Research Article

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Stable integration and expression of a *cry1Ia* gene conferring resistance to fall armyworm and boll weevil in cotton plants

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Abstract

BACKGROUND: Boll weevil is a serious pest of cotton crop. Effective control involves applications of chemical insecticides, increasing the cost of production and environmental pollution. The current genetically modified *Bt* crops have allowed great benefits to farmers but show activity limited to lepidopteran pests. This work reports on procedures adopted for integration and expression of a *cry* transgene conferring resistance to boll weevil and fall armyworm by using molecular tools.

RESULTS: Four Brazilian cotton cultivars were microinjected with a minimal linear cassette generating 1248 putative lines. Complete gene integration was found in only one line (T0-34) containing one copy of *cry1Ia* detected by Southern blot. Protein was expressed in high concentration at 45 days after emergence (dae), decreasing by approximately 50% at 90 dae. Toxicity of the cry protein was demonstrated in feeding bioassays revealing 56.7% mortality to boll weevil fed buds and 88.1% mortality to fall armyworm fed leaves. A binding of cry1Ia antibody was found in the midgut of boll weevils fed on T0-34 buds in an immunodetection assay.

CONCLUSION: The gene introduced into plants confers resistance to boll weevil and fall armyworm. Transmission of the transgene occurred normally to T1 progeny. All plants showed phenotypically normal growth, with fertile flowers and abundant seeds.

Keywords: *Gossypium hirsutum*; *Anthonomus grandis*; *Spodoptera frugiperda*; Resistance; *Bt-cry* gene; inheritance

1 INTRODUCTION

Cotton is an important world agricultural commodity. Brazil is a leading global producer of cotton, with an annual lint production exceeding 4 million t (IBGE, http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/lspa/lspa_201407_2.shtm). The crop system is based on high technology and robust cultivars adapted to the tropical environment.[1,2] A challenge for cotton production in Brazil is the lack of natural traits for tolerance against important insect pests, which limits the efficacy of conventional breeding. The annual costs for insect pest control in cotton fields are around $US 2–3 billion, and they account for approximately 10% of the production costs and up to 50% of the chemical pesticides used in developing countries.[3,4]

The barriers of susceptibility were overcome after the development of genetically modified (GM) crops containing exogenous genes conferring resistance to several insect species. The management of these GM crops is less dependent on pesticides; however, so far, most genes currently inserted in commercial GM crops are from the *Bacillus thuringiensis* (*Bt*) *cry* family, conferring resistance only to lepidopteran insects.[5]

Bt is an entomopathogen bacterium that forms inclusions (crystals) produced by one or more insecticidal proteins, which exhibit specific toxicity towards several insect orders.[6] In the midgut lumen of the insect, crystals (Cry protein) are actived, destroying the brush border membrane integrity by pore formation, resulting in cell swelling, eventual cell rupture and insect death.[6,7]

More than 200 *cry* genes have been described and classified into a large number of groups according to their amino acid sequence homology (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html). Cry1Ia, Cry1Ib, Cry1Id and Cry1Ie have been described, exhibiting toxicity against both Lepidoptera and Coleoptera, with LC_{50} values varying from 0.20 to 140 µg mL^{-1} and from 250 to 2600 µg mL^{-1} respectively.[8–11] The Cry1Ia protein is toxic to both lepidopteran and coleopteran insects.[11,12]

Boll weevil, *Anthonomus grandis* Boheman, 1843 (Coleoptera: Curculionidae), is the more serious pest of the Brazilian cotton belt owing to its high capacity for survival, reproduction and

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dispersal: more than 1.5 million adult insects can be found in a single hectare.[13,14] Immature life stages are found inside squares or bolls. Moreover, larvae and adults feed preferentially on fruiting structures. Feeding and oviposition injuries usually result in abscission of squares and small bolls, causing direct damage to fibre production.[15–17] Another serious insect pest in the Brazilian cotton belt is fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), which causes damage by cutting the young plants, defoliating and punching buds, flowers and bolls.[18] Larvae of first and second instars feed primarily on the underside leaf surface of plants; later instars feed preferentially on fruiting structures and finally move downwards within the plant canopy to feed on bolls before pupating.[19]

Control of these insect pests has been done through the application of broad-spectrum insecticides; the high economic investment, the increase in resistant insect populations and environmental pollution have stimulated the development of new technologies to minimise damage.[20–22] Investment in identifying genes with multiple insecticide action is a more promising strategy in the development of GM crops because it adds value and can minimise the relative cost of technology.

Herein, we report on procedures adopted for introduction and expression of a new *cry1Ia* gene, previously isolated by our team at the Brazilian Company of Agricultural Research (Embrapa), the toxin of which has exhibited toxicity against fall armyworm and cotton boll weevil. This gene has been stably integrated into cotton plants, as demonstrated by molecular and entomological assays.

2 EXPERIMENTAL METHODS

2.1 Gene constructs

The gene used in the construction (*cry1Ia*) was previously isolated from the *Bacillus thuringiensis* S1451 strain, an accession from Embrapa – Genetic Resources and Biotechnology (CENARGEN) collection (*Bt* germoplasm bank, Brasília, Brazil, http://sicol.cria.org.br/crb/BGB) that exhibited toxicity against boll weevil (*A. grandis*) and fall armyworm (*S. frugiperda*) in feeding bioassays using recombinant Cry1Ia protein, with LC_{50} values of 21.5 μg mL^{-1} to boll weevil and only 0.29 μg mL^{-1} to fall armyworm.[23]

The original sequence of the *cry1Ia* gene was edited with 40% changes based on the cotton codon usage, and a linear cassette (~3 kb) (Fig. 1) was synthesised (promoter + open reading frame + terminator) by the GenScript Corporation (Piscataway, NJ) for further use in transformation procedures.

