate degradation (constant amount) with different quantities of midgut proteinases and with different incubation times. In both cases, only r values higher than 0.995 were considered as predictive.

Results

Gene cloning

PCR amplification of the target genes was performed using forward and reverse primers designed on the basis of published sequences (Table 1 in Materials and methods). Since agarose gel electrophoresis indicated that the amplified fragments had approximately the same size as the full-length genes (651 bp for KTi_3, and 252 bp for both *C-II* and *PI-IV*), the fragments were ligated into pGEM-T vectors. When phagemid DNAs were extracted and analysed, the nucleotide sequences of KTi_3 and *PI-IV* were found to be identical to those published. In contrast, the amplified *C-II* sequence (eventually submitted to EMBL and accepted as X76727) differed from EMBL M20732 at four positions; the base changes in the new version did not alter the deduced primary structure of the inhibitor, rather they characterized the most frequent *C-II* allele, as indicated by restriction fragment length polymorphic (RFLP) analysis carried out on randomly chosen soybean varieties (results not shown).

Plant transformation

After mobilisation of the genes to *Agrobacterium tumefaciens* and infection of tobacco leaf and potato tuber discs, more than 240 tobacco and potato shoots were obtained. These shoots survived levels of kanamycin (selective agent) as high as 150 mg l^{-1} and, without exception, were found to be PCR-positive for the gene whose insertion was attempted. In contrast, no shoots were regenerated in the presence of kanamycin in uninfected tobacco or potato; furthermore, no amplification was observed in untransformed shoots belonging to the same variety grown *in vivo* or *in vitro*. Southern blot hybridizations carried out on regenerated plants and their progenies confirmed that transgenic plants were produced for all genes (Fig. 1).

A preliminary evidence about transgene expression was obtained using RT-PCR (Fig. 2). To further demonstrate that the target genes were correctly expressed in both tobacco and potato, we performed Northern blots on puta-

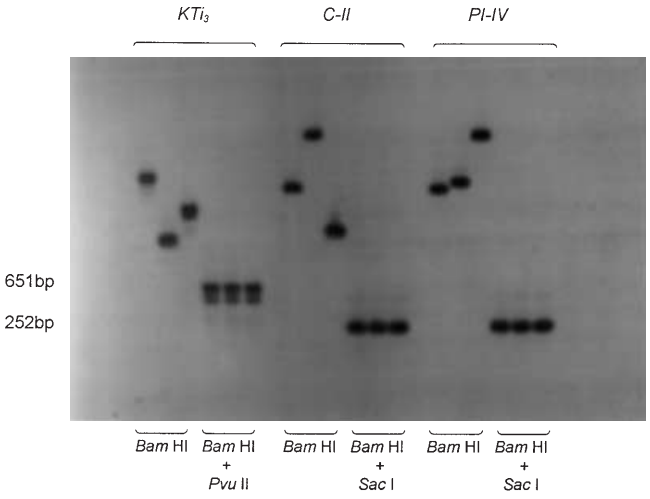

Fig. 1 Southern blot analysis of transgenic potato. Genomic DNA was extracted and cleaved with *Bam*HI or *Bam*HI and *Sac*I (*Pvu*II in the case of KTi_3); endonuclease-treated DNA was hybridized with radioactive probes corresponding to the KTi_3, *C-II* and *PI-IV* genes. *Bam*HI is expected to cleave DNA between the promoter and the start of the coding sequence as well as in an unknown position downstream of the terminator. *Bam*HI in combination with *Sac*I (or *Pvu*II for KTi_3) should release the transgene by cutting DNA at the 3' and 5' transgene cloning site; the expected length of the fragment should be 651 bp for KTi_3-tranformants and 252 bp for plants harbouring the *C-II* or *PI-IV* gene. For ease of presentation, potato clones carrying single copies of the transgene were selected

