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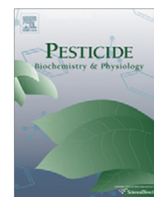

Investigation of insecticidal activity of rye α-amylase inhibitor gene expressed in transgenic tobacco (*Nicotiana tabacum*) toward cotton boll weevil (*Anthonomus grandis*)

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ABSTRACT

Innumerable proteinaceous α-amylase inhibitors have been isolated and identified from different plant species. Among them, an α-amylase inhibitor gene with bioinsecticidal potential toward *Anthonomus grandis* (cotton boll weevil) was previously identified in rye seeds (*Secale cereale*). This cereal inhibitor was expressed in tobacco plants (*Nicotiana tabacum*) under control of phytohemaglutinin promoter by using *Agrobacterium tumefasciens* – mediated transformation. Presence of *αBIII-rye* gene and further protein expression were confirmed by PCR and Western blot analysis, respectively. Immunological assays indicated that the recombinant inhibitor was expressed in concentration range from 0.1% to 0.28% (w:w) of the total protein in tobacco seeds of R_0 plants. From 14 independent transformants, five plants with expression levels between 0.20% and 0.28% in seeds were *in vitro assayed* against *A. grandis* amylolytic enzymes causing clear inhibition. Moreover, bioassays using transgenic seed flour mixture for artificial diet produced 74% mortality in *A. grandis* first larval instar. These data suggest that rye inhibitor could be a promising biotechnological tool for produce transgenic cotton plants with an increased resistance to cotton boll weevil. Moreover, *αBIII-rye* gene should be considered a potential compound for a pyramiding strategy aiming to delay insect-resistance.

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1. Introduction

Anthonomus grandis is a major pest of cultivated cotton, *Gossypium hirsutum* L. [3,42], having endophytic behavior that makes larvae control difficult with chemical insecticides [45]. Major efforts have been applied in order to find better alternative to control, including transgenic strategy. Plant genetic transformation using exogenous genes encoding defense factors for phytophagous insect–pests is a successful alternative strategy to using synthetic chemical insecticides, mainly thanks to the low costs and reduced hazardous components [22,37,41,54]. Since GM plants appeared, relevant results were obtained by engineered plants expressing crystal protein genes from *Bacillus thuringiensis* and several variants of *cry* genes encoding toxic Bt crystal proteins were widely used to produce insect-resistant transgenic crops [6,18,52]. Another alternative for increasing plant‘ resistance to herbivorous insects consists of genes encoding enzyme inhibitors [27,47]. In this field, a wide number of heterologous inhibitor genes have been expressed in plants [13,19,38] and some of them have clearly reduced insect damages [4,22,37,49,51].

Among digestive enzyme inhibitors commonly used to control insect-pests, α-amylase inhibitors have usually been focused as molecular tools for plant defense [25,37,41,54]. Indeed, a chimeric gene encoding a bean lectin-like α-amylase inhibitor (*α-AI-1*) expressed in tobacco plants produced a protein that inhibits digestive α-amylases from yellow mealworm (*Tenebrio molitor*) [1]. Moreover, the expression of this inhibitor in pea seeds at a level comparable to that found in bean seeds conferred resistance to cowpea, pea and azuki bean weevils [37,41,54]. On the other hand, only a partial protection of transgenic peas expressing the *α-AI2* gene was obtained against the pea weevil, *Bruchus pisorum* [37]. Until

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now, only legume inhibitors have been expressed in transgenic plants, aiming to improve bruchid resistance, although cereals are also described as a rich source of amylase inhibitors. These inhibitors are known as cereal-type inhibitors, containing different members isolated from wheat, barley, maize and several others [12,34]. An inhibitor belonging to this family, named BIII, was purified from rye (*Secale cereale*) kernels [26]. This 13.6 kDa protein showed enhanced *in vitro* activity against *Acanthoscelides obtectus*, *Z. subfasciatus* and *A. grandis* α-amylases [26,42]. Recently the molecular cloning and characterization of the *αBIII-rye* gene was described. A 311 bp fragment of this inhibitor was expressed in *Escherichia coli* and *in vivo* effects of the recombinant protein were evaluated against *A. grandis*, confirming its potential against this important devastating cotton insect–pest [8].

