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N. Bohorova · R. Frutos · M. Royer · P. Estañol
M. Pacheco · Q. Rascón · S. McLean · D. Hoisington

Novel synthetic *Bacillus thuringiensis cry1B* gene and the *cry1B-cry1Ab* translational fusion confer resistance to southwestern corn borer, sugarcane borer and fall armyworm in transgenic tropical maize

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Abstract In order to develop a resistance management strategy to control tropical pests based on the co-expression of different toxins, a fully modified *Bacillus thuringiensis cry1B* gene and the translational fusion *cry1B-cry1Ab* gene have been developed. Both constructs were cloned under the control of a maize ubiquitin-1 or a rice actin-1 promoter and linked to the *bar* gene driven by the CaMV 35S promoter. Immature embryos from the tropical lines CML72, CML216, and their hybrids, were used as the target for transformation by microprojectile bombardment. Twenty five percent of the transformed maize plants with *cry1B* expressed a protein that is active against southwestern corn borer and sugarcane borer. Ten percent of the transgenic maize expressed single fusion proteins from the translational fusion gene *cry1B-1Ab* and showed resistance to these two pests as well as to the fall armyworm. Transgenic maize plants that carried the *cry1B* gene in T1 to T3 progenies transmitted trangenes with expected Mendelian segregation and conferred resistance to the two target insects. Molecular analyses confirmed the *cry* genes integration, the copy number, the size of protein(s) expressed in maize plants, the transmission, and the inheritance of the introduced *cry* gene. These new transgenic products will provide another recourse for reducing the build-up of resistance in pest populations.

Keywords Tropical maize · Transformation · Plant regeneration · Insect resistance · *Bacillus thuringiensis* (Bt)

N. Bohorova · P. Estañol · M. Pacheco · Q. Rascón · S. McLean
D. Hoisington (✉)
CIMMYT, Apdo. Postal 6-641, 06600 Mexico, D. F., Mexico
e-mail: d.hoisington@cgiar.org
Tel.: +52-5804-7575, Fax: +52-5804-7567

R. Frutos · M. Royer
CIRAD, TA 40/02, 34398 Montpellier, cedex 5, France

Present address:
N. Bohorova, Research Director, Epicyte Pharmaceutical Inc.,
5810 Nancy Ridge Drive, Suite 150, San Diego, CA 92121, USA

Introduction

Transgenic maize has been bioengineered to protect the crop against several corn borers by the expression in different plant tissues of insecticidal crystal proteins (Cry) derived from the soil bacterium *Bacillus thuringiensis* (Bt) (Koziel et al. 1993; Armstrong et al. 1995; Willims et al. 1997; Bohorova et al. 1999). Synthetic *cry* genes, by increasing the G/C content of their encoding genes using plant preferred codons, have been developed and enhance the production of toxins in transgenic plants (Perlak et al. 1991; Estruch et al. 1997; Fearing et al. 1997). Published field trials of transgenic maize containing the *cry1Ab* gene have demonstrated the effectiveness against the temperate stem borer *Ostrinia nubilalis* (Fromm et al 1990; Koziel et al. 1993; Armstrong et al. 1995; McKenzie 1997; Peferoen 1997). These transgenic maize varieties have also demonstrated good control of the tropical stem borer *Diatraea grandiosella* (southwestern corn borer, SWCB) and *Spodoptera frugiperda* (the fall armyworm, FAW) (Bergvinson et al. 1997; Williams et al. 1997) and reduce Fusarium ear rot (Munkvold et al. 1997). However, several insect species have evolved resistance to *Bt* toxins, which could seriously compromise the success of *Bt*-transformed crops in controlling pests. Successful pesticide resistance management involves several strategies: tissue- or time-specific expression of the Cry toxin; a combination of different *cry* genes (gene stacking strategy); a combination of toxins with different modes of action; low doses of toxins in combination with natural enemies; mixtures, rotation or mosaics of transgenic plants; and the use of refugia (Tabashnik et al. 1991; Perlak et al. 1993; Bergvinson et al. 1997; McGaughey et al. 1998; Frutos et al. 1999). In addition, a strategy employing *cry* gene translational fusions for transfer and expression in plants to simultaneously prevent or postpone the development of resistant insect population has been developed (Van der Salm et al. 1994; Rang et al. 1997). The analysis of toxic activity through bioassay allowed the identification of the Cry1Ab, Cry1Ac and Cry1B toxins against *D. grandiosella* and

Diatraea saccharalis (Sugarcane borer, SCB) (Bohorova et al. 1997). Synthetic *cry1Ac* genes have been introduced, stably expressed, and conferred resistance to southwestern corn borer (SWCB) in tropical maize (Bohorova et al. 1999). In order to develop a resistance management strategy to control SWCB, based on co-expression of different toxins, a fully modified *cry1B* gene and the translational fusion *cry1B-cry1Ab* have been developed. We report here the search for effective *B. thuringiensis* toxins against the major lepidopteran pests; the synthesis of *B. thuringiensis* modified *cry1B* and *cry1B-1Ab* genes and the development of gene constructs for transformation; as well as gene transfer, integration, expression and inheritance in tropical maize.

Materials and methods

Testing the toxicity of *Bt* CryIB proteins on individual lepidopteran species

Insecticidal crystals containing Cry1B protoxin were purified from the Mexican isolated *B. thuringiensis subsp. thuringiensis* IB43 strain. This strain was characterized by the enzyme-linked immunosorbent assay (ELISA), using a specific monoclonal antibody against the Cry1B toxin and a polymerase chain reaction (PCR) analysis using specific primers for the *cry1B* gene described in Cerón et al. (1994). The insecticidal proteins produced in the recombinant *Escherichia coli* strains were purified as described (Bohorova et al. 1997). Larvae of *S. frugiperda, D. grandiosella, D. saccharalis*, and *H. zea* were obtained from colonies established in the Entomological Rearing Facility at CIMMYT (Mexico), where the bioassay was conducted, as described by Bohorova et al. (1996, 1997).

