RESEARCH

A barley cystatin stably expressed in rice exhibits strong in vitro *inhibitory activity against gut proteinases of rice water weevil*

✍ Raúl Armas[1], Carlos Hernández[1], Daymi Abreu[1], Maylin Pérez[1], Yeosvany Cabrera[1], Merardo Pujol[2], Julio Alfonso-Rubi[1]

[1]Center for Genetic Engineering and Biotechnology, CIGB
PO Box 83, Sancti Spíritus, Cuba
[2]Center for Genetic Engineering and Biotechnology, CIGB
Ave. 31 / 158 and 190, Cubanacán, Playa, PO Box 6162, Havana, Cuba
E-mail: raul.armas@cigb.edu.cu

ABSTRACT

Rice water weevil, *Lissorhoptrus brevirostris* Suffrian (Coleoptera: Curculionidae), is one of the most important rice pests in Cuba. Here, we describe the production of transgenic rice plants expressing barley cystatin HvCPI-1 (*Icy1* gene) to explore the potential of this protein for the control of rice water weevil. Rice plants (*Oryza sativa* L. cv IACuba-28) were transformed via *Agrobacterium tumefaciens* with a plasmid carrying the *Icy1* gene fused to the 35S promoter and the first exon/intron/exon from rice actin-1 gene. From 65 independent transgenic lines, 62 were positive in the PCR-Southern blot analyses. The transgene was correctly translated as indicated by western- and dot-blot assays with level of expression in T_1 plants of up to 2% of the total extracted protein. The functional integrity of the protein was confirmed in vitro by a reduction of up to 90% of the cysteine-proteinase activity in the gut of rice water weevils exposed to rice leaf extracts. Moreover, proteins extracted from T_2 transgenic rice roots showed a significant inhibition of up to 70% at pH 4.5 and 45% at pH 6.0 of the cathepsin B-like activity in the *L. brevirostris* larvae gut. These results demonstrate the potential of barley cystatin as an effective compound that may be combined with other pest control methods as an alternative in the struggle against insect resistance.

Keywords: *Lissorhoptrus brevirostris*, cystatin, transgenic rice, gut proteinase activity, curculionids

Biotecnología Aplicada 2009;26:321-327

RESUMEN

La expresión en arroz de una cistatina de cebada inhibe significativamente la actividad proteinasa digestiva del picudo acuático del arroz *in vitro*. El picudo acuático del arroz, *Lissorhoptrus brevirostris* Suffrian (Coleoptera: Curculionidae), es una de las principales plagas del cultivo en Cuba. En este trabajo describimos la obtención de plantas transgénicas de arroz que expresan la cistatina de cebada HvCPI-1 (gene *Icy1*) como un modelo para explorar la potencialidad de esta proteína en el control del picudo acuático. Se transformó arroz (*Oryza sativa* L. cv IACuba-28) vía *Agrobacterium tumefaciens* conteniendo un plásmido que porta el gen *Icy1* fusionado al promotor 35S del CaMV y al primer exón/intrón/exón del gen Act-1 de arroz. De 65 líneas transgénicas independientes, 62 resultaron positivas al análisis por PCR-Southern blot. El transgen se expresó correctamente según se pudo apreciar por western y dot blot con un nivel de expresión superior al 2% de las proteínas totales extraídas en plantas T_1. La integridad funcional fue confirmada por la reducción hasta un 90% de la actividad cisteino proteinasa en el extracto digestivo de larvas del picudo acuático por extractos de hojas de arroz. Además, extractos de raices de plantas transgénicas de generación T_2 produjeron una inhibición significativa, 70% a pH 4.5 y 45% a pH 6.0, de la actividad tipo catepsina B en extractos del intestino de larvas de *L. brevirostris*. Estos resultados demuestran el potencial de la cistatina de cebada como un efectivo componente para ser usado en combinación con otras estrategias para el control de esta plaga como una alternativa contra el desarrollo de insecto resistencia

Palabras clave: *Lissorhoptrus brevirostris*, cistatina, arroz transgénico, actividad proteinasa digestiva, curculiónidos

*I*ntroduction

Water weevils are widely distributed and economically important because they feed on rice roots. Particularly, during its larval stage the *Lissorhoptrus brevirostris* (Coleoptera: Curculionidae), the most destructive rice pest in Cuba, may reduce yields by 30-60% with a sever infestation [1]. The semi-aquatic adults of this insect feed on the leaves of rice and other aquatic grasses but do not cause economic losses. The larval and pupal stages of the rice water weevil (RWW) take place, almost completely, in flooded or water-saturated soils, where they feed on rice roots causing important economic losses by affecting the labor and plant growth in the infested fields. The ecology and behavioral habits of RWW make it difficult to combat this pest. Chemical applications combined with a biological control of entomo-pathogenic fungi against adults are being used to control the RWW in Cuba. However, due to their potential risk on the environment, alternative approaches should be included in conventional plant-protection programs. We have studied digestive endoprotease activities of *L. brevirostris* larvae and found that the anterior and middle sections of the gut express mainly the cysteine-proteinase type, essentially cathepsin B-like [2]. This stron-

✍ Corresponding author

1. Meneses R, Gutiérrez A, García A, Antigua G, Gómez J. Guía para el trabajo de campo en el manejo integrado de plagas del arroz. Cuarta edición revisada y ampliada en 2001; [http://www.ciat.cgiar.org/riceweb/pdfs/guia_trabajo_campo.pdf, active August 2009]

2. Hernández C, Pujol M, Alfonso-Rubi J, Armas R, Coll Y, Pérez M, *et al*. Proteolytic gut activities in the rice water weevil, *Lissorhoptrus brevirostris* Sufrian (Coleoptera: Curculionidae). Arch Insect Biochem Physiol 2003;53:19-29.

gly suggests that digestive cysteine-proteinases are required for their digestion and that plants expressing cystatins would be useful for pest control.

Cystatins are a group of proteins specifically inhibiting cysteine-proteinases of the papain family C1A [3]. Most of the plant cystatins, referred to as phytocystatins (PhyCys), are small proteins with a molecular mass ranging from 11 to 16 kDa, although some of them contain a carboxy-terminal extension with a molecular size of ~23 kDa [4-6]. Martinez *et al.* [7] have demonstrated that these extended PhyCys are bifunctional inhibitors of papain and legumain cysteine-proteinases. Several multi-PhyCys of 87 kDa have also been described [4, 8]. Physiologically, PhyCys play a double role, as regulators of the protein turnover [9] and programmed cell death [10], and in protein defense, being able to inhibit proteinases from heterologous predators and pathogens. This protective function is supported by *in vitro* data on the inhibition of digestive proteinases of insects and nematodes [8, 11] and by the enhanced resistance against insects, nematodes, slugs and viruses obtained in transgenic plants over-expressing PhyCys [12-15]. Moreover, antifungal and antimite activities have also been described for several PhyCys [5, 6, 16]. The characterization of barley cystatin HvCPI-1 and variants derived from it, and the analysis of their inhibitory capacity against both cysteine-proteinases of different origin and the growth of phytopathogenic fungi have been reported [17, 18].

