Inhibition of Heliothis virescens *larvae growth in transgenic tobacco plants expressing cowpea trypsin inhibitor*

RESEARCH

✉ Merardo Pujol[1], Carlos A Hernández[2], Raúl Armas[2], Yamilet Coll[1], Julio Alfonso-Rubí[2], Maylin Pérez[2], Camilo Ayra[1], Annerys González[2]

[1] Center for Genetic Engineering and Biotechnology, CIGB
Ave 31 e/ 158 and 190, Playa, PO Box 6162, Havana 10 600, Cuba
E-mail: merardo.pujol@cigb.edu.cu
[2] Center for Genetic Engineering and Biotechnology, CIGB
PO Box 83, Sancti Spiritus 60 200, Cuba

ABSTRACT

Fall armyworm (*Spodoptera frugiperda*) is an important pest for corn and rice in Cuba. Here, we describe the obtainment of transgenic tobacco plants expressing the cowpea trypsin inhibitor (cpti) gene as a model to explore the efficiency of this protein for fall armyworm control. Tobacco plants were transformed with *Agrobacterium tumefaciens* containing a plasmid conferring kanamycin resistance and carrying the cpti gene fused to the 35S promoter and the enhancer of the Ω fragment from the TMV. From the regenerated tobacco clones, lines 19 and 26 showed higher trypsin inhibitory activity and were chosen for molecular characterization and bioassays against insects. Tobacco budworm larvae feeding on leaf discs from clone 19 showed marked inhibition growth as a consequence of cpti in the plants. This demonstrates the usefulness of the cpti protein in the control of insects with trypsin-like protease activity and supports its potential to be combined with insecticidal *Bacillus thuringiensis* Cry proteins in transgenic plants as an alternative against the development of insect resistance.

Key words: cowpea trypsin inhibitor, *Spodoptera frugiperda*, transgenic tobacco plants

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RESUMEN

Interferencia del crecimiento de larvas de *Heliothis virescens* en plantas transgénicas de tabaco que expresan el inhibidor de tripsina del frijol caupí. La palomilla del maíz (*Spodoptera frugiperda*) es una plaga importante en los cultivos de maíz y arroz en Cuba. En este trabajo, describimos la obtención de plantas transgénicas de tabaco que expresan el inhibidor de tripsina del frijol caupí (cpti), como un modelo para evaluar la potencialidad de esta proteína en el control de la palomilla del maíz. Se transformó el tabaco via *Agrobacterium tumefaciens* conteniendo un plásmido que confiere resistencia a la kanamicina y que porta el gen cpti fusionado al promotor 35S del CaMV y al activador transcripcional Ω del TMV. Entre los clones regenerados, las líneas transgénicas 19 y 26, mostraron la mayor actividad inhibitoria de la tripsina y fueron escogidas para su caracterización molecular y para ensayos frente a insectos. Las larvas del cogollero del tabaco que fueron alimentadas con discos de hojas del clon 19 mostraron una marcada afectación de su crecimiento como consecuencia de la expresión del cpti en las plantas. Esto demuestra la utilidad de la proteína cpti para en el control de insectos con actividad proteasa tipo tripsina, y apoya su potencialidad de combinarla con proteínas insecticidas Cry de *Bacillus thuringiensis* en plantas transgénicas, como una alternativa contra el desarrollo de insecto resistencia.

Palabras clave: inhibidor de tripsina del frijol caupí, *Spodoptera frugiperda*, plantas transgénicas de tabaco

Introducción

A number of proteinase inhibitors from many plant species have been isolated and characterized by their biochemistry and genetics [1]. With the advent of plant genetic engineering, the introduction of foreign genes allowed the expression of recombinant proteinase inhibitors from several sources in transgenic tobacco [2, 3] and other species, including monocotyledons as rice, providing resistance to major crop pests [4, 5]. However, the only pesticidal transgenic plants that have reached the market so far express the insecticidal *cry* genes from the *Bacillus thuringiensis* (Bt). As occurs with other substances used for pest control [6], insects may develop resistance to Cry proteins [7]. An alternative is the alteration of insect gutproteases that inactivate or reduce Cry toxin potency. Protease inhibitors could play a major role in the neutralization of insect gut proteases, hence increasing the potency of any Bt product. In the case of Bt-plants, the expression of a protease inhibitor in addition to the Cry toxin could result in better control of target pests, and give a longer lasting resistance of the transgenic plants. The use of this approach, however, is not completely straightforward, since some reports have recently suggested the importance of devising refined strategies for the effective inhibition of insect digestive proteinases *in vivo*, based on the use of recombinant hybrid inhibitors active against different protease classes [8, 9].

