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Production of transgenic tropical maize with *cryIAb* and *cryIAc* genes via microprojectile bombardment of immature embryos

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Abstract To enhance the level of resistance to insects in tropical maize germplasm we have developed techniques to successfully transform elite tropical maize inbred based on the activity of specific *cryI* proteins against four major maize pests – corn earworm, fall armyworm, southwestern corn borer and sugarcane borer. Constructs containing *cryIAb* or *cryIAc* synthetic genes were used. To generate transgenic plants we have established methods for biolistic bombardment and the selection and regeneration of immature embryos and calli from the elite tropical lines CML72, CML216, CML323, CML327 and hybrids. Transgenic plants resistant to the herbicide Basta™ contained the bands for the *cry*, *bar* and *gus* genes as detected by Southern blot analyses. A simple leaf bioassay presented varying levels of resistance to Southwestern corn borer of transgenic tropical maize carrying the *cryIAc* gene. Analyses of the progenies confirmed the sexual transmission of the introduced genes and their stable expression.

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

Introduction

The impact of insect pests such as corn earworm (CEW), fall armyworm (FEW), southwestern corn borer (SWCB), and sugarcane borer (SCB) on maize production and storage worldwide is considerable (Dicke and Guthrie 1988). Host plant resistance is an effective and environmentally safe means for controlling such pests. In recent years, CIMMYT's maize program has generated source populations of maize with multiple borer resistance through recurrent selection under infestation with different insect pests and has produced elite germplasm with multiple resistance to pest complexes (Mihm 1985; Bergvinson et al. 1997). The ability to transfer genes into agronomically important inbred lines offers the potential to improve important traits such as insect resistance. Recent studies on the field performance of transgenic maize plants demonstrated that genes derived from insecticidal genes of bacterium origin, in particular *Bacillus thuringiesis* (*Bt*), express an insecticidal protein in plant cells (Koziel et al. 1993; Armstrong et al. 1995; Estruch et al. 1997). *Bt* has been used as an insecticide for many years, and the expression of insecticidal proteins from cloned genes has raised the prospect of using these insecticidal proteins in transgenic plants.

Maize has been one of the prime targets for genetic manipulation in monocotyledonous grains. Demonstrations of the progress made are the various reports on the successful production of transgenic plants by microprojectile bombardment (Klein et al. 1989; Fromm et al. 1990; Gordon-Kamm et l. 1990; Genovesi et al. 1992; Waters et al. 1992; Frame et al. 1994; Register et al. 1994; Wan et al. 1995; Brettschneider et al. 1997) and by helium blasting (Pareddy et al. 1997), followed by successful hygromycin (Walters et al. 1992) and bialaphos (Spencer et al. 1990) selection of stable transformants. More recently, *Agrobacterium*-mediated gene insertion in maize plants has been reported (Ishida et al. 1996). Most studies on maize transformation have utilized genotypes adapted to temperature zones (Fromm et al. 1990; Gordon-Kamm et al. 1990; Walters et al. 1992; Armstrong et al. 1995), and plants regenerated from these lines were shown to transmit the recombinant DNA to their progeny. Little or no attention, however, has been focused on the transformation potential of maize

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germplasm and inbred lines adapted to tropical and subtropical regions. The production of genetically transformed plants depends both on the ability to integrate foreign genes into target cells and the efficiency with which plants are regenerated from genetically transformed cells. Embryogenic calli and plant regeneration were obtained from 50% of tropical and subtropical lines, 87% of mid-altitude lines and 75% of highland lines tested (Bohorova et al. 1995); type-II callus with a high potential for plant regeneration from tropical maize was also reported (Prioli and Silva 1989; Carvalho et al. 1997). These studies serve as the basis for developing transgenic technology for maize inbreds adapted to tropical conditions. We report here techniques for transforming elite CIMMYT tropical, subtropical, and mid-altitute inbreds and for stably integrating and expressing insecticidal proteins in tropical and subtropical maize germplasm.