2.2 Transformation procedures

In the greenhouse, cotton seeds of four Brazilian cultivars were sown in pots (40 cm diameter) filled with nutrient-rich soil and watered daily. Each pot contained only two seedlings. At full blooming, 50–60 days after emergence (dae), plants were transformed by the microinjection technique as described in Zhou *et al.*[24] A 10 μL drip of DNA (100 ng μL^{-1}) was introduced onto the ovaries of young bolls (24 h after pollination) using a glass microcapillary injection pipette (Hamilton, Reno, NV).[24] Ten bolls were used per plant. All putative transformed seeds (T0 generation) were collected and sown in the greenhouse for further assays.

2.3 Polymerase chain reaction and sequencing of amplicons

All putative transgenic lines (PTLs) were tested by polymerase chain reaction (PCR) assays. Genomic DNA from plants was obtained by using the Illustra DNA extraction kit Phytopure (GE Healthcare, Little Chalfont, UK), following the manufacturer's instructions. PCR assays were performed in reaction of 25 μL containing 1 μL of DNA (20 ng μL^{-1}), 2.5 μL of 10× buffer, 1.5 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTP (10 mM), 1 μL each of forward and reverse primer at 10 μM and 0.3 μL of *Taq* polymerase (5 U μL^{-1}). Three specific primer combinations were used in reactions (Fig. 1A): 1 F (5′-ATCTTCGGAAAAGAATGGGG-3′)/3R (5′-AGGAGCATTGTTGTTATACC-3′), 0.44 kb; 3 F (5′-GGGGAATAGAG AAGATTTGG-3′)/2R (5′-TCCTGAAGACAAATTGAAAGC-3′), 0.52 kb; 5 F (5′-CAGAAGCCAAAGGGCTAT-3′)/2R, 2.1 kb.

The PCR conditions (Mastercycler Gradient, Eppendorf, Germany) were: initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 96 °C for 1 min, annealing at 56 °C for 45 s and extension at 72 °C for 1 min. For the 5 F/2 F combination, the extension was at 72 °C for 2 min. A final extension was added at 72 °C for 10 min for all primer combinations. A 15 μL aliquot of PCR products was resolved by electrophoresis on 0.8% agarose gel and further photodocumented.

For sequencing analysis, PCR amplicons were purified using Illustra GFX PCR DNA and gel band purification kit (GE Healthcare) and analysed on a MegaBace 1000 sequence analyser (GE Healthcare) using DyEnamic™ ET-Terminators kit. Five replications were used for each sample. The sequences obtained were compared with the *cry1Ia* gene sequence deposited in BlastN (Basic Local Alignment Search Tool), available at http://www.ncbi.nlm.nih.gov, and further aligned using the *MultipleM sequence alignment* program (http://bioinfo.genotoul.fr/multalin/multalin.html).

2.4 Southern blot analysis

Genomic DNA was isolated from leaves (Illustra DNA extraction kit Phytopure) and then digested (15 μg) with *Bam*HI (Promega), separated by electrophoresis on 0.8% agarose gel and transferred to a nylon membrane (Hybond N+; GE Healthcare) by capillarity. The membrane was hybridised with a DIG-labelled *cry1Ia* probe (1 F/2R, 0.96 kb) using DIG High Prime DNA labelling and detection starter kit I (Roche Diagnostics Brasil Ltda., São Paulo, Brazil), following the manufacturer's instructions. Immunological detection was performed using the CDP-Star detection solution (Roche Applied Science). The membrane was exposed to X-ray (Kodak) for 30 min and developed for further blotting analysis. The assay was performed in three replications.

2.5 Immunodetection by enzyme-linked immunosorbent assay

Leaves from PTLs and non-transformed plants, collected at 45, 60 and 90 days, were freeze dried, finely ground and stored at −80 °C. Next, total proteins were extracted according to Xie *et al.*,[25] and concentrations were estimated according to Bradford.[26] Expression of the Cry1Ia protein in putative transgenes was evaluated by indirect enzyme-linked immunosorbent assay (ELISA) according to the methodology described by Koenig,[27] with some modifications. Briefly, 100 μL (500 ng) of capture antibody (Cry1Ia) produced by our team was coated onto ELISA plates by incubation at 4 °C for 2 h. Unbound antibody was washed with phosphate-buffered saline (PBS), and non-specific binding sites were blocked by incubation with 1% BSA in PBS for 1 h at 23 °C. Samples of cotton leaf protein (1 μg well^{-1}) were loaded onto plates and allowed to complex with the bound antibody for 4 h at room temperature (RT). Unbound products were washed, and anti-Cry1Ia IgG labelled with biotin was allowed to bind to the antigen for 2 h at

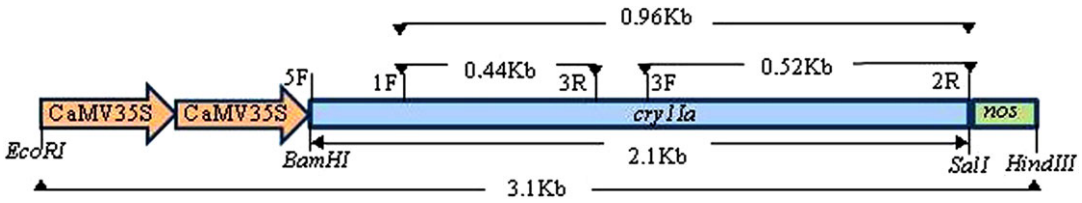

Figure 1. Diagramatic scheme of the construction used for microinjection into cotton bolls via ovary drip.