Fall armyworm (*Spodoptera frugiperda* Smith) is one of the most important pests in tropical regions, where it affects major crops such as rice, corn and tomato [10]. Previously, we have characterized the digestive activity of fall armyworm larvae as being trypsin-like and demonstrated its inhibition by various inhibitors of plant origin [11, 12]. We are especially interested in the study of the *cpti* trypsin and its inhibitory efficacy against this pest or others with

✉ Corresponding author

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similar digestive activity for use in future transgenic Bt-rice and-corn plants [13].

Here, we describe the transformation of tobacco plants with the cowpea trypsin inhibitor (*cpti*) gene and the CPTI biological activity against the tobacco budworm *Heliothis virescens* and for the potential of this gene for fall armyworm control. It was previously reported that insects of the *Heliothis* genus have the same type of digestive activity as the fall armyworms [14]. This makes it possible to use this insect as a model for the potential of this inhibitor for transgenic rice and corn plants against fall armyworm attacks. In addition, tobacco requires less laborious and time-consuming tissue cultures and genetic transformation procedures than rice and corn plants.

Materials and methods

General methods

All DNA procedures, including double-strand DNA sequencing were performed according to Manniatis *et al* [15].

Cloning the cowpea trypsin inhibitor gene

A 350bp *Nco*I-*Xba*I fragment comprising the *cpti* gene (Genbank accession AY204562) was excised from plasmid pBlueL3 [16] and inserted into plasmid pBPFΩ8 [17] to obtain plasmid pBPFΩ*cpti*. This was *Hin*dIII digested to obtain the cassette containing the 35S promoter, the non-translatable tobacco mosaic virus (TMV) Ω enhancer, the *cpti* gene and the NOS terminator, which was inserted in the *Hin*dIII site of the plasmid pBin19 [18] to yield the pBinΩ*cpti* plasmid (Figure 1).

Plant transformation

Leaf discs from Petit Havana SR-1 tobacco plants grown *in vitro* were infected with the *Agrobacterium tumefaciens* LBA 4404 strain carrying the binary plasmid pBinΩ*cpti* according to the procedure described by Horsch *et al*. [19]. Seeds from self-pollinated plants were germinated on an MS medium [20] containing 50 mg/L of kanamycin. F_1 plants resistant to kanamycin were transplanted to pots and grown in greenhouse conditions for further analysis.

Protein extraction

Leaves of the same developmental stage from transgenic and non-transgenic tobacco plants were macerated in liquid nitrogen. The resulting powder was homogenized at a 1:2 ratio in the extraction buffer (100 mmol L^{-1} Tris-HCl pH 8.2, 1.5% polyvinylpirro-lidone (m/v), 5% glycerol (v/v), and 77 mmol L^{-1} β-mercaptoethanol) and then sieved through cheesecloth and centrifuged (13 000 g, 10 min, 4 °C) to remove cell debris. The supernatant was stored at -70 °C for later use. Protein concentration was determined by the Bradford method [21] using bovine albumin protein as the standard.

Trypsin inhibition assays

Total protein extracts from tobacco plants were used for assaying inhibitory activity against bovine pancreas trypsin [22]. Briefly, protein extracts (75 µg) from transgenic and non transgenic plants were incubated with 2.8 µg of trypsin in 100 µL of 100 mmol L^{-1} Tris buffer for 15 min at 25 °C. N-benzoil-L-p-nitroanilide (L-BapNA) substrate was added to 1 mmol L^{-1} in a final volume of 700 µL and the enzyme activity was measured in a spectrophotometer at 410 nm. Enzymatic activity was expressed in nmol min^{-1} with an extinction coefficient of 8.8 cm^2 μmol^{-1} for L-BapNA. Each assay was repeated three times.

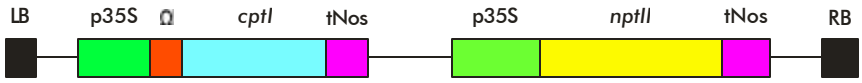

Figure 1. Schematic representation of the T-DNA region of pBinΩ*cpti* plasmid, harboring the *cpti* gene. LB=left border, RB=right border, p35S=35 S promoter from Cauliflower Mosaic Virus, Ω=TMV transcriptional enhancer, *cptI*=cowpea trypsin inhibitor gene, tNos=nopaline synthase terminator from *A. tumefaciens*, *nptII*=neomycin phosphotransferase gene.

DNA analysis

DNA from transformed and wild type tobacco plants was extracted [23], and PCR amplification was performed on genomic DNA templates using the *cpti* primers described in figure 3, which amplify a *cpti* gene fragment of 225 bp. The amplified products were electrophoresed on 0.8% agarose gel and then transferred onto a nitrocellulose membrane. Southern blot hybridization was carried out using the *Nco*I-*Xba*I *cpti* fragment from pBlueL3 as the probe. This DNA was radiolabeled by incorporating alpha ^{32}P-dATP using the Klenow fragment of DNA polymerase I and random hexanucleotide primers [24].