This paper reports the expression of the *αBIII-rye* gene into transgenic tobacco through *Agrobacterium*-mediated transformation in order to evaluate its processing and ability to inhibit cotton boll weevil α-amylases under *in vitro* and *in vivo* bioassays. The gene has been introduced under a strong seed-specific promoter, phytohemaglutinin (PHA), which directs gene expression to tobacco seeds. The expression of this inhibitor in seeds is part of a larger project to validate the potential of proteinaceus factors to control the cotton boll weevil [10,11,16,42].

2. Materials and methods

2.1. Transformation vector construction

The *α-BIII*-rye gene was cloned from total DNA of rye leaves [8], which was used as a template in PCR with the oligonucleotide primers: ForBSMRAI (5′ CCC GAA TGC ATG GCT TCC TCC AAC TTA CTC ACC CTA GCC CTC TTC CTT GTG CTT CTC ACC CAC GCA AAC TCA ATG GAA GAT TGC ACC CCA 3′) that contains a *BsmI* restriction site and signal peptide from α-AI1(α-amylase inhibitor present in *Phaseolus vulgaris*), and RevPSTIRAI (5′ CCC CTG CAG TCA GTT GTG AAC GGT CGT 3′) that included a *Pst*1 restriction site and stop codon. A proof-reading DNA polymerase, *Pfu* (Stratagene, CA, USA), was used for the PCR (94 °C, 30 s denaturation, 45 °C, 30 s annealing, and 72 °C, 40 s elongation, 35 cycles). The PCR product (approximately 428 bp) was digested with *Eco*RI, gel purified and ligated into the pGEMT-easy vector (Promega Corporation, Madison, Wis., USA), producing pGEMT- *αBIII-rye* vector In the intermediate step, aiming to subclone *αBIII-rye* gene under control of PHA-L promoter, the last vector was digested in *Bsm*I and *Pst*I enzymes sites and introduced by PCR into plasmid pTA-2 [15] flanked by the same sites and downstream of the seed-specific promoter phytohemaglutinin PHA-L. Finally, the resulting construction (pTA-2 – *αBIII-rye*) containing the PHA-L promoter, the αAI-1 signal peptide and the *αBIII*-rye gene sequence was subcloned into the *Hind*III and *Eco*RI sites of the plasmid vector pCAMBIA 1390 (Cambia GPO, Canberra, Australia), containing the nopaline synthase transcriptional (NOS) terminator, originating the binary vector pCAMBIA 1390/*αBIII-rye*, which was used for plant transformation (Fig. 1).

2.2. *Agrobacterium tumefaciens* mediated transfer of *αBIII-rye* into tobacco

Tobacco (*Nicotiana tabacum* cv. *xanthi*) plant transformation was performed using the leaf disk method [20] and *A. tumefaciens* strain GV3101 [28] carrying the pCambia1390/*αBIII-rye* construct or the standard pCambia1390 vector (*αBIII-rye* gene without to be used as negative control). The binary vectors were transferred to *A. tumefaciens* using heat-shock procedure. *Agrobacterium tumefasciens* strain harboring the construct was grown in 5 mL YEB medium, supplemented with rifampycin (100 μg mL^{-1}), gentamicin (50 μg mL^{-1}) and kanamycin (100 μg mL^{-1}), for 16 h at 28 ± 2 °C until OD_{600} reached to 0.8. Sterilized leaf explants of tobacco plants (*N. tabacum* cv. *xanthi*) were incubated with the *A. tumefasciens* culture (A_{600nm} = 0.8) in a Petri dish with MS [39] (Sigma) liquid medium for 20 min, at room temperature. For co-cultivation experiments, leaf discs were put a Petri disk containing MS solid medium (0.7% agar) and maintained for 2 days in darkness at 28 ± 2 °C. For regeneration and selection, the explants were transferred to MS solid medium (0.65% agar) containing 1 μg mL^{-1} benzylaminopurine (BAP), 500 μg L^{-1} cefotaxime and 30 μg mL^{-1} hygromycin and maintained under 16 h photoperiod 25 ± 2 °C in the same medium. Untransformed explants were placed onto the same medium with or without hygromycin as the negative and positive controls, respectively. Shoots regenerated on selection medium were excised at the base and placed in Magenta GA7 boxes containing rooting medium (the same medium as the regeneration medium but without BAP). The hygromycin-resistant and PCR positive plants were transferred to soil and grown in a greenhouse at 25 ± 10 °C and 50% humidity. The T_0 mature seeds were collected after 4 months.