Table 1. Number of putative transgenic lines (PTLs) based on PCR amplicons of DNA extracted from four cotton cultivars

	Lines identified by PCR[a]			
Cultivars	Number of PTLs	1 F/3R	3 F/2R	5 F/2R
BRS Antares	301	T0-27, T0-42	T0-226	–
CNPA Precoce I	336	T0-58, T0-77, T0-80, T0-85	–	–
BRS Araçá	315	–	T0-75	0
BRS 293	296	T0-21, T0-25, T0-34	T0-34, T0-46, T0-65	T0-34

[a] Primers: 1 F/3R: 0.44 kb; 3 F/2R: 0.52 kb; 5 F/2R: 2.1 kb.

room temperature. The anti-Cry1Ia IgG was kindly provided by the Biologic Control team[23] from CENARGEN. The secondary antibody was subsequently detected using an antibiotin labelled with alkaline phosphatase (Sigma, St Louis, MO) by incubation for 4 h at RT. Then, antibody–antigen complexes were incubated with *p*-nitrophenyl-disodium phosphatase (1 mg mL^{-1}) diluted in 10% diethanolamine buffer for 20 min at RT. The colorimetric reaction was stopped by adding 3 N NaOH. Absorbance values were read in the ELISA reader (Mark, Thermo Plate; Model, TP-READER) at 405 nm. Leaf proteins of cv. Bollgard I (Monsanto, St Louis, MO) were used as a positive control because either Cry1Ia or Cry1Ac (from Bollgard) have structural similarity and therefore the antibody used binds to both. A previous serial dilution assay was carried out via dot-blot, setting up 1:1000 dilution, and this was therefore used in assays. Protein concentrations obtained by the ELISA reader were estimated from the standard curve established by dilutions of Cry1Ia protein. All treatments were performed in five replications. Data were subjected to analysis of variance using the SISVAR program v.5.1.[28] The Scott–Knott test ($P < 0.05$) was used for mean comparisons.

2.6 Immunodetection assay by confocal microscopy using boll weevil larvae

Larvae (2nd) of boll weevil were fed on artificial diet containing 50 mg mL^{-1} of dried and crushed young buds, collected from PTLs and non-transformed plants, at 45 days.[29] Next, guts were collected and cells were fixed overnight at 4 °C using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 1 mM of $CaCl_2$, pH 7.3. Following six further washings in 1× PBS buffer solution, cells were dehydrated with a graded series of acetone (30–100%) prior to embedment in paraffin.[30,31] Infiltration was performed with successive exchanges of paraffin every 16 h [2 xylol:1 paraffin, 1 xylene:1 paraffin, 1 xylene:2 paraffin, pure paraffin wax (2×)]. Then, samples were embedded for further sectioning (10 µm) using an automatic microtome.

For analysis by confocal microscopy, samples were prepared by deparaffinising sections in serial baths of xylene/ethanol, followed by blockage in PBS containing 1% bovine serum albumin (BSA, w/v) for 1 h at 25 °C and further incubation in biotinylated anti-Cry1Ia at 25 °C for 1 h. The sections were washed 3 times in PBS for 30 s and incubated in avidin-conjugated Texas Red fluorophore and 4′,6-diamidino-2-phenylindole (DAPI) at 25 °C for 1 h according to the manufacturer's instructions. Sections were analysed using an LSM410 confocal fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with lasers Air (ex 488 nm) and He/Ne (ex 543 nm). Digital images were acquired using the filters LP 515–488 nm and BP 590–610 to 543 nm.

2.7 Feeding bioassays with fall armyworm and boll weevil

Fall armyworm colonies were maintained on an artificial diet under laboratory conditions at 28 ± 2 °C and 65 ± 5% relative humidity under a 14:10 h (light:dark) photoperiod. Fresh leaf discs (1 cm diameter) were collected from PTLs and control (non-transformed) plants at 45 dae and deposited in 24-well trays. Then, one second-instar larva was placed individually in each well and incubated at 28 °C in a biochemical oxygen demand (BOD) growth chamber for 7 days. Leaf discs were changed every 2 days. The assay was completely randomised with six replications (six 24-well trays), totalling 144 insects tested in each line. Measurements were performed daily, recording the number of cadavers present in each well. The mortality rate was estimated from the total number of live larvae present in the control treatment at 7 days after trial establishment.

For the boll weevil bioassays, adults (12 days from the pupal stage) were fed on leaves and buds collected from PTLs and non-transformed plants at 45 dae. Five adults were maintained in pots (0.5 kg) containing, separately, fresh young leaf tissue (250 mg) or young 10 cm buds. Pots were incubated in the BOD growth chamber programmed for a 14:10 h (light:dark) photoperiod at 28 ± 2 °C and 65 ± 5% relative humidity.[29] The experiment was carried out with 21 repetitions for each line, giving a total of 105 adult insects evaluated over a period of 2 months.

