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Transformation of Maize Elite Lines with *cry1Ca* of *Bacillus thuringiensis* to Control *Spodoptera frugiperda*

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Fall armyworm, *Spodoptera frugiperda*, is the most important insect pest of corn in Brazil. *Bacillus thuringiensis* (Bt) is an insect pathogen that may be used to control this insect in the field. Strain 1644, discovered in a soil sample in the south of Brazil, was found to be very efficient in controlling this insect in laboratory bioassays. This strain harbors the *cry1C* gene (2.7 Kb). Twelve primers were used to perform the sequencing along the gene. After sequencing, *cry1C* gene was cloned in the pCAMBIA 3301 vector, under the control of the ubiquitin promoter and terminator NOS. The plasmid was amplified in *Escherichia coli* strain DH5α, purified in CsCl gradient and used to transform tropical maize (*Zea mays L.*) calli via biobalistic process. The integration of *cry1C* gene was confirmed by PCR in 13 maize plants generated. Leaves of transgenic plants regenerated directly from callus lines were bioassayed with S. frugiperda and showed different levels of resistance to this insect pest, and one inbred line, L3, inhibited 100% of larval growth (L3 – Elite line transgenic corn belongs to Embrapa Maize and Sorghum Research Center).

Introduction

Fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is one of the most important insect pests in Brazil. Its feeding damage (Figure 1) may reduce corn yield by up to 34% (2). Currently, chemical insecticides such as organophosphates, chlorpyrifos, and spinosad are used to control this pest in Brazil to reduce damage.

FIG. 1. Fall armyworm, *Spodoptera frugiperda*, damage on non-transgenic corn (*Zea mays* L.) leaves.

Material and methods

Expression Cassete: *Ubiquitine Promoter* + *gene* cry1Ca + *terminator NOS*. The terminator NOS was amplified using PCR from the vector pCAMBIA 3301, using the primers listed in Table 1. Ubiquitin promoter was isolated from the vector pAHC17 using restriction enzymes *Hind* III and *Bam* HI. The fragment corresponding to the promoter was isolated from the gel using the Qiagen kit (Valencia, CA, USA) and added to the vector pCAMBIA 3301 cleaved with the same enzymes. The map of the vector pCAMBIA 3301 containing the promoter, the *cry1Ca* gene and NOS terminator is described in Figure 2. The *Bt* gene *cry1Ca* was isolated from *Bacillus thuringiensis* strain 1644, collected from soil sample in southern Brazil, and it was deposited in the Microorganism Bank of the Embrapa Maize and Sorghum Research Center. The band containing the gene was cut out of the gel and cloned in a vector TOPO-TA (Invitrogen) and sequenced according to the instructions of the manufacturer (BigDye 3.1 – Sequencer 3100 – Applied Biosystem, Foster City, CA, USA). AdvantageTaqPol (Clontech Inc. Palo Alto, CA, USA) was used in the PCR reaction and the amplification consisted of 5 ng of DNA. A predenaturation step of 15 sec at 95°C was followed by 30 PCR cycles of 95°C for 15 sec (denaturation), 60°C (annealing) for 30 sec, 72°C for 3 min (elongation) and final elongation step of 10 min at 72°C. The final product representing the *cry1Ca*

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TABLE 1. Primers used in the isolation of the terminator NOS and *cry1Ca* gene.

Name	Sequence
cry1Ca - 5'	TTA GGA TCC TTA TGG AGG AAA ATA ATC AAA TC
cry1Ca - 3'	TTA GGA TCC TGC AGG AAC AAT CTA GAT C
NOS - 5'	GAT AAG CTT CCC CGA TCG TTC AAA C
NOS - 3'	GAT AAG CTT GAA TTC CGC CAA TAT ATC CTG TC

GGA TCC – *Bam* HI, **AAG CTT** – *Hind* III, **GAA TTC** –*Eco* RI

gene was isolated from the gel and cloned in the vector TOPO-TA. The *cry1Ca* gene was cleaved from TOPO-TA with *Bam* HI and linked in the vector pCAMBIA 3301, and cut with the same enzymes. Sequencing reactions to confirm this construction used primers corresponding to different regions of the promoter, the *cry1Ca* gene and the flanking regions of the polylinker of the pCAMBIA 3301 (M13 universal forward and reverse primers).

Maize genetic transformation. Immature maize embryos of the inbred line L3 (*Zea mays* L.), 1.0 - 1.5 mm long, were isolated and cultivated during 28 days in callus induction medium (3) and bombarded according to Carneiro et al. (1). Calli are tissues that form over the surface abrasions in plants. Transgenic plants selection started 15 days after the bombardment, when calli were transferred to SM medium (without proline) supplemented with ammonium glufosinate. Calli were sub-cultivated every 2 weeks with increasing dosages of ammonium glufosinate (3 mg/L and 9 mg/L). When the regenerated plantlets were 5 cm high they were transferred to the greenhouse (26°C ± 2°C, 75% R.H. ± 5% and 14L:10D). Thirteen transformed plants, from the inbred line L3, were used as treatment, and 3 plants, from the same line but not transformed, served as control in the feeding test conducted against the fall armyworm.

Feeding tests. Pieces of leaves (25 cm^2 in size) of each of the 13 transgenic and 3 non-transgenic (control) maize were provided to five 1-day-old, first instar larvae. A total of 65 larvae were fed transgenic plants and 15 larvae were fed control plants. Maize leaves were changed every 2 days and visual observations of feeding damage and larval mortality were recorded daily. Fall armyworm larvae, because of their cannibalistic habit, were kept in individual containers with their food.

