Detection of GMOs in food products using PCR

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Received January 11, 2018

This project aimed at developing a method to detect genetic modifications, specifically in GMO (genetically modified organism) maize (*Zea mays*). The MON810 maize has been chosen as a model since it is most often used to combat crop loss due to insects. It has been genetically modified with a gene coming from the bacterium *Bacillus thuringiensis* and coding for the cry1Ab toxin, efficient against insects. Therefore, the strategy of GMO detection is to identify the cry1Ab gene. The method of detection was based on DNA extraction and the supposition that the cry1Ab gene could be detected by amplification through PCR (Polymerase Chain Reaction). Several primers were designed to realize the amplification: primers targeting the cry1Ab gene and control primers, targeting reference genes for *Zea Mays* (hmga gene, NCBI Acc. n° AJ131373) and plant (chloroplast gene, NCBI Acc n°X15901, Z00044). After PCR, the genetic material was then quantified by gel electrophoresis to confirm the presence of the target sequences. In parallel, a preparation of a monoculture of *Bacillus thuringiensis* was produced from a bio-insecticide. The sequence responsible for 'cry' protein synthesis was extracted from the BT spore and used as a positive control.

Nowadays, 32% of maize (*Zea mays)* crops are genetically modified worldwide, making it the second most genetically modified species behind soy **[1]**. Most of the genetically modified maize strains express resistance to insect pests. For example, the MON810 corn was designed to combat crop loss due to insects in the Lepidoptera order (butterflies and moths), including the European Corn Borer **[2]**. It has been genetically modified with a gene coming from the bacterium *Bacillus thuringiensis* (BT) and coding for the toxin cry1Ab, efficient against insects **[3]**.

Bacillus thuringiensis is a Gram-positive, aerobic, endospore-forming bacterium. The insecticidal properties of BT are due to crystalline protein inclusions produced during sporulation **[4]**. The genetic modification of maize aims at providing it the ability to produce these insecticidal proteins. Indeed, when they are ingested by insect larvae, a protoxin is dissolved and activated under alkaline conditions present in the midgut of target insects, releasing the cry1AB active toxin. It then binds to a specific receptor in the insect's midgut epithelial cells resulting in pore formation in the membrane which eventually leads to disruption of osmotic balance, cell lysis and death **[5]**.

This paper describes a method to detect genetic modifications, specifically in maize, through the identification of the cry1Ab gene. The method of detection is based on DNA extraction with CTAB, a powerful surfactant, and amplification through PCR (Polymerase Chain Reaction). Additionally, a preparation of a monoculture of *Bacillus thuringiensis kurstaki* was produced from a bio-insecticide to extract the sequence responsible for the cry protein synthesis from the BT spore which can be used as a positive control for the presence of the cry1ab fragment in maize.

MATERIALS AND METHODS

Maize types. Several types of maize were used: a French raw corn on the cob coming from the Auchan supermarket, bought the 3th of December (named MS) and another one coming directly from a French field in the Seine-et-Marne department 77 (named MC).

Culture and identification of *Bacillus thuringiensis (BT)* from the insecticide. The bacteria were extracted from a commercial bio-insecticide (Delfin® Jardin). The acetate selection method was used to selectively grow *Bacillus thuringiensis* **[6]**. Briefly, 0,5g of bio-insecticide was inoculated in LB broth buffered with sodium acetate (0.25 M, pH 6.8) in a 125-ml flask. This mix was placed at 30°C and 250 rpm for 4 hours. A 1-ml aliquot was then heated to 65°C for 10 min in a pre-warmed 5-ml test tube, and undiluted aliquots were spread on LB-agar plates and incubated for 48 h at 30°C. Bacteria were then stored at 4°C for future experimentations.

For identification, bacteria were gram-stained using classical protocol from Bartholomew *et al.* **[7]**.

Genomic DNA extraction. The preparation of CTAB (Cetyl trimethylammonium bromide) buffer required for the maize & leaf DNA extraction involves preparation of TE (Tris-EDTA) buffer 1X. To prepare the TE buffer, Trizma-Hydrochloride (Tris-HCl, Sigma, T5941) and Ethylenediaminetetraacetic acid disodium (EDTA, Sigma, E5134) were used at pH 8.