Evaluations were performed daily, recording the number of dead insects in each pot. The mortality rate was estimated from the total number of live adults compared with the control treatment at 7 days after trial establishment. The corrected mortality of insects was estimated by the Schneider–Orelli formula,[32] which estimates the corrected percentage mortality as follows:

$$\%\ \text{Mortality} = (A - B/100 - B) \times 100$$

A = Percentage mortality in treated plot
B = Percentage mortality in control plot

2.8 Genetic analysis of T1 lines

The T1 generation of transgenes was analysed through amplification by PCR assays (1 F/2R primer combination, 0.96 kb) using

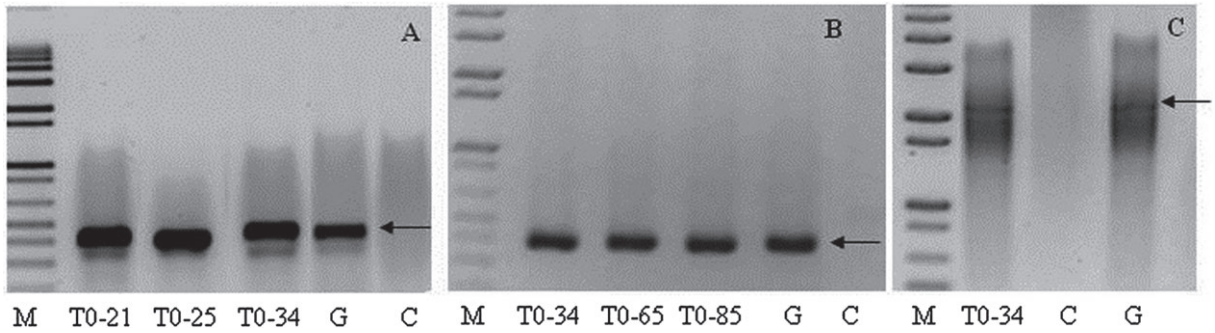

Figure 2. Amplicons obtained by PCR assays in T0 lines separated by 0.8% agarose gel. Primer combinations for *cry1Ia*-CNPA: (A) 3 F/2R (0.52 kb); (B) 1 F/3R (0.44 kb); (C) 5 F/2R (2.1 kb). M – 1 kb molecular marker (Ladder Plus; Invitrogen, Carlsbad, Califórnia, USA); G – positive control (*cry1Ia*-CNPA); C – negative control (non-transformed plant, BRS 293).

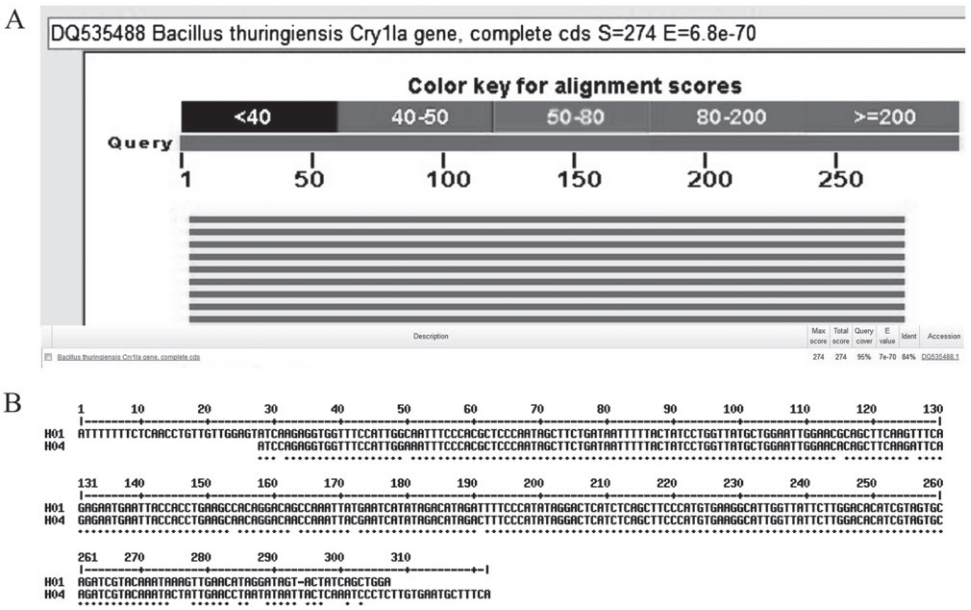

Figure 3. Comparison of the nucleotide sequences encoded by the *cry1Ia* (DQ535488) gene with 0.44 bp *cry1Ia*-CNPA deposited in the NCBI gene bank, by using BLAST analysis (A) and alignment of both sequences using the *MultipleM sequence alignment* program (B). H01 – *cry1Ia* (DQ535488); H04 – *cry1Ia*-CNPA. The asterisk (*) below each nucleotide indicates the conserved sequence.

leaves of self-pollinated plants. Self-pollination proceeded in five pre-anthesis squares (2 mm) of each selected line. The top of the squares was tied with copper wire, and further seeds (T1) were collected. An average of 150 seeds were collected from each line, and 10% of them were used for PCR assays. Seeds were sown in the greenhouse in 20 cm diameter pots filled with nutrient-rich soil and watered daily. Each pot contained only one seedling. From 15 dae, young leaves (100 mg) were collected for DNA extraction and PCR assays, as previously described. Chi-square (χ^2) analysis was performed to determine the segregation ratio and consistency with Mendel's laws.

