Identification of Recombinant DNA from Genetically Modified BT-11 Maize

Md Shahidul Islam[1]*, Md Nahid Hossain[2], Mst Naima Afrin Eliyana[3], Amitabh Shuva Chakma[4], Sanjit Chandra Barman[5] and Md Mydul Islam[6]

[1]Department of Plant Pathology, Yunnan Agricultural University, Kunming, China
[2]Department of Food Technology and Rural Industries, Bangladesh Agricultural University, Mymensingh, Bangladesh
[3]Department of Fisheries Management, Hajee Mohammad Danesh Science & Technology University, Bangladesh
[4]Department of Environmental Science, Bangladesh Agricultural University, Mymensingh, Bangladesh
[5]Department of Agronomy, Bangladesh Agricultural University, Mymensingh, Bangladesh
[6]Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, Bangladesh
Corresponding author: islam_ynau@163.com

***Abstract*—** Detection of recombinant DNA segment from foods/feeds/kernels is important for the labelling and safety assessment of genetically modified (GM) foods. In this study, polymerase chain reaction (PCR) assay was performed to detect the recombinant DNA and maize intrinsic gene sequence from the genetically modified Btll and non- Bt maize. DNA was extracted from freeze dried GM Btll (N58, Novartis Seed Company) maize powder and freeze-dried powder from Non-GM isoline of the Btll maize. Primer pair IVO11-5' and CRO11-3' was designed to amplify a part of the region of the insect resistant Cry 1 Ab gene sequence that was inserted in GM Btll maize. A primer pair ZEO1-ZEO2 was also designed to detect the maize intrinsic zein (ze 7) gene to assess the efficiency of all reactions, thereby eliminating any false negatives. To confirm the reproducibility, specificity and sensitivity of the designed primers, PCR was performed on diluted genomic DNAs extracted from GM and source of non-GM maize. A parallel negative control was run each time to avoid the contamination. DNA extraction kits were able to extract good quality DNA from the crushed maize samples. Recombinant Cry 1 Ab gene (437 bp) and maize intrinsic zein (ze 1) gene (242 bp) sequence were successfully amplified from the freeze dried Btll and non-Bt maize powder in this investigation. The reproducibility, specificity and sensitivity of the detection were high and therefore, can be concluded that this method could be used for fast and easy screening of Bt gene in the food products and GM Bt crops and could be used in the safety assessment procedure of Bt crops.

***Keywords*—** GMO detection, PCR, RNA, DNA, biotechnology, bio-engineering, molecular biology.

I. INTRODUCTION

Food biotechnology focuses on the use of biological techniques to agriculture and microorganisms to enhance the value, yield, safety, processing and production system of food [1]. It for this reason consists of the traditional meals production approaches used for bread, beer, cheese and various fermented milk products [2]. A extraordinarily greater current (i.e. beginning approximately 25 years in the past) utility of biotechnology to food is genetic modification (GM), also called genetic engineering, genetic manipulation, gene technology and/or recombinant DNA technology [3]. The collective time period "Genetically changed Organisms" or GMOs is used regularly in regulatory documents and within the clinical literature to explain vegetation, animals and microorganisms which have had DNA introduced into them by means of way other than by aggregate of an egg and a sperm or by using natural bacterial conjugation [4].

Recent years have been tremendous advances in food biotechnology, including transgenic crop breeding and genetic modifications of organisms used in food production [5]. For centuries cross-breeding techniques have been used to modify or improve the quality, yield and taste characteristics of food [6]. Now, with new technology, it is possible to identify and transfer particular characteristics of living organisms [7] and alter them in a specific and direct way [8]. By introducing a new segment of genetic material coming from other living organisms, whether plant, animal or microbe, the resultant plant or animal is what is called “a genetically modified organism" or GMO [9].

Genetically modified organisms (GMOs) may be described as organisms wherein the genetic cloth (DNA) has been altered in a manner that doesn't arise evidently through mating or natural recombination [10]. The usage of "recombinant DNA generation" or "genetic engineering" lets in decided on person genes to be transferred from one organism into every other, every now and then among non-related species [11]. For example, GM maize is insect resistant maize that has been developed by inserting gene sequence from the bacterial organism[12]. The new GM food and feed

regulation provides a harmonized procedure for the scientific assessment and authorization of GMOs and GM food and feed. The assessment process is centralized with the European food safety Authority (EFSA) taking duty for venture this technique.