It has been shown that the ingestion of cystatins in natural or artificial diets increases mortality and delays the development of certain curculionids such as the alfalfa weevil *Hypera postica* and monocot weevils *Sitophilus oryzae* [19, 20]. Furthermore, protein extracts from transgenic rice seeds expressing corn cystatin I inhibit digestive proteinases from *Sitophilus zeamais* [21]. Similarly, the gut proteinase activity in two strains of the cabbage seed weevil *Ceutorrhynchus assimilis*, was affected *in vitro* by the oryzacystatin I (OC-I), but surprisingly the inhibition of gut enzymes by the ingestion of OC-I transgenically expressed *in planta* was only detected in one of the curculionid strains [22]. Bonade-Bottino *et al.* [23] observed an important reduction in the gut cysteine-proteinase activity in another curculionid, *Baris coerulescens,* reared with transgenic rapeseed expressing the OC-I; however, the partial compensation of this inhibition by increasing the digestive serine-proteinase activity allowed the larvae to overcome the effects of OC-I consumption. On the other hand, the over-expression in rice plants of genes encoding inhibitors of proteinases of different mechanistic classes has been successful in many cases, producing increased resistance against insects feeding on vegetative tissues and storage pests [21, 24-29].

This paper describes the inhibitory properties of barley cystatin HvCP-1 expressed in *indica* rice plants cv IACuba-28 on the gut cysteine-proteinases of the RWW *L. brevirostris*. Additionally, we report the integration of the barley *Icy1* gene into the rice plants and the analysis of their stable expression at the protein level of this cystatin in rice leaves and roots. These results, together with the demonstration of the ability of transgenic rice leaves and roots to inhibit the digestive proteinases of *L. brevirostris* at the larval stage, make barley cystatin a possible insecticidal protein that can be combined with other control methods for an integrated pest management system against the RWW in Cuba.

Materials and methods

Materials from plants and insects

Cultures of embryogenic calluses of *Oryza sativa* L. (cv IACuba-28) derived from seeds were produced and maintained as described by Coll *et al.* [30]. They were routinely sub-cultured and used to transform and generate rice plants.

Larvae of rice water weevil *L. brevirostris* Suffrian (Coleoptera: Curculionidae) used in the experiments were obtained from the rice roots collected in a rice paddy in Sancti-Spíritus (Cuba). Larvae stages at third and fourth instars were isolated and the gut was dissected, frozen in liquid nitrogen and stored at -70 ºC for later use in enzymatic inhibition assays.

Plasmid constructs

A 450 bp *BamHI-NcoI* fragment containing the complete ORF of the barley cystatin gene (*Icy1*) was excised from the fusion expression vector pRSETA, where this gene had been cloned by Gaddour *et al.* [18], and inserted into vector pBPFA9 [31] to obtain the plasmid pBPFA-HvCPI-1. This construction was digested with *PstI* to obtain the *Icy1* gene expression cassette that was then inserted in the *PstI* site of the pCAMBIA 1300 (CAMBIA BioForge) plasmid to yield the final pC1300-HvCPI-1 construct used in further transformation experiments (Figure 1).

Plant transformation

The *A. tumefaciens,* strain EHA-105 that had been transformed with plasmid pC1300-HvCPI-1, was used for the transformation of embryogenic calluses obtained from mature seeds of *Oryza sativa* L. (cv IACuba-28) following the procedure described by Hiei *et al*. [32], with minor modifications. After a selection on 50 mg/L hygromycin, surviving calluses were transferred onto a KIBAN regeneration media [30] containing 30 g/L maltose instead of sucrose [33] plus 50 mg/L hygromycin. Regenerated plants were individualized on an MS medium [34] containing 50 mg/L hygromycin to induce root formation. Putative primary transformants (T_0 generation) were acclimatized in a greenhouse, transplanted to big plastic pots filled with soil, watered daily and grown to maturity.

3. Rawlings ND, Morton FR, Barrett AJ. MEROPS: the peptidase database. Nucleic Acids Res 2008;36:320-25.

4. Girard C, Rivard D, Kiggundu A, Kunert K, Gleddie SC, Cloutier C, *et al*. A multicomponent, elicitor-inducible cystatin complex in tomato, *Solanum lycopersicum*. New Phytol 2006;173:841-51.

5. Martínez M, Abraham Z, Gambardella M, Echaide M, Carbonero P, Diaz I. The strawberry gene *Cyf1* encodes a phytocystatin with antifungal properties. J Exp Bot 2005;56:1821-29.

6. Abraham Z, Martínez M, Carbonero P, Díaz I. Structural and functional diversity within the cystatin gene family of *Hordeum vulgare*. J Exp Bot 2006;57:4245-55.

7. Martínez M, Díaz-Mendoza M, Carrillo L, Díaz I. Carboxy terminal extended phytocystatins are bifunctional inhibitors of papain and legumain cysteine proteinases. FEBS Lett 2007;581:2914-18.

8. Siqueira-Junior CL, Fernandez KVS, Machado OLT, Cunha M, Gómez VM, Moura D, *et al*. 87 kDa tomato cystatin exhibits properties of a defence protein and forms crystals in prosystemin overexpressing transgenic plants. Plant Physiol Biochem 2002;40:247-54.

9. Kiyosaki T, Matsumoto I, Asakura T, Funaki J, Kuroda M, Misaka T, *et al*. Gliadain, a gibberellin-inducible cysteine proteinase occurring in germinating seeds of wheat, *Triticum aestivum* L., specifically digests gliadin and is regulated by intrinsic cystatins. FEBS J 2007;164:470-77.

10. Solomon M, Belenghi B, Delledonne M, Levine A. The involvement of cysteine proteases and protease inhibitor genes in programmed cell death in plants. Plant Cell 1999;11:431-44.

11. Haq SK, Atif SM, Khan RH. Protein proteinase inhibitor genes in combat against insects, pests, and pathogens; natural and engineering phytoprotection. Arch Biochem Biophys 2004;431:145-59.

12. Gutiérrez-Campos R, Torres Acosta J, Saucedo-Arias LJ, Gómez-Lim MA. The use of cysteine proteinase inhibitors to engineer resistance against potyviruses in transgenic tobacco plants. Nature Biotechnol 1999;17:1223-26.