2.3. PCR analysis of transgene incorporation

Genomic DNA was isolated from leaf tissue of both untransformed and putative transformed plants using a CTAB method [40]. The integrated fragment was detected in the PCR using the same oligonucleotide primers ForBSMRAI and RevPSTIRAI (described above), amplifying an expected 428 bp fragment. The PCR mixture (50 μl) was composed of 10 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxibonucleotide dATP, dGTP, dCTP and dTTP, 0.2 mM each primer, 100 ng template DNA and 2.5 units AmpliTaq DNA Polymerase (Perkin Elmer). A positive control was utilized comprising the *αBIII-rye* gene cloned in *E. coli* strain BL21 [8] with 480 bp. In addition, to confirm the presence of the *αBIII-rye* gene in transgenic plants and to obtain F1 plants, mature seeds were germinated on MS medium supplemented with hygromycin (30 μg mL^{-1}).

2.4. Preparation of seed extracts

The soluble recombinant proteins were extracted from untransformed and transformed tobacco seeds by grinding 250 mg of dry tobacco seeds, in an ice-cold mortar containing 1 mL of extraction buffer (50 mM Tris pH 8.0, 30 mM NaCl added, 0.1% Triton X-100 and 2% β-mercaptoethanol). For inhibitory enzyme assays, proteins were extracted in the same buffer in the absence of β-mercaptoethanol. Extraction was carried out for 2 h at 4 °C with agitation. The extract was cleared by centrifugation at 12,000*g* for 10 min and the supernatant was used as a source of inhibitor for α-amylase assays [15,53]. The cleared homogenates were stored at −20 °C for use in immunoblotting or immediately used to measure *in vitro* and *in vivo* inhibitory activities. Protein concentration was determined according to Bradford method [5] using bovine serum albumin as standard protein in calibration curve.

2.5. Immunoanalysis

Protein extracts (150 μg) precipitated with 70% trichloroacetic acid and separated using 12% denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli [29]. Immunoblots were performed as described by Sambrook and Maniatis [48]. Proteins were transferred to nitrocellulose membranes (Hybond-C, Amersham) using a semi-dry TransBlot Cell Unit (Bio-Rad) and Tris–glycine transfer buffer [55]. Initially the membrane was incubated with mouse polyclonal primary antibody raised against recombinant BIII [8]. Penta–His,

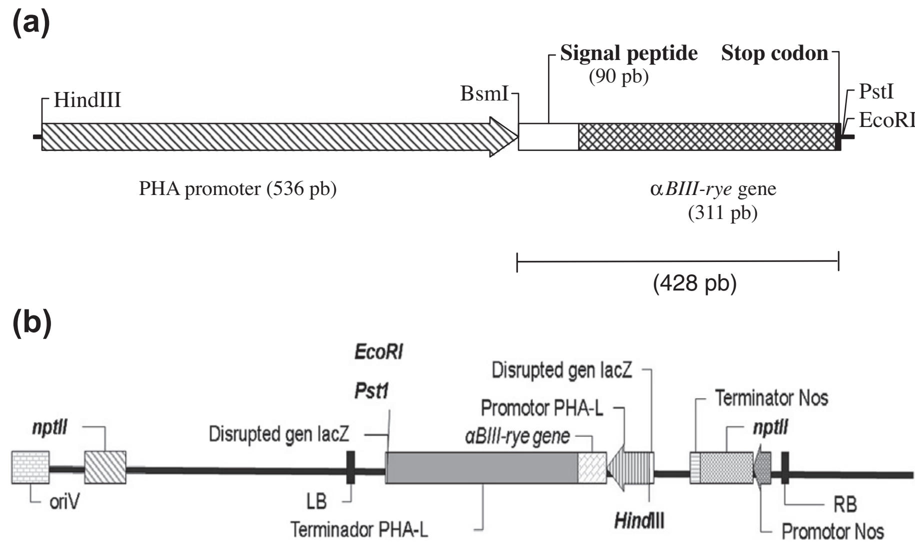

Fig. 1. Schematic representation of constructs for seed-specific expression of the *αBIII-rye* gene in tobacco plants. (a) Construction of the pTA-2 – *αBIII-rye* vector containing a *αBIII-rye gene*, *Bsm*I and *Pst*1 restriction site, stop codon and signal peptide from αAI-1(α-amylase inhibitor present in *Phaseolus vulgaris*) (b) T-DNA region of the pCAMBIA1390/*αBIII*. RB right border; LB left border, NOS nopaline synthase terminator, hph phosphotransferase; PHA phytohemagglutinin promoter, *αBIII-rye gene*. Also highlighted are the positions of *Eco*RI, *Pst*1 and *Hind*III enzyme sites.