Materials and methods

Preparation and culture of immature embryos

Twelve-to-fifteen-day-old immature embryos were taken from plants produced in CIMMYT's greenhouse, screenhouse or Tlaltizapan experimental station and used for transformation experiments. Maize inbred lines CML67, CML72, CML216 and CML323 and the hybrids from these lines were used in the experiments. Immature embryos (1.0–1.5 mm) were aseptically removed from the kernels and placed, scutellum up, on initiation medium in the center of a petri dish (100×15 mm). The N6C1 medium used for embryogenic callus initiation and maintenance consisted of modified N6 basal medium (N6) (Chu et al. 1975) supplemented with 200 mg/l casein hydrolysate, 2.302 mg/l L-proline, 3% sucrose and 2 mg/l dicamba (Bohorova et al. 1995). For callus initiation and maintenance, the cultures were incubated in darkness at 28°C, and embryogenic tissue was subcultured every 21 days. Plants were regenerated from embryogenic calli by transferring tissue to Magenta boxes containing basal MS medium (Murashige and Skoog 1962) with 2% sucrose, 0.5 mg/l indol-3-acetic acid (IAA) and 1 mg/l 6-benzylamino purine (6-BAP) (MSR medium). They were then transferred to MSE medium for root formation (Bohorova et al. 1995).

Gene identification and DNA bombardment

In order to study which genes are most appropriate for the generation of transgenic plants, we screened the toxic activity of native isolates of *Bacillus thuringiensis* (*Bt*) and specific *cryI* proteins against four major tropical maize pests (Bohorova et al. 1996, 1997). Based on these results, we initially used plasmids containing synthetic *cryIAb* and *cryIAc* genes (Sardana et al. 1996) under the control of a maize ubiquitin promoter (plasmid was kindly supplied by Dr. I. Alsosaar, Canada) and *bar/gus* (kindly supplied by Dr. S. Maddock; Pioneer Hi-Bred, USA) carrying the β-glucuronidase (*gus*) and the selectable *bar* genes under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

For microprojectile bombarment, prewashed 50-µl aliquots of gold particles (40 mg/ml distilled water using a procedure from the Bio-Rad Instruction Manual) were coated with 5 µl plasmid DNA (1 µg/µl) on ice. Fifty-microgram sterile aliquots of 2.5 *M* $CaCl_2$ were mixed with 20 µl of 0.1 *M* spermidine in a microfuge tube (1.5 ml) and added to the solution of particles with DNA. The DNA was adjusted to a concentration of 1 µg/µl in TE buffer (1 m*M* TRIS, pH 7.8; 0.1 m*M* Na_2EDTA and stored at –20°C). The mixture was vortexed for 3 min at room temperature and centrifuged in a microfuge for 1 min. The supernatant was removed and discarded. The DNA particles were washed with 240 µl ethanol (75%), resuspended in 240 µl absolute ethanol, and 3–5 µl of the suspension was spread onto the center of each macrocarrier and air-dried. Fifty immature embryos (aseptically removed from maize caryopses ore pre-cultured on callus initiation medium for 4 days) were placed on disposable petri plates (100×15 mm) with 10 ml N6C1 medium and arranged in a circle about 2 cm in diameter in the center of the plates. For osmotic pre-bombardment treatments (Vain et al. 1993), the material was kept on N6C1 medium with 12% maltose for 4 h. Bombardments were performed using the Bio-Rad PDS-1000 helium-driven biolistics particle delivery system. Each plate of tissue was bombarded once or twice at a rupture pressue of 900, 1,100, 1,350 and 1,500 psi. Different bombardment parameters were evaluated: particles with diameters of 0.4–1.0 µm (gold powder, spherical, Aldrich Chem) and 1.0 µm, 1.5 µm, 1.9 µm (Bio-Red gold powder); particles per bombardment (14–900 µg per shot); particle suspension volume per bombardment (3–5 µl); DNA per bombardment (5–20 µl); and number of shots per target (1–2 shots).

Histochemical GUS activity assay

GUS activity was detected histochemically as described by McCabe (1988). About 20 immature embryos or calli from every transformed plate were used for *gus* assays. *Gus*-expressing cells were routinely visualized 48 h after microprojectile bombardment by incubating bombarded immature embryos or calli in 400 µl of the 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc; Sigma) solution. Plant tissue was incubated 48 h in the above mixture at 37°C, then the X-Gluc solution was replaced with ethanol (75%) for 2–3 days and β-glucuronidase activity determined microscopically.