13. Walker AJ, Urwin PF, Atkinson HJ, Brain P, Glen DM, Shewry PR. Transgenic *Arabidopsis* leaf tissue expressing a modified oryzacystatin shows resistance to the field *Deroceras retuculatum* (*Mull*). Transgenic Res 1999;8:95-103.

14. Atkinson HJ, Grimwood S, Johnston K, Green J. Prototype demonstration of transgenic resistance to the nematode *Radopholus simies* conferred on banana by a cystatin. Transgenic Res 2004;13:135-42.

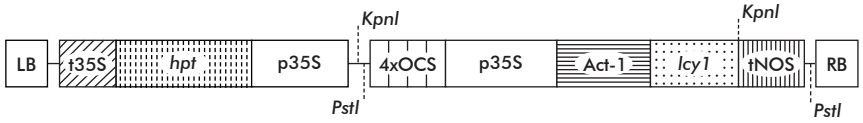

Figure 1. Schematic representation of the T-DNA region of the pC1300-HvCPI-1 plasmid harbouring the barley cystatin (*Icy1*) gene expression cassette. LB - leaf border, RB - right border, p35S - 35S promoter from the Cauliflower Mosaic Virus, 4xOCS-quadruplicated octopine synthase enhancer element from *A. tumefaciens*, Act-1 - first exon/intron/exon from rice Actin1 gene, *Icy* - barley cystatin inhibitor gene, tNOS - nopaline synthase terminator from *A. tumefaciens*, *hpt* - hygromicin phosphotransferase gene, t35S - 35S terminator from Cauliflower Mosaic Virus. *PstI* and *KpnI* restriction sites of the whole expression cassette are marked.

Seeds from self-pollinated plants were always germinated in a hygromycin-MS medium and T_1 plants were transplanted to pots and grown under greenhouse conditions (16 h light: 8 h dark photoperiod and a temperature of 28-30 °C) for further assays.

DNA analysis

Total DNA was isolated from leaves of transgenic and wild type rice plants [35] and PCR amplification was performed on genomic DNA templates, using the internal primers ctgcctgaaaccgaactgc (5´) and cttctgcgggcgatttgtg (3´), to amplify a *hpt* selective gene fragment of 632 bp. The amplified products were separated by electrophoresis in 0.8% agarose gels containing ethidium bromide for further DNA visualization and transferred onto Hybond-N+ membranes (Amersham Biosciences). For PCR-Southern blot we used a DIG-11-dUTP-labelled entire *hpt* gene. The presence of the *Icy1* gene in the rice genome was tested by Southern blot following the procedure described by Potrykus and Spangenberg [36]. Purified rice DNA (10 µg) was restricted with *KpnI*, electrophoresed in 0.8% agarose gels and blotted onto Hybond-N+ membranes (Amersham Biosciences). The complete *Icy1* coding region labeled with DIG-11-dUTP was used as a probe. Hybridization, post-hybridization washes and immunological detection of the hybridized DIG-labeled probes were performed following procedures recommended by manufactures of the DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim).

Rice protein extraction and immunodetection

Rice leaves (100 mg) from 3 week-old plants were ground in liquid nitrogen, homogenized in 150 µL of Laemmli [37] buffer and heated at 100 ºC for 5 minutes. After centrifugation for 5 minutes at 12 000 rpm, soluble protein concentration in the supernatant was estimated following the method proposed by Bradford [38] using BSA as a standard. Proteins extracted from leaves of T_1 transgenic lines (30 µg each sample) were separated by SDS-PAGE in 12% polyacrylamide gels according to Laemmli [37] and transferred onto nitrocellulose membranes (Amersham Bioscience) with an Electro Transblot apparatus (BioRad).

Total protein extracts from roots were obtained from axenic cultures of transgenic and non-transgenic rice plants after germinating seeds for 3 weeks in an MS medium. Roots were isolated, cut into small pieces, ground in a mortar at 4 ºC and homogenized in 100 mM phosphate buffer, pH 6.0. Protein was quantified according to Bradford [38], using BSA as a standard. Aliquots of 10 µg of proteins extracted from T_2 rice roots were directly applied to nitrocellulose membranes (Amersham Bioscience) using a Hybrid-Dot-Manifold apparatus (Life Technologies Inc).

Immunological staining was carried out with the anti-HvCPI-1 antibody (produced in rabbits) at a 1: 2000 dilution, followed by washes and the addition of the second antibody (goat anti-rabbit IgG conjugated alkaline phosphatase; Sigma) at a dilution of 1: 30 000. Color was developed by the reaction of the nitroblue tetrazoilum and 5-bromo-4-chloro-3-indolyl phosphate. Recombinant HvCPI-1 protein purified from *E. coli* was prepared as a control.

Inhibitory activity of recombinant barley cystatin

The *Icy* gene encoding the barley cystatin HvCPI-1 was expressed as a fusion protein in *E. coli* following Martinez *et al*. [17] and purified using His-Band resin and elution conditions from a Ni^{2+}-column according to the manufacture's instructions (Novagen). Guts from third and fourth instar larvae of *L. brevirostris* were dissected and prepared as described by Hernández *et al*. [2] to produce an enriched midgut proteinase fraction. Protein was quantified according to Bradford [38] using BSA as the standard.

The inhibitory activity of the barley cystatin against insect digestive proteinases was assayed using 0.1% sulfanilamide-azocasein solution as the substrate. Different concentrations of recombinant barley cystatin were pre-incubated with gut extracts (100 µg) from RWW in the assay buffer (100 mM acetate buffer, pH 4.5) at 30 °C for 30 min, before adding the substrate. The reaction was incubated for 24 h and stopped with 5% TCA (trichloroacetic acid). Undigested azocasein was removed by centrifugation at 12 000 rpm for 5 minutes and the absorbance of the supernatant was read at 405 nm. All assays were carried out in triplicate and blanks were used for the spontaneous breakdown of substrates.

Effects of transgenic plant extracts on the gut proteinase activity of *L. brevirostris*

Total protein extracts from T_1 leaves and T_2 roots were obtained from axenic cultures of 3-week-old transgenic and non-transgenic rice plants grown on a hygromycin-MS medium. Leaves were ground in liquid nitrogen and homogenized in 50 mM citrate buffer, pH 5.0 containing 3.1 mM DTT. Similarly, rice roots were isolated, cut in small pieces, ground in a mortar at 4 ºC and homogenized in 100 mM acetate buffer, pH 4.5 or in 100 mM phosphate buffer, pH 6.0, depending on the conditions of the further inhibitory assays, plus 3.1 mM DTT in both buffers. Soluble proteins from root and leaf tissues were recovered from supernatants after a centrifugation for 10 minutes at 12 000 rpm at 4 ºC. In parallel, guts from the third and fourth instar larvae of RWW were dissected and prepared as described above.