The CTAB buffer was prepared with 0.5g of CTAB (H6269, Sigma), 2.05g of NaCl (S3014, Sigma) and 0.25g of PVP (Polyvinylpyrrolidone, PVP40, Sigma) in 25 mL of TE buffer 1X (previously prepared).

200mg of maize or leaf was ground with a mortar and pestle, then gently dispersed in 500mL of CTAB extraction buffer. The mixture was placed in a 65°C water bath for 5 minutes. Following this step, 200 µl of chloroformisoamyl-alcohol (C0549, Sigma) were added. After centrifugation (12 000 rpm, 5 min), the upper aqueous phase was discarded. 200µL of isopropanol were added to precipitate the DNA. After centrifugation (12 000 rpm, 15 min), the isopropanol is removed and the pellet was washed with 200µL of ethanol 70%. After centrifugation (12 000 rpm, 5 min), the ethanol was completely removed, and the pellet was re-suspended in 30 µL of TE overnight.

Regarding the bacterial genomic DNA extraction, it was followed as described in Valicente *et al*. **[8]**. After the extraction, samples were stored at -20°C until further use.

Quantification of extracted DNA. The quantification of DNA after extraction was realized by two methods: spectrophotometry and agarose gel electrophoresis. Spectrophotometry was conducted using a Microdrop (Thermo Scientific µDrop Plate, AN-MRµDrop_0911). In brief, 3 µL of the DNA samples in the microdrop plate was deposited, in comparison with 3 µL of TE buffer (blank). The ratio A260/A280 gives an estimate of the protein contamination of the sample. For a good quality sample, the value should be between 1.8 and 2.0. The ratio A260/A230 estimates the chemical contamination such as phenols, carbohydrates or a high salt concentration. The expected 260/230nm ratio is approximately 2. The DNA concentration can be calculated by using this formula: [DNA] (µg/mL) = Abs260 x 50 µg /mL x (10mm/0,5mm) = Abs260 x 50 x 20. In parallel, a 1 % agarose gel electrophoresis was performed with TAE buffer 1X and 0,5 of 1Kb DNA ladder, at 120V for one hour.

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Table 1. Sequences of primers designed to amplify plant, maize, and bacterial regions of DNA (Eurofins Genomics)

Name	Sequence (5' -> 3')	% GC	Tm (°C)
Singh-plantf	CGAAATCGGTAGACGCTACG (20)	55	59.4
Singh-plantr	GGGGATAGAGGGACTTGAAC (20)	55	59.4
Singh-maizef	GAAATCCCTGAGCGAGTCGGTA (22)	54.5	62.1
Singh-maizer	GCGATGGCCTTGTTGTTGTACTCGA (25)	52	64.6
Singh-Cry1abf	CGCAGGCCCTTCAACATCGGTATC (24)	58.3	66.1
Singh-Cry1abr	GGTCGGCGCCCAACAACAAGA (21)	61.9	63.7

Primer Design. Three pairs of primers (Table 1) were ordered to Eurofins following the primers design from Singh, C. K., *et al.* **[9]**, to amplify a 550 bp fragment of a plant gene (chloroplast gene, NCBI Acc n°X15901, Z00044), a 175 bp fragment of a maize gene (hmga gene, NCBI Acc. n° AJ131373) and a 1533 bp fragment of the cry1AB gene.

Polymerase Chain Reaction. An annealing temperature optimization for each primer set was performed using the temperature gradient setting. Each 25 µL PCR reaction contained: 1 µM forward and reverse primers, 250 ng DNA, 0.25mM dNTP mixture (Taq Ozyme), 1.25U DNA Taq Polymerase (Taq Ozyme), 1X PCR Amplification buffer (RB), 1.5 mM MgCl2 and sterile distilled water. PCR settings are listed in Table 2.

Table 2. PCR conditions for amplification of extracted DNA

	Temperature (°C)	Time (s)
1	94.0	120
2	94.0	30
3	Gradient 52.5 – 60.0°C	30
4	72.0	60
Steps 2-4 were repeated for 30 cycles		
5	72.0	300
6	10.0	Pause

DNA electrophoretic analysis. 10µL of PCR DNA samples were deposited with loading buffer 6X and analyzed in 1% agarose gel with TAE buffer 1X, in comparison with 1kb and 100bp DNA ladders. The gel was run at 120 V for one hour. DNA was then observed under the UV light.