Construction of the synthetic *cry1B* gene

Oligonucleotide design

The sequence encoding the first 641 amino acids of the *cry1Ba2* gene originated from *B. thuringiensis* HD-110 and was divided into seven blocks ranging from 101 bp to 452 bp with appropriate restriction sites. These restriction sites were used to assemble the different blocks after synthesis and to clone the synthetic gene under the control of the selected promoters. The restriction site *Pst*I at position 979 was mutated after assembly because it was incompatible with the correct encoding sequence. For each fragment 2 to 8 oligonucleotides, 64 to 76 in length, were synthesized. For each block, one oligonucleotide complements the coding-strand sequence. This complementary oligonucleotide is called x1 (x corresponding to the block number) and is indicated by a thick arrow in Fig. 2. Others oligonucleotides, called x2 to x8, correspond to the coding-strand sequence. Oligonucleotides were designed using the oligo-4 program. Oligonucleotides were determined so that they do not form secondary structures; they show an overlap of 15 bases with the contiguous oligonucleotides (as shown in Fig. 2), and they do not anneal with other oligonucleotides except for oligonucleotides x1 and x2, which anneal together at the level of the 15-base overlap. Oligonucleotides were prepared by the solid-phase phosphoramidite triester coupling approach, using an Applied Biosystems synthetiser (Oligo express, Paris). Crude oligonucleotides mixtures were purified by polyacrylamide gel-electrophoresis.

PCR reactions

PCR reactions were carried out sequentially to synthesize the complete block with one pair of oligonucleotides. For one block with *n* oligonucleotides, *n*-1 PCR phases were performed. The first-phase PCR reaction was conducted with 20 pmol of x1 and 1 pmol of x2; the second phase with 20 pmol of x1 and 1 pmol of x3; the *n*th phase with 20 pmol of x1 and 1 pmol of x *n*+1; and the last phase with 20 pmol of x1 and 20 pmol of the last oligonucleotide, to obtain a complete block. PCR reactions contained oligonucleotides, 5 µl of 10X Vent buffer, 200 µM of each of the dNTPs, 6 mM of $MgSO_4$, two units of Vent polymerase (New England Biolabs) and a 8-µl aliquot (from the next phase) with a 50-µl final volume. PCR conditions involved an initial denaturation of 7 min at 95°C and four cycles of 1.5 min at 95°C, 2 min at 55°C and 3 min at 72°C. For the last phase, there were six cycles and a final extension of 10 min at 72°C.

For DNA block-cloning, sequencing, and assembly of the gene the amplified fragments were separated in a 2% agarose gel (Nusieve 3:1; FMC) and cloned into the *Eco*RV site of pBluescript KS (+) (Stratagene). For each block, ten clones were sequenced on both strands. For blocks 1, 3, 4 and 6, one clone was identified as having no misincorporation. For blocks 2 and 3, one clone with one misincorporation each was detected and then repaired by single-strand site-directed mutagenesis. The repaired block was totally sequenced again. The full-length coding sequence was assembled using the different restriction, and the *Pst*I restriction at position 979 was mutated by single-strand site-directed mutagenesis to restore the correct coding sequence.

The cloning and site-directed mutagenesis were done using standard procedures and kits from commercial manufacturers. Clones were sequenced using the ABI Perkin-Elmer sequencer and the *Taq* dideoxy terminator, according to the manufacturer's procedure (Genome Express, Grenoble).

Cloning of the *bar* gene and synthetic *cry1B* gene on a single plasmid

pUbi1B/35Sbar plasmid

The synthetic *cry1B* gene was cloned under the control of the entire 5′ untranslated region of the maize polyubiquitin gene Ubi-1 (promoter, exon-1 and intron-1) and the polyadenylation sequence from the *Agrobacterium tumefaciens nos* gene into the *Bam*HI site of the pAHC25 plasmid constructed by Christensen et al. (1992). A *Hin*dIII fragment containing the synthetic *cry1B* gene under the control of the 5′Ubi-1 and 3′ *nos* regions was cloned into the *Hin*dIII restriction site of the p35SAc plasmid (constructed by Dr Eckes, Biol. Res. C, H 872N, HOECHST AG, 65926, Frankfurt, Germany), which contains the synthetic *Streptomyces viridochromogenes bar* gene under the control of the CaMV 35S promoter and terminator inserted into pUC18.

PAct1B/35Sbar plasmid

The vector pAct-1 (Zhang et al. 1997) was digested by *Xho*I and *Sma*I and the fragment containing the 5′untranslated region of the rice actin-1 gene (promoter, exon-1 and intron) was cloned in the *Sma*I and *Sal*I sites of the vector pALTER (Promega). The plasmid pALTER-Act was used for mutagenesis to introduce a *Nco*I site at the level of the translation initiation codon of the Act-1 gene. Mutagenesis was achieved using the oligonucleotide 5′ggCgTCAgCCATggTCTACCTACA3′ and has been checked by DNA sequencing. The synthetic *cry1B* gene was cloned under the control of the 5′Act-1 and 3′ nos regions into the *Nco*I site of the pALTER-Act. A *Hin*dIII fragment containing the *cry1B* gene under the control of the 5′Act-1 and 3′nos regions was cloned into the *Hin*dIII site of pAc35S.