The inhibition of the proteolytic activity of the gut of *L. brevirostris* by the protein extract from transgenic rice tissues was analysed *in vitro* using two different assays. First, the inhibition of non-specific proteinase activity was assayed using 0.1% sulfanilamide-azocasein solution as a substrate, and leaf proteins extracted from 65 independent transgenic lines and from non-transgenic rice. Then, the ZAA_2MNA (N-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide) substrate at a concentration of 50 µM was used to determine the specific inhibition of gut cathepsin B-like activity by root protein extracts. The specific inhibitory assay was only carried out with roots extracted from three of those transgenic lines that showed the highest inhibitory effects when leaf protein extracts were tested.

The azocasein assay was performed with 160 µg of protein extracted from the *L. brevirotris* gut and 100 µg of protein extracted from rice leaves plus 200 µg of sulfanilamide-azocasein in a final volume

15. Alvarez-Alfageme F, Martínez M, Pascual-Ruíz S, Castañera P, Díaz I, Ortego F. Effects of potato plants expressing a barley cystatin on the predatory bug *Podisus maculiventris* via herbivorous prey feeding on the plant. Transgenic Res 2007; 16:1-13.

16. Pernas M, Sánchez-Monge R, Lombardero M, Ateaga C, Castañera P, Salcedo G. *Der* p1 and *Der* F1, the highly related and major allergens from house mites, are differentially affected by a plant cystatin. Clinic Exp Allergy 2000;30:972-78.

17. Martínez M, López-Solanilla E, Rodríguez-Palenzuela P, Carbonero P, Díaz I. Inhibition of plant-pathogenic fungi by the barley cystatin Hv-CPI (gene *Icy*) is not associated with its cysteine-proteinase inhibitory properties. Mol Plant Microbe Interact 2003;16:876-83.

18. Gaddour K, Vicente-Carbajosa J, Lara P, Isabel-Lamoneda I, Díaz I, Carbonero P. A constitutive cystatin-encoding gene from barley (*Icy*) responds differentially to abiotic stimuli. Plant Mol Biol 2001;45:599-08.

19. Elden TC. Selected proteinase inhibitor effects on alfalfa weevil (Coleoptera: Curculionidae) growth and development. J Econ Entomol 1995;88:1586-90.

20. Pittendrigh BR, Huesing JE, Shade RE, Murdock LL. Effects of lectins, CRY1A/CRY1B Bt δ-endotoxin, PAPA, protease and α-amylase inhibitors on the development of the rice weevil, *Sitophilus oryzae*, using an artificial seed bioassay. Entomol Exp Appl 1997;82:201-11.

21. Irie K, Hosoyama H, Takeuchi T, Iwabuchi K, Watanabe H, Abe M, *et al*. Transgenic rice established to express corn cystatin exhibits strong inhibitory activity against insect gut proteinases. Plant Mol Biol 1996;30:149-57.

22. Girard C, Bonade-Bottino M, Pham-Delegue MH, Jouanin L. Two strains of cabbage seed weevil (Coleoptera: Curculionidae) exhibit differential susceptibility to a transgenic oilseed rape expressing oryzacystatin I. J Insect Physiol 1998;44:569-77.

23. Bonade-Bottino M, Lerin J, Zaccomer B, Jouanin L. Physiological adaptation explains the insensitivity of *Baris coerulescens* to transgenic oilseed rape expressing oryzacystain I. Insect Biochem Mol Biol 1999;29:131-38.

24. Duan X, Li X, Xue Q, Abo-El-Saad M, Xu D, Wu R. Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. Nat Biotechnol 1996;14:494-98.

25. Xu D, Xue Q, McElroy D, Mawai Y, Líder V, Wu R. Constitutive expression of a cowpea trypsin inhibitor gene, *CpTi*, in transgenic rice plants confers resistance to two major insect pests. Mol Breed 1996;2:167-73.

26. Lee AI, Lee SH, Koo JC, Chum HJ, Lim CO, Mun JH, *et al*. Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown planthopper (*Nilaparvata lugens* Stal) in transgenic rice. Mol Breed 1999;5:1-9

27. Mochizuki A, Nishizawa Y, Onodera H, Tabei Y, Toki S, Habu Y, *et al*. Transgenic rice plants expressing a trypsin inhibitor are resistant against rice stem borer, *Chilo suppressalis*. Entomol Exp Applicata 1999;93:171-8

of 160 µL of the assay buffer (50 mM citrate buffer, pH 5.0,) containing 2.5 mM DTT. The reaction was incubated for 24 h at 37 ºC and stopped with 5% TCA. Undigested azocasein was removed by centrifugation at 12 000 rpm for 5 minutes and the absorbance was read at 405 nm with a multiskan plate reader (SUMA, Cuba).

The inhibition of cathepsin B-like activity from the rice water weevil guts was determined essentially as described by Novillo *et al.* [39] with minor modifications. Fifty micrograms of the crude gut extracts were incubated with 20 µg of proteins extracted from rice roots in 150 µL of assay buffer (100 mM acetate for pH 4.5 or 100 mM phosphate for pH 6.0) containing 2.5 mM DTT, and adding 10 µL of 800 µM ZAA_2MNA substrate. After 24h of incubation at 30 ºC, the mersalyl-Fast Garnet-Brij reagent was added and the absorbance was monitored at 520 nm with a multiskan plate reader (SUMA, Cuba).

The percentage of inhibition was calculated according to the equation: (1-Ai/Ac) x 100, where Ai is the absorbance value of each sample and Ac the absorbance value of the control sample containing non-transformed rice proteins. Gut extracts were preincubated with the rice proteins for 30 min at room temperature before adding the corresponding substrate. All proteinase activities were measured after 24 h of reaction at their optimum pH and temperature. The assays were carried out in triplicate, and the blanks used to account the spontaneous breakdown of substrates without plant protein extracts were subtracted from each sample.

Results and discussion

It is well known that most curculionid species use cysteine-proteinases for digestion [28, 40-42]. Our previous results had shown cathepsin B-like activity as the main proteolytic activity located in the anterior and middle gut sections of *L. brevirostris* larvae [2]. Based on this, it seemed sensible to select cysteine-proteinases from larval guts as potential targets of cystatins in order to develop a new control system against weevil attack. To explore this approach against RWW, the most destructive insect pest of rice in Cuba, we first carried out *in vitro* inhibitory assays with the recombinant barley cystatin HvCPI-1 and proteins extracted from larval midgut from the RWW demonstrating the ability of cystatin for inhibiting the proteinase activity in this coleopteran species (Table 1). As shown in table 1, similar levels of inhibition were obtained when the cystatin was tested at three different concentrations, indicating that the amount of HvCPI-1 used in the inhibitory assay was excessive compared to the amount of proteinases in the gut extracts.