RESULTS

Identification of the biological material. Bacteria BT extracted from the biological pesticide were cultured on LB agar plate as described in the methods section.

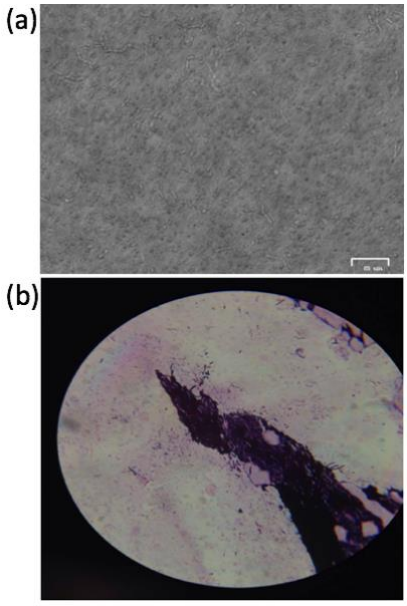

Fig 1. Fresh state *Bacillus thuringiensis* observed with optical microscope X1000 (a) Gram stained *Bacillus thuringiensis* observed with optical microscope X400 (b)

Bacteria were first observed without any coloration to test their mobility and shape. The bacillus shape was confirmed. Figure 1a showed colonial and microscopic morphology identical to *B. thuringiensis*. Figure 1b shows result of the gram- staining, bacillus shape and purple coloration were observed displaying the gram-positive structure expected for *Bacillus thuringiensis*.

Assessment of the quantity of extracted DNA by Microdrop. Three replicates were used for each sample. To examine the purity of the DNA extract, the UV absorption at 230, 260, and 280 nm were measured and the A260/280 and A260/230 ratios were calculated (Table 3). The two types of maize did not have a significant effect on the DNA concentration, there were high concentration of DNA in all tubes. Regarding the ratios of contaminations, there were no protein contamination in the samples (the value of A260/A280 was between 1.8 and 2.0). However, the ration A260/230 of the maize from the field (MC) sample suggested the presence of chemical contamination such as phenols, carbohydrates or a high salt concentration.

Concerning the bacterial genomic DNA extraction, the sample was not contaminated by proteins but indicated the presence of co-purified contaminants (phenols, salt or carbohydrates).

Table 3. DNA quantification by Microdrop (MS: maize from the supermarket; MC: maize from the field)

	MS	MC	*Bacillus thuringiensis*
[DNA] (µg/mL)	1 831	1 395	238
A260/A280	1,93	1,71	1,87
A260/A230	2,09	1,39	1,79

Visualizing extracted DNA by agarose gel electrophoresis. Three replicates were used for each sample of maize and two tree's leaves as positive controls. The extracted maize DNAs are presented in Figure 2. The gel electrophoresis revealed a single, high molecular weight DNA band up to 1kb for the MS (maize from the supermarket) and the MC (maize from the field) samples. The visualization confirms the presence of DNA.

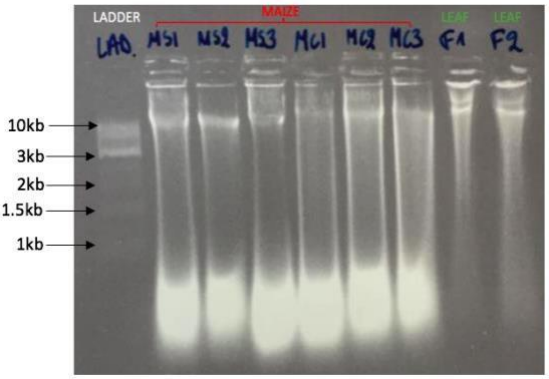

Fig 2. Electrophoresis of extracted maize DNA on 1% agarose gel. MS: Maize coming from the supermarket - MC: Maize coming directly from the field - Ladder: 1kb

The extracted bacteria DNA is presented in the two wells in Figure 3. The gel electrophoresis revealed an expected single, high molecular weight DNA band up to 10kb. Therefore, the visualization verified the presence of DNA in the sample.