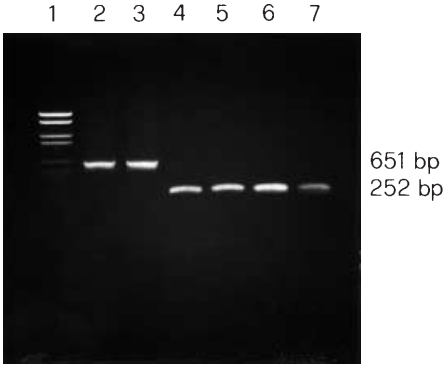

Fig. 2 Results of the RT-PCR on kanamycin-resistant shoots of tobacco and potato. *Lane 1* Molecular-weight marker VI (Boehringer Mannheim), *lanes 2, 4, 6* tobacco shoots putatively transformed with the KTi_3, *C-II* and *PI-IV* genes, respectively, *lanes 3, 5, 7* potato shoots putatively transformed with the KTi_3, *C-II* and *PI-IV* genes, respectively

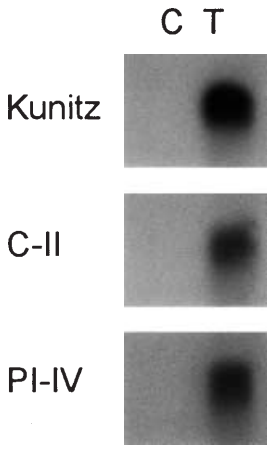

Fig. 3 Northern blot: comparison between untransformed control (*C*) and transformed tobacco plants (*T*)

Table 2 Trypsin inhibitory activities (TIAs) in crude saps from untransformed controls and from tobacco and potato plants expressing different proteinase inhibitor genes

Inhibitor	Tobacco plant	TIA[a]	Potato plant	TIA[a]
Kunitz	K12	165 a	K2	85 a
	K14	159 b	K149	59 b
	K10	86 e	K57	55 b
	K8	84 e	K55	43 c
	K7	70 f		
	K5	58 g		
C-II	C6	98 d	C68	57 b
	C4	97 d	C37	55 b
	C9	73 f	C51	54 b
			C71	38 c
PI-IV	P4	85 e	P29	82 a
	P8	74 f	P28	79 a
	P5	69 f	P31	41 c
Control		27 h	Control	24 d

[a] Micrograms SBTI-A_2 equivalent per milliliter crude sap; means with a letter in common within a column are not significantly different at P=0.01

tively transformed plants using the full-length gene as a probe. As shown in Fig. 3, the probes strongly hybridized with RNAs extracted from kanamycin-resistant plants, whereas no signal was obtained with untransformed controls. The extent of expression was evaluated on 32 tobacco and 19 potato plants by measuring the trypsin inhibitory activity (TIA) displayed by known amounts of the crude sap. In both species, a range of values was obtained, irrespective of the gene considered (Table 2). However, it should be considered that the transgenic plants had significantly higher TIA values than the controls (untransformed plants of the same variety). Through the adoption of standard proteinases and specific chromogenic substrates, it was demonstrated that only serine-proteinases were affected by the crude sap of transgenic plants.

After two vegetative (potato) or reproductive (tobacco) cycles, plants still retained and expressed the transgenes, as indicated by PCR and biochemical assays. In some tobacco progeny, "nulls" appeared at every cycle due to segregation.

Insect resistance

When first-instar larvae were transferred to untransformed tobacco or potato leaf tissue, they survived and grew quickly to maturity. In contrast, feeding on several of

Table 3 Percentage of larval survival (*Spodoptera littoralis*, first instar) observed by using tobacco plants transformed with different proteinase inhibitor genes as the sole food source. Each plant was infested with 200 larvae (4 replicates of 50 larvae each); data were recorded 48 h after infestation

Inhibitor	Tobacco plant[a]	Survivors[b] (% on total)
	Control	97 a
Kunitz	K14–18	0 g
	K8–3	1 g
	K7–2	27 e
	K6–8	48 d
C-II	C4–6	8 f, g
	C6–1	14 f
	C8–20	45 d
	C9–17	48 d
	C5–9	77 b
PI-IV	P14–3	11 f
	P7–8	62 c
	P2–5	71 b