3 RESULTS

3.1 Identification of putative transgenes and integration analysis

A total of 1248 putative transgenic seeds were collected from microinjected bolls of four Brazilian cultivars and sown in the greenhouse. At first, PTLs were analysed by PCR using three primer combinations. A few amplicons were verified in the cultivars and, even so, in one or two primer combinations, indicating incomplete integration of the gene (Table 1). Only in cv. BRS 293 was at least one PTL (T0-34) identified in all primer combinations, indicating possible complete integration of *cry1Ia* based on PCR reactions, mainly with the 5 F/2R primer combination, which generated an amplicon corresponding to the complete gene sequence (2.1 kb) (Fig. 2). A fragment of 0.44 kb (1 F/3R) collected from this line was purified, sequenced and analysed using BLAST tools. The results showed high homology between the sequences of *cry1Ia* from BRS 293 T0-34 and the *Bt cry1Ia* gene deposited in the NCBI gene bank (DQ535488, E-value: $3e^{-63}$ to $1e^{-12}$), as can be seen in Fig. 3.

In order to confirm the number of copies of *cry1Ia* integrated to the genome of the T0-34 line, Southern blot assays were carried out using a DIG-labelled probe from the *cry1Ia* gene (0.96 kb). Only one hybridisation signal was seen at ~5.0 kb (Fig. 4), indicating that complete gene construction was successfully introduced.

3.2 Immunodetection of Cry1Ia protein by ELISA and histological assays

Based on the number of copies of the gene detected in the Southern blot, a battery of ELISA assays was carried out in order to estimate the concentration of Cry1Ia protein in leaves of T0-34

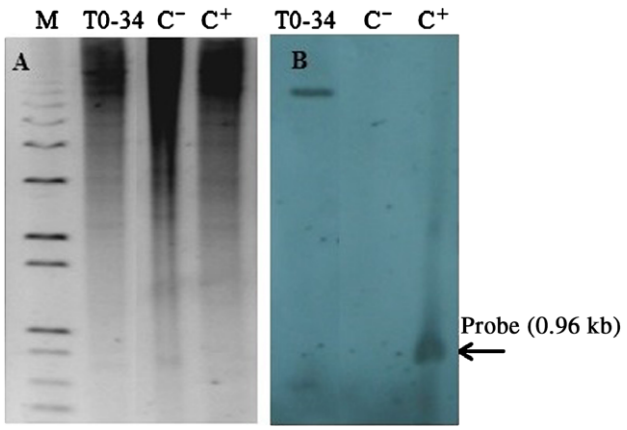

Figure 4. Southern blot hybridisation analysis of genomic DNA extracted from leaves of two transgenic cotton lines and the non-transformed plant (BRS 293). (A) DNA digested with *HindIII*; (B) blot exposition in X-ray. C^- – negative control: DNA from BRS 293 digested with *Hind III*; C^+ – positive control: DNA from BRS 293 digested with *Hind III* + probe (0.93 kb probe, 1 ng μL^{-1}); M – 1 kb molecular marker (Ladder Plus; Invitrogen).

Table 2. Cry1Ia concentrations ($\mu g\ g^{-1}$ dry weight) in cotton leaves from non-transformed (BRS 293), T0-34 and Bollgard I genotypes, estimated by indirect ELISA, during three phenological periods

	Concentration[a]		
Genotypes	45 dae	60 dae	90 dae
T0-34	2.7(±0.61) aA	1.9(±0.71) aB	1.4(±0.59) aC
Bollgard I	2.9(±0.58) aA	1.9(±0.68) aB	1.5(±0.61) aC
Control (BRS 293)	0.8(±0.32) bA	0.6(±0.26) bA	0.6(±0.24) bA

[a] Means followed by the same letter are not significantly different ($P \leq 0.05$; Scott–Knott test). Upper-case letters compare periods; lower-case letters compare genotypes. Standard deviations of the mean are given in parentheses.

during three growth periods. A positive control (Bollgard I) was used as the expression reference. As can be seen in Table 2, the patterns of protein ($\mu g\ g^{-1}$ dry weight) in T0-34 and Bollgard I were similar during the periods evaluated, showing peak expression at 45 dae (2.7 $\mu g\ g^{-1}$ dry weight) and decreasing by approximately 50% at 90 dae.

By confocal microscopy, the binding of anti-Cry1Ia antibody to midgut cells of boll weevil larvae was verified in samples of the T0-34 line (Figs 5B to D). The conjugation reaction of complex protein × antibody that was revealed with avidin conjugated to Texas Red showed emission of fluorophore only in epithelial cells of larvae fed on artificial diet + young buds of the transgenic line. Disruptions of microvilli were also seen in T0-34 samples. No emission of Texas Red was seen in cells from non-transformed samples (BRS 293) (Figs 5A to C).

3.3 Toxicity of Cry1Ia to fall armyworm and boll weevil

In feeding bioassays, fall armyworm larvae and boll weevil adults were fed on tissues of T0-34 plants over a period of 7 days in order to estimate the mortality rates due to crystal ingestion. For fall armyworm, mortality of larvae was verified 24 h after the beginning of feeding. In most cases, more than 50% of the larvae had died after the 4th day (Fig. 6B). The mortality rate after 7 days was 88.1% (Table 3).