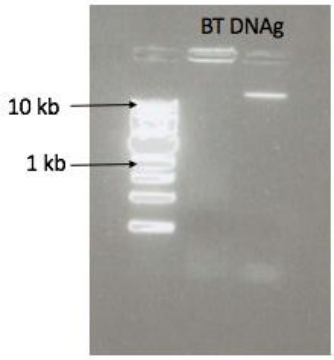

Fig 3. Agarose gel (1%) results showing total DNA extracted from *Bacillus thuringiensis*

PCR Optimization. The optimization of the annealing temperature for maize primers and plant primers was done with DNA from the MS and the MC sample. The primer sets Singh-maizef/Singh-maizer hybridized well within the annealing temperature range of 56-60°C. However, the primer sets were optimal at 60°C (data not shown). The primer sets Singh- plantf/Singh-plantr performed well within the annealing temperature range of 52- 56,8°C with an optimal annealing temperature at 56,8°C (data not shown).

Amplification of a plant gene (chloroplast) and a maize gene (hmga). Two replicates were used for each sample, one positive control (the leaf) and one negative control (TE buffer). The maize primers and the plant primers were used to confirm the presence and quality of DNA extracted from the maize sample. The maize primers and the plant primers successfully amplified the expected regions from the MS and MC DNA samples (Figure 4). Indeed, a band is observed at 175bp which corresponds to the maize fragment (Figure 4a). Regarding the plant gene (chloroplast gene), a band is observed at 550bp for the two different maize samples representing the specific gene (Figure 4b). Therefore, the results confirm the presence of maize DNA in the MS and MC samples.

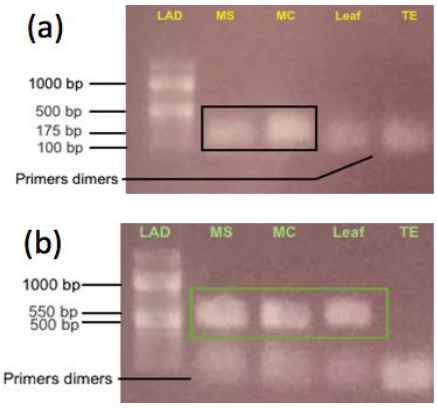

Fig 4. PCR amplification of a maize gene (a) and a plant gene (b)

Amplification of the cry1AB gene. Three different samples were used: maize DNA form the supermarket (MS), maize DNA from the field (MC) and the bacteria DNA (BT). Three positive control (maize DNA with maize primers for the hmga gene) and a negative control (TE buffer) were utilized. The amplification is presented in Figure 5. All the positive controls were as expected, a 175 bp fragment is presented. However, the 1533 bp fragment expected in the samples is not observed. Therefore, it seems that the maize samples and the bacteria did not have the cry1AB sequence.

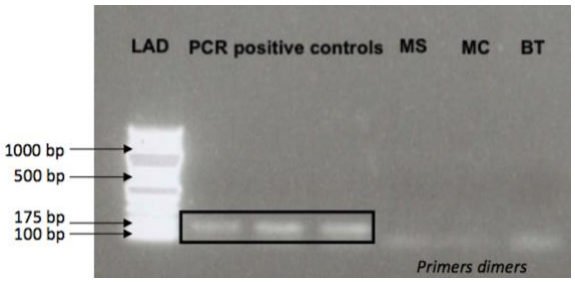

Fig 5. The agarose gel analysis of the amplification products, using the primers specific for cry1AB gene

DISCUSSION

This work aimed at developing a method to detect GMO, specifically in maize, by detecting the presence of the gene cry1Ab. The method of detection is based on DNA extraction and amplification through PCR (Polymerase Chain Reaction), with specific primers that target the cry1Ab gene. Successful PCR products were obtained but a small amount of primers dimers was also observed, suggesting that concentration maize should be optimized, indeed, an overload of primers is suspected.

The first step was the maize DNA extraction using the CTAB method.

