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Full Length Research Paper

Detection of *Bacillus thuringiensis* genes in transgenic maize by the PCR method and FTA paper technology

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We optimized the PCR method to detect genetically engineered *Bacillus thuringiensis* (Bt) maize in open quarantine fields in Kenya. Many factors affect the extraction of the DNA from plants, such as the amount of tissue available, the condition of the plant material, the numbers of steps involved in the extraction procedure, and the required purity needed. We tested the application of the FTA paper technology for field sampling whereby leaf materials are not removed and transported from the site. We also applied the PCR method, a technique that is widely used for detection of genetically modified organism (GMOs). We used primers specific to the Bt genes present in the transgenic maize to screen for the Bt genes Cry 1Ab and Cry 1Ba.

Key words: Genetically modified organism (GMO) detection, maize, *Bacillus thuringiensis* (Bt), PCR method, FTA paper technology.

INTRODUCTION

More than 62 million ha world wide are covered with transgenic crops that produce insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt) (Tabashnik et al., 2003). *B. thuringiensis* (Bt) is a bacterium of great importance that produces an arsenal of crystal proteins during sporulation whose toxins kill a large variety of host insects and even nematodes (Maagd et al., 2001). The development of Bt transgenic maize is on going in Kenya. Currently the maize is being grown under restriction at an open quarantine station. The introduction of Bt maize was necessitated by the loss in yield to the farmers occasioned by six major stem borer species *Chilo partellus, Chilo orichalcociliellus, Eldana saccharina, Sesamia calamistis* and the economically important *Busseola fusca* (Mugo et al., 2005). The IRMA (Insect resistant maize for Africa) project was hence launched in 1999 to develop locally adapted maize with Cry 1 delta-endotoxins of *B. thuringiensis* which are generally active against lepidopteran insects. A comprehensive overview of the IRMA project can be found at http://www.cimmyt.org/emglish/wpp/gen_res/irma/htm.

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The BT maize under development has been genetically engineered for resistance to stem borers using two genes: Cry 1Ab and Cry 1Ba (Bohorova et al., 1999). These genes code for a toxin that crystallizes in the digestive tract of insect larvae, leading to its starvation. Cry 1Ab is active against Lepidoptera while Cry 1Ba, is active against both coleopteran and lepidopteran larvae (Bradley et al., 1995). There is grow-ing concerns about the impact of GM crops on the envi-ronment such as vertical or horizontal gene flow, related ecological impacts especially on non-target insects, effects on biodiversity and the impact of presence of GM material in other maize products such as baby corn. Insect pests have not evolved resistance to Bt crops yet although studies with Diamondback moth (*Plutella xylost-ella*) show evolvement of resistance to Bt sprays in the field (Fierr and Rie, 2002). Therefore developing reliable methods for detecting the gene either in the breeding pro-gram or in neighboring corn fields and refuges is impor-tant.

MATERIALS AND METHODS

Maize plants

There were 24 crosses planted by the breeder at the Kiboko open quarantine station, Kenya. Most of those crosses were lines derived

from crossing Bt events developed from (CML216xCML72) and backcrossed to conventional CML216 thereby developing various Bt maize inbred line carriers of the Bt genes (Mugo et al., 2005). To produce locally adapted Bt maize lines, a Bt line containing any of the events was crossed to a conventional non-Bt locally adapted line. The objective was to produce homozygous locally adapted lines with the Bt gene. The materials were at different selection cycles and had all undergone Bt gene screening using leaf bioassays within a few weeks after planting. The plants were expected to have the Cry 1Ab or Cry 1Ba gene in 75% of their seeds (50% hemizygous and 25% homozygous) (Chilcutt and Tabashnik 2004). Non Bt maize plants were sampled for control experiments.