[a] The first number after the capital letter indicates the primary transformant whereas the second number identifies the plant within the progeny
[b] Means with a letter in common are not significantly different at P=0.01

Table 4 High levels of larval mortality (*Spodoptera littoralis*, first instar) achieved by using different KTi_3-expressing tobacco plants as the sole food source. Data were recorded 48 h after infestation

Tobacco plant[a]	Survivors	Number of larvae tested
K4–9	0	60
K8–3	0	60
K14–18	0	60
K6–12	0	60
K1–1	0	60
K7–5	0	60
K5–15	1	60
K13–15	1	60
K11–9	1	60
Control	59	60

[a] The first number after the capital letter indicates the primary transformant whereas the second number identifies the plant within the progeny

the tobacco transformants often led to larval death within 2 days. Owing to its frequency, first-instar mortality rate appeared to be the most suitable parameter to evaluate insect resistance in transgenic tobacco. Interestingly, resistance was achieved with any one of the transgenes and was maintained in the PCR-positive progeny of the transformants (Table 3). In the best tobacco plants, many larvae died as soon as 16 h after infestation and only very few of them survived longer than 48 h (an insight referring to the best KTi_3-transformants is provided in Table 4). Upon microscopic inspection, leaves were found to be only slightly damaged, suggesting that larvae died even after little feeding. Since the amount of debris found at the end of the trial was nearly absent, it can be deduced that leaf material remained in the gut and prevented the larvae from further feeding. Before dying, larvae gradually lost their turgor and became shrunken; apparently food avoidance had a dramatic effect also on the water balance of the first-instar larvae.

Table 5 Reduction in larval weight (*Spodoptera littoralis*, first instar) achieved using transformed potato as the sole food source

Inhibitor	Potato plant	Larval weight[a] (% on control)
	Control	100
Kunitz	K2	48 a
	K55	62 b, c, d
	K57	70 d
	K149	71 d
C-II	C68	56 a, b
	C46	63 b, c, d
	C45	67 b, c, d
	C70	69 b, c, d
PI-IV	P57	58 a, b, c
	P28	64 b, c, d
	P26	67 b, c, d
	P22	73 d

[a] Means with a letter in common are not significantly different at P=0.01

In potatoes, larval mortality was much less frequently achieved, apparently because of the higher nutritional value of potato leaves; when *Spodoptera* instars were fed untransformed tobacco or potato leaves for 5 consecutive days, larval weight was 3.9 times greater with potato. Therefore, for testing insect resistance of transgenic potatoes, we considered the mean live weight 5 days after artificial infestation. As shown in Table 5, all inhibitors were partly effective against *Spodoptera*; in the best plants a 50% reduction in larval weight was observed and leaf damage also appeared reduced.

Correlation between inhibitor content and level of insect resistance

When 10 KTi_3 tobacco plants plus an untransformed control were analysed to quantify the crude sap TIA and then used as food for 8-day-old third-instar larvae, a highly significant correlation coefficient (r = -0.86, $P<0.001$) between inhibitor content and live weight of the larvae was found (Fig. 4). Through repeated weighings it was possible to demonstrate that 1 day was sufficient to discriminate between transformed and untransformed plants and that selection of the best transformants could be applied as soon as 4–5 days after infestation (Table 6). Even when the inhibitor content of the transgenic leaf tissue was not much higher than that of the control, leaf consumption was reduced by 60–65%. With TIA values above 165 µg SBTI-A_2 ml^{-1} crude sap, leaf consumption was only 16–19% that of the control. Similarly to what observed on first-instar larvae, the