coupled with horseradish peroxidase (HRP), was used as a secondary antibody and the reaction was visualized using 0.1% (v/v) H_2O_2 in TBS buffer mixed with 3% (w/v) HRP color development reagent in methanol. A positive control comprised the recombinant *αBIII-rye* gene expressed in the *E. coli* system, produced as an N-terminal fusion to thioredoxin with a 6× His tag at the C-terminal, resulting in a protein of 27.5 kDa [8]. Furthermore, in order to quantify BIII α-amylase inhibitor in tobacco seeds, procedures for sandwich enzyme-linked-immunosorbent assay (ELISA) were done following the procedure described by Ausubel et al. [2]. A calibration curve was previously prepared with purified recombinant protein BIII inhibitor produced in *E. coli* diluted in PBS (0–100 ng). Wells of microtiter plates were coated with 100 μl of protein extract preparations of transformed and untransformed tobacco seeds and incubated at 4 °C overnight. After four washes with blocking solution (137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4, 0.03% (v/v) skimmed milk, 0.02% (v/v) Tween 20), 100 μl of the mouse polyclonal antibody was added at 1:500 dilution. After 1 h of incubation at 37 °C and followed by three washes with PBS buffer, 100 μl (1:2000 dilution) of the secondary antibody (anti-mouse IgG coupled to alkaline phosphatase) (Bio-Rad) was added. The plate was incubated for 1 h at 37 °C and washed four times with wash solutions (500 μl Tween 20; 1000 ml PBS buffer solution). Finally, the plate was developed with 100 μl *p*-nitrophenyl phosphate substrate (Sigma) (prepared for 1 μg mL^{-1} according to manufacturer's instructions). Color development was stopped after 30 min and absorbance was then read at 405 nm.

2.6. Extraction of larval α-amylase and inhibitory enzyme assays

α-Amylase inhibitory activities were measured by using the dinitrosalicylic acid (DNS) method adapted by Bernfeld [24] using 1% soluble starch as substrate. Midguts from third instar larvae of *A. grandis* were excised from cold-anesthetized larvae and macerated in 0.1 M phosphate buffer, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$ using a 1:10 w/v dilution ratio. Crude extract was cleared by centrifugation at 12,000*g* for 20 min. After centrifugation, the supernatant was stored at −20 °C and used as a enzyme source for α-amylase inhibition assays. For the inhibitory assays, 250 mg of crude protein extract from tobacco seeds expressing the BIII α-amylase inhibitor were pre-incubated for 20 min at 37 °C with one unit of α-amylase activity of PPA (porcine pancreatic α-amylase) or *A. grandis*. Each assay contained 1.0 UI of α-amylase activity, prior to the addition of 250 μl of 1% starch as substrate followed by incubation for 10 min at 37 °C. One α-amylase unit was defined as the amount of enzyme that increases the absorbance by 0.1. The reactions were stopped by the addition of 500 μl of DNS reagent followed by color development placing the tubes in boiling water for 10 min. After addition of 5 ml distilled water, the absorbance was read at 546 nm. Assays were carried out in triplicate, where sample variation was no more than 12%.

2.7. Anthonomus grandis bioassays

Bioassays were carried out in six-well plates containing 5 ml of artificial diet sterilized in each well [7]. The recombinant protein was extracted from tobacco seeds as previously described, further dialyzed and incorporated into the diet at three standard concentrations: 0.5%; 1.0% and 1.5% w/v. Protein concentrations were determined according to Bradford [5] and the level of recombinant BIII was estimated by ELISA as described above. Ten larvae from first instar were fed on artificial diets per treatment. Two negative controls were used: (i) distilled water and (ii) proteins extracted from non-transformed tobacco seeds. After 7 days of incubation at 28 ± 2 °C, 55% relative humidity and photoperiod of 14 h, the dead larvae were counted. Each treatment was carried out four replicates. Mortality was calculated as the percentage of neonate larvae that no completed their development.