Field maize sampling

The samples for DNA analysis were taken from each individual plant in a row. The plants were at the early grain filling stage. The sampling protocol was done according to the modified protocols of FTA paper technology (Mbogori et al., 2006). FTA ® classic card (Whatman Inc., Clifton, NJ) is a Whatman paper that has been impregnated with a patented chemical formulation that lyses cells, captures and immobilizes nucleic acids in the paper matrix, in addition to having denaturing, chelating and free radical trap all geared towards preventing nucleic acids damage (http://www.whatman.com). The second or third leaf was excised from the plant, wrapped round the FTA paper strip, and placed in a small polythene bag. A pair of pliers was used to press the leaf sample extract on to the FTA paper until both sides of the FTA were soaked. Placing the material directly onto the FTA paper and applying moderate pounding/pressure with a blunt object such as a pestle (Lange et al., 1998; Lin et al., 2000) ensures enough amount of sap is soaked onto the paper. Ethanol (70%) was used to clean pliers in between cases where the polythene bag had cut, exposing the pliers to the plant tissue. The FTA card was then air dried on temporary constructed drying line using a paper clip for 2 to 5 h, and later stored in an air tight plastic container. It is important to ensure proper drying of the FTA papers as presence of moisture content was observed to cause further degradation of the DNA. All the leaf matter was put in biosafety plastic bags and containers for disposal according to laid down procedures.

Sample preparation

This protocol was modified from that of Whatman FTA® for Plant DNA (http://www.whatman.com/products). Briefly, FTA discs measuring 1.2 mm each were punched from the FTA disc using 1.2 mm Harris Micro punch (Whatman, Inc. US), and placed in a 96-well or 384-well PCR plate containing 50 µl and 35 µl of FTA wash solution, respectively. The PCR plate was incubated at room temperature for 15 min with shaking, and the solution removed with pipettor. FTA purification reagent (50 µl) was used twice, followed by one rinse with 100 µl of double distilled water for 5 min, and once with 50 µl absolute ethanol for 5 min. The FTA discs were dried in an oven for 15 min at 56°C and ready for PCR amplification. Washing was carried out directly in the PCR tubes/plates to minimize the tedious step of transferring discs from wash tubes / plate to new PCR tubes/plate. For extracted genomic DNA, a few transgenic plants from the green house were sampled and 5 µl used as template (Saghai-Maroof et al., 1994).

PCR amplification and product analysis

The PCR mix contained 20 µl of PCR mix (1X Reddymix, 3 mM MgCl, 1.25 U Taq, 0.2 mM dNTPs, 40 pM each primer) and 2 FTA disc. PCR amplification was carried out using a GeneAmp PCR system 9700, Applied Biosystems. Amplification conditions for PCR were 94°C, 2 min; then 94°C, 1 min; 60°C, 2 min; 72°C, 2 min for 40 cycles and a final extension of 72°C, 10 min. Oligonucleotide PCR primers and sequence were kindly received from Alessandro Pellegrineschi (CIMMYT, Mexico);

Cry 1Ab*:*
5'-ACCATCAACAGCCGCTACAACGACC-3'
5'-TGGGGAACAGGCTCACGATGTCCAG-3'
Cry 1Ba:
5'-CCATGGTTACCTCCAACCGT-3'
5'-GGATGATCTCGATCTTGTCGA-3'

PCR product analyses were visualized by UV transillumination on a 1% agarose and TAE buffer. The gel was stained with ethidium bromide (Sambrook et al., 1989).

RESULTS AND DISCUSSION

The major success of this experiment was the ability to use FTA paper and obtain sufficient amounts of DNA samples from old maize plants. Secondly the convenience of transporting the samples over a long distance after three days of sample taking without the risk of degradation and contamination. This method avoids the tedious way of transporting leaves in iceboxes which cannot be left overnight in most cases. The FTA procedure conforms to biosafety regulations by minimizing the risk of contamination in transit while maintaining the condi-tion of DNA samples. The control non Bt plants were also sampled and showed high infestation incidence as shown in Figure 1. The plants are usually artificially infested at about 2 weeks after emergence and scored two weeks later when the plants are about a month old. The application of the PCR detection method will make detection convenient by screening for resistant plants containing the Bt genes through DNA analysis at any plant growth stage where enough sap can be obtained.