Construction of the translational fusion cry1B-cry1Ab

A pAct1B/35S/bar plasmid was used for mutagenesis to remove the synthetic *cry1B* stop codon, to re-create the two codons encoding the Cry1B activation site, and to create an *Apa*I site. Mutagenesis was achieved using oligonucleotide 5′CTgCAggAATTCgggCCCTCTCCAAgTC3′ and was checked

by DNA sequencing. A 96-bp DNA fragment that contains a sequence encoding for the 28 amino acids located downstream from the *Cry1B* activation site and a *Hpa*I site was synthesized and inserted into the *Apa*I site created by mutagenesis. The synthetic *cry1Ab* gene (Sardana et al. 1996) was cloned into the *Hpa*I site in the same open reading frame as synthetic *cry1B*. The resulting pAct1B-1Ab plasmid contains the translational fusion *cry1B-cry1Ab* under the control of the 5′Act-1 and 3′nos regions.

Cloning of the bar gene and translational fusion *cry1B-cry1Ab* onto a single plasmid

pUbi1B-1Ab/35Sbar plasmid

The translational fusion *cry1B-cry1Ab* was cloned under the control of the 5′Ubi-1 and 3′ nos regions into the *Bam*HI site of the pAHC25 plasmid. A *Hin*dIII fragment containing the translational fusion *cry1B-cry1Ab* under the control of the 5'Ubi-1 and 3' nos regions was cloned into the *Hin*dIII site of p35SAc.

PAct1B-1Ab/35Sbar plasmid

A *Hin*dIII fragment isolated from *pAct1B-1Ab* containing the translational fusion *cry1B-cry1Ab* under the control of the 5′Act-1 and 3′nos regions was cloned into the *Hin*dIII site of pAc35S.

Transformation of tropical maize with the synthetic *cry1B* and the fusion *cry1B-cry1Ab* genes

For the transformation experiments, the inbred lines CML216, CML323, CML327 and the hybrids between maize inbred lines CML72 and CML216 were used. Immature embryos (1.0–1.5 mm) were aseptically removed from kernels (12–15 days after pollination) and placed, scutellum-up, on the callus initiation medium. The medium used for embryogenic callus initiation and maintenance, consisted of modified N6 basal medium supplemented with 200 mg/l of casein hydrolysate, 2.302 mg/l of L-proline, 3% sucrose, and 2 mg/l of dicamba (N6C1) as reported Bohorova et al. (1995). A 4-h pre-bombardment osmotic treatment of the embryos was performed on the N6C1 medium with 12% maltose. Gold particles (Bio-Red, 0.6 µm in diameter) were coated with 5 µl of plasmid DNA, mixed with spermidine and $CaCl_2$, re-suspended twice in 240 µl of absolute ethanol and pipetted onto the center of a macrocarrier, previously positioned in a macrocarrier holder, and air-dried (Bohorova et al. 1999). Bombardments were applied using the Bio-Rad PDS-1000 helium-driven biolistics-particle delivery system. Selection of transformed cells during callus-formation was achieved using N6C1 callus formation medium containing 3 mg/l of bialaphos, and the embryos were maintainined for 4 weeks (at a temperature of 28°C darkness) in a growth-culture cabinet. The selection process continued during the regeneration stage when a concentration of 7 mg of l PPT was added to MSR medium (Bohorova et al. 1995) and the selected calli were maintained for another 2 weeks at a temperature of 28°C under a photoperiod regime of 16 h fluorescent light. The selected plantlets were transferred to the MSE medium (Bohorova et al. 1995) supplemented with 1 mg/l of bialaphos for root formation. Within 1 week, putative transformed maize plants were transferred to soil and grown under greenhouse conditions. Regenerated plantlets were healthy and successfully grew into fertile plants.

Phenotypic confirmation of transgenic plants carrying the *cry1B* gene

Plants were further selected by painting the fifth or sixth leaf near the tip of the youngest fully extended leaf with 2% Basta solution containing 0.1% Tween 20. A simple leaf bioassay (in 5-cm Petri dishes) was performed by testing all herbicide-resistant transgenic and control lines of maize with five neonate larvae and then observing insect mortality. The leaves of the transgenic plants were classified as resistant according to the damage caused by the larvae formed. All insect-resistant plants were crossed with the respective CML216 line. From each event, at least 16 plants were tested in the T1 generation, and 32 plants in T2 and T3, by three infestations of 30 larvae (per infestation). Plants were scored for feeding damage 2 weeks after the infestation and were divided into two classes: resistant and susceptible.

Molecular confirmation of transgenics

For molecular confirmation, genomic DNA was isolated from leaf samples of each putative transformant as well as the untransformed tropical lines. Plant genomic DNA was extracted from freeze-dried young leaf tissue (0.5 g) of primary transformants and progeny plants using a modification of the procedure described by Shure et al. (1983). Each sample was digested with a restriction enzyme(s), *Hin*dIII and/or *Eco*RI, separated on 0.8% agarose (FMC Corporation), and transferred to nylon membranes. Probes from the transgene *cry1B* were labeled with digoxigenin-dUTP and used for Southern hybridizations for transgenic plants carrying *cry1B* and *cry1B-1Ab* genes. Southern blots were probed with 3.5-kb (for Act:*cry1B*) and 4.1-kb (for Ubi:*cry1B*) labeled *cry1B* coding-region fragments. Pre-hybridization and hybridization protocols, as well copy number determination and non-radioactive procedures for detecting DNA sequences, were applied as described by Bohorova et al. (1999).

Results

Test of the toxicity of *B. thuringiensis cry1B* proteins on individual lepidopteran species

Bioassays with neonate lepidopteran larvae were performed with Cry1B insecticidal crystal proteins. It has been reported that Cry1-type proteins are highly specific against lepidopteran insects. There is little information regarding the control of *D. grandiosella* or *D. saccharalis* by *B. thuringiensis* ICPs. We found that both types of larvae are highly susceptible to Cry1B toxin with a LC50 of 5.1 ng/g (artificial diet) (Fig. 1). Cry1B toxin was found to be 20-times less active against *D. saccharalis* than against *D. grandiosella*. (Bohorova et al. 1997).