Bioassays

Second instar *Heliothis virescens* larvae from a laboratory colony [25] were used for the study. Leaf discs of 1.5 cm diameter from transgenic and non transgenic tobacco plants were collected, washed in distilled water, blotted on filter paper, and transferred to vials containing a gel prepared with 2% agar, 0.5 mg/mL fundazol and 0.2 mg/L streptomycin. Vials containing one disc and one larva were incubated at 27 °C. The experiments consisted of ten replicates of each treatment. Leaf discs were changed daily with new ones and the weight of the larva was recorded at 0, 2, 4, 7 and 9 days. Data was used to measure the effect of the feedings on the growth of each larva by calculating the ratio of its weights recorded at the different time points and the weight at day 0.

Results and discussion

Broadway and Duffey [14] showed that proteinase inhibitor activity in tobacco leaves accounted for a reduction in the growth rate of the beet armyworm *Spodoptera exigua* larvae when compared with controls. In another report, McManus and Burguess [26] reported a significant reduction in growth of the Egyptian armyworm *Spodoptera litura* fed with a purified soybean trypsin inhibitor as compared with insects fed an inhibitor-free diet. However, similar studies are not available for *S. frugiperda*, the predominant crop pest of this genus in the western hemisphere. This pest is polyphagous and causes major damage in corn, rice and vegetables. Previous reports have established that gut protease digestive activity of fall armyworm larvae is mainly trypsin-like and inhibited by various inhibitors of plant origin [11, 12]. We describe the obtainment of transgenic tobacco plants expressing

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the cowpea trypsin inhibitor and use them to evaluate the effectiveness of this protein against *Heliothis virescens*, a Lepidopteran defoliating pest of the same family (*Noctuidae*) carrying a digestive proteinase activity comparable to *S. frugiperda* [14, 27].

Figure 2 shows the results obtained from *in vitro* evaluation of the inhibitory activity against bovine trypsin protease extracts from four randomly selected transgenic tobacco F_1 lines compared with extracts from a non-transformed line. Extracts from lines 19 and 26 showed the highest inhibition of enzymatic activity. These lines were able to inhibit 78 and 63% of the trypsin activity respectively. Others [3] have reported a 70% inhibition of the bovine pancreas trypsin activity with 90 µg of total protein extracts from transgenic tobacco plants expressing the tomato type II inhibitor. As shown in figure 2, we obtained 78% of *in vitro* bovine trypsin activity inhibition with 75 µg of total protein extract from clone 19. Although the *cpti* expression levels were not quantified in the transgenic plants, greater inhibition was achieved using less total protein extracts, as compared with that reported by Johnson *et al*. [3], which might be explained by higher expression levels of the inhibitor because of the inclusion of the TMV Ω fragment in the constructs. This sequence has been previously reported as an enhancer of gene expression in transgenic tobacco plants when combined with the CaMV 35S promoter [17, 28]. Also, a recent report argues that the inhibitor expression levels will determine the insecticidal effectiveness of the protein or the development of the adaptive capacity of the target pest [29].

To confirm the transgenic nature of the plants, we designed oligonucleotide primers and a band of 225 nucleotides from the *cpti* gene was amplified by PCR from transgenic tobacco clones. This band was later hybridized (PCR-Southern) using a radiolabeled fragment of the *cpti* gene. A single band corresponding to the *cpti* gene amplified product at the correct molecular size was visualized in a PCR-Southern experiment of clones 19 and 26 but not in non-transformed plants after probing the membrane with the *Nco*I-*Xba*I *cpti* fragment, thus confirming the transgenic nature of clones 19 and 26 (Figure 3).

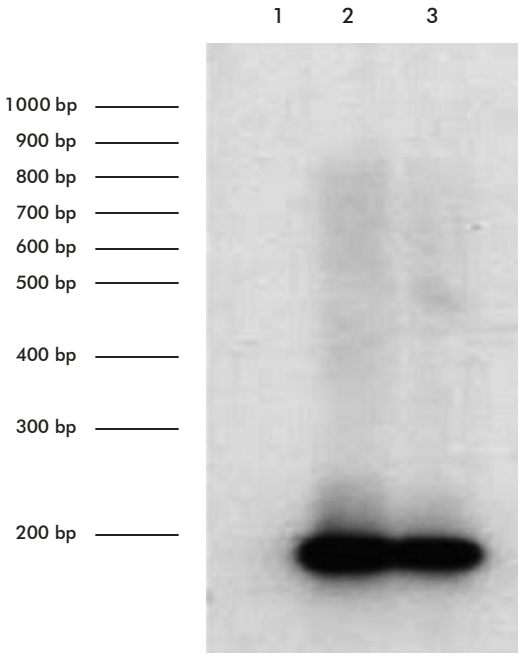

Figure 3. PCR-Southern showing the presence of the *cpti* gene in the transgenic tobacco plants genome. The internal primers gggtcactactgcagccatgg (5´) and tcaaggcaacgacacttgcc (3´) were used to detect the *cptI* gene in transgenic plants. Lane 1, non-transformed plant; lane 2, clone 19; lane 3, clone 23. 100 bp DNA ladder was used as a molecular weight marker (Promega, Madison, USA).