The law calls for labelling of all GM meals and feed, which include or encompass GMOs or are constituted of or incorporate substances constructed from GMOs no matter the presence or absence of GM fabric inside the final food or feed.

The peoples also worried about the potential risk of transgene transfer from GMOs to mammalian cells, potential transfer of antibiotic resistance of bacteria, potential chronic toxicity from GM protein to man and animals etc. Various international organizations, e.g. FAO, OECD, and FDA were also concern and they formed different AdHoc working groups to develop the safety assessment protocols for safe transfer, handling and safe use of GMOs, to solve this problem [13].

In 2000, the international protocol on biosafety has been finalized in Cartagena, Columbia. The protocol is named as "Cartagena Protocol" on biosafety which has also given emphasis on labelling of GMOs [14].

In these regards, detection of genetically modified trait is very important and it's the first step of the safety assessment procedure and the labeling that separates GMOs from non-GMOs. effective regulatory manage over GMOs is crucially depending on the lifestyles of reliable analytical methodology for detection and identity of particular genes, and for quantification where, for instance, a threshold restrict is ready [15]. Till the mid-1990s, inside the absence of dependable analytical methods it become impossible to decide whether or not a meals or food aspect have been genetically changed [16].

More recently, however, new methods have been developed polymerase chain reaction (PCR) - a method which can amplify *in vitro* of specific DNA sequences known as nucleotides. As a result, it can detect very tiny amounts of organism-specific DNA sequences.

Recently Bangladesh has been evaluating agronomical performance of GMOs in contained condition in BRRI and BARI, Gazipur. Bangladesh National Biosafety Framework (BNSF) has been already passed and emphasized on the labelling of GMOs.

Therefore, development of methods required for the detection of transgene / genetically modified trait is important [17]. This study intends to develop specific PCR method to detect *cry 1 Ab* gene from GM (Genetically Modified Btll) maize that will allow identifying *Bt* maize from non-*Bt* maize and will also be applicable in safety assessment and labelling procedure of *Bt* crops.

II. MATERIALS AND METHOD

A. Study Materials

This experiment was carried out at the molecular biology laboratory, Department of Plant Pathology, Faculty of Agriculture, Bangladesh Agricultural University, Bangladesh.

B. DNA Extraction

Wizard^{R} Genomic DNA Purification Kit (Promega Corporation, 2800, Woods Hollow Road. Madison, USA) was used for DNA extraction. Main reagents were Cell Lysis Solution, Nuclei Lysis Solution, Protein Precipitation Solution, RNase Solution, DNA Rehydration Solution, 1.5 ml micro centrifuge tubes, Water bath at 65° C, Water bath at 37° C, Isopropanol at room temperature, and 70% ethanol at room temperature.

Forty (40) mg freeze dried powder was taken to a 1.5 ml micro centrifuge tubes. 600 µl Nuclei Lysis Solution was added and vortexed 1-3 seconds to wet the tissue. Incubated at 65° C for 15 minutes. RNase Solution of 3 µl was added to the cell lysate and mixed the sample by inverting the tube 2-5 times. The mixture was incubated at 37°C for 15 minutes. The sample was allowed to cool to room temperature for 5 minutes before proceeding. 200 µl Protein Precipitation Solution was added and vortexed vigorously at high speed for 20 seconds. The solution was centrifuged for 3 minutes at 13,000-16,000 xg. The precipitated proteins were formed a tight pillate. The supernatant containing the DNA (leaving the protein pellet behind) was carefully removed and transferred it to a clean 1.5 micro centrifuge tube containing 600 µl of room temperature isopropanol. The solution was mixed gently by inversion until thread-like strands of DNA from a visible mass [18]. The solution was centrifuged at 13,000-16,000 x g for 2 minutes at room temperature. The supernatant was carefully decanted, 600 µl, 70% ethanol was added at room temperature and gently inverted the tube several times to wish the DNA. The solution was again centrifuged at 13,000-16,000 x g for 2 minutes at room temperature. The ethanol changed into carefully aspirated using either a drawn Pasteur pipette or a sequencing pipette tip. The DNA pellet could be very unfastened at this point and care must be used to keep away from aspirating the pellet into the pipette. The tube was inverted into clean absorbent paper and air dries the pellet for 15 minutes. DNA re-hydration solution was added dehydrated the DNA by incubating at 65° C for 1 hour. Periodically the solution was mixed by gently tapping the tube. The DNA was stored at 4° C until use.