Although the plants were older, enough sap was obtained using FTA paper technology to carry out DNA analysis (Figures 2a and 2b). Unlike in the application of other DNA protocols where the recommendation is to use young leaves to obtain sufficient amounts of DNA, the FTA method offered not only convenience in sample handling but also in obtaining enough sap. Using a temperature gradient, the best annealing temperature for both Cry 1Ab and Cry 1Ba genes was found to be 60°C. Only data for Cry 1Ab is shown in Figure 3. The primers were first tested on genomic DNA using plants grown in the green house. Both primers were shown to give positive amplifycation (Figure 4). There was no amplification for non Bt control plants. The primers were specific for the maize Cry 1Ab Bt gene (Tengel et al., 2002) as shown in Figure 5. The samples taken from Cry 1Ab were amplified with both primers for Cry 1Ab and Cry 1Ba. As the results show, all Bt plants from Cry 1Ab did not amplify with the Cry 1Ba primer. The results for Cry 1Ba were similar.

A total of 400 plants were screened and the results confirmed most of the plants contained either of the Bt gene. Negative plants had already been destroyed during the leaf bioassays analysis. Absence of a band was an indication that the Bt gene was absent. The negative pla-

Figure 1. Stem borer damage on non Bt control maize plants. High infestation and damage from stem borer results in total yield loss on all infested ears.

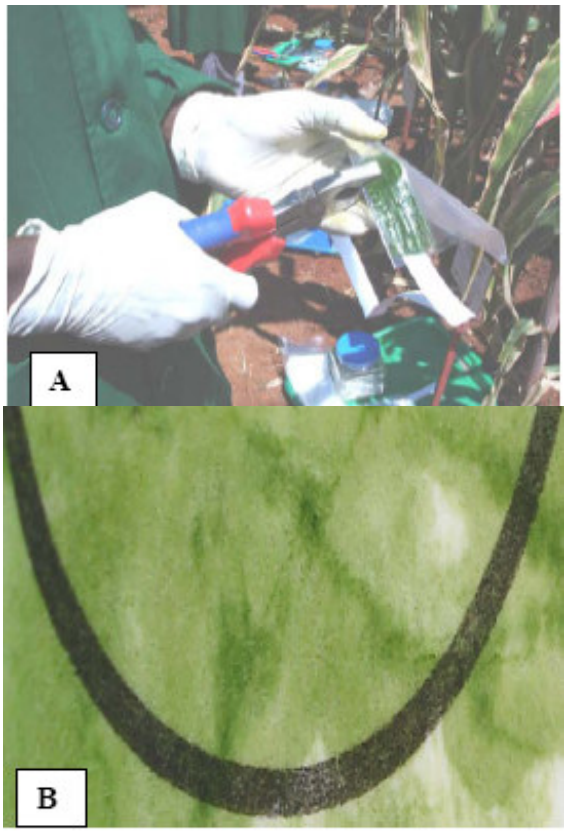

Figure 2. Sap being introduced into the FTA paper (A). Enough sap is pressed onto the FTA paper from mature maize plants at the grain filling stage (B).

nts found after the PCR amplifications were destroyed. There were ninety-five percent of positive plants detected by the PCR method, a good indication of the reliability of both leaf bioassays and PCR analysis (Figure 6). FTA paper technology shows a clear advantage over other DNA extraction methods when the objective is to obtain sufficient amounts of DNA for PCR analysis. However, the FTA paper technology does not allow for DNA quantification and can be limiting where this information is crucial to the analysis being conducted.

The maize plants analyzed had all been selected using leaf bioassays and the PCR data confirmed the reliability of both methods. Apart from being applicable in the breeding process, the PCR method and FTA technology offer an economical and less intensive screening method. In the protocol developed by Tozzini et al. (2002), primers for the CAMV promoter were used as a marker. The team developed a routine PCR protocol for detection of GMOs in a semi-quantitative way giving good estimations of the concentration of GMO DNA. Among the different techniques currently used, the real-time quantitative PCR is also a powerful technology well adapted to the mandatory labeling requirements in the European Union (EU). And has been used in the detection of Bt gene in the event MON810 (Hernandez et al., 2003).

Although not tested during this study, we believe that the PCR method can also be used to detect the Bt genes in non target insects and refuges. This may be a tool to monitor evolving resistance of non-target insects to the Bt genes and movement of transgenes to wild relatives of crops and landraces of maize being primarily wind-pollinated (Luna et al., 2001). There is also a growing risk of refuge contamination by Bt genes which may interfere with the important strategy of delaying pest resistance development by promoting survival of susceptible pests (Chilcutt and Tabashnik, 2004).