Table 1. Inhibition of protease activity from *L. brevirostris* digestive extracts by the recombinant barley cystatin HvCPI-1

HvCPI-1 protein	% Inhibition ± SE[a]
10 µg	56.8 ± 1.3
50 µg	57.2 ± 1.5
70 µg	56.1 ± 2.3

[a] Values are the mean ± standard error (SE) of triplicate measurements from unique pools of gut extracts.

Based on these results, we decided to stably express the barley cystatin in the *indica* rice cv IACuba-28. A total of 200 embryogenic calluses were co-cultured with *A. tumefaciens* transformed with the pC1300-HvCPI-1 plasmid. Forty-four independent calluses were obtained (22% of callus formation) after the selection of proliferating cells on the selective medium. The selected calluses were transferred to a KIBAN regeneration medium where 71 independent transgenic lines were regenerated, but only 65 of them grew to maturity under greenhouse conditions. The presence of the *hpt* selective gene was determined by PCR-Southern blot assays and the results revealed that 62 out of the 65 transgenic lines showed a clear 632 bp DNA band corresponding to the selective gene (Figure 2A). In addition, using Southern blot of genomic DNA purified from the T_1 generation we demonstra-

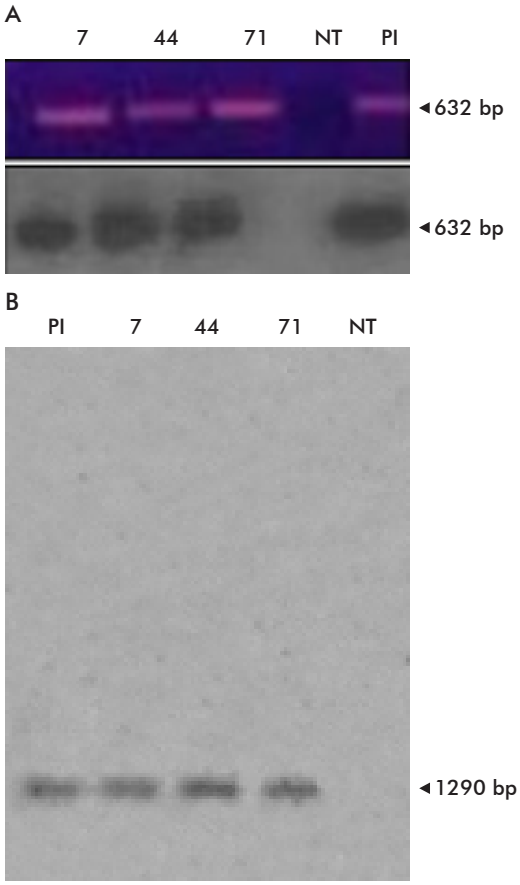

Figure 2. DNA hybridization analysis of transgenic rice plants. (a) PCR analysis (upper panel) and hybridization of PCR reaction products (lower panel) were obtained by the amplification of DNA (200 ng) isolated from T1 rice transformed leaves (lines 7, 44 and 71) after hybridization with the DIG-labelled *hpt* gene. DNA isolated from non-transformed rice leaves (NT: 200 ng) and from the plasmid pC1300-HvCPI-1 (PI: 20 ng) were used as the negative and positive controls, respectively. (b) Southern blot analysis of DNA (10 µg) isolated from transgenic T1 rice plants, digested with *KpnI* and hybridized with the DIG-labelled *Icy1* gene. DNA isolated from non-transformed rice leaves (NT: 10 µg) and from the plasmid pC1300-HvCPI-1 (PI: 50 pg) were used as negative and positive controls, respectively. Arrows indicate the hybridization bands and their corresponding sizes in base pairs.

28. Alfonso-Rubí J, Ortego F, Castañera P, Carbonero P, Díaz I. Transgenic expression of trypsin inhibitor CMe from barley in *indica* and *japonica* rice, confers resistance to the rice weevil *Sitophilus oryzae*. Transgenic Res 2003;12:23-31.

29. Vila L, Quilis J, Meynard D, Britler JC, Marfa V, Murillo I, *et al*. Expression of the maize proteinase inhibitor (*mpi*) gene in rice plants enhances resistance against the striped stem borer (*Chilo suppressalis*): effects on larval growth and insect gut proteinases. Plant Biotechnol J 2005;3: 187-02.

30. Coll Y, Pujol M, Castillo D, González A, Alfonso J, Armas R. Improvement of indica rice (*Oryza sativa*) *in vitro* regeneration efficiency from callus mediated by stress. Cereal Res Commu 1998;26:153-60.

ted the stable integration of the barley *Icy1* gene into the transformed rice plants. As shown in figure 2B, a single hybridization band of 1 290 Kb appeared in the samples analyzed after restriction with *KpnI* endonuclease.

To confirm the expression of the HvCPI-1 protein encoded by the *Icy1* gene we analyzed the proteins extracted from rice leaves with western-blot and a single band, corresponding to the estimated molecular size of 11.8 kDa of the HvCPI-1 protein [18], was observed with a different intensity. This band was absent in extracts from leaves of non-transformed rice (Figure 3A). The range of the HvCPI-1 protein expression level was estimated on 0.25 to 2.0% of the total soluble proteins in leaves by scanning densitometry analyses (data not shown), showing that the transgenic rice line TL-7 had the highest amount of cystatin (Figure 3A). Furthermore, a stronger larger size band was detected when barley cystain was purified as a recombinant protein from *E. coli* cultures. The larger protein size of approximately 16.04 kDa resulted from the fusion of the cystatin with the histidine tail to facilitate the recombinant protein purification procedure. The expression of HvCPI-1 in the root of the transgenic lines was also confirmed by dot blot (Figure 3B).

The amount of HvCPI-1 protein detected in these transgenic rice leaves showed an expression level at a similar range to those quantified in rice leaves transformed with other proteinase inhibitors that have been shown to confer resistance against insects [24-26].