Selection of transformed embryos/calli and recovery of transgenic plants

Selection of transformed cells was achieved using either phosphinothricine (PPT) (Sigma) or bialaphos (B) (Meiji Seika Kaisha, Yokohama, Japan). Phosphinothricine inhibits glutamine synthesis, causing a rapid accumulation of ammonia that leads to plant cell death (Spencer et al. 1992). Bialaphos is a tripeptide antibiotic consisting of PPT and two L-alanine-residues (Thompson et al. 1987). Concentrations ranging from 1 to 10 mg/l of PPT or bialaphos were tested with non-transformed calli to derive an effective selection system. Two different approaches were followed for the selection of transformants. In the first approach, the embryos or calli were transferred to either N6C1 medium containing all of the medium components as described by Bohorova et al. (1995) or N6C1 medium containing 1 mg/l PPT/B (N6C1B1) and then cultured in darkness for 7 days. In the second selection experiment, the bombarded material was transferred to N6C1 medium that excluded L-proline and casein hydrolysate (N6C2) but did contain1 mg/l PPT/bialaphos. In both cases the duration of the selection process was 50–75 days on the media with 1, 3, 5 and 10 mg/l PPT/B, depending of the genotype used, with the selection medium being refreshed every 2 weeks. All cultures were kept at 28°C under dark conditions in the growth culture chamber.

Plant regeneration and Basta™ testing

All the PPT/bialaphos-resistant callus tissue, which grew uniformly on the selection medium, were transferred to the regeneration medium supplemented with 5 mg/l bialaphos at a temperature of 28°C under a 16-h photoperiod provided by fluorescent light. Somatic embryos capable of developing into green shoots within 2–4 weeks were characterized as putative transformants. The selected plantlets were transferred to the MSE medium (Bohorova et al. 1995) supplemented with 1 mg/l bialaphos for root formation.

Plantlets that developed on this medium were transferred to soil in environmentally controlled growth chambers and greenhouse conditions and grown for further analyses. Plants were further selected by painting the fifth or sixth leaf near the tip of the youngest fully extended leaf with a 2% Basta™ solution containing 0.1% Tween 20. Each regenerated plant was tested for its response to Basta™. One week after the first Basta™ treatment, the plants were painted again with a second Basta™ treatment and again assessed for damage 1 week after herbicide application.

Southern hybridization

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 according the procedure described by Hoisington et al. (1994). Each sample was digested with a restriction enzym(s), *Hin*dIII and/or *Eco*RI, separated on 0.8% agarose gels and transferred to nylon membranes. Probes from the transgenes *bar, cryIAb* and *cryIAc* were labeled with digoxigenin-dUTP and used for Southern hybridizations. Prehybridization and hybridization protocols were as described by Hoisington et al. (1994).

Leaf bioassay

For phenotypic confirmation, a simple leaf bioassay was performed in 5-cm petri dishes. Leaf samples (approximately 10 cm^2) were taken from the latest fully-emerged leaf of each plant. Five first-instar larvae of southwestern corn borer were placed on the leaf tissue with a small camel hair brush. The dishes were covered with Parafilm and incubated at room temperature for 4 days. The leaves of the transgenic plants were classified according to the number of live larvae and the degree of damage (1=best, 9=worst). All Southern-positive plants for *cry* genes were crossed with the respective CML216 to produce T1 seeds. From each event at least 30 plants were tested in the T1 generation. In order to assess resistance to the herbicide Basta™, we manually painted all plants with 2% Basta™ and then infested them with neonate SWCB larvae. Three infestations of 30 larvae (per infestation) were applied during the test period. Plants were scored for feeding damage 2 weeks after the final infestation using the previously described 1–9 scale and were subsequently divided into two classes: resistant and susceptible.