In the boll weevil bioassays, mortality of adults was seen at 48 h after boll feeding. At first, the insects moved slowly in the corner of the pots (Figs 6F and G), and they died the next day in the 'head up' position (Fig. 6H). The mortality rate was higher in leaves (83.7%) than in buds (56.7%). These results were expected, as the promoter used in construction (CaMV 35S) has limited expression in flower buds.[33,34] On the other hand, the LC_{50} of the recombinant Cry1Ia protein tested in previous feeding bioassays was lower for fall armyworm (0.29 $\mu g\ mL^{-1}$) than for boll weevil (21.5 $\mu g\ mL^{-1}$).[23]

3.4 Transgene descendent analysis

Twenty self-fertilised seeds, all descendents from the T0-34 line, were sown in the greenhouse, and leaves of plants were used in PCR assays in order to estimate *cry1Ia* gene inheritance by descendents. Seventeen plants showed expected amplicons in the T1 generation (Fig. 7), exhibiting a segregating ratio consistent with Mendel's laws (3:1; $\chi^2 = 0.39$, df = 1, $P = 0.47$), and indicating integration of the transgene at a single locus. During the development, all plants showed phenotypically normal growth, with fertile flowers and boll yields similar to BRS 293 (non-transformed plant). Some agronomical traits are given in Table 4. Although no fibre traits were evaluated, no pleiotropic effect resulting from insertion of *cry1Ia* was identified in the T1 lines, at least for the traits recorded.

4 DISCUSSION

Insect pests are a serious problem in all crop systems. In management involving agricultural commodities such as soybean, maize, cotton and canola, the strategy of control should be decided quickly in order to avoid further losses. Lepidopteran and coleopteran insects attack a large number of agricultural species. In most cases, chemical insecticides are the only means of control.

The boll weevil is a major pest of cotton fields in several countries, accounting for yield losses and control costs of the order of several billion US dollars. Insects provoke high levels of bud abscission in plants owing to feeding or oviposition punctures. The abscission is mainly due to enzymes present in the insect's saliva, but mechanical damage could also contribute to square abscission.[16,35]

In Brazil, since the introduction of the boll weevil in 1983, several cotton fields have been destroyed, resulting in serious damage to farmers, especially to those located in the semi-arid north-east region. Adopting a proactive approach, Embrapa Cotton (Embrapa Algodão) has developed several technologies to minimise production losses, including those of early cultivars with a concentrated fruit set and short cycle. Such technologies, however, have not been sufficient to contain the evolution of the pest, given its dynamic life cycle with several overlapping generations. In each cotton cycle (120–170 days), 5–6 boll weevil cycles take place, generating approximately 100 000 adults at the cotton harvest, so that cotton management is only practicable with the adoption of several applications of chemical insecticides to control larvae and adults.[36]

The Embrapa research team has made efforts to minimise boll weevil damage by developing research in genetic engineering and areas of conventional breeding. The *Bt* collection from Embrapa Genetic Resources and Biotechnology holds over 2000 accessions, which are periodically surveyed for Cry toxin activity against