3. Results and discussion

Several studies demonstrated the efficiency of proteinaceous inhibitors against digestive enzymes of important economic Lepidopteran and Coleopteran pests [33,57], especially when utilized in genetically engineered plants [17,32,37]. In the last few years, studies have reported the potential use of proteinase inhibitors against the boll weevil [10,11]. Moreover, previous reports [8,26,42] demonstrated that purified and recombinant BIII

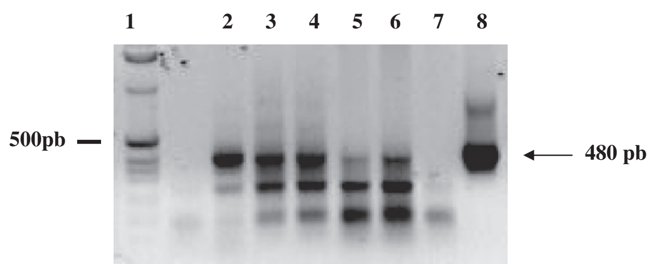

Fig. 2. PCR amplification of *αBIII-rye* gene (480 bp) in the putative transgenic tobacco plants. Lane 1: 1.0 kb marker; lanes 2–6: putative transgenic plants P3, P9, P8, P12 and P14, respectively; lane 7: negative (untransformed) control; and lane 8: positive control (pCAMBIA 1390/*αBIII-rye* vector).

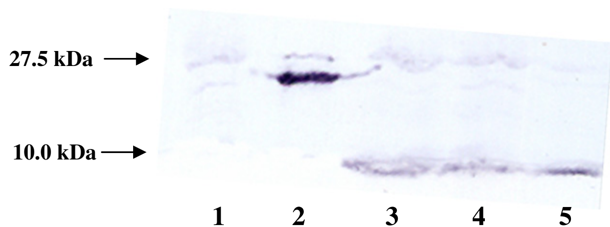

Fig. 3. Western blot analysis of protein expressed in transgenic tobacco plant seeds. Lane 1: negative control (untransformed tobacco); lane 2: positive control: recombinant αBIII-rye gene expressed in the *E. coli* system, produced as an N-terminal fusion to thioredoxin with a 6× His tag at the C-terminal, resulting in a protein of 27. 5 kDa: lane 3: positive transgenic P3; lane 4: positive transgenic P12; and lane 5: positive transgenic P8.

inhibitor was able to inhibit up to 90% α-amylase activity of *A. obtectus, Z. subfasciatus* and *A. grandis*, indicating that BIII inhibitor could be potentially useful in producing transgenic crop plants resistant to the attack by *A. grandis*.

Aiming to evaluate this hypothesis, 150 leaf explants in three different experiments produced only 14 transformants or 9.3%. All transformants obtained were mediated by *A. tumefasciens* by using seed-specific promoter of phytohemaglutinin. The expression of α-amylase inhibitor in plants was described by several authors [1,44,54] with similar transformation rates. In the present work, the transformation efficiency rate calculated on the basis of PCR analysis using genomic DNAs as a template of the transformed plants showed that the α*BIII* gene (amplicon of 428 bp) was present in 14 plants out of 109. These results are presented for five T_o plants; namely, P3, P9, P8, P12 and P14 (Fig. 2). Moreover, mature seeds of transgenic plants germinated in hygromycin (30 μg mL^{-1}) selective medium, confirming PCR analysis. Germination frequencies of plants were consistent with the expected rate (3:1) of Mendellian segregation (data not shown). Furthermore, the presence of BIII inhibitor in seed extracts was detected by immunoblotting. A single reactive band in the transgenic seed extracts was observed in the Western blot (Fig. 3), absent in negative control (untransformed seeds extracts), confirming that synthesis of BIII protein occurred in transgenic tobacco seeds.