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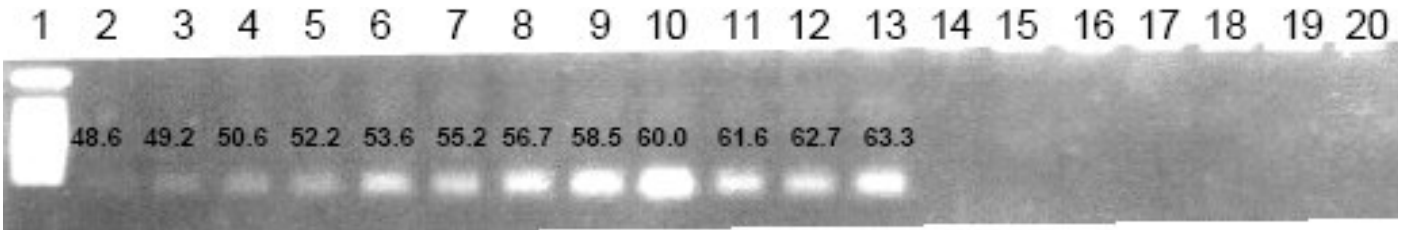

Figure 3. Experimental determination of optimal annealing temperature of DNA samples prepared using the FTA method. The temperature ranged from 48ºC to 64ºC (Lane 2-13) for Cry 1Ab. Control non Bt plants are in lane 14 -20. Lane 1 contains the standards. The optimal temperature was found to be at 60ºC.

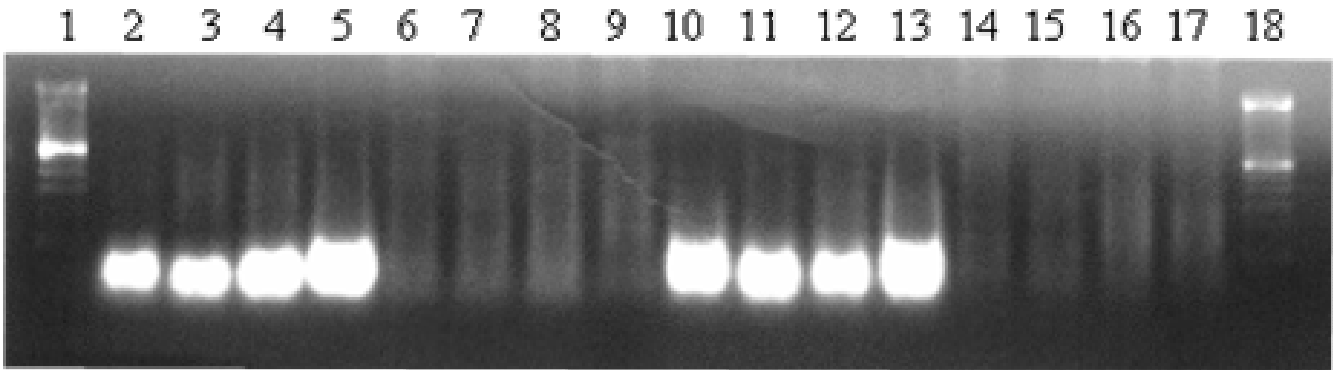

Figure 4. The amplification of the Cry 1Ab and Cry 1Ba primers using genomic DNA. The leaves were harvested from the biosafety greenhouse and immediately frozen in liquid nitrogen before DNA extraction. Lanes 2-5: Cry 1Ab plants amplified with the Cry 1Ab primer. Lanes 6-9: non Bt plants using the Cry 1Ab primer. Lanes 10-13: Cry 1Ba plants amplified with the Cry 1Ba primer. Lanes 14-17 are non Bt control plants amplified with Cry 1Ba primer. Lanes 1 and 18 are standards.