C. Confirmation of DNA Preparation

DNA extracted following the above protocol often contains genomic DNA contains a large amount of RNA

and pigments, which usually cause over estimation of DNA concentration on a spectrophotometer [19]. Thus, the DNA samples were evaluated both quantitatively and qualitatively using spectrophotometer and agarose gel electrophoresis. The main reagents were Agarose powder (Takara, LO3 grade) and 5X TBE Buffer (pH8.3). The major components of the composition for 1000 ml were 54g Tris, 27.5g Boric Acid, 4.65g EDTA and Ethidium Bromide. At first, 0.40 g agarose powder was weighted out and placed into a 500ml Erlenmeyer flask. 50ml electrophoresis buffer (1X TBE buffer) was added into the flask. The flask was cooked into a micro wave oven for about 1 minute with occasional swirling to generate uniform suspension until no agarose particle was seen and the agarose solution became transparent [20]. 2.5 µl ethidium bromides (10mg/ µl) were added into the flask. Gel casting tray (15 x 15x2 cm^3 in size) was assembled with gel comb of appropriate teeth size and number [21]. Molten agarose was poured on to the gel casting tray and allowed to solidify on the gel casting tray and allowed to solidify on the bench).

D. DNA Sample Preparation and Electrophoresis

The main reagents were loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and mM EDTA) and DNA marker [22]**.** The comb was gently removed. The hardened gel was transferred to the electrophoresis chamber and maintaining the gel horizontal and containing sufficient 1X TBE buffer to cowl the gel ~lmm [23].

Loading dye (as required) placed on a piece of aluminum foil paper using adjustable micropipette (0.5 to 10µl). The dye molecules provide a visual tracking method. 7 µl extracted DNA sample was added to it and mixed well using the same micropipette and samples were loaded to an appropriate well of the gel. The known DNA marker (as required) was loaded in the first lane of the gel. The gel tank was covered and the electrophoresis apparatus were connected to the power supply and turned on to migrate the DNA from negative to positive electrode (Black to Red) [24]. Electrophoresis was carried out at 100v for 35 minutes to get the dye 3/4 of the gel length. When DNA migrated sufficiently, as judged from the migration of Bromophenol blue of loading buffer, the power supply was switched off [25].

E. Documentation of the DNA Sample

For documentation the gel was taken out carefully from the gel chamber and the gel placed on the UV Tran illuminator in the dark chamber. The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in a discatte, as well as printed on thermal paper. As Ethidium Bromide (EtBr) is a powerful mutagen and carcinogen, hand gloves were used. A transillumination produces UV radiation which can cause eye damage thus eye protector should be used while working with it [8].

F. Quantification of DNA Concentration

Quantification is necessary to optimize the DNA concentration which is important variable for PCR amplification [26]. The DNA concentration of the solutions was determined by measuring the UV absorption at 260nm. Excessive genomic DNA may result in smears or in a lack of clearly defined bands in the gel [14]. On the other hand, too little DNA gives non-reproducible patterns [27].

In the course of the quantification of DNA concentration, a rectangular cuvette became filled with 2 ml sterile distilled water and location within the cuvette chamber and the absorbance reading was adjusted to 0 for standardization [28]. The take a look at samples had been organized by taking 2 µl of each DNA sample inside the cuvette containing 2 ml sterile distilled and through blending by pipetting. After recording the absorbance reading, the cuvette was rinsed out with sterile water. The absorbance readings of extracted DNA samples of two maize cultivars are listed in Table 1.

$$\text{DNA concentration } \left(\frac{\text{ng}}{\mu\text{l}}\right) = \text{absorbance} \times \frac{\text{Volume of distilled water } (\mu\text{l})}{\text{Amount of DNA sample (ml)}} \times \text{Conversion factor } (0.05) \times 1000$$

Note: The results of the DNA concentration were obtained in µg/ µl, this result containing a fraction. So, to avoid fraction was converted it in ng/ µl. (1 µg = 10^{-3}ng). Therefore, the result was multiplied with 1000 (Table 1).