Results

Optimization of DNA delivery parameters

Highly regenerable embryos were bombarded with gold particles coated with plasmid DNA (*gus/bar*) to optimize delivery of DNA-coated microprojectiles. Different microprojectile bombardment parameters were evaluated using transient β-glucuronidase (*gus*) assays. The highest number of transient *gus* expression events were observed with embryogenic immature embryos. For bombardment of 20 petri plates, each with rings of about 50 embryos, the following procedures were used: (1) freshly isolated immature embryos (1–1.5 mm) or 4-day precultured immature embryos were obtained; (2) a 4-h pre-bombardment osmotic treatment of the embryos was performed on N6C1 medium with 12% maltose; (3) 50 µg of gold particles (1.0 µm in diameter) coated with 5 µl plasmid DNA (2.5 µl DNA from two different plasmids) were put on ice; (4) a mixture of 20 µl spermidine and 50 µl $CaCl_2$ was added and mixed using a vortex shaker with a multiple platform head for 3 min at room temperature; (5) the mixture was centrifuged in a microfuge for 1 min at 13,000 *g*, and the supernatant was removed and discarded; (6) the solution was resuspended twice in 240 µl absolute ethanol; (7) 5 µl of suspension was pipetted onto the center of a macrocarrier previously positioned in a macrocarrier holder, and air-dried; (8) two bombardments were applied with 1,350 psi rupture discs, using particle densities of 30 µg per shot; (9) target materials were positioned 8 cm below the microprojectile stopping plate. Major differences in transient gene expression were not observed between the particle diameters and densities. An average number of 60–100 transient *gus* signals per embryo was recorded from maize embryos using particle densities of 14, 30, and 75 µg per shot Fig. 1a). Each blue spot of *gus*-expressing cells had a densely stained blue central core, with a less densely stained surrounding region. With particle densities of 150, 300, 600, and 900 µg per shot fewer blue spots appeared, but more of a diffused blue color was detected on the surface of the embryos or callus. Christou et al. (1991) observed in rice tissue that only the core cells received the particles and that the *gus* enzyme or its reaction products diffused from the core cells to the surrounding cells. Acceleration pressure was optimized in a range of 900–1,500 psi. For compact calli established from CML72 and CML216, 1,350 psi was used, and for friable tissue, such as that originating from CML67 embryos, 1,100 psi was used. In stable transformation experiments we found transformation rates were higher at 1,350 psi than at 900 psi.

The concentration of DNA needs to be carefully considered. Currently used methods of coating DNA onto particles do not lend themselves to uniform coating, while a higher volume of DNA may cause agglomeration of the particles. To eliminate this problem the particles should not be prepared prior to the day of use, or a low concentration of coating DNA (5 µl for transformation and 2,5 µl for co-transformation) should be used.

Selection and regeneration of transgenic plants

An important component of the transformation system is the selection of transgenic tissue. We used herbicides such as phosphinothricin (PPT) or bialaphos (B) that were incorporated into the culture media at concentrations of 1, 3, 5 and 10 mg/l, for 3–4 months. To ascertain which of these selection regimes was the most efficient for different maize genotypes, we determined the sensivity of maize tissue to the two toxic compounds. The lethal concentration of bialaphos for non-transformed calli for CML67 and CML72 was determined to be 5 mg/l and for CML216 and CML323, 10 mg/l. According to our observations, selection with bialaphos was better than with PPT; consequently, only results from the selections using bialaphos will be presented.