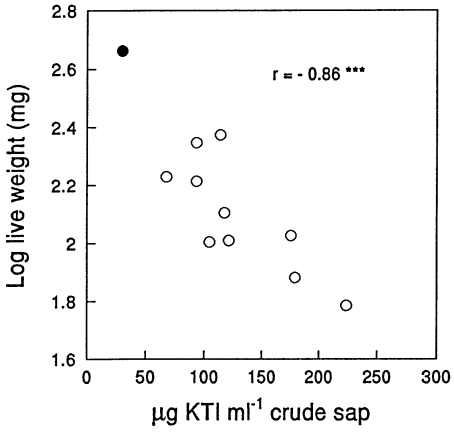

Fig. 4 Correlation between Kunitz trypsin inhibitor (KTI) content and live weight of *Spodoptera* larvae fed different transgenic tobacco plants plus an untransformed control for 5 consecutive days. The *black marker* in the *upper left corner* identifies the untransformed control; a highly significant correlation was still found when the control was not considered during the statistical analysis. KTI content was determined analytically (Smith et al. 1980) on plants at the stage of anthesis. At the start of the experiment, *Spodoptera* larvae were 8 days old

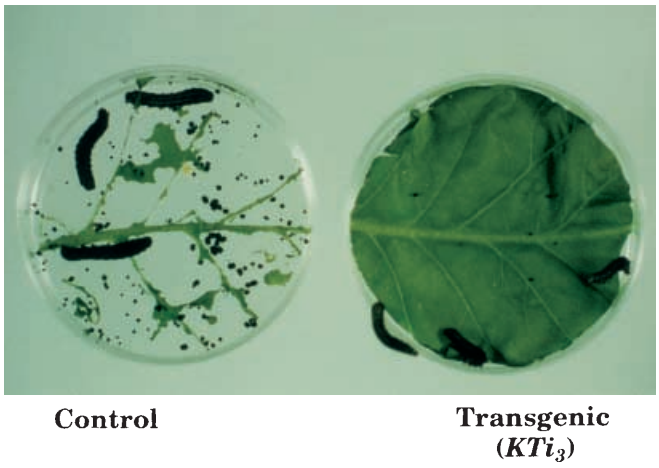

Fig. 5 Feeding behaviour of *S. littoralis* larvae with the tobacco untransformed control and with the KTi_3-expressing plant, K11–7. The presence of the transgene product in the leaf induced a sort of food avoidance in the larvae. As the amount of faecal debris was greatly reduced and the gut of the sacrified larvae was found to be filled with material, food avoidance was apparently caused by a reduced flow of food throughout the midgut

Table 6 Trends of live weight shown by contemporary larvae (*Spodoptera littoralis*) fed untransformed control or different KTi_3-expressing tobacco plants for 5 consecutive days. At the start of the experiment, larvae were 8 days old

Plant[a]	Live weight[b] (mg)					
	Time from tobacco leaf infestation (h)					
	0	24	48	72	96	120
Control	51 a	90 a	143 a	196 a	315 a	459 a
K19–10	49 a	61 b	103 b	121 b, c	148 b	236 b
K18–8	49 a	60 b	108 a, b	123 b	153 b	222 b, c
K3–7	45 a	58 b	83 b, c	126 b	141 b	169 b, c, d
K3–2	46 a	62 b	99 b	98 b, c, d	110 b, c	163 b, c, d
K2–1	49 a	53 b	76 b, c	100 b, c, d	107 b, c	127 b, c, d
K18–9	47 a	58 b	81 b, c	93 b, c, d	86 b, c	106 b, c, d
K16–5	51 a	58 b	68 b, c	88 b, c, d	94 b, c	102 b, c, d
K12–4	48 a	56 b	68 b, c	85 b, c, d	96 b, c	101 b, c, d
K4–1	43 a	49 b	70 b, c	82 b, c, d	88 b, c	98 b, c, d
K5–10	44 a	47 b	53 c	56 d	54 c	76 c, d
K4–9	55 a	62 b	84 b, c	99 b, c, d	66 c	73 c, d
K11–7	45 a	48 b	52 c	67 c, d	57 c	61 d

[a] The first number after the capital letter indicates the primary transformant whereas the second number identifies the plant within the progeny

[b] Means with a letter in common within a column are not significantly different at P=0.01

presence of the inhibitor reduced the flow of food in the midgut and this led to a food refusal (Fig. 5). Due to the altered feeding behaviour, larvae grew irregularly on inhibitor-rich plants and sometimes weight losses occurred between two consecutive weighings (Table 6). The comparison between the proteolytic activities in larvae fed transformed or untransformed tobacco leaves revealed a clear reduction in the former. Using a plant producing approximately 165 µg SBTI-A_2 ml^{-1} crude sap, the proteolytic activity in 35-mg larvae was decreased by 68%; the dietary effect upon the midgut proteolytic activity was less pronounced when the inhibitor content in the transgenic leaf tissue was also lower.