Fig. 1 Insect bioassays on artificial diet for the identification of insecticidal activity of the Cry proteins against southwestern corn borer and sugarcane borer

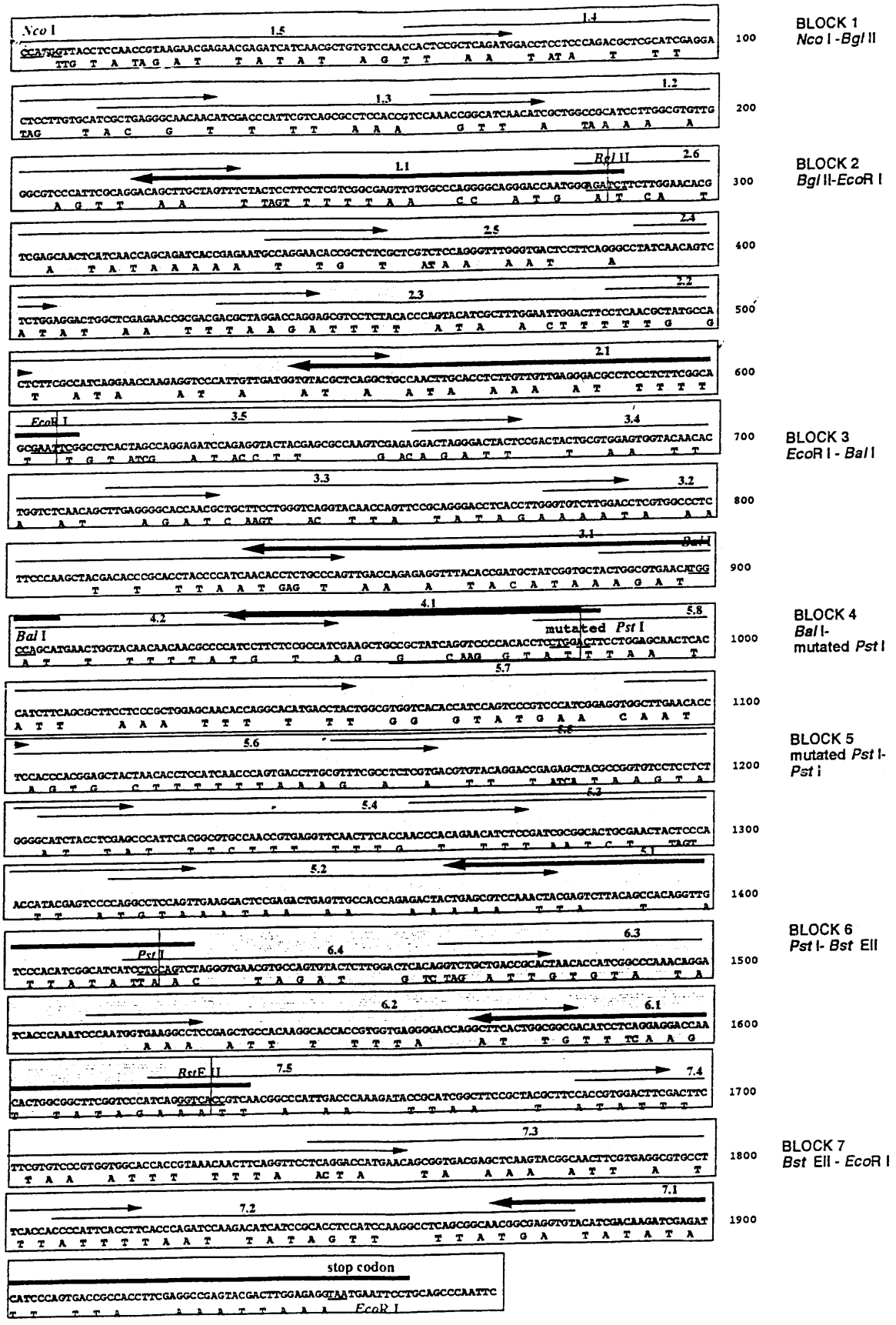

Fig. 2 Nucleotide sequence of the synthetic *cry1B* gene. The sequence of the wild-type *cry1B* gene was fully modified from nucleotide 1 to 1941 (amino acid 1 to 647), which corresponds to the sequence coding for the active toxin plus 29 amino acids on the N-terminal end

Fig. 3a–c Biolistic transformation of tropical maize using the *cry1B* gene. **a** Embryogenic callus on N6C1 medium under selection condition using 3 mg/l of bialaphos for 4 weeks; **b** plant regeneration on MSR medium under selection using 7 mg/l of PPT for 3 weeks; **c** transgenic plants carrying the *cry1B* gene showing resistance for SWCB and SCB

Table 1 Number of transgenic tropical maize plants carrying *cry1B* and *cry1B-1Ab* genes linked with the *bar* gene, showing resistance to herbicide Basta and SWCB/ SCB

Construct used	Genotype used	Total number of tested plants	Plants resistant to Basta	Plants resistant to insect(s)
Total *cry1B*		237	231 (97%/237)	60 (25%/ 237)
Ubi:*cry1B*	CML72×CML216 or CML216×CML72	61	61 (26%/237)	14 (6%/237)
Act: *cry1B*	CML72×CML216 or CML216×CML72	165	159 (67%/237)	42 (18%/237)
	CML216 CML323 CML327	11	11 (5%/237)	4 (1%/237)
Total *cry1B-1Ab*		172	162 (90%/172)	17 (10%/172)
Ubi: *cry1B-1Ab*	CML72×CML216 or CML216×CML72	35	35 (20%/172)	8 (5%/172)
	CML327	1	1 (1%/172)	1 (1%/172)
Act: *cry1B-1Ab*	CML72×CML216 or CML216×CML72	122	112 (63%/172)	8 (5%/172)
	CML327	14	14 (8%/172)	0