According to the double-antibody sandwich ELISA analysis, the expression levels of BIII recombinant proteins varied greatly among transformed plants. Expression levels ranged from 0.10% to 0.29% of total soluble protein. In three plants (P5, P10 and P13), the recombinant protein was not detected. Expression levels varied between 0.28% and 0.26% (P3, P8, P12 and P1) and an intermediate expression level was observed in four of the other transformed plants, varying between 0.10% and 0.18% (Fig. 4). The expression level of the recombinant BIII inhibitor are considerably lower when compared to the 1.0–1.3% of α-AIs found in cultivated beans [51], or the expression of 1.5–3.0% of total soluble proteins of α-AI1, expressed in transgenic pea seeds [50] and 0.82% in transgenic azuki bean seeds [25]. However, Pereira et al. [44] also showed expression level ranging from 0.02 to 0.05% of the total soluble proteins when αAI-Pc1 inhibitor, from *Phaseolus coccineus*, were expressed in tobacco seeds under PHA-L promoter control, indicating that protein expression levels in different transgenic plants can be variable.

Plants provide a promising expression vehicle for numerous proteins such as antibodies, vaccines and hormones [43]. Compared to the use of conventional steel tank bioreactors and mammalian cells or microorganisms, the cost of producing a protein under good manufacturing conditions is reduced [31]. Transgenic plants have many advantages as a source of proteins compared

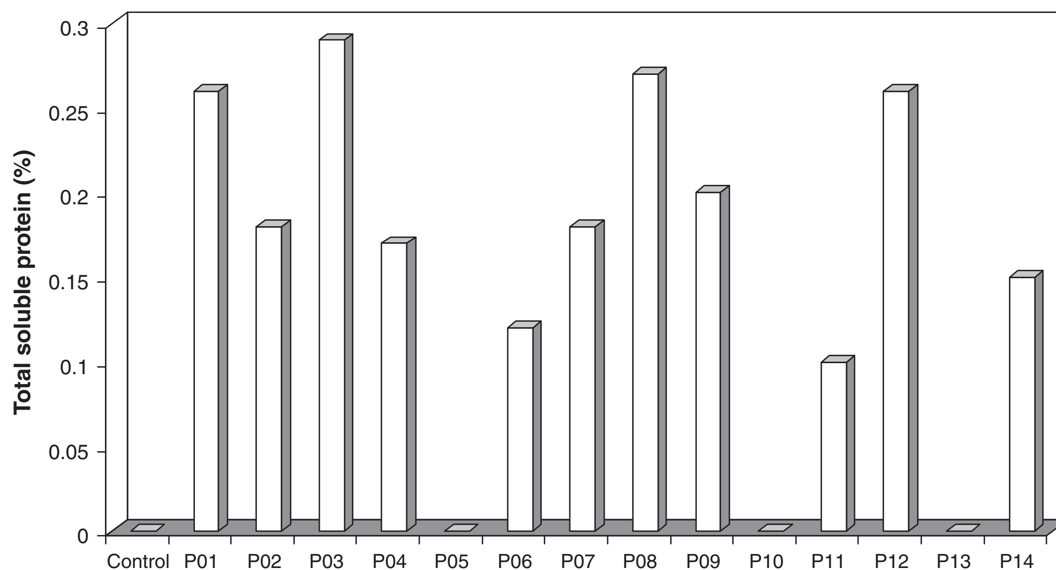

Fig. 4. Quantitative estimation of the αBIII-rye inhibitor content in control and transformed tobacco seeds using ELISA. P3–P13, transgenic tobacco plant lines expressing the αBIII-rye inhibitor. Control (untransformed plant) Amounts are indicated as the percentage of total soluble protein. One hundred micrograms of seed total extract was used in each analysis. Each measurement was done in triplicate.

with animal tissues, recombinant microbes, transfected animal cell lines or transgenic animals, where a rapid scale-up of production and correct eukaryotic assembly of proteins is possible. Depending upon the promoters used, transgenic proteins can be deposited throughout the plant, in specific parts of the plant (e.g. seeds) or in specific cell organelles, such as chloroplasts. Numerous laboratories have shown transgenic protein accumulation in seeds of corn [46], soybean [58], tobacco [9] or barley [21]. A wide number of inhibitor genes have been expressed in tobacco plants under the control of constitutive promoters such as CaMV 35S, in which a wide range of foreign protein expression levels is found. For example a trypsin inhibitor BTI-CMe protein from barley was expressed in tobacco leaves, exhibiting a total soluble protein content of 0.03–0.14% and further causing a remarkable reduction in *Spodoptera exigua* digestive serine-proteinase activities [30]. Moreover, a corn bifunctional inhibitor with ability to inhibit trypsin and α-amylase, was expressed in tobacco plants in amounts of up to 0.05% of the total protein in young leaves of R1 plants [35]. When phytohemagglutinin promoter (PHA) was used to express α-amylase inhibitor (α-AI) in tobacco seeds [1,44], beans [25,54] and peas [37,50] soluble protein levels expressed were between 0.8% and 3.0%.