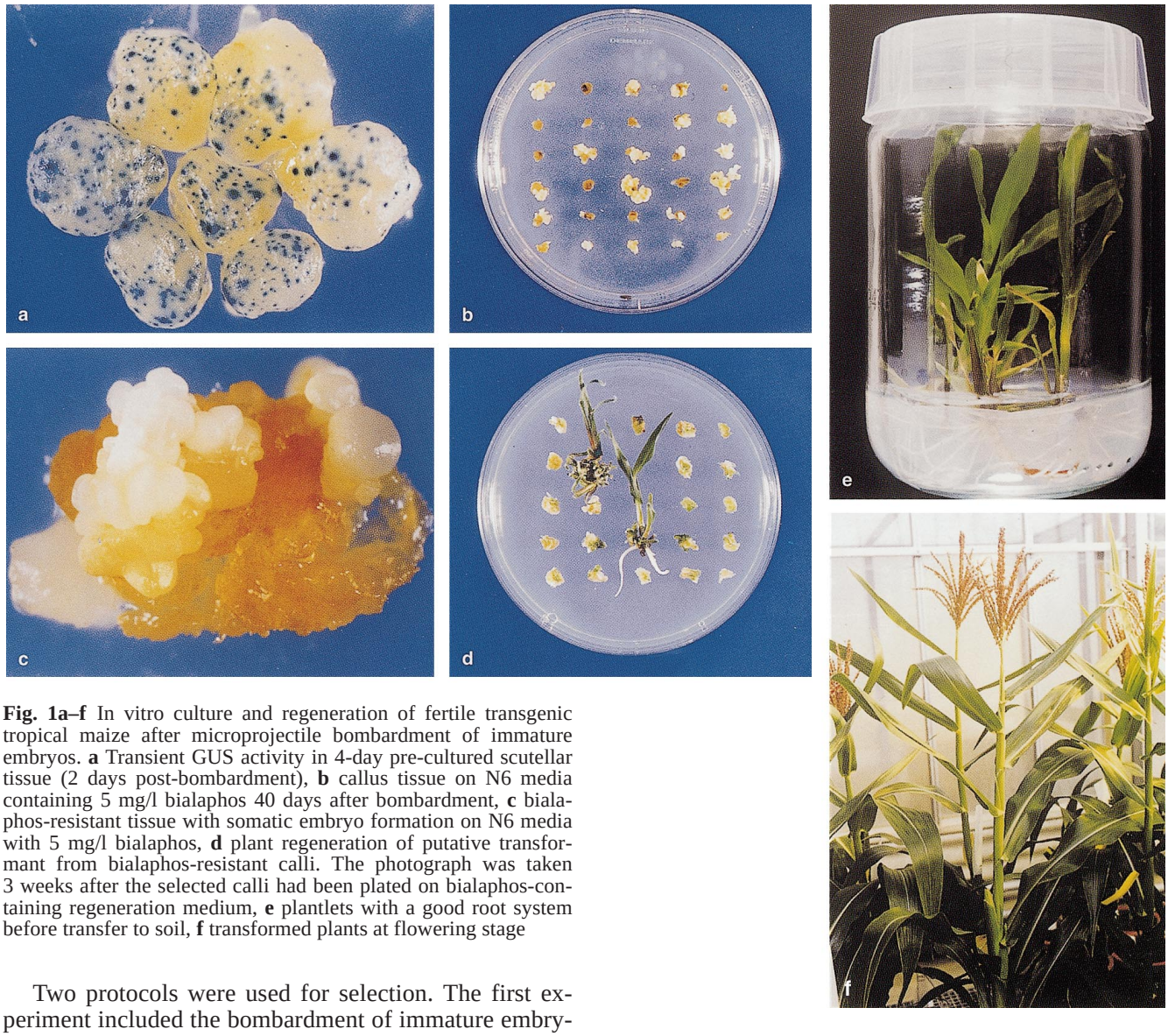

Fig. 1a–f In vitro culture and regeneration of fertile transgenic tropical maize after microprojectile bombardment of immature embryos. **a** Transient GUS activity in 4-day pre-cultured scutellar tissue (2 days post-bombardment), **b** callus tissue on N6 media containing 5 mg/l bialaphos 40 days after bombardment, **c** bialaphos-resistant tissue with somatic embryo formation on N6 media with 5 mg/l bialaphos, **d** plant regeneration of putative transformant from bialaphos-resistant calli. The photograph was taken 3 weeks after the selected calli had been plated on bialaphos-containing regeneration medium, **e** plantlets with a good root system before transfer to soil, **f** transformed plants at flowering stage

Two protocols were used for selection. The first experiment included the bombardment of immature embryos or 4-day-old callus produced from immature embryos. In this experiment, cells were grown on callus initiation medium (N6C1) for 7 days post-bombardment or on N6C1 with 1 mg/l bialahos for 7–10 days. The proliferating explants were subsequently transferred to N6C1 medium with 5 or 10 mg/l bialaphos, depending on the genotypes used. Four weeks later, the difference between the resistant explants and the control explants became more pronounced (Fig. 1b). Resistant colonies appeared at 6–8 weeks (Fig. 1c).