Because of the difficulties in rearing the RWW under environment-controlled conditions [43] it is almost impossible to carry out feeding bioassays with this insect at the laboratory level. We therefore decided to analyze whether the cystatin activity acquired by the transgenic rice plants affects the digestive proteinases of *L. brevirostris* larvae *in vitro*. In a first approach we

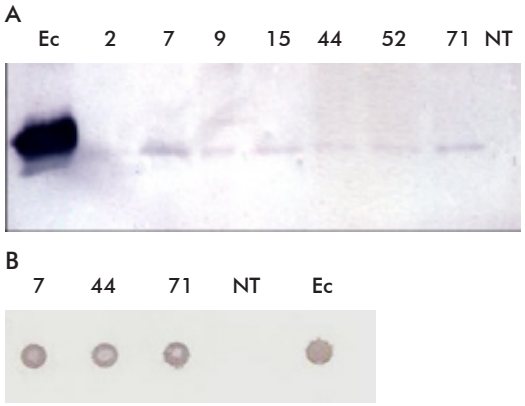

Figure 3. Immunodetection of the HvCPI-1 protein expressed in transgenic rice plants. A. Western blot analysis of proteins (30 μg) extracted from leaves of T1 transgenic rice (lines 2, 7, 9, 15, 44, 52, and 71) in SDS-PAGE gel. B. Dot blot analysis of proteins (10 μg) extracted from roots of T2 transgenic rice (lines 7, 44 and 71) directly applied to membranes. Proteins from leaves (30 μg) and roots (10 μg) of non-transformed rice (NT) and the pHvCPI-1 recombinant protein purified from *E. coli* (Ec; 5 μg and 0.8 μg for Western- and dot-blot, respectively) were used as negative and positive controls, respectively. Immunological detection was done with the anti-HvCPI-1 antibody and staining was developed by the alkaline phosphatase reaction.

analyzed the inhibition of the total proteolytic activity of the RWW larvae guts by protein extracts from leaves of the 65 independent transgenic rice lines, as well as from non-transformed rice leaves. Sixty two of the 65 transgenic lines showed an inhibitory activity that was absent in protein extracts from the non-transformed control (Figure 4). The three negative rice lines (TL-6, TL-24 and TL-69) corresponded to those that were negative in the PCR-Southern blot

31. González-Cabrera J, Coego-Gonzáles A, Martínez-Gil A, de la Riva GA, Vázquez-Padrón RI. Optimization of transgene expression in sugar-cane cells. Biotechnol Tech 1998;12:793-96.

32. Hiei Y, Ohta S, Komari T, Kumashiro T. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 1994;6:271-82.

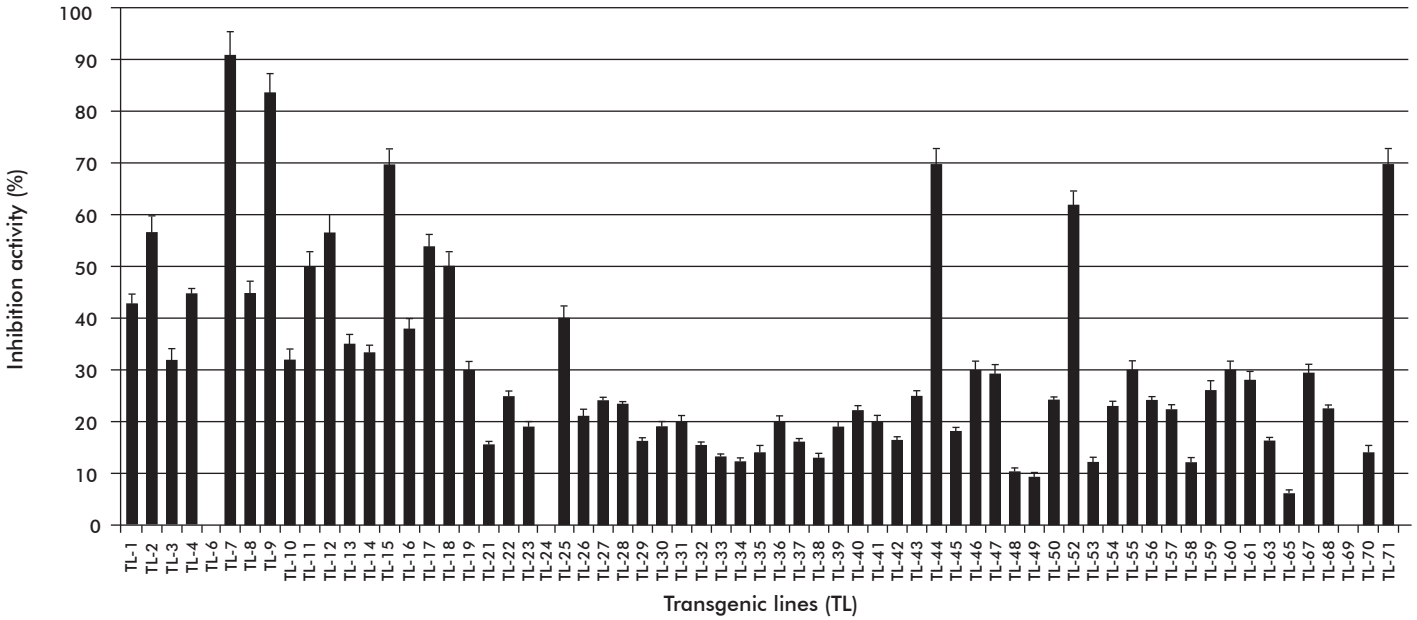

Figure 4. Inhibitory activity of protein extracts from leaves of transgenic rice lines against total digestive proteinases from *L. brevirostris* larvae. Protein extracts (100 μg) from leaves of 65 transgenic lines and from non-transformed leaves were incubated with 160 μg of gut extracts and the activity measured using azocasein as the substrate. Percentage of inhibition was calculated according to the equation: (1-Ai/Ac) x 100. Ai and Ac were the absorbance values of each sample and the control sample of non-transformed rice, respectively. Data are means ± standard errors of triplicate measurements from a unique pool of gut extracts.

assays. As shown in Figure 4, more than 50% of the gut proteinase activity of the *L. brevirostris* larvae was inhibited by 100 μg of the total soluble protein extracted from leaves in 11 lines (TL-2, TL-7, TL-9, TL-11, TL-12, TL-15, TL-17, TL-18, TL-44, TL-52 and Tl-71) from the 62 PCR positive transgenic plants. Protein extracts from lines TL-15, TL-44 and TL-71 inhibited 70% of the gut proteinase activity of the insect and particularly interesting were the transgenic lines TL-7 and TL-9 due to their ability of inhibiting 91 ± 5% and 84 ± 4% of the gut proteinase activity of *L. brevirostris,* respectively.