In our work total protein extracted from seeds of the 14 transgenic tobacco plants and from untransformed tobacco seeds was used to measure *in vitro* inhibitory activity against *A. grandis* α-amylases. The α-amylase inhibition assays showed that recombinant protein produced in the transgenic plants was active, being accumulated in the seed endosperm. *In vitro* assays revealed that seeds from three transgenic plants showed inhibitory activity against the α-amylases tested. An inhibition of 41% was observed in P3 and a weak but discernible inhibition of around 23% was observed in P2 and P6. Similar inhibition has already been described with purified protein from rye seeds [26,42]. The results indicate that the inhibition level is probably related to the expression level of recombinant protein, since plants with high levels of recombinant protein (0.29–0.26%) show higher levels of inhibition. In order to evaluate insecticidal activity of BIII inhibitor toward insect-pests, protein extracted from tobacco seeds was challenged against *A. grandis* by incorporating three different protein amounts (0.5%, 1.0% and 1.5% w/v) into an artificial diet. The recombinant protein was able to cause mortality in all concentrations tested (Table 1). Significant mortality (74%) was observed at the highest concentration of transformed seed extracts tested. Bioassay showed that BIII adversely affected boll weevil mortality at all concentrations tested (Table 1). Similar results were observed in bioassays using α-AI1 against *Callosobruchus maculatus* and *C. chinensis*, in which artificial seeds containing inhibitor at a concentration of 0.2% adversely influenced the larval development and an inhibitor concentration of 1.0% caused complete mortality [23]. Furthermore, transgenic pea plants expressing α-AI1 levels in the range of 0.8–1.0% of total protein in seeds, produce complete protection against the three Old World bruchids: the pea weevil *Bruchus pisorum*, the cowpea weevil and the adzuki bean weevil [24]. Otherwise α-AI2 was also partially effective against pea weevil at the same concentrations [37].

The primary reason for producing insect-resistant transgenic crops is to reduce the use of chemical pesticides and, thereby, the cost to the farmer and the consumer and to reduce the insecticide loads on the environment. At present, the boll weevil is controlled by a range of management strategies, including use of cultivar selection, crop control and extensive chemical sprays on adult weevils in the field. The presence of α-amylases in the *A. grandis* alimentary tract [42] and its reliance upon these enzymes for feeding may represent a new strategy to control this pest with proteinaceous α-amylase inhibitors. The results reported that the BIII α-amylase inhibitor expressed in tobacco showed *in vitro* and *in vivo* capacity to provide protection against *A. grandis*. These facts means there are prospects for the development of boll weevil-resistant cotton and consequent reduction in the use of insecticidal chemical sprays. Finally it was also important to remember that a transgene with enhanced effectiveness may result in selection pressure that causes the rapid emergence of resistant insects. In this respect, an inhibitor that simply reduces the boll weevil population to below economically important levels may be more desirable. Several studies have demonstrated the potential of proteinase inhibitors [11,12] and Cry toxins [16,36] against the boll weevil and another strategy for slowing the rate of resistance development is use of a pyramid of genes encoding insecticidal proteins [14]. Pyramiding involves the incorporation of more than one insecticidal gene with different modes of action into a single plant line. On the other hand insect selection pressure at this modified genetic locus could generate novel inhibitors with different specificities, being clearly affected by co-evolution [56]. Considering the results presented in this paper, we suggest that *αBIII-rye* gene could be a candidate to be tested in potential combinations of the compounds, in the future aiming to obtain transgenic cotton presenting delayed insect-resistance.

Table 1
Evaluation of larval mortality of boll weevil developed in artificial diet in the presence of different concentrations (w/v) of tobacco seed transformed of *αBIII-rye* gene into artificial diet. Two negative controls were utilized: artificial diet containing distilled water and artificial diet containing non-transgenic plant extract.

Treatments	% Mortality
0.5%	32
1.0%	66
1.5%	74
Distilled water	20
Non-transformed seeds	20

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