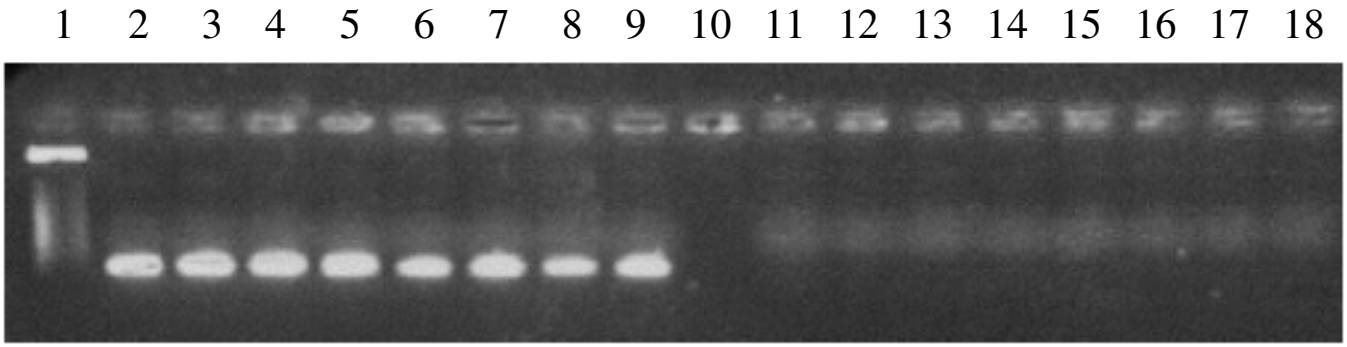

Figure 5. Cry 1Ab specific amplifications tested on both Cry 1Ab and Cry 1Ba plants with DNA samples prepared using the FTA paper technology. Lanes 2-9 shows positive results of Cry 1 Ab plants amplified with Cry 1Ab primer. Lanes 11 -18 show negative results of Cry 1Ab plants amplified with Cry 1Ba primer. Lane 1 is standard.

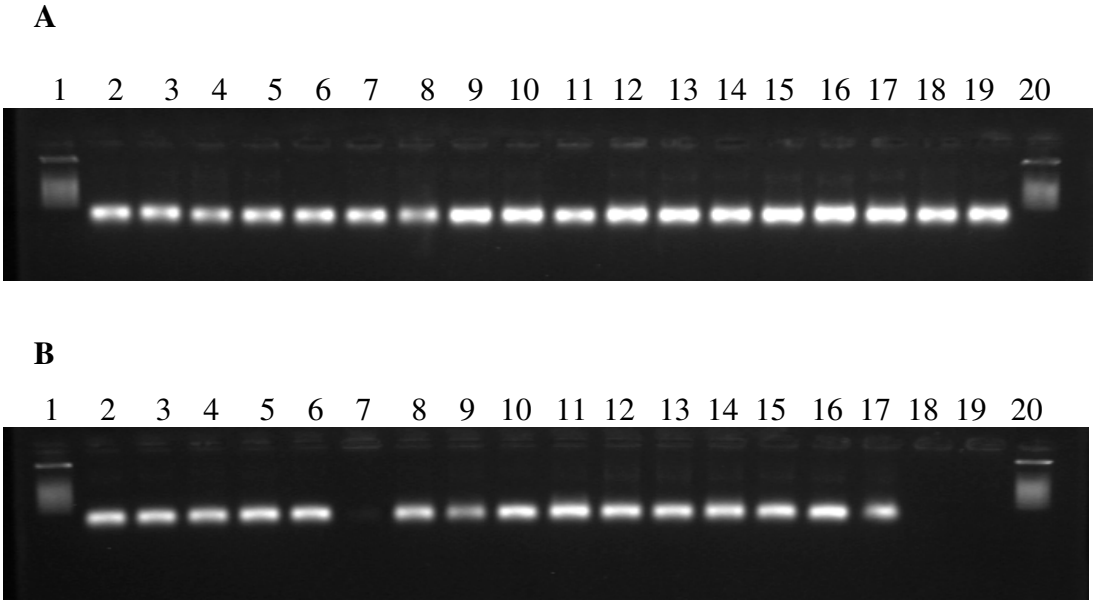

Figure 6. A. Results of some of the Cry 1Ab plants all showing presence of the Cry 1Ab gene. **B**. Results of Cry 1Ba plants showing negative results for Cry 1Ba gene as missing bands in lanes 6, 17, 18. Lanes 1 and 20 are standards.

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