Considering that the economic losses in rice are attributable primarily to larvae that feed on rice roots, we selected transgenic lines TL-7, TL-44 and TL-71 to study the effect of the T_2 root protein extract on the proteolytic activity present in the guts of the RWW larvae. Our previous results had revealed that the digestive endoprotease activities of RWW larvae are mainly of the cysteine-proteinase type, essentially the cathepsin B-like. We had also found that the maximum hydrolysis of the ZAA_2MNA, the cathepsin B-like specific substrate, occurred at pH 4.5 and 6.0, respectively [2]. Taking into account these results, we carried out the inhibitory assays under both pH conditions, using the ZAA_2MNA specific substrate. Protein extracts from the three transgenic roots were good inhibitors of the gut cathepsin B-like activity, particularly when the reaction took place in 100 mM acetate under pH 4.5 (Figure 5). Regardless of the pH tested, extracts from transgenic line 7 showed the maximum inhibitory effects, being able to reduce the cathepsin B-like activity of the gut up to 70.3 ± 0.8% and 44.9 ± 1.5%, at pH 4.5 and 6.0, respectively.

The two peaks of maximum activity at pH 4.5 and 6.0 resulting from the hydrolysis of the specific substrate ZAA_2MNA by digestive endoproteases of *L. brevirostris* larvae suggest the presence of at least two cysteine-proteinases. Moreover, the gelatine-PAGE gels confirmed at least three proteinase forms at pH 5.0 [2]. These results were consistent with the differences detected on the inhibitory ability of the root protein extracts tested a pH 4.5 and 6.0, confirming the coexistence of more than one cathepsin B-like activity in the gut extracts of the RWW.

In vivo assays will definitively confirm the potential of the barley cystatin gene as a defense protein to combat RWW, but unfortunately the complex feeding habits of this pest makes it almost impossible to develop this kind of feeding test. At this state of the art, it is important to mention that several transgenic rice plants expressing proteinase inhibitor encoding genes have been already proven to have an effect against lepidopteran [24, 25] and coleopteran rice pests [21, 28]. Duan *et al.* [24] introduced the potato trypsin/chymotrypsin inhibitor PINII into several japonica rice varieties and showed an increased resistance to *S. inferens*; in pot experiment they found that the number of plants with *S. inferens* symptoms in transgenic plants were significantly lower (16-17%) than those of non transformed plants (72-100%). Xu *et al.* [25] also reported transgenic rice carrying the cowpea trypsin inhibitor *(CpTi)* gene with enhanced resistance to two species of rice stem borers, *C. suppressalis* and *S. infestans,* which are major rice insect pests. The corn

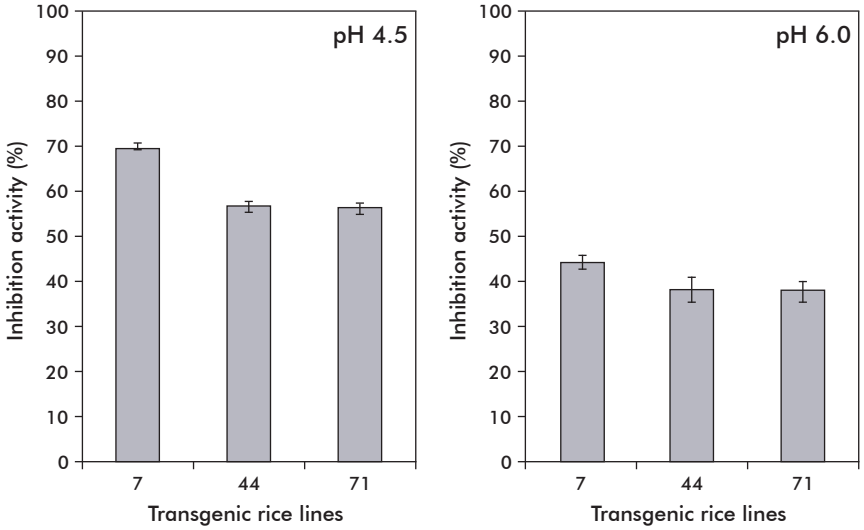

Figure 5. Inhibitory activity of protein extracts from roots of transgenic rice lines against the specific cathepsin B-like proteinases from the gut of L. brevirostris larvae. Protein extracts (20 μg) from roots of the transgenic lines (7, 44 and 71) and from non-transformed roots were incubated with 50 μg of gut extracts and the activity was measured using ZAA2MNA as the substrate. The assays were carried out at pH 4.5 and 6.0 under the conditions indicated in materials and methods. Percentage of inhibition was calculated according to the equation: (1-Ai/Ac) x 100. Ai and Ac were the absorbance values of each sample and the control sample of non-transformed rice, respectively. Data are means ± standard errors of triplicate measurements from a unique pool of gut extracts.

cystatin CC-I expressed in rice seeds inhibited the digestive proteinases of the *Sitophilus zeamais* [21] and the trypsin inhibitor BTI-CMe from barley expressed in *indica* and *japonica* rice seeds conferred resistance to *S. oryzae* [28]. However, as far as we know, there are no reports where plant proteinase inhibitors had been used as transgenes to fight insect pests feeding on rice roots. We have only found one paper where roots of transgenic rice plants expressing the modified oryzacystatin OC-IΔD86 conferred resistance to root predators such as the nematode *Meloidogyne* spp [44]. It is known that it is difficult to study and combat insect pests specifically feeding on roots and it is even more difficult if the roots are growing under water.

In conclusion, the recombinant cystatin HvCPI-1 from barley is a good inhibitor of the cysteine-proteinases present in the gut of the RWW and it also functions properly as an inhibitor when it is stably expressed in rice. Although we successfully expressed the HvCPI-1 gene under a constitutive promoter and confirmed its active expression in rice roots it will be of outstanding interest to express the gene under the control of a strong root specific promoter.

Furthermore, the antifungal properties expressed by the barley cystatin HvCPI [17] combined with its ability to inhibit the cysteine-proteinases of the gut of the RWW (shown in this paper) also suggest that a leaf- and root-specific promoter could be used to direct, simultaneously, the expression of the *Icy* gene in organs attacked by pathogens and pests without altering seed quality.

Insect herbivores have developed effective strategies to elude the inhibitory effects of plant protease inhibitors, including the use of complex digestive protease systems with proteases of different mechanistic classes acting in a coordinated manner; the production of alternative, insensitive protease forms following the

33. Pérez M, Coll Y, González A, Alfonso-Rubí J, Armas R, Hernández C, *et al*. Influencia de la fuente de carbono y el agente gelificante sobre la regeneración de arroz indica variedad IACuba-28 a partir de callos. Biotecnol Vegetal 2002; 2:183-86.

34. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissues cultures. Physiol Plant 1962;15:473-97.