Table 2 Transgenic maize plants with resistance in the progenies to the herbicide Basta and Southwestern corn borer

Plant #	Construct used for transformation	Genotype	Resistance to Basta in T0 (R:S)	Segregation for Basta resistance in T1 (R:S)	Resistance to SWCB/SCB/ FAW in T0 (R:S)	Segregation for SVCB resistance in T1 (R:S)	Segregation for SWCB resistance in T2 (R:S)	Southern-blot analyses for cryIB integration
1978	Act/cry1B	CML72×CML216	R	8:8	R to SWCB	8:8		Act/1B+
2647	Act/cry1B	CML72×CML216	R	4:2	R to SWCB	3:3		Act/1B+
2111	Act/cry1B	CML72×CML216	R	10:6	R to SWCB	9:7		Act/1B+
2618	Act/cry1B	CML72×CML216	R	8:8	R to SWCB	7:9		Act/1B+
2625	Act/cry1B	CML72×CML216	R	6:9	R to SWCB	5:10		Act/1B+
2726	Act/cry1B	CML72×CML216	R	9:6	R to SWCB	5:10		Act/1B+
2549	Act/cry1B	CML216×CML72	R	7:9	R to SWCB	5:11		Act/1B+
2590	Act/cry1B	CML72×CML216	R	4:4	R to SWCB	2:6		Act/1B+
5601	Act/cry1B	CML72×CML216	R	1:15	R to SWCB	1:15	17:32	Act/1B+
6256	Act/cry1B	CML72×CML216	R	0:34	R to SWCB	0:34	2:11	Act/1B+
2417	Act/cry1B	CML216×CML72	R	3:11	R to SWCB	0:14		Act/1B+
2489	Act/cry1B	CML72×CML216	R	9:6	R to SWCB	0:15		Act/1B+
2545	Act/cry1B	CML72×CML216	R	10:6	R to SWCB	0:16		Act/1B+
2554	Act/cry1B	CML72×CML216	R	0:15	R to SWCB	0.15		Act/1B+
2456	Act/cry1B	CML72×CML216	R	8:8	R to SWCB	0:16		Act/1B+
2706	Act/cry1B	CML72×CML216	R	2:12	R to SWCB	0:14		Act/1B+
1796	Act/cry1B	CML216×CML72	R	10:6	S to SWCB	10:6		Act/1B+
2226	Act/cry1B	CML72×CML216	R	10:6	S to SWCB	10:6		Act/1B+
2170	Act/cry1B	CML72×CML216	R	9:7	S to SWCB	9:7		Act/1B+
2230	Act/cry1B	CML72×CML216	R	9:7	S to SWCB	9:7		Act/1B+
2622	Act/cry1B	CML72×CML216	R	10:6	S to SWCB	0:16		Act/1B+
2193	Act/cry1B	CML216×CML72	R	5:11	S to SWCB	0:16		Act/1B+
6118	Ubi/cry1B	CML216×CML72	R	0:37	R to SWCB	9:0		Ubi/1B+
6117	Ubi/cry1B	CML216×CML72	R	0:25	R to SWCB	0:25		Ubi/1B+
6355	Ubi/cry1B	CML72×CML216	R	0:44	R to SWCB	0:44	10:17	Ubi/1B+
6122	Ubi/cry1B	CML216×CML72	R	0:3	S to SWCB	0:23		Ubi/1B+
7	Ubi/cry1B-1Ab	CML216×CML72	R	n.d.	R to SWCB/SCB	5:9		Ubi/1B+
8	Ubi/cry1B-1Ab	CML216×CML72	R	n.d.	R to SWCB/SCB	11:5		Ubi/1B+
34	Ubi/cry1B-1Ab	CML216×CML72	R	n.d.	R to SWCB/SCB	8:8		Ubi/1B+
261	Ubi/cry1B-1Ab	CML72×CML216	R	16:0	R to SWCB/SCB	0:16		Ubi/1B+
333	Ubi/cry1B-1Ab	CML72×CML216	R	10:6	R to SWCB	1:15		Ubi/1B+
442	Ubi/cry1B-1Ab	CML72×CML216	S	1:15	R to SWCB	16:0		Ubi/1B+
19	Act/cry1B-1Ab	CML72×CML216	R	7:7	R to SWCB	0:14		Act/1B+
20	Act/cry1B-1Ab	CML72×CML216	R	9:7	S	0:16		Act/1B+
22	Act/cry1B-1Ab	CML72×CML216	R	10:6	S	0:16		Act/1B+
23	Act/cry1B-1Ab	CML72×CML216	R	6:8	S	0:14		Act/1B+
27	Act/cry1B-1Ab	CML72×CML216	R	3:13	S	0:16		Act/1B+
342	Act/cry1B-1Ab	CML323	R	0:16	R to FAW	0:16		Act/1B+
344	Act/cry1B-1Ab	CML72×CML216	R	8:8	R to FAW	8:8		Act/1B+
351	Act/cry1B-1Ab	CML72×CML216	R	9:7	R to FAW	16:0		Act/1B+
352	Act/cry1B-1Ab	CML72×CML216	R	1:15	S	16:0		Act/1B+
979	Act/cry1B-1Ab	CML216×CML72	R	0:16	R to FAW	0:16		Act/1B+

Sequence modification of a truncated *cry1B* gene

The sequence of the wild-type *cry1B* gene was fully modified from nucleotide 1 to 1941 (amino acid 1 to 647), which corresponds to the sequence coding for the active toxin plus 29 amino acids at the N-terminal end (Fig. 2). The optimized sequence starts after the wild-type *cry1B* start-codon, which is a Leu codon (TTG), and ends after the codon encoding the last amino acid of the active toxin. We set out to modify the start-codon, which could not be functional in plants, to adapt the codon usage to that of the monocotyledonous plants, and to eliminate all AT-rich sequences. The wild-type *cry1B* start-codon was replaced by an ATG codon and a G nucleotide was added after the start-codon to improve expression in plants. Addition of this nucleotide explains the presence of a Val codon (GTT) after the start-codon that is not present in the native sequence. Other *Bt* codons were replaced whenever possible by the monocotyledonous codons (Murray et al. 1989) without changing the amino-acid composition of the encoded polypeptide.