Molecular analysis. Genomic DNA from 13 transformed plants was used to assure the presence

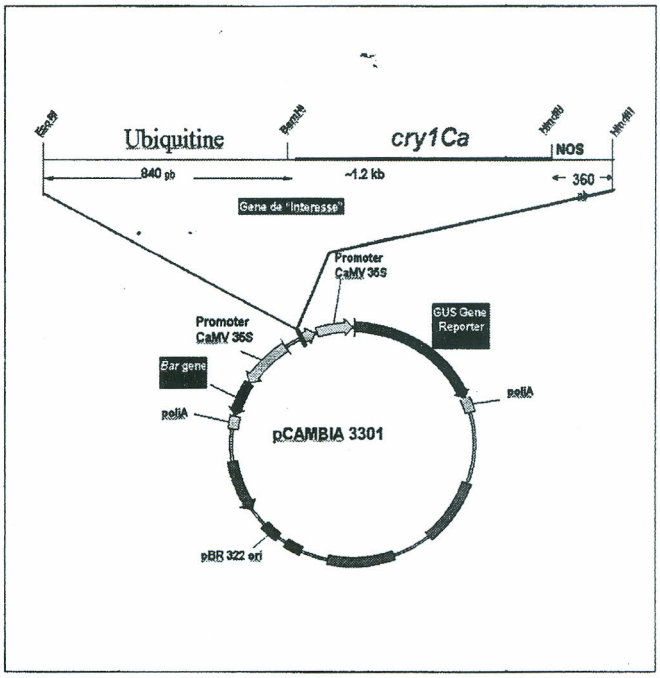

FIG. 2. Vector pCAMBIA 3301 with the Ubiquitin promoter, *cry1C* gene and the terminator NOS.

of the ubiquitin - *cry1C* construction in the regenerated plants, confirmed by PCR using specific primers. Each 25 µl of the reaction consisted of 1X PCR buffer, 2 mM $MgCl_2$, 100 µM dNTP, 0.2 µM of each primer, 25 ng of DNA, and 1 UI of *Taq* DNA Polimerase. Thermocycler model 9600 (Perkin-Elmer Cetus, Norwalk, CT, USA) was used for amplification as follows: a predenaturation step of 15 sec at 94°C was followed by 30 PCR cycles of 94°C for 15 sec (denaturation), 55°C (annealing) for 1 min, 72°C for 3 sec (elongation) and final elongation step of 7 min at 72°C.

Results and discussion

The results showed a considerable difference in the feeding damage among leaves of transgenic and

TABLE 2. Larval mortality of *Spodoptera frugiperda* and estimated damage to test plants during laboratory feeding bioassay.

Treatment or Host type	No. of plants	Total no. of insescts	Estimated percent damage of corn-leaf area	No. of insects that died
Transgenic L3 containing *cry1C*	13	65	10	20
L3 line without *cry1C* (Control)	3	15	90	0

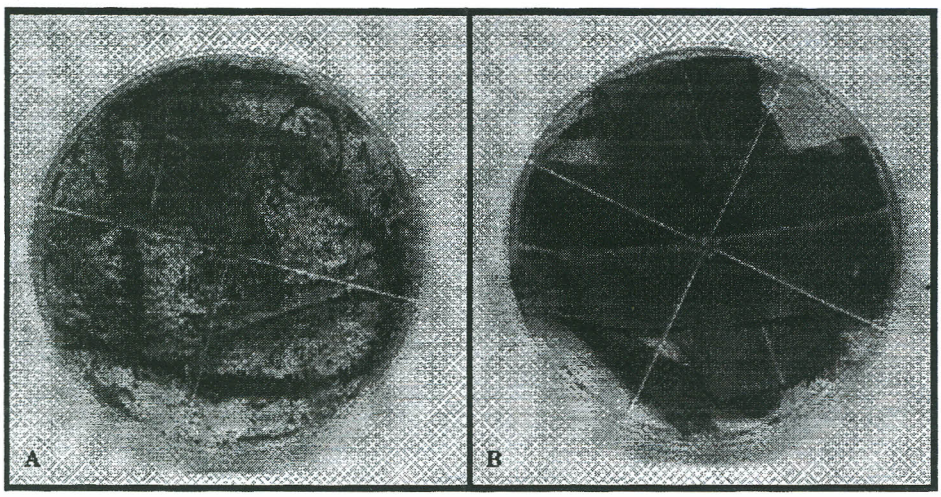

FIG. 3. Feeding bioassays using fall armyworm, *Spodoptera frugiperda*, larvae. (A) Control - Non-transformed L3 maize inbred line. (B) Transformed maize L3 inbred line containing *cry1C* gene.

control plants provided to the insects (Table 2). Out of the 13 transgenic L3 inbred lines, 5 plants were able to kill fall armyworm larvae. Four out of 5 larvae died after feeding on each of the 5 transformed plants. All dead larvae were tested to check the presence of *Bt* toxin via PCR using Cry1C-specific primers and phase contrast microscope, after reisolation from dead bodies in culture media. All transformed maize plants showed no more than 10% of leaf damage while non-transformed plants showed an average damage of 90%. Transformed maize plants showed only some scratching on the leaf surface, however larvae regularly fed on non-transformed maize plants caused the usual leaf damage as shown in Figure 1.

Conclusion

Laboratory bioassays, using transgenic corn plants, containg *cry1C* gene, showed that the expressed toxin is very effective against fall armyworm. Future experimental goals are: 1. Transfer the gene to Embrapa's inbred elite maize line using traditional plant breeding methodology. 2. Conduct several controlled field trials with and without artificial infestation with fall armyworm larvae, to evaluate the insecticidal performance of the transgenic plants.

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