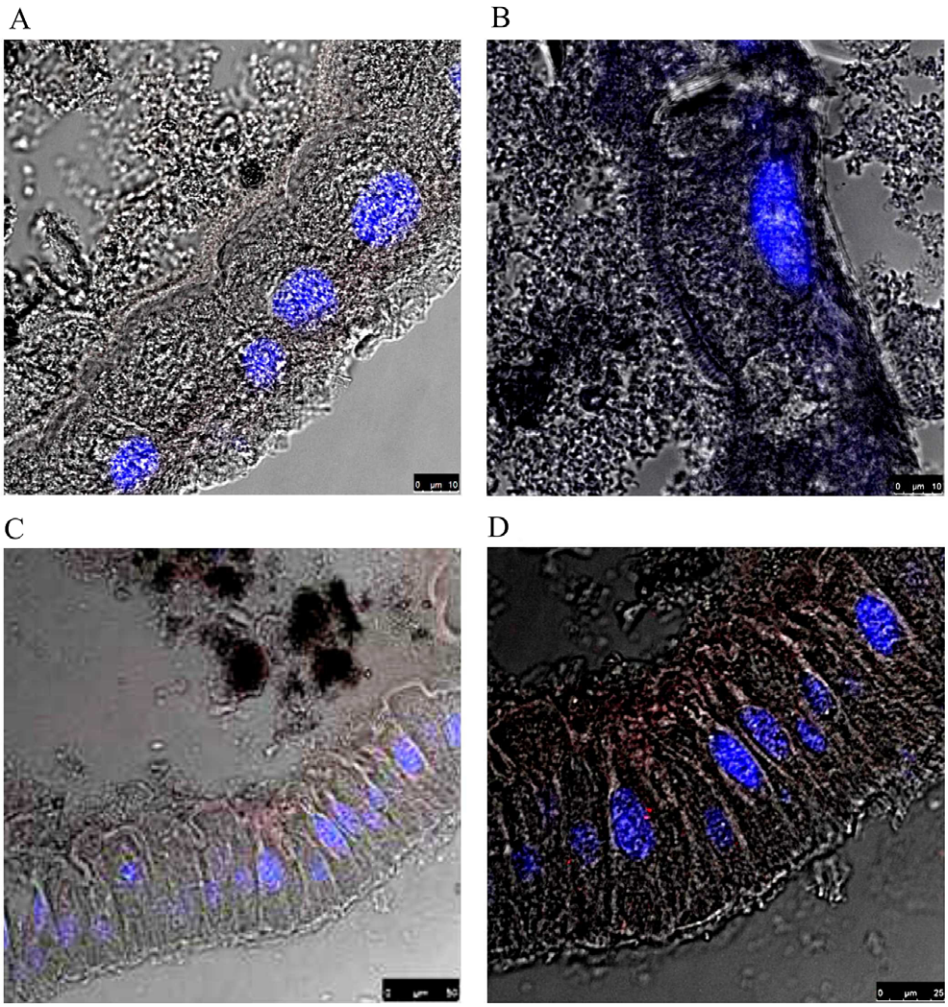

Figure 5. Epithelial cells of boll weevil guts analysed by confocal microscopy. Gut sections of insects fed on non-transformed plants (A and B) and T0-34 line (C and D). Conjugation reaction with biotinylated anti-Cry1Ia antibody revealed with avidin conjugated to Texas Red and DAPI. Magnification 10 µm. Arrow in (A), cell nuclei DAPI labelled.

dipteran, coleopteran and lepidopteran insects.[37] As a result, several isolates have been identified, and genes from the *cry* family have been isolated for further use in transgenics for insect pest control.[23,38–41]

The gene used in this work (*cry1Ia*) was previously isolated by our team and confers toxicity against two important cotton pests, *S. frugiperda* and *A. grandis*.[23] Molecular and immunodetection assays confirmed the integration of the gene and the toxicity of the Cry1Ia protein in a unique line generated from cv. BRS 293, representing a transformation rate of 0.34%, based on 296 lines tested from this cotton cultivar (Table 1). This rate was close to those found by other methodologies. Aragão *et al.*[42] achieved rates of between 0.45 and 0.71% by biolistic procedures in Brazilian cotton cultivars.

The results obtained here show the tangible possibility of controlling fall armyworm and boll weevil in a non-chemical way. The mortality rates observed in entomological assays for fall armyworm (88.1%) and boll weevil (83.7%) fed on cotton leaves over a period of 7 days were high, but the mortality rate was moderate (56.7%) for boll weevil fed on floral buds (Table 3). This value is justified, given that the promoter used in our gene construction, CaMV 35S, has been reported to have broad expression in various plant organs but limited expression in reproductive structures.[33,34] However, considering the damage to epithelial cells of larval midguts seen in confocal microscopy (Figs 5B to D), it is possible that continuous feeding will contribute to raising the mortality rate of insects in a period longer than that observed here. This could make a valuable agronomic contribution during the reproductive period, when cotton plants are prone to both pests.[43,44] A way to promote higher mortality in cotton fruits is to use tissue-specific promoters in gene construction. This strategy is being implemented by our team, and some promoter regions have already been identified.[45]

The T1 lines with positive amplicons shown in Fig. 6 were all self-fertilised, and T2 plants were submitted to feeding bioassays with fall armyworm and boll weevil insects. Seven lines have been identified with mortality rates close to those presented in Table 3 (data not given). The progress in selection procedures in these lines by the genetic improvement team at Embrapa Cotton has helped to identify materials that show great promise for the further development of cultivars resistant to these target insects.

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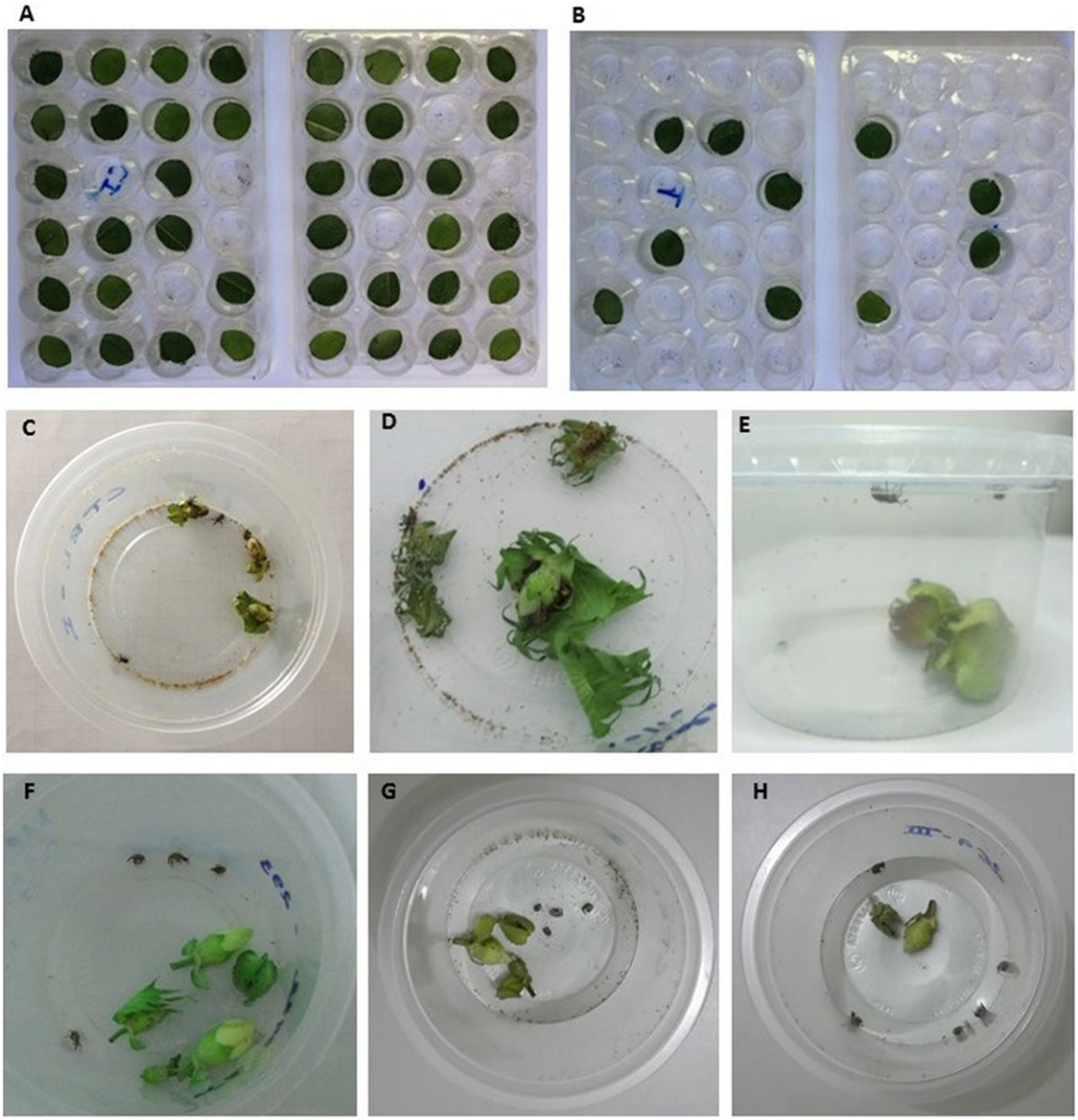