To have an effective phosphinothricin selection it is important to omit not only glutamine from the selective medium but also several other amino acids. For this reason, in the second experiment we excluded both L-proline and casein hydrolysate from the selective medium (N6C2) during the transformation experiment and increased the concentration of bialophos to 10 mg/l. After bombardment, embryos/calli were plated immediately onto medium with the selective agent bialaphos at a concentration of 1 mg/l. They were kept on this medium for 1 week, then transferred to a selection medium containing 5 or 10 mg/l bialaphos for 4 more weeks. At the concentration of 10 mg/l bialaphos, the growth of transformed calli was reduced and the growth of untransformed calli fully inhibited. However, embryogenic calli in the continuous selection medium with 10 mg/l bialaphos resulted in the appearance of both transformed and non-transformed plants. We assume that at a certain stage in their development the embryos are less sensitive to the herbicide or, as Christou and Ford (1995) observed, that transformed rice tissue of a particular explant is capable of detoxifying the selective agent to efficiently that non-transformed tissue in close proximity can survive and regenerate plants.

For the analysis of transient *gus* expression, we separated one experiment of 20 petri plates, each containing 50 embryos, and sacrificed calli at 48 h after bombardment, after 4 weeks on the selection medium with 5 mg/l of bialaphos and after another 4 weeks on the selection

Table 1 Summary of co-transformation experiments with plasmids carrying *cryIAb*, *cryIAc* and *bar/gus* genes

Event	Total no. of plants	Total no. embryos bombarded	Genotype	Plasmids	T0 generation[a]			
					Basta R/S	Insect R/S	*Bar* +/–	*Cry* +/–
1	1	343	CML216	Ubi:CryIAb+35S:bargus	R	S	+	–
2	2	150	CML72×CML216	Ubi:CryIAb+35S:bargus	R	S	+	+
3	10	150	CML72×CML216	Ubi:CryIAb+35S:bargus	R	S	+	+
4	5	150	CML72×CML216	Ubi:CryIAb+35S:bargus	R	S	+	+
5	12	337	CML72×CML216	Ubi:CryIAb+35S:bargus	R	S	+	+
6	1	96	CML72	Ubi:CryIAc+35S:bargus	R	S	+	+
7	1	96	CML216	Ubi:CryIAc+35S:bargus	R	S	+	+
8	3	175	CML216	Ubi:CryIAc+35S:bargus	R	S	+	+
9	1	37	CML216	Ubi:CryIAc+35S:bargus	R	S	+	–
10	1	92	CML216	Ubi:CryIAc+35S:bargus	R	R	+	+
11	3	400	CML323	Ubi:CryIAc+35S:bargus	R	S	+	+
12	1	215	CML216×CML72	Ubi:CryIAc+35S:bargus	R	R	+	+
13	1	204	CML216×CML72	Ubi:CryIAc+35S:bargus	R	R	+	+
14	1	200	CML216×CML72	Ubi:CryIAc+35S:bargus	R	S	+	–
15	3	571	CML72×CML216	Ubi:CryIAc+35S:bargus	R	R	+	+
16	1	415	CML72×CML216	Ubi:CryIAc+35S:bargus	R	R	+	+
17	1	107	CML67×CML216	Ubi:CryIA+35S:bargus	R	S	+	–
18	1	92	CML216×CML72	Ubi:CryIAc+35S:bargus	R	S	+	–

[a] R, Resistant; S, Susceptible; +, gene presence; –, gene absence

medium with 5 mg/l bialaphos. The results showed that the 2-day post-bombardment embryos presented 5–80 blue spots on the scutellar part of all the embryos used for transformation. After 4 weeks on selection with 5 mg/l bialaphos, 14% of the calli showed blue regions and after a second 4 weeks on the selection medium with 5 mg/l bialaphos only 2% of the calli showed blue regions in the selected calli. To avoid competition from non-transformed cells, we prefered to apply selective pressure early in escalated the steps – starting with a low concentration of bialaphos (1 mg/l), and then 3 or 5 mg/l bialaphos for CML67 and CML72 and 10 mg/l bialophos for CML216. All transformed callus lines were highly regenerable, and a large number of plants have been regenerated from each line. The concentration of 5 mg/l bialaphos in the regeneration medium and 1–3 mg/l in rooting medium resulted in the recovery of transformed plantlets (Fig. 1d). Within 4–5 months, putative transformed maize plants (Fig. 1e) were transferred to soil and grown under greenhouse conditions. All the plantlets were healthy and successfully grew into fertile plants (Fig. 1f). All 49 transgenic plants were regenerated from bialahos-resistant calli, which were derived from 18 independent immature embryos and therefore presented independent events. Among all of the CIMMYT maize inbreds used for transformation, only CML216, CML72 and the hybrids between them and CML323 could be transformed. The hybrid CML72×CML216 gave the most successful transformation efficiency.