The modified *cry1B* gene was synthesized using an improved procedure based on recursives principles (Promodou and Pearl 1992) except for the oligonucleotide design, which was adapted from the principles described by Fujimoto et al. (1993).

Construction of a *cry1B-cry1Ab* translational fusion gene

The *cry1B-cry1Ab* translational fusion gene encodes a single Cry1B-Cry1Ab fusion protein that is activated by protease in the insect midgut to provide Cry1B and Cry1Ab toxins. To reconstitute functional Cry1B and

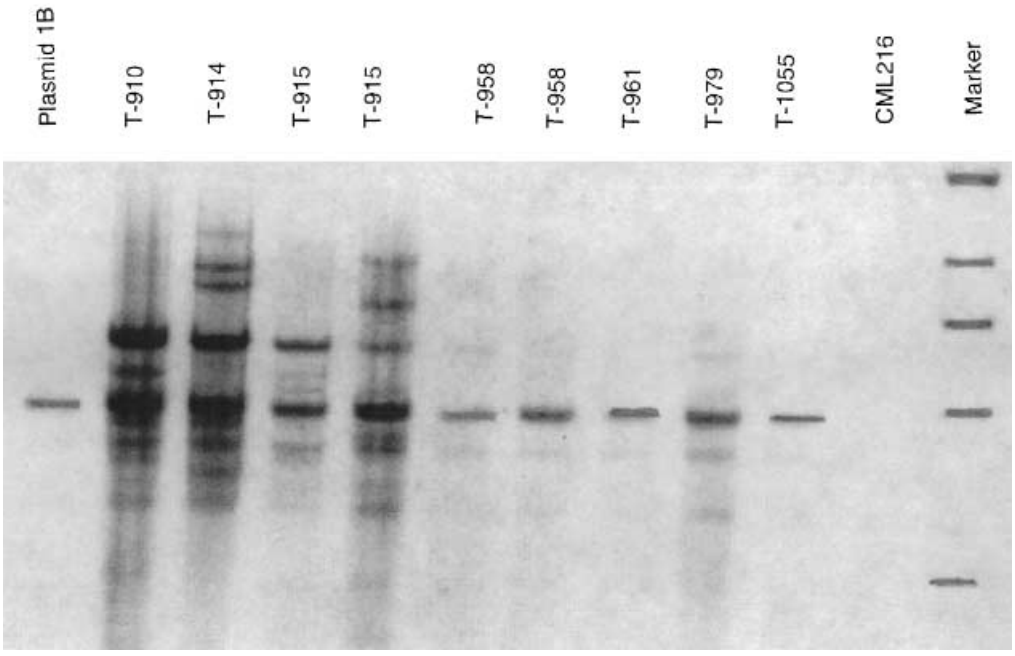

Fig. 4 Southern blot of nine T0 transgenic plants carring the *cry1B* gene in T0 presented resistance to SWCB and SCB. Each plant is *Hin*dIII-digested and probed with *cry1B*. The line labelled CML216 is from non-transformed lines, and *Pst*I is a molecular-weight marker

Cry1Ab activation sites in the fusion protein, synthetic *cry1B* and *cry1Ab* genes were fused at the level of 28th codon downstream from the Cry1B activation-site codons and the 29th codon upstream from the Cry1Ab activition-site codons (see Fig. 5a).

Cloning of the bar gene and *Bt* genes onto a single plasmid

To improve the coexpression efficiency of synthetic *cry1B* and *cry1B-cry1Ab* genes and the selective gene (PTT gene, Hoesht) we transformed both selective and *cry* genes linked into a single plasmid. This strategy is based on the use of a single plasmid to direct the introduction of foreign sequences into a plant's chromosomes.

Integration and expression of the synthetic *cryIB* gene in transgenic tropical maize

Experiments addressing key parameters in the development of the delivery system and selection procedures specifically for transgenic tropical maize have been presented previously (Bohorova et al. 1999). Following particle bombardment and regeneration, transformed embryogenic calli and plants were recovered through selection for resistance to the herbicide bialaphos (under darkness) with plant regeneration under light conditions (Fig. 3a, b).

The expression of the *cry1B* gene was studied in CIMMYT inbred lines with different susceptibilities to SWCB and SCB, and the hybrid between these lines. Plasmids contained the synthetic *cry1B* and fusion *cry1B-1Ab* gene under the control of a maize ubiquitin-1 promoter or rice actin-1 promoter and the selectable *bar* gene under the control of the cauliflower mosaic virus (CaMV) 35 S promoter. Most transgenic plants were morphologically normal; some regenerated with phenotypic modifications that reverted to the original genotype after 2–4 weeks. The tested 237 plants carried the *cry1B* gene, and 172 transgenics with *cry1B-1Ab* showed resistance to the herbicide Basta. These plants were selected in the biosafety greenhouse for further bioassay analyses (Table 1). After 5 days, results from the insect bioassay with SWCB and SCB showed that 60 plants out of the 237 (25%) tested presented insect mortality and no leaf damage when compared to the control plants. The score for resistance was based on the area eaten on the leaf surfaces (Fig. 3c).