Figure 6. Feeding bioassays with fall armyworm and boll weevil. Detail of 24-well trays containing fall armyworm larvae fed on leaves of non-transformed (A) and T0-34 (B) plants after 7 days. Small black spots are larval cadavers. Boll weevil adults fed on buds of non-transformed (C, D, E) and T0-34 (F, G, H) plants after 7 days.

Table 3. Mortality rate (%) of fall armyworm larvae and boll weevil adults fed on tissues from control and T0-34 plants after 7 days[a]

	Fall armyworm larvae			Boll weevil adults				
Genotypes	*N*	Leaves[b]	CM(%)	*N*	Leaves[c]	CM(%)	Buds[d]	CM(%)
Control[e]	144	4.5(±1.4)	–	105	12.3(±1.8)	–	9.1(±1.1)	–
T0-34	144	88.6(±2.3)	88.1	105	85.7(±1.2)	83.7	60.7(±1.6)	56.7

[a] *N*: number of insects tested; CM: corrected mortality estimated by the Schneider–Orelli formula.[29] Standard deviations of the mean are given in parentheses.
[b] Fresh leaf discs (1 cm diameter) deposited in 24-well trays.
[c] Fresh young leaves (250 mg).
[d] Young 10 cm buds.
[e] BRS 293 (non-transformed plants).

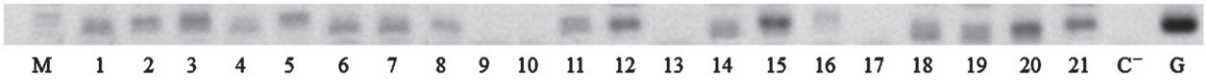

Figure 7. Amplicons obtained by PCR in T1 lines separated by 0.8% agarose gel. 1–21 – T1 lines descendent from T0-34; G – *cryI1a* probe (0.96 kb); C⁻ – negative control (BRS 293); M – 1 kb molecular marker (Ladder Plus; Invitrogen).

Table 4. Agronomic traits recorded in T1 lines during the growth cycle in the greenhouse

T1 line	Height (cm)	Blooming (dae)	Boll number	Boll weight (g)	Seed oil[a] (%)	Open boll weight (g)
1	78	55	15	21.98	19.7	5.6
2	75	55	15	21.87	19.8	5.8
3	74	56	13	22.11	20.1	5.6
4	75	56	15	22.56	20.6	5.8
5	77	55	14	22.35	20.2	6.1
6	78	56	14	21.96	21.2	5.9
7	75	57	15	21.87	20.5	6.1
8	74	56	15	22.03	21.5	6.2
11	79	57	16	22.35	20.7	5.9
12	78	56	15	22.15	20.5	5.8
14	78	57	16	21.99	19.8	5.7
15	76	56	15	22.12	19.9	5.6
16	75	58	16	22.21	21.0	5.7
18	76	56	15	21.95	21.1	5.8
19	78	57	15	22.42	20.5	5.8
20	76	58	14	21.85	20.4	5.9
21	75	58	14	22.05	19.7	6.1
T1 mean[b]	76.3(±1.6)	56.4(±1.0)	14.8(±0.8)	22.1(±0.2)	2.4(±0.5)	5.8(±0.2)
BRS 293[c]	77.2(±1.3)	56.1(±1.1)	15.2(±0.8)	22.3(±0.3)	20.3(±0.7)	5.7(±0.2)

[a] Data obtained from three repetitions by nuclear magnetic resonance.
[b] Standard deviations of the mean are given in parentheses.
[c] Means of ten plants.

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