Cetyltrimethylammonium bromide (CTAB) is a powerful cationic surfactant that has been established as the best detergent to use during the extraction/isolation of highly polymerized DNA from plant material. This detergent simultaneously solubilizes the plant cell wall and lipid membranes of internal organelles, and denatures proteins. It thus promotes the cell lysis **[10]**. However, some difficulties were encountered, making the DNA extraction challenging to reproduce. Indeed, plant tissues are rich in complex polysaccharides and secondary metabolites like polyphenols. These metabolites interfere and co-precipitate with DNA during the isolation procedure. Thus, some other chemicals like Polyvinylpyrrolidone (PVP) were used to minimize the interference effects of these metabolites **[11]**. On another hand, chloroformisoamyl-alcohol was used to simultaneously remove glass beads, cell wall debris, denatured proteins, and most polysaccharides. Following these steps, isopropanol was added to precipitate the DNA. The bands obtained after migration of genomic DNA confirmed that this procedure can be validated and allows to have correct concentrations of maize DNA.

Bacteria extraction was performed from the germinated spore of *Bacillus Thuringiensis*, leading to difficulties. Bacteria spore are a resistant form and do not display same characteristics and physiology as the native one, and so do not have the same extraction protocol. In the current literature, there are various existing methods which did not seem to present difficulties. An acetate culturing protocol allowed us to isolate and germinate the spores after several attempts. The DNA extraction in Valicente et al. **[8]** also presented some imperfections since the DNA yield was not important and with impurities. Proteinase K was suspected to be the cause of these impurities since it's an enzyme having digestive activity of proteins **[12]**. Proteinase K step has been optimized by heating at 55°C instead of a possible inactivation at 65°C and could be done in 5 hours instead of an overnight culture. Spectrophotometric assay indicated an increase yield by 4 and less contaminants, allowing to suppose that proteinase K is more active at 55°C.

Microscope observation with and without gram coloration confirmed partially the nature of our bacteria. Indeed, since the bacterial support is from a commercial bio-pesticide (Delfin® Jardin), identification of the strain could have been investigated more precisely. Common techniques are available such as 16S-rDNA **[13]**, RFLP (Restriction Fragment Length Polymorphism)**[14]**, and RAPD (Random Amplified Polymorphic DNA) **[15]**. These PCR-based techniques are dependent on DNA extraction which has been difficult to proceed and so we were unable to provide a precise and specific identification. However, RFLP and RAPD are known for a lack of reproducibility and reliability, producing false positive and false negative **[16]**. API Gallery is also a simple and well-documented method to identify specific micro-organism **[17]**, but in our case *B. thuringiensis spp. kurstaki* would not have been completely identifiable by this method since there is a lack of specificity in this assay. In Logan and Berkley **[17]**, *B. thuringiensis* API signature is not even distinct from *B. cereus* and *B. mycoides* which are also considered as plant resistance-promoting bacteria.

Successful PCR products were obtained with maize and plant primers, validating the correct PCR protocol. However, no amplifications were observed for the gene cry1Ab. Several facts could explain these negative results. First, we are not totally sure

of the bacteria type as it comes from a commercially available insecticide. Then, the gene may have been modified from the native *B. thuringiensis* gene in the process of obtaining the GMO, which could explain the non-specificity of the primers chosen. For instance, as described in Jabeen R. et al. **[17]**, codon optimization has probably been done to modify the maize DNA. However, DNA sequencing could be done to know the location of mutagenesis points.

FUTURE EXPERIMENTS

More strain identification techniques such as 16S-rDNA should be attempted to identify precisely the bacteria. This technique is reliable enough to identify the genus, the species and even the subspecies of the bacteria tested.

To definitely validate our PCR method to detect GMO in maize, several experiments can be performed. For example, other genetically modified maize could be tested, with the same control primers (maize and plant). Moreover, as we did not obtain any amplification after PCR with cry1ab primers, the use of other sets of primers to detect the cry1ab sequence could also constitute further studies. They could be designed after bacterial DNA sequencing.

CONCLUSION

In this study, we used the PCR method to detect maize GMO. The results showed that the technique could be a potential step in a prototype Do-It-Yourself for the diagnostic of GMO.

ACKNOWLEDGEMENT

We would like to thank Dr. Frank Yates for his insightful guidance on the project, as well as Mrs. Rafika Jarray, Mrs. Jacqueline Bert and Mrs. Annabelle Planques for their assistance in the laboratory. We also would like to acknowledge Mrs. Estelle Mogensen for her supervision and finally, our fellow classmates for their cooperation.

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