Discussion

Transformation of tobacco and potato plants with soybean proteinase inhibitor genes induced a variable degree of resistance towards *S. littoralis*, an insect with proteolytic enzymes of the serine type (Marchetti et al. 1998). Although the Kunitz inhibitor gene seemed to give the best results, ranking of inhibitors was not related to their activity against a standard proteinase such as bovine trypsin. In fact, PI-IV, which is a weak inhibitor of this enzyme (Odani and Ikenaka 1978) hindered larval growth at least as much as C-II, an inhibitor to which all mammalian trypsins are very sensitive (Odani and Ikenaka 1977). The *PI-IV* gene must therefore be regarded as an important defence element and not merely the ancestor sequence from which the classic Bowman-Birk inhibitor probably originated.

In general terms, the evidence collected in the present study is similar to that reported by Hilder et al. (1987) and Johnson et al. (1989), who also showed that inhibitors play an important role in limiting and defining the range of insect pests potentially harmful to plants. However, when working with the same array of genes on two crops, it was possible to demonstrate that different levels of insect resistance can be achieved by using different plant species as targets for transformation. While transgene products displayed a strong insecticidal activity in tobacco, all of them acted as growth retardants when inserted in potato. It should be pointed out that a different rate of gene expression cannot be claimed as the only reason for this: tobacco and potato plants, transformed with the same construct and sharing the same crude sap TIA, still affected larval growth in very distinct ways. For example, tobacco plant K8–5 and potato plant K2 had identical trypsin inhibitor contents (85 µg per milliliter crude sap), but the former killed 95% of *Spodoptera* first-instar larvae within 48 h, whereas the latter caused only a 36% growth reduction over a 5-day period. Similar gaps between tobacco and potato plants were recorded regardless of the transgene considered. It can therefore be concluded that results in the two species differed also because transgenes were added to native defence mechanisms of different types and strength. Actually, we found that the nutritional value of untransformed tobacco leaf tissue is only 26% of that of potato.

Secondly, our results clearly indicate that transferring proteinase inhibitor genes from one plant to another without knowing the intimate nature of the molecular targets is an approach whose response is left primarily to chance. Maybe it is for this reason that a general disappointment over the value of proteinase inhibitors has accumulated over time. None of the transgenic potato plants produced in this study was really resistant to *S. littoralis*; parallel experiments, designed to isolate and characterize the main proteolytic enzymes of this insect, revealed that results achieved with transgenic potatoes are just those expected on the basis of insect proteinase susceptibility to Kunitz- as well as Bowman-Birk-type inhibitors. In fact, two trypsin-like enzymes were found in the Sudan strain of *S. littoralis*, but only one was inactivated by soybean inhibitors (Marchetti et al. 1998). From the evidence collected, it appears that the overall strategy of building insect resistance should be modified, giving more emphasis to the isolation and characterization of insect proteolytic enzymes. Their availability would allow the application of methods for selecting more powerful and more specific inhibitors before plant transformation.

In a previous study (Gatehouse et al. 1994) the decrease in larval biomass was only roughly proportional to the level of transgene expression. To our knowledge, we provide here the first evidence of such a relationship; it is noteworthy that this result was achieved by measuring the inhibitor content in less relative terms (microgram of inhibitor per milliliter of crude sap) than the ones commonly used (% soluble proteins). Insect resistance may therefore be increased through selection, and the best transformants can be screened out by measuring the inhibitory activity of the crude sap. Since the analytical method only requires 120 mg leaf tissue, plants can be selected early, and the bioassay is consequently no longer conducted on plants with little or no value, thus saving considerable time and labour.

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