A large number of transgenic plants was obtained and selected for herbicide resistance and, consequently, with expression levels of the translational fusions containing the coding sequence for the crystal protein toxic fragments. The 172 maize plants resistant/susceptible to the herbicide Basta were obtained and analyzed for insect resistance. Portions of this analysis are shown in Table 2. The plasmid carrying the *cry1B-1Ab* gene produced 172 transgenic plants, of which 162 plants expressed the *bar* gene and 17 plants (10% of the 172 transgenic plants tested) showed resistance for SWCB, SCB and FAW (see Fig. 5b). Transformed maize plants with the fusion gene resulted in 53% (out of 17 resistant plants) of the individual transgenic plants being protected against SWCB and SCB, and 35% (out of the total 17 insect-resistant) were protected against the less-sensitive FAW and SWCB. Event 351 was resistant to FAW only, while event 915 was protected against SWCB.

The recovery of large numbers of maize transformants, confirmed by Southern-blot analysis, indicated that these plants contained restriction fragments corresponding to intact copies of the coding region of the relevant enzyme

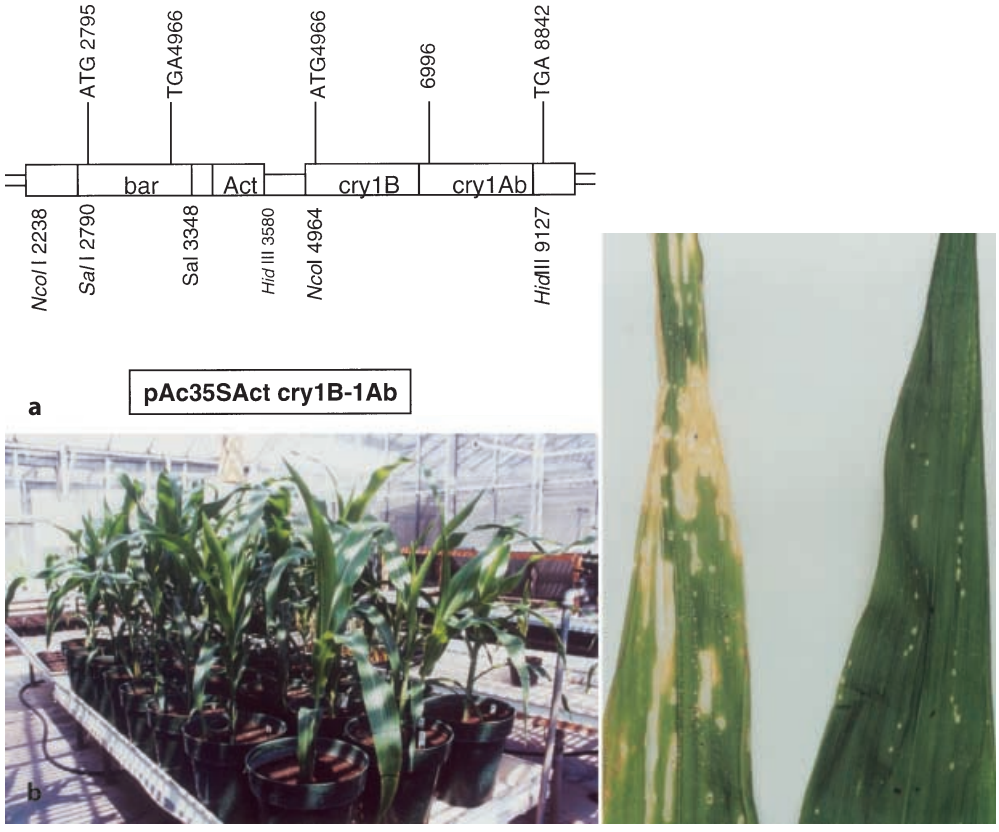

Fig. 5a, b Construct pAc35SbarActcry1B-1Ab. **b** The translational fusion *cry1B-1Ab* gene expressed single fusion proteins in transgenic tropical maize and showed resistance to SWCB, SCB and FAW

from the introduced plasmid (Fig. 4). Genomic DNA isolated from each line and digested with *Hin*dIII realeases the 3.5-kb *cry1B* expression cassette (gene-coding region) with the actin promoter, and 4.1 kb with the ubiquitin promoter. The detection of the corresponding band in DNA digested with *Hin*dIII is shown, suggesting the presence of at least one intact *cry1B* expression cassette. Since multiple transgenic plants were recovered from the embryogenic callus that originated from the same explant, it was of interest to determine whether those plants were derived from independent transformation. For example, in experiment number 241, 14 plants regenerated from the same embryo, 13 plants plants were resistant to the herbicide Basta, nine of them presented high resistance to SWCB (Southern bloting confirmed the presence of the transgene *cry1B*) and one was nontransgenic.

Screening of T1-T3 progenies for inheritance of the *cry1B* and *cry1B-1Ab* genes

Inheritance of the transgenes was monitored in T1-T3 individuals in order to identify events demonstrating resistance to the herbicide Basta and resistance to SWCB and SCB. Inheritance and the segregation ratios for *bar* and *cry* genes in T1 progeny and for a separate event in T3 (due to the number of different transformed lines tested, it was not possible to run bioassays for all transgenic plants) are shown in Table 2. The results of the T0-T2 progeny test from 26 selected events carrying the *bar* and *cry1B* gene (confirmed by plants resistant to the insects, and the Southern-blot test for *cryIB* gene presence) show that for the bar gene, 42% of the T1 progeny segregate at or near the expected Mendelian ratio (1:1) for the resistant (R) /susceptible (S) response, and 27% of transgenic plants with the *cry1B* gene present the same ratio. About 23% were susceptible to the herbicide in T1, and 38% of the T1 progeny were completely damaged after infestation with SWCB and/or SCB larvae. Six of the transgenic plants expressed resistance to the herbicide in T0 but did not confer resistance to insect infestation. In T2 and T3 progenies, event 5601 continued segregation for resistance/ susceptiblity to SWCB and SCB in ratios of 17R:32S and 23R:26S, respectively. After three backcrosses the *cry1B* gene has been integrated into CIMMYT inbred lines and will be further evaluated under field conditions.