Analysis of T0, T1 and T2 plants

The present results from co-transformation of different maize inbred lines and the hybrids with *Bt* genes and *bar/gus* constructs are summarized in Table 1.

As reported previously, we chose the constructs in order to have effective herbicide selectable markers containing the *bar* and *gus* genes to use for co-transformation with the *cryIAb* and *cryIAc* genes. This allowed us to screen putative transgenic plants by means of a Basta™ bioassay (Fig. 2a). Most of the T0 plants were morphologically normal and werecrossed with CML216. Plants showing moderate a high resistance to Basta™ herbicide were further checked using Southern blot analyses. The T0 plants regenerated from non-transformed calli were susceptible to the herbicide treatment, as expected. All T0 plants were analyzed for the presence/absence of introduced genes by DNA hybridization. Southern hybridization analysis confirmed that these 49 plants were derived from 18 independent transformation events (Table 1, Fig. 2b). The estimated copy number of the intact fragment varied from approximately1 to 10 for these independently produced transgenic plants.

For phenotypic confirmation, a simple insect bioassay was performed using leaf samples from each transgenic plant carrying the *cryIAb* gene, the transgenic state having already been confirmed by Southern blot. For example, from 150 bombarded immature embryos in experiment no. 77 (lab list), 60 embryos were recovered after selection, which subsequently produced 75 plants that were transferred to the greenhouse. Seventeen plants were classified as fully resistant to Basta™, none were resistant to SWCB in T0 progeny and none displayed resistance in T1 progeny. The 17 plants originated from 3 different embryos belonging to 3 different events that carried *bar*, *gus* and *cryIAb* genes. The segregation analysis for the *bar* gene in T1 was close to the expected Mendelian segregation ratio (1:1). Resistance to larvae of SWCB was shown by T0 plants from 5 events which carried *bar, gus* and *cryIAc* genes (Fig. 2c, Table 1). In T1 progeny, only transgenic plants from event no. 16

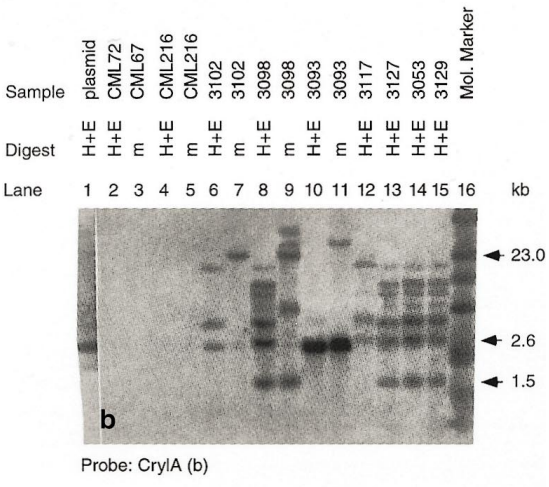

Fig. 2a–c Analysis of the putative transformants. **a** Test of the putative transformants for resistance to Basta™. Young leaves of non-transformed (*left*) and transformed (*right*) plants were planted with a 2% Basta™ solution. **b** Southern blot analysis of transformed plants (T0 generation). DNA extracted from Basta™-positive plants was digested with *Bam*HI and/or *Eco*RI and allowed to hybridize with CryIA(b) probe. *Lane 1* Plasmid DNA, *lanes 2–5* non-transformed control plants, *lanes 6–15* transformed plants nos. 3102 and 3098; 3093; 3117, 3127, 3053 and 3129 regenerated from bialaphos-resistant calli, which were derived from three separate immature embryos. **c** Simple leaf bioassay test with first-instar larvae for southwestern corn borer (SWCB) resistance. Young leaves from a non-transformed (*left*) and transformed (*right*) plant carrying the *cryIAc* gene, the transformed state already confirmed by Southern blot

presented resistance to Basta™ and SWCB larvae, and these segregated 19:17 (resistant:susceptible), which is close to the expected Mendelian ratio (1:1). In T2 progeny the segregation ratio for Basta™ and SWCB was as expected 1:1 (24 resistant:24 susceptible), and all positive plants were blotted and probed with *cryIAc* fragments. Analyses of the T2 progeny confirmed the sexual transmission of the two introduced genes and their stable expression.