Table1. Absorbance reading and concentration of different DNA samples

Items	Sample No.	Absorbance (260 nm)A	DNA Concentration (ng/pl)
Genetically Modified (GM) maize	GM-1	0.025	1250
	GM-2	0.025	1350
	GM-3	0.027	1350
Non-Genetically modified (Non-GM) maize	NGM-1	0.011	550
	NGM-2	0.017	850
	NGM-3	0.014	700

G. Preparation of Working Solution

Preparation of working solution (50, 25 and 10 ng/ µl) from different DNA samples were prepared by using the following formula

$$V_1 \times S_1 = V_2 \times S_2$$

$$V_1 = \frac{V_2 \times S_2}{S_1}$$

Where,

V_1= Final volume of DNA solution (µl)

S_1= Final DNA concentration (25 ng/ µl)

V_2 = Initial volume of DNA solution (µl)

S_2 = Initial DNA concentration (ng/ µl)

III. AMPLIFICATION OF DNA BY PCR

A. Oligonucleotide Primer

Two pairs of oligonucleotide primers were used to detect the intrinsic maize genes and the recombinant *crylAb* gene [29]. The *ZE01* and *ZE02* primer pair was used to detect of intrinsic zein gene. The *IV01* and *CR01* primer pair was used to detect recombinant *cry 1 Ab* gene [30]. Details of the primer can be seen in Table 2. Primers were synthesized by the Singapore Chemical Co., Ltd. Singapore and then diluted with an appropriate volume of water to final concentration of 50 µmol/L and stored at -20° c until use. The r-DNA components introduced into the GM maize lines and an intrinsic gene are shown in Figure 2. Two pairs of oligonucleotide primers are shown in Table 2. Oligonucleotide primers for GM maize were designed based on *cry Ab* and the promoter regions of P-35S and the intron *adh 1/1S/IVS6* using the DNA sequence information [31]. The oligonucleotide primer pair *IV01* and *CR01* was synthesized to detect the part of the *cry 1 Ab* gene cassette of *Bt 11* maize (Figure 2a). The oligonucleotide primer *ZE01- ZE02* was used for detection of the intrinsic zein (*ze 1*) gene to assess the efficiency of all reactions, thereby eliminating any false negatives as shown in Figure 2b.

Table 2: Primers and their sequences used in the study

Primer	Sequence	Specificity
ZE01	5'-TGC-TTG-C AT-TGT-TCG-CTC-TCC-TAG-3'	Ze 1/sense
ZE02	5'-GTC-GCA-GTG-ACA-TTG-TGG-C AT-3'	Ze 1/antisense
IV01	5'-GGT- ACA-GTA-CAC-ACA-CAT-GTA-T-3'	Adh 1-1S/sense
CR01	5'-GAT-GTT-TGG-GTT-GTT-GTC-CAT-3'	Cry 1(b)/ antisense

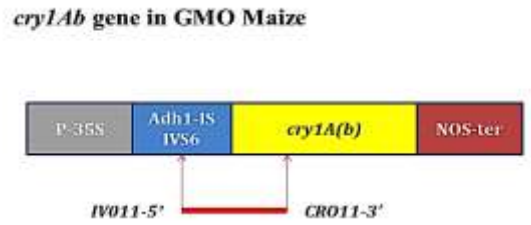

Figure 2a. Shows schematic diagram of the CrylAb gene cassette of Bt11 maize. Indicates part of the cassette that is amplified by the IVOl 1-5'- CRO11-3' primer pair. The unit of an insect resistant triat consists of [CMV P-35S] - [DNA fragment containing the No.6 intron sequence (IVS6) from maize alcohol dehydrogenase 1 gene (Adh1-IS)]-[synthetic cry 1Ab]- [NOS-ter derived from agro-bacterium tumefaciens]

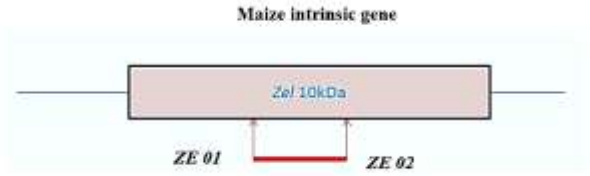

Figure 2b. Schematic diagram of zein (Zel) gene showing part of the gene that was amplified by ZE01-ZE02 primer pair.