Transgenic maize plants carrying the *cry1B-1Ab* gene were tested in T1 for insect tolerance by feeding leaves to neonate larvae of the three insects. The results showed that in seven families out of 17 screened (see Table 2 b) 81 plants produced 100% mortality of larvae, and in 169 transgenic plants larvae cause damage on the leaves. In the same families 80 plants were found to be resistant to 2% Basta and 125 were susceptible.

Discussion

In this study, maize plants transformed with genes encoding proteins from *cry1B* and *cry1B-1Ab* showed that

the foreign proteins were expressed throughout the leaf tissue in tropical maize and that they afford high levels of protection against significant insect pests. It has been suggested that the low expression of crystal protein genes in plants is a consequence of both poor transcription and translation (Perlak et al. 1990; Murray et al. 1991). The wild-type *cry1B* gene was modified with the aim of obtaining a highly expressed synthetic gene in maize. The full modification of the *cry1B* gene was based on eliminating known de-stabilizing sequences in the eucaryotic system, such as ATTTA sequences (Shaw and Kamen 1986, Laird-Offringa 1992), putative polyadenylation signals such as AATAAT which are normally encountered in the 3′ flanking region of plant genes (Dean et al. 1986), cryptic splicing sequences, and A+T strings. It was also based on adjusting the codon usage to that of highly expressed monocotyledonous genes (Murray et al. 1989). As a result, 647 codons (78.3%) are now modified; the overall G+C content of the modified gene is 58% while that of the original *cry1B* is 39.3%. These modifications are similar to the modified *cry1Ab* genes introduced into maize (65%; Koziel et al. 1993) and into rice (59.2%; Fujimoto et al. 1993). This is the first highly detailed report of totally PCR-directed *Bt* gene synthesis. In other studies, synthesis of three different fully modified *cry1Ab* genes were reported but oligonucleotide design was not described (Sardana et al. 1996; Koziel et al. 1993) and detailed reaction conditions were not reported (Fujimoto et al. 1993 Koziel et al. 1993). Construction of a synthetic *cry1C* gene (Strizhov et al. 1996) and a synthetic *cry3A* (Adang et al. 1993) have been reported using ligation-approach methods.

Previous reports on fusion genes involving two *cry1* genes indicated that a fusion at the level of the 28th codon downstream from the activate site was fully functional (Honee et al. 1990; Van der Salm et al. 1994). Our results confirm this observation. We demonstrated that the Cry1B-Cry1Ab fusion protein is functional and toxic against tropical pests.

In Black Mexican Sweet Corn, the co-expression frequency of maize cells transformed by particle gun bombardment is reported as 100% with a single plasmid containing both the selected and non-selected genes (Klein et al. 1989), and as 50% with separate plasmids (Spencer et al. 1990). In stable transformants from embryogenic maize cultures of A188×B73 and A188 x B84 using selected and non-selected genes on two separate plasmids, co-integration frequency was 77% and co-expression frequency was 18% (Gordon-Kamm et al. 1990). Depending on the mechanism(s) involved, co-expression frequency may improve if both selected and non-selected genes are on the same plasmid. We obtained a co-expression frequency of 97% for resistance to the herbicide Basta™ and 25% to two insects, SWCB and SCB. These results are in agreement with a study of the effects of the Cry IB toxin protein in an artificial diet that highly reduced feeding by tropical insects (Bohorova et al. 1997).

The successful use of the *cry1B-cry1Ab* fusion to confer resistance to SWCB, SCB and FAW in tropical maize opens up opportunities to apply the simultaneous delivery of Cry1B and Cry1Ab toxins to delay the appearance of resistance to Cry1Ab and/or Cry1B in the three pests examined. Development of this strategy requires determination of Cry1B and Cry1Ab binding sites in SWCB SCB, and FAW. In particular, binding sites determined for Cry1B and Cry1Ab in other insect species cannot be applied to our target insects because binding domains recognized by a particular toxin vary between species. The successful expression of the *cryIB-IAb* fusion allows us to apply the strategy of translational fusions to broaden the insect resistance of transgenic plants, but also and more importantly to simultaneously employ different gene classes in resistance-management strategies to control pest insects.

For application of maize transformation in breeding programs, it is necessary to produce a number of transgenic lines for each gene. Transformation efficiency will be a fundamental consideration identifying the transgenic line associated with the capability of gene integration, the stability of expression of the inserted gene, and the stability of inheritance of the transgene. The ability to engineer organized and potentially regenerable tissue by microprojectile bombardment permits the introduction of foreign genes into elite inbred lines. Consequently, backcrossing is not required to restore the original line. The embryogenic pathway of cell division using highly vigorous embryos from hybrids between CML216 and CML72 indicates that more competent cells have been activated. Results showed that 25% of the transgenic maize carrying *cry1B* genes presented resistance to the larvae of SWCB and SCB. The co-expression of the *cry1B-1Ab* gene has been obtained in 17% of the transgenic plants and confers resistance to the three insects.

Whole-plant assays confirmed the insecticidal activity of those genes, which were expressed in the transgenic progenies. In these progenies, transgenic lines demonstrated Mendelian inheritance and expression of the introduced *cry1B* genes over three/four successive backcross generations.

The present study shows that in small-scale experiments carried out under controlled environmental conditions, expression of *cry1B* (in three generations) and *cry1B-1Ab* in transgenic maize plants presented reduced leaf damage and was effective against three important tropical insects. The relevance of these results to crop protection in the field needs to be tested. However, the results are encouraging and the strategy of employing different *cry* genes as well as a translational fusion gene makes a valuable contribution to crop protection, particularly when used as part of an integrated pest management system.

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