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 cry1 proteins against four major maize pests – corn earworm, fall armyworm, southwestern corn borer and sugarcane borer. Constructs containing cry1Ab or cry1Ac 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 cry1Ac 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 pro-duction 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 thuringiensis (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 al. 1990; Walters et al. 1992; Armstrong et al. 1995; Waters et al. 1992; Frame et al. 1994; Register et al. 1994; Lang et al. 1995; Brettschneider et al. 1997) and by helium blasting (Paredy 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; Waters 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...
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-altitude 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, greenhouse or Tihlizapan 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, scuttleum 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 and 2 mg/l dicamba (Bohorova et al. 1995). Plants were maintained on Magenta boxes containing basal MS medium 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 pressure 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 glucuronic acid (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 synthetase, 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 contain 1 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 TM 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 MSEM medium (Bohorova et al. 1995) supplemented with 1 mg/l bialaphos for root formation.

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. Alsosaa, 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 bombardment, 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 mg CaCl2 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 mM Tris, pH 7.8; 0.1 mM Na2EDTA 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 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 pressure 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).