B. Reaction Mix Preparation to Perform Polymerase Chain Reaction (PCR)

PCR reactions were performed on each DNA sample in a 10 µl reaction mix containing the reagents such as Ampli Taq polymerase buffer (10 x) = 1 µl, Primer = 0.5 µl each of forward and reverse 0.5 µl each, dNTPs = (250 µM) = 1 µl, Taq DNA polymerase = 0.2 µl, Genomic DNA (50 ng/ µl, 25 ng/ µl and 10 ng/ µl) = 1 µl and Nuclease Free Water = 5.8 µl. For the duration of the experiment, PCR buffer, dNTPs, and primer solutions had been thawed from frozen stocks, blended by using vortexing and placed on ice [32]. DNA samples have been also thawed out and combined lightly. The primers were pipetted first into 0.2 ml PCR tubes. Instead of extracted DNA, water was used as negative control [33]. For each DNA sample being tested, a pre-mix was then prepared including, in the following order: buffer, dNTPs, primer and distilled water. Taq polymerase enzyme was then added to the pre-mix [5]. The pre-mix was then mixed well then aliquoted into the tubes 1 µl of DNA sample was added that contained 50ng/ µl or 25ng/ µl or 10 ng/ µl DNA. The tubes were then sealed and placed in a thermo cycler and the cycling was started immediately. Care was taken during pipetting. Care was taken during usage of the enzyme Taq polymerase. Taq polymerase, primer and reaction mix were always kept on ice.

C. Thermal Profile

Different thermal profile was tried and finally the following protocol was found suitable. The reaction mix

was preheated at 94°C, for 10 minutes followed by 45 cycles of 1-minute denaturation at 94° C, 1 min annealing at 60°C and elongation or extension at 72°C for 1 minute [26]. After the ultimate cycle, a very last step of 7 minutes at 72°C turned into introduced to permit complete extension of all amplified fragments. After of entirety of biking software, reaction changed into held at 4°C.

D. Electrophoresis of the Amplified Products and Documentation

The amplified products were separated electrophoretically on 2% agarose gel containing ethidium bromide. The gel was prepared using 1 g agarose powder (Fisher Bioteh, New Jersey, and USA), 100 ml 1X TBE buffer and 5 µl ethidium bromide. Agarose gel electrophoresis was conducted in 1TBE buffer at 92 V for 35 minutes. One molecular weight marker 100 bp DNA ladder size was electrophoresed alongside the PCR reactions [14]. DNA bands were observed under UV light on a Trans illuminator and photographed by Image Documentation System (Labortechnik, Germany).

IV. CONCLUSIONS

Freeze dried powder was received from National Institute of Animal Health Tsukuba, Japan as GMO and non-GMO reference material were used in this study. DNA was extracted from freeze dried GM *Bt11* and non-GM maize powder as reference material using Wizard ^{R} Genomic DNA purification Kit (Promega Corporation, 2800 Woods Hollow Road, Madison, USA). Detection of recombinant DNA segment from food/feed kernels are important for the safety assessment procedure and labeling to differentiate GMOs from non-GMOs. Polymerase Chain Reaction (PCR) assay was developed to detect and identify the genetically modified maize *Bt11*. We designed primer pair *IVO1 1-5'* and *CRO1 1-3'* to detect the region of the insect resistant *cry 1 Ab* gene sequence inserted in GM *Bt11* maize. A primer pair *ZEO1-ZEO2* was also used for the maize intrinsic zein gene (Ze 1) to assess the efficiency of all reactions, thereby eliminating any false negatives. To confirm the specificities of the designed primers, PCR was performed on genomic DNAs extracted from GM and non-GM maize. We were able to detect successfully the *cryAb* gene sequence from the GM *Bt11* maize but not from non-GM maize. Absence of *cryAb* specific band on negative control and in non-GM maize samples indicated high specificity of this PCR protocol. Presence of specific band in diluted DNA samples indicated high sensitivity of the reaction. Because the presence of the corresponding DNA segment was specifically detected in GM maize by the designed primer, then it is concluded that this method is useful for fast and easy screening of *Bt* gene in the maize/corn food products.

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