Discussion

In our experiments to develop transgenic technology for tropical and subtropical maize, we focused on optimizing biolistics-mediated gene delivery into cell cultures, applying enhanced gene expression vectors for the efficient selection of transformed tissue and using various parameters and selection regimes for the recovery of transgenic maize. Our goals were to establish a replicable plant genetic transformation system for tropical maize, to introduce gene(s) into intact cells and to have these adequately express in maize plants.

The primary requirement for an optimal target is that the tissue or cells receiving exogenous DNA are culturable in vitro, actively dividing and capable of giving rise to fertile plants. The fundamental problems of transformation methods for cereals are: the loss of embryogenic capacity during long-term culture based on protoplast or suspension cultures (Jähne et al. 1991; Golovkin et al. 1993), genotype dependence (Potrykus et al. 1990), occurrence of somaclonal variation (Wang et al. 1992) and a relatively high input of labor and energy (Brettschneider et al. 1997). Transgenic plants can only be produced when cells are competent for both integrative transformation and regeneration. Not all plant cells are totipotent, and cells differ in their capacity to respond to triggers (Potrykus 1990). Pre-culture immature embryos or isolated scutella with competent cells for somatic embryogenesis have proven to be excellent targets for microprojectile bombardment and for subsequent rapid recovery of transgenic plants (Bimmineni and Jauhar 1997). The usefulness of maize scutellar tissue for transformation has already been demonstrated by tissue electroporation (D'Halluin et al. 1992) and by biolistic transformation (Fromm et al. 1990; Gordon-Kamm et al. 1990; Register et al. 1994; Brettschneider et al. 1997). Compact type-I callus, which can be obtained from the scutellum of immature embryos, is an excellent bombardment target for generating transgenic maize in comparison with type-II callus, which came highly recommended based on the first transformation experiments with maize

(D'Halluin et al. 1992; Wan et al. 1995). Between 10 and 20 days after pollination, immature embryos provide several distinct developmental stages of meristematic tissue competent for transformation (Schläppi and Hohn 1992). In our experiments, the tropical maize inbred lines and hybrids CML72, CML216, CML323, CML216×CML72 and CML72×CML216 formed type-I calli and were successfully transformed. The optimization of the parameters directly led to a comparatively stable transformation frequency. Although the development of transgenic plants is becoming more routine, the transformation frequency is still very low. The transformation frequency for temperate maize H99 and A188 reported by Brettschneider et al. (1997) is 3%; Koziel et al. (1993) obtained a transformation frequency of 1%. In our experiments, the transformation frequency was 1–2%. Each parameter has been the subject of individual optimization experiments to improve the conditions for particle bombardment. The concentration of DNA in our experiments was 2.5 µl for co-transformation and 5 µl for transformation using a single plasmid. Not every transiently expressing cell becomes a stable transformant, and an increase in the number of transiently expressing cells will not necessarily lead to an increase in stable transformants (Sauter et al. 1991). Preliminary observations indicated that particles could penetrate up to five cell layers, and *gus*-expressing cells have been observed one or two cell layers below the surface of maize embryo scutella (Klein et al. 1987).

Approximately 40 different genes conferring insect resistance have been incomporated into crops, and the first insect-resistant crops have been produced (Perlak et al. 1990; Estruch et al. 1997; James and Krattiger 1996) and commercialized in several countries (Schuler et al. 1998; Jouanin et al. 1998). The expression of *Bt* genes in maize, resulting in excellent protection against European corn borer (Koziel et al. 1993; Armstrong et al. 1995) and against SWCB/FEW (Williams et al. 1997) was advanced when elite cultivars of maize were transformed with a truncated *cryIAb* gene. With the use of transformation techniques to obtain new cereal crop products, we expect genetically improved tropical maize containing *Bt* genes to play a vital role in integrated pest management in tropical areas. The new products should provide breeders with anothers resource for enhancing the genetic composition of their germplasm. However, the actualization of genetically modified plants' potential, particularly tropical maize upon which so many of the world's poor rely, depends on a broad array of non-technical issues, especially intellectual property rights and related regulatory developments (patent structures and guides for the deployment of genetically modified organisms).

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