Given the results of the *in vitro* trypsin inhibition experiments, we used clone 19 to further test its *in vivo* biological activity against *Heliothis virescens* lar-

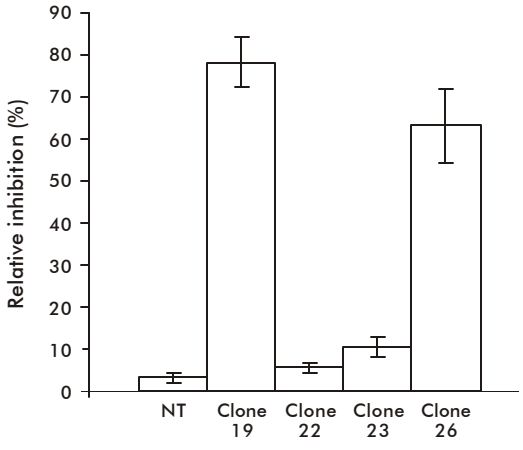

Figure 2. *In vitro* inhibition of trypsin activity by protein extracts from transgenic tobacco plants. Gut extracts from larva were assayed for protease activity in the presence of protein extracts from transgenic and non transgenic tobacco clones. Values are expressed as percentage of inhibition of protease activity of control larvae not assayed versus plant extracts. Thin bars represent the standard deviations. NT=non-transgenic plant.

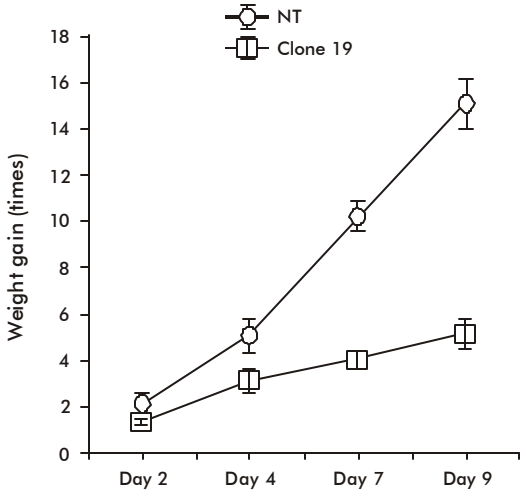

Figure 4. Growth of larvae fed with leaf discs from transgenic and non-transgenic tobacco plants. Larvae were weighted at 0, 2, 4, 7 and 9 days after starting the feeding on tobacco leaves. Values of the curve express the fold average of larvae weight gain compared to the weight recorded in the preceding evaluation. Thin bars on each point represent the standard deviations.

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vae. Figure 4 shows the weight gain of larvae fed leaf discs from transgenic or non-transgenic plants during the course of the experiment. The effects of *cpti* substantially delayed normal larvae growth compared to larvae fed on non-transformed plants, causing 5 fold lower weights. Coincidentally, a report by Chao-Yang, *et al* [16] using the same proteinase inhibitor found comparable effects on insect growth when tested against the cotton bollworm.

A recent report [30] from Zavala and Baldwin have shown that the expression of trypsin proteinase inhibitors interfering with insect metabolism account for be-nefits greater than their cost when the plants are attacked. This suggests that the proteinase inhibitor approach might result in a positive balance for plant defense.

The simultaneous use of insect-protease inhibitors and insecticidal Cry proteins in transgenic plants could be a strategy for insect-resistance management in the field. While *B. thuringiensis* Cry proteins have shown to be effective in insect control and in autologous-pesticide transgenic plants, their use may be restricted in the future by the appearance of insect-resistance, as occurring with chemical insecticides. Indeed, any of the steps in the complex multi-step mechanism of insect killing of Cry proteins could explain insect-resistance, and there are several reports on the inactivation of the Cry toxin or the decline of its potency caused by insect protease activities [31-33].

The results of this work demonstrate the feasibility of controlling insect pests with trypsin-like digestive activity by expressing trypsin inhibitors like *cpti* in transgenic plants. The combined expression with specific Cry proteins is a promising strategy for the control of plant insect-pests.

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