35. Taylor B, Powell A. Isolation of plant DNA and RNA. Focus 1982;4:4-6.

36. Potrykus I, Spangenberg G, editors. Gene transfer to plants. Springer: Berlin-Heidelberg-New York; 1995.

37. Laemmli UK. Cleavege of structural proteins during assembly of the head of bacteriophage T4. Nature 1970;277:680-85.

38. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

39. Novillo C, Castañera P, Ortego F. Characterization and distribution of chymotrypsin-like and other digestive proteases in Colorado potato beetle larvae. Arch Insect Biochem Physiol 1997;36:181-201.

40. Baker KJE. Digestive proteinases of *Sitophilus* weevils (Coleoptera: Curculionidae) and their response to inhibitors from wheat and corn flour. Can J Zool 1982; 60:3206-14.

41. Matsumoto I, Emori Y, Abe K, Arai S. Characterization of a gene family encoding cysteine proteinases of *Sitophilus zeamais* (maize weevil), and analysis of the protein distribution in various tissues including alimentary tarot and germ cells. J Biochem 1997;121:464-76.

ingestion of protease inhibitors; and the degradation of defensive protease inhibitors using non-target, insensitive digestive proteases [45]. In this context, the development of recombinant protease inhibitors with strong inhibitory effects that are specific to the targeted organism represents a challenging task for the future of this strategy. From a biotechnology viewpoint, one challenge now is to generate insect-specific cystatin variants with improved activity against the digestive protease of the target herbivore but decreased activity against non-target cysteine-proteases in the surrounding environment. Recently, Goulet *et al*. [46] were able to obtain four mutants of the tomato muticystatin unit *Sl*CYS8 that exhibited improved inhibitory activity against both cystatin-sensitive and cystatin-insensitive digestive cysteine protease of Colorado potato beetle, while also exhibiting lowered, or unaltered, activity against cysteine proteases of potato leaves and protease I, the main digestive cysteine protease of the insect predator *P. bioculatus.*

In general, cystatins are not new to human and animal diets. They occur naturally in seeds such as those of rice and maize [47] and are present in potato tubers [48]. Cystatins also occur in human saliva [49] and are present at a high level in egg-whites [50]. Finally, the absence of target cysteine proteases in the human gut and the negligible negative effects expected for these proteins in foods [51, 52] turn cystains into a good candidates for the design of pest-resistant transgenic crops intended for human use.

For future perspectives and in order to avoid their adaptation, the pyramiding approach will be an alternative to control this rice weevil by the co-expression of genes encoding cystatins and the *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionis*, which has been reported as an effective control of other curculionids [53, 54], or with other genes with insecticidal properties. In fact, the expression of multiple insecticidal transgenes is currently being used by different Chinese groups to increase the efficacy of transgenic rice against insect pests [55-57].

Acknowledgments

This study was supported by the Cuban Council of State. The authors acknowledge the kind gift of the *Icy-1* gene by the Dr. Isabel Diaz, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid. We are also grateful to Manuel Delgado from the Estación Experimental del Arroz Sur del Jíbaro (Sancti-Spíritus) for his technical assistance in the collection of rice water weevil larvae and to Jorge Salcedo for his general assistance.

42. Wilhite SE, Elden TC, Brzin J, Smigocki AC. Inhibition of cysteine and aspartyl proteinases in the alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors. Insect Biochem Mol Biol 2000;30:1181-88.

43. Zhang Z, Stout MJ, Shang H, Pousson RC. A method for rearing the rice water weevil, *Lissorhoptrus oryzophilus* (Coleoptera: Curclionidae), in the laboratory. The Coleopterists Bulletin 2004;58:644-51.

44. Vain P, Worland B, Clarke MC, Richard G, Beavis M, Liu H, Kholi A, *et al*. Expression of an engineered cysteine proteinase inhibitor (Oryzacystatin-IΔD86) for nematode resistance in transgenic rice plants. Theor Appl Genet 1998;96:260-71.

45. Broadway RM. The adaptation of insects to protease inhibitors. In: Michaud D, editor. Recombinant Protease Inhibitors in Plants. Landes Bioscience, Georgetown TX; 2000. p.80-8.

46. Goulet MC, Dallaire C, Vaillancourt LP, Khalf M, Badri AM, Preradov A, *et al*. Tailoring the specificity of a plant cystatin toward herbivorous insect digestive proteases by single mutations at positively selected amino acid sites. Plant Physiol 2008;146:1010-9.

47. Richardson M. Seed storage proteins: the enzyme inhibitors. Methods Plant Biochem 1991;5:259-305.

48. Rodis P, Hoff JE. Naturally occurring protein crystals in the potato. Plant Physiol 1984;74:907-11.

49. Veerman ECI, VandenKeybus PAM, Vissink A, Amerongen AVN. Human glandular salivas: their separate collection and analysis. Eur J Oral Sci 1996;104:346-52.

50. Awade AC. On hen egg fractionation: applications of liquid chromatography to the isolation and the purification of hen egg white and egg yolk proteins. Z. Lebensm.-Unters-Forsch 1996;202:1-14.

51. Arai S, Abe K. Cystatin-based control of insects, with special reference to oryzacystatin. In: Michaud D, editor. Recombinant protease inhibitors in plants. Landes Bioscience, Georgetown TX; 2000. p. 27-42.

52. Atkinson HJ, Johnston KA, Robbins M. Prima facie evidence that a phytocystatin for transgenic plant resistance to nematodes is not a toxic risk in the human diet. J Nutr 2004;134:431-4.

53. Herrnstadt C, Soares G, inventors; Mycogen Corporation, assignee. Control of cotton boll weevil, alfalfa weevil, and corn rootworm via contact with a strain of *Bacillus thuringiensis*. US patent 4,797,276. 1989 Jan 10.

54. Bradfisch GA, Schnepf HE, Kim L, inventors; Mycogen Corporation, assignee. *Bacillus thuringiensis* isolates active against weevils. US patent 5,707,619. 1998 Jan 13.

55. Li G, Xu X, Xing H, Zhu H, Fan Q. Insect resistance to *Nilaparvata lugens* and *Cnaphalocorcis medinalis* in transgenic indica rice and the inheritance of gna+sbit transgenes. Pest Manag Sci 2005;61:390-6.

56. Han L, Wu K, Peng Y, Wang F, Guo Y. Evaluation of transgenic rice expressing *Cry1Ac* and *CpTI* against *Chilo suppressalis* and intrapopulation variation susceptibility to *Cry1Ac*. Environ Entomol 2006;35:1453-9.

57. Han L, Wu K, Peng Y, Wang F, Guo Y. Efficacy of transgenic rice expressing *Cry1Ac* and *CpTI* against the rice leaffolder, *Cnaphalocrocis medinalis* (Guenee). J Invertebrate Pathol 2007;96:71-9.

Received in November, 2009. Accepted